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The ^{103}Pd -DOTMP complex for conversion electron and Auger electron therapy of bone metastatic tumor cells

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Abstract

The radionuclide palladium-103 (^{103}Pd) has significant potential for use in targeted conversion electron and Auger electron radionuclide therapy. In this study, we synthesized a complex of ^{103}Pd with the macrocyclic ligand DOTMP (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylenephosphonic acid). In the complex, the Pd^{2+} ions are coordinated by the macrocyclic nitrogen and carboxylic oxygen atoms. While ^{103}Pd remains stably bound to the ligand, our results indicate that approximately 10% of the decay product, $^{103\text{m}}\text{Rh}$, is released from the $[^{103}\text{Pd}]\text{Pd}$ -DOTMP complex in PBS buffer. We evaluated the cytotoxicity of $[^{103}\text{Pd}]\text{Pd}$ -DOTMP and compared it with those of $[^{177}\text{Lu}]\text{Lu}$ -DOTMP, which emits β^- particles, and $[^{161}\text{Tb}]\text{Tb}$ -DOTMP, which emits both β^- particles and Auger electrons. Since DOTMP complexes do not exhibit internalization and lack nuclear localization within cells, the cytotoxic effect of $[^{103}\text{Pd}]\text{Pd}$ -DOTMP is significantly lower than that of DOTMP labeled with the beta emitters ^{177}Lu and ^{161}Tb , which emit β^- particles over a longer range.

Keywords: Palladium-103; Auger electron emitter, Bone radionuclide therapy

1. Introduction

Primary bone cancers account for less than 1% of all cancer cases. However, bones are commonly preferred sites for the spread of many other cancers, including those of the breast, prostate, and lung. These metastases can lead to severe pain, increased bone fragility, and frequent fractures. During metastasis, cancer cells activate osteoclasts, which increase abnormal bone resorption and disrupted bone turnover. Additionally, osteoblast activity at metastatic sites leads to significant new bone formation. The primary goal of treating bone metastases is to manage pain and improve quality of life. Additionally, reducing the tumor size is highly desirable. Bone metastases are treated using various methods, including strong pain relief medications such as opioids, cytotoxic chemotherapy, hormonal therapy, radiotherapy, bisphosphonates, and bone-targeting radiopharmaceuticals. Among these methods, bone radiopharmaceuticals are preferred because patients generally tolerate them well and experience minimal side effects. There are currently two classes of therapeutic radiopharmaceuticals

used to treat bone metastases. These include calcium analogue-based radiopharmaceuticals, such as $[^{89}\text{Sr}]\text{SrCl}_2$ and $[^{223}\text{Ra}]\text{RaCl}_2$, as well as bisphosphonates that are labeled with radionuclides, primarily beta emitters like ^{153}Sm , ^{177}Lu , and ^{166}Ho .

The radiation doses used in treatment are limited due to the sensitivity of bone marrow to radiation. This does not allow the use of higher doses. As a result, better therapeutic outcomes can be achieved by using ^{223}Ra , which emits short-range alpha particles. Also, there has been significant interest in the use of low-energy conversion electron-emitting radioisotopes due to their reduced tissue penetration and the potential benefits for bone marrow dose reduction. Using conversion electron emitters allows for the administration of significantly higher therapeutic doses while minimizing harm to bone marrow. Recent studies on ^{161}Tb -based radiobioconjugates have shown their exceptional effectiveness compared to similar ^{177}Lu -labeled analogues. The initially observed higher efficacy was attributed to the additional emission of Auger electrons from ^{161}Tb . However, last reports indicate that the IC electrons, rather than Auger electrons, emitted by ^{161}Tb are responsible for a higher absorbed dose in the cell nucleus and a lower clonogenic survival rate for ^{161}Tb -labelled bioconjugates compared to their ^{177}Lu -labeled counterparts [1].

The only conversion electron emitter studied so far for bone pain therapy is the radionuclide $^{117\text{m}}\text{Sn}$, which has a half-life of 14.5 days [2]. Unlike β^- radionuclides and ^{223}Ra , which is an alpha emitter, $^{117\text{m}}\text{Sn}$ emits short-range, low-energy conversion electrons, effectively destroying tumors while minimizing damage to bone marrow and other healthy tissues. A phase 1/2 dose-ranging study examined the impact of the $[^{117\text{m}}\text{Sn}]\text{Sn-DTPA}$ complex on pain relief [3]. Approximately 25% of the patients in the study reported complete pain relief, while another third experienced more than 50% pain relief. The average duration of response was 3.3 months, with a maximum reported duration of 13.4 months. Unfortunately, the $[^{117\text{m}}\text{Sn}]\text{Sn-DTPA}$ complex has several disadvantages, including a low affinity for bone and the tendency for tin to be easily detached from the DTPA complex [4]. Additionally, the production of $^{117\text{m}}\text{Sn}$ is both challenging and inefficient. This radionuclide can be produced only by irradiating ^{116}Cd with 18 MeV alpha particles in the reaction $^{114}\text{Cd}(\alpha, n)^{117\text{m}}\text{Sn}$, or through a less efficient reaction involving fast neutrons, represented by the equation $^{117}\text{Sn}(n, n'\gamma)^{117\text{m}}\text{Sn}$ [5]. In this reaction, $^{117\text{m}}\text{Sn}$ is produced with a very low specific activity, making its use difficult.

The high emission of conversion and Auger electrons and the greater availability than $^{117\text{m}}\text{Sn}$ make the palladium radionuclide ^{103}Pd an interesting candidate for low-energy electron therapy. ^{103}Pd is widely used in seed form for prostate brachytherapy because it also emits X-ray radiation in the 20–23 keV range. The widespread use of ^{103}Pd in brachytherapy has led to the development of methods for its large-scale production. ^{103}Pd can be produced in a reactor by irradiating a ^{102}Pd target with neutrons or in a cyclotron in non-carrier-added form by irradiating a natural monoisotopic rhodium target with protons in a p,n reaction.

^{103}Pd ($T_{1/2} = 16.991$ d) decays by electron capture (EC), which is a β transition in which no charged particle is ejected from the nucleus. ^{103}Pd emits a limited number of Auger electrons and X-rays. As a result of ^{103}Pd decay, $^{103\text{m}}\text{Rh}$ is created ($T_{1/2} = 56.12$ m), which is an attractive candidate for targeted therapy utilising conversion and Auger electron emissions. In the case of $^{103}\text{Pd}/^{103\text{m}}\text{Rh}$ *in vivo* generator, both ^{103}Pd and $^{103\text{m}}\text{Rh}$ emit multiple low-energy Auger electrons (7.44 for ^{103}Pd and 5.88 for $^{103\text{m}}\text{Rh}$), so that the total number of Auger electrons emitted by the $^{103}\text{Pd}/^{103\text{m}}\text{Rh}$ *in vivo* generator is 13.3. Additionally, an average of 0.95 conversion electrons is emitted by $^{103\text{m}}\text{Rh}$ per decay [6]. However, the short half-life of $^{103\text{m}}\text{Rh}$ presents a challenge, making it difficult to prepare radiopharmaceuticals based on this isotope. Despite this, $^{103\text{m}}\text{Rh}$ can still be utilized as an *in-vivo* generator for targeted therapy applications.

To date, no radiopharmaceuticals based on ^{103}Pd have been synthesized or studied. Some research has been conducted using β^- emitting ^{109}Pd to label antibodies, porphyrins, and nanostructure-based conjugates [7,8]. Recently, our team published papers on the synthesis of bisphosphonate-bipyridine complexes labeled with ^{103}Pd , which are intended for the treatment of bone metastases in cancer patients [9]. The compound $[^{103}\text{Pd}]\text{Pd}_2(\text{bpy})_2\text{ale}$ demonstrated significant multimodal toxicity via Auger and conversion electrons and exhibited chemotoxicity levels comparable to those of cisplatin, highlighting its therapeutic potential.

Table 1
Comparison of conversion electron emission from the $^{117\text{m}}\text{Sn}$ and $^{103}\text{Pd}(^{103\text{m}}\text{Rh})$

$^{117\text{m}}\text{Sn}$ [10]		$^{103}\text{Pd}(^{103\text{m}}\text{Rh})$ [11]	
Energy (keV)	Intensity (%)	Energy (keV)	Intensity (%)
126.8	64.9	16.54	19.0
129.4	11.7	36.34	142.3
151.6	26.2	39.13	28.8
		39.67	9.42

Table 1 compares the energy and intensity of conversion electrons emitted by $^{117\text{m}}\text{Sn}$ and $^{103}\text{Pd}(^{103\text{m}}\text{Rh})$. It can be seen that $^{103}\text{Pd}/^{103\text{m}}\text{Rh}$ is a very efficient source of conversion electrons. For $^{117\text{m}}\text{Sn}$, the tissue range of conversion electrons is approximately 220 to 290 μm , which corresponds to about 10 cell layers. In contrast, $^{103}\text{Pd}(^{103\text{m}}\text{Rh})$ emits conversion electrons that have a range of 22–27 μm , which corresponds to the diameter of approximately 2–3 typical mammalian cells. This enables a 'micro-crossfire' effect, potentially damaging cells directly adjacent to the one targeted by the radiopharmaceutical. This is important because bisphosphonates accumulate on the mineral component of the bone in areas where cancer cells stimulate skeletal remodeling, i.e., in the vicinity of metastatic tumor cells. Therefore, the range of conversion electrons emitted by $^{103}\text{Pd}/^{103\text{m}}\text{Rh}$ indicates its greater potential effectiveness against

micrometastases. It can therefore be expected that ^{103}Pd bisphosphonates may complement or replace $^{117\text{m}}\text{Sn}$ in bone palliation therapy.

As mentioned earlier, we synthesized and investigated a mixed complex of ^{103}Pd with bipyridine and alendronate [9]. However, the stability of these complexes was limited due to the bidentate bond formation of the alendronate and bipyridine with Pd^{2+} . Here, we propose the use of a ^{103}Pd -labeled cyclic phosphonate ligand, DOTMP (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylene phosphonic acid) for bone therapy of cancer metastases.

Two studies have shown a very high affinity of cyclam and its derivatives for Pd^{2+} [12,13]. However, no cyclam derivatives with alkylphosphonate side arms have been synthesized and studied so far. On the other hand, cyclene-based phosphonate, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylene phosphonic acid (DOTMP) has attractive properties for radionuclide bone therapy. The first synthesis of DOTMP was reported in 1984 [14]. Since then, DOTMP has been extensively studied for its potential applications as an MRI contrast agent and for clinical uses as an NMR shift and relaxation reagent [15]. This interest stems from its structural similarity to the widely used ligand DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(acetic acid)). Due to its cyclic structure, it forms strong and kinetically inert complexes with metal cations, thereby reducing the risk of off-target release. DOTMP also exhibits an affinity for hydroxyapatite, the main component of bone. So far, DOTMP has been labeled with various radionuclides in the 3+ oxidation state, primarily radiolanthanides, and its potential applications as a radiolabeled phosphonate have been investigated in animal models and some clinical trials involving the β^- emitting radionuclide ^{177}Lu [16]. However, a thermodynamically stable divalent ^{64}Cu complex has also been obtained. By spectrophotometry and electron paramagnetic resonance (EPR) spectroscopy, the structure of the Cu(II)-DOTMP complex in solution was determined to be a square-pyramidal geometry with four ring-nitrogen atoms in the equatorial plane, while the axial position is occupied by an oxygen from one methanephosphonate side-arm [17]. The complexes of Pd^{2+} with the DOTMP ligand have not yet been synthesized and studied [18]. Recent research has only indicated the formation of a ^{103}Pd complex with the ligand DOTA, which is similar to DOTMP [19]. The stability of this complex has not been determined.

In the present work, we propose using $^{103}\text{Pd}/^{103\text{m}}\text{Rh}$ in vivo generator to label the cyclic phosphonate ligand DOTMP for bone metastasis therapy, considering the high emission of conversion and Auger electrons from $^{103}\text{Pd}/^{103\text{m}}\text{Rh}$ and the expected stability of the complex.

2. Results

2.1 Reactor production of ^{103}Pd and ^{109}Pd

Due to the easier production of ^{109}Pd , some experiments, such as studies of affinity to hydroxyapatite, were carried out with this radionuclide. High activities of ^{109}Pd can be obtained in a nuclear reactor through the n,γ reaction of ^{108}Pd with thermal neutrons, due to the relatively high cross section of 12.2 b. Using natural palladium as a target material, after 7 hours of irradiation in the Maria reactor in Świerk

at a flux of $10^{14} \text{ n cm}^{-2} \text{ s}^{-1}$, over 500 MBq/mg of ^{109}Pd was obtained. In the case of a 98% enriched ^{108}Pd target, the obtained radioactivity exceeded 2 GBq/mg. In the gamma radiation spectrum of the irradiated natural target recorded before radiochemical treatment, photopeaks characteristic for ^{109}Pd (22.1 and 24.8 keV) and $^{109\text{m}}\text{Ag}$ (88.2 keV) can be observed. Additionally, small peaks from the ^{111}Ag dopant (96, 245, and 342 keV) formed in the $^{110}\text{Pd}(n,\gamma)^{111}\text{Pd} \rightarrow ^{111}\text{Ag}$ reaction can be observed. The ^{103}Pd impurity formed from the $^{102}\text{Pd}(n,\gamma)$ reaction is negligible due to the low ^{102}Pd abundance (1%) and low neutron cross section. Separation of ^{111}Ag by co-precipitation method with AgCl [8] was very efficient, and the gamma radiation spectrum of the ^{109}Pd solution after radiochemical treatment did not show gamma peaks characteristic of ^{111}Ag . This indicates that ^{109}Pd was obtained with almost 100% radionuclide purity. The average radiochemical yield of ^{109}Pd after radiochemical separation of ^{111}Ag was about 80%.

Due to the great difficulties in dissolving the rhodium target used in the cyclotron method, we decided to apply reactor method for ^{103}Pd production. To achieve sufficient activity for the planned research, a high neutron flux of $10^{15} \text{ n cm}^{-2} \text{ s}^{-1}$ is necessary. Within the framework of the European PRISMAP project, we had the opportunity to irradiate three ^{102}Pd targets, producing three batches of ^{103}Pd at the high-flux reactor in Grenoble at the Institut Laue-Langevin (ILL). In the high-flux neutron channel ($> 10^{15} \text{ n cm}^{-2} \text{ s}^{-1}$), one target weighing 1.5 mg and two targets each weighing 2.5 mg were irradiated for a duration of 7 days. The results showed that the first sample produced 1.94 GBq of ^{103}Pd , while each of the other two samples produced 5.0 GBq. The specific activities were 1.3 GBq/mg for the first sample and 2.0 GBq/mg for the other two samples. The irradiated metallic targets were dissolved in 0.5 mL of aqua regia. The X-ray spectrum of the obtained ^{103}Pd is presented in Fig. 1.

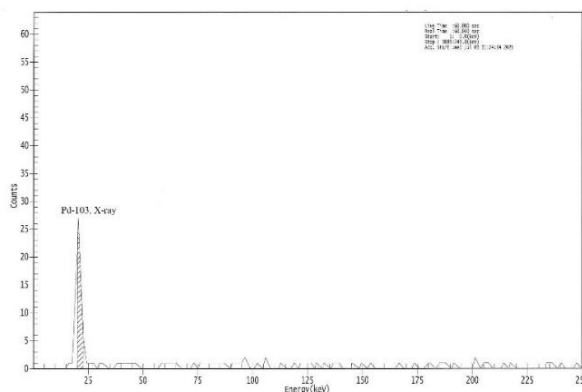


Fig. 1. X-ray spectrum of ^{103}Pd sample.

On the spectrum, only the X-ray lines from $^{103}\text{Pd}/^{103\text{m}}\text{Rh}$ are observed. The γ line at 39.75 keV from the de-excitation of the daughter nuclide $^{103\text{m}}\text{Rh}$ is not observed due to very low intensity (0.0684%).

2.2 Synthesis and characterization of non-radioactive Pd-DOTMP complex

By complexing Pd^{2+} cations with the DOTMP ligand, we obtained a yellow, water-soluble Pd-DOTMP complex. Successful complex formation was confirmed by mass spectrometry. The mass-to-charge ratio

of the complex was recorded at $M+H^+$ 653; the spectral data correspond to the calculated value (Pd-DOTMP- $C_{12}H_{32}N_4O_{12}P_4Pd$ – 653.7). Isotopic peaks in the spectrum align with the simulated spectrum. Comparison of the FT-IR spectra of DOTMP ligand and the complex of Pd-DOTMP (Fig. 2) reveals noticeable changes in the regions characteristic of phosphonate group vibrations (1100 – 950 cm^{-1}) and $\nu(P=O)$ and $\nu_{as}(P-O)$ vibrations (1250 – 1100 cm^{-1}). In the spectrum of the complex, these bands are shifted and their profiles are modified relative to those of the free ligand, indicating changes in the electronic environment of the phosphonate groups upon complex formation. However, the spectral region between 1250 and 1100 cm^{-1} also contains contributions from the C–N stretching vibrations of the DOTMP ring. Changes observed in this region after complexation are consistent with the coordination of Pd^{2+} to the nitrogen atoms of the macrocycle, although overlap with phosphonate vibrations prevents an unambiguous assignment based solely on the IR spectra. Furthermore, two new weak bands appearing in the 500 – 400 cm^{-1} region may be tentatively assigned to Pd–N and/or Pd–O stretching vibrations, as reported for related palladium and platinum coordination compounds [26].

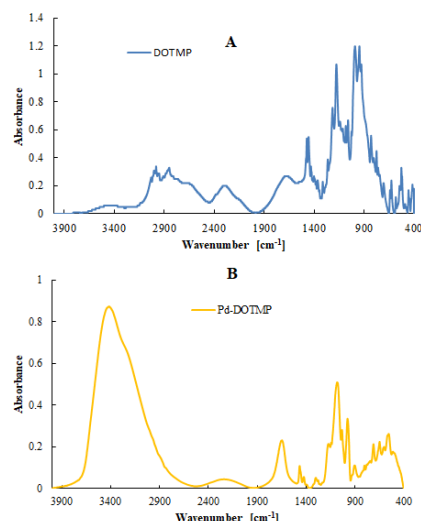


Fig. 2. FTIR spectrum of DOTMP ligand (A) and Pd-DOTMP complex (B)

2.3 Radiolabeling and stability of the obtained complexes

To determine the optimal amount of DOTMP needed for effective complexation of ^{103}Pd , we studied the labeling efficiency as a function of the amount of the ligand used. After the labeling process, we assessed the labeling yield using ITLC analysis. Table 1 presents the complexation yields of ^{103}Pd –DOTMP at different ligand-to-metal ratios.

Table 1. Complexation yields of $[^{103}Pd]Pd$ –DOTMP complex at different [ligand]:[metal] ratios

[ligand]:[metal] ratios	Labeling yield (%)
2:1	30
10:1	32

20:1	89
50:1	98

The results indicated that labeling yield with DOTMP was achieved with over 90% at metal/ligand ratios greater than 1:20. For a metal/ligand ratio of 1:50 labeling yield exceeded 98%, which indicates that for 100 MBq of ^{103}Pd , 100 nmol of DOTMP should be used. Labeling DOTMP with ^{177}Lu resulted in an efficiency of 98.2% at a ligand-to-metal ratio of 2:1, while the specific activity of ^{177}Lu was significantly higher. This indicates the formation of weaker complexes by Pd^{2+} than Lu^{3+} . However, as shown in Fig. 3, the complex exhibits sufficient stability in PBS buffer and human serum over a long period of time.

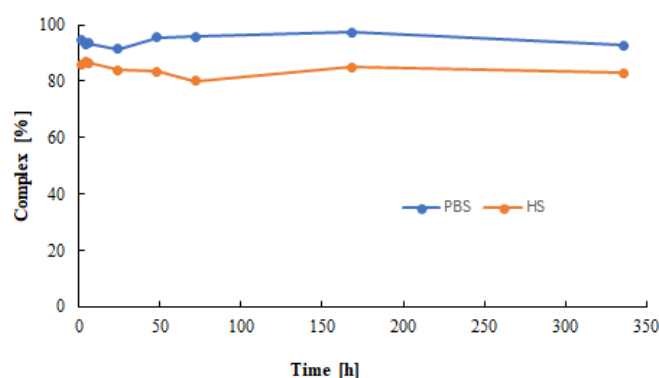


Fig. 3. Stability of the $[^{103}\text{Pd}]\text{Pd}$ -DOTMP complex in HS and PBS buffer.

2.4 Liberation of $^{103\text{m}}\text{Rh}$ from $[^{103}\text{Pd}]\text{Pd}$ -DOTMP complex

An important issue when using *in-vivo* generators is the possibility of release of isotopes derived from the complex. Our studies, which utilized ITLC analysis, demonstrate the presence of free $^{103\text{m}}\text{Rh}^{3+}$ that is released from the ^{103}Pd -DOTMP complex in PBS buffer. Fig. 4 shows examples of TLC strips measured immediately after sample development and one hour later.

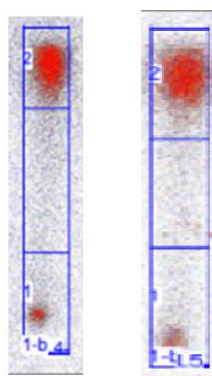


Fig. 4. TLC strips measured immediately after sample development (left) and one hour later (right).

As shown, the spot at the starting point, which indicates the presence of the free cation, decreases by half after one hour, indicating the presence of the free $^{103}\text{mRh}^{3+}$ cation. The ITLC method allows for only estimating the percentage of ^{103}mRh release; approximately 10% of ^{103}mRh was released from the ^{103}Pd -DOTMP complex in PBS buffer.

2.5 Adsorption on hydroxyapatite

The $[^{103}\text{Pd}]\text{Pd}$ -DOTMP complex was evaluated for sorption on hydroxyapatite grains, which mimic the mineral component of bone. Fig. 5 shows that approximately 95% of the complex was absorbed by hydroxyapatite, indicating a strong affinity of the DOTMP complex for bone tissues.

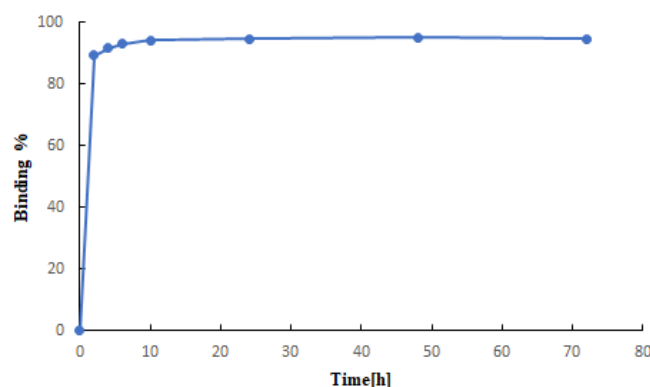


Fig. 5. Kinetics of $[^{103}\text{Pd}]\text{Pd}$ -DOTMP complex adsorption on HA surface.

2.6 *In vitro* cytotoxicity studies

Metastatic cancer cells exhibit characteristics similar to those of primary cancer when examined under a microscope and analyzed in other ways, but not to those of the cells in the location where the metastatic cancer originated. Since prostate and ovarian cancers are among the frequent bone metastatic neoplasms, we chose the cells of these tumors to study the cytotoxicity of the proposed complexes. The cytotoxicity of ^{103}Pd -DOTMP was evaluated against cancer cells that are capable of forming bone metastases. In our studies, we selected SKOV-3 ovarian cancer and DU-145 prostate cancer cells.

Initially, we studied the effect of the non-radioactive Pd-DOTMP complex on cell viability. The results for SKOV-3 and DU-145 cells are shown in Fig. 6.

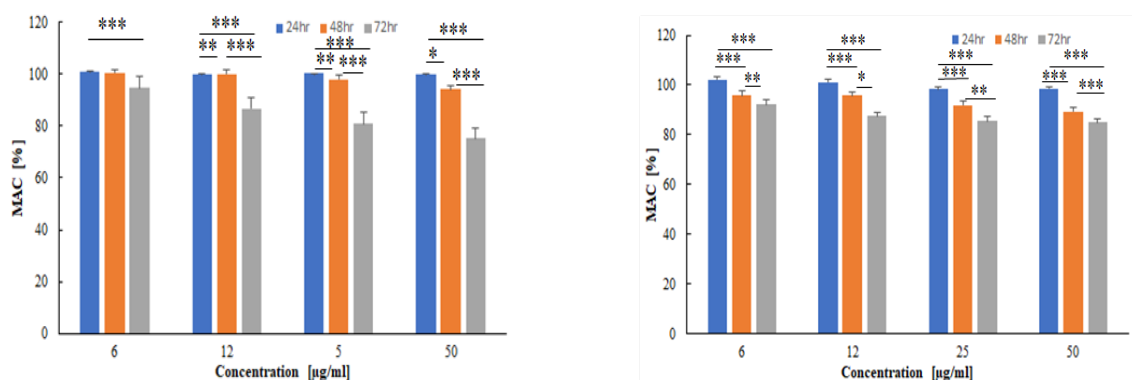


Fig. 6. Metabolic active cells (MAC) of DU145 (left) and SKOV-3 after treatment with different concentrations of non-radioactive Pd-DOTMP.

As shown in Fig. 6, the cytotoxicity of Pd-DOTMP is notably low, especially when compared to the previously tested Pd-bipyridyl-bisphosphonate complex (Pd(bpy)ale) [9].

The effects of different radioactivity of ^{103}Pd -DOTMP on the metabolic viability of DU-145 and SKOV-3 over 24, 48, and 72 hours of incubation are shown in Fig. 7.

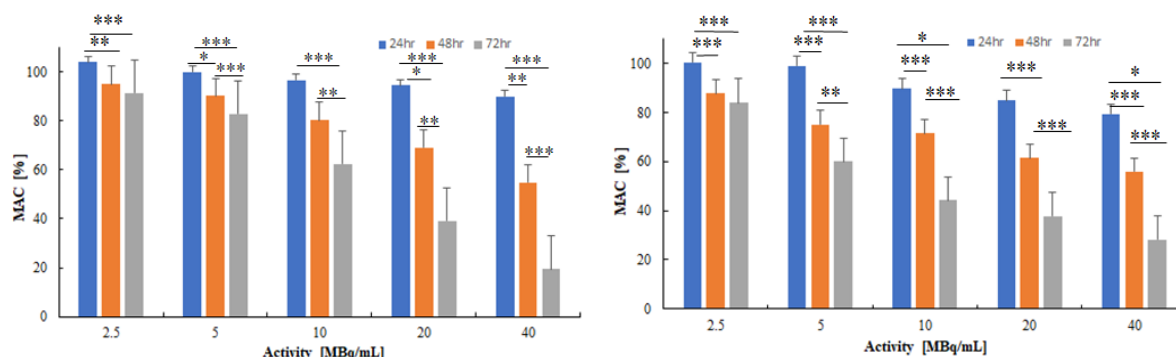


Fig. 7. The effects of different radioactivity of $[^{103}\text{Pd}]\text{Pd}$ -DOTMP on the metabolic viability of DU-145 and SKOV-3 cells.

It is evident that, for the two cell lines studied, the use of the ^{103}Pd -DOTMP complex significantly reduced cell metabolic viability, with the extent of the reduction depending on the activity and the time interval after administration. Increasing the radioactivity of the radiobioconjugate resulted in a gradual reduction in mitochondrial activity to 20% (DU-145) and 28% (SKOV-3) at 40 MBq/ml ^{103}Pd -DOTMP after 72 h.

To provide a comparison with other used DOTMP radioactive complexes, we conducted cytotoxicity studies on $[^{177}\text{Lu}]\text{Lu}$ -DOTMP, which emits β^- particles, and $[^{161}\text{Tb}]\text{Tb}$ -DOTMP, β^- and Auger electrons emitter (Fig. 8). This comparison allows us to assess how the type of emitted radiation influences the cytotoxicity of radiolabeled DOTMP complexes. The results clearly indicate that the cytotoxicity of $[^{103}\text{Pd}]\text{Pd}$ -DOTMP, which emits conversion and Auger electrons, is significantly lower than that of DOTMP complexes labeled with the β^- emitters ^{177}Lu and ^{161}Tb .

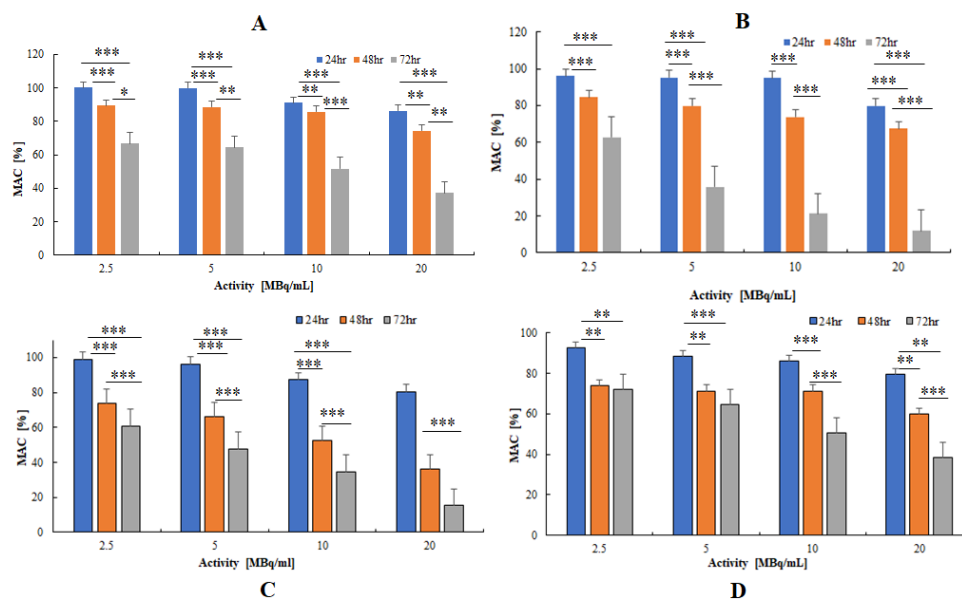


Fig. 8. Cytotoxicity of $[^{177}\text{Lu}]$ Lu-DOTMP (a,b) and $[^{161}\text{Tb}]$ Tb-DOTMP (c,d) for DU-145 (a, c) and SKOV-3 (b, d) cell.

2.7 Double strand break

A double-strand break experiment was performed on the DU145 prostate cancer cell line treated with the $[^{103}\text{Pd}]$ Pd-DOTMP complex at a concentration of 5 MBq/mL. DNA double-strand breaks are visualized in Fig. 9, along with the quantification of these foci, presented as the integrated density of γH2AX foci per nucleus area. Cells with untreated doses showed an average of about 1 double-strand break up to 24 h. Only 2 γH2AX foci were recorded after 4 hours of treatment, but in the longer incubation time, (24 h) the number of γH2AX foci increased to 5. The presence of DSBs in the DU145 cells treated with $[^{103}\text{Pd}]$ Pd-DOTMP complex is consistent with the cytotoxicity results shown in Fig. 8.

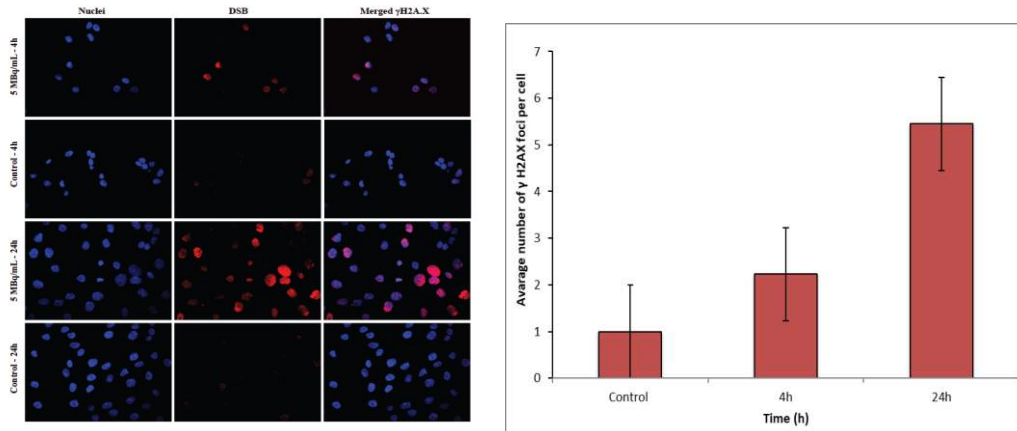


Fig. 9. DNA double-strand breaks visualized by γH2AX foci after incubation with ^{103}Pd -DOTMP. On left confocal microscopy images of cells stained with Hoechst 33258 (blue) and anti-phospho-Histone

H2A.X (Ser139) antibody (red). On the right, the average number of γ H2AX foci per cell. Images were analyzed with Fiji software.

3. Discussion

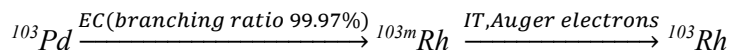
Currently, complexes of β^- emitter radionuclides with the DOTMP ligand are being extensively studied for their potential use in treating cancer metastases to bone. Radionuclides of trivalent metal cations, such as ^{90}Y and the lanthanides (like ^{153}Sm , ^{177}Lu , and ^{166}Ho), form very stable complexes that exhibit square antiprismatic geometry. In these complexes, the DOTMP ligand acts as an octadentate chelator, surrounding the metal ion by forming two parallel coordination planes consisting of nitrogens (N_4) and oxygens (O_4) planes. Among the divalent cations, a stable ^{64}Cu complex with square-pyramidal geometry was also synthesized.[23]. When DOTMP complexes are compared to other acyclic bisphosphonates, such as alendronate or zoledronic acid, they demonstrate enhanced thermodynamic and kinetic stability, while also reducing the trans chelation effect under biological conditions. [28,29]. In the case of Pd^{2+} , the situation differs significantly due to its tendency to form square planar geometries in its complexes. Unfortunately, our FTIR spectroscopy studies did not yield clear structural results, as the signals from the vibrations of Pd–O and C–N bonds overlap, making it difficult to assign peaks based solely on the IR spectra. Unlike Cu^{2+} , Pd^{2+} does not fit into the N_4 cyclen ring, a finding that is supported by the results of Grim et al. [30]. However, the attachment of methyl carboxyl groups to the nitrogen atoms in the ring led to the complexation of Pd^{2+} [30]. Therefore, it can be concluded that in the complex with DOTMP, Pd^{2+} forms bonds with both the nitrogen atoms in the ring and the oxygen atoms of the carboxyl groups. This conclusion is supported by our findings on the stability of the DOTMP and cyclam - ^{103}Pd complexes.

Labeling and stability studies conducted in this work showed that the ^{103}Pd complex was less stable than its ^{177}Lu counterpart. However, its stability was comparable to that of the ^{177}Lu complex used in therapy [31]. We also found that its stability was lower than that of the recently investigated by us ^{103}Pd complex, which maintained a stability exceeding 99.8% in saline, PBS, and HS over a 21-day incubation period [32]. This may be explained by their different accommodation of the Pd^{2+} cation in ligands. The Pd^{2+} ionic radius perfectly fits the 14-membered cavity of the cyclam ring, allowing for coordination without distorting the metal–nitrogen bonds. In contrast, the 12-membered cyclen ring in DOTMP is too small to accommodate the Pd^{2+} metal center [18]. The mixed complex formed with nitrogen and oxygen atoms outside the ring is less stable due to the absence of a macrocyclic effect.

As mentioned, the N ring and O phosphonate donor atoms are involved in the formation of the Pd-DOTMP complex; therefore, some phosphonate arms remain uncoordinated with Pd^{2+} , which leads to a strong affinity for the bone matrix. Sorption on hydroxyapatite occurs through the coordination of the phosphonate oxygen atom with the calcium ions on the hydroxyapatite surface. With available non-

metal coordinated phosphonate arms, the Pd-DOTMP as a Cu-DOTMP complex can establish stronger interactions with Ca^{2+} than the coordination-saturated Lu-DOTMP complex.

Although ^{103}Pd emits an average of 7.44 of Auger electrons, it decays to a radionuclide $^{103\text{m}}\text{Rh}$, one of the most promising candidates for Auger electron radiotherapy, according to the reaction [33]:



$^{103\text{m}}\text{Rh}$ additionally emits an average of 5.88 Auger electrons and, most importantly, conversion electrons with an average energy of 34.97 keV. Unfortunately, the short half-life of $^{103\text{m}}\text{Rh}$ ($t_{1/2}=56.11$ min.) makes it difficult and almost inapplicable to prepare the $^{103\text{m}}\text{Rh}$ radiopharmaceuticals for bone therapy. Therefore, the use of an *in vivo* generator is very beneficial. On the other hand, the release of $^{103\text{m}}\text{Rh}$ can cause unnecessary toxicity in healthy tissues while decreasing therapeutic efficacy in targeted cells. It is challenging to predict whether the $^{103\text{m}}\text{Rh}$ formed after decay will remain in the complex or escape from it. The release of $^{103\text{m}}\text{Rh}$ from the ^{103}Pd complexes as a result of the $^{103}\text{Pd} \xrightarrow{\text{EC}} ^{103\text{m}}\text{Rh}$ nuclear reaction was theoretically investigated by Zeevaart et al. [34]. Comprehensive estimates of the recoil energy associated with the emission of Auger electrons, photons, and neutrinos were conducted. It was noted that the decay energy is less than the bond strength of the ligand-metal complexes, indicating that there should be no escape of $^{103\text{m}}\text{Rh}$ from the complex. However, further experimental studies showed release of $5.81 \pm 0.14\%$ of $^{103\text{m}}\text{Rh}$ from ^{103}Pd complexed with 16aneS4-O-octyl macrocyclic ligand [35]. Recent studies using ^{103}Pd -labelled DOTATATE, phthalocyanine-TATE, and DOTA-TOC bioconjugates show release of $9.8 \pm 3\%$, $9.6 \pm 2.7\%$, and $12.0 \pm 0.5\%$ of $^{103\text{m}}\text{Rh}$, respectively [19]. Thus, the approximately 10% liberation of $^{103\text{m}}\text{Rh}$ from the ^{103}Pd -DOTMP complex observed by us is similar to these values.

According Zeevaart et al. [33], the observed partial release of $^{103\text{m}}\text{Rh}$ is probably due to the formation of the highly charged $^{103\text{m}}\text{Rh}^{n+}$ cation after the nuclear decay $^{103}\text{Pd} \xrightarrow{\text{EC}} ^{103\text{m}}\text{Rh}$. As explained, the highly charged $^{103\text{m}}\text{Rh}^{n+}$ ion extracts electrons from the surrounding donor atoms of the chelators, resulting in the formation of a Rh^{3+} cation. As a result of electron transfer to these highly charged atoms, the donor atoms of the chelators also gain a positive charge. This induced charge creates a repulsive force between the positively charged atoms, which causes the metal-ligand bonds to break. Consequently, this allows for the release of $^{103\text{m}}\text{Rh}$ as free cations.

In our previous publications, we found that preventing the release of $^{103\text{m}}\text{Rh}$ from ^{103}Pd and $^{109\text{m}}\text{Ag}$ from ^{109}Pd is achieved by the deposition of ^{103}Pd on the surface of gold nanoparticles [36,37]. The free metallic electrons can rapidly neutralize the highly charged Rh^{n+} cations, preventing their diffusion. Also, the complete retention of Rh^{3+} in the $[\text{}^{103}\text{Pd}]\text{Pd}(\text{bpy})\text{ald}$ complex observed by Annamalaisamy et al. [9] is related to the presence of a bipyridyl ligand containing delocalized electrons. According to Nath et al. [38], these ligands, such as for example heme, show the ability to quench the observed phenomenon

known as the "Coulomb explosion" by transporting delocalized electrons to the metal core within time scales equivalent to the Auger cascade.

Cytotoxicity studies did not reveal the high cytotoxicity expected from the emission of Auger electrons by ^{103}Pd complexed with DOTMP. The metabolic viability of both cell types incubated with ^{103}Pd -DOTMP was significantly higher than that of cells treated with the previously evaluated $^{103}\text{Pd}[\text{Pd}_2(\text{bpy})_2\text{ale}]$ complex. As reported by Fathy et al. [39], the $\text{Pd}_2(\text{bpy})_2\text{ale}$ complex dissociates upon adsorption onto hydroxyapatite, releasing the small $^{103}\text{Pd}[\text{Pd}(\text{bpy})\text{Cl}_2]$ complex. This smaller entity can internalize into the cell nucleus and bind directly to DNA, which is essential for the cytotoxic action of Auger electrons. These electrons possess a nanometer-scale range and a high linear energy transfer (LET) of 4–26 keV/ μm , inducing irreparable double-strand DNA breaks. Consequently, the low cytotoxicity of ^{103}Pd -DOTMP is justified; DOTMP complexes, similar to other bisphosphonates, exhibit extracellular binding and localize predominantly via adsorption onto the mineralized bone matrix rather than penetrating the cell membrane. In this scenario, the nanometer range of Auger electrons is insufficient to induce cell death. To exploit the cytotoxic potential of Auger electrons, the $^{103}\text{Pd}/^{103\text{m}}\text{Rh}$ complex must either be internalized or immobilized directly onto the cell surface. Therefore, the moderate cytotoxic effect observed for ^{103}Pd -DOTMP was likely mediated solely by the emission of conversion electrons from $^{103\text{m}}\text{Rh}$, which features a longer therapeutic range in the body of approximately 25 μm .

In the case of DOTMP labeled with ^{161}Tb and ^{177}Lu emitters, the range of β^- particles in experimental conditions is sufficient to destroy cancer cells. Lack of significant differences in cytotoxicity between the β^- emitter ^{177}Lu and the β^- and Auger electron emitter ^{161}Tb complexes (Fig. 8) confirms this explanation. As has been repeatedly observed [40] for radiobioconjugates that internalize into the cell nucleus, the cytotoxicity of ^{161}Tb -labeled radioconjugates, which combine the favorable β^- properties of ^{177}Lu with additional high-LET electron emission of Auger and conversion electrons, shows significantly higher cytotoxicity than that of the corresponding ^{177}Lu -labeled conjugates.

5. Conclusions

In summary, a stable radioactive ^{103}Pd -DOTMP complex was synthesized with a high yield. To our knowledge, following ^{117}Sn -DTPA, this is the second potential bone radiopharmaceutical utilizing conversion electron emitters described in the literature. In contrast to $^{117\text{m}}\text{Sn}$, ^{103}Pd is simpler to produce and is commercially available. The resulting complex showed a strong affinity for the surface of hydroxyapatite grains, the main mineral component of bone. Consistent with previous studies, we observed that - similar to other macrocyclic ^{103}Pd complexes such as cyclam, 16-S4-diol, and DOTA - there is only a minor release (<10%) of the daughter isotope $^{103\text{m}}\text{Rh}$ from the complex. The tested ^{103}Pd -DOTMP complex exhibits cytotoxicity against human prostate cancer cells (DU145) and Her2+ ovarian cancer cells (SKOV-3). However, because of its low internalization, the cytotoxicity of ^{103}Pd -DOTMP, which emits low-range conversion and Auger electrons, is significantly lower than that of

DOTMP complexes labeled with the β^- emitters, ^{177}Lu and ^{161}Tb . Also, the ^{103}Pd]Pd-DOTMP complex demonstrates significantly lower toxicity compared to the previously studied ^{103}Pd]Pd(bpy)ale complex, which has the potential to enter the cell and additionally bind to DNA. The location within the cell or its surroundings is essential for the cytotoxic effects of conversion electrons, while binding to DNA is critical for the effective cytotoxic action of Auger electrons.

Based on the results of our study, we can conclude that bisphosphonate complexes of Auger electron emitters must be capable of either internalizing into the cell or binding to the cell membrane in order to fulfil a therapeutic role.

6. Materials and Methods

6.1 Chemical reagents

Potassium Tetrachloropalladium (K_2PdCl_4) and DOTMP (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrayltetrakis(methylene)]tetrakis(phosphonic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid and sodium hydroxide were purchased from POCH (Gliwice, Poland). For cell and other biological studies Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA), Cell Titer® 96 Aqueous One solution reagent (MTS assay) from Promega (Mannheim, Germany). SKOV-3 and DU145 cell line bought from American Type Tissue culture collection (ATCC, Rockville, MD, USA) and were cultured in Mc Coy's and MEM-Eagles medium supplemented with 10%Fetal Bovine serum (FBS) and 1% Penicillin-Streptomycin (Beth Haemek, Isreal).

6.2 Radionuclides

To obtain ^{109}Pd , enriched ^{108}Pd (>99%) was irradiated with thermal neutrons flux $1.5 \times 10^{14} \text{ n cm}^{-2} \text{ s}^{-1}$ for 7 hours in the Maria nuclear reactor located in Otwock-Świerk, Poland, using approximately 1 mg of metal target. After a 3-hour cooling period, the radioactive palladium was dissolved in 200-400 μL of aqua regia (a mixture of nitric acid and hydrochloric acid in a 1:3 ratio) and heated to 130 °C until nearly all the liquid evaporated. The remaining nitrates were then removed by dissolving the residues in 100 μL of 0.1 M hydrochloric acid three times and heating at 130 °C until almost complete evaporation occurred. Finally, the residual material was reconstituted in 1 mL of 6 M hydrochloric acid, resulting in the formation of H_2PdCl_4 .

Since the neutron irradiation of the natural Pd target can result in the formation of ^{111}Ag as an impurity in the reaction of $^{110}\text{Pd}(\text{n},\gamma)^{111}\text{Pd} \rightarrow ^{111}\text{Ag}$, it is essential to remove it from the solution before application [20]. The removal of ^{111}Ag can be accomplished by precipitating it as AgCl using AgNO_3 in the procedure detailed described in our previous paper [9].

^{103}Pd was obtained as part of the European PRISMAP project from the Institute Laue–Langevin in Grenoble. The ^{103}Pd was produced by 168 h irradiating 2.5 mg of an enriched ^{102}Pd target (90.7%) in ILL High-Flux Reactor, with a neutron flux of $>10^{15} \text{ n cm}^{-2} \text{ s}^{-1}$. To dissolve the irradiated target, we

followed the same procedure used for obtaining ^{109}Pd . Following dissolution, we obtained 0.5 ml of solution with a concentration of 10.0 GBq/ml and a specific activity of 2 GBq/mg.

The ^{177}Lu (carrier added) and ^{161}Tb (non-carrier added) were obtained from NCBJ-Polatom, Świerk, Poland.

6.3 Radioactivity measurement

The activity and radionuclidic purity of the obtained ^{103}Pd and ^{109}Pd were determined by gamma-ray spectrometry. Diluted solutions gained after radiochemical processing of the irradiated target were measured with an HPGe detector connected to a PC-based Multichannel Analyzer (MCA, Canberra). The Canberra GX1018 detector is perfectly suited for measurement selected X-ray peaks. As mentioned in Canberra's official technical specifications for the GX1018 model, the manufacturer guarantees resolution (FWHM) values at 22 keV around 0.7 keV. The GX series features an ultra-thin front contact and a specialized carbon composite window. This design allows soft X-rays and low-energy gamma emissions to pass through to the active volume without significant attenuation.

The ^{109}Pd was measured using an 88 keV (3.67%) gamma peak originating from the $^{109\text{m}}\text{Ag}$, decay product of ^{109}Pd . For ^{103}Pd , X-ray peaks of 20.07 keV (22.3%), 20.21 (42.4%) and 22.71 (10.4%) and for $^{103\text{m}}\text{Rh}$, 20.07 keV (2.14%) and 20.21 (4.06%) were applied [21]. The total X-ray intensity for $^{103\text{m}}\text{Rh}$ was determined to be 8.25% [22].

Thin-layer chromatography was performed to calculate the radiochemical purity using Watman Chromatographic paper. The strips were developed by a water/acetonitrile mixture (1:3 v/v). The activity distribution was measured using radiometric TLC strip reader Cyclone® Plus Storage Phosphor System (Perkin Elmer). This system allows the measurement of γ , X-ray, and β radiations. Low-energy Auger electrons are not detected because their short range causes complete absorption within the micrometer-thick polymer protective layer.

6.4 Structural studies of non-radioactive Pd^{2+} - DOTMP complex

The complex was synthesized using the procedure for synthesizing $[\text{Cu}^{2+}]\text{Cu}$ -DOTMP [23] by mixing 0.075 mmol of K_2PdCl_4 with 0.15 mmol of DOTMP. The mixture was then gently stirred and heated at 60°C for 1 hour. The pH was maintained at 7 and the product was precipitated by adding acetone.

IR spectra were performed on the NICOLETE iS10 Thermo Scientific spectrometer (Waltham, Massachusetts, USA). 1 mg of the complex was finely ground with KBr, and pellets were formed using a hydraulic press. Then the pellets were placed in a sample holder and spectra were recorded in the 4000-400 cm^{-1} region. In this study, FTIR was used to analyse the chemical structure of the studied palladium-phosphonate compounds, aiming to determine the donor atoms and functional groups present in these complexes.

6.5 Labelling of DOTMP with ^{103}Pd , ^{177}Lu and ^{161}Tb

The radiolabeling of DOTMP with ^{103}Pd was conducted in an ammonia acetate buffer at a pH of 5.5. To investigate labeling efficiency, the following ligand-to-palladium ratios were employed: 1:2, 1:10, 1:20,

and 1:50. For the kinetics and recoil studies, as well as for adsorption on hydroxyapatite, 1 MBq/ml of ^{109}Pd was utilized, while cytotoxicity studies involved the application of 100 MBq/ml of ^{103}Pd . After the addition of the radionuclide to acetate buffer, the pH was adjusted to 7.0 using 0.1 M NaOH. The solution was then heated to 60°C and vortexed for 30 minutes.

Since Lu^{3+} and Tb^{3+} cations exhibit nearly identical chemical properties, the same labeling procedure was applied to ^{177}Lu and ^{161}Tb . Labeling was performed using a modified procedure elaborated by Das et al. [24] for ^{177}Lu . DOTMP was dissolved in 100 μL of ammonia acetate buffer. To this, around 50 MBq of either ^{161}Tb or ^{177}Lu was added, and the pH was maintained at 7. The solution was then heated at 60°C for 15 minutes.

6.6 Complex stability

The stability of the complex was analyzed at 37°C in human serum (HS) and PBS over 24 hours (~2 half-lives of ^{109}Pd). 50 μL of a radioactive complex was added to freshly prepared HS and PBS, and incubated at 37 °C for 24 hours. The stability was analyzed at specific time intervals using radio ITLC.

6.7 Release of $^{103\text{m}}\text{Rh}$ from ^{103}Pd -DOTMP complex

To investigate the release of $^{103\text{m}}\text{Rh}$ from the ^{103}Pd -DOTMP complex, the ITLC technique was employed. After synthesizing the ^{103}Pd -DOTMP complex and allowing it to reach radioactive equilibrium of $^{103}\text{Pd}/^{103\text{m}}\text{Rh}$ over a period of 10 hours, the complex was applied to Whatman chromatographic paper strips. These strips were then developed using a water/acetonitrile mixture (1:3 v/v). Under these conditions, the complex moves along with the eluent, while the released $^{103\text{m}}\text{Rh}^{3+}$ cation remains at the starting point. The Cyclone® Plus Storage Phosphor System was utilized to measure the X-ray and conversion electron radiation emitted by ^{103}Pd and $^{103\text{m}}\text{Rh}$ with high sensitivity.

6.8 Sorption on hydroxyapatite (HAP)

Sorption on HAP was performed according to our previous work [25]. Briefly, 80 mg of HAP was weighed and equilibrated with 2 ml saline, and next 50 μL of [^{109}Pd]Pd-DOTMP was added and rotated at room temperature. After 1 h, the mixture was centrifuged, and the supernatant was carefully removed. The radioactivity in both supernatant and HAP was measured using radio TLC. The percentage sorption of ^{109}Pd -DOTMP complex was calculated using the formulae

$$\% \text{ Sorption} = (1 - A/B) * 100\%,$$

where A is the average activity of the sample after equilibration and B is the initial activity of the solution.

6.9 Cytotoxicity Studies

The MTS assay was employed to evaluate the ability of cells to proliferate. Tests were conducted on SKOV-3 (ovarian adenocarcinoma) and DU 145 (prostate cancer) cells. SKOV-3 and DU145 cells were maintained in McCoy's and MEM Eagle's media, respectively. Both cell types were seeded in 96-well plates at a density of 3×10^3 cells per well, suspended in 100 μL of culture medium. After 24 hours of seeding, the ^{103}Pd -DOTMP complex with the desired activities (2.5 MBq/mL, 5 MBq/mL, 10

MBq/mL, 20 MBq/mL, 40 MBq/mL) was suspended in the medium and incubated for 24, 48 and 72 hours. Before adding 20 µl of MTS, the cells were washed with PBS, and fresh medium was added (100 µl). Absorbance was measured at 490 nm.

6.10 DNA break studies

Prostate cancer cell DU145 cells were seeded in a 6-well plate at a density of 0.25 million cells per well in 1.5 mL media. Each well contains five sterile cover slips, which were washed with ethanol and PBS. Cells were incubated for a period of 24 h at 37°C and 5% CO₂. After 24h, the medium was removed and cells were treated with [¹⁰³Pd]Pd-DOTMP (5 MBq/mL). At a particular time interval, the cover slips were transferred to 24-well plates and washed with PBS. Around 350 µL of primary antibody was added to each well (anti-phospho-histone H2A.X (ser139)), clone JBW301, with a dilution of 1:100 in blocking buffer and left at 4°C overnight. After 24 hours of incubation with primary antibody in the fridge, the wells were washed with PBS and treated with 250 µL of anti-mouse IgG(H + L) conjugated with CF633 in each well. The plates were then shaken and incubated at room temperature for 2 hours. After 2 h, nuclei were stained using Hoechst 33258. The stained nuclei and breaks were analyzed by FV-1000 confocal microscopy (Olympus Corporation, Tokyo, Japan) with excitation/em maxima of 630/650 nm for CF633 and 325/454 nm for Hoechst 33258. The images were then analysed by Fiji ImageJ.

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Declarations

Conflict of interest: The authors declare that they have no competing interests.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material: Data will be available upon reasonable request from corresponding author.

Author contributions

Conceptualization: A. B. and M. L. formal analysis: G. P. A., M. L. and A. B; funding acquisition: A. B.; investigation: G. P. A., M. L., U. K., K. Z. M. P.; methodology: G. P. A., M. L., K. Z. and A. B.; supervision: A. B.; visualization: G. P. A., M. L., M. P.; writing—original draft A. B., G. P. A., M. L.; writing—review and editing: G. P. A. M. L. and A. B. All authors have read and agreed to the published version of the manuscript.

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