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Selective Cu(I) complex with phosphine-peptide (**SarGly**) conjugate contra breast cancer: Synthesis, spectroscopic characterization and insight into cytotoxic action

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ABSTRACT

The main disadvantage of conventional anticancer chemotherapy is the inability to deliver the correct amount of drug directly to cancer. Those molecular delivering systems are very important to destroy cancer cells selectively. Herein we report synthesis of phosphine-peptide conjugate (Ph_2PCH_2-Sar-Gly-OH, **PSG**) derived from **SarGly** (sarcosine-glycine), which can be easily exchanged to other peptide carriers, its oxide (OPh_2PCH_2-Sar-Gly-OH, O**PSG**) and the first copper(I) complex ([CuI(dmp)$(P(Ph)_2CH_2$-Sar-Gly-OH)], **1-PSG**, where dmp stands for 2,9-dimethyl-1,10-phenanthroline). The compounds were characterized by elemental analysis, NMR (1D, 2D), UV–Vis spectroscopy and DFT (Density Functional Theory) methods. **PSG** and **1-PSG** proved to be stable in biological medium in the presence of atmospheric oxygen for several days. The cytotoxicity of the compounds and cisplatin was tested against cancer cell lines: mouse colon carcinoma (CT26; $^{1\text{-PSG}}IC_{50} = 3.12 \pm 0.1$), human lung adenocarcinoma (A549; $^{1\text{-PSG}}IC_{50} = 2.01 \pm 0.2$) and human breast adenocarcinoma (MCF7; $^{1\text{-PSG}}IC_{50} = 0.98 \pm 0.2$) as well as against primary line of human pulmonary fibroblasts (MRC-5; $^{1\text{-PSG}}IC_{50} = 78.56 \pm 1.1$). Therapeutic index for **1-PSG** (MCF7) equals 80. Intracellular accumulation of **1-PSG** complex increased with time and was much higher (96%) inside MCF7 cancer cells than in normal MRC5 cells (20%). Attachment of **SarGly** to cytotoxic copper(I) complex *via* phosphine motif improved selectivity of copper (I) complex **1-PSG** into the cancer cells. Precise mechanistic study revealed that the **1-PSG** complex causes apoptotic cells MCF7 death with simultaneous decrease of mitochondrial membrane potential and increase of caspase-9 and -3 activities. Additionally, **1-PSG** generated high level of reactive oxygen species that was the reason for oxidative damages to the sugar–phosphate backbone of plasmid DNA.

1. Introduction

Mortality caused by cancer is about to exceed that from cardiovascular diseases in near future. Approximately 7 million people die from cancer-related cases per year, and it is estimated that there will be > 16 million new cancer cases every year by 2020 [1,2]. Chemotherapy is still one of the major approaches to treat cancer by delivery of a cytotoxic agent to cancer cells. However, the main disadvantage of conventional chemotherapy is the inability to deliver the correct amount of drug directly to cancer cells without affecting healthy cells [3]. Drug resistance, altered biodistribution, biotransformation, and drug clearance are also considered common problems [3]. This shows the importance of development of systems that will selectively destroys cancer cells [4–7].

Various opportunities for the design of therapeutic agents can be offered by medicinal inorganic chemistry (very often not accessible to organic compounds). Coordinated chemistry of metal-based drugs has been placed in the frontline in the fight against cancer by widespread use of cisplatin. Unfortunately, the use of this drug is very limited due to its high toxicity [8]. Copper-based complexes could be less toxic for normal cells with respect to cancer cells, despite copper toxicity related to its redox activity. Also, a different response of normal and tumor cells to copper ions can be a platform for development of copper complexes endowed with antineoplastic characteristic [8–10].

Copper(I) complexes constitute a group of compounds that is still not exploited enough, but over the last several years, we can observe a significant interest increase of their anticancer [11–17], antibacterial [18], antiviral [19,20], antifungal [21], and inflammatory [14] activity. Furthermore, the phosphine ligands forms a strong bond with copper(I) ion which prevents oxidation of the phosphine ligand and

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copper(I) to copper(II) [12], what was also proven in our previous studies [13–17,22–24]. Additionally, phosphine ligands can be easily functionalized, which is remarkable. In particular, aminomethylphosphanes derived from amino acids [25–28] or prepared from the highly water-soluble aliphatic secondary amines [29,30], seem to be interesting in terms of the formation of potential conjugates with a wide range of biomolecules. This makes them good candidates for drugs.

Linking of the peptides (described as a carriers) *via* phosphine motif to copper(I) complexes [13,22–24,31–34], may enable selective delivery of these coordination compounds to the tumor cells. Examples of such peptides carriers are RGD (Arg-Gly-Asp) motif and other NGR peptide (Asn-Gly-Arg) selectively recognize integrins - proteins responsible for the growth, division, adhesion and migration of cancer cells (peptides that have entered clinical trials). It is worth noting that mentioned motifs combined with doxorubicin, paclitaxel and fluorouracil cause significant decrease of *in vivo* toxicity of these drugs [35–40]. In this paper we propose a novel approach – connecting peptides, in our case: sarcosine-glycine (**SarGly**) with copper(I) complexes. We decided to choose this peptide **SarGly**, because of several reasons. Firstly, **SarGly** is small and cheap molecule and we could establish synthetic conditions before applying it to our research peptides as RGD or NRG (very expensive). Initial, presented here, results were surprisingly good, so we decided to continue research with this compound **SarGly**. Secondly, motif GlySar was reported as a PET tracer targeted to the PEPTs (H+/peptide transporters – functionally expressed in some human cancer cell lines) for cancer detection in mice [41,42]. It is worth mentioning that peptides possess well-known advantages as drugs, such as specificity, potency, and low toxicity [43]. To the best of knowledge, in the literature there are no such systems reported so far.

This article is continuation of our previous projects describing copper(I) complexes bearing phosphine ligands derived from fluoroquinolone antibiotics [14–17,50,51,58,65]. We demonstrated high cytotoxic activity towards view cancer lines of inorganic derivatives of fluoroquinolones. It was proven that mentioned above compounds caused apoptotic cancer cell death *via* caspase-dependent mitochondrial pathway. Unfortunately, we were struggling with high toxicity of those complexes. That is why we decided to exchange fluoroquinolone molecule to some simple, cheap and lipophilic peptides.

In this paper we describe synthesis of phosphine ligand (PPh_2CH_2-Sar-Gly-OH; **PSG**) derived from sarcosine-glycine (**SarGly**), its oxide ($OPPh_2CH_2$-Sar-Gly-OH; **OPSG**) and copper(I) complex [CuI(2,9-dimethyl-1,10-phenanthroline)PSG] (**1-PSG**). Physicochemical properties of all these compounds were detected using elemental analysis, NMR (1D and 2D), UV–Vis spectroscopy and theoretical calculations. Cytotoxic activity *in vitro* of **SarGly** and its organic (**PSG**, **OPSG**) and inorganic (**1-PSG**) derivatives was tested against three cancer cell lines: mouse colon carcinoma (CT26), human lung adenocarcinoma (A549) and human breast adenocarcinoma (MCF7) as well as one primary line of human pulmonary fibroblasts (MRC5). Herein, we also try to approach the mechanism of **1-PSG** cytotoxic action towards human breast adenocarcinoma (MCF7). To realize our goal we undertook a series of experiments: (i) intracellular uptake of the tested complex was studied by ICP-MS spectrometry; (ii) the mode of cell death was examined by flow cytometric analysis; (iii) level of mitochondrial membrane potential was measured; (iv) activity of caspases 3 and 9 was determined; (v) the ability of the complexes to generate reactive oxygen species (ROS) in the cells was examined using two different fluorescent probes; (vi) generation of oxidative DNA cleavage was studied by gel electrophoresis.

2. Experimental part

2.1. Materials

All reactions were carried out under a dinitrogen atmosphere using standard Schlenk techniques. $PPh_2(CH_2OH)_2Cl$ was synthesized according to a literature procedures [44]. Peptide **SarGly** was purchased from Bachem (Switzerland). Ph_2PH, dmp, CuI, other small chemicals and solvents were purchased from Sigma-Aldrich (Germany) and used without further purifications. All solvents were deaerated before use.

2.2. Methods

Elemental analyses were performed on a Vario EL3 CHN analyser for C, H, and N, and they were within 0.3% of the theoretical values. NMR spectra were recorded on a Bruker AMX 500 spectrometer (at 298 K) with traces of solvent as an internal reference for ^{1}H (DMSO-d_6: 2.50 ppm) and ^{13}C spectra (DMSO-d_6: 39.51 ppm) and 85% H_3PO_4 in H_2O as an external standard for ^{31}P. The signals in the spectra are defined as: s = singlet (* – strongly broadened signal), d = doublet, dd – doublet of doublets, t = triplet and m = multiplet. Chemical shifts are reported in ppm and coupling constants are reported in Hz. Absorption spectra were recorded on a Cary 50 Bio spectrophotometer (Varian Inc., Palo Alto, CA) in the 800–200 nm range.

2.3. Synthesis

2.3.1. Preparation of Ph_2P-CH_2-Sar-Gly-OH (PSG)

$PPh_2(CH_2OH)_2Cl$ (0.6434 g, 2.27 mmol) was dissolved in 20 mL of methanol and cooled down using water bath (T = 8 °C). Then, a slight excess of NEt_3 (triethylamine; 2.60 mmol) was added. Mixture was stirred and with time reached room temperature (RT). After 40 min **SarGly** (0.3320 g, 2.27 mmol) in water (10 mL) was added dropwise into the mixture (RT). After 1 h of stirring, clear solution was observed. Mixture was dried under reduced pressure for few hours. White, crude product was dissolved in water (30 mL; milky solution was observed) and extracted four times with $CHCl_3$ (10 mL) using cannula. Chloroform phase was dried under pressure and white solid of **PSG** was formed. It is well soluble in $CHCl_3$, DMSO, CH_2Cl_2, CH_3CN, moderately in ethanol, methanol and water.

Yield: 90%, **Molar mass**: 344.35 g/mol. **Anal. Calcd** for PC18H21N2O3: C, 62.78; H, 6.15; N, 8.14%. Found: C, 62.77; H, 6.16; N, 8.13%.

NMR (DMSO-d_6, 298 K): ^{31}**P{^{1}H}**: -27.08 s, ^{1}**H**: H^{Ph}: 7.34–7.55; H^{NH}: 4.41 s; H^1: 3.07 s; H^2: 2.31 s; H^3: 3.30 d (J = 3.05); H^5: 3.51d (J = 5.53); ^{13}**C{^{1}H}**: $C^{Ph(i)}$: 137.65 d (J = 12.72); $C^{Ph(o)}$: 132.72 d (J = 19.07); $C^{Ph(m)}$: 128.62 d (J = 6.36); $C^{Ph(p)}$: 128.76 s; C^1: 61.86 d (J = 9.10); C^2: 44.29 d (J = 10.0); C^3: 60.03 d (J = 4.50); C^4: 169.37 s; C^5: 41.99 s; C^6: 171.62 s.

2.3.2. Preparation of $Ph_2P(O)CH_2SG$ (OPSG)

The oxide derivative was prepared in the reaction of **PSG** (0.4258 g; 1.24 mmol) dissolved in chloroform (20 mL) with equimolar amount of H_2O_2 (35% solution in water). After 1 h of stirring (RT) the solution was evaporated to dryness. White solid well soluble in $CHCl_3$, DMSO, CH_2Cl_2, CH_3CN, moderately in ethanol, methanol and water was obtained.

Yield: 100%, **Molar mass**: 360.34 g/mol **Anal. Calcd for** PC18H21N2O4: C, 60.00; H, 5.87; N, 7.77%. Found: C, 59.99; H, 5.88; N, 7.76%.

NMR (DMSO-d_6, 298 K): ^{31}**P{^{1}H}**: 26.94 s ^{1}H: H^{Ph}: 7.76–7.42; **H^1**: 3.14 s; H^2: 2.31 s; H^3: 3.52 d (J = 5.34); H^5: 3.56 d (J = 5.91); ^{13}**C{^{1}H}**: $C^{Ph(i)}$: 132.82 d (J = 94.46); $C^{Ph(o)}$: 131.68 d (J = 1.82); $C^{Ph(m)}$: 130.74 d (J = 9.08); $C^{Ph(p)}$: 131.03 d (J = 8.08) s; C^1: 59.89 d (J = 87.19); C^2: 44.68 d (J = 6.36); C^3: 55. 88 d (84.47); C^4: 169.57 s; C^5: 40.77 s; C^6: 171.22 s.

2.3.3. Preparation of the complex with phosphine-peptide conjugate (1-PSG)

Neocuproine (0.1540 g; 0.7396 mmol) and copper iodide (0.1409 g; 0.7396 mmol) were added in equimolar ratios to the phosphine