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The redox status of human breast cancer cell lines (MCF-7 and MDA-MB231) treated with novel dinuclear berenil-platinum(II) complexes

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This study compared the effects of cisplatinum and novel berenil-platinum(II) complexes on the redox status of breast cancer cells that were estrogen receptor-positive (MCF-7) or estrogen receptor-negative (MDA-MB231). Both cell lines were treated with cisplatinum or the following berenil-platinum(II) complexes: Pt_2(isopropylamine)$_4$(berenil)$_2$, Pt_2(piperidine)$_4$(berenil)$_2$, Pt_2(2-picoline)$_4$(berenil)$_2$, Pt_2(3-picoline)$_4$(berenil)$_2$, and Pt_2(4-picoline)$_4$(berenil)$_2$. Changes in levels of reactive oxygen species, levels and activities of antioxidants, and lipid peroxidation products levels were measured. All investigated compounds enhanced ROS generation, reduced the activity of antioxidant enzymes (e.g., glutathione peroxidase and glutathione reductase), and decreased levels of small-molecule antioxidants (GSH, vitamins E and A). Such conditions are conducive to generating oxidative stress and phospholipids peroxidation. Cellular phospholipids in MCF-7 cells were most sensitive to the Pt_2(isopropylamine)$_4$(berenil)$_2$ complex, whereas MDA-MB231 cells were not particularly sensitive to any berenil-platinum(II) complex. These findings will facilitate future anticancer drug design strategy for breast cancer pharmacotherapy.

1. Introduction

Breast cancer is the most commonly diagnosed cancer in women. One of the major challenges to achieving better prognoses for breast cancer patients is the ineffectiveness of chemotherapy, due to a lack of drug selectivity and the development of drug resistance. Consequently, additional effective therapies are needed to reduce the mortality rate associated with breast cancer. Clinical data suggest that platinum-based chemotherapy is an effective treatment for patients with advanced breast cancer (Ott and Gust 2007).

Cisplatinum is a DNA-binding compound that is used to inhibit proliferation of tumor cells and induce apoptosis (Wang et al. 2005). It is hypothesized that cisplatinum-induced apoptosis is mediated by alterations in the intracellular redox status. These changes lead to activation of downstream signaling pathways. Physiologically, the cellular redox status reflects a balance between the generation of oxidants and the antioxidant capacity of the cell. A number of studies have shown that cisplatinum affects the intracellular redox status of a cell, based on its ability to promote oxidation (Gęgotek et al. 2013; Hu et al. 2010). Moreover, cisplatinum toxicity is closely related to increased levels of reactive oxygen species (ROS) (Lu and Cederbaum 2006; Kim et al. 2010). Growing evidence also suggests that cisplatinum induces phase I and phase II metabolic enzymes (Nakata et al. 2006). This observation may contribute to the beneficial antioxidant activity associated with cisplatinum, however, it may also be responsible for its own activation. Taken together, these findings suggest that cisplatinum generates oxidative stress, which leads to cellular toxicity through modification of DNA, lipids, and proteins (Koberle et al. 2010).

Although cisplatinum is cytotoxic to cancer cells, the lack of selectivity and drug resistance associated with it have sparked investigations into novel agents that have similar biochemical/clinical profiles to cisplatinum, but that cells cannot become as resistant to (Popławska et al. 2009). Therefore dinuclear platinum(II) complexes with berenil (1,3-bis(4'-amidinophenyl)triazene), an aromatic bisamidyn with antiprotozoan and antitumor activity, were synthesized (Bielawski et al. 2008). These compounds exhibited significantly greater anticancer activity compared to cisplatinum (Bielawska et al. 2010). Even though DNA platination has been identified as essential and the first step in the cytotoxic activity of these drugs, the mechanism(s) by which these novel platinum-based compounds cause cell death is not fully understood. A proposed mechanism is that these complexes induce apoptosis by enhancing ROS generation and in consequence oxidative stress formation (Pabla et al. 2008).

As cellular redox status may play an important role in the anticancer pathways of berenil-platinum(II) complexes, the goal of this study was to investigate changes in levels of ROS and in the levels or activities of enzymatic and non-enzymatic antioxidants that lead to phospholipids peroxidation in human breast cancer cell lines. Both estrogen receptor (ER)-positive (MCF-7) and ER-negative (MDA-MB231) cell types were investigated. The novel dinuclear berenil-platinum (II) complexes examined had the general structure of $Pt_2L_4B_2$ and included: Pt_2(isopropylamine)$_4$(berenil)$_2$, Pt_2(piperidine)$_4$(berenil)$_2$, Pt_2(2-picoline)$_4$(berenil)$_2$, Pt_2(3-picoline)$_4$(berenil)$_2$, and Pt_2(4-picoline)$_4$ (berenil)$_2$ (Fig. 1).

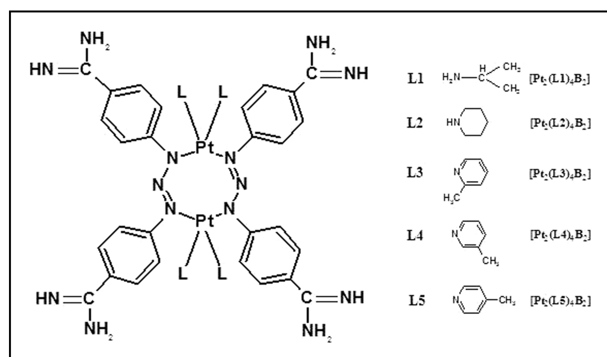

Fig. 1: Structure of novel berenil-platinum(II) complexes.

2. Investigations and results

Dinuclear berenil-platinum(II) complexes were more cytotoxic to both cell lines than cisplatinum. LC_{50} values for each compound in MCF-7 cells were as follows: Pt_2(isopropylamine)$_4$(berenil)$_2$, 16 μM; Pt_2(2-picoline)$_4$(berenil)$_2$, 21 μM; Pt_2(3-picoline)$_4$(berenil)$_2$, 36 μM; Pt_2(piperidine)$_4$(berenil)$_2$, 61 μM; Pt_2(4-picoline)$_4$(berenil)$_2$, 74 μM; and cisplatinum, 76 μM. LC_{50} values for each compound in MDA-MB231 cells were as follows: Pt_2(isopropylamine)$_4$(berenil)$_2$, 18 μM; Pt_2(2-picoline)$_4$(berenil)$_2$, 26 μM; Pt_2(3-picoline)$_4$(berenil)$_2$, 38 μM; Pt_2(piperidine)$_4$(berenil)$_2$, 74 μM; Pt_2(4-picoline)$_4$(berenil)$_2$, 94 μM; and cisplatinum, 96 μM.

Treating MCF-7 and MDA-MB231 cell lines with cisplatinum or dinuclear berenil-platinum(II) complexes increased the total ROS levels compared to controls. Moreover, extended treatment (up to 24 h) further increased ROS levels (Fig. 2). Among the complexes tested, Pt_2(isopropylamine)$_4$(berenil)$_2$ had the greatest effect in both cell lines after 12 and 24 h.

Treating MCF-7 and MDA-MB231 with cisplatinum or dinuclear berenil-platinum (II) complexes altered the activities of antioxidant enzymes and levels of non-enzymatic antioxidants compared to untreated cells (Table). Glutathione peroxidase (GSH-Px) activity was increased and glutathione reductase (GSSG-R) activity was decreased in both MCF-7 and MDA-MB231 cells after incubation with cisplatinum for 24 h. For MCF-7 cells, each of the five platinum-containing complexes reduced GSH-Px activity more than cisplatinum. Only Pt_2(piperidine)$_4$(berenil)$_2$ did not affect GSSG-R activity. Other derivatives altered enzyme activity in the same manner as cisplatinum. Only Pt_2(piperidine)$_4$(berenil)$_2$ increased GSH-Px activity in MDA-MB231 cells. In addition, reductions in GSH-Px activity by the other four complexes were comparable to those induced by cisplatinum. All tested compounds decreased GSSG-R activity in MDA-MB231 cells. Moreover, berenil-platinum(II) complexes were found to be more potent than cisplatinum.

In both MCF-7 and MDA-MB231 cells, treatment with cisplatinum or any of the five platinum complexes altered levels of non-enzymatic antioxidants. Treating MCF-7 cells with Pt_2(isopropylamine)$_4$(berenil)$_2$, Pt_2(piperidine)$_4$(berenil)$_2$, Pt_2(3-picoline)$_4$(berenil)$_2$, or Pt_2(4-picoline)$_4$(berenil)$_2$ decreased glutathione levels, but glutathione was unaltered by treatment with cisplatinum or Pt_2(2-picoline)$_4$(berenil)$_2$ (Table). Cisplatinum also reduced the concentrations of vitamins A and E in MCF-7 cells (Fig. 3). In comparison, Pt_2(piperidine)$_4$(berenil)$_2$ and Pt_2(2-picoline)$_4$(berenil)$_2$ reduced levels of vitamin A, but not to the same extent as cisplatinum. Pt_2(3-picoline)$_4$(berenil)$_2$ and Pt_2(4-picoline)$_4$(berenil)$_2$ reduced vitamin A levels more than cisplatinum. Pt_2(isopropylamine)$_4$(berenil)$_2$ reduced the con-

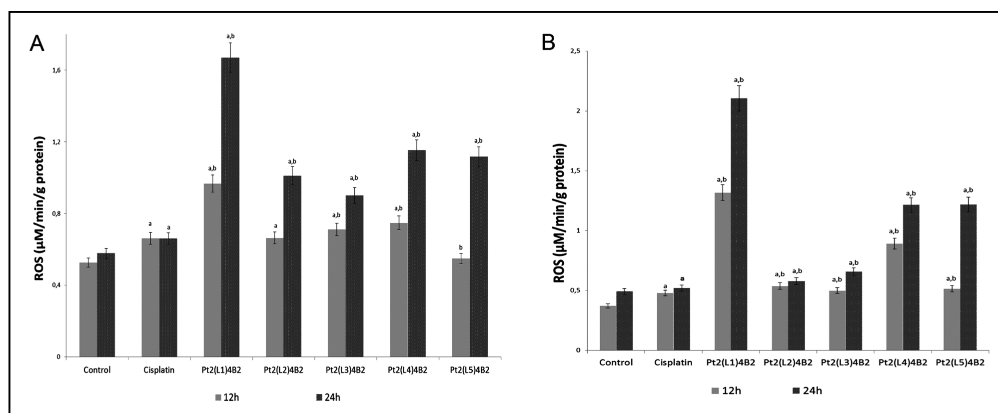

Fig. 2: Comparison of ROS levels generated in MCF7 [A] and MDA-MB231 [B] cells after incubation with cisplatin and berenil-platinum (II) complexes for 0 h (control), 12 h, or 24 h. Mean values ± SD of five independent experiments are presented. a $p < 0.05$ vs. control group; b $p < 0.05$ vs. cisplatin group.

Table: DNA damage marker N7-MetG and the activity of glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-R) and levels of glutathione (GSH) in MCF-7 and MDA-MB231 cells - control and after 24 hours incubation with cisplatin and berenil-platinum(II) complexes

Cell line	Analysed parameter	Control	Cisplatin	$Pt_2(L1)_4B_2$	$Pt_2(L2)_4B_2$	$Pt_2(L3)_4B_2$	$Pt_2(L4)_4B_2$	$Pt_2(L5)_4B_2$
MCF-7	N7-MetG pmol/mg DNA	n.d.	412 ± 14	208 ± 6 ^{b}	156 ± 5 ^{b}	354 ± 12 ^{b}	257 ± 8 ^{b}	199 ± 6 ^{b}
	GSH-Px mU/mg protein	30.9 ± 1.7	37.2 ± 2.0 ^{a}	19.1 ± 1.3 ^{ab}	16.9 ± 1.1 ^{ab}	19.9 ± 1.6 ^{ab}	18.3 ± 1.1 ^{ab}	13.4 ± 1.0 ^{ab}
	GSSG-R mU/mg protein	27.4 ± 2.2	23.3 ± 1.7 ^{a}	17.9 ± 1.2 ^{ab}	26.1 ± 2.0 ^{b}	23.5 ± 1.4 ^{a}	22.4 ± 1.7 ^{a}	24.3 ± 1.8 ^{a}
	GSH pmol/mg protein	60.1 ± 4.0	62.6 ± 3.9	50.9 ± 3.0 ^{ab}	54.3 ± 3.1 ^{ab}	56.1 ± 3.2 ^{b}	49.7 ± 3.1 ^{ab}	50.5 ± 2.9 ^{ab}
MDA-MB231	N7-MetG pmol/mg DNA	n.d.	497 ± 19	278 ± 11^{b}	389 ± 16 ^{b}	169 ± 5 ^{b}	324 ± 12 ^{b}	n.d.
	GSH-Px mU/mg protein	34.8 ± 2.0	38.1 ± 2.4 ^{a}	31.9 ± 2.0 ^{b}	38.5 ± 2.3 ^{a}	31.6 ± 2.1 ^{b}	34.1 ± 2.2 ^{b}	36.2 ± 2.4
	GSSG-R mU/mg protein	20.9 ± 1.2	18.9 ± 1.2 ^{a}	15.1 ± 1.1 ^{ab}	16.9 ± 1.0 ^{ab}	15.6 ± 1.0 ^{ab}	15.0 ± 1.2 ^{ab}	16.0 ± 1.1 ^{ab}
	GSH pmol/mg protein	95.1 ± 5.2	91.4 ± 5.2	89.0 ± 5.0	91.9 ± 5.2	92.8 ± 5.2	88.7 ± 5.0	91.2 ± 5.1

Mean values ± SD of five independent experiments are presented. ^{a} $p < 0.05$ vs. control group; ^{b} $p < 0.05$ vs. cisplatin group.

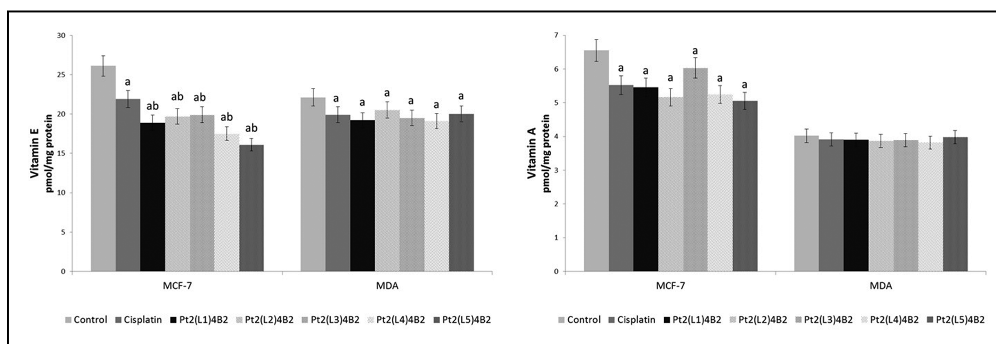

Fig. 3: The level of non-enzymatic lipophilic antioxidants [vitamin A and E] in MCF-7 and MDA-MB231 cells - control and after 24 hours incubation with cisplatin and berenil-platinum(II) complexes. Mean values ± SD of five independent experiments are presented. a $p < 0.05$ vs. control group; b $p < 0.05$ vs. cisplatin group.

centration of vitamin A in MCF-7 cells to the same extent as cisplatinum. Treatment of MCF-7 cells with cisplatinum or the platinum complexes decreased vitamin E concentrations. However, the berenil-platinum(II) complexes reduced vitamin E to lower levels than cisplatinum. Similarly, cisplatinum and the five platinum (II) complexes reduced vitamin E levels in MDA-MB231 cells (Fig. 3).
The effect of cisplatinum and berenil-platinum(II) complexes on the redox status of MCF-7 and MDA-MB231 cells was estimated by measuring DNA, lipid, and protein modifications. Cisplatinum, and the platinum complexes to a lesser degree, increased levels of N7-methyldeoxyguanine in MCF-7 cells. Similar results were observed with MDA-MB231 cells, except that $Pt_2(4\text{-picoline})_4(\text{berenil})_2$ did not increase levels of N7-methyldeoxyguanine (Table).
Incubating MCF-7 and MDA-MB231 cells with the platinum derivatives enhanced lipid peroxidation (Fig. 4). Changes in the level of arachidonic acid were observed after treatment with cisplatinum and the platinum complexes. However, only $Pt_2(\text{isopropylamine})_4(\text{berenil})_2$ induced greater decrease than cisplatin in both MCF-7 and MDA-MB231 cells. Increases in LOOH and 8-isoprostanes were observed in both MCF-7 and MDA-MB231 cells after treatment with cisplatinum and the platinum complexes. The greatest increase was observed after $Pt_2(\text{isopropylamine})_4(\text{berenil})_2$ treatment. When MCF-7 and MDA-MB231 cells were incubated with cisplatinum or other platinum compounds, levels of prostaglandin derivatives were also enhanced. The $Pt_2(\text{isopropylamine})_4(\text{berenil})_2$ complex induced the largest increase among the tested platinum complexes and was similar to cisplatinum treatment.

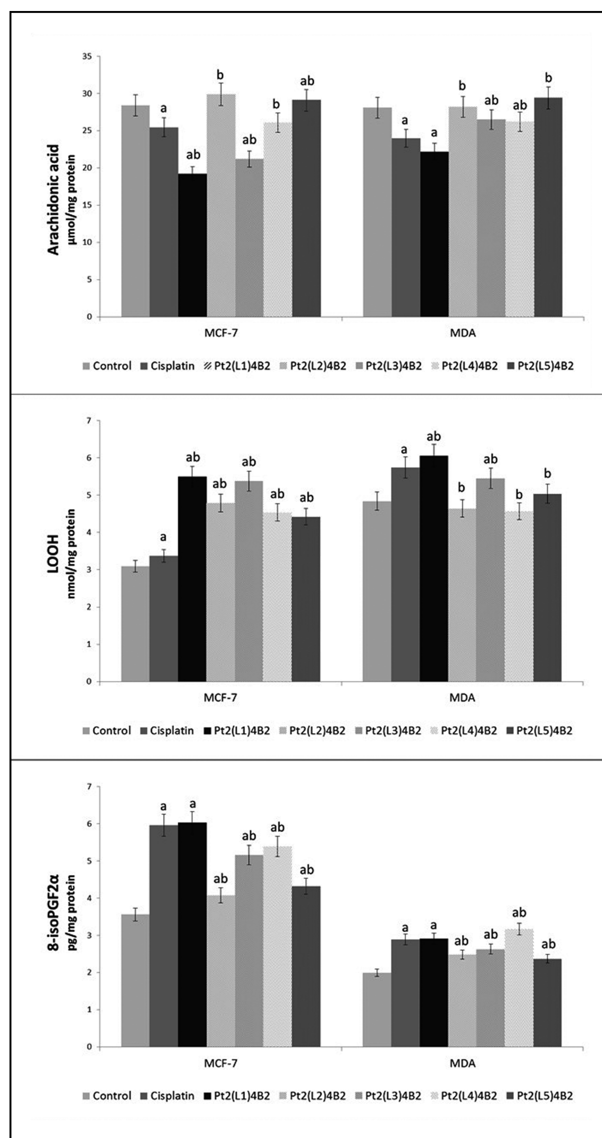

Fig. 4: The level of arachidonic acid, isoprostanes (8-isoPGF2α) and lipid hydroperoxides (LOOH), in MCF-7 and MDA-MB231 cells - control and after 24 hours incubation with cisplatin and berenil-platinum(II) complexes. Mean values ± SD of five independent experiments are presented. a $p < 0.05$ vs. control group; b $p < 0.05$ vs. cisplatin group.

3. Discussion

This study demonstrates that dinuclear berenil-platinum(II) complexes alter the redox status and enhance lipid peroxidation in human breast cancer cells (MCF-7 and MDA-MB231). ROS, including superoxide radicals and hydrogen peroxide, are potentially harmful by-products of normal cellular metabolism that oxidize and damage cellular components. Despite the potential damage from such reactions, growing evidence indicates that they may be required for signal transduction to regulate cell growth and intracellular redox status. As a result, ROS may directly affect cellular functions that are crucial to physiological function and survival of cells (Wang et al. 2012).
Previous studies demonstrated that many anticancer agents, including vinblastine, cisplatinum, mitomycin C, doxorubicin, camptothecin, inostamycin, and neocarzinostatin, exhibit antitumor activity through the ROS-dependent activation of apoptosis (Fang et al. 2007). The reaction between cisplatinum and genomic DNA primarily accounts for its cytotoxic and anticancer properties. Dinuclear platinum (II) berenil complexes are slightly more cytotoxic to breast cancer cells than cisplatinum, even though cisplatinum induces more DNA methylation. This finding suggests that platinum-berenil complexes act through different mechanisms than cisplatinum.
The berenil amidino moiety is a strong base and totally protonated at physiological pH (Brown et al. 1990). This aspect may be a key consideration for both DNA binding and bioavail-

ability. The presence of aromatic amines with extended alkyl substituents at position 3 also improves the stability of these complexes. As a result, higher binding affinities for DNA and greater cytotoxicity are achieved due to multiple interactions of the berenil amidino moiety with the minor groove of DNA (Bielawska et al. 2010; Bielawski et al. 2008). Moreover, this is consistent with the increased cytotoxicity observed for these compounds in the present study. DNA damage and the subsequent induction of apoptosis represent the primary cytotoxic mechanisms of cisplatinum and other DNA-binding anticancer drugs (Rebillard et al. 2010; Rousseau et al. 2010).

The ROS generated during altered mitochondrial function and/or drug metabolism after exposure to these platinum complexes also led to oxidative modifications of unsaturated fatty acids, thereby inducing peroxidation with increased levels of hydrogen peroxide and a prostaglandin derivative, 8-isoprostane. The most effective compound to generate ROS and lipid peroxidation was Pt_2(isopropylamine)$_4$berenil$_2$. Moreover, the ER-positive cells (MCF7) were more susceptible to its action than the ER-negative cells (MDA-MB231).

The extent of lipid peroxidation is dependent on the activities of enzymes involved in glutathione metabolism, particularly PSH-Px and GSSG-R. Cisplatinum increases the activity of GSH-Px, an antioxidant enzyme in eukaryotic cells that converts hydrogen peroxide, lipid peroxides, and other peroxides into nontoxic molecules (Handy et al. 2009; Ozgocmen et al. 2007). In contrast, the novel platinum(II) complexes reduced the activity of this enzyme, thereby raising the level of ROS in the tested cells. GSH-Px is a glutathione-dependent enzyme (Masella et al. 2005), and glutathione levels are notably reduced in MCF-7 cells. Selenosuphydryl peroxidase can be inactivated by 4-HNE, a reactive aldehyde generated during lipid peroxidation (Bosh-Morell et al. 1998).

Furthermore, GSSG-R activity is reduced by these compounds. GSSG-R helps maintain levels of reduced GSH in the cytoplasm. Greater changes in GSSG-R activity were observed in MCF-7 cells than in MDA-MB231 cells. The Pt_2(isopropylamine)$_4$berenil$_2$ complex induced the greatest change in MCF7 cells. If GSSG-R activity is inhibited, cells accumulate hydrogen peroxide, which can oxidize glutathione, among other compounds (Caramelo-Nunes et al. 2011). As the disulfide form of glutathione increases and reduced glutathione decreases, protein synthesis and GSH-Px activity are inhibited. Disturbances in the glutathione system decrease levels of arachidonic acid. Decreases in arachidonic acid are further enhanced by low levels of fat-soluble vitamins (E and A), which also have antioxidant activity. This condition leaves cell membranes susceptible to oxidative damage. The interactions of the platinum(II) complexes with phospholipids change phospholipids conformation and the properties of biomembranes (Dobrzyńska et al. 2013).

The estrogen-dependent MCF-7 cell line showed more pronounced decreases of non-enzymatic antioxidants, such as GSH, vitamin E, and vitamin A, compared to estrogen-independent cells (MDA-MB231). In addition to its antioxidant properties, reduced glutathione helps neutralize electrophilic compounds, such as alkylating drugs, by forming inactive conjugates (Peklak-Scott et al. 2008). It is hypothesized that depleted GSH may be associated with a high concentration of drugs inside cells. Moreover, cancer cells deprived of basic non-enzymatic antioxidants may be more sensitive to oxidative stress (Brabec and Kasparkova 2005). Thus, when GSH and other antioxidants are depleted and high concentrations of anticancer drugs are present, ROS accumulate intracellularly to cause irreversible damage and apoptosis.

In conclusion, this study demonstrates that cisplatinum and berenil-platinum(II) complexes enhance oxidative stress, increasing ROS production and decreasing the antioxidant capacity of breast cancer cells. Pt_2(isopropylamine)$_4$berenil$_2$ had stronger effects on ER-positive breast cancer cells than on ER-negative breast cancer cells. These findings could facilitate future anticancer drug design strategies.

4. Experimental

4.1. Cell culture and treatment

The human breast cancer cell lines MCF-7 and MDA-MB231 (American Type Culture Collection), were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. When the cells reached confluency were used for assays. Cells (1×10^6 cells/ml) were suspended in 6 ml DMEM and incubated with or without test compounds in cell culture plates. Platinum (II) complexes: Pt_2(isopropylamine)$_4$(berenil)$_2$ ($Pt_2(L1)_4B_2$), Pt_2(piperidine)$_4$(berenil)$_2$ ($Pt_2(L2)_4B_2$), Pt_2(2-picoline)$_4$(berenil)$_2$ ($Pt_2(L3)_4B_2$), Pt_2(3-picoline)$_4$(berenil)$_2$ ($Pt_2(L4)_4B_2$), Pt_2(4-picoline)$_4$(berenil)$_2$ ($Pt_2(L5)_4B_2$), and cisplatin were added to the cultured cells at a final concentration 20 μM. Control cells were incubated without test compounds. After 12 or 24 h, cells were rinsed with PBS, collected by scraping into cold PBS and centrifuged. Cells were then resuspended in 1 ml PBS and subjected to two freeze/thaw cycles. Total protein content was measured using a Bradford assay and reagents (Bradford 1976).

4.2. Measuring cell viability/cytotoxicity

Measurement of cytotoxicity of the test compounds was performed according to the MTT assay (Mosmann 1983). All experiments were run eight times, and lethal concentration 50 (LC_{50}) was determined after 24 h.

4.3. Estimation of DNA methylation

To estimate DNA modifications, genomic DNA was isolated using Sigma's GenElute Mammalian Genomic DNA Miniprep kit. Purified DNA was then subjected to neutral thermal hydrolysis at 100 °C for 30 min and precipitated. N7-Methyldeoxyguanosine (N7-MetG) was also assayed using a modified liquid chromatography - mass spectrometry method using an Agilent 1290 LC instrument with triple quadruple mass spectrometer with electrospray ionization source LC-MS method of Chao et al. (2007). Samples were analyzed in the positive ion multiple reaction monitoring mode and the transitions of the precursors to the product ions were as follows: m/z 166 → 124 (qualifier ion) for N7-MetG, m/z 171 → 153.

4.4. Detection of ROS

The generation of total ROS was detected using an electron spin resonance (ESR) spectrometer e-scan (Noxygen GmbH/Bruker Biospin GmbH, Germany), where selective interaction of the spin probes CMH (1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetrame-thylpyrrolidine, 200 μM) with ROS forms a stable nitroxide CM-radical with a half-life of 4 h. Thus, ROS formation was measured from the kinetics of nitroxide accumulation according to the electron spin resonance (ESR) amplitude of the low field component of ESR spectra (Kuzkaya et al. 2003).

4.5. Detection of enzyme activity

Glutathione peroxidase (GSH-Px – EC.1.11.1.6) activity was assessed spectrophotometrically using the technique of Paglia and Valentine (1967). According to this technique, glutathione (GSH) formation was assayed by measuring the conversion of NADPH to $NADP^+$. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADPH min^{-1} at 25 °C and pH 7.4. Enzyme specific activity was expressed in units per mg of protein.

Glutathione reductase (GSSG-R – EC.1.6.4.2) activity was measured according to the method of Mize and Langdon (1962) by monitoring the oxidation of NADPH at 340 nm at pH 7.4. Enzyme activity was expressed in units per mg of protein. One unit of GSSG-R oxidized 1 mmol of NADPH/min at 25 °C and pH 7.4. Enzyme specific activity was expressed in units per mg of protein.

4.6. Detection of non-enzymatic antioxidants level

Total glutathione was determined using high performance liquid chromatography (HPLC) to detect reduction of the disulfide group of oxidized glutathione with dithiothreitol (Cereser et al. 2001). Derivatization of GSH with o-phthalaldehyde, which reacts with both the sulfhydryl and the primary amino group of glutathione, forms a highly fluorescent product. Oxidized glutathione was then measured by HPLC after elimination of

GSH with N-ethylmaleimide, followed by reduction of disulfides with dithiothreitol, and derivatization with o-phthalaldehyde (Neuschwander-Tetri and Roll 1989). GSH concentrations were calculated by subtracting GSSG from the total glutathione value.
HPLC methods were used to detect levels of vitamins A and E (Vataserry et al. 1988). Briefly, cell homogenates were centrifuged (1000 × g, 10 min) to remove precipitated protein. Vitamins A and E were extracted from cell lysates using hexane containing 0.025% butylated hydroxytoluene. The hexane phase was removed and dried with sodium sulfate, and 50 μl of the hexane extract was injected on the column. UV detection at 250 nm was applied.

4.7. Detection of phospholipids modifications

Lipid peroxidation was estimated by measuring of lipid hydroperoxides and 8-isoprostanes levels as well as determination of phospholipid arachidonic acid level was done.
Lipid hydroperoxides (LOOH was estimated by the technique of Tokumaru et al. (1995). The method involved the oxidation of 1- naphthyldiphenylphosphine (NDPP) into its oxide. The separation was performed on octadecyl column RP C18 and UV detection at 292 nm was applied. As a eluent was used methanol and water (80:20)
8-Isoprostaglandine F2α (8-isoPGF2α) was assayed according to a modified LC-MS method of Coolen et al. (2005). Briefly, samples were purified using a SEP-PAK C18 column containing octadecylsilyl silica gel. 8-IsoPGF2α was analyzed by HPLC and detected using ESI-MS. Samples were analyzed in the negative multiple reaction mode for MS. Target ions with a m/z 353→193 were selected.
Fatty acids profile was determined by gas chromatography (Christie 1993). Phospholipids components were isolated by Folch extraction using chloroform/methanol mixture (2:1, v/v). Using TLC total phospholipids were separated with the mobile phase heptane – diisoprophyl ether – acetic acid (60:40:3, v/v/v) and transmetylated to fatty acid methyl esters (FAMEs) with boron trifluoride in methanol reagent under nitrogen atmosphere without previous separation from the layer material at 100 °C for 30 min. FAME's were analyzed by gas chromatography with a flame ionization detector. Separation of FAME was carried out on capillary column coated with Varian CP-Sil88 stationary phase.

4.8. Statistical analysis

Data were analyzed using standard statistical analyses, including one-way Student's test for multiple comparisons, and the results are expressed as the mean ± standard deviation (SD) for n = 5. P-values less than 0.05 were considered significant.

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