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Full Length Research Paper

Antiproliferative activity of some novel platinum complexes on C6 glioma and MCF-7 breast cancer cells

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The anti-cancer chemotherapeutic potential of novel platinum(II) complexes of salicylate derivatives [Pt(dppe)$(SA)_2$, Pt(dppm)$(SA)_2$] and fumaric acid [Pt(dppe)$(FU)_2$] were determined, using two cancer cell lines, breast cancer (MCF-7) and glioma cells (C6). IC_{50} values of the three compounds were lower in the cisplatin-resistant cell type C6 cell lines than in MCF-7 cells.

Key words: Cisplatin, antiproliferative activity, breast cancer cells (MCF-7), glioma cells (C6), $IC_{50.}$

INTRODUCTION

Cisplatin [cis-diamminedichloroplatinum(II)] was introduced into clinical practice in the early 1970s and it is one of the most active antineoplastic agents currently used in medical oncology (Barbara et al., 2006). Over the years, thousands of cisplatin analogues have been synthesized, varying the nature of the leaving groups and the carrier ligand (Matesanza and Souza, 2007; Rosenberg et al., 1969). Despite the large amount of compounds synthesized and tested, only a small number have advanced to the late stages of clinical development, that is the second-generation platinum drug carboplatin is in routine clinical use (Wang et al., 2002).

Cisplatin is the best example of metal complexes and binds to DNA by coordination of the Pt atom to the N_7 positions of two guanine bases or to adenine and guanine, which then block replication and/or prevent transcription. Non-covalent interactions between metal complexes and DNA include hydrogen bonding, electrostatic interaction and intercalation (Rebolledo et al., 2005; Kovala-Demertzi et al., 2003; Padhye et al., 2005). Cisplatin has a broad spectrum of activity against epithelial cancers and become the foundation of curative regimens in testicular and ovarian cancers, demonstrating significant activity also against lung, head and neck, esophagus, bladder, cervix, and endometrial cancers (Elwell et al., 2006). Although cisplatin is one of the most frequently used chemotherapeutics in the treatment of many tumors, the clinical application of cisplatin is greatly limited by its toxicity, narrow range of activity, both intrinsic and acquired resistance, and low aqueous solubility (Cui et al., 2006).

In recent years, there has been an emergence of new structural types of compounds often with promising activity and able to circumvent cisplatin resistance (Van der Schilden et al., 2004; Perez et al., 2001; Kasparkova et al., 2003). The majority of platinum complexes exhibiting anti-tumor activity have two cis-leaving ligands, such as chlorides and two strongly bonded, relatively inert amine type systems (non-leaving ligands) (Ciesielska et al., 2006; Reedijk,1992). A few different liposomal formulations of cisplatin have also been prepared and biologically evaluated since the introduction of cisplatin. Nevertheless, so far none of liposomal formulations of cisplatin have been approved for the clinical use in the world. The key reasons are the poor water solubility and low lipophilicity of cisplatin, which make it difficult to efficiently encapsulate the drug in a liposome. An alternative approach is to synthesize

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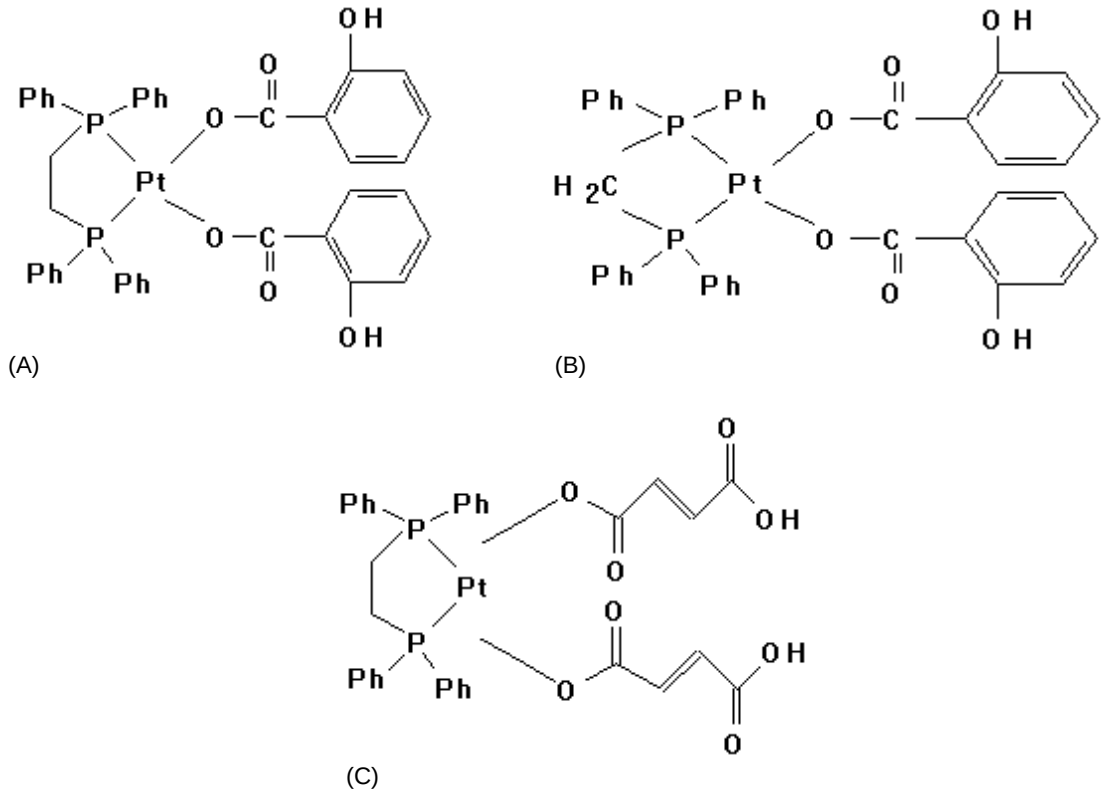

Figure 1. Schematic representation of Pt(II) complexes. (A) Pt(dppe)(SA)2 ; (B) Pt(dppm)(SA)2 and (C) Pt(dppe)$(FU)_2$.

lipophilic complexes. In addition, any work carried out in this field can be concluded that even small variations in the amine ligands of cisplatin analogues have already huge effects on the activity of the complexes. Zutphen et al. (2005) reported that new cisplatin compound composed of methylamine located cis to aminoethanol that posses promising cytotoxic activity (Abine Van Rijta et al., 2006; Van Zutphen et al., 2005). Some studies have demonstrated that Dppe as non leaving ligand has *in vitro* cytotoxic and *in vivo* antitumor activity for silver (Au-based) drugs (Berners-Price et al., 1986; Snyder et al., 1986; Mirabelli et al., 1985).

Therefore, the reduction of essential cellular components or the generation of reactive radical species during phosphine oxidation, perhaps involving redox-active metal ions such as copper and iron may play a role in the cytotoxic activity of Dppe.

MATERIALS AND METHODS

Synthesis of complexes

For preparing Platinum(II) complexes, $PtCl_2COD$ (where COD is 1.5 cyclooctadiene) was used as the main starting material and $PtCl_2$(dppx) (where dppx is bis (diphenyl phosphino) methane and 1,2 bis (diphenyl phosphino) ethane) complexes were also used as the following starting materials. All the compounds synthesized and tested for *in vitro* antitumor activity were given in Figure 1. $Pt(dppe)(SA)_2$ (Figure 1A), $Pt(dppm)(SA)_2$ (Figure 1B) and $Pt(dppe)(FU)_2$ (Figure 1C) were synthesized under the nitrogen atmosphere according to reported methods (Nakiboglu, 2006). All complexes were characterized by elemental analysis and spectroscopic techniques namely FTIR and 31P{H} NMR (δ ^{31}P, $J_{31P-195Pt}$). Formula weights and melting points for complex 1 (Figure 1A) ($PtC_{40}H_{34}O_6P_2.1/2CH_2Cl_2$) is: 909.74 g and 188ºC. Analysis Calculated for complex 1 is: C, 53.42 and; H, 3.88. Found: C, 54.34 and; H, 3.90. Formula weights and melting points for complex 2 (Figure 1B) ($PtC_{39}H_{32}O_6P_2.1.5CH_2Cl_2$) is: 980.73 g and 206ºC. Analysis Calculated for complex 2 is: C, 49.59 and; H, 3.57. Found: C, 49.67 and; H, 3.40. Formula weights and melting points for complex 3 (Figure 1c) ($PtC_{34}H_{30}O_8P_2$) is: 823.89 g and 204ºC. Analysis Calculated for complex 3 is: C, 49.56 and; H, 3.64. Found: C, 49.41and; H, 4.04. IR spectra demonstrated the characteristic patterns expected from carboxylate ligands.

The $\upsilon_{a_1}(COO^-)$ and $\upsilon_s(COO^-)$ bands for complex 1 is: 1530 to 1610 cm^{-1} and 1390 cm^{-1}; for complex 2 is 1560 to 1650 cm^{-1} and 1390 cm^{-1}; for complex 3 is 1540 to 1660 cm^{-1} and 1410 cm^{-1}. $^{31}P\{^1H\}$ NMR spectral data of the platinum complexes further support the structure of aforementioned complexes. $^{31}P\{^1H\}$ NMR spectra were recorded on a JOEL JNM-Ex400 spectrometer. They were measured in methanol (CD_3OD) solutions and 85% H_3PO_4 served as an external reference. NMR grade solvents were used for

nuclear magnetic resonance (NMR) analysis. All platinum complexes give a triplet on the NMR spectra. This triplet of the ^{31}P spectrum is due to the coupling of platinum atom with two phosphorus atoms. The coupling constant, $J_{P\text{-}Pt}$, ranges from 3200 to 3985 Hz, which is typical for platinum(II) complexes containing two phosphorus and two oxygen donors. ^{31}P spectra of the carboxylate complexes are very similar to those of chlorides starting complexes, but the carboxylate complexes exhibit a large shift of the ^{31}P resonance relative to that of the chlorides. Because of the platinum-oxygen bond being weaker than platinum-chloride bond, the chemical shift values of starting complexes shifted to higher field. δP (ppm) and $J_{P\text{-}Pt}$ value for complex 1 is: +32.0 ppm and 3985.5 Hz; for complex 2 is: -67.0 ppm and 3390.0 Hz and; complex 3 is: +41.7 ppm and 3607.8 Hz.

Cell lines and cytotoxicity

MCF-7 mammary cancer cells and C6 rat glioma cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml Penicilin, 10 ug/ml Streptomycin, 2mM L-glutamine and 10% HI- Fetal Calf Serum (FCS) at 37ºC in a humidified 5% CO_2 incubator.

Cell viability was determined by MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] assay. 5×10^3 cells per well were plated onto 96-well sterile for 24 h. Cells were incubated with various concentrations, ranging from 0 to 400 µM of the compounds tested freshly, and dissolved in dimethyl sulfoxide (DMSO) for 24, 48 and 72 h at 37°C. Then, the cells were fixed by adding 20 µl of MTT (5 mg/ml) per well, and incubated for 4 h at 37°C. Subsequently, the medium containing MTT was removed, and 100 µl of acidified isopropanol (0.04 N HCl) added. Spectrophotometric absorbance of each sample was measured at 550 nm, using a micro plate reader (Bio-Tek, model powerwave XS).

All cytotoxicity experiments were performed in triplicate. The 50% inhibitory dose (IC_{50}) was defined as the drug concentration that reduced the number of living cells to 50%:

$$\text{Inhibition (\%)} = [1 - (T/C)] \times 100$$

Where, T is the mean absorbance of the treated cells and C is the mean absorbance in the controls.

For comparison purposes, the cytotoxicity of cisplatin was evaluated under the same experimental conditions.

Statistical analysis

Statistical analysis was evaluated by one-way ANOVA. $P < 0.05$ was accepted as significant difference.

RESULTS AND DISCUSSION

The ligand exchange reactions of leaving groups of platinum complexes with biological nucleophiles are likely governing their antitumour and toxic properties. The nephrotoxicity, gastrointestinal toxicity and possible bone narrow suppression, induced by platinum based antitumour agents, might be attributed to ligand exchange reactions of platinum with sulfydryl groups, and the subsequent inactivation of essential enzymes and other proteins. The platinum complexes have been shown to react with the series of peptide and proteins from glutathione, metallothionein to plasma proteins by binding to sulfhydryl groups (Hall and Hambley, 2002). In this respect, some research proposes that fumaric acid esters also react with sulfhydryl groups in proteins. Therefore, besides others, fumaric acid esters are interesting for their antipsoriatic activity (Frycak et al., 2005). Complex 3 was synthesized using fumaric acid as a leaving group. Another leaving group was salicylate that is important nonsteroidal anti-inflammatory agents (Figure 1A and B). It has been well known that they can block metastasis of cancer cells by inhibiting synthesis of the prostaglandins (Ye et al., 2007).

Sodium salicylate is a drug widely used all over the world, initially in the form of aspirin (acetyl salicylate) which is rapidly converted to salicylate in the organism. Although it has few side effects, it can cause changes in the hearing system (Jastreboff and Sasaki, 1994; Garetz and Schacht, 1996). Particularly, Li et al. (2002) using a rat experimental model, observed significant oto-protection by sodium salicylate against the cytotoxic effects of cisplatin, as demonstrated by the brain stem auditory potentials, without affecting the antitumoral potency of cisplatin. As for non-leaving group, 1,2-bis(diphenylphosphino)ethane (dppe) and 1,2-bis(diphenylphosphino)methane were used. Dppe and a number of closely related diphosphines have potent cytotoxicity to tumor cells in culture including P388 leukemia, M5076 reticulum cell sarcoma, B16 melanoma, and the mammary adenocarcinoma and have reproducible activity in animal tumor models. Some investigator has reported that some gold-phospine compounds exhibit potent ligand-mediated cytotoxicity (Li et al., 2006). All the design strategies in our research are to develop platinum complexes with expectations of higher liposolubility and chemical stability, along with higher anti-tumor activities.

The solubility of the complexes, both in water and organic solvents such as ethanol, acetone and ether was determined. Three compounds, Pt(dppe)$(SA)_2$, Pt(dppm)$(SA)_2$, and Pt(dppe)$(FU)_2$, were not water soluble. Dimethyl sulfoxide (DMSO) was used for dissolving the complex, and in each experiment, controls with appropriate concentrations of vehicle were performed. Concentrations of compounds were ranging from 0, 1 to 100 µM. The compounds were then filter-sterilized and stored at room temperature. The new platinum(II) complexes were tested for their cytotoxicity by MTT assay on cancer cell lines, human breast carcinoma cell line, MCF-7 and rat C6 glioma cells as a model of astro-cytomas (Mosmann, 1983). In attempting to evaluate the cyto-selective nature of these test agents and provide the best possible means of comparison, we selected two different model cell lines. In addition, it is worth noting that these two neoplastic cells are not identical in origin, since their media requirements and growth kinetics

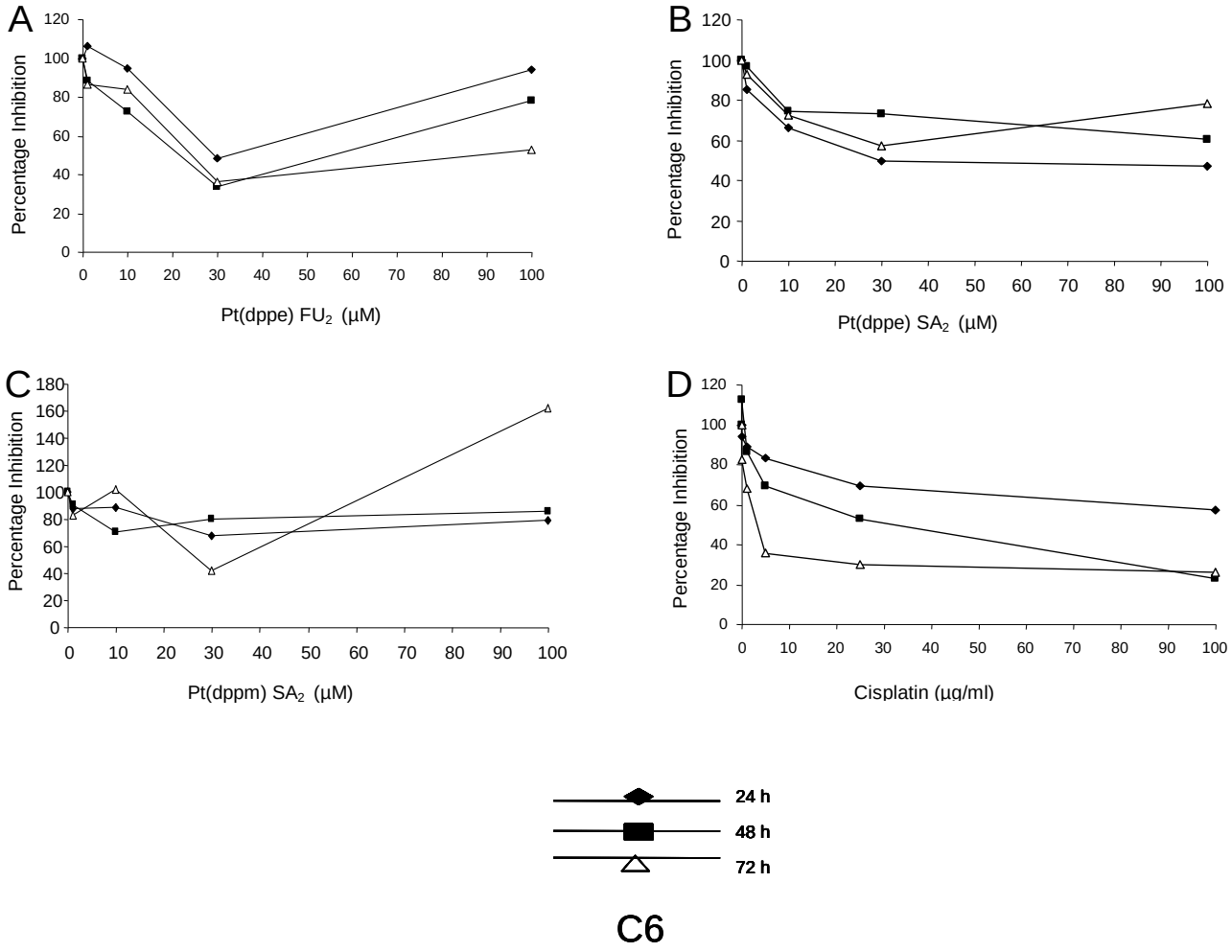

Figure 2. Time and dose dependent cytotoxic effects of cisplatin and newly synthesized cisplatin complexes Pt(dppe)$(FU)_2$, Pt(dppe)$(SA)_2$ and Pt(dppm)$(SA)_2$ on C6 cells for 24, 48 and 72 h. Cell viability was evaluated by the MTT colorimetric assay at every 24 h.

differ. C6 cells represent a model of astrocytomas that are the most common tumors of the central nervous system (CNS), usually with a poor prognosis, and extremely difficult to treat (Poppenborg, 1999). Otherwise, MCF-7 cells represent breast cancer cell type. Hence, there remains the possibility that these factors may contribute to the apparent non-selective nature of the test agents.

MCF-7 and C6 cells were exposed to four different concentrations of the compounds [Pt(dppe)$(SA)_2$, Pt(dppm)$(SA)_2$, and Pt(dppe)$(FU)_2$] for 24, 48 and 72 h, respectively (Figures 2 and 3). The relative potency of these test agents was determined by the inclusion of one of the best known and most biologically active metal-based anti-cancer agent, cisplatin.

Pt(dppe)$(FU)_2$, in C6 and MCF-7 cells exhibit dose dependent cytotoxicities in all time intervals (Figures 2A and 3A). The cell growth inhibition in C6 cells diminished at the level of 30 µM since the doses above this concentration led to DMSO or compound dependent cell death. As seen IC_{50} values in Table 1, C6 cells is more sensitive than MCF-7 cells for this compound. Similarly, IC_{50} values of Pt(dppe)$(SA)_2$ exhibited the most cytotoxic effect in C6 cells compared to MCF-7 cells (Table 1). However, at 72 h incubation time, 30 µM of the compound reached the maximum cytotoxic effect as the cells went to death due to high DMSO concentration (Figure 2B). In MCF-7, at the beginning of the incubation, Pt(dppe)$(SA)_2$ has led to the cell proliferation at 24 h incubation and, then, this effect turned to inhibition effect (Figure 3B).

Pt(dppm)$(SA)_2$ is different from the complex 1 as non leaving group (Figure 1A). Therefore, the effect of “dppm” could be specifically evaluated. This compound inhibited cell proliferation up to 50% at 72 h incubation period in C6 cells (Figure 2C). Similarly with other compounds,

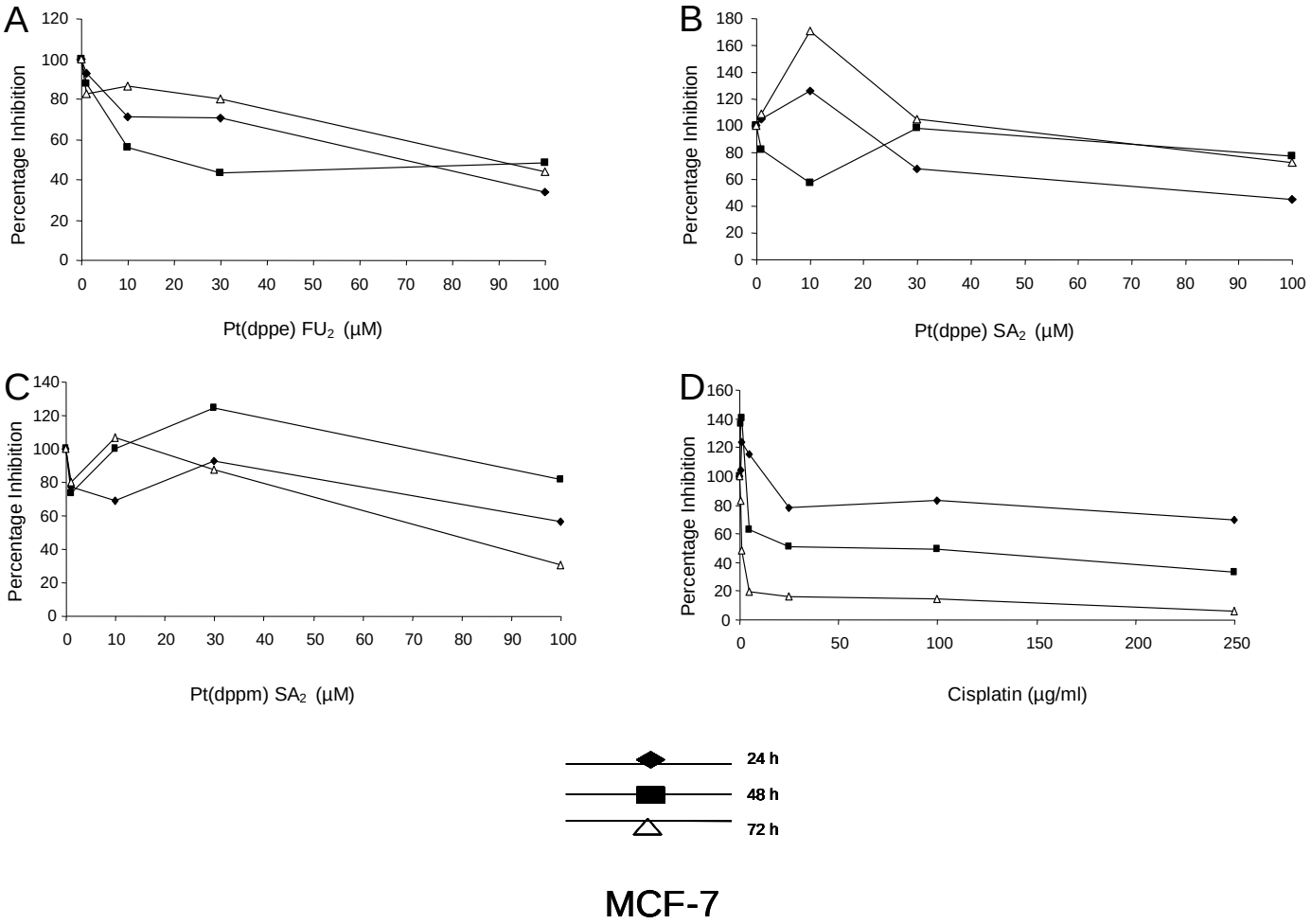

Figure 3. Time and dose dependent cytotoxic effects of cisplatin and newly synthesized cisplatin complexes Pt(dppe)$(FU)_2$, Pt(dppe)$(SA)_2$ and Pt(dppm)$(SA)_2$ on MCF-7 cells for 24, 48 and 72 h. Cell viability was evaluated by the MTT colorimetric assay at every 24 h.

Table 1. IC_{50} values of cisplatin and synthesized Platinum(II) complexes at 72 h.

Compound	Cytotoxicity in MCF-7 (IC_{50}, µg/ml)	Cytotoxicity in C6 (IC_{50}, µg/ml)
Cisplatin	1.56	16.75
Pt(dppe)$(SA)_2$	129.96	27.74
Pt(dppm)$(SA)_2$	81.90	23.97
Pt(dppe)$(FU)_2$	66.20	28.95

upon 72 h incubation of Pt(dppm)$(SA)_2$, inhibition effect was not able to be detected anymore, due to the excess cell death after 30 µM (Figure 2C). In MCF-7 cells, the inhibition effect of Pt(dppm)$(SA)_2$ compound could be obtained up to 100 µM (Figure 3C). The corresponding IC_{50} values demonstrated that this compound is more cytotoxic in C6 cells than those of MCF-7 cells (Table 1). Table 1 shows that the cytotoxicity of Pt(dppm)$(SA)_2$ is very close to that of cisplatin against C6 cells. In MCF-7 cells, Pt(dppm)$(SA)_2$ has the more cytotoxicity than compound Pt(dppe)$(SA)_2$. This could be due to the difference of a non leaving group. As a non leaving group, phospine ligands have been used for the all three compounds. Our compounds with salicylic acid (Figure 1A and B) exhibited the most cytotoxic effect to C6 cells. From the above biological results, it can be concluded

that C6 glioma cell was most sensitive to our compounds. The cytotoxicity of compounds Figures 1A, B and C) against C6 cells was comparable to that of cisplatin (Figure 1; Table 1).

The most effective inhibition of cisplatin was found in MCF-7 cells suggesting that, in general, cells of breast origin may be more sensitive than glial cells for cisplatin. Interestingly, IC_{50} values for the three compounds were lower in the cisplatin resistant cell type C6 cell lines, than in MCF-7cells. Therefore, it may be possible that our test agents do display a degree of selectivity for one cancer cell type over another. In fact, cyto-selectivity is a highly desirable characteristic for all anti-cancer agents. It is often difficult to achieve, even with cisplatin, a drug currently in clinical use to the patient's needs.

In conclusion, all three compounds, $Pt(dppe)(SA)_2$, $Pt(dppm)(SA)_2$, and $Pt(dppe)(FU)_2$, seem to be very efficient as a cytotoxic agent against astrocytomas *in vitro*. Further investigation about its mechanism of action, as well as exploration of its capability to restrain tumor formation *in vivo* would be our future goals.

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