A COMPLEX STUDY ON ANTICANCER COPPER CHELATORS

PhD thesis

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1. Introduction

Cancer is the second most frequent cause of death. Treatment of advanced stage diseases is still a major clinical problem despite the availability of a broad range of therapeutic agents with different mechanisms of anticancer activity. The discovery of cisplatin ([cis-diammine-dichloroplatinum(II) complex]) laid the foundation for the use of metal-based compounds in the treatment of several cancers, including testicular cancer, ovarian cancer, or breast cancer. Second and third generation platinum (Pt) drugs were developed in order to reduce the dose-limiting toxicity of cisplatin and overcome the intrinsic or acquired cisplatin resistance of cancer cells. The development of further Pt-based compounds has been the subject of intensive research. In parallel, alternative metal-based complexes are also considered. Copper (Cu) chelating compounds are primary candidates for development, given the strong *in vitro* toxicity of copper complexes. Common examples of copper chelating ligands include derivates of dithiocarbamate, 8-hydroxyquinoline, thiosemicarbazone and phenantroline structures. These ligands form stable and highly toxic copper complexes with persistent toxicity, even in multidrug resistant cell lines.

The essentiality of copper (Cu) is partially linked to its involvement in basic electron transfer processes through the reduction of Cu(II) to Cu(I)). However, the same one electron exchange reaction also generates free radicals that can be harmful to cells. Cells efficiently protect their intracellular milieu by hiding copper from cellular constituents, thereby preventing the accumulation of free ionic copper. Copper chelator structures can circumvent these defensive processes. The exact mechanism by which copper increases the toxicity of certain chelator compounds is not known. The toxicity of copper chelating ionophores is usually accompanied by the increased cellular accumulation of copper, which is primarily responsible for cell death.

Drug delivery systems (DDSs), such as liposomes, can be utilized to formulate Cu complexes. Liposomes can be manufactured in various sizes, depending on the desired in vivo behavior, and they can encapsulate a wide range of molecules. Liposomes with an average diameter of 100 nm have been found to remain in circulation for a significantly longer period than liposomes of a larger size. Most DDSs target malignant tissues rather than healthy organs, even in the absence of a targeting ligand, due to the EPR effect.

Although many Cu complexes have been synthesized as anticancer agents, their in vivo efficacy remains questionable, and there are currently no promising copper complexes under clinical investigation. The main challenge in the clinical development is the high toxicity and low selectivity of copper complexes. Both selective targeting and toxicity reduction can be

achieved by formulation strategies coupled with a homing (targeting) system. Liposomal encapsulation provides a solution to increase the concentration of cytotoxic copper complexes in the tumor, while decreasing the blood concentration and, thus, reducing general toxicity. Active targeting can be achieved by using thermosensitive liposomes and mild hyperthermia to further reduce unwanted toxicity and to enhance drug delivery.

1. Objectives

The aim of this thesis is to investigate the copper accumulation ability of different chelating structures, and their role in cytotoxic effect. Due to their poor *in vivo* selectivity and high toxicity, a drug delivery system, namely, the liposomal delivery of these drugs, appears to be at the forefront of overcoming the pharmaceutical challenge of developing metal-based therapeutics. One of the main aims of the present research is to show the applicability of copper chelating compounds *in vivo* using liposomal formulations. The goal of this PhD was to formulate liposomes that will achieve acceptable loading of an ionophore structure in the presence of Cu(II) ions, while the formulation allows drug targeting using mild hyperthermia.

2. Methods

3.1. Chemicals

Throughout the experiments, deionized Milli-Q (MQ, Millipore, Molsheim, France) water with a relative resistivity of 18.2 MΩ·cm was used. All the chemicals were of analytical grade. The various chelator-like structures (**Table 1**) are products of Sigma-Aldrich (St. Louis, MO, USA). The Q4 chelator was donated by Dr. Gergely Szakács. Metal salts were dissolved and applied in aqueous solutions, while the chelators were dissolved in DMSO, except neocuproine hydrochloric salt for the preparation of liposomes, which was taken up in Milli-Q water. Synthetic high-purity 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), hydrogenated soybean phosphatidylcholine (HSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG 2000) were obtained from Avanti Polar Lipids (Alabaster, AL, US) or from Sigma Aldrich.

3.2. *In vitro* experiments

3.2.1. Cell lines

The following cancer cell lines were used: HT-29, HCT-15, HCT-116 colon adenocarcinoma were obtained from ATCC (LGC Standards GmbH, Wesel, Germany) and C-26 (also referred to as Colon-26) mouse colon carcinoma cell lines from CLS (Cell Lines Service GmbH, Germany). The A-375 malignant melanoma; MCF-7, MDA-MB-231 and ZR-75-1 breast adenocarcinomas; Caov-3 and OVCAR-3 human ovarian carcinoma cell lines; PANC-1 pancreatic cancer cell line; H358 lung (bronchioalveolar) carcinoma; and HT-1080 fibrosarcoma cell were also purchased from ATCC.

3.2.2. Cell culture conditions

Cell lines were cultured in RPMI-1640 (developed by Moore et al. at Roswell Park Memorial Institute, hence its acronym RPMI) or in Dulbecco's Modified Eagle's Medium (DMEM) media according to manufacturer's instructions. The RPMI and DMEM (Sigma Aldrich) were supplemented with 10% FBS (fetal bovine serum, Gibco, purchased from Thermo Fisher Scientific, Waltham, Massachusetts, US), 5 mM glutamine, and 50 unit/mL penicillin and streptomycin (Life Technologies, Waltham, Massachusetts, US). Cell cultures were kept in a humidified incubator at 37 °C, and in 5% CO2 atmosphere. Washing steps were executed by Dulbecco's Phosphate Buffered Saline solution (DPBS, without Ca^{2+} and Mg^{2+}, Gibco, purchased from Thermo Fisher Scientific); cells were trypsinized by the required concentration of 10x Trypsin–EDTA solution (5.0 g/L porcine trypsin and 2.0 g/L EDTA·4 Na in 0.9% v/v

sodium chloride solution purchased from Sigma Aldrich). The cell number was counted with either a Bürker counting chamber (Marienfeld Superior - Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) or TC20 Automated Cell Counter (Bio-Rad Laboratories, Budapest, Hungary) using Trypan Blue (Gibco, purchased from Thermo Fisher Scientific).

3.2.3. Evaluation of in vitro toxicity of investigated chelators, complexes and liposomes

The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) viability assays, Sulforhodamine B (SRB) assays and PrestoBlue assays were performed. Clonogenic assay and spheroid generation and treatment were also implemented.

3.2.4. Determination of the in vitro apoptotic effect by flow cytometry (FACS)

The nonspecific cell death and apoptosis were determined by staining with allophycocyanin labeled Annexin-V (APC-Annexin-V, Invitrogen, Thermo Fisher Scientific).

3.2.5. Analysis of ROS by flow cytometry

To assess ROS production of investigated chelators in the presence of Cu(II) ions, an oxidation-sensitive marker, 2’,7’-dichlorodihydrofluorescein diacetate (H_2DCF-DA).

3.3. Multi-element analysis

3.3.1. Total Reflection X-ray fluorescence (TXRF) analysis for the determination of intracellular Cu, Fe and Zn

For the determination of the intracellular Cu, Fe and Zn content of cells, TXRF method was used. Briefly, all determinations were performed on an Atomika 8030C TXRF spectrometer (Atomika Instruments GmbH, Oberschleissheim, Germany). Gallium (Ga) was used as an internal standard. The stock solution of 1000 mg/L Ga was purchased from Merck (Darmstadt, Germany). The Kα lines were used for Cu, Fe, and Zn determinations at 8.047 keV, 6.403 keV, 8.638 keV, respectively.

3.3.2. Sample preparation for determination of intracellular Cu levels

Cells were seeded into 6-well culture plates (106 cells/well) in 2 mL completed media. Cells were incubated overnight and the medium was changed to 2 mL FBS-free medium before the treatment (or to fresh completed media in some experiments). Cells were treated with the different chelators with or without varied metal ions. After a 4-hour-long incubation, cells were harvested with a trypsin–EDTA solution. After 5 minutes incubation the trypsinization was

stopped using the usual media completed by 10% FBS and was transferred to 1.5 mL Eppendorf tubes (VWR International Ltd., Debrecen, Hungary). After centrifugation (300 g, 5 min), cells were washed twice with 1 mL of DPBS. The cell number was counted with either a Bürker counting chamber or a TC20 Automated Cell Counter using Trypan Blue. After the last centrifugation, the DPBS was removed completely and 20 µL of 30% H2O2, 80 µL of 65% HNO_3 and 15 µL of 10 µg/mL Ga were added to the cells and digested for 24hours at room temperature. From the resulting solutions, 2-10 µL was pipetted on the quartz reflectors used for TXRF analysis. Data normalized to counted cell numbers was in good agreement with the determined sulfur content.

3.4. Preparation of Drug-loaded Liposomes

PEGylated liposomes were prepared by lipid film hydration technique using a pH gradient.

Thermosensitive liposomes suitable for hyperthermic treatment were also optimized using different lipid ratios (different weight percentages of DPPC and HSPC), namely HEAT SENS LIPO. The antitumor effect of thermosensitive and non-thermosensitive liposomes, HEAT SENS LIPO and HEAT RES LIPO was compared, in addition to the effect of the hyperthermic treatment was also tested.

To characterize the prepared liposomes, dynamic light scattering (DLS), microfluidic resistive pulse sensing (MRPS), differential scanning calorimetry (DSC), infrared (IR) spectroscopy, ultraviolet–visible (UV-Vis) spectrophotometry and TXRF were used.

3.5. *In vivo* studies

3.5.1. In vivo anti-tumor efficacy of copper and neocuproine-containing liposomes

The $2x10^6$ mouse colon carcinoma (C-26) cells were injected into the left flank of 6-to-9-week-old male BALB/c mice from our specific pathogen-free colony subcutaneously (s.c.) in a volume of 0.2 mL serum-free media. Two weeks after injection (when tumors were detectable), the mice were randomly and evenly divided into groups (10 mice/group). Treatment groups received 10 µL liposome / 1g body weight intravenously (i.v.) on the first and eighth day of the treatment. The concentration of the complex (Cu:neocuproine) was taken as 1 mM, resulting in an amount of 2.8 mg/kg of “active ingredient” calculated for the thermosensitive formulation. The dose of the encapsulated drug for the “thermoresitant” formulation was 2.6 mg/kg. Controls received equivalent volumes of sterile NaCl 0.9%. All animals were included in the analysis. Changes in their body weight were also determined throughout the study. No adverse events were observed during the experiment. The antitumor effects were registered twice a week by

measuring the tumor size with a caliper. The absolute tumor volume was calculated with the formula for an ellipsoid shape (L (length)× W (width)^2 × (π/6). Eighteen days after the first treatment, the mice were euthanized, and the tumors were extracted and dried in an oven to obtain a constant weight. The samples were weighed on an analytical balance (Mettler Toledo XS105, Greifensee, Switzerland). Statistical analysis was performed using Graph Pad Prism 8 software using One-Way ANOVA analyses. Animal experiments were carried out at the Department of Experimental Pharmacology, National Institute of Oncology, Budapest, Hungary and the animal-model experiments were conducted following the standards and procedures approved by the Animal Care and Use Committee of the National Institute of Oncology, Budapest (license number: PEI/001/2574–6/2015). All animal protocols were approved by the Hungarian Animal Health and Animal Welfare Directorate, according to the directives of the European Commission.

3.5.2. Tumor hyperthermia

Tumors were heated to test the temperature dependent release of liposomes in two treated animal groups. Animals were treated under anesthesia with desflurane (9% desflurane in 30% oxygen/air). Local mild hyperthermia (41-42ºC ± 0.5°C) was performed under anesthesia with a custom-made contact heating device based on direct heat conduction using a metal rod connected to a temperature-controlled water bath. Intratumoral temperature was measured with optical sensors (Luxtron FOT Lab Kit, LumaSense Technologies, Inc., Milpitas, CA, US) and kept between 41-42°C (± 0.5°C) for 20 min. The applied temperature for mild hyperthermia is above the phase transition temperature of the thermosensitive liposome (HEAT SENS LIPO). The permeability of the lipid bilayer is known to be enhanced above this temperature, so using hyperthermic treatment causes pronounced drug release.

Table 1. *Investigated chelator-like structures and their chemical nomenclature. Tested compounds include thiosemicarbazone, phenanthroline, 8-hydroxyquinoline, thiocarbazone, dithiocarbamate, biquinoline derivatives. D-penicillamine is used in chelation therapy to remove toxic metals from the body, not because of cytotoxicity, so it was achieved as control chelators.*

Dp44mT	**DpC**	**Triapine**	**APDTC**	**2,2'-biquinoline**
Di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone	Di-2-pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone	3-Aminopyridine-2-carboxaldehyde thiosemicarbazone, 3-AP, PAN-811	Ammonium pyrrolidinedithiocarbamate	2,2′-Diquinolyl, Cuproin
neocuproine	**oxine**	**Q4**	**dithizone**	**D-Penicillamine**
2,9-Dimethyl-1,10-phenanthroline	8-Hydroxyquinoline	5-Chloro-7-((2-fluorobenzylamino)methyl)quinolin-8-ol	Diphenylthiocarbazone	3,3-Dimethyl-D-cysteine

3. Results

4.1. Intracellular Cu content increased under the influence of 50 μM Dp44mT and increased more than 30 times in 8 different cancer cells in the presence of 5 μM Dp44mT and 2 μM $CuSO_4$ solution

The percentage of the intracellular Fe, Cu and Zn concentration of cancer cell lines and PBMC were determined incubated them with growth medium and 50 μM Dp44mT. The Cu accumulation was much higher when the concentration of Cu, Fe and Zn of the pool was increased to 2 μM. In the case of five different cancer cell lines, – colon (HT-29), melanoma (A-375), lung (H358) and ovarian (OVCAR-3 and Caov-3) – the intracellular Cu content increased more than 30-times than in the control, when Dp44mT was added to the culture solution. The increase observed for Cu was at least 10-fold compared to the control growth cell culture for all cancer cell lines investigated. However, it should be mentioned that the intracellular Cu concentration of the PBMC cells also increased approximately 30-fold. A rise in the concentration of copper(II) in the presence of Dp44mT appears to derive a saturation-like curve in terms of intracellular Cu content. Nevertheless, competition between Cu, Fe and Zn was not observed.

4.2. Co(II) decreases Dp44mT mediated Cu accumulation and reduces the toxicity of Dp44mT

Competition studies were conducted considering formation constants for Fe(II), Co(II) and Ni(II) and Dp44mT. The Cu(II) concentration in the culture cell was fixed at 2 μM and the concentration of Fe(II), Co(II) or Ni(II) was increased to 5 μM, 25 μM and, finally, to 100 μM, each prior to the addition of Dp44mT at a concentration of 5 μM. Thereafter, the intracellular elemental concentration was determined by means of TXRF. The intracellular content of Cu was considered to be 100% and the Cu concentrations applied in the subsequent experiments conducted with the increased Fe(II), Co(II) and Ni(II) treatments were related to this value. Considering as an important result: in the case of 100 μM Co(II), the intracellular Cu content decreased to the levels of control cells in spite of the presence of 5 μM Dp44mT.

Performing an MTT test, based on the previous metal uptake result, the viability of HT-29 cells was very low in the presence of 5 μM Dp44mT, even if Fe(II) was added at an increasing concentration, e.g., up to 100 μM. By elevating the Fe(II) concentration of the culture medium to 200 μM, the viability hardly exceeded that of the control. Addition of Ni(II) in the concentration range of 6.25 μM to 50 μM steadily increased the viability of HT-29 cell lines to about 50 % compared to the control level. However, above 50 μM Ni(II), no more positive

effect was observed. Among the investigated metal ions, Co(II) considerably inhibited the toxicity of Dp44mT, even if it was applied at low concentrations, i.e., 6.25 μM. When the experiment was repeated by adding 2 μM Cu(II) to the culture medium, neither Ni(II) nor Fe(II) could change the cell viability in the applied concentration range, while at least 25 μM Co(II) is required in order to increase the viability.

4.3. The uptake and cytotoxicity of Cu mediated by Dp44mT depends on extracellular Cu(II) concentration

The Cu content of cells as a function of increasing treatment with Cu(II) from 0.1 μM to 100 μM and 5 μM chelator concentration after 4 hours was measured. The Cu content of cells showed an increase up to 20 μM on treatment with solutions of increasing Cu(II) concentration. Above this concentration, the Cu content of the cells increased negligibly, and it seemed to reach a saturation plateau. The Cu content in the control sample was 4.5 ng/10^6 cells. Therefore, an extremely high increase of 20,000% was observed as compared to the control in the case of the treatment with 20-100 μM Cu(II). According to MTT assays, increasing the Cu(II) concentration from 0.78 μM to 100 μM, cell viability drastically decreased from about 70% to 5% when Dp44mT was applied at a concentration of 0.0625 μM.

4.4. Low concentration (0.1 μM) of Dp44mT can deliver high amounts of copper inside the cells

Cu accumulation far exceeded the base level of 0.1 μM chelator which is the maximum amount of copper that a chelator can transport. The question arises of whether either the ligands or Cu is accumulated in the cells. In order to decide this, low ligand concentration (0.1 μM) and relatively high Cu(II) concentrations (2-10 μM) were applied. It could be calculated that the chelator in 0.1 μM concentration would allow only 12.7 ng Cu in the cells in 2 mL cell media if Cu accumulated in the form of complex species. We demonstrated that Cu levels were far exceeding this base level in the cells. Thus, the chelator is responsible for the intracellular Cu accumulation. In the case of effective chelators (Dp44mT, neocuproine, APDTC), accumulation of Cu in the presence of low chelator concentration (0.1 μM) is observed in all tested cell lines.

4.5. Detailed *in vitro* cytotoxicity, intracellular copper uptake and depletion data in the case of Dp44mT, neocuproine, APDTC, oxine, 2,2'-biquinoline and dithizone chelators are presented. Moreover, cellular localization of copper is shown using Dp44mT and oxine

2 μM of Cu(II) alone did not affect the cell viability, even for five days. Dp44mT was the most efficient chelating agent. For the remainder of the chelating agents, the following toxicity order could be established: Dp44mT > neocuproine > APDTC > oxine > 2,2´-biquinoline > dithizone. D-penicillamine did not show any toxicity.

The IC_{50} values decreased drastically in the presence of Cu(II). In the present study, the difference in the IC_{50} values for the investigated ligands could even be of two orders of magnitude. However, the cytotoxicity order was not altered by Cu(II) addition. Thus, the increase in cytotoxicity of Dp44mT was 4.8-fold. For the other Cu chelating agents, the increased cytotoxicity ranged between 4.2 (oxine) and 13.9 (neocuproine).

About metal transport cababilities of the investigated chelators can be stated:

- Accumulation of Cu can be observed for all tested chelators, closely related to the amount of available external copper
- Cu accumulation rate could be about 2,000% compared to the control
- Cu accumulation was far exceeding the base level of 0.1 μM chelator the maximum amount of copper that a chelator can enter
- Cu accumulation arise similar values in the presence of a reducing agent (ascorbic acid).

4.6. Copper localization and its colocalization with Zn were determined by μ-XRF imaging. Colocalization of Cu and Zn in the nucleus of HT-29 cells was observed for Dp44mT (correlation coefficient as high as 0.85)

Colocalization of Cu and Zn was observed in several cases measured by Micro X-ray fluorescence spectroscopy and pixel-by-pixel by Pearson correlation setting in the case of Dp44mT chelator and Cu(II) ions.

4.7. Cu uptake with respect to different chelators has been shown to be a relatively universal process and that among all possible divalent metals, only cadmium will accumulate in the same way

No metal accumulation was measured for Co, Ni and Hg, unlikely some Pb, Zn and Fe accumulation was observed.

4.8. Compounds with similar intracellular Cu uptake have been shown to induce very different values of cytotoxicity

I have proved that compounds with different toxicity induced similar intracellular Cu concentrations. The Dp44mT, neocuproine and APDTC are each capable of forming 1–1 metal to ligand complexes of similar stability. Therefore, these ligands can accumulate Cu in the same way. However, considerable differences could be observed in terms of their toxicity.

4.9. The Cu uptake results in a cell type dependent intracellular Zn depletion, which is in correlation with the toxicity of the chelator

Intracellular Zn content slightly decreased for MCF-7 cells, while dramatically decreasing for MDA-MB-231. The same trends can be observed on HT-29 and HCT-116 cell line pair Simultaneously, with Cu uptake, another reproducible phenomenon could be observed, namely intracellular Zn depletion. In contrast, all other detectable element (e.g., Ca, K, Fe) levels were not altered substantially. Interestingly, the extent of Zn depletion was cell line dependent. Intracellular Zn content slightly decreased for MCF-7 cells, while a dramatic decrease was observed for MDA-MB-231 cells. This effect was slightly dependent on the type of the applied chelator. A similar decrease was observed for HCT-116, while Zn depletion for HT-29 corresponded to an intermediate level.

4.10. The Cu(II) concentration-dependent, fast apoptosis could be detected with the application of Dp44mT and oxine chelators

The number of apoptotic cells was low and comparable with that of the control and 0.5 μM Cu(II) when 5 μM Dp44mT and 5 μM oxine as representatives of stable coordination compounds of 1:1 and 1:2 Cu(II)-to-ligand molar ratio respectively were incubated for 20 min with 0.5 μM Cu(II) solution. The number of apoptotic cells increased dramatically with a 10- and 100-fold increase of the added Cu(II) concentration. Interestingly, the ratio of the apoptotic cells was very similar for these two representatives. However, the fluorescent signals characterizing apoptosis increased gradually with the increment of the Cu(II) concentration for oxine, while the plateau was achieved already at a combined 5 μM Cu(II) and 5 μM Dp44mT treatment.

4.11. The chelating agents – except for neocuproine – restored the DNA damage induced by free Cu(II)

When Cu was added to DNA and then titration was performed with each chelating agent, change in the CD spectra of DNA indicating intercalation was observed only for the Cu(II) and neocuproine system. In this case, the intensity increase of the positive band was significant. A strong intercalation was observed when the Cu(II):ligand molar ratio was 1:2. It can be supposed that this coordination compound has a planar structure. Next to the maximum value (at 283 nm), a shoulder appeared in the spectra at 300 nm, presumably due to an extended conjugation causing chiral disturbance. The maximum value of the negative band at 246 nm suffered a bathochromic shift of about 3 nm.

4.12. The feasibility of using a liposome preparation containing a copper nanotoxin and excess of copper ion in a water-soluble form for tumor targeting has been demonstrated. I have developed liposomal formulation of copper-neocuproine complex HEAT SENS LIPO as thermosensitive and HEAT RES LIPO as „thermoresistant", which are suitable for *in vivo* application

Both formulations resulted monodisperse liposomes, measured by DLS and MRPS measurements, suitable for in vivo administration. Thermosensitive PEGylated liposomes were prepared with different molar ratios of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) and hydrogenated soybean phosphatidylcholine (HSPC) in the presence of copper(II) ions. Optimal, temperature dependent drug release was obtained at 70:30 DPPC to HSPC weight ratio. Neocuproine (applied at 0.2 mol to 1 mol phospholipid) was encapsulated through a pH gradient while using unbuffered solution at pH 4.5 inside the liposomes, and 100 mM HEPES buffer pH 7.8 outside the liposomes. Copper ions were present in excess, yielding 0.5 mM copper-(neocuproine)$_2$ complex and 0.5 mM free copper.

4.13. I have shown that both the thermosensitive and the "thermoresistant" formulations induce intracellular copper accumulation and related in vitro cytotoxic effects. In vivo antitumor activity could be detected with both liposomes, but the effect of HEAT SENS LIPO was found to be more pronounced. Mild hyperthermia treatment combined with the HEAT SENS LIPO formulation allowed the reduction of the applied dose

After 24-hour-long treatments, both of the liposomal formulations were more toxic than neocuproine, but less toxic than neocuproine-copper complexes. TXRF measurements indicate that HEAT SENS LIPO transmits copper more efficiently than the HEAT RES LIPO without using mild hyperthermia. *In vivo* administration of the liposomal formulations was designed to achieve the maximum dose of neocuproine-copper(II) complex (2.8 mg/kg), which was still tolerable for the studied animals. In the first experiment, the efficacy of HEAT SENS LIPO and HEAT RES LIPO was compared without applying heat treatments. Treatments with the liposomes showed a profound antitumor effect, reaching significance (p=0.046) in the HEAT SENS LIPO group. The antitumor effect was detectable in the weight of the dried tumor in both of the treated groups, in terms of 65% and 50% tumor mass reduction in the HEAT SENS LIPO and HEAT RES LIPO groups, respectively. In the second experiment, our aim was to investigate whether using a smaller dose of thermosensitive liposome formulation combined with local heating of the tumor could show appropriate antitumor effects. Hence, the thermosensitive liposome formulation (HEAT SENS LIPO) was administered in parallel with mild heat treatment in two doses (2.8 mg/kg and 1.4 mg/kg) and compared to the group receiving higher dose treatment without mild heating. The mild hyperthermia by itself does not show any antitumor effect (data not shown here). All three treated groups showed reduced tumor growth as compared to the control group. Surprisingly, the strongest antitumor effect was observed as a result of treatment with 1.4 mg/kg liposome combined with mild heat treatment (p=0.0145). The imaging (PET/CT) results suggest efficient tumor uptake of the drug; however, further studies are required to accurately determine the amount of released free or neocuproine-bound copper in the circulation and how it can be affected by means of hyperthermic treatment.

5. Conclusions

Since the initial clinical success of platinum-containing anticancer compounds, considerable attention has been paid to the research of various transition metal-containing complexes and chelator-like structures. Many of these compounds have also been the subjects of clinical trials. During my doctoral research, I investigated antitumor chelators, including thiosemicarbazone, thiocarbazone, thiocarbamate, quinoline, and phenanthroline structures. I have shown that the investigated chelators cause high cellular accumulation of copper, even at low chelator concentrations. They are able to accumulate multiples of their own amounts in the presence of free copper, and the copper content appears in the nuclei in a diffuse manner. The amount of accumulated copper within the cell can be up to thirty times higher than that of the control. I have also demonstrated that there is no strong correlation between copper accumulation and toxicity alone. I observed that, for more potent compounds, copper content could not be removed from cells, and zinc depletion could also be observed. I have demonstrated that Dp44mT, which is known as an iron chelator, also causes copper accumulation in the presence of copper, which can be suspended with cobalt ions. The localization of copper in the case of Dp44mT shows significant colocalization with zinc. I have shown that their cytotoxicity is greatly increased in the presence of free copper(II) and fast (20 min) copper-dependent apoptosis can be detected. I have pointed out that the *in vitro* cytotoxic effect does not depend on the cell line type; they are similarly toxic in several different tumor cell lines and also in resistant tumor models. I have shown that when examining toxic metals, a process similar to copper accumulation occurs only in the case of cadmium, but also to a much lesser extent. Many antitumor complex compounds and chelators do not have appropriate pharmacological parameters for successful *in vivo* applicability. Liposomal formulations have shown several advantages in terms of the *in vivo* applicability of chemotherapeutic agents. I have developed the preparation method of liposomes containing copper ionophore (neocuproine). I have recognized the formulation of a thermosensitive liposome for the purpose of targeted drug release (combination with hyperthermia treatment). Liposomes containing neocuproine Cu(II) were shown to be effective *in vivo* (BALB/c C-26 colon adenocarcinoma) in a mouse tumor model, with significant tumor growth inhibition observed in all cases. Hyperthermia treatment with the use of thermosensitive liposomes allowed dose reduction.

6. Publication list

6.1. Publications related to the thesis

Gaál A, Orgován G, Polgári Z, Réti A, Mihucz VG, Bősze, S, Szoboszlai N, Streli C (2014) Complex forming competition and *in-vitro* toxicity studies on the applicability of di-2-pyridylketone-4, 4,-dimethyl-3-thiosemicarbazone (Dp44mT) as a metal chelator. Journal of Inorganic Biochemistry, 130: 52–58. IF: 3.444

Gaál A, Mihucz VG, Bősze S, Szabó I, Baranyi M, Horváth P, Streli C, Szoboszlai N (2018) Comparative *in vitro* investigation of anticancer copper chelating agents. Microchemical Journal, 136: 227–235. IF: 3.206

Gaál A, Orgován G, Mihucz VG, Pape I, Ingerle D, Streli C, Szoboszlai N (2018) Metal transport capabilities of anticancer copper chelators. Journal of Trace Elements in Medicine and Biology, 47: 79–88. IF: 2.895

Gaál A, Garay TM, Horváth I, Máthé D, Szöllősi D, Veres DS, Mbuotidem J, Kovács T, Tóvári J, Bergmann R, Streli C, Szakács G, Mihály J, Varga Z, Szoboszlai N (2020) Development and *in vivo* application of a water-soluble anticancer copper ionophore system using a temperature-sensitive liposome formulation. Pharmaceutics, 12: 466. IF: 4.773

6.2. Further publications in peer-review journals not related to the thesis

Majer Z, Bősze S, Szabó I, Mihucz VG, **Gaál A**, Szilvágyi G, Pepponi G, Meirer F, Wobrauschek P, Szoboszlai N, Ingerle D, Streli C (2015) Study of dinuclear Rh(II) complexes of phenylalanine derivatives as potential anticancer agents by using X-ray fluorescence and X-ray absorption. Microchemical Journal, 120: 51–57. IF: 2.893

Cserepes M, Türk D, Tóth S, Pape VFS, **Gaál A**, Gera M, Szabó JE, Kucsma N, Várady G, Vértessy BG, Streli C, Szabó PT, Tovari J, Szoboszlai N, Szakács G (2020) Unshielding Multidrug Resistant Cancer through Selective Iron Depletion of P-Glycoprotein-Expressing Cells. Cancer Research, 80: 663–674. IF: 8.378