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Analysis of the effect of transition metal complexes on tumor cell lines

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Abstract

The current knowledge connected with biological activity of complexes of transition metals was summarized in the theoretical part. In the experimental part, the results of seven mentioned expert publications concerning with determination of cytostatic activity of newly prepared compounds of vanadium, molybdenum and platinum, with study of mechanism of effect of chosen complexes and with study of possible use of graphene structures for aimed transport of drug were summarized and discussed. Obtained results confirmed high cytotoxic activity against wide spectrum of cancer lines, including line resistant against impact of *cis*-platin, and also high cytotoxicity of tested nanoparticles to non-cancer cells.

Abstrakt

V teoretické části byly shrnuty aktuální poznatky spojené s biologickou aktivitou komplexů přechodných kovů. V experimentální části byly sumarizovány a diskutovány výsledky sedmi uvedených odborných publikací, zabývajících se stanovením cytostatické aktivity nově připravených sloučenin vanadu, molybdenu a platiny, studiem mechanizmu účinku vybraných komplexů a možností využití grafenových struktur pro cílený transport léčiv. Získané výsledky potvrdily vysokou cytotoxickou aktivitu proti širokému spektru nádorových linií, včetně linie rezistentních vůči působení *cis*-platiny, a vysokou cytotoxicitu testovaných nanočástic k nenádorovým buňkám.

Keywords

transition metals, metallocenes, cytotoxicity, apoptosis, mechanism of action, graphene

Klíčová slova

přechodné kovy, metaloceny, cytotoxicita, apoptóza, mechanizmus účinku, grafen

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Introduction

Despite the continuous decline in associated mortality rates since the early 1990s, cancer remains one of the leading causes of death worldwide[1]. Chemotherapy represents the principal form of cancer treatment, together with surgical removal and radiotherapy[2].

The use of transition metal complexes as chemotherapeutics in oncology has increased since the discovery of the cytotoxic activity of *cis*-diamminedichloroplatinum (*cis*-platin) by Rosenberg et al. in 1965[3]. In the seventies and eighties, platinum complex became very popular antineoplastic agents with high efficiency against human testicular, ovarian, bladder, head and neck carcinomas. However, toxic side effects such as nephrotoxicity and myelotoxicity are major drawbacks of this complex for clinical applications[4-6].

The development of new metallotherapeutics continues in two directions. Based on identical geometric parameters with *cis*-platin, novel modifications of *cis*-platin are constantly researched in effort to minimize development of resistance and undesirable side effects of currently used derivatives while maintaining potent antitumor activity. However, although the thousands of compounds were tested, only a few platinum complexes have been successfully used clinically (carboplatin, oxaliplatin …)[7,8]. The other direction are non-platinum complexes from IV, V and VI group of periodic table. Use of the modification of the ligand sphere of transition metal complexes can have notable effects on important pharmacological characteristics, such as solubility, stability and cytotoxicity[9-12].

Presented Doctoral Thesis is aiming to determine cytotoxic activity of newly prepared compounds of vanadium, molybdenum, platinum with bidendate bonded ligands and to describe mechanism of their cytotoxic effect.

1. Goals of the thesis

- Summarize current knowledge about cytotoxic effect and mechanism of effect of complexes of transitional metals (V, Mo, Pt) on cancer lines.

- Evaluate impact of presence of different central metals and different ligands on cytotoxic activity based on results of WST-1 assay of newly prepared complexes.

- Study biological effect for selected compounds leading to sketch mechanism of effect for this type of complexes. Use suitable spectral methods, methods of flow cytometry, qRT-PCR and western blot method with imunoanalytics detection.

- Compare obtained results with own or published results for *cis*-platin or newly prepared platinum complexes.

2. Methods

Rough description of methods is written bellow. Detailed description is present in publication.

2.1. WST-1 assay

The cells were seeded into 96-wells/plate (TPP, Poland). The cells were cultivated overnight and then was added 100 μL solution of tested complex. The final concentrations were in ranging from 0.1 to 50 μM. The cytotoxicity expressed as a half of inhibition concentration, IC_{50} value, was determined after incubation time using WST-1 assay (SigmaAldrich, St. Lous, USA). The IC_{50} values for each cell line were calculated and expressed as mean ± standard deviation.

2.2. Cell viability and proliferation

The cells were seeded at a concentration 1 x 10^6 cells in 5 mL culture medium into culture flash (TPP, Poland), incubated overnight and treated with tested complex in final tested concentrations. The incubation time was 24, 48 and 72 hours. The proliferation activity and viability of cells were determined using couting in Bürker chamber after coloring with Trypan Blue (Sigma-Aldrich, USA). The result are expressed as mean ± standard deviation from three independent biological replicates.

2.3. Activity of caspases

The cells were prepared in amount 1 x 10^6 cells in 5 mL culture medium in culture flash (TPP, Poland), incubated overnight and then was added tested complex in final tested concentrations. The incubation lasted 48 hours. The activity of caspases was measured after generation of luminescent signal using commercial kit CaspasesGlo3/7, CaspasesGlo8 a CaspasesGlo9 (Promega, Madison, Wisconsin, USA). The result are expressed as mean ± standard deviation from three independent biological replicates.

2.4. Distribution of cell cycle

Cell cycle was measured using flow cytometer CyAn flow (Beckman Coulter, Miami, FL, USA) with an excitation wavelength of 488 nm. The 1 x 106 cells in 5 mL culture medium were treated tested complex in final tested concentrations during incubation time. The cells were harvested, washed in phosphate buffer and fixed in 70% ethanol for at least 48 hours. It was used Vindel solution for detection of low molecular fragments. The data were analyzed using Multicycle AV software (Phoenix Flow Systems, San Diego, CA, USA). The results were determined from three independent experiments as mean ± standard deviation.

2.5. RNA izolation, qRT-PCR

The cells were seeded in concentration 1 x 10^6 cells in 5 mL culture medium, incubated overnight and followed by treatment with tested complex in final tested concentrations for incubation time. The total amount of RNA was extracted using

RNeasy Mini Kit according manufacture´s instruction (Qiagen, Hilden, Germany). RNA in amount 1 μg was reversibly transcribed using cDNA Reverse Transcription Kit and quantified with TagMan Gene Expresion Assays. ID numbers used in this study were (CCNB1 Hs01030097_m1, MDM2 Hs00234753_m1, CDKN1A Hs00355782_m1 and p53(TP53) Hs_00153349_m1). Relative expression was measured using Quantstudio 6 RealTime PCR system (all from Applied Biosystems, CA). The amount RNA was calculated using comparative Ct method (ΔΔCt method) and expressed as mean ± standard deviation from three independent experiments.

2.6. Western blot analyses

The 5 x 10^6 cells in 10 mL culture medium cells were harvested after incubation with tested complex in final tested concentrations after incubation time. As positive control was used *cis*-platin or doxorubicine. The cells were washed in phosphate buffer and the proteins were extracted using Cell lysis buffer (Cell Signaling Technology, Danvers, USA). The total amount of proteins were determined using Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, St. Louis, USA). Western blot analysis was proved according to standard protocol. The result are expressed as representative blot from three independent experiments. β-actin was determined as control of the same amount of loading.

3. Results and discussion

Already from nineties a group of chemists works on Department of General and Inorganic Chemistry, University of Pardubice. This group concerns with prepare of organometallic compounds with catalytic and currently biological activity. Their first article describing the microbiological effect of bent metallocene compounds on Escherichia coli[13] cells was published in 2005. Other expert publications and works were made yet in cooperation with Department of Medical Biochemistry, Charles University in Hradec Králové. My active work in this significant group started in 2010. My focus was to bring in the standard methods and evaluation of the cytotoxic effect of prepared complexes with the WST-1 assay. In my Bachelor's and subsequently my Master's thesis, I tested and evaluated more than 100 samples. Obtained and published results[14-20] became a base for the following research and the assignment of this Doctoral thesis. Doctoral thesis is therefore focused on purposely prepared and until now not yet described complexes of vanadium, molybdenum and platinum. The cytostatic activity of these complexes was determined. For the most perspective complexes, the mechanism of the effect on selected cell lines was studied. The attention was aimed also to the possible use of carbon nanostructures for transport of these complexes into the organism.

3.1. Determination of cytotoxicity of complexes use WST-1 assay

Based on previous results, the attention for bent metallocenes was aimed towards the study of vanadocene complexes stabilized with N,N- or S,S-chelate ligands, containing non-substituted or methyl-substituted cyclopentadienyl (Cp) ligand. New results showed that substitution on cyclopentadienyl ring on complexes of vanadium (IV) (Figure 1) does not have any significant impact on cytostatic activity of this type of complexes. Contrarily, the presence of the chelated ligand and its type have a fundamental effect.

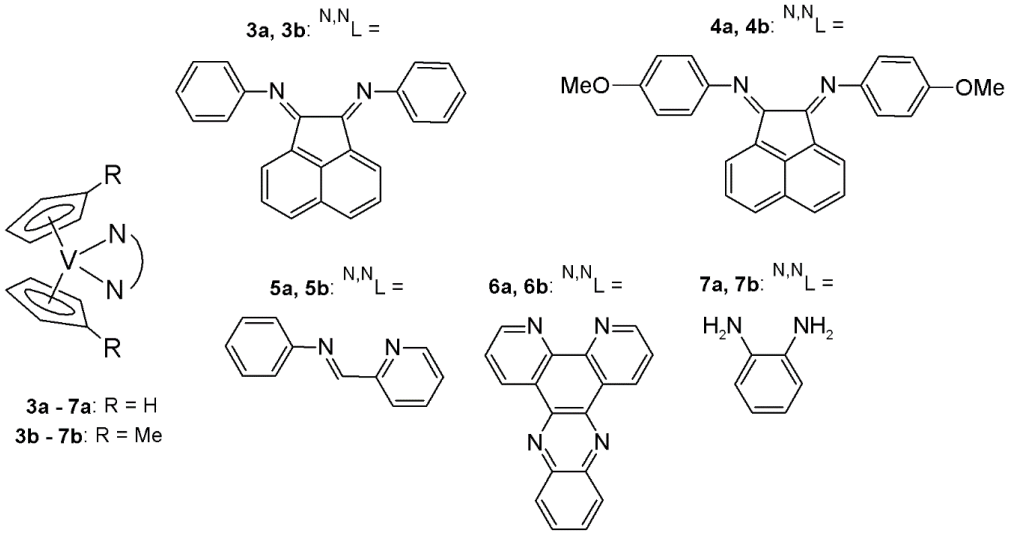


Figure 1 - Structure of tested vanadocene, where R is a substituent, L is N,N-chelate.

Complexes **3a – 7a** and **3b** – **7b** were tested with primary screening on panel of cells containing different cancer lines. After one-time dose of 10 μM was found out that complexes containing 1,2-bis(phenylimino)acenaphtene (bian) (**3a**, **3b**) or 4-MeO-bian (**4a**, **4b**) have significantly higher cytotoxic effect than complexes carrying (E)-N-((pyridin-2-yl)methylen)benzenamine (**5a**, **5b**), dipyridol[3,2-a:2',3'-c]phenazine (**6a**, **6b**) or benzen-1,2-diamine (**7a**, **7b**) after 48 h incubation. The cytotoxicity effect of the tested complexes on the cell lines is also dependent on the origin of the cell line. The complexes **3a**, **3b**, **4a** and **4b** evince a very good inhibitory effect on epithelial cells HeLa and A549 or cells Saos2. However, studying these complexes, we observe also a significant cytotoxic effect on the human breast cancer MCF-7, ovarian cancer A2780 and pancreatic cancer PANC-1 cell lines. The complexes with ligand pyma (**5a**, **5b**) are the least sensitive ones to the cell lines. To investigate the biological activity, the complexes **3b** and **4b**, which inhibited growth of all tested cancer cell lines, were selected. Collectively, most efficient inhibition was observed in A549 cells of non-small cell lung cancer. Thus, for this cell line, the dose dependency was evaluated and the value of 50% inhibition concentration (IC_{50}) was calculated from the independent measured results. In the A549 cells, the determined IC_{50} was 3.3 ± 0.6 μM after 24 h for and 2.9 ± 0.2 μM after 48 h for **3b**, 2.1 ± 0.2 μM after 24 h and 1.6 ± 0.1 μM after 48 h for **4b**. Value IC_{50} was determined also for *cis*-platin, and that was for *cis*-platin to 43.5 ± 1.5 μM after 24 h and 24.0 ± 1.9 μM after 48 h. Result show that cytotoxicity of tested complexes is not significantly influenced by time of effect on A549 cells and that tested complexes have several times higher activity than *cis*-platin. According to obtained

results was proven that cytotoxicity of *cis*-platin on A549 cells is dependent on incubation time, where IC_{50} value after 48 h decreases down to one half.

Next tested group of vanadocene complexes is series of bis(cyclopentadienyl) complexes V(IV) with different dithiocarbamate ligand (Figure 2).

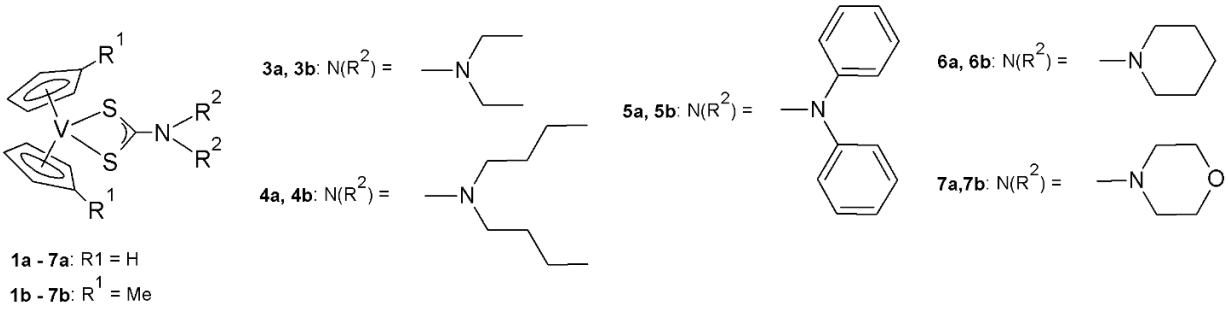
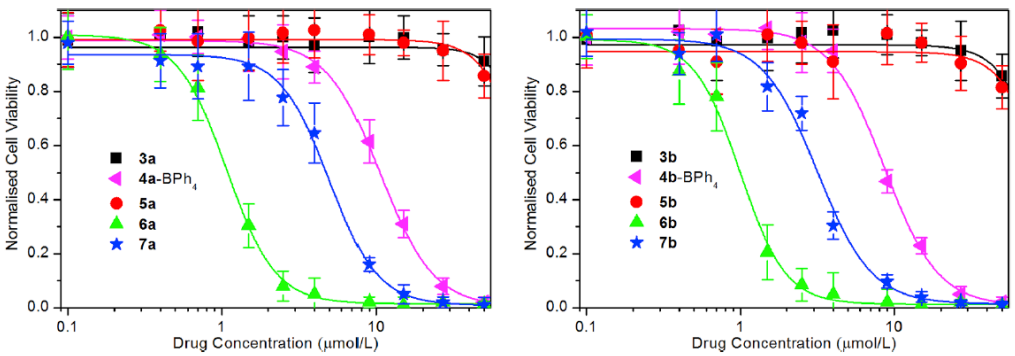


Figure 2 – Structure of bis(cyclopentadienyl) complexes of vanadium(IV) with dithiocarbamate ligands.

Test on cytotoxic effect (Graph 1) studied on human cells MOLT-4 after 24 h showed that the effect is dependent on type of ligand. IC_{50} values of tested complexes occur in wide range from 0.9 μM up to more than maximal test limit 50 μM similar to vanadocene complexes with N,N-chelates[14,17,21]. On the other hand, effect of substitution with methyl group on cyclopentadienyl ring is negligible and differences in values of cytotoxicity are minimal. Very high cytotoxic effect was observed at complexes of dithiocarbamate with piperidine ring **6a** (IC_{50} = 1.0 ± 0.1 μM) and **6b** (IC_{50} = 1.0 ± 0.1 μM). They are 15 times more active than *cis*-platin (15.8 ± 1.9 μM) on MOLT-4 cells[22]. High activity was observed also at derivatives carrying morpholine ring **7a** (IC_{50} = 5.1 ± 0.4 μM) and **7b** (IC_{50} = 3.2 ± 0.3 μM) and at dibutyldithiocarbamate complex **4a-BPh$_4$** (IC_{50} = 10.4 ± 0.5 μM) a **4b-BPh$_4$** (IC_{50} = 8.9 ± 0.7 μM). Remaining complexes **3a** and **3b** with ethyl and **5a** and **5b** with phenyl groups prove IC_{50} values higher than 50 μM.

In general, activity of vanadocene complexes containing S,S-ligands is similar to N,N-chelate ligands[14]. Therefore, this type of compounds is suitable for further biological study.



Graph 1 - Cytotoxic effect of tested vanadocene complexes with dithiocarbamate ligands on the MOLT-4 cells. Determined using WST-1 assay 24 h after treatment. Results are the mean values ± SD of three independent replications.

Next series of tested complexes were half-sandwich complexes of molybdenum. This group of complexes is not so well studied in the expert literature. The series of compounds containing molybdenum(II) as a central atom was purposely prepared, these compounds contained two carbonyl ligands in basal plane and two other monodentate ligands or one N,N-chelate ligand. The position in the peak of the deformed pyramid is occupied by η^5-bonded cyclopentadienyl or indenyl ligand.

Cyclopentadienyl complexes **15 - 20** and indenyl complexes **21 - 23** substituted with amino group were tested (Figure 3).

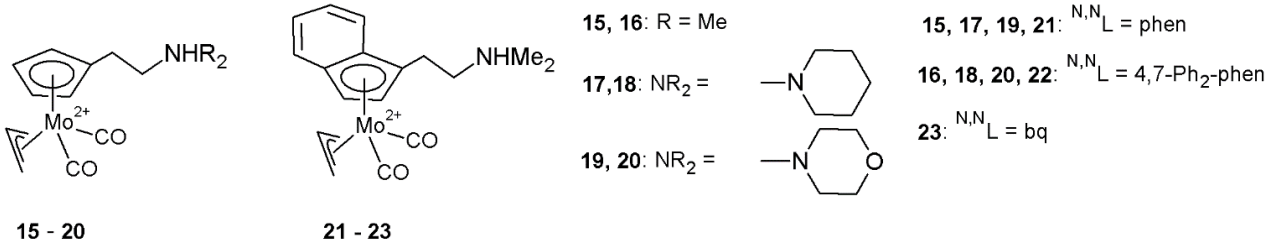


Figure 1 - Structure of half-sandwich indenyl and cyclopentadienyl complex substituted with amino group. (phen is 1,10-phenanthroline, 4,7-Ph$_2$-phen is 4,7-phenyl-1,10-phenantroline).

For all tested groups containing N,N-chelate ligand on MOLT-4 cells and HL-60 was discovered (Table 1) that their activity is about 10 times higher than *cis*-platin. The results show that there is not big difference in activity of cyclopentadienyl or indenyl compounds. Additionally, the results indicate that modification of cyclopentadienyl ring with more hydrophilic groups lead in this case to more noticeable increase of cytotoxic effect both for substituted cyclopentadienyl complexes **15**–**20** against non-substituted cyclopentadienyl complex $[(\eta^5\text{-}C_5H_4)Mo(CO)_2(phen)][BF_4]$ [IC_{50} (MOLT-4) = 19.9 ± 0.7 μM][13], and substituted indenyl complexes **21**–**23** against non-substituted indenyl complex $[(\eta^5\text{-}C_9H_6)Mo(CO)_2(phen)][BF_4]$ [IC_{50} (MOLT-4) = 4.9 ± 0.7 μM][15].

*Table 1 – IC_{50} value for complexes of molybdenum substituted with amino group **15** – **23** determined in the MOLT-4 and HL-60 cells after 24 h of treatment using WST-1 assay. Results shown are mean values ± SD of three independent replications. IC_{50} for cis-platin (DDP) was published[14].*

	MOLT-4	HL-60		MOLT-4	HL-60
15	2.4 ± 0.1	6.9 ± 0.7	**20**	2.2 ± 0.1	2.3 ± 0.3
16	6.6 ± 0.6	8.3 ± 0.5	**21**	2.0 ± 0.1	6.1 ± 0.9
17	3.7 ± 0.7	6.1 ± 0.5	**22**	1.4 ± 0.1	1.5 ± 0.2
18	1.2 ± 0.1	3.4 ± 0.2	**23**	1.2 ± 0.1	2.6 ± 0.3
19	4.6 ± 0.5	5.4 ± 0.4	DDP[b]	15.8 ± 1.9	11.3 ± 2.5

Cytostatic activity was increased also by substitution with thiophene group. The cytotoxicity of prepared series of compounds **3** - **7** (Figure 4) was firstly tested on standardly used cells MOLT-4.

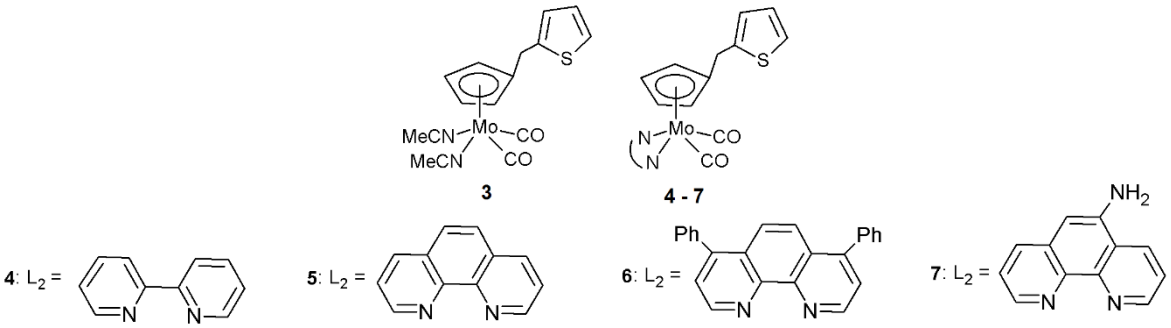
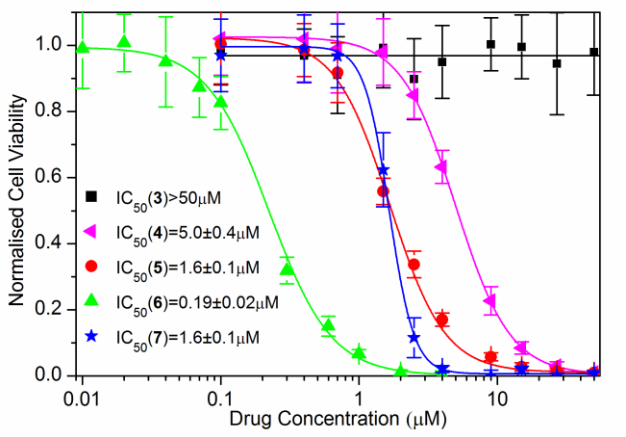


Figure 2 - Structure of half-sandwich indenyl complex substituted thiophene group.

The results of cytotoxic determination (Graph 2) showed that cytotoxic effect of all N,N-chelate complexes **4**-**7** significantly increase (IC_{50} = 0.19 to 5.0 μM) against initial non-chelate compound **3** (IC_{50} > 50 μM). The highest activity was found at complex **6** carrying 4,7-Ph_2phen (IC_{50} = 0.19 ± 0.02 μM) that's about 100 times less than for *cis*-platin[14].



*Graph 2 - Cytotoxicity effect of indenyl complexes substituted with thiophene group **3** - **7** determined in the MOLT-4 cells after 24 h of treatment using WST-1 assay. Results shown are mean values ± SD of three independent replications.*

The activity of complex **6** was in second step tested in concentrations 0.1, 2, and 5 μM on six different human tumor cell lines (MOLT-4, A2780, A549, HT-29 and A2780cis) and non-tumor cell lines MRC-5. It was discovered that complex **6** has selective effect to cancer cells; the highest one to cells of lung adenocarcinoma A549 meanwhile non-cancer cells of lung fibroblasts MRC-5 are less sensitive to complex **6**.

Next tested group were monocyclopentadienyl complexes of molybdenum substituted with carboxyl group (Figure 5). Substitution with carboxyl group should ensure increase of solubility in application and therapeutic environment. They were tested on cell lines MOLT-4. Complexes containing N,N-ligands 4,5-diazafluoren-9-

one (**21**) and 2,2'-bichinolin (**22**) didn't prove cytostatic activity in observed range of concentrations and with IC_{50} > 50 μM were evaluated as ineffective. This result is unexpected especially for ligand bq that was one of the most effective in case of complex $[\{\eta^5\text{-}C_9H_6CH_2CH_2NHMe_2\}Mo(CO)_2(bq)][BF_4]$[22].

The most effective complex of this series became complex **19** containing non-substituted phenanthroline $[(\eta^5\text{-}C_5H_4COOH)Mo(CO)_2(phen)][BF_4]$ with value IC_{50} = 10.5 ± 0.5 μM. Established value is lower than that for analogical complex containing non-substituted cyclopentadiene $[(\eta^5\text{-}C_5H_5)Mo(CO)_2(phen)][BF_4]$ (IC_{50} = 19.9 ± 0.7 μM)[15], yet roughly 10 times more than in case of the most effective complexes substituted with tertiary amine[22].

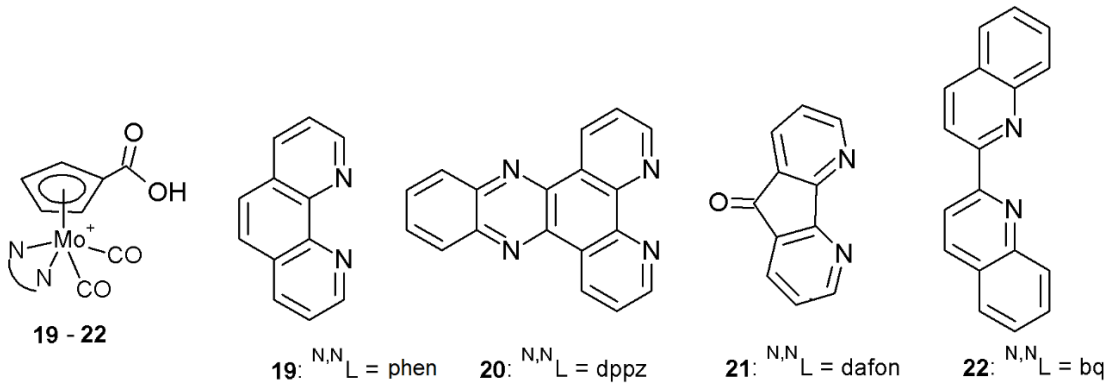


Figure 3 – Structure of half-sandwich cyclopentadienyl complex substituted with carboxylic group.

It was discovered in cytotoxic study that definitely positive impact on biological activity hat implementation of N,N-chelate ligand. Activity increased more than 50 times in most cases compared to non-chelate analog. 7 types of N,N-ligands were tested. High activity showed especially phenanthroline complexes containing amine IC_{50} (MOLT-4) ~ 1.0 μM and 4,7-phenyl$_2$-phenanthroline IC_{50} (MOLT-4) ~ 1.0 μM. These structural types were chosen for further advanced studies related with revealing of mechanism of effect.

The possibility of comparison of methods contributing to describe the mechanism of effect led us to use of newly prepared platinum complexes to this study (Figure 6). Tested pseudotetrahedral complexes containing two sterically demanding PNH ligands are structural analogs of *cis*-platin. The complex chosen for further study proved high cytostatic activity IC_{50} (A2780) = 0.9 ± 0.1 μM and for A2780cis was IC_{50} = 1.8 ± 0.7 μM. Cytotoxic effectivity on non-canter cell line MRC-5 is 23.9 ± 1.9 μM (Graph 3).

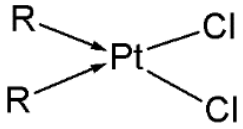
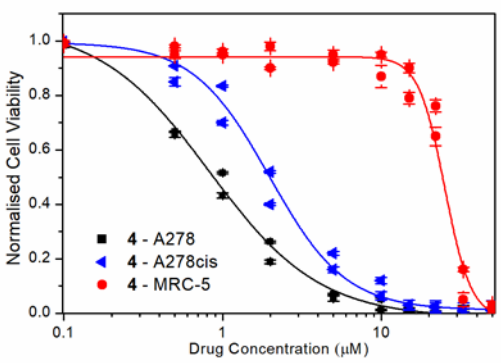


Figure 4 - Structure of newly prepared platinum complexes, where R is phosphine ligand.



Graph 3 - Cytotoxicity effect of platinum *complexes **4** on the A2780, A2780cis and MRC-5 cells after 48 h of treatment using WST-1 assay. Results shown are mean values ± SD of three independent replications.*

3.2. Analyses of mechanism action of selected complexes

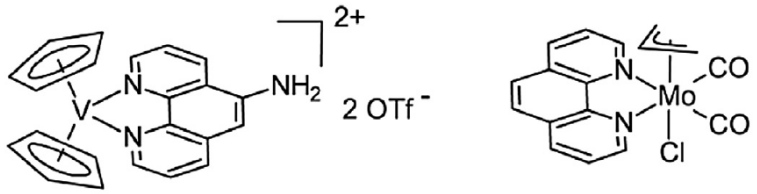
Complexes with high cytostatic activity, different central metal, structure and stabilizing N,N-chelate ligands were chosen for study of mechanism of effect of metalotherapeutics. Modern technics of spectrophotometric analysis, flow cytometry, qRT-PCR and western blot methods with imunoanalytic detection were used.

Expert literature describing the effect of *cis*-platin on cancer lines implicate that it is not possible to use any general concept for all types of tumor or cell cultures for explaining mechanism of its effect. The effect is assigned mainly to intracellular cleavage off chlorides and hydration with following irreversible covalent bond on DNA N7-guanin and adenine[23,24] with formation of intrachain netted aducts[23]. Nevertheless up to 95 % of cases end with preferred bond to the nucleophile component in cell, like peptides, proteins, enzymes and RNA[26,27]. Total anticarcinogenic effect is very often a combination of more mechanisms and is influenced by specificity of the cancer cell. Yet the cancer cells usually react with the arrest of replication and transcription, block in G2-phase of the cell cycle and apoptotic death[28,29] at the end of the reaction on *cis*-platin, usually with connection of AKT path (i.e. thymomas of aldo-keto reductase mice), c-Abl (i.e. Abelson tyrosine kinase), p53 and activation of MAPK (i.e. mitogen-activated protein kinases), especially JNK (i.e. c-Jun N-terminal kinase) and p38[30-34].

Literature describes as well that for example treatment of leukemic attacks with *cis*-platin causes creation of reactive oxygen species (ROS), increase peroxidation of lipids and decrease of negative charge on cell surface. This disrupts the amount of phospholipids in cell membrane[35,36].

It is known from results described in our previous publication[16] that complexes with N,N-chelates (Figure 7) **V1** ([(η^5-C_5H_5)$_2$V(5-NH_2-phen)]OTf and **Mo1** [(η^3-C_3H_5)Mo(CO)$_2$(phen)Cl] containing vanadium and molybdenum as central transitional metal have mechanism of effect similar to *cis*-platin on the leukemic lines MOLT-4 and HL-60, regardless presence of different central atom. Proliferation activity decreases depending on increasing concentration of complex and incubation time. This effect is accompanied with activation of cytotoxic path, induced on MOLT-4 through p53 protein that results into induction of caspase chain initiated through inner path by activation of

caspase 9. At the end of cascade reaction, the caspase 9 activates caspase 3 and 7 that induces apoptotic cell death and increase of amount of death cells MOLT-4 and HL-60.



V-1 **Mo-1**

Figure 7 - Structure of complexes V1 and Mo1.

It was found out that leukemic cells react with a very similar mechanism also after influencing by molybdenum complex **20** [{η^5-$C_5H_4CH_2CH_2NH(CH_2)_4O$}$Mo(CO)_2$(4,7-$Ph_2$-phen)][$BF_4$]$_2$ containing space-demanding N,N-ligand and tertiary amino group. Cytotoxic effect shows concentration and time reliance inhibiting cell proliferation with increase of death cells in late phase of apoptosis. Because of activation of p53 on MOLT-4 cells the cell cycle is arrested in G1 phase accompanied by apoptotic cells in early phase. It is known from literature that this tumor-suppressor gen p53 is not exceptionally present in human cancer cells HL-60[37]. Nevertheless apoptosis in these cells connected with cytotoxic effect of tested complexes can be induced independently on protein p53, for example by excessive expression E2F (i.e. E2 transcription factor), release of cyclin D1 and E and induction of apoptosis because of hyperphosprhorylation pRB (i.e. phosphorylated retinoblastoma protein) as mentioned in literature[38]. Results indicate that effect of complex **20** on leukemic lines MOLT-4 and HL-60 will be probably multifactorial process, where the main difference in sensitivity will be given by activation of p53 followed by proved posttranslational phosphorylation on serine 15 at MOLT-4.

The cell line MOLT-4 derived from T-lymphoblastic leukemia proves in cytotoxic tests to be a suitable model of first choice for study of cytotoxic activity of newly prepared complexes because of its properties (short doubling time 29h[39], passaging without need to trypsinize cells, immediate influence without need of cell adhesion or undemanding character on supplements contain in cultivating medium). However, it is important to know the level of selectivity to more cancer cell types to obtain a more detailed knowledge about analysis of mechanism of cytotoxic effect and focus on study of perspective compounds to most sensitive cancer cell line. One of the most sensitive lines not only from our tested complexes are cells A549. It is human cells derived from adenocarcinoma of alveolar basal epithelial lung cells. This cancer type belongs to histological subtype of adenocarcinoma non-small lung carcinoma. Because of late diagnosis the amount of survival for 5 years for lung adenocarcinoma is still very low from 4 to 17 % depending on stadium of illness and regional differences[40,41]. Cells A549 create blending monolayers with characteristic morphology and they feature cytochrome P450 IA1 and P450 IIB6 important for biotransformation of drugs[42].

Complex **6** [(η^5-$C_9H_6CH_2C_4H_3S$)$Mo(CO)_2$(4,7-Ph_2-phen)][BF_4] was chosen for analysis of reaction of cells A549 on molybdenum complex, it contains 4,7-Ph2-phen N,N-chelate and indenyl similarly to complex **20**, but here it is substituted by thiophene.

It was discovered that complex **6** causes arrest of cell proliferation activity related with down-regulation of gene CCNB1 (i.e. G2/mitotic-specific cycline-B1) for cyclin B, up-expression of protein p21 and CHK1 (i.e. checkpoint kinase) stabilized with phosphorylation on serine 345 and inhibition of protein PCNA (i.e. proliferating cell nuclear antigen). Combination of these effects leads to arrest of cell cycle in G2-phase and it was additionally found out that distribution of cell cycle in cells A549 is independent on used concentration. Moreover, it was proven that impact of complex **6** does not cause induction of apoptotic process on cancer cells. The tests with complex **6** were done also for non-cancer lung fibroblast cells MRC-5 to compare the sensitivity of effect on cancer tissue. The effect of complex **6** on non-cancer cells is different and shows same signs as previously tested complex **20** and *cis*-platin on cancer cells. On the MRC-5, the apoptosis is induced through activation of the inner apoptotic path through caspase 9 and decrease of amount of protein MDM2 (i.e. mouse double minute 2) important for stabilization of p53. Decrease of proliferation of cells A549 is given by decrease of S-phase accompanied by G2-block of the cell cycle with up-regulation of p21, CHK1 and down-regulation of PCNA. Changes in regulation of cell cycle are not observed at cells MRC-5. Activation of p21 and small increase of amount of CHK1 phosphorylated on serine 345 occurs at lower tested concentration of complex **6**. This advantage in a different effect of complex on cancer line against cells of healthy lung tissue is very important for future research that could discover possibilities of selective anticancer therapy.

Lung adenocarcinoma cells A549 were treated also with complexes containing vanadium as central atom. Namely highly effective complexes $[(\eta^5\text{-}C_5H_4Me)_2V(bian)][OTf]_2$ **3b**, $[(\eta^5\text{-}C_5H_4Me)_2V(4\text{-}MeO\text{-}bian)][OTf]_2$ **4b** were chosen. Mechanism of effect of **3b** and **4b** is dependent on p53 analogically as p53 and different from complex **6**. Corresponding to effect of *cis*-platin in MAP kinase activity on A549, same cell answers were found also after treatment with **3b** and **4b**. Increase of p38 correlates with proapoptotic path, contrarily during up-regulation of proteins JNK and ERK1/2 (i.e. extracellular signal-regulated kinases) occurs proapoptotic reaction. Apoptosis on cells A549 is supported by activation of MAP kinase activity through protein p38 similar to *cis*-platin[41]. Nevertheless, it was observed that apoptosis for tested complexes is partially inhibited by increase of amount of antiapoptotic factor Bcl-2 (i.e. B-cell lymphoma 2). A549 cells treated with **3b** and **4b** demonstrated up-regulation of protein ERK1/2, that increase share of cells in S phase. Decrease of viability of A549 and release of lactate dehydrogenase is given by activation of apoptotic process, this time with involving outer apoptotic path of caspase 8 followed by activation of powerful caspases 3/7. Both tested complexes **3b** and **4b** have significant antiproliferative effect dose-dependent with up-regulation of p21 and activation of CHK1 together with CHK2 lead to accumulation of cells in G2 phase. Anyway, the cytotoxic effect of both complexes is achieved in lower concentrations compared to *cis*-platin and it occurs already after 24 h of incubation. This can be used in therapy of cancer disease of lung adenocarcinoma.

Increased amount of ERK1/2 protein after treatment of A549 cells with *cis*-platin is also in literature described in relation with development of resistance of A549 cells to *cis*-platin[43]. This effect is amplified with insufficient activation of p38[44]. Same dependence of MAP kinase activity is described also during effect of this drug on *cis*-platin resistant cells of ovarian carcinoma A2780cis[45]. For this reason, our attention

during mapping of mechanism of effect of new organometallic complexes was aimed to cells of ovarian carcinoma. Besides that, patients with this carcinoma suffer from relapse of disease during 6 - 24 months already connected with development of chemoresistance to *cis*-platin. That is related with very low share of 5-year survival, around 20 to 25 %[46].

During development of metallotherapeutics remain efforts to develop analog to *cis*-platin to minimize development of chemoresistance and keeping strong anticancer activity. Our next work described a synthesis of new heterobimetallic Pt-Sn and Pt-Ag complexes bearing aminophosphine ligands. Measured IC_{50} value for these complexes was several times higher than for *cis*-platine[47]. The initial cytotoxicity study by WST-1 revealed a very promising activity against ovarian cancer cell lines A2780 and A2780cis for both Pt-Ag complexes (**4** and **5**) while Pt-Sn compounds **2** and **3** are inactive.

Literature describes different mechanism of effect of phosphine complexes of platinum compared to *cis*-platin[48-51]. Most of them pass through without arrest of cell proliferation probably because of bad solubility[52]. Described antitumor activity based on induction of apoptosis, loss of mitochondrial potential and accumulation of cells in G1-phase of cell cycle. Other Pt(II) complexes containing phosphine groups showed antitumor effect with intercalation into DNA and activity splitting DNA. Biologically active complexes were found predominantly in cytoplasm. Cytotoxic activity was function of lipophility and cell accumulation of complexes leading to cell apoptosis facilitated by creation of ROS[53]. In addition, the topic of heterobimetallic Pt(II) complexes is supposed as attractive field, since they can perform as dualmode anticancer agents with improved pharmaco-chemical properties[54].

Antiproliferative effect with activation of cell apoptosis occurs at our tested complex **4** on cells A2780 and A2780cis already after 24 h of treatment. Apoptosis is however so dependent on protein p53 compared to *cis*-platin. Nevertheless, proapoptotic protein p38 is activated also on *cis*-platin resistant cells A2780cis. That can be probably one of factors decreasing resistance to *cis*-platin complexes and induction of apoptosis. Increased amount of p38 is further connected with depolarization of mitochondrial membrane and activation of BAX protein (i.e. Bcl-2-associated X protein) followed by getting caspase 9 into action. This implicates increase in activity of effector caspases 3 and 7. Higher amount of p21 and check point kinase activity in cells A2780 lead to G2-block of cells, cells A2780 reacts by accumulation in S-phase. For comparison, non-cancer cells MRC-5 react cytotoxically to complex **4** only after use of higher concentrations and after longer time of treatment. Apoptotic death in cells of non-cancer fibroblasts is not activated after short time of treatment.

Obtained results indicate that selective effect on tumor cells occurs also in case of platinum complex **4** after short time of treatment. Moreover, complex **4** affects also *cis*-platin resistant cells with high cytotoxicity. Analysis of mechanism of effect further shows that it is similar to effect of *cis*-platin but presence of phosphine groups in molecule bring differences that can positively influence the treatment of cancer diseases and can be used in perspective of complex to be a new potential antitumor drug.

Question of selective effect of chemotherapeutics to ensure maximal effect with its minimal side effects could be solved with aimed transport. This is topic of last part of presented doctoral thesis. Graphene nanostructures are one of perspective materials that could be used as carrier for aimed transport of drugs in future. Unfortunately, literature mention several publications describing its high cytotoxicity[55,56].

In cooperation with Institute of Inorganic Chemistry of Academy of Science of Czech Republic, it was discovered that cytotoxicity of graphene particles could be related with presence of different contaminants. Created graphene oxide (GO) therefore underwent wash process that creates salt-washed GO (sw-GO). This was further reduced to reduced sw-GO (rsw-GO). The surface of (r)sw-GO was coated with Pluronic F127 (GO-F127) in next step. Structure of graphene particles is shown on figure 8.

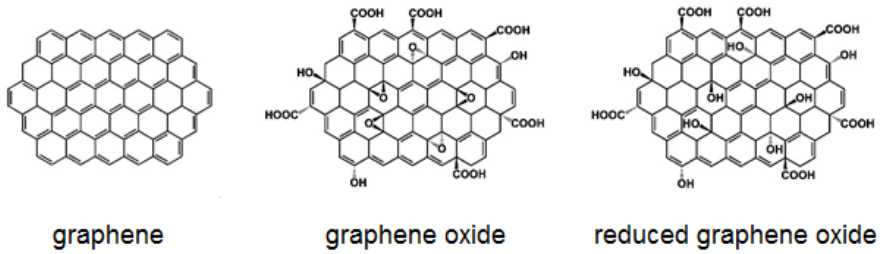


Figure 5 – Structure of graphene nanoparticles. Taken over and modified[57].

It was proven during tests of cytotoxic effect on non-cancer cell lines of human dermal fibroblasts HDF and cell lines MRC-5 that all studied graphene structures have cytotoxic effect on both cell lines, especially on MRC-5. For MRC-5 it was measured [IC_{50} (GO) = 1.9 ± 0.4 μg/ml, IC_{50} (swGO) = 5.2 ± 0.7 μg/ml, IC_{50} (swGO-F127) = 6.5 ± 1.5 μg/ml, IC_{50} (rswGO) = 9.9 ± 1.2 μg/ml] after 48 h of treatment. Values for HDF are [IC_{50} (GO) = 4.9 ± 0.3 μg/ml, IC_{50} (swGO) = 17.1 ± 4.2 μg/ml, IC_{50} (swGO-F127) = 28.2 ± 6.4 μg/ml, IC_{50} (rswGO) = 50.6 ± 8.3 μg/ml].

IC_{50} values on cells obtained from healthy lung and dermal fibroblasts MRC-5 and HDF showed that the wash process described in our contribution has positive effect on decrease of cytotoxic effect. It was found out that the cytotoxicity decreased radically in case of modified graphene oxide (rswGO) compared to standardly prepared graphene oxide (GO).

Nevertheless, it was observed that cytotoxicity would probably be a property of graphene oxide particles themselves. It came out that on graphene oxide rid of contaminants, and when stabilizing polymer Pluronic-F127 is used to prevent aggregation of particles on cell surface and therefore their direct damage, the viability is significantly influenced.

In statement that toxicity of nanoparticles will play significant restriction in biomedicine, we prop up on last study[58] that was created on Department of Medical Biochemistry, Charles University in Hradec Králové cooperation with Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice. Thiol-Functionalized Silica-Coated Iron Oxide Nanoparticles on cell line A549 were studied. Study revealed that nanoparticles will be negatively influenced grow and adhesion of cells. Collapse of microfilament actin net and disruption of microtubular cytoskeleton in direct interaction with activation of FAK protein (i.e. focal adhesion kinase) up-regulating ERK and p38 was proven.

These discoveries can represent serious restrictions in use of graphene oxide and other nanoparticles as transport medium of drugs for use in biomedicine applications.

4. Conclusion

Unfortunately, chemotherapeutics used until now cannot distinguish cancer cells and rapidly proliferating healthy cells. Therefore effort to find better cytostatics keeps going. Presented doctoral thesis also concerns with this topic.

Knowledge about cytotoxic effect and mechanism of effect of complexes of transitional metals are summarized in theoretical part. Based on this information, our attention focused on complexes containing vanadium and molybdenum as central metal.

Cytostatic activity of various structural types of organometallic complexes of vanadium and molybdenum was estimated with WST-1 assay in practical part. Results confirmed that these complexes are suitable adepts for treatment of cancer diseases. It was definitely proven that high cytotoxicity is connected with presence of stabilizing S,S- or N,N-chelate ligand. The most effective ones were phenanthroline complexes containing amine or phenyl-phenanthroline. Effectivity of these compounds was many times higher against standardly used drug *cis*-platin. Higher therapeutic effect was observed also at newly prepared platinum complex with phosphine ligands. This complex was used for further study of mechanism of effect too.

Molecular changes in tumor cells after treatment with chosen complexes of vanadium, molybdenum and new platinum complexes were comparable with changes of *cis*-platin. Antiproliferative effect occurs with induce of apoptotic process accompanied by accumulation of cells in G1-phase of cell cycle. It was found out that cytotoxic effect is induced independently on used cancer cells but it is dependent on used concentration and time of treatment. Significant difference was found at thiophene complex of molybdenum that induced on adenocarcinoma cell-line inhibition of cell growth with G2 block of the cell cycle without induction of apoptotic death. The preferential effect at this concept was proven to the cancer tissue compared to cell line derived from healthy tissue. Similar selectivity of the effect was confirmed also at Pt-Ag complex with phosphine ligands that. This complex used its potential in tests with cells resistant to *cis*-platin.

Over the scope of established aims of the doctoral thesis, the possibility of reduction of side effects of cytostatics using aimed transport of potential drugs bonded on surface of nanoparticles based on graphene oxide was studied. Unfortunately, it was proven during analysis of biocompatible effect that tested nanoparticles themselves dispose with high cytotoxicity to non-cancer cells and therefore are not suitable for use in biomedicine.

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