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# MASTERARBEIT

Titel der Masterarbeit

# Cellular and molecular effects of antitumoral organometallic ruthenium and osmium complexes containing Cdk inhibitors


Verfasserin

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# Abstract

Recently, the successful cooperation of medicinal chemistry and biology provides exciting opportunities in cancer treatment. A detailed and deep understanding of the mechanism of action of anticancer drugs based on metals makes it possible to change and improve the structural properties that are relevant for the potency of these drugs.

In this work we have investigated two groups of metal complexes. The first group are new ruthenium-arene complexes [RuCl($\eta^6$-arene)(L)]Cl (**1c**–**5c**) with a modified arene ligand, 4-formylphenoxyacetyl-$\eta^6$-benzylamide, that may be tethered to rHSA, and L = 3-(1*H*-benzimidazol-2-yl)-pyrazolo[3,4-*b*]pyridines (**L1**–**L3**) or indolo-[3,2-*d*]benzazepines (Paullones) (**L4**, **L5**), which are known CDK inhibitors. The second group consist of organometallic complexes [$M^{II}$Cl($\eta^6$-cymene)(L)]Cl (where M = Ru (**11a**, **12a**, **13a**), Os (**11b**, **12b**, **13b**) and L = **L1**–**L3**).

The cytotoxic activity of these complexes was detected by the colometric MTT assay in three human cancer cell lines: CH1 (ovarian carcinoma), A549 (non-small cell lung carcinoma) and SW480 (colon carcinoma) cells. In the cytotoxicity test the compounds showed the strongest effects in the chemosensitive ovarian carcinoma cell line CH1, whereas the generally chemoresistant non-small cell lung cancer cell line A549 is the least sensitive to this series of compounds. The most active of the complexes bearing a 4-formylphenoxyacetylbenzylamide ligand is the Paullone complex **5c**, which shows higher antiproliferative activity in all three cell lines, as was previously reported for [RuCl($\eta^6$–*p*-cymene)(L)]Cl complexes (as well as their osmium analogues) with Paullones **L4** and **L5**.

The complexes were screened for effects on the cell cycle by means of flow cytometry with propidium iodide staining. These experiments showed that Os complexes have lesser effect than Ru analogues on the cell cycle. In particular, ruthenium complexes **12a** and **13a** with ligands **L2** and **L3** have pronounced effects on the cell cycle, with varying intensity depending on the concentration. Among the Ru complexes with a 4-formylphenoxyacetylbenzylamid ligand, **5c** with Paullone ligand **L5** showed the strongest effect (G2/M phase arrest).

With Western blotting we have investigated the apoptosis-associated protein (PARP) in CH1 and SW480 cell lines treated with the bromo-substituted ruthenium complexes **2c** and **5c**. Cleavage of PARP was significantly detected only in CH1 cells in a dose-dependent manner after **5c** exposure.

Furthermore, we have studied the capacity of these compounds to inhibit cyclin-dependent kinases. Measurements of kinase activity of recombinant Cdk/cyclin complexes showed high inhibitory activity of the ligands, in particular Cdk2/cyclin E, compared with their related complexes.

A comparison of structure–activity relationships with regard to cytotoxicity, cell cycle effects and kinase inhibition does not support Cdk1 and Cdk2 as the general principal targets of these compounds. In cell-free experiments only several compounds proved to be capable of inhibiting Cdks in relevant concentrations, and the results of the kinase inhibition experiments do not correlate with cell cycle analysis data. In sharp contrast, ligands exerting minor effects on the cell cycle show high Cdk inhibitory potency and, vice versa, the ruthenium complexes with these ligands cause stronger inhibition of the cell cycle than Cdk inhibition, which might point to principal differences between cell-free and cell culture settings.

# Zusammenfassung

Das erfolgreiche Zusammenspiel von Medizinischer Chemie und Biologie ermöglicht vielversprechende Behandlungsansätze in der Krebstherapie. Ein detailliertes und umfangreiches Verständnis der Wirkmechanismen von metall-basierten Krebsmedikamenten ist notwendig, um die Wirkung dieser Substanzen durch strukturelle Variationen zu verbessern.

In dieser Arbeit wurden zwei verschiedene Gruppen metallbasierter Komplexe untersucht. Die erste Gruppe umfasst die neuen Ruthenium-Aren-Komplexe [RuCl($\eta^6$-Aren)(L)]Cl (**1c–5c**) mit dem modifizierten Aren-Liganden 4-Formylphenoxyacetyl-$\eta^6$-benzylamid, der an rHSA gebunden werden kann, und L = 3-(1*H*-Benzimidazol-2-yl)-pyrazolo[3,4-*b*]pyridin (**L1–L3**) oder Indolo-[3,2-*d*]benzazepines (Paullones) (**L4**, **L5**), die bekannte CDK inhibitoren sind. Die zweite Gruppe besteht aus organometallischen Komplexe [$M^{II}$Cl($\eta^6$-Cymen)(L)]Cl (wobei M = Ru (**11a**, **12a**, **13a**), Os (**11b**, **12b**, **13b**) und L = **L1–L3**).

Die zytotoxische Aktivität dieser Komplexe wurde mit Hilfe des kolorimetrischen MTT-Tests in drei humanen Krebszelllinien bestimmt: CH1 (Ovarialkarzinom), A549 (nicht-kleinzelliges Bronchialkarzinom) und SW480 (Dickdarmkarzinom). Den stärksten Effekt erzielten die Verbindungen in der chemo-sensitiven Ovarialkarzinom-Zelllinie CH1, während sie in der chemoresistenten nicht-kleinzelligen Lungenkrebs-Zelllinie A549 die schwächste Aktivität zeigten. Der aktivste Komplex ist der Paullonekomplex **5c** mit dem 4-Formylphenoxyacetyl-$\eta^6$-benzylamid-Liganden, der in allen drei Zelllinien antiproliferative Aktivität aufwies. Solche Aktivität wurde bereits für [RuCl($\eta^6$–*p*-cymene)(L)]Cl-Komplexe (und deren Osmium-Analoga) mit den Paullon-Liganden **L4** und **L5** berichtet.

Der Effekt der Komplexe auf den Zellzyklus wurde mittels Propidiumiodidfärbung und Durchflusszytometrie untersucht. Dabei stellte sich heraus, dass die Osmiumkomplexe weniger Einfluss auf den Zellzyklus haben als die Ruthenium-Analoga. Die Rutheniumkomplexe **12a** und **13a** mit den Liganden **L2** und **L3** beeinflussen den Zellzyklus konzentrationsabhängig. Unter den Rutheniumkomplexen mit dem 4-Formylphenoxyacetyl-$\eta^6$-benzylamid-Liganden zeigte **5c** mit dem Paulloneligand **L5** den stärksten Effekt (G2/M Phase-Arrest).

Die Wirkung auf das apoptose-assoziierte Protein (PARP) wurde mit Hilfe der Western Blot-Technik in CH1-, und SW480-Zellen überprüft, die mit den brom-substituierten Komplexen **2c** und **5c** behandelt wurden. Eine deutliche Spaltung des PARP wurde nur in CH1-Zellen festgestellt, die mit **5c** inkubiert worden waren.

Des Weiteren wurde die Kapazität der Komplexe, Cyclin-abhängige Kinasen zu inhibieren, untersucht. Die Messung der Kinase-Aktivität von rekombinanten Cdk/Cyclin Komplexen, besonders Cdk2/Cyclin E, zeigte eine hohe inhibitorische Aktivität der Liganden im Vergleich zu den entsprechenden Komplexen.

Ein Vergleich der Struktur-Wirkungs-Beziehungen dieser Komplexe in Bezug auf die Zytotoxozität, Effekte auf den Zellzyklus und die Kinase-Inhibition spricht gegen Cdk1 und Cdk2 als primäre Targets dieser Verbindungen. In den zellfreien Versuchen waren nur wenige Komplexe in der Lage, in relevanten Konzentrationen Cdks zu inhibieren, und die Ergebnisse dieser Experimente korrelieren nicht mit den Zellzyklus-Studien. Im Gegensatz dazu zeigten die Liganden, die nur wenig Einfluss auf den Zellzyklus hatten, starkes Cdk-inhibierendes Potenzial, während, vice versa, die Ruthenium-Komplexe mit diesen Liganden einen stärkeren Einfluss auf den Zellzyklus haben, und weniger effektiv in der Cdk-Inhibierung sind. Diese Ergebnisse sind möglicherweise auf Unterschied zwischen zellfreien Versuchen und Experimenten in der Zellkultur grundlegenden.

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# 1. Introduction

## 1.1 Cancer and carcinogenesis

Cancer is the common term for malignant neoplastic diseases. They are characterized by the occurrence of tumors which, in contrast to benign tumors, invade and actively destroy the surrounding tissue and frequently disseminate via the circulatory and lymphatic system to distant sites where they can form secondary tumors (metastases). Especially the metastatic stage bears a high risk of fatal events due to the destruction of essential organs and poses a continuing challenge to medicine, because it is very difficult (too often even impossible) to control.

According to global cancer statistics, cancer is one of the leading causes of death in economically developed countries and the second leading cause of mortality in developing countries. Cancer cases are increasing in economically developing countries as a result of increasing life expectancy, population growth, and changing lifestyle, including smoking, physical inactivity, unhealthy diet and many other reasons [22].

There are certain models of carcinogenesis, supported and proved by experimental observations. Any model of carcinogenesis should include several basic principles. The first principle is that a tumor is the result of genetic defects in normal cells that have the potential to become cancerous. The genetic basis of carcinogenesis was first postulated by Theodor Boveri in 1914 in his theory of somatic mutations. Recent advances in molecular biology of cancer, such as the discovery of oncogenes and tumor suppressor genes, have provided definitive evidence for this theory [7, 46].

The second basic principle of carcinogenesis is multistage development, meaning that cancer development requires more than one genetic mutation. Currently it is not clear completely which specific genetic mutations must occur or how they occur, but the multievent development of cancer is well confirmed in both animal models and human tumors [52, 6].

The third principle of carcinogenesis is based on a well-known observation of geneticists. No DNA replication is 100% accurate [32]. In other words, with every cell division, there is a certain probability of error in any gene of the daughter cells, including genes responsible for cancer development. Any DNA damage and replication error that remains unrepaired during the mitotic cycle is fixed in the following cell generations [12].

Quite a long time ago the cancer research community came to the conclusion that cancer is not a disease for which there will be a universal method of treatment. Most forms of tumor therapy carry the risk of selection for resistance; this problem can be exacerbated by the genomic

plasticity inherent in most cancers. Another problem is not sufficiently selective activity, resulting in damage to healthy cells of the body [15].

### 1.2 Anticancer activity of metal complexes

Since ancient times, metal compounds have been used successfully to treat various diseases and their application is still expanding. Already the ancient Egyptians discovered and successfully applied the therapeutic potential of gold salts. In traditional Chinese medicine, preparations of arsenic compounds such as arsenic trioxide have been successfully used as antiseptics, for treatment of rheumatoid diseases, syphilis and psoriasis and indeed, arsenic trioxide was among the first compounds that have been proposed for anticancer therapy (leukemia) [23].

The modern era of anticancer drugs based on metals began with the discovery of tumor-inhibiting effects of *cis*-diamminedichloroplatinum(II) (cisplatin) by Barnett Rosenberg in the 1960s. Currently, cisplatin and its analogues carboplatin and oxaliplatin are widely used in chemotherapy against various cancers.

In particular, cisplatin has become one of the most widely used anticancer drugs and has a strong effect in the treatment of certain tumors such as ovarian cancer and testicular cancer. At the same time, cisplatin shows two major drawbacks: severe toxicity and limited applicability in a narrow range of tumors because of inherent resistance of many forms of cancer [38, 39, 51]. In search of new treatment methods to avoid these drawbacks, other metals were considered as an alternative to platinum. Coordination compounds based on ruthenium, gold, titanium, copper, rhodium, vanadium, cobalt, osmium, zink, gallium and germanium were tested for their anticancer activity, and some of these metals have been promising candidates for anticancer agents [23]. Particular attention was paid to the compounds of ruthenium, as they have shown cytotoxicity against cancer cells, a ligand exchange capacity similar to platinum complexes, no cross-resistance with cisplatin, and lower toxicity in healthy tissues [10, 11].

The ruthenium complexes are among the most effective non-platinum metal complexes with regard to their antitumor activity. There are two drug candidates (KP1019 and NAMI-A) that are currently being studied in clinical trials. Originally KP1019 was developed for treatment of solid tumors in general, while NAMI-A was designed specifically as an antimetastatic drug. Both compounds were well tolerated and have only minor side effects, especially KP1019. Promising results were obtained in patients with solid tumors after treatment with KP1019 – disease stabilization was observed in 5 of 6 patients [19].

It is important to understand the mechanism of action of ruthenium complexes. Despite the fact that Ru was detected in the nuclei of cells, there is reason to assume that the antitumor activity of some ruthenium compounds is not based on DNA damage only. In particular, Ru(III) compounds are characterized by high affinity to (serum) proteins, which presumably are critical for drug accumulation in tumor tissue and may explain the minor side effects observed in clinical trials with KP1019 [23].

The ability of ruthenium to mimic iron in binding to various biomolecules, such as human serum transferrin (iron-transport protein), may contribute to more effective delivery of ruthenium complexes to cancer cells. Cancer cells as rapidly dividing cells have a greater demand for iron and over-express transferrin receptors. This property may explain the significantly lower toxicity of ruthenium-based anticancer drugs, compared with platinum drugs.

Furthermore, the rich and well-established synthetic and coordination chemistry of ruthenium in combination with the fact that the metal has several oxidation states accessible under physiological conditions, namely, Ru(II), Ru(III) and Ru(IV), makes ruthenium compounds interesting and suitable for use in medical applications [34]. The «activation by reduction» hypothesis suggests that Ru(III) complexes may be prodrugs, which are activated by reduction in the tumor microenvironment with rapid coordination to biomolecules. There is enabling low oxygen content (hypoxia) in tumor tissue as the tumor uses oxygen quickly during the fast grow but is not optimally vascularized. Under hypoxic conditions cancer cells use glycolysis to produce energy, which generates lactic acid that lowers the pH. These metabolic differences in tumor relative to normal cell metabolism should foster the production of Ru(II) relative to Ru(III) in tumors, compared with normal tissue [10, 55].

There are interesting studies of the reaction with the sulfur-containing amino acids L-cysteine and L-methionine, which are likely to play an important role in biochemistry of Pt(II), Pt(IV) and Ru(III) anticancer drugs. These amino acids and protein interactions may be taken for explanation of low level of side effects of this class of anticancer drugs [8].

In our work we compare the Ru-based drugs with Os-based analogues. Speaking about the mechanism of action of Os drugs, we should mention several reports on the electrophoretic mobility of DNA duplex oligonucleotides with osmium adducts. With these experiments it was shown that DNA bending is induced by osmium binding. The binding of an osmium-arene complex leads to a large degree of unwinding which is stronger than for ruthenium(II) complexes.

This large unwinding angle can be explained by additional interactions of the arene ligand with the duplex by a strong connection with osmium. This suggests that Os and Ru complexes interact with DNA in different ways [9].

Although there is no clear understanding of the mode of action of ruthenium compounds, there is already enough evidence that the redox reactions and interference with cellular redox balance and binding to different cellular sites play an important role in the antitumor activity of many compounds of ruthenium. Ruthenium has been studied deeply and widely for its anticancer potential in the past two decades, from both the chemical and biological point of view. Several unique characteristics were shown for ruthenium complexes: (a) selective accumulation in tumor cells via interaction with transferrin, (b) activation by reduction of inert Ru(III) to the active Ru(II) in more hypoxic and acidic tumor microenvironment, (c) favorable kinetics of ligand exchange, (d) antimetastatic effects (some ruthenium agents) by inhibition of tumor cell detachment, invasion/migration and (e) unique DNA binding patterns different from those of platinum due to the different coordination geometry [2].

In summary, ruthenium compounds are second generation (post-platinum) investigational anticancer metallopharmaceuticals, which have unique properties, at least in preclinical studies, in particular more selective entry into tumor cells with fewer toxic effects on normal cells. In the future, elucidation of specific mechanisms of action of ruthenium drugs will be needed to clarify the actual therapeutic potentials of ruthenium complexes in the treatment of cancer.

### 1.3 Cdk inhibition as a therapeutic approach

The cell cycle is controlled in general by the interaction of several types of proteins: cyclin-dependent kinase (Cdk), cyclins - proteins that interact with the Cdk inhibitors, forming complexes – and complexes of Cdk-cyclin.

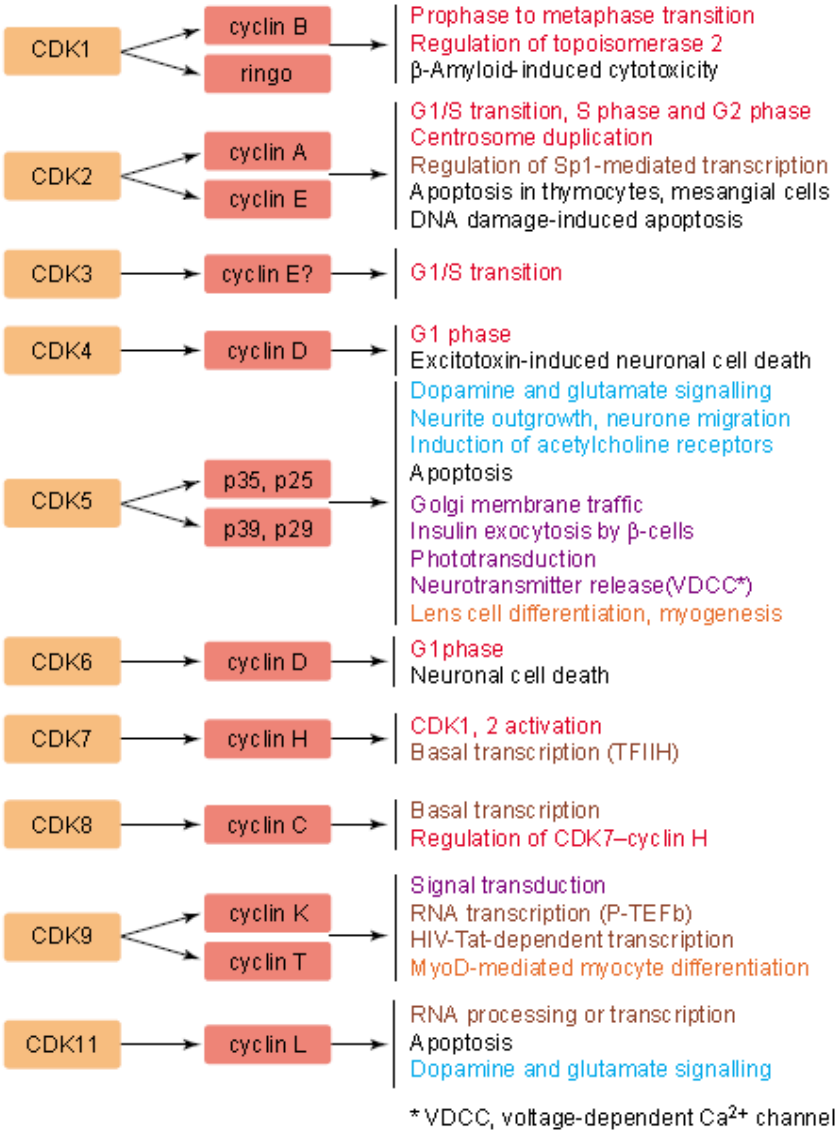



Figure 1.1. Overview of cyclin-dependent kinases [24].

Cyclin-dependent kinases (Cdks) phosphorylate other proteins, changing their function. The cell cycle is controlled by changing activity of Cdks, which are regulated by the periodic formation and the collapse of their regulatory subunits – the cyclins. A cyclin-dependent kinase (Cdk) is

activated specifically by a cyclin, which binds to it. The cyclin component binds the target protein, and this protein is then phosphorylated by the kinase component (Figure 1.2).

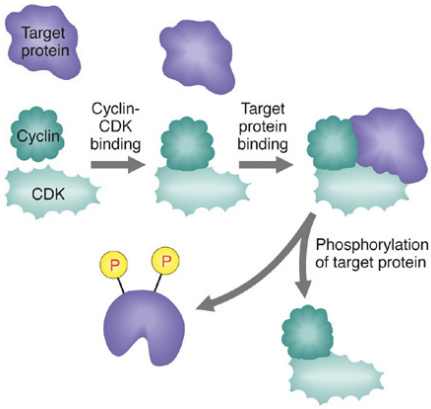



Figure 1.2. The binding of Cdk-cyclin complex [www.bio.miami.edu].

Different cyclins are involved in the different phases of the cell cycle. They are binding to Cdks with the purpose of forming various Cdk-cyclin complexes, and these complexes regulate different phases of the cell cycle.

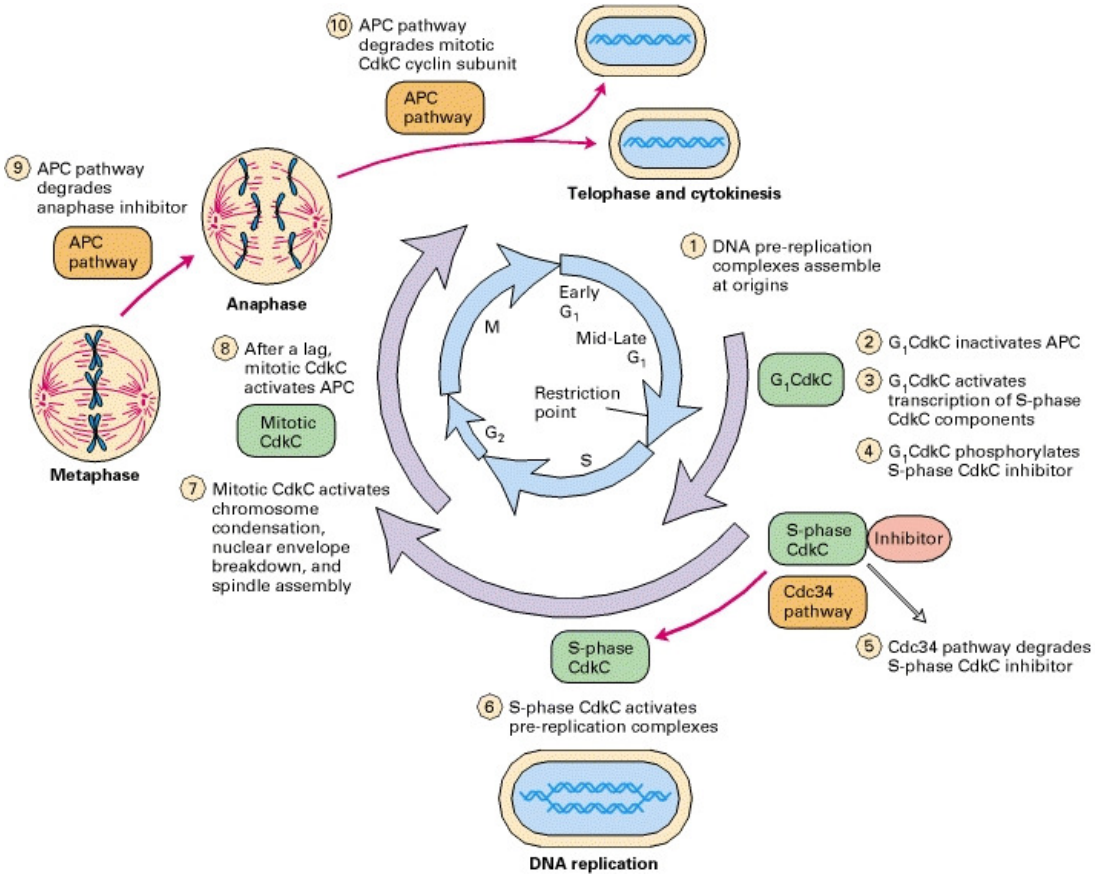



Figure 1.3. Current model for regulation of the eukaryotic cell cycle [28].

### 1.3.1 The cell cycle

Cyclin-dependent kinases (Cdks) are the catalytic subunits of a family of mammalian heterodimeric serine/threonine kinases are central for the control of cell-cycle progression, transcription and neuronal function [29].

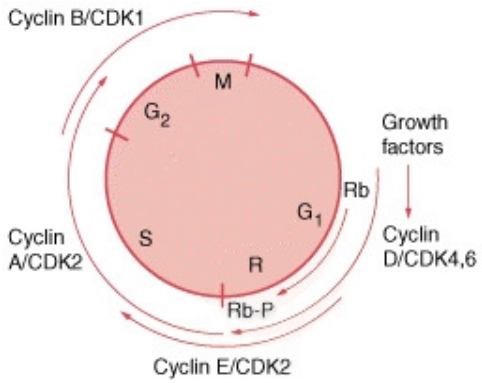



Figure 1.4. Cyclin-dependent kinases. The major Cdks involved in controlling the cell cycle [26].

In response to a mitogenic stimulus, cells in the G0 or early G1 phase, begin their passage through the cell cycle. As a result, the induction of gene expression of cyclins D and E, which are a group of cyclins controlling the processes in the G1 phase, increases their intracellular concentration. Cyclins D1, D2 and D3 form a complex with kinases Cdk4 and Cdk6. Activation of Cdk2/4/6 leads to phosphorylation of the RB protein (retinoblastoma gene product pRb) and its associated proteins p107 and p130. In the early G1 phase pRb is phosphorylated poorly, allowing it to be in a complex with transcription factor E2F, which plays a key role in the induction of DNA synthesis, and blocks its activity. The fully phosphorylated form of pRb releases E2F from the complex, which leads to activation of genes controlling DNA replication. The concentration of D-cyclins increases during the G1 phase and reaches a maximum value immediately before the S phase. Phosphorylation of pRb is completed under the influence of Cdk2, activated by cyclin E. The intracellular concentration of cyclin E reaches its maximum at the transition from G1 phase to S phase. The Cdk2/cyclin E complex activates DNA synthesis through the phosphorylation of proteins involved in initiating of replication. As a result, the cell enters the S phase of the cell cycle and begins to synthesize DNA.

The entry of the S phase is accompanied by rapid degradation of cyclin E and Cdk2 activation by cyclin A. Signal for completion of the S phase and transition to the G2 phase is the activation of Cdk1/cyclin A and decreasing the activity of Cdk2 [37].

The signal for cell division (mitosis) is based on the factor MPF (M phase promoting factor). MPF is a complex of Cdk1 with its activating cyclins A or B. Cyclins B1 and B2 are present in very low concentrations in the G1 phase. Their concentration begins to increase at the end of the S and during the G2 phase, reaching its peak during mitosis, and replaces cyclin A in the complex with Cdk1. Cell division begins only after Cdk1 (bound in a complex with cyclin B) is phosphorylated on residues Thr-14 and Tyr-16 and then dephosphorylated at residues Thr-14 and Tyr-15. Activated by this process, Cdk1 phosphorylates structural proteins in the nucleus, including nucleolin, nuclear lamins and vimentin. The first stage of mitosis – prophase – begins after Cdk1 is fully phosphorylated, followed by metaphase, anaphase and telophase, ending with cell division – cytokinesis. Cyclin B is destructed after the end of cytokinesis, followed by inactivation of Cdk1, which leads to the entry of cells into G1 or G0 phase of the cell cycle [18].

### 1.3.2 Cdks as targets for cancer therapy

There is evidence that Cdks, their regulators and substrates are susceptible to genetic changes in many kinds of human tumors. Thus, the cyclin dependent kinase pathways have become a target for anticancer therapy [17].

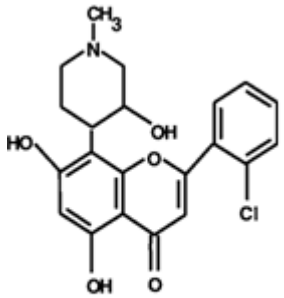
Effects of Cdk inhibitors on the cell cycle and their high potency for cancer treatment have been extensively studied. There are several properties that make Cdk inhibitors attractive as potential agents in the fight against cancer [45]. Firstly, they are powerful antiproliferative agents capable of arresting cells in G1 or G2/M phase. Secondly, they cause apoptosis, alone or in combination with other treatments. However, it is important to note that the therapeutic value of inhibition of specific Cdks is highly dependent on genetic conditions and the activation of specific signaling pathways that control proliferation and distribution of tumor cells. For example, inhibition of Cdk4 may be very effective in HER2-positive breast cancer, but shows absolutely no effect on MYC-induced breast cancer [30].

Application of highly specific inhibitors will require a balance between accurate knowledge of the therapeutic value of the target kinase and the possible specific genetic changes in each of the primary tumor or metastatic clone. Toxicity may become an obstacle in such situations, too.

According to publications, six classes of inhibitors of Cdks have been identified: the purine-based compound olomoucine and its analogues, butyrolactone, flavopiridol, staurosporine and the related compound UCN-01, suramin and 9-hydroxyellipticine. All of them occupy the ATP-binding pocket of the enzyme and compete with ATP [17].

For effective cancer chemotherapy it is important to know all details of the cell cycle processes and apoptotic events. In this regard, the effects of inhibitors of Cdks is multistage and complex, because they induce apoptosis in dividing cells, but they protect normal cells from apoptosis induced by some but not all cytotoxic agents [24].

The discovery of the cyclin-dependent kinases (Cdks) allowed the investigators to study different possibilities of modulations of Cdk activity. Flavopiridol, a flavonoid derived from an indigenous plant *Dysoxylum binectariferum* [43] from India (Figure 1.5), demonstrated specific and potent inhibition of Cdks (Cdks 1/2/4/7) *in vitro*. Furthermore, preclinical studies showed the capacity of flavopiridol to induce apoptotic mechanisms, modulate transcriptional events, promote differentiation and inhibit angiogenic activity. The primary testing in clinical studies in human patients with infusion of flavopiridol showed effect on some patients with non-Hodgkin's lymphoma, renal, prostate, colon and gastric carcinomas. Secretory diarrhea and a pro-inflammatory syndrome associated with hypotension were observed as major side effects [44].




**Flavopiridol**

Figure 1.5. Chemical structure of flavopiridol [44].

Flavopiridol is interesting for further studies because of its high capacity of inhibiting the proliferation of a wide range of human tumor cell lines: experiments show the correlation between high cytoxicity and potency to inhibit certain kinases, both tyrosine and serine kinases. It shows the capacity to inhibit a wide range of human tumors *in vivo* experiments [43].

The expression and function of Cdks and their substrates are regulated in a multistage and complex way. In cancer cells this control is dysregulated by loss of proapoptotic proteins or by enhancement of functions of proteins inhibiting apoptosis. Flavopiridol seems to interfere with this dysregulated system in several ways, e.g., by directly inhibiting Cdks involved in the cell cycle or by inhibition of different cyclins (cyclin D1/D3 and possibly decreasing expression of Cdk4). As a result, by inhibition of Cdks flavopiridol may decrease/block the function of antiapoptotic proteins, which allows the inhibition of cell proliferation and induces apoptosis [42]. The arrest of cells by flavopiridol can be achieved in different stages. For example, the arrest of the cell in G1 phase by flavopiridol can be related to the inhibition of Cdk2 and Cdk4. The G2 arrest by flavopiridol may be related to Cdk2 or Cdk1 inhibition. Flavopiridol inhibits Cdks by binding of adenosine triphosphate (ATP). Cocrystallization studies using des-chloro-flavopiridol and CDK2 have shown that flavopiridol binds to the ATP-binding pocket of CDK2. In addition to inhibiting these CDKs directly, flavopiridol may interfere with the regulatory phosphorylations of CDKs to inactivate them [54]. Thus, flavopiridol is the first cyclin-dependent kinase inhibitor studied in clinical trials and demonstrates clear effects on cell cycle progression. Obtained positive results will enhance the development of novel Cdk modulators for cancer therapy [44].

Significant efforts and successes of many research groups have led to an understanding and optimization of powerful and effective inhibitors of Cdks. Studies show that the majority of Cdk inhibitors with antiproliferative properties are associated with apoptosis-inducing activity and display antitumoral activities. Most efforts have concentrated on creating drugs against Cdk2, since this enzyme was shown to be essential for the early phases of the cell cycle, which are most frequently misregulated in cancer cells. These studies should provide important information for developing the best strategies and high-potential drug to target these kinases for therapeutic purposes in patients with cancer.

### 1.4 Cytotoxicity assay

Many biological assays require the measurement of surviving and proliferating mammalian cells. Multiwell scanning spectrophotometers (ELISA readers) can measure large numbers of samples with a high degree of precision [33]. The original MTT method involves reading the intensity of a purplish blue color resulting from the conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to formazan crystals by the dehydrogenase activity of mitochondria of viable cells. The MTT assay is simple, accurate and yields reproducible results [27]. The mitochondrial succinate dehydrogenases (and probably other reducing agents) of viable cells cleave the tetrazolium ring, resulting in formation of formazan crystals (Figure 1.6). The crystals can be dissolved in isopropyl alcohol, methanol or dimethyl sulfoxide (DMSO). When the formazan crystals are dissolved (e.g. in DMSO), the optical density of the purplish solution is quantified using a scanning multiwell spectrophotometer (ELISA reader). An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity of the tested compounds.

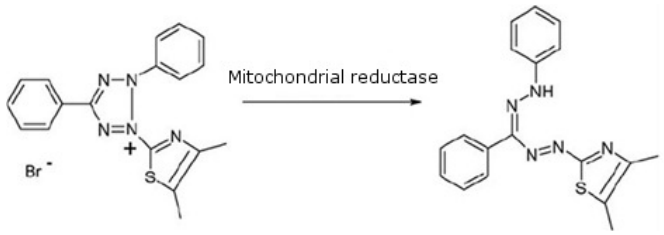



Figure 1.6. Metabolization of MTT to formazan product in viable cells [www.chemie.uni-hamburg.de].

The reduction of tetrazolium salts to formazan products occurs only in living cells. Thus, the detection of the optical density of the samples allows determining cell viability. The quantity of viable cells is usually expressed in terms of T/C values by comparison with untreated control microcultures, and 50% inhibitory concentrations ($IC_{50}$) are calculated from concentration-effect curves by interpolation (Figure 1.7).

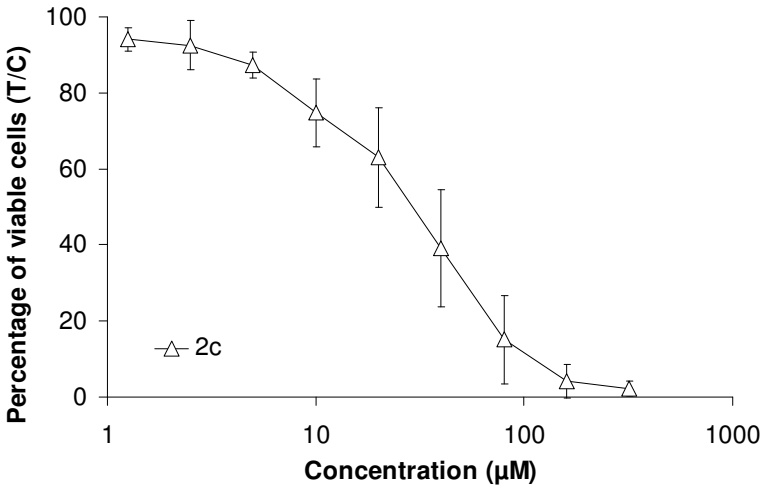



Figure 1.7. Concentration-effect curve showing the effect of complex **2c** on ovarian carcinoma cells (CH1).

### 1.5 Fluorescence-activated cell sorting (FACS)

The Fluorescence-Activated Cell Sorter (FACS) was invented in the late 1960s by Bonner, Sweet, Hulett, Herzenberg, and others. Thus it became possible to do flow cytometry and sorting of viable cells [21]. The sorting process is based upon the specific light scattering and fluorescent characteristics of each cell (Figure 1.8). The cell suspension enters the system in the center of a narrow and rapidly flowing stream of liquid. A vibrating mechanism makes the cell flow divide into separate drops, each containing not more than one cell. The flow passes through the fluorescence measuring station where the fluorescent characteristics of each cell are measured. The fluorochromes attached to the cells absorb light and then emit light of a specific wavelength, depending on the type of fluorochrome. The detectors collect the light emitted by the fluorochromes [20]. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge (Figure 1.9). The data from all the detectors is sent to a computer and plotted in histograms.

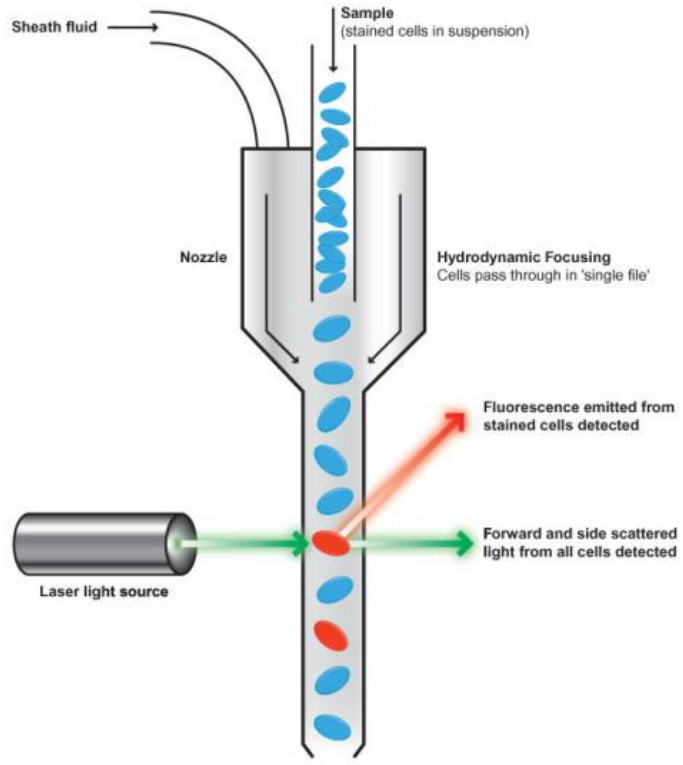



Figure 1.8. Flow cytometry principle [www.abcam.com].

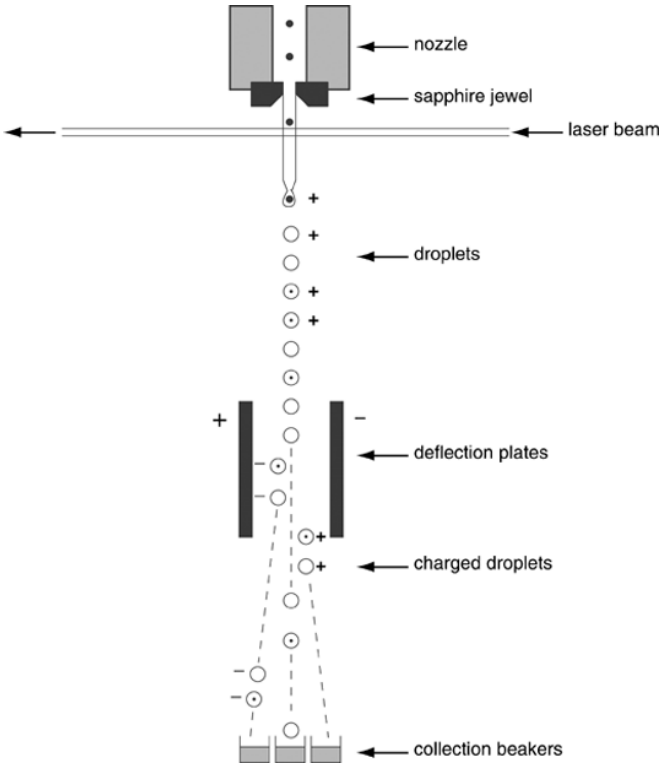



Figure 1.9. The principle of cell sorting [13].

This flow cytometric method can be used for the detection of intact cells, cells undergoing apoptosis, and dead cells resulting from apoptotic processes by the differences in cell compartments and chromatin condensation [5]. The detection of dead cells is possible with propidium iodide staining as a probe for membrane damage. Propidium iodide (Figure 1.10) binds to DNA and this binding significantly increase the fluorescence of the dye. The dye can not enter the viable cell as it is prevented by the cell membrane. Thus, the laser-excited fluorescence is observed only when PI is associated with the nuclei of the dead cells [25, 40].

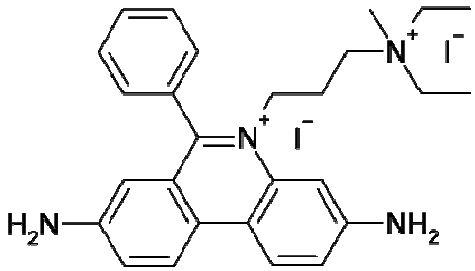



Figure 1.10. Chemical structure of propidium iodide.

By allowing the determination of the relative cellular DNA content upon staining with a DNA-binding fluorochrome such as propidium iodide (after permeabilization of cell membranes), flow cytometry also enables the analysis of cell populations with regard to their distribution to the phases of the cell cycle. Cells in G2 or M (mitosis) phase contain the double amount of DNA compared to cells in G1 or G0 phase, with the S (DNA synthesis) phase forming the transition. However, G2 and M phase, which both have an identical DNA content, cannot be discriminated based on their differences in DNA content. Diverse software containing mathematical models that fit the DNA histogram have been developed in order to calculate the percentages of cells occupying the different phases of the cell cycle [36].

In addition to the above, the apoptotic processes are well-controlled and tightly regulated physiological mechanisms which lead to self-destruction of cells. Most studies suggest that apoptosis is a central mechanism in embryogenesis and morphogenesis, is involved in regulation of the immune system and controls normal turnover of tissues. At the same time, it is also involved in various pathological processes/diseases such as cancer [3, 50]. In this regard, developing reliable and sensitive methods is very important for detection of cell distribution and cell death, e.g., fluorescence-activated cell sorting [4]. FACS was found suitable to identify cell

cycle distribution of the tested cells in the sample with human ovarian carcinoma cells (CH1), treated with ruthenium and osmium anticancer drugs.

### 1.6 Western blotting

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is used to specifically detect an antigen) was introduced by Towbin et al. in 1979 and is now a routine technique for protein analysis [49]. Western blotting can produce qualitative and semi-quantitative data about proteins.

The first step in a Western blotting procedure is to separate proteins by gel electrophoresis. Gel electrophoresis is a technique in which charged molecules such as proteins or DNA, are separated according to their physical properties, while they are forced through the gel by an electric current. Proteins are usually separated by electrophoresis with polyacrylamide gel electrophoresis (PAGE). PAGE combined with Western blotting is a powerful analytical tool to provide information on the mass, charge, purity and presence of a protein. After electrophoresis, the separated molecules are transferred onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane (Figure 1.11). Membrane supports used in Western blotting have a high affinity for proteins. Thus, after transfer of proteins from the gel, it is important to block the remaining surface of the membrane to prevent nonspecific binding of antibody in subsequent steps. Blocking the free surfaces of the membrane is usually accomplished by use of different buffers, from milk or normal serum to highly purified proteins. Blocking with special buffers should improve the assay sensitivity by reducing background noise and improving signal/noise ratio [35]. Unfortunately, no blocking agent is ideal for all settings, as each pair of antibody-antigen has unique characteristics. In this case the blocking buffer should be chosen according to the conditions of the experiment.

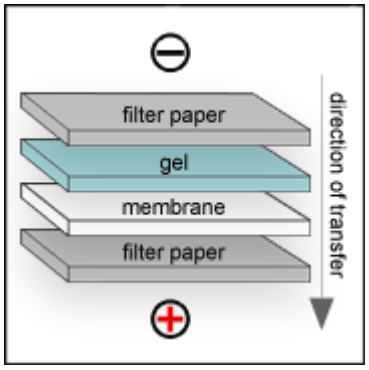



Figure 1.11. Western blot sandwich [www.cardio-research.com]

When the membrane is blocked to prevent nonspecific antibody binding to the membrane surface, the next stage is the detection of proteins. The direct method of detection is based on primary antibodies marked with an enzyme or a fluorescent dye which are used to detect antigens on the spot. This detection method is not widely used, since most researchers prefer the indirect method of detection because of its high efficiency.

In the indirect method of detection, primary antibody is added first to bind to the antigen. This is accompanied by a labeled secondary antibody that is directed against the primary antibody. Labels can include biotin, fluorescent probes such as rhodamine and conjugated enzymes, such as horseradish peroxidase or alkaline phosphatase (Figure 1.12). The indirect method offers many advantages over the direct method.

The final stage of the procedure is the detection of the signal from the secondary antibodies associated with the membrane proteins using, for example, chemiluminescent blotting substrates.

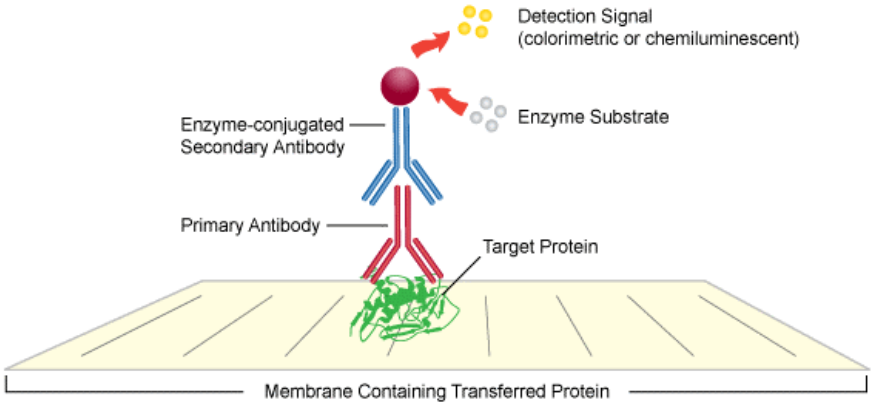



Figure 1.12. Scheme of the indirect method of detection [www.leinco.com].

Chemiluminescent substrates retain the signal only as long as the reaction proceeds. If the substrate is used up or the enzyme loses its activity, the reaction will be terminated and the signal will be lost. However, in a well-optimized protocol using the correct dilution of antibodies the reaction can produce a stable output of light for 1 to 24 hours depending on the substrate. Such a sequence of stages increases the detection sensitivity and allows the detection of a protein by X-ray film or digital imaging equipment [www.piercenet.com].

In our work we have investigated poly(ADP-ribose) polymerase (PARP-1) by Western blotting. PARP-1 is the first and one of the best studied members of the PARP family of protein modifying enzymes. PARP-1 is an enzyme responsible for the poly(ADP)-ribosylation of itself or other proteins after exposure to single/double-stranded nicked DNA [14, 53]. This enzyme enhances the target protein activity by the covalent attachment of oligo/poly(ADP-ribose) (PAR) chains to it (e.g., p53, waf-1, DNA ligase III, histones, telomerase). Covalently binding PARs change of the stability, activity and DNA-binding capacities of the targets. PARP-1 is an abundant protein, whose capacity is stimulated up to 500-fold by single/double-strand DNA breaks. PARP-1 immediately binds to the points of DNA breaks and starts to modify a wide range of nuclear proteins. PARP-1 may poly(ADP-ribosyl)ate itself and then leaves the breaks allowing other repair proteins to access the site of damage. When the damage is too strong, PARP-1 may deplete the cell of NAD+, leading to ATP drain, and with others modifications this may enhance apoptosis. Thus, PARP-1 can be cleaved by activated caspase-3 and the cleaved product of the protein (89 kDa) is commonly used as an indicator for apoptotic processes [53].

# 2. Materials and methods

## 2.1 Characterizations of tested complexes

In this work we investigated two groups of metal complexes. The first group includes new ruthenium-arene complexes of the formula [RuCl($\eta^6$-arene)(L)]Cl (**1c**–**5c**) (Figure 2.1), with a modified arene ligand, 4-formylphenoxyacetyl-$\eta^6$-benzylamide, that may be tethered to rHSA, and L = 3-(1*H*-benzimidazol-2-yl)-pyrazolo[3,4-*b*]pyridines (**L1**–**L3**) and indolo-[3,2-*d*]benzazepines (**L4**, **L5**), which are potential CDK inhibitors.

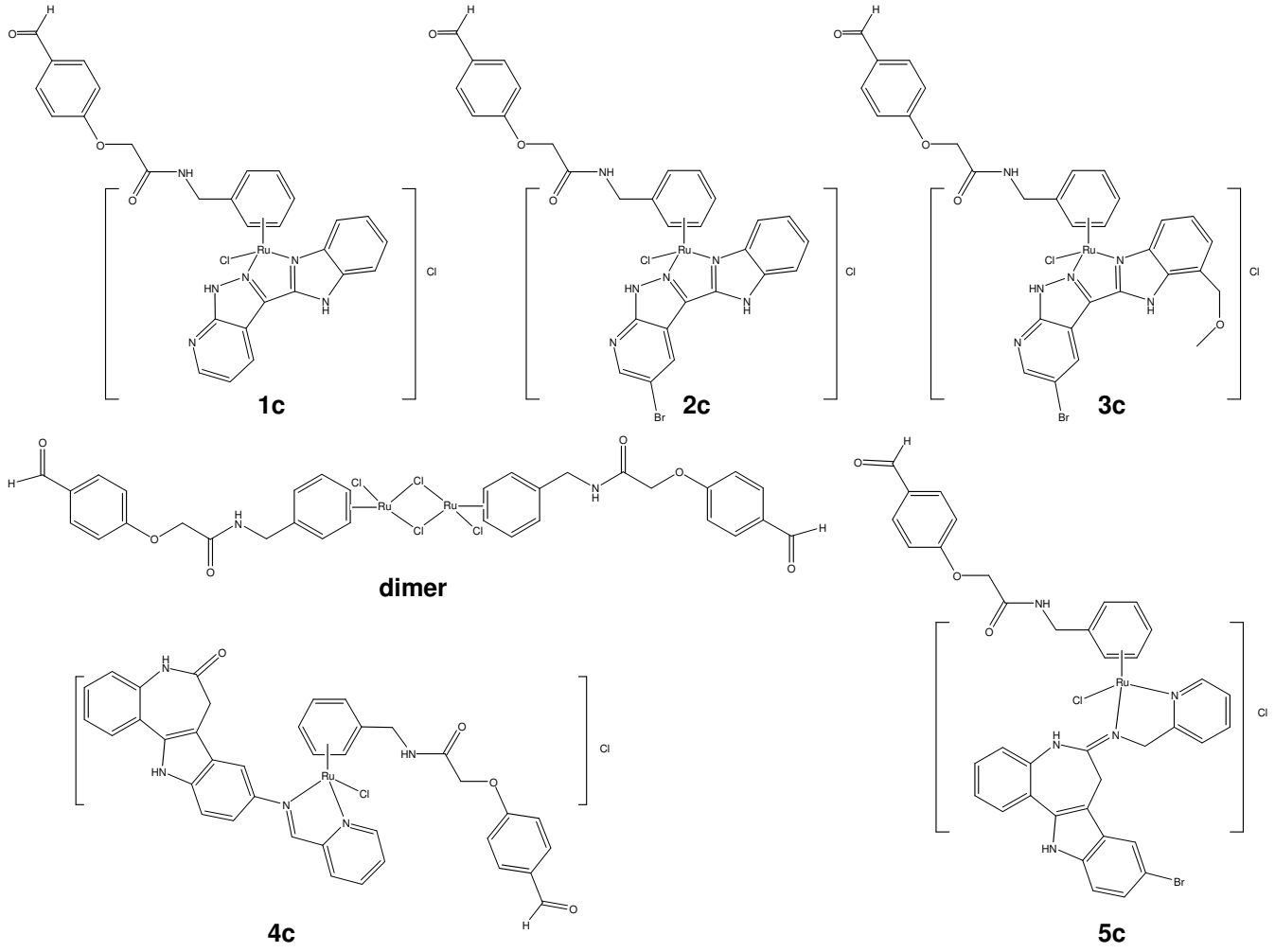



Figure 2.1. Compounds **1c**–**5c** with atom numbering [48].

The second group (Figure 2.2) consists of organometallic complexes [$M^{II}Cl(\eta^6$-cymene)(L)]Cl (where M = Ru (**11a**, **12a**, **13a**), Os (**11b**, **12b**, **13b**)) with L = 3-(1*H*-benzimidazol-2-yl)-pyrazolo[3,4-*b*]pyridines (**L1**–**L3**). The latter ligands are also known as potential cyclin-dependent kinase (Cdk) inhibitors.

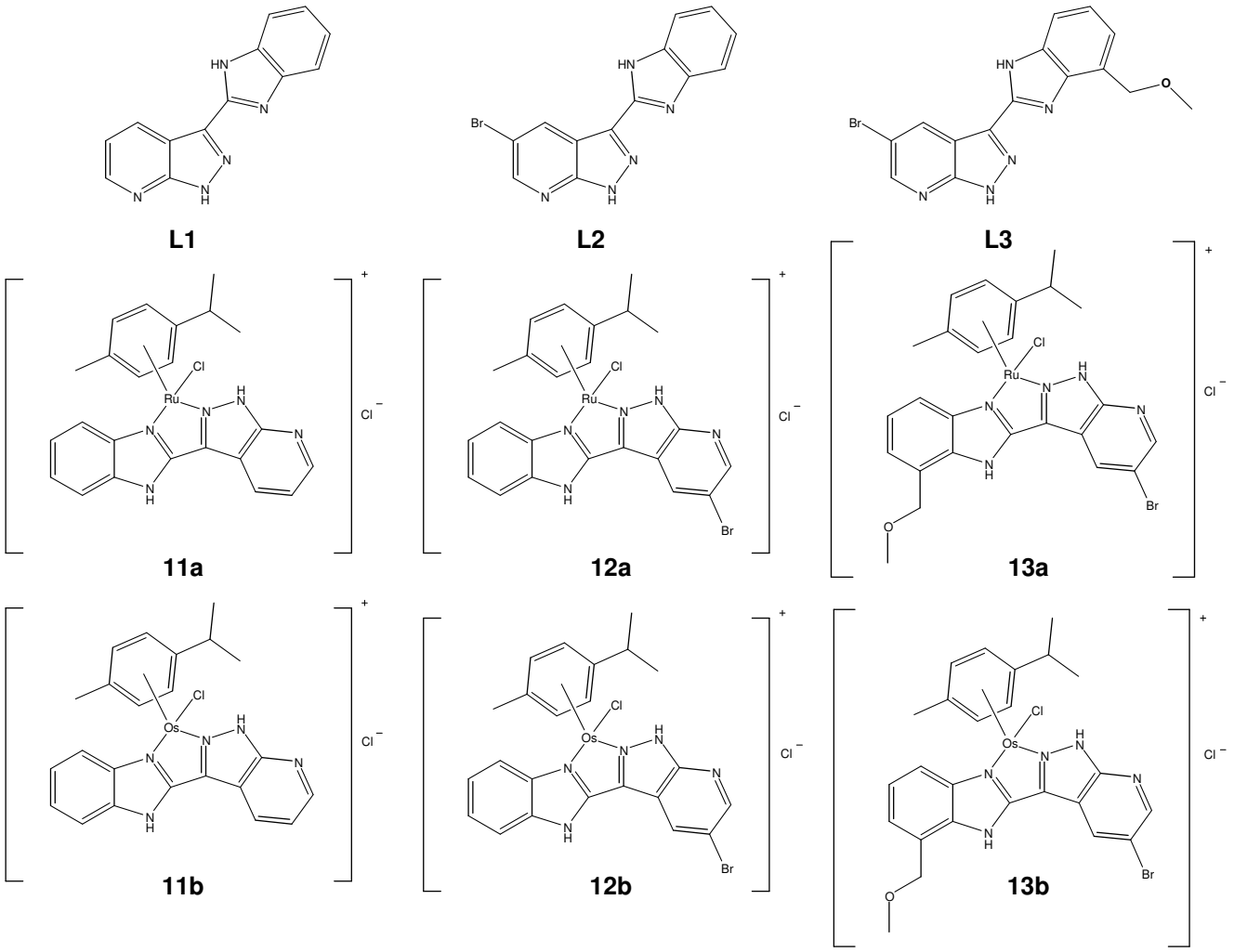



Figure 2.2. Ligands **L1**–**L3** and complexes **11a–13a**, **11b**–**13b** reported in this work with atom numbering schemes [47].

## 2.2 Cell culture and inhibition of cell growth

A549 (non-small cell lung carcinoma, human) and SW480 (colon carcinoma, human) cells were kindly provided by Brigitte Marian (Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria). CH1 (ovarian carcinoma, human) cells were a gift from Lloyd R. Kelland (CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK). All cell culture media and supplements were purchased from Sigma-Aldrich. Cells were grown in 75 $cm^2$ flasks (Iwaki) in complete medium (i.e., Minimal Essential Medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 4 mM L-glutamine and 1% non-essential amino acids from 100× stock) as adherent monolayer cultures. Cultures were grown at 37 ºC under a humidified atmosphere containing 5% $CO_2$ and 95% air.

Antiproliferative activity in vitro was determined by the MTT assay (MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Fluka). For this purpose, cells were harvested from culture flasks by use of trypsin and seeded in complete medium (100 µL/well) into 96-well plates (Iwaki) in densities of $4 \times 10^3$ (A549), $1.5 \times 10^3$ (CH1) and $2.5 \times 10^3$ (SW480) viable cells per well. Cells were allowed for 24 h to settle and resume proliferation. Test compounds were dissolved in DMSO first, appropriately diluted in complete medium and instantly added to the plates (100 µL/well), where the DMSO content did not exceed 0.4% and 1% for ligands and complexes, respectively. After exposure for 96 h, medium was replaced with 100 µL/well RPMI 1640 medium (supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine) plus 20 µL/well MTT solution in phosphate-buffered saline (5 mg/mL), followed by incubation for 4 h. Subsequently, the medium/MTT mixture was removed, and the formazan product formed by viable cells was dissolved in DMSO (150 µL/well). Optical densities were measured with a microplate reader (Tecan Spectra Classic) at 550 nm (and a reference wavelength of 690 nm) to yield relative quantities of viable cells as percentages of untreated controls, and 50% inhibitory concentrations ($IC_{50}$) were interpolated from concentration-effect curves. Calculations are based on at least three independent experiments with triplicates for each concentration level.

### 2.3 Western blotting

Based on the results of the cytotoxicity test, the human ovarian carcinoma cell line (CH1) and human colon carcinoma cells (SW480) were chosen for the Western blot with the purpose to detect the cleavage of apoptosis-associated protein PARP after drug treatment.
Cells were seeded in densities of $2 \times 10^5$ cell/well (CH1) and $3 \times 10^5$ cell/well (SW480) into 6-well plates (Iwaki). The cells were incubated for 24 h at 37 ºC with the compounds **2c** and **5c** in different concentrations. After the treatment the cells were lysed by adding SDS sample buffer [62.5 mM Tris-HCl (pH 6.8 at 25 °C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue]. The proteins in the cell extract were separated by 8% SDS-polyacrylamide gel electrophoresis. Subsequently the proteins were transferred onto nitrocellulose membranes (Millipore) using a semi-dry blotting apparatus (Biorad). The membranes were blocked with blocking buffer [1x Tris-buffered saline, 0.1% Tween-20 with 5% w/v nonfat dry milk] and immunoblotted with the relevant primary antibodies [PARP-rabbit (1:1000)]. Primary antibodies were detected using peroxide-conjugated secondary antibody [anti-rabbit and anti-biotin antibodies (1:3000)] and visualized with a chemiluminescence detection system using luminol reagent [500 µl LumiGLO 20x, 500 µl peroxide 20x and 9 ml Milli-Q water]. Successful results are based on repetition and comparison of different protocols which require optimization according to the experiment conditions [modified after www.cellsignal.com].

### 2.4 Cell cycle analysis

To study effects on the cell cycle of exponentially growing CH1 cells by flow cytometric analysis of their relative DNA content, cells were harvested from culture flasks, seeded in complete medium into 90-mm Petri dishes ($1 \times 10^6$ cells/dish) and, after recovery for 24 h, exposed to various concentrations of the test compounds for 24 h. For this purpose, test compounds were diluted from DMSO stocks with complete medium (see above) such that the effective DMSO content did not exceed 0.5%. After exposure, treated and control cells were collected by scratching, washed with PBS and stained with hypotonic fluorochrome solution (HFS buffer) which contains propidium iodide for staining the DNA [sodium citrate 0.1% (w/v), Triton X-100 0.1% (v/v), PBS; shortly before use, add 5 µg/mL propidium iodide]. Their fluorescence was measured with a FACS Calibur instrument (Becton Dickinson), and the obtained histograms were

analyzed with Cell Quest Pro software (Becton Dickinson). At least two independent experiments were performed for each setting, and 2.5 or $3.0 \times 10^4$ cells were measured per sample.

### 2.5 Kinase assay

The Cdk-inhibitory capacities of test compounds were studied by a radiochemical assay using recombinant Cdk1/cyclin B and Cdk2/cyclin E isolated from Sf21 insect cells and histone H1 as the substrate for phosphorylation, as described by Marko et al. [31]. Briefly, MOPS-buffered assay mixtures containing the test compound (with a maximum of 1% DMSO), the respective kinase/cyclin complex, histone H1, and 0.4 μCi ($\gamma$-$^{32}$P) ATP per sample were incubated for 10 min at 30 °C. Following a short centrifugation, the aliquots of the solution were added onto phosphocellulose squares, which had been washed three times with 0.75% phosphoric acid followed by acetone. The dried squares were measured in scintillation vials by β-counting (PerkinElmer Tri-Carb 2800TR; Quanta Smart software). Results were obtained in duplicates in at least two independent experiments, and $IC_{50}$ values were calculated by interpolation [16].

# 3. Results and discussion

## 3.1 Cytotoxicity in cancer cells

Antiproliferative capacity of the ligands **L1**–**L3** and corresponding [MCl($\eta^6$–*p*–cymene)L]Cl complexes containing either Ru(II) (**11a**–**13a**) or Os(II) (**11b–13b**) as a metal center were studied for their influence on cell grow in human cancer cells (A549, CH1, SW480). Obtained $IC_{50}$ values are listed in Table 3.1. All compounds show the strongest effect in the generally chemosensitive ovarian carcinoma cells, whereas the more chemoresistant non-small cell lung cancer cells are also less affected by the tested compounds. The cytotoxicity of the metal-free ligands decreases in the following order: **L3** > **L2** > **L1**, indicating that both the bromo and the methoxymethyl substituents are advantageous for cytotoxic potency. These structure-activity relationships were also shown for the corresponding cymene ruthenium and osmium complexes: **13a>12a>11a** and **13b>12b>11b**, but $IC_{50}$ values are shifted to higher concentrations (Figure 3.1). Differences between ruthenium and osmium analogues are not dramatic, osmium complexes being at least as active their ruthenium analogues.

Table 3.1. Antiproliferative activity of metal-free ligands (**L1–L2**), their ruthenium(II) (**11a–13a**) and osmium(II) (**11b–13b**) arene complexes in three human cancer cell lines.

| Compound | $IC_{50}$, μM [a] | | |
|---|---|---|---|
| | **CH1** | **SW480** | **A549** |
| **L1** | 11 ± 3 | 23 ± 6 | 29 ± 7 |
| **11a** | 96 ± 18 | > 320 | 525 ± 102 |
| **11b** | 64 ± 19 | 223 ± 29 | > 640 |
| **L2** | 1.5 ± 0.6 | 5.1 ± 1.0 | 6.7 ± 0.3 |
| **12a** | 21 ± 3 | 70 ± 8 | 268 ± 35 |
| **12b** | 22 ± 3 | 29 ± 2 | 123 ± 21 |
| **L3** | 0.63 ± 0.09 | 0.74 ± 0.26 | 5.2 ± 0.5 |
| **13a** | 11 ± 1 | 11 ± 2 | 68 ± 12 |
| **13b** | 7.9 ± 2.2 | 12 ± 2 | 89 ± 11 |

[a] 50% inhibitory concentrations (means ± standard deviation from at least three independent experiments), as obtained by the MTT assay (exposure time: 96 h)

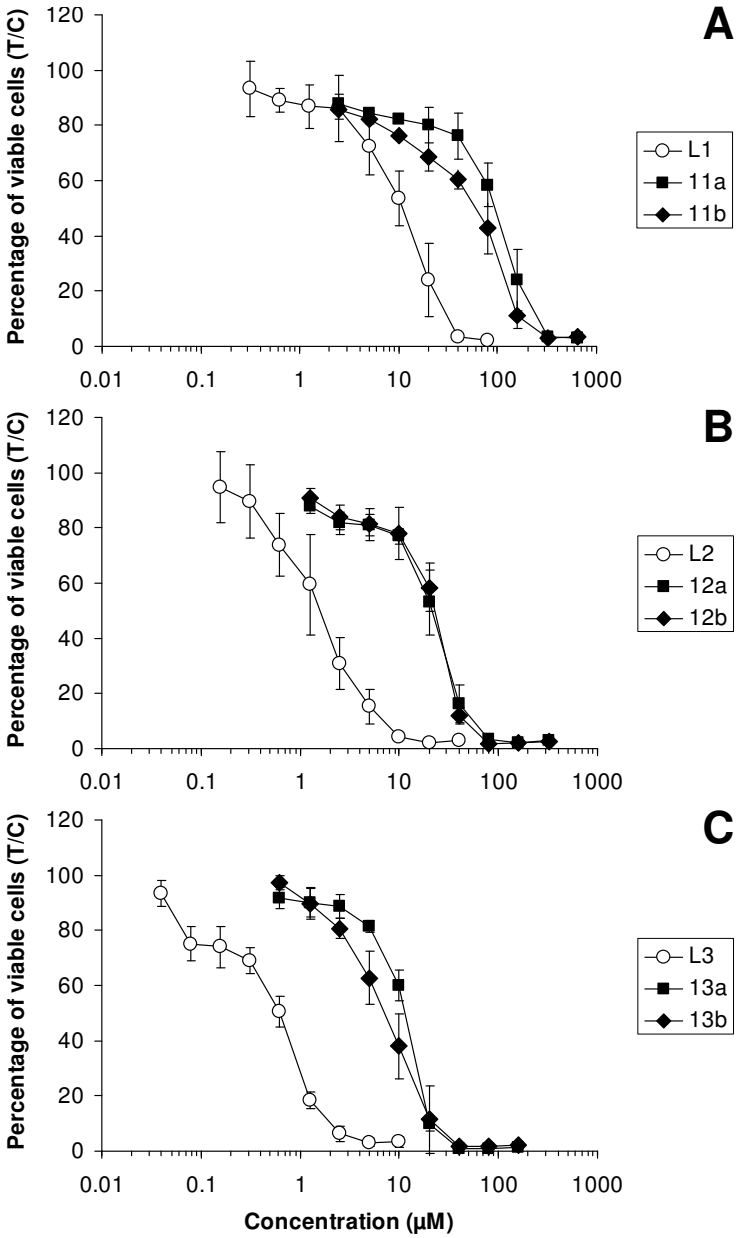



Figure 3.1. Concentration–effect curves of each ligand compared to the corresponding ruthenium and osmium complexes in CH1 ovarian cancer cells (MTT assay, 96 h exposure): (A) **L1**, **11a**, **11b**; (B) **L2**, **12a**, **12b**; (C) **L3**, **13a**, **13b**. Both the presence of substituents in the ligands and complexation result in a marked shift of antiproliferative activity.

The second group of compounds was tested for antiproliferative capacities on A549, CH1 and SW480 cells as well. The $IC_{50}$ values of **1c**–**5c** are compared to those of $[RuCl(\mu\text{–}Cl)(\eta^6\text{–arene})]_2$ (**Dimer**) in Table 3.2. The compounds were active on the chemosensitive ovarian carcinoma cells and at least some of them showed effect on the chemoresistant non-small cell lung cancer cells. Concentration–effect curves of **1c**–**5c** and $[RuCl(\mu\text{–}Cl)(\eta^6\text{–arene})]_2$ (**Dimer**) in cancer cells are depicted in Figure 3.2. The structure–activity relationship of complexes **1c–3c** is less clear-cut, which may be caused by the borderline solubility associated with the presence of the 4-formylphenoxyacetylbenzylamide ligand. In SW480 and A549 cells, complexes **1c**–**3c** show no antiproliferative activity in concentrations up to 320 μM, and neither do **4c** and $[RuCl(\mu\text{–}Cl)(\eta^6\text{–arene})]_2$ (**Dimer**) in A549 cells (Figure 3.2). The most active of the complexes bearing a 4-formylphenoxyacetylbenzylamide ligand is the Paullone complex **5c** with $IC_{50}$ values of 29 μM in CH1 cells, 49 μM in SW480 and 123 μM in A549 cells. This Paullone complex (**5c**) with a derivatized lactam unit shows higher antiproliferative activity than the Paullone complex with unmodified lactam group (**4c**) in all three cell lines, as was reported for $[RuCl(\eta^6\text{–}p\text{-cymene})(L)]Cl$ complexes **4a** and **5a** (as well as their osmium analogues) with Paullones **L4** and **L5** [41].

Table 3.2. Antiproliferative activity of ruthenium(II) (**1c–5c**) and **Dimer** arene complexes in three human cancer cell lines.

| Compound | $IC_{50}$, μM [a] | | |
|---|---|---|---|
| | **CH1** | **SW480** | **A549** |
| **1c** | 142 ± 33 | > 320 | > 320 |
| **2c** | 32 ± 13 | > 320 | > 320 |
| **3c** | 153 ± 42 | > 320 | > 320 |
| **dimer** | 65 ± 21 | 215 ± 35 | 420 ± 11 |
| **4c** | 55 ± 15 | 179 ± 24 | > 320 |
| **5c** | 29 ± 2 | 49 ± 2 | 123 ± 20 |

[a] 50% inhibitory concentrations (means ± standard deviation from at least three independent experiments), as obtained by the MTT assay (exposure time: 96 h)

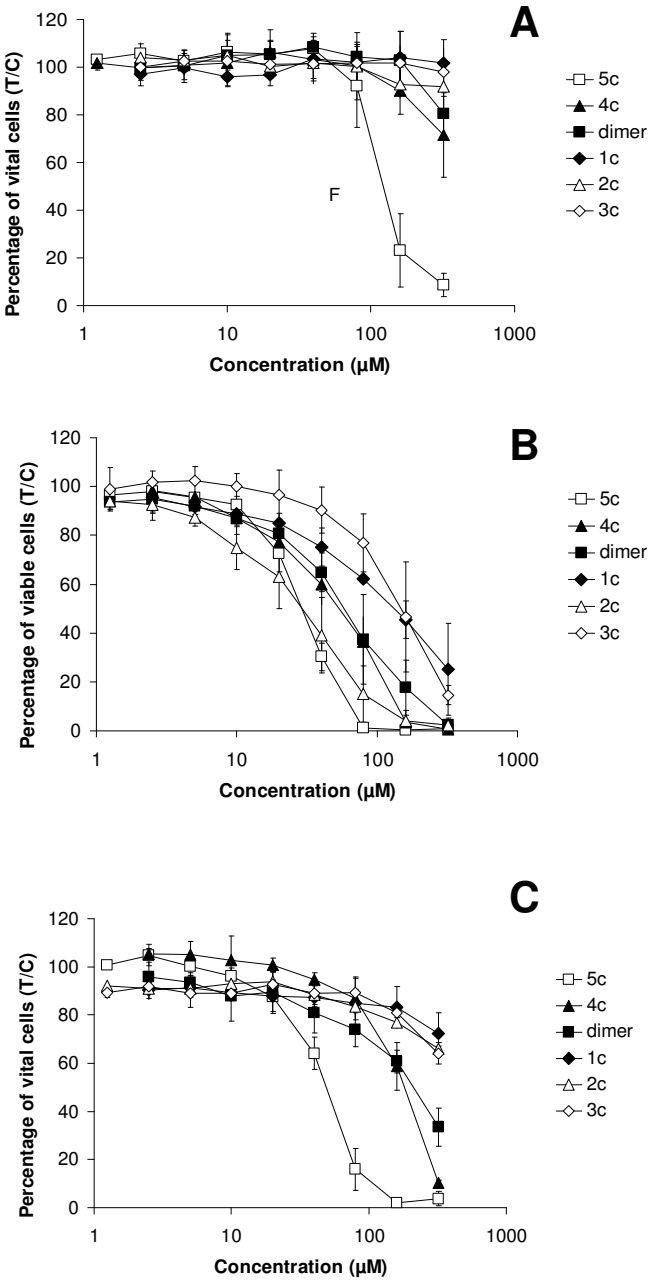



Figure 3.2. Concentration–effect curves of **1c**–**5c** and [RuCl($\mu$–Cl)($\eta^6$–arene)]$_2$ (**Dimer**) in A549 non-small cell lung cancer cells (A), CH1 ovarian carcinoma cells (B) and in SW480 colon carcinoma cells (C) obtained by the MTT assay (96 h exposure).

The impact of conjugation **1c**–**5c** to rHSA on their antitumor potency in vitro was studied in ovarian carcinoma cell lines A2780 and A2780cisR with sensitivity and resistance to cisplatin, respectively, by P. Dyson [1]. As expected from the cytotoxicity data mentioned above, the ruthenium complexes alone did not show significant effect on cell growth in the tested

concentrations range. Upon conjugation with rHSA, $IC_{50}$ values of 26 and 28 µM were observed in the two cell lines. This indicates that the conjugation strategy overcomes the resistance mechanism that blocks entry and/or increases efflux of cisplatin from the cells.

### 3.2 Effects on the cell cycle

Since 3-(1*H*-benzimidazol-2-yl)-1*H*-pyrazolo[3,4-*b*]pyridines have been reported to be potent Cdk inhibitors, we expected these ligands and complexes to inhibit the cell cycle as well. Human ovarian carcinoma cells (CH1) were treated with these compounds in various concentrations for 24 h, then stained with propidium iodide and analyzed for their DNA content by fluorescence-activated cell sorting (FACS).

Unexpectedly, within the metal-free ligands **L1**–**L3** only slight effects on the cell cycle distribution were observed. Only a slight increase of the G2/M fraction was observed, from 32% in the untreated control to 53% by 20 µM **L2** as the strongest effect (Figure 3.3).

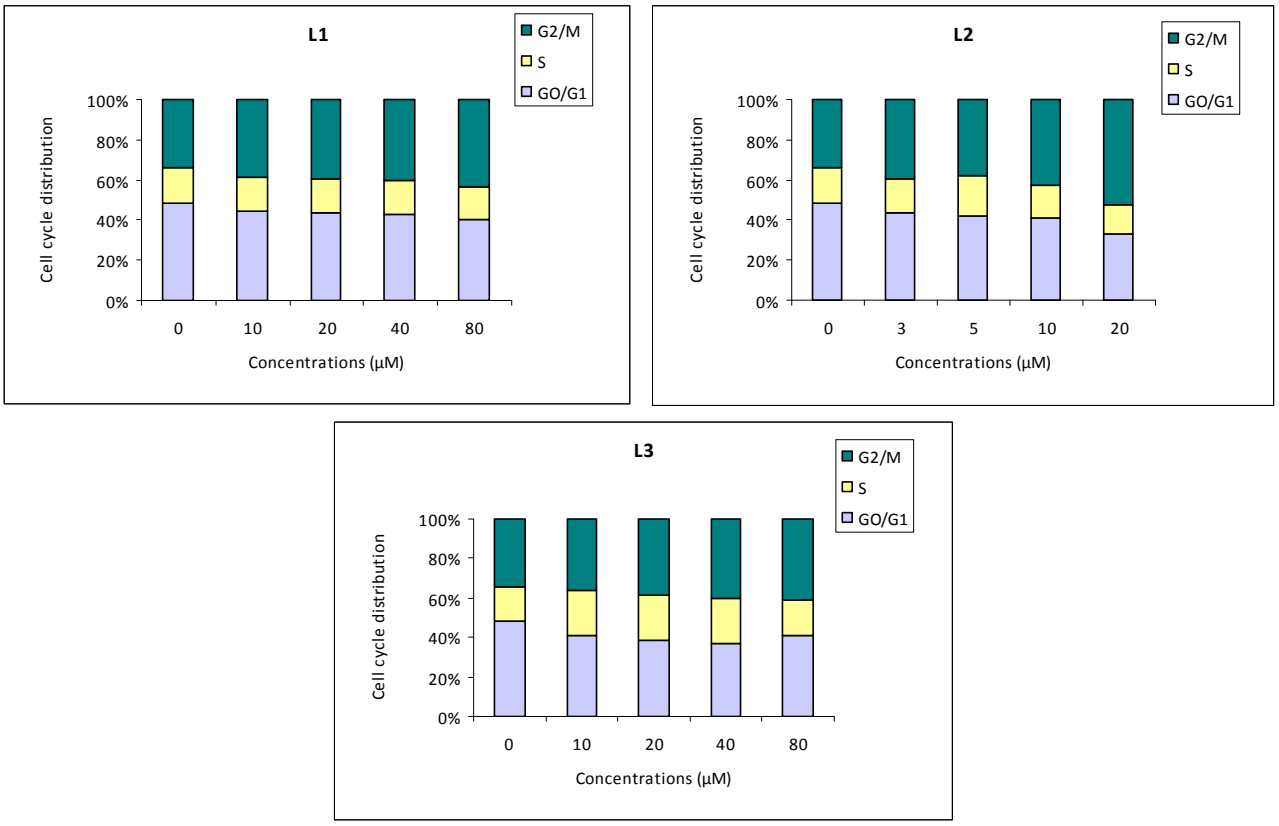



Figure 3.3. Concentration-dependent impact of **L1**, **L2** and **L3** on the cell cycle distribution of CH1 cells after exposure for 24 h. The DNA content of cells stained with propidium iodide was analyzed by flow cytometry.

The complexes showed stronger effect on the cell cycle distribution, especially those with a ruthenium center. These experiments showed that the ruthenium complex **12a** (with the bromo benzimidazolyl-pyrazolopyridin ligand) induces a strong G2/M phase arrest with 65% of all cells at 80 µM, followed by **13a** (with bromo-methoxymethyl benzimidazolyl-pyrazolopyridin ligand) with 59% at 40 µM (Figure 3.4). The least effect was observed for the ruthenium complex with the unsubstituted ligand (**11a**).

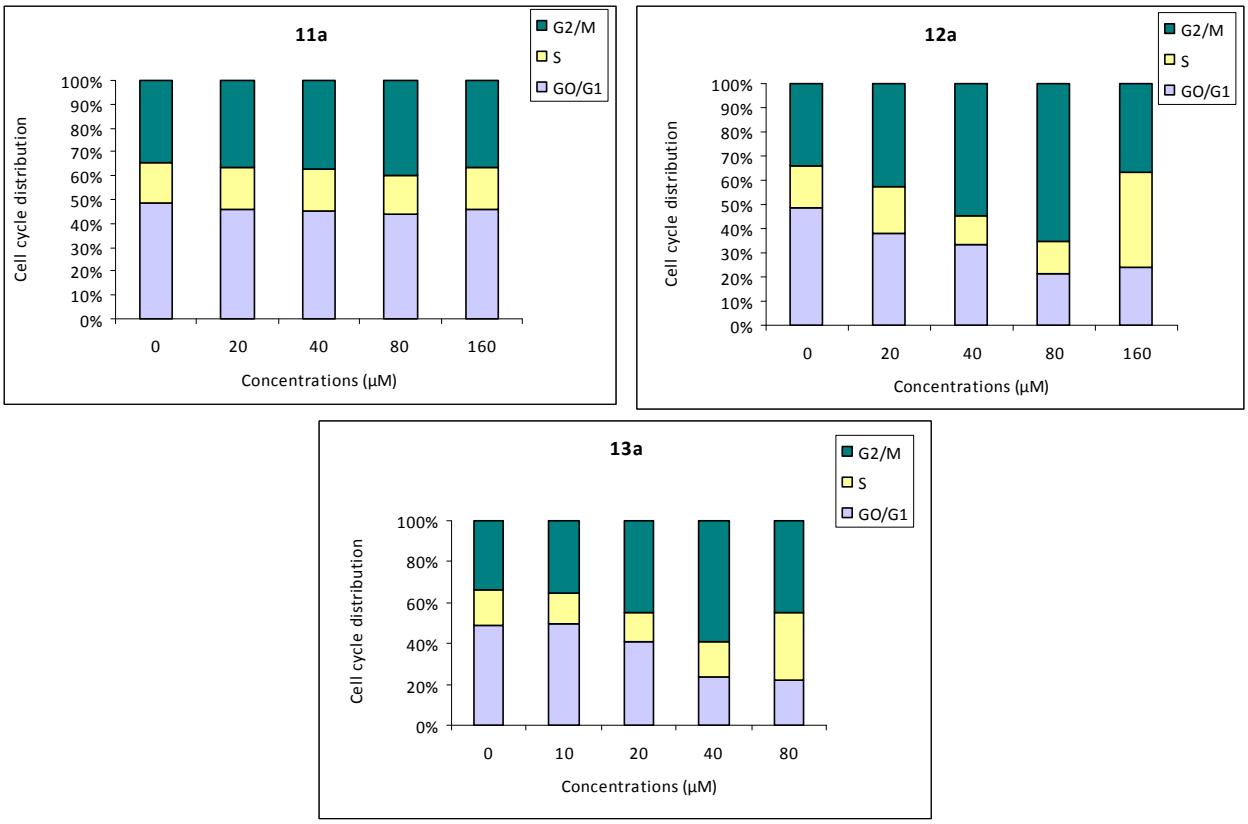



Figure 3.4. Concentration-dependent impact of **11a**, **12a** and **13a** on the cell cycle distribution of CH1 cells after exposure for 24 h. The DNA content of cells stained with propidium iodide was analyzed by flow cytometry.

Osmium complexes do not generally show stronger effect on the cell cycle than the metal-free ligands. The osmium complex **13b** showed effect in the G2/M phase, arresting up to 53% at 80 µM. (Figure 3.5).

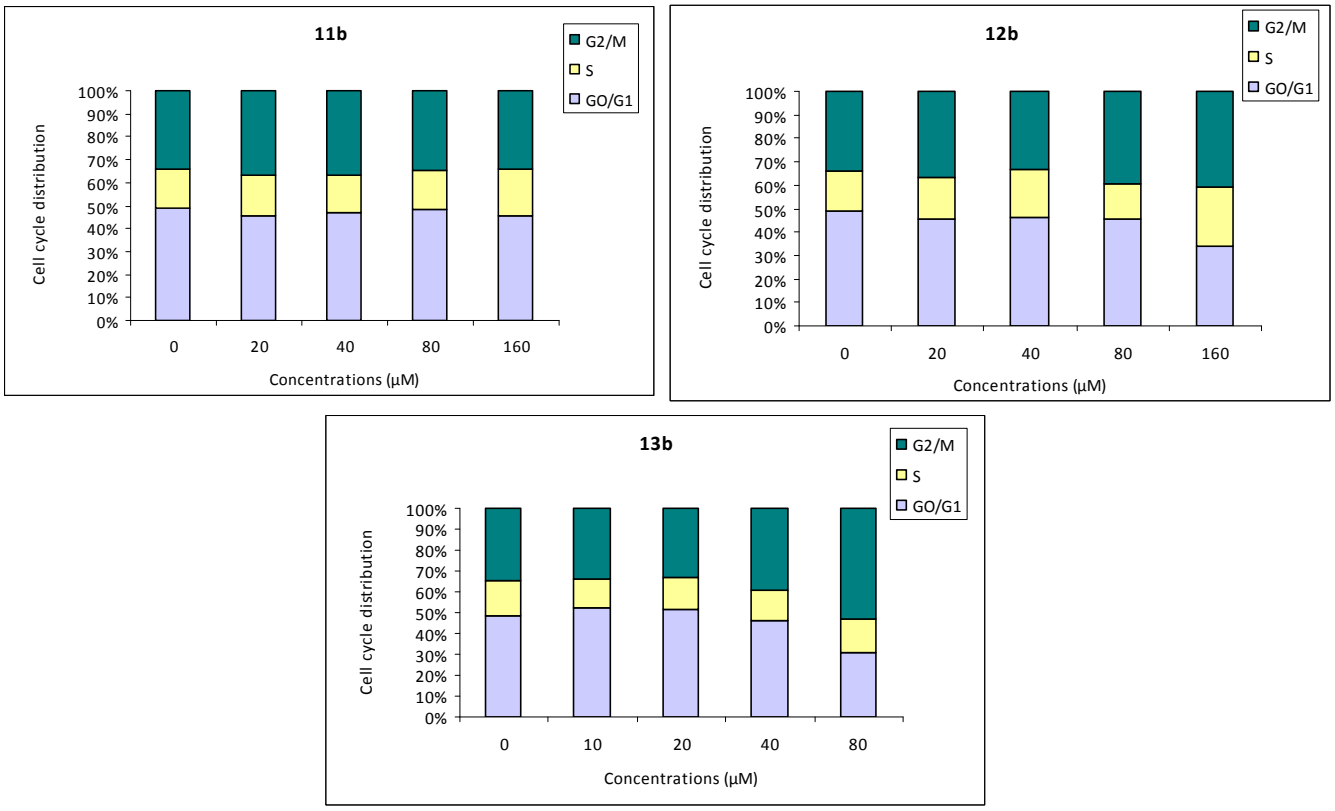



Figure 3.5. Concentration-dependent impact of **11b**, **12b** and **13b** on the cell cycle distribution of CH1 cells after exposure for 24 h. The DNA content of cells stained with propidium iodide was analyzed by flow cytometry.

The impact of ruthenium compounds from the other group on the cell cycle was analyzed by FACS as well. These experiments showed that complexes **4c** and **5c** with indolobenzapine-derived ligands **L4** and **L5**, respectively, induce the cell phase arrest more effectively than **2c** with a pyrazolopyridine-derived ligand (**L2**). Figure 3.6 depicted that the ruthenium complex **5c** (**L=L5**) induces the strongest G2/M phase arrest of 81% of all cells at 80 µM, followed by **4c** (**L=L4**) with 55% at 80 µM. The least effect showed the ruthenium complex **2c** with 42% of the arrested cells at 160 µM (**L=L2, X=Br, Y=H**).

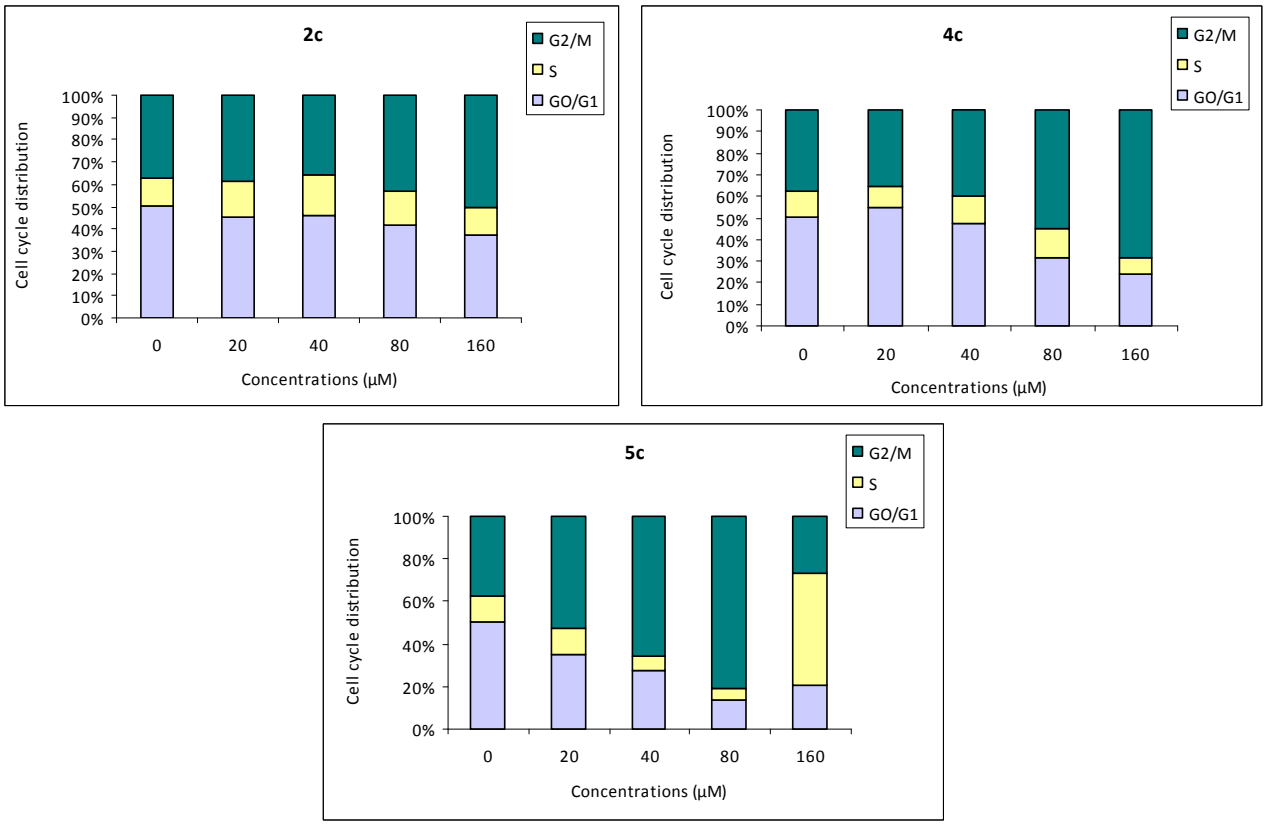



Figure 3.6. Concentration-dependent impact of **2c**, **4c** and **5c** on the cell cycle distribution of CH1 cells after 24 h exposure. The DNA content of the cells stained with propidium iodide was analyzed by flow cytometry.

With the example of **4c** and **5c**, we can assert that the differences in the position of the chelating fragment (e.g. lactam ring) modulate the antiproliferative capacity of the compounds and, correspondingly, change the capacity to inhibit the cell-cycle progression.

### 3.3 Cdk inhibition

Inhibitory potencies of the ligands **L1**–**L3** and corresponding [RuCl($\eta^6$−*p*−cymene)L]Cl complexes containing Ru(II) (**11a**–**13a**) or Os(II) (**11b–13b**) as a metal center were studied in cell-free experiments by the cyclin-dependent kinase assay in comparison to flavopiridol as a positive control (Tables 3.3, 3.4).

Table 3.3. Concentration-dependent inhibition of Cdk1/cyclin B and Cdk2/cyclin E activity (means ± standard deviations) by flavopiridol (FP) as a positive control.

| | Cdk1/cyclin B | | Cdk2/cyclin E | |
|---|---|---|---|---|
| | Conc. (μM) | Inhibit. (%) | Conc. (μM) | Inhibit. (%) |
| FP | 1 | 10.2 ± 2.3 | 1 | 37.5 ± 4.5 |
| | 10 | 35.3 ± 9.1 | 10 | 66.2 ± 5.5 |
| | 50 | 67.0 ± 7.5 | 50 | 93.1 ± 0.7 |
| | 100 | 95.4 ± 0.8 | 100 | 97.3 ± 2.0 |

Table 3.4. Concentration-dependent inhibition of Cdk1/cyclin B and Cdk2/cyclin E activity (means ± standard deviations) by the 3−(1*H*−benzimidazol−2−yl) pyrazolo[3,4−*b*]pyridines ligands (**L1** – **L3**).

| | Cdk1/cyclin B | | | Cdk2/cyclin E | |
|---|---|---|---|---|---|
| Subst. | Conc. (μM) | Inhibit. (%) | Subst. | Conc. (μM) | Inhibit. (%) |
| **L1** | 1 | 7.1 ± 1.3 | **L1** | 1 | 55.7 ± 2.4 |
| | 10 | 36.2 ± 3.0 | | 10 | 80.9 ± 4.1 |
| | 50 | 51.0 ± 5.8 | | 50 | 87.0 ± 2.8 |
| | 100 | 69.5 ± 10.4 | | 100 | 87.7 ± 2.5 |
| **L2** | 1 | 17.5 ± 10.2 | **L2** | 1 | 69.6 ± 4.1 |
| | 10 | 43.3 ± 2.8 | | 10 | 76.5 ± 2.7 |
| | 50 | 46.1 ± 3.3 | | 50 | 81.6 ± 4.2 |
| | 100 | 51.4 ± 4.0 | | 100 | 90.7 ± 4.4 |
| **L3** | 1 | 22.5 ± 0.9 | **L3** | 1 | 24.4 ± 0.1 |
| | 10 | 37.1 ± 5.8 | | 10 | 71.1 ± 6.3 |
| | 50 | 48.4 ± 5.3 | | 50 | 80.2 ± 5.5 |
| | 100 | 55.9 ± 2.8 | | 100 | 91.8 ± 0.0 |

Measurements of kinase activity of recombinant Cdk/cyclin complexes showed that all compounds are capable of inhibiting kinase activities in a concentration-dependent manner. Moreover, this assay revealed a higher inhibitory potency of the metal-free ligands compared

with their related complexes (Table 3.4). Also, the inhibitory capacity of these ligands was more effective in tests with Cdk2/cyclin E than with Cdk1/cyclin B.

The ruthenium(II) and osmium(II) complexes possess lower inhibitory potency compared to the ligands (Table 3.5). The inhibitory potencies of ruthenium and osmium analogues show no dramatic differences in these settings, but all complexes inhibit Cdk2/cyclin E more efficiently than Cdk1/cyclin B, as was observed for all ligands (Figure 3.7).

Table 3.5. Concentration-dependent inhibition of Cdk1/cyclin B and Cdk2/cyclin E activity (means ± standard deviations) by Ru/Os complexes [$M^{II}Cl(\eta^6$–cymene)(L)]Cl (**11a**, **12a**, **13a/ 11b**, **12b**, **13b**).

| | Cdk1/cyclin B | | | Cdk2/cyclin E | |
|---|---|---|---|---|---|
| Subst. | Conc. (µM) | Inhibit. (%) | Subst. | Conc. (µM) | Inhibit. (%) |
| **11a** | 1 | 5.1 ± 1.7 | **11a** | 1 | 6.7 ± 3.2 |
| | 10 | 6.6 ± 1.9 | | 10 | 6.7 ± 1.4 |
| | 50 | 25.1 ± 6.2 | | 50 | 60.5 ± 7.7 |
| | 100 | 30.3 ± 4.2 | | 100 | 65.5 ± 5.7 |
| **11b** | 1 | 9.1 ± 0.5 | **11b** | 1 | 12.7 ± 2.5 |
| | 10 | 12.4 ± 1.9 | | 10 | 14.1 ± 3.4 |
| | 50 | 30.8 ± 0.6 | | 50 | 44.9 ± 3.2 |
| | 100 | 38.0 ± 3.9 | | 100 | 66.9 ± 7.0 |
| **12a** | 1 | 6.2 ± 1.1 | **12a** | 1 | 7.9 ± 2.0 |
| | 10 | 9.1 ± 0.5 | | 10 | 13.7 ± 1.1 |
| | 50 | 34.6 ± 1.8 | | 50 | 51.2 ± 6.7 |
| | 100 | 45.3 ± 6.6 | | 100 | 74.7 ± 3.6 |
| **12b** | 1 | 11.3 ± 3.7 | **12b** | 1 | 12.7 ± 1.6 |
| | 10 | 15.0 ± 2.7 | | 10 | 22.5 ± 4.6 |
| | 50 | 32.4 ± 9.7 | | 50 | 68.8 ± 5.2 |
| | 100 | 42.5 ± 9.0 | | 100 | 81.6 ± 4.0 |
| **13a** | 1 | 7.8 ± 0.1 | **13a** | 1 | 19.2 ± 4.0 |
| | 10 | 13.2 ± 2.0 | | 10 | 36.8 ± 1.5 |
| | 50 | 19.1 ± 0.4 | | 50 | 52.8 ± 3.9 |
| | 100 | 24.2 ± 1.3 | | 100 | 61.1 ± 3.3 |
| **13b** | 1 | 3.4 ± 0.2 | **13b** | 1 | 9.4 ± 2.5 |
| | 10 | 11.7 ± 2.2 | | 10 | 45.4 ± 0.4 |
| | 50 | 21.5 ± 5.5 | | 50 | 51.7 ± 4.7 |
| | 100 | 23.5 ± 4.2 | | 100 | 52.3 ± 1.8 |

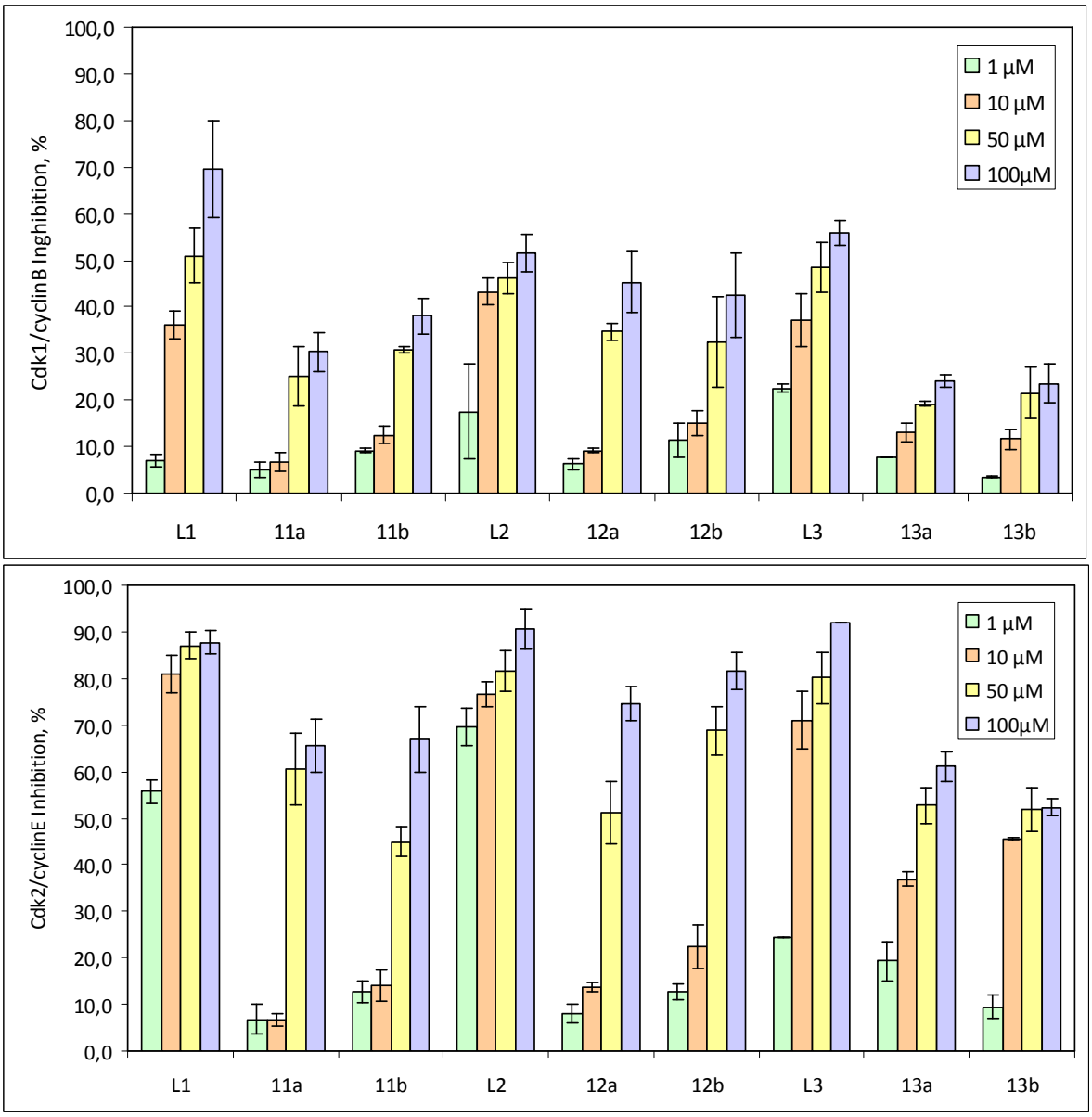



Figure 3.7. Activity of recombinant enzyme Cdk1/cyclin B and Cdk2/cyclin E measured by phosphorylation of histone H1 using 0.4 µCi ($\gamma$-$^{32}$P) ATP.

After analyzing our data, we can assert that only **L1**–**L3** effectively inhibit Cdk2/cyclin E in low concentrations. According to the results, it can be stated that the complexes have lower inhibitory ability than the corresponding free ligands, in accordance with MTT results.

On one hand, the results of the kinase inhibition experiments do not correlate strictly with cell cycle analysis data. The ligands exerting minor effects on the cell cycle show high Cdk inhibitory potency, and vice versa the corresponding ruthenium complexes cause relatively high inhibition of the cell cycle and weak Cdk inhibition.

On the other hand, we can compare results of Cdk inhibition experiments with the results of the cytotoxity tests. The most potent inhibitors are **L1**–**L3** for both Cdks, and these ligands have shown the strongest cytotoxic effect on all cell lines as well. However, ligands have shown only slight effects on the cell cycle distribution. This fact may be connected with settings of the experiments. Cdk experiments were conducted in a cell-free setting, unlike cytotoxicity tests and cell cycle distribution, only when the compound enters the cell, it may influence at the cell cycle mechanisms. These studies could explain why some compounds show pronounced Cdk inhibition bur weak or no effects on the cell cycle.

### 3.4 Induction of apoptosis

We have investigated poly(ADP-ribose) polymerase (PARP1) cleavage with Western blotting. Cleavage of this apoptosis-associated protein was analyzed in CH1 and SW480 cells treated with bromo-substituted ruthenium complexes **2c** and **5c**. Cleavage of PARP1 was significantly detected in CH1 cells only after **5c** exposure (Figure 3.8).

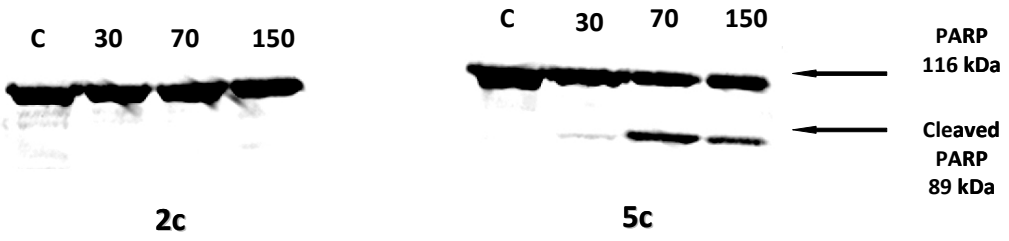



Figure 3.8. PARP cleavage was indicated by Western blotting after treatment with compound **2c** and **5c** (0, 30, 70 and 150 μM) in **CH1** cells.

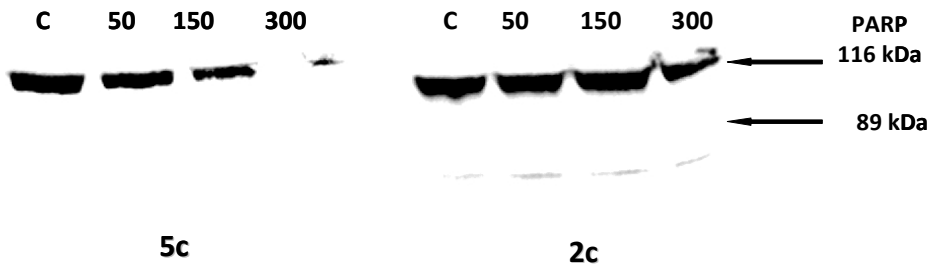



Figure 3.9. PARP cleavage was not indicated by Western blotting after treatment with compounds **2c** and **5c** (0, 50, 150 and 300 μM) in **SW480** cells.

PARP cleavage was indicated in the Western blots by the decrease in intensity of the 116 kDa bands and presence of an 89 kDa band. Ovarian carcinoma and colon cancer cells were treated with ruthenium complexes at various concentrations. Cleaved PARP, indicating the induction of apoptosis, was found after **5c** treatment in CH1 cells only. With increasing concentration of **5c** PARP fragmentation increased in a dose-dependent mode. Cleavage of PARP under the influence of ruthenium compounds with indolobenzapine-derived ligands means these complexes induce an apoptotic cascade in the ovarian carcinoma cells.

# 4. Conclusions

The cytotoxicity of the cymene complexes with 3−(1*H*−benzimidazol−2−yl)-pyrazolo[3,4−*b*]pyridines ligands has been characterized by several biological methods. The structure–activity relationship of the ruthenium and osmium complexes asserts that modulation of biological activities by substitution at the ligands can be achieved in the metal-free compounds and their metal complexes in a similar way. The Cdk inhibitory potency of the ligands and their higher capacity of inhibiting cell growth were compared to their metal complexes. However, the metal complexes especially those with the ruthenium center show stronger effects than ligands on cell cycle distribution in human ovarian carcinoma cells than ligands. At the same time, cell cycle experiments indicated that both the bromo and the methoxymethyl substituents are advantageous for cytotoxic potency. The structure–activity relationship of the ruthenium complexes designed for conjugation to rHSA is less clear-cut, which can be caused by the borderline solubility associated with the presence of the 4-formylphenoxyacetylbenzylamide ligand. The most active of the complexes bearing a 4-formylphenoxyacetylbenzylamide ligand is the Paullone complex. At the same time, this Paullone complex with a derivatized lactame unit and the second Paullone complex with unmodified lactame group did not pose any solubility difficulties in comparison with other complexes of this group. The ruthenium complexes with Paullone ligands can induce the arrest of G2/M and S phase. At the same time this complex induces the cleavage of apoptosis-associated protein (PARP1) in CH1 cell line.

According to the investigations of ruthenium complexes from both groups, we can assert they have a noticeable effect on cancer cells. Knowledge and understanding of the details of the mechanism of action could help in creating new drugs, improving their potency and selectivity. Future investigations should be focused on apoptotic mechanisms associated with Cdk pathways as well. It is necessary to clarify the mode of action for the ruthenium complexes which showed stronger impact than osmium analogues on the cell cycle distribution, since these complexes showed a low potency to inhibit cell grow and kinases activity. At the same time, DNA damage/repair and apoptosis induction under the influence of such complexes can be characterized as well as studied by means of Comet Assay and flow cytometry with double-staining (propidium iodide and Annexin V) respectively.

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# 6. Appendix

## 6.1 Curriculum vitae

**Maria S. Novak**

Born: January 4th, 1988

Place of birth: Saint-Petersburg

Nationality: Russian

Education

- Student, primary school № 630, St.Petersburg, 1995-1996;
- Student, primary school, secondary school and high school № 163 (specialization in humanitarian disciplines), St.Petersburg, 1996-2005;
- Undergraduate Student the faculty of Biology and Pedology, Department of Cytology and Histology, St.Petersburg State University, Russia, 2005-2009;
- Bachelor Degree, the faculty of Biology and Pedology, Department of Cytology and Histology, St.Petersburg State University, 2009;
- Magister Student of the Department of Cytology and Histology, St.Petersburg State University, 2009-2010.

Additional training

- Trainee, Laboratory of Genosystematics "TAXON", Zoological Institute of Russian Academy of Sciences, St. Petersburg, Russia, 2008. Practiced methods of manipulations with DNA.
- Trainee, Laboratory of hybridomical technologies, Institute of Radiology and Surgical technologies, St.Petersburg, Russia, 2008. Practiced Immunology methods.
- Summer student, Institute of Molecular Biotechnology, Austrian Academy of Sciences, Josef Penninger's group, Vienna, Austria (July–October, 2009).

Research experience

- Bachelor Graduation Work «Polymorphisms of genes of renine-angiotensin system, serotonin system and antioncogene *P53* and their correlation with psychological strategies of stress defense» under the supervision of PhD Irina M. Spivak, in Institute of Cytology

of the Russian Academy of Science (Laboratory of Radiation Cytology), St.Petersburg, Russia, 2007-2009;

- Summer Research Work «The E3 ligase HACE1 is a tumor suppressor involved in multiple cancers» under the supervision of Prof. Josef M. Penninger and postdoc Roberto Nitsch, Institute of Molecular Biotechnology Austrian Academy of Sciences, Josef Penninger's group, Vienna, Austria, 2009.

Honors and Awards

- The Central district of St.Petersburg Biology Olympiad, Certificate for the second place, 2002;

- The Central district of St.Petersburg Biology Olympiad, Certificate of honor for the first place, 2003.

Conferences

- Poster presentation at the «The 13th international conference of young scientists» Puschino, Russia, 2009;

- Oral presentation at the conference «Psychiatry consultation and interaction», Saint-Petersburg, Russia, 2009;

- Oral presentation at the 3rd International Youth Medical Congress, Section of Obstetrics and Gynecology, Saint-Petersburg, Russia, 2009.

- Co-author of the poster presentation «Organoruthenium Cdk inhibitors with a chance to be delivered by a carrier protein molecule (rHSA) to cancer cells» at the 24th International Conference on Organometallic Chemistry (24th ICOMC); July 18–23, 2010, Taipeh, Taiwan (Presented by Iryna Stepanenko).

Publications

- M.S. Novak, T.Ju. Smirnova, I.M. Spivak, D.L. Spivak. « Polymorphisms of genes of renine-angiotensin system, serotonin system and antioncogene *P53* and their correlation with psychological strategies of stress defense». «Biology and science» 2009: 34;

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References


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