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Collation of Anticancer Activities for Benzo/Hydroxypyrone Ligands and Copper(II) Complex with Maltol on 2D Cell Models *In Vitro*

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Abstract

Heterocyclic structures are the basic building blocks of many naturally occurring organic compounds that are important for the development of essential biological processes in plants and animals, including carbohydrates and vitamins. They are also widely used as raw material sources to produce pharmaceuticals. In this study, three ligands from the groups of benzopyrones (chromone-2-carboxylic acid) and hydroxypyrones (maltol and coumalic acid), and a newly prepared complex of copper(II) nitrate with maltol as a ligand were investigated for their ability to inhibit cell proliferation. For research purposes, a series of solutions of the tested materials were prepared at different final concentrations (10^{-5} mol dm^{-3}, 10^{-6} mol dm^{-3}, and 10^{-7} mol dm^{-3}), and then applied to a total of 7 selected cell lines, including one healthy cell line, while the others were tumour cell lines. The results of MTT cytotoxicity assay on the selected 2D cell models showed that none of the selected ligands exhibited antiproliferative activity at any concentration on any of the tested cell lines. The complex of copper(II) nitrate and maltol, in contrast to the tested ligands at the 10^{-5} mol dm^{-3} concentration, exhibited significant cytotoxicity as follows: KATO III > HT-29 > Hep G2 > NCI-H358 > MDA-MB-231 > Caco-2 > MRC-5. The healthy cell line (MRC-5) had a survival rate higher than 90.0 % at all tested concentrations, which led to the conclusion of the selectivity of the compound towards tumour cell lines.

Keywords

Maltol, chromone-2-carboxylic acid, coumalic acid, copper(II) complex, cell proliferation, MTT assay, cell lines, tumour

1 Introduction

Chromone and its derivatives are found mainly in plants, with algae and conifers having the highest concentrations.[1] They have been shown to perform important biological functions in plants, including promotion of growth and absorption of the oxygen molecules into plant tissue. Chromone structures are also found in some other biologically important substances, such as flavones and isoflavones. Chromones are classified into simple and complicated chromones (pyranochromones and furanochromones) based on their structural properties.[1–2] Due to the presence of an oxygen atom in the pyrone ring and the functionality of two exocyclic oxygen atoms in *ortho* position, they are excellent ligands for bonding with ions of various metals (trivalent such as Fe^{3+}, Al^{3+} and divalent such as: Ni^{2+}, Cu^{2+}, Zn^{2+} or $[VO]^{2+}$, $[MoO_2]^{2+}$, *etc.*) and the formation of complexes. Unlike benzopyrone (chromone), hydroxypyrones are cyclic compounds that, in their structure, contain a pyrone ring to which other functional groups such as hydroxyl or carboxyl are attached. They are O- and N-(hydroxypyridinones) heterocyclic compounds that form a group of ligands with exceptional application in medicinal chemistry. The 3-hydroxy-4(1*H*)-pyrones and their analogues serve as building blocks for the synthesis of biologically active compounds.[3]

From the literature, chromones are known to be cytotoxic. For example, the compound 4′-methoxy-2-styrylchromone showed cell proliferation activity of 70 % in HeLa cells (human cervical tumour), and 25 % in CHO (Chinese hamster ovary cells).[4] Chromone derivatives bearing different dithiocarbamate (234-237)[5] or styryl (238)[6,7] groups have emerged as promising candidates due to their high efficacy and broad spectrum against HCCLM-7, HeLa, MDA-MB-435S, SW-480, Hep-2, MCF-7 (breast adenocarcinoma), and NCl-H460 (non-small cell lung cancer). Further analysis of flow-activated cell sorting revealed that these compounds arrested the cell cycle of SW-480 and MDA-MB -435S in a dose-dependent manner at G2/M phase, and could have an apoptosis-inducing effect on these tumour cell lines. It was found that 6-(3-methylbut-2-enyl) chromone-3-carbaldehyde 239 had antiproliferative and cytotoxic activities against all human carcinoma cell lines studied from a concentration of 2.5 μg cm^{-1}.[3–4]

Various *in vitro*, *in vivo,* and clinical studies have demonstrated the role of chromones in alleviating allergies, reducing oxidative damage, inhibiting cancer, infections and inflammation, and treating neurological and psychiatric disorders.[5–7] Of all the ligands used in this study, maltol is the best known. Nowadays, compounds of maltol and some transition metals such as vanadium are known to have excellent insulinomimetic properties, while their antitumour properties are almost unknown. Chromone-2-carboxylic acid is known as a vasoprotective agent used in the treatment of venous disorders and microvascular diseases.

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Oral treatment of rats with this acid showed a significant decrease in the degradation of the blood vessel wall by intravenous collagenase. Treatment with this compound resulted in a smaller increase in blood-brain barrier permeability, shorter recovery time, lower hydroxyproline levels in cerebrospinal fluid, and a slight decrease in collagen content in the basal lamina of brain capillaries.[8–9] Literature indicates that coumalic acid is also a known phytotoxin and has shown strong toxic side effects when exposed to rats and mice.[10] The biological activity of copper results from its ability to generate reactive oxygen species (ROS), replace binding sites with other metal cations, peroxidise lipids, and directly cleave deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).[11] To date, numerous copper complexes have been synthesised and subjected to biological tests, showing significant antitumour activity in several *in vitro* studies, as well as in several *in vivo* experiments in mice. Elevated copper concentrations have been observed in many human cancers, including lymphoma, retinal cell sarcoma, laryngeal carcinoma, cervical, breast, pancreatic, gastric, and lung cancers.[12] So far, not a single paper has been published in the literature on complexes with chromone-2-carboxylic acid and coumalic acid as ligands. The aim of this research was to study the biological activity of ligands from the group of chromones, and compare it with the biological activity of a complex of copper and maltol, and determine whether the biological activity increases due to the binding of the ligand to copper ions, since literature data show that copper compounds have been intensively studied as antitumour agents after the discovery of cisplatin.

The goal of today's modern inorganic medicinal chemistry is to design and develop new compounds that have metals in their active site, such as copper, vanadium, silver, gold, with emphasis on compound selectivity.[13]

2 Experimental section

2.1 Materials and methods

2.1.2 Preparation of [Cu·(C$_6$H$_6$O$_3$)$_2$] (Complex)

An amount of 5 cm^3 of a 0.05 mmol aqueous (ultrapure water) solution of copper(II) nitrate was mixed with 10 cm^3 of a 0.05 mmol warm water solution of maltol. After mixing, the final solution was neutralised to pH 7.2 with 1 mol dm^{-3} sodium hydroxide (Scheme 1). The obtained reaction mixture contained a pale blue precipitate that was filtered off, washed with cold water (5 cm^3), and air-dried. All chemicals were reagent grade and used without further purification. Copper(II) nitrate (99 %) was obtained from T.T.T., Croatia, and maltol was obtained from Acros Organics, USA.

2.1.3 Characterisation techniques

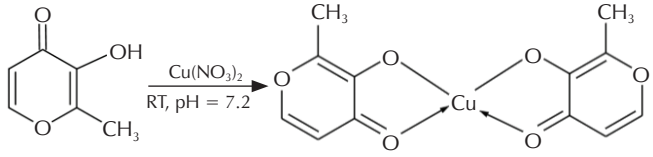
C, H, N, S analysis was determined using Elementar Vario Macro CHNS. Detection range: C: 0.002 % – 100 %; H: 0.015 % – 100 %; N: 0.004 % – 100 %; S: 0.004 % – 100 %.

FT–IR Spectra were recorded using a Shimadzu FTIR 8400S spectrophotometer with a DRS 8000 attachment in the range 4000 – 400 cm^{-1}.

IR (DRS, cm^{-1}): 3500 (*s*), 3360 (*s*), 1749 (*vs*), 1700 (*s*), 1650 (*s*), 1550 (*s*), 1350 (*w-m*), 1050 (*w*), 850 (*w*), 750 (*w*), 500 (*m*)

Analytical calculation for [Cu·(C$_6$H$_6$O$_3$)$_2$]: C (45.86 %), H (2.64 %).

Found: C (45.67 %), H (2.72 %).



Ligand 2 (Maltol) Complex [Cu · (C$_6$H$_6$O$_3$)$_2$]

Scheme 1 – Synthesis of copper(II) complex with maltol
Shema 1 – Sinteza kompleksa bakra(II) s maltolom

2.1.4 Cell lines

The anticancer activity of the ligands and the complex was studied on 7 cell lines obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The malignant cell lines were: Caco-2 and HT-29 (colorectal adenocarcinoma), MDA-MB-231 (human breast adenocarcinoma), KATO III (human gastric adenocarcinoma), Hep G2 (hepatocellular carcinoma), NCI-H358 (bronchioalveolar carcinoma). The normal cell line was MRC-5 (human lung fibroblasts). Cells were grown in a monolayer and cultured in Dulbecco's modified Eagle medium – DMEM with high glucose, 10 % FBS, and 2 mmol dm^{-3} Glutamax. NCI-H358 was grown in Rosswell Park Memorial Institute (RMPI 1640) medium containing 10 % FBS, 2 mmol dm^{-3} Glutamax, 10 mmol dm^{-3} sodium pyruvate, and 2 mmol dm^{-3} HEPES in aerated tissue culture flasks (BD Falcon, Germany) in humidified atmosphere under the conditions of 37 °C and 5 % CO_2 in a CO_2 incubator (IGO 150 CELLlife TM, JOUAN, ThermoFisher Scientific, Waltham, MA, USA). Cell viability was confirmed by erythrosin B. The chemicals and media used for cell cultivation and cell assay were from Sigma Aldrich, St. Louis, USA. DMSO was manufactured by Acros Organics (USA).

2.1.5 Chemicals

The tested ligands: chromone-2-carboxylic acid – **Ligand 1** (Alfa Aesar, USA), maltol – **Ligand 2** (Acros Organics, USA), and coumalic acid – **Ligand 3** (Alfa Aesar, USA) (Fig. 1) were dissolved in DMSO as 10^{-3} mol dm^{-3} stock solution. Final concentrations of 10^{-5} mol dm^3, 10^{-6} mol dm^{-3}, 10^{-7} mol dm^{-3} were prepared by diluting the stock solution in DMEM.

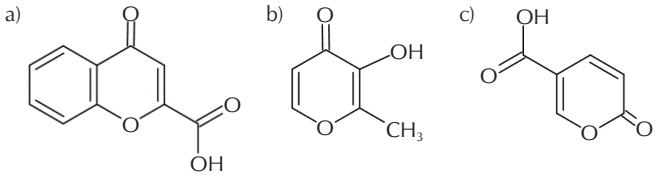


Fig. 1 – Structure formulas of ligands
Slika 1 – Strukturne formule liganada

2.1.6 MTT assay on 2D cell models

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay[14] is used to determine cellular metabolic activity as an indicator of cell proliferation, and cytotoxicity. Cells were seeded in 96-well culture plates at a density of $2 \cdot 10^4$ cells/cm^3 and grown overnight. After 24 h, cells were treated with freshly prepared dilutions of ligands, and incubated for 72 h. On the day of measurement, the medium was discarded and replaced with fresh 1·MTT/PBS solution (5 mg cm^{-3}) followed by incubation at 37 °C for 4 h. DMSO was used to dissolve the water-insoluble MTT formazan crystals. Absorbance was measured at 595 nm using an Elisa microplate reader (iMark, BIO RAD, Hercules, CA, USA). All experiments were performed in triplicate. Data were analysed by STATISTICA™ software version 14.0. for Windows. A nonparametric Mann-Whitney test was applied to evaluate differences between controls and treatments. Results with $P < 0.05$ were considered significant.

3 Results and discussion

The results of FT-IR spectroscopy helped to identify the structure of the Cu(II) complex. Two of the most characteristic bands of free ligand 2 (maltol) are the (O−H) stretching vibration, which is found as a very strong IR band at 3360 cm^{-1}, and the (C−OH) vibration, which is seen as a very weak feature at 1350 cm^{-1}. Peaks at 1350 cm^{-1} and 1650 cm^{-1} were observed, indicating deprotonation of the OH group and binding of the metal cation, in this case Cu^{2+}, to the ligand. After analysis, the complex was subjected to a cytotoxicity test. Compound cytotoxicity is primarily defined as the potential of a substance to cause cell death. Most *in vitro* cytotoxicity tests measure necrosis. *In vitro* assays offer many advantages and allow us to predict human effects by testing compounds on cells. They serve as a way for us to "screen" and establish comprehensive toxicological profiles. The advantages of *in vitro* toxicity testing involve lower costs compared to *in vivo* testing, a high degree of standardisation, reproducibility, and speed of performance, with a lower amount of toxic waste generated, and, of course, the welfare of the test animals. In addition, the use of cell cultures allows a large number of substances to be analysed in a wide range of concentrations in a short time, which is certainly a good default for the design of *in vivo* studies.[15,16]

At the evaluated concentration range, MTT assay showed that the complex of maltol and copper(II) ions at the highest concentration (10^{-5} mol dm^{-3}), exhibited the greatest anti-tumour effect on the KATO III (32.4 %), Hep G2 (35.6 %), and HT-29 (38.1 %) cell lines, respectively. NCI-H358 is less sensitive to the presence of the complex indicating survival rate of 55.1 %. The two remaining tumour cell lines (MDA-MB 231 and Caco-2) were resistant to the presence of the complex, as shown at the Fig. 2d. MRC-5 cells were not affected by the presence of the complex. Their survival rate was above 90 % (90.2 %), indicating selectivity of the complex toward KATO III, Hep G2 and HT-29 cell lines. At the other two examined concentrations (10^{-6} and 10^{-7} mol dm^{-3}), the most sensitive cell lines (KATO III; Hep G2 and HT-29) were less affected, with a proliferation status above 50 %. MRC-5 cells demonstrate equal proliferation ability (90.9 and 94.4 %) regardless of the applied complex concentration.

Unlike the complex, benzo/hydroxypyrone ligands (chromone-2-carboxylic acid – **Ligand 1**; maltol – **Ligand 2**; coumalic acid – **Ligand 3**), do not supress cell growth of investigated cell lines regardless of the tested concentration (Fig. 2a-c). Our results are in contradiction to some previous research findings.[17–19]

One of the most important essential metals, without which our cells cannot live, is copper. The ability to switch between oxidation states (+1) and (+2) is one of the properties that allow copper to form various compounds or complexes, including potential antitumour agents of the future. The toxicity of copper is the result of its redox capacity (conversion of redox states of Cu(I) and Cu(II) in oxidation-reduction reactions), property to displace other ions from enzyme binding sites, high binding affinity to DNA, and ability to induce DNA chain breakage. In most cases, copper modifies the backbone of the complexed ligand and provides better affinity, specificity, and DNA stability.[20] The change in oxidation state is related to the cytotoxicity of the copper complexes synthesised so far, which have been tested *in vitro* and *in vivo* on a number of different cell lines, such as Hela, HT1080, SW872, MCF-7, A 549, Hep-G2; HL-60, normal human fibroblasts.[21] In general, the toxic effect of copper complexes is associated with the induction of cell death by activation of apoptosis or necrosis, which was found for copper complexes with phenanthroline.[22]

A ligand that complexes with the copper ion contributes to the overall efficiency of the copper complex, because it can modulate the targeting and toxicity properties of the metals: sulphur and phosphorus donor ligands stabilise Cu(I), while nitrogen and oxygen donor ligands tend to stabilise Cu(II). In addition, the ligand significantly affects the lipophilic/hydrophilic nature of the resulting complex and can determine its solubility in extracellular fluids as well as its ability to pass through the lipid bilayer of the cell membrane.[23] The hydrophobic or hydrophilic nature of a molecule is indicated by the partition coefficient – logP. LogP is the logarithmic value of the partition coefficient of a solute between octanol and water at nearly infinite dilution.[24] It is often used in drug discovery and development as an indicator of a solute's potential usefulness as a drug. A negative

value for logP means that the compound has a higher affinity for the aqueous phase (it is more hydrophilic); when logP = 0, the compound is evenly distributed between the lipid and aqueous phases; a positive value for logP means a higher concentration in the lipid phase (*i.e.*, the compound is more lipophilic).

Lipophilicity is an important physicochemical parameter that contributes to the absorption, distribution, metabolism, excretion, and toxicity of a drug. For **Ligand 2**, we calculated the logP value of 0.35 using the software HSPiP, edition 5.2.02, which indicated that some parts of the ligand molecule were hydrophilic, whereas others were hydrophobic, thus, facilitating permeability through the lipid barrier of the cell. Drug molecules transport through a lipid bilayer and movement from one body compartment to another requires structural properties that allow solubility in a hydrophobic medium as well as in water.[25] In this study, unlike the complex, the ligand exhibited no antiproliferative effect in any cell line at any concentration (Fig. 2b). Our results indicate that the association of the copper ion with the ligand molecules significantly increases the antiproliferative activity. One of the functions of ligands described in the literature is that of a metal ion carrier, and it can be assumed that they potentially bring copper ions to specific sites in the cells.[26]

The purpose of modern inorganic medicinal chemistry is to design and develop new compounds that have metals other than platinum or palladium at their site of action, such as copper, vanadium, silver, or gold, with an emphasis on maximising selectivity, *i.e.*, minimising the risk to surrounding normal tissue.[13] The selectivity of compounds can be expressed by the selectivity indices. The selectivity index (SI) can be defined as the ratio between the IC_{50} value (concentration which results with 50 % suppression of the cell growth) of the normal cell line and the IC_{50} value of the cancer cell line.[27] The higher the ratio, the more effective and safer a drug would theoretically be in an *in vivo* treatment. A favourable SI > 1.0 indicates a drug with greater efficacy against tumour cells than toxicity against normal cells.[28] The calculated selectivity indices (SI) for the synthesised complex can be found in Table 1. It shows that the newly prepared complex is very selective towards KATO III, Hep G2, and HT-29 cell lines. The selectivity index was calculated by Eq. (1).

$$SI = \frac{IC_{50} \text{ value of normal cell line}}{IC_{50} \text{ value of cancer cell line}} \quad (1)$$

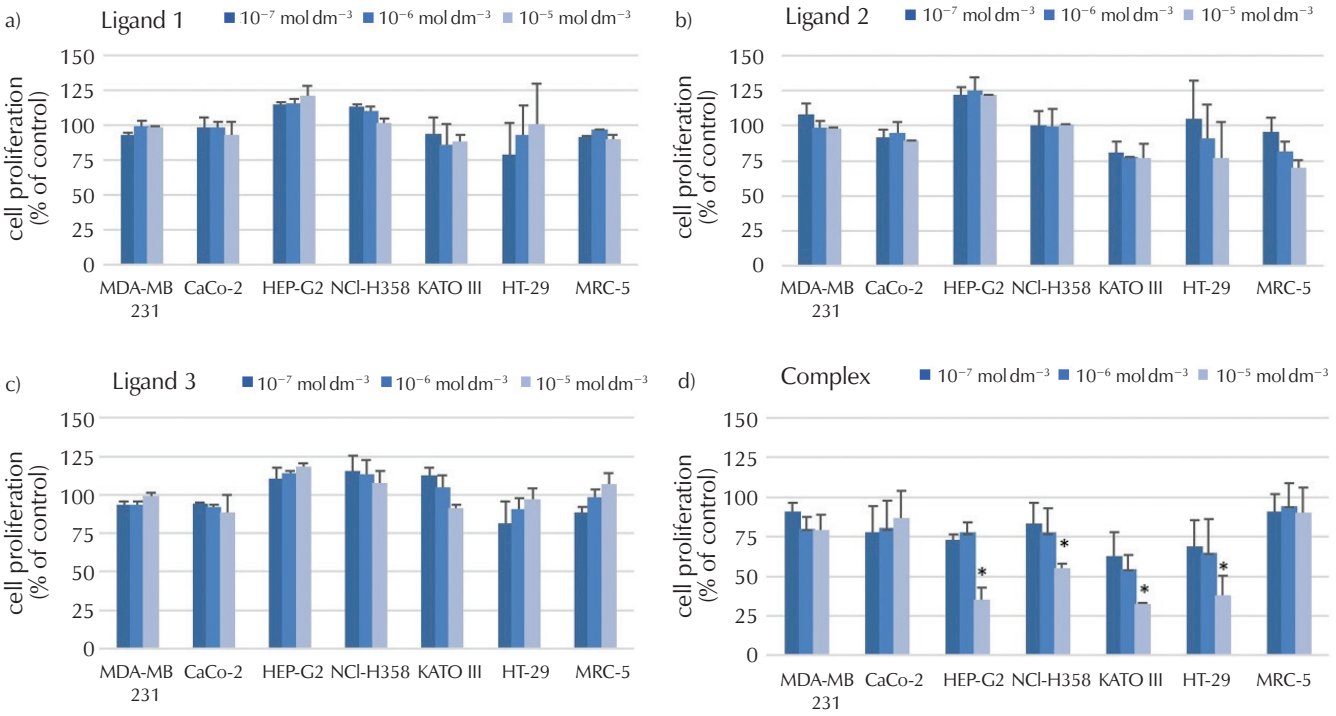


Fig. 2 – Antiproliferative effect of ligands (**Ligand 1** – **Ligand 3**) (a-c) and **Complex** (copper(II) nitrate and maltol) (d). Data are presented as a percentage of cell proliferation (%) as a function of concentration range. Histogram bars represent the mean of three independent experiments performed in triplicate and error bars correspond to SD. **L1** – chromone-2-carboxylic acid; **L2** – maltol; **L3** – coumalic acid
$P < 0.05$; Mann-Whitney U test

Slika 2 – Antiproliferativni učinak liganada (**Ligand 1** – **Ligand 3**) (a-c) i **Kompleks** (bakrov(II) nitrat i maltol) (d). Podatci su prikazani kao postotak stanične proliferacije (%) kao funkcija raspona koncentracije. Stupci histograma predstavljaju srednju vrijednost tri neovisna eksperimenta izvedena u tri primjerka, a stupci pogrešaka odgovaraju SD. **L1** – kromon-2-karboksilna kiselina; **L2** – maltol; **L3** – kumalična kiselina
$P < 0,05$; Mann-Whitney U test

Table 1 – Selectivity indices (SI) of the tested complex
Tablica 1 – Indeksi selektivnosti (IS) ispitivanog kompleksa

Cell lines	SI
KATO III	2.769
HT-29	2.368
Hep G2	2.535
NCI-H358	1.637
MDA-MB-231	1.343
Caco-2	1.059

4 Conclusion

Selected ligands from the group of benzopyrones and hydroxypyrones did not exhibit statistically significant antiproliferative activity in any cell line at any concentration. The survival rate of all cell lines after treatment with the selected ligands was > 80 %. The structural features described in the literature indicate some functionality of the ligands as well as their complexation potential. The newly synthesised complex of copper(II) nitrate and maltol shows significant selectivity towards gastric (KATO III), hepatic (Hep G2) and colon (HT-29) malignant cell lines. Normal cells exposed to the complex of copper(II) nitrate and maltol are not sensitive, and survive more than 90 % irrespective of applied concentrations. The calculated selectivity indices (SI) for the newly synthesised compound were > 1.0 for all tested cell lines, indicating that the synthesis of the complex was successful, and that the developed complex was biologically active in contrast to the pure ligand, maltol. Since no complexes of chromone-2-carboxylic acid and coumalic acid are known in the literature, further research should be conducted on the preparation of such compounds and *in vitro* assays.

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Conflict of interest statement

All authors declare no conflict of interest.

Author contributions

K. M. Š. and B. M designed the experiment. N. F. performed the experiments. K. M. Š and N. F. analysed the data and wrote the paper. B. M. supervised the chemical part of the study. All authors read the paper and contributed in their area of expertise.

List of abbreviations and symbols
Popis kratica i simbola

ATCC – American Type Culture Collection
– Američka zbirka staničnih linija

Caco-2 – colorectal adenocarcinoma
– stanična linija izolirana iz kolorektalnog adenokarcinoma

DMEM – Dulbecco's modified Eagle medium
– Dulbeccov modificirani hranjivi medij

DMSO – dimethyl sulfoxide
– dimetil sulfoksid

Hep G2 – hepatocellular carcinoma
– stanična linija izolirana iz hepatocelularnog adenokarcinoma

HT-29 – colorectal adenocarcinoma
– stanična linija izolirana iz kolorektalnog adenocarcinoma (primarnog)

KATO III – human gastric adenocarcinoma
– stanična linija izolirana iz adenokarcinoma želudca

MDA-MB-231 – human breast adenocarcinoma
– stanična linija izolirana iz adenokarcinoma dojke

MRC-5 – human fibroblasts from lung
– stanična linija izolirana iz zdravih plućnih fibroblasta

MTT test – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test
– 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazolijev bromid

NCI-H358 – bronchioalveolar carcinoma
– stanična linija izolirana iz bronhioalveolarnog karcinoma

PBS – phosphate-buffered saline
– fosfatni pufer

RPMI 1640 – Rosswell Park Memorial Institute
– Rosswell Park Memorial Institute

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SAŽETAK

Usporedba protutumorske aktivnosti liganada iz skupine benzo/hidrokispirona i bakrova(II) kompleksa s maltolom na 2D staničnim modelima *in vitro*

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Heterocikličke strukture osnovne su gradivne jedinice mnogih prirodnih organskih spojeva ključnih za odvijanje životnih funkcija u biljnom i životinjskom svijetu kao što su ugljikohidrati i vitamini, a nije strana niti njihova uloga kao sirovina za proizvodnju lijekova. U ovom su radu ispitana antiproliferativna svojstva triju odabranih liganada iz skupine benzopirona (kromon-2-karboksilna kiselina) i hidroksipirona (maltol i kumalična kiselina) kao i novog kompleksnog spoja pripravljenog iz bakrova(II) nitrata s maltolom kao ligandom. Za potrebe istraživanja pripravljene su serije otopina ispitivanih tvari različitih finalnih koncentracija (10^{-5} mol dm^{-3}, 10^{-6} mol dm^{-3} i 10^{-7} mol dm^{-3}), koje su potom aplicirane na ukupno 7 odabranih humanih staničnih linija od kojih je jedna bila zdrava, a ostale tumorske stanične linije. Rezultati MTT testa citotoksičnosti na odabranim 2D staničnim modelima pokazali su da niti jedan od testiranih liganada ne pokazuje antiproliferativnu aktivnost ni pri jednoj koncentraciji niti na jednoj ispitanoj staničnoj liniji. Za razliku od liganada, kompleksni spoj pripravljen iz bakrova(II) nitrata i maltola pokazuje značajnu citotoksičnost pri koncentraciji 10^{-5} mol dm^{-3} na odabranim staničnim linijama kako slijedi: KATO III > HT-29 > Hep G2 > NCI-H358 > MDA-MB-231 > Caco-2 > MRC-5. Zdrava stanična linija (MRC-5) je pri svim ispitivanim koncentracijama imala stopu preživljenja višu od 90,0 %, što je uputilo na zaključak o selektivnosti spoja prema tumorskim staničnim linijama.

Ključne riječi
Maltol, kromon-2-karboksilna kiselina, kumalična kiselina, bakrov(II) kompleks, proliferacija stanica, MTT test, stanične linije, tumor

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