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1. Danalev, D., Borisova, D., Yaneva, S., Georgieva, M., Balacheva, A., Dzimbova, T., **Iliev, I**., Pajpanova, T., Zaharieva, Z., Givechev, I., Naydenova, E. Synthesis, *in vitro* biological activity, hydrolytic stability and docking of new analogs of BIM-23052 containing halogenated amino acids. Amino Acids, 2020, 52(11-12): 1581-1592, ISSN: 0939-4451, **IF: 3.520, Q1**

Abstract
One of the potent somatostatin analogs, BIM-23052 (DC-23-99) d-Phe-Phe-Phe-d-Trp-Lys-Thr-Phe-Thr-NH2, has established in vitro growth hormone inhibitory activity in nM concentrations. It is also characterized by high affinity to some somatostatin receptors which are largely distributed in the cell membranes of many tumor cells. Herein, we report the synthesis of a series of analogs of BIM-23052 containing halogenated Phe residues using standard solid-phase peptide method Fmoc/ OtBu-strategy. The cytotoxic effects of the compounds were tested in vitro against two human tumor cell lines—breast cancer cell line and hepatocellular cancer cell line, as well as on human non-tumorigenic epithelial cell line. Analogs contain ing fluoro-phenylalanines are cytotoxic in μM range, as the analog containing Phe (2-F) showed better selectivity against human hepatocellular cancer cell line. The presented study also reveals that accumulation of halogenated Phe residues does not increase the cytotoxicity according to tested cell lines. The calculated selective index reveals different mechanisms of antitumor activity of the parent compound BIM-23052 and target halogenated analogs for examined breast tumor cell lines. All peptides tested have high antitumor activity against the HepG2 cell line (IC50 ≈ 100 μM and SI > 5) compared to breast cells. This is probably due to the high permeability of the cell membrane and the higher metabolic activity of hepatocytes. In silico docking studies confirmed that all obtained analogs bind well with the somatostatin receptors with preference to ssrt3 and ssrt5. All target compounds showed high hydrolytic stability at acid and neutral pH, which mimic physiological condition in stomach and human plasma.

2. Georgieva, M., Balacheva, A., Detcheva, R., **Iliev, I**., Nives, G., Pajpanova, T. New aroyl hidrazones combined with specific peptide analogues: looking for possible enhanced cytotoxic effects. Journal of Chemical Technology and Metallurgy, 2020, 55(6): 1994-1998, ISSN: 1314-7471, **SJR: 0.220, Q3**

Abstract
Aroyl hydrazones have been intensively investigated over the last decades due to their various biological properties such as anticancer, antibacterial and antimicrobial effects. Herein, we examine the anti-proliferative effect of two new aroyl hydrazones used in a combination with AVPI- and RGD – peptides. The anti-proliferative activity is checked by MTT assay after 72h of treatment on MDA-MB-231 breast cancer cells. Though the aroyl hydrazone derivatives used as single agents shows a high activity, the combination is found not beneficial.

3. Jaber, S., **Iliev, I**., Angelova, T., Nemska, V., Sulikovska, I., Naydenova, E., Georgieva, N., Givechev, I., Grabchev, I., Danalev, D. Synthesis, Antitumor and Antibacterial Studies of New Shortened Analogues of $(KLAKLAK)_2$-NH_2 and Their Conjugates Containing Unnatural Amino Acids. Molecules, 2021, 26(4): 898, ISSN: 1420-3049, **IF: 4.927, Q1**

Abstract
(1) Background: $(KLAKLAK)_2$ is a representative of the antimicrobial peptide group which also shows good anticancer properties. (2) Methods: Herein, we report synthesis using SPPS and characterization by HPLC/MS of a series of shortened analogues of $(KLAKLAK)_2$. They contain single sequence KLAKLAK as C-terminal amides. In addition, substitution of some natural amino acids with unnatural β-Ala and nor-Leu is realized. In addition, these structures are conjugated with second pharmacophore with well proven anticancer properties 1,8-naphthalimide or caffeic acid. Cytotoxicity, antiproliferative effect and antimicrobial activity of newly synthesized structures were studied. (3) Results: The obtained experimental results reveal significant selective index for substances with common chemical structure KLβAKLβAK-NH_2. The antibacterial properties of newly synthesized analogues at two different concentrations 10 μM and 20 μM, were tested against Gram-negative microorganisms Escherichia coli K12 407. Only two of the studied compounds KLAKLAK-NH_2 and the one conjugated with second pharmacophore 1,8-naphthalimide and unnatural amino acid nor-Leu showed moderate activity against tested strains at concentration of 20 μM. (4) Conclusions: The obtained results reveal that the introducing of 1,8-naphthalimideGly- and Caf- increase the cytotoxicity and antiproliferative activity of the peptides but not their selectivity. Only two compounds KLAKLAK-NH_2 and 1,8-naphthalimideGKnLAKnLAK-NH2 show moderate activity against Escherichia coli K12 at low concentration of 20 μM.

4. Jaber, S., Nemska, V., **Iliev, I**., Ivanova, E., Foteva, T., Georgieva, N., Givechev, I., Naydenova, E., Karadjova, V., Danalev, D. Synthesis and Biological Studies on (KLAKLAK)2-NH2 Analog Containing Unnatural Amino Acid b-Ala and Conjugates with Second Pharmacophore. Molecules, 2021, 26(23): 7321, ISSN: 1420-3049, **IF: 4.927, Q1**

Abstract
(1) Background: Peptides are good candidates for anticancer drugs due to their natural existence in the body and lack of secondary effects. $(KLAKLAK)_2$ is an antimicrobial peptide that also shows good anticancer properties. (2) Methods: The Solid Phase Peptide Synthesis (Fmoc-strategy) was used for the synthesis of target molecules, analogs of $(KLAKLAK)_2$-NH_2. The purity of all compounds was monitored by HPLC, and their structures were proven using mass spectrometry. Cytotoxicity and antiproliferative effects were studied using 3T3 NRU and MTT tests, respectively. For determination of antimicrobial activity, the disc-diffusion method was used. Hydrolytic stability at three pH values, which mimic the physiological pH in the body, was investigated by means of the HPLCtechnique. (3) Results: A good selective index against MCF-7 tumor cell lines, combined with good cytotoxicity and antiproliferative properties, was revealed for conjugates NphtG-$(KLAKLAK)_2$ NH_2 and Caf-$(KLAKLAK)_2$-NH_2. The same compounds showed very good antifungal properties and complete hydrolytic stability for 72 h. The compound Caf-$(KLβ\text{-}AKLβ\text{-}AK)_2$-$NH_2$ containing β-Ala in its structures exhibited good antimicrobial

activity against *Escherichia coli* K12 407 and Bacillus subtilis 3562, in combination with very good antiproliferative and cytotoxic properties, as well as hydrolytic stability. (4) Conclusions: The obtained results reveal that all synthesized conjugates could be useful for medical practice as anticancer or antimicrobial agents.

5. Danalev, D., **Iliev, I**., Borisova, D., Dzimbova, T., Pajpanova, T., Zaharieva, Z., Karadjova, V., Foteva, T., Naydenova, E. Synthesis, Anticancer Activity, Docking Calculations and Hydrolytic Stability Studies of Bioconjugates of Monofluorenated Analogue of BIM-23052. Protein & Peptide Letters, 2022, 29(8): 721-731, ISSN: 0929-8665, **IF: 1.600, Q3**

Abstract
Background: The fight against cancer has started since its discovery and has not subsided to nowadays. Currently, the hybrid molecules have become a promising alternative to the standard chemotherapeutics for the treatment of multi-causal diseases, including cancers. Objective: Herein, we report the synthesis, biological evaluation, mathematical docking calculations and hydrolytic stability of the new bioconjugates of monofluorinated analogues of BIM-23052, containing second pharmacophore naphthalimide, caffeic acid or the tripeptide Arg-Gly-Asp. Methods: All new molecules are obtained using standard peptide synthesis on solid support. Anticancer potential is studied against a panel of tumor cell lines included human mammary carcinoma cell lines MCF-7 (ER+, PR+ and Her-2-); MDA-MB-231 (ER-, PR- and Her-2-), as well as cell lines BALB 3T3 (mouse embryonic fibroblasts) and MCF-10A (human breast epithelial cell line). Results: The IC_{50} values found in the MCF-10A cell line assay were used to calculate the selective index (SI). The highest SI relative to MCF-7, with a value of 2.62 is shown by the compound Npht- Gly-D-Phe-Phe(4-F)-Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2. In MCF-10 cells, the weakest antiproliferative effect was caused by the same compound (IC_{50} = 622.9 ± 23.91 μM), which makes this analogue a good candidate for the new anticancer medical drug. Unfortunately, the hydrolytic stability studies reveal that this bioconjugate is the most unstable of hydrolysis under physiological conditions in the body. Conclusion: Even with lower anticancer activity and selectivity in comparison with Npht-Gly-DPhe- Phe(4-F)-Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2, the compound Arg-Gly-Asp-D-Phe-Phe(4-F)-Phe- D-Trp-Lys-Thr-Phe-Thr-NH_2 is the best candidate between three investigated bioconjugates for practical application due to combination of activity and stability profiles. Mathematical docking calculation also reveals that synthesized bioconjugates show selectivity according to different somatostatin receptors on the surface of different cell lines.

6. Danalev, D., **Iliev, I**., Dobrev, S., Angelova, S., Petrin, S., Dzimbova, T., Ivanova, E., Borisova, D., Naydenova, E. Synthesis, Antiproliferative Effect and In Silico LogP Prediction of BIM-23052 Analogs Containing Tyr Instead of Phe. Pharmaceutics, 2023, 15(4): 1123, ISSN: 1999-4923, **IF: 4.900, Q1**

Abstract
Background: Hydrophobicity (or lipophilicity) is a limiting factor in the ability of molecules to pass through cell membranes and to perform their function. The ability to efficiently access cytosol is especially important when a synthetic compound has the potential to become a drug sub stance. D-Phe-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2 (BIM-23052) is a linear analog of somatostatin with

established in vitro GH-inhibitory activity in nanomolar (nM) concentrations and high affinity to different somatostatin receptors. (2) Methods: Series of analogs of BIM-23052 were synthesized where Phe residue(s) in the BIM-23052 molecule were replaced with Tyr using standard SPPS, Fmoc/t-Bu strategy. Analyses of target compounds were performed using HPLC/MS technique. Toxicity and antiproliferative activity were studied using *in vitro* NRU and MTT assays. The values of logP (partition coefficient in octanol/water) for BIM-23052 and its analogs were calculated. (3) Results: The obtained data show the best antiproliferative effect against studied cancer cells for compound D-Phe-Phe-Phe-D-Trp-Lys-Thr-Tyr7-Thr-NH_2 (DD8), the most lipophilic compound according to the predicted logP values. (4) Conclusions: Multiple analyses of the obtained data reveal that compound D-Phe-Phe-Phe-D-Trp-Lys-Thr-Tyr7-Thr-NH_2 (DD8) where one Phe is replaced by Tyr has the best combination of cytotoxicity, antiproliferative effect and hydrolytic stability.

7. Jaber, S., Nemska, V., **Iliev, I**., Ivanova, E., Foteva, T., Georgieva, N., Givechev, I., Tanev, D., Naydenova, E., Danalev, D. Synthesis, antiproliferative and antimicrobial activities of (KLAKLAK)2-NH2 analogue containing nor-Leu and its conjugates with a second pharmacophore. Biotechnology & Biotechnological Equipment, 2023, 37(1): 151-158, ISSN: 1310-2818, **IF: 1.500, Q3**

Abstract
Peptides are a promising alternative of conventional medical drugs for the treatment of different diseases because they have no or have very few side effects owing to the natural mechanisms for their elimination. There are a lot of examples of drugs on the pharmaceutical market based on modified amino acids and peptides. Herein, we report the synthesis and studies on the antimicrobial peptide $(KLAKLAK)_2$-NH_2 where Leu is replaced by the unnatural amino acid nor-Leu. In addition, a second pharmacophore with well proven anticancer properties is introduced to the peptide moiety. All structures were synthesized by conventional solid phase peptide synthesis. The antiproliferative and antimicrobial activities were studied using MTT-dye reduction assay and disk-diffusion test, respectively. Biological activity assays showed that the introduction of nor-Leu in the primary structure of the parent compound does not lead to an increase in the antiproliferative activity. However, the combination with the second pharmacophore 1,8-naphtalimide in a hybrid structure 1,8-NphtG-$(KNleAKNleAK)_2$-NH_2 leads to a significant increase in the antiproliferative properties. The antimicrobial tests showed that all tested compounds exhibit antimicrobial activity. The peptide and the second pharmacophore had a synergistic effect. In combination with complete hydrolytic stability for 72 h in model systems, the compound 1,8-NphtG-$(KNleAKNleAK)_2$-NH_2 is the best candidate for a medical drug in the treatment of mammary gland type A adenocarcinoma (MCF-7) in combination with antimicrobial properties.

8. Borisova, B., Nocheva, H., **Iliev, I**., Laronze-Cochard, M., Gerard, S., Petrin, S., Danalev, D. Synthesis and analgesic activity of new analogs of FELL tetrapeptide containing D-Phe in the first position. Current Research in Biotechnology, 2024, 8: 100249, ISSN: 2590-2628, **IF: 4.000, Q2**

Abstract
Pain, whether acute or chronic, is one of the most unpleasant experiences. It can have different origins and long- term effects on the body starting from the trivial once such as physical discomfort, accompanying by emotional distress and going to the more serious like depression, anxiety, and social isolation. The removal and proper treatment of the pain is a problem highly dependent on both the source and the individual features of each organism. Herein the view is turned on investigation of activity of new analogs of natural FELL peptide as a promising alternative of the existing antipain molecules. All targeted compounds are obtained by means of conventional peptide synthesis on solid support using standard Fmoc/OtBu approach and their analgesic activity was evaluated by Paw-pressure (Randall-Selitto) test. Determination of the *in vivo* analgesic activity of the newly synthesized substances showed that the substitution of both Leu (BB11) with Val residues (BB8) increased PPT of the experimental animals on the 10th min, compared to the values after the nonmodified parent molecule in jection. On the 20th and the 30th min, BB8 analgesic activity was comparable to BB11 and further a decrease in the PPT was observed. In addition, compared to the controls, analgesia exists until the end of the monitored period of 50 min. The other three newly synthesized substances including Nle (BB6), Ile (BB7) and triple Leu (BB5) instead of double Leu residues showed time-varying short-term analgesic activity, which did not reach that of the parent molecule BB11. Final results show that D-Phe in a first position of the molecule, combined with both Leu residues in the third and fourth positions are the best combination concerning analgesic activity. In addition, lengthening the peptide chain by adding one more hydrophobic residue has also a positive effect on the obtained analgesia. Cytotoxicity of final molecules is significantly lower than those of the positive control SLS, combined with complete hydrolytic stability, which allows their safety use in pharmacy.

9. Angelova, N., **Iliev, I**., Nemska, V., Dzimbova, T., Georgieva, N., Danalev, D., Naydenova, E. Design, Synthesis, and Biological Evaluation of New Analogs of Aurein 1.2 Containing Non-Proteinogenic Amino Acids. Molecules, 2025, 30(9): 2050, ISSN: 1420-3049, **IF: 4.600, Q1**

Abstract
Extensive use of classical antibiotics has led to the growing emergence of many resistant strains of pathogenic bacteria. To combat this challenge, researchers have turned to the antimicrobial peptides (AMPs). Aurein 1.2 (GLFDIIKKIAESF-NH_2) was demonstrated to have broad spectrum bi-functionality against bacterial and cancer cells. The Solid Phase Peptide Synthesis (Fmoc-strategy) was used for the synthesis of new analogs of aurein 1.2. The purity of all compounds was monitored by HPLC, and their structures were proven using mass spectrometry. Cytotoxicity and antiproliferative effects were studied using 3T3 NRU and MTT tests, respectively. The antibacterial activity was estimated against Gram-positive and Gram-negative bacteria using broth microdilution method in concentrations from 0 to 320 μg/mL to determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The antiproliferative activity test shows that the peptide analog EH $[Orn]^8$ has the highest activity (IC_{50} = 44 ± 38 μM) for the three cell lines studied (MCF-12F, MCF-7, and MDA-MB-231). The same compound exhibited good antimicrobial activity. The obtained results reveal that replacement of Lys with non-proteinogenic amino acids can increase both the potency and activity spectra of natural template peptides, making them suitable candidates for new drug development.

10. Dimitrova, D., Nemska, V., **Iliev, I**., Petrin, S., Georgieva, N., Danalev, D. New Temporin A Analogues Modified in Positions 1 and 10—Synthesis and Biological Studies. Pharmaceutics, 2025, 17(4): 396, ISSN: 1999-4923, **IF: 5.500, Q1**

Abstract
Background: With growing antimicrobial resistance, the overuse of antibiotics, and stagnation in the discovery of new antibiotics, a novel alternative is required to overcome hard-to-treat infections. Antimicrobial peptides (AMPs) show great potential as a possible alternative to standard chemotherapeutics. Temporins are a group of AMPs that have been under the spotlight in numerous studies. Herein, we report the design and synthesis of Temporin A modified in position 1, where the proteinogenic amino acid Phe is replaced by Tyr or fluorinated Phe. In addition, in other analogues, in position 10, the Ser residue is replaced by Tyr or Thr. The aim of all modifications in the primary structure of the native Temporin A is to study the influence of the changes made on the antibacterial properties, antiproliferative activity, and hydrolytic stability of the newly synthesized molecules. Methods: The Fmoc/OBu^t SPPS strategy was employed for the synthesis of the novel-designed analogues. The antibacterial activity was evaluated with both disk diffusion and broth microdilution methods. The BALB 3T3 NRU test and MTT dye reduction assay were used to determine safety and antiproliferative activity. Results: The investigated analogues have low toxicity and are photosafe. The greatest selectivity was shown by DTTyr10 towards MCF-7 cells. DT4F, containing fluorinated Phe in position 1, was the most effective antibacterial agent among the new compounds. The incorporation of Thr in position 10, in comparison with the natural Ser residue, led to an increase in the antiproliferative effect of the new peptide. Conclusions: The obtained structure–activity relationship data show that the most promising compound in the tested series is FLPLIGRVL-**Y**-GILNH_2, where the Ser residue in position 10 is replaced by a more hydrophobic OH-containing Tyr residue. The analogue containing fluorinated Phe in position 1, DT4F, has the highest antiproliferative effect against both tested tumor cell lines, combined with good antibacterial properties at the lowest MIC (80 μg/mL), but it is more cyto- and phototoxic than the parent DTA molecule and is not stable at pH 9 for a 24 h period.

11. **Iliev, I**., Kontrec, D., Detcheva, R., Georgieva, M., Balacheva, A., Galic, N., Pajpanova, T. Cancer cell growth inhibition by aroylhydrazone derivatives. Biotechnology & Biotechnological Equipment, 2019, 33(1): 756-763, ISSN: 1310-2818, **IF: 1.186, Q3**

Abstract
Hydrazones have versatile properties that make them promising for a range of possible applications. In this study, we examined a library of 17 aroylhydrazones derived from nicotinic acid hydrazide (1-12) and isonicotinic acid hydrazide (A-E) created by us for their biological activity. The antiproliferative activity of the compounds was investigated on non-tumour MCF-10A cells and cancer cell lines, MCF-7 and MDA-MB-231. Four compounds were selected as most active in cell growth inhibition of the tumour cell lines. These compounds, 5, 11, C and E,weretestedon four additional cell lines: non-tumour BJ and cancer cell lines, HeLa, HepG2 and HT-29. Compounds 5 and E exhibited the highest selectivity index on cancer cell lines MDA-MB-231, HeLa and HepG2. High selectivity to MCF-7 cells was demonstrated with compound 5. Compound C was very selective to HepG2 cells as well as to MDA-MB-231 but to a lesser degree.

Compound 11 showed selectivity against MDA-MB-231. The obtained results allow assessing the structure–activity relationship of the compounds and provide insight into the further develop ment of this group of aroylhydrazones as more potent and selective anti-neoplastic agents.

12. Danailova, A., Krumova, S., **Iliev, I**., Gartcheva, L., Taneva, S., Todinova, S. Calorimetric markers for inflammation in *in vivo* experimental models. International Journal Bioautomation, 2019, 23(4): 479-488, ISSN: 1314-1902, **SJR: 0.242, Q3**

Abstract
In this work differential scanning calorimetry was applied to determine the specific calorimetric features of blood plasma proteome associated with immune response stimulation in experimental model (albino Wistar rats). The thermodynamic behavior of the blood plasma of male and female animals subjected to egg albumin (EA) treatment was investigated. The calorimetric profiles of blood plasma from EA treated rats exhibited reduced heat capacity of the albumin-assigned transition and up-shifted weighted average center of the thermogram as compared to healthy controls, the effect being more pronounced for male animals. Increase in the amplitude of the main transition at 70 °C was observed for female rats after EA treatment, which resulted in higher calorimetric enthalpy. Common feature of the thermograms of EA treated males and females was the broadening of the transitions above 75 °C and the appearance of exothermic transition above 90 °C due to protein aggregation. Our study clearly revealed gender-specific immune response in rats and contributes to better understanding of the correlation between the calorimetric features of blood plasma and the immunological conditions in the experimental animals.

13. Mateva, R., Georgieva, A., **Iliev, I**., Toshkova, R., Pajpanova, T. Antiproliferative and apoptogenic effects of myosmine on erythroleukemia and hepatocellular carcinoma cells. Biotechnology & Biotechnological Equipment, 2019, 33(1): 613-619, ISSN: 1310-2818, **IF: 1.186, Q3**

Abstract
Myosmine, 3-(1-pyrroline-2-yl) pyridine is a minor tobacco alkaloid that has also been found in various widely used foods. Recently, this phytochemical has been gaining an increasing interest as a potential risk factor for the development of oesophageal adenocarcinoma. This study aimed to examine the effects of myosmine on the cell viability and proliferative activity of erythroleu kemia and hepatocellular carcinoma cells and to obtain additional information about the mecha nisms underlying its cytotoxic activity. The in vitro cytotoxic effect of myosmine on the HepG2 and MEL tumour cell lines was assessed by MTT dye reduction and trypan blue dye exclusion assays. The alterations in the tumour cell morphology induced by myosmine were analysed by fluorescent microscopy after staining with acridine orange (AO)/ethidium bromide (EtBr) and 40,6-diamidine-20-phenylindole dihydrochloride (DAPI). Annexin V-FITC/propidium iodide (PI) staining was used to assess the apoptosis-inducing ability of myosmine. The modulating action of antioxidant treatment on myosmine-induced cytotoxicity against the HepG2 tumour cell line was also examined. The cell viability tests indicated that myosmine induced a significant dose-depend ent reduction of the viability and proliferative activity of both tumour cell lines. Fluorescent micros copy studies revealed marked alterations in the morphology of myosmine-treated tumour cells with

signs of cell cycle arrest and apoptosis. The results of the simultaneous treatment with myos mine and vitamin C showed modulating activity of vitamin C on the cytotoxic effect of myosmine with concentration- and time-dependent variations. The presented results could contribute to the assessment of the potential health risks associated with the dietary myosmine exposure.

14. Vasileva, A., **Iliev, I**., Lozanov, V., Dimitrova, M., Mitev, V., Ivanov, I. *In vitro* study on the antitumor activity of Tanacetum vulgare L. extracts. Bulgarian Chemical Communications, 2019, 51(2): 249-255, ISSN: 0861-9808, **SJR: 0.142, Q4**

Abstract
The major nonvolatile compounds derived from extracts and fractions from *Tanacetum vulgare* L. flowers were determined by LC–HRMS. Major compounds in the crude extract were determined to be: six hydroxycinnamoyl quinic acids with 4,5-dicaffeoylquinic acid and twelve flavonoids and their derivatives, six of which were in the form of flavonoid-O-glucuronides. Generally, the major flavonoid aglycone in tansy was luteolin. Extracts and fractions were tested under *in vitro* conditions in nine cell lines - one control non-tumorogenic and eight tumor lines, whereby antitumor activity was observed after 72 hours of incubation with the aforementioned substances as determined by an MTT assay. The obtained results show the highest selectivity index for the ethyl acetate extract from Flores Tanaceti (EAFT) and for the ethyl acetate fraction of the crude extract (EACE). EAFT extract was found to exert the highest antitumor effect, followed by EACE. From the above results it becomes evident that ethyl acetate extracts of *T. vulgare* contain substances with high selective activity against tumor cells.

15. Georgieva, A., Todorova, K., **Iliev, I**., Dilcheva, V., Vladov, I., Petkova, S., Toshkova, R., Velkova, L., Dolashki, A., Dolashka, P. Hemocyanins from Helix and Rapana snails exhibit in vitro antitumor effects in human colorectal adenocarcinoma. Biomedicines, 2020, 8(7): 194, ISSN: 2227-9059, **IF: 6.081, Q1**

Abstract
Hemocyanins are oxygen-transporting glycoproteins in the hemolymph of arthropods and molluskshatattract scientific interest with their diverse biological activities and potential applications in pharmacy and medicine. The aim of the present study was to assess the in vitro antitumor activity of hemocyanins isolated from marine snail *Rapana venosa* (RvH) and garden snails *Helix lucorum* (HlH) and *Helix aspersa* (HaH), as well the mucus of *H. aspersa* snails, in the HT-29 human colorectal carcinomacell line. The effects of the hemocyanins on the cell viability and proliferation were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the alterations in the tumor cell morphology were examined by fluorescent and transmission electron microscopy. The results of the MTT assay showed that the mucus and α-subunit of hemocyanin from the snail *H. aspersa* had the most significant antiproliferative activity of the tested samples. Cytomorphological analysis revealed that the observed antitumor effects were associated with induction of apoptosis in the tumor cells. The presented data indicate that hemocyanins and mucus from *H. aspersa* have an antineoplastic activity and potential for development of novel therapeutics for treatment of colorectal carcinoma.

16. Georgieva, A., Todorova, K., **Iliev, I**., Dilcheva, V., Vladov, I., Petkova, S., Toshkova, R., Velkova, L., Atanasov, V., Dolashki, A., Dolashka, P. *In vitro* antitumor activity of hemocyanins isolated from Helix aspersa and Helix lucorum in human bladder carcinoma cells. Compt. rend. Acad. bulg. Sci., 2021, 74(9): 1346-1353, ISSN: 1310-1331, **IF: 0.326, Q3**

Abstract
The present study aims to assess the *in vitro* antineoplastic potential of total hemocyanins isolated from *Helix aspersa* and *Helix lucorum* (HaH-total; HlH-total), their structural subunits (βc-HaH; α-HaH; βc-HlH; α-HlH) and *Helix aspersa* mucus in human urinary bladder carcinoma cell line 5637. The effects of the hemocyanins on the cell viability and proliferative activity was determined by MTT test. The morphological changes induced by hemocyanins in the tumour cells were analyzed by fluorescent microscopy after staining with acridine orange/ethidium bromide and DAPI. The results of the MTT test showed a significant antiproliferative effect of all tested hemocyanin samples. The antitumour effects of subunits α-HaH and βc-HlH were most clearly pro nounced. Microscopic analysis of the hemocyanin-treated bladder carcinoma cells revealed typical morphological features of apoptosis. The results of our study indicate that in addition to the known immunogenic effects, the mollus can hemocyanins also have a direct antitumour activity against urinary bladder cancer.

17. Dimitrov, M., **Iliev, I**., Bardarov, K., Georgieva, D., Todorova, T. Phytochemical characterization and biological activity of apricot kernels' extract in yeast-cell based tests and hepatocellular and colorectal carcinoma cell line. Journal of Ethnopharmacology, 2021, 279: 114333, ISSN: 0378-8741, **IF: 5.195, Q1**

Abstract
Ethnopharmacological relevance: Bitter apricot kernels' extract contains a broad spectrum of biologically active substances with a lot of attention to amygdalin – cyanogenic glycoside. The extract has been used in the pharmaceutical industry for years as an ingredient of different pharmaceuticals with anti-inflammatory, anti microbial, or regenerative properties. In traditional medicine, the bitter apricot kernels are known as a remedy for respiratory disorders and skin diseases. The apricot kernels and amygdalin are often prescribed by practi tioners for the prevention and treatment of various medical conditions, including colorectal cancer. The present study aims: to evaluate the phytochemical composition and the potential antimutagenic, anti recombinogenic, and antitumor effect of apricot kernels' extract at very low concentrations in yeast cell-based tests and mammalian hepatocellular and colon carcinoma cell lines. Materials and methods: Phytochemical analysis was performed by LC-MS profiling. Reverse-phase HPLC and UV detection were applied for the determination of amygdalin quantity in the extract. Biological activity was evaluated by Zimmermann's mutagenicity and Ty1 retrotransposition test. Cytotoxic/antiproliferative activity of apricot kernels' extract was performed on four types of cell lines – HepG2, HT-29, BALB/3T3, clone A31, and BJ using the standard MTT-dye reduction assay. Results: Data revealed the presence of more than 1000 compounds and 4 cyanogenic glycosides among them – Amygdalin, Deidaclin, Linamarin and Prulaurasin. The Amygdalin concentration was measured to be 57.8 g/mL. All extract concentrations demonstrated a strong antigenotoxic, antirecombinogenic, antimutagenic, and anti carcinogenic effect in the yeast cell-

based tests. High selectivity of the extract action is established among different mammalian cell lines. Normal cell line BJ is found to be resistant to the extract action. HepG2 was found to be the most sensitive to apricot kernels' action. μ Conclusion: The present study provides the first phytochemical analysis of Bulgarian bitter apricot kernels. Three new cyanogenic glycosides were reported. Evidence is obtained that the apricot kernels' extract at low con centrations is not able to induce some of the events related to the initial steps of tumorigenesis. Additionally, a high selectivity of the extract action is established among different cell lines. The most sensitive cell line was found to be HepG2.

18. Semkova, S., Antov, G., **Iliev, I**., Tsoneva, I., Lefterov, P., Christova, N., Nacheva, L., Stoineva, I., Kabaivanova, L., Staneva, G., Nikolova, B. Rhamnolipid Biosurfactants—Possible Natural Anticancer Agents and Autophagy Inhibitors. Separations, 2021, 8(7): 92, ISSN: 2297-8739, **IF: 3.344, Q2**

Abstract
Background: A number of biologically active substances were proved as an alternative to conventional anticancer medicines. The aim of the study is in vitro investigation of the anticancer activity of mono- and di-Rhamnolipids (RL-1 and RL-2) against human breast cancer. Additionally, the combination with Cisplatin was analyzed. Materials and Methods: Breast cell lines (MCF-10A, MCF-7 and MDA-MB-231) were treated with RLs and in combination with Cisplatin. The viability was analyzed using MTT assay, and investigation of autophagy was performed via acridine orange staining. Results: In contrast to the healthy cells, both tested cancer lines exhibited sensitivity to RLs treatment. This effect was accompanied by an influence on the autophagy-related acidic formation process. Only for the triple-negative breast cancer cell line (MDA-MB-231) the synergistic effect of the combined treatment (10 μM Cisplatin and 1 μg/mL RL-2) was observed. Conclusion: Based on studies on the reorganization of membrane models in the presence of RL and the data about a higher amount of lipid rafts in cancer cell membranes than in non-tumorigenic, we suggest a possible mechanism of membrane remodelling by formation of endosomes. Shortly, in order to have a synergistic effect, it is necessary to have Cisplatin and RL-2 as RL2 is a molecule inducing positive membrane curvature.

19. Todinova, S., Nikolova, B., **Iliev, I**., Semkova, S., Krumova, S., Taneva, S. Thermodynamic behaviour of breast cancer cell lines after miltefosine and cisplatin treatment. Journal of Thermal Analysis and Calorimetry, 2021, 147(14): 7819–7828, ISSN: 1388-6150, **IF: 4.755, Q1**

Abstract
Breast cancers exhibit different response to drug treatment. In this work, we analyze and compare the effect of two anticancer drugs differing in their primary action, miltefosine and cisplatin (cis-Pt), on two different breast cancer (the low—(MCF-7) and high—(MDA-MB-231) metastatic) cell lines, and one normal epithelial (MCF-10A) breast cell lines. The effect of cip-Pt and miltefosine on the thermodynamic behavior of the cancer cell lines was analyzed by differential scanning calorimetry, the cell morphology and viability were determined by optical microscopy and MTT test. We revealed distinct effects of miltefosine and cis-Pt on the thermodynamic behavior and

viability of the cancer and normal cells. Importantly, the normal MCF-10A cells were drastically affected by miltefosine, while not by cis-Pt. MDA-MB-231 cell line, on the other hand, is more susceptible to cis-Pt than MCF-7 cells, while both cancer cell lines are equally affected by miltefosine. The drug associated alteration of the thermal unfolding of the cells constituents correlated with the changes in the cell viability. The altered thermodynamic behavior of the cancer cells upon the drug treatment strongly indicates altered conformations of the proteins in cancer cell membrane and cellular matrix, and the DNA-containing structures.

20. Yankova, I., Ivanova, E., Todorova, K., Georgieva, A., Dilcheva, V., Vladov, I., Petkova, S., Toshkova, R., Velkova, L., Dolashka, P., **Iliev, I**. Assessment of the toxicity and antiproliferative activity of hemocyanins from Helix lucorum, Helix aspersa and Rapana venosa. Bulgarian Chemical Communications, 2021, 53(Special Issue A): 15-21, ISSN: 0861-9808, **SJR: 0.168, Q4**

Abstract
Hemocyanins (Hcs) are respiratory, oxygen-carrying metalloproteins that are freely dissolved in the hemolymph of many molluscs and arthropods. The interest in hemocyanins has grown significantly since it was found that they can be successfully used in immunotherapy of neoplastic diseases as non-specific or active stimulators of the immune system. The present study aims to assess the cytotoxicity, *in vivo* toxicity and antiproliferative activity of hemocyanins isolated from marine snail *Rapana venosa* (RvH), garden snails *Helix lucorum* (HlH) and *Helix aspersa* (HaH). For *in vitro* safety testing, 3T3 Neutral Red Uptake (NRU) test was used. The experiments for antiproliferative activity of the hemocyanins were performed by MTT assay on a panel of cell lines - a model of breast cancer. The *in vivo* toxicological assessment was performed by regular clinical examinations of hemocyanin-treated laboratory mice and histopathological analysis of hematoxylin/eosin stained preparations of parenchymal organs. The evaluation of the *in vitro* cytotoxicity showed that the tested hemocyanins does not induce toxic effects in nontumorigenic epithelial cell lines. In contrast, significant reduction of the viability of human breast carcinoma cell lines was found after treatment with high concentrations of hemocyanins. The *in vivo* experiments showed no signs of organ and systemic toxicity in the hemocyanin-treated animals. The presented data indicate that Hcs show a potential for development of novel anticancer therapeutics due to their beneficial properties, biosafety and lack of toxicity or side effects.

21. Kolev, I., Stoeva, S., **Iliev, I**., Marinov, P. A small-scale method of sample preparation suitable for simultaneous HPLC-UV assay of capecitabine and its 5'-DFCR metabolite in mouse blood plasma. Brazilian Journal of Pharmaceutical Sciences, 2022, 58: e201043, ISSN: 1984-8250, **IF: 1.300, Q2**

Abstract
The objective of the study was to develop an easy, cheap, effective, and safe, small-scale method for sample preparation suitable for the simultaneous high-performance liquid chromatography (HPLC)-ultraviolet (UV) assay of capecitabine and its 5′-deoxy-5-fluorocytidine (5′-DFCR) metabolite in mouse blood plasma. The suitability of the proposed method of sample preparation was verified by the optimal effectiveness and efficiency achieved in the overall analytical

workflow. The chromatographic separation of capecitabine and its first metabolite was performed on a Hypersil GOLD aQ column with a mobile phase consisting of 1% formic acid, methanol, and water, and run in a gradient elution mode. The absence of interfering endogenous components at the retention times of each analyte was confirmed by the chromatographic analysis of blank matrices and matrices spiked with the corresponding standards. The absence of any tactile matrix effect was also recorded. For the first time, the effect of the vacutainer's anticoagulant on the extraction efficiency of both analytes was evaluated. The method was found to be accurate, precise, and specific. The estimated mean "extraction" efficiencies were ≥ 90 % for each analyte. The lower limit of quantitation for both capecitabine and 5′-DFCR was 0.05 μg/mL.

22. Komsa-Penkova, R., Danailova, A., Krumova, S., Georgieva, G., Giosheva, I., Gartcheva, L., **Iliev, I**., Gartchev, E., Kercheva, K., Savov, A., Todinova, S. Altered Thermal Behavior of Blood Plasma Proteome Related to Inflammatory Cytokines in Early Pregnancy Loss. International Journal of Molecular Sciences, 2022, 23(15): 8764, ISSN: 1422-0067, **IF: 5.600, Q1**

Abstract
Early pregnancy loss (EPL) is a relatively common pathology of which almost 50% of cases remain idiopathic. In the search for novel biomarkers, differential scanning calorimetry (DSC) is intensively used to characterize the thermodynamic behavior of blood plasma/serum proteome in health and disease. Herein, for the first time, we investigate the DSC denaturation profiles of blood plasma derived from patients suffering EPL compared to healthy pregnant and non-pregnant women. Data analysis reveals that 58% of the EPL thermograms differ significantly from those of healthy pregnant women. Thermal stabilization of a fraction of albumin-assigned transition with concomitant suppression of the major and enhancement of the globulin-assigned transition are characteristic features of EPL calorimetric profiles that could be used as a new indicator of a risk pregnancy. The presented results suggest an altered composition or intermolecular interactions of the plasma proteome of women with EPL. In addition, the alterations of the EPL thermograms correlate with the increased blood levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and a higher prevalence of the polymorphism in the plasminogen activator inhibitor type-1 (PAI-1) gene, suggesting an expression of an overall enhanced immune response. The concomitant changes in plasma thermograms confirm the potential of the DSC approach for distinguishing changes in the pathological state of the blood plasma proteome.

23. Radoeva, R., Yankova, I., Enchev, B., Karsheva, M., Ivanova, E., **Iliev, I**. Polyphenols of grape pomace from local bulgarian variety mavrud. Antioxidant and antitumor effect against breast cancer. Journal of Chemical Technology and Metallurgy, 2022, 57(3): 508-521, ISSN: 1314-7471, **SJR: 0.196, Q3**

Abstract
Grape pomace is the main by-product of winemaking, a valuable source of polyphenols with antimicrobial, antioxidant and anti-cancer effect. Grape seeds and marcs extracts are of strategic importance for the development of new therapeutic approaches against certain cancer diseases, including breast cancer. In this study the antioxidant and antitumor potential of the polyphenolic

fraction of grape pomace obtained from the vinification of the local Bulgarian grape variety Mavrud is reported. After Soxhlet extraction with 50 % water solution of ethanol, the total polyphenol content of the extracts by the Folin-Ciocalteu colorimetric method was determined. The obtained extracts were characterized by HPLC-DAD and their antioxidant activity was studied with DPPH assay. The cytotoxic effect was tested on 3T3 cell line, while MCF-7 and MDA-MB-231 breast cancer cell lines were selected to determine antitumor activity. The results showed higher total polyphenol content in the marc compared to the seed extracts and prevalence of gallic acid, catechin and epigallocatechin. A correlation between antioxidant activity and total polyphenol content was established. The studied extracts had a low cytotoxic effect, as the seed extracts showed a stronger antitumor activity compared to the extracts of marcs and potential for treatment of luminal breast cancer.

24. Stoichev, S., Danailova, A., **Iliev, I**., Sulikovska, I., Strijkova, V., Mladenova, K., Andreeva, T. Fabrication and Biocompatibility of Layer-by-layer Assembled Composite Graphene Oxide-polysaccharide Microcapsules. Int. J. BIOautomation, 2022, 26(3): 225-240, ISSN: 1314-1902, **SJR: 0.159, Q4**

Abstract
The present study is focused on the construction and characterization of the morphology and biocompatibility of polysaccharide multilayered microcapsules (PMC) composed of natural polyelectrolytes (chitosan/alginate/hyaluronic acid), and on the effect of graphene oxide (GO) incorporation in the polymer matrix. The insertion of GO in the polymer matrix is an innovative and still evolving strategy used to modify the properties of the polyelectrolyte microcapsules. We have fabricated a number of hybrid GO-polysaccharide multilayered capsules by layer-by-layer assembling technique onto a CaCO3 core, followed by core decomposition in mild conditions. Hybrid microcapsules with different composition were constructed by varying the number or localization of the incorporated GO-layers. It was found that the thickness of the hybrid microcapsules, evaluated by atomic force microscopy, decreases after incorporation of GO nanosheets in the polymer matrix. Analysis of the viability and proliferation of fibroblasts after incubation with hybrid PMC revealed pronounced concentration-dependent cytotoxic and antiproliferative effect. Based on the results, we can conclude that the hybrid multilayered microcapsules made of natural polysaccharides and graphene oxide could be used for biomedical applications.

25. Georgieva, A., Todorova, K., **Iliev, I**., Dilcheva, V., Vladov, I., Petkova, S., Dolashki, A., Velkova, L., Dolashka, P., Toshkova, R. Assessment of the *In Vitro* and *In Vivo* Antitumor Activity of Hemocyanins from *Helix aspersa*, *Helix lucorum*, and *Rapana venosa* in a Graffi Myeloid Tumor Model. Biomedicines, 2023, 11(6): 1545, ISSN: 2227-9059, **IF: 3.900, Q1**

Abstract
Hemocyanins are oxygen-transporting glycoproteins in the hemolymph of some invertebrate species that attracted scientific interest as potential anticancer agents. The present study aims to assess the *in vitro* and *in vivo* anticancer activity of hemocyanins isolated from *Helix aspersa*,

Helix lucorum, and *Rapana venosa* in the Graffi myeloid tumor model. The *in vitro* antitumor activity of the hemocyanins was determined by a MTT test and cytomorphological analysis by fluorescent and transmission electron microscopy. The *in vivo* effects of the hemocyanins were examined in hamsters transplanted with Graffi tumor. The serum antibody titers against the tested hemocyanins and tumor antigen were determined by ELISA. Histopathological assessment of the morphological features related to antitumor effect, immune system response, and toxicity in some internal organs was performed. The results of *in vitro* studies indicated that the tested hemocyanins induced significant antiproliferative and apoptogenic effects. The *in vivo* investigations demonstrated a protective antitumor effect, expressed in reduced transplantability, suppression of tumor growth and metastasis, reduced mortality, prolonged survival time, and absence of toxic side effects. The present study indicated that the antitumor activity of the studied hemocyanins was due to both immune stimulation and direct effects on the tumor cells, and they displayed their potential as therapeutic agents against hematological malignances.

26. **Iliev, I**., Tsoneva, I., Nesheva, A., Staneva, G., Robev, B., Momchilova, A., Nikolova, B. Complementary Treatment of Breast Cancer Cells with Different Metastatic Potential with Iscador Qu in the Presence of Clinically Approved Anticancer Drugs. Current Issues in Molecular Biology, 2024, 46(11): 12457-12480, ISSN: 1467-3037, **IF: 3.000, Q2**

Abstract
European mistletoe extract (Iscador Qu) has been studied for decades, but it has not ceased to arouse scientific interest. The purpose was to investigate the impact of Iscador Qu on the antiproliferative potential of 11 standard chemotherapeutic agents on two breast cancer cell lines: MCF-7 low-metastatic and MDA-MB-231 high-metastatic and control cell lines (MCF-10A). MTT-dye reduction assay, FACS analysis, and PI staining were utilized. The most promising combinations acting against the MDA-MB-231 cell line were observed upon the simultaneous application of Iscador Qu (80 µg/mL) and Docetaxel, with 4-fold reduction in IC_{50}. An antagonistic effect was found under treatment with Cisplatin and Iscador Qu (1.5-fold increase in IC_{50}). The response of the low-metastatic breast cancer cell line MCF-7 to the tested combinations was different compared to the high-metastatic one. The most pronounced cytotoxic effect was found for the combination of Oxaliplatin and Iscador Qu (20 µg/mL) (5.2-fold IC_{50} reduction). An antagonistic effect for MCF-7 line was also observed when combinations with Olaparib and Tamoxifen were applied. This *in vitro* study offers new combinations between Iscador Qu and standard chemotherapeutic agents that hold great promise in establishing breast cancer therapeutic protocols compared to traditional monotherapies.

27. Andreeva, T., Rudt, A., Fabian, L., Ayaydin, F., **Iliev, I**., Jung, O., Barbeck, M., Der, A., Krastev, R., Taneva, S. Control of Cell Adhesion and Growth on Polysaccharide-Based Multilayer Coatings by Incorporation of Graphene Oxide. Coatings, 2024, 14(5):570, ISSN: 2079-6412, **IF: 2.800, Q2**

Abstract
Controlling cell adhesion, viability, and proliferation on solid surfaces is critical for the successful implantation and proper functioning of temporary and permanent medical devices. While, with

temporary or removable implants as well as surgical instruments, even slight cellular adhesion leads to an increased risk of secondary infections, bleeding and other complications, good cellular adhesion and viability are essential for the rapid healing and successful integration of permanent implants. This work was motivated by the growing interest in the construction of biocompatible and biodegradable coatings for the biofunctionalization of medical devices. Polysaccharide-based coatings are well known for their biocompatibility, but they are non-cell-adhesive, which hinders their application as implant coatings. In this study, we demonstrate that the incorporation of one or more graphene oxide layers in hyaluronic acid/chitosan multilayers is one avenue to regulate the degree of unspecific adhesion and growth of different cells (human umbilical vein endothelial cells, HUVEC, and mouse embryonic fibroblasts, 3T3). Furthermore, we demonstrate that this approach allows cell adhesion to be regulated across the entire range between completely prevented and highly promoted cell adhesion without introducing systemic cytotoxicity. These findings may contribute to the establishment of a new approach to adapt medical devices to cells and tissues.

28. Vladimirova, S., Hristova, R., **Iliev, I**. Synthesis, Cytotoxicity and Antiproliferative Effect of New Pyrrole Hydrazones. Molecules, 2024, 29(23): 5499, ISSN: 1420-3049, **IF: 4.600, Q1**

Abstract
Novel pyrrole-based carbohydrazide (1) and hydrazones (1A–D) were synthesized, charac terized, and subjected to spectroscopic studies. The hydrazones were obtained by reacting a pyrrole hydrazide with substituted pyrrole aldehydes. The initial carbohydrazide was prepared by selective hydrazinolysis of the obtained N-pyrrolylcarboxylic acid ethyl ester. The biological activity of the newly synthesized compounds was investigated *in vitro* on a panel of tumor and non-tumor cell lines. Mouse embryonic fibroblasts BALB 3T3 clone A31 were used in the safety test (BALB 3T3NRU-assay). Antiproliferative activity was determined on keratinocytes (HaCaT) and melanoma (SH-4) cells by MTT-dye reduction assay. The safety test of the compounds showed low cytotoxicity and absence of phototoxic potential. Among our novel pyrrole hydrazones, 1C was the most selective (SI = 3.83) in human melanoma cells and exhibited very good antiproliferative activity (IC_{50} = 44.63 ± 3.51 μM). The cytotoxic effect of 1C correlates with its ability to induce apoptosis and to cause cell cycle ar rest in the S phase. In addition, the results show that hydrazones obtained by condensation with β-aldehydes are more bioactive than those obtained by condensation with α-aldehydes.

29. Kamenova, K., **Iliev, I**., Prancheva, A., Tuleshkov, P., Rusanov, K., Atanassov, I., Petrov, P. Hydroxypropyl Cellulose Hydrogel Containing Origanum vulgare ssp. hirtum Essential-Oil-Loaded Polymeric Micelles for Enhanced Treatment of Melanoma. Gels, 2024, 10(10): 627, ISSN: 2310-2861, **IF: 5.300, Q1**

Abstract
Origanum vulgare ssp. *hirtum* essential oil (OEO) is a natural oil with high therapeutic potential. For some applications, however, the development of novel formulations is still needed to improve the bioavailability and stability of OEO. In this study, we describe the fabrication of an original

nanocomposite hydroxypropyl cellulose (HPC) physical hydrogel, containing OEO-loaded polymeric micelles, for topical delivery. The concentration of the main active compounds of OEO—carvacol and thymol—was determined using gas chromatography (GC) analysis. OEO was first encapsulated into Pluronic F127 micelles, and then embedded into HPC gel. Micellar and gel formulations of pure polymers and OEO-containing systems were characterized by dynamic light scattering (DLS) and rheology measurements, respectively. Selected formulations were evaluated for cytotoxicity and antiproliferative activity. The hydrogel formulation of HPC with micellar OEO (8% HPC, 2% F127, 1% OEO) exhibited sustained release of the oil and selectivity towards SH-4 tumor cells (an *in vitro* model of melanoma).

30. Zaharieva, L., Stoyanova, M., Dimova, V., Genova, T., Antonov, L., Markovski, A., **Iliev, I**., Andreeva, C. Absorbance measurement for interdisciplinary educational experiment on cytotoxicity. Physics Education, 2024, 59(6): 065018, ISSN: 0031-9120, **SJR: 0.523**

Abstract
We propose a multidisciplinary educational experiment, linking knowledge and skills in physics, biology and information technology through the training and application of the research method by students. From a physics point of view, the experiment consists in light absorbtion measurement, and in terms of biology, it demonstrates the process of cytotoxicity evaluation by means of the colorimetric method Neutral Red Uptake in vitro test (NRU assay). The test allows to determine the concentration of a test drug at which 50% cytotoxicity (CC_{50} values) of the cells is observed. After initial exposure to the tested drug, Neutral Red is added to the cell culture medium, it is absorbed by the living cells and subsequently released in a desorb buffer. The cell culture medium becomes coloured, and the more intensive colour corresponds to higher cell viability, while more transparent solution corresponds to lower number of living cells. The NR concentration in the solution is estimated by the absorbance value. We measure the absorbance of NR by utilizing a very affordable educational-grade spectrometer and compare its output with that of a professional microplate reader. We show that the educational spectrometer gives a result on the CC_{50} value that is in agreement with the acceptable values of the official protocol. We include a comment on the results when performing this laboratory exercise in one class of 18-year old students (in their last year of secondary education). The experiment can be also successfully applied in the laboratory practicum of first year university study.

31. Mihaylova, V., **Iliev, I**., Vasileva, A., Mazzio, E., Mochona, B., Mateeva, N., Tasheva, D. Synthesis and Evaluation of the Antiproliferative Activity of the Derivatives of 3,5-Diaryl-3,4-dihydro-2H-pyrrole-2-carboxylic Acids. Molecules, 2025, 30(7): 1602, ISSN: 1420-3049, **IF: 4.600, Q1**

Abstract
The metabolic cycle of L-proline plays a crucial role in cancer cell survival, proliferation, and metastasis. A key intermediate in the biosynthesis and degradation of proline is 3,4-dihydro-2*H*-pyrrole-2-carboxylic acid. A direct route for synthesizing substituted derivatives of this acid involves the cyclization of 2-amino-5-oxonitriles. Michael additions of [(diphenylmethylene)amino]acetonitrile to enones in a basic medium—either with aqueous

sodium hydroxide or under solid–liquid phase-transfer catalysis conditions using CaO as a base—enable the synthesis of substituted 2-amino-5-oxonitriles as single diastereoisomers or as diastereoisomeric mixtures. Selective removal of the diphenylmethylene-protecting group, followed by in situ cyclization in acidic conditions, yields *trans*- and *cis*-3,5-diaryl-3,4-dihydro-2*H*-pyrrole-2-carbonitriles. The reaction of nitriles with HCl / dioxane / methanol followed by treatment with water produces esters and amides as by-products. In vitro screening of the synthesized compounds against multiple human cancer cell lines revealed that some compounds exhibit a good or high selectivity index. In conclusion, the synthetic schemes presented offer simple and efficient routes for the preparation of the derivatives of substituted 3,4-dihydro-2*H*-pyrrole-2-carboxylic acids, with some compounds exhibiting promising antiproliferative activity.

32. Vilhelmova-Ilieva, N., Nenova, R., Kalinov, K., Dobreva, A., **Iliev, I**. Anti-Herpes Simplex Virus Type 1 Activity of Rosa damascena Mill Essential Oil and Floral Water in Retinal Infection *In Vitro* and *In Silico*. International Journal of Molecular Sciences, 2025, 26(15): 7521, ISSN: 1422-0067, **IF: 4.900, Q1**

Abstract
Recently, essential rose oils and rose products have gained increasing importance in both the cosmetic and food industries, as well as in the composition of medicinal products. We investigated the *in vitro* antiviral activity of essential oil and floral water from *Rosa damascena* Mill against herpes simplex virus type 1 (HSV-1) infection in rabbit retinal cells (RRCs). The composition of the main chemical components in the rose essential oil was determined by means of gas chromatographic analysis. The effect on the viral replication cycle was determined using the cytopathic effect (CPE) inhibition assay. The virucidal activity, the effect on the adsorption stage of the virus to the host cell, and the protective effect on healthy cells were evaluated using the endpoint dilution method. The effects were determined as deviation in the viral titer, Δlg, for the treated cells from the one for the untreated viral control. The identified main active components of rose oil are geraniol (28.73%), citronellol (21.50%), nonadecane (13.13%), nerol (5.51%), heneicosane (4.87%), nonadecene (3.93), heptadecane (2.29), farnesol (2.11%), tricosane (1.29%), eicosane (1.01%), and eugenol (0.85%). The results demonstrated that both rose products do not have a significant effect on the virus replication but directly affect the viral particles and reduce the viral titer by Δlg = 3.25 for floral water and by Δlg = 3.0 for essential oil. Significant inhibition of the viral adsorption stage was also observed, leading to a decrease in the viral titers by Δlg = 2.25 for floral water and by Δlg = 2.0 for essential oil. When pretreating healthy cells with rose products, both samples significantly protected them from subsequent infection with HSV-1. This protective effect was more pronounced for the oil (Δlg = 2.5) compared to the one for the floral water (Δlg = 2.0). We used the in silico molecular docking method to gain insight into the mechanism of hindrance of viral adsorption by the main rose oil compounds (geraniol, citronellol, nerol). These components targeted the HSV-1 gD interaction surface with nectin-1 and HVEM (Herpesvirus Entry Mediator) host cell receptors, at N-, C-ends, and N-end, respectively. These findings could provide a structural framework for further development of anti-HSV-1 therapeutics.

33. Vladimirova, S., Hristova, R., **Iliev, I**. Novel methyl-substituted pyrrole hydrazones as selective melanoma agents. Current Research in Biotechnology, 2025, 10: 100354, ISSN: 2590-2628, **IF: 4.000, Q2**

Abstract

Melanoma represents the most aggressive form of skin cancer, derived from melanocytes the pigment- producing cells located in the epidermis. Effective treatment of melanoma remains a major clinical challenge, largely due to its capacity for immune evasion, rapid metastatic spread, and the development of resistance to therapeutic interventions. Based on our previously developed compounds with high selectivity towards mela noma SH-4 cell line, we created a new series of compounds in which we replaced the halogen atom with a methyl group in the precursor pyrrole-based carbohydrazide 2. All derivative hydrazones (2A–D) were synthesized by the condensation of carbohydrazide 2 with various substituted pyrrole aldehydes, subsequently characterized, and subjected to detailed spectroscopic analysis. *In vitro* evaluation of these newly developed compounds was performed across a diverse set of cancerous and non-cancerous cell lines to determine their biological effects. A safety profile was established using the BALB 3 T3 NRU-assay with mouse embryonic fibroblasts (BALB 3 T3 clone A31), revealing minimal cytotoxicity and an absence of phototoxic effects. Antiproliferative effects were quantified in keratinocytes (HaCaT) and melanoma (SH-4) cells using the MTT dye reduction assay. Of particular note, compound 2C emerged as the most selective agent (SI = 5.51) against human melanoma cells, displaying significant antiproliferative activity (IC_{50} = 31.93 ± 2.59). Further investigation revealed that the cytotoxic action of 2C is mediated by the induction of cell accumulation in the S/G2 phase. Consistent with our prior research, the current results further confirm that hydrazones synthesized via condensation with β-aldehydes exhibit enhanced bioactivity relative to those obtained from α-aldehydes.