1ST INTERNATIONAL SCIENTIFIC CONFERENCE

ADVANCES IN BIOMEDICAL RESEARCH WITH USE OF IN VITRO METHODS

ABSTRACTS

Editors:
Beata Bujalska
Monika Iwaniuk

Lublin, 17.06.2021 r.

1st International Scientific Conference "Advances in biomedical research with use of *in vitro* methods"

Book of abstracts

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Table of content:

Plenary Speakers

Participants

Plenary Speakers

In vitro cultured tissue-engineered product in skin regeneration – clinical application

***Prof. Justyna Drukała**, Head of CELL BANK – ATMP Manufacturing, the Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Cracow*

Improvement of methods of human cell isolation and cultures and the use of biomaterials to create biocompatible scaffolds have allowed the development of tissue engineering and regenerative medicine. It is a promising alternative for conventional methods of organ transplantation.

Skin is a specialized barrier that separates the internal organs of our body from the external environment and determines homeostasis of the whole organism. Epidermis, the most outer layer of the skin, ensures fast and efficient wound closure in the case of injury. The isolation of epidermal stem and progenitor cells residing in the germinal layer enables the reconstruction of epidermis *in vitro* and its application in clinics to enhance wound healing process.

Laboratory manufacturing advanced therapy medicinal products (ATMP) namely, cells cultured for clinical purposes, is obliged to develop and implement the quality assurance system to obtain a consent of the Main Pharmaceutical Inspector after Committee of Advanced Therapies recommendation on ATMP classification.

For over 20 years our laboratory has been using cultured keratinocytes derived from the skin biopsy to provide permanent wound coverage for extensive burn treatment. The suspension of cultured epithelial autografts (CEA) in a fibrin glue is transplanted onto the wound bed. The ability of CEA to ensure efficient wound healing with minimal hypertrophy makes it an attractive alternative to split-thickness skin graft. The use of CEA directly on a simple granulation tissue or other vascularized wound bed is also possible in the case of deep wounds, however, this procedure usually leads to

substantial scar formation and thus, not satisfactory results. The previous application of INTEGRA Dermal Regeneration Template® is of a great importance in such cases. INTEGRA is an advanced biocompatible wound care product, a "device" that facilitates regeneration of the dermal layer by patient's organism. In our laboratory, we use combined ATMP product – Integra with adipose derived mesenchymal stem cells to accelerate its integration. We apply this innovative approach as local treatment of post-burn scars in an ongoing clinical trial in Malopolska Burns and Plastic Surgery Centre, The Rydygier Memorial Hospital in Krakow.

This research is Sponsored by National Program for the Development of Transplant Medicine 2011-2021.

The use of fetal bovine serum in *in vitro* methods: Blessing or curse?

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Foetal Bovine Serum (FBS), also known as Foetal Calf Serum (FCS), is still being applied as the universal medium supplement to grow and maintain most cells and tissues. But, the use of FBS has also been regarded critically for decades. The use of FBS presents four significant issues:

i. the degree of suffering experienced by the calf during blood collection;
ii. inappropriate cellular growth profiles and physiological responses of cells;
iii. FBS contamination with viruses, prions, etc.;
iv. the large variability of FBS such that it is very difficult to even ensure consistent and well controlled in vitro cell culture between batches.

Since, being a biological product, FBS differs in composition between different batches dependent on origin, moment of collection and composition of the herd, the use of FBS is a serious factor that contributes to the reproducibility crisis. Nevertheless, to date, FBS is used at a large scale, despite many successful attempts to develop FBS-free media.

For many years, xeno-free cell culturing, in particular when applied in human therapeutic research, is stimulated for above mentioned reasons. One attractive solution is the use of human platelet lysates (hPL's) as a valuable alternative to FBS as cell culture supplement when cells and tissues of human origin are cultured. But, as hPL's are undefined and, thus, also show batch-to-batch variations, these are not always the best solution.

The use of chemically-defined media appeared to be the best approach. In contrast to FBS, the chemically-defined media are generally cell-type specific. For several cell types, chemically-defined media have now been developed. To quickly identify whether and which medium is available for a specific cell type, the FCS-free database (fcs-free.org) was established. This

database provides an overview of commercially available serum-free media for cell and tissue culture, as well as medium formulations for specific cell types obtained from scientific literature.

But also strategies to develop a serum-free medium for specific cells are available and will be discussed.

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SARS-CoV-2 RBD *in vitro* evolution parrots and predicts contagious mutation spread

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SARS-CoV-2 is continually evolving, with more contagious mutations spreading rapidly. Using *in vitro* evolution to affinity maturate the receptor-binding domain (RBD) of the spike protein towards ACE2 resulted in the more contagious mutations, S477N, E484K, and N501Y, to be among the first selected, explaining the convergent evolution of the "European" (20E-EU1), "British" (501.V1), "South African" (501.V2), and Brazilian variants (501.V3). Plotting the binding affinity to ACE2 of all RBD mutations against their incidence in the population shows a strong correlation between the two. Further *in vitro* evolution enhancing binding by 600-fold provides guidelines towards potentially new evolving mutations with even higher infectivity. For example, Q498R epistatic to N501Y. Nevertheless, the high-affinity RBD is also an efficient drug, inhibiting SARS-CoV-2 infection. The 2.9Å Cryo-EM structure of the high-affinity complex, including all rapidly spreading mutations, provides a structural basis for future drug and vaccine development and for *in silico* evaluation of known antibodies.

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Using mesenchymal stem cells in COVID-19 therapy

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The COVID-19 disease pandemic, caused by the SARS-CoV-2 coronavirus, still remains a major public health problem, as well as a therapeutic challenge. The virus triggers a "cytokine storm" in some hosts' tissues resulting in overproduction of various cytokines and stimulation of the immune system to an inadequate immunological response, involving many kinds of mutually-catalyzing inflammatory processes. The effects of this response include a severe damage within the respiratory system and other vital organs, that can lead to multi-organ failure and to patient's death. As for now, there are no effective medicines, that would be able to stop that damage, and save severely ill COVID-19 patients. The numerous medicines that have been used so far are useful as supportive therapy, but not quite able to suppress all the pathological processes accompanying the disease. The promising alternative for COVID-19 pharmacotherapy seem to be mesenchymal stem cells (MSC) that have been introduced into the regenerative medicine. These multipotent stem cells existing in various types of human tissues, secrete some immunomodulatory factors and might be found helpful in inhibiting a "cytokine storm" and repairing damages resulting from this process. Among others, human amniotic cells (hAC) could be used as a source of immunomodulatory factors. Isolated hAC have shown constitutive expression of several markers commonly measured on pluripotent stem cells, multipotent differentiation capacity, and upon transplantation they never form cancer tumors. hAC have been successfully

used in tissue engineering or regenerative approaches. The unique multifactorial activity provides MSC with a major advantage in comparison to the classic pharmaceuticals. The outcomes of clinical trials that have been conducted so far, are promising in the effective and safe MSC-based COVID-19 therapy.

The effect of *Nigella sativa* essential oil on human T cell proliferation, activation, and apoptosis *in vitro*

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Nigella sativa, also known as black cumin, is an annual flowering plant in the Ranunculaceae family with well-documented health potential. According to research papers, Its seeds, oil, or extracts present antibacterial, antifungal, antioxidant, and anti-inflammatory properties. Also, it has been shown that black cumin reduces the symptoms of asthma, allergic rhinitis, or rheumatoid arthritis in humans, but the mechanism of its action is not fully understood. Allergies and autoimmune diseases are related to lymphocytes' abnormal activity in response to allergens or autoantigens. Therefore, the aim of this study was to assess the *in vitro* influence of Nigella sativa essential oil on human CD4+ and CD8+ lymphocytes, especially their proliferation and their susceptibility to apoptosis. The study group consisted of 10 prescreened healthy people of mean age of 27. Peripheral blood mononuclear cells were isolated, stained with Violet Proliferation Dye 450, and stimulated with an immobilized monoclonal anti-CD3 antibody in the presence of serial ethanol dilutions of *N. sativa* essential oil in standard

culture conditions (5% CO_2, 100% humidity at 37°C) for five days. Cells were then collected after 72 and 120 hours and stained with monoclonal antibodies directed against CD4, CD8, CD28, or CD25 antigens. Cells also were stained with annexin V or 7-AAD and analyzed with flow cytometry. *Nigella sativa* essential oil has been shown to inhibit proliferation of CD4+ and CD8+ lymphocytes in a dose-dependent manner – the percentage of proliferating cells was significantly decreased in the presence of 1:10, 1:50, and 1:100 oil dilutions. At the same time, the percentage of living cells was significantly decreased, while the percentage of cells apoptotic and necrotic was increased. The results have shown that *N. sativa* essential oil has a cytotoxic effect on human lymphocytes *in vitro*.

Biological activity of synthetic sulfonamide derivatives of pyrazolo[4,3-e]tetrazolo[4,5-b][1,2,4]triazine in normal and cancer cells *in vitro*

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Conventional chemotherapy is non-specific and exhibits toxic effects both in cancer and normal cells. Many commonly used drugs in medicine contain a heterocyclic core or substituent of this type. One important approach to antineoplastic agent development is the design of antimetabolite drugs whose structure is similar to those of pyrimidines and purines involved in the biosynthesis of DNA. Here we present possible biological activities of a novel class of sulfonamide pyrazolo[4,3-e]tetrazolo[4,5-b][1,2,4]triazine derivatives.

The objective of the work: The main objective of the presented research was to evaluate the cytotoxic and genotoxic potential of sulfonamide derivatives of pyrazole-triazine in normal and cancer cells in vitro.

Materials and methods: BXPC3 (pancreas adenocarcinoma), PC3 (prostate adenocarcinoma) human cancer cell lines, and normal lung fibroblast cell line – WI-38, were used in the study. MTT assay was used to estimate IC50 values for tested compounds. The genotoxicity of sulfonamide derivatives was estimated using alkaline comet assay and compared with bleomycin-induced DNA damage.

Results: Mean IC50 values after a 72 h incubation of cells with the studied compounds ranged from 0,11-0,33 μM in cancer cells and 0,27-0,65 μM in

normal fibroblasts. Genotoxicity of tested compounds used in nanomolar concentrations varied with median tail DNA percent of 0,17-18,81% in cancer cells or 0,01-1,5% in normal fibroblasts, and 19,31-50,79% and 29,13% respectively for 20 μM bleomycin.

Conclusions: Tested compounds were more cytotoxic in cancer cell lines compared with normal cell line, the compounds were more cytotoxic towards prostate cancer cell line (PC3) compared with other cell lines, in all cell lines MM137 derivative was the most cytotoxic agent, compounds exhibited varied genotoxicity across cell lines used in the assay with little effect on normal cell line and profound effect on pancreas adenocarcinoma (BXPC3) cells.

Characterization of murine alternatively activated macrophages (M2) under the influence of interleukin 6

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Macrophages are important cells of the innate immune response. These cells are extremely plastic, in the changing conditions of the surrounding microenvironment macrophages show two opposite phenotypes: classically activated M1 phenotype and alternatively activated M2 phenotype. The activity of M1 macrophages, due to their cytotoxic activity, should be strictly controlled, e.g. through their polarization into M2 immunosuppressive macrophages. The mechanism responsible for macrophages phenotypic transition still remains unknown.

The aim of this work was to obtain macrophages with the M2 phenotype under the influence of Interleukin 6.

Bone marrow was isolated from femurs and tibiae of C57BL/6 mice 6-10 weeks of age. Isolated cells were differentiated into macrophages using

M-CSF for 7 days. After one week Bone Marrow-Derived Macrophages (BMDM) were incubated for 48 h with four different concentrations of Interleukin 6 (25, 50, 100, or 200 ng/mL). The control M2 macrophages were incubated for 48 h with IL-4 (10 ng/mL) or with IL-10 (10 ng/mL). The morphology of differentiated macrophages was analyzed using phalloidin staining. Fluorescence imaging of the stained cells was performed using an LSM710 confocal microscope. Macrophages growth during in vitro differentiation was assessed using the MTS assay after 48 hours. The macrophages phenotype was determined after 48 hours by flow cytometry.

IL-6 greatly increased the viability of isolated Bone Marrow-Derived Macrophages. IL-6 increased the expression of CD206 (characteristic for M2 macrophages) in BMDM in all used concentrations. The obtained macrophages also differed in morphology.

We hope our work will provide new information on the properties of M2 macrophages obtained under the influence of IL-6.

Cytotoxicity, DNA- and albumin-binding of the cobalt(II), iron(III) and nickel(II) complexes with the non-steroidal antiinflammatory drug niflumic acid

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After the accidental discovery of cis-platinum, extensive attempts have centralized on the rational design of metallic compounds for cancer treatment. The interaction of compounds with nucleic acids and proteins has been extensively studied in recent years since many drugs owe their biological effects to various reversible or irreversible interactions. Such research is important not only in understanding the mechanism of interaction but can also be used in the design of new drugs. The aim of the study was to evaluate the cytotoxicity of niflumic acid (HNif) and its new complexes with Co^{2+}, Fe^{3+} and Ni^{2+} ions, as well as to investigate the properties of DNA and protein binding with the use of UV-Vis and fluorescence spectroscopy. The in vitro cytotoxicity of the complexes against two human cancer (DU145 and MCF-7) cell lines and one non-cancerous human fibroblasts (BJ) cell line was evaluated using a neutral red assay. The results indicated the specificity of HNif-complexes to cancer cells and demonstrated that the complexation of niflumic acid with metal ions increases its biological activity. The concentration of each complex required for 50% inhibition of cell growth was higher in comparison with cisplatin. The UV-Vis spectra confirmed the formation of complexes between tested compounds and: i) calf thymus DNA (ct-DNA); ii) bovine serum albumin (BSA). Competitive displacement assay has shown that all compounds are

able to displace Hoechst 33342 dye from ct-DNA–Hoechst complex suggesting the minor groove binding mode. Experiments using warfarin and flufenamic acid site markers lead to the conclusion that HNif can interact with BSA only at site I, while all three metal ion complexes interact both at a site I and II. Investigation of the mechanism/s of cell death following exposure to complexes of HNif needs further performance of different staining techniques, flow cytometry, and gene expression analysis, as well as *in vivo* absorption, distribution, and excretion evaluation.

Simultaneous Inhibition of Protein Kinase CK2 and Thymidylate Synthase or Focal Adhesion Kinase Results In Synergistic Effect on Hormone Dependent Breast Cancer Cells

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Protein kinase CK2 has been considered an attractive drug target for anticancer therapy. CK2 belongs to a serine/threonine kinase family and phosphorylates numerous proteins associated with the regulation of many signal pathways involved in cell cycle regulation, cell proliferation, and apoptosis. The purpose of our work was to test the anticancer effect of simultaneous inhibition of protein kinase CK2 and thymidylate synthase (TS) or FAK (focal adhesion kinase). The combination effect of 5-fluorouracil (5-FU) with either a new inhibitor of protein kinase CK2, namely 3-(4,5,6,7-Tetrabromo-2-methyl-1H-benzimidazole-1-yl)propyl octanoate (6c) or FAK inhibitor 14 on the viability of MCF-7 breast cancer cell line was studied. Combination index (CI) values were determined using MTT-based assay and the Chou-Talalay model. The effect of the tested drug combinations on pro-apoptotic properties and cell cycle progression was examined using flow cytometry. CK2-mediated phosphorylation of NF-κB p65 after the combined treatment was evaluated by the western blot method. Our results showed that a synergistic effect (CI < 1) occurred in MCF-7 after treatment with both combinations of 5-FU with 6c or inhibitor 14. We concluded that the synergistic interactions observed for both tested combinations correlated with a significant decrease of CK2-mediated phosphorylation of NF-κB p65

after the combined treatment. We also detected prolongation of the S-phase of the cell cycle and an increase of apoptosis in cells treated with combinations. The obtained results support the recent observation that CK2 inhibitors can improve 5-FU-based anticancer therapy and FAK kinase can be an attractive molecular target in breast cancer therapy.

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Methodological aspects of the measurement of human Mas-Related G Protein-Coupled Receptor-X2 levels in human serum

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Mas-Related G Protein-Coupled Receptor-X2 (MRGPRX2)-dependent reactions are currently one of the most extensively studied new possible mechanisms responsible for clinical manifestations of immediate hypersensitivity. Preliminary reports from human studies suggest that MRGPRX2 concentration is increased in patients with allergic asthma. To date, our research has not confirmed this. To clarify discrepancies in reports, by means of re-validation of existing results with an alternative laboratory kit and additional negative and positive controls. Real-time PCR was used to determine MRGPRX2 transcript levels in human cells and tissues including skin and dermal fibroblasts. MRGPRX2 levels in serum samples of 12 individuals (6 healthy controls, 2 allergic asthmatics, 3 patients with a history of anaphylactic reactions due to Hymenoptera stinging, and 1 patient with urticaria) were measured by ELISA (Abbexa) and were re-tested using an alternative ELISA kit (MyBioSource). Real-time PCR showed that MRGPRX2 transcripts are present in the whole dermis but not in dermal fibroblasts. Therefore, human dermis and dermal fibroblasts lysates can be used as positive and negative controls, respectively. Comparison of commercial ELISA assays for quantification of MRGPRX2 serum levels revealed enormous variability of total MRGPRX2 protein in the same serum samples, independently from the clinical background of tested individuals. The ELISA kit provided by MyBioSource showed low specificity since there

were no differences in MRGPRX2 protein levels between positive and negative controls. MRGPRX2 serum levels are not increased in patients suffering from immediate hypersensitivity reactions. Caution should be exercised while selecting an ELISA kit targeting MRGPRX2 because of the potential risk of unspecific bindings.

Effects of different forms of vitamin C on epigenetic DNA modification levels in the cellular model of chronic myeloid leukemia

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Introduction: Molecular diagnosis of cancer is primarily based on analysis of gene mutations and chromosome rearrangements. However, mutations are not the only possible cause of carcinogenesis. Changes in gene expression caused by impairments in epigenetic mechanisms are present in every type of cancer, especially in hematological malignancies. 5-methylcytosine (5-mC) is an epigenetic marker often linked with gene silencing and is oxidized by TET (Ten-Eleven Translocation) dioxygenases in a 2-ketoglutarate, Fe^{2+} dependent reaction to 5-hydroxymethylcytosine (5-hmC) and further to 5-formylcytosine (5-fC) and 5-carboxycytosine (5-caC), which are removed

from DNA by BER pathway. Vitamin C is a potent antioxidant that enhances TET protein activity by restoring the pool of available iron ions, therefore its level may affect global epigenetic make-up and gene expression. Methods: HAP1 (chronic myeloid leukemia cells) were cultured in accordance with manufacturer recommendations and exposed for 24 h to different vitamin C forms: ascorbic acid, ascorbic acid sodium salt, ascorbic acid palmitate, and ascorbic acid sodium salt encased in a liposomic carrier. Levels of epigenetic derivatives of 5-mC in cellular DNA were analyzed by two-dimensional ultraperformance liquid chromatography with tandem mass spectrometry. Results: Each form of vitamin C enhanced TET protein activity in a similar, time and dose-dependent manner. 100 uM concentration of vitamin C, regardless of its form, was sufficient to increase levels of 5-hmC, 5-fC, and 5-caC in leukemia cells. Conclusions: It seems that the concentration of vitamin C rather than its form is crucial for enhancing the activity of TET proteins. Our findings indicate that vitamin C supplementation may normalize an aberrant pattern of epigenetic modifications and may be considered as an adjuvant treatment of chronic myeloid leukemias.

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Bioluminescence as a sensitive marker for detection of electroporation *in vitro*: comparison with fluorescent labeling

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High-intensity pulsed electric fields (PEF) can cause electropermeabilization of the cell membrane, also known as electroporation, which is employed in gene and drug delivery, cancer treatment, and biotechnology. For the successful development of parametric protocols and standardization, quantification of the cell membrane permeability is required. Therefore, propidium iodide (PI) and YO-PRO-1 (YP) can be highlighted as one of the most popular fluorescent probes used to detect permeabilization after electroporation. In this work, a methodology based on cell bioluminescence was employed for the analysis of the electroporation phenomenon in vitro. As a model, mice myeloma cell line (Sp2/0) has been transfected with Luciferase-pcDNA3 plasmid and it was shown that oxidation of D-luciferin sodium salt can be successfully utilized to assay electroporation via the

expression of the luciferase gene. Electroporation has been studied using the 0.1-5 μs × 250 and 100 μs × 1-8 pulsing protocols in 1-2.5 kV/cm range. Also, sequences of 12.5 kV/cm x 100 ns pulses have been used to grasp the differences between nanosecond and microsecond pulses. Lastly, the effects of high (MHz range) and low (kHz range) frequency pulsing have been covered. It was shown that electroporation triggers a significant increase in the bioluminescence signal, which scales with the increase of the number of pulses. The sensitivity of the methodology is on par with PI electrotransfer, however, when calcium is added to the buffer (2-10 mM), the sensitivity improves even further – the effects of 100 ns pulses can be detected. The improvement of sensitivity due to extracellular calcium is likely associated with increased metabolism and/or increased ATP consumption due to calcium entry when cells are electroporated.

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The electrodesensitization effect on cells after electroporation using microsecond pulses

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Electroporation nowadays is a well-known phenomenon that has gained broad applications from food processing to gene therapy and cancer treatment. The fundamental mechanism of such phenomenon is based on the induction of pore formation in the plasma membrane of the cells as a result of external electric field application. The pores formed allow electrotransfer through the membrane of various molecules, that otherwise cannot permeate through the membrane. Under well-controlled conditions, the pores can reseal, and cells preserve viability. Nevertheless, in comparison to unaffected cells, previously electroporated cells respond differently to electric field treatment. Thus, this phenomenon, that is related to cell sensitization or desensitization can be regarded as cell memory. This study aimed to investigate cell sensitization/desensitization following electroporation. CHO cells were electroporated using 2 pulses with the strength of 1200-1400 V/cm duration 100 µs, at 1 Hz. Electrotransfer of PI into control or previously electroporated cells (10 min before the second electroporation) was done by 1 pulse with the strength of 1200-1400 V/cm duration 100 µs. Electrotransfer efficiency was evaluated using fluorescence microscopy and flow cytometry.

Fluorescence microscopy images were processed by using ImageJ. Flow cytometry analysis was performed by using FLOWJO software. Cell viability was assessed by using a clonogenic assay. The obtained results showed that PI electrotransfer into cells that were previously electroporated was less efficient in comparison to unaffected cells. Results also showed that at least partially this electrodesensitization effect can be explained by a decrease in cell size following electroporation.

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Exploring the pharmacokinetic and pharmacogenetic properties of haloperidol using *in-vitro* methods: a review

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Haloperidol ($C_{21}H_{23}ClFNO_2$; 375,9 g/mol) is a drug with antipsychotic and antiemetic properties, that is widely used in modern psychiatry. From a chemical point of view, it is a phenylbutylpiperadine derivative, composed of a central piperidine structure with hydroxy and p-chlorophenyl substituents at position 4 and an N-linked p-fluorobutyrophenone moiety. Moreover, it is an aromatic ketone, organofluorine compound, classified as a member of monochlorobenzenes. After oral administration, it is absorbed from the gastrointestinal tract. It can also be administered intramuscularly (i.m.), which results in better bioavailability. The main pharmacological mechanism is that it is antagonistic to D2 dopamine receptors. Haloperidol has also a less antagonistic effect on the following receptors: 5-HT2, α1, and D1. Recent studies indicate that it may affect the functioning of microglia. Haloperidol inhibits interferon-gamma-induced (IFN-γ) microglial activation by reducing inducible nitric oxide synthase (INOS) and TNF-α expression in mice microglial cells. However, this inhibition is lesser than those induced by risperidone and aripiprazole. Haloperidol increases the glycolytic activity of cells, while aripiprazole reduces it. The pharmacokinetic properties may be improved by conjugating the drug with polymers: poly(ethylene glycol) and polyamidoamine dendrimers (PAMAM). Certain genotypes can influence the metabolism and toxicity of haloperidol. For example, CYP2D6 polymorphism may affect drug concentration levels in patients with alcohol use disorder. SCL6A3 gene polymorphism may affect the safety of haloperidol.

In vitro study on the interaction of veterinary drugs

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Improper husbandry practices lead to the presence of veterinary drugs in food. The problem of its safety still remains unresolved due to the lack of data on consumer exposure to mixtures of various veterinary drugs. Enrofloxacin (ENRO) is the most commonly used fluoroquinolone in Poland due to its broad spectrum of antibacterial activity. Fipronil (FIPRO) is approved for the control of ectoparasites in companion animals. However, due to its broad spectrum of antibacterial activity, it is used illegally by farmers on animal farms. This study was conducted to evaluate the interaction between the most commonly detected veterinary drugs in food: ENRO and FIPRO on three cell cultures HepG2, Caco-2, and Balb/c 3T3. The cytotoxic effects of ENRO and FIPRO and their mixtures (1:1; 2:1 and 1:2) on basic cellular parameters i.e.: cell metabolism (MTT assay), lysosomal activity (NRU assay), proliferation (TPC assay), and cell membrane integrity (LDH assay) were evaluated. The nature of the fluoroquinolone-pesticide interaction was determined using the combination index (CI) after 72 h exposure. The toxicity of the study drugs and their mixtures was shown to depend mainly on the cell culture. The highest sensitivity was observed in Balb/c 3T3 > HepG2 > Caco2 cells. The toxic effect was proportionally dependent on the concentrations of the drugs in their mixtures. The mechanism of action was mixture-dependent. The EC20 concentrations for the mixtures (0.08-0.28 µg/ml – Ba lb/c 3T3; 1.6-4.5µg/ml – HepG2 and 3.3-4.75 µg/ml – Caco-2) were lower compared to the concentrations for ENRO. The nature of the interaction between ENRO and FIPRO was dependent on cell culture, method, and concentrations used. Synergistic and antagonistic effects of both drugs were observed in Balb/c 3T3, HepG2, and Caco-2 cells, respectively. The results obtained suggest that the mixtures of veterinary

drugs i.e. ENRO and FIPRO are a toxicological problem and pose a health risk to consumers.

The study was funded under the in-house project "Veterinary drug interactions *in vitro* studies" (No. F/106) carried out at the National Veterinary Institute – National Research Institute in Puławy.

Electrochemical methods for studies of the cell suspensions

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Most of the researchers use electrochemical analysis just for one purpose – measurements of pH. However, potentiometric analysis using selective electrodes, amperometry, or conductometry can be used for very different assays of biological micro-objects. In my talk will analyze, how electro-chemical measurements using electrodes selective to different compounds can be used for the determination of the physiological activity and/or viability of cells, characterization of the viral infections, or assays of the sensitivity of cells to antimicrobial compounds. Examples will be presented, how the viral infection cycle can be directly monitored using different electrodes. Determination of multidrug resistance of microorganisms and the search of the inhibitors of efflux is another research area to be discussed in my talk. Electrochemical monitoring allows additional analyses of samples taken directly from the reaction vessels, i.e., measurements of absorbance or fluorescence, determinations of ATP content, or counting of cells and viruses. During electrochemical monitoring, aeration of the cell suspension can be controlled by selecting the speed of the magnetic stirring, cell suspensions in the incubation vessels can be thermostated. Even monitoring of pH of the cell suspension allows to evaluate the intensity of the energy metabolism or to detect changes in the plasma membrane permeability to H+. Investigations in the area of development of PVC membrane-based ion-selective electrodes will be also discussed.

Antiviral capacity of doxycycline against SARS-CoV-2 *in vitro*

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Coronavirus disease (COVID-19) is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), against whose entry or genomic replication there are no effective drugs. Thus, finding an effective treatment that combats this pathogen is an urgent priority. Doxycycline exhibits broad-spectrum activities and has antimicrobial, antimalarial, and anti-inflammatory properties. However, studies on doxycycline activity against SARS-CoV-2 are very limited. This study aimed to investigate the antiviral activity of doxycycline against SARS-CoV-2 in vitro. Doxycycline (0.1-100 μM) was administered 2 h before infection. Human nasal epithelial cells (hNECs) were then infected with the clinically isolated SARS-CoV-2 strain, IHUMI-3, at a multiplicity of infection of 0.5 μM. Replication was evaluated using real-time RT-qPCR 48 h before infection. The 50% cytotoxicity concentration (CC50) and half-maximal effective concentration (EC50) were determined using sigEmax (inhibitory) and evaluated using nonlinear regression; the final values were presented as means of 5-8 separate assessments, followed by evaluation of doxycycline activity against IHUMI-3 (5 μM). Treatments were as follows: full-time, wherein hNECs underwent pre-treatment for 2 h followed by doxycycline administration for 48 h; entry, wherein hNECs were treated with doxycycline for 2 h before infection and replenished with fresh

medium without doxycycline at 4 h after infection; and post-entry, wherein doxycycline was administered 4 h after infection. The peak serum concentration (Cmax) of doxycycline was evaluated for oral and intravenous administrations. Cytotoxicity analysis of doxycycline revealed a CC50 > 100 µM (48 h). The EC50 for doxycycline was 5.2+3.3 µM, and the Cmax/EC50 ratio in blood was 0.81, 2.32, and 1.36 for 100 and 200 mg oral and 100 mg intravenous administration, respectively. These study results showed acceptable doxycycline levels before and after SARS-CoV-2 infection in hNECs. Our study showed a significant increase in in vitro antiviral activity of oral and intravenous doxycycline against SARS-CoV-2. Doxycycline was effective against SARS-CoV-2 both, at entry and post-entry. Compared with other available antiviral drugs, doxycycline might be a significant adjunct to COVID-19 treatment owing to its affordability and minimal side effects.

Index of Authors

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