Session 4: Pharmaceutical Biotechnology

Lectures

L4.1

Nucleic Acid Therapy — from off target effects to metabolic networks

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In biological studies, the "antisense effect" of oligonucleotides is one of the most important tools to regulate gene expression. Surprisingly, after more than 20 years of research, therapeutic application of this approach is still far away from reaching its tremendous perspectives. Obviously, in the first "heroic" phase of gene therapy, the perfect target specificity of oligonucleotides has drawn the attention of the researchers too far away from important criteria of drug development, such as toxicity and pharmacokinetic ADME parameters.
Focused on bcl-2 down regulation in a melanoma cell line, effected either by antisense oligonucleotides (AONs) or by siRNA, the lecture will deal with the detection of "off target" effects by means of 2D-gel electrophoresis. Although the concept is straightforward in principle, technical details — such as validation of methods or staining techniques for quantitative analysis — are prerequisites to come up with relevant interpretations. In general — at the same level of down regulation — AONs induce clearly more significant differences in the expression pattern of the proteins than siRNA does. A set of proteins has been identified, whose level had changed along with the down regulation of bcl-2. This set opens up the opportunity to study physiological effects associated with the down regulation of the target protein — both related to wanted effects and to unwanted side effects. In a more general perspective, this method provides an efficient experimental approach towards pharmaceutical systems biology.

L4.2

Drug loaded immunonanoparticles in the combat of cancer stem cells – safety and efficacy considerations

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Monoclonal antibodies (MAbs) provide an important strategy in the combat of cancer. Since the introduction of rituximab for the treatment of non-Hodgkin lymphoma in 1997 as well as trastuzumab for inhibiton of epidermal growth factor receptor activity in 1998, the FDA had approved 9 monoclonal antibodies to treat various cancers with ofatumumab being the recent addition for the treatment of chronic lymphocytic leukemia. In the past the pharmaceutical industry had focused its efforts to improve treatment efficiency by using either humanized or fully human MAbs engineered for selectivity and efficacy. By now it is clear, that the therapeutic activity of many, if not most naked anticancer MAbs is considerably enhanced if either combined with chemotherapy or attached to radioactive material or a toxin or chemotherapeutic agent. A promising strategy for improving cancer therapy is the conjugation of MAbs to nanoparticles wherein MAbs are the targeting moieties and the nanoparticle carriers high drug payloads for local drug delivery at the tumour site despite its systemic application.
In this regard antigens expressed on tumour stem cells provide unprecedented opportunities to selectively eradicate otherwise recalcitrant cell population that due to their ability of self renewal provide a constant mean for the production of tumour progenitor cells. While it is also possible to conjugate more than one antibody to the same delivery system for the purpose of enhancing therapeutic activity the development of ImmunoNPs to carry cytotoxic agents to tumours, with favourable residence time, bio-degradability, biocompatibility, high drug payload and adequate release kinetics represent a major breakthrough. For its clinical use the safety and tolerability of drug-loaded nanocarrier-based formulations still needs to be evaluated. In my presentation I will address the great opportunities but also the hurdles still to overcome for immunonanoparticle targeted cancer therapies.

Reference:

Harush-Frenkel O *et al.* (2010) *Toxicol Appl Pharmacol* **246:** 83–90.

L4.3

Cancer stem cells in drug resistance and *in vitro* drug screening models

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During last few years a revival of a relatively old idea is observed that dysregulated stem cells are at the origin of most cancers. This hypothesis implies that heterogenous tumor cell population contains a small minority of cells, called cancer stem cells (CSC), that is tumorigenic and produces more differentiated tumor cell progeny. The existence of CSC has been experimentally confirmed in many different tumor types, however, it is still broadly unclear which properties are shared between normal stem cells and CSCs.
It is freqeuntly observed that a small fraction of cells in a tumor survives anticancer treatment when exposed to radiation and cytotoxic drugs. This resistance phenotype of some tumor cells may be associated with increased expression of drug transporters, enhanced DNA repair capacity, and defects in cell cycle checkpoints or cell death pathways. Inefficiency of anticancer treatment can be also explained by drug resistant phenotype of CSCs. These cells may have lower proliferation potential, and anticancer treatment, that targets actively proliferating cells, such as DNA damaging agents, mitotic spindle poisons or antimetabolites, is less effective in killing CSCs than more differentiated cancer cells. CSCs may also acquired genomic instability that enables them to modify gene expression profiles in response to anticancer treatment and activate mechanisms involved in drug resistance. During this lecture the most controversial issues related to the cancer stem cell paradigm will be discussed. In addition, based on the available literature data and our own results possible defects in drug response in CSC will be presented.
CSCs have also been identified in cell populations from established tumor cell lines maintained *in vitro*. This discovery has important implications as it opens a possibility to use tumor cells cultivated *in vitro* in drug screening to find new compounds or drug combinations, which are able to kill CSCs. The most obvious approach to use CSCs sorted based on their membrane markers by flow cytometry seems not to be very practical. Thus, new screening systems are needed to be proposed and evaluated. If successful, this will greatly improve anticancer therapy and may revolutionize drug screening and drug evaluation assays and procedures. To do this, however, we need a detailed understanding about the biology of CSCs.

L4.4

Transcription factors as drug targets

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Although many traditional drugs target cell surface receptors, there is growing need for looking for new possible drugs objectives.
Transcription factors play an important role in the regulation of cell growth, differentiation and responses to environmental signals. Moreover, level of a diversity of such proteins is altered in cancer and they may modulate susceptibility to carcinogenesis. Therefore they might provide selective targets for novel pharmaceutical intervention.
From many different transcription factors, NF-E2-related factor 2 (Nrf2) may be an excellent example of the factor for selective drug treatment. It up-regulates transcription of a number of antioxidant and detoxication genes through an antioxidant response element (ARE) present in their transcriptional promoters. It is a target of synthetic triterpenoids, sulforaphane or oltipraz, and nowadays, all of them are widely studied for anti-cancer or chemopreventive activities. Our recent results show that Nrf2 may be a direct target of another transcription factor, hypoxia inducible factor-1 (HIF-1), the main factor responsible for the regulation of genes in hypoxic conditions, which are inextricably linked to cancer development. The inhibition of Nrf2 by HIF-1 may have potential implications in clinics, especially for the anti-angiogenic and anti-cancer therapies. The understanding of the mechanisms responsible for drug — transcripiton factor interaction may improve design of drugs directed against specific transcription factors. This will result in the production of new, potent agents which could be used in a plethora of clinically important situations.

L4.5

Clinically relevant interactions between monoclonal antibodies and anticancer drugs

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Monoclonal antibodies (mAbs), of which 10 have been approved for clinical use in oncology, become the mainstay in the treatment of a number of tumors. Their antitumor effects results from either blocking of the activity of target molecules or from the triggering of indirect effector mechanisms of the immune system that include activation of complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), or phagocytosis. Moreover, some studies indicate direct influence of mAbs on tumor cells that leads to induction of various types of cell death. Despite the wealth of data on the mechanisms of cytotoxicity that accumulated over the last two decades their relative contribution to therapeutic outcome of mAbs is still difficult to predict in individual patients. Elucidation of molecular mechanisms of mAbs action is necessary to deliver their maximal activity in rationally deigned combinations with other therapeutic approaches and to design next generation mAb with improved ability to eliminate tumor cells.

L4.6

Targeted lipidomics of arachidonic acid mediators in human diseases

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Polyunsaturated fatty acids are not only constituents of biological membranes but also substrates for hundreds of highly biologically active oxylipids. Arachidonic acid is the precursor of eicosanoids, the best described family of about 400 bioactive compounds. Specific oxidation of arachidonic acid is achieved by a tightly regulated cyclooxygenase pathway, 5-, 12-, and 15-lipoxygenase pathways and other enzymes belonging to the oxygenases family. Some eicosanoids are ligands for a single receptor, others exert their various activities activating several receptors, still another require coupling with glutathione to achieve their biological activity. Eicosanoids are in general mediators of inflammation, but they participate in homeostasis of the most basic functions of the organism. Mass spectrometry occupies a leading position in the characterization, identification and quantitation of lipids. Its use has led to emergence of lipidomics, defined as the large-scale study of cellular lipids (i.e. the lipidome). Mass spectrometry was applied sporadically for measurement of single lipid mediators. We developed recently high performance liquid chromatography — tandem mass spectrometry (HPLC-MS/MS) and gas chromatography — mass spectrometry (GC-MS) techniques focused on the "targeted" lipidomic analysis of multiple derivatives of arachidonic acid. This analytical approach proved effective in clinical studies on common diseases. Using easily available non-invasive biological samples, eicosanoids compounds showed involvement into pathological processes of asthma and coronary artery disease of the heart [1–3]. This identified potential targets for the future therapeutic intervention.

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L4.7

Scientific collaboration with biotech companies: advantages and limitations

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The studies on synthesis of *P*-chiral non-ionic analogues of nucleic acids, with potential applications as sequence specific inhibitors of protein synthesis *(antisense drugs)* and tools for biological studies will be presented, with a special emphasis on stereocontrolled and stereoconvergent methods of the internucleotide bond formation.
These studies were performed within a long-term research collaboration between PAS and biopharmaceutical company.
The advantages and limitations of such collaboration will be discussed.

L4.8

Efficacy evaluation of novel Pim kinase inhibitors with anticancer activity

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Pim-1, -2, and -3 serine-threonine kinases play an important role in intracellular signaling and contribute to pathways involved in cell survival, proliferation, stress response and cellular motility. Pim kinases emerged as a novel and interesting target of significant potential for therapeutic intervention in cancer.
Overexpression of Pim kinases was reported for a variety of cancer types of both hematological and solid tumor type origin such as diffuse B cell lymphoma, chronic lymphocytic leukemia, Flt3-mediated acute myelogenous leukemia, prostate, pancreatic and hepatic cancers. Selvita is presenting results of the currently performing lead optimization program of novel, small molecule Pim kinase inhibitors.
Among the newly synthesized compounds we have identified molecules exerting substantial specificity and superior potency in inhibition of all three Pim kinase isoforms with IC_{50} values in low nanomolar range. Anticancer effect of our new derivatives was investigated in several cancer cell lines of hematological and solid tumor origin where the compounds induced cell death with low micromolar ED_{50} values. Additionally, synergistic toxic effect was observed in combination with standard therapeutics. Biomarker analysis of Pim kinase downstream targets confirmed Pim-dependent mechanism of action of tested compounds. We have observed a potent inhibition of 4EBP1 and S6 phosphorylation, as well as downregulation of c-Myc levels already after 4h of the treatment with our Pim kinase inhibitors. Following the results obtained in vitro in cell culture, compounds were further profiled for their ADMET properties like permeability, metabolic stability and bioavailability. Oral administration of our small molecule Pim kinases inhibitors in a subcutaneous leukemia *in vivo* xenograft model revealed strong inhibition of tumor growth with almost 90% TGI. Moreover, histological analysis of the organs from the animals dosed sub-chronic for over 2 weeks with the compounds, did not show any significant organ deleterious effects.

L4.9

New phenylpropanoic acid derivative with antidiabetic potential acting as a partial PPAR gamma agonist — preclinical studies

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Due to the explosive increase in the number of people diagnosed with diabetes world-wide in the past two decades, we can now speak of diabetes epidemic even if this word seems inappropriate in conjunction with a chronic disease. Diabetes is now considered as one of main threats to human health in the 21-st century. It is a metabolic disorder primarily characterized by insulin resistance and elevated blood glucose levels. Insulin resistance and glucose intolerance are key elements of the metabolic syndrome, which is currently associated with impaired function of PPAR gamma nuclear receptor and excessive production of fat tissue hormones. PPAR gamma is critical transcription factor in regulating lipid and glucose metabolism as well as insulin sensitivity, thus PPAR gamma receptor is considered as a very promising molecular target for developing new antidiabetic compounds. New investigated compound is a phenylpropanoic acid derivative (non-thiazolidinedione), selective, partial agonist of PPAR gamma nuclear receptor. As a partial PPAR gamma agonist, new compound has less than 30% of the activity associated with currently marketed full PPAR gamma agonist, rosiglitazone. Theoretically, our partial agonist should minimize side effects associates with rosiglitazone treatment by limiting the spectrum of activation of PPAR gamma. Consistent with this prediction, animal studies have revealed that the compound is effective in lowering blood glucose levels and demonstrates a relatively benign adverse event profile at putative therapeutic dose ranges. Doses used in animal models of diabetes such as the db/db mouse and the ZDF rat showed significant efficacy in the control of blood glucose level with minimized body weight gain, when compared to rosiglitazone. Moreover safety pharmacology and toxicology studies with mice, rats, dogs and monkeys suggest a broad safety window in which to study new compound's benefits in humans.

Posters

P4.1

Nephroprotective activity of PRL as ingredient of kidney perfusion and preservation solutions

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The perfusion and preservation solutions are the significant element of successful transplantation. Thus, finding a suitable composition, which ensures optimal organ preservation, is necessary. PRL, the hormone with a broad action spectrum, was used in formulation of the investigated solution. The results of our earlier research works prove that PRL is effective on liver preservation, as well [1-3]. This effect comes from PRL action on the membrane receptors of the Kupffer's cells in liver and on the receptors located directly on hepatic cells. PRL receptors are located also in nephrons, wherethrough PRL demonstrates an immunostimulating and proinflammatory action on kidney preservation. In this study the following solutions: HTK solution and modified HTK solution with addition of 1 μg/l PRL were used. The degree of kidneys damage during perfusion and preservation based on the biochemical markers concentration assay, such as alanine aminotransferase, aspartic aminotransferase and lactic dehydrogenase, was determined. The results analysis indicates that kidney preservation in HTK-PRL solution during 48 hours slow down Alat release in 50% compared with HTK solution. In turn, release of Aspat persist practically on the same level, whereas release of LDH reduced in 50% related to original HTK solution. Obtained results prove nephroprotective activity of PRL, when it is added to HTK solution.

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P4.2

The effect of propylene glycol on the biosynthesis of tacrolimus by *Streptomyces tsukubaensis*

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Introduction: Tacrolimus (also FK-506), an immunosuppressive drug from a group of calcineurin inhibitors, is widely used in transplantology as well as dermatology and many other branches of medicine for different clinical applications.
This drug is isolated from the whole fermentation broth of bacteria *Streptomyces tsukubaensis* cultivated in submerged culture since it was first discovered as the product of this strain.
Aim: The objective of our research was to investigate the influence of propylene glycol supplementation of culture media on tacrolimus biosynthesis by *Streptomyces tsukubaensis* strain.
Materials and methods: For this research shake flask cultures of *Streptomyces tsukubaensis strain* (FERM BP-927) were used. The cultures were enriched with different concentrations of propylene glycol and cultivated in submarged culture. We investigated the response of tacrolimus biosynthesis to 0.25%, 0.5%, 0.75%, and 1.0% (v/v) concentrations of propylene glycol in fermentation medium.
Results: The addition of propylene glycol to culture medium results in higher tacrolimus biosynthesis.
The best productivity of 8.97 mg/L was recorded for the concentration of propylene glycol in culture media equal to 0.75%.
We obtained more then 100% increase of the strain growth and tacrolimus biosynthesis as compared with the control.
The higher the concentration of propylene glycol, the higher the productivity of tacrolimus, reaching its maximum with the concentration of propylene glycol equal to 0.75% (v/v). However, with higher concentrations than 0.75% the productivity of tacrolimus decreases.
Conclusions: The obtained results indicate that there is a strong correlation between the concentration of propylene glycol and the productivity of tacrolimus by *Streptomyces tsukubaensis*. Propylene glycol seems to be a promising precursor of FK-506 biosynthesis path way. Its aplication for the biosythesis process can help to optimise the productivity of this clinically important immunosuppressive agent.

References:

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P4.3

Possibilities of mint cultivation and regeneration *in vitro* conditions

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Secondary metabolites, like volatile oils, often are present in intact plants at very low concentration, and often are present in endangered plant species. These facts lead to tendency of their production by using alternative ways. In tissue cultures the production of secondary metabolites is possible to increase by optimizing the cultivation conditions, the type and concentration of growth regulators, by regulation of the osmotic value of cultivation medium, the temperature and suitable pH of the medium.
The aim of our work was to set up mint primocultures followed by multiplication of the plants and by developing protocols for callus cultures. In first case, we have tested various combinations of growth regulators and cultivation media to promote growth and multiplication of the plant material. Using *in vitro* cloning we have achieved sufficient amount of the plant material. This approach secured the genetic stability of our plant material, even in long cultivation procedure. With the aim to obtain callus we have tested the influence of temperature, the light intensity and sub-cultivation intervals. On selected media we also tested the influence of the length of inter-nodal explants for callus tissue induction.
Direct regeneration of mint has shown to be optimal on solid MS medium (Murashige & Skoog, 1962) [1] supplemented with 30 g L^{-1} of sucrose, without any growth regulators.
For callus culture induction in mint, the most suitable has been the MS medium containing higher concentration of cytokinin and lower level of auxin. Medium MS supplemented with 30 g L^{-1} sucrose + 5 mg L^{-1} 6-benzylaminopurin (BAP) and 1 mg L^{-1} α-naftylacetic acid (NAA) optimally initiated dediferentiation and cell division in the area of wounded tissue by cutting of the explants, followed by appearance of green fragile callus. Calogenesis of mint on the above described medium has been realized only in the case when internodal segments (5–10 mm of leght) have been cultured at the temperature 25ºC, at light intensity 36 $\mu mol \times m^{-2} \times s^{-1}$ and at 7 day sub-cultivation interval. Cultivation in the dark, or at lower temperature or longer cultivation intervals has induced necrosis of explants.

References:

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P4.4

Luteolin as a factor decreasing expression of drug transporters in human adenocarcinoma cell lines

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Flavonoids are plant secondary metabolites that are organized into few classes of compounds according to chemical structure. Many studies have suggested that flavonoids may play an important role in medical treatment because of their biological activity including antioxidant, anty-inflammatory as well as anticancer properties.
Luteolin, 3',4',5,7-tetrahydroxyflavone, belongs to flavones and it was found in many vegetables and fruits. There is some preliminary evidence showing that luteolin inhibits proliferation many types of cancer cells including human breast and ovarian [1] and oral squamous cancer cell lines. It was documented that luteolin induces G1 cell-cycle arrest by inhibition of cyclin- dependent kinase and retinoblastoma tumor suppression protein (pRb) expression [2].
The pretreatment with luteolin sensitizes cancer cells to apoptosis. Many biochemical targets of this compound is unknown however it was proved that luteolin is involved in mitochondria translocation of Bax/Bak and activation of c-jun NH_2 –terminal kinase (JNK) in human hepatoma cells [3]. Luteolin decreases anti-apoptotic genes expression by inhibition of NFκβ activation. It is connected with sensitizing effect of this compound on tumor necrosis factor-induced apoptosis in human tumor cells [4].
In our research we used LoVo and LoVo/Dx human adenocarcinoma cell lines that are sensitive and resistant to doxorubicin respectively. To determine an influence of luteolin on cancer cells growth SRB assay was applied. The results showed that 3',4',5,7-tetrahydroxyflavone effectively inhibits proliferation both LoVo and LoVo/Dx cancer cells.
The main aim of our studies was to investigate the relationship between inhibition of cancer cells growth in these cell lines and resistance to chemotherapeutic agent. The genes expression level of ABC transporters (ABCB1, ABCG2, ABCC1) involved in developing of multidrug resistance was determined. RT-PCR data confirmed the pattern of ABC expression and suggested that luteolin significantly decreases expression of each investigated drug transporters gene in LoVo as well as LoVo/Dx cell line. These results suggest that luteolin may play an important role in decrease of multidrug resistance level in human adenocarcinoma cell lines.

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P4.5

Berries of various cultivars of sea buckthorn (*Hippophae rhamnoides* L.) as sources of health-beneficial carotenoids

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Sea buckthorn berries are not widely eaten in Poland, but they can be a valuable source of health-beneficial carotenoids. The paper compares the carotenoid content of eight cultivars of sea buckthorn (originating in Russia), grown as part of a comparative study of cultivar differences conducted at The Fruit Experiment Station in Brzezna. Berry extracts were analysed using a UV/Vis Spectrophotometer JASCO V-530 and HPLC Shimadzu LC10AS. High carotenoid content was found in the berries, which allows them to be classified as functional food. The highest carotenoid content was found in berries of the sea buckthorn cultivars 'Arumnyi' and 'Podorok Sadu'. Carotenoids play a very important role in the human diet, providing components essential to our health. It is now known that, in addition to the role of the provitamin A and xanthophylls in the process of seeing, their action as antioxidants is also important in protecting lipids and other chemical compounds against free radicals. They play an important role as antimutagenic factors in cancer prevention and their action hinders the development of some types of cancer. A carotenoid-rich diet is attributed a role in reducing cardiovascular diseases, excessive blood pressure, osteoporosis and neurodegenerative diseases. A diet properly balanced with carotenoids shows a favourable effect on the human immune system, so it can be expected to have a positive effect in the prevention of many other diseases. Our studies showed that the most valuable cultivars for the Malopolska region, in terms of their carotenoid content, are 'Arumnyi', 'Podorok Sadu' 'Aromatnaya' and 'Botanicheskaya'.

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P4.6

The influence of Ca(II) ions from calcium gluconate origin on absorption of calcium ions in *in vitro* conditions

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Insufficient calcium intake in the diet leads very often to systemic and cellular disorders of calcium homeostasis. Thus, the research works focus on the conditions in which absorption process of calcium ions is the most effective. The aim of this investigation was to trace the influence of calcium ions concentration (1; 10; 20 mmol/l) on the absorption of calcium ions in *in vitro* conditions. The Ca(II) ions which were studied permeated from the environment which was imitating a stomach (donor) to the environment which corresponded to the natural condition at further sections of digestive track - intestines (acceptor). The amount of permeated (acceptor) and impermeated (donor) Ca(II) ions through natural membrane - intestine was determined. The amount of absorbed Ca(II) ions was calculated from concentrations differences. The experiment was carried out within 5 h. An organic calcium salt, which is one of most often used salt in calcium supplementation and fortification, was used in the research. The most of Ca(II) ions — 81.90% were absorbed at calcium concentration of 10 mmol/l and the least — 26.15% at calcium concentration of 1mmol/l. At concentration of 20 mmol/l the amount of absorbed Ca(II) ions was 55.68%. The transport process of Ca(II) ions from calcium gluconate proceeded with different intensity. The most intensive was in the first 2.5 h of the experiment. After that time the permeation of Ca(II) ions proceeded much more slowly.

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P4.7

The influence of curcumin and quercetin on the oxidative DNA damage induced by etoposide in the slenocytes of the rats with promyelocytic leukemia

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Curcumin (C) and quercetin (Q) are polyphenols with chemopreventive and antioxidative properties. They can protect the cells from DNA damage induced by some cancerogens. It was proved in the *in vitro* study that these polyphenols reduce the level of oxidative DNA damage induced by etoposide (Eto). The myeloid cells are sensitive to oxidative stress induced by this cytostatic. Phenoxyl radicals of etoposide created under the influence of myeloperoxidase led to oxidative DNA damage in normal myeloid cells. Oxidative action of etoposide in these cells can, in turn, lead to malignant transformation and secondary leukemia.
The aim of the study was to investigate the effects of antioxidant therapy with curcumin and quercetin on the level of oxidized purine bases, induced by etoposide in the splenocytes of rats with promyelocytic leukemia.
Brown Norway rats were injected (i.v.) with leukemic cells and then treated with: 1) C (200 mg/kg b.w. by gavage) for 22 consecutive days, 2) Q (100mg/kg b.w. by gavage) for 22 consecutive days, 3) Eto (14 mg/kg b.w., i.p.) for 3 consecutive days. 3) C+Eto, 4) Q+Eto, 5) solvents for examined compounds (control groups). The rats were killed 2 hours after the last dose of polyphenol and/or 1 hour after Eto administration. At this stage of the disease, the leukemic cells constituted about 40% of the splenocytes. The oxidative DNA damage was measured by alkaline comet assay using formamidopyrimidine glycosylase (Fpg) which recognises oxidised purine bases. The amount of Fpg-labile sites was estimated using Comet Score software.
The level of Fpg-labile sites increased significantly in the splenocytes under the influence of etoposide administration to the rats in comparison to the controls. The antioxidant therapy with curcumin reduced significantly the level of etoposide-induced oxidized purine bases in the splenocytes of leukemic rats. Quercetin did not exert significant influence on the oxidative DNA damage induced by the examined cytostatic.
Curcumin is an effective tool for the reduction of prooxidative effects in DNA induced by etoposide.

P4.8

Epicatechin reduces the level of oxidative DNA damage induced by etoposide in the bone marrow cells of the rats with promyelocytic leukemia

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In our earlier research we have proved that epicatechin (EC) sinergistically cooperates with etoposide (Eto) in reduction of leukemic cells in the bone marrow of the rats. As an antioxidant, EC could also diminish the side effects of etoposide which are oxidative DNA damage in the precursor cells of myeloid lineage with high constitutive myeloperoxidase (MPO) activity. Chemotherapeutic activities of Eto is based on inducing single and double DNA strand breaks triggering apoptosis in cancer cells. Eto also undergos one-electron oxidation by MPO, which lead to production of phenoxyl radical of this cytostatic. Prooxidant action of its phenoxyl radicals may lead to the development of secondary myeloid leukemia in patients treated with high doses of this chemotherapeutic.
The aim of the study was to examine the influence of EC on the level of oxidized purine bases in the bone marrow cells of the rats with promyelocytic leukemia.
Brown Norway rats were injected (i.v.) with leukemic cells and then treated with: 1) EC (40 mg/kg b.w. by gavage) for 22 consecutive days, 2) Eto (14 mg/kg b.w., i.p.) for 3 consecutive days. 3) EC+Eto, or 4) solvent for examined compounds (control group). The rats were killed 2 hours after the last dose of EC and/or 1 hour after Eto administration, when leukemic promyelocytes constituted about 35% of all bone marrow cells. The oxidative DNA damage was measured by alkaline comet assay using formamidopyrimidine glycosylase (Fpg) which recognises oxidised purine bases. The amount of Fpg-labile sites was estimated using Comet Score software.
Eto induced a significant increase in the amount of oxidized purine bases in the cells of leukemic rats. EC significantly protected the rat bone marrow cells from Eto-induced oxidative DNA damage. EC alone did not affect the amount of Fpg-labile sites in the examined cells.
The obtained results indicate that EC effectively protects DNA against oxidative stress induced by Eto in the bone marrow cells. The examined polyphenol could be a useful object for future study of complementary treatment.

P4.9

Optimization of yeast protein hydrolysis process

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Yeast are widely used as a source of protein in both food industry and animal feed production. Different aminoacid composition of yeast protein can be obtained depending on species of the yeast used and culture conditions. Although yeast contain rich intracellular protein content, tough and rigid cell wall is a serious disadvantage. In order to incerase digestibility of yeast protein many different treatments are used. Among the most commonly used methods are: freeze-drying and crushing, drum drying, enzymatic treatment and organic solvents. In search for convenient and economic methods of inceasing yeast protein's digestibility autolysis at elevated temperatures was tested. **Aims:** 1. Testing the efficiency of *Saccharomyces cerevisiae* cell autolysis at 50ºC2. Comparing the results with enzymatic treatment using pepsin and papain. **Methods:** Experiment 1. Yeast were incubated in liquid medium at 3 different temperatures: 20, 35 and 50ºC for 6, 12 and 18 h. Then cells were collected by centrifugation and the supernatant was examined for protein content using spectrophotometric method. Experiment 2. Yeast were incubated with either papain or pepsin solution (1%) for 24 h at 40ºC. Cells were collected by centrifugation and the supernatant was examined for protein concentration.
Results: Significant autolysis was observed at 50ºC in all experiments. Incubation at 50ºC for 12 h results in release of 69% of initial incubation mass into the supernatant.Experiment 2. Enzymatic treatment was not as effective as the autolysis. Protein concentration in the supernatant was 12,5 mg/ml for pepsin and 23.7 mg/ml for papain treatment. **Conclusions:** Autolysis at 50ºC is an effective method of obtaining yeast cell lysate. In comparison with incubation at 20 or 35ºC, incubation at 50ºC yields 14 to over 20 fold increase in supernatant protein concentration. Enzymatic treatment is less effective.

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P4.10

Oxidative DNA damage in blood of CVD patients taking Detralex

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There are many available studies showing occurrence of oxidative DNA damages during the progression of various disorders, where phenomenon of oxidative stress is involved. The main goal of the work reported here was to determine the degree of oxidative/alkali-labile DNA damages in peripheral blood as well as in the blood stasis from varicose vain of CVD patients. Moreover, determination of the impact of Detralex usage on the level of (oxidative) DNA damages in CVD patients was evaluated as well. The degree of oxidative DNA damages was studied in a group consisted of ten patients with diagnosed chronic venous insufficiency (CVI) in the 2nd and 3rd degree, according to clinical state, etiology, anatomy and pathophysiology (CEAP), and qualified to surgical procedure from February, 2010 through May, 2010 in the II Department of General Surgery UJ CM and in the 5th Army Hospital with Polyclinic in Cracow. Patient qualification for the study was carried out by phlebologist during ultrasonography (USG) examination of venous vessels of lower limbs (DOPPLER). The Bioethical Committee of the Jagiellonian University in Cracow (KBET/162/B/2009) expressed positive approval about the study. The control group consisted of normal volunteers (blood donors) qualified during standard examinations at Regional Centers of Blood Donation and Blood Therapy. The comet assay, electrophoresis of individual cells on agarose gels, was used for determination of DNA damages. The cell viability assay with fluorescein diacetate (FDA) and ethidium bromide (EtBr) was used to evaluate the viability of cells before each study. Cell viability was above 95%. Analyses of the obtained results showed increase in the level of oxidative/alkali-labile DNA damages in lymphocytes originating from antebrachial blood and blood stasis of CVD patients as compared to the control group (Control) ($p<0.002$; ANOVA). In addition, it was demonstrated that the usage of Detralex® resulted in decrease of the level of oxidative/alkali-labile DNA damages in CVD patients as compared to patients without Detralex® treatment ($p<0.001$; ANOVA). Based on findings from the study, it may be hypothesized about occurrence of significant oxidative DNA damages as the consequence of strong oxidative stress in CVD. In addition, antioxidative effectiveness of Detralexu® was observed at the recommended dose, 1 tablet twice daily. Correlation between DNA damages and administration of drug justify effectiveness of micronized purified flavonoid fraction in prevention and treatment of chronic venous disease.

P4.11

Optimization of biotransformation processes towards pharmacologically useful products: a case of testolactone

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Steroidal lactones frequently exhibit various useful biological properties, such as anticancer, antiandrogenic and antihypercholesterolemic activity [1-4]. Among these lactones, testolactone (17α-oxa-D-homo-androsta-1,4-diene-3,17-dione), an aromatase inhibitor, is used as pharmaceutical factor which controls conversion of androgens into estrogens, e.g. in case of gynecomastia [2] or precocious puberty [5]. Our previous studies confirmed the fact that fungi from *Penicillium* species exhibit ability to carry out Baeyer-Villiger oxidation of various steroid substrates [6] and thus can hold out a potential new route to novel biologically active steroids. Herewith, we attempted to transform 1-dehydrotestosterone acetate and propionate employing two strains of *Penicillium* species: *Penicillium lilacinum* AM111 and *Penicillium urticae* AM84. In the case of *Penicillium urticae* AM84 the main product of biotransformation of both substrates turned out to be 15α-hydroxyandrosta-1,4-diene-3,17-dione – resulting from hydroxylation of the ketone formed after hydrolysis of the ester to the alcohol. Testolactone – a residue of microbial Baeyer-Villiger D ring oxidation – was in this case obtained in decisively smaller amount. On the other hand, *Penicillium lilacinum* AM111 was show to be a microorganism much more selective towards the process of our interest: the main product of the transformation of the investigated steroid esters was the desired testolactone. The studies carried out during this project consisted of analysis of the transformation broth after various periods of incubation, in order to show the time evolution of the ongoing processes. The compounds were identified on the basis of GC and spectroscopic (NMR) data.

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P4.12

The influence of extraction parameters on the recovery of antioxidant compounds from *Sambucus nigra* and *Chaenomeles japonica* fruits

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Fruits contain many valuable ingredients such as polyphenolic compounds, which have antiradical properties. A substantial technological problem is the recovery of these compounds before their usage in different branches of industry.
The aim of the research was to optimize the parameters of the extraction from plant raw material allowing the efficient recovery of antioxidant compounds.
Sambucus nigra (elder) and *Chaenomeles japonica* (Japanese quince) fruits were used in the study. A maceration (magnetic stirrer, 6 hours) with different ethanol concentrations (40, 60, 80, 96% v/v) was performed. An extraction was conducted with the high-speed homogenizer (at 11000, 19000, 24000 rpm; for 2, 5, or 10 min). Preliminary treating of plant material by the microwave radiation (50, 100, 200, 300, 600 W; 0,5 i 1 min) followed by extraction using the high-speed homogenizer (19 000 rpm; 5 min) was one of experiment variants. The antioxidant activity (in reaction with the ABTS radical) and total polyphenol content in extracts were assessed by spectrophotometric methods.
It has been shown that the antioxidant activity of macerates obtained with different concentrations of ethanol ranged from 110 to 332 mg Trolox/100 ml of extract and the total polyphenols content from 24 to 58 mg catechin/100 ml of extract. Higher values of the evaluated parameters were achieved for *Chaenomeles japonica* fruits. It was found that extracts prepared with 80% ethanol were characterized by the highest polyphenols concentration and antioxidant activity. High-speed homogenization significantly increased the recovery of phenolic compounds in comparison with the maceration. The highest values of antioxidant activity and total polyphenol content were found in samples extracted with the rotation speed of 19000 rpm for 5 min (439.65 mg Trolox/100 ml and 86.68 mg catechin /100 ml for elderberry, and 363.74 mg Trolox/100 ml and 76.72 mg catechin /100 ml for Japanese quince fruits). Tests connected with the inactivation of enzymes from the oxidases group were also performed. Before homogenization, the samples had been treated by microwave radiation. However, obtained values of antioxidant activity and total polyphenols content were lower than values received for extracts made without microwave treatment.
The results of the study showed that the best way for the extraction of *Sambucus nigra* and *Chaenomeles japonica* fruits is the homogenization with 80% ethanol at 19 000 rpm for 5 min.

P4.13

The possibility to enhance flavonoids production from selected sorts of *Carthamus* L. in form of callus cultures

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Selected cultivars of *Carthamus* L. genus are characterized by numerous biological effects, which are due in particular to the activity of flavonoids and other secondary metabolites.
Carthamus tinctorius L. is used as natural dye in various industries due to the presence of safflor dyes like safflomin A, safflor yellow B and carthamin red, and also as oil and spice in the food industry. However, its important use is also known in pharmacy and medicine for its pharmacological effect. Extract of *Carthamus tinctorius L.* is used because of its sedative and anti-inflammatory effects in gynecological diseases and in treatment of cardiovascular diseases, trombosis and high cholesterol. *Carthamus tinctorius L.* petals contain flavonoid compounds like kempferol, quercetin, rutin, and their glycosides.
Production of flavonoids in Safflower callus culture (*Carthamus tinctorius* L.) was dependent on culture conditions and culture media composition. The content of flavonoids increased in callus cultures maintained on media supplemented with NAA (4 mg×l^{-1}) or NAA:BAP (4 mg×l^{-1} and 1 mg×l^{-1}) in 16h photoperiod. Flavonoids represented 2.17–2.53% of callus dry mass.

P4.14

Multicellular Tumor Spheroid Formation inside polymeric microwells: a prospective system for anticancer drug screening

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Nowadays, most of drug screening tests are performed using a monolayer cellular model. However, monolayer cultured cells lack interactions present *in vivo*, which cause the inability of referring results obtained to the drug effect on living organism. Therefore, the drug screening method more closely mimicking *in vivo* environment is required. The best cellular model for anticancer therapy testing developed so far is Multicellular Tumor Spheroid (MCTS). The MCTS presents morphology and physiology similar to tumor *in vivo* with the network of cell-cell interactions, 3D structure and nutrients, metabolites and oxygen gradients [1]. Number of methods of MCTS formation were described in literature and several found their final applications. However, most of them cause variation in size or are cost and energy consuming.

The "Lab-on-a-chip" technology offers a lot of advantages for cell engineering. [2]. In this work a 3D microfluidic system for MCTS cultivation is presented. The poly(dimethylsiloxane) was chosen for the fabrication, due to its gas permeability and hydrophobicity preventing cell adhesion. Separate layers were fabricated using low-cost soft lithography and replica molding method [3]. An array of microwells (volume of 0.2 μL each) was fabricated in the middle layer, while upper and lower layers consisted microchannels for medium supply. Alignment and plasma bonding of the three layers resulted in the 3D structure which enabled cultivation of MCTS of diameters up to 300 μm with the medium perfusion around.

HT-29 cells suspended in cell culture medium were introduced into the microsystem. After 16 hours of incubation cell aggregation was observed. Unaggregated cells were washed out from microchannels with the medium flow. Within the next 24 hours loose cell aggregates remained in the microwells formed compact spheroids. The growth rate of MCTS observed in the microsystem was lower than in the culture plates, which most likely corresponds with the tumor growth *in vivo*.

The presented microsystem can be an inexpensive and easy to handle alternative for current MCTS cultivation methods. The microfluidic array can be easily coupled with the concentration gradient generator [4] forming an integrated system for anticancer drug screening.

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