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Conference Report

Report of the Third Hungarian Cell Analysis Conference: 16–18 May 2002, Budapest, Hungary

Attila Tárnok and Harry Crissman

This conference was a successful continuation of previous conferences such as the Hungarian Cell Analysis Conference, Budapest, 1998 and 2000, and the ISAC-Sponsored International Conference for Flow Cytometry and Image Analysis, Epona, 1999. The Cell Analysis Section of the Hungarian Biophysical Society organized all four conferences. The popularity of the conference was hallmarked by the large number of participants (close to 300).

The form of the conference proved to be very attractive: In the mornings, experts from specific fields delivered scientific lectures. In addition to Hungarian scientists, such as Margit Balázs, Gyula Hadlacky, Béla Molnár, László G. Puskas, János Szöllösi, and György Vereb, experts from abroad significantly raised the scientific standards of the conference. The international speakers included Peter Adorjan, Francis Mandy, Abe Schwartz, Howard M. Shapiro, and the authors of this introduction. In the afternoons, practical demonstrations were presented, ranging from basic techniques to state-of-the-art instrumentation. Practical training included cell culturing; mechanical cell separation for flow cytometry; methods for detection of cell proliferation; detection of apoptosis; fluorescence microscopy, image acquisition, and processing; confocal laser microscopy; fluorescence in situ hybridization; magnetic cell separation; magnetic mRNA isolation; real-time and traditional polymerase chain reaction; and detection of mutation. During the conference the attendees were able to choose six sessions of 11 topics. These demonstrations were headed by one of the speakers and sponsored by commercial companies that provided special kits and/or instruments to ensure a stable basis for successful practical training. An impressive selection of commercial companies provided contacts and improved awareness of advanced analytical cytology for the participants.

The purpose of the conference was multifaceted. It served as a forum for presentations from Hungarian scientists, and, with the active participation of foreign experts, it opened a window to state-of-the art methods and techniques. The conference also facilitated the research cooperation between well-established scientists and Ph.D. graduate students, with the major purpose of the conference being education. The average age of the attendees was approximately 30 as more than 50% of the participants were Ph.D. students. Graduate students were able to earn credit points toward their final examination.

The venue for the conference was the Medical University of Semmelweis in Budapest, the capital of Hungary. The university has great traditions, with its name reflecting the rich history of scholastic activity in Budapest. It was over 150 years ago that Ignaz Semmelweis completed his classic epidemiologic study in Budapest, studies that led to effective prophylaxis against childbed fever that still stands as the hallmark of evidence-based studies in epidemiology.

At the conference both the lectures and the posters proved to be of high quality, demonstrating the high scientific standards of the community. The topics of the posters ranged from oncology (basic and applied research) to signal transduction, genetics, fertility research, and investigation in lower organisms such as *Drosophila* and earthworm. The methods applied were varied and highly sophisticated. Of 29 poster presentations, three prizes were awarded. Originally the poster awards committee (Margit Balázs and Attila Tárnok) was supposed to give only one award, but the decision was not easy because of the large number of excellent posters. With the financial help of commercial companies, the committee was able to present three poster awards instead of one. These awards were:

First prize: Generation and Characterization by Flow Cytometry of Dendritic Cells, by Gizella Veszely, János Fent, Ágnes Nagy, and Furész József, Department of Pathophysiology, Institute for Health Protection of HDF, Budapest.

Second prize: Hemocyte-Specific Molecular Markers in the Hematopoiesis and Innate Immunity of *Drosophila melanogaster*, by István Nagy, Éva Kurucz, and István Andó, Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged.

Third prize: Astrocytes Support the Neuronal Differentiation of Neuroectodermal Progenitor Cells, by Vanda Szlávik, Zsuzsanna Környei, and Emília Madarász, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest.

On the following pages, the reader will find the rich program of the Third Hungarian Cell Analysis Conference through the lecture and poster abstracts.

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LECTURE ABSTRACTS
IN ALPHABETIC ORDER

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METHYLATION BASED CLASS PREDICTION USING SUPPORT VECTOR MACHINES

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Molecular portraits, such as mRNA expression or DNA methylation patterns, have been shown to be strongly correlated with phenotypical parameters. These molecular patterns can be revealed routinely on a genomic scale. This means that the several hundreds or thousands of variables are measured in parallel in a single experiment. The major goal of these experiments is to identify those genes whose expression or methylation pattern correlates strongly with the investigated tissue classes because these genes have a crucial importance for diagnostic or pharmaceutical development. Here we demonstrate novel machine learning techniques to visualize and interpret these high dimensional microarray data sets.

In order to perform a methylation based class prediction we use the well known support vector machine algorithm. This algorithm has shown outstanding performance in several areas of application and has already been successfully used to classify mRNA expression data. The major problem of all classification algorithms for methylation and expression data analysis alike is the high dimension of input space compared to the small number of available samples. Although the support vector machine is designed to overcome this problem it still suffers from these extreme conditions. Therefore feature selection is of crucial importance for good performance and we give special consideration to it by comparing several methods on our methylation data.

The data set consists of cell lines and primary tissue obtained from patients with acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML). A total of 17 ALL and 8 AML samples were included. The methylation status of these samples was evaluated at 81 CpG dinucleotide positions located in CpG rich regions of the promoters, intronic and coding sequences of 11 genes. These were randomly selected from a panel of genes representing different pathways associated with tumor genesis.

Our results clearly demonstrate that microarray based methylation analysis combined with supervised learning techniques can reliably predict known tumor classes. Classification results were comparable to mRNA expression data and our results suggest, that methylation analysis should be applied to other kinds of tissue. Well-documented tissue samples with patient history can be obtained only as archived specimens. This strongly limits the amount and number of tissues available for expression analysis. The methylation approach has the potential to overcome this fundamental limitation: through the mere fact that the stable DNA is the object of study, extraction of material is possible form archived samples. This enables the examination of methylation patterns in large numbers of archived specimen with comprehensive clinical records and removes one of the major limitations for the discovery of complex biological processes by statistical means.

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APPLICATION OF FLUORESCENCE IN SITU HYBRIDIZATION IN THE DIAGNOSIS OF MALIGNANT DISEASES

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Genetic alterations of malignant diseases or premalignant lesions are in many cases associated with the prognosis of the disease. Identification of chromosomal alterations that are involved in the initiation and progression of the malignant process may allow not only the better prediction and monitoring of the disease but it can lead to the development of new therapeutic strategies. Conventional banding analyses are not easy to perform because metaphase chromosomes of sufficient quality and quantity are often difficult to obtain from many solid tumors. Over the last decade, fluorescence *in situ* hybridization (FISH) has become a powerful and essential technique to detect chromosome copy number changes and structural alterations in metaphase and interphase cells. It can be used in different field of biology, including the study of chromatin organization, gene mapping, karyotype analysis, radiation dosimetry and clinical diagnosis of malignant diseases. For the detection of numerical and structural chromosome alterations several different types of probes are now commercially available. DNA probes recognizing repeat sequence targets, such as alphoid and satellite DNA, mostly present in the centromeric and telomeric regions, are used routinely to detect chromosome aneuploidy. Locus-specific probes are usually collections of one or a few cloned DNA sequences ranging from just one or less than one kb to over 1 Mb and are applied to study gene amplifications and deletions. For targets of much larger scale, from chromosome bands up to the entire genomes, more complex mixtures of DNA sequences are used as probes. With the availability of an increasing number of spectrally distinct fluorophores it is possible to visualize all chromosomes with different colors using painting probes for all 11chromosomes in one experiment. This multiplex-FISH (M-FISH) technique relies on digital image analysis and allows the rapid detection of numerical and structural alterations of metaphase chromosomes obtained from tumor cells. Another

methodological breakthrough in FISH technology is comparative genomic hybridization (CGH). CGH has the advantage that allows the tumor genome to be screened for copy number changes without the need to obtain metaphase spreads from the tumor cells, chromosome copy number alterations can be detected and mapped throughout the tumour genome in a single hybridization. The CGH technique is based on dual color, competitive FISH and is performed using differentially labeled test DNA obtained from tumor cells (e.g. green fluorescence) and reference normal DNA (e.g. red fluorescence) co-hybridized to normal human chromosomes (counterstained with a blue fluorescent DNA specific dye). If over-represented or amplified sequences are present in the test DNA, that region of the normal chromosomes will hybridize increased amount of tumor DNA and will result in an increase of the green to red fluorescence intensity ratio. Under-represented or deleted regions will be represented by the decrease in the green to red ratio on the normal chromosomes.

The different FISH techniques have been applied for many solid and haematological tumor types, cell lines and archival materials to characterize chromosomal alterations. The use of FISH methods in molecular pathology will be of great value in the early detection of malignant lesions and monitoring the effect of different therapies in cancer. (OTKA 32587, ETT587/2000)

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ARTIFICIAL CHROMOSOMES IN GENE THERAPY

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Satellite DNA-based artificial chromosomes (SATACs) can be generated by induced *de novo* chromosome formation, in cells of different mammalian species including humans. These artificially generated stable accessory chromosomes are composed of predictable DNA sequences and they contain defined genetic information.

Human Satellite DNA-based artificial chromosomes (SATACs) developed in our laboratory represent a potential non-integrating vector with megabasepair size carrying capacity. SATACs may serve as stable neutral platform for persistent or controlled expression of therapeutic gene(s).

To become a potential vector for use in gene therapy, SATACs have already passed several hurdles:

1. Generation of SATACs in a reproducible manner from predictable DNA sequences. Over the recent years, a number of different mouse, mouse/hamster, hamster, hamster/human, and human SATACs were made.

2. Large-scale purification of SATACs by flow cytometry with fluorescence activated dual laser-beam cell sorter (FACS).

3. Stable transfer of SATACs into different cells (mouse, hamster bovine, human) and embryos (mouse, bovine) while at the same time preserving their structural integrity and function. FACS purified SATACs have successfully been delivered to recipient cells by microinjection, microcell-mediated mitotic chromosome transfer, with cationic lipids and dendrimers, sonoporation, direct chromosome uptake, etc.

4. Generation of transgenic animals with purified SATACs and germline transmission of these mammalian artificial chromosomes have been demonstrated.

5. Tissue specific expression of a therapeutic gene from SATACs in transgenic animals (mouse).

Based on the above achievements, in the long term, carefully designed artificial chromosomes may play an important role in human germline gene therapy, while in the short term they offer great potential for *ex vivo* somatic gene therapy.

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FLOW CYTOMETRIC FLUORESCENCE LIFETIME ANALYSIS OF NUCLEIC ACID BINDING FLUOROCHROMES

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A new dimension has been added to multiparameter flow cytometric analysis through development of the Los Alamos Phase Sensitive Flow Cytometer with capabilities for performing fluorescence lifetime measurements as well as conventional FCM measurements. Monitoring the changes in the absolute lifetime value of the probe yields information relating to the changes in molecular conformation and the functional activity of the molecular target. Lifetime values also provide unique signatures for resolving the emissions of multiple fluorochrome labels with overlapping spectra, thereby increasing the number of fluorochrome combinations using a single excitation source.

Lifetime analysis of cells stained with different nucleic acid-binding fluorochromes revealed several other unique observations and demonstrated the accuracy of the PS-FCM methodology. Our lifetime studies provided the discrimination of DNA and dsRNA based on differences in the lifetime value of either PI or EB bound to the respective nucleic acids. Differences in lifetime values relate to the differences in the structure of the nucleic acid complexes, as well as the dissimilarities in the dye-intercalation into DNA or dsRNA. Similar lifetime data were obtained with fluorescent chemotherapeutic agents, including ellipticine and adriamycin, thereby allowing, potentially, for discriminating and quantitating binding of these drugs to either DNA or RNA. Bivariate profiles of lifetime versus DNA content, obtained from analysis of EB stained, HL-60 cell populations induced into apoptosis showed a 3.0 ns reduction in the lifetime of EB bound to apoptotic cells compared to the non-apoptotic subpopulation. DNA content and lifetime analysis revealed a unique subpopulation of human skin fibroblasts cells in very early S phase with a significantly reduced EB-lifetime. Multiparameter DNA content, EB lifetime and immunofluorescent antibody analysis of cyclin D and cyclin E levels in asynchronous HSF cells demonstrated that the subpopulation of cells contained elevated levels of both cyclin D and cyclin E, characteristic of cells in very early S phase. Following release of

synchronized cells from G1/S phase, the subpopulation entered mid-S phase with EB lifetime values elevated above G1 phase cells and a progressive increase in EB lifetime was noted as cells proceed to the G2/M phase.

These studies demonstrate applications of lifetime measurements for the analysis of the binding of different fluorochromes to DNA or RNA in single cells. Data also show applications of lifetime measurements for monitoring changes in chromatin structure associated with cell cycle progression, cellular differentiation, or DNA damage, as in the early stages of apoptosis. Potential modifications of the PS-FCM will provide for simultaneous measurement of multiple lifetimes, thereby enhancing detection and quantitation of fluorescent compounds, including chemotherapeutic agents, bound to multiple subcellular complexes in viable cells.

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T-CELL SUBSET ENUMERATION: PAST, PRESENT AND FUTURE

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Flow cytometry impacted HIV disease monitoring more than any other clinical condition. Therefore, it seems appropriate to review the evolution of immunophenotyping in the context of following the fight against AIDS over the past 20 years. Contrary to some of the original expectations, it was AIDS, not some frequently performed oncological test that was responsible for the massive and rapid worldwide mobilization of flow cytometers into clinical immunology laboratories. In 1981, reports appeared from various parts of the USA about young gay men who had unusual immunosuppression manifesting as opportunistic infections. Soon it was discovered that the hallmark of this new disease, acquired immunodeficiency syndrome (AIDS), was a decrease in numbers of CD4 T-cells in peripheral blood.

Clinical flow cytometers handle 5 or 6 distinct parameters: forward scatter (FS), side scatter (SS), and three or four fluorescent light (FL) signals. The two light scatters are intrinsic parameters that define morphological features of leukocytes: size and granularity, respectively. The FL parameters measure extrinsic attributes, such as identification of surface antigens; via fluorescent scattering from fluorochromes coupled to monoclonal antibodies (MAbs). Fluorescein isothiocyanate (FITC) is the most universal fluorochrome. The second common dye is, R-phycoerythrin (PE). Both dyes excite at 488 nm. As third and fourth dyes, both natural and man-made tandem dyes are utilized. Currently, simultaneous four-color immunophenotyping is the advanced clinical method. Multi-color application is accomplished either by adding a fourth PMT for the detection in the far red (Beckman Coulter), or by adding an additional laser as well as a fourth PMT (BD Biosciences). The dye APC is used as the fourth fluorochrome with the red diode laser that emits at 635 nm.

In the quest to eradicate the AIDS pandemic, perhaps the next generation of multi-laser, multi-parameter instruments will accelerate discoveries in cellular immunology and that in turn will lead to breakthroughs in the fundamental understanding of events in adaptive and innate immunity. It is predicted that cytometers will continue to shrink in size and cost. That engineers that develop instruments to comply with the visions incorporating cytomics will also design low cost instruments to deal with the realities of the resource poor parts of the globe.

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RARE CELL ANALYSIS AND MULTIFUNCTIONAL EVALUATION BY CHIP TECHNOLOGIES

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Background: Chip and array technologies are used more and more for the multifunctional analysis of pathologically altered cells. Today there are DNA methylation and polymorphism chips, mRNA expression and protein chips commercially available. The required cell amount for a single chip analysis is changing from 10 000 to 200 000 cells. The focused interest of clinical researchers in rare cell applications is limited by the discrepancy between the amount of isolable cells (from 1 to several thousands) and the RNA, DNA or protein amount that is necessary for reliable array analysis.

Aims: Evaluation of the available multiplication techniques for the increase of the rare cells' cell component amount for routine chip analysis.

Results: Basically one can choose between different methods. These can include the increase of the amount of the isolated cells, the multiplication of the isolated cell number by culturing or the multiplication of the isolated cell components.

The first alternative is the application of increased amount of sample volume for rare cell isolation. Isolation efficiency can be enhanced in the case of magnetic isolation using increased amount of magnetic antibodies. The isolated cells can be cultured, too. However in this case the cell function can be altered due to different culturing conditions. The RNA amplification can be performed by T7 RNA amplification protocol. DNA amplification can be performed by using RAP-PCR (random access primed PCR), DOP-PCR (degenerate oligonucleotid primer PCR) or PEP-PCR (primer extension protocol PCR) techniques. This way not only DNA, but cDNA can be amplified, too.

The amount of available proteins isolated from in vivo cells cannot be multiplied according to our knowledge, until now. In single cases, after the determination of the protein structure, in vitro translation systems can be used.

For the increase of the chip sensitivity tyramin signal amplification protocol can be used.

Discussion: Today the commercially available chip arrays require intermediate amplification techniques for rare cell applications. Further technological improvements are required in the chip sensitivity and standardization efforts are necessary to have comparable results from different laboratories.

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FABRICATION AND APPLICATION OF DNA-MICROARRAYS

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Large-scale and simultaneous measurement of gene expression using hybridization of complex probes, representing the active genes of a certain biological sample to arrays of cDNA fragments or oligonucleotides is becoming a widely used technique in functional molecular biology. Using DNA-microarrays or DNA-chips, global gene expression changes of diverse physiologic and pathologic states, single nucleotide polymorphisms or mutations can be followed. In recent years, this analysis is based on hybridization of fluorescent labeled probes prepared from mRNA, total RNA, or DNA obtained from diverse biological samples to microarrays having complementary sequences as targets on their surfaces. In the case of transcriptome analysis for each hybridization usually a mixture of two fluorescent labeled probes is applied onto a cDNA-microarray, where one labeled probe is obtained from a control (untreated or unaffected) and the other is from a treated or affected sample. This direct comparative hybridization method allows a quantitative comparison of the relative abundance of individual sequences. Of great interest recently has been the potential application of microarray technology to follow the effects of disease-inducing elements, determine new disease subclasses, and predict the outcome of drug treatment based on exclusively the gene expression pattern of the patient.

To obtain appropriate amounts of RNA for standard labeling techniques, milligrams of tissue or millions of cells are needed, or alternatively one can apply different sample or signal amplification methods. We developed a novel amplification technique, which combines PCR amplification and *in vitro* transcription to obtain high-quality RNA for labeling starting from micrograms of total RNA. We have also investigated the reproducibility, reliability and sensitivity of the method.

To monitor the gene expression of homogenous sample isolated from a patient or other clinical sample appropriate separation techniques, such as fluorescent activated cell sorter have to be used to obtain pure cell population as starting material. In combination of these methods precise and valuable information can be gained on differentiation, malfunction and disease progress arisen from specific cell types.

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QUANTITATIVE IMMUNOFLUORESCENCE BY FLOW CYTOMETRY

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One of the major goals of quantitative immunofluorescence is to determine the average number of specific receptors on a cell population. Flow cytometry is a powerful tool that enables the operator, through multi-parameters, to select cell populations and measure the fluorescence signal arising from antibodies or other probes bound to that population.

The problem is that each step in such a determination contains many unknown variables. Although fluorescence intensity is a linear relationship within certain ranges, it is dependent on energy transfers dependent on both the absorption and emission properties of the fluorochrome, which in turn the resulting signal is dependent on instrument, environmental and molecular properties. These relationships may be described by equations such as:

$$i_F = [geΩεϕ\int Q(\lambda)s(\lambda)T(\lambda\ )d\lambda]c$$

These factors can all be removed from consideration by the introduction of a fluorescence unit that is independent of the instrument, environmental and molecular properties. This unit is Molecules of Equivalent Soluble Fluorochrome (MESF). As implied by the name, this unit indicates the intensity of the standard or sample relative to the intensity of a gravimetric primary solution of the same fluorochrome. Solutions and/or particle suspensions may be calibrated in MESF units against such a primary solution using spectrofluorometry, and in turn used as standards, so long as the following criteria are observed:

The excitation and emission spectra of the standards have to match those of the sample.

The environmental response of the fluorochrome of the standards must match those of the sample.

The standards and the sample must be run on the same instrument at the same settings.

Care must be taken since the number of receptors on a cell may not be reflected by measure the number of receptors available to bind to a particular monoclonal antibody due to steric hindrance may affect antibody binding, as well as binding stoichiometry where additional Scatchard analysis may be required. In summary, MESF units allow for determinations of the number of antibodies binding to cells independent of the instrument and the environment that provides a unique tool for comparison of data across different laboratories and extended periods of time.

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THE EVOLUTION OF CYTOMETRY

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Until the mid-20th Century, determining whether cells were present in a specimen, how many there were, what kinds of cells were represented and what their functional characteristics might be required that a human interpret a microscope image. The same tasks remain for modern cytometers, specialized microscopes in which technology improves on what could be obtained "by eye" alone.

From the 1930's on, Caspersson developed microspectrophotometers to quantify abnormalities in DNA, RNA, and protein content in tumors based on UV absorption. The first applications of flow cytometry, beginning in the 1940's, were to counting and sizing cells in liquid suspension and aerosols,

using measurements of light scattering or electrical impedance (Coulter principle). By the 1960's, investigators were attempting to automate analysis of the Papanicolaou smear and the differential white blood cell count. Kamentsky concluded that limitations of existing hardware and software made it impossible to develop a practical high-resolution scanning instrument; he built a flow cytometer, with a dedicated computer, that could measure as many as four parameters. The problem of isolating cells for identification and further analysis was solved in the mid 1960's, when Fulwyler and Kamentsky, respectively, demonstrated droplet deflection-based and fluidic cell sorters. By the late 1960's, fluorescence measurements using reagents such as nucleic acid dyes and labeled antibodies were providing information about a large variety of cellular constituents. Groups led by Göhde, Herzenberg, Kamentsky, and Van Dilla developed apparatus for fluorescence flow cytometry and sorting that was produced commercially in the early 1970's. A flow cytometric differential leukocyte counter also reached the market at this time.

Herzenberg's fluorescence-activated cell sorter attracted immunologists, who used the instrument to separate various cells of the immune system. The technology aided in the development of monoclonal antibodies as reagents in the 1980's, and was made immensely more powerful thereby. As it became practical to simultaneously measure multiple antigens in or on a single cell, additional fluorescent labels, including new dyes, phycobiliproteins, and tandem conjugates were developed. By the mid-1980's, bench-top analyzers with improved optics, which could make sensitive measurements using air-cooled lasers, began to appear. Their adaptation in research and clinical laboratories was catalyzed by the emergence of AIDS, in which CD4+ lymphocyte counts provided valuable prognostic information. Analysis of tumor DNA content by flow cytometry also became clinically important. The application of multiparameter measurement and analysis was greatly facilitated by developments in personal computers. Flow cytometers were also used to identify and sort individual human chromosomes and sperm, and methods for measurement of physiologic parameters, such as membrane potential, intracellular calcium content, and pH were developed. By the mid-1990's, it was recognized that the *Aequorea* green fluorescent protein (GFP) and related proteins, produced after cotransfection with other genes of interest, could act as reporters, indicating whether transfection had been successful in an individual cell.

Today, practical diode and solid state sources at wavelengths ranging from the UV to the infrared make even multibeam, multiparameter flow cytometers smaller, less power-hungry, and less expensive, enabling them to be used for a wider range of applications. Kamentsky's Laser Scanning Cytometer, an instrument that uses low resolution imaging to extract much of the same information from cells as is now conventionally obtained by flow cytometry. Even simpler static cytometry apparatus has been described to facilitate CD4+ T-cell counting in resource-poor countries heavily affected by the HIV epidemic. Flow cytometry has taken the differential count beyond the wildest dreams of the pioneers in the field, enabling precise analysis of normal and abnormal cells from the blood, bone marrow, and immune system. Image analyzing systems have been approved for automated screening of Papanicolaou smears. The range of particles amenable to cytometric analysis and sorting now runs from single molecules to multicellular organisms, and the pace of progress is, if anything, becoming more rapid.

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ROLE OF LIPID RAFTS IN ORGANIZING RECEPTOR COMPLEXES ON THE CELL SURFACE

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Molecular-scale physical associations among ErbB family members (ErbB1/EGFR, ErbB2, ErbB3 and ErbB4) have been studied by classical biochemical, molecular biological and biophysical methods. When isolated from cells, members of the ErbB family self-associate (homoassociate) and associate with other family members. However, experiments on isolated proteins are inherently unable to detect interactions in cellular environments *in vivo* and *in situ*, and cannot detect heterogeneity within or among cells. Fluorescence resonance energy transfer (FRET) between membrane proteins labeled with fluorescent antibodies or their Fab fragments measures the monomer-dimer distribution of ErbB1 and ErbB2 receptors in fixed and living cells. FRET is a physical process by which energy is transferred non-radiatively from an excited fluorophore (donor) to second chromophore (acceptor) via long-range dipole-dipole interactions. FRET efficiency (E) decreases with the 6^{th} power of the donor-acceptor separation over a range of 1-10 nm – and this distance is particularly relevant to interacting biological macromolecules. We have used the fluorescence microscope to visualize FRET within single cells with spatial resolution limited only by diffraction in the optical microscope, which allows detailed analysis of the spatial heterogeneity of molecular interactions. We also used the flow cytometric energy transfer measurements (FCET) that reveal cell-to-cell heterogeneity within a cell population. Plasma membrane microdomains (lipid rafts, caveolae) gained an especially high interest in biology during the past few years. Lipid rafts contain abundant saturated fatty acids, cholesterol and sphingolipids, and present a markedly different environment than the bulk lipid phase in ordinary membranes. It is believed that transmembrane tyrosine kinases such as the epidermal growth factor receptor are enriched in caveolae - special lipid rafts characterized by the expression of caveolin. It has also been suggested that ErbB2 might also be present in caveolae. We investigated lipid rafts for colocalization of the glycosphingolipid GM1 ganglioside (labeled with fluoresceinated subunit B of cholera toxin (CTX-B)) and ErbB2 clusters (labeled with Cy5-conjugated Fab fragments of the 4D5 antibody) using a CLSM equipped with three lasers. The results suggest that ErbB2 is localized mostly in lipid rafts, similar to ErbB1. Since stimulating ErbB2 increases the size of ErbB2 clusters and lipid rafts, the amount of ErbB2 concentrated in rafts is very likely related to the

function of the protein. The lipid raft environment could alter the association properties of ErbB2 - similar to our findings regarding other membrane receptors. ErbB2's localization in lipid rafts is dynamic, since it can be dislodged from rafts by cholera toxin-induced crosslinking of rafts. The association properties and biological activity of ErbB2 expelled from rafts differ from those inside rafts. For example, internalization of ErbB2 mediated by 4D5 monoclonal antibody was blocked in cholera toxin-pretreated cells, while its antiproliferative effect was not. These results emphasize that alterations in the local environment of ErbB2 strongly influence its association properties, which are reflected in its biological activity and in its behavior as a target for therapy.

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MULTICOLOR IMMUNO-PHENOTYPING BY SLIDE BASED CYTOMETRY

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Slide based cytometry or cytometry on the slide combines the advantage of flow cytometry (FCM, rapid and accurate measurement of cells on a single cell basis) and microscopy (morphological evaluation, no cell loss). The Laser Scanning Cytometer (LSC) is one of the first commercial slide based cytometry systems. Since its appearance an exponentially increasing number of publications show the broad versatility of LSC. Several techniques have been developed to quantify cell proliferation, immunophenotype, and cell function, among others, by analyzing cells, cellular compartments, and tissues. Fluorochrome-labeled specimens are immobilized on microscopic slides which are placed on a conventional epi-fluorescence microscope and are analyzed by one or two lasers. Data comparable to flow cytometry are generated. But in addition, the position of each individual event is recorded, a feature that allows to re-localize and to visualize each event that has been measured. Therefore, the major advantage of LSC compared with other cytometric methods is the combination of two features: a) the minimal clinical sample volume needed, and b) the connection of fluorescence data and morphological information about the measured event. State of the art techniques allow five-color immunophenotyping by LSC using commercially available fluorochrome labeled antibodies. Nevertheless, the majority of authors apply only 2-3 colors. However, by changing the filter settings of the LSC even the newly available infrared emitting tandem dyes PE-Cy7 and APC-Cy7 have been adapted to the LSC allowing up to seven-color immunophenotyping. An even more sophisticated assay using eleven different fluorochromes was recently described for FCM. Assays like these may in future be important for the characterization and quantification of rare cells such as antigen-specific T-cells, memory-T-cells, dendritic cells, and other functionally distinct leukocyte subsets as well as minimal residual disease. An alternative approach that is absolutely unique to slide based cytometry is the repeated staining of surface antigens on lymphocytes and subsequent analyses and merging of the data files. This method enlarges the scope of the LSC: adding a previously used color for a second or a third time generates virtual "new colors" without the need for new dyes, lasers, and filters. Multiple color immunophenotyping and restaining techniques make the LSC an "n-color phenotyping" system.

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DIGITAL MICROSCOPY

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Digital image acquisition and processing is becoming the standard data handling method in modern microscopy. A summary of its background and of up-to-date approaches is provided.

1. Image formation in the traditional light microscope and ways of storing this image.

The light sensitive material (film, CCD) is placed in the back focal plane of the objective. Resolution is affected by the objective, wavelength and the pixel/grain size of the detector. Absorption of live material is low, thus contrast must be enhanced (phase contrast, polarization, dark field microscopy, fluorescence)

2. Fluorescence is a phenomenon frequently used to enhance contrast in microscopy as (i) every photon emitted is 100% intensity gain against the dark background, (ii) molecule-specific labeling can be applied, and (iii) fluorescent labeling can easily preserve cell viability. It is mostly observed in an epifluorescent setup.

3. Imaging modes in the microscope.

(i) In full field mode, the illumination covers the field of view (Köhler optics is often applied), detection is by eye, SLR camera, or electronic camera. (ii) In point scanning mode, illumination is with a point source, (often a laser beam), detection is with PMTs or avalanche photodiodes, that are sensitive, but not coding spatial information. Spatial information is defined by the location of the volume element that is the source of photons emitted and detected. Scanning can be done by the stage or the beam. (iii) Slit scanning can be viewed as an interim solution more related to the point scanning mode.

4. Electronic detectors for full field imaging

The functioning of the most widely used CCD (charge coupled device) detectors is discussed. In addition, SIT (silicon intensified target) and CID (charge injection device) detectors are to be mentioned. We differentiate between video rate and slow scan scientific CCDs. Digitization is done with a frame grabber in the case of video CCDs. Slow scan devices are read and digitized on a pixel by pixel basis. The latest cameras have sometimes more A/D converters, running at 8, 12 or 16 bits and accordingly faster and slower speeds,

offering fast and noisy vs. slower and high S/N modes. Signal to noise principles (Poisson and instrument noise, binning, full well capacity, spillage and blooming) and spectral sensitivity of various types of chips relevant to specific applications (GFP, red-shifted Cy dies, etc.) are discussed. The consecutive generations of image intensifiers are described, detailing their advantage in photon gain and disadvantage in sacrificing spatial resolution.

5. Color CCD-s use the same CCD chips, but with filters. The major types are the single chip CCDs that have a rotating filter wheels, the single chip – microlens combination cameras that use tiny filters and microlenses to project the R, G and B components on neighboring pixels, thus offering high speed but lower spatial resolution, and the 3-chip cameras that offer the best in both speed and resolution but at a higher price.

6. Digital image processing offers the quantitative characterization of data, including corrections for background, dark current of the chip, uneven illumination (flat field). At the same time, documentation should be judicious and processing logged in detail, avoiding the blow-up of insignificant differences. Some examples of advanced digital image processing are provided, including time resolved microscopy in the time and frequency domain, photobleaching energy transfer microscopy, and spectral microscopies utilizing various transforms (Hadamard, Fourier).

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POSTER ABSTRACTS

1

EXAMINATION OF A HERCEPTIN RESISTANT BREAST CANCER CELL LINE IN SCID MOUSE MODEL

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ErbB2 is an important prognostic factor in human breast cancer. Its overexpression, which is mostly activated by gene amplification, occurs in 20–30% of breast tumors and is associated with poor prognosis and rapid relapse.

Herceptin—a humanized monoclonal antibody against erbB2—has anticancer effect alone, but in combination with chemotherapy is especially effective. Unfortunately, in about 30% of erbB2 positive patients Herceptin has no effect, and in some cases it becomes ineffective during the treatment. To compare Herceptin sensitive and resistant breast tumor cells in order to find the possible molecular background of Herceptin resistance, we need appropriate model cell lines. Many breast tumor cell lines that are sensitive are known to grow in culture. However, there are only a few resistant ones, none, to our knowledge readily available to be grown in vitro. The B585 line is one of these, it cannot grow in cell culture, only in nude or SCID mice. We have studied the growth of the B585 cell line in SCID mice, and its erbB genotype and phenotype.

B585 breast cancer cells were injected subcutaneously into young female SCID mice. 0.2 ml–0.3 ml of minced tumor tissue was used, suspended in 1:1 mixture of Hank's buffer and Matrigel. Tumors were observed 3–4 weeks after inoculation. From this time onwards we have measured tumor size and the weight of mice. The first tumor was taken out after 10 weeks. A part of the retrieved tumor was inoculated in other mice; another part was used for the following examinations:

Fast frozen samples were labeled with Cy3 and Cy5 conjugated monoclonal antibodies against erbB1, erbB2, erbB3 and erbB4 (Abs 528, 4D5, HER3-105.5, HER4-77.16). Fluorescent images were analyzed with a Zeiss LSM 510 confocal microscope.

We tested the production of single-cell suspension from excised tumors with a Medimachine grinder, and with dispase digestion so that flow cytometry can be used for analysis. We have made touch preparations to examine the copy number of erbB2 gene using fluorescent in situ hybridization. We attempted to make adherent cultures from cells of the excised tumor in various media and substrates.

According to our results, the Medimachine tissue grinder is inappropriate for producing living cell suspensions. The B585 cells do not grow in adherent culture in MEM, DMEM, F-12, SHEM, either on tissue culture grade surfaces, or on collagen, or Matrigel coatings. Addition of EGF and Cholera-toxin to the medium also does not promote growth. We found that B585 cells had lower erbB2 expression level than SKBR-3 cells, although the erbB2 gene was amplified in both cell lines. The resistance cannot be interpreted only by these differences, since there are sensitive breast cancer cell lines with erbB2 expression level similar to that of the B585.

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2

REPAIR PROCESSES AFTER LOW DOSE IONISING RADIATION AND BYSTANDER EFFECT

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It is generally accepted that one of the radiation-induced effects is the DNA damage. Recent results showed that the genetic changes, such as mutation and transformation can be generated after low dose high-LET ionizing radiation (neutron, alpha) not only in the cells directly hit but also in the neighboring cells. This phenomenon is the bystander effect.

The aim of our study was to examine the radio-adaptive responses induced by low dose neutron irradiation. One of our main objectives was to verify whether culture medium removed from the irradiated cells is able to transmit signals to non-irradiated cells.

CHO (Chinese hamster ovary) and V79 (Chinese hamster fibroblast) cells were irradiated with neutron (0.5-2-10 mGy with 1.59 mGy/min dose rate). In order to demonstrate adaptive response, repair and bystander effect the mutation on the HPRT locus was analyzed. After irradiation the cells were incubated 6 days to allow the development of $hprt^-$ mutant phenotype, then they were treated with 6-thioguanine, a selective agent. The treated cells were plated on Petri-dishes and incubated 8-10 days for colony formation. The colonies were stained with methylene-blue, counted and the mutation frequency was calculated. After different adaptation time they were irradiated with challenge doses (2 Gy gamma irradiation) and assayed for mutation.

Our results show that survival of the cells is inversely related to the radiation dose. Directly irradiated cells with low (0.5-10 mGy) and after that a higher (2 Gy) dose showed 58-65% lower mutation frequency than cells irradiated with 2 Gy alone. After the adapting dose (low dose) the repair capacity of the cells increased and they adapted to the subsequent higher dose. In non-irradiated cells incubated in the transferred medium from the irradiated cells mutation was also induced. This mutation frequency was lower than after 2 Gy only but much higher than in the non-irradiated control. Thus it is shown that the irradiated cells released a "signal" to the medium which caused mutation in the non-irradiated cells.. Better understanding of cell mechanisms involved in bystander effect will lead us to more realistic risk estimators of low dose exposure.

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3

HYPERFRACTIONATED IRRADIATION INDUCED APOPTOSIS IN VITRO

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Purpose: Recent studies show that radiation hyperfractionation with very low doses, where hypoxic cells lose their radioresistance or even can become more sensitive than oxic cells can be clinically useful. In this study survival of tumor cell lines was measured in vitro. Hyperfractionated irradiation was combined with treatment with a bifunctional chemotherapeutic agent. The aim of the study was to examine whether hyperfractionation can influence the radiosensitizing effect of the bioreductive agent. A further aim was to determine whether apoptosis have a role in low dose hypersensitivity in hypoxia

Methods and Materials: Clonogenicity of B16 mouse and RPMI 8322 human melanoma cell lines were studied after final doses up to 4 Gy. Irradiation was applied in 1-8 fractions 6 hours between them. The bifunctional alkylating agent dibromo-dulcitol (DBD, Elobromol) was used as radiation modifier. Radiosensitizing effect of DBD was examined by incubation of the cells for 1 hour in a fresh medium containing 5 μg/ml DBD. All the treatments were applied both in hypoxic and aerobic conditions. Hypoxia was carried out by equilibrating the cells with 95% N_2 and 5% CO_2 mixed gas before one hour and during irradiation. Survival of cells was examined by determination of the living cells and by colony forming assay at various time points. Apoptotic cells were determined 6h, 24h, 48h and 72h after the final treatment by *In situ apoptosis cell detection kit* (Boehringer Mannheim).

Results: Hypoxic cells showed higher sensitivity than the oxic cells after 0.5 Gy irradiation Radiosensitizing effect of DBD depended on the radiation dose and this was more characteristic in hypoxia. Radiosensitizing enhancement of DBD was as much higher with fractionated irradiation with 0.5 Gy fraction/dose as with single high dose treatment. Higher fraction dose or lower fraction number was not effective enough. The apoptotic index was significantly higher 18 h after treatment and it can be shown also after 72 h, especially after hyperfractionated treatment. Combination of radiation treatment with DBD as radiosensitizer further increased frequency of apoptotic cells.

Conclusion: Our results showed the significance of the hyperfractionated irradiation with 0.5 Gy per fraction in cancer therapy. The role of apoptosis was a determining factor in the mechanism of cell death after the combined modality hyperfractionated treatments.

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4

EARTHWORM COELOMOCYTES EXAMINED BY CONFOCAL MICROSCOPY AND REVERSE TRANSCRIPTION PCR

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For the better understanding of innate immunity our attention turned towards invertebrate animals, recently. The annelids have an effective cellular and humoral defense system against the environmental pathogens. Some evolutionary conserved molecules (eg. cytokines, peptide hormones and enzymes) may have essential role in invertebrate defence mechanisms. Our flow cytometric analysis distinguished three coelomocyte populations by physical parameters. These cells reacted with conserved molecule specific monoclonal antibodies differently. Anti-TNF-α monoclonal antibody stained both the surface and intracellular structures of the cells. In order to get further information about the presence of TNF-α in coelomocytes we made RT-PCR with human TNF-α primers. These primers amplified a 300 and 500 bp product. The human β-actin as house-keeping gene, could also be amplified. The isolated PCR products are now under sequencing and their evaluation is in progress. Monoclonal antibodies were raised by hybridoma technology for the better characterization of coelomocytes. Until now two clones specific for coelomocytes were found with different intensity

and specificity. Cytoplasmic granules characteristic for a type of coelomocytes (presumably chloragocytes) are stained by one of the clones. The other clone reacted with membrane in all type of cells. The antibodies did not show cross-reactions with mammalian antigens (e.g. human peripheral lymphocytes, mouse thymocytes). Our further goal is to characterize the coelomocytes with other specific monoclonal antibodies and molecular biological techniques.

5

EFFECTS OF INOSITOL 1,4,5-TRISPHOSPHATE CONCENTRATION ON INTRACELLULAR CALCIUM WAVES CHARACTERISTICS

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Numerical simulations with a developed Tang and Othmer model was used to investigate the effects of the second messenger inositol 1,4,5-trisphosphate's ($InsP_3$) concentration on intracellular calcium waves based on Ca^{2+} release by $InsP_3$. The model assumes that increasing the concentration of InsP3 ([$InsP_3$]), as a parameter value, the cell moves from a stable state, to an excitable state, to a self-oscillatory state.

Waves were evoked by local perturbation of Ca^{2+} concentration in a homogeneous medium. Calcium waves were also evoked in the stable regime in the classical sense. The value of perturbation needed for wave initiation depended on the level of $InsP_3$, and on the amount and space extension of the perturbation at that certain $InsP_3$ level. The initiated Ca^{2+} wave front amplitude remained relatively constant in function of [$InsP^3$], whereas, we found wave propagation velocity to be an increasing function of [$InsP^3$].

Our study showed that calcium wave characteristics were considerably affected by the intracellular concentration of $InsP_3$. Therefore, it could be important to take the deviations caused by the level of $InsP_3$ or $InsP_3$ receptor sensitivity into account in calcium wave modeling.

6

LOCALIZATION OF ANTI-ERBB2 ANTIBODY BINDING SITES AND EFFICACY OF IMMUNOTHERAPY

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In breast cancer and other tumorous malignancies, important regulatory functions are assigned to the EGFR family, of which the over-expression of erbB2 protein is accompanied by poor prognosis. In the last few years, immunotherapies have been developed in which anti-erbB2 antibodies have the possibility to diminish or stop malignant proliferation, thus increasing the efficacy of other chemotherapies. So far, the most effective antibody is Herceptin (4D5), which is already being used in clinical treatments. However, there are other anti-erbB2 antibodies that have different biological effects, and do not lead to the arrest of malignant growth. We investigated three other antibodies, of which 2C4 inhibits the effect of a regulatory ligand, Heregulin, 7C2 induces apoptosis and the single-chain antibody F5-cys causes internalization of erbB2 without decreasing proliferation.

The structure of extracellular domains of the EGFR family was modeled by assuming analogy to the IGFR-1 receptor, and the specific antibody binding sites were determined by phage-display. We supplied further molecular distances based on flow cytometric fluorescence resonance energy transfer measurements (FCET), where the distance (proximity) of two, spectrally overlapping fluorochromes can be determined in the range of 1–10 nm. We have used monoclonal antibodies and lipid probes labeled with the carbocyanine dyes Cy3 and Cy5 as donor-acceptor pair. In these measurements we were able to determine the molecular distances between the antibody Fab fragments binding to different epitopes of erbB2, their distance from the lipid membrane and the proximity of erbB2 dimers.

According to our data, 4D5 and F5-cys bind closest to the membrane and there is a protruding linker segment between the transmembrane and the extracellular domain of erbB2. So it seems that any ligands binding to the juxtamembrane domain can induce internalization, however other factors are also essential in signal trafficking for more prominent biological effects.

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DETECTION OF CD10 EXPRESSION IN THE CASE OF LYMPHOMAS: SIGNIFICANCE OF CHOOSING THE RIGHT FLUOROCHROME-ANTIBODY COMBINATION

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Introduction: In the course of immunophenotyping of haematological diseases determination of the appropriate combination of antibodies in the applied panels has crucial importance in the flow cytometry laboratory. During the construction of such panels one should consider the following points: 1. relevant antibody combinations according to the diagnostic process, 2. gating strategy, 3. choosing the right antibody-fluorochrome conjugate according to the expected antigen expression intensity.

The expression of CD10 is typical in common acute lymphoblastic leukemia (c-ALL), follicular lymphoma (FL) and also characteristic for normal cells like germinal center cells in the lymph nodes, B-cell precursors in the bone marrow

and mature neutrophil granulocytes. The intensity of CD10 expression is the strongest in c-ALL and normal B-precursors followed by mature neutrophils and weak in FL and germinal center cells. In the last two cases the selection of the best conjugate is critical in order not to miss the weak expression of the antigen.

In this study we compared the sensitivity of CD10 detection using FITC as the „dimmest" fluorochome and PE as the „brightest" conjugate staining the same samples.

Materials and methods: Bone marrow and lymph node fine needle aspiration samples from patients with FL and follicular hyperplasia were investigated. The flow cytometric immunophenotyping were done using 3-color labeling. Identical clones of CD10 specific monoclonal antibodies labeled either with FITC or PE were used. The staining protocol was carried out as recommended by the manufacturer (DAKO AS, Denmark). We applied CD19 vs. SSC or CD45 vs. SSC gating during the analysis.

Results and conclusion: The increase of the geometric mean of the signal intensity compared to the isotype matched negative control stain was 2 to 3 fold using CD10 FITC and 10 to 50 fold using CD10-PE in the cases of the gated B-cells. In general the average increment of the brightness was approximately one order of magnitude higher in the case of the CD10-PE staining regardless of the CD10 positive cells population investigated (e.g. FL cells, normal germinal center cells, mature neutrophils or bone marrow B-precursors). Based on our findings we concluded that in the cases of FL and follicular hyperplasia only the CD10 PE conjugate makes the differentiation between the negative and the positive cells reliable and therefore this conjugate can be suggested for using in the course of the flow cytometric immunophenotyping.

8

LOCALIZATION OF PHOSPHATIDYL-INOSITOL 4-KINASE ISOFORM PI4K230 IN THE NUCLEOLUS OF NEURONAL CELLS

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Enzymes of the phophoinositide metabolism, as well as their products including PtdIns-4P, PtdIns-4,5P_2, diacylglycerol and IP_3 are, in addition to the cytoplasm and the cell membrane, also present in the nucleus of eukaryotic cells. While this hints at the possibility of a distinct, phosphoinositide-based signaling mechanism inside the nucleus, hardly anything is known about the role of phospholipids and lipid kinases in this cell compartment. We have studied the distribution of phosphatidylinositol 4-kinase isoform PI4K230 by immunofluorescence in rat brain, and in cell lines of neuronal origin. Using paraformaldehyde (PFA) fixation, PI4K230 can be detected by immunofluorescence only in the cytoplasm of neurons in rat brain and spinal cord. However, after fixation of native cryosections with ethanol, PI4K230 can also be detected in the nucleolus of neuronal cells. PFA treatment of native cryosections prevents the immunoreaction of PI4K230 in the nucleolus, but if PFA fixation is preceded by incubation of the cryosections with PBS for 5-10 min, the nucleoli show PI4K230 immunoreactivity. These results demonstrate an indirect masking effect of PFA suggesting the association of PI4K230 with a nucleolar component which, when crosslinked with PI4K230, possibly buries or hides its immunoreactive epitopes. Cultured cell lines of neuronal origin, such as HCN-1A and HCN-2 human cortical neurons also exhibit cytoplasmic and nucleolar PI4K230 positivity, and the latter can be masked with PFA fixation. However, in undifferentiated cells of neural origin, such as NE-4C mouse embryonic neurons, PC12 rat pheochromocytoma and A172 human glioblastoma, we detected PI4K230 primarily in the cytoplasm, and not at all in the nucleolus. Differentiation induced with nerve growth factor, isobutilmethylxanthine, and dibutiryl-cAMP did not affect the subcellular distribution of PI4K230 in any of the cells examined. Overexpressed HA-tagged PI4K230 was seen only in the cytoplasm of COS-7 cells that normally do not show PI4K230 immunoreactivity. However, after 2 h treatment with 5 ng/ml Leptomycin B, an inhibitor of nuclear export, the enzyme partly translocated to the nucleus, though, it was not localized to the nucleolus even then. These results, taken together with nuclear localization and nuclear export signals in the amino acid sequence of PI4K230, indicate regulated transport of PI4K230 between the cytoplasm and the nucleus. In addition, the unique accumulation of PI4K230 in the nucleolus of mature neuronal cells suggests a specifically associating component that can be involved in some nucleolar function of PI4K230.

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CHEMOPREVENTIVE ROLE OF AVEMAR IN DECREASING THE CYTOTOXIC EFFECT OF XENOBIOTICS

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The chemopreventive effect of AVEMAR, a new natural medicinal product dominantly composed of substituted benzochinon (2,6-dimethoxy-p-benzochinon), extracted from fermented wheat germ was studied. Earlier studies demonstrated that AVEMAR exhibits anti-metastatic, immunrestitutive, antioxidant properties, and able to increase apoptosis.

The *in vitro* cytotoxic effect of AVEMAR was determined using the MTT assay on both normal (MRC-5) and transformed (Vero, HepG2, MCF-7, MDA-MB-231) cell lines. MCF-7 and HepG2 cell lines are estrogen receptor positive (ER+), MDA-MB-231 does not express the estrogen receptor

(ER-). Studies were performed how AVEMAR influences the cytotoxic effect of xenobiotic herbicides Atrazine, Alachlor, and Acetochlor. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-3-triazine) is one of the most widely applied herbicide in the agriculture. Its stability is high therefore its persistence in the environment is long. It can be detected in the groundwater as well as in drinking water so there is a high chance of human exposition. Atrazine is known as a hormone disrupter. Genotoxicology testing of Atrazine resulted negatively but further investigation of its biological activity is necessary. On the basis of earlier data the toxic biological effect of Atrazine is developed through the disassembling of the mitochondrial electron transport system. Atrazine—and other triazines—raise the plasma progesterone levels through dopaminerg pathway so they may contribute to the initiation/development of mammary tumors. The oncogenic impact of Acetochlor is realized through non-genotoxic pathways. Formaldehyde released during the O-demethylation of Acetochlor and Alachor is supposed to contribute to the enhanced nasal tumor incidence induced by chloracetanilides.

In our experiments AVEMAR was found to exhibit estrogen-like stimulatory activity in ER+ cells whereas no effect was detected on ER- cell lines. AVEMAR was found to decrease the cytotoxicity Atrazine applied in high doses on normal, non-transformed cell line. The proliferation stimulation caused by AVEMAR treatment was not influenced by low doses of Atrazine. Our results support the hypothesis that the hormone disrupter activity of Atrazine is developed through an estrogen receptor-independent pathway. In the background of Atrazine-induced increase of mammary carcinoma incidence also seems to be independent of the estrogen receptor pathways but rather due to the increase of plasma prolactin levels. The xenoestrogenic Alachlor and Acetochlor were found to produce significant proliferation enhancement of ER+ cell line and this effect was not modified considerably by AVEMAR. The proliferative effect of the xenoestrogens tested was prevented by AVEMAR on the ER- cell line Vero.

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IDENTIFICATION OF THE FIRST LAMELLOCYTE-SPECIFIC CELL SURFACE RECEPTOR IN DROSOPHILA MELANOGASTER

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One of the basic features of the immune system is the recognition of microbes, parasites and tumour cells. The recognition is mediated by transmembrane receptors expressed in the cellular elements of the immune system. The *Drosophila*'s innate immune system protects the integrity of the body by antimicrobial peptide production, phagocytosis and the encapsulation reaction. The latter reaction resembles the granuloma formation in vertebrates and is mediated by a highly specialized cell for this function, the lamellocyte. In *Drosophila* lamellocytes take care of the particles which are too large to be phagocytosed, by separating them in the body cavity thus inactivating them. After recognition of the foreign particles by so far unidentified cell surface receptors, cell-cell interactions follow and lamellocytes isolate and cause the death of the intruder. The aim of our work is to identify the cell surface receptors and the signal transduction pathways, which are involved in the encapsulation reaction. In the course of our studies we have identified several lamellocyte specific transmembrane proteins, among them a very abundant, 16 kDa antigen, designated as L1. This molecule has at least three immunological epitopes. By the aid of a mixture of monoclonal antibodies to these three epitopes we purified the protein then analyzed it by MALDI-TOF. Two short peptides have been identified. Both of them fit into a single protein sequence encoded by a *Drosophila* gene, which occurs in one copy in the genome and consists of three exons. The encoded protein is made up of 143 amino acids, contains a signal sequence, an extracellular region and a transmembrane domain at the C-terminal. None of the available algorithms predict a cytoplasmic tail. Molecules with similar structure take part in creating receptor complexes by lateral interactions, organizing multi-molecular complexes involved in recognition. Our further aim is a detailed structural and functional analysis of the gene and its protein. The upstream region will be used to produce transgenic flies for studies on differentiation and function of lamellocytes. In addition there is a P-element near the 3' end of the gene, which will help to produce loss of function type mutants by jumping the P-element.

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DIAGNOSIS OF BIGH3 GENE MUTATIONS IN CORNEAL DYSTROPHIES

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Corneal distrophies linked to 5q31 are autosomal dominant hereditary diseases. Clinically they are characterized by the progressive decrease of corneal transparency in young adulthood, eventually leading to blindness. Histological examination reveals a deposit of protein origin in the diseased corneas. In recent years, several mutations of the BIGH3 gene, positioned on 5q31, were described. In this study we have undertaken the molecular genetic examination of patients with autosomal dominant corneal dystrophies treated since 1994 at the Dept. of Ophthalmology, University of Debrecen. 15 members of 7 families were examined. 2 patients were diagnosed with granular corneal dystrophy (Groenouw I, or GCD), 9 with lattice type I dystrophy (Haab-Dimmer, or LCDI), 4 apparently healthy relatives were also examined. Exons 4., 12. and 14. of the BIGH3 gene in the DNA isolated from peripheral blood were amplified with PCR and analyzed with automatic sequencing. All 9 patients with

LCDI were heterozygous for the most frequent mutation, Arg124Cys, of exon 4 in the BIGH3 gene (CGC→TGC). An SSCP protocol was optimized for the quick detection of this mutation. A grown-up healthy relative carried wild type alleles. The other 3 healthy relatives were under 3 years of age, hence no clinical symptoms could not yet be seen. Two of them were homozygous for the wild allele, while the third carried the Arg124Cys mutation. The most frequent mutations of GCD were not present in our 2 patients. In conclusion, since inheritance of corneal dystrophies cannot be clinically diagnosed at a young age, in these cases demonstrating or excluding the mutant allele is only possible using molecular genetics. This is of great prognostic value regarding patient expectations, necessity of follow-up examinations and adequate choices of future profession for the child concerned. Regarding granular dystrophies, it is possible that in the Hungarian population they are caused by an as yet unknown mutation.

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QUANTITATIVE DETECTION OF INTERPHASE NUCLEI WITH BALANCED CHROMOSOMAL TRANSLOCATIONS BY AUTOMATED SPOT DISTANCE MEASUREMENTS

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Introduction: Fluorescence in situ hybridization (FISH) became a powerful routine approach for the demonstration of specific chromosomal aberrations in interphase cells. The analysis relies on the statistical evaluation of count and relative topography of FISH signals, which is complicated by several problems. Random signal colocalization is especially limiting in the demonstration of low frequency cytogenetic events. To improve its diagnostic value, numerous DNA-probe combinations have been introduced to interphase cytogenetics the evaluation of which is based on spot count, size and multiple signal colocalisations. Unfortunately, at this complexity the statistical evaluation of samples is hardly possible without the use of computer assisted automated scanning systems.

Aims: Our aim was to elaborate an automated image processing search protocol for the quantitative evaluation of cells with balanced translocations by accurate multicolor spot distance measurements.

Methods: Tissue imprints from follicular lymphomas and lymph nodes with follicular hyperplasia were analyzed for the presence of t(14;18) by FISH using a IgH/BCL2 probe (Vysis Inc., Dovers Grove, IL). The major criteria for the translocation were the dual colocalization of the red and green hybridization signals on both involved alleles. For the evaluation, the automatic image analysis station Metafer4 (MetaSystems, Altlussheim, Germany) was used. All spot distances were automatically measured in nuclear areas segmented on the DAPI staining for at least 1000 cells per sample. The two shortest spot distances in each cell, representing most probably the signal fusions were displayed on a histogram. By the use of a control probe pair of similar size on the same slide, the normal random distribution of the two shortest red/green spot distances could be graphically presented.

Results: The spot distances were comparable to the control probe in the non-neoplastic lymph node samples, when applying the IgH/BCL2 probe. As expected, the two shortest spot distances were significantly reduced (0.01–0.18 μm, respectively) as a result of the translocation in follicular lymphomas with t(14;18). Whereas the shortest spot distance frequently reflected random signal colocalization, the low value of the second shortest spot distance was clearly specific for translocation. Differences between control and translocated histograms suggested a distinctive value for translocated cells. Cells with less than 0.12 μm second shortest spot distance could be all interpreted as translocated cells. The frequency of cells with t(14;18) in the analyzed follicular lymphoma imprints ranged between 2.2 and 67.6%, as determined by the presented method.

Conclusion: Balanced translocations can be specifically presented by signal fusions at both involved alleles. Spot distance based automatic measurements, therefore, highly improve the power of dual-fusion FISH approaches. The presented method enables a sensitive statistical evaluation of large cell numbers and is clinically applicable for the rapid demonstration of small cell populations with balanced chromosomal translocations.

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REVERSAL OF MULTIDRUG-RESISTANCE IN TUMOR CELLS BY PHENOTIAZINE, PERPHENAZINE AND CYCLOSPORIN DERIVATIVES

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Resistance arising during cancer treatment is the major source of chemotherapeutic failure and it is an important problem worldwide. In human cancer cells common phenomena the cross resistance between structurally unrelated cytostatic agents called multidrug-resistance (mdr). The best characterized mdr mechanism is the overexpression of p-glycoprotein (p-gp). P-gp the member of the ABC transporter family is an integral plasma membrane drug efflux pump, which extrudes the drugs from the cytoplasma thus decreasing the concentration of the chemotherapeutic compounds in the tumor cells. One possible way to combat mdr is the use of mdr reversing agents, which are capable of binding and inactivating directly to the p-gp molecule thus preventing the chemotherapeutic drug efflux.

In our in vitro experiments mouse T cell lymphoma cell line (L5178Y; par) and its multidrug resistant sub-line (L5178; mdr) transfected with the human MDR1 gene was used to study the effect of different resistance modulators. The effect of the tested compounds on mdr reversal effect was studied

by Rhodamine 123 extrusion assay and in flow cytometry. The objective of our experiments was to test some phenotiazine, perphenazine derivatives, cyclosporins and some well-known mdr reversing agents, such as verapamil, trifluoperazine. The conclusion of our study was that the cyclosporin-D analogue was more effective than cyclosporin-A on mdr reversing, while the biological activity of the other compounds was depending upon the chemical structure.

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HEMOCYTE-SPECIFIC MOLECULAR MARKERS IN THE HEMATOPOIESIS AND INNATE IMMUNITY OF DROSOPHILA MELANOGASTER

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Hemocytes represent the cellular elements of the innate immune defense in insects. These cells recognize, attack and inactivate the microorganisms and parasites invading the body cavity of the insect larvae. Although remarkable advances have been made in the past few years in understanding the hematopoiesis of *Drosophila*, little is known about the origin of hemocytes and mechanisms by which these cells act. To understand the differentiation and the immune response mediated by these cells we have identified molecular markers for *Drosophila* hemocytes. The number of the so far identified antigens is over thirty. On the basis of their expression pattern these molecules fell into seven main clusters. By the aid of these markers we have detected molecular heterogeneity among morphologically identical cell types, re-defined the lineages of hemocyte subsets identified new, so far undefined subpopulations. The three main cell types are: the phagocytes (plasmatocytes), flattened cells (lamellocytes) and crystal cells. We recognized the immediate and distant precursors of these cell types and also distinguished the hematopoietic stem cells. Using these newly identified molecular markers it is possible to separate hemocyte subsets. In the presence of growth factor/s the separated subpopulations differentiate in vitro.

By the aid of these markers we have also characterized the differentiation and function of hemocytes. The hematopoietic stem cells show mitotic activity and they phagocytose foreign objects. The plasmatocytes phagocytose bacteria and other foreign objects and they have the ability to produce antimicrobial peptides. Both plasmatocytes and their immediate precursors—proplasmatocytes—show mitotic activity. The function of the terminally differentiated flattened lamellocytes and their precursors is to encapsulate foreign objects and abnormally developing tissues, which are too large to be phagocytosed. This subpopulation of hemocytes does not phagocytose and does not show mitotic activity. In contrast to the previous hypothesis we have demonstrated that the plasmatocyte and the lamellocyte differentiation lineage are clearly separated from each another. The procrystal- and the crystal cells, which are involved in the melanization and coagulation reactions - do not phagocytose. There are no crystalline inclusions in the procrystal cells, which show mitotic activity, while mature crystal cells contain crystals but they don't divide. The procrystal- and crystal cells form a separate differentiation lineage from the plasmatocyte and the lamellocyte lineages.

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THE ACTIVITY OF PDGF-RECEPTOR-B ON THE SURFACE OF A172 GLIOBLASTOMA CELLS IS RELATED TO LIPID RAFTS

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The autocrine loops formed by PDGF and its receptor bearing tyrosine kinase activity probably play a part in the proliferation of several glia tumors. Our previous studies on the A172 glioblastoma cell line revealed that during the PDGF-induced signal transduction process cells released calcium from the intracellular stores followed by influx from the extracellular space. In our present studies we have examined the types of PDGF- receptors expressed on these cells and the conditions for their proper functioning with special respect to the lipid rafts— detergent resistant microdomains that appear to have an organizing role in signaling. The α- and β-type PDGF receptors were labeled by indirect immunofluorescence. Their positions in space and their relationships to lipid rafts decorated by Choleratoxin B (CTX) subunit were determined by confocal laser scanning microscopy. The changes of intracellular free calcium concentration upon PDGF treatment were studied by videomicroscopy using fura-2 calcium indicator. Since in the maintenance of lipid rafts an important role is attributed to the cholesterol content of the membrane, we also modulated this factor with methyl-β-cyclodextrine (MBCD).

According to our results, mainly the β-type PDGF receptors that are expressed on A172 gliobastoma cells, $\sim 1\text{–}4 \times 10^5$ per cell, depending on the confluence of the cell culture: membrane concentration of receptors increases with cell density. The non-random distribution of PDGF receptors on the cell surface results in a freckled (clustered) distribution, which to a large extent overlaps with the similarly patchy CTX label marking the lipid rafts. The depletion of the amount of cholesterol in the membrane by MBCD treatment led to the deterioration of lipid rafts. Moreover, the calcium transient arising in control cells upon PDGF treatment almost entirely disappeared after the destruction of lipid rafts.

We conclude that PDGFRβ exhibits a clustered distribution on A172 glioblastoma cells and its significant colocalization with lipid rafts has functional importance.

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ANALYSIS OF THE EFFECT OF KINASE INHIBITORS ON PROTEIN DEGRADATION AND PROTEIN SYNTHESIS IN ISOLATED PANCREATIC ACINI OF THE RAT

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The continual intracellular protein turnover that includes protein synthesis and protein degradation is a basic feature of cell function. Protein degradation can be carried out by two major ways: non-lysosomal and lysosomal. Stimulated protein breakdown is mostly carried out by lysosomal macroautophagy, a process that was discovered more than forty years ago. In spite of this relatively long time many important aspects of its role and regulation is still unknown.

Recent results, primarily in the yeast and in mammalian liver cells, with various inhibitors of kinases have indicated the involvement of these types of enzymes in the regulation of macroautophagy. The new method of the isolation of pancreatic acini developed in our laboratory has made it possible to expand our studies and investigate the effect of kinase inhibitors on pancreatic acinar cells in vitro.

Our results show, that inhibitors of phosphatidylinositol 3-kinases (wortmannin, LY294002, 3-methyladenin) as well as the cAMP-related inhibitor theophyllin and the phosphoprotein phosphatase inhibitor ocadaic acid inhibit the autophagic protein degradation completely. Our electron microscopic investigations reveal that the treatment with wortmannin, LY294002, 3-methyladenine and theophyllin results in the lack of autophagic vacuoles in pancreatic acinar cells, therefore, their inhibitory mechanism seems to be carried out at the level of the formation of autophagic vacuoles i.e. sequestration. However, ocadaic acid treated cells contain numerous autophagic vacuoles indicating a post sequestrational effect of this drug, a result that is in contrast with those in liver cells.

The phosphatidylinositol 3-kinase inhibitors were less effective on protein synthesis. 10^{-6} M wortmannin that was maximally effective on protein degradation inhibited protein synthesis only by 20%. The insulin-stimulated elevation of protein synthesis, however, was reversed by 35% by wortmannin.

The phosphatidylinositol 3-kinase related TOR-inhibitor rapamycin did not stimulate autophagic protein degradation above the control level in isolated pancreatic acini. At the same time, rapamycin inhibited protein synthesis by about 20%. We also observed the suppression of insulin-stimulated protein synthesis by 38% by rapamycin.

Our results show that phosphatidylinositol 3-kinases are involved in the regulation of both protein degradation and protein synthesis in pancreatic acinar cells. In addition to the results that show similarities with those obtained in liver, we have also observed significant differences indicating special features of regulation in different cell types.

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IONIZING RADIATION AND HEAVY METALS EFFECT ON CELLS

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Cells respond to different stress events (like radiation, heat, hypoxia, drugs, heavy metals, etc.) with the initiation of defense mechanisms, which determine various biochemical changes. Depending on the intensity of stress there will be changes in the life of the cell, which modifies their survival, decreases the protein- and DNA-RNA synthesis, likewise the glutathione content. Also some newly synthesized proteins appear or their expression is changed, playing an important role in modifying the cell's radiation- and drug sensitivity.

The aim of our study was to investigate whether ionizing radiation lead to stress protein appearance and to determine its molecular weight. We also studied the role of low-dose neutron irradiation in cell survival and the effect of heavy metals.

We used Chinese Hamster Ovary (CHO) and lung fibroblast (V79) cells. The irradiated cells were lysed and electrophoresed with a protein standard on a 5–20% gradient gel. The protein's molecular weight was calculated after construction of a calibration curve related to the standard's migration distance. For the study of the effect of heavy metals we treated the cells with different concentrations (from 10 mM to 1 μM) and for different time periods (1-72h). Cytotoxicity was measured using the MTT assay. The essence of this is that only living cells can reduce the reagent and lead to formation of colored MTT-formazan cristals, which is solubilized in HCl-isopropanol solution. The absorbance was measured spectrophotometrically and from the results we calculated the LC_{50} value.

After low dose ionizing radiation we have detected 8 stress proteins. Their molecular weight ranges between 18–109 kDa and their appearance was different in time. The LC_{50} value in CHO cells was 0.05 mM (at 24 h treatment) and 0.005 mM (at 48 h), while in V79 cells these were 0.1 mM after 24 h treatment and 0.01 mM after 48 h.

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SIMULTANEOUS DETECTION OF VIABILITY AND SEX OF BOVINE SPERMATOZOA

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Viability and sex of bovine spermatozoa were simultaneously evaluated. After viability and acrosome staining with

trypan blue/Giemsa, only live bovine spermatozoa became decondensed by a modified papain-dithiothreitol method. Due to this specific effect, live sperm heads were easily distinguished by their enlarged size and dark violet colour from small and light blue dead ones after fluorescence in situ hybridization (FISH) with a yak XY paint set and DAPI counterstaining. Morphological evaluation of bovine spermatozoa before FISH provides extra information, and the combined staining and sexing of live sperm cells may be an effective tool in evaluating sex-oriented or sex-determined semen samples.

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INDIVIDUAL DISTRIBUTION AND COLOCALIZATION OF VIP, NOS, GABA, GLUTAMATE AND NMDA RECEPTORS IN THE DEVELOPING HUMAN ENTERIC NERVOUS SYSTEM

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Immunoreactivity for glutamate and NMDA receptors in the enteric nervous system (ENS) of guinea-pig strongly supports the hypothesis that glutamate is an enteric neurotransmitter. Glutamatergic transmission through NMDA receptors is proposed to be important in visceral nociception and control of food intake in rat. The coexistence of mRNA for NMDA receptors and VIP in the enteric neurons of rat suggests that NMDA-mediated glutamate responsivity in the intestine is modulated by VIPergic neurons. Several population of enteric VIPergic neurons have been defined in the guinea-pig intestine where VIP was costored with GABA and NOS. Information about the distribution of nerve fibres and somata of these neuronal subpopulations and about the presence of glutamate and NMDA receptors in the developing human ENS is scarce. The first aim of this work was therefore to determine the individual distribution of VIP, NOS, GABA, glutamate and NMDA receptors in the ENS of the human fetal intestine. The second aim was to investigate the possible colocalization of these substances in order to find the particular neuronal population expressing NMDA receptors and serving as a target of glutamatergic excitatory input.

Human fetuses at week 18 of gestation were obtained after legally approved abortions in accordance with the declaration of the Medical World Federation (Helsinki, 1964). The dissected whole intestines were flushed with phosphate buffer and immersed in the fixative for 4 h. Wholemounts were prepared from selected intestinal segment. After preincubation in normal goat serum, wholemounts were incubated overnight in the primary antisera (against NOS, VIP, glutamate, GABA, NMDA NR1). Simultaneous incubations were applied in double-labelling experiments. The species-specific secondary antibodies used for visualizing immunopositivity were conjugated to Cy3, TRITC and FITC. Preparations were viewed and photographed with a Zeiss Axioscope fluorescent microscope with an AxioCam digital camera.

Enteric neuronal elements displayed immunoreactivity for VIP, NOS, GABA, glutamate and NMDA NR1 in the myenteric plexus of the developing human ENS. NOS-positive neurons were numerous whereas glutamate-, GABA- and VIP-immunoreactive cells were rarely seen. Double-labeling experiments revealed a limited coexistence of NMDA NR1 with VIP and GABA with NOS. In addition, these experiments demonstrated VIP-immunopositive pericellular baskets around a given population of NOS- and NMDA NR1-positive myenteric neurons. These results give the first immunocytochemical evidence that NMDA receptor-mediated glutamate responsivity and transmission might be modulated by VIPergic neurons. The question that the VIPergic neurons, which modulate glutamatergic function do or do not coexpress other substances like NOS or GABA is under investigation.

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NONCLASSICAL FUNCTIONS OF MHC II ON A HUMAN LYMPHOMA CELL LINE

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In immune cells, main histocompatibility complex (MHC) II molecules play a role in the activation of several cell functions. This feature is called "nonclassical" to make a difference from their "classical" function of antigen-presenting. The nonclassical pathways can be activated by bindings of antibodies, superantigens and CD4 molecules to MHC II molecules. The activated cell function can be cytokine-secretion, the activation of nuclear factors, proliferation or apoptosis.

According to recent publications, MHC II molecules can induce two different types of intracellular signaling pathways, depending on the level of differentiation of the B-cell on which they are expressed. One of these cascades results the translocation of the serine/threonine kinase PKC and the elevation of the cAMP-level. The alternative way involves tyrosine-kinase activation and Ca^{2+} mobilization.

Kv1.3 voltage-dependent potassium channels have a high level of expression in several immune cells. It takes part in lymphocyte activation processes that influence the rate of proliferation, Ca^{2+} dependent signal transduction, interleukin-secretion and cytotoxicity. It was already shown that in human T-lymphocytes the phosphorylation of Kv1.3 by PKC activates the channel. Nevertheless, tyrosine-phosphorylation of Kv1.3 channel causes the inactivation of the channels, furthermore it also modulates the kinetics of the current.

The aim was to investigate how antibody binding to MHC II modulates the voltage-dependent potassium current. We also wanted to examine if the ligation of golden beads to the receptor-antibody system causes an alteration in the modulation of the current. Our experiments were performed on a Kit 225 K6 human lymphoma cell line. For the indirect labeling of the cells, we used anti-(human MHC II) L234 as primary antibody and goat anti-mouse antibody conjugated 30 nm golden beads as secondary antibody. After labeling, we measured the whole-cell potassium current using the patch-clamp technique. The holding potential was set to –120 mV and depolarizing pulse was applied to +50 mV for 80 ms.

According to our results the incubation with L243 decreased the whole-cell peak potassium current to 33.8 ± 16.8% (RF). However, cells which were also labeled secondarily by the goat anti-mouse immunogold antibody, showed a decreased fall in peak currents, to 70.3 ± 28.2% (RF) of the original level (n = 8). We also examined the effect of antibodies different from L243: The anti-(human MHC I) W6/32 and the anti-(human VLA-4 integrin) TS2-711 did not influence the potassium currents.

The obtained results support the idea that the L243 binding to MHC II elicits tyrosine phosphorylation, which leads to channel inactivation and current fall. Nevertheless immunogold particle binding induces a greater change in MHC II conformation, which causes PKC activation and also channel activation with current increase.

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HOW TO CIRCUMVENT THE LOW SIGNAL-TO-NOISE RATIO IN FLOW CYTOMETRIC ENERGY TRANSFER MEASUREMENTS?

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Background: Flow cytometric fluorescence resonance energy transfer (FCET) is an efficient method for mapping associations of biomolecules due to its high sensitivity to changes in molecular distances in the range of 1–10 nm. Wide application of the method is limited by the requirement for an advanced instrument and the need for a relatively high signal-to-noise system (i.e. high expression level of the molecules). FRET applications might be limited by the lack of appropriate directly labeled monoclonal antibodies to serve as a donor/acceptor pair. The use of fluorophore-conjugated secondary antibodies or Fabs may overcome this problem, although the altered geometry of indirect labeling compared to direct labeling can significantly affect the observed FRET efficiency values.

Methods: Cy3- and Cy5-conjugated antibodies were used to label membrane proteins on the cell surface. FCET measurements were performed on a widely used flow cytometer, FACSCalibur, using cell-by-cell analysis of energy transfer efficiency.

Results: In an attempt to increase the accuracy of FRET measurements we applied a long wavelength donor/acceptor pair, Cy3 and Cy5, which had a beneficial effect on the signal-to-noise ratio. A new algorithm for cell-by-cell correction of autofluorescence further improved the sensitivity of the technique; cell subpopulations with only slightly different FRET efficiencies could be identified. Several direct and indirect immunofluorescent labeling strategies were tested on both the donor and/or acceptor side yielding significantly different FRET efficiencies: the application of a larger antibody complex causes a decrease in intramolecular FRET efficiency due to the geometry of the antibody complexes, i.e. when antibody or Fab complexes get larger, the actual distance between the donor and acceptor fluorophores increases.

Conclusions: We developed a new FCET method by applying long wavelength excitation and detection of fluorescence and perfecting autofluorescence correction. Increased accuracy of the new method makes cells with low receptor expression amenable to FRET investigation. As a practical advantage, the new approach can be easily implemented on a commercially available flow cytometer.

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FORENSIC APPLICATION OF MTDNA POLYMORPHISM

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Besides the nucleus, the mitochondria also contain genetic material. The human mitochondrial DNA (mtDNA) is a double-stranded, circular molecule and is 16569 bp long. There are 1000–10000 copies of mtDNA per cell. It has a maternal inheritance, it's non-recombining and has a greater mutation rate compared to the nucleus.

In forensic samples one often has to face the problem of degraded DNA (disintegrated tissues, ancient bones) or a minimal amount of sample material (e.g. cigarette butts, stamps). In these cases (according to pertinent literature) mitochondrial markers prove to be more successful. In other cases— e.g., hair without follicle, cuticle cells without nucleus— only these markers can be used. The mitochondrial genome is highly polymorphic. Most of the variable locations are found within the single non-coding region, the "D-loop", in which there are three hyper-variable regions.

In a crime case in Budapest, we compared the mtDNA profile found on the criminal object with the profile of the suspected person. We isolated DNA and amplified the HVI. region. We cycle-sequenced the amplified fragment. The product of the sequencing reaction was separated and detected with the ABIprism 310 Genetic Analyzer. The results were compared to the reference sequence.

We found a mixed trace in the sample which indicates the presence of the DNA profile of more persons, including that of the suspect.

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ELEVATED VON WILLEBRAND FACTOR LEVEL AND THROMBUS FORMATION

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Background According to clinical observations elevated plasma level of von Willebrand factor (VWF) carries a higher risk of thrombosis. The VWF is a highly multimerized plasma glycoprotein which promotes platelet adhesion and aggregation at high shear rate. It also functions as a carrier of coagulation factor VIII. Normal range of plasma VWF is 0.6-1.5 U/ml. Thrombotic disorders; vasculitises, diabetes, obesity, hypertension, renal failure, liver-, and malignant diseases are associated with the elevation of plasma VWF concentra-

tion. In the present work we constructed an in vitro model system—using a modified cone and plate viscometer—to study the influence of high VWF level on thrombus formation under well defined flow conditions.

Methods Platelet adhesion and aggregation in response of VWF concentrations was carried out at 1000 s^{-1} shear rate on human collagen type III surface with low molecular weight heparin anticoagulated blood. We have evaluated the surface coverage and size of the objects with Virginia image analysis software controlled light microscope. Platelet count was measured by Sysmex K4500. VWF multimers were analyzed by SDS-agarose gel electrophoresis. Plasma levels of VWF antigen were determined by a sandwich enzyme-linked immunosorbent assay.

Results We found, that the surface coverage did not increase significantly with the amount of VWF added to the blood until approximately 5 U/ml. Just as in the control experiments the thrombus was oriented in the direction of flow. Above 5 U/ml VWF concentrations, the surface coverage decreased, tiny aggregates and several giant thrombi were formed which were not orientated in the direction of flow. The average area of giant thrombi was 1100 μm^2 with the largest thrombi areas up to 6000 μm^2. We also measured the height of these thrombi using confocal laser microscope and found to be 30-40 μm. The multimer structure of VWF was not changed, but the VWF:Ag level decreased with 18–22%. The number of platelets remained in the blood after adhesion was reduced with 32–56%.

Conclusion According to the disappearance of single platelets and the calculation based upon the surface coverage, the platelet aggregation process has to be dominant. The elevated level of VWF does not result in increased adhesion, but induces the formation of giant thrombi. We have assumed that the increased thrombotic activity at higher VWF level is caused by the platelet-platelet interaction mediated by the VWF.

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A_1 AND A_2 ADENOSINE RECEPTOR ACTIVATION INVERSELY MODULATES MEMBRANE POTENTIAL OF DDT_1 MF-2 CELLS

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Adenosine is an endogenous modulator of cellular functions in the central nervous system and peripheral tissues. Many of the effects of adenosine are coupled to conductance changes, particularly the modification of K^+ and Ca^{2+} channels, involving an alteration also in membrane potential. Cell surface adenosine receptors mediate physiological effects of adenosine. These receptors were classified by Burnstock as P_1 and P_2 purinergic receptors, depending on their preferential interaction with adenosine (P_1) or ATP (P_2). The P_1 sites are further subdivided into A_1, A_{2a}, A_{2b} and A_3 adenosine receptors, on the basis of their differential selectivity for a series of adenosine analogs. Voltage dependent potassium conductance has also been proven to be present on these cells using patch-clamp technique. Mature follicular oocytes (stages V and VI) of *Xenopus laevis* are known to possess receptors for adenosine (P_1) and ATP (P_2). A correlation was found between the levels of adenosine or ATP treatment and the measure of the outward rectifying potassium current in these cells. Most possibly adenosine receptors stimulate this adenylate cyclase system and potassium current is modified via cAMP level alterations. The aim of our study was to investigate the effect of the interaction of A_1 and A_2 adenosine receptors and their ligands on the potassium conductance and membrane potential of the cells. We intended to describe a P_1 mediated potassium channel activation in the above DDT_1 MF-2 model.

Methods: The effect of A_1 and A_{2a} adenosine receptor ligands on transmembrane potential was measured with flow cytometry using the negatively charged bis oxonol dye. The ion currents were measured by patch-clamp technique in whole-cell configuration, voltage-clamp mode. The extracellular solution was Normal Ringer, pH 7.35; the pipettes were filled with KF intracellular solution, pH 7.23. Materials were solved in Normal Ringer and transported to cells by perfusion. Holding potential was kept on -120 mV, the time of depolarization was 2 s, levels of depolarization ranged from -80 to + 60 mV by 20 mV steps. All patch-clamp measurements were carried out at 20 °C. Clampex 8.0 software was used for measuring and Clampfit 8.0 for data analysis. For each result, we calculated average and standard deviation.

Results: A_1 adenosine receptor agonist CPA (50 nM) elicited a rapid and maintained increase in the potassium conductance and a concomitant hyperpolarization of the membrane. A_{2a} agonist CGS 21680 (50 nM) caused decrease of the potassium current and depolarized the cell membrane in the same way. These effects were eliminated by subtype-selective adenosine receptor antagonists (DPCPX [A_1], CSC [A_{2a}], ZM 41385 [A_{2a}], all 1 μM). The ligand induced membrane potential changes were reversible.

Conclusions: It can be concluded that used adenosine agonists and antagonists take part in adenosine receptor-mediated processes and prove presence and activity of A_1 and A_{2a} adenosine receptors in the studied cell line. We suggest that stimulation or inhibition of the adenylyl cyclase system by A_1 and A_{2a} receptor activation can occur by a mechanism involving a ligand induced change in transmembrane potential among the first elementary steps as alterations in the membrane polarity may lead to voltage-dependent conformational change of enzymes.

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ASTROCYTES SUPPORT THE NEURONAL DIFFERENTIATION OF NEUROECTODERMAL PROGENITOR CELLS

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Multipotential neural progenitor cells and the already committed young neuronal precursors are connected with

glial cells in their close neighborhood. Astrocytes guide and regulate the migration of neuronal precursors during cortical development. Glial cells within the subventricular zone of adult might to be involved in the maintenance of the undifferentiated neural stem cell population. Multipotential stem cells transplanted into the mature, damaged brain in the hope of cell replacement and better functional regeneration, get into the environment of reactivated astrocytes.

To examine the influence of astroglial cells on the fate of uncomitted neuroectodermal progenitors, we performed *in vitro* - astroglia / neuroectodermal progenitor - *co-culture* experiments.

Neuroectodermal cells of the NE-4C cell line, derived from the forebrain vesicles of 9 day old, p53 deficient mouse embryos, give rise to neurons and astroglia upon induction with all trans-retinoic acid (RA). The RA-induced neuronal differentiation of NE-4C cells proceeds through well-characterized steps of morphological and functional maturation.

Subclones of the NE-4C cells were labeled by vectors encoding modified green fluorescent protein (GFP) or placental alkaline phosphatase enzyme (PLAP). GFP/NE-4C or PLAP/NE-4C cells were planted onto monolayers of astroglial cells, derived from perinatal mouse or rat forebrains. Proliferation of the individual progenitor cells on the surface of the glial monolayer resulted either in the appearance of undifferentiated cell clusters, or in the development of foci of abundant neuron production. The co-localization of GFP or the substrates of the PLAP enzyme with neuronal markers indicated that the multitudinous neuronal differentiation observed at distinct culture areas was indeed due to the differentiation of the cells of the NE-4C cell line.

Glial conditioned media did not induce neuronal differentiation of the NE-4C cells. However, considerable neuronal induction could be observed, if progenitors were cultured in the liquid environment of live astrocytes. Our data indicate, that the success of neuronal induction of neuroectodermal progenitors by astrocytes is due to a close cell to cell contact or due to some easily degradable, short range acting factors released into the medium by astroglial cells.

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SIGNALLING BY THE THE EGFP-EGFR FUSED PROTEIN STUDIED BY FLUORESCENCE CORRELATION MICROSCOPY

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Characteristics of the diffusional motion of cell surface receptors are indicators of interactions affecting the translational mobility of these molecules including aggregation/association, immobilization by cytoskeletal elements, compartmentalization in lipid domains, etc. We studied the mobility of green fluorescent protein - epidermal growth factor receptor fusion proteins (eGFP-EGFR) transfected into Chinese hamster ovary cells, and the first steps of signal transduction induced by stimulation with EGF using fluorescence correlation spectroscopy (FCS) and fluorescence recovery after photobleaching (FRAP). The effectiveness of the signal transduction cascade was checked by measuring tyrosine phosphorylation and intracellular calcium transients. Since EGFR diffusion has previously been measured only using labeled ligands, our measurements are the first ones on unstimulated cells. FCS revealed two main diffusing species: a mainly intracellular fast component with $D_1 = (2 \pm 0.8) \times 10^{-7}$ cm^2/s, and a slower component with $D_2 = (1.2 \pm 0.5) \times 10^{-9}$ cm^2/s dominant in the plasma membrane. Data were fitted by an anomalous subdiffusion model, which assumes diffusional motion among randomly located obstacles or transient binding processes hindering free diffusion. Triplet state formation and protonation dependent GFP blinking were also considered. The diffusion coefficient of the slow component decreased to $(0.66 \pm 0.3) \times 10^{-9}$ cm^2/s after stimulation with 50 nM EGF. Analysis of the amplitude of autocorrelation function and fluorescence intensities revealed that the specific fluorescence per molecule decreased to 94 ± 36% of the original value, implying that there was no significant change in the aggregation state of receptors in the first few minutes after stimulation. These data support the model of conformational activation - the decrease in mobility is probably due to binding of the receptor to molecular complexes having low mobility. Our FCS measurements report on the microheterogeneity of local diffusion conditions both in terms of mobility and the effect of obstacles or binding sites hindering diffusion. This is an indication of the microdomain structure of the cell membrane and its influence on the motion of cell surface receptors. Diffusion coefficients derived from FRAP are in accordance with earlier mobility data on EGFR, but are lower than those determined by FCS. Time fractionation of the analyzed data reveals that the short-term diffusion coefficient is larger than the long-term value, in accordance with the microdomain structure of the cell membrane. The sensitivity of the FCS method is illustrated by experiments on cells with such low expression level that the mean number of molecules residing simultaneously in the sensitive volume is less than one. A method to measure the axial and lateral dimensions of the detection volume directly is presented.

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CO-LOCALIZATION OF CB1 RECEPTOR IMMUNOREACTIVITY WITH NEU-N EXPRESSION AND IB4 BINDING IN THE SPINAL DORSAL HORN AND DORSAL ROOT GANGLIA OF RATS

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It is well established that cannabinoids, both exogenous and endogenous, act through two types of specific receptors, CB1 and CB2. It is also demonstrated that the two receptors show contrasting distribution patterns in various tissues. The

CB1 receptor is located dominantly in the central nervous system, whileCB2 expression is characteristic for immune-competent cells. A long line of experimental evidence shows that CB1 receptor mediated endogenous cannabinoid mechanisms play a substantial role in various sensory, motor and higher brain functions including motivational affective behaviors like pain. The distribution of CB1 receptors has also been extensively studied in higher brain centers. However, our present knowledge concerning the localization of CB1 receptor molecules in the spinal dorsal horn and dorsal root ganglia (DRG) is quite limited, although it has been shown that endogenous cannabinoids are strongly involved in spinal nociceptive-antinociceptive information processing mechanisms. Accordingly, by using immunohistochemical methods, we have studied the localization of CB1 receptors in the spinal cord and DRG. In addition, the co-localization of CB1 receptors with neural markers that have already been reported to label specific populations of primary afferents and spinal neurons was also investigated. In dorsal root ganglia, we demonstrated that the CB1 receptor is expressed by a number of small and medium sized neurons. We also showed that some of the CB1 immunoreactive DRG cells also bind isolectin-B4 (IB4), indicating that the CB1 receptor is expressed by some non-peptidergic nociceptive primary sensory neurons. Confirming this result, we found a substantial co-localization between CB1-immunoreactivity and IB4-binding also in axon terminals scattered in the superficial spinal dorsal horn. In addition to axon terminals of DRG neurons, we also encountered CB1 immunoreactive neurons in the superficial spinal dorsal horn. Some of them also showed immunoreactivity for the neural marker Neu-N, whereas others were negative for this nuclear protein. This finding suggests that the CB1 receptor is expressed by both neurons and glial cells in the spinal dorsal horn.

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GENERATION AND CHARACTERIZATION BY FLOW CYTOMETRY OF DENDRITIC CELLS

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Dendritic cells (DC) represent a special population of immune system (DC). The main function of immature DCs is the uptake and processing of antigens. After migration to lymphoid organ mature DCs are able to prime naive T cells for antigen presentation. The expression of major histocompatibility (MHC) and co-stimulatory molecules (CD80, CD86, CD54, LFA) on the cell surface is up regulated upon maturation for effective T cell activation. Monocyte- and CD34+ progenitor-derived DCs are generated with cytokines (GM-CSF, IL-4, Flt-3, TNF-alpha) supplemented culture medium.

In this study CD14+ cells were isolated from peripheral blood of 8 healthy donors by magnetic separation (Miltenyi). One part of cells were generated in present the presence of cytokine cocktail (GM-CSF, IL-4, Flt-3), the other part of cells were cultured without cytokines. The cell culture was maintained during 12 days. On 7th day TNF-alpha was added to medium that contained cytokine cocktail. In every second day a sample was taken a sample from the cell culture. The cells were determined viability of cells was determined and the expression of the following cell surface antigens that were characterized (characteristic for monocytes and dendritic cells) was measured by flow cytometry: CD1a, CD11c, CD14, CD16, CD86, HLA-DR, mannose receptor. The cell cultures were examined by invertoscope, and the cells were stained by MGG on the 12th day.

During the generation the number of viable cells was decreased by degrees. From the 7th day of culture the cells number—that were treated by cytokines—were higher than the cell culture without cytokines. The treated cells have become clumps and have developed typical shape. The cultured cells were characterized by flow cytometric analysis, results were showed that FS, SS and autofluorescence was increased similar to generation time. Expression of CD14 on monocytes were increased till 5th day, after than decreased slowly, but the cells were expressed this marker to end of generation. In the presence of cytokines on the cells were increased expression of CD14 till 3rd day and later decreased intensively, and lots of cells were lost the CD14 marker. TNF-alpha was not chosen expression on the cell surface had not influence on the expression of the examined cell surface antigens. CD16 was expressed low level on monocytes at 5th day and remained long time. The cells were not expressed CD16 in the presence of cytokines. The mannose receptor was expressed on cells the same level in the presence or absence of cytokines from 3–5 day of culture. CD64 was showed low level on monocytes and on cells presence of cytokines. CD1a and CD86 are characteristic DC markers, these have became significant on cells with cytokines treated, the monocytes were expressed the special markers on very low level. In contrast the HLA-DR and CD11c were increased till 10th day, but after than expression of monocytes were stained high, while the cells in the presence of cytokines were decreased a little.

Monocyte-derived dendritic cells were generated in presence of cytokines can be differentiated more mature phase on base of antigen expression of cells. These results indicate of strategies using DCs to induce T cells type immune response only from 10th day of culture on cells treated with cytokines, the monocytes were expressed these special markers on very low level. Opposite to others' observations the HLA-DR and CD11c were increased till 10th day, but after than the expression of monocytes were stayed high, while that of the cells in the presence of cytokines were decreased a little. Several mature phase of the monocyte-derived dendritic cells generated in presence of cytokines can be differentiated on the base of the antigen expression of cells. These results indicate of strategies using DCs to induce T cells type immune response.

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MULTIDRUG-RESISTANCE REVERSAL OF NAPHTIRIDINE DERIVATIVES

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During the chemotherapy of tumours the multidrug resistance caused by active efflux of the cytostatic agent from the cells means a serious problem. The 170kD p-glycoprotein (pgp 170) coded by *mdr1* gene is an energy dependent ABC-type efflux pump. Reversal of multidrug resistance can be achieved by inhibiting efflux pumps. In our experiments mouse T lymphoma cell line transfected with human *mdr-1* gene was treated with naphtiridine derivatives and the accumulation of Rhodamine-123 in the cells measured by flow cytometer was characteristics of possible inhibiting of efflux pump. Among naphtiridines the BG1045, BG1028, BG1046, BG1055 and BG691 exerted strong MDR reversal effect, BG1022, BG1048, BG1044 and BG691 were moderately effective at inhibiting pgp-170 and further four BG compounds were ineffective compared to the positive control verapamil. The inhibition of pgp-170 can be achieved by complex formation between the pump protein and the chemical agents. Some of our results shows that certain naphtiridine derivatives have a strong affinity to the protein existing in the membrane of tumour cells, however other of our experiments (UV spectral shift and melting point alteration of DNA treated with naphtiridines) suggest that some of the naphtiridine derivatives can influence the expression of *mdr-1* gene through the physically interaction of DNA and drugs inhibiting the expression of the proper gene.