

National Institute of Biology
 Department for Genetic Toxicology and Cancer
 Biology

From Cancer Prevention to Cancer Development

Metka Filipič

Introduction

The Department for Genetic Toxicology and Cancer Biology at the National Institute of Biology was established in 1995 by Prof Tamara Lah as the Department for Molecular Biology. At that time, the research was mainly focused on studies of basic mechanisms of cancer invasion and metastasis, in particular the role of cystein proteinases in these processes. A year later, when Dr Metka Filipič joined the group, the research was expanded to the field of genetic toxicology, which deals with the study of environmental and lifestyle



Doc. dr. Metka Filipič
 Head Department for Genetic Toxicology and Cancer Biology
 National Institute of Biology



Figure 1: Department for Genetic Toxicology and Cancer Biology.

From the left in the front row: Anja Pucer, Saša Kenig, Jana Petković, Katja Kološa, Tina Eleršek; middle row: Tamara Lah, Maria Beatriz Duran Alonzo, Irena Zajc; back row: Bojan Sedmak, Irena Hreljac, Boris Gole, Mihael Bricelj, Karmen Stanič and Metka Filipič.

factors which induce genetic alterations leading to cancer initiation. In 2001, researchers from the group for ecotoxicology and ecotoxinology, whose research is focused on the impact of environmental pollution on water ecosystems, joined the department. Currently, the department is headed by Dr Metka Filipič and has nine researchers with PhDs, six PhD students and two technical assistants (Figure 1). As environment and human health are closely related, the unique interdisciplinary expertise of the researchers in our department enables us to apply new approaches to study complex interactions between environmental and genetic factors in the development of cancer and other degenerative diseases, as well as how these interactions affect ecosystems.

Environmental contamination due to human activities is a major threat to ecosystems as a whole and to human health. Therefore it is of major importance to recognise these threats in order to introduce efficient preventive and protective measures. Of particular concern is exposure of living organisms to genotoxic contaminants, which induce damage to genetic material and mutations, and which in humans lead to heritable diseases and cancer. Since genetic function and structure in all organisms is basically the same, genotoxins in exposed ecosystems can cause severe changes in biodiversity.

Cancer development is an extremely complex multistage process. Initiation of cancer starts with DNA damage, which leads to mutations. Mutations can occur spontaneously at a very low rate and, much more frequently, as a result of DNA damage caused by chemicals or ionising radiation. Cells accumulate mutations, and some cells, which acquire mutations that silence tumour suppressor genes or activate oncogenes, eventually gain the ability to escape cell cycle checkpoints and divide without control. Further mutations

and tumour-promoting chemicals, which stimulate cell division by different mechanisms, lead to abnormal proliferation of transformed cells and the development of cancer. Part of the basic and applied research of the Department of Genetic Toxicology and Cancer Biology is focused on studying the effects of genotoxic contaminants on rodent and human cells, as well as the effects of these pollutants and toxins on water ecosystems. The aim of these studies is to identify genotoxins and to explore their mechanisms of action with the goal being to find effective preventive measures against cancer initiation and adverse effects on ecosystems. However, exposure to genotoxins is unavoidable, and therefore human cancer is also unavoidable. The other part of our research is focused on exploring the mechanisms of tumour progression and the increased invasiveness of tumour cells and other biochemical alterations, with the goal of identifying new targets and strategies for prevention of cancer development, and for therapy.

Genetic toxicology, ecotoxicology and ecotoxinology

In our everyday life, we are exposed to different carcinogens from different sources (Figure 2). For human risk assessment and risk management, it is very important to characterise DNA-damaging (genotoxic) chemicals and to clarify their exact mechanisms of action. Our research in the field of genetic toxicology is focused on exploring the mechanisms of genotoxicity of some ubiquitous environmental carcinogens such as heavy metals, certain organic pollutants (particularly food contaminants), and pesticides, as well as emerging contaminants such as nanoparticles and natural toxins. For these studies, we are using different standard genotoxicity assays such as bacterial mutagenicity (Ames) and SOS/umu assay as well as more complex mammalian cell and tissue models. For measuring genotoxic effects, we use different methods for detection of mutations, chromosomal and primary DNA damage. One of the most sensitive methods for measuring



Prof Dr Tamara Lah, Director

Who are we?

Within our department, we are discovering new natural compounds and investigating and explaining mysterious laws of nature. We are able to determine the mechanisms of action of natural and artificial toxic and genotoxic substances by inventing new detection methods. We also offer services in toxicology by routinely using a battery of detection assays – from microbial-based toxicity and genotoxicity tests all the way up to fish embryo tests, human cell cultures and tissue models.

As is well known, cancer is in many cases avoidable, and cancer prevention is an increasingly important area of interest. Our research is focused in environmental aspects that can lead to cancer development – how strong is the link between the lack of care for the environment and the increasing frequency of such damaging diseases? On the other hand, many natural compounds consumed every day may reverse carcinogenesis, and we are seeking to investigate some of them.

Following carcinogenesis, it is important to understand how cancer progresses; we are researching how to predict tumour progression and regression – how to predict the results of applied therapy and how to improve it.

We are researching answers to all these questions, proposing new prognostic and prediction markers, and are up-to-date with new trends in global scientific research, as well as with the most recent guidelines and legislation in the field of toxicology and environmental policy.

If you have questions in these fields – believe us, we can provide answers!

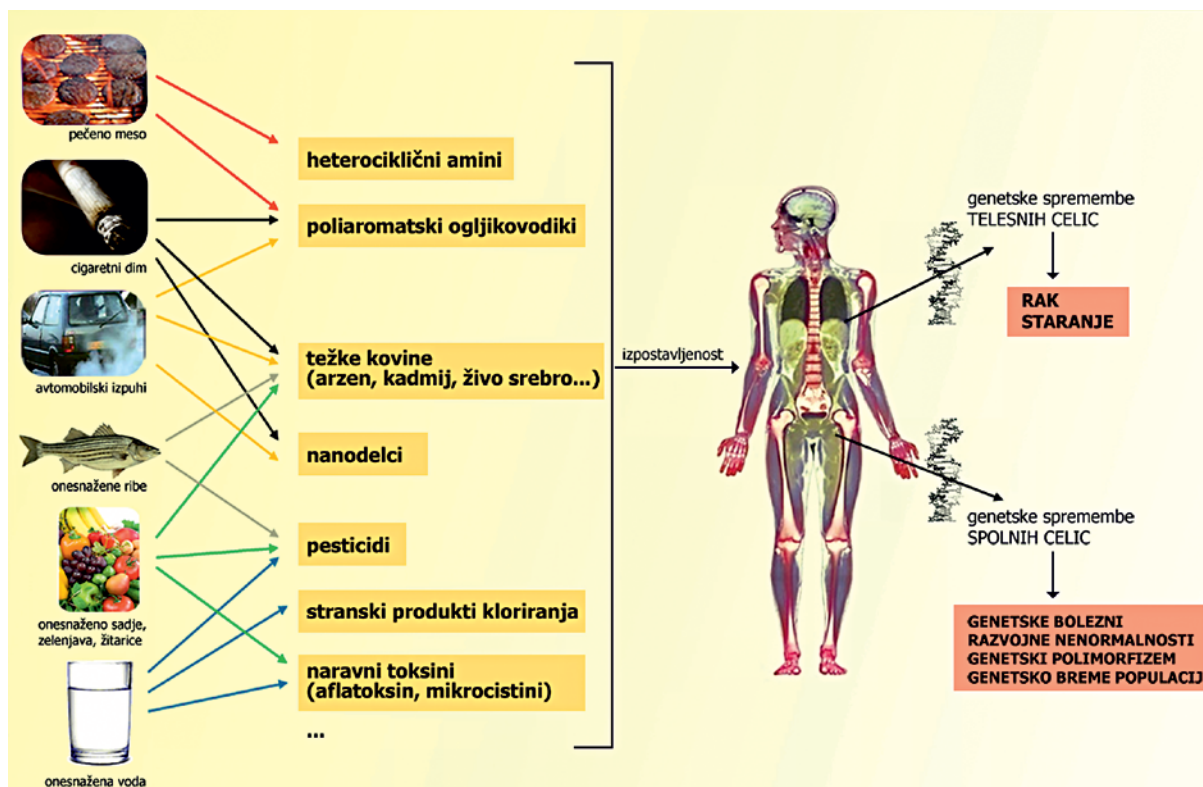


Figure 2: Major sources of human exposure to genotoxic carcinogens [diagram: Irena Hreljac]. In exposed humans, genotoxic carcinogens induce DNA damage, which, if not repaired, leads to mutations and consequently to increased probability of cancer development and genetic diseases.

DNA damage is single cell gel electrophoresis, also called Comet assay (Figure 3).

Genotoxic chemicals may react with DNA directly, but most need metabolic activation in order to damage DNA. Therefore, metabolically active experimental models that reliably reflect metabolism in humans are needed. Within the 5th European Framework project, HEPDNA, we collaborated on the development of an experimental model with metabolically active human hepatoma HepG2 cells for genotoxicity and antigenotoxicity studies. In collabo-

ration with the University of Groningen, we recently introduced a model with precision-cut liver slices. We were the first to apply the Comet assay for studying DNA damage in human and rat precision-cut liver slices, and this new method has been recently published in the journal Toxicology in Vitro. Its major advantage is that it allows study of the mechanisms of genotoxicity in whole-tissue samples, which better reflect processes in intact organs than isolated primary and permanent cells.

analysis of different parameters i.e. cell cycle analysis, apoptosis, etc.

Heavy metals are ubiquitous environmental contaminants: within Slovenia areas around Mežica and Celje are heavily polluted with cadmium. Cadmium is classified as a human carcinogen, however the mechanisms of its carcinogenicity are not fully understood. Cadmium has been considered as non-genotoxic carcinogen, but our studies showed that, at the low concentrations that are relevant for environmental exposure, it induces DNA

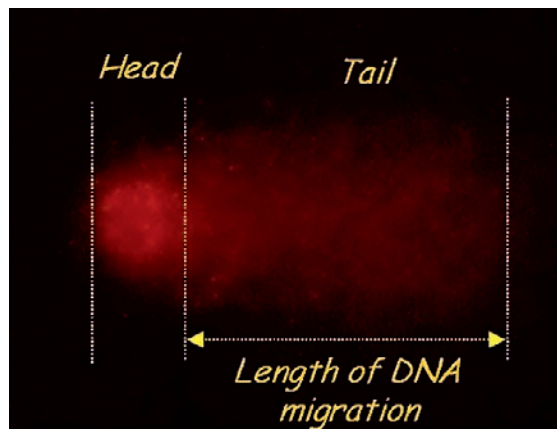


Figure 3: Comet images of the nuclei with undamaged (left) and damaged (right) DNA in liver cells released from precision-cut liver slices [photo: Janja Plazar]. Comet assay, also known as single cell gel electrophoresis (SCG), is a microgel electrophoresis technique which detects DNA damage and repair in individual cells. The damage is represented by an increase of DNA strand breaks that have, due to their negative charge, migrated out of the cell nucleus towards an anode, in the form of a characteristic streak similar to the tail of a comet. The length and DNA content of the tail is directly proportional to the amount of DNA damage.

As a response to the requirement of replacing animal tests with in vitro methods, we recently introduced in our laboratory a fish embryo test (FET test) using zebrafish (Danio rerio) (Figure 4). The method is suitable for determination of the acute lethal, sublethal and teratogenic effects of environmental pollutants and chemicals. In addition, zebrafish is genetically well characterised, which enables mechanistic studies at molecular level. To clarify the mechanisms of actions of different chemicals, we use various state-of-the-art molecular and cell-based methods such as gene expression analysis using quantitative RT-PCR and PCR-arrays, fluorescent microscopy with immunostaining, flow cytometry for analy-

sis of different parameters i.e. cell cycle analysis, apoptosis, etc. Heavy metals are ubiquitous environmental contaminants: within Slovenia areas around Mežica and Celje are heavily polluted with cadmium. Cadmium is classified as a human carcinogen, however the mechanisms of its carcinogenicity are not fully understood. Cadmium has been considered as non-genotoxic carcinogen, but our studies showed that, at the low concentrations that are relevant for environmental exposure, it induces DNA damage and mutations, and that it inhibits repair of DNA damage. To protect the health of exposed humans it is necessary to recognise early effects of exposure to cadmium, and our studies indicate that the DNA repair efficiency of lymphocytes might be a reliable biomarker of the effects of cadmium exposure.

An important group of environmental pollutants is organophosphorous pesticides (OPs), which are the most widely used insecticides. OPs are nerve toxins; their primary mechanism of toxicity is inhibition of acetylcholine esterase in the nerve synapses.



Figure 4: The laboratory aquaria with adult zebrafish (*Danio rerio*, Hamilton-Buchanan) [photo: Aleksander Gnjiđić] (left) and zebrafish embryo, 48 h after fertilization, under light microscope, 100 × magnification [photo: Tina Eleršek] (right). Use of vertebrates at early developmental stages is recognised as an alternative ecotoxicological method. Replacement of currently used whole-animal toxicity tests has the potential to save a large number of adult fish from suffering acute toxicity.

Chronic exposure to low non-acutely toxic doses of these pesticides has been linked to increased risk for certain forms of cancer. However, not much is known about their mechanisms of carcinogenesis. Using the HepG2 cells experimental model, we studied the mechanisms of carcinogenicity of three model OPs: parathion, paraoxon and dimefox. Investigation showed that parathion and paraoxon are genotoxic and may act as tumour initiators, while dimefox induces increased cell proliferation and can be considered a tumour promoter. The most important finding is that parathion, which in humans shows lower acute toxicity than paraoxon, and is therefore used in commercial pesticide preparations, has higher genotoxic activity, indicating that at chronic exposure it represents a higher risk for human health. We also found that all three OPs exert very strong synergistic genotoxic effects in combination with carcinogenic polycyclic aromatic hydrocarbon benz(a)pyrene. As humans are typically exposed to multiple contaminants, this synergistic activity can be considered a mechanism that contributes to the carcinogenic potential of OPs, and should therefore be taken into account for risk assessment.

One of the fastest developing technologies currently is nanotechnology. Professional and public exposure to nanomaterials is expected to increase dramatically in the coming years. Thus, it is necessary to know the health hazards related to this exposure. Nanoparticles (defined as particles with at least one dimension smaller than 100nm) have nanostructure-dependent properties (e.g. chemical, mechanical, electrical, optical, mag-

netic, biological), which make them suitable for numerous commercial or medical applications. However, these same properties may potentially lead to nanostructure-dependent biological activity with adverse effects on humans and the environment, and which are currently relatively unknown. This is why our group recently decided to start investigating mechanisms of toxicity and genotoxicity of nanoparticles. Our current studies aim to establish appropriate in vitro experimental model for nanoparticle toxicity studies; current results indicate that the model with human hepatoma HepG2 cells is a sensitive indicator of cytotoxic and genotoxic effects of model nanoparticles.

An important part of our research is centred on finding and exploring the mechanisms of activity of potential cancer-preventive agents of natural origin. We studied the antigenotoxicity of mushroom extracts, certain medicinal plant constituents and hops extracts. Our most important finding is that xanthohumol, the principal prenylated flavonoid present in the hop plant, *Humulus lupulus* L., inhibited genotoxic effects of indirect acting carcinogens and oxidants at very low, nanomolar concentrations (Figure 5). These effects were observed in HepG2 cells and also in precision-cut liver slices. In collaboration with the Medical University Vienna, we also found that xanthohumol prevented formation of preneoplastic lesions in rat liver induced by heterocyclic amine (a carcinogen present in cooked meat and fish). These results provide additional evidence for the strong cancer-preventive potential of xanthohumol, which warrants further investigations and

Figure 5: The hop plant (*Humulus lupulus* L.); the source of the prenylated flavonoid, xanthohumol, with extremely promising cancer-preventive properties.



development of xanthohumol-based food or pharmaceutical supplements for chemoprevention of cancer.

Within ecotoxicology, our major area of research is cyanobacteria (blue green algae). Cyanobacteria capable of bloom formation produce different toxins including cyclic peptides, some of which i.e. microcystins, are highly toxic for humans and animals (Figure 6). Microcystins (MCs) are cyclic heptapeptides that are considered to be liver-specific toxins. They are suspected carcinogens; in animals they have been shown to be potent tumour promoters acting via inhibition of alkaline phosphatases. We were the first to show that microcystins are also genotoxic, and that the underlying mechanism of their genotoxicity is induction or reactive oxygen species formation. In collaboration with colleagues from the Medical Faculty, Institute for Pathophysiology, we showed that at chronic exposure to nonlethal doses, microcystins induce DNA damage, not only in liver tissue but also in other tissue, including brain tissue. These results indicate that exposure to microcystins represents a serious health threat, particularly if they are present in drinking water to which a large human population can be exposed. Our results also indicate that application of antioxidants can efficiently counteract their genotoxic effects.

With studies of the effects of cyanobacterial toxins on water ecosystems,

we have recently discovered that non-toxic peptides, which have the ability of serine protease inhibition, can be cytotoxic to different phytoplankton species in a cyanobacterial bloom. With this activity, they can have an essential influence on biodiversity in water bodies, and have an impact on cyanobacterial bloom decay.

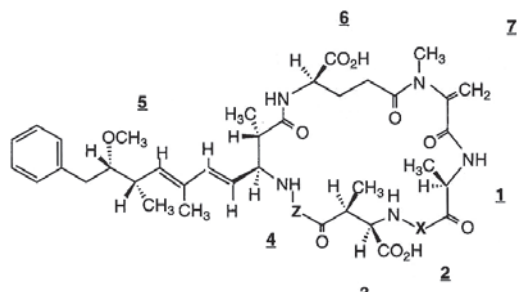


Figure 6: Cyanobacterial blooms are becoming a serious problem worldwide due to anthropogenic water contamination and global warming. Cyanobacterial toxins – microcystins – are hepatotoxic and carcinogenic, and represent a serious health threat to exposed human populations.

Tumour Biology

Research in the field of Tumour Biology is currently in a great part focused on gliomas, the most abundant primary brain tumours in adults, of which glioblastoma multiforme (GBM) is the deadliest. The name “multiforme” (i.e. “many forms”) derives from the vast morphological heterogeneity of these tumours. GBM express extensive neo-angiogenesis (i.e. formation of new blood vessels) and diffuse invasion of tumour cells into healthy brain tissue, making complete surgical removal impossible. In addition, due to a high cell proliferation rate and high cell death resistance, other types of treatment, such as radiotherapy and chemotherapy, are also unsuccessful. Most patients die within a year of diagnosis. Improving current therapies and developing new ones is therefore highly important. To enable that, the basic characteristics of GBM need to be thoroughly investigated.

Cysteine cathepsins are highly important enzymes in glioma progression – their specific and controlled inhibi-

tion could therefore be efficiently used in cancer therapy. In the invasion process, cysteine cathepsins can act at the beginning of a so-called “proteolytic cascade”, enabling GBM cells to invade the surrounding brain tissue by degrading the extracellular matrix. Using the spheroid invasion model (Figure 7), we have shown the activity of at least two cysteine cathepsins, B and L, to be highly elevated in the cells invading the surrounding matrix. We found that specific inhibition of cathepsin B decreased the invasion of GBM cells, confirming its role in the invasion process. This result is consistent with other studies showing a correlation between high cathepsin B expression and tumour invasiveness.

On the other hand, inhibition of cathepsin L by chemical inhibitors, antisense RNA or siRNA (Figure 8) showed no effect on invasion. Increased cathepsin L thus confers some other advantage to the invading GBM cells. Resistance of tumour cells to cell death is one of the main reasons for unsuccessful treatment of GBM. We have found increased activity of cathepsin L to increase cell death resistance in GBM cells, while its decrease has an opposite effect. Thus, cathepsin L acts in an anti-apoptotic manner – preventing GBM cells from going to apoptosis-programmed cell death. Its inhibition would therefore sensitise cells for cell death induction and would increase the efficiency of chemotherapy. Although the mechanisms of cathepsin L action are not yet entirely clear, current results suggest cathepsin L inhibitors as possible candidates for adjuvant therapy. These studies are part of the 6th Framework Programme integrated project, CANCERDEGRADOME.

A promising new GBM therapeutic is arsenic trioxide, As₂O₃. In 2000, the first chemotherapeutic drug containing As₂O₃ was registered by the Food and Drug Administration for treatment of acute promyelocytic leukaemia.

Currently it is also in clinical studies for treatment of GBM. To predict how an individual patient might respond to As₂O₃ treatment, the mechanisms of its action must be thoroughly investigated. It was shown that arsenic can induce apoptosis and autophagy, another type of cell death. We are studying these two types of cell death, and especially the possibility of sensitising cells to arsenic by modulating the expression of cysteine cathepsins, which have been shown to have an important role in cell death.

Apart from tumour cells, other cell types, such as immune and endothelial cells, commonly named stromal cells, are present in all tumours. Promising new therapies are aimed at the stroma because of its important contribution to cancer development and progression. Stromal cells are also genetically more stable and therefore less likely to become resistant to therapy. In our group, we are investigating the communication of GBM cells with endothelial cells and macrophages, two of the most important stromal cell types present in GBM. By growing cells in co-cultures we are able to follow changes in processes such as invasion, proliferation, and resistance to apoptosis of both tumour and stromal cells. Again, we are especially interested in the role of cysteine cathepsins.

Applied research

Management of water resources in Slovenia and worldwide is becoming more and more demanding. Changes in climate have adverse effects on the quality and quantity of water resources. We are conducting a number of applied projects oriented towards the biological properties of surface waters (using indicators such as zooplankton, phytoplankton, macrophyte populations, etc.), to support recognition of the general quality conditions of water bodies in Slovenia,



Figure 7: Glioblastoma multiforme cell line U87 spheroid invasion into collagen gel – day 0, 1, 3 and 7 (left to right). To study GBM invasion, we facilitate a “classical” one-dimensional Transwell invasion assay and recently developed 3D-spheroid invasion assay. While the first is easier and quicker to perform, and is therefore more suitable for routine work, the more complex spheroid invasion model reflects the real tumours more closely and also enables long-term studies of the invasion process.

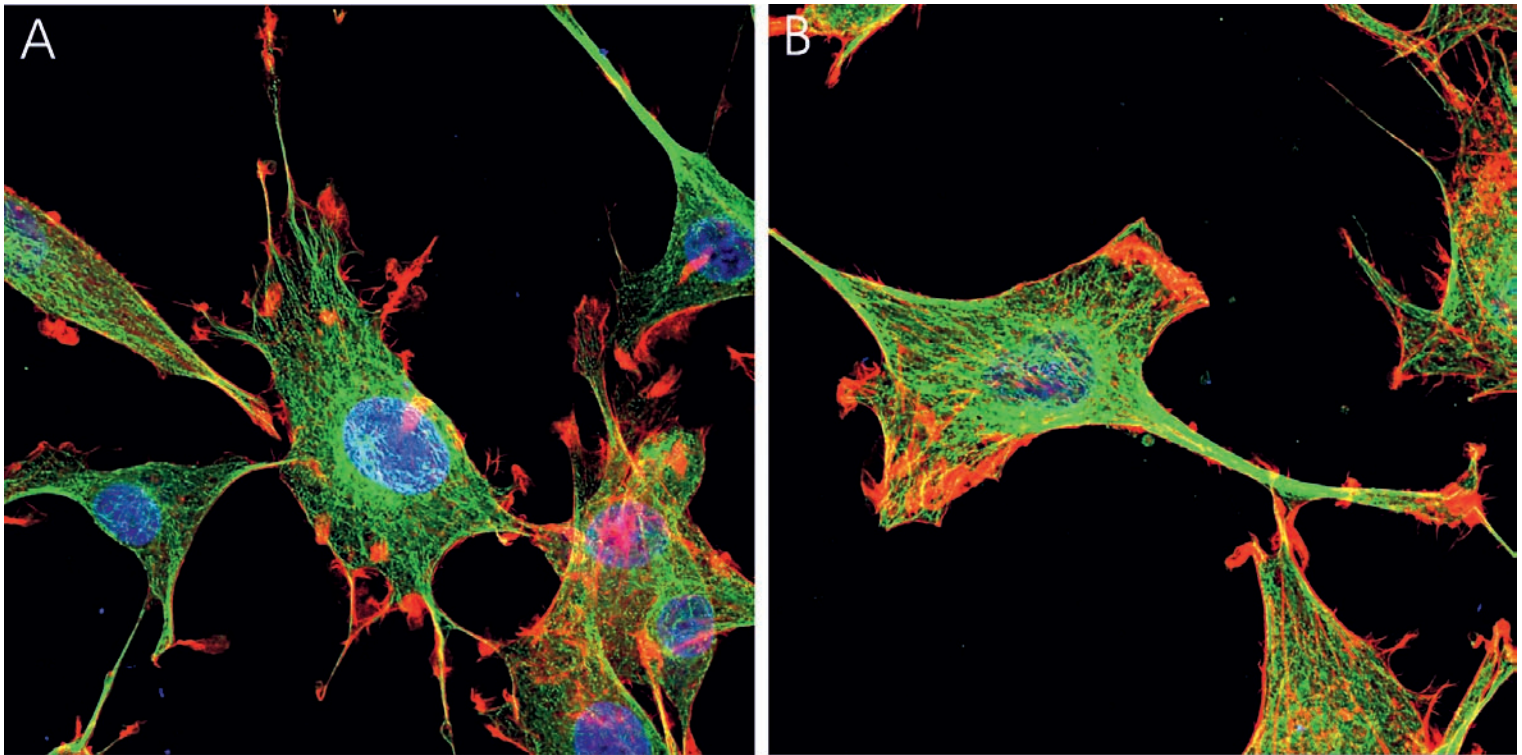


Figure 9: The effect of cyclic peptide planktopeptin BL 1125 from cyanobacteria *Planktotrix rubescens* on the cytoskeleton of glioblastoma cells U87. Confocal images show the organization of actin filaments (in red) and microtubules (in green) in U87 cells exposed to planktopeptin BL 1125. (a) Control U87 cells with normal distribution of actin filaments and microtubules. (b) Reorganization of cytoskeleton – collapse of actin filaments after 24 h exposure to 10 μM planktopeptin BL1125. (Photos: Prof. Robert Frangež)

identification of sources of water pollution and classification of surface waters into quality classes. We are conducting numerous contracted projects. For the Environmental Agency of the Republic of Slovenia we are undertaking “Monitoring the quality of lake waters” and “Monitoring the quality of surface waters”. We have developed non-invasive methods of tracing, with which we can trace directions of the pollution of water resources and mixing of surface and ground waters.

The presence of genotoxic contaminants in surface, ground and drinking water is of special concern. In our laboratories, we are conducting several genotoxicity tests according to the standard methods. A fish embryo test has been introduced for the purposes of monitoring of wastewater toxicity prior to release into the environment. We are partners in the Centre of Excellence: Environmental Technologies, where our main role is developing new biological methods for detection of toxins and monitoring the effectiveness of wastewater treatment plants.

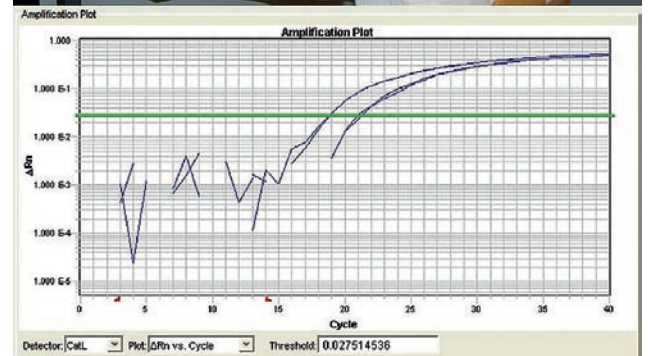


Figure 9: Efficient inhibition (silencing) of cathepsin L is confirmed by quantitative real-time PCR – the delayed signal (the right curve) means lower expression of the gene.

To study the role of cathepsin L, its expression was inhibited using siRNA. These are short RNA fragments complementary to the target mRNA (cathepsin L mRNA in our case), which specifically bind to target mRNA. The double-stranded RNA so formed is then degraded. Gene expressions (of cathepsin L and other genes) are analyzed by quantitative real-time PCR.

Taken together, our research contributes to basic knowledge of the processes and mechanisms involved in human carcinogenesis, which are important for the identification of new targets for cancer prevention and treatment, as well as for reliable risk assessments on exposure to potential carcinogens. Our research is also in line with the objectives of the recently adopted European chemical safety legislation, REACH (Registration, Evaluation and Authorisation of Chemicals; Official Journal No L 396, 30/12/2006), which calls for the testing of all chemicals produced in amounts greater than 10 tonnes, and requires reductions in animal use in toxicity testing. We provide consultation, as well as toxicity and genotoxicity testing, for industry and other stakeholders who are required to provide toxicological data for the chemicals they are producing or using. With the development of new in vitro toxicity testing approaches, we are also contributing to reduction of the use of animals in toxicity testing.