UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

DEVELOPMENT OF WHOLE CELL BIOSENSOR SYSTEMS FOR DETECTION OF GENETIC DAMAGE

DISSERTATION

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ABBREVIATIONS AND SYMBOLS

2-(AAF)	Acetylaminofluoren
AFB_1	Aflatoxin B1
ALS	Alkali labile sites
AMP	Adenosine monophosphate
ATM	Ataxia telangiectasia mutated protein
ATR	Ataxia telangiectasia and Rad3-related protein
β-GAL	β-galactosidase
BaP	Benzo[a]pyrene
BPDE	Benzo(a)pyrene Diolepoxide
B_2O_3	Boric acid
CAT	Chloramphenicol acetyl transferase
$CdCl_2$	Cadmium chloride
C_2H_5OH	Ethanol
CDKN1A	Cyclin-dependent kinase 1A
CisPt	Cisplatin
CO_2	Carbon dioxide
CMV	Cytomegalovirus
DAPI	4',6-diamidino-2-phenylindole
dEGFP	Destabilized EGFP
DIN	German Institute for Standardization
DMSO	Dimethyl sulphoxide
DMF	N, N-dimethylformamide
DNA	Deoxyribonucleic acid
DsRed	Red fluorescent protein
DSBs	Double-strand breaks
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbant assay
FCS	Foetal calf serum
FRET	Fluorescence resonance energy transfer

GFP	Green fluorescent protein
GMP	Guanosine monophosphate
GST	Glutathione-S transferase
HepG2	Human hepatoma cells
HPRT	Hypoxanthine phosphoribosyltransferase
HTTS	High throughput test system
IARC	International Agency for Research on Cancer
IPTG	Isopropyl-β-D-thiogalactoside
IQ	2-Amino-3-methylimidazo[4,5-f] quinoline
$K_2Cr_2O_7$	Potassium dichromate
LOEC	Lowest Observed Effect Concentration
LOEL	Lowest Observed Effect level
MEM	Minimum essential medium
MLA	Mouse lymphoma assay
MODC	Mouse ornithine decarboxylase
mRNA	Messenger Ribonucleic acid
MMS	Methyl methane sulphonate
MTS	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NaCl	Sodium chloride
NER	Nucleotide excision repair
OECD	Organisation for Economic Co-operation and Development
PCNA	Proliferating cell nuclear antigen
PRPP	5-phosphoribosyl-1-pyrophosphate
REACH	Registration, evaluation and authorization of chemicals
SEAP	Secreted Placental Alkaline Phosphatase
SSBs	Single-strand breaks
ssDNA	Pure single-stranded (ss) DNA
SCGE	Single cell gel electrophoresis
VLB	Vinblastine
UDS	Unscheduled DNA synthesis

1 INTRODUCTION

Pollution of natural environments is a common and serious problem in our society. Many new industrial compounds are beening synthesized for commercial and industrial purpose, which generates health and environmental concerns. Therefore the potential harmful effects on human and environmental health should be identified for the safe use of these chemicals. The new European Community Regulation on chemicals and their safe use REACH (EC 1907/2006) deals with the registration, evaluation, authorization, and restriction of chemical substances (Foth and Hayes, 2008). The new law entered into force on 1 June 2007 and aims to improve the protection of human health and the environment through better and earlier identification of the intrinsic toxicological properties of chemical substances. Toxicological properties play a central role in (1) evaluating health hazards associated with the exposure of humans to these products, (2) making crucial decisions on whether or not to invest valuable resources in developing a new lead molecule, and (3) to maintain a product that is already on the market by providing data from up-to-date state of the art study designs.

Toxicological data are gaining increasing importance also in the fields of environmental pollution monitoring and protection. Although many chemical/physical methods are currently being employed for the monitoring of environmental pollution, the effects of environmental chemicals, including the potential effects of unknown or chemically undetected substances, as well as the synergistic and antagonistic effects of chemical mixtures, cannot be adequately estimated solely on the basis of the analyte concentrations. These characteristics can be assessed, however, through the use of bioassays. At present, biomonitoring is an essential tool for complete implementation of the European Union (EU) Directives (e.g. Water Framework Directive and the Marine Strategy Framework Directive).

Genotoxic chemicals are of particular concern because they induce damage to genetic material that can lead to mutations. Mutations are in exposed humans associated with increased risk for cancer development, degenerative diseases, and can contribute to genetic load and heritable disease. In the environment genotoxic

pollutants may lead to changes in natural communities and affect biodiversity. For protection of human health as well as general protection of the environment it is important to identify such agents. Regulatory requirements for genotoxicity testing of chemicals and products such as pharmaceuticals, pesticides, food additives, and cosmetics rely on a battery of genotoxicity tests, which generally consist of an *in vitro* test for gene mutations in bacteria and mammalian cells, an *in vitro* test for chromosomal damage and an *in vivo* test for chromosomal damage in rodent hematopoetic cells. However these same methods are unsatisfactory for rapid screening for several reasons: testing can take many weeks, when it is desirable to obtain genotoxic data in a shorter time frame, and large quantities of a tested compound are needed, when only limited quantities are available, such as during drug development or in environmental monitoring when concentrated samples are tested. Therefore, simple, fast and relaible genotoxicty tests are needed.

1.1 Conventional in vitro genotoxicity tests

Genotoxicity is the property of an agent to interact with DNA and other cellular targets that control the integrity of the genetic material. These interactions include induction of DNA adducts, strand breaks, point mutations, and structural and numerical chromosomal changes. Tests for genotoxicity are considered short-term in nature and are an integral part of product safety assessment.

Genotoxicity tests can be defined as *in vitro* or *in vivo* tests designed to detect compounds that induce genetic damage directly or indirectly by various mechanisms. These tests should enable hazard identification with respect to damage to DNA and its fixation. Fixation of damage to DNA in the form of gene mutations, larger scale chromosomal damage, recombination, and numerical chromosome changes are generally considered as essential for heritable effects (Dearfield, 1995). Compounds or environmental samples which are positive in tests that detect such kinds of damage have the potential to be rodent and/or human carcinogens and/or mutagens (Dearfield, 1995; Monro, 1996; Battershill and Fielder, 1998; Goodman and Wilson, 1999, Kirkland *et al*., 2005, 2006).

Based on the endpoint that different genotoxicity assays detects, we can divide them into:

a) Assays that detect mutations,
b) Assays that detect chromosomal aberrations,
c) Assays that detect DNA damage and cellular response to DNA damage.

While the first two groups of assay reflect the final outcome, the latter group assays are used as markers for genotoxic effects and are of great importance also for mechanistic studies.

1.1.1 Assays that detect induction of mutations

The Ames assay (Ames *et al.,* 1973), originally developed by Ames in 1973 is the most widely used genotoxicity assay. It uses mutant *Salmonella typhimurium* strains that have lost their ability to grow in the absence of histidine. Reverse mutations caused by exposure to mutagenic compounds can reactivate their ability to synthesise histidine and thus can grow in the absence of histidine. For the evaluation of chemicals according to OECD and EC standards five tester strains are needed. Although the results are obtained in a relatively short time the assay is still time consuming and inappropriate for screening large series of compounds or samples. An adapted version of the Ames test is used for waste water evaluation (DIN, 1999), and a commercial microplate version of the Ames-test based on colour changes has been developed (Hubbard *et al*., 1984). Although Ames test is highly sensitive for detection of mutagens and correlates well with potential carcinogenicity, its main disadvantage is, that as a bacterial assay it is less relevant for human and environmental risk assessment.

In vitro mammalian cell gene mutation assays with different cell lines and using different genetic loci for detection of mutations are available. However, only forward mutations in three genetic loci are extensively used for mutation detection: hypoxantine phosphoribosyltransferase (hprt), thymidine kinase (tk), and the cell

membrane Na^+/K^+ ATPase. The mammalian cell mutation assays using hprt, tk and Na^+/K^+ ATPase locuses are standardized by OECD and EC.

The hprt enzyme is a member of a family of phosphoribosyltransferases, that constitutes purine salvage or reutilization pathway that utilizes hypoxanthine and 5-phosphoribosyl-1-pyrophosphate (PRPP) to form adenosine monophosphate (AMP), and guanosine monophosphate (GMP). The hprt gene is located on the X chromosome, and cells are relatively tolerant to genetic changes in this gene, as they can use a second de novo pathway for purine synthesis. In male cells there is a hemizygous situation and, accordingly, recessive mutations can be assessed. Whether or not the enzyme is expressed provides the basis for the sensitive selection system that permits selection of mutated hprt deficient cells using the analogue 6-thioguanine (O'Neill *et al*., 2009). While non-mutated cells die in presence of the selective agent, mutated cells survive.

Human lymphoblastoid TK6 and mouse lymphoma $L5178Y^{TK+/-}$ cells detect gene mutations (point mutations) and chromosomal events (deletions, translocations, mitotic recombination/gene conversion, and aneuploidy) at the tk locus (Mitchell *et al.,* 1997, Wang *et al.,* 2007). Mutant cells, which are deficient in the enzyme tk necessary for phosphorylation of thymidine to thymidine monophosphate, are resistant to the cytotoxic effect of pyrimidine analogues, such as trifluorothymidine. The mouse lymphoma assay (MLA) detects mutations known to be important in the etiology of cancer and other human genetically mediated illnesses.

The third marker used for mutagenicity studies is the gene coding for Na^+/K^+ ATPase. In nonmutated cells, the Na^+/K^+ ATPase mediates the active transport of Na^+ and K^+ across the plasma membrane, a function that can be inhibited specifically by the cardiac glycoside ouabain, which is toxic to mammalian cells in culture. Mutation affecting Na^+/K^+ ATPase results in lower affinity for ouabain and thus to resistance to its cytotoxic effects (Corsaro and Migeon, 1977).

The MLA based on the tk gene and the V79-hprt assay effectively measure specific types of mutations but is limited in sensitivity (Moore *et al*., 1989) due to the requirement that flanking genes on the chromosome remain functional for cell

survival. If the mutation extends beyond the mutated gene location, it may then cause cell death and the mutation is not scored. This is especially true in the hprt assay because the gene is located on the X-chromosome and flanking genes may not be rescued by a homologous chromosome. Large deletions, for example, are likely to kill the cell and alter the accurate mutant yield induced by a genotoxic agent, thereby reducing the assay sensitivity (Li *et al*., 1991).

Because of these problems, a mammalian cell mutation assay was designed with human-hamster hybrid CHO_{AL} cell line containing a single copy of human chromosome 11, which encodes several cell surface proteins including the glycosylphosphatidylinositol (GPI)-anchored cell surface protein CD59 (Waldren *et al*., 1979). As a consequence of a mutation in the CD59 gene, its expression is lost which is expressed as resistance to the rabbit serum complement. The assay efficiently detects small and large deletion mutations induced by metals that are difficult to detect with other assays (Filipič and Hei, 2004). The newest versions of the CHO_{AL} test measures the CD59-mutant cell yields by quantifying the fluorescence of cells labelled with phycoerythrin-conjugated mouse monoclonal anti-CD59 antibody using flow cytometry (Zhou *et al.,* 2006).

1.1.2 Assays that detect chromosomal aberrations

Chromosome aberration include structural aberrations such as fragments or intercalations and numerical aberrations ((unequal segregation of homologous chromosomes during cell divisions, which leads to a loss or surplus of chromosomes (aneuploidy and polyploidy)). Cytogenetic effects can be studied either in whole animals or in cells *in vitro*. Generally the cells are exposed to the test substance and then afterwards treated with a metaphase-arresting substance (i.e. colcemide). Following suitable staining the metaphase cells are analysed microscopically for the presence of chromosomal aberrations. The main limitation of these assays is requirement of skilled personnel and time consuming analysis which limits the applicability of this assay in routine measurements. The *in vitro* and *in vivo* chromosomal aberration assays are standardized by OECD.

Micronucelus assay has been developed as an alternative for metaphase chromosomal aberration assay (Miller *et al*., 1998). Micronuclei are chromosome fragments or whole chromosomes that were not incorporated in the daughter cell nuclei and appear in the cytoplasm. For the measurement of micronuclei cell division must be allowed to continue up to the interphase. The assay is still time consuming but less than chromosomal aberration assay and recently an automatized version has been developed. A mammalian erythrocyte micronucleus assay with bone marrow has been standardized by OECD and EC and recently OECD adopted also the draft for *in vitro* micronucleus assay. Flow cytometric measurement of micronuclei is also possible (Bryce *et al*., 2007) but equipment costs are very high.

1.1.3 Assays that detect DNA damage

Primary DNA damage is an early indicator of the ability of an agent to interact with DNA although it does not necessarily result in mutation or chromosomal aberration. A series of mammalian test systems is currently being used to demonstrate an agent's ability to interact with cellular DNA and a variety of experimental techniques have been developed to detect DNA damage in cell populations. The most important are

measurement of DNA adducts and modified bases and measurement of DNA strand breaks.

The DNA adducts and modified bases are measured using either antibodies for detection of specific DNA adducts such as O^6-methyl- and O^6-ethylguanine, 7-methylguanine, N^6-methyladenine, bulky adducts such as N-2-acetylaminofluorene and BPDE or modified bases such as cyclobutane pirimidine dimers (Baan *et al.,* 1985) or ^{32}P-post-labelling that allows for detection of chemically not characterized adducts (Reddy, 2000).

Other types of DNA lesions are breaks occurring in the sugar phosphate backbone of DNA. DNA single-strand breaks (SSBs) are the commonest DNA breaks that arise directly from the attack of deoxyribose by reactive oxygen species in living cells (Thompson and West, 2000). Double-strand breaks (DSBs), in which both strands in the double helix are broken in close proximity, are particularly hazardous to the cell because they can lead to genome rearrangements (Morgan *et al.,* 1998). Several methods for quantitative measurements of strand breaks have been described, which assess DNA damage either under neutral or alkaline conditions. In cases of quasi-neutral conditions (pH 7.0–7.6), DNA remains in its double-stranded (ds) form, thus allowing only DSBs detection (Iliakis *et al.,* 1991). Methods based on alkaline conditions make use of the fact that dsDNA unwinds in alkaline solutions, allowing the detection of DSBs, SSBs, and alkali-labile sites. The free DNA ends of broken DNA are multiplied in cells that have been exposed to radiation or DNA-reactive agents, and are the starting points from which unwinding begins (Dusinska and Slamenova, 1992). The extent of denaturation depends on the experimental conditions. Under stringent alkaline conditions (pH > 12.6), DNA unwinding is completed within short time, resulting in pure single-stranded (ss) DNA. The reduction of molecular length reflects the amount of all induced DNA strand breaks. If damaged DNA is only partially unwound, ssDNA and dsDNA fractions can be separated by hydroxyapatite chromatography (Ahnstrom and Erixon, 1973) or differentially stained using fluorescence dyes (ethidium bromide, DAPI, bisbenzimide, or picogreen) with preferential binding to dsDNA (fluorometric analysis of DNA unwinding - FADU assay) (Baumstark-Khan *et al.,* 1992;

Baumstark-Khan *et al.,* 1999; Baumstark-Khan *et al.,* 2000; Birnboim and Jevcak, 1981; Elmendorff- Dreikorn *et al.,* 1999).

Complete DNA denaturation is required for analytical methods which determine molecular size of fragments such as alkaline elution (Kohn and Grimek-Ewig, 1973), alkaline version of the pulsed-field gel electrophoresis (Sutherland *et al.,* 1987) or sucrose density gradient centrifugation (Korba *et al.,* 1981). These methods may be performed in the alkaline as well as in the neutral version in order to detect SSBs and DSBs, respectively (Lu *et al.,* 1996).

However, none of these methods is able to give absolute number for strand breaks without calibration. Neither are any of these methods, with the exception of immune histochemical assays using antibodies, able to locate definitively the lesions in individual cells or in tissue made up from a variety of cell subpopulations which represent different target sensitivities.

Over the past few decades, single cell gel electrophoresis (SCGE), also known as comet assay has become a method of choice for assessing DNA damage (Olive, 2002; Olive, 2009; Singh *et al.,* 1988). The method is very sensitive and detects SSBs, DSBs, alkali-labile sites, DNA–DNA/DNA–protein cross-links and SSBs associated with incomplete excision repair at the level of single cells (Tice *et al.,* 2000). It is technically simple, cheap, and DNA breaks can be investigated in virtually all mammalian cell types. Nevertheless, is a laborious method, because many cells have to be monitored and the data have to be related to graded calibration curves (De Boeck *et al.,* 2000).

A modified comet assay with lesion-specific enzymes such as endonuclease (EndoIII) or T4 endonuclease V (EndoV), which are specific for oxidized pyrimidines, formamido pyrimidine glycosylase (Fpg), recognizing ring opened purines and 8-oxoguanine glycosylase (hOGG1) specific for 8-oxoguanine that introduce breaks at sites of damage has been developed to quantify these specific lesions (Collins, 2009).

1.1.4 Assays that detect cellular response to DNA damage

It is well known that organism react to DNA damage with the activation of defence mechanisms that include cell cycle arrest and activation of DNA repair, thus indicators of response to DNA damage are useful markers of genotoxic insult. The best known assays that detect response to DNA damage are bacterial tests that detect induction of a well characterized SOS-response that comprises about 20 inducible genes (Quillardet *et al.,* 1982; Oda *et al.,* 1985; Ptitsyn *et al.,* 1997; Ben-Israel *et al.,* 1998; Sutton *et al.,* 2000).

One of the key molecules in DNA damage signaling is the histone variant H2AX, which is involved in DNA repair and the maintenance of genomic stability. H2AX becomes phosphorylated on serine 139 by Ataxia telangiectasia mutated (ATM) protein or Ataxia telangiectasia and Rad3-related (ATR) protein early in the response to DNA ds breaks. It plays role in DSB repair, both in homologous recombination and nonhomologous end joining DNA repair pathways (Solier *et al.,* 2009). H2AX is thought to have a critical function in the recruitment of DNA repair factors and DNA damage-signalling proteins, while hyperphosphorylation of H2AX may be linked to chromatin fragmentation prior to apoptosis. Antibodies directed against the phosphorylated variant of H2AX are used to visualize DNA damage loci by immune-fluorescent techniques (Pilch *et al.,* 2003). Recent studies indicate that the γH2AX focus assay, based on phosphorylation of the variant histone protein H2AX could be used as a biomarker of genotoxicity, which could predict the outcome of *in vitro* mammalian cell genotoxicity assays (Watters *et al.,* 2009).

A well know assay that has been extensively used and is also standardized by OECD is unscheduled DNA synthesis (UDS) that is based on the fact that, during certain repair steps, DNA precursors are integrated in damaged DNA. Cells treated with a genotoxic agent are supplemented with labeled DNA precursors, preferably with tritiated thymidine, which is incorporated into the damaged DNA in the course of DNA repair. In nonproliferating cells, the amount of the incorporated precursor is a direct measure for the repair capacity of the cells (Madle *et al.,* 1994). The method is sensitive and detects broad variety of lesions which are removed by the nucleotide

excision repair (NER) pathway, where the repair of the damage results in the de novo synthesis of DNA stretches.

1.2 Definitions and applications of biosensors

'Biosensor' is a general term that refers to any system that detects the presence of a substrate by use of a biological component which then provides a signal that can be quantified (Gu *et al.,* 2004).

The beginning of biosensors dates back to year 1962 when the first biosensor was developed by Clark and Lyons (Clark and Lyons, 1962). A Clark oxygen electrode was combined with the enzyme glucose oxidase to monitor glucose levels. The first commercially produced biosensor Springs Instruments (Yellow Spring, OH, USA) was placed on the market in 1975. This device was applied as a fast glucose assay in blood samples from diabetics. At present, there are many proposed and already commercialized devices based on the biosensor principle including those for pathogens and toxins (Pohanka and Skladal, 2008).

In a traditional sense a biosensor is defined as: bioanalitical device incorporating a biological material or a biomimic (e.g., tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc.), intimately associated with or integrated within a device - physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric or magnetic. Tranducer convert a biochemical signal into a quantifiable electrical signal. The usual aim of a biosensor is to produce either discrete or continuous signals, which are proportional to a single analyte or a related group of analytes (Soper et al., 2006).

Application of biosensor systems has advantages and weaknesses. The main advantages of biosensors are:

- short times of analysis,
- short response time,

- high specificity
- low cost of assays,
- miniaturization and integration into portable equipment,
- real-time measurements and,
- usage as remote device (on-site measurements).

They can be considerably more time-effective and cost-effective and constitute a good tool for monitoring changes or on-line processes. Important advantage of biosensors is also that they detect only the biologically active pollutants, and the response is proportional to the level of toxicity (Ron, 2007). These new technologies have been applied in quantitative analysis of target analytes and eco toxicological measurements.

The main drawback of biosensors is that, because they depend on biological systems, they are less reproducible than chemical methods and the values obtained are usually relative and not absolute. The biological material can also be of poor stability under harsh or sub-optimal conditions, which may be a reason for the relatively slow commercialisation of many biosensor systems (Belkin, 2003).

1.3 Biosensors in toxicology and genetic toxicology

Environmental biosensors represent a significant breakthrough for the monitoring of pollutants in contaminated matrices since they have the unique ability to measure the interaction of specific compounds with biological systems through highly sensitive biorecognition processes (Keane *et al.,* 2001). They are defined as monitoring systems based on the use of biological organisms or biologically derived reactions (Figure 1). For environmental biosensors it is important to enable long storage at room temperature (Ron, 2007).

The main classes of bio-receptors applied in the environmental analysis of organic pollution are:

- DNA biosensors,
- Immunosensors,
- Enzyme biosensors and
- Whole cell biosensors.

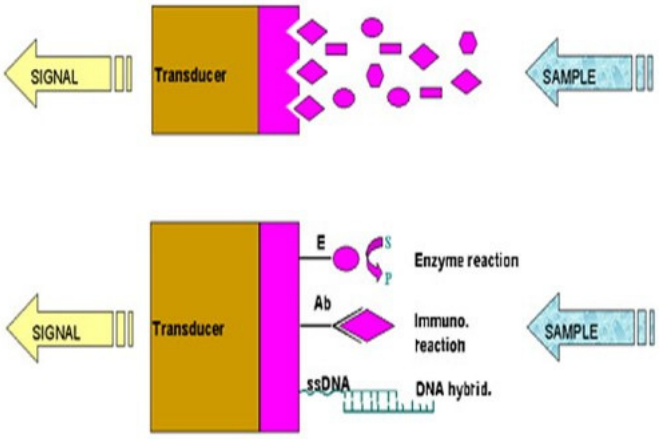


Figure 1: *Example of schematic diagram of a biosensor device. (www.iitk.ac.in/.../newhtml/storyoftheweek55.htm)*

1.3.1 DNA biosensors

The structure of DNA is very sensitive to the influence of environmental pollutants and chemicals which may cause mutagenic and carcinogenic activity. DNA biosensors can be used as a genotoxicity assay, for rapid testing of pollutants and other chemicals (Scheller *et al.,* 2001). The decoding of the human genome has significantly promoted this development but development of biosensors that exploit nucleic acid binding events (DNA sensors) has been still more limited than antibody-based analysis (Pancrazio *et al.,* 1999).

Nucleid acid-based biosensors represent a promising tool for gene sequence analysis and for mutation detection (Jayarajah and Thompson, 2002). Nucleic acid technology relies on the hybridization of known molecular DNA probes (single-stranded

oligonucleotides) or sequences with complementary strands in a test sample. The transduction principle is the optical detection of fluorescence-labeled oligonucleotides. The parallel analysis of a large number of DNA fragments can be provided by array techniques leading to Bio - or DNA chips. New development trends in DNA sensors have focused on: the mediated oxidation of guanine within the DNA, amplification of the hybridization event by an enzyme label and impedance analysis, and the electron transport through the DNA double helix (Scheller *et al.,* 2001, Zhou *et al.,* 2001).

1.3.2 Immunosensors

Immunosensors are based on immunochemical antibody-antigen (Ab–Ag) interactions. Antibodies are the traditional recognition elements for detecting analytes in the sub-nanomolar concentration region in combination with optical or electrochemical sensors to produce analytical tools for the determination of the concentration of an analyte in real time without any subsidiary reagent (Scheller *et al.,* 2001). Antibody-based biosensors that require additional reagents for each measurement fall into the category of traditional immunoassays such as enzyme linked immunosorbant assay (ELISA), colorimetric "pregnancy" test strips and so on. This type of device combines the principles of solid-phase immunoassay with physico-chemical-transduction elements (electrochemical, optical, piezzoelectric). The main limitation of these techniques is the electrochemical detection of the immunoreaction, because it is necessary to use enzymes that will generate electrochemically active compounds.

Studies for new separation- and reagent- free immunoassays and immunosensors are based on recombinant technologies which allow the site-directed incorporation of reporter molecules into a protein, leading to fluorescent-protein biosensors described by Giuliano and Taylor (Giuliano and Taylor, 1998). Fuorescent-protein biosensors can transform conformational changes during the ligand binding into a signal, for example, via fluorescence quenching or fluorescence resonance energy transfer (FRET) (Marvin *et al.,* 1997; Miyawaki *et al.,* 1997).

1.3.3 Enzyme biosensors

Enzyme biosensors use enzymes which are very appropriate recognition elements because they combine high chemical specificity and inherent biocatalytic signal amplification. For example, enzyme sensor utilized glucose oxidase attached onto the surface of an amperometric oxygen electrode and was used to directly quantify the amount of glucose in a sample (Clark and Lyons, 1962).

Enzyme-based technology relies on a natural specificity of given enzymatic protein to react biochemically with a target substrates. Many enzymes participate in cellular signalling and sometimes are targeted by compounds associated with environmental toxicity. Enzyme biosensors can be categorized into two groups:

- Enzyme biosensor which measure inhibition of a specific enzyme due to the presence of target analytes and,
- Enzime biosensor which measure catalytic transformation of target analytes by a specific enzyme.

Most transduction elements associated with enzyme-based biosensors are electrochemical (i.e. amperometric or potentiometric). The main advantage of this class of transducer is low cost, a high degree of reproducibility and disposable electrodes (which are often available). The instrumentation is also very easy to obtain and can be inexpensive and compact; this allows for the possibility of on-site measurements. Limitations for amperometric measurements include potential interferences with the response if electroactive compounds are present in the sample (Farre *et al.,* 2009). The new trends of enzyme biosensors also gained from novel enzymes and engineered proteins. Sode *et al.* investigated in their study fructosyl amine oxidase which has been introduced for biosensing glycated hemoglobin. The enzyme reacts with fructosyl valine liberated by the proteolytic digestion of the glycated protein (Sode *et al.,* 2000).

1.3.4 Whole cell biosensors

Because of their characteristics, most whole cell biosensors have been implemented in the area of environmental toxicity analysis and monitoring including studies on water, air, and soil quality. Whole cell biosensors are measurement systems combining analytical devices and whole cells that produce biological signals as the recognition element. Whole cell biosensor systems, developed for environmental monitoring of pollutants are mostly using microorganisms. Pollutants can activate microorganism pathways involved in metabolism or non-specific cell stress, resulting in the expression of one or more genes (Belkin *et al.,* 1997). They can respond to various ranges of changes in their environment or conditions and are suitable for use in eco-toxicity tests and environmental monitoring where the nature of toxicants and pollutants cannot be predicted.

Whole cell biosensors utilizing microorganisms address and overcome many of the concerns, raised with other conventional methods, because they are usually cheap and easy to maintain while offering a sensitive response to the toxicity of a sample (Gu *et al.,* 2004). Bacterial whole cell biosensors produce measurable gene products encoded by reporter genes, which are either present naturally in the bacterial strain or introduced by genetic manipulation (Sørensen *et al.,* 2006). To this group of biosensors belong also already mentioned bacterial genotoxicity assays (see 1.1.4).

Genetically modified yeast cells and human cell lines are being used for estrogen activity measurements by transcription activation of reporters' genes (Bovee *et al.,* 2003). Although human cell lines are more sensitive than yeast and may be able to identify estrogenic compounds that require human metabolism, for activation into their estrogenic state (Legler *et al.,* 1999 and Hoogenboom *et al.,* 2001), yeast-based assays have several advantages. These include robustness, low costs, lack of known endogenous receptors and the use of media that devoid of steroids.

The use of mammalian cells have some important advantages over other eukaryotic systems such as yeast, insect or fish cells or over prokaryotic systems based on Escherichia coli or Salmonella species. The use of a mammalian cell system is

preferable over yeast cells because yeast cells have more rigid cell wall than mammalian cells and the fact that chemical interactions via transcription factors can be monitored only in mammalian cells unless the relevant transcription factors are transfected into yeast cells. Eukaryotic systems compared to prokaryotic ones have the advantage of an appropriate membrane environment, post-translational processing of proteins and easier extrapolation to the humans. A prokaryotic bioassay would offer the advantage of faster growth, which means faster results (Hellweg *et al.,* 2000).

Biosensors incorporating mammalian cells have also a distinct advantage of responding in a manner that can offer insight into the physiological effect of an analyte (Pancrazio *et al.,* 1999). O'Connell mentioned that integrating live mammalian cells with specialized miniature biosensors hold enormous promise for rapidly assessing cell based responses (O'Connell *et al.,* 2007). It might be said that any alteration of a microorganism-based biosensor response is important and that insufficient selectivity actually offers advantage in providing generic detection. But in this case it could be better to use whole cell-based biosensors derived from the biological system of interest – mammalian cells.

1.4 Whole cell biosensors based on reporter gene technologies

The whole cell biosensors based on the reporter genes represent highly flexible technology, which is the subject of worldwide granted patents. The ground of this technology is to place reporter genes under the control of promoters of constitutively or inducible expressed genes.

1.4.1 Constitutive gene expression system

Constitutive expression typically uses a promoter that is highly expressed under normal conditions, leading to a high basal level expression of the reporter gene fused to promoter. Under harmful or toxic conditions this basal level is reduced and the reduction correlates with the toxicity of the sample (Gu *et al.,* 2004). For constitutive expression it is typical that it gives information about total toxicity but no information about specific cells toxicity (Figure 2A). One of the great advantages of constitutive gene expression system is that they can be used to measure mixed toxicants. They can detect unpredictable additive effects between chemicals in complex mixtures and in environmental samples.

1.4.2 Inducible gene expression system

Low-level of basal expression is typical for inducible expression systems. This one is increased in presence of stress-inducible activator protein and the response is measured as an increase in the expression of reporter gene fused to promoter. A higher expression level indicates the presence of the inducer, which indicates that the cells or culture are experiencing stressful conditions or are responding to the presence of a chemical inducer (Gu *et al.,* 2004).

Reporter genes are used to monitor the changes in gene expression in living cells since they produce a measurable phenotype that can be easily distinguished over a background of endogenous proteins (Alam and Cook, 1990) (Figure 2B and 2C).

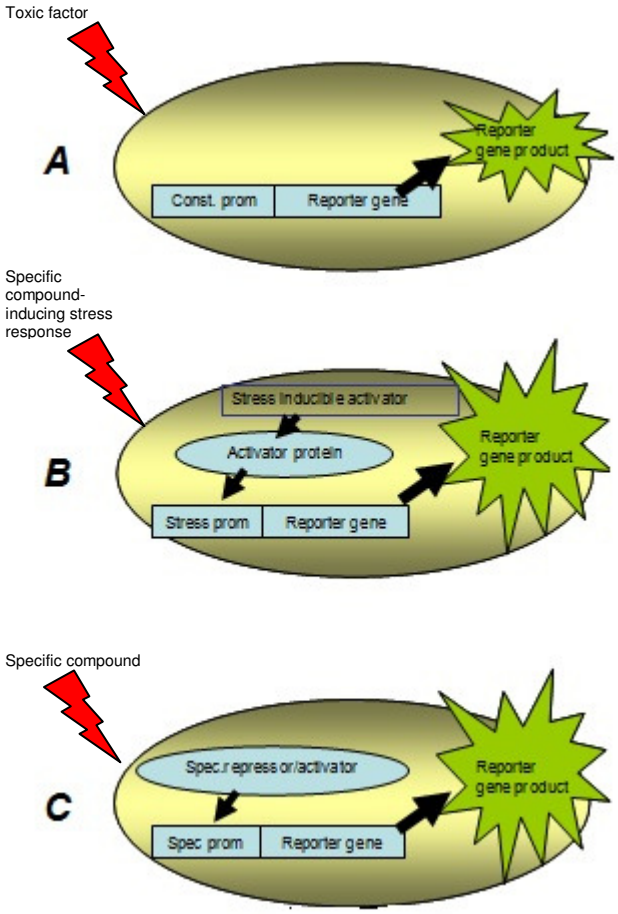


Figure 2: *Different approaches in development of cell biosensor system.*
If the reporter gene is placed downstream of a constitutively expressed promoter, the biosensor reports a decrease in metabolic activity through a decline in the intensity of the signal produced (A). In some biosensors, the reporter gene is fused to a stress-responsive promoter, resulting in reporter gene expression when the biosensor is exposed to conditions triggering a stress response; for example, DNA damage (SOS response) or protein damage (heat shock response) (B). The specific biosensors (C) respond to the presence of a certain compound or condition (Sørensen *et al.,* 2006).

1.4.3 Reporter proteins

Quantification of the reporter protein yield indirectly provides information on the activity of the sample under investigation. Quantification can take place by detecting the corresponding mRNA, the reporter protein, or by measuring the enzyme activity of the reporter protein. When the reporter system is selected, care must be taken to ensure that the reporter gene is not already endogenously expressed in the examined cells and that the gene does not influence the physiology of the transfected cells. Frequently used reporter genes include the *E. coli* enzyme chloramphenicol acetyl transferase (CAT), and β-galactosidase (β-GAL), and secreted placental alkaline phosphatase (SEAP) (Arnone *et al.,* 2004, Schlaeger *et al.,* 2003). CAT can be quantitated by an ELISA assay or by radioactive acetyl group releases, β-GAL activity can be quantified by enzymatic conversion of a substrate such as X-gal, or by an ELISA assay. SEAP has the advantage over β-GAL and CAT in being a protein that is secreted from the cells into the culture medium, allowing monitoring over time without the need for cell lysis. SEAP detection offers substantial benefits over classical assays through the use of the chemiluminescent 1, 2-dioxetane substrates.

The firefly luciferase gene has been used because of its high sensitivity compared with that of colorimetrical reporter enzymes. The recently described Gaussia luciferase (Gluc) has several advantages over previous luciferases as it possesses a natural secretory signal of 16 amino acids that drives its secretion into cell medium (Michelini *et al.,* 2008), thus allowing luminescence measurements without cell lysis. Furthermore, its codon-humanized version produces a 100-fold higher luminescent signal intensity compared to firefly luciferase (Tannous *et al.,* 2005). Fluorescent proteins, such as enhanced green fluorescent protein (EGFP) and its multiple colored variants as well as coral reef fluorescent proteins (Baumstark-Khan *et al.,* 2002; Hellweg *et al.,* 2001) are the reporters of choice, especially for high-throughput screening.

1.4.3.1 Green fluorescent protein

The green fluorescent protein (GFP) has revolutionized many areas of cell biology and biotechnology. GFP is involved in the bioluminiscence of cnidarians, and was cloned from the jellyfish *Aequorea victoria* (Prashel *et al.,* 1992), where it is responsible for the emission of green light along the margin of the jellyfish's bell.

GFP is a 238-amino acid polypeptide that is unique among light-emitting proteins in that it does not require the presence of any cofactors or substrates for emitting fluorescence. The crystal structure of the molecule consists of a light-emitting chromophore located in the centre of a barrel-like basket. This structure provides the proper environment for chromophore to fluoresce by excluding solvent and oxygen (Arun *et al.,* 2005). GFP has been fused to a variety of proteins in both eukaryotic and bacterial systems. The localization and spatial dynamics of such fusion proteins can be monitored non-invasively (the exposure of the GFP to UV light generates visible fluorescence) by fluorescence microscopy in living cells (Jakobs *et al.,* 2000). GFP has a large excitation maximum at 395 nm (near ultraviolet light), a second-smaller excitation peak at 457 nm (blue light) and an emissin maximum at 509 nm (Chalfie *et al.,* 1994; Misteli and Spector, 1997; Jakobs *et al.,* 2000).


A

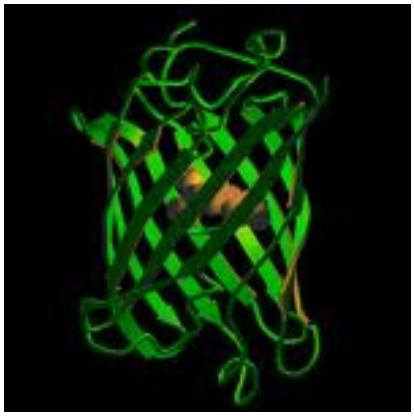

B

Figure 3: *The bioluminiscent jellyfish Aequorea victoria, which emits a green fluorescent light (A) and the crystal structure of GFP (B). (brainwindows.wordpress.com/category/gfp/www.nigms.nih.gov/News/Results/nobel_chemistr... <2.7.10>)*

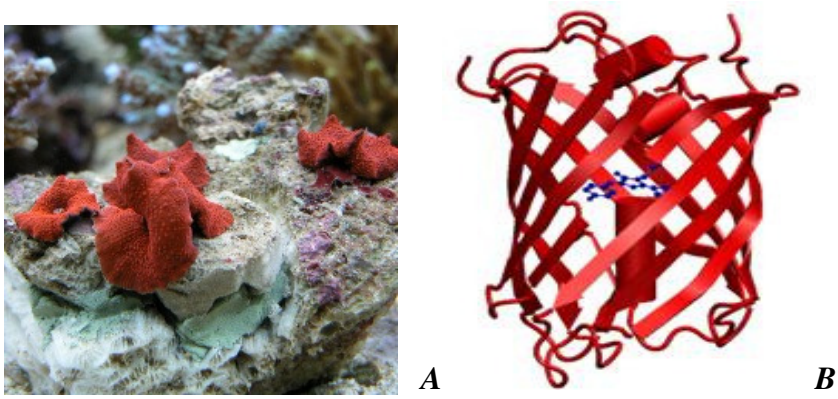
GFP is very robust and has no cell-based energy requirement. Once formed, GFP persists for hours, and its fluorescence can be detected even after cell death (Keane *et al.,* 2001).

New versions of GFP have been developed such as enhanced green fluorescent protein (EGFP), which has increased synthesis of the product in human cells. This EGFP carries a mutation in its chromophore which shifts the excitation peak to 488 nm and enhances its fluorescence intensity (Zhang *et al.,* 1996). EGFP-based systems are very useful for looking at induction of a promoter in question by a variety of stimuli over time. It also allows the examination of the same cells for repeated times (Hellweg *et al.,* 2001). Destabilized EGFP (dEGFP) is a modified form of EGFP that features a fast rate of turnover in mammalian cells. Arun *et al.* (2005) created in their study destabilized variants of dEGFP and fused EGFP to amino acid residues 422-461 of mouse ornithine decarboxylase (MODC). They noticed that the fluorescence intensity and spectral properties dEGFP were the same to the original EGFP chromophore, but with the shortened half-life (1-4 hours). Consequently dEGFP variants can be used to precisely measure the kinetics of promoter activity or the temporary expression of a protein to which it is fused (Arun *et al.,* 2005).

Important disadvantages of GFP reporter protein-based systems are autofluorescence of cells, media constituents and components of cell culture dishes. Fluorescence intensities resulting from these factors are not posing a problem when using cells expressing EGFP from a strong promoter. On the hand, weak EGFP expression from inducible promoters and for low transfection efficiencies the background from cells, plates and media components may be limiting (Amsterdam *et al.,* 1996; Hellweg *et al.,* 2001). For an optimal signal-to-noise ratio, it is necessarily that the fluorescence of the confounding factors at the excitation wavelength of reporter protein is minimal.

1.4.3.2 Red fluorescent protein

To overcome problems associated with autofluorescence when using EGFP, search for the other fluorescence protein was done. One class of the proteins is red fluorescent proteins (DsRed). They have been isolated from the Indo Pacific reef corals. Protein, named drFP583 or DsRed as it is known commercially, was first cloned from a red reef coral Discosoma species and has excitation at 558 and emission maximum at 583 (Baird *et al.,* 2000; Jakobs *et al.,* 2000). It was recognized that it has the longest excitation end emission maximal for a wild-type fluorescent protein. DsRed is a homomeric tetramer *in vitro*, as well as in living cells and each monomer has a form of beta barrel in which the pigment is located at the center of barrel. These red fluorescent proteins form chromophores internally and share structural similarities with GFP (Baird *et al.,* 2000; Verkhuska and Lukyanov, 2004; Yarbrough *et al.,* 2001).



A ***B***

Figure 4: *Red Discosoma Coral (A) and chemical structure of chromophore of DsRed (B).*
brainwindows.wordpress.com/.../web.aibn.uq.edu.au/cbn/research_biomolecular.htm

Therefore, a combination of EGFP and DsRed has been shown to be promising for double labeling studies with negligible cross-talk. For expressing either EGFP and DsRed imaged by one-photon confocal and by two-photon microscopy, Jakobs *et al.* (2000) used in their study genetically engineered bacterium *E. coli.* They expressed both fluorescent proteins separately in *E. coli,* induced by addition of 1mM isopropyl-β-D-thiogalactoside (IPTG). Clear EGFP fluorescence has been detected 4 h after induction. On the other hand, DsRed fluorescence required aproximatelly 20 h

after induction for efficient microscopic visualization. They noticed that delay was not due to inefficient expression of the DsRed protein, since the protein was detectable in high quantities already 2 h after induction. Also, measurements of the overall fluorescence begin approximately 10 h after induction. The difference between the appearance of DsRed protein and DsRed fluorescence indicates on extended maturation time of the protein. They concluded also, that DsRed expressing bacterial cells were markedly smaller than EGFP expressing *E.coli* cells due to the aggregation of DsRed within the cytoplasm over time (Jakobs *et al.,* 2000).

Like green fluorescent proteins, red fluorescent proteins have been mainly employed for cellular applications as the expression tracer and gene transfer. DsRed has generally some advantages compared to EGFP for use as a single color fluorescent marker, since it provides a higher signal-to-noise ratio and it is relatively resistant to photobleaching (Baird *et al.*, 2000). It has also been described by Bowen and Woodbury (Bowen and Woodbury, 2003) that DsRed has an increased photostability, in comparison with the commonly used EGFP and one of the reasons of higher stability is probably tetramerization of DsRed under mildy and alkaline conditions (Lauf *et al.*, 2001, Verkhuska *et al.*, 2003). With those characteristics DsRed has opened new fields for multicolor labeling and FRET applications (Mizuno *et al.*, 2001).

1.4.4 Currently used reporter systems in genetic toxicology

The most widely used are bacterial systems in which genotoxic effects are identified based on the changes in expression of SOS response genes. Vollmer et al. (Vollmer *et al.,* 1997) described a sensor system in which DNA damage-inducible promoters recA, uvrA, alkA from *E. coli* were fused to luxABCDE of *Vibrio fischeri*. Also based on monitoring SOS activation is the VITOTOX® test, where *E. coli* recN promoter was fused to the lux operon of *V. fischeri* and introduced into *S. typhimurium* (Van der Lelie *et al.,* 1997). Another assay is SOS chromotest that is based on the induction of the SOS gene sfiA, monitored by means of a lacZ fusion in *E. coli* (Quillardet *et al.,* 1982) and SOS/umu test is based on induction of umuC

fused to lacZ reporter in *S. typhimurium* (Oda *et al.,* 1985). Recently yeast *Saccharomyces cerevisiae* DNA reporter assays in which the *RAD54* promoter is fused to green fluorescent protein (GFP) (Walmsley *et al.,* 2003) and *RAD51* promoter fused to *Renilla* luciferase (Liu *et al.,* 2008) have been developed.

Recently, attempts are being made to develop and validate the induction of stress pathways/proteins as end-points in genotoxicity assays to be used for high throughput screening approaches. The choice of the pathways was mostly based on microarray experiments with genotoxic chemicals. In mammalian cells the most prominent pathway of cellular response to DNA damage is activation of the tumor suppressor and transcription factor p53 through phosphorylation by DNA damage-responsive kinases (Zhou and Elledge, 2000). Activated p53 then induces the expression of genes involved in DNA repair, cell cycle arrest, or apoptosis (Sionov and Haupt, 1999).

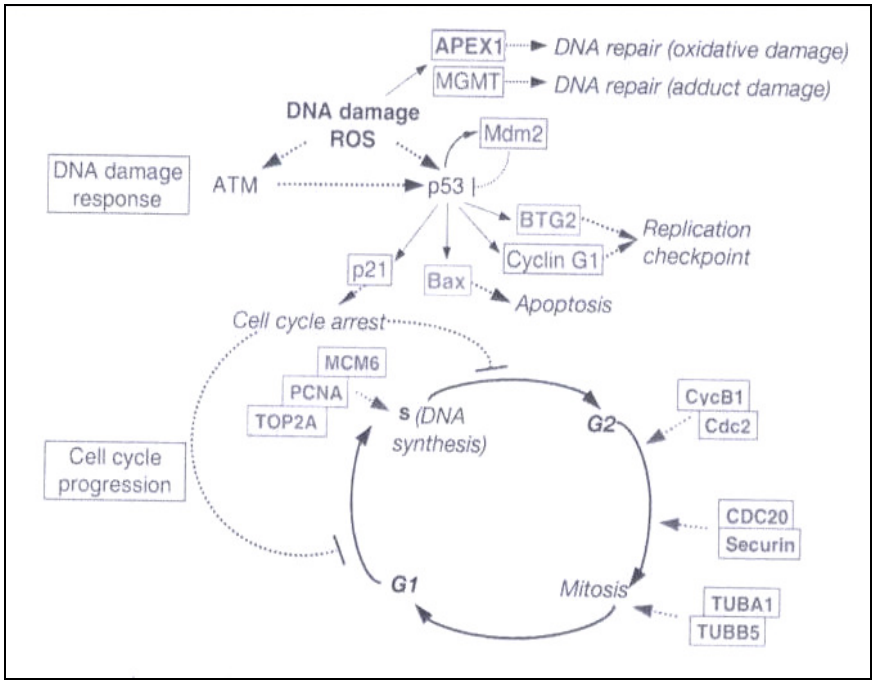


Figure 5: *Components of the signalling pathway of p53 (Ellinger-Ziegelbauer et al., 2005)*

Several reporter genotoxicity assays using mammalian cells and DNA damage responsive genes that under the control of p53 as the biomarkers of genotoxic injury has been described. That *p53* gene plays an important role in early transcriptional responses was first described in 1979, and it was the first tumour-suppresor gene to

be identified. p53 is a transcription factor essential for cell cycle arrest and apoptosis after the onset of DNA damage. p53 protein is normally in inactivated stage and is regulated mainly by various posttranslational modifications (Vogelstein *et al.,* 2000), but also transcriptionally (Wang and El-Deiry, 2006) when the cells are stressed or damaged. Such cells pose a threat to the organism: they are more likely than undamaged cells to contain mutations and exhibit abnormal cell-cycle control, and present a greater risk of becoming cancerous. Therefore p53 is also called "the guardian of the genome".

Todd *et al*, (Todd *et al.,* 1995) were the first who exploited DNA damage responsive genes: *p53R2*, *GADD45a* and *GADD153* for construction of CAT reporter that was stably integrated into HepG2 cells. However, there is very little data published from this assay. The *p53R2*, one of the p53 target genes that encode a subunit of ribonucleotide reductase, which is expressed mainly in response to DNA damage (Tanaka *et al.,* 2000; Guittet *et al.,* 2001) has been used more recently for construction of reporter assay with MCF7 and HepG2 cells using luciferase as reporter gene (Ohno *et al.,* 2005, 2008). The growth arrest and DNA damage (*GADD*)-inducible gene family is another group of target genes regulated by p53 that are expressed in response to various environmental stresses including DNA damage. In response to DNA-damage *GADD* genes induce arrest in cell cycle progression at G1/S or G2/M checkpoints (Siafakas and Richardson, 2009). Hastwell *et al.* (Hastwell *et al.,* 2006), developed an assay that exploits a reporter system in which the expression of EGFP is controlled by regulatory elements of *GADD45a* gene hosted in p53-competent human lymphoblastoid TK6 cell line. The thorough validation of this assay showed its high sensitivity and specificity (Birrell *et al.,* 2010). The assay is commercially available as GreenScreen HC assay provided by Gentronics Ltd (UK). Recently Zhang *et al.* (Zhang *et al.,* 2009) developed a stably transfected HepG2 cell line containing *GADD153* promoter region coupled to luciferase reporter gene.

1.4.5 Upregulation of tumor suppressor p21 as biomarker of genotoxic insult

The cyclin-dependent kinase 1A (CDKN1A) inhibitor *p21* (*Waf1/Cip1*) is the major downstream target gene of activated p53 and is responsible to a variety of stress stimuly, like causing cell cycle arrest following DNA damage (Waldman *et al.,* 1995). This was a major discovery in the early 1990's that revealed how cells stop dividing after being exposed to different damaging agents like chemical agents and irradiation. In addition to growth arrest, p21 can mediate cellular senescence. The activated p53 protein directly stimulates expression of *p21*, which through its negative effect on various CDKs inhibits both the G1 to S and the G2 to mitosis transition (Vogelstein *et al.,* 2000, Khan-Baumstark *et al.,* 2010). In addition, by binding to proliferating cell nuclear antigen (PCNA), p21 interferes with PCNA-dependent DNA polymerase activity; thereby inhibiting DNA replication and modulating various PCNA - dependent DNA repair processes (Moldovan *et al.,* 2007). Up-regulation of *p21* expression upon exposure to irradiation or genotoxic chemicals has been reported in several *in vitro* and *in vivo* studies (Park *et al.,* 2006; Zegura *et al.,* 2008; Hreljac *et al.,* 2008; Ellinger – Ziegelbauer *et al.,* 2005).

1.5 The aim of the dissertation and the hypothesis

1.5.1 Aim

The aim of the dissertation was to develop a method for rapid and sensitive detection of agents that cause DNA damage using stably transfected metabolically active human hepatoma HepG2 cells.

Plasmids containing promoter of DNA damage responsive gene *p21* fused to gene coding for reporter EGFP and DsRed were constructed and transfected to HepG2 cells. Stably transfected HepG2 clones expressing inducible EGFP or DsRed were isolated and were tested for their sensitivity to detect genotoxic agents by exposure to genotoxic and non-genotoxic compounds with known mechanisms of action.

1.5.2 Hypothesis

We assumed that exposure of stably transfected human cell line HepG2 containing reporter gene for EGFP or DsRed fluorescent protein fused to promoter of DNA damage responsive gene *p21* to genotoxic agents will result in increased production of EGFP and DsRed proteins. Their fluorescence can be measured and used for quantification of DNA damaging potential. The system will be suitable for the development of high throughput test system (HTTS) for rapid detection of genotoxic compounds and complex samples.

1.5.3 Specific tasks

To confirm the hypothesis the following specific tasks were implemented:

- Preparation of plasmid containing *p21* gene promoter fused with reporter gene coding for EGFP.
- Preparation of plasmid containing promoter for *p21* gene fused with reporter gene coding for DsRed.
- Optimisation of electroporation protocol for optimal transfection of plasmids encoding EGFP and DsRed to the HepG2 cell line.
- Preparation and selection of stably transfected cell line HepG2 with inducible p21 promoter mediated expression of reporter genes.
- Optimization of exposure conditions and data collection.
- Validation of the test system with model genotoxic and non-genotoxic chemicals.

2 EXPERIMENTAL

2.1 Materials

2.1.1 Host cell line

HepG2, metabolically active human hepatoma cells were used in experiments for preparation of stably transfected cell line. The human hepatoma HepG2 cell line was obtained from 85011430 ECACC (Wiltshire, UK), and was grown in minimum essential medium (MEM, GIBCO, Invitrogen, Paisley, UK) without phenol red and supplemented with 10% heat inactivated fetal calf serum (FCS, SIGMA, St. Louis, MO, USA). Cells were routinely subcultured twice per week and were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

The liver is known to be the main site of xenobiotics biotransformation due to the ability of this organ to express a plethora of enzymes, both quantitatively and qualitatively (Mersch-Sundermann *et al.,* 2004). The HepG2 cells were chosen because of their human origin and their retained activities of xenobiotic-metabolizing enzymes, which make them a good model for reflecting the processes in intact liver (Knasmuller *et al.,* 2004; Doostdar *et al.,* 1990). It is characteristic for HepG2 cells that posses a wide range of phase I enzymes such as cytochrome P450, CYP1A1, CYP1A2, CYP2B, CYP2C, CYP3A and CYP2E1, arylhydrocarbon, hydrolase, nitroreductase, *N*-demethylase, catalase, peroxidase, NAD(P)H: cytochrome *c* reductase, cytochrome P450 reductase, and NAD(P)H, quinone oxidoreductase and phase II enzymes such as glutathione-S transferase (GST), uridine glucuronosyl transferase, and *N*- acetyl transferase in an inducible form (Knasmüller *et al.,* 1998). Numerous studies showed high sensitivity of HepG2 cells for the detection genotoxic agents (Winter *et al.,* 2008; Mersch-Sundermann *et al.,* 2004; Knasmüller *et al.,* 2004; Uhl *et al.,* 1999; Zegura *et al.,* 2004, 2008; Plazar *et al.,* 2007, 2008; Hreljac and Filipič, 2009).

Additionally, HepG2 cells express wild-type tumor suppressor *p53* (Bressac *et al.,*. 1990), making them an appropriate model for the development of the test system based on p53-mediated DNA damage response.

2.1.2 Plasmids

For construction of the plasmids encoding reporter genes EGFP and DsRed2 under the promoter of *p21* gene several different plasmids were used. The plasmid pEGFP-N1, encoding EGFP controlled by CMV promoter (Clontech, Basingstoke, UK) was used as a source of coding sequence of the EGFP gene. The source of the coding sequence of p21 promoter was the WWP-LUC plasmid, which was a gift from Prof. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, Maryland, USA). The plasmid has pBluescript (KS+) vector as a backbone and was first described by El-Deiry *et al.* 1993 (El-Deiry *et al.,* 1993). The plasmid pORF-mIL12 (inVivoGen, Toulouse, France) was a source for ORF promoter and pCLEF35DsRed2 plasmid (InVivoGen) was a source for DsRed2 sequence.

2.1.3 Chemicals

William's medium E, penicillin/streptomycin, phosphate buffered saline, trypsin, L-glutamine, MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Methyl methane sulphonate (MMS), Benzo[a]pyrene (BaP), 2-Acetylaminofluoren, Aflatoxin B1, Cadmium chloride ($CdCl_2$), Mannitol, Boric acid, EDTA, N,N-dimethylformamide, 8-hydroxyquinoline, Vitamine C were from Sigma (St. Louis, USA). Normal melting point (NMP) and low melting point (LMP) agarose were from Gibco BRL (Paisley, UK). Cisplatin (CisPt) was from Medac (Hamburg, Germany). Vinblastine (VLB) was from France S.A Lilly (Fagersheim, France). 2-Amino-3-methylimidazol[4,5-f] quinoline (IQ) was from Wako (Osaka, Japan). Potassium dichromate ($K_2Cr_2O_7$), NaCl and Ethanol were from Merck (Darmstadt, Germany). *O*-toluidine and Saccharose was from Kemika (Zagreb, Croatia) and Xanthohumol was from N.I.C. (Hamburg, Germany).

Model genotoxic and non-genotoxic chemicals with their diluents, stock solutions and concentration range used in the experiments for development and validation of test system are listed in the Table 1.

Table 1: *Chemicals* ***employed*** *in optimization and validation experiments of the whole cell biosensor system*

Chemicals	Dissolved in	Stock Solution		Tested Concentrations Range (µg/mL)
		mg/mL	mM	
Methyl methane sulphonate (MMS)	dH2O	50 mg/mL	454 mM	5 - 50 µg/mL
Benzo[a]pyrene (BaP)	DMSO	2.52 mg/mL	10 mM	0.05 – 1.26 µg/mL
Cisplatin (CisPt)	dH2O	2 mg/mL	6.7 mM	0.412 - 6.6 µg/mL
Vinblastine (VLB)	NaCl (0.9%)	1 mg/mL	1.1 mM	0.05 – 5 µg/mL
2-amino-3-imidazo[4,5-f] quinoline (IQ)	DMSO	19.82 mg/mL	100 mM	12.39 – 198.2 µg/mL
2-acetylaminofluoren (2-AAF)	DMSO	22.33 mg/mL	100 mM	0.22 – 220 µg/mL
Aflatoxin B1 (AFB_1)	DMSO	1 mg/mL	3.2 mM	0.008 - 5 µg/mL
Cadmium chloride ($CdCl_2$)	dH2O	18.3 mg/mL	100 mM	0.000114 – 0.00183 µg/mL
Potassium dichromate ($K_2Cr_2O_7$)	dH2O	29.4 mg/mL	100 mM	0.184 – 2.94 µg/mL
O-toluidine	DMSO	10.72 mg/mL	100 mM	0.0011 – 10.72 µg/mL
Xanthohumol	DMSO	24.8 mg/mL	70 mM	0.0354 – 7.086 µg/mL
Mannitol	dH2O	60 mg/mL	330 mM	112.5 - 1800 µg/mL
Boric acid (B_2O_3)	dH2O	20 mg/mL	323 mM	37.5 - 600 µg/mL
Ethylenediaminetetraacetic acid (EDTA)	dH2O	100 mg/mL	342 mM	122.5 - 3500 µg/mL
Saccharoze (Sucrose)	dH2O	100 mg/mL	292 mM	214 - 3420 µg/mL
N,N-dimethylformamide (DMF)	dH2O	25 mg/mL	342 mM	45.6 - 730 µg/mL
Sodium chloride (NaCl)	dH2O	20 mg/mL	342 mM	36.25 - 580 µg/mL
Ethanol (C_2H_5OH)	dH2O	15 mg/mL	325 mM	28.75 - 460 µg/mL
8-hydroxiquinoline	DMSO	14.5 mg/mL	100 mM	0.00145 – 14.5 µg/mL
Vitamine C (Ascorbic acid)	dH2O	60 mg/mL	340 mM	110 - 1760 µg/mL

2.2 Methods

2.2.1 Construction of plasmids

The construction of recombinant vector containing *p21* promoter reporter cassette and EGFP was done in several steps using the Clontech pEGFP-N1noCMV plasmid (gift from Dr. Claudie Karl, University of Regensburg, Germany) as a back bone and standard molecular biology techniques of restriction and ligation. In addition, the gene for neomycin resistance was included into the plasmid, which enabled the isolation of HepG2 cells with stable expression of reporter gene under the pressure of Geneticin® (neomycin, GIBCO) (Figure 6). The constructed plasmid pp21-EGFP was cloned into *E. coli* (strain DH5α, Invitrogen, UK).

The construction of recombinant vector containing p21 promoter reporter cassette and DsRed2 was done using pp21-EGFP as a backbone and a sequence of DsRed2 excised from pORFDsRed2 plasmid. The plasmid pORFDsRed2 was prepared from plasmids pORFmIL-12 and pCLEF35DsRed2 with standard molecular biology techniques. The plasmid pORFDsRed2 was prepared in order to obtain the appropriate restriction sites for inclusion of DsRed2 sequence into the pp21-EGFP plasmid (Figure 7). The plasmid was cloned into DH5α *E.coli* strain.

Plasmids were isolated using the Qiagen Maxi Endo-Free kit (Qiagen, Hilden, Germany), according to manufacturer's instructions and diluted to concentration of 1 mg/mL. Purified plasmid DNA was subjected to quality control and quantity determinations, performed by agarose gel electrophoresis and by means of spectrophotometry. The plasmid pp21-EGFP was prepared in collaboration with Dr. Irena Hreljac from the National Institute of Biology Ljubljana and p21DsRed2 in collaboration with Dr. Urška Kamenšek from the Institute of Oncology Ljubljana.

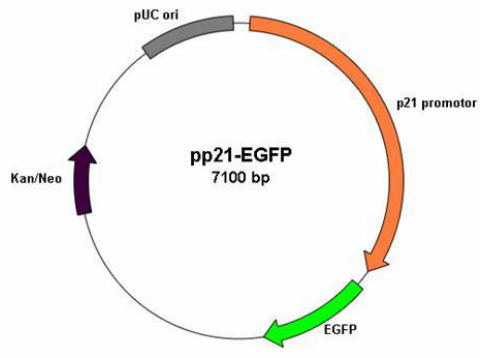


Figure 6: *Scheme of pp21-EGFP plasmid*

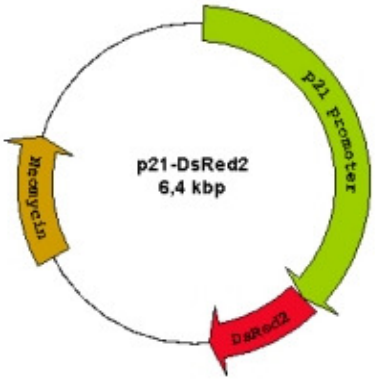


Figure 7: *Scheme of p21-DsRed2 plasmid*

2.2.2 Determination of inhibitory concentration of neomycin for HepG2 cell line

To determine the inhibitory concentration of neomycin for HepG2 cells, which was used for preparation of stably transformed cells, the 20 000 cells per well were plated onto 24-well plates (Corning Costar, Acton, USA) in 1.5 mL of culture medium containing different neomycin concentrations ranging from 300 µg/mL to 4000 µg/mL. The cells were incubated in a 5% CO_2 humidified incubator at 37°C.

The cells were cultured for at least 14 days replacing the antibiotic-containing medium every 3 days. During the 14 days of cell culturing, examination of the viability of the cells was performed every 2 days.

The identification of the lowest neomycin concentration, which killed all cells, was determined within 14 days and was 1.3 mg/mL. This neomycin concentration was

used for initial selection of stably transfected HepG2 with plasmids pp21-EGFP and p21-DsRed2.

2.2.3 Preparation of stably transfected HepG2 cell lines

The HepG2 cells were transfected with pp21-EGFP and p21-DsRed2 plasmids. Physical method, electroporation was used for transfection of cells. Selection of stably transfected clones was done by culturing the cells in the medium containing neomycin. The stably transformed cell lines were named **p21HepG2 EGFP** (cells transfected with plasmid encoding EGFP under the control of p21 promoter) and **p21HepG2 DsRed** (cells transfected with plasmid encoding DsRed under the control of p21 promoter).

2.2.3.1 Electroporation

In the experiment physical transfection technology – electroporation (electropermeabilization) was used. Already forty years ago reversible membrane characteristic of permeability was described under the exposure of cells to high electric fields, in the early 70's (Neumann *et al.,* 1972) (Figure 8). After 10 years it was reported that gene transfection could be obtained by electroporation of a plasmid-cells mixture (Neumann *et al.,* 1982). This procedure is now routinely used in cell biology for transfection of different bacterial and eucaryotic cells. To achieve successful transfection efficiency of the electroporation following conditions has to be fulfilled: the field strength must be high enough to trigger membrane electropermebilization (Rols and Teissie, 1990) and DNA must be present during the field application on cells. A field dependent electrophoresis pushes the DNA in the bulk towards the cell surface, but the movement of DNA across the cell membrane is very slow and is a post pulse event (Eynard *et al.,* 1997). The level of expression is dependent on the amount of added DNA. Pulse duration must be long to obtain an efficient level of expression. Limits in field strength and pulse duration must be set,

as high values can cause damage to the cell membrane and lead to cell death (Cemazar *et al.,* 2006).

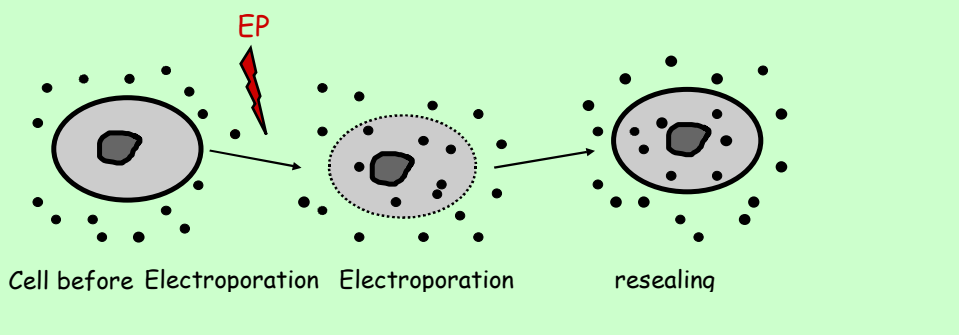


Figure 8: *Principle of electroporation of cells*

For electrotransfection of HepG2 cells, a stock cell suspension at a concentration $2.5x10^7$ cells/mL was prepared. This dense cell suspension (40 µL) was mixed with 10 µg of plasmid DNA (pp21EGFP or p21DsRed2) and placed between two flat parallel stainless steel electrodes with 2-mm gap and subjected to 8 square-wave shaped electric pulses with 5 ms duration, repetition frequency 1 Hz. Different electric field intensity were tested, 600 V/cm, 700 V/cm, 800 V/cm. The electric pulses were generated by an electroporator (GT-1, electroporator, Faculty of Electrical Engineering, University of Ljubljana, Slovenia). After exposure to electric pulses, the cells were incubated for 5 min at room temperature. Figure 9 shows an electroporator and the electrodes, which are necessary equipment for electrotransfection.

Thereafter, cells were maintained in non-selective medium for 1-2 days after the transfection. The selection of stably transfected clones was performed by culturing the cells in the medium containing 1.3 mg/mL Geneticin®. The cultivation in the selective medium was continued for 14-21 days with frequent changes of medium with purpose of eliminating dead cells and debris, until distinct colonies with stable transformed cells were be visualized under the fluorescent microscope. During this period the cells without the plasmid died off while the cells containing stably incorporated plasmid were able to replicate and form colonies. Untransfected cells do not make green or red fluorescent protein. Separate colonies were picked and transferred into wells of 96 well micro titer plates and cultivated further under the

pressure of 0.5 mg/mL Geneticin® for p21HepG2 EGFP cells and 2.4 mg/mL Geneticin® for p21HepG2 DsRed cells. After reaching sufficient number, the cells were transferred to bigger plates for further propagation and further selection of the most responsive clones. The clones with visible morphological and/or replication changes were discharged.

Figure 9: *Electroporator and flat parallel electrodes used for electrotransfection*

2.2.4 Determination of calibration curves for p21HepG2 EGFP cells

To determine the most appropriate number of cells for measurements of fluorescence and viability in the experiments with chemical agents, the calibration curves of relationship between cell number and absorbance for p21HepG2 EGFP cell line were determined.

To determine the appropriate number of cells per well for measurement of cell viability MTS (3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, USA) was used. The cells were plated in different numbers from 1000 cells per well to 5000 cells per well. For each cell number four wells were used. The microtiter plates were incubated at 37°C in an incubator containing humidified atmosphere and 5% CO_2. MTS assay was performed 72, 120, and 168 hours after seeding by addition of 20 µL of MTS solution to each well of 96-well microtiter plates and incubated for 2 h in a humidified atmosphere with 5% CO_2 at 37°C. After the incubation with MTS the microtiter plates were

shaken for 30 s and the absorbance of the resulting solution was measured at 492 nm using a Labtec HT2 micro plate reader (Anthos, Wals, Austria).

2.3 Optimization of exposure conditions of reporter cell lines for 96-well microplate format

In the first part of the study, genotoxic agents with known mechanisms of action were used to test and validate the cell biosensor system. Prior to the testing, stock solutions of chemicals were prepared: MMS, and CisPt were dissolved in distilled water at concentrations of 50 mg/mL (454 mM) and 2 mg/mL (6.7 mM), respectively. BaP was dissolved in DMSO at a concentration of 2.52 mg/mL (10 mM) and VLB in 0.9% NaCl at a concentration of 1 mg/mL (1.1 mM). Further dilutions were made in cell culture media.

A suspension of exponentially growing p21HepG2 EGFP cells ($3x10^5$cells/mL) in MEM without phenol red and with 10% FCS was distributed in 3 mL aliquots to plastic test tubes. To each tube 30 µL of test chemical of appropriate concentration (100 fold higher concentrations from final treatment concentrations) or 30 µL of vehicle for controls were added. The following final concentrations were used: MMS: 5, 10, 20, 40, 50 µg/mL; CisPt: 0.41, 0.82, 1.65, 3.3, 6.6 µg/mL; BaP: 0.05, 0.13, 0.25, 0.5, 1.26 µg/mL, and VLB: 0.05, 0.1, 0.5, 1.0, 2.5, 5.0 µg/mL. For the EGFP fluorescence measurements from each test tube 100 µL aliquots were distributed to 6 wells of 96-well black microtiter plates with clear bottom (Greiner BIO-ONE, Nuernberg, Germany) and for viability measurements 100 µL aliquots from each test tube of treated or control cells were distributed into 4 wells of clear 96 well microtiter plates (TPP, Switzerland) and incubated for 24, 48, 72, 120 and 168 h. For each of the 5 time point measurements a separate microtiter plate was prepared.

The same experiments were repeated with p21HepG2 DsRed cells. A suspension of exponentially growing p21HepG2 DsRed cells ($4x10^4$ cell/mL) in MEM without

phenol red with 10% FCS was distributed in 1 mL aliquots to plastic test tubes. To each tube 10 µL of test chemical of appropriate concentration (100 fold higher concentrations from final treatment concentrations) or 10 µL of vehicle for controls were added. The final tested concentration ranges are presented in Table 1. From each tube 100 µL aliquots were distributed to 6 wells of 96-well black micro titer plates with clear bottom (Greiner BIO-ONE) for fluorescence measurements and for MTS assay 100 µL aliquots were distributed to clear microtiter plates (TPP). The experiments were repeated 3-times.

In the second part of the dissertation (validation of the system) different chemicals with different effects on cells were used.

2.3.1 Measurements of reporter gene expression

Fluorescence intensity of transfected cells was measured with Microplate reader (Tecan Infinite F200). The Tecan Infinite 200 is a microplate reader for a variety of applications that are quantified by Fluorescence, Absorbance or Luminiscence. The instrument is suitable to handle assays in microplate formats from 6 to 384 well plates, PCR tubes and cuvettes (Figure 10).

Figure 10: *Microplate reader (Tecan Infinite 200)*

For fluorescence measurements black flat clear bottom 96-well microtiter plates (Greiner BIO-ONE) were used. The plates containing cells treated with different chemicals were incubated at 37°C, 5% CO_2. Intensity of fluorescence of p21HepG2

EGFP cell line was measured at different time intervals (24, 48, 72, 120 and 168 h) after treatment of cells. Measurement parameters were: excitation wavelength 485 nm and emission wavelength 535 nm for p21HepG2 EGFP cells.

For measurement of fluorescence intensity of p21HepG2 DsRed cells after treatment several parameters, such as excitation and emission peak, suitable filter pair and type of microtiter plate reader were first evaluated to determine the appropriate excitation and emission wavelength for optimised measurement. For these experiments, the cells were plated in black flat clear bottom 96-well microtiter plates and exposed to MMS or CisPt for 48 h. One column of six wells was utilized for each selected concentration of the chemical agent, control cells and background. The experiments were repeated three times.

Tecan Infinite M200, monochromator - based multimode reader, was used to determine excitation and emission peak for DsRed2. Different filter pairs for measurement of DsRed fluorescence were tested using Tecan F200 and to determine which type of microplate reader is the most sensitive and suitbale, the measurements of fluorescence intensity of DsRed2 were also performed on Synergy fluorimeter (Biotek, Winooski, USA).

From fluorescence intensity measurements, a relative EGFP and DsRed induction ratio was calculated. Fluorescence intensities of the treated cells were divided by the fluorescence intensity of control cells and normalized to the relative cells' viability determined with the MTS assay (see below).

2.3.2 Determination of cell viability (MTS assay)

At the end of incubation period (24, 48, 72, 120 and 168 h) with chemical agents, 20 µL of MTS solution was added to each well of 96-well microtiter plates and incubated for 2 h in a humidified atmosphere with 5% CO_2 at 37°C. After the incubation with MTS the microtiter plates were shaken for 30 s and the absorbance of the resulting solution was measured at 492 nm using a Labtec HT2 micro plate

reader (Anthos, Wals, Austria). The experiments were performed in quadruplets and repeated 3-times.

The MTS assay that measures the conversion of MTS to formazan product by dehydrogenase enzymes of the intact mitochondria of living cells correlates with the number of viable cells. This assay was used to indirectly determine the relative changes in cell numbers during the exposure to tested chemicals. For each treatment in parallel to the plate for EGFP and DsRed fluorescence measurements five plates for the measurement of cell viability (one plate for each time point) was prepared. The correlation analysis of the proliferation of p21HepG2 EGFP cells showed that absorbance of the formed formazan crystals correlated to the cell proliferation ($r = 0.96$). The data also indicate that during the exponential growth phase the doubling time of the p21HepG2 EGFP cells is about 48 hours and of the p21HepG2 DsRed is about the same (49 h). At each time point the relative cell viability compared to non-treated control cells was calculated by dividing the absorbance of the treated cells with the absorbance of the control cells and the factor was used for the normalization of the relative EGFP induction ratio to the number of viable cells. The reduction of relative cell viability by more than 30% (reduction factor 0.7) was considered as cytotoxic.

2.3.3 Determination of the expression of reporter protein DsRed by flow cytometry

In optimization processes of whole cell biosensor system, fluorescence intensity of p21HepG2 DsRed cells was determined by flow cytometry after treatment with, MMS, BaP, CisPt and VLB because of unique feature of flow cytometry that it measures fluorescence per cell or particle in contrary to spectrophotometry in which the percent absorption and transmission of specific wavelengths of light is measured for a bulk volume of sample. Flow cytometry is a technique for counting and examining microscopic particles, such as cells and chromosomes. These particles are suspended in a stream of fluid and passing them by an electronic detection apparatus. Detecting system allows simultaneous multiparametric analysis of the physical

and/or chemical characteristics. Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data. The size of the particles and cells are ranging from 0.5 µm to 40 µm diameter.

Cells were treated with MMS, BaP, CisPt and VLB for 48 h, trypsinized and prepared in 0.9% NaCl at a concentration $1x10^5$ cells/mL for flow cytometry measurements. The percentage of fluorescent cells was determined for the histograms using non-transfected HepG2 parental cell line as a negative control.

2.3.4 Comet assay

Genotoxic effects of MMS, CisPt, BaP and VLB were evaluated by Comet assay as described by Singh *et.al.,* 1988 with modifications which have been described by Žegura and Filipič, 2004 (Žegura and Filipič, 2004; Singh *et.al.,* 1988). The Comet assay, also known as single cell gel electrophoresis (SCGE), is a very sensitive microgel electrophoresis technique, which detects DNA damage and repair in individual cells. Types of DNA damage that can be detected by comet assay include SSBs and DSBs, ALS, like apurinic/apyrimidinic (AP) sites, DNA-DNA and DNA-protein cross-links and protein - SSB interactions, the latter being formed transiently as an intermediate phase of DNA repair process (Tice *et al.,* 2000). DNA strand breaks induced by chemical agents as well as those generated in DNA repair processes can be evaluated by Comet assay.

Cells (40 000 cells/well) treated for 24 h with MMS, BaP, CisPt and VLB were embedded in thin agarose gel and placed on microscope slide. The cells were lysed to remove all cellular proteins and the DNA subsequently allowed unwinding under alkaline conditions. Following unwinding the DNA was electrophoresed and stained with a fluorescent dye (Figure 11). Degree of DNA damage was determined by data analysis software (Perspective Instruments, UK) of 50 images captured by fluorescent microscope (Nikon Eclipse E800, Japan).

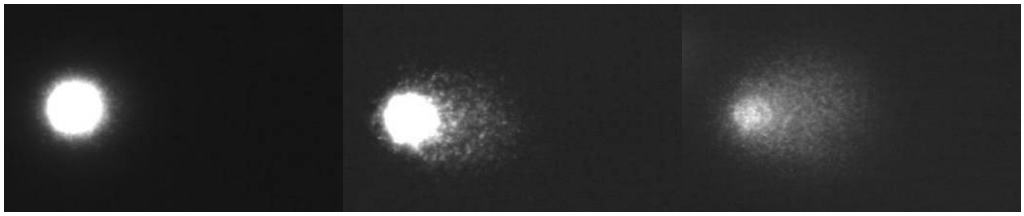


Figure 11: *Fluorescence photomicrographs of HepG2 cells after comet assay (Zegura and Filipic, 2004)*

2.4 Validation of p21HepG2 DsRed test system with genotoxic and non-genotoxic chemicals

Further optimisation and validation of whole cell biosensor system included the preparation of test system that would enable the genotoxicity testing of the compound on single microtiter plate enabling measurement of fluorescence and viability of the cells on the same microtiter plate. Based on results obtained with 4 model genotoxic compounds, 48 h exposure was selected as suitable time point for both measurements.

In order to determine the appropriate number of cell for plating for 48 h exposure that would allow high enough signal for fluorescence measurement and will at the same time not overgrow the cell culture space, different number of cells (30 000, 40 000 and 50 000) were plated in 96-welll microtiter plates for 48 h and fluorescence and viability measured thereafter.

Furthermore, a layout for viability measurements by MTS and fluorescence intensity of the cells on the single microtitre plate was prepared for testing 2 chemicals with up to 6 concentrations and 6 parallels at the same time. MMS is used as the positive control for DsRed induction. As a negative control, untreated p21HepG2 DsRed cells are used. The layout of the plate is presented in Figure 12.

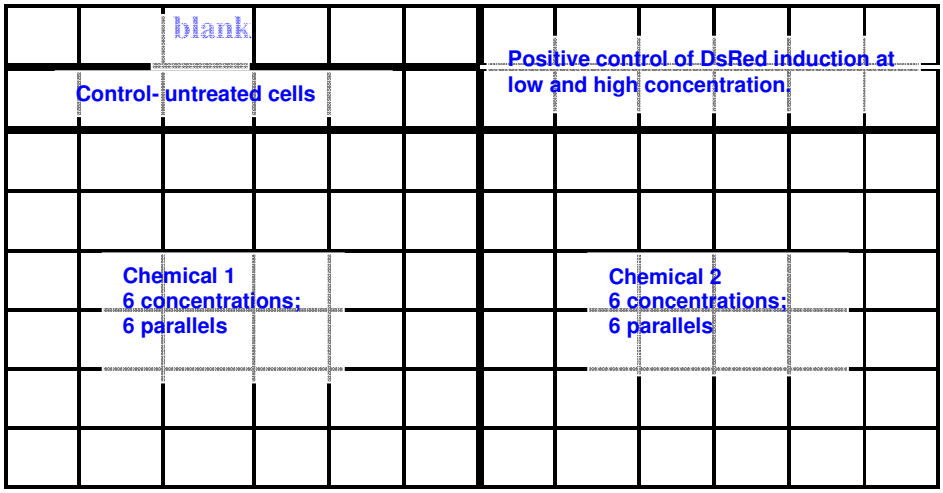


***Figure 12**: The layout of 96-well microtiter plate for testing 2 compounds on single microtiter plate*

For these experiments, a suspension of exponentially growing p21HepG2 DsRed cells ($4x10^4$ cells/mL) in MEM without phenol red with 10% FCS was distributed in 1 mL aliquots to plastic test tubes. To each tube 10 µL of test chemical of appropriate concentration (100 fold higher concentrations form final treatment concentrations) or 10 µL of vehicle for controls were added. Then from each tube 100 µL aliquots of reaction mixture were distributed to 6 wells of 96 well black microtiter plates with clear bottom (Greiner BIO-ONE, Nuernberg, Germany) and incubated for 48 h at 37°C, 5% CO_2. At the end of incubation first the DsRed fluorescence was measured, after that MTS reagent was added and cell viability was determined 2 h later by measuring optical density of the formed formazan product.

2.5 Data collection, calculation of relative cell viability and reporter gene expression, and statistical analysis

Following incubation (24, 48, 72, 120 and 168 h), EGFP or DsRed reporter fluorescence data and cell-culture absorbance data were collected from the same population on the same microplate, while this was not possible for the determination of cell viability. Therefore, in these experiments the cell viability was measured by

MTS assay at each time point on separate microtiter plate. Absorbance data from MTS assay were used to give an indication of the toxicity or reduction in relative cell growth, and these data were normalized to the untreated control (=100% viability). Fluorescence data were divided by absorbance data to give 'fluorescence units', as the measure of the average EGFP or DsRed induction per cell. These data were normalized to the untreated control (=1) as relative EGFP or DsRed fluorescence induction factor.

In the experiments with exposure of p21HepG2 DsRed cells to up to 48 h the fluorescence and cell viability were measured on the same plate as described in cell viability and relative DsRed fluorescence inducing were calculated as described above.

Statistical analysis was performed using SigmaStat software (Systat Software, Inc., Richmond CA). All data were first tested for normality with the Kolmorogov-Smirnov normality test. Significance tests were carried out using analysis of variance (ANOVA) and two-tailed Student's t-test. Values of $p < 0.05$ were considered significant.

The response was considered as genotoxic when significant increase ($p < 0.05$) in fluorescence expression compared to control was observed calculated by ANOVA and two-tailed Student's t-test from 6 parallels on the microtitre plate.

In all experiments the reduction of relative cell viability by more than 30% was considered as cytotoxic. The increase of reporter gene expression at concentrations that reduced the viability by more than 30% was not considered as indicator of genotoxic effect.

3 RESULTS AND DISCUSSION

In the present study we developed whole cell biosensor system for identifying genotoxic agents by detection of up-regulation of the gene expression of DNA damage responsive gene *p21* in cells stably transfected with *p21* gene promoter region linked to a DNA sequence that encodes reporter protein. To produce such cell line a chimeric gene containing the p21 promoter region linked to coding region of either EGFP or DsRed was stably integrated into genome of human metabolically active hepatoma HepG2 cells.

p21 belongs to p53 mediated DNA damage responsive genes that has not been previously used as an indicator of genotoxic injury. For the construction of the reporter system we have chosen p21 promoter, because recently Ellinger-Ziegelbaure *et al.,* 2005, reported that in liver of rats exposed to genotoxic and non-genotoxic carcinogens *p21* was up-regulated only by genotoxic carcinogens (Ellinger-Ziegelbaure *et al.,* 2005). The HepG2 cells were chosen because of their human origin and their retained activities of xenobiotic-metabolizing enzymes, which make them a better model for reflecting the processes in an intact liver than other *in vitro* test systems. In addition, HepG2 cells express wild-type tumor suppressor p53, making them an appropriate model for development of the test system based on the p53-mediated DNA damage response.

3.1 Construction of reporter gene plasmid and stable transformed HepG2 cells

For genotoxicity screening system a plasmid pp21-EGFP with *p21* promoter inserted in front of the EGFP reporter gene was constructed. Successful construction and isolation of pp21-EGFP was confirmed with the restriction analysis. The pp21-EGFP plasmid was then transfected to HepG2 cells.

In the final step HepG2 cell clones expressing low basal and high inducible EGFP expression were isolated. For the isolation of DNA damage responsive clones we

used MMS as a test genotoxic chemical. After measuring the basal and MMS induced EGFP levels in 36 independent clones the one with the highest inducible and the lowest basal level of EGFP expression was selected for further propagation, characterization and the experiments with the known model genotoxic compounds. The clone was named p21HepG2 EGFP. Microscopic observations of p21HepG2 EGFP cells demonstrated clear increase of EGFP fluorescence intensity induced by 50 µg/mL MMS after 48 h exposure (Figure 13).

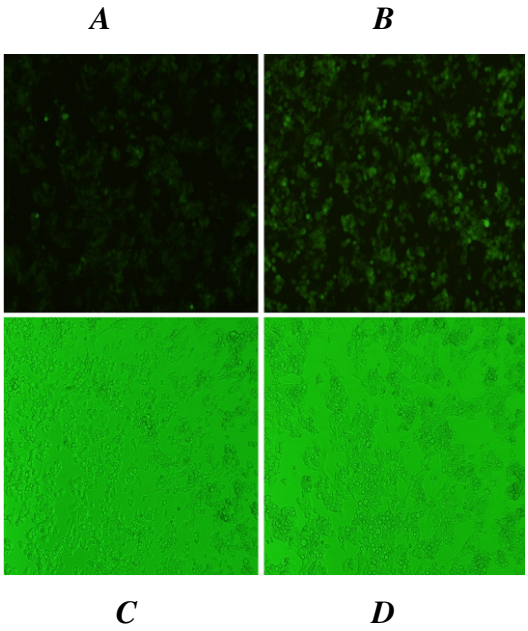


***Figure 13**: Photomicrographs of control (A, C) and p21HepG2 EGFP cells exposed to 50 µg/mL MMS for 48 hours (B, D).*
Images were taken under visible light condition (C, D) and under fluorescence epi-illumination (A, B).

3.2 Determination of calibration curve for p21HepG2 EGFP cell line

In order to use the new developed p21HepG2 EGFP cells for measuring the response to genotoxicity it was necessary to establish the optimal experimental conditions. In the first step we determined optimal cell density per well of 96 well microplate for the measurement of the induced EGFP fluorescence. As HepG2 cells are growing in monolayer it is essential that during the exposure cells are exponentially growing and do not reach confluence. The cells density was determined indirectly using MTS assay therefore a calibration curves were established at different initial plating densities.

Figure 14 shows the results of the MTS assay 72, 120 and 168 h after plating of different number of cells. Absorbance as a measure of cell density increased with the time of incubation. p21HepG2 EGFP cells did not reach full confluence and absorbance maximum after 168 h even when 5000 cells per well were seeded. The correlation analysis showed that the increase of absorbance of the formed formazan product correlated with the time of incubation (r = 0.96) and the data indicate that during the exponential growth phase the doubling time of the p21HepG2 EGFP cells is about 48 h indicating cell proliferation (Figure 15).

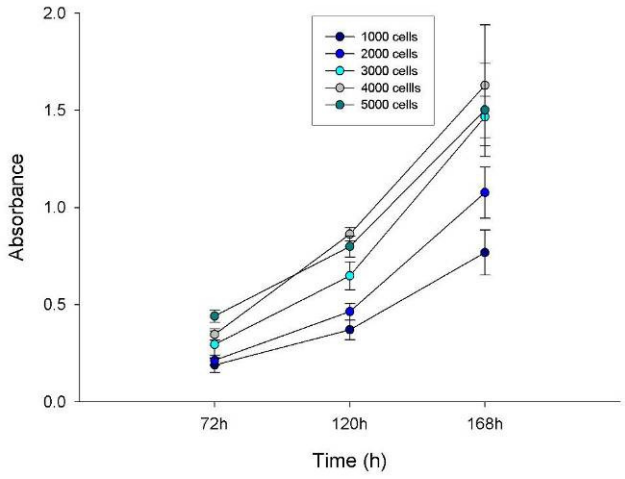


Figure 14: *Results of MTS assay for p21HepG2 EGFP cells after 72, 120 and 168 h after seeding different number of cells.*

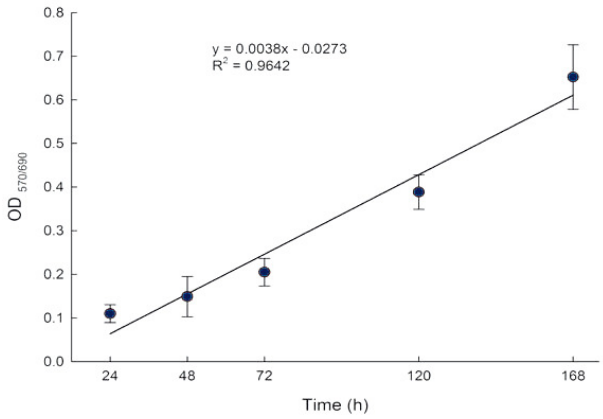


Figure 15: *Proliferation of p21HepG2 EGFP cells measured with the MTS assay. 5000 cells/well were plated on 96-well microtiter plates in triplicate and incubated for 24, 48, 72, 120 and 168 h. The values represent means of four independent experiments ± SD. OD - optical density*

Since it is known that genotoxic chemicals are at certain concentrations cytotoxic or can suppress cell growth during the exposure, it was necessary to normalize the observed level of EGFP fluorescence to the number of viable cells. Thus MTS absorbance data were used to give an indication of the reduction of cell viability, and were normalized to the untreated control (=100% growth). Fluorescence data were then multiplied by normalized absorbance data to give 'relative fluorescence units', as the measure of the average fluorescence induction per cell. These data were finally normalized to the untreated control (=1) to give relative induction of EGFP expression. While induction of EGFP fluorescence was measured after 24, 48, 72, 120 and 168 h on the same cell population, this was not possible for the determination of cell viability, because no appropriate method that would allow for determination of cell viability without termination of the cell culturing is available. Therefore for the viability measurements separate plates for each time point were prepared.

3.3 Optimization of whole cell biosensor system - p21HepG2 EGFP cells

To optimize the test system and demonstrate its sensitivity for detection of genotoxic chemicals we used four genotoxic chemicals with different known mechanism of genotoxic action: MMS, BaP, CisPt and VLB. The time and dose dependence of EGFP fluorescence induced by model genotoxic agents was measured at 24 h intervals up to 168 h (7 day) exposure.

3.3.1 MMS

MMS a direct acting genotoxic agent that induces alkylation of DNA bases, is a known mutagen and rodent carcinogen (Lawley, 1989; Beranek, 1990). Recently it has been reported that MMS induces phosphorylation of p53 protein and increases its DNA-binding properties to cause an increased expression of *p21* (Jaiswal and Narayan, 2002). It was tested at concentrations ranging from 5 µg/mL to 50 µg/mL, which are known to produce DNA damage to exposed mammalian cells.

MMS induced statistically significant increase in EGFP fluorescence after 24 h at 50 µg/mL and after 48 h exposure at 20, 40 and 50 µg/mL (Figure 16b, Annex A). The MMS induced increase of EGFP fluorescence was time and dose dependent, which was reflected in the increasing values of relative EGFP induction ratio (Figure 16b, Annex A). After 120 and 168 h exposure significant increase in EGFP fluorescence associated with the increase in relative EGFP induction ratio was observed at all concentrations (Annex A).

The parallel measurement of cell viability during the exposure to MMS showed that it was not significantly affected during the initial 72 h of exposure (Figure 16a), while after 120 and 168 h it was reduced by more than 30% compared to non-treated control cells (Annex A).

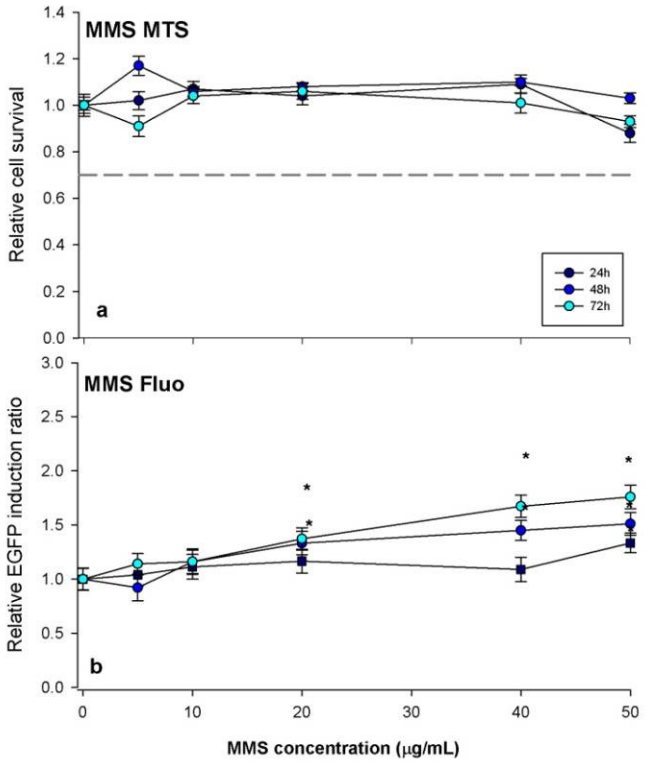


Figure 16: *Results of MTS assay (a), and relative EGFP induction ratio 24, 48, 72 h (b) in p21HepG2 EGFP cells after treatment with 5, 10, 20, 40 and 50 µg/mL MMS. * p<0.05. Dash line represents reduction of cell viability to 70%.*

The results show that MMS induced dose dependent increase of EGFP fluorescence with the LOEC 20 µg/mL. The sensitivity of our system for MMS genotoxicity detection is similar to that of GreenScreen HC assay with *GADD45a* promoter fused to an EGFP gene, in which the LOEC was 25 µg/mL (Hastwell *et al.,* 2006) and to that with *p53R2* promoter fused to luciferase reporter in MCF-7 cells in which the LOEC was around 10 µg/mL (Ohno *et al.,* 2008).

3.3.2 BaP

BaP is an indirect acting genotoxic carcinogen that is metabolized by cytochrome P450 enzymes to diol epoxide (BPDE), which binds covalently to guanine bases (Perlow *et al.,* 2002). Exposure of cells to BaP is known to induce the activation of p53 protein and its downstream regulated genes including *p21* (Wang *et al.,* 2003;

Sadikovic and Rodenhiser, 2006). BaP was tested in concentration range from 0.05 to 1.26 µg/mL. BaP induced significant dose dependent increase in EGFP fluorescence at all exposure times and all concentrations except the lowest one (0.05 µg/mL). However, the relative EGFP induction ratio did not increase with the prolonged exposure. (Figure 17b, Annex A).

BaP did not significantly reduce the cell viability during the exposure up to 72 h (Figure 17a, Annex A), while with further exposure the viability was reduced for more than 30% at all doses of BaP (Annex A).

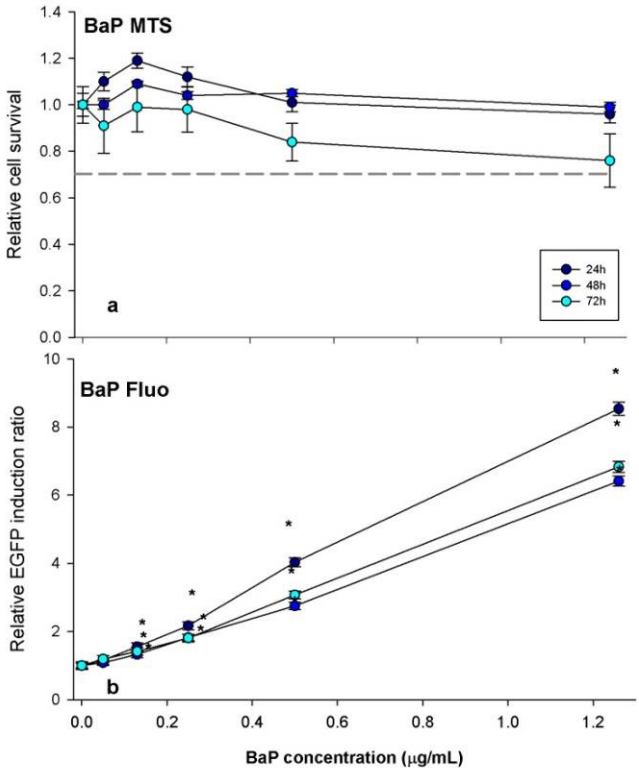


Figure 17: *Results of MTS assay (a), relative EGFP induction ratio 24, 48, 72 h (b) of p21HepG2 EGFP cells after treatment with 0.05, 0.13, 0.25, 0.5 and 1.26 µg/mL BaP.*

** $p < 0.05$. Dash line represents reduction of cell viability to 70%.*

Photographs in Figure 18 show p21HepG2 EGFP cells observed under visible light (A, B, C and D), and under fluorescence light (E, F, G and H) after different times of incubation with BaP at concentration 0.5 µg/mL, demonstrating an increase in the number of cells expressing green fluorescence with prolonged exposure to BaP.

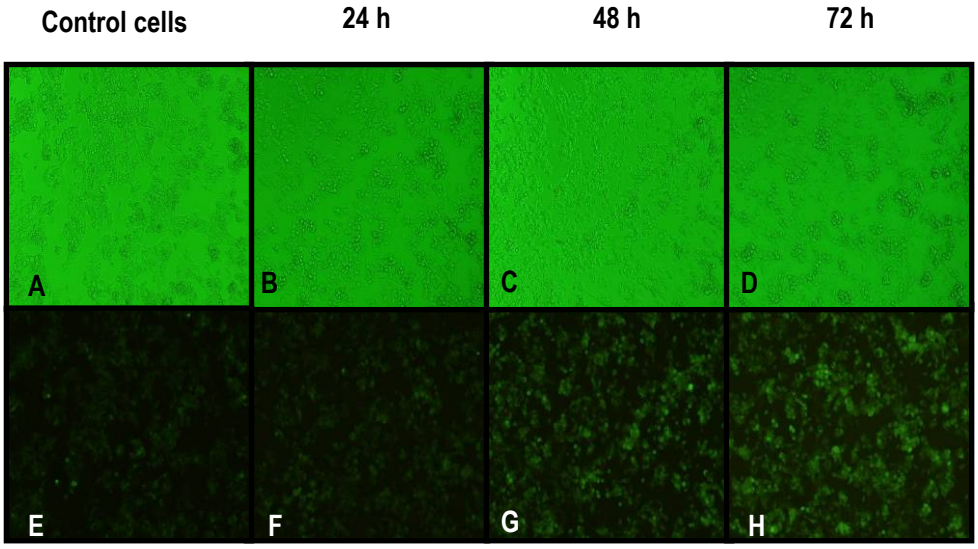


Figure 18: *Images of control untreated p21HepG2 EGFP cells (A, E) and cells exposed to 0.5 µg/mL BaP concentration (B, C, D, F, G and H).*
Images were taken under visible light condition (A, B, C and D) and under fluorescence epi-illumination (E, F, G and H) 24, 48 and 72 hours after treatment. Images of control cells were taken 48 h after the start of experiment. The photographs were taken by Olympus DP50 camera attached to Olympus inverted fluorescence microscope at 40x magnification with excitation filter 460-490 nm and emission filter 510- nm.

The LOEC for BaP was at 0.13 µg/mL (0.5 µM), and at the highest tested concentration 1.26 µg/mL (5 µM) the relative EGFP induction ratio was 8.54 after 24 h exposure. HepG2 cells transfected with GADD153 fused to luciferase were significantly more sensitive for BaP genotoxicity detection; the LOEC was 0.0025 µg/mL (10 nM) (Zhang *et al.,* 2009). The authors ascribed the high sensitivity of their assay compared to other reporter systems to the sensitivity of luciferase, which seems to be higher than that of EGFP (Zhang *et al.,* 2009). In MCF-7 cells transfected with *p532R* coupled to luciferase reporter gene, LOEC for BaP was 0.26 µg/mL when tested without metabolic activation and 0.12 µg/mL in the presence of metabolic activation (Ohno *et al.,* 2008). Lower sensitivity of MCF-7 cells in the absence of metabolic activation compared to HepG2 cells can be ascribed to their lower expression of metabolic enzymes. When using metabolically incompetent cells

the indirect acting genotoxic agents have to be tested in the presence of exogenous metabolic activation, usually S9 liver extracts. However, S9 is light absorbing and fluorescent that can confound spectrophotometric measurements of fluorescence, which is the main limitation of the reporter systems based on EGFP. For the GreenScreen HC test system a protocol based on flow cytometry (FCM) has been developed for the detection of indirect acting genotoxic chemicals, and the LOEC for BaP was 1.25 µg/mL (Jagger *et al.,* 2009). Thus our test system with HepG2 cells represents a great potential for direct detection of the indirect acting genotoxic agents.

3.3.3 CisPt

CisPt a well known chemotherapeutic agent, is a direct acting genotoxic agent that induces alkylation of DNA and DNA cross-links. CisPt induced lesions known to block DNA transcription *in vitro* (Corda *et al.,* 1993). In cells the response to CisPt induced DNA damage has been shown to activate *p53* through ATR-Chk2 pathway (Pabla *et al.,* 2008). The bulky DNA damage induced by different genotoxic chemicals such as DNA cross-linkers or BaP are repaired by the nucleotide excision repair (NER). The studies showed that triggering of the signal transduction cascade that leads phosphorylation of *p53* or *p21* requires recognition and processing of the lesions by the NER (Marini *et al.,* 2006).

CisPt induced significant increase of EGFP fluorescence already after 24 h exposure at all concentrations. With further exposure the relative EGFP induction ratio tended to increase with the time of exposure (Annex A). In cells exposed to 3.3 µg/mL CisPt the relative EGFP induction ratio increased from 1.40, determined after 24 h, to 2.67 determined after 48 h of exposure (Figure 19b, Annex A). CisPt did not reduce cell viability after 24 h of exposure. After 48 and 72 h exposure the viability of the cells was significantly reduced at the two highest concentrations (3.3 and 6.6 µg/mL) (Figure 19a), while after 120 and 168 h exposure CisPt reduced the viability of the cells by more than 30% at all tested concentrations (Annex A).

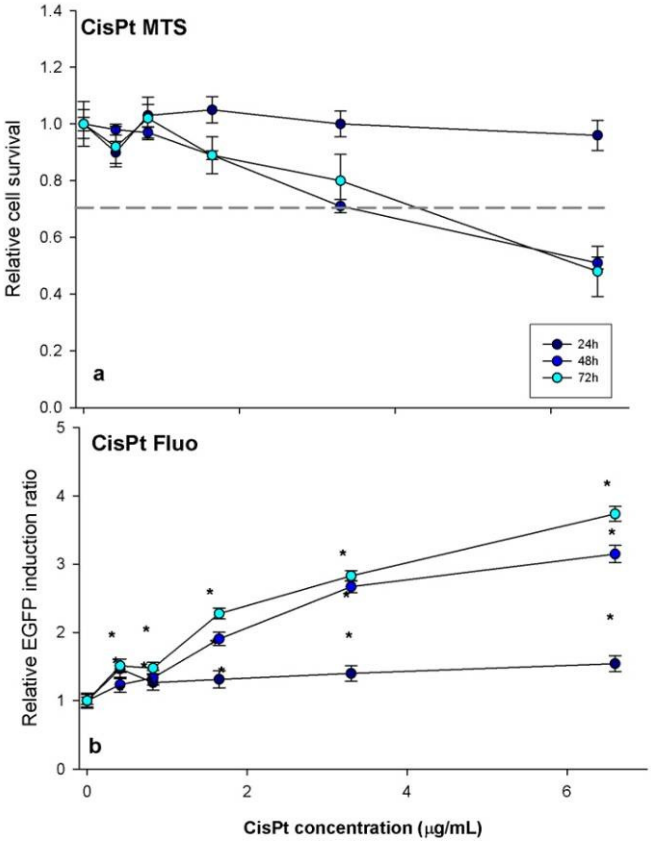


Figure 19: *Results of MTS assay (a), relative EGFP induction ratio 24, 48, 72h (b) of p21HepG2 EGFP cells after treatment with 0.41, 0.82, 1.65, 3.3, 6.6 µg/mL CisPt. * $p < 0.05$. Dash line represents values of cell viability below 70% compare to control - untreated cells*

In p21HepG2 EGFP cells CisPt induced a dose dependent induction of EGFP fluorescence. The LOEC was 0.41 µg/mL. This is more sensitive compared to the response, observed with GreenScreen HC assay in which the LOEC was 1 µg/mL (Hastwell *et al.,* 2006). The MCF-7 cells carrying *p53R2* promoter linked to luciferase reporter were even less sensitive; the LOEC was around 10 µg/mL (Ohno et al., 2008).

3.3.4 VLB

VLB is a chemotherapeutic that does not induce directly DNA damage. VLB belongs to spindle poisons that block polymerization of tubulin into microtubules and inhibit cell division without directly damaging DNA (Owellen *et al.,* 1976). These chemicals induce activation of *p53* and cell cycle arrest mediated by *p21* (Tishler *et al.,* 1995), although the details of this process are not clear. VLB induced significant increase of EGFP fluorescence after 24 h exposure at all concentrations, except the highest (5.0 µg/mL). After 48 h exposure significant increase of EGFP fluorescence was detected at the lowest three concentrations (0.1, 0.5 and 1.0 µg/mL), while at higher concentrations and with prolonged exposure the EGFP fluorescence intensity was reduced (Figure 20b, Annex A).

The viability measurements showed that VLB was highly cytotoxic. Although after 24 and 48 h exposure the relative cell viability was not reduced by more than 30%, except at the highest concentration, after prolonged exposure it rapidly decreased. After 72 h exposure the relative viability was reduced by more than 40% at all concentrations and after 168 h exposure it decreased by more than 90% compared to the viability of non treated control cells (Figure 20a, Annex A).

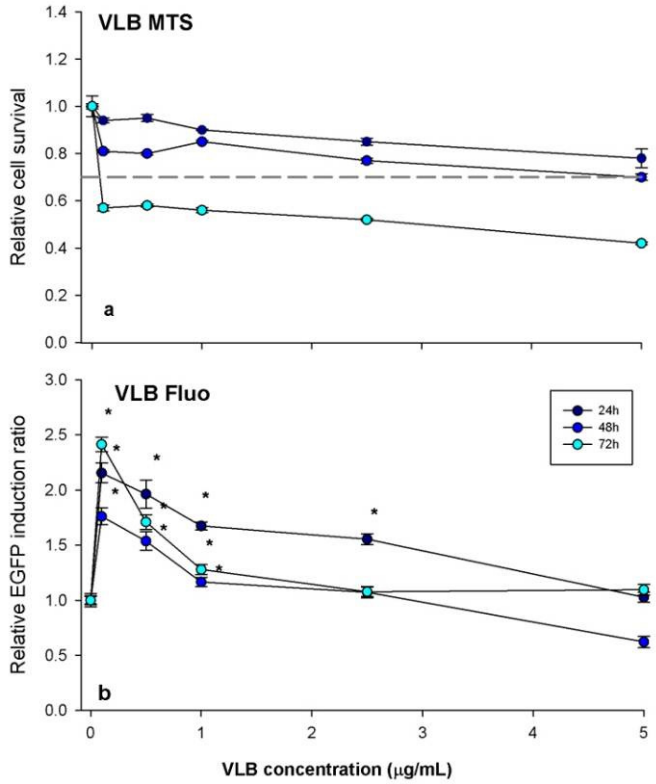


Figure 20: *Results of MTS assay (a), relative EGFP induction ratio 24, 48, 72h (b) of p21HepG2 EGFP cells after treatment with 0.1, 0.5, 1.0, 2.5 and 5.0 μg/mL VLB. * $p < 0.05$. Dash line represents values of cell viability below 70% compare to control - untreated cells*

VLB induced significant increase of EGFP fluorescence at the lowest tested concentration 0.1 μg/mL, which decreased at higher concentrations. VLB showed cytostatic effect, which is reflected in rapid decrease of relative cell viability during the prolonged exposure. Lower induction of *p21* mediated EGFP expression at higher concentrations of VLB may be explained by its toxicity. In MCF-7 cells with *p53R2* mediated luciferase reporter VLB induced comparable cytotoxicity and induction of reporter gene (Ohno *et al.,* 2008) as we observed in our test system. VLB was highly cytotoxic also in the GreenScreen HC test with LOEC for growth inhibition and EGFP induction at 0.02 μg/mL (Hastwell *et al.,* 2006).

Taken together, the results showed that the new biosensor system with human hepatoma cell line p21HepG2 EGFP efficiently detects different types of genotoxic agents. Use of metabolically competent human cells allow direct detection of indirect acting genotoxic chemicals and spectrofluorimetric measurements of reporter genes on micro plate format ensuring easy handling and rapid data acquisition.

3.4 Cell-based biosensor system with red fluorescent protein - p21HepG2 DsRed cells

One of the disadvantages of the EGFP as a reporter protein is its fluorescence spectrum which overlaps with autofluorescence of cells as well as with numerous fluorophores in the cell medium (Hellweg *et al.,* 2001). In addition, tested compounds and/or their metabolites may be fluorescent at wavelengths used for the measurement of EGFP fluorescence. Therefore we attempted to improve the method for genotoxicity testing with replacement of EGFP with DsRed. For this purpose, the indicator HepG2 cells were transfected with p21 promoter region linked to DNA sequence encoding DsRed reporter protein.

3.4.1 Selection of fluorescence filters

In order to determine the optimal excitation and emission wavelength for measurement of fluorescence intensity of DsRed protein, microplate reader with monochromator was used. Due to the technical limitations of the microplate reader a 30 nm wavelength gap had to be present between the excitation and emission wavelengths in order to measure fluorescence intensity. The excitation and emission peaks of fluorescence intensity of DsRed protein were determined using two chemicals, CisPt and MMS at different concentrations at 48 h post-exposure. According to the literature data, two different excitation wavelengths were used 535 and 560 nm and emission was measured in the interval ranging from 570 or 590 to 670 nm (Figures 21, 22). In both tested conditions, a clear dose dependence of

increased fluorescence intensity was observed with increasing concentrations of both chemicals. The increase was more pronounced for CisPt that for MMS. The increase in fluorescence intensity was higher for both chemicals when excitation was fixed to 535 nm compared to 560 nm. However in both cases, the emission peak was at 590 (Figure 21, 22).

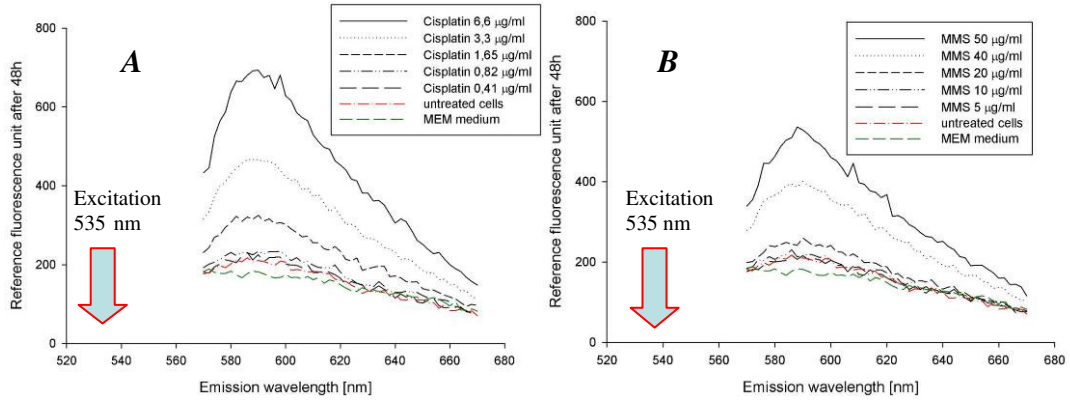


Figure 21: *p21hepG2 DsRed cells treated with 0.41, 0.82, 1.65, 3.3 and 6.6 µg/mL CisPt (Figure 21A) and with 5, 10, 20, 40 and 50 µg/mL MMS (Figure 21B) after 48 h.* ***Excitation 535 nm, emission 570-670 nm***

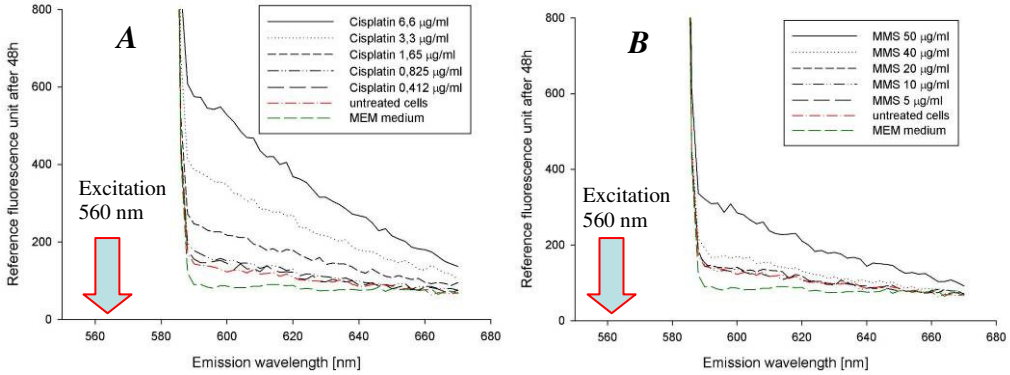


Figure 22: *p21hepG2 DsRed cells treated with 0.41, 0.82, 1.65, 3.3 and 6.6 µg/mL CisPt (Figure 22A) and with 5, 10, 20, 40 and 50 µg/mL MMS (Figure 22B) after 48 h.* ***Excitation 560 nm, emission 590-670 nm***

Then, to determine the optimal excitation wavelength, the emission was set to 590 nm and the fluorescence intensity for excitation wavelengths were measured in the range from 430 to 560 nm. Fluoresence intensity of DsRed protein, measured at 590

nm, started to increase above excitation wavelength 540 nm for the highest concentration of chemicals used and was the highest at excitation wavelength of 560 nm (Figure 23). Again, the increase was more pronounced for CisPt than for MMS.

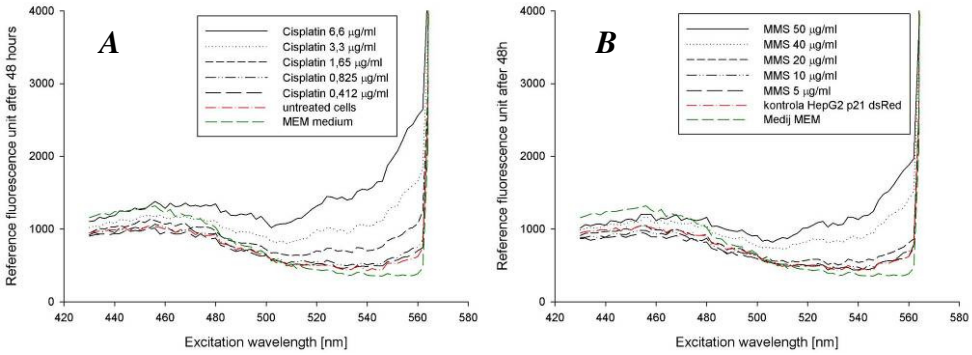


Figure 23: *p21hepG2 DsRed cells treated with 0.41, 0.82, 1.65, 3.3 and 6.6 µg/mL CisPt (Figure 23A) and with 5, 10, 20, 40 and 50 µg/mL MMS (Figure 23B) -* ***Excitation 430-560 nm, emission 590 nm***

3.4.2 Excitation – absorption filter pair comparison

Selection of the most appropriate filters for the measurements of the fluorescence intensity of DsRed protein was performed between three different filter combinations based on the results obtained by fluorescence intensity measured at different excitation and emission wavelengths. The three different filter pairs selected were 535-590 nm, 535-612 nm and 560-590 nm. The experiments were made with CisPt (Figure 24) at different concentrations. The fluorescence intensity was measured with Microplate reader (Tecan Infinite 200). The highest induction ratio was obtained with filter pair 535 nm for excitation and 590 nm for emission. The induction ratio of DsRed measured by this filter pair was significantly higher than induction ratio determined by other two filter pairs, although according to the reported excitation and emission peak reported in the literature and our results obtained with range of different wavelenghts, the filter pair 560-590 nm should be more appropriate (Patterson *et al.,* 2001). Therefore, the filter pair 535-590 nm was further used in all subsequent experiments.

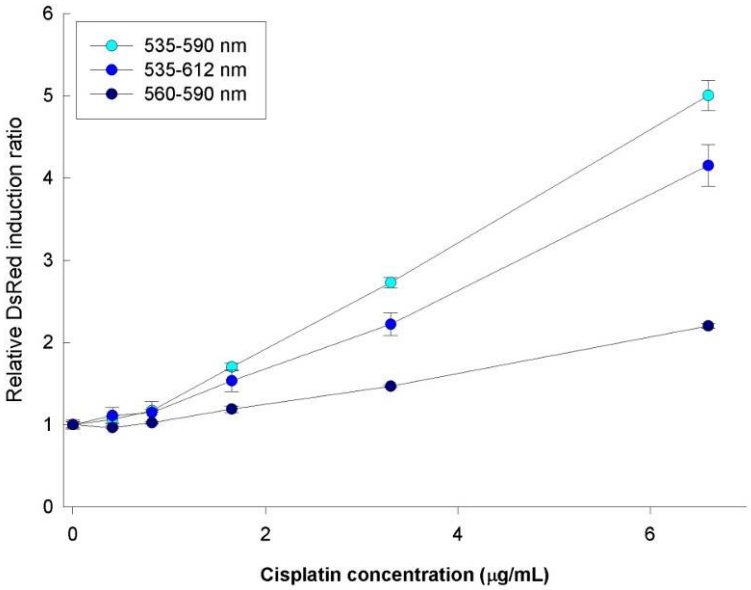


Figure 24: *Excitation – Absorption Filter pair comparison. The experiments were made with 0.41, 0.82, 1.65, 3.3 and 6.6 μg/mL CisPt.*
**Statistically significant difference ($p < 0.01$).*

3.5 Comparison of microplate readers

In optimization processes we also compared two different microplate readers: Tecan Infinite 200 (535-590 nm) and Synergy 2 (BioTek, USA) (530-590 nm). Synergy apparatus enables measurements from bottom or the top of the microplates, therefore this comparison was performed, too. Different concentrations of CisPt were used in this optimization.

Fluorescence intensity was measured 48 h after exposure to the CisPt. Although not statistically significant, the highest increase in induction ratio was obtained with Tecan microplate reader (Figure 25). Based on these results, Tecan microplate reader was used for further experiments.

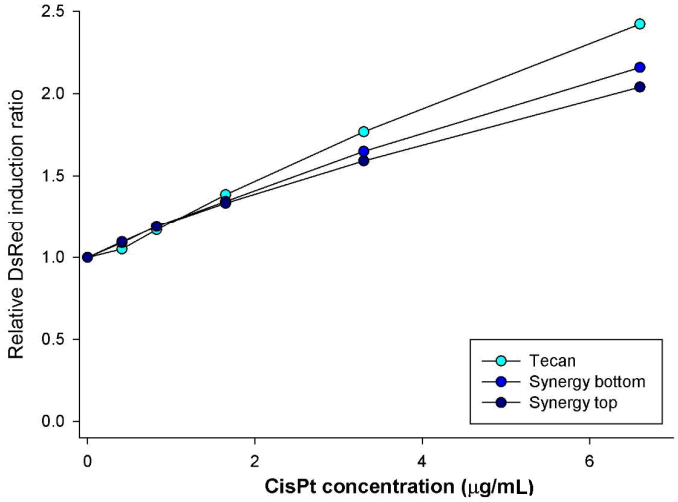


Figure 25: *p21HepG2 DsRed cells treated with 0.41, 0.82, 1.65, 3.3 and 6.6 µg/mL CisPt. Fluorescence intensity was measured with two different microplate readers. With Synergy apparatus the measurements were performed from the bottom and the top of the microplates*

3.5.1 Responses of p21HepG2 DsRed cells to the exposure to model genotoxic agents

The sensitivity of p21HepG2 DsRed cells has been evaluated by the same model genotoxic agents as were used for the evaluation of p21HepG2 EGFP cells: MMS, BaP, CisPt and VLB. The same exposure conditions were used as described in the experiments with p21HepG2 EGFP cells.

3.5.1.1 MMS

The measurement of relative DsRed induction ratio after MMS exposure showed statistically significant increase in DsRed fluorescence at all exposure times and concentrations (Figure 26b, Annex B). Already at the lowest tested concentration (5 µg/mL) the increase in relative fluorescence was 1.9 fold after 24 h exposure and

after 48 h 2.7 fold higher than in the control cells, while with further exposure it did not increase. Compared to the results with p21HepG2 EGFP cells in which significant increase in fluorescence was after 24 h exposure detected at 50 µg/mL and after 48 h at 20 µg/mL, in p21HepG2 DsRed cells significant increase in fluorescence was detected at 5 µg/mL at all exposure times. This indicates higher sensitivity of p21HepG2 DsRed cells compared to p21HepG2 EGFP cells. The measurement of the viability of p21HepG2 DsRed during the exposure to MMS showed similar sensitivity as p21HepG2 EGFP cells. During the exposure up to 48 h the cell viability was not reduced, while after 72 h exposure the viability of the cells was significantly reduced at the highest concentration 50 µg/mL (Figure 26a). After 120 and 168 h exposure it was reduced by more than 30% at concentration above 10 µg/mL, respectively (Annex B).

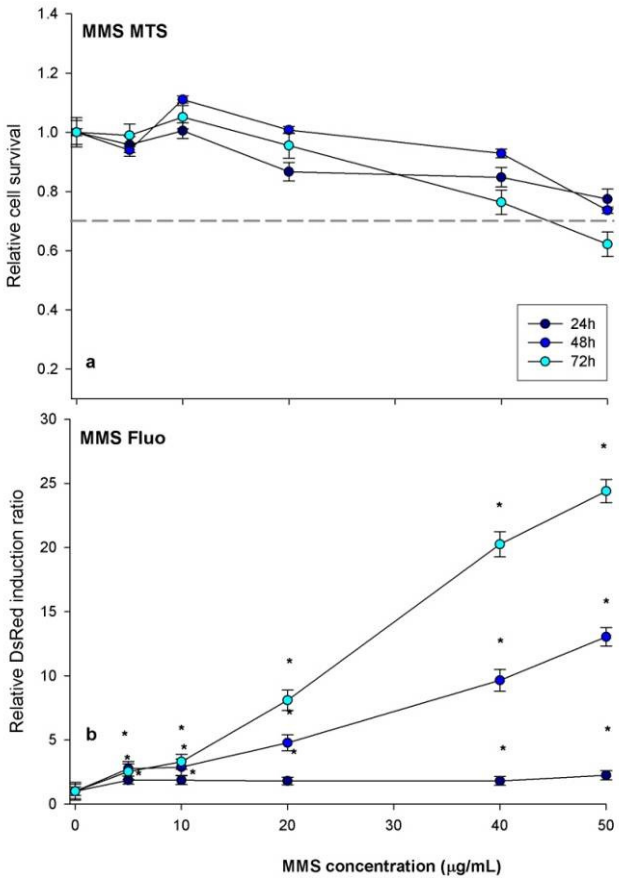


Figure 26: *Results of MTS assay (a), relative DsRed induction ratio 24, 48, 72h (b) of p21HepG2 DsRed cells after treatment with 5, 10, 20, 40 and 50 µg/mL MMS.*
** $p < 0.05$. Dash line represents values of cell viability below 70% compare to control - untreated cells.*

3.5.1.2 BaP

BaP induced dose dependent increase in DsRed fluorescence, which was significant at the highest concentration (1.26 µg/mL) after 24 h treatment. After 48 h exposure the significant increase was observed at concentrations ≥ 0.25 µg/mL and after 72 h exposure at concentrations ≥ 0.05 µg/mL, which was the lowest tested concentration (Figure 27, Annex B).

BaP did not significantly reduce the cell viability during the exposure up to 72 h (Figure 27, Annex B). Further exposure (120 h and 168 h) of cells to BaP was reduced the viability for more than 30 % at all doses of BaP (Annex B).

Compared to the results obtained with p21HepG2 EGFP cells the sensitivity of p21HepG2 DsRed cells for detection of BaP induced increase in fluorescence of reporter gene seems to be lower. In p21HepG2 EGFP cells significant increase in EGFP fluorescence has been after 24 h exposure at concentration 0.13 µg/mL, and also the relative induction ratios were higher. However, it has been reported that BaP metabolites are fluorescent (Jagger *et al.,* 2009). Therefore, in p21HepG2 EGFP cells the effect of fluorescence of BaP metabolites cannot be ruled out, and may thus contribute to high fluorescence readouts, that are not reliable.

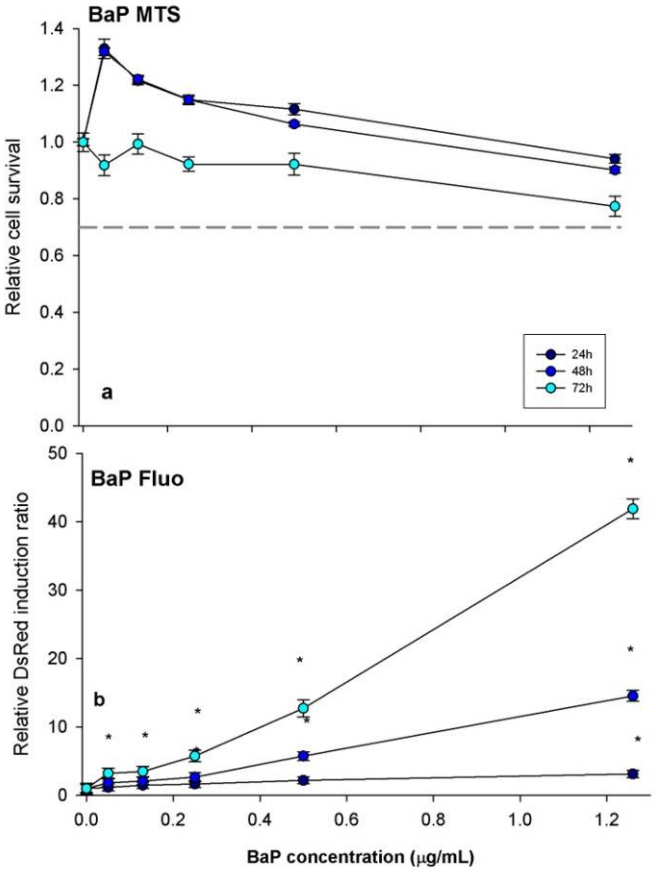


Figure 27: *Results of MTS assay (a), relative DsRed induction ratio 24, 48, 72 h (b) of p21HepG2 DsRed cells after treatment with 0.05, 0.13, 0.25, 0.5 and 1.26 µg/mL BaP.*

*Dash line represents values of cell viability below 70% compare to control - untreated cells. * $p < 0.05$*

3.5.1.3 CisPt

No increase of relative DsRed fluorescence induction was observed after 24 hour exposure. After 48 h CisPt induced significant increase of DsRed fluorescence exposure at the two highest concentrations. With further exposure the relative DsRed induction ratio tended to increase with the time of exposure (Figure 28b, Annex B). In cells exposed to 1.65 µg/mL CisPt the relative DsRed induction ratio increased from 2.34, determined after 48 h, to 15.54 determined after 72 h of exposure (Figure 28b, Annex B).

CisPt did not reduce cell viability after 24 h of exposure. After 48 h exposure the viability of the cells was reduced by more than 30% at the highest concentration (6.6 µg/mL), and after 72 h at 3.3 and 6.6 µg/mL. After 120 and 168 h exposure CisPt reduced the viability of the cells by more than 30% at all tested concentrations (Figure 28a, Annex B).

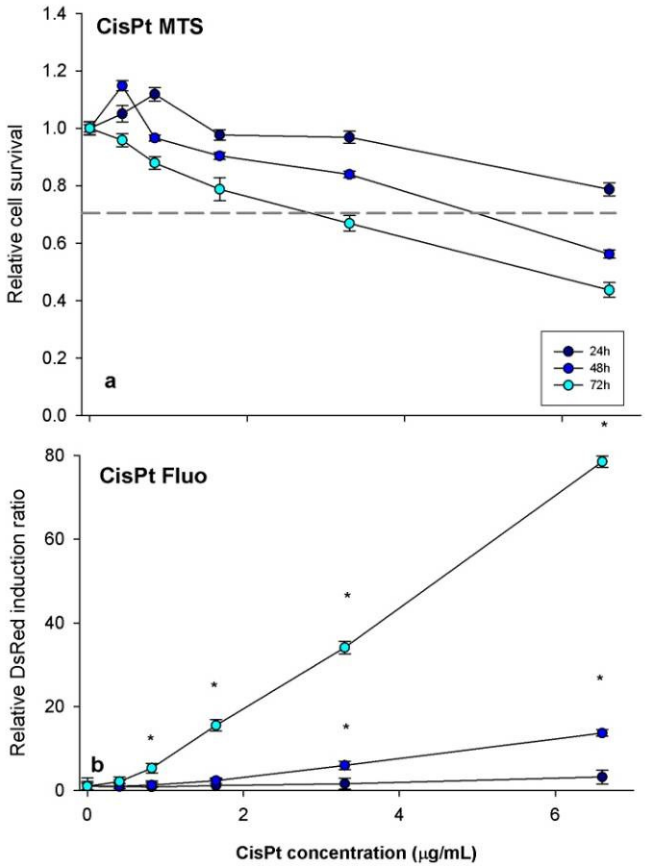


Figure 28: *Results of MTS assay (a), relative DsRed induction ratio 24, 48, 72 h (b) of p21HepG2 DsRed cells after treatment with 0.41, 0.82, 1.65, 3.3 and 6.6 µg/mL CisPt.*

** $p < 0.05$. Dash line represents values of cell viability below 70% compare to control - untreated cells.*

In p21HepG2 DsRed cells significant increase in fluorescence of reporter protein was observed at 6.6 µg/mL CisPt, while in p21HepG2 EGFP cells significant increase in the expression of reporter protein was after 24 h exposure detected at 1.65 µg/mL CisPt. After 48 h exposure with both cell lines we detected CisPt mediated increase

in fluorescence of reporter protein at 1.65 µg/mL, and after 72 h exposure at the lowest tested concentration (0.41 µg/mL), indicating comparable sensitivity.

3.5.1.4 VLB

VLB induced significant increase of DsRed fluorescence at all exposure times and concentrations (Figure 29 b, Annex B) which decreased with higher concentrations. VLB showed cytostatic effect, which was reflected in rapid decrease of relative cell viability during the prolonged exposure (Annex B).

At all tested concentration the relative cell viability was reduced by more than 30% already after 72 h exposure therefore only the effect observed after 24 h and 48 h exposure was considered (Figure 29a).

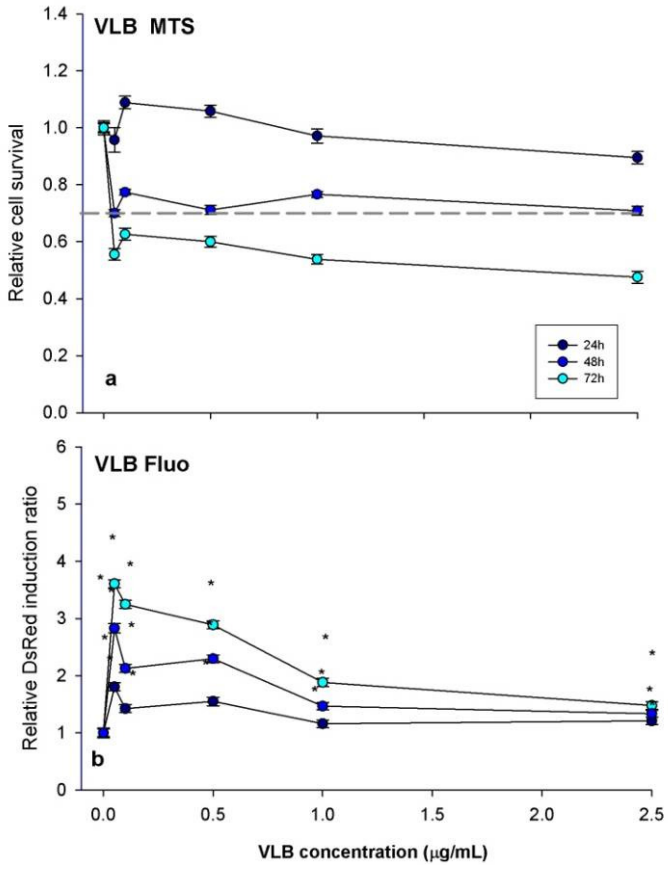


Figure 29: *Results of MTS assay (a), relative DsRed induction ratio 24, 48, 72h (b) of p21HepG2 DsRed cells after treatment with 0.05, 0.1, 0.5, 1.0, 2.5 µg/mL VLB.*

** $p < 0.05$. Dash line represents values of cell viability below 70% compare to control - untreated cells.*

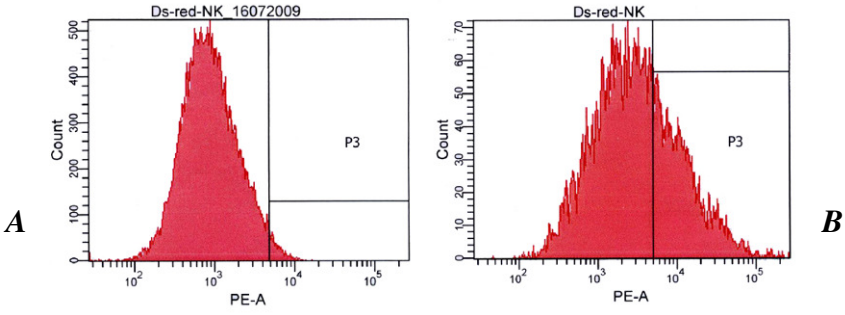
p21HepG2 DsRed and p21HepG2 EGFP showed comparable sensitivity to VLB regarding induction of reporter protein expression as well as the cytostatic effect.

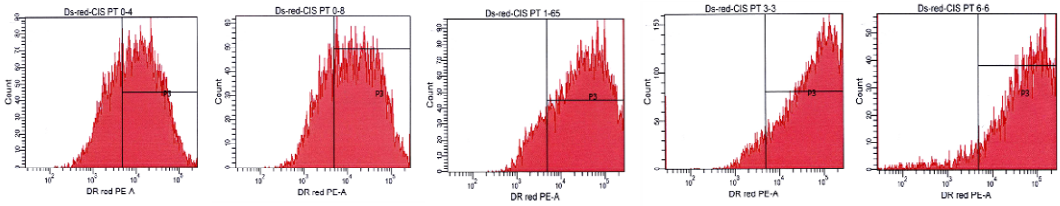
3.5.2 Validation of the biosensor system by flow cytometry

The biosensor system with p21HepG2 DsRed cells was further validated by flow cytometry measurement of the expression of reporter protein in cells exposed to MMS, BaP, CisPt and VLB.

Based on the results obtained by microplate reader, incubation time of 48 hours was selected, because at this time the fluorescence intensity was already high enough to measure statistically significant differences. The results are presented as fluorescence intensity histograms for CisPt and MMS, showing number of cells in relation to fluorescence intensity (Figure 30). Parental HepG2 cells were used as a negative control (Figure 30A) to determine the position of non-fluorescent cell population and to place the boundary for fluorescent cells (P3 label in histograms). The median of fluorescence intensity of control, untreated p21HepG2 DsRed cells was 77 (Figure 30B).

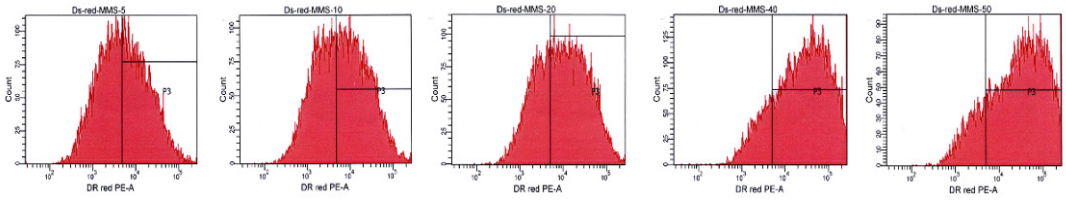
Histograms of p21HepG2 DsRed cells after treatment with CisPt and MMS showed clear dose dependent increase of fluorescence intensity with increasing concentration of selected chemicals. Higher increase of fluorescence intensity was observed for CisPt than for MMS (Figure 30C, 30D), with median of fluorescence intensity increasing from 156 for the lowest CisPt concentration to 317 at the 3.3 µg/mL CisPt concentration, where it reached a plateau value. The increase in fluorescence intensity ranged from 105 at the lowest concentration to 175 at the 40 µg/mL concentration in the case of MMS.





CisPt

0.41 µg/mL *0.82 µg/mL* *1.65 µg/mL* *3.30 µg/mL* *6.60µg/mL*



MMS

5 µg/mL *10 µg/mL* *20 µg/mL* *40 µg/mL* *50µg/mL*

Figure 30: *Histograms of HepG2 and p21HepG2 DsRed cells fluorescence intensity after treatment with CisPt and MMS*

Histograms of HepG2 cells (Figure 30A) and p21HepG2 DsRed cells (Figure 30B) – control cells. Figure 30C represent histograms of fluorescence intensity of p21HepG2 DsRed cells after treatment with 0.41, 0.82, 1.65, 3.3 and 6.6 µg/mL CisPt. Figure 30D represent histograms of fluorescence intensity of p21HepG2 DsRed cells after treatment with 5, 10, 20, 40 and 50 µg/mL MMS.

Table 2: *Comparison of increase in DsRed fluorescence ratio in p21HepG2 DsRed cells exposed to model genotoxic agents measured with flow cytometry and spectrofluorimetricaly*

Relative Fluorescence induction		
	Flow cytometry	Spectro-fluorimetry
MMS µg/mL		
0	1.00	1.00
5	1.36	2.75
10	1.44	2.89
20	1.74	4.78
BaP µg/mL		
0	1.00	1.00
0.05	0.97	1.83
0.13	1.94	2.10
0.25	2.59	2.67
CisPt µg/mL		
0	1.00	1.00
0.41	2.03	1.00
0.83	2.05	1.29
1.65	2.86	2.34
VLB µg/mL		
0	1.00	1.00
0.05	1.15	3.61
0.1	1.28	3.25
0.5	1.21	2.89

From Table 2 it can be seen, that measurement of DsRed expression by flow cytometry and spectrofluorimeter on 96-well microplates gave comparable results with BaP and CisPt, whereas with MMS and VLB lower induction was detected when measured with flow cytometer than with spectrofluorimeter. This result confirms high sensitivity of spectrofluorimetric detection of induction of reporter protein DsRed.

3.5.3 Validation of the p21HepG2 DsRed system by comet test

Comet assay is very sensitive method for detection of SSBs, DSBs, alkali-labile sites, DNA–DNA/DNA–protein cross-links and SSBs associated with incomplete excision repair at the level of single cells (Tice *et al.,* 2000). We compared the sensitivity of this method to detect DNA damage induced by model genotoxic agents with the sensitivity of measurement of the induction of expression of reporter gene. In all experiments BaP was used as a positive control.

The p21HepG2 DsRed cells were exposed to MMS, BaP, CisPt and VLB and the DNA damage was assessed with the comet assay after 24 h of exposure (Figures 31-34). For the comet assay only 24 h exposure has been used as the strand breaks detected with this method are transient and may be during the prolonged exposure repaired.

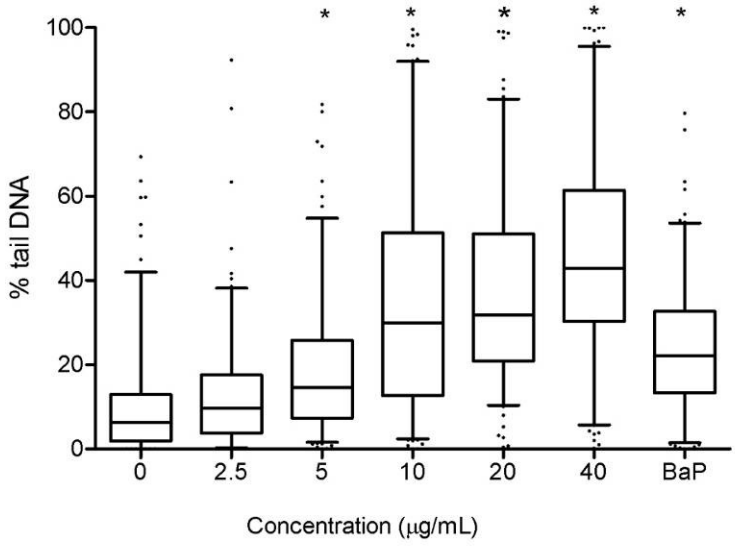


Figure 31: *Comet assay after 24 h of exposure to MMS induced DNA damage in p21HepG2 DsRed cells. The cells were exposed to 2.5, 5, 10, 20 and 40 μg/mL MMS for 24 h. The level of DNA strand breaks is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. The data are presented as quartile box plots. The edges of the box represent the 25th and the 75th percentiles, a line in the box present the median value, and the bars represent the 95% confidence intervals.* Significantly different from intreated control: p<0.05 (One Way ANOVA).*

A clear dose dependant increase in % tail DNA can be observed, which was significantly different from % tail DNA in control cells at concentrations ≥ 5 µg/mL MMS. The Lowest Observed Effect level (LOEL) concentration is 5 µg/mL MMS. At the same concentration also significant increase in expression of DsRed was observed.

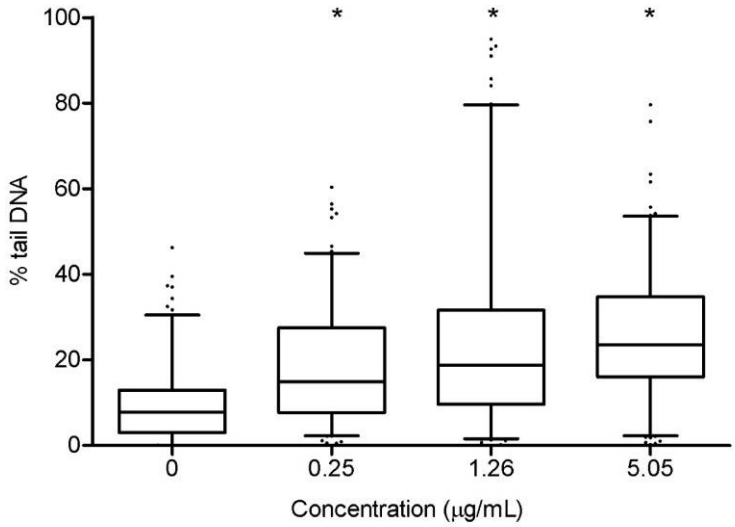


Figure 32: *Comet assay after 24 h of exposure to BaP induced DNA damage in p21HepG2 DsRed cells. The cells were exposed to 0.25, 1.26 and 5.05 µg/mL BaP for 24 h. The level of DNA strand breaks is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. The data are presented as quartile box plots. The edges of the box represent the 25th and the 75th percentiles, a line in the box present the median value, and the bars represent the 95% confidence intervals. * Significantly different from untreated control: $p < 0.05$ (One Way ANOVA).*

The LOEL at which BaP induced DNA strand breaks were significantly elevated was 0.25 µg/mL which is the same as was obtained with the measurement of DsRed fluorescence induction.

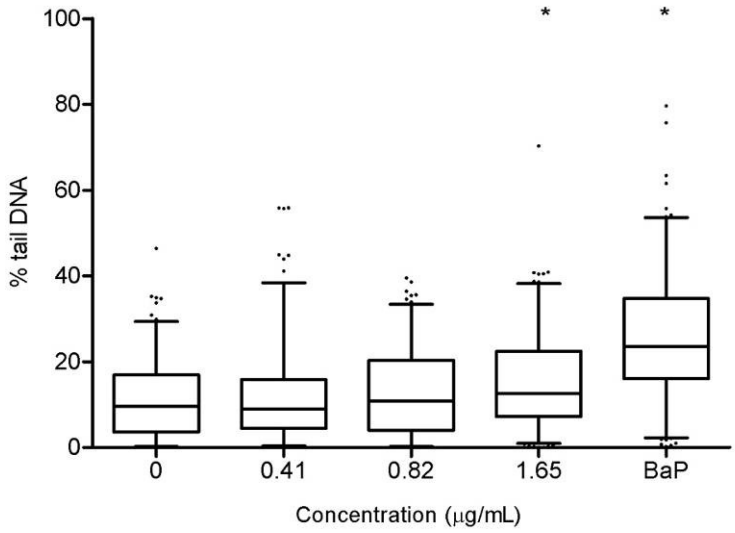


***Figure 33**: Comet assay after 24 h of exposure to CisPt induced DNA damage in p21HepG2 DsRed cells. The cells were exposed to 0.41, 0.82, and 1.65 µg/mL CisPt for 24 h. The level of DNA strand breaks is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. The data are presented as quartile box plots. The edges of the box represent the 25th and the 75th percentiles, a line in the box present the median value, and the bars represent the 95% confidence intervals. * Significantly different from untreated control: $p < 0.05$ (One Way ANOVA).*

Slight, although significant increase in % tail DNA was detected only at the highest tested concentration (1.65 µg/mL). With the measurement of the expression of DsRed fluorescence its significant increase was also observed at 1.65 µg/mL.

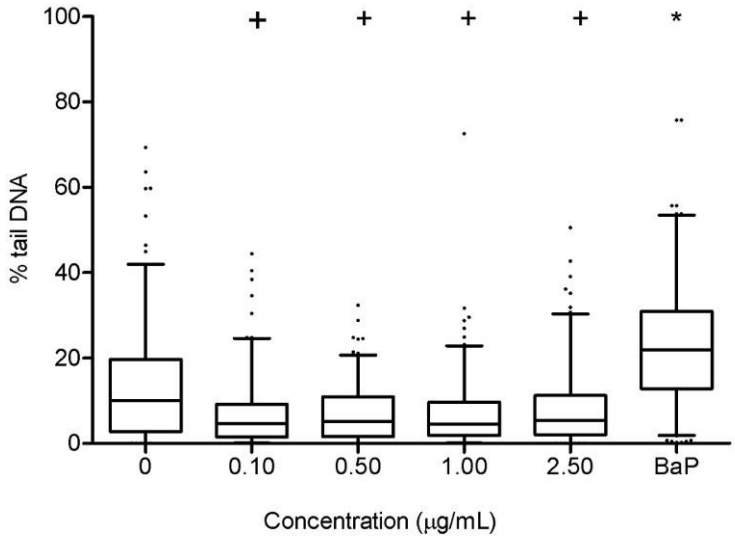


Figure 34: *Comet assay after 24 h of exposure to VLB did not induced DNA damage. The p21HepG2 DsRed cells were exposed to 0.10, 0.50, 1.00 and 2.50 μg/mL VLB for 24 h. The level of DNA strand breaks is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. The data are presented as quartile box plots. The edges of the box represent the 25th and the 75th percentiles, a line in the box present the median value, and the bars represent the 95 % confidence intervals. * Significantly different from untreated control: $p < 0.05$ (One Way ANOVA).*

VLB did not induce DNA strand breaks, which was expected as it is known to induce numerical chromosomal aberrations due through inhibition of polymerization of tubulin into microtubules and thus inhibits cell division without directly damaging DNA.

3.6 Comparison of the responses of the model genotoxic agents determined by EGFP and DsRed relative induction ratio, flow cytometry and by the comet assay

Comparison of the results EGFP vs. DsRed induction ratio showed some important data. In Table 3 showed the data from measurements of cell viability, relative EGFP

and DsRed induction ratio after 48 h, comet test after 24 h and flow cytometry determined induction ratio after 48 h post treatment with MMS, BaP, CisPt and VLB.

Table 3: *Comparison of the relative induction of EGFP in p21HepG2 EGFP and relative induction of DsRed fluorescence and DNA strand breaks in p21HepG2 DsRed cells, after exposure to model genotoxic agents.*

	EGFP		**DsRed**		**Flow**	**Comet**
	Viability (%)	EGFP ind. ratio	Viability (%)	DsRed ind. ratio	DsRed ind. ratio	DNA damage ind.
MMS						
0	100	1.00	100	1.00	1.00	1.00
5	117	0.92	94	2.75*	1.36	1.28*
10	106	1.16	111	2.89*	1.44	2.05*
20	109	1.33*	101	4.78*	1.74	1.86*
40	110	1.45*	93	9.65*	2.27	2.14*
50	104	1.51*	74*	13.04*	2.12	ND
BaP						
0	100	1.00	100	1.00	1.00	1.00
0.05	100	1.08	132	1.83	0.97	ND
0.13	109	1.33*	122	2.10	1.94	ND
0.25	104	1.82*	115	2.67*	2.59	1.43*
0.50	105	2.75*	106	5.75*	4.12	ND
1.26	99	6.41*	90	14.55*	2.32	2.52*
CisPt						
0	100	1.00	100	1.00	1.00	1.00
0.41	98	1.23	115	1.00	2.03	1.38
0.83	97	1.34	97	1.29	2.05	1.13
1.65	89	1.90*	90	2.34*	2.86	1.37*
3.30	71*	2.67*	84*	6.00*	ND	ND
6.60	51*	3.15*	56*	13.72*	ND	ND
VLB						
0	100	1.00	100	1.00	1.00	1.00
0.05	ND	ND	70*	3.61*	1.15	ND
0.10	81*	1.76*	77*	3.25*	1.28	0.55
0.50	80*	1.54*	71*	2.89*	1.21	0.47
1.00	85*	1.17	77*	1.88	ND	0.55
2.50	77*	1.07	71*	1.48	ND	0.71
5.00	70*	0.62	ND	ND	ND	ND

ND – not determined

From the Table 3 it can be seen, that p21HepG2 DsRed cells detected genotoxic effect induced by MMS and VLB at lower concentration than p21HepG2 EGFP cells. The genotoxic effect of CisPt was by both cell lines detected at the same concentration by both cell lines, whereas for detection of genotoxicity of BaP

p21HepG2 DsRed cells appear to be less sensitive. However, it cannot be excluded, that EGPF fluorescence in p21HepG2 EGFP cells observed at low concentration of BaP was not due to the fluorescence of BaP metabolites.

The measurement of the DsRed fluorescence by flow cytometry showed lower relative increase in DsRed fluorescence compared to spectrofluorimetric measurements. Significant increase of DsRed fluorescence was observed at higher concentrations as when measured with the spectrofluorimeter, indicating that detection of changes in DsRed fluorescence by flow cytometry is less sensitive than detection by spectrofluorimeter. The results also indicate that the sensitivity of measurement of DsRed expression is comparable to the sensitivity of detection of DNA strand breaks by the comet assay, except in the case of VLB, which is known that does not induce direct DNA damage.

3.7 Optimization and further validation of whole cell based biosensor system with p21HepG2 DsRed cells

Based on the obtained results with 4 model genotoxic compounds we decided to further validate the p21HepG2 DsRed cells and to optimize treatment conditions in a way to allow the use of single 96-well microtitre plate for both, DsRed fluorescence measurement and viability assessment. 48 h exposure was selected as suitable time point for both measurements.

3.7.1 Optimization of cell density for measurement of fluorescence and viability on the same microtiter plate at 48 h post-treatment

The prerequisite for measurement of both fluorescence and viability on the same microtitre plate is that the cell density is optimized in a way, that it is high enough to enable fluorescence detection and low enough, so that the cells do not overgrow the

available space. Therefore, different number of cells (30 000, 40 000 and 50 000) were plated on microtiter plates and changes in fluorescence intensity and viability of the cells were determined at 48 h post-treatment with BaP (Figure 35).

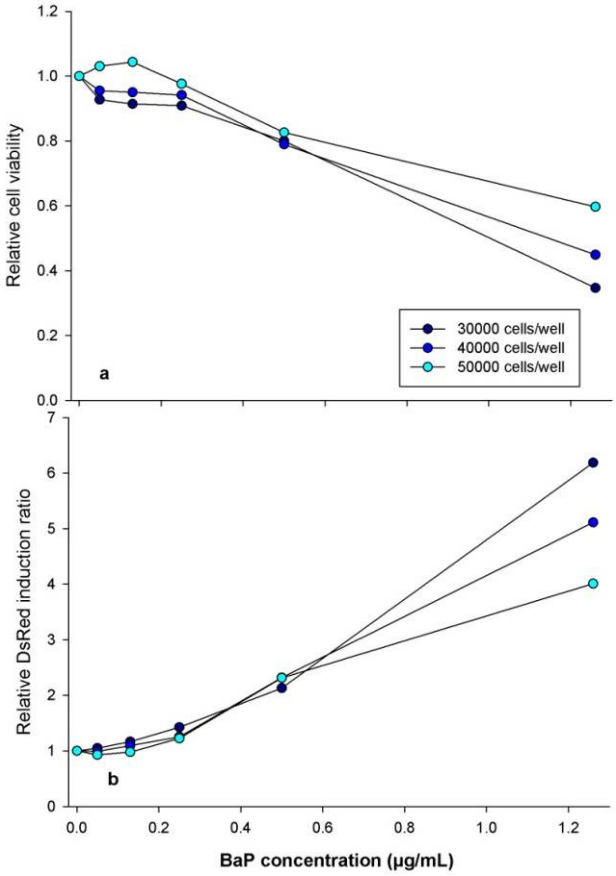


Figure 35: *Results of relative cell viability (a), and relative DsRed induction ratio 48 h (b) of p21HepG2 DsRed cells after treatment with 0.05, 0.13, 0.25, 0.5 and 1.26 µg/mL BaP at 30 000, 40 000 and 50 000 cells/well.*

The cell density did not significantly affect the changes in DsRed fluorescence detection. In line with previous experiments significant increase was observed at concentrations ≥ 0.25 µg/mL. The cell viability was reduced in a dose dependent manner, but there was no statistically significant difference between different cell densities.

However, because at cell density 50 000 cells/well control cells sometimes reached confluent stage already after 48 hours (Figure 38), 40 000 cells/well was chosen for further studies.

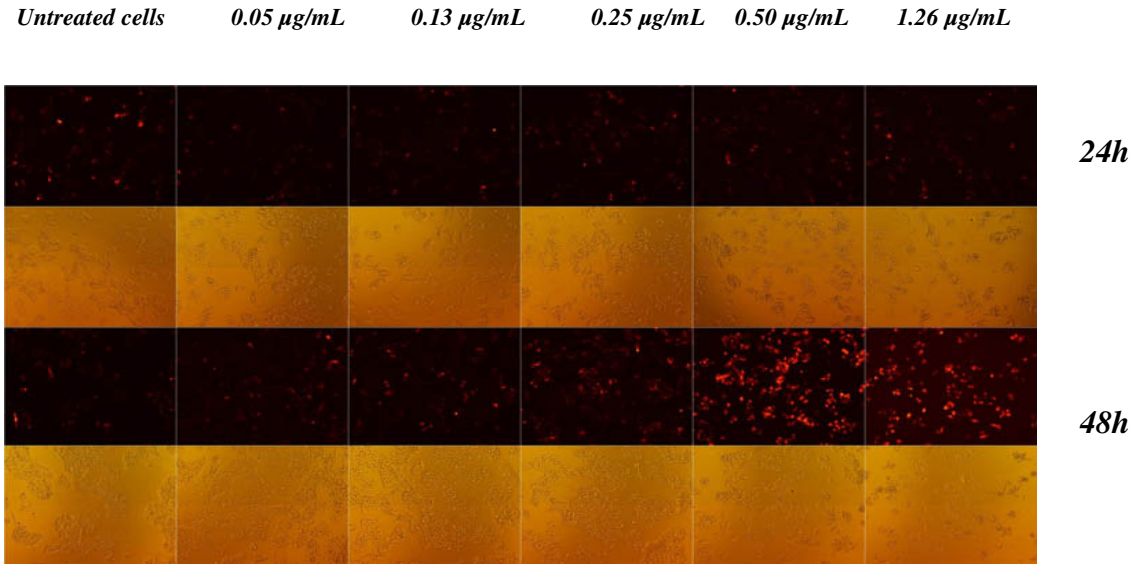


Figure36: *Relative p21HepG2 DsRed cells induction ratio at 30 000 cells/well after 24 h and 48 h and after treatment with 0.05, 0.13, 0.25, 0.5 and 1.26 µg/mL BaP.*

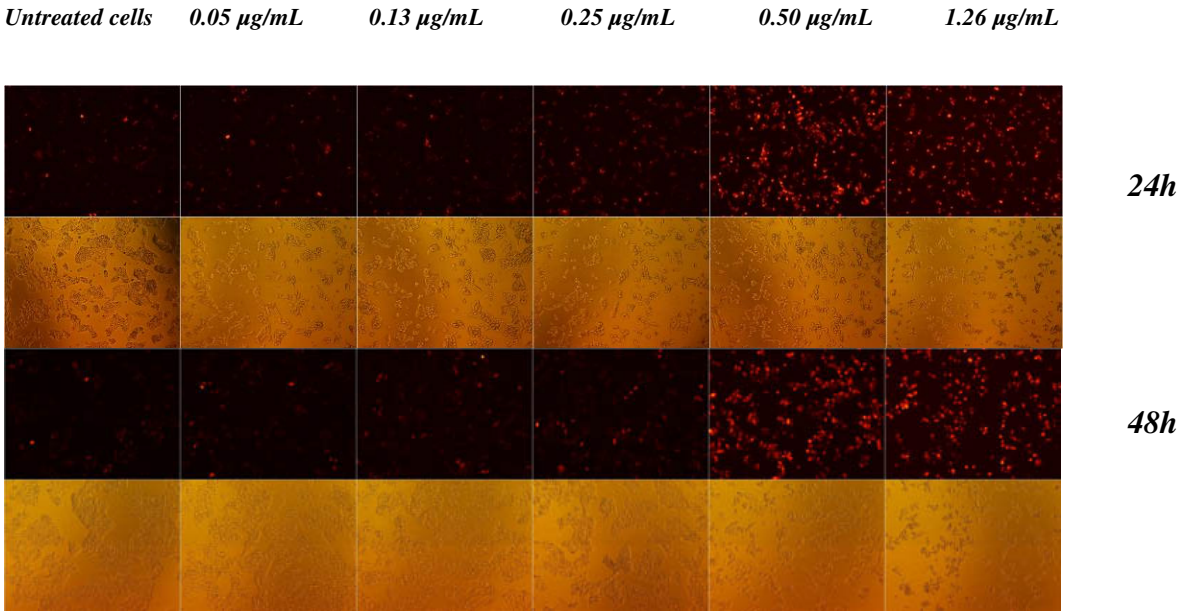


Figure 37: *Relative p21HepG2 DsRed cells induction ratio at 40 000 cells/well after 24 h and 48 h after treatment with 0.05, 0.13, 0.25, 0.5 and 1.26 µg/mL BaP.*

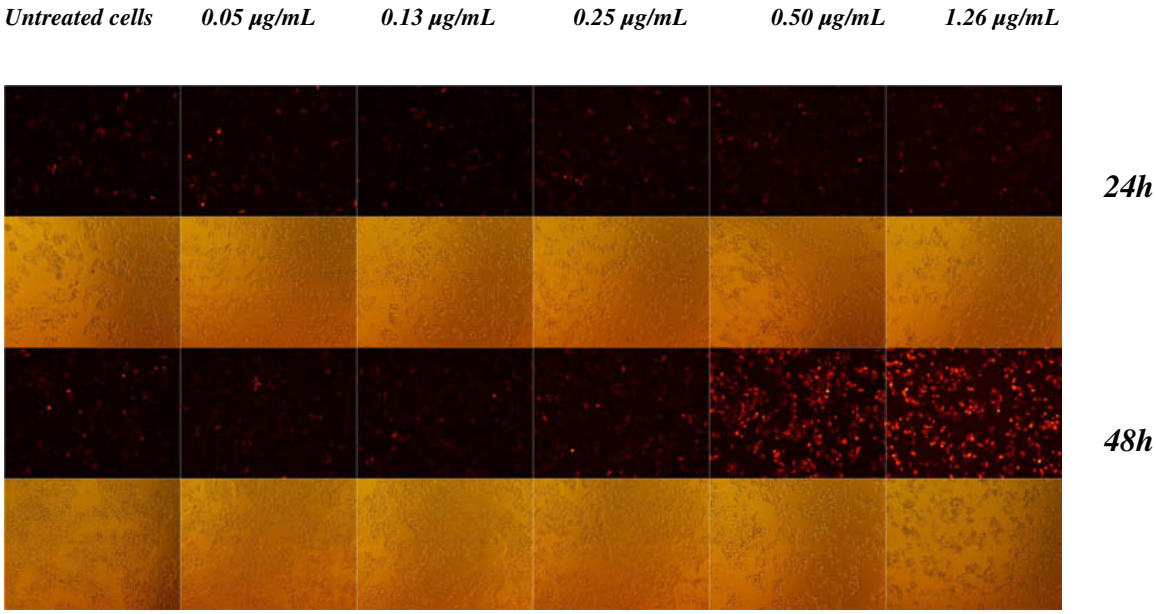


Figure 38: *Relative p21HepG2 DsRed cells induction ratio at 50 000 cells/well after 24 h and 48 h after treatment with 0.05, 0.13, 0.25, 0.5 and 1.26 µg/mL BaP.*

In figures from 36 to 38, increased fluorescence intensity of cells treated with BaP could be seen. Pictures were captured under visible and fluorescence light. Fluorescence intensity of cells increased with higher concentrations of BaP and with prolonged exposure time (24 h and 48 h).

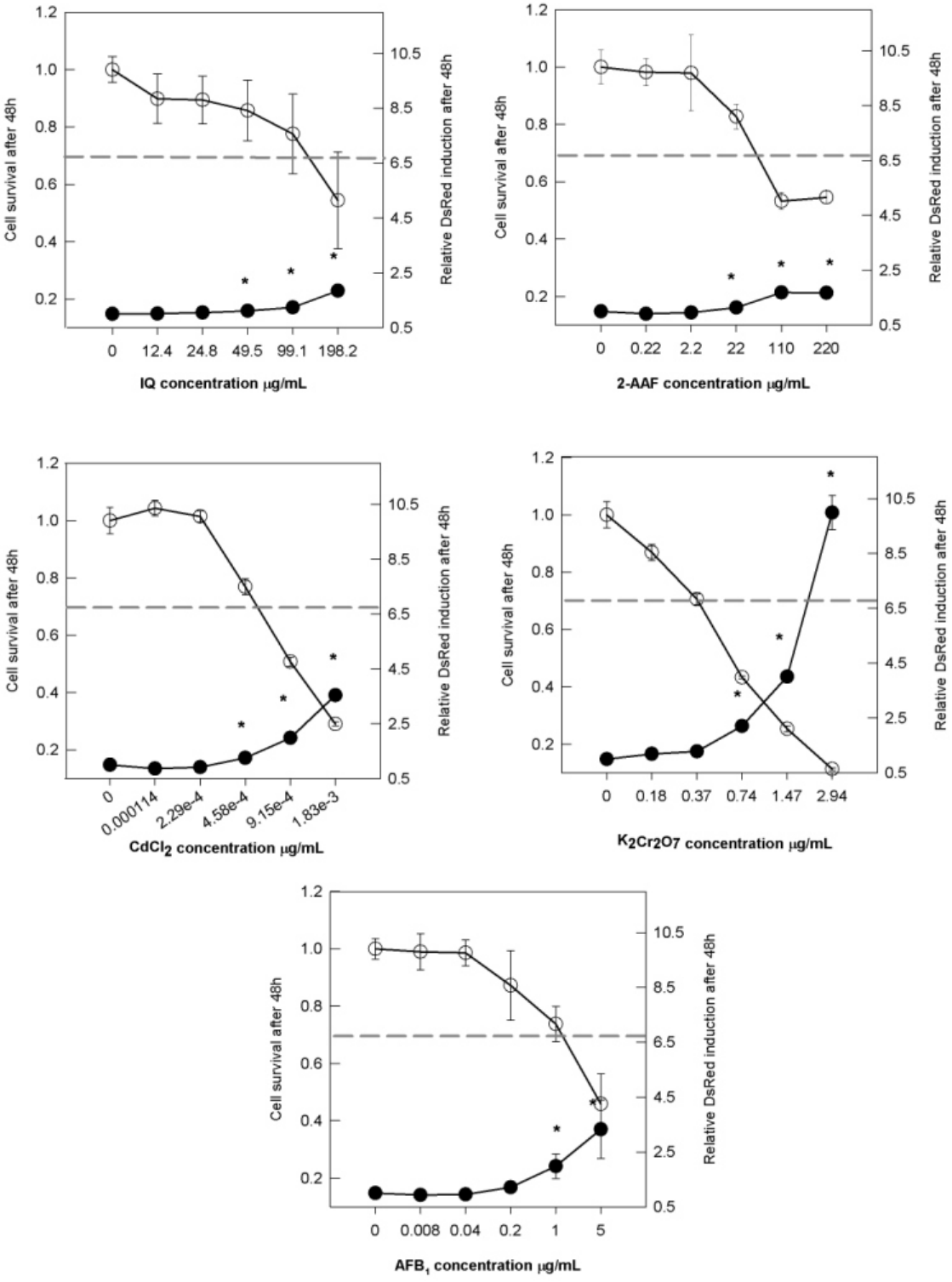
3.7.2 Further validation of the p21HepG2 DsRed whole cell based biosensor system with known genotoxic and non-genotoxic agents

In the validation process we included additional 16 chemicals that included genotoxic carcinogens, non-genotoxic carcinogen, genotoxic compounds that are not classified as carcinogens and non-genotoxic compounds.

3.7.2.1 The group of genotoxic chemicals

The group of genotoxic chemicals included: 2-Amino-3-methylimidazo[4,5-f] quinoline (IQ), 2-Acetylaminofluoren, Aflatoxin B1, Cadmium chloride ($CdCl_2$),

Potassium dichromate ($K_2Cr_2O_7$), *o*-toluidine and 8-hydroxyquinoline. The obtained results are shown on Figure 39.



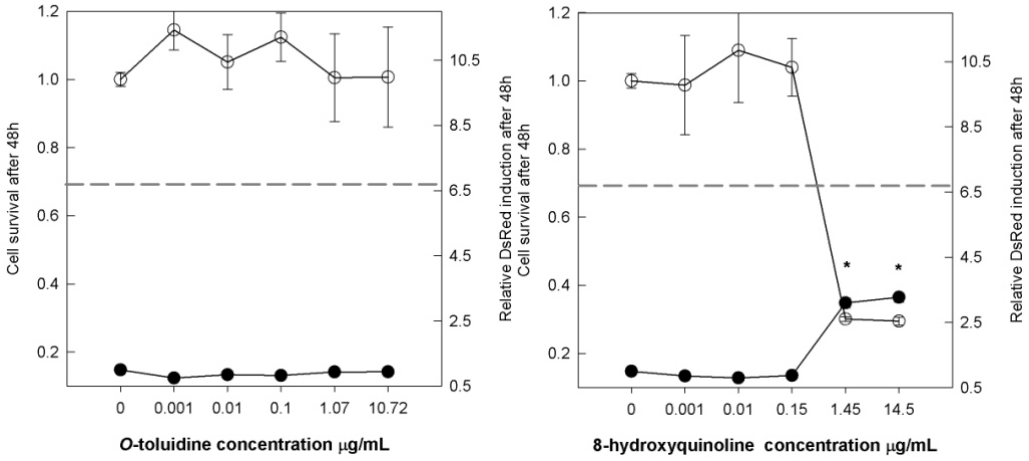


Figure 39. *Results of cell survival and relative DsRed induction after 48 h exposure of p21HepG2 DsRed cells to group of genotoxic chemicals*
Exposed concentrations were as follows: 12.4, 24.8, 49.5, 99.1, 198.2 μg/mL ***(IQ)****, 0.22, 2.2, 22, 110, 220 μg/mL* ***2-(AAF)****, , 0.000114, 0.000229, 0.000458, 0.000915, 0.00183 μg/mL* $CdCl_2$*, 0.18, 0.37, 0.74, 1.47, 2.94 μg/mL* $K_2Cr_2O_7$*, 0.008, 0.04, 0.2, 1, 5 μg/mL* AFB_1*, 0.001, 0.01, 0.1, 1.07, 10.72 μg/mL* ***o-toluidine*** *and 0.001, 0.01, 0.15, 1.45, 14.5 μg/mL* ***8-hydroxyquinoline****. Open circles (O) represent cell survival after 48 h. Closed circles represent (●) relative DsRed induction after 48 h. * $p < 0.05$ (One Way ANOVA).*

IQ is classified by IARC as *probably carcinogenic to humans (Group 2A)* based on sufficient evidence of benign and malignant tumor formation at multiple tissue sites in multiple species of experimental animals (IARC, 1993). Studies of the genotoxicity of IQ have given uniformly positive results in a wide variety of bacterial, plant, and animal assays, in systems providing metabolic activation. The genotoxicity of IQ depends on its metabolic activation to reactive intermediates by a two-step process involving N-hydroxylation by CYP1A2, followed by esterification of N-hydroxylamine by N-acetyltransferase or sulfotransferase to reactive ester derivatives that covalently modify DNA (Schut and Snyderwine, 1999). IQ induced mutations, chromosomal aberrations, sister chromatid exchanges, micronuclei and unscheduled DNA synthesis in various human cells in culture.

In p21HepG2 DsRed cells IQ induced significant increase of DsRed fluorescence at the three highest concentrations, whereas the cell viability was reduced by more than

30% only at the highest concentration (198.2 µg/mL). IQ gave clear positive response in p21HepG2 DsRed cells based genotoxicity test system.

2-AAF is a carcinogenic and mutagenic derivate of fluorene. 2-AAF is indirect acting genotoxic carcinogen that requires metabolic activation with CYP450 enzymes. 2-AAF is a bacterial mutagen (Dunkel *et al.,* 1984) in vitro it is positive in the MLA (Myhr and Caspary, 1988; Mitchell *et al.,* 1988), whereas in vivo in mice it induced chromosome aberrations and sister chromatid exchange (Luke *et al.,* 1988). 2-AAF is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals however IARC does not classify it. It used as a positive control by toxicologists to study the carcinogenicity and mutagenicity of aromatic amines. Shirai, 1997, used 2-AAF as potent hepatocarcinogen in his study for development a bioassay system for rapid detection of hepatocarcinogenicity of chemicals (Shirai, 1997).

In the present study, results of the exposure of p21HepG2 DsRed cells showed that 2-AAF induced significant dose dependant increase of relative DsRed fluorescence at the three highest concentrations (22 µg/mL, 110 µg/mL and 220 µg/mL). The cell viability was reduced by more than 30% at two the highest concentrations, thus we consider that 2-AAF was positive in the system with p21HepG2 DsRed cells.

Aflatoxins are secondary toxic fungal metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. They are known for their hepatotoxic and hepatocarcinogenic effects and are by IARC classified into Group 1 (IARC, 1993). **AFB_1** has consistently been found to be genotoxic. It causes DNA damage and mutation in bacteria. In cultured human and animal cells, it induces DNA damage, gene mutation and chromosomal damage. It has been shown to produce DNA-adducts and chromosomal damage in rodents following *in vivo* administration and also to produce DNA-adducts in humans *in vivo* (IARC, 1993). The genotoxicity of AFB_1 depends on its metabolic activation by hepatic cytochrome P450 enzyme system to produce a highly reactive intermediate, AFB_1-8,9-epoxide that covalently bind to N7 of guanine (Sharma and Farmer, 2004; Klein *et al.,* 2002).
In p21HepG2 DsRed cells AFB_1 induced significant dose dependant increase of relative DsRed expression at the two highest concentrations (1 µg/mL and 5 µg/mL).

The cell viability was reduced by more than 30% at the highest concentration (5 µg/mL). Thus p21HepG2 DsRed cells detected genotoxic effect of AFB_1.

Cadmium - Cd compounds are classified as human carcinogens by several regulatory agencies incuding International Agency for Research on Cancer (IARC, 1993). Cadmium affects cell proliferation, differentiation, apoptosis and other cellular activities and can cause numerous molecular lesions that would be relevant to carcinogenesis. For a long time cadmium has been considered as a non-genotoxic carcinogen, as it is only weakly mutagenic in bacterial and mammalian cell test systems. More recent experimental evidence suggests that cadmium at low, for environmental exposure relevant concentrations, induces mutations by inducing oxidative DNA damage and that it decreases genetic stability by inhibiting the repair of endogenous and exogenous DNA lesions, which in turn increase the probability of mutations and consequently cancer initiation by this metal (Filipic *et al.,* 2006).

In present study $CdCl_2$ induced significant increase of DsRed fluorescence at three highest concentrations post-treatment (Figure 39). At the two highest tested concentrations $CdCl_2$ reduced cell viability by about 50%, however a significant induction of DsRed fluorescence was observed at concentration that did not reduce cell viability by more than 30%, therefore $CdCl_2$ can be considered as positive in this assay.

$K_2Cr_2O_7$ belongs to hexavalent chromium compounds that are by IARC classified as group 1 human carcinogens (IARC, 1990). It has been shown to induce mutations in the Ames Salmonella assay, in yeast, and in mammalian cells, including sister chromatid exchanges, increased chromosome aberrations, inhibition of DNA synthesis and repair, and induction of dominant lethal mutations in mice. This material or product has not been evaluated for carcinogenicity in long term rodent carcinogenicity studies. In rats, $K_2Cr_2O_7$ by bronchial implantation did not produce an increase in lung tumors. However, epidemiological studies from around the world have consistently shown excess risks of lung cancer in workers involved in chromate and chromate pigment production. The studies do not clearly implicate specific chromium compounds, but implicate the class of compounds, chromium [VI] compounds, to which potassium dichromate belongs. IARC has concluded that there

is sufficient evidence in humans for the carcinogenicity of chromium [VI] compounds (IARC, 1990). The studies showed that the damage to living tissues exposed to chromium (Cr) (VI) is caused mainly by the production of reactive oxygen species or free radicals as soon as Cr (VI) is metabolized by the body into its penta-, tetra- and trivalent forms (Kimura, 2007). $K_2Cr_2O_7$ makes complexes with nucleic acids.

In p21HepG2 DsRed cells $K_2Cr_2O_7$ induced significant dose dependent increase in DsRed fluorescence at three highest concentrations (0.73 µg/mL, 1.47 µg/mL and 2.94 µg/mL) however at these concentrations the cell viability was reduced by more than 60% (Figure 39). $K_2Cr_2O_7$ is highly toxic for p21HepG2 DsRed cells therefore they are not suitable for evaluation of the genotoxicity of this compound.

***O*-toluidine** is aromatic amine. In bacterial assay system it showed negative or inconsistent data or at most, weakly positive results. In the yeast Saccharomyces cerevisiae, *o*-toluidine caused reverse mutation at some loci and occasionally recombinational events. It caused gain or loss of whole chromosomes and mutation of mitochondrial DNA. In cultured mammalian cells, it generally caused sister chromatid exchanges and sometimes also increased gene mutations, chromosomal aberrations and micronuclei. It induced aneuploidy and increased cell transformation in such cells. *O*-toluidine may inhibit intercellular communication. It has been demonstrated to be a mutagen but not a recombinogen in Drosophila melanogaster. In rodent models *in vivo*, it enhanced sister chromatid exchanges but gave equivocal results for micronuclei induction and sperm morphology. After oral administration to mice, it induced an increased incidence of haemangiomas and haemangiosarcomas and hepatocellular carcinomas or adenomas. In rats, oral administration of *o*-toluidine increased the incidence of tumours in multiple organs, including fibromas, sarcomas, mesotheliomas, mammary fibroadenomas and transitional-cell carcinomas of the urinary bladder. *O*-toluidine is classified as probably carcinogenic to humans (Group 2A) by IARC (IARC, 2000).

In p21HepG2 DsRed cells exposure to *o*-toluidine to concentrations up to 10 µg/mL (100 µM) did not induce increase in DsRed fluorescence neither affected the cell viability (Figure 39). Based on the available data on genotoxic activity of *o*-toluidine

we expected positive response in p21HepG2 DsRed test system. It is possible, that it was not activated by HepG2 cells due to their limited metabolic capacity. *O*-toluidine like other aromatic amines, it is thought to undergo metabolic activation initially via N-hydroxylation, leading to covalent binding to tissue macromolecules, including DNA. It is also possible that the lack of response is due to the use of to low concentrations of *o*-toluidine. In the comet assay with MCL-5 cells significant increase in DNA strandbreaks was observed at concentrations ≥ 1.7 mM (Martin *et al.,* 1999). It is also possible that the reason for the lack of positive response is in limited metabolic capacity of HepG2 cells to activate *o*-toluidine.

8-hydroxyquinoline was mutagenic in strain TA100 of Salmonella only in the presence of S9. *In vitro* in Chinese hamster ovary cells 8-hyfroxyquinoline induced sister chromatid exchange and gave a weak positive response in inducing chromosomal aberrations (Loveday *et al.,* 1990), whereas it was positive in the *in vitro* L5178Y tk+/tk- MLA (McGregor *et al.,* 1988). *In vivo* 8-hydroxyquinoline induced neither micronuclei (Shelby *et al.,* 1993) nor sister chromatid exchange (McFee, 1989). Carcinogenicity studies of 8-hydroxyquinoline in mice and rats by oral administration and subcutaneous injection or in mice by skin application gave positive or negative results of borderline significance. Positive results were obtained in bladder implantation experiments when 8-hydroxyquinoline was incorporated in cholesterol pellets but were negative when paraffin wax pellets were employed. Thus IARC concluded that no evaluation of the carcinogenicity of 8-ydroxyquinoline can be made on the basis of the available data and classified it into Group 3 (IARC, 1987).

Results in the present study showed that 8-hydroxyquinoline induced significant dose dependent of relative DsRed induction only at the two highest concentration (1.45 and 14.5 µg/mL) at which the cell viability was reduced by about 70% (Figure 39). Thus 8-hydroxiquinoline should be considered as negative in the test system with p21HepG2 DsRed cells.

3.7.2.2 The group of non-genotoxic chemicals

In group of chemicals that are considered as non-genotoxic we included: Xanthohumol, d-Mannitol, Boric acid (B_2O_3), Ethylenediaminetetraacetic acid (EDTA), Sodium chloride (NaCl), Ethanol (C_2H_5OH), N,N-dimethylformamide (DMF), Saccharose (Sucrose) and Vitamine C (Ascorbic acid). The results are shown in Figure 40.

Xanthohumol is a prenylated flavonoid derived from the female flowers of the hops plant (Humulus lupulus L.). It has been shown to have protective effect against genotoxic effects of IQ, while it was not mutagenic in Salmonella reverse mutation assay and did not induce DNA stran breaks in HepG2 cells (Plazar *et al.,* 2007). In p21HepG2 DsRed cells xanthohumol did not induce increase in DsRed expression, neither decreased the cell viability.

D-mannitol is a naturally occurring six-carbon sugar alcohol or polyol. It is the most abundant polyol in nature occurring in bacteria, yeasts, fungi, algae, lichens and several plants (Wisselink *et al.,* 2002). It is used as sugar replacement. Genotoxicity studies showed negative results in Salmonella reverse mutation assay (Haworth *et al.,* 1983) *in vitro* chromosome aberration assay (Gulati *et al.,* 1989), *in vitro* MLA (Myhr and Caspary, 1991) and in *in vivo* micronucleus assay (Shelby *et al.,* 1993). In present study relative d-manitol did not induce increase in DsRed fluorescence and did not affect cell viability.

Boric acid is used for different medicinal and nonmedicinal purposes (Heindel *et al.,* 1997). It is also used as a food preservative (E284). The limited number of studies showed that it was not mutagenic in the Salmonella/microsome test system, did not increase the sister chromatid exchanges in Chinese hamster ovary (CHO) cells (National Toxicology Program, 1987) and was not mutagenic in MLA (McGregor et al., 1988).

In p21HepG2 DsRed cells boric acid induced increase in DsRed fluorescence at three highest concentrations, while it reduced cell viability by more than 30% only at the highest tested concentration. Therefore it showed clear positive response in this test

system. Although in previous genotoxicity assays boric acid was negative, it was genotoxic in the bacterial SOS assay with *E. coli* PQ37 strain in the presence and absence of the S9 mix (Odunola, 1997), and recent study showed that it clearly induced chromosomal aberration in peripheral human lymphocytes at similar concentrations (Arslan *et al.,* 2008). This may explain the observed positive response in the system with p21HepG2 DsRed.

EDTA is polyamino carboxylic acid, a colourless, water-soluble solid and a widely applied synthetic agent, characterized by highly persistence. EDTA is in widespread use and known as a persistent organic pollutant (Zhiwen *et al.,* 2006) and is used in many laboratory applications. In test system with p21HepG2 DsRed cells EDTA induced significant dose dependent increase in DsRed fluorescence at two highest concentrations (490 µg/mL and 980 µg/mL) at which cell viability was reduced by more than 30%.

Sodium chloride or salt is an ionic compound. It has an interesting property of the solubility in water that changes very little with temperature. It is used industrially as the starting point for a range of sodium-based products. Commonly is sodium chloride used as food preservative. It has a key role in biological systems in maintaining electrolyte balances. In experiments for validation of the new cell based biosensor system it did not induce increase in DsRed fluorescence neither affected the cell viability.

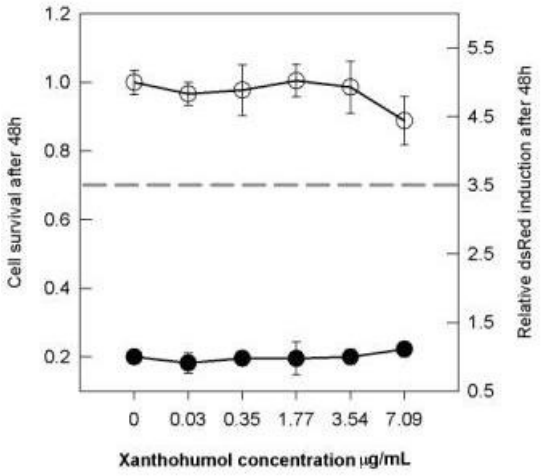
Ethanol is a volatile, flammable, colorless liquid. Ethanol has widespread use as a solvent of substances intended for human contact, including scents, flavorings, colorings, and medicine. It is not a carcinogen but the first metabolic product of ethanol, acetaldehyde is toxic, mutagenic and carcinogenic. In Salmonella reverse mutation assay ethanol did not induce mutations (Zeiger *et al.,* 1992). In experiments for validation of the new cell based biosensor system ethanol did not induce increase in DsRed fluorescence and did not reduce cell viability.

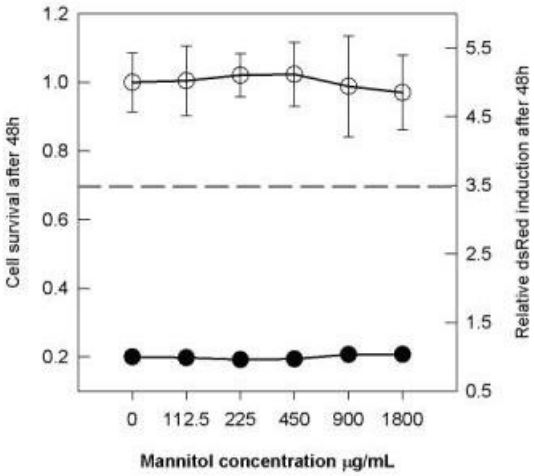
DMF is the organic compound, colorless to very slightly yellow liquid. Primarily is DMF used as a solvent in the production of polyurethane products and acrlylic fibers. It is also used in the pharmaceutical industry, in the formulation of pesticides, and in

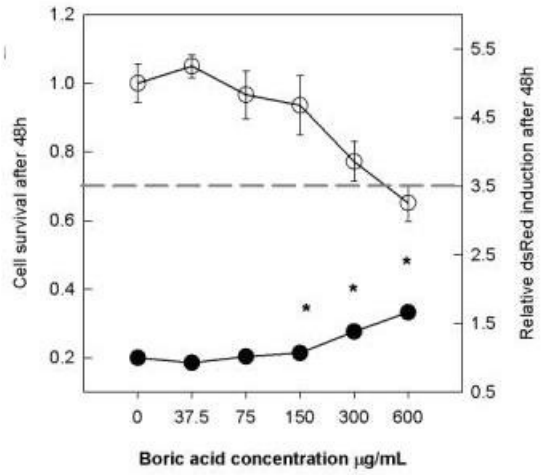
the manufacture of synthetic leathers, fibers and films (Gescher, 1993). DMF is not a bacterial mutagen (Mortelmans *et al.,* 1986), *in vitro* it did not induce chromosome aberrations, while in MLA two studies showed negative result (Mitchell *et al.,* 1988; Myhr and Caspary, 1988), and one showed positive response (McGregor *et al.,* 1988). In the test system with p21HepG2 DsRed cells DMF was negative.

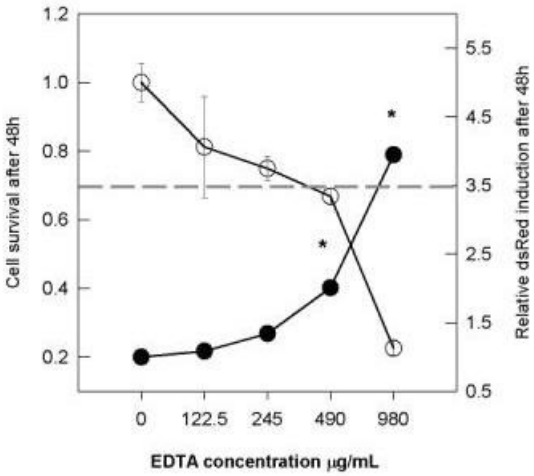
Saccharose is an organic compound and the molecule of saccharose exists as a single isomer. Saccharose has been included in three *in vitro* mouse lymphoma genotoxicity studies and was in all three studies negative (Mitchell et al., 1988; Myhr and Caspary, 1988; McGregor et al., 1987). Saccharose was negative also in the test system with p21HepG2 DsRed cells.

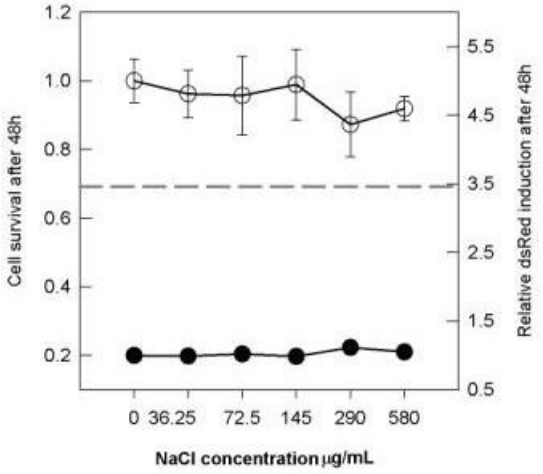
Vitamine C is an important dietary antioxidant and significantly decreases the adverse effect of reactive species such as reactive oxygen that can cause oxidative damage to macro-molecules such as lipids, DNA, and proteins, which are implicated in chronic diseases including neurodegenerative diseases (Halliwell and Gutteridge, 1999; Packer *et al.,* 2002). Vitamin C was negative in Salmonella reverse mutation assay (Zieger *et al.,* 1988), *in vitro* it did not induce chromosome aberrations, but induced sister chromatid exchange (Gulati *et al.,* 1989), and gave equivocal result *in vitro* MLA (Myhr and Caspary, 1991). *In vivo* vitamin C induced micronuclei formation (Shelby *et al.,* 1993). In p21HepG2 DsRed cells vitamin C did not induce increase in DsRed fluorescence and did not affect cell viability.

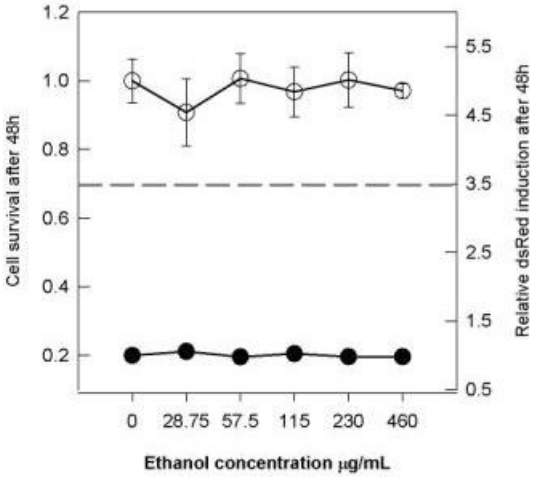

Cell survival after 48h
Relative dsRed induction after 48h
0 0.03 0.35 1.77 3.54 7.09
Xanthohumol concentration μg/mL


Cell survival after 48h
Relative dsRed induction after 48h
0 112.5 225 450 900 1800
Mannitol concentration μg/mL


Cell survival after 48h
Relative dsRed induction after 48h
0 37.5 75 150 300 600
Boric acid concentration μg/mL


Cell survival after 48h
Relative dsRed induction after 48h
0 122.5 245 490 980
EDTA concentration μg/mL


Cell survival after 48h
Relative dsRed induction after 48h
0 36.25 72.5 145 290 580
NaCl concentration μg/mL


Cell survival after 48h
Relative dsRed induction after 48h
0 28.75 57.5 115 230 460
Ethanol concentration μg/mL

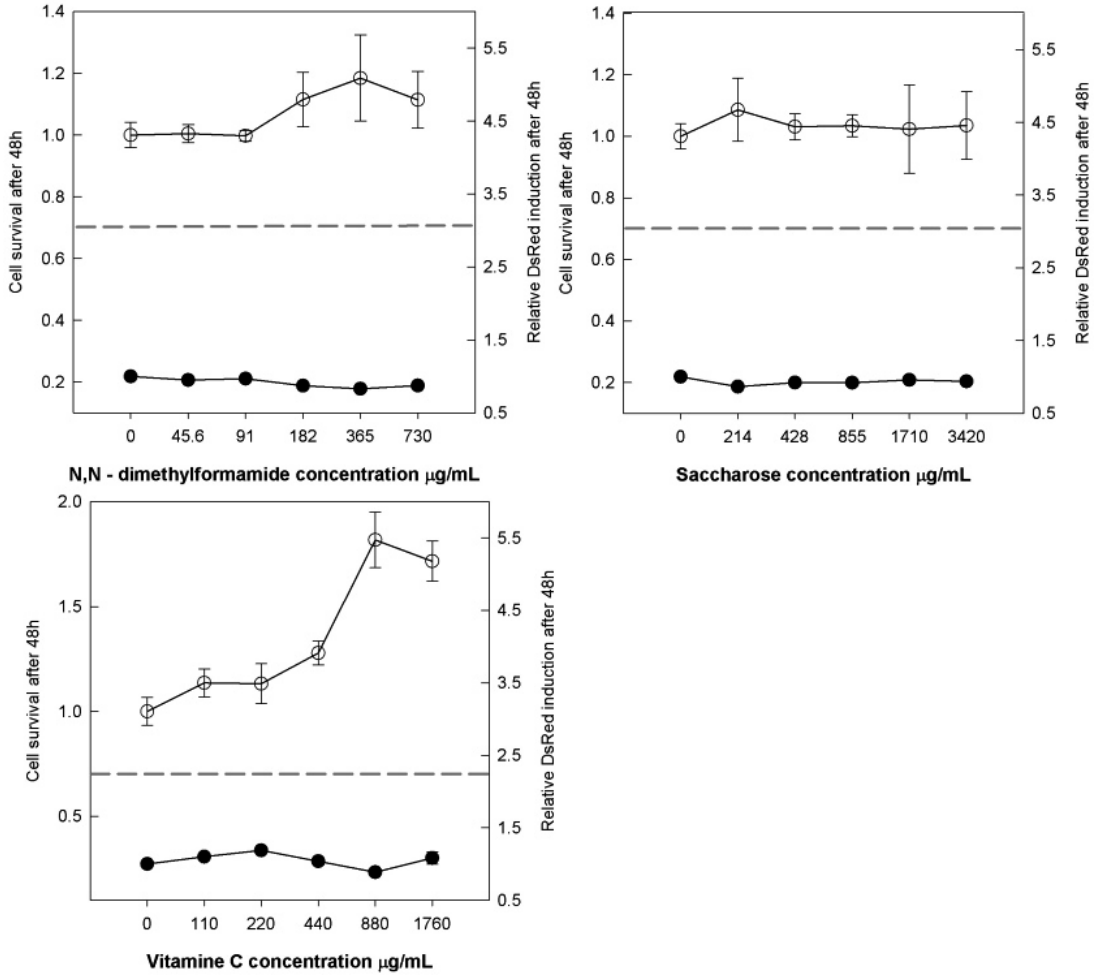


Figure 40: *Results of cell survival and relative DsRed induction after 48 h exposure of p21HepG2 DsRed cells to group of non-genotoxic chemicals.*

Exposed concentrations were as follows: 0.03, 0.35, 1.77, 3.54, 7.09 μg/mL ***Xanthohumol****, 112.5, 225, 450, 900, 1800 μg/mL* ***Mannitol****, 37.5, 75, 150, 300, 600 μg/mL* ***Boric acid****, 122.5, 245, 490, 980 μg/mL* ***EDTA****, 36.25, 72.5, 145, 290, 580 μg/mL* ***NaCl****, 28.75, 57.5, 115, 230, 460 μg/mL* ***Ethanol****, 45.6, 91, 182, 365, 730 μg/mL* ***DMF****, 214, 428, 855, 1710, 3420 μg/mL* ***Saccharose*** *and 110, 220, 440, 880, 1760 μg/mL* ***Vitamine C****. Open circles (O) represent cell survival after 48 h. Closed circles represent (●) relative DsRed induction after 48 h.* p<0.05 (One Way ANOVA).*

3.7.3 Overall performance of the genotoxicity test system with p21HepG2 DsRed cells

The results of the testing of 20 compounds of which 11 are considered genotoxic and 9 non-genotoxic show that all genotoxic compounds were detected as positive while of the non-genotoxic chemicals one gave positive response (Table 4). Although the number of tested chemicals is not high enough to drive any final conclusion on the specificity and sensitivity of the developed test sytem these data are promising. It is important, that with p21HepG2 DsRed cells we detected positive response with indirect acting genotoxins: BaP, IQ, 2-AAF and AFB_1 in the absence of exogenous metabolic activation. Currently best validated *in vitro* mammalian cell based reporter gene tests system GreenScreen that uses metabolically inactive human lyphoblastoid TK6 cell line is not able to detect indirect genotoxins without metabolic activation. Due to the problems with interference of S9 mixture with EGFP fluorescence, it is not possible to use this assay in microtiter plate format. Thus for the detection of indirect acting genotoxins EGFP fluorescence is measured by flow cytometry, which is more complicated and also costly method. Regarding the sensitivity for detection indirect acting genotoxins with p21HepG2 DsRed cells genotoxic response was detected at 0.13 µg/mL, whereas with GreenScreen it was detected at nearly tenfold higher concentration (1.25 µg/mL). The sensitivity for detection the genotoxicity of IQ and 2-AAF were comparable, whereas genotoxicity of AFB_1 was with GreenScren detected at 25 fold lower concentration (0.04 µg/mL) than with p21HepG2 DsRed cells (1 µg/mL).

Table 4: *Results of 20 compounds tested with p21HepG2 DsRed cells and data from GreenScreen HC, standard genotoxicity tests and carcinogenicity*

Compound	Max. test. conc		p21HepG2 DsRed				GreenScreen HC				Standard genotoxicity tests				Rodent carcino-genicity (IARC)
			Cytotoxicity		Genotoxicity		Cytotoxicity		Genotoxicity						
	mM	µg/mL		LEC µg/mL		LEC µg/mL		LEC µg/mL		LEC µg/mL	Ames test	In vitro CA/MN	MLA	In vivo CA/MN	
Genotoxic compounds															
Methyl methansulphonate	0.454	50	NT		P	10	T	25	P[1]	25	P	P	P	P	P (2A)
Benzo[a]piren	0.005	1.26	NT		P	0.13		-	P[2]	1.25	P	P	P	P	P (1)
Cisplatin	0.03	6.6	T	6.6	P	1.65	T	4	P[1]	1	P	P	P	P	P (2A)
Vinblastine	0.005	5	T	0.05	P	0.05	T	0.02	P[1]	0.02	N	P	P	P	N/P (3)
2-amino-3-imidazo[4,5-f] quinoline	1	198	T	198	P	50	-		P[2]	30	P	P	-	N	P (2A)
2-Acetylaminofluorene	1	220	T	110	P	22	-		P[2]	50	P	-	P	P	P
Aflatoxin B1	0.016	5	T	5	P	1	-		P[2]	0.04	P	P	P	P	P (1)
Cadmium chloride	0.01	1.83	T	0.91	P	0.45	-		P[2]	1.8	N	P	nd	P	P (1)
Potasium dichromate	0.01	2.94	T	0.36	P	0.73	-		-		P	P	nd	nd	P (1)
8-hydroxyquinoline	0.1	14.5	T	1.45	P/N	1.45	-		-		P	P	P	N	N/P (3)
o-toluidine	0.1	10.7	NT		N		-		-		N/P	N/P	-	N/P	P (2B)
Non-genotoxic compounds															
N,N-dimethylformamide	10	730	NT		N		-		-		N	N	N/P	-	N/P (3)
Xanthohumol	0.02	7.1	NT		N		-		-		N	N	-	-	-
d-manitol	10	1800	NT		N		NT		N[1]		N	N	N	N	-
Boric acid	10	600	T	600	P	300	T	620	N[1]		N/P	N	N	-	N
EDTA	10	2922	T	490	P/N	490	T	895	N[1]	2061	N	N	N	P	-
NaCl	10	584	NT		N		NT		N[1]		N	N	N	N	-
Ethanol	10	460	NT		N		NT		N[1]		N	N	N	N	-
Vitamine C	10	1761	NT		N						N/P	N	N/P	-	-
Saccharose	10	3423	NT		N		NT		N[1]		N	N	N	N	-

[1] Data from Hastwell et al. (*Hastwell et al.,* 2006).

[2] Data from Jagger et. al., (Jagger et al., 2009). In the presence of S9 mix, EGFP expression has been detected by flow cytometry. NT-non-toxic; T-toxic; N-negative; P-positive; N/P-negative and positive results.

4 CONCLUSIONS

We develop a method for rapid and sensitive detection of agents that cause DNA damage using stably transfected metabolically active human hepatoma HepG2 cells. Cells were transfected with plasmids containing promoter of DNA damage responsive gene *p21* fused to gene coding for EGFP and DsRed.

We confirmed our hypothesis that exposure of stably transfected human cell line HepG2 containing reporter gene for EGFP or DsRed fused to promoter of DNA damage responsive gene *p21* to genotoxic agents will result in increased production of EGFP and DsRed proteins, which we measured by increase in fluorescence intensity.
Furthermore, in order to establish the whole cell biosensor system we achieved the following specific tasks:

- Plasmid containing CMV promoter and promoters for *p21* fused with reporter gene coding for EGFP was prepared.
- Plasmid containing promoter for *p21* fused with reporter gene coding for DsRed was prepared.
- Optimisation processes of electroporation protocol for optimal transfection of the HepG2 cell line was performed.
- Stably transfected cell line HepG2 with inducible expression of reporter genes EGFP and DsRed were made.
- The responsiveness of p21HepG2 EGFP and p21hepG2 DsRed cells to genotoxic insult was first evaluated by exposure to model genotoxic agents: MMS, CisPt, BaP and VLB.
- The responsiveness of p21hepG2 DsRed cells (the sensitivity of the induction of reporter gene expression after exposure to genotoxic agents) was compared with the induction of DNA damage measured with the comet assay, which is one of the most sensitive methods for detection of genotoxic agents and the results showed good correlation.
- The increase in fluorescence intensity after exposure to model genotoxic agents obtained by the spectrofluorimetric measurement on the microplate

was compared to the fluorescence intensity after measured with the flow cytometry, which is one of the most sensitive methods for measuring fluorescence intensity per cell and the results showed good correlation.

- The test system was then developed into a 96-well microtiter plate format that allows for simultaneous testing of 2 compounds at 6 different concentrations and 6 parallels using the same plate for reporter gene DsRed quanitification and assessment of cell viability.
- Using this microtitre plate format the biosensor system with stably transfected cell p21HepG2 DsRed cells was further validated by exposure to additional 16 different compounds (carcinogenic and non-carcinogenic) and the results showed high sensitivity and specificity.

The major advantage of p21HepG2 DsRed test system over other currently available mammalian cell based reporter gene test systems is its ability of to detect indirect acting genotoxic agents.

After further validation of the test system, which is currently in progress, this cell based biosensor system based on *p21* gene expression can become a valuable tool with potential applications in the fields of chemical and drug safety evaluation as well as for environmental and occupational monitoring of exposure to chemical agents.

5 SUMMARY

Human exposure to genotoxic chemicals is a common and serious problem in our society. The harmful effects on human and environmental health should be identified for the safe use of new chemicals which are used for commercial and industrial purpose. The aim of this dissertation was to develop a human cell based biosensor system for simple and fast detection of genotoxic chemicals.

The metabolically active human hepatoma HepG2 cell line was used for preparation of stably transfected cell lines, with plasmids encoding EGFP and DsRed under the control of *p21* promoter. The stably transfected cell lines were named p21HepG2 EGFP and p21HepG2 DsRed. The cell based biosensor system was tested by induction of DNA damage with genotoxic chemicals with known mechanism of action. Fluorescence intensity of transfected cells, due to EGFP and DsRed expression after treatment with genotoxic chemicals, normalized to viability of treated cells (fluorescence induction ratio) was used as a measure of genotoxicity.

Exposure of cells to MMS and VLB showed that p21HepG2 DsRed cells detected genotoxic effect at lower concentration than p21HepG2 EGFP cells. The genotoxic effect at CisPt was detected at the same concentration in both cell lines, whereas p21HepG2 DsRed cells seem to be less sensitive for detection of genotoxicity of BaP. Furthermore, in the validation processes, p21HepG2 DsRed cells were exposed to 16 other genotoxic and non-genotoxic chemicals. A positive response with indirect acting genotoxins: BaP, IQ, 2-AAF and AFB_1 in the absence of exogenous metabolic activation was detected.

The results of our study demonstrated that a stably transformed cell lines p21HepG2 EGFP and p21HepG2 DsRed can be used as a fast and simple biosensor system for detection of genetic damage caused by genotoxic chemicals.

Key words: HepG2 cells, stable transformed cell lines, biosensor system

POVZETEK

Izpostavljenost človeka genotoksičnim snovem predstavlja resen problem v današnji družbi. Za varno uporabo novih kemikalij, namenjenih tako v komercialne kot tudi industrijske namene, je potrebno ugotavljati njihove škodljive učinke, če govorimo o zdravju človeka ter skrbi za okolje. Cilj doktorske naloge je bil razviti celični biosenzorski sistem za enostavno in hitro odkrivanje genotoksičnih snovi.

Metabolno aktivne človeške jetrne celice (HepG2) smo uporabili za pripravo genetsko modificiranih (transfeciranih) celičnih linij. V celice smo z elektroporacijo vnesli plazmida pod kontrolo promotorja za gen *p21* (pp21HepG2 EGFP in p21HepG2 DsRed), ki nosita zapis za zeleno in rdeče fluorescirajoči protein. Stabilno transfecirane celične linije smo poimenovali p21HepG2 EGFP in p21HepG2 DsRed. Celice smo testirali tako, da smo poškodbe DNA povzročili z genotoksičnimi snovmi s poznanimi mehanizmi delovanja. Povečana intenziteta fluorescence (EGFP in DsRed) transfeciranih celic je bila po izpostavljenosti genotoksičnim snovem normalizirana na preživetje tretiranih celic (stopnja indukcije fluorescence) in uporabljena kot merilo za genotoksičnost.

Izspostavljenost celic MMS in VLB je pokazala, da so p21HepG2 DsRed celice zaznale genotoksični učinek pri nižji koncentraciji kot pa p21HepG2 EGFP celice. Genotoksični učinek pri CisPt je bil zabeležen pri enaki koncentraciji pri obeh celičnih linijah. Celice p21HepG2 DsRed so bile manj občutljive na zaznavanje genotoksičnih sprememb pri BaP. Za dodatno potrditev delovanja novega biosenzorskega sistema, so bile celice p21HepG2 DsRed izpostavljene tudi 16 drugim genotoksičnim in negenotoksičnim snovem. Pozitiven odgovor je bil dokazan pri posredno delujočih genotoksičnih snoveh : BaP, IQ, 2-AAF in AFB_1 ob odsotnosti eksogene metabolične aktivacije.

Rezultati naše študije so pokazali, da stabilno preoblikovane celične linije p21HepG2 EGFP in p21HepG2 DsRed lahko uporabimo kot hiter in enostaven biosenzorski sistem za odkrivanje genetskih poškodb, ki jih povzročijo genotoksične snovi.

Ključne besede: HepG2 celice, stabilno transfecirane celične linije, biosenzorski sistem

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ANNEX A: Laboratory results (EGFP)

ANNEX A: Cell viability and induction of EGFP fluorescence in p21 HepG2 EGFP cells exposed to MMS, BaP, CisPt and VLB for 24, 48, 72, 120 and 168 h.

MMS

	24 hours			48 hours			72 hours			120 hours			168 hours		
Conc. µg/ml	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]
0	100 ± 0.05	8.8 ± 0.87	1.00 ± 0.10	100 ± 0.02	12.8 ± 1.29	1.00 ± 0.10	100 ± 0.03	15.4 ± 1.58	1.00 ± 0.10	100 ± 0.06	10.5 ± 1.42	1.00 ± 0.14	100 ± 0.06	13.4 ± 1.94	1.00 ± 0.14
5.00	102 ± 0.04	9.4 ± 0.98	1.04 ± 0.10	117 ± 0.04	13.8 ± 1.79	0.92 ± 0.12	91 ± 0.04	16.1 ± 1.34	1.14 ± 0.09	79 ± 0.06	12.9 ± 1.03	1.56 ± 0.12	67 ± 0.08	16.7 ± 1.11	1.85 ± 0.11
10.00	108 ± 0.03	10.6 ± 1.17	1.11 ± 0.12	106 ± 0.02	15.8 ± 1.48	1.16 ± 0.11	104 ± 0.03	18.7 ± 2.08	1.16 ± 0.12	87 ± 0.05	18.5 ± 2.16	2.02 ± 0.17	63 ± 0.05	20.6 ± 1.13	2.43 ± 0.11
20.00	104 ± 0.04	10.8 ± 1.06	1.17 ± 0.11	109 ± 0.02	18.6 ± 1.47	1.33 ± 0.11	107 ± 0.03	22.6 ± 1.58	1.37 ± 0.10	79 ± 0.06	23.3 ± 0.87	2.82 ± 0.11	60 ± 0.06	25.1 ± 2.47	3.13 ± 0.16
40.00	110 ± 0.04	10.6 ± 1.13	1.09 ± 0.11	110 ± 0.02	20.5 ± 1.07	1.45 ± 0.09	102 ± 0.04	26.3 ± 1.58	1.67 ± 0.10	59 ± 0.05	24.8 ± 1.56	4.04 ± 0.14	37 ± 0.05	25.3 ± 2.17	5.03 ± 0.15
50.00	88 ± 0.04	10.4 ± 0.75	1.33 ± 0.09	104 ± 0.02	20.1 ± 1.35	1.51 ± 0.10	94 ± 0.03	25.5 ± 1.78	1.76 ± 0.11	51 ± 0.04	23.4 ± 0.81	4.34 ± 0.11	27 ± 0.04	24.5 ± 0.81	6.74 ± 0.10

BaP

	24 hours			48 hours			72 hours			120 hours			168 hours		
Conc. µg/ml	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]
0	100 ± 0.05	9.8 ± 1.12	1.00 ± 0.11	100 ± 0.01	14.3 ± 0.98	1.00 ± 0.07	100 ± 0.08	17.6 ± 1.60	1.00 ± 0.09	100 ± 0.04	13.0 ± 1.30	1.00 ± 0.10	100 ± 0.04	17.1 ± 1.60	1.00 ± 0.09
0.05	110 ± 0.04	12.5 ± 1.42	1.16 ± 0.13	100 ± 0.02	15.5 ± 1.38	1.08 ± 0.08	91 ± 0.12	19.1 ± 1.87	1.19 ± 0.10	66 ± 0.04	16.2 ± 2.06	1.89 ± 0.13	55 ± 0.05	22.2 ± 1.77	2.36 ± 0.10
0.13	119 ± 0.03	18.1 ± 1.06	1.55 ± 0.11	109 ± 0.01	20.7 ± 1.73	1.33 ± 0.09	99 ± 0.11	24.7 ± 2.40	1.42 ± 0.11	58 ± 0.05	21.0 ± 1.30	2.79 ± 0.10	32 ± 0.04	26.2 ± 1.69	4.79 ± 0.10
0.25	112 ± 0.04	23.8 ± 1.04	2.17 ± 0.11	104 ± 0.02	27.1 ± 1.88	1.82 ± 0.10	98 ± 0.10	31.2 ± 2.50	1.81 ± 0.12	50 ± 0.03	28.0 ± 1.15	4.31 ± 0.09	23 ± 0.03	33.6 ± 0.56	8.54 ± 0.06
0.50	101 ± 0.04	39.9 ± 1.46	4.03 ± 0.13	105 ± 0.02	41.3 ± 2.13	2.75 ± 0.11	84 ± 0.08	45.4 ± 2.38	3.07 ± 0.11	46 ± 0.03	43.0 ± 1.50	7.19 ± 0.11	20 ± 0.03	45.7 ± 2.98	13.36 ± 0.13
1.26	96 ± 0.04	80.3 ± 2.55	8.54 ± 0.19	99 ± 0.02	90.8 ± 3.18	6.41 ± 0.14	76 ± 0.11	91.4 ± 4.14	6.83 ± 0.16	41 ± 0.03	80.0 ± 4.39	15.01 ± 0.22	17 ± 0.02	79.3 ± 2.01	27.28 ± 0.11

CisPt

	24 hours			48 hours			72 hours			120 hours			168 hours		
Conc. µg/ml	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]
0	100 ± 0.05	13.5 ± 1.50	1.00 ± 0.11	100 ± 0.02	21.0 ± 1.89	1.00 ± 0.09	100 ± 0.08	24.1 ± 1.25	1.00 ± 0.05	100 ± 0.01	16.4 ± 2.26	1.00 ± 0.04	100 ± 0.11	11.3 ± 0.97	1.00 ± 0.02
0.41	90 ± 0.04	17.8 ± 2.28	1.47 ± 0.14	98 ± 0.02	25.4 ± 2.75	1.23 ± 0.11	92 ± 0.07	33.5 ± 3.48	1.51 ± 0.10	70 ± 0.01	26.8 ± 2.63	2.33 ± 0.05	41 ± 0.08	18.6 ± 0.79	4.01 ± 0.02
0.83	103 ± 0.04	17.6 ± 1.55	1.27 ± 0.11	97 ± 0.02	27.2 ± 2.56	1.34 ± 0.11	102 ± 0.08	36.3 ± 2.87	1.48 ± 0.09	71 ± 0.01	30.8 ± 2.46	2.65 ± 0.04	37 ± 0.06	20.6 ± 0.59	4.93 ± 0.02
1.65	105 ± 0.05	18.6 ± 1.85	1.31 ± 0.12	89 ± 0.02	35.6 ± 2.26	1.90 ± 0.10	89 ± 0.07	48.8 ± 2.59	2.28 ± 0.08	56 ± 0.01	38.2 ± 1.34	4.16 ± 0.03	27 ± 0.06	25.7 ± 0.90	8.42 ± 0.02
3.30	100 ± 0.05	18.9 ± 1.57	1.40 ± 0.11	71 ± 0.02	39.8 ± 1.83	2.67 ± 0.09	80 ± 0.09	54.5 ± 2.48	2.83 ± 0.08	34 ± 0.01	41.5 ± 1.23	7.44 ± 0.03	22 ± 0.06	31.7 ± 0.85	12.75 ± 0.02
6.60	96 ± 0.05	20.0 ± 1.63	1.54 ± 0.12	51 ± 0.02	33.7 ± 3.35	3.15 ± 0.12	48 ± 0.09	43.2 ± 3.97	3.73 ± 0.11	21 ± 0.02	38.2 ± 2.43	11.09 ± 0.04	14 ± 0.07	32.8 ± 1.90	20.73 ± 0.03

VLB

	24 hours			48 hours			72 hours			120 hours			168 hours		
Conc. µg/ml	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]	Viab. (%) ± SD[a]	GFP int. ± SD[b]	GFP ind. ± SD[c]
0	100 ± 0.01	13.0 ± 0.45	1.00 ± 0.03	100 ± 0.00	18.2 ± 0.78	1.00 ± 0.04	100 ± 0.04	20.8 ± 1.27	1.00 ± 0.06	100 ± 0.03	18.5 ± 1.06	1.00 ± 0.06	100 ± 0.10	20.8 ± 1.17	1.00 ± 0.06
0.10	94 ± 0.01	26.3 ± 1.89	2.15 ± 0.09	81 ± 0.01	25.9 ± 2.01	1.76 ± 0.08	57 ± 0.01	28.7 ± 1.50	2.41 ± 0.07	18 ± 0.02	18.2 ± 1.12	5.46 ± 0.06	5 ± 0.01	17.2 ± 1.22	16.55 ± 0.05
0.50	95 ± 0.01	24.3 ± 2.87	1.96 ± 0.13	80 ± 0.01	22.3 ± 2.27	1.54 ± 0.08	58 ± 0.01	20.7 ± 1.50	1.71 ± 0.07	14 ± 0.00	12.2 ± 0.92	4.70 ± 0.05	4 ± 0.00	11.2 ± 1.22	13.45 ± 0.05
1.00	90 ± 0.00	19.6 ± 0.49	1.67 ± 0.04	85 ± 0.00	18.0 ± 0.75	1.17 ± 0.04	56 ± 0.01	14.9 ± 0.58	1.28 ± 0.04	16 ± 0.02	9.6 ± 1.01	3.24 ± 0.06	5 ± 0.01	8.3 ± 0.57	8.03 ± 0.04
2.50	85 ± 0.01	17.2 ± 0.82	1.55 ± 0.05	77 ± 0.01	15.0 ± 1.08	1.07 ± 0.05	52 ± 0.00	11.7 ± 0.47	1.08 ± 0.04	16 ± 0.01	7.3 ± 0.78	2.45 ± 0.05	5 ± 0.01	7.3 ± 0.80	7.07 ± 0.05
5.00	78 ± 0.04	10.4 ± 0.76	1.03 ± 0.05	70 ± 0.01	7.9 ± 1.07	0.62 ± 0.05	42 ± 0.01	9.6 ± 0.75	1.10 ± 0.05	15 ± 0.00	3.3 ± 0.56	1.17 ± 0.04	6 ± 0.01	3.7 ± 0.67	2.95 ± 0.04

[a] Cell viability was measured with the MTS assay and is expressed as % of viable p21 HepG2 GFP cells treated with MMS, BaP, CisPt and VLB compared to control, non-treated cells.

[b] Intensity of EGFP fluorescence measured at 485 nm excitation and 535 nm emission wavelengths.

[c] Relative EGFP induction expressed as the ratio between the EGFP fluorescence intensity of the treated cells and non-treated control cells, normalized to cell viability.

Light grey areas represent significantly different values compared to control ($P<0.001$)

Dark grey areas represent values of cell viability below 70% of control.

ANNEX B: Laboratory results (DsRed)

ANNEX B: Cell viability and induction of DsRed fluorescence in p21HepG2 DsRed cells exposed to MMS, BaP, CisPt and VLB for 24, 48, 72, 120 and 168 h.

MMS

	24 hours			48 hours			72 hours			120 hours			168 hours		
Conc. µg/ml	Viab. (%) ± SD[a]	DsReD int. ± SD[b]	DsReD ind. ± SD[c]	Viab. (%) ± SD[a]	DsReD int. ± SD[b]	DsReD ind. ± SD[c]	Viab. (%) ± SD[a]	DsReD int. ± SD[b]	DsReD ind. ± SD[c]	Viab. (%) ± SD[a]	DsReD int. ± SD[b]	DsReD ind. ± SD[c]	Viab. (%) ± SD[a]	DsReD int. ± SD[b]	DsReD ind. ± SD[c]
0	100 ± 0.04	4.1 ± 1.96	1.00 ± 0.32	100 ± 0.01	1.9 ± 1.60	1.00 ± 0.57	100 ± 0.05	2.7 ± 2.82	1.00 ± 0.69	100 ± 0.03	4.8 ± 3.10	1.00 ± 0.43	100 ± 0.05	4.3 ± 1.51	1.00 ± 0.23
5.00	96 ± 0.03	7.3 ± 1.74	1.87 ± 0.30	94 ± 0.02	4.9 ± 1.60	2.75 ± 0.56	99 ± 0.04	6.8 ± 2.11	2.54 ± 0.60	82 ± 0.03	6.9 ± 2.09	1.75 ± 0.36	78 ± 0.05	6.6 ± 1.74	1.96 ± 0.25
10.00	100 ± 0.03	7.6 ± 2.23	1.86 ± 0.34	111 ± 0.01	6.1 ± 2.03	2.89 ± 0.64	105 ± 0.04	9.4 ± 1.95	3.30 ± 0.58	87 ± 0.03	10.3 ± 2.62	2.45 ± 0.39	66 ± 0.04	9.8 ± 2.03	3.44 ± 0.27
20.00	87 ± 0.03	6.4 ± 1.73	1.81 ± 0.30	101 ± 0.01	9.1 ± 1.93	4.78 ± 0.62	96 ± 0.04	21.2 ± 3.64	8.10 ± 0.79	62 ± 0.03	23.3 ± 2.95	7.77 ± 0.42	38 ± 0.04	20.7 ± 2.97	12.58 ± 0.34
40.00	85 ± 0.03	6.2 ± 2.22	1.81 ± 0.34	93 ± 0.01	16.9 ± 3.21	9.65 ± 0.85	76 ± 0.04	41.9 ± 5.22	20.25 ± 0.98	46 ± 0.03	46.7 ± 5.93	20.99 ± 0.62	22 ± 0.03	33.3 ± 3.62	34.97 ± 0.39
50.00	77 ± 0.03	7.0 ± 2.26	2.25 ± 0.35	74 ± 0.01	18.2 ± 2.51	13.04 ± 0.73	62 ± 0.04	41.2 ± 4.65	24.39 ± 0.91	38 ± 0.03	44.4 ± 4.55	24.20 ± 0.53	15 ± 0.03	33.8 ± 5.78	51.97 ± 0.56

BaP

	24 hours			48 hours			72 hours			120 hours			168 hours		
Conc. µg/ml	Viab. (%) ± SD[a]	DsReD int. ± SD[b]	DsREd ind. ± SD[c]	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsRed ind. ± SD[c]	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsRed ind. ± SD[c]	Viab. (%) ± SD[a]	DsReD int. ± SD[b]	DsReD ind. ± SD[c]	Viab. (%) ± SD[a]	DsReD int. ± SD[b]	DsReD ind. ± SD[c]
0	100 ± 0.01	3.1 ± 2.71	1.00 ± 0.58	100 ± 0.01	2.4 ± 2.11	1.00 ± 0.59	100 ± 0.03	1.7 ± 2.11	1.00 ± 0.80	100 ± 0.03	2.2 ± 1.93	1.00 ± 0.59	100 ± 0.07	1.89 ± 1.62	1.00 ± 0.57
0.05	133 ± 0.03	4.8 ± 1.90	1.17 ± 0.49	132 ± 0.01	5.8 ± 1.84	1.83 ± 0.55	92 ± 0.04	5.1 ± 1.84	3.23 ± 0.73	57 ± 0.02	5.1 ± 2.65	4.09 ± 0.70	38 ± 0.05	3.94 ± 1.23	5.50 ± 0.50
0.13	122 ± 0.01	5.6 ± 1.92	1.48 ± 0.50	122 ± 0.01	6.1 ± 1.67	2.10 ± 0.53	99 ± 0.04	5.9 ± 1.67	3.49 ± 0.76	57 ± 0.02	6.0 ± 1.78	4.86 ± 0.57	27 ± 0.04	6.44 ± 2.08	12.64 ± 0.65
0.25	115 ± 0.02	5.9 ± 2.06	1.66 ± 0.51	115 ± 0.01	7.3 ± 2.59	2.67 ± 0.66	92 ± 0.03	9.1 ± 2.59	5.75 ± 0.85	49 ± 0.02	8.8 ± 2.83	8.27 ± 0.73	21 ± 0.04	9.50 ± 2.68	23.95 ± 0.76
0.50	112 ± 0.02	7.7 ± 1.77	2.20 ± 0.48	106 ± 0.01	14.6 ± 2.55	5.75 ± 0.65	92 ± 0.04	20.2 ± 2.55	12.73 ± 1.28	48 ± 0.03	26.0 ± 5.31	25.00 ± 1.11	19 ± 0.04	22.61 ± 3.27	63.00 ± 0.86
1.26	94 ± 0.02	9.2 ± 2.20	3.13 ± 0.53	90 ± 0.01	31.3 ± 3.51	14.55 ± 0.78	77 ± 0.04	55.6 ± 3.51	41.89 ± 1.47	38 ± 0.03	67.7 ± 5.79	82.19 ± 1.19	14 ± 0.04	68.28 ± 5.20	258.19 ± 1.20

CisPt

	24 hours			48 hours			72 hours			120 hours			168 hours		
Conc. µg/ml	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsReD ind. ± SD[c]	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsRed ind. ± SD[c]	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsRed ind. ± SD[c]	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsRed ind. ± SD[c]	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsRed ind. ± SD[c]
0	100 ± 0.02	1.1 ± 3.28	1.00 ± 1.97	100 ± 0.01	2.6 ± 2.97	1.00 ± 0.76	100 ± 0.02	1.2 ± 2.16	1.00 ± 1.18	100 ± 0.03	1.6 ± 1.86	1.00 ± 0.77	100 ± 0.06	1.4 ± 1.63	1.00 ± 0.78
0.41	105 ± 0.03	1.1 ± 2.09	0.90 ± 1.61	115 ± 0.02	3.0 ± 1.86	1.00 ± 0.62	96 ± 0.02	2.5 ± 1.85	2.13 ± 1.09	61 ± 0.03	4.7 ± 2.09	4.80 ± 0.82	36 ± 0.04	4.3 ± 2.13	8.56 ± 0.90
0.83	112 ± 0.02	1.1 ± 1.46	0.86 ± 1.42	97 ± 0.01	3.3 ± 1.55	1.29 ± 0.58	88 ± 0.02	5.7 ± 2.03	5.32 ± 1.14	52 ± 0.03	9.0 ± 1.84	10.74 ± 0.76	24 ± 0.04	8.7 ± 2.48	26.17 ± 0.99
1.65	98 ± 0.02	1.3 ± 1.82	1.17 ± 1.53	90 ± 0.01	5.5 ± 2.00	2.34 ± 0.63	79 ± 0.04	15.0 ± 2.91	15.54 ± 1.38	40 ± 0.02	26.4 ± 2.70	41.03 ± 0.94	16 ± 0.03	20.9 ± 2.74	94.00 ± 1.05
3.30	97 ± 0.02	1.7 ± 1.11	1.60 ± 1.32	84 ± 0.01	13.2 ± 4.67	6.00 ± 0.98	67 ± 0.03	27.9 ± 3.43	34.12 ± 1.53	34 ± 0.02	45.4 ± 3.57	82.96 ± 1.12	11 ± 0.03	40.3 ± 4.12	263.64 ± 1.38
6.60	79 ± 0.02	2.8 ± 2.28	3.23 ± 1.67	56 ± 0.01	20.1 ± 3.26	13.72 ± 0.80	44 ± 0.03	42.2 ± 2.67	78.51 ± 1.32	22 ± 0.03	71.1 ± 5.46	200.47 ± 1.51	6 ± 0.03	69.1 ± 6.00	828.67 ± 1.83

VLB

	24 hours			48 hours			72 hours			120 hours			168 hours		
Conc. µg/ml	Viab. (%) ± SD[a]	DsReD int. ± SD[b]	DsRed ind. ± SD[c]	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsRed ind. ± SD[c]	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsRed ind. ± SD[c]	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsRed ind. ± SD[c]	Viab. (%) ± SD[a]	DsRed int. ± SD[b]	DsRed ind. ± SD[c]
0	100 ± 0.02	3.1 ± 2.05	1.00 ± 0.07	100 ± 0.01	2.2 ± 1.86	1.00 ± 0.07	100 ± 0.02	2.9 ± 2.51	1.00 ± 0.09	100 ± 0.03	1.6 ± 2.02	1.00 ± 0.07	100 ± 0.05	1.5 ± 1.41	1.00 ± 0.04
0.05	96 ± 0.04	5.4 ± 2.34	1.80 ± 0.07	70 ± 0.01	5.6 ± 2.09	3.61 ± 0.07	56 ± 0.02	4.7 ± 2.13	2.83 ± 0.08	29 ± 0.02	3.9 ± 1.35	8.32 ± 0.06	15 ± 0.03	3.1 ± 1.50	13.83 ± 0.05
0.10	109 ± 0.02	4.8 ± 1.95	1.43 ± 0.07	77 ± 0.01	5.6 ± 2.19	3.25 ± 0.07	63 ± 0.02	3.9 ± 1.44	2.13 ± 0.07	35 ± 0.02	3.0 ± 1.92	5.32 ± 0.06	16 ± 0.03	1.9 ± 1.39	8.10 ± 0.04
0.50	106 ± 0.02	5.1 ± 2.51	1.55 ± 0.07	71 ± 0.02	4.6 ± 1.86	2.89 ± 0.07	60 ± 0.02	4.1 ± 1.38	2.30 ± 0.07	28 ± 0.02	1.9 ± 1.71	4.19 ± 0.06	12 ± 0.03	1.2 ± 1.73	6.48 ± 0.05
1.00	97 ± 0.02	3.5 ± 1.89	1.16 ± 0.06	77 ± 0.01	3.2 ± 2.27	1.88 ± 0.07	54 ± 0.02	2.3 ± 1.14	1.47 ± 0.06	27 ± 0.02	1.4 ± 1.56	3.19 ± 0.06	11 ± 0.03	0.6 ± 1.45	3.37 ± 0.04
2.50	90 ± 0.02	3.4 ± 1.71	1.21 ± 0.06	71 ± 0.02	2.3 ± 1.79	1.48 ± 0.06	48 ± 0.02	1.9 ± 0.91	1.34 ± 0.06	23 ± 0.03	0.8 ± 1.86	2.25 ± 0.06	10 ± 0.03	0.3 ± 1.57	1.85 ± 0.05

[a] Cell viability was measured with the MTS assay and is expressed as % of viable p21HepG2 DsRed cells treated with MMS, BaP, CisPt and VLB compared to control, non-treated cells.

[b] Intensity of DsRed fluorescence measured at 535 nm excitation and 590 nm emission wavelengths.

[c] Relative DsRed induction expressed as the ratio between the DsRed fluorescence intensity of the treated cells and non-treted control cells, normalized to cell viability.

Light grey areas represent significantly different values compared to control ($P<0.001$)

Dark grey areas represent values of cell viability below 70% of control.