



(43) International Publication Date  
19 December 2013 (19.12.2013)

(10) International Publication Number  
**WO 2013/185884 A1**

- (51) **International Patent Classification:**  
*G01N 33/50* (2006.01)
- (21) **International Application Number:**  
PCT/EP2013/001575
- (22) **International Filing Date:**  
28 May 2013 (28.05.2013)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**  
61/659,024 13 June 2012 (13.06.2012) US
- (71) **Applicant:** MERCK PATENT GMBH [DE/DE]; Frankfurter Strasse 250, 64293 Darmstadt (DE).
- (72) **Inventors:** MUELLER, Stefan Otto; Am Winkelbach 6, 64625 Bensheim (DE). DIETZ, Yasmin; Nuernberger Strasse 39, 65462 Gustavsburg (DE).
- (81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Published:**

— with international search report (Art. 21(3))

(54) **Title:** PROTEIN EXPRESSION ANALYSES FOR IDENTIFYING GENOTOXIC COMPOUNDS

(57) **Abstract:** The invention relates to a method for screening compounds with (pro-)genotoxic activity by providing a system being capable of expressing a panel of defined proteins, incubating at least a portion of the system with compounds to be screened, and comparing the expression of the proteins in the system with the protein expression in a control system, thereby detecting the (pro-)genotoxic activity. Another object of the invention concerns a method for monitoring the likelihood of developing a physiological and/or pathological condition, which is caused, mediated and/or propagated by the genetic deregulation of proliferation, differentiation and/or damage repair, in response to a compound administered to a mammal in need of such treatment by determining an expression level of defined proteins in a biological sample withdrawn from the mammal. The invention also relates to a kit for screening compounds with (pro-)genotoxic activity comprising antibodies that specifically bind to marker proteins.



**WO 2013/185884 A1**

## Protein expression analyses for identifying genotoxic compounds

The invention relates to a method for screening compounds with (pro-)genotoxic activity by providing a system being capable of expressing a panel of defined proteins, incubating at least a portion of the system with compounds to be screened, and comparing the expression of the proteins in the system with the protein expression in a control system, thereby detecting the (pro-)genotoxic activity. Another object of the invention concerns a method for monitoring the likelihood of developing a physiological and/or pathological condition, which is caused, mediated and/or propagated by the genetic deregulation of proliferation, differentiation and/or damage repair, in response to a compound administered to a mammal in need of such treatment by determining an expression level of defined proteins in a biological sample withdrawn from the mammal. The invention also relates to a kit for screening compounds with (pro-)genotoxic activity comprising antibodies that specifically bind to marker proteins.

A comprehensive evaluation of a compound's genotoxic potential is an important part during chemical risk assessment and pharmaceutical development. The current standard testing battery involves in-vitro and in-vivo tests designed to detect compounds that induce DNA damage by a variety of different mechanisms of action. A bacterial reverse mutation assay as well as a genotoxicity test with mammalian cells in in-vitro and/or in-vivo is required. Genotoxicity testing systems gain in importance because of the fact that compounds tested positive in these tests are potential human carcinogens. However, the standard testing battery has a low throughput and need a comparatively large amount of compound. For this reason, these assays are less suitable in the early drug discovery phase (Westerink et al., 2011, *Mutat. Res.* 724, 7-21).

Frequently, chemicals that turn out not to be in-vivo genotoxins, or not to induce tumors via a mutagenic or DNA-reactive mechanism, give false positive results in mammalian cell tests, which complicate human hazard extrapolation.

Additionally, in terms of the chemical characterization within the EU REACH legislation and modern risk evaluation, mechanistic analyses were getting more important.

The difficulties are now forcing the development of new in-vitro tools. Novel genotoxicity test systems with enhanced specificity (i.e. less false positive results) without losing the

sensitivity (i.e. detecting DNA-reactive carcinogens and in-vivo genotoxins) are needed (Kirkland et al., 2005, Mutat. Res. 584, 1-256; Kirkland et al., 2006, Mutat. Res. 608, 29-42; Kirkland et al., 2008, Mutat. Res. 653, 99-108).

5 Therefore, the technical problem forming the basis of the present invention is to provide a method for screening compounds, which effectively allows the identification and characterization of their genotoxic and/or pro-genotoxic properties. It is another problem of the invention to provide a kit for the detection of genotoxic and/or pro-genotoxic activity.

10 The present invention solves the problem by providing a method for screening compounds with genotoxic and/or pro-genotoxic activity comprising the step of determining expression levels of active forms of at least two proteins selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68) in a system incubated with one or more  
15 compounds in comparison with expression levels of said proteins in the system substantially not incubated with the compounds or the same concentration of the compounds.

It has been surprisingly demonstrated by the inventors that the combination of at least two  
20 of the said proteins is correlated with genotoxicity. Consequently, the aforementioned plurality of marker proteins represents novel genotoxicity markers, which are well suited for differentiating the stage of genotoxicity. The underlying proteins are selected as result of differential expression analysis. All protein expressions are characterized by a distinct difference to untreated cells, wherein the treated cells show the difference by up-regulation.

25 The determined proteins are already described in the art by sequence and other features, but lacking a linkage to genotoxicity in the defined combination of the invention. Either of the pairwise marker protein combinations, optionally supplemented with further marker proteins, can be used for the utmost test reliability. The proteins may be named in another way, but are uniquely defined by the accession number, which is generally accepted and  
30 registered in numerous data bases, such as the UniProt, SwissProt and the like (cf. Table 1; Zhou and Bartek, 2004, Nat Rev Cancer 4, 216-25).

Although the identified nine proteins are involved in DNA-damage-response signal-transduction network, they are not inevitably associated by function or location in their  
35 entity as presently known, but it is not excluded that such relations appear between a single member or more members of the group.

The DNA-damage-response signal-transduction network is composed of at least two different pathways: Ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia-mutated and Rad3-related (ATR) pathway. In response to damaged DNA, the ATM pathway is initiated by autophosphorylation of ATM on Ser1981. ATM regulates cell cycle checkpoints and DNA repair via phosphorylation of a couple of proteins. The ATR pathway responds to DNA-double strand breaks (DSB) - although slower than ATM - and to chemicals that interfere with the DNA replication fork. There are two phospho-sites (p-ATR (Ser428) and p-ATR (Thr1989)) which may lead to the activation of ATR. The sensors that initially recognize aberrant DNA structure and activate the DNA-damage-response network are unclear.

A very early event of DNA-damage response is the phosphorylation of histone H2A.X via ATM at Ser139. Phosphorylated H2A.X accumulates at sites of DNA damage and recruits a variety of DNA-damage response and DNA repair signals.

DNA double-strand breaks induce Chk2 phosphorylation at Thr68 and other sites in this region by ATM which leads to p53-dependent or p53-independent cell cycle arrest and/or apoptosis.

The phosphorylation of p53 at position Ser15 in particular via ATM prevents the ability of mdm2 to bind and detains its degradation. Phosphorylated and therefore activated p53 acts as transcription factor for a couple of genes including the gene for the protein p21 and growth arrest and DNA damage-inducible protein a (Gadd45a). P21 inhibits different cyclin-dependant kinases which lead to a cell cycle arrest in G1-phase. Furthermore, p21 binds the PCNA and interrupts the DNA-replication. Induced nuclear Gadd45a interacts with the cyclin-dependant kinase 2 (cdc2) and leads to its dissociation from the cdc2/cyclin B1 complex. Free cyclin B1 is exported into the cytoplasm and degraded via ubiquitination. Gadd45a therefore inhibits the cdc2 kinase activity and the cell remains at the transition into mitosis.

Besides the Gadd45a-mediated inhibition of the cdc2 kinase, mediation via Chk1 also occurs. Chk1 can be activated by the kinases ATM and ATR, but the phosphorylation of Chk1 at Ser345 and Ser317 are performed by ATR. Chk1 is able to phosphorylate the phosphatase Cdc25C at Ser216 which disables the loss inhibitory phosphorylation of cdc2 at Thr14 and Tyr15 and the cell remains at the G2/M phase transition.

The analysis of the proteins and associated molecules, as discussed above, demonstrates the complex regulation of cell death and survival, e.g. in response to the exposure of HepG2 cells with (pro-)genotoxicants. Mainly affected processes comprise cell cycle regulation, cell proliferation and apoptosis. Proteins found to be differentially regulated can be predominantly assigned to pro-apoptotic and anti-proliferative functions. The data indicate an induction of cell cycle arrest and programmed cell death in response to compound-induced DNA damage.

The linkage of genotoxicity to distinct proteins is utilized for the in-vitro detection of mutagens and pro-mutagens, which are able to interfere with signaling in proliferation, differentiation or damage repair. Building a compound-specific protein expression profile, which is based on the plurality of proteins of the invention, is of unexpected benefit in establishing a genotoxic mechanism of action and, therefore, supports the evaluation of potential hazards or benefits of novel compounds either alternatively or supplementary to the classical screening methods. The inventive principle underlying the present method comprises detecting the defined proteins. The gene product is chosen in respect of both its absolute and relative amount as well as the specificity for a certain cell type.

A "protein" refers to a biochemical compound comprising one or more polypeptides. A polypeptide is a single linear polymer chain of amino acids bonded together by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. It is typically folded into a globular or fibrous form to facilitate a biological function. An "active form" of a protein refers to the fact that the residues in a protein are often chemically modified by posttranslational modification, which alters the physical and chemical properties, folding, stability, activity, and ultimately, the function of the protein. For example, the activity of the transcription factor p53 is crucially regulated by means of protein phosphorylation which can be induced by multiple signals, including general cellular stress, DNA damage or interferons.

A "plurality of proteins" as used herein refers to a group of identified or isolated proteins whose levels of expression vary in different tissues, cells or under different conditions or biological states. The different conditions may be caused by exposure to certain agent(s) – whether exogenous or endogenous – which include hormones, receptor ligands, chemical compounds and the like. The expression of a plurality of proteins demonstrates certain patterns. That is, each protein in the plurality is expressed differently in different conditions, or with or without exposure to certain endogenous or exogenous agents. The extent or level of differential expression of each protein vary in the plurality and may be determined

qualitatively and/or quantitatively according to this invention. A protein expression profile, as used herein, refers to a plurality of proteins that are differentially expressed at different levels, which constitutes a "pattern" or a "profile." As used herein, the term "expression profile", "profile", "expression pattern", "pattern", "protein expression profile" and "protein  
5 expression pattern" are used interchangeably.

As used herein, a "compound with genotoxic activity", also referred to as "mutagen", is a physical or chemical agent that changes the genetic material, usually DNA, of an organism and thus increases the frequency of mutations above the natural background level. The  
10 skilled artisan would know that, for instance, one of the biological effects of mutagens is to promote the development of cancer. Other biological effects of mutagens are well documented and discussed. The changes in nucleic acid sequences by mutations comprise substitution of nucleotide base-pairs and insertions and deletions of one or more nucleotides in DNA sequences.

15

First, a system is provided and incubated in total or in part(s). The system is also referred to as test system hereunder. Although a cellular system is preferred in the scope of the invention, an in-vitro translation system can be alternatively used which is based on the protein synthesis without living cells. The cellular system is defined to be any subject  
20 provided that the subject comprises cells. Hence, the cellular system can be selected from the group of single cells, cell cultures, tissues, organs, plants and animals. The scope of the cellular system also comprises parts of such biological entities, i.e. samples of tissues, organs, plants and animals. Barring plants, it shall be understood that each cellular system in the aforementioned order could represent a sample of the respective following system.

25 Cell samples, non-human animals, mammals or non-human mammals are preferred embodiments of the cellular system according to the invention. A cell sample refers to any type of primary cells or genetically engineered cells, either in the isolated status, in culture or as cell line. Particularly, the cell sample is taken in-vivo or in-situ from a mammal to be tested. The withdrawal of the cell sample follows good medical practice. Biological samples  
30 may be taken from any kind of biological species, but the sample is especially taken from a human, rat or a mouse.

In the present invention, the cellular system may also comprise a biological fluid, wherein the sample of body fluid preferably consists of blood, serum, plasma, saliva or urine. It is  
35 also preferred to gather a tissue sample by biopsy, especially taken close to the location of ailment. The biological samples can be originated from any tissue, including the liver, kidney, intestine, bone marrow, etc. In preferred embodiments, the biological samples are

from the kidney, liver, and the intestine. The sample may be purified to remove disturbing substances, such as inhibitors for the formation of hydrogen bonds.

The system is capable of expressing, or expressing, several proteins of p53, p21, H2A.X, ATM, Chk1, ATR, cdc2, Gadd45a or Chk2. In an embodiment of the invention, the system is additionally capable of expressing, or expressing, MDM2. The system is also capable of activating, or activating, several proteins of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68). The system is preferably capable of expressing, or expressing, active forms of at least two proteins selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68). More preferably, the system is capable of expressing, or expressing, active forms of at least two proteins selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139) and p-Chk1 (Ser345). Most preferably, the system is capable of expressing, or expressing, active forms of at least two proteins selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981) and p-Chk1 (Ser345).

It shall be understood that variants, mutants, parts or homologous protein sequences having the same function, are included in the scope of definition as well as protection. Preferably, the homology amounts to at least 85 %. Possible alterations comprise deletion, insertion, substitution, modification and addition of at least one amino acid, or the fusion with another peptide or protein. The engineered cells are capable of expressing these proteins after transfection with appropriate vectors harboring the underlying nucleic acid sequence or parts thereof. Preferably, the recombinant cells are of eukaryotic origin.

25

In a more preferred embodiment of the present invention, HepG2 cells are provided for the screening method. The dose of S9 used has an influence on the experimental outcome in spite of being negative in cytotoxicity evaluations. It should be noted that the dose is comparable to the dose used within regulatory standard genotoxicity testing. The significantly S9-regulated genes differ from those induced by the (pro-)genotoxic agents and the S9-effect can be adjusted by comparison to the proper controls. In any case, a cellular system having sufficient intrinsic metabolic activity would certainly be appropriate, but currently no such metabolically competent cellular system exists for genotoxicity evaluation. Primary hepatocytes are the current gold standard for drug metabolism and CYP induction/ inhibition studies in-vitro. Even though adult differentiated hepatocytes lack proliferation competency making them an unsuitable model for genotoxicity investigations, and they also exhibit significant changes in morphology as well as protein and gene

35

expression during cultivation, limited availability of human hepatocytes and often marked donor/batch variability complicating standardization of such systems, a major advantage of HepG2 cells are their human molecular characteristics. For instance, specific targets such as topoisomerases and eukaryotic repair enzymes are expressed and prevent the overestimation of genotoxicity and therefore, contribute to a reduction of false positives.

The cell sample is stored, such as frozen, cultivated for a certain period or immediately subjected to the next step. Before incubating it with compounds to be screened, the cell sample could be divided into multiple portions. If doing so, at least two portions are provided; one is used for screening while the other one serves as control. Preferably, the number of portions for screening exceeds the number of control portions. Usually, numerous portions are subjected to a high-throughput screening.

The compounds are composed of biological and/or chemical structures capable to interact with a target molecule. Herein, any component of genomics or proteomics signaling shall be considered as "target molecule", which is not limited to genes, or a regulator protein or a gene product thereof, or a component of a signal transduction pathway comprising a gene or gene products thereof. Consequently, the specific interaction of compounds may involve either the mere targeting or the induction of alterations in cell function, or it may even include both effects simultaneously.

The compounds to be screened in the inventive method are not restricted anyway. In particular, the compounds are selected from the group of nucleic acids, peptides, carbohydrates, polymers, small molecules having a molecular weight between 50 and 1.000 Da and proteins. These compounds are often available in libraries. It is preferred to incubate a single compound within a distinct portion of the cell sample. However, it is also possible to investigate the cooperative effect of compounds by incubating at least two compounds within one portion. While a fraction of the cellular system is incubated with one or more (pro-)genotoxic compounds to be analyzed, a further portion of cells is incubated in the absence of the compounds and this additional non-treated fraction of the system is used as negative control. It is also possible that the system acts simultaneously as test and control system by determining the status before exposing genotoxic stress and comparing it with the status upon genotoxic stress. Further control systems are outlined below.

The term "incubation" denotes the contacting of the compounds with the cells for a distinct period, which depends on the kind of compounds and/or target. The incubation process also depends on various other parameters, e.g. the cell type and the sensitivity of



detection, which optimization follows routine procedures known to those skilled in the art. The incubation procedure can be realized without a chemical conversion of mutagens or may involve a metabolic conversion of pro-mutagens. Adding chemical solutions and/or applying physical procedures, e.g. impact of heat, can improve the accessibility of the target structures in the sample. Specific incubation products are formed as result of the incubation.

The identification of effective compounds in the meaning of the invention is indirectly performed by determining the expression pattern of at least two defined proteins, which the system is capable of expressing. The determination is performed at a specified moment and correlated to the signal strength at the beginning of the experiment and the control. For example, the control system is not incubated with the compounds (negative control), or the control system is incubated with a standard compound having no genotoxic activity (negative control). The control system can also be incubated with a standard compound having (pro-)genotoxic activity (positive control). The activity is revealed by a change in expression. Preferably, the proteins expressed in cells with mutagen exposure are compared to the proteins expressed in cells that were not exposed to mutagens. Pairwise comparisons are made between each of the treatments. A pairwise comparison involves that the expression data for a given protein under a given treatment condition are compared to the expression data for this protein under a second treatment condition. The comparison is performed using suitable statistical technique with the assistance of known and commercially available programs.

It is another preferred aspect of the invention that the inherent (pro-)genotoxic activity is detected if the expression of proteins is up-regulated in the system in comparison with a negative, positive or negative control system, or if the expression of proteins is substantially identical in the system and a positive control system. In detail, (i) a higher protein expression in the test system in comparison with a negative control system, which is not incubated with any compound, indicates said activity, or (ii) a substantially identical or higher protein expression in the test system in comparison with a positive control system, which is incubated with a standard compound having genotoxic and/or pro-genotoxic activity, indicates said activity, or (iii) a higher protein expression in a relative control system, which is incubated with compounds having a concentration other than that in the test system with the proviso that a lower concentration is assigned to the relative control system, indicates said activity. It shall be understood that it is not required to determine the expression by two or all comparisons set forth above but the indication of (pro-)genotoxic

activity resulting from any comparison of expression levels under (i), (ii) or (iii) inevitably implies said activity in any other comparative determination (whether performed or not).

It is more preferred that an increase in the expression levels of at least one of said proteins in the system incubated with the compounds in comparison with the system not incubated with the compounds indicate said activity. It is most preferred embodiment of the invention that the expression levels of at least three, four, five, six, seven, eight or nine of said proteins are increased. Highly preferably, the existing activity is detected by differential protein expression analysis with the negative control system.

In another preferred aspect of the invention, the expression levels are increased by a factor of at least 1.5, more preferably at least 1.6, most preferably at least 1.8, highly preferably at least 1.9, and particularly highly preferably at least 2.1.

It is another most particular aspect of the invention that a decreased concentration of the compounds is administered to provide the expression levels having a factor from 1 to 1.1 compared to those of the control system. The decreased compound concentration refers to a decrease of at least 10%, preferably at least 20%, more preferably at least 30%, most preferably at least 40%, highly preferably at least 50%, particularly highly preferably at least 60%.

In another more preferred aspect of the invention, substantially identical expression levels of at least one of said proteins in the system incubated with the compounds in comparison with the system incubated with a standard compound having genotoxic and/or pro-genotoxic activity indicate said activity.

Suitable tests for monitoring protein expression, determination and variant analysis of amino acid sequences are known to those skilled in the art or can be easily designed as a matter of routine. The assay according to the invention may be any assay suitable to detect and/or quantify protein expression. It is a preferred embodiment of the invention that the expression levels are determined by immunofluorescence staining.

The selected markers can be particularly used to establish screening tools with a higher throughput, preferably High Content Imaging (HCI) or Luminex xMAP technology. Luminex color-codes tiny beads, called microspheres, into 500 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. The Luminex technology particularly combines a

sandwich ELISA immobilized on microparticle beads and flow cytometry. It allows simultaneous quantitative measurement of several proteins in one single sample. Inside the Luminex analyzer, a light source excites the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Dual lasers are employed to  
5 detect identity of the beads (based on the spectral properties of the beads specific for each analyte) and the amount of associated Phycoerythrin (PE) fluorescence (Hoffmann et al, 2010, Toxicology 277, 49-58). Automated imaging platforms combining fluorescence microscopy with image analyses algorithms and informatics tools enable the analyses of fluorescent images from millions of cells with a high-resolution examination of the  
10 localization of cellular components, cellular macromolecular structures and the temporal dynamics of cellular functions. The application of the HCI technology for toxicological evaluation shows that in-vitro predictions can fortify the performance of traditional preclinical tests by identifying human hepatotoxicants (Xu et al., 2008, Toxicol Sci 105, 97-105). Furthermore, the HCI technology can be used for genotoxicity evaluation by detecting  
15 micronuclei (MN). The in-vitro MN test detects compound-induced clastogenic (chromosome damage) and aneugenic (chromosome loss) effects, by counting the chromosomal fragments called micronuclei in the cytoplasm of the cells. A HCI-based assay is able to detect genotoxic potential with a high performance score and also allow the differentiation between aneugenic and clastogenic compounds (Westerink et al., 2011,  
20 Mutat. Res. 724, 7-21).

Moreover, the technology allows the combination of several selected endpoints, preserving biological complexity and molecular interactions to a certain extent. HCI offers the possibility to combine classical genotoxic endpoints (e.g. MN induction) and the analysis of  
25 cellular markers with the simultaneous acquisition of cell viability/cytotoxicity. Cell viability is an important parameter to consider for genotoxicity testing because false positives in standard assays can be generated among others via cytotoxicity. The same holds true for measuring molecular marker, such as p53. Although p53 reacts extremely sensitive to DNA damage, nutrient deprivation and hypoxia could also induce activation. Consideration of  
30 cytotoxicity for dose selection, together with multiple endpoint measurements may prevent or reduce false positives.

Consequently, this invention relates to a method for predicting the cellular effect of a compound having genotoxic activity by preparing a protein sample from a cell to be  
35 evaluated, contacting the protein sample with an antibody, detecting a protein binding to the antibody, and comparing a detected result with a result detected using a protein sample prepared from a control cell.

The detection may be performed by applying the intact cell to a detection method of choice. It is preferred, however, to provide cellular extracts first. Cell lysis can be performed in suitable, well-known lysis buffers, which may cause an osmotic shock and perforate the cell membrane. The stability of the cell structure can also be destroyed by mechanical forces, such as ball mill, French press, ultrasonic, etc., by enzymatic degradation of cell wall and cell membrane, respectively, and/or by the action of tensides. The biomarkers may be further purified to remove disturbing substances or the biomarkers can be concentrated in the sample. Downstream-processing and/or concentrating are preferably performed by the method of precipitation, dialysis, gel filtration, gel elution, or chromatography, such as HPLC or ion exchange chromatography. It is recommended to combine several methods for better yields.

Suitable tests for detecting genotoxicity are known to those skilled in the art or can be easily designed as a matter of routine. Many different types of assays are known, examples of which are set forth below. Although the assay according to the invention may be any assay suitable to detect and/or quantify gene expression, genotoxicity is preferably determined by means of substances specifically interacting with the proteins p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68).

The term "specific substances" as used herein comprises biological and/or chemical structures capable to interact with the proteins of the invention in such a manner that makes a recognition, binding and interaction possible. In particular, the substances are selected from the group of nucleic acids, peptides, carbohydrates, polymers, small molecules having a molecular weight between 50 and 1.000 Da and proteins, preferably nucleic acids and proteins. The specific substances express a sufficient sensitivity and specificity in order to ensure a reliable binding and detection. Other biomolecules present in the sample do not significantly bind to the substance specific for the protein marker of the invention. Preferably, the level of binding to a biomolecule other than the marker protein results in a binding affinity of only 10 % of the affinity of the marker protein, more preferably only 5 % or less. A specific substance has at least an affinity of  $10^{-7}$  M for the corresponding marker protein. The specific substance preferably has an affinity of  $10^{-8}$  M or even more preferred of  $10^{-9}$  M for the marker protein. A most preferred specific substance will fulfill both the above minimum criteria for affinity as well as for specificity.

The substances are preferably specific to parts of the protein. Such parts represent a restriction to those regions which are sufficient for the expression of a specific function, i.e. the provision of a structural determinant for recognition. All truncations are inevitably limited by the requirement of preserving the function, e.g. unique recognition. However, the protein  
5 fragments can be very small. Preferably, the substances are mono-specific in order to guarantee an exclusive and directed interaction with the chosen single target.

The recognition of the proteins according to the invention can be realized by a specific interaction with substances on the primary, secondary and/or tertiary structure level of an  
10 amino acid sequence. In the context of the present invention, the term "recognition" - without being limited thereto - relates to any type of interaction between the specific substances and the target, particularly covalent or non-covalent binding or association, such as a covalent bond, hydrophobic/ hydrophilic interactions, van der Waals forces, ion pairs, hydrogen bonds, ligand-receptor interactions, interactions between epitope and  
15 antibody binding site, nucleotide base pairing, and the like. Such association may also encompass the presence of other molecules such as peptides, proteins or other nucleotide sequences.

The specific substances are preferably selected from the group consisting of antibodies, cytokines, lipocalins, receptors, lectins, avidins, lipoproteins, glycoproteins, oligopeptides,  
20 peptide ligands and peptide hormones. More preferably, antibodies are used as specific substance. "Antibody" denotes a polypeptide essentially encoded by an immunoglobulin gene or fragments thereof. According to the invention, antibodies are present as intact immunoglobulins or a number of well-characterized fragments. Fragments are preferably  
25 selected from the group consisting of  $F_{ab}$  fragments,  $F_c$  fragments, single chain antibodies (scFv), variable regions, constant regions, H chain ( $V_H$ ), and L chain ( $V_L$ ), more preferably  $F_{ab}$  fragments and scFv. Fragments, such as  $F_{ab}$  fragments and  $F_c$  fragments, can be produced by cleavage using various peptidases. Furthermore, fragments can be engineered and recombinantly expressed, preferably scFv.

30

DNA aptamers and RNA aptamers have been found to express a high affinity for a wide variety of target molecules. Target structures may comprise proteins, peptides and small molecules, such as organic dyes, nucleotides, amino acids, vitamins, alkaloids, etc. More preferred are RNA aptamers since the 2'-hydroxyl group available in RNA promotes a  
35 couple of intra- and intermolecular contacts, the latter being between molecules of the same sequence, different sequences, or between RNA and any other molecule which is not composed of RNA. These nucleic acid ligands can be identified by an efficient in-vitro

selection procedure – the so-called SELEX process (systematic evolution of ligands by exponential enrichment). Since RNA is very susceptible to nucleolytic degradation in biological solutions, RNA aptamers should be chemically modified using phosphorothioates, locked nucleic acids, or Spiegelmers, for instance. L-RNA versions of aptamers called Spiegelmers are especially long-lived as they are essentially impervious to natural degradation processes. Because of their high affinity for a broad spectrum of structural targets, aptamers act very similar to antibodies. Aptamers can be synthesized using standard phosphoramidite chemistry. In addition, RNA aptamers having more than approximately 30 nucleotides can be favorably synthesized in large amounts by in-vitro transcription. Selection, synthesis, and purification of aptamers are well-known to those skilled in the art.

The specific substances can be labeled, in doing so the labeling depends on their inherent features and the detection method to be applied, i.e. the required sensitivity, ease of conjugation, stability requirements, and available instrumentation and disposal provisions. For the detection of specific incubation products, the applied methods depend on the specific incubation products to be monitored and are well-known to the skilled artisan. Preferred examples of suitable detection methods according to the present invention are luminescence, particularly fluorescence, furthermore VIS coloring and/or radioactive emission.

Luminescence concerns the emission of light as a result of chemiluminescence, bioluminescence or photoluminescence. Chemiluminescence involves the emission of visible light as a result of a chemical reaction, whereas bioluminescence requires the activity of luciferase. The presently preferred photoluminescence, which is also known as fluorescence stimulation, is caused by the absorption of photons, preferably provided by radiation, which is released again as photon with a shift in wavelength of 30 to 50 nm and within a period of approximately  $10^{-8}$  seconds. The instruments for fluorescence detection include, but are not limited to typical benchtop fluorometers, fluorescence multi-well plate readers, fiber optic fluorometers, fluorescence microscopes and microchips/microfluidics systems coupled with fluorescence detection.

VIS coloring denotes the visualization of any achromatic substance in order to be visible to the naked eye. Preferably, the intensity of coloring is measured by a photometer.

Radioactive radiation of isotopes is measured by scintillation. The process of liquid scintillation involves the detection of beta decay within a sample via capture of beta

emissions in a system of organic solvents and solutes referred to as the scintillation cocktail. The beta decay electron emitted by radioactive isotopes such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$  and  $^{35}\text{S}$  in the sample excites the solvent molecule, which in turn transfers the energy to the solute. The energy emission of the solute (the light photon) is converted into an electrical  
5 signal by a photo-multiplier tube within a scintillation counter. The cocktail must also act as a solubilizing agent keeping a uniform suspension of the sample. Gamma ray photons often arise as a result of other decay processes (series decay) to rid the newly formed nucleus of excess energy. They have no mass and produce little if any direct ionization by collision along their path. Gamma photons are absorbed for detection and quantization by  
10 one or more of three mechanisms: The Compton effect, the photoelectric effect and pair production. A favorable gamma decay isotope of the present invention is  $^{125}\text{I}$ .

A labeling method is not particularly limited as long as a label is easily detected. A "labeled specific substance" is one that is bound, either covalently through a linker or a chemical  
15 bond, or non-covalently through ionic, van der Waals, electrostatic, hydrophobic interactions or hydrogen bonds, to a label such that the presence of the marker proteins may be detected by detecting the presence of the label bound to them.

The covalent linkage of an antibody to an enzyme may be performed by different methods,  
20 such as the coupling with glutaraldehyde. Both, the enzyme and the antibody are interlinked with glutaraldehyde via free amino groups, and the by-products of networked enzymes and antibodies are removed. In another method, the enzyme is coupled to the antibody via sugar residues if it is a glycoprotein, such as the peroxidase. The enzyme is oxidized by sodium periodate and directly interlinked with amino groups of the antibody.  
25 Other enzyme containing carbohydrates can also be coupled to the antibody in this manner, however sometimes a loss in activity is observed due to the oxidation, e.g. a diminished activity of alkaline phosphatase. Enzyme coupling may also be performed by interlinking the amino groups of the antibody with free thiol groups of an enzyme, such as  $\beta$ -galactosidase, using a heterobifunctional linker, such as succinimidyl 6-(N-maleimido)  
30 hexanoate.

Specific immunological binding of an antibody to a protein can be detected directly or indirectly. Direct labels include fluorescent or luminescent tags, metals, dyes, radionuclides, and the like, attached to the antibody. An antibody labeled with iodine-125 ( $^{125}\text{I}$ ) can be  
35 used. A chemiluminescence assay using a chemiluminescent antibody specific for the protein marker is suitable for sensitive, non-radioactive detection of protein levels. An antibody labeled with fluorochrome is also suitable. Examples of fluorochromes include,

without limitation, DAPI, fluorescein, fluorescein isothiocyanate (FITC), Oregon Green, Hoechst 33258, R-phycoerythrin, green fluorescent protein (GFP), B-phycoerythrin, R-phycoerythrin, rhodamine, Texas red, tetrahydroimidine isothiocyanate (TRITC), Cy3, Cy5 and lissamine. Indirect labels include various enzymes well known in the art, such as

5 horseradish peroxidase (HRP), alkaline phosphatase (AP),  $\beta$ -galactosidase, urease and the like. A horseradish-peroxidase detection system can be used, for example, with the chromogenic substrate tetramethylbenzidine (TMB), which yields a soluble product in the presence of hydrogen peroxide that is detectable at 450 nm. An alkaline phosphatase detection system can be used with the chromogenic substrate p-nitrophenyl phosphate, for

10 example, which yields a soluble product readily detectable at 405 nm. Similarly, a  $\beta$ -galactosidase detection system can be used with the chromogenic substrate o-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG), which yields a soluble product detectable at 410 nm. A urease detection system can be used with a substrate, such as urea-bromocresol purple. Auto-quenched fluorescent compounds are activated by tumor-associated proteases,

15 enzymes, e.g. luciferase, nanoparticles, biotin, digoxigenin and the like.

In another preferred embodiment of the present invention, the aptamers are labeled with digoxigenin, biotin, chemiluminescence substances, fluorescence dyes, magnetic beads, metallic beads, colloidal particles, electron-dense reagents, enzymes, all of them are well-

20 known in the art, or radioactive isotopes. Preferred isotopes for labeling nucleic acids in the scope of the invention are  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , more preferred  $^{32}\text{P}$ ,  $^{33}\text{P}$ , or  $^{125}\text{I}$ .

A variety of immunoassay techniques, including competitive and non-competitive immunoassays, can be used. The term "immunoassay" encompasses techniques including,

25 without limitation, enzyme immunoassays (EIA), such as enzyme multiplied immunoassay technique (EMIT), enzyme-linked immunosorbent assay (ELISA), IgM antibody capture ELISA (MAC ELISA) and microparticle enzyme immunoassay (MEIA), furthermore capillary electrophoresis immunoassays (CEIA), radio-immunoassays (RIA); immunoradiometric assays (IRMA), fluorescence polarization immunoassays (FPIA) and chemiluminescence

30 assays (CL). If desired, such immunoassays can be automated. Immunoassays can also be used in conjunction with laser induced fluorescence such as the Luminex technology. Liposome immunoassays, such as flow-injection liposome immunoassays and liposome immunosensors, are also suitable for use in the present invention. In addition, nephelometry assays, in which the formation of protein/antibody complexes results in

35 increased light scatter that is converted to a peak rate signal as a function of the marker concentration, are suitable for use in the methods of the present invention.



In an embodiment of the present invention, antibodies are used as specific substances and the incubation products are detected by the labeling of the antibodies, preferably by ELISA, RIA, fluoro immunoassay (FIA), soluble particle immune assay (SPIA) or western blotting.

5 In a preferred embodiment of the invention, an ELISA is used for the detection of the incubation products. Component of ELISAs are enzymes which are bound to one partner of the immunological reaction. The tracer antigen (analyte derivative) of a marker protein is preferably labeled in the competitive ELISA using a single capture antibody (herein after referred to as primary), whereas the antibody is preferably labeled in the non-competitive  
10 ELISA preferably comprising the precipitation of the antigen-antibody complex by a second antibody (herein after referred to as secondary) which is directed to another epitope of said marker protein than the primary antibody. Complexes consisting of antigen and two antibodies are also called sandwich complexes. The detection comprises the subsequent enzymatic conversion of a substrate to a product, preferably a colored product, which is  
15 recognized by visual coloring, bioluminescence, fluorescence or the measurement of electrical signals (enzyme electrode). Favorable enzymes for labeling in the present invention are known to the skilled artisan, such as peroxidase (e.g. HRP), chloramphenicol acetyl transferase (CAT), green fluorescent protein (GFP), glutathione S-transferase (GST), luciferase,  $\beta$ -galactosidase and AP.

20

Additionally preferred are radioactive immunoassays utilizing radioactive isotopes which are either incorporated into an immune reagent during synthesis, or subsequently coupled to an immune reagent of the assay, preferably to an antibody. Preferred radioactive isotopes in the inventive method are  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^{35}\text{S}$ , and  $^{125}\text{I}$ , and more preferred  $^{14}\text{C}$ ,  
25  $^{35}\text{S}$ , and  $^{125}\text{I}$ . A favorite method follows the competitive principle of binding. A constant amount of radioactive protein marker and a variable amount of said marker of the sample to be analyzed compete for a defined amount of antibody which is present in excess. The displacement of tracer is directly proportional to the marker concentration which can be evaluated by a calibration curve.

30

Antigens or antibodies, respectively, which are favorably labeled with fluorophores, are used in FIAs.

35

SPIA utilizes the color change of silver particle as result of agglutination. Neither a secondary antibody nor an indicator reaction are required making it particularly useful in the scope of the present invention. Similarly favorably is the latex agglutination test using antibodies which are bound to colored latex particles.

Another favorite detection method for specific incubation products of the invention is western blotting. Firstly, a gel is mixed and cast, samples previously prepared are loaded onto the gel and fractionated by electrophoresis. The proteins present in the

5 polyacrylamide gel are blotted onto a nitrocellulose membrane to which antibodies may be applied to detect the specific protein of interest. Western blotting is simply performed and advantageously when an exact determination of the concentration is dispensable.

A distinct number of specific antibodies against the protein markers of the invention is  
10 available. Antibodies are usually produced in mammal organisms when an immune response is caused by antigens being strange to the organism and having a molecular weight which exceeds 3.000 g/mol. Favorable host species for antibody production comprise goat, rabbit, and mouse. Further polyclonal and monoclonal antibodies can be selected originated from different species and fragments thereof. Popular techniques, such  
15 as the hybridoma technology, are well-known to the skilled artisan. The antibodies are applied as specific substances in the inventive method.

The antibodies can be immobilized onto a variety of solid supports, such as magnetic or chromatographic matrix particles, the surface of an assay plate {e.g. microtiter wells},  
20 pieces of a solid substrate material or membrane {e.g. plastic, nylon, paper) and the like. An assay strip can be prepared by coating the antibody or a plurality of antibodies in an array on a solid support. This strip can then be dipped into the test sample and processed quickly through washes and detection steps to generate a measurable signal, such as a colored spot.

25

The analysis can be carried out in a variety of physical formats. For example, the use of microtiter plates or automation could be used to facilitate the processing of large numbers of test samples. Alternatively, single sample formats could be developed to facilitate diagnosis or prognosis in a timely fashion. Useful physical formats comprise surfaces  
30 having a plurality of discrete, addressable locations for the detection of a plurality of different biomarkers. Such formats include protein microarrays or protein chips. In these embodiments, each discrete surface location may comprise antibodies to bind one or more protein markers for detection at each location. Surfaces may alternatively comprise one or more discrete particles (e.g. microparticles or nanoparticles) immobilized at discrete  
35 locations of a surface, where the microparticles comprise antibodies to immobilize one or more protein markers for detection. In one embodiment, a matrix is provided, in which each position represents a discrete binding site for a protein, and in which binding sites are

present for all proteins p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68).

A signal from the direct or indirect label can be analyzed, for example, using a  
5 spectrophotometer to detect color from a chromogenic substrate, using a radiation counter to detect radiation, such as a gamma counter for detection of  $^{125}\text{I}$ , or using a fluorometer to detect fluorescence in the presence of light of a certain wavelength. For detection of enzyme-linked antibodies, a quantitative analysis can be made using a spectrophotometer, such as an EMAX Microplate Reader (Molecular Devices; Menlo Park, CA) in accordance  
10 with the manufacturer's instructions. If desired, the assays of the present invention can be automated or performed robotically, and the signal from multiple samples can be detected simultaneously. In detail, the proteins can be detected by fluorescence which is recorded, for example, with a fluorescence laser microscope and a CCD camera, and the fluorescence intensity is analyzed with a computer. Optical images viewed and optionally  
15 recorded by a camera or other recording device (e.g. a photodiode and data storage device) are optionally further processed in any of the embodiments herein, e.g. by digitizing the image and storing and analyzing the image on a computer. A variety of commercially available peripheral equipment and software is available for digitizing, storing and analyzing a digitized video or digitized optical image. One conventional system carries light  
20 from the specimen field to a cooled charge-coupled device (CCD) camera, in common use in the art. A CCD camera includes an array of picture elements (pixels). The light from the specimen is imaged on the CCD. Particular pixels corresponding to regions of the specimen are sampled to obtain light intensity readings for each position. Multiple pixels are processed in parallel to increase speed. The apparatus and methods of the invention  
25 are easily used for viewing any sample, e.g. by fluorescent or dark field microscopic techniques.

In another embodiment of the screening method, the detection of mutagenic and/or pro-mutagenic activity can be additionally refined. For this purpose, the protein expression is  
30 determined by detecting several proteins selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68), and correlating an amount of signal, or change in signal, with the protein expression in the system. In other words, the expression levels of the active forms of the proteins correlate with an amount of an emitted physical  
35 signal, or change in an emitted physical signal. The cellular system of the invention is incubated with various concentrations of an identified (pro-)genotoxic compound. The amount of emitted signal, or change in signal, observed in the presence of the mutagenic

compound is indicative of the change in protein expression experienced by the compound. The change can be then related to the concentration of the mutagen in the sample, i.e. the calibration curve enables the meter-reading of a matching concentration. Preferably, the calibration curve is based on the Lambert-Beer equation if using UV/VIS coloring or luminescence. Genotoxicity of compounds is diagnosed by comparing the concentration of the protein in the sample with known protein concentration levels of cells treated with mutagens and/or not. It shall be understood that the known concentrations are statistically proven, therefore representing a certain level or range, respectively. The direction and strength of protein expression have also been figured out by the differential expression analysis of the marker proteins of the invention such that a distinct up-regulation with a certain factor has been recognized. Any measured concentration, which differs from the protein concentration level of non-stimulated cells, indicates an abnormality of the tested cell sample, whereas a compound cannot be classified as mutagen at a protein concentration which is comparable to the concentration level of non-stimulated cells. It is preferred to measure concentrations, which are higher than the protein concentration level of non-stimulated cells, for detecting genotoxicity. Using this method, the inventors demonstrated sensitivity to submicromolar or even nanomolar concentrations. The calibration plot reveals that the method can be applied in a dynamic range that spans over a couple of magnitude.

Therefore, the method of the invention includes that a level of genotoxic and/or pro-genotoxic activity is screened by comparing the protein expression level in the test system with the protein expression level in the control system.

Although the biomarker panel of the invention exhibits a sensitivity that allows the use of only two marker proteins in the scope of the screening method, it is preferred to apply more than these marker proteins for detecting genotoxicity. The inventors have illustrated that analyzing multiple mutagen-responsive proteins increases screening stability and reduces error rates by covering a broader spectrum of genotoxic responses than low-plurality-protein reporter assays. In a preferred embodiment of the present invention, the expression levels of at least three, four, five, six, seven, eight or nine of said proteins are determined, more preferably at least five proteins, most preferably nine proteins.

It is most preferred that the expression levels of at least the proteins p-p53 (Ser15) and p21 are determined. It is highly preferred that the expression levels of at least the proteins p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981) and p-Chk1 (Ser345) are determined. In addition to the expression of said five proteins, the expression levels of one

or more proteins selected from the group of p-ATR (Ser428), p-cdc2 (Thr14/Tyr15) and p-Chk2 (Thr68) can be determined, particularly selected from the group of p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68).

5 In another most preferred embodiment of the invention, the expression levels of at least the proteins p-p53 (Ser15), p21, p-H2A.X (Ser139), p-Chk1 (Ser345), p-ATR (Ser428), p-Chk2 (Thr68) and optionally MDM2 are determined. It is still another most preferred embodiment of the invention that the expression levels of at least the proteins p-p53 (Ser15), p-H2A.X  
10 (Ser139), p-Chk1 (Ser345) and p-Chk2 (Thr68) as well as total protein levels of p21 and optionally ATR and MDM2 are determined.

It is highly preferred that the expression levels of the proteins p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATR (Ser428), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15),  
15 Gadd45a and p-Chk2 (Thr68) are determined.

It goes without saying that the expression of said respective preferred marker proteins in any preferred embodiment is compared with the protein expression in the control system.

20 If the system or the sample thereof is capable of expressing multiple proteins and an expression pattern of multiple proteins is compared with the expression pattern in the control system, the genotoxicity can be characterized compound-specifically. Particularly, the expression pattern is determined by a correlation of the multiple proteins and/or a magnitude of altered regulation. The screening method of this invention does not only  
25 evaluate the effect of chemical substances having genotoxic activity on cells to be evaluated, but can also indicate the details of this effect. By individually evaluating the expression level of categorized proteins, it is possible to distinguish how chemical compounds having genotoxic activity that affect the cells to be evaluated.

30 The invention also teaches an embodiment of the screening method, wherein the expression levels are determined in a biological sample withdrawn from a mammal, preferably a laboratory mammal, administered with one or more compounds to be screened, in comparison with a mammal showing non-genotoxic effects, wherein an increase in the expression levels indicates an increased likelihood that the compound has a  
35 therapeutic effect for a genotoxicity-susceptible pathological condition. With the therapeutic effect, the qualitative level is incorporated. A "therapeutic effect" relieves to some extent one or more symptoms of a disease or returns to normality, either partially or completely,

one or more physiological or biochemical parameters associated with or causative of the disease or pathological conditions. In addition, the expression "therapeutically effective amount" denotes an amount which, compared with a corresponding subject who has not received this amount, has the following consequence: improved treatment, healing,  
5 prevention or elimination of a disease, syndrome, condition, complaint, disorder or side-effects or also the reduction in the advance of a disease, complaint or disorder. The expression "therapeutically effective amount" also encompasses the amounts which are effective for increasing normal physiological function. Testing of several compounds makes the selection of that compound possible that is best suited for the treatment of the mammal  
10 subject. The in-vivo dose rate of the chosen compound is advantageously pre-adjusted to the specific cells with regard to their in-vitro data. Therefore, the therapeutic efficacy is remarkably enhanced.

In another aspect of the invention, the least (pro-)genotoxic compound or a non-(pro-)genotoxic compound is identified, e.g. within a series of compounds, wherein the least  
15 increase or no increase in the expression levels indicates said compound, which is suitable for its intended use. The use could be therapeutic or non-therapeutic and is not limited to any particular purpose but complies with the intended use as known in the art (e.g. manual, paper, etc.). In a preferred aspect of the invention, a method for administering a therapeutic  
20 compound to a patient is provided, comprising performing the screening method of the invention with a series of compounds, identifying the compound having no genotoxic and pro-genotoxic activity, and administering said compound to a patient in need of the compound's intended use. It shall be understood that the method does not aim the identification of any use and/or the most active compound for said use but the elimination  
25 of adversary (pro-)genotoxic effects in the course of the intended use.

The invention also relates to a method for monitoring the likelihood of developing cancer, tumor, metastasis and/or disorder of angiogenesis, which are caused, mediated and/or propagated by deregulation of proliferation, differentiation and/or damage repair, in  
30 response to a treatment with a compound, wherein the expression levels of at least two proteins selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68) are determined in a biological sample withdrawn from a mammal in need of such treatment with said compound administered to said mammal, wherein an increase in the  
35 expression levels of at least one of said proteins indicates that said compound has genotoxic and/or pro-genotoxic activity and said likelihood is increased. The compound is preferably obtained by the screening method of the invention as set forth above. Thus, the

prior teaching of the present specification concerning the screening method is valid and applicable without restrictions to method of monitoring if expedient.

5 The identification of the plurality of genes described above provides a powerful tool for assessing the progression of a state, condition or treatment. Specifically, a plurality of genes can be identified in a patient prior to an event, such as surgery, the onset of a therapeutic regime, or the completion of a therapeutic regime, to provide a base line result. This base-line can then be compared with the result obtained using identical methods either during or after such event. This information can be used for both diagnostic and  
10 prognostic purposes.

The inventive method of monitoring can be employed in human and veterinary medicine. The mammal is preferably a laboratory animal and/or a non-human organism. Herein, the compounds can be administered before or following an onset of disease once or several  
15 times acting as therapy. The terms "effective amount" or "effective dose" or "dose" are interchangeably used herein and denote an amount of the pharmaceutical compound having a prophylactically or therapeutically relevant effect on a disease or pathological conditions, i.e. which causes in a tissue, system, animal or human a biological or medical response which is sought or desired, for example, by a researcher or physician.

20 The aforementioned medical products of the inventive use are particularly used for the therapeutic treatment. Monitoring is considered as a kind of treatment, wherein the compounds are preferably administered in distinct intervals, e.g. in order to booster the response and eradicate the pathogens and/or symptoms of the genotoxicity-mediated  
25 disease completely. Either the identical compound or different compounds can be applied. The medicament can also be used to reduce the likelihood of developing a disease or even prevent the initiation of those diseases in advance that are associated with proliferation, differentiation and/or damage repair because of a genotoxic impact, or to treat the arising and continuing symptoms. In the meaning of the invention, prophylactic treatment is  
30 advisable if the subject possesses any preconditions for the aforementioned physiological or pathological conditions, such as a familial disposition, a genetic defect, or a previously passed disease. The diseases as concerned by the invention are preferably cancer, tumors, metastasis and/or disorders of angiogenesis.

35 The said compounds according to the invention can be used in their final non-salt form. On the other hand, the present invention also encompasses the use of these compounds in the form of their pharmaceutically acceptable salts, which can be derived from various organic

and inorganic acids and bases by procedures known in the art. The expressions  
“pharmaceutically acceptable salt” and “physiologically acceptable salt”, which are used  
interchangeable herein, in the present connection are taken to mean an active ingredient  
which comprises a compound according to the invention in the form of one of its salts, in  
5 particular if this salt form imparts improved pharmacokinetic properties on the active  
ingredient compared with the free form of the active ingredient or any other salt form of the  
active ingredient used earlier. The pharmaceutically acceptable salt form of the active  
ingredient can also provide this active ingredient for the first time with a desired  
pharmacokinetic property which it did not have earlier and can even have a positive  
10 influence on the pharmacodynamics of this active ingredient with respect to its therapeutic  
efficacy in the body.

Moreover, the invention relates to an in-vitro method for predicting the likelihood that a  
patient will suffer from a tumor in response to a therapeutic treatment with a drug,  
15 comprising the steps of (i) measuring in a biopsy sample from tissue or plasma of said  
patient expression levels of at least two proteins selected from the group of p-p53 (Ser15),  
p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2  
(Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68), (ii) exposing ex-vivo a tissue sample from  
tissue or plasma of said patient to said drug, and (iii) measuring in said exposed sample of  
20 step (ii) the expression levels of said proteins specified in step (i) along with calculating the  
differences in expression levels measured in steps (i) and (iii), wherein an increase in the  
expression levels of at least one of said proteins obtained in this step (iii) compared to step  
(i) indicates that said drug has genotoxic and/or progenotoxic activity and said likelihood  
is increased.

25  
Object of the invention is also the use of at least two proteins selected from the group of p-  
p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428),  
p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68) as marker proteins for screening  
compounds with genotoxic and/or pro-genotoxic activity. The prior teaching of the present  
30 specification concerning the screening method is valid and applicable without restrictions to  
said uses if expedient.

It is still another object of the present invention to use substances specifically interacting  
with at least one marker protein for detecting genotoxic or pro-genotoxic activity. It is a  
35 preferred aspect of the present invention to use at least two antibodies, each with specific  
binding to a different protein selected from the group of p-p53 (Ser15), p21, p-H2A.X  
(Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15),



Gadd45a and p-Chk2 (Thr68), for detecting genotoxic and/or pro-genotoxic activity. The antibodies are preferably directly labeled with isotopes, e.g. chromophores, luminophores or chromogens, or indirectly labeled with biotin to which a streptavidin complex may later bind. By assaying the presence or absence of the antibodies, one can detect the presence or  
5 absence of a marker protein of interest. The prior teaching of the present specification concerning the screening method is considered as valid and applicable without restrictions to said use of said anti-marker antibodies if expedient.

The invention may also be practiced as a kit for use in the detection of genotoxic and/or  
10 pro-genotoxic activity comprising at least two antibodies, each with specific binding to a different protein selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68). The kit is particularly designed to perform the inventive method for screening compounds with genotoxic and/or pro-genotoxic activity. The kit of the invention may  
15 include an article that comprises written instructions or directs the user to written instructions for how to practice the method of the invention. The prior teaching of the present specification concerning the screening method is considered as valid and applicable without restrictions to the kit if expedient.

20 In the scope of the present invention, a method for screening compounds with genotoxic activity, which applies unique protein expression patterns of up to nine proteins, is provided for the first time. The present invention teaches characteristic expression fingerprints of marker proteins that are associated with genotoxicity. The data support that mechanistic profiling in-vitro is a powerful tool compared to single endpoint detections to predict  
25 genotoxicity. Mechanistic profiling is of benefit during interpretation of such data, and mechanistic investigations are a powerful tool facilitating classification of genotoxic compounds. Furthermore, mechanistic data will improve chemical characterization and risk assessment for genotoxic compounds. Applying protein profiling to early screening during pharmaceutical development helps to rank different molecules and highlight compounds  
30 with genotoxic characteristics early in development, saving costs and animals by preventing follow-up testing in-vivo.

The nine putative marker proteins found can be used for screening unknown compounds, which effectively allows a specific and sensitive identification and characterization of their  
35 genotoxic and/or pro-genotoxic potential in the early phase of drug development. Genotoxic stress of the cellular system, which is able to functionally express up to nine marker proteins, leads to an increased expression and activation of these marker proteins

compared to a control of this cellular system. The aforementioned proteins in combination have a synergistic effect, and therefore allow detecting genotoxins with a high performance. The analysis of the differentially expressed proteins is particularly suitable for high throughput test systems. High Content Imaging (HCI)-based protein expression analyses can be favorably applied to genotoxicants with an unknown mode of action and predict their potential to exert genotoxic effects early in the drug discovery process. In addition to that, a cell-based model using HCI, has the advantage of generating data rapidly with small compound needs. The detection method as well as arising monitoring method of the invention can be performed in a simple, cost-efficient and reliable manner.

Compounds having (pro-)genotoxic activity are screened with a sensitivity and/or specificity of at least 75%, more preferably at least 80%, most preferably at least 90%. Herein, "sensitivity" denotes the number of compounds that are correctly tested to be positive in relation to all positive tested compounds, and "specificity" denotes the number of compounds that are tested to be negative in relation to all negative compounds. This specific marker set can be combined with other genotoxicity endpoints like micronucleus assay to generate a highly predictive screening system for a broad range of potentially genotoxic compounds.

All references cited herein are incorporated by reference in their entirety in the disclosure of the invention.

It is to be understood that this invention is not limited to the particular methods, specific substances, uses and arrays described herein, as such matter may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention, which is only defined by the appended claims. As used herein, including the appended claims, singular forms of words such as "a," "an," and "the" include their corresponding plural referents unless the context clearly dictates otherwise. Thus, e.g. reference to "a compound" includes a single or several different compounds, and reference to "a method" includes reference to equivalent steps and methods known to a person of ordinary skill in the art, and so forth. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art to which this invention belongs.

The techniques that are essential according to the invention are described in detail in the specification. Other techniques which are not described in detail correspond to known standard methods that are well known to a person skilled in the art, or the techniques are

described in more detail in cited references, patent applications or standard literature. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable examples are described below. The following examples are provided by way of illustration and not by way of  
5 limitation. Within the examples, standard reagents and buffers that are free from contaminating activities (whenever practical) are used. The example are particularly to be construed such that they are not limited to the explicitly demonstrated combinations of features, but the exemplified features may be unrestrictedly combined again if the technical problem of the invention is solved.

10

Table 1 lists the five proteins analyzed and the four supplementary markers with their post translational modifications, (alternative) protein names, (alternative) gene names and accession numbers, which are unique for each protein.

15

Table 2 lists the results for the nine protein markers (p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68)) tested with (pro-)genotoxins (cyclophosphamide, 7,12-dimethylbenzanthracene, aflatoxin B<sub>1</sub>, 2-acetylaminofluorene, actinomycin D, methyl methanesulfonate, etoposide) and non-genotoxins (D-mannitol, phenfomin HCl,  
20 progesterone). Positive/negative results are displayed by the colors red/green, respectively.

25

Table 3 lists the group 1 chemicals ("in-vivo genotoxins which should be detected as positive in in-vitro mammalian cell genotoxicity tests") recommended by the European Center for the Validation of Alternative Methods (ECVAM) in order to judge the  
performance of in-vitro genotoxicity tests (Kirkland et al., 2008, Mutat. Res. 653, 99-108).

30

Table 4 lists the group 2 chemicals ("non-DNA-reactive chemicals, including non-genotoxic carcinogens, which should give negative results in in-vitro mammalian cell genotoxicity tests") recommended by the European Center for the Validation of Alternative Methods (ECVAM) in order to judge the performance of in-vitro genotoxicity tests (Kirkland et al.,  
2008, Mutat. Res. 653, 99-108).

35

Table 5 lists the group 3 chemicals ("Chemicals that should give negative results in in-vitro mammalian cell genotoxicity tests but have been reported to induce chromosomal  
aberrations or tk mutations in mouse lymphoma cells, often at high concentrations or at  
high levels of cytotoxicity.") recommended by the European Center for the Validation of

Alternative Methods (ECVAM) in order to judge the performance of in-vitro genotoxicity tests (Kirkland et al., 2008, Mutat. Res. 653, 99-108).

Table 6 lists the group 1 chemicals ("in-vivo genotoxins which should be detected as positive in in-vitro mammalian cell genotoxicity tests") recommended by the ECVAM and their highest concentration tested limited by autofluorescence (AF), cytotoxicity based on previous experiments (Cyto) and precipitations (Prec) as well as the highest concentration <50% for the two cytotoxicity parameters selected cell count per valid field (SCC) and CMFDA cytoplasm average intensity (CMFDA).

Table 7 lists the group 2 chemicals ("non-DNA-reactive chemicals, including non-genotoxic carcinogens, that should give negative results in in-vitro mammalian cell genotoxicity tests") recommended by the ECVAM and their highest concentration tested limited by autofluorescence (AF), cytotoxicity based on previous experiments (Cyto) and precipitations (Prec) as well as the highest concentration <50% for the two cytotoxicity parameters selected cell count per valid field (SCC) and CMFDA cytoplasm average intensity (CMFDA).

Table 8 lists the group 3 chemicals ("Chemicals that should give negative results in in-vitro mammalian cell genotoxicity tests but have been reported to induce chromosomal aberrations or tk mutations in mouse lymphoma cells, often at high concentrations or at high levels of cytotoxicity.") recommended by the ECVAM and their highest concentration tested limited by autofluorescence (AF), cytotoxicity based on previous experiments (Cyto) and precipitations (Prec) as well as the highest concentration <50% for the two cytotoxicity parameters selected cell count per valid field (SCC) and CMFDA cytoplasm average intensity (CMFDA).

Table 9 lists the results for the five protein markers (p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345)) tested with the group 1 chemicals ("in-vivo genotoxins which should be detected as positive in in-vitro mammalian cell genotoxicity tests") recommended by the ECVAM. Positive/ negative results are displayed by the colors red/green, respectively.

Table 10 lists the results for the five protein markers (p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345)) tested with the group 2 chemicals ("non-DNA-reactive chemicals, including non-genotoxic carcinogens, that should give negative results in in-vitro

mammalian cell genotoxicity tests”) recommended by the ECVAM. Positive/negative results are displayed by the colors red/green, respectively.

5 Table 11 lists the results for the five protein markers (p-p53 (Ser15), p21, p-H2A.X(Ser139), p-ATM (Ser1981), p-Chk1 (Ser345)) tested with the group 3 chemicals (“Chemicals that should give negative results in in-vitro mammalian cell genotoxicity tests but have been reported to induce chromosomal aberrations or tk mutations in mouse lymphoma cells, often at high concentrations or at high levels of cytotoxicity.”) recommended by the ECVAM. Positive/negative results are displayed by the colors red/green, respectively.

10

Table 12 lists the results of changes in phosphorylated p53 (Ser15) and total protein level of p21 in cell lysates using a MILLIPLEX MAP Magnetic Bead kit and Luminex system.

#### EXAMPLE

##### 15 • Chemicals and cell culture media supplements

The compound set tested is recommended by the European Center for the Validation of Alternative Methods (ECVAM) in order to judge the performance of in-vitro genotoxicity tests: The two out of three groups of compounds which were already tested are listed (Table 3 , 4 and 5) (Kirkland et al., 2008, Mutat. Res. 653, 99-108). All compounds tested  
20 were obtained at a minimum purity of 97%. Most of the compounds were ordered from Sigma-Aldrich (Taufkirchen, Germany) except Cyclophosphamide which was from Calbiochem (Darmstadt, Germany); 2-Acetylaminofluorene and Taxol were from Acros Organics (Geel, Belgium), Dimethylnitrosamine and Fluometuron were from Dr. Ehrenstorfer GmbH (Augsburg, Germany), 2-Amino-3methylimidazo[4,5-f]quinolone and 2-  
25 Amino-1-methyl-6-phenylimidazo[4,5-f]pyridine were from Apollo Scientific Ltd. (Bredbury, UK), N,N-Dicyclohexylthiourea and Amitrole were from TCI Europe (Zwijndrecht, Belgium) and Ephidrin sulfate was from LGC (Teddington, UK). DMEM/F12, gentamicin, and sodium pyruvate were purchased from Invitrogen Corp. (Karlsruhe, Germany). Foetal bovine serum (FBS) was obtained from HyClone (Order No. SV30160.03, Lot No. RSJ30856, HyClone  
30 UK Ltd., Cramlington, UK). B-naphthoflavone/ phenobarbital-induced rat liver S9 (Order No. R1081.S9, Lot No. 0710507) was ordered from Tebu-bio (Offenbach, Germany). Trypsin,  $\beta$ -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH) and penicillin/streptomycin solution were obtained from Sigma-Aldrich (Taufkirchen, Germany). Magnesium chloride, potassium chloride, sodium phosphate monobasic monohydrate and sodium phosphate dibasic heptahydrate were purchased  
35 from Merck KGaA (Darmstadt, Germany).

- Cell culture

HepG2 cells (Order No. HB-8065, Lot. 58483209, ATCC, Manassas, USA) were cultured in DMEM/F12 with L-Glutamine and 15 mM Hepes supplemented with 10% (v/v) FBS, 1% (v/v) penicillin (10 kU/ml)/ streptomycin (10 mg/ml) solution, 0.1% (v/v) gentamicin (50 mg/ml), and 1 mM sodium pyruvate at 37°C and 5% CO<sub>2</sub> in culture flasks. Depending on the experiment, an appropriate number of cells were seeded onto plates after detaching of the cells using trypsin and cells were cultured at 37°C and 5% CO<sub>2</sub> for 24 h prior to treatment. All experiments were performed three times with cells of passages 4-20.

10    Testing of nine putative marker proteins

- Cell treatment and dose selection

Cells were seeded onto black Poly-D-Lysin coated 96-well-plates (BD Biocoat, Franklin Lakes, USA) ( $0.02 \times 10^6$  cells/well) and left 24 h for adherence and then treated with test compounds. For each compound, seven concentrations were tested with 2-fold serial dilutions. DMSO 1 % (v/v) served as vehicle control for the experiments. The treatment procedure was different for direct-genotoxic and pro-genotoxic compounds. The treatment for direct genotoxic compounds was ongoing and was repeated daily over 48 h. Pro-genotoxic compounds were incubated for 6 h together with metabolic activation system (to limit the cytotoxic effect of the S9-mixture). After this period, cells were washed with cell media followed by 18-repeated daily over 48 h. The metabolic activation system used consisted of the following components and concentrations: 8 mM MgCl<sub>2</sub>, 32.8 mM KCl, 12 mM NADPH, 124 mM phosphate buffer and 2500 pmol/ml cytochrome P450 (CYP) content in the pre-mixture corresponding to 2.4 mM MgCl<sub>2</sub>, 9.8 mM KCl, 3.6 mM NADPH, 37.2 mM phosphate buffer, and 750 pmol/ml CYP as final concentrations after dilution 1:3.33 with cell culture media.

- Immunofluorescence staining

- Staining protocol for cytotoxicity parameters:

After treatment, cells were rinsed with PBS (Gibco Invitrogen, Karlsruhe, Germany), and then stained with 10 µM CellTracker Green 5-Chloromethylfluorescein Diacetate (CMFDA) (Invitrogen, Karlsruhe, Germany) in cell culture media. CMFDA is per se non-fluorescent and is hydrolyzed by esterase in viable cells to a fluorescent-dye. The reaction with thiol groups leads to a cell-impermeable fluorescent-dye-adduct. The esterase activity can be used as an indicator for viability of cells (Papadopoulos et al., 1994, J Immunol Methods 177, 101-111). After an incubation period of 30 min at 37°C, cells were washed with PBS and then fixed with 3.7% formaldehyde (Sigma-Aldrich, Taufkirchen, Germany) in PBS for

20 min. Afterwards, two additional washing steps with 0.05% Tween 20 followed, then cells were washed with PBS and finally PBS was added and cells were measured.

- Staining protocol for putative marker proteins:

- 5 After treatment, cells were rinsed with PBS (Gibco Invitrogen, Karlsruhe, Germany), and then fixed with 3.7% formaldehyde (Sigma-Aldrich, Taufkirchen, Germany) in PBS for 20 min. After washing twice with PBS, cells were blocked and permeabilized with 7.5% goat serum (Sigma-Aldrich, Taufkirchen, Germany) and 0.3% Triton X-100 (Sigma-Aldrich, Taufkirchen, Germany) in PBS for 30 min. Each primary antibodies were diluted separately
- 10 to final concentration of 10 µg/ml anti-Gadd45α rabbit monoclonal antibody IgG (Cell Signaling, Danvers, USA), 10 µg/ml anti-p21 rabbit monoclonal antibody IgG (Cell Signaling, Danvers, USA); 5 µg/ml anti-p-ATM (Ser1981) rabbit monoclonal antibody IgG (Cell Signaling, Danvers, USA); 10 µg/ml anti-p-ATR (Ser428) rabbit monoclonal antibody IgG (Cell Signaling, Danvers, USA); 4 µg/ml anti-p-cdc2 (Tyr15/Thr14) rabbit polyclonal
- 15 antibody IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, USA); 20 µg/ml anti-p-Chk1 (Ser345) rabbit monoclonal antibody IgG (Cell Signaling, Danvers, USA); 20 µg/ml anti-p-Chk2 (Thr68) rabbit monoclonal antibody IgG (Cell Signaling, Danvers, USA); 2.5 µg/ml anti-p-Histone H2A.X (Ser139) rabbit monoclonal antibody IgG1 (Cell Signaling, Danvers, USA) or 2 µg/ml anti-p-p53 (Ser15) rabbit monoclonal antibody IgG (Invitrogen, Karlsruhe,
- 20 Germany) in Blocking/Permeabilization solution were added afterwards. After incubation for 16-18 h, cells were washed twice with 0.05% Tween 20 (Calbiochem, Darmstadt, Germany) in PBS and the secondary antibody/16 µM Hoechst staining solution in Blocking/Permeabilization solution was added. Secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen, Karlsruhe, Germany) was diluted to a final concentration
- 25 2 µg/ml. After an incubation of 1 h, followed by two additional washing steps with 0.05% Tween 20, cells were washed with PBS and finally PBS was added and cells were measured.

• Image acquisition and analysis for cytotoxicity parameters:

- 30 Image acquisition was performed on the ArrayScan VTI HCS Reader (Cellomics, Pittsburgh, USA) using a 10x objective. To detect enough cells, 20 images starting at the middle of each well were gathered.

The filter for were chosen according to the excitation/ emission wavelengths for each dye:

- 35 - Protocol for cytotoxicity parameters:

Channel 1: 365 ± 25 and 515 ± 10 nm (XF93- Hoechst) for Hoescht 33342

Channel 2: 475 ± 20 and 515 ± 10 nm (XF93- FITC) for CMFDA

- Image acquisition and analysis for putative marker proteins:

Image acquisition was performed on the ArrayScan VTI HCS Reader (Cellomics, Pittsburgh, USA) using a 20x objective. To detect enough cells, 20 images starting at the middle of each well were gathered.

The filter for were chosen according to the excitation/ emission wavelengths for each dye:

- Protocol for putative marker proteins:

Channel 1:  $365 \pm 25$  and  $515 \pm 10$  nm (XF93- Hoechst) for Hoescht 33342

10 Channel 2:  $475 \pm 20$  and  $515 \pm 10$  nm (XF93- FITC) for Alexa Fluor 488 goat anti-rabbit

The analysis of the images was performed with the software iDev and the Bioapplication Version 4 (Cellomics, Pittsburgh, USA). The stained nuclei were used for localization of the nucleus and the cytoplasm (ring around the nucleus).

15

The following readout parameters were generated:

- Protocol for cytotoxicity parameters:

Channel 1: selected cell count per valid field

Channel 2: CMFDA cytoplasm average intensity

20

- Protocol for putative marker proteins:

Channel 2: protein nucleus average intensity

- Data analysis and interpretation

25 Fold-regulations of treated samples against the vehicle control were calculated for each compound and concentration. Statistical significance ( $p$ -value  $< 0.05$ ) was determined using the Student's t-test. The thresholds for each parameter were chosen based on the control value and addition of three times the standard deviation compared to the control value. For p-p53 (Ser15), p-H2A.X (Ser139) and p-ATM (Ser1981) the thresholds were set to 1.5, for p21, p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Tyr15/Thr14) and Gadd45  $\alpha$  to 1.6 and p-Chk2 (Thr68) to 1.9. Concentrations with precipitation or autofluorescence were excluded as well as concentrations which showed at least in one of the two cytotoxicity parameters (selected cell count per valid field and CMFDA intensity in the cytoplasm) a cytotoxic effect  $\geq 50\%$ .

35

Testing of five marker proteins



- Cell treatment and dose selection

Cells were seeded onto black Poly-D-Lysin coated 96-well-plates (BD Biocoat, Franklin Lakes, USA) ( $0.02 \times 10^6$  cells/well) and left 24 h for adherence and then treated with test compounds at a maximum concentration of 1 mM which is recommended by the ICH

5 S2(R1) guideline (EMA, 2011). For each compound, nine concentrations were tested with 2-fold serial dilutions. DMSO 1 % (v/v) served as vehicle control for the experiments. The treatment procedure was different for direct-genotoxic and pro-genotoxic compounds. The treatment for direct genotoxic compounds was ongoing and was repeated daily over 48 h. Pro-genotoxic compounds were incubated for 6 h together with metabolic activation system  
10 (to limit the cytotoxic effect of the S9-mixture). After this period, cells were washed with cell media followed by 18-repeated daily over 48 h. The metabolic activation system used consisted of the following components and concentrations: 8 mM  $MgCl_2$ , 32.8 mM KCl, 12 mM NADPH, 124 mM phosphate buffer and 2500 pmol/ml cytochrome P450 (CYP) content in the pre-mixture corresponding to 2.4 mM  $MgCl_2$ , 9.8 mM KCl, 3.6 mM NADPH,  
15 37.2 mM phosphate buffer, and 750 pmol/ml CYP as final concentrations after dilution 1:3.33 with cell culture media.

- Immunofluorescence staining

Two different staining procedures were performed for each compound with and without  
20 metabolic activation system:

- Staining protocol 1:

After treatment, cells were rinsed with PBS (Gibco Invitrogen, Karlsruhe, Germany), and then fixed with 3.7% formaldehyde (Sigma-Aldrich, Taufkirchen, Germany) in PBS for  
25 20 min. After an additional washing step with PBS, cells were blocked and permeabilized with 2% donkey serum (Millipore, Schwalbach, Germany) and 0.25% Triton X-100 (Sigma-Aldrich, Taufkirchen, Germany) in PBS for 30 min. Primary antibodies were diluted to final concentrations of 2  $\mu$ g/ml anti-p-p53 (Ser15) rabbit monoclonal antibody IgG (Invitrogen, Karlsruhe, Germany), 0.8  $\mu$ g/ml anti-p21 goat polyclonal antibody IgG, (R&D systems,  
30 Minneapolis, USA); 4  $\mu$ g/ml anti-p-Histone H2A.X (Ser139) mouse monoclonal antibody IgG1 (Millipore, Schwalbach, Germany) in Blocking/Permeabilization solution were added afterwards. After incubation for 16-18 h, cells were washed with 0.05% Tween 20 (Calbiochem, Darmstadt, Germany) in PBS and the secondary antibody/16  $\mu$ M Hoechst staining solution in Blocking/Permeabilization solution was added. Secondary antibodies  
35 were diluted 1:400 (Alexa Fluor 488 donkey anti-rabbit, Alexa Fluor 555 donkey anti-goat, Alexa Fluor 647 donkey anti-mouse, Invitrogen, Karlsruhe, Germany). After an incubation

of 1 h, followed by two additional washing steps with 0.05% Tween 20, cells were washed with PBS and finally PBS was added and cells were measured.

- Staining protocol 2:

5 After treatment, cells were rinsed with PBS (Gibco Invitrogen, Karlsruhe, Germany), and then stained with 10  $\mu$ M CellTracker Green 5-Chloromethylfluorescein Diacetate (CMFDA) (Invitrogen, Karlsruhe, Germany) in cell culture media for 30 min. CMFDA is per se non-fluorescent and is hydrolyzed by esterase in viable cells to a fluorescent-dye. The reaction with thiol groups leads to a cell-impermeable fluorescent-dye-adduct. The esterase activity  
10 can be used as an indicator for viability of cells (Papadopoulos et al., 1994, J Immunol Methods 177, 101-11). Afterwards, cells were washed with PBS and fixed with 3.7% Formaldehyde (Sigma-Aldrich, Taufkirchen, Germany) in PBS for 20 min. After that, cells were washed with PBS and blocked and permeabilized with 2% donkey serum (Millipore, Schwalbach, Germany) and 0.25% Triton X-100 (Sigma-Aldrich, Taufkirchen, Germany) in  
15 PBS for 30 min. Primary antibodies were diluted in Blocking/Permeabilization solution to final concentrations of 4  $\mu$ g/ml anti-p-Chk1 (Ser345) goat polyclonal antibody IgG (santa cruz, Santa Cruz, USA), 4  $\mu$ g/ml anti-p-ATM (Ser1981) mouse monoclonal antibody IgG1k (Millipore, Schwalbach, Germany) and added to the cells. After incubation for 16-18 h, cells were washed with 0.05% Tween 20 (Calbiochem, Darmstadt, Germany) in PBS, and the  
20 secondary antibody/ 16  $\mu$ M Hoechst staining solution Blocking/ Permeabilization solution was added. Secondary antibodies were diluted 1:400 (Alexa Fluor 555 donkey anti-goat, Alexa Fluor 647 donkey anti-mouse, Invitrogen, Karlsruhe, Germany). After an incubation of 1 h, followed by two additional washing steps with 0.05% Tween 20, cells were washed with PBS and finally PBS was added and cells were measured.

25

• Image acquisition and analysis

Image acquisition was performed on the ArrayScan VTI HCS Reader (Cellomics, Pittsburgh, USA) using a 20x objective. To detect enough cells, 50 images starting at the middle of each well were gathered.

30

The filter for were chosen according to the excitation/ emission wavelengths for each dye:

- Protocol 1:

Channel 1:  $365 \pm 25$  and  $515 \pm 10$  nm (XF93- Hoechst) for Hoescht 33342

Channel 2:  $475 \pm 20$  and  $515 \pm 10$  nm (XF93- FITC) for Alexa Fluor 488 donkey anti-rabbit

35 Channel 3:  $549 \pm 4$  and  $600 \pm 12.5$  nm (XF93-TRITC) for Alexa Fluor 555 donkey anti-goat

Channel 4:  $655 \pm 15$  and  $730 \pm 25$  nm (XF110-Cy5 (sensitive)) for Alexa Fluor 647 donkey anti-mouse

- Protocol 2:

Channel 1:  $365 \pm 25$  and  $515 \pm 10$  nm (XF93- Hoechst) for Hoescht 33342

Channel 2:  $475 \pm 20$  and  $515 \pm 10$  nm (XF93- FITC) for CMFDA

5 Channel 3:  $549 \pm 4$  and  $600 \pm 12.5$  nm (XF93-TRITC) for Alexa Fluor 555 donkey anti-goat

Channel 4:  $655 \pm 15$  and  $730 \pm 25$  nm (XF110-Cy5 (sensitive)) for Alexa Fluor 647 donkey anti-mouse.

The analysis of the images was performed with the software iDev and the Bioapplication

10 Version 4 (Cellomics, Pittsburgh, USA). The stained nuclei in both assays were used for localization of the nucleus and the cytoplasm (ring around the nucleus).

The following readout parameters were generated for each well:

15 - Protocol 1:

Channel 2: p-p53 (Ser15) nucleus average intensity

Channel 3: p21 nucleus average intensity

Channel 4: p-H2A.X (Ser139) nucleus average intensity

20 - Protocol 2:

Channel 1: selected cell count per valid field

Channel 2: CMFDA cytoplasm average intensity

Channel 3: p-Chk1 (Ser345) nucleus average intensity

Channel 4: p-ATM (Ser1981) nucleus average intensity

25

• Data analysis and interpretation

Fold-regulations of treated samples against the vehicle control were calculated for each compound and concentration. Statistical significance ( $p$ -value  $< 0.05$ ) was determined using the Student's  $t$ -test. The thresholds for each parameter were chosen based on the

30 control value and addition of three times the standard deviation compared to the control value. For p-Chk1 (Ser345) and p-H2A.X (Ser139), the thresholds were set to 2.1 for p21,

p-ATM (Ser1981) to 1.8 and p-p53 (Ser15) to 1.9. If at least one of the five proteins showed a fold-change above this threshold, the compound was set as positive. Otherwise the compound was set as negative. Concentrations with precipitation or immunofluorescence

35 were excluded as well as concentrations which showed at least in one of the two cytotoxicity parameters (selected cell count per valid field and CMFDA intensity in the cytoplasm) a cytotoxic effect  $\geq 50\%$ .

The study aimed to develop a novel, specific and sensitive high content imaging-based test system for mutagens and promutagens in-vitro using HepG2 cells. Due to their limited metabolic capacity, a combined system of HepG2 cells and a metabolic activation system (MAS – rat liver S9) was established for promutagen testing. Up to nine different proteins involved in DNA-damage response served as putative markers for compound-induced genotoxicity. The protein expression- and activation changes were quantified 48h after treatment with (pro-)genotoxins (Cyclophosphamide, 7,12-Dimethylbenzanthracene, Aflatoxin B<sub>1</sub>, 2-Acetylaminofluorene, Actinomycin D, Methyl methanesulfonate, Etoposide) and non-genotoxins (D-mannitol, Phenfomin HCl, Progesterone) using the HCl technology.

The best classification was achieved using five out of nine putative marker proteins. The five most predictive markers out of the aforementioned nine markers (p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/ Tyr15), Gadd45a, p-Chk2 (Thr68)) were selected. While the experiments were predicated on the five markers p-p53 (Ser15), p21, p-H2A.X(Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), an additional analysis of p-ATR (Ser428), p-cdc2 (Thr14/ Tyr15), Gadd45a, p-Chk2 (Thr68) allowed a more detailed characterization of the compound's mode of action, and they were used as supplementary markers.

The analysis of the nine putative markers p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/ Tyr15), Gadd45a and p-Chk2 (Thr68) was quantified 48 h after treatment with (pro-)genotoxins (Cyclophosphamide, 7,12-Dimethylbenzanthracene, Aflatoxin B<sub>1</sub>, 2-Acetylaminofluorene, Actinomycin D, Methyl methanesulfonate, Etoposide) and non-genotoxins (D-mannitol, Phenfomin HCl, Progesterone) using the HCl technology. The best classification was achieved using the five markers (p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981) and p-Chk1 (Ser345)) out of nine putative marker proteins (Table 2).

The analysis of the group 1 chemicals ("in-vivo genotoxins which should be detected as positive in in-vitro mammalian cell genotoxicity tests") recommended by the ECVAM led to four false negative compounds. Ethylnitrosourea, dimethylnitrosamine, 2,4-diaminotoluene and cadmiumchlorid conducted no positive result for any of the protein markers tested. Therefore, the sensitivity of this test system with 18 compounds classified as positive out of 20 in-vivo genotoxins tested is 80% (Table 6 and 9).

The group 2 chemicals ("non-DNA-reactive chemicals, including non-genotoxic carcinogens, which should give negative results in in-vitro mammalian cell genotoxicity tests") led to two false positive tested compounds. Progesterone and Phenanthracene both gave a positive result for p-ATM (Ser1981) resulting in two false positive tested compounds  
5 out of 23 tested compounds. As a consequence, a specificity of 91.3% could be calculated (Table 7 and 10).

The group 3 chemicals ("non-DNA-reactive chemicals, including non-genotoxic carcinogens, metabolic poisons and others that should give negative results in-vitro in  
10 mammalian cell genotoxicity tests, but have been reported to induce chromosomal aberrations or tk mutations in mouse lymphoma cells, often at concentrations or at high levels of cytotoxicity") led to four false positive tested compounds. Propyl gallate, p-nitrophenol, Eugenol and 2,4-dichlorophenol gave positive results resulting in four false positive tested compounds out of 19 tested compounds. As a consequence, a specificity of  
15 78.9% could be calculated (Table 8 and 11).

These five proteins were potential candidates for the identification of potential genotoxicants early in the drug discovery process.

20 • Luminex analysis

A Magnetic Bead kit (Milliplex MAP) was used to detect changes in phosphorylated p53 (Ser15) as well as total protein level of p21 in cell lysates using the Luminex system.

MILLIPLEX MAP is based on the Luminex xMAP technology. Luminex uses proprietary  
25 techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly colored bead sets can be created, each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin-Phycoerythrin (SAPE) conjugate, the reporter molecule,  
30 to complete the reaction on the surface of each microsphere. The microspheres are illuminated, and the internal dyes fluoresce, marking the microsphere set(s) used in a particular assay. A second illumination source excites PE, the fluorescent dye on the reporter molecule. Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals.

35

The performance of the assay was done as described below and reagents were used from the MILLIPLEX MAP kits (EMD Millipore Cat. # 46-662 and 46-621).

- Cell lysis protocol

Adherent or non-adherent cells grown in sterile 96-well tissue culture grade plates could be treated, washed and lysed in the same plate, but needed to be filtered in a separate 96-well filter plate. The protocol steps were as follows: For non-adherent cells, the tissue culture plate was centrifuged for 2 minutes at 500 x g to pellet cells. If adherent cells were used, it was started with the next step. The media was removed via aspiration, and 100 µL ice-cold PBS or TBS were added. For non-adherent cells, the first step was repeated. The wash was removed via aspiration. It was added 35 µL/well ice-cold 1X MILLIPLEX MAP Lysis Buffer containing phosphatase inhibitors (including 1 mM sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ )) and freshly prepared protease inhibitors. The plate was placed on an orbital shaker (600-800 rpm) for 10-15 minutes at 4°C. The lysate was stored at -70°C until ready for use.

- Immunoassay protocol

The filtered lysates were diluted at least 1:1 in MILLIPLEX® MAP Assay Buffer. The suggested working range of protein concentration for the assay was 1 to 25 µg of total protein/well (25 µL/well at 40 to 1000 µg/mL). 50 µL of Assay Buffer were added into each well of the plate and covered and mixed on a plate shaker for 10 minutes at room temperature (20-25°C). The Assay Buffer was decanted, and the residual amount was removed from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. The 1X bead suspension was vortexed for 10 seconds, and 25 µL of 1X bead suspension were added to each well. 25 µL of Assay Buffer, reconstituted control cell lysates and sample lysates were added to appropriate wells and incubated overnight (16-20 hours) at 2-8°C on a plate shaker (600-800 rpm) protected from light. A handheld magnetic separation block was attached to the plate, and 60 seconds were allowed for beads to settle before the samples and controls were decanted. The plate was removed from the magnetic separation block, and washed with 100 µL Assay Buffer per well. It was repeated for a total of two washes. 25 µL/well of 1X MILLIPLEX MAP Detection Antibody were added. The plate was sealed, covered with lid and incubated with agitation on a plate shaker for 1 hour at room temperature (20-25°C). The Magnetic Separation Block was attached, and it was waited for 60 seconds before the Detection Antibody was decanted. 25 µL of 1X MILLIPLEX MAP Streptavidin-Phycoerythrin (SAPE) were added. The plate was sealed, covered with lid and incubated with agitation on a plate shaker for 15 minutes at room temperature (20-25°C). 25 µL of MILLIPLEX MAP Amplification Buffer were added to each well. The plate was sealed, covered with lid and incubated with agitation on a plate shaker for 15 minutes at room temperature (20-25°C). The Magnetic Separation Block was attached, and it was waited for 60 seconds before the SAPE /Amplification buffer was

decanted. The beads were suspended in 150  $\mu$ L of MILLIPLEX MAP Assay Buffer, and mixed on plate shaker for 5 minutes before analysis using the Luminex system.

- Data analysis and interpretation

- 5    Fold-regulations of treated samples against the vehicle control were calculated for each compound and concentration. The thresholds for each parameter were chosen based on the control value and addition of three times the standard deviation compared to the control value. For p-p53 (Ser 15) and p21 the threshold was set to 1.5. If at least one of the proteins showed a fold-change above this threshold, the compound was set as positive.
- 10   Otherwise the compound was set as negative.

The results of the MILLIPLEX MAP Magnetic Bead kit, which was used to detect changes in phosphorylated p53 (Ser15) as well as total protein level of p21 in cell lysates using the Luminex system are shown in Table 12. The genotoxic compounds Methylmethane

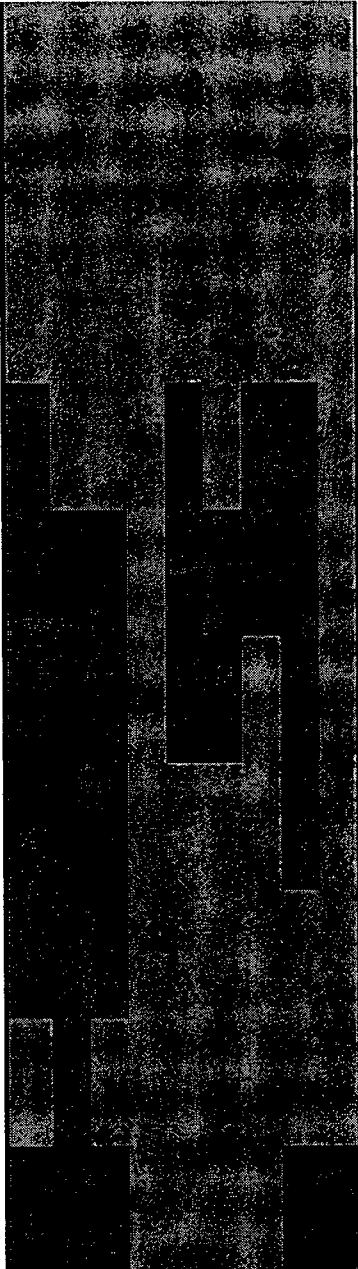
- 15   sulfonate and Etoposide as well as non-genotoxic D-mannitol were tested.

Table 1

<b>Protein</b> (with post translational modification)	<b>Protein name</b> (alternative name)	<b>Gene name</b> (alternative name)	<b>Accession number</b>
p-p53 (Ser15)	Cellular tumor antigen p53, (Alternative name(s): Antigen NY-CO-13 Phosphoprotein p53 Tumor suppressor p53)	TP53 (P53)	P04637
p21	Cyclin-dependent kinase inhibitor 1, (CDK-interacting protein 1 Melanoma differentiation-associated protein 6, p21)	CDKN1A, (CAP20, CDKN1, CIP1, MDA6, PIC1, SDI1, WAF1)	P38936
H2A.X (Ser139)	Histone H2A.X	H2AFX, (H2AX)	P16104
p-Chk1 (Ser345)	Serine/threonine-protein kinase Chk1, (CHK1 checkpoint homolog Cell cycle checkpoint kinase Checkpoint kinase-1)	CHEK1, (CHK1)	O14757
p-ATM(Ser1981)	Serine-protein kinase ATM, (Ataxia telangiectasia mutated)	ATM	Q13315
<b>Supplementary marker</b>			
p-ATR (Ser428)	Serine/threonine-protein kinase ATR, (Ataxia telangiectasia and Rad3-related protein FRAP-related protein 1)	ATR, (FRP1)	Q13535
p-cdc2 (Thr14/ Tyr15)	Cyclin-dependent kinase 1, (Cell division control protein 2 homolog Cell division protein kinase 1 p34 protein kinase)	CDK1, (CDC2, CDC28A, CDKN1, P34CDC2)	P06493
Gadd45a	Growth arrest and DNA damage-inducible protein GADD45 alpha, (DNA damage-inducible transcript 1 protein)	GADD45A, (DDIT1, GADD45)	P24522
p-Chk2 (Thr68)	Serine/threonine-protein kinase Chk2, (CHK2 checkpoint homolog Cds1 homolog, Checkpoint kinase 2)	CHEK2, (CDS1, CHK2, RAD53)	O96017



Table 2

endpoint	I. direct genotoxic			II. progenotoxic + MAS				III. non-genotoxic		
	Eto	ActD	MMS	CPA	DMBA	AFB1	AAF	D-Man	Prog	Phen
highest test concentration [ $\mu$ M]	2	0.2	1000	200	400	200	1000	1000	1000	1000
highest conc. Tested	2	0.013	250	100	400	200	1000	1000	15.63	125
<50% cytotoxicity [ $\mu$ M]	2	0.2	500	50	400	200	1000	1000	31.25	1000
p-p53 (Ser15)										
p21										
p-Histon H2A.X (Ser139)										
Gadd45a										
p-ATM (Ser1981)										
p-ATR (Ser428)										
p-cdc2 (Thr 14/Tyr 15)										
p-Chk1 (Ser345)										
p-Chk2 (Thr68)										

classification

Table 3

compound	abbreviation	CAS number
<b>I. Ames-positive in vivo genotoxins</b>		
(i) O6 and N7 alkylators		
Cyclophosphamide	CPA	6055-19-2
Ethyl nitrosourea	ENU	759-73-9
Methylmethane sulfonate	MMS	66-27-3
(ii) Polycyclic aromatic hydrocarbons		
Benzo[a]pyrene	BaP	50-32-8
7,12-Dimethylbenz[a]anthracene	DMBA	57-97-6
(iii) Aromatic amines		
Dimethylnitrosamine	DMN	62-75-9
2-Acetylaminofluorene	AAF	53-96-3
2,4-diaminotoluene	DAT	95-80-7
IQ (2-Amino-3-methylimidazo[4,5-f]quinoline)	IQ	76180-96-6
PhIP.HCl (2-Amino-1-methyl-6-phenylimidazo[4,5-f]pyridine)	PhIP	105650-23-5
(iv) Others		
Aflatoxin B1	AFB1	1162-65-8
Cadmium chloride	CC	10108-64-2
Cisplatin	CP	15663-27-1
p-chloroaniline	CA	106-47-8
<b>II. In vivo genotoxins negative or equivocal in Ames</b>		
Etoposide	Eto	33419-42-0
Hydroquinone	HQ	123-31-9
Azidothymidine	AZT	30516-87-1
Sodium arsenite	SA	7784-46-5
Taxol	T	33069-62-4
Chloramphenicol	CIA	56-75-7

Table 4

<b>compound</b>	<b>abbreviation</b>	<b>CAS number</b>
<b>(i) Non-carcinogens with negative in vivo genotoxicity data</b>		
Ampicillin trihydrate	AmpT	7177-48-2
D-mannitol	D-Man	69-65-8
<b>(ii) Non-carcinogens with no in vivo genotoxicity data</b>		
Phenformin HCl	Phen	834-28-6
n-butyl chloride	n-BCl	109-69-3
(2-chloroethyl)trimethyl-ammonium chloride	TAC	999-81-5
Cyclohexanone	CyH	108-94-1
N,N-dicyclohexyl thiourea	DCHT	1212-29-9
Trisodium EDTA trihydrate	TET	150-38-9
Ephidrine sulphate	EpS	134-72-5
Erythromycin stearate	EyS	643-22-1
Fluometron	FM	2164-17-2
Phenanthrene	PhA	85-01-8
<b>(iii) Non-genotoxic carcinogens</b>		
D-limonene	DL	5989-27-5
Di-(2-ethylhexyl)phthalate	DEPh	117-81-7
Amitrole	AT	61-82-5
Tert-butyl alcohol	TBA	75-65-0
Diethanolamine	DA	111-42-2
Melamine	Mel	108-78-1
Methyl carbamate	MeC	598-55-0
Progesterone	Pro	57-83-0
Pyridine	Pyr	110-86-1
Tris(2-ethylhexyl)phosphate	TEP	78-42-2
Hexachloroethane	HCE	67-72-1

Table 5

<b>(i) Non-carcinogens that are negative or equivocal for genotoxicity in vivo</b>		
D,L-menthol	Men	15356-70-4
Phthalic anhydride	PhAh	85-44-9
Tertiary-butylhydroquinone	TBHQ	1948-33-0
o-Anthranilic acid	AA	118-92-3
1,3-Dihydroxybenzene (resorcinol)	DB	108-46-3
2-Ethyl-1,3-hexanediol	EH	94-96-2
Sulfisoxazole	SO	127-69-5
<b>(ii) Non-carcinogens with no in vivo genotoxicity data</b>		
Ethionamide	EtAm	536-33-4
Curcumin	Cu	458-37-7
Benzyl alcohol	BA	100-51-6
Urea	U	57-13-6
<b>(iii) Non-genotoxic carcinogens or carcinogenic by irrelevant (for humans) mechanism</b>		
Sodium saccharin	Ssa	128-44-9
<b>(iv) Supplementary list (prediction of in vitro genotoxicity results less clear)</b>		
Propyl gallate	PG	121-79-9
p-Nitrophenol	NPh	100-02-7
Sodium xylene sulfonate	SXS	1300-72-7
Ethyl acrylate	EtAc	140-88-5
Eugenol	Eu	97-53-0
Isobutyraldehyde	IBA	78-84-2
2,4-Dichlorophenol	DP	120-83-2

Table 6

			SCC		CMFDA	
	-S9	+S9	-S9	+S9	-S9	+S9
<b>I. Ames-positive in vivo genotoxins</b>	highest conc.		highest conc. <50%			
(i) O6 and N7 alkylators	mM	limit	cytotox. over control [ $\mu$ M]			
Cyclophosphamide	1.00	mM	1000	1000	1000	62.5
EthylNitrosourea	1.00	mM	1000	1000	1000	1000
Methylmethane sulfonate	1.00	mM	250	500	500	250
(ii) Polycyclic aromatic hydrocarbons						
Benzo[a]pyrene	0.125	AF	3.91	125	125	125
7,12-Dimethylbenz[a]anthracene	1.00	mM	125	500	250	1000
(iii) Aromatic amines						
Dimethylnitrosamine	1.00	mM	1000	1000	1000	1000
2-Acetylaminofluorene	1.00	mM	1000	1000	1000	1000
2,4-diaminotoluene	1.00	mM	1000	1000	1000	1000
IQ (2-Amino-3-methylimidazo[4,5-f]quinoline)	1.00	mM	1000	125	1000	125
PhIP.HCl (2-Amino-1-methyl-6-phenylimidazo[4,5-f]pyridine)	1.00	mM	500	1000	500	1000
(iv) Others						
Aflatoxin B1	1.00	mM	31.25	500	500	500
Cadmium chloride	1.00	mM	31.25	125	62.5	62.5
Cisplatin	1.00	mM	1000	1000	1000	1000
p-chloroaniline	1.00	mM	1000	1000	1000	1000
<b>II. In vivo genotoxins negative or equivocal in Ames</b>						
Etoposide	1/0.005	mM	-/2.5	1000/5	1000/5	1000/5
Hydroquinone	1.00	mM	250	1000	250	1000
Azidothymidine	1.00	mM	1000	1000	1000	1000
Sodium arsenite	1.00	mM	7.81	125	62.5	125
Taxol	1.00	mM	1000	500	1000	1000
Chloramphenicol	1.00	mM	1000	1000	1000	1000

Table 7

			SCC		CMFDA	
			-S9	+S9	-S9	+S9
(i) Non-carcinogens with negative in vivo genotoxicity data	highest conc. mM	limit	highest conc. <50% cytotox. over control [µM]			
Ampicillin trihydrate	1.00	mM	1000	1000	1000	1000
D-mannitol	1.00	mM	1000	1000	1000	1000
(ii) Non-carcinogens with no in vivo genotoxicity data						
Phenformin HCl	1.00	mM	500	1000	1000	1000
n-butyl chloride	1.00	mM	1000	1000	1000	1000
(2-chloroethyl)trimethyl-ammonium chloride	1.00	mM	1000	1000	1000	1000
Cyclohexanone	1.00	mM	1000	1000	1000	1000
N,N-dicyclohexyl thiourea	1.00	mM	500	1000	1000	500
Trisodium EDTA trihydrate	1.00	mM	500	1000	1000	1000
Ephidrine sulphate	1.00	mM	1000	1000	1000	1000
Erythromycin stearate	1.00	mM	31.25	125	62.5	125
Fluometron	1.00	mM	500	1000	1000	1000
Phenanthrene	1.00	mM	1000	1000	1000	1000
(iii) Non-genotoxic carcinogens						
D-limonene	1.00	mM	1000	1000	1000	1000
Di-(2-ethylhexyl)phthalate	1.00	mM	1000	1000	1000	1000
Amitrole	1.00	mM	1000	1000	1000	1000
Tert-butyl alcohol	1.00	mM	1000	1000	1000	1000
Diethanolamine	1.00	mM	1000	1000	1000	1000
Melamine	1.00	mM	1000	1000	1000	1000
Methyl carbamate	1.00	mM	1000	1000	1000	1000
Progesterone	1.00	mM	125	500	62.5	250
Pyridine	1.00	mM	1000	1000	1000	1000
Tris(2-ethylhexyl)phosphate	1.00	mM	500	1000	7.813	1000
Hexachloroethane	1.00	mM	1000	1000	1000	1000

Table 8

			SCC		CMFDA	
			-S9	+S9	-S9	+S9
(i) Non-carcinogens that are negative or equivocal for genotoxicity in vivo	highest conc.		highest conc. <50%			
	mM	limit	cytotox. over control [µM]			
D,L-menthol	1.00	mM	1000	1000	1000	1000
Phthalic anhydride	1.00	mM	1000	1000	1000	1000
Tertiary-butylhydroquinone	1.00	mM	125	1000	125	1000
o-Anthranilic acid	1.00	mM	1000	1000	1000	1000
1,3-Dihydroxybenzene (resorcinol)	1.00	mM	1000	1000	1000	500
2-Ethyl-1,3-hexanediol	1.00	mM	1000	1000	1000	1000
Sulfisoxazole	1.00	mM	1000	1000	1000	1000
(ii) Non-carcinogens with no in vivo genotoxicity data						
Ethionamide	1.00	mM	1000	1000	1000	1000
Curcumin	0.125	AF	31.25	125	31.25	125
Benzyl alcohol	1.00	mM	1000	1000	1000	1000
Urea	1.00	mM	1000	1000	1000	1000
(iii) Non-genotoxic carcinogens or carcinogenic by irrelevant (for humans) mechanism						
Sodium saccharin	1.00	mM	1000	1000	1000	1000
(iv) Supplementary list (prediction of in vitro genotoxicity results less clear)						
Propyl gallate	1.00	mM	500	1000	1000	1000
p-Nitrophenol	1.00	mM	500	1000	500	1000
Sodium xylene sulfonate	1.00	mM	1000	1000	1000	1000
Ethyl acrylate	1.00	mM	1000	1000	1000	1000
Eugenol	1.00	mM	1000	125	1000	125
Isobutyraldehyde	1.00	mM	1000	1000	1000	1000
2,4-Dichlorophenol	1.00	mM	250	500	1000	1000

Table 9

## I. Ames-positive in vivo genotoxins

## (i) O6 and N7 alkylators

	p-p53 (Ser15)	p21	p-H2A.X (Ser139)	p-Chk1 (Ser345)	p-ATM (Ser1981)
Cyclophosphamide	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
Ethyl nitrosourea	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
Methylmethane sulfonate	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9

## (ii) Polycyclic aromatic hydrocarbons

Benzo[a]pyrene	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
7,12-Dimethylbenz[a]anthracene	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9

## (iii) Aromatic amines

Dimethylnitrosamine	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
2-Acetylaminofluorene	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
2,4-diaminotoluene	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
IQ (2-Amino-3-methylimidazo[4,5-f]quinoline)	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
PhIP.HCl (2-Amino-1-methyl-6-phenylimidazo[4,5-f]pyridine)	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9

## (iv) Others

Aflatoxin B1	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
Cadmium chloride	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
Cisplatin	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
p-chloroaniline	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9

## II. In vivo genotoxins negative or equivocal in Ames



Etoposide	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
Hydroquinone	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
Azidothymidine	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
Sodium arsenite	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
Taxol	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9
Chloramphenicol	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9	-S9 +S9



(i) Non-carcinogens with negative in vivo genotoxicity data						
Ampicillin trihydrate						
D-mannitol						
(ii) Non-carcinogens with no in vivo genotoxicity data						
Phenformin HCl						
n-butyl chloride						
(2-chloroethyl)trimethyl-ammonium chloride						
Cyclohexanone						
N,N-dicyclohexyl thiourea						
Trisodium EDTA trihydrate						
Ephidrine sulphate						
Erythromycin stearate						
Fluometron						
Phenanthrene						
(iii) Non-genotoxic carcinogens						
D-limonene						
Di-(2-ethylhexyl)phthalate						
Amitrole						
Tert-butyl alcohol						
Diethanolamine						
Melamine						
Methyl carbamate						
Progesterone						
Pyridine						
Tris(2-ethylhexyl)phosphate						
Hexachloroethane						

(i) Non-carcinogens that are negative or equivocal for genotoxicity in vivo	p-p53 (Ser15) -S9 +S9	p21 -S9 +S9	p-H2A.X (Ser139) -S9 +S9	p-Chk1 (Ser345) -S9 +S9	p-ATM (Ser1981) -S9 +S9
D,L-menthol					
Phthalic anhydride					
Tertiary-butylhydroquinone					
o-Anthranilic acid					
1,3-Dihydroxybenzene (resorcinol)					
2-Ethyl-1,3-hexanediol					
Sulfisoxazole					
(ii) Non-carcinogens with no in vivo genotoxicity data					
Ethionamide					
Curcumin					
Benzyl alcohol					
Urea					
(iii) Non-genotoxic carcinogens or carcinogenic by irrelevant (for humans) mechanism					
Sodium saccharin					
(iv) Supplementary list (prediction of in vitro genotoxicity results less clear)					
Propyl gallate					
p-Nitrophenol					
Sodium xylene sulfonate					
Ethyl acrylate					
Eugenol					
Isobutyraldehyde					
2,4-Dichlorophenol					

Table 12

Endpoint	p-p53 (Ser15)	P21
Genotoxic Test compound		
Methylmethane sulfonate		
Etoposide		
Non- Genotoxic Test compound		
D-Mannitol		

## CLAIMS

1. A method for screening compounds for genotoxic and/or pro-genotoxic activity  
5 comprising the step of determining expression levels of active forms of at least two proteins selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68) in a system incubated with one or more compounds in comparison with the expression levels in a system not incubated with the compounds.
- 10 2. The method according to claim 1, wherein the expression levels of at least the proteins p-p53 (Ser15) and p21 are determined.
3. The method according to claim 1 or 2, wherein the expression levels of at least the  
15 proteins p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981) and p-Chk1 (Ser345) are determined.
4. The method according to any of claims 1 to 3, wherein the expression levels of the  
20 proteins p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68) are determined.
5. The method according to any of claims 1 to 4, wherein the expression levels are determined by immunofluorescence staining or Luminex technology.
- 25 6. The method according to any of claims 1 to 5, wherein an increase in the expression levels of at least one of said proteins in the system incubated with the compounds in comparison with the system not incubated with the compounds indicate said activity.
7. The method according to claim 6, wherein the expression levels are increased by a  
30 factor of at least 1.5.
8. The method according to claim 7, wherein a decreased concentration of the compounds is administered to provide the expression levels with the factor between 1 and 1.1.

9. The method according to any of claims 1 to 8, wherein the compounds with said activity are screened with a sensitivity and/or specificity of at least 80%.
- 5 10. The method according to any of claims 1 to 9, wherein the least (pro-)genotoxic compound or a non-(pro-)genotoxic compound is identified, and wherein the least increase or no increase in the expression levels indicates said compound, which is suitable for its intended use.
- 10 11. A method for administering a therapeutic compound to a patient, comprising performing the method of any of claims 1 to 10 with a series of compounds, identifying the compound having no genotoxic and pro-genotoxic activity, and administering said compound to a patient in need of the compound's intended use.
- 15 12. A method for monitoring the likelihood of developing cancer, tumor, metastasis and/or disorder of angiogenesis, which are caused, mediated and/or propagated by deregulation of proliferation, differentiation and/or damage repair, in response to a treatment with a compound, wherein the expression levels of at least two proteins selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-  
20 Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68) are determined in a biological sample withdrawn from a mammal in need of such treatment with said compound administered to said mammal, wherein an increase in the expression levels of at least one of said proteins indicates that said compound has genotoxic and/or pro-genotoxic activity and said likelihood is increased.
- 25
13. An in-vitro method for predicting the likelihood that a patient will suffer from a tumor in response to a therapeutic treatment with a drug, comprising the steps of (i) measuring in a biopsy sample from tissue or plasma of said patient expression levels of at least two proteins selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM  
30 (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68), (ii) exposing ex-vivo a sample from tissue or plasma of said patient to said drug, and (iii) measuring in said exposed sample of step (ii) the expression levels of said proteins specified in step (i) along with calculating the differences in expression levels measured in steps (i) and (iii), wherein an increase in the expression levels of at

least one of said proteins obtained in this step (iii) compared to step (i) indicates that said drug has genotoxic and/or pro-genotoxic activity and said likelihood is increased.

14. Use of at least two proteins selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68) as marker proteins for screening compounds with genotoxic and/or pro-genotoxic activity.
15. Kit for use in the detection of genotoxic and/or pro-genotoxic activity comprising at least two antibodies, each with specific binding to a different protein selected from the group of p-p53 (Ser15), p21, p-H2A.X (Ser139), p-ATM (Ser1981), p-Chk1 (Ser345), p-ATR (Ser428), p-cdc2 (Thr14/Tyr15), Gadd45a and p-Chk2 (Thr68).

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP2013/001575

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-15(partially)

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/001575

A. CLASSIFICATION OF SUBJECT MATTER  
INV. G01N33/50  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STÉPHANIE SOLIER ET AL: "The apoptotic ring", CELL CYCLE, vol. 8, no. 12, 15 June 2009 (2009-06-15), pages 1853-1859, XP055066877, figures 4c,4d	1
A	M. MALMLOF ET AL: "Mdm2 as a Sensitive and Mechanistically Informative Marker for Genotoxicity Induced by Benzo[a]pyrene and Dibenzo[a,l]pyrene", TOXICOLOGICAL SCIENCES, vol. 102, no. 2, 18 October 2007 (2007-10-18), pages 232-240, XP055066879, ISSN: 1096-6080, DOI: 10.1093/toxsci/kfm305 abstract; figure 5	1
	----- -/--	



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 June 2013

Date of mailing of the international search report

17/09/2013

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040,  
Fax: (+31-70) 340-3016

Authorized officer

Rosin, Oliver



## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/001575

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WEN-WEN CHOU ET AL: "Arecoline-induced phosphorylated p53 and p21 WAF1 protein expression is dependent on ATM/ATR and phosphatidylinositol-3-kinase in clone-9 cells", JOURNAL OF CELLULAR BIOCHEMISTRY, vol. 107, no. 3, 1 June 2009 (2009-06-01), pages 408-417, XP055066909, ISSN: 0730-2312, DOI: 10.1002/jcb.22137 abstract figures -----	1,2,6-15
X	T. THOMPSON ET AL: "Phosphorylation of p53 on Key Serines Is Dispensable for Transcriptional Activation and Apoptosis", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 279, no. 51, 6 October 2004 (2004-10-06), pages 53015-53022, XP055067470, ISSN: 0021-9258, DOI: 10.1074/jbc.M410233200 abstract; figures 1-3 -----	1,2,6-15
X	ZOE A STEWART ET AL: "Increased p53 phosphorylation after microtubule disruption is mediated in a microtubule inhibitor- and cell-specific manner", ONCOGENE, vol. 20, 15 December 2000 (2000-12-15), pages 113-124, XP055067473, figure 2 -----	1,2,6-15
X	XIAOBING LIANG ET AL: "The Role of Wild-Type p53 in Cisplatin-Induced Chk2 Phosphorylation and the Inhibition of Platinum Resistance with a Chk2 Inhibitor", CHEMOTHERAPY RESEARCH AND PRACTICE, vol. 4, no. 3, 1 January 2011 (2011-01-01) , pages 216-8, XP055067516, ISSN: 2090-2107, DOI: 10.1158/1535-7163.MCT-08-0492 abstract; figure 1 -----	1,2,6-15
X	Y. ZHU ET AL: "Intra-S-Phase Checkpoint Activation by Direct CDK2 Inhibition", MOLECULAR AND CELLULAR BIOLOGY, vol. 24, no. 14, 29 June 2004 (2004-06-29) , pages 6268-6277, XP055067572, ISSN: 0270-7306, DOI: 10.1128/MCB.24.14.6268-6277.2004 figures ----- -/--	1-3,6-15

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/001575

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. S. PALII ET AL: "DNA Methylation Inhibitor 5-Aza-2'-Deoxycytidine Induces Reversible Genome-Wide DNA Damage That Is Distinctly Influenced by DNA Methyltransferases 1 and 3B", MOLECULAR AND CELLULAR BIOLOGY, vol. 28, no. 2, 8 November 2007 (2007-11-08), pages 752-771, XP055067587, ISSN: 0270-7306, DOI: 10.1128/MCB.01799-07 figures 2,4	1-15
T	----- K. BOEHME ET AL: "Genomic Profiling Uncovers a Molecular Pattern for Toxicological Characterization of Mutagens and Promutagens In Vitro", TOXICOLOGICAL SCIENCES, vol. 122, no. 1, 28 April 2011 (2011-04-28), pages 185-197, XP055066869, ISSN: 1096-6080, DOI: 10.1093/toxsci/kfr090 figures 4C,4D -----	

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-15(partially)

Methods, uses and kits for screening compounds with genotoxic activity comprising p-p53(Ser15) and a second claimed biomarker.

---

2. claims: 1-15(partially)

Methods, uses and kits for screening compounds with genotoxic activity comprising p21 and a second claimed biomarker.

---

3. claims: 1, 3-15(all partially)

Methods, uses and kits for screening compounds with genotoxic activity comprising p-H2A.X(Ser139) and a second claimed biomarker.

---

4. claims: 1, 3-15(all partially)

Methods, uses and kits for screening compounds with genotoxic activity comprising p-ATM(Ser1981) and a second claimed biomarker.

---

5. claims: 1, 3-15(all partially)

Methods, uses and kits for screening compounds with genotoxic activity comprising p-Chk1(Ser345) and a second claimed biomarker.

---

6. claims: 1, 4-15(all partially)

Methods, uses and kits for screening compounds with genotoxic activity comprising p-ATR(Ser428) and a second claimed biomarker.

---

7. claims: 1, 4-15(all partially)

Methods, uses and kits for screening compounds with genotoxic activity comprising p-cdc2(Thr14/Tyr15) and a second claimed biomarker.

---

8. claims: 1, 4-15(all partially)

Methods, uses and kits for screening compounds with

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

genotoxic activity comprising Gadd45a and a second claimed biomarker.

---

9. claims: 1, 4-15(all partially)

Methods, uses and kits for screening compounds with genotoxic activity comprising p-Chk2(Thr68) and a second claimed biomarker.

---