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(57) Abstract: The invention concerns novel methods for determining the toxic potential of test compounds, as well as equipment and kits for implementing said methods. The invention also concerns methods for generating nucleic acid sequences for use as genetic markers of toxicity. The invention is based in particular on the constitution of differential libraries of nucleic acids, characteristic of situations wherein the viability and/or cell proliferation are disrupted, and for demonstrating that said libraries are useful for very dependably assessing with high degree of sensitivity the toxic profile of compounds. The invention is particularly useful in the pharmaceutical industry, for analysing the toxic profile of molecules to be used for pharmaceutical preparation and/or in pharmaceutical compositions.

(57) Abrégé : La présente invention décrit de nouvelles méthodes permettant de déterminer le potentiel toxique de composés tests, ainsi que des outils et kits pour la mise en oeuvre de ces méthodes. L'invention décrit également des méthodes pour générer des séquences d'acides nucléiques utilisables comme marqueurs génétiques de la toxicité. L'invention repose notamment sur la constitution de banques différentielles d'acides nucléiques, caractéristiques de situations dans lesquelles la viabilité et/ou la prolifération cellulaire sont dérégulées, et dans la mise en évidence que ces banques sont utilisable pour évaluer avec une fiabilité et une sensibilité importante le profil toxique de composés. L'invention est particulièrement utile dans l'industrie pharmaceutique, pour l'analyse du profil toxique de molécules qui pourront entrer en développement pharmaceutique et/ou dans des compositions pharmaceutiques.

PATENT

GENETIC MARKERS OF TOXICITY, PREPARATION AND USES

DESCRIPTIVE SUMMARY

The present invention describes new methods for the determination of the potential toxicity of test compounds, as well as the kits and tools for the implementation of these methods. The invention also describes methods for generating nucleic acid sequences that can be used as genetic markers of toxicity. The invention is based in particular on the creation of differential nucleic acid banks characteristic of situations in which cell viability and/or proliferation are deregulated, and on the demonstration that these banks can be used to evaluate the toxicity profile of compounds with reliability and high sensitivity. The invention is of special utility in the pharmaceutical industry for analysis of the toxicity profile of compounds involved in drug development and/or in pharmaceutical compositions.

GENETIC MARKERS OF TOXICITY
PREPARATION AND USES

5   The present invention is related to the technical areas of biotechnology, medicine, biology and biochemistry. Its applications concern the fields of human, animal and plant health. More particularly, the invention sets forth new methods for determining the potential toxicity of test compounds or for predicting the sensitivity or response of patients to compounds, as well as the

10   kits and tools for the implementation of these methods. The invention also describes methods for obtaining nucleic acid sequences that can be used as genetic markers of toxicity.   The invention is of special utility in the pharmaceutical industry for analysis of the toxicity profile of compounds involved in drug development and/or in pharmaceutical compositions.

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Toxicity is the major reason for abandoning candidate therapeutic molecules during preclinical and clinical development. To our knowledge, at the present time there are no tests by which to rapidly determine the toxicity profile of a compound in man. Yet the regulatory authorities require that new candidate

20   drugs undergo toxicity, mutagenicity, carcinogenicity and teratogenicity testing in animals as well as clinical trials in man. These tests are long and costly and are only partially satisfactory. For example, animal toxicity is far from being a reflection of human toxicity.   Furthermore, the small number of patients enrolled in clinical trials does not systematically allow identification of toxicities

25   associated with a small, specific population. The development, perfection and use of such tests should make it possible to identify and remove toxic compounds from the development process as far upstream as possible. In this manner new drugs could be marketed sooner and at a lesser cost to drug companies and, in turn, to health care organizations and consumers.   In

30   addition, such tests might also make it possible to detect some toxicities which currently come to light only during the post-marketing period.

The tests which are currently available do not provide sufficient characterization of toxicity markers or the potential toxicity of compounds. Some tests in bacteria, such as the Ames test, or in yeast, such as the test described in patent US 4,997,757, evaluate the mutagenic potential of compounds. These tests can only detect damage at the level of the DNA. Yet many drugs exert toxic effects without being mutagens and cannot therefore be flagged by tests such as these. Other tests, such as that described in application WO 94/17208, make use of certain known eukaryotic gene promoters or response elements from these promoters, induced under different conditions of stress, to characterize drug toxicity. However, the small number of such genetic markers and the process being measured (promoter activity) do not allow prediction of the potential toxicity of the compounds. What's more, these tests are difficult to implement because they involve the culturing of transformed cell lines comprising one or more genetic constructions.

The present invention now describes rapid, effective methods by which to determine the potential toxicity of test compounds, as well as the tools and kits for the implementation of such methods. In the context of the invention, the term "toxicity" refers to any adverse and/or side effect of a compound on the metabolism of a cell or a tissue such as, in particular, its mutagenic, carcinogenic or teratogenic potential, and more generally any alteration in metabolism that can result in a harmful effect of the compound on the cell or the tissue. The present invention is based more specifically on genomics and on the development of genetic markers of toxicity that can be used to predict the toxic potential of any type of compound on any type of cell. The present invention also sets forth new methods for obtaining nucleic acid sequences that allow determination of the toxicity of compounds (eg., genetic markers of toxicity), particularly compounds entering into drug development and/or pharmaceutical compositions.

The present invention is based in part on the demonstration that genetic markers can be created and used to evaluate the toxicity of test compounds. In particular, and in an advantageous manner, these markers can be used
5 regardless of the toxic compound being tested, and regardless of the type of cell in which the test compound is being studied. Such markers, as well as the supports, kits and methods of the invention advantageously lead to the rapid generation of toxicity profiles that are particularly thorough and reliable. Furthermore, these markers, supports, kits and methods of the invention also
10 make it possible to determine and assign a toxicity index to the test compounds.

In particular, the present invention demonstrates that there exist genetic events that are common to situations of toxicity and to cellular metabolic
15 pathways induced in the absence of toxic compounds. Such genetic events can therefore be induced and then used as markers of toxicity. As will be described in detail herein, such markers can further be selected or modified for the constitution of improved banks allowing a more highly predictive diagnosis of the toxicity of a compound. More specifically, the present invention now
20 shows that genetic markers induced in a cell in a situation where cell signalling pathways are deregulated, particularly a cell in which cell viability and/or proliferation are deregulated (for example, in a situation of apoptosis), can be efficiently used to characterize the toxicity profile of test compounds. In an advantageous manner, the invention also shows that these markers can be
25 used in toxicity tests irrespectively of the type of compound and the type of cell used. The invention also provides for the constitution of differential banks of nucleic acids characteristic of deregulated cell signalling pathway(s), particularly situations in which cell viability and/or proliferation are deregulated, and demonstrates that these banks can be used for a reliable, highly sensitive
30 evaluation of the toxicity profile of compounds.

The invention also allows the identification and/or the characterization of mutations or polymorphisms (in particular of SNPs "Single Nucleotide Polymorphisms") which are characteristic of the profile of subjects, particularly of the response profile to a compound or treatment, of a sensitivity profile to a

5   toxic effect, etc. In this regard, the libraries produced represent an extraction of sequences which are mobilised during deregulations of cell signalling and, particularly, during stress that can lead to apoptosis. The invention shows that the expression of these sequences is modified in a specific manner in response to given compounds. Mutations or polymorphisms, also termed

10   SNPs (Single Nucleotide Polymorphisms), can affect genes from which these sequences derive. Individuals presenting such polymorphisms are thus susceptible to exhibit altered responses to compounds that mobilise or recruit the expression of these genes. One aspect of this invention is thus also to enable a pertinent selection of the genes involved in toxic phenomenon. More

15   precisely, the invention allows to further select, within this selection, genes that are involved in the response to a given compound. The invention provides a basis for the identification of polymorphisms in a limited (10 to 50) number of genes whose expression modifications are linked to the toxic impact of a given compound, said compound being of any pharmacological class and of any

20   chemical family.

One object of the invention is therefore based more specifically on the use of genetic markers characteristic of situation(s) of deregulation of cell signalling pathway(s) (situation(s) of "deregulation") to characterize the toxicity profile of

25   test compounds. The invention more preferably concerns the use of genetic markers induced in a situation of deregulation to characterize the toxicity profile of test compounds. The invention more preferably concerns the use of nucleic acid clones corresponding to genetic events, such as transcriptional and/or splicing events, that are characteristic of situations of deregulation, as

30   genetic markers of toxicity.

The invention also concerns methods for analysis of the potential toxicity of a test compound, comprising at least one hybridization step between a) a sample of nucleic acids from cells treated with this compound and b) a preparation (for example, a bank) of nucleic acids corresponding to genetic events characteristic of situation(s) of deregulation, the hybridization profile indicating the toxic potential of the test compound.

The invention further concerns the kits for the implementation of these methods, as well as the compositions and nucleic acid banks comprising genetic markers of deregulation(s) of cell signalling pathways, and also the methods by which to generate such markers.

The invention also relates to the use of clones or nucleic acids as disclosed, for the identification of mutations or polymorphisms, particularly SNPs, that are correlated to the response of subjects to pharmaceutical compounds. The invention indeed allows the identification of a set of genes whose expression is altered in response to a given compound. These genes may present mutations or polymorphisms (including SNPs) in human populations. The invention allows the selection of genes and their polymorphisms to be studied in order to segregate patients with respect to their genotype for their capacity to respond in a more or less optimal manner to a compound and, more particularly, with limited risks of toxicity.

In this respect, the invention relates, more generally, to the use of nucleic acids representative of splicing events characteristic of a given physio-pathological situation, for the identification or the characterisation of SNPs correlated to said situation.

The invention also concerns a method for the identification of SNPs or other mutations or polymorphisms that allow the assessment of the response of a subject to a given compound, the method comprising (i) the identification in

vitro of nucleic acids characteristic of splicing events induced in a cell treated with said compound and (ii) the identification of SNPs or other mutations or polymorphisms in the gene or genes corresponding to nucleic acids identified in (i). The invention thus provides methods and means to select genes for which polymorphisms are associated to a toxicity for a given compound.

The invention also relates to a population of nucleic acids specific for polymorphisms or SNPs thus identified, in particular of nucleotide primers for the selective amplification of said polymorphisms, or of probes for the selective hybridisation to said polymorphisms.

The invention also concerns methods for the analysis (e.g., the prediction, the evaluation or the determination) of the response of a subject to a test compound, comprising the analysis of polymorphisms, in particular SNPs, as defined above.

The present invention is therefore based in particular on the identification and development of genetic markers of toxicity that can be used for predictive toxicity testing of test compounds, or as targets for the study of the mechanisms of cell death or the research and development of therapeutic agents.

1. Identification and development of genetic markers of toxicity

Cell viability and differentiation are regulated by the balance that exists between mitosis and apoptosis. In the body, tissue homeostasis is also regulated by this equilibrium between cell proliferation and cell death. Alterations in this equilibrium form the basis of disease, whether they be related to an excess (neurodegenerative disorders) or to a defect in apoptosis (rheumatoid arthritis, cancer). The present invention is based in particular on the hypothesis that disturbances in this equilibrium can also be involved in

toxic phenomena and that genetic markers characteristic of these events of deregulation of cell signalling pathways (especially cell viability and/or proliferation) might represent efficient markers of toxicity.

5   The present invention now shows that there are genetic events common to these situations of deregulation of cell signalling pathways, and to situations of toxicity induced by compounds. The present invention also provides for methods by which to identify, characterize, select and isolate nucleic acid clones corresponding to these genetic events, and shows that they can be
10 efficiently used as genetic markers of toxicity. The invention also describes the establishment of banks of such clones, particularly on solid supports, and their use in methods for evaluation of the toxicity of test compounds.

2. Definitions

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In the context of the present invention, the term situation of deregulation, or "deregulation", refers to any situation in which one or more cell signalling pathways are deregulated or altered, and in particular any situation in which cell growth and/or viability are deregulated. This therefore concerns any cell in
20 which one or more cell signalling pathways have been altered, in particular induced or repressed, for example a situation of apoptosis, as will be described hereafter in further detail.

In the context of the present invention, the term "genetic event characteristic of
25 a situation of deregulation" refers more specifically to any modification of gene activity, particularly of gene expression (enhancement or repression, inhibition or induction, etc.), post-transcriptional regulation (particularly splicing), replication, appearance of deletions, etc., characteristic of deregulation, i.e. induced in a cell in a situation of deregulation. It is understood that each
30 individual genetic event characteristic of deregulation is not necessarily specific to this situation, in so far as some cell signalling pathways participate

in a number of cellular processes. However, the nucleic acid banks described by the invention contain different clones and therefore effectively represent the genetic signatures characteristic of such situations.

The clones described by the invention more preferably correspond to transcriptional and/or splicing genetic events characteristic of deregulation(s). In the context of the invention, the term "transcriptional event" encompasses more specifically any activity of gene expression, i.e. the production of primary transcripts, premessengers and messengers from coding regions. The term "splicing event" refers more particularly to any splicing event related to a situation of deregulation, i.e. the appearance of specific splicing forms, the disappearance of specific splicing forms, or possibly the modulation of the quantity of splicing forms, etc.

The invention therefore describes the production of nucleic acid clones corresponding to such genetic events characteristic of situations where one or more cell signalling pathways are altered, and their use as genetic markers of toxicity. In the context of the invention, a nucleic acid clone corresponding to such a genetic event refers to any nucleic acid or nucleic acid fragment comprising at least one region whose sequence is specific to this event. For example, it may be any nucleic acid comprising a sequence specific for a splicing form or a deletant characterizing a situation of deregulation, or even a gene which is specifically regulated or expressed in a situation of deregulation. Such clones are therefore nucleic acids that can hybridize, in the nucleic acid test sample, with splicing forms characteristic of the situation of deregulation or with genes preferentially or specifically expressed in a situation of deregulation. Through hybridization, these clones thereby reveal the presence of targeted genetic events in a test nucleic acid population.

3. Preparation of genetic markers of toxicity

As noted above, the present invention shows that genetic markers characteristic of situations of deregulation can be used as markers of toxicity, and in this regard it describes the development of methods by which to generate and isolate such markers.

One subject of the invention is therefore based on the methods by which to generate genetic markers of toxicity. The methods of the invention more specifically comprise the establishment of clones and nucleic acid banks characteristic of deregulation(s) in cell signalling pathway(s). These clones and banks can be generated in different ways, as described hereafter. Furthermore, the banks of the invention can contain variable quantities of nucleic acid clones. In addition, these banks are advantageously deposited on supports to facilitate hybridization and analysis of the hybridization profile.

The starting material used for the production of the clones and banks primarily comprises populations of nucleic acids derived from cells in a situation of deregulation, and populations of nucleic acids derived from cells in a control situation. In a more specific embodiment, the process of production of genetic markers of toxicity according to the invention advantageously comprises a hybridization step between a nucleic acid sample derived from a cell in a situation of deregulation, and a nucleic acid sample derived from a cell in a control situation. This hybridization step enables the generation of (banks of) nucleic acid clones characteristic of the cell in the situation of deregulation relative to the control situation.

The nucleic acid populations used are, for example, total RNA or messenger RNA from cells in a situation of deregulation and a control situation, or nucleic acids derived from this total or messenger RNA by reverse transcription, amplification, cloning into vectors, etc. These populations can be prepared by conventional methods known to those skilled in the art, such as reverse transcription, amplification, etc.

The choice of the situation or situations of deregulation, the control situation or situations, and the methods for production and isolation of the markers, is important because it determines the relevance of the markers generated, and therefore of the banks, and also their ease of use.

3.1. Starting material

As indicated above, the genetic markers of toxicity according to the invention are generally prepared initially from nucleic acid preparations derived from cells in a situation of deregulation. It is understood that these markers, once isolated and characterized, can be reproduced or amplified by artificial means such as PCR, artificial synthesis (when the sequences are available), amplification in bacteria, replication of banks, etc., as illustrated hereafter.

As noted above, in the context of the present invention the term "situation of deregulation" refers to any situation in which cell growth and/or viability are deregulated, for example any cell in which at least one cell signalling pathway has been altered.

According to a specific embodiment of the invention, the cell in the situation of deregulation is a cell in which the expression of all or part of a gene involved in cell growth or viability has been modified.

According to another specific embodiment of the invention, the cell in the situation of deregulation is a cell in which the expression of all or part of a relevant oncogene or anti-oncogene has been modified.

According to another specific embodiment of the invention, the cell in the situation of deregulation is a cell in which the expression of all or part of a gene involved in apoptosis has been modified.

Apoptosis is a term familiar to those skilled in the art; it refers to the process of programmed cell death. Apoptosis encompasses all the mechanisms, genes and metabolic pathways that lead to cell death. Examples of factors that

5 initiate or carry out apoptosis include the anti-oncogenes, Fas and TNF receptors, adaptors (particularly "death domain"), members of the bcl2 family, or caspases, which are involved in ontogenic morphogenesis, pathological deregulations and cell death.

10 Within the scope of the invention, a situation of apoptosis can be any situation involving initiation, execution or termination of apoptosis, i.e. a situation of apoptosis at any stage in the process of cell death, including very early stages in which only some metabolic pathways (or cell signalling pathways) have been altered, for example by activation, repression, stimulation, etc. The

15 clones of the invention can therefore be produced from any cell in which at least one cell signalling pathway has been altered, particularly in which at least one apoptotic pathway has been initiated. These can be cells in which a metabolic pathway involved in apoptosis has been induced or stimulated, even if this induction or stimulation is carried out in sublethal conditions (i.e. not

20 resulting in cell death).

In this regard, the cells can be in a natural situation of apoptosis, as for example a population of tumor cells, certain nerve cells in pathological situations, etc., or cells in which apoptosis has been artificially induced. In the latter case, the situation of apoptosis can be induced by different types of

25 treatments, particularly by modulation of the activity, preferably of the expression of genes that initiate or carry out apoptosis.

In a particular embodiment, the genetic markers of the invention are produced initially from a population of cells in which a situation of apoptosis has been

30 artificially induced, by the induction or repression of the activity, preferably of the expression of all or part of a gene that initiates or carries out apoptosis.

According to a preferred embodiment of the invention, the cell in a situation of deregulation is a cell in which the activation, preferably the expression of all or part of an anti-oncogene (or tumor suppressor gene) has been induced or

5   enhanced, for instance by over-expression, post-translational modification(s) (phosphorylation, glycosylation, etc.), deregulated intra-cellular localisation, association with different functional partners, etc.

In an especially preferred embodiment of the present invention, the genetic

10   events characteristic of a situation of deregulation are genetic events induced by modification of the expression of one or more genes that initiate or carry out apoptosis, particularly one or more tumor suppressor genes (or a functional variant of such genes).

15   This can particularly be any cell in which overexpression of an anti-oncogene has been induced. In particular, this can be a cell in which a gene construct has been introduced to induce or enhance the quantity of an anti-oncogene in this cell. Specific examples of anti-oncogenes (also called tumor suppressor genes) include p53 (Eliyahu et al., Proc Natl Acad Sci USA (1989) 86: 8763-7;

20   Finlay et al., Cell (1989) 57: 1083-93); Rb (Lee et al., Science (1987) 235: 1394-9); p73 (Kaghad et al., Cell (1997) 90: 809-19); TUPRO-2 (US5,849,899); NHTS (US5,892,016); p15 (Hannon et al., Nature 371 (1994) 257); p16 (Ivison et al., Nature Genet. 371 (1994) 180); etc., but this list is not exhaustive.

25

A typical example of a situation of deregulation according to the invention is generated by the induction or enhancement of p53 activity, preferably of p53 expression. The p53 tumor suppressor gene, or anti-oncogene, is situated at the crossroads between cell life and death, negatively regulating cell growth by

30   inducing cell cycle arrest at the end of the G1 phase. This cell cycle arrest is characterized by repression of the expression of genes involved in G1 to S

progression and by induction of specific genes. Abolition of these p53 functions is one of the mechanisms involved in the development of a large number of tumors in which p53 deletion or mutations are observed. Overexpression of p53 can induce apoptosis in different cell types. This overexpression induced by infection with a virus permitting expression of wild type p53 forms is the basis for the gene therapy of cancer. Induction of p53 expression can also occur physiologically, particularly during restructuring of the mammary glands after lactation has ceased. Apoptosis induction by removal of serum, a situation which affects a large number of metabolic pathways, has also been reported to involve p53 stabilization.

p53 induction triggers programmed cell death in cells when DNA damage saturates cell repair systems. This apoptosis induction involves the Fas system and the caspases in particular, whose messengers must be spliced to isoforms that are competent for cell death.

Furthermore, p53 is known to regulate the expression of genes involved in other various cell processes, such as cell cycle regulation, angiogenesis, response to oxidative stress, DNA alteration or cell growth. P53 also plays a direct role on the cell response to DNA alterations, hypoxia, cellular redox potential, or cell adhesion. In addition, it has been shown that p53 can migrate from the cell nucleus towards mitochondria, where it can interact with hsp70 protein, leading to cell death. The signatures obtained with the present invention upon induction of p53 thus enable the study of all of these signalling pathways and to provide a complete and predictive response on the toxic potential of compounds.

The invention more specifically describes the use of genetic markers induced by p53 activation, preferably by p53 expression (in particular wild type p53) to characterize the toxicity profile of test compounds. The invention more preferably concerns the use of nucleic acid clones corresponding to genetic

events, such as transcriptional and/or splicing events, induced by p53 activation, preferably p53 expression (in particular wild type p53), as genetic markers of toxicity.

The invention more specifically describes the obtaining and use of cDNA clones corresponding to qualitative and/or quantitative genetic events that differ between control cells (for example growing cells) and cells engaged in apoptosis through induction of wild type p53.

It is understood that there are other possibilities by which to subject cells to a stress that can engage them in programmed cell death (apoptosis) or alter cell signalling pathways. The invention therefore also concerns the use of other cell models such as those based on inducible (or constitutive) expression of all or part of anti-oncogenes, promotors, initiators and mediators of apoptosis. Among these genes can be advantageously chosen RB, the retinoblastoma gene product; p73, a p53 homolog; myc; or any other protein or protein fragment capable of interfering with cell growth and viability. As noted above, this can also be a tumor cell or a degenerated nerve cell, for example.

Other types of starting material consist, for example, of samples containing cells in which proliferation has been deregulated by activation of oncogene cascades (such as the ras signalling pathway), inactivation of tumor suppressor genes, inhibition of apoptotic cascades, inhibition or activation or kinase-mediated pathways (such as p13 kinase which stimulates Akt protein), inhibition of gene expression by antisense RNA or oligonucleotides, etc. A specific example of cells consists of tumor cells obtained from tumor biopsies, in which case the genetic markers according to the invention are those characteristic of the tumor cell relative to a specimen of control tissue, particularly healthy tissue, obtained by biopsy.

Furthermore, the situation of deregulation can also be induced by one or more selected toxic compounds that act on cell viability via processes that alter

genome integrity (genotoxicity), cell redox potential, cell surface receptor response, protein conformation or energy status. In particular, such compounds can be used in combination with the aforementioned induction methods based on modification of gene expression.

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The cell population used to induce a situation of deregulation can be of diverse origin and nature, such as epithelial, hepatic, pulmonary, nerve, muscle, fibroblastic cells, etc. Preferably these are human cells. They can be primary cultures or established lines, in which deregulation is induced under the
10   aforementioned conditions. They can also be tumor specimens which will contain cells in a situation of deregulation, or serum-deprived cells. As indicated below, the genetic markers of deregulation for use as markers of toxicity according to the invention can be produced from different cell types.

15   The control situation used generally corresponds to a population of cells of the same type as the cells used for the situation of deregulation, although different cell types can be used. The control situation can be a normal, quiescent or proliferative situation or even a situation of deregulation, but at a degree or stage less advanced than the reference situation of deregulation.

20

The situation of deregulation is typically a situation of induction of the activation, preferably of the expression of a tumor suppressor gene such as p53 or Rb, and the control situation is a situation in which induction of this activation, preferably of this expression is absent, or at a lower level. In
25   another example, the situation of deregulation is a population of tumor cells and the control situation is a population of the same but healthy cells. Specific examples of cells are given in the experimental section.

3.2. Production of markers and banks

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As noted above, the process of production of genetic markers of toxicity according to the invention advantageously comprises a hybridization step between a nucleic acid sample derived from a cell in a situation of deregulation, and a nucleic acid sample derived from a cell in a control

5   situation. This hybridization step enables the generation of nucleic acid clones characteristic of the cell in the situation of deregulation relative to the control situation.

The nucleic acid populations are, for example, total RNA or messenger RNA

10   from cells in a situation of deregulation and a control situation, or nucleic acids derived from this total or messenger RNA by reverse transcription, amplification, cloning into vectors, etc. These nucleic acids can be prepared according to methods familiar to those skilled in the art. Briefly, these methods generally comprise lysis of the cells, tissue or sample and isolation of

15   the RNA by extraction. In particular, this can consist of a treatment with chaotropic agents such as guanidium thiocyanate, which destroys the cells and protects the RNA, followed by RNA extraction with solvents such as phenol or chloroform. Such methods are well known to those skilled in the art (see Maniatis et al., Chomczynski et al., Anal. Biochem. 162 (1987) 156), and

20   can be easily put into practice by using commercially available kits such as the kit US73750 (Amersham) for total RNA. The RNA used does not have to be perfectly pure, and in particular the presence in the preparation of traces of genomic DNA or other cell components such as protein, etc. is not a problem so long as they do not significantly affect RNA stability. In addition, in an

25   optional manner it is also possible to use not total RNA preparations but rather messenger RNA preparations. These can be isolated either directly from the biological sample or from total RNA by means of polyT sequences, according to conventional methods. In this regard, messenger RNA can be obtained through the use of commercially available kits such as the kit US72700

30   (Amersham). The RNA can also be obtained directly from banks or from other

samples prepared in advance and/or available in collections, and stored under suitable conditions.

The hybridization can be carried out under different conditions which can be adjusted by those skilled in the art. The hybridization preferably uses an excess of the nucleic acid population derived from the situation of deregulation relative to the nucleic acid population derived from the control situation.

Using the product of the hybridization reaction, two main types of approaches can be used to isolate the clones characteristic of deregulation according to the invention. The first, which is strictly quantitative, enables the generation of a nucleic acid preparation comprising all (or a significant part) of the clones resulting from a difference in the level of expression between the two situations. Such clones (and banks) are obtained by known subtractive hybridization methods consisting primarily of eliminating the hybrids formed during the hybridization step and keeping only non-hybridized clones characteristic of the deregulated situation relative to the chosen control situation.

In a preferred embodiment, however, a qualitative process is used for the generation of a nucleic acid preparation comprising all (or a large part) of the clones resulting from functional gene mutations characteristic of the deregulated situation relative to the chosen control situation. More particularly, such a qualitative bank comprises not the entire group of clones whose expression is modified, but for example clones corresponding to splicing or deletion events that differ between the two situations. Considering the role of alternative splicing in cell regulatory and transformation pathways, such preparations (and banks) advantageously comprise clones having an important functional value and therefore likely to reflect the genetic modifications involved in the situation of deregulation. Such clones therefore enable the constitution of banks with greater predictive power and the generation of more representative genetic markers.

Such qualitative banks can be constituted by the isolation from the hybrids formed during the hybridization step of nucleic acid regions corresponding to differential splicing or to deletions. Depending on the methods used, these regions correspond either to unpaired regions or to paired regions.

5

These two approaches are described below in more detail.

3.2.1. Production and use of differential quantitative banks

In a first embodiment, the present invention therefore makes use of a
10 differential quantitative bank, i.e. a bank comprising nucleic acid clones corresponding to genes whose level of expression is modified in cells in the situation of deregulation relative to a control situation. Such banks can also be derived from differential quantitative analyses, pooling sequences whose expression is increased or decreased in cellular deregulation phenomena.
15 The methods to establish this type of bank are familiar to those skilled in the art and can be broken down into the following categories:

High flow sequencing electronic subtraction

This process is based on the random sequencing of a certain number of
20 cDNAs. A computer search engine can then be used to perform a subtraction between the two situations under analysis.

Serial Analysis of Gene Expression (SAGE)

This process is based on the recognition of a signature associated with each
25 cDNA by using restriction enzymes and oligonucleotide adaptors. This label corresponds to a part of the cDNA sequence (10 nucleotides long so as to unambiguously identify the corresponding cDNA). The labels are then assembled for sequencing and analysis (Velculescu et al., Science, 1995, 270: 484-487). This approach therefore represents a short-cut to systematic
30 sequencing.

Nucleic Acid Arrays

This method is based on the application at more or less high density of nucleic acids such as oligonucleotides, PCR fragments or cDNAs on solid supports such as membranes, glass plates or bio-chips. Messenger RNA probes from the healthy or pathological samples are then used in hybridization to identify
5   messengers that are overexpressed or underexpressed.

Differential Display

This method makes use of an oligo-dT primer and random primers to perform PCR on cDNA populations. The PCR products are then compared on very
10   high resolution gels. Differentially expressed fragments are then isolated and their presence confirmed by northern blot analysis prior to sequencing.
Several variants of this method have been described (Prashar and Weissman, PNAS, 1996, 93: 659-663). These variants differ in terms of the primer and restriction enzymes and adaptor used. Like the SAGE method, they make use
15   of the 3'-ends of cDNAs. This approach is made accessible by the existence of several commercially available kits.

Subtractive cloning

This method is based on the elimination of cDNAs that are common to the two
20   samples under comparison. Thus, different kits in which the "tester" cDNA is hybridized with an excess of "driver" cDNA are available (Clontech). The final product consists of a pool of PCR-amplified fragments derived from differentially expressed cDNAs, which can be cloned in a suitable vector for subsequent analysis. RDA (Representational Difference Analysis) is another
25   method based on this principle of subtraction (Lisitsyn et al., Science, 1993, 259: 946-951).

The implementation of these differential analytical methods therefore enables the generation of quantitative banks and clones, i.e. comprising all the
30   sequences whose expression is increased or decreased in cellular deregulation phenomenon or phenomena.

3.2.2. Production and use of differential qualitative banks

In another embodiment, the present invention advantageously uses a differential qualitative bank, i.e. a bank comprising nucleic acid clones of which
5 at least part of the sequence corresponds to the sequence of the genes differentially spliced in the cells in a situation of deregulation. This type of bank therefore comprises sequences that are differentially spliced in deregulatory processes.

10 The use of this type of bank is particularly advantageous. In fact, the different cellular stresses leading to cell death or to other deregulatory phenomena involve initiators and mediators of signalling pathways and regulate key cell cycle components, including p53. Regulation of the level of expression of this protein occurs mainly through stabilization and transcriptional enhancement.
15 While Fas receptors, members of the bcl2 family and caspases are regulated at the transcriptional level, they are also regulated at the level of premessenger RNA maturation and splicing. This post-transcriptional regulation is critical because, from a same transcription event, it determines the creation of a pro- or anti-apoptotic form of each member of the concerned
20 protein families. These transcriptional and splicing modifications are regulated by cell signalling and metabolism. The transcription and splicing regulatory factors which provide "trans" regulation of these key steps of gene expression are proteins whose own activity is regulated, notably by phosphorylation, according to the state of proliferation, differentiation and viability of the cell.
25 The splicing program which is engaged during cell deregulation and which mobilizes different initiators and mediators of cell death therefore reflects modifications in cell signalling due to different damage. By affecting the transcriptional and splicing machinery, these modifications affect the expression of many genes which are not limited to the genes known to play a
30 role as initiators or mediators of deregulation, but participate at different levels of the signalling cascades. Likewise, the cascades altered during

oncogenesis, particularly by expression of splicing variants, mobilize markers whose expression is not restricted to tumors.

To take into account these phenomena and this complexity, and to thereby provide a more predictive evaluation of the toxic potential of a test compound, the process of the invention advantageously uses splicing events characteristic of deregulated situations, as genetic markers of toxicity.

To do so, the present invention uses, for example, differential qualitative nucleic acid banks produced according to "DATAS" methodology described in international patent application n° WO99/46403, incorporated in the present by reference. In particular, such banks can be prepared by hybridization between the nucleic acid population derived from cells in a situation of deregulation, and the nucleic acid population derived from cells in a control situation, and isolation, from the hybrids formed, of the nucleic acids corresponding to differential splicing.

In this approach, hybridization is preferably carried out in liquid phase. Furthermore, it can be carried out in any suitable device such as tubes (Eppendorf tubes, for example), plates or any other suitable support commonly used in molecular biology. The hybridization is advantageously carried out in volumes of between 10 and 1000 µl, for example between 10 and 500 µl. It is understood that the device and volumes used can be easily adapted by those skilled in the art. The quantities of nucleic acids used for the hybridization are also known to those skilled in the art. In general, microgram quantities of nucleic acids suffice, for example between approximately 0.1 and 100 µg. Furthermore, it is possible to use the nucleic acids in a driver/tester ratio ranging from approximately 50 to 0.02, preferably from 40 to 0.1. Even more advantageously, this ratio is preferably close to or greater than 1, advantageously between approximately 1 and approximately 10. It is understood that this ratio can be adapted by those skilled in the art according to the conditions of the process (available quantities of nucleic acids, physiological situations, purpose, etc.). The other hybridization parameters,

including time, temperature and ionic strength, can also be adapted by those skilled in the art. As a general rule, following denaturation of the tester and driver, for example by heating, the hybridization is carried out for approximately 2 to 24 hours at a temperature of approximately 37°C (possibly submitted to temperature spikes), and in standard conditions of ionic strength, which can range from 0.1 to 5 M NaCl, for example. Ionic strength is known to be one of the factors that determines the stringency of a hybridization, especially in the case of hybridization on a solid support.

According to a specific embodiment of the invention, the hybridization is carried out in a phenol emulsion, for example according to the PERT method ("Phenol Emulsion DNA Reassociation Technique) described by Kohne D.E. et al. (Biochemistry, Vol. 16, No. 24, pp 5329-5341, 1977). The hybridization is avantageously carried out in a phenol emulsion maintained by thermocycling (temperature increase from approximately 37°C to approximately 60-65°C) and not by agitation, according to the method described by Miller and Riblet (NAR 23 (1995) 2339).

Any other hybridization method in liquid phase, preferably in emulsion, can be used within the scope of the present invention. Furthermore, the hybridization can also be done with one of the strands immobilized on a support. Advantageously, it is the driver that is immobilized. This can be done notably thanks to biotinylated primers or by any other immobilization technique known to those skilled in the art.

Using the nucleic acid populations generated by hybridization, the genetic markers of the invention (the clones characteristic of qualitative genomic alterations) can be identified by any method familiar to those skilled in the art. In the case of RNA/DNA heteroduplex, these regions are mainly regions of unpaired RNA (RNA loops) and can be identified and cloned by separation of the heteroduplex and the single-stranded nucleic acids (excess nucleic acids which did not react), selective digestion of the double-stranded RNA (domains participating in the heteroduplex), followed by separation of the resulting single-stranded RNA and the single-stranded DNA. In the case of

heterotriplex, these differential splicing regions consist mainly of regions of double-stranded DNA and can be identified and cloned by treatment with suitable enzymes such as an enzyme that digests RNA, followed by an enzyme that digests single-stranded DNA. The nucleic acids so obtained are directly in the form of double-stranded DNA and can be cloned in any suitable vector.

It is understood that other specific variants and conditions for the isolation of nucleic acids, hybridization and obtaining of qualitative clones, are indicated in WO99/46403, incorporated in the present by reference.

These methods enable the generation of nucleic acid banks and clones corresponding to qualitative genetic markers of a situation(s) of deregulation. As indicated in the experimental section, these nucleic acid preparations are especially useful markers to characterize the toxicity profile of compounds.

4.   Diversity of the banks

The aforementioned methods therefore enable the generation of groups of nucleic acid clones characteristic of situations of deregulation. Each method of preparation generates many clones which constitute banks. These banks can be used as is, deposited on supports, modified by the addition or removal of clones, or different banks can be pooled or control clones added, etc.

The banks provided for by the invention can comprise 10 to 50,000 clones, more generally 10 to 10,000 clones, and even more preferably 50 to 5,000 clones. The clones are generally deposited in a well-ordered fashion on one or more supports so as to facilitate analysis of the hybridization results. The support can be composed of glass, nylon, plastic, fiber, etc. or generally be any solid support suitable for the deposit of nucleic acids. The banks can be deposited on the supports by conventional methods known to those skilled in the art, as described for example in the international application No. WO99/46403.

The banks used can comprise both the nucleic acid clones corresponding to genes whose level of expression is altered in cells in a situation of deregulation (quantitative genetic markers) and the nucleic acid clones of which at least part of the sequence corresponds to genes that are differentially spliced in cells in a situation of deregulation (qualitative genetic markers). Thus, the genetic markers can be generated by different approaches, then pooled to obtain a response that is as predictive as possible.

In this regard, it is also possible to use banks comprising clones derived from different situations of deregulation. For example, as noted above, the situations of deregulation can be induced in different manners (increased p53 gene expression, increased Rb gene expression, use of different cell populations, use of tumor cells, etc.). These different situations of deregulation do not always induce exactly the same genetic events, and therefore do not always produce exactly the same nucleic acid banks according to the invention. Although each of these banks can be used separately within the scope of the present invention, it is also possible to pool them on the same support (or on separate supports) so as to further increase the number of genetic markers of toxicity and thereby improve the predictability of the methods of the invention.

A subject of the present invention is therefore also based on a nucleic acid preparation comprising qualitative and quantitative genetic markers characteristic of cellular deregulation(s). A specific subject concerns a bank comprising genetic markers characteristic of different situations of deregulation. Another subject of the invention is any solid support on which at least two banks of nucleic acids characteristic of two situations of deregulation have been deposited. In this regard, the invention further concerns a process of preparation of a DNA chip used to determine the toxic potential of a test compound, comprising the application, on a solid support, of one or more nucleic acid preparations characteristic of situation(s) of deregulation.

Furthermore, in a preferred embodiment, use is made of nucleic acid banks refined through use by the selection of clones based on their actual involvement in toxicity phenomena. The initial banks can in fact comprise all the clones characteristic of genetic events of a situation of deregulation.

5 Implementation of the diagnostic process set forth by the invention then makes it possible to observe that some of the clones hybridize very rarely, if ever, with the nucleic acid test samples. Such clones can eventually be removed from the bank in order to generate more specific, and therefore more universal and predictive banks.

10

Another advantageous subject of the invention is therefore based on a nucleic acid bank comprising nucleic acid clones corresponding to genetic events common to a situation of deregulation and a situation of toxicity.

15 Another subject concerns a method of preparation of banks of genetic markers of toxicity, comprising hybridization between a nucleic acid population derived from cells in a situation of deregulation, and a nucleic acid population derived from cells in a control situation, isolation from the hybridization product of clones characteristic of the situation of deregulation, and hybridization of the

20 clones obtained with a nucleic acid sample derived from cells in a situation of toxicity. The above process advantageously further comprises a selection step, comprising hybridizing the bank with different nucleic acid samples derived from cells in a situation of toxicity induced by different compounds, and identification of the clones in the bank that hybridize most frequently with

25 said samples.

More specifically, the present invention now describes the identification and characterization of such clones, which can be used as genetic markers of toxicity.

30

In this regard, the invention discloses the identification, preparation, cloning and characterization of particular clones, whose sequences are represented

by SEQ ID NOs: 1 to 37. These clones can be used as genetic markers of toxicity, according to the present invention. These clones have been isolated by qualitative methods and comprise sequences corresponding to qualitative genetic alterations characteristic of deregulation events that affect different

5 cellular genes, such as genes coding for proteins, enzymes, mitochondrial genes, transcription factors, pathological genes, stress-response genes, genes involved in signal(s) transduction, etc. Such clones, the preparation and libraries containing the same, as well as their uses, represent particular objects of this invention.

10

In this regard, a particular object of this invention resides in a nucleic acid preparation or library, comprising a plurality of nucleic acid clones that are genetic markers of toxicity, said preparation or library comprising at least one clone of sequence selected from SEQ ID Nos: 1 to 37.

15

A particular object of the invention resides, more generally, in any library of nucleic acids comprising at least one clone of sequence selected from SEQ ID n° : 1 to 37.

20 A clone of sequence SEQ ID n° : X designates, within the context of this invention, (i) any clone comprising all of said sequence SEQ ID n° : X (or of a sequence complementary thereto), optionally flanked by additional sequences, preferably a clone consisting in all of said sequence SEQ ID n° : X (or a sequence complementary thereto) ; as well as (ii) any clone comprising a part

25 of said SED ID n° : X (or of a sequence complementary thereto), optionally flanked at one end by additional sequence(s) (in particular originating from the sequence of the corresponding gene or messenger), said part of the sequence being sufficient to allow a specific hybridization (for instance at least 10 bases) ; or (iii) any other nucleic acid sequence specific for the gene or for a genetic

30 event of deregulation of the gene identified by SEQ ID n° : X, without necessarily overlapping with SEQ ID n° : X. Sequences (iii) are, for instance, sequences from a distinct region of said gene, allowing its detection in similar

conditions. Clones (i) to (iii) of this invention typically comprise between 10 and 1000 bases, more generally between 20 and 500 bases, a portion of which at least being specific for the genetic event under consideration.

5   The preparations or libraries of the invention comprise more preferably at least 5, more preferably at least 10, even more preferably at least 15, even further preferably at least 20 clones of sequence selected from SEQ ID n° : 1 to 37. As indicated before, the libraries comprise, typically, between 10 and 50 000 clones, more generally between 10 and 5 000 clones, in particular between 50 10   and 1 000 clones, a part thereof at least being represented by one or several clones of sequence selected from SEQ ID n° : 1 to 37.

The libraries and preparations of this invention can be deposited on supports and used as disclosed in this application.

15

One of the major applications of the identification and cloning of these genetic markers concerns the evaluation of the toxic potential of a test compound. This evaluation can be carried out by hybridizing a probe corresponding to the messenger RNA of a cell culture treated with this product, with one or more 20   banks of signatures characteristic of situation(s) of deregulation, such as those described above. An other application resides in the identification of genomic alterations (in particular SNPs) that are correlated to a response of a subject to a given compound, for instance the response to a treatment, the sensitivity to a compound, etc. These applications are described in more detail below.

25

5. Methods of analysis and diagnosis of toxicity

The invention therefore also concerns a method for analysis of the toxic potential of test compounds, using the aforementioned genetic markers. In 30   particular, the invention allows to determine the toxic potential of any compound by hybridizing a sample of nucleic acids from cells treated with this compound, with the aforementioned genetic markers, the observed

hybridization profile indicating the toxic potential of the compound. To this end, the genetic markers used are preferably combined into banks so that the response can be as predictive as possible. One of the advantages of the present invention also concerns the large number of markers used, which makes the information obtained even more predictive. The predictive nature of the information is further strengthened by the type of markers used and prepared.

A specific subject of the invention is based on a method of analysis of the toxic potential of a test compound, comprising at least one hybridization step between a) a nucleic acid sample from cells treated with this compound and b) a nucleic acid bank corresponding to genetic events characteristic of situation(s) of deregulation of cell signalling pathway(s), the hybridization profile indicating the toxic potential of the test compound. In particular, the greater the number of hybridization-positive clones, the greater the potential toxicity of the test compound.

The analysis of the toxicity profile of the compound is more generally done by comparing the hybridization profile on the bank of a nucleic acid sample from cells treated with this compound, with that of a nucleic acid sample from cells not treated with the compound. This comparison makes it possible to demonstrate a difference between the number of hybridizing clones in the toxic situation and in the control situation, by which a toxicity index for the test compound can be established.

More specifically, the invention therefore concerns a method of analysis of the toxic potential of a test compound, comprising at least

- a hybridization step between a) a nucleic acid sample from cells treated with this compound and b) a nucleic acid bank corresponding to genetic events characteristic of situation(s) of deregulation of cell signalling pathway(s),

- a hybridization step between c) a nucleic acid sample from cells not treated with this compound and b) a nucleic acid bank corresponding to genetic events characteristic of situation(s) of deregulation of cell signalling pathway(s),

5   - comparison of the hybridization profiles obtained indicates the toxic potential of the test compound.

The hybridization profile can be detected by any method familiar to those skilled in the art, and particularly by labelling of the nucleic acid test samples

10   and detection of the label in the bank. Thus, the invention more specifically concerns a method of analysis of the toxic potential of a test compound comprising at least the separate hybridization between a) labelled nucleic acid probes corresponding to RNA from cells not treated and cells treated with said test compound and b) a nucleic acid bank corresponding to genetic events,

15   particularly transcriptional and/or splicing events, characteristic of deregulation(s), the hybridization profile indicating the toxic potential of the test compound.

The nucleic acids can be labelled by any method known to those skilled in the

20   art, particularly through the use of radioactive, fluorescent, enzymatic or colorimetric labels. In general, radioactive labelling is used and the hybridization is visualized by detection of radioactivity on the support. Fluorescent labelling can also be used, in which case the hybridization is visualized by detection of the fluorescence emitted on the support.

25

As indicated above, in the methods set forth by the invention, the nucleic acid banks corresponding to genetic events characteristic of deregulation(s) can be, in particular, nucleic acid banks characteristic of situations of deregulation of cell growth and/or viability. More specifically, these are nucleic acid banks

30   characteristic of cells in a situation of deregulation of cell growth, notably transformed cells, tumor cells in particular.

In the methods of the invention described above, the nucleic acid banks are more preferably nucleic acid banks corresponding to genetic events characteristic of alterations in signalling pathways induced by the activation, preferably the expression of all or part of a tumor suppressor gene, preferably p53. In particular these are banks characteristic of apoptosis induced by a tumor suppressor gene, preferably p53. The results presented in the examples demonstrate in particular that, through the use of a specific differential qualitative screening strategy, a situation of apoptosis resulting from induction of p53 protein expression leads to many modifications in splicing, in addition to quantitative modifications related to transcriptional regulation of different genes. The invention shows that probes from cells treated with different toxic compounds hybridize with clones from banks derived from quantitative and qualitative screening prepared from control cells, on the one hand, and from cells in which wild type p53 expression is induced, on the other hand. This indicates that the transcriptional and splicing events are common to toxic situations and to situations where wild type p53 protein expression is induced.

Moreover, the results obtained also show that when the treated cells are of the same type as those in which p53 expression has been induced, the probes prepared from the messenger RNA from cells treated with increasing concentrations of the toxic compounds hybridize a larger number of clones in the quantitative and qualitative banks when the concentrations are high, and therefore the toxicity important. This demonstrates that there is a correlation between the level of toxicity and the hybridization profile on the banks of the invention.

It is clear however that the application of the methods, kits and banks of the invention is not restricted to a specific cell type. In particular, it is not restricted to tests of products on the same cells as those from which the quantitative or qualitative banks and clones are derived. In fact, the results presented show

that the clones of the invention advantageously enable a diagnosis of toxicity independent of the toxic compound and the type of test cell.

In a specific embodiment, the nucleic acid library is a library comprising at least one clone of sequence selected from SEQ ID Nos: 1 to 37, as defined above.

In a specific embodiment of the methods of the invention, the cells treated or not treated with the test compound and the cells used to generate the banks are of a different type.

More specifically, the cells treated or not treated with the test compound can be of different origin and type. They are preferably mammalian cells, even more preferably human cells. They can be primary cultures or cell lines. The cell type used, its concentration, density and physiological state (resting, stimulated, etc.) can be chosen by the user of the methods of the invention according to the test compounds and desired applications. The cells can also be cells extracted from organs or tissues of animals treated or not treated with the compound.

The test compound can also be highly varied in nature. For example, it can be an individual compound or a mixture of compounds. The compound can be chemical or biological. In particular, it can be a peptide, polypeptide, nucleic acid, lipid, carbohydrate, a chemical molecule, plant extract, combinatorial libraries, etc. The test compound can also be a treatment applied to the cells, such as irradiation or UV. To implement the process of the invention, the test compound can be applied at different concentrations chosen by the user. Furthermore, the nucleic acid samples derived from the treated cells can be prepared at various time points after treatment with the compound. In this way, kinetic studies can be carried out as needed.

The nucleic acids and corresponding probes are prepared by conventional methods. Hybridization can then be carried out, also under conditions that can be adapted by those skilled in the art and by the user. In this regard, the hybridization can be carried out under high, intermediate or low stringency,

5 depending on the desired level of sensitivity, quantity of available material, etc. Furthermore, according to the present invention the hybridizations between the bank and the probes can be homologous (when the bank and probes are composed of nucleic acids from the same species, for example human) or heterologous (when the bank and probes are composed of nucleic acids from

10 different species, for example a human bank and probes prepared from material derived from another mammalian species). Although the banks and probes are preferably constructed from material of human origin, it is in fact possible to use other sources, particularly for the probes (for example, murine origin). For such heterologous hybridizations, the hybridization conditions can

15 be adjusted by those skilled in the art according to conventional techniques, particularly by decreasing the hybridization temperature and/or by increasing the salt concentration of the hybridization buffer.

6. Determination of a toxicity index

20

An advantage of the methods of the invention is based on the possibility of determining a toxicity index for a given compound on any cell type. For this application, the clones corresponding to elements identified from cells used to establish banks specific for cell viability, as well as those specific for the

25 induced deregulation, are hybridized with a probe derived from the untreated cells under study. Their hybridization serves as the reference RNA expression common to both the model of deregulation and the cell model under study. Comparison of RNA expression in the untreated cell situation with that of situations treated with different products at different concentrations and

30 treatment times can be easily done by comparing the hybridization profiles of each probe from each situation under study. The toxicty index of a situation

can be evaluated by assigning a value of 1 to clones that hybridize with the untreated probe and do not hybridize with the treated situation, on the one hand, and by also assigning a value of 1 to clones not hybridizing with the untreated probe but hybridizing with the treated probe, on the other hand. The

5   index is then the sum of the individual 1 values expressed as a percentage of the number of clones subjected to hybridization. This index can therefore range from 0 to 100. This example of calculation of the toxicity index is not limiting. Other mathematical methods, algorithms or calculation processes such as neuronal networks can also be used.

10

The value of determining such an index by hybridizing clones derived from differential qualitative screening between two states of a same cell type (proliferation and apoptosis-induced) with probes from other cell types, is confirmed by the ability to determine an index that is all the greater for a given

15   compound when the concentration applied to the test cells is high.
Naturally, the value of the index depends on the threshold at which a clone is assigned a value of 1 or 0. The index is all the greater when the factor of repression or induction that the clone requires for consideration is low. The robustness of the method is nonetheless illustrated by the fact that there is a

20   linear relation between the chosen factor and the calculated toxicity index. This linearity reflects the number of independent events which constitute the qualitative bank, which makes it possible to avoid giving too much weight to a particular clone with an overly high differential expression between the treated and untreated situation.

25

7.Identification of polymorphisms linked to the toxicity for a given compound

The determination of the toxic potential of a compound according to the procedure disclosed in 5. and in the examples allows the identification of the

30   sequences which derive from genes involved in mechanisms of stress and cell death, whose expression is altered by this compound.

These genes represent candidates of choice for the identification of polymorphisms that may affect the response to said compound. These polymorphisms allow to segregate candidate patients for the administration of this compound in different groups that are more or less susceptible of developing a pronounced toxicity, in particular hepatic.

For example, it can be envisioned that a polymorphism which affects a gene whose expression is decreased by a compound renders the patient more susceptible to the toxic activity of this compound. Without being limited to such particular example, the importance of polymorphisms in the genes selected according to the invention is assessed by determining the correlation existing between said polymorphisms and the toxicities observed during clinical trials conducted with the compound under consideration.

An object of the invention is thus the use of clones or nucleic acids disclosed, for the identification of mutations or polymorphisms, in particular SNPs, correlated to the response of subjects to pharmaceutical compounds.

The invention concerns, more generally, the use of nucleic acids representative of splicing events characteristic of a given physiopathological situation, for the identification or the characterization of SNPs correlated to said situation.

The identification may be performed using different methods or approaches known to the skilled person. In particular, it can be performed by sequencing, from biological samples derived from subjects that respond and from subjects that do not respond to a compound, the genes corresponding to the selected clones or nucleic acids, followed by the identification of polymorphisms in said genes. The identification can also be performed from SNP libraries, by searching for SNPs present in said genes.

The invention also concerns a method for the identification of SNPs or other mutations or polymorphisms that allow the assessment of the response of a subject to a given compound, the method comprising (i) the identification in

vitro of nucleic acids characteristic of splicing events induced in a cell treated with said compound and (ii) the identification of SNPs or other mutations or polymorphisms in the gene or genes corresponding to nucleic acids identified in (i), said SNPs or other mutations or polymorphisms allowing the assessment of the response of a subject to said given compound. The invention thus provides methods and means to select genes for which polymorphisms are linked to a toxicity for a given compound.

The invention also relates to a population of nucleic acids specific for polymorphisms or SNPs thus identified, in particular a population of nucleotide primers for the selective amplification of said polymorphisms, or of probes for the selective hybridization to the considered polymorphisms.

The invention also relates to methods for the analysis (e.g., the prediction, evaluation or determination) of the response of a subject to a test compound, comprising the study of polymorphisms, in particular the analysis of SNPs, as defined above. A particular method relates to the analysis of the sensitivity or of the response of a subject to a compound, in particular to the toxicity of a compound, comprising the analysis, from a biological sample comprising DNA from said subject, of the presence in the subject's DNA of polymorphisms, SNPs or other genomic alternations present in genes whose splicing is modified in response to said compound. As indicated above, the presence of polymorphisms, SNPs or other genomic alterations in a subject's DNA may be determined by different techniques known to the skilled person, such as sequencing, amplification, hybridization, separation or chromatographic methods, etc. A preferred approach consists in the amplification of the subject's DNA using primers specific for the alteration (in particular the SNP) under consideration. An other approach consists in the use of a nucleotide probe whose sequence is specific for the alteration (in particular the SNP) under consideration. It should be understood that the invention is not to be limited to particular methods.

8. Kits

The present invention also concerns kits for the implementation of the aforementioned methods. The invention more specifically concerns kits for the study of the toxic potential of a test compound, comprising at least :

5   -   a nucleic acid bank corresponding to genetic events (transcriptional and/or splicing) characteristic of alteration(s) in one or more cell signalling pathways, notably situation(s) of deregulation of cell growth and/or viability.

10   The kits of the invention more generally comprise a bank of genetic markers characteristic of deregulation(s), for example apoptosis, such as defined in the invention, deposited on a solid support.

The kits of the invention more preferably comprise a nucleic acid bank
15   characteristic of several situations of deregulation induced in different conditions.

According to another specific embodiment, the kits of the invention comprise at least one nucleic acid bank comprising clones corresponding to genetic
20   events common to cells in a situation of deregulation and in a situation of toxicity.

According to a particular embodiment, the invention relates to any kit comprising a library of nucleic acids, the library comprising at least one clone
25   of sequence selected from SEQ ID Nos: 1 to 37, as defined above. Advantageously, the library is deposited on a support, preferably a solid support, in particular, glass, silica, filter, membrane, nylon, plastic, etc.

In the kits according to the invention, the banks further advantageously
30   comprise control clones for calibration of the detected signals. These controls can more particularly comprise three types of material :

- genomic DNA clones,
- cDNA used for construction of probes that will hybridize the banks, and/or
- cDNA corresponding to mRNA encoding control proteins, for example housekeeping proteins (especially enzymes) such as β-actin or GAPDH (Glyceraldehyde Phosphate DeHydrogenase).

The controls provided by the housekeeping enzymes are classically used by those skilled in the art in cDNA display experiments However, during the deregulation phenomena according to the invention, the quantity of mRNA of these enzymes can vary. Therefore, to normalize the hybridizing power (and therefore the signal) of each probe, the present invention proposes the use of one or more other control systems. In this regard, the use of genomic DNA and/or cDNA (unlabelled) corresponding to the probe (i.e. to the DNA population from the treated or untreated biological sample) makes it possible, according to the invention, to obtain hybridization controls independent of any qualitative or quantitative variations in gene expression. Equivalent quantities of cDNA with the same specific activity after radiolabelling should in principle generate equivalent signals by hybridizing with both genomic DNA and with themselves.

The kits of the invention can also comprise :
- oligonucleotides for use in the preparation of the probes, and/or
- a hybridization protocol, and/or
- items and information for establishment of a toxicity index for each test product, notably a signal processing software program.

The oligonucleotides are preferably semi-random, such as oligonucleotides with the formula $X-N_n-AGGT$, where X is a stabilizing sequence such as GAGAAGCGTTATG or CCACGCTACAAG(C), N is a base, and n is a whole number between 3 and 8 inclusive.

The feasibility, realization and other advantages of the invention are depicted in further detail in the examples that follow, which should be considered illustrative and non-limiting.

5 Legends to figures :

Figure 1. Differential hybridization of probes from HepG2 cells untreated (A), treated with ethanol (B) or treated with cyclosporine on filters containing PCR fragments corresponding to clones from the qualitative screening of a p53-
10 induced apoptosis system.

Figure 2. Toxicity index calculated for different toxic compounds on the p53 apoptosis filter.

15 Figure 3. Differential hybridization of probes from HepG2 cells untreated (A), treated with clenbuterol (B), R-propranolol (C) or D,L-propranolol (D) on filters containing PCR fragments corresponding to clones from the qualitative screening of tumor biopsies.

20 9. Examples

9.1. Identification of genetic markers of toxicity from qualitative screening on a cellular apoptosis system

25 An example of the contribution of differential qualitative analysis to the identification of genetic markers associated with drug toxicity is given by the use of DATAS in a model of apoptosis induction via induction of wild type p53 expression. This cell model was established by transfection of a system of inducible expression of the p53 tumor suppressor gene. So as to identify the
30 qualitative differences specifically related to p53-induced apoptosis, qualitative screening was implemented on messenger RNA extracted from induced (ml)

and non-induced (mNI) cells. This messenger RNA is converted to the complementary DNA (cI) and (cNI) using reverse transcriptase (RT) and biotinylated oligonucleotide primers. mI/cNI and cI/mNI hybrids are then obtained in liquid phase.

5

The required quantities of RNA and cDNA (in this case, 200 ng of each) are combined and ethanol-precipitated. The samples are taken up in 30 μl of hybridization buffer (40 mM Hepes pH 7.2, 400 mM NaCl, 1 mM EDTA) supplemented with deionized formamide (80 % V/V). After denaturation for 5

10   min at 70°C, samples are incubated overnight at 40°C.

Streptavidin beads (Dynal) are washed and then reconditioned in binding buffer (2X= 10 mM Tris-HCl pH 7.5, 2 M NaCl, 1 mM EDTA). Hybridization samples are adjusted to a volume of 200 μl with water and added to 200 μl of

15   beads, then incubated at 30°C for 60 min. The beads are captured on a magnet and washed, then suspended in 150 μl of RnaseH buffer and incubated at 37°C for 20 min. After capture on the magnet, non-hybridized regions are released in the supernatant which is treated with Dnase, then extracted with acidic phenol/chloroform and ethanol-precipitated. Ethanol

20   precipitations of small quantities of nucleic acids are done with the commercial polymer SeeDNA (Amersham Pharmacia Biotech) for quantitative recovery of nucleic acids from very dilute solutions (ng/ml concentrations).

cDNA synthesis from the RNA samples derived via the action of RnaseH is

25   carried out by using random hexanucleotides and Superscript II Reverse Transcriptase. The RNA is then degraded in a mixture of RnaseH and Rnase T1. The primer, unincorporated nucleotides and enzymes are separated from the cDNA on a GlassMAX Spin cartridge. The cDNA corresponding to splicing loops is then used for PCR with semi-random oligonucleotides with

30   the following structure : (FS3)N7AGGT, where (FS3) = CCACGCTACAAG.

PCR is performed with taq polymerase for 30 cycles :

- Initial denaturation : 94°C for 1 min.
- 94°C for 30 s
- 55°C for 30 s
- 72°C for 30 s
- Final elongation : 72°C for 5 min.

The PCR products are cloned in a pGEM-T vector (Promega) with a floating T at the 3' ends to facilitate cloning of fragments derived from the action of taq polymerase. Following transformation in competent JM109 bacteria (Promega), colonies are stored on 96 well plates. The PCR products generated with the T7 and Sp6 oligonucleotides located on the cloning plasmid and surrounding the insertion cassettes are deposited on nitrocellulose filters.

HepG2 cells are then individually treated with different compounds with different levels of toxicity. In this example, the concentration used was calculated by a cell viability test (MTT) and corresponds to an IC80. Table 1 gives the concentrations of the different compounds and the exposure times.

Table 1 :

Compound	Concentration	Time
Aspirin	2 mM	18h
Paracetamol	1 mM	18h
Cyclosporine	2.5 µM	18h
Diclofenac	50 µg/ml	18h
Beclomethazone	50 nM	18h
Methotrexate	500 nM	18h
Erythromycin	2.5 µg/ml	18h
Vinblastine Sulfate	30 µM	18h
Clonidine	150 µM	18h
Valinomycin	15 nM	18h
Etoposide	50 µg/ml	18h
Camptothecin	1 µg/ml	4h
PMA	1 µg/ml	18h
Ethanol	0.1 M / 0.5 M	4h
5 FU	10 µM	24h
Cycloheximide	5 µg/ml	18h
FCCP	5 µM	18h
Staurosporine	50 nM	18h
Terbutaline Sulfate	200 ng/ml	18h
Epinephrine Hydrate	50 µg/ml	18h
AZT	50 µg/ml	18h
H2O2	0.1 mM	18h
Actinomycin D	0.02 µg/ml	18h

Labelled RNA probes are prepared from these situations. To do so, the first cDNA strand obtained from total RNA by methods known to those skilled in the art serves as matrix for the PCR reaction using the oligonucleotides (FS4)GN3AGGT and FS3CN3AGGT, in which (FS4) = GAGAAGCGTTAT,
5   according to the following program :

Initial denaturation : 95°C for 5 min
5 cycles with :   denaturation : 94°C for 30 sec
                     annealing : 30°C for 30 sec
10   temperature increase from 30 to 72°C in 30 sec
                     elongation : 72°C for 30 sec
35 cycles with :   denaturation : 94°C for 30 sec
                     annealing : 57°C for 30 sec
                     elongation : 72°C for 30 sec
15 Final elongation: 72°C for 7 min

The PCR products are labelled with the Redyprime kit (Amersham).

Filters are prehybridized in hybridization buffer (Rapid Hybrid Buffer,
20 Amersham) containing 100 µg/ml of salmon sperm DNA at 65°C for 30 min. The PCR probe is then applied to the membrane (0.5×10^6 to 1×10^6 cpm/ml) and incubated at 65°C for 2 to 18 hours. Filters are washed in 5X SSC buffer, 0.1% SDS at 65°C for 30 min then in 0.2X SSC buffer, 0.1% SDS. The hybridization profiles are analyzed by measuring the radioactivity with an
25 InstantImager (Packard Instruments) (Figure 1). Quantification of the individual hybridization intensities allows calculation of an index according to the procedure described above (Figure 2). This result shows that it is possible to classify different products according to their ability to induce the expression of markers that are differentially spliced during p53-induced toxicity.   For
30 example, in HepG2 cells, diclofenac generates more splicing events identical to those induced by p53-related toxicity than aspirin.   The use of banks

containing cDNA sequences differentially spliced in a reference (healthy) situation and a situation involving a major route of toxicity, therefore enables the classification of products whose actions are not distinguishable using standard toxicity tests such as MTT.

9.2. Identification of genetic markers of toxicity from qualitative screening on tumor biopsies

A second example of the use of the invention for the identification of genetic markers of toxicity consists in the generation of differential nucleic acid banks for healthy tissue and tumor tissue. The value of identifying sequences specifically expressed in control (healthy) tissue on the one hand, and in tumor tissue on the other hand, is related to the fact that within a tumor, information characteristic of deregulated cell growth and cell death is present in different cells but also within the same cells. As compared to a model of apoptosis induced by deregulated expression of p53 or any other protein with a negative effect on cell growth and viability, the use of tumor specimens therefore additionally provides filters for use in predictive toxicology bearing sequences whose expression is characteristic of cellular hyperproliferation or carcinogenesis.

The working material comprised tumor biopsy specimens from 7 patients with head and neck cancers together with biopsy specimens from healthy uvula of these same patients. The procedure was identical to that described in example 9.1 although this is not a quantitative considering the scarcity of material available from human biopsy specimens.

The nucleic acid fragments generated by application of the invention to tumor and normal tissues were deposited on nitrocellulose filters. These filters were hybridized with the same radioactive probes as those used in example 9.1, i.e. generated from the genetic material of HepG2 cells treated with different

compounds displaying different toxic potential. Differential hybridization profiles were observed for the different compounds, thereby demonstrating the utility of these nucleic acid banks for evaluation of the toxicity of a given compound.

5

Figure 3 depicts the hybridization signals obtained with probes derived from normal HepG2 cells, or cells treated with clenbuterol, the inactive propranolol enantiomer, or a racemic mixture of the active and inactive propranolol enantiomers. The compounds were used at the concentrations indicated in

10 Table 1 and the hybridization probes and protocols were identical to those in the paragraph describing the use of filters containing specific signatures of p53-induced apoptosis (example 9.1).

The hybridization signals shown in Figure 3 can be quantified and serve as a

15 basis for determining a toxicity index for each compound using the same procedure as that used to obtain the indices shown in Figure 2. Thus, the hybridizations shown in Figure 3 reveal that the effects of the different compounds on HepG2 cells, which cannot be distinguished by standard toxicological methods such as MTT staining, can be classified according to

20 their ability to mobilize splicing events specific for the differences that exist between cells from healthy tissue and tumor cells.

9.3. Description of genetic markers of toxicity

25 The implementation of the techniques disclosed in examples 9.1 and 9.2 allowed the constitution and use of libraries of clones, genetic markers of toxicity. The clones of these libraries have been sequenced and analyzed, and the sequence of a portion thereof is presented in the sequence listing included in the present application (SEQ ID n° : 1 to 37).

30

The analysis of these clones shows that they correspond to qualitative genetic

events that affect varied cellular genes, thus confirming (i) the efficacy of the methods of this invention, (ii) the existence of "universal" genetic markers within the meaning of this invention and (iii) the importance of the diversity of the clones to obtain a predictive response.

5

In particular, the sequences identified enable the detection, through hybridization, of expression variations or of alterations in the following human cellular genes : Aldolase A (exon 9) (SEQ ID NO: 1) ; S4 subunit of proteasome 26S (SEQ ID NO: 2) ; Alpha-tubulin (SEQ ID NO: 3) ;

10 Glucosidase II (SEQ ID NO: 4) ; lamin B receptor homologue (SEQ ID NO: 5) ; EF1-alpha (SEQ ID NO: 6) ; Fra-1 (SEQ ID NO: 7) ; tyrosine kinase AX1 receptor (SEQ ID NO: 8) ; spliceosomale Protein SAP62 (SEQ ID NO: 9) ; TRAF-3 (SEQ ID NO: 10); EF2 (SEQ ID NO: 11); TEF-5 (SEQ ID NO: 12) ; CDC25b (SEQ ID NO: 13) ; interleukine-1 receptor-associated kinase

15 (« IRAK ») (SEQ ID NO: 14) ; WAF-1 (SEQ ID NO: 15) ; c-fos (exon 4) (SEQ ID NO: 16); ckshs1 (SEQ ID NO: 17) ; PL16 (SEQ ID NO: 18) ; NFAR-2 (SEQ ID NO: 19) ; phosphatidylinositol4-kinase (SEQ ID NO: 20), ERF (SEQ ID NO: 21), Eph type receptor tyrosine kinase (hEphB1b) (SEQ ID NO: 22) ; BAF60b protein of the SWI/SNF complex (SEQ ID NO: 23) ; EB1 (SEQ ID NO:

20 24) ; MSS1 (SEQ ID NO: 25) ; retinoïc acid alpha receptor (RARa) (SEQ ID NO: 26) ; translation initiation factor eiF4A (SEQ ID NO: 27) ; STE20 type kinase (SEQ ID NO:28) ; protein HSP 90kda (SEQ ID NO:29) ; Lipocortin II (SEQ ID NO:30) ; protein TPT1 (« translationally controlled tumor proteon ») (SEQ ID NO:31) ; Hsc70 (SEQ ID NO:32) ; Cytokeratin 18 (SEQ ID NO:33) ;

25 2-oxoglutarate dehydrogenase (SEQ ID NO:34) ; mitochondrial gene NADH6 (SEQ ID NO:35) ; mitochondrial gene NADH deshydrogenase 4 (SEQ ID NO:36) ; alpha subunit of mitochondrial ATP synthase (SEQ ID NO:37).

A particular object of the invention resides in the use of a nucleic probe

30 specific for all or part of a gene coding for a protein or a factor as mentioned above, for the detection or the evaluation of the toxic potential of a compound,

- 46 -

and for the detection of a toxicity situation in a biological sample.

Preferably, the probe comprises less than 1 000 bases, more particularly less than 500 bases, and comprises a sequence complementary to a portion of an above-
5   mentioned gene. More preferably, the probe is labeled.

Advantageously, the invention relates to a set of probes, in particular of 5 probes or more, preferably of 10 probes or more, each being complementary to a portion of a gene selected from the above-mentioned genes. Even more preferably, the
10   probes are immobilized on a support, as disclosed in the present application.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or
15   group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of
20   the common general knowledge in Australia.

SEQUENCE LISTING

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<120> Genetic Markers of Toxicity, preparation and uses thereof

<130> B0001WO

<140>
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<170> PatentIn Ver. 2.1

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      GenBank Acc: AF037332.


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      (RARalpha) mRNA cds, nucleotides 567-859. GenBank
      Acc: X06614.

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agctgttgtt cgtagtgtat ttgcccagct ggcagagggc agggaaggtt tcctggtgcg 180
ctttgcgcac cttctcaatg agctccccca cctccggcgt cagcgtgtag ctctcagagc 240
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gaccaccttc tgtatctgct gagccaattc tcgagtgggt gctaggacca aggcctgggt 180
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catcctcatc gccctcgaga ggggggatct matcaggaac tgcagcattg ggttcctctg 180
ctgccacttc atyttcatca atacctaaac ctagcttgat catgcaataa atgcggttgg 240
agtgggtctg gggatcctca ggggaaaagc cagaagatag cagggcggtt tcaaacagca 300
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tggtcttgat ggctgtttca atgttcaaag catcccgctc agcatcaaag ttagtatagg 240
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ggtgtcgata ttgtcatgaa ccatcacctg caggaaacaa gtttcacaaa agaagcctac 180
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gaaagagtaa aacctttat gacaggggct gcagaacaaa tcaagcacat ccttgctaat 300
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ttggactacc gtgaggatgg tgtgccccat aatgatttct ttaaggtggt taaaaatgga 420
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cacaagcagt acggaggcgt cttacagctc tcttgttctc actgatgtcc ttcttatgct 180
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<210> 34
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<210> 35
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<210> 36
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tggcaccatc ttgtcctgac ctccccggat acgctttcct catcatcaat cactagtgcg 180
gcgctgcagg tcgaccatat gggagagctc ccaacgcgtt ggatgcatag cttgagtatc 240
tatagtgtca cctaaata   258

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of analysis of the toxic potential of a test compound, comprising at least one hybridization step between a) a nucleic acid sample from cells treated with this compound and b) a nucleic acid bank, said bank comprising clones specific for (portions of) genes or transcripts characteristic of apoptosis, the hybridization profile indicating the toxic potential of the test compound.

2. A method of analysis of the toxic potential of a test compound, comprising at least a separate hybridization step between a) labelled nucleic acid probes corresponding to RNA from untreated cells and from cells treated with said test compound and b) a nucleic acid bank, said bank comprising clones specific for (portions of) genes or transcripts characteristic of apoptosis, the hybridization profile indicating the toxic potential of the test compound.

3. Method according to claim 1 or 2, wherein the nucleic probes a) correspond to messenger RNAs from treated and untreated cells.

4. Method according to one of claims 1 to 3, wherein the nucleic probes a) are cDNA or cDNA fragments prepared from the RNA of treated and untreated cells.

5. Method according to any one of the previous claims, wherein the nucleic probes a) are amplification products.

6. Method according to any one of the previous claims, wherein the nucleic probes a) are labelled by radioactive, fluorescent, enzymatic or colorimetric labels.

7.   Method according to any one of the previous claims, wherein the test compound is an individual compound or is present in a mixture with other substances.

8.   A method according to any one of the previous claims, wherein the bank b) comprises nucleic acids corresponding to genes whose level of expression is modified in a situation of apoptosis.

9.   Method according to one of claims 1 to 7, wherein the bank b) comprises nucleic acids of which at least part of the sequence corresponds to the sequence of genes that are differentially spliced during the situation of apoptosis.

10.   Method according to one of claims 1 or 2, wherein the bank b) comprises nucleic acids according to claim 8 and nucleic acids according to claim 9.

11.   Method according to claim 9, wherein the bank b) is prepared by hybridization between a nucleic acid population from a cell in a situation of apoptosis and a nucleic acid population from a cell in a control situation, and separation, from the hybrids formed, of nucleic acids corresponding to differential splicings.

12.   Method according to any one of the previous claims, wherein the situation of apoptosis is induced by modification of the activation, preferably of the expression of a gene that initiates or carries out apoptosis.

13.   Method according to claim 12, wherein the situation of apoptosis is produced by induction or enhancement of the activation, preferably of the expression of an anti-oncogene.

14.   Method according to claim 13, wherein the anti-oncogene is selected from

p53, Rb, p73, myc, TUPRO-2 and NHTS.

15. Method according to claim 12, wherein the situation of apoptosis is induced by modification of the activation, preferably of the expression of a gene involved in cell growth or viability.

16. Method according to claim 12, wherein the situation of apoptosis is induced by constitutive or inducible activation, preferably expression of all or part of a gene involved in cell growth, cell viability or apoptosis.

17. Method according to any one of claims 1 to 7, wherein the bank b) comprises at least 1 clone of sequence selected from SEQ ID Nos: 1 to 37, more preferably at least 5.

18. Method according to any one of claims 1 to 7, wherein the bank b) comprises a set of probes, in particular 5 probes or more, preferably 10 probes or more, each of said probes being complementary to a part of a gene selected from the following genes : Aldolase A; S4 subunit of proteasome 26S; Alpha-tubulin; Glucosidase II; lamin B receptor homologue; EF1-alpha; Fra-1; tyrosine kinase AX1 receptor; spliceosomale Protein SAP62; TRAF-3; EF2; TEF-5; CDC25b; interleukine-1 receptor-associated kinase («IRAK»); WAF-1; c-fos (exon 4); ckshs1; PL16; NFAR-2; phosphatidylinositol4-kinase, ERF, Eph type receptor tyrosine kinase (hEphB1b); BAF60b protein of the SWI/SNF complex; EB1; MSS1; retinoïc acid alpha receptor (RARa); translation initiation factor eiF4A; STE20 type kinase; protein HSP 90kda; Lipocortin II; protein TPT1 («translationally controlled tumor proteon»); Hsc70; Cytokeratin 18; 2-oxoglutarate dehydrogenase; mitochondrial gene NADH6; mitochondrial gene HADH deshydrogenase 4; alpha subunit of mitochondrial ATP synthase.

19. Method according to any one of the previous claims, wherein the treated or

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untreated cells a) and the cells in a situation of apoptosis b) are of a different type.

20. Method according to any one of the previous claims, wherein the treated or untreated cells are mammalian cells, preferably of human origin.

21. Method according to any one of the previous claims, wherein the treated or untreated cells are cell lines.

22. Method according to any one of the previous claims, wherein the treated or untreated cells are primary cultures.

23. Method according to any one of the previous claims, wherein the treated or untreated cells are cells extracted from the organs or tissues of treated or untreated animals.

24. A method of diagnosis of the toxic potential of a test compound, comprising at least the hybridization between one of the one hand, labelled nucleic probes corresponding to mRNA from untreated cells and a nucleic acid bank comprising clones specific for (portions of) genes or transcripts characteristic of apoptosis and, on the other hand, labelled nucleic probes corresponding to mRNA from cells treated with said test compound and said nucleic acid bank comprising clones specific for (portions of) genes or transcripts characteristic of apoptosis, the hybridisation profile indicating the toxic potential of the test compound.

25. Method according to claim 24, wherein the bank comprises clones specific for (portions of) genes or transcripts characteristic of deregulation in p53 signalling pathway.

26. Method according to claim 24 or 25, wherein the nucleic acid bank is a

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nucleic acid bank characteristic of transformed cells, in particular tumor cells.

27.   Method according to claim 24, wherein the nucleic acid bank comprises at least 1 clone of sequence selected from SEQ ID Nos: 1 to 37, more preferably at least 5.

28.   Use of nucleic acid clones specific for (potions of) genes or transcripts characteristic of apoptosis in a mammalian cell, as genetic markers of the toxicity of test compounds on mammalian cells.

29.   Use according to claim 28, of a clone of sequence selected from SEQ ID Nos: 1 to 37.

30.   A kit when used for the study of the toxic potential of a test compound, comprising a nucleic acid bank deposited on a support, the bank comprising at least 5 distinct clones having a sequence selected from SEQ ID Nos: 1 to 37.

31.   A nucleic acid composition comprising at least 5 distinct clones having a sequence selected from SEQ ID Nos: 1 to 37 when used to study the toxic potential of a test compound.

32.   A process of production of genetic markers of toxicity, comprising (i) a hybridization between a nucleic acid population derived from cells in a situation of apoptosis and a nucleic acid population derived from cells in a control situation, (ii) the isolation, from the hybridization product of step (i), of clones characteristic of the situation of apoptosis, and (iii) the hybridization of the clones obtained in step (ii) with a nucleic acid sample derived from cells in a situation of toxicity, the hybridization products thus formed representing genetic markers of toxicity.

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33. Process of preparation of a DNA chip that is used to diagnose the potential toxicity of a test compound, the process comprising the application on a solid support of one or more nucleic acid compositions according to claim 31.

34. A method according to any one of claims 1 to 27, a use according to any one of claims 28 to 29, a kit according to claim 30, a nucleic acid composition according to claim 31 or a process according to any one of claims 32 to 33 substantially as hereinbefore described with reference to the Figures and/or Examples.

DATED this 31st day of August, 2005
EXONHIT THERAPEUTICS S.A.
by its Patent Attorneys
DAVIES COLLISON CAVE

A)
Untreated
Cells
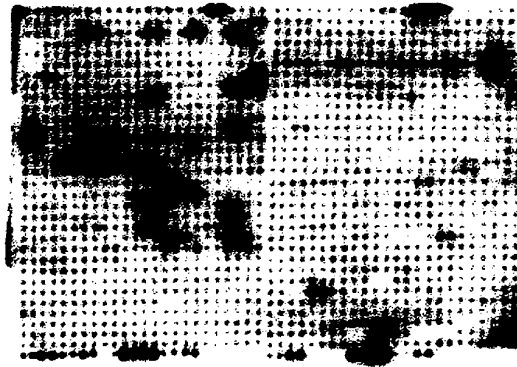
B)
Cells treated
with Ethanol
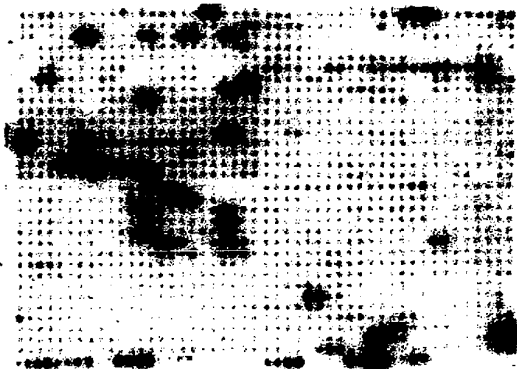
C)
Cells treated
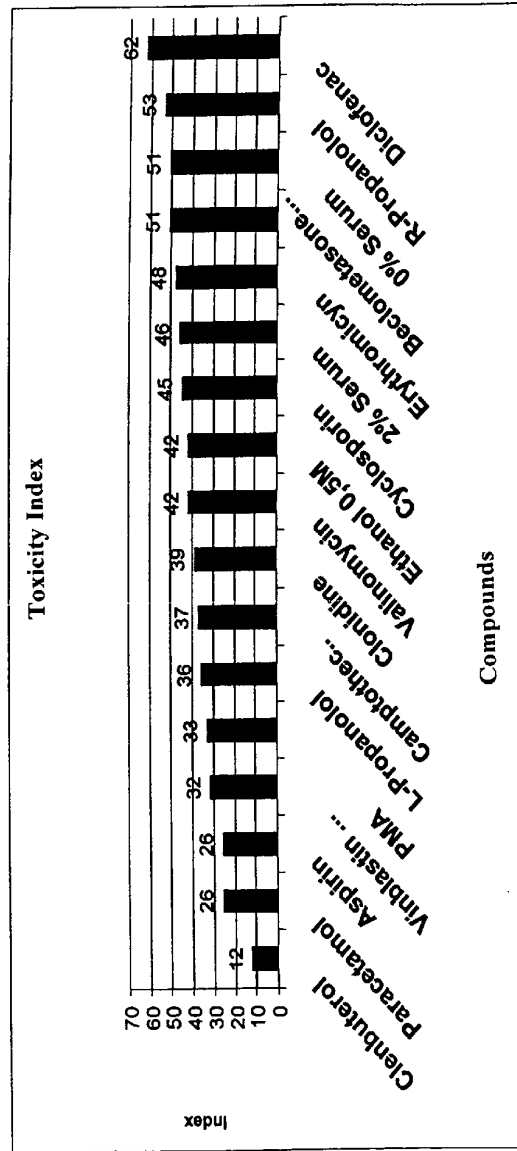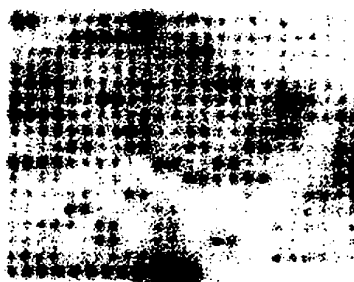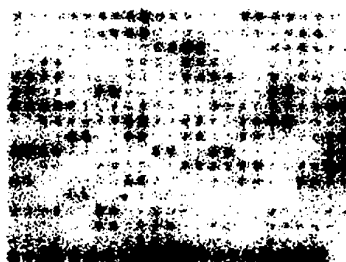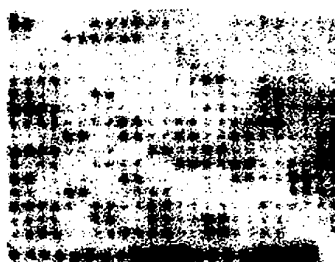with Cyclosporin
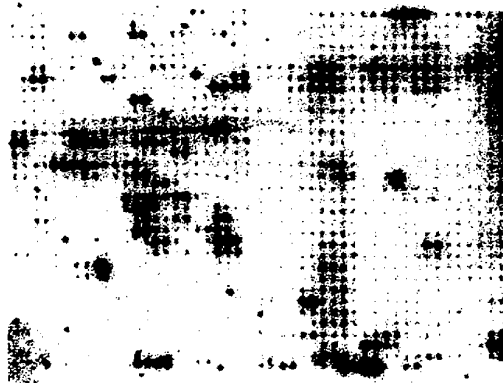
Figure 1

Figure 2

A)
Untreated
Cells

B)
Cells treated
with Clenbuterol

C)
Cells treated
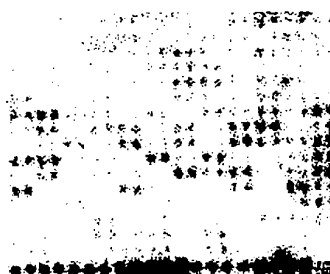with R-propanolol

D)
Cells treated
with DL-propanolol

Figure 3