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Review of current and "omics" methods for assessing the toxicity (genotoxicity, teratogenicity and nephrotoxicity) of herbal medicines and mushrooms

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ABSTRACT

Ethnopharmacological relevance: The increasing use of traditional herbal medicines around the world requires more scientific evidence for their putative harmlessness. To this end, a plethora of methods exist, more or less satisfying. In this post-genome era, recent reviews are however scarce, not only on the use of new "omics" methods (transcriptomics, proteomics, metabonomics) for genotoxicity, teratogenicity, and nephrotoxicity assessment, but also on conventional ones.
Methods: The present work aims (i) to review conventional methods used to assess genotoxicity, teratogenicity and nephrotoxicity of medicinal plants and mushrooms; (ii) to report recent progress in the use of "omics" technologies in this field; (iii) to underline advantages and limitations of promising methods; and lastly (iv) to suggest ways whereby the genotoxicity, teratogenicity, and nephrotoxicity assessment of traditional herbal medicines could be more predictive.
Results: Literature and safety reports show that structural alerts, *in silico* and classical *in vitro* and *in vivo* predictive methods are often used. The current trend to develop "omics" technologies to assess genotoxicity, teratogenicity and nephrotoxicity is promising but most often relies on methods that are still not standardized and validated.
Conclusion: Hence, it is critical that toxicologists in industry, regulatory agencies and academic institutions develop a consensus, based on rigorous methods, about the reliability and interpretation of endpoints. It will also be important to regulate the integration of conventional methods for toxicity assessments with new "omics" technologies.

1. Introduction

Herbal medicines have a long history of use for the prevention and treatment of diseases; their use can be traced back to the first written testimonies of humanity, through antiquity and middle ages till modern time (Williamson, 2003). They have always been part of human culture. According to the World Health Organization (WHO), nearly 80% of the world populations still rely on medicinal herbs for their primary health care. Herbal medicines are then widely used around the world, and increasingly so in Western nations (Winslow and Kroll, 1998; Jordan et al., 2010). For instance, 71% of the population in Canada (IPSOS-Reid, 2005) and 80% in Germany (Thomas et al., 2001) used, in their lifetime, traditional medicines under the wording "*complementary and alternative medicine*". In the United States, about 19% of the adult populations are using herbal medicinal products (Kennedy, 2005; Patwardhan et al., 2005); the herb supplement sales have increased in USA by 23% from 2000 to 2010, reaching a market of more than 5 billion dollars (NBJ, 2011). Europe was estimated to import in 2004 about

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400,000 tons of medicinal plants per annum, with an average market value of US$ 1 billion from Africa and Asia (Wakdikar, 2004). Besides, in developing countries, WHO strongly encourages the use of traditional herbal medicines in primary health care delivery system (Atsamo et al., 2011).

Even for efficient and documented herbal medicinal products, the toxicity can be relatively unexplored; indeed, in contrast with conventional drugs research and development, the toxicity of traditional herbal medicines is not often evaluated (Hartmann et al., 2004; Suter, 2006; Smart et al., 2011). Most of the population however does not pay attention, believing that, if these products have been used so far, they should be devoid of toxicity (Cosyns et al., 1994; Tanaka et al., 1997a,b; Stengel and Jones, 1998; Lord et al., 1999; Luyckx and Naicker, 2008). In the era of post-genome and bioinformatics, innovations in genomics, proteomics and metabonomics (Cho, 2007; Li, 2007; Kang, 2008; Lao et al., 2009) can play an important role in assessment of the genotoxicity, teratogenicity and nephrotoxicity of plant-based medicinal products. However, recent reviews are scarce, not only for post-genomic era but also for conventional methods.

The present work aims to (i) review conventional methods used to assess genotoxicity, teratogenicity and nephrotoxicity and their application to herbal medicinal products; (ii) report recent progress in the use of "omics" technologies to detect these toxicities, with emphasis on the application to herbal products and mushrooms; (iii) analyze the advantages and limitations of each approach; and lastly (iv) suggest ways whereby the genotoxicity, teratogenicity, and nephrotoxicity assessment of plants-based medicinal products could be more predictive. The different methods discussed are presented in Fig. 1.

2. Part A: methods for genotoxicity assessment

Genotoxicity can be due to many physico-chemical agents that result in a wide variety of possible damages to the genetic material, ranging from various DNA adducts to single- and double-strand breakages, DNA–DNA and DNA–protein cross-links or even chromosomal breakage (Ogura et al., 2008; Cavalcanti et al., 2010; Wang et al., 2012). The major challenge in genotoxicity testing resides in developing methods that can reliably and sensibly detect either such a vast array of damages or a general cellular response to genotoxic insult. It is recognized that no single test can detect every genotoxin, therefore the concept of tests battery has been implemented in many regulatory guidelines (Billinton et al., 2008).

In the last decades, numerous damage signalization and repair mechanisms, complex and extremely efficient, have been unravelled, both in prokaryote, eukaryote and mammalian systems (Moller and Wallin, 1998; Bootsma et al., 2001). Although DNA alterations, both in the sequence of nucleotides and in the arrangement of DNA strands, can also arise from mistakes in the repair process, agents interfering with damage signalization and repair mechanism are generally not considered in safety testing. They should however be detected as they could impair indirect genotoxicity by facilitating the activity of genotoxic agents such as direct genotoxins, reactive oxygen species, and radiations (UV, γ) (Johnson and Loo, 2000; Kelly et al., 2001; Azqueta et al., 2009).

2.1. Conventional methods

The Organization for Economic Co-operation and Development (OECD, 2012) and the European Centre for the Validation of Alternative Methods (ECVAM, 2012) have largely investigated the validation of mutagenicity tests and should be refered to for more details.

2.1.1. Detection of phytochemical compounds bearing structural alerts for genotoxicity activity

Structural alerts or "toxicophores" are defined as molecules or molecular functionalities that are associated with toxicity. Their presence in compounds or a molecular structure alerts the investigator to their potential toxicity (Jacobson-Kram and Contrera, 2007). A few well-characterized compounds include (i) 1–2 unsaturated pyrrolizidine alkaloids esters from many *Boraginaceae*, *Asteraceae* and *Fabaceae* (Prakash et al., 1999b; Fang et al., 2011) that exhibit a large variety of genotoxicities, including DNA binding, DNA cross-linking, DNA–protein cross-linking, sister chromatid exchange, and chromosomal aberrations (Roeder, 2000; Fu et al., 2001, 2002a, 2004); (ii) aristolochic acids (AA), nitro-polyaromatic compounds responsible for terminal nephropathies observed after intoxication by many *Aristolochia* species (Fang et al., 2011); a series of studies confirmed that they are genotoxic in both bacterial and mammalian cells, yielding highly persistent and non-repaired DNA adducts; (iii) allylalkoxybenzenes (*e.g.* eugenol, methyleugenol, estragole), safrole (4-allyl-1,2-methylenedioxybenzene) and β-asarone, potentially genotoxic components from some essential oils (Liu et al., 2004; Munerato et al., 2005; Zhang et al., 2005; Smith et al., 2010). The notion of threshold for genotoxic insult is still a matter of heavy debates; consequently such compounds should be proscribed from herbal medicines or at least severely limited.

2.1.1.1. Analytical methods. Analytical methods with suitable detection limits have been developed for the characterization and quantification of many known molecules or molecular functionalities associated with genotoxicity; they are now being implemented in official pharmacopeias. Such methods are based on spectrophotometry (the Ehrlich reagent for pyrrolizidine alkaloids), thin layer chromatography (TLC), gas chromatography/mass spectroscopy (GC/MS) and liquid chromatography/mass spectroscopy (LC/MS and LC/MS/MS) (Fu et al., 2002b, 2007; Zenga et al., 2007; Napoli et al., 2010).

2.1.1.2. In silico methods. These predictive methods generally refer to a computational experiment, mathematical calculation, or scientific analysis of substances data through a computer-based analysis (Valerio-Jr, 2009). Computer models used for genotoxicity prediction fall into three principal categories (Votano et al., 2004): (i) rule-based expert systems such as DEREK that estimates the presence of a DNA-reactive moiety in a given molecule (Greene, 2002); (ii) quantitative structure–activity relationship models (QSAR) such as TOPKAT that uses "electro-topological" descriptors (atom-type, bond-type and group-type E-state) rather than chemical structure to predict mutagenic reactivity with DNA (Wolfgang, 2000; Mattioni et al., 2003; Serra et al., 2003; Votano et al., 2004; Snyder and Smith, 2005); and (iii) three-dimensional computational DNA-docking model to identify molecules capable of non-covalent DNA interaction (Snyder et al., 2004; Snyder and Smith, 2005).

In silico prediction systems are cheaper, rapid, have higher reproducibility, have low compound synthesis requirements, can undergo constant optimization, and have potential to reduce or replace the use of animals (Hofer et al., 2004; Valerio-Jr, 2009). Their limitations are the lack of available toxicity data, inappropriate (simplistic) modeling of some endpoints, and poor domain applicability of models (Cronin, 2002). The application of *in silico* methods to complex mixtures such as herbal extracts is by evidence limited to the detection of known phytochemical compounds bearing known or new structural alerts for genotoxicity activity; they could however help to elucidate which compounds are responsible for a proven effect.

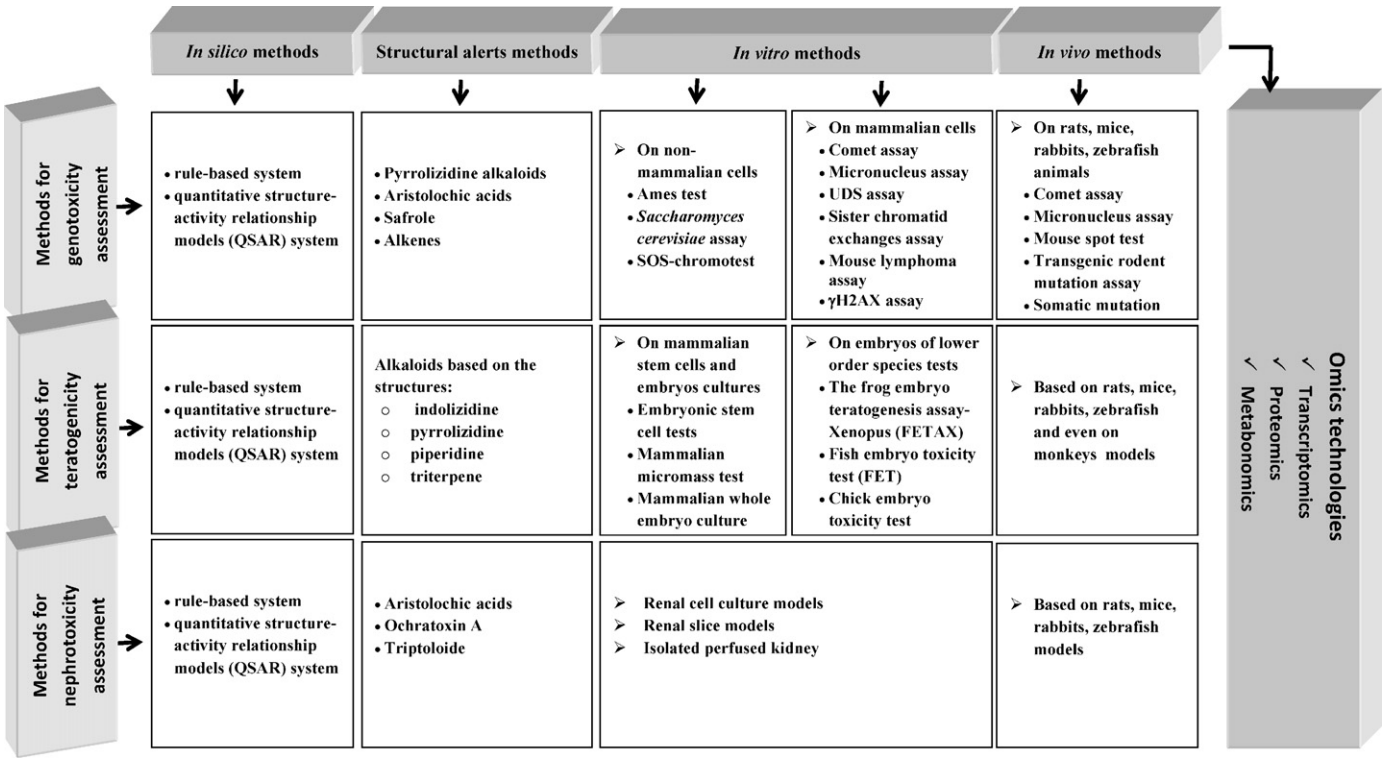

Fig. 1. Comparative graphic of different approaches used by genotoxicity, teratogenicity and nephrotoxicity assessment methods.

2.1.2. In vitro methods for genotoxicity assessment

By evidence, the absence of known phytochemical compounds bearing structural alerts for genotoxic activity in an herbal medicine does not mean the absence of potential genotoxicity. It is highly possible that many genotoxic natural molecules have not been identified yet and, therefore, complementary testing methods have been developed.

2.1.2.1. Investigations on non-mammalian cells. All these tests can be performed both in the absence and in the presence of an exogenous metabolizing system, often a rat liver S9 (microsomal) suspension.

2.1.2.1.1. Prokaryotic organisms: the Ames test. The Ames test, a bacterial reverse mutation assay, is performed with histidine-dependent auxotrophic mutants of *Salmonella typhimurium* (strains TA97, TA98, TA100, TA102, TA1535, TA1537, *etc.*) or tryptophan dependent auxotrophic mutants of *Escherichia coli* (WP2 isogenic strains uvr) (Maron and Ames, 1983). In the presence of a mutagenic agent, a selective pressure, from a medium depleted in the essential amino acid, results in reverse mutations and the growth of colonies that are simply counted. Several different strains of *Salmonella* must be used, because each strain individually assays for a particular type of mutagen (*i.e.*, one strain for base pair substitutions and a separate strain for frameshift mutations) (Ramos et al., 2002; Zhang et al., 2004; Marshall, 2007; Ogura et al., 2008).

2.1.2.1.2. Prokaryotic organisms: tests based on the SOS response. The response of *Escherichia coli* and *Salmonella typhimurium* to genotoxic agents involves the triggering of a complex system of genes known as the "*SOS response*". The SOS-chromotest procedure is based on the *Escherichia coli* PQ37 strain in which the β-galactosidase (β-Gal) gene, *lacZ*, is placed under the control of *sfiA*, one of the SOS genes, through an operon fusion (Quillardet and Hofnung, 1985; Emig et al., 1996; Nair et al., 2000; Nieminen et al., 2002). The Vitotox assay involves genetically modified *Salmonella typhimurium* that harbor a luciferase gene under control of the recN or pr1 promoter. In the presence of a DNA damaging compound, the SOS response leads to β-Gal or luciferase expression, respectively, which is then an indirect measure of the genotoxic activity of a test compound. The bacterial toxicity needs to be assessed, *e.g.* by measurement of alkaline phosphatase (Nieminen et al., 2002). The Vitotox test has a high predictivity for bacterial mutagenicity and the number of false-positive scores due to cytotoxicity is relatively low (Westerink et al., 2009, 2010).

2.1.2.1.3. Eukaryotic organisms: Saccharomyces cerevisiae. The most widely used *Saccharomyces cerevisiae* assay, proposed by Zimmermann et al. in 1975 (Sanchez-Lamar et al., 2002; Nohynek et al., 2004), relies on the diploid D7 strain. When compounds mutate the DNA of this yeast strain, easily scorable phenotypes (color of colonies, growth on particular media) are produced at three separate genomic sites. Additionally, each one of these phenotypic changes specifies a separate type of mutation and repair mechanism (Marshall, 2007). The RadarScreen assay is based on a RAD54 promoter-linked β-Gal reporter assay in yeast; RAD54 is involved in DNA recombination events and repair mechanisms, especially those involving double-stranded DNA breaks during both mitosis and meiosis. Upon DNA damage and β-Gal expression, the substrate d-luciferin-o-β-galactopyranoside liberates luciferin that is luminometrically measured (Westerink et al., 2009). The RadarScreen assay has a high predictivity for clastogenicity; the number of false-positive scores due to cytotoxicity is relatively low (Westerink et al., 2009, 2010).

2.1.2.1.4. Eukaryotic organisms: Aspergillus nidulans. Because of its parasexual cycle, the filamentous fungus *Aspergillus nidulans* constitutes an excellent system for studying mitotic crossing-over, since its cells spend a substantial part of their cell cycle in the G2 phase during the germination period; two copies of each chromosome during that period of the cell cycle significantly favors a mitotic recombination event, visually detected by simple plating tests (Osman et al., 1993; Souza-Júnior et al., 2004).

2.1.2.1.5. Investigations on mammalian cells. There are circumstances where the performance of the above tests does not provide appropriate or sufficient information for the assessment of genotoxicity. The first circumstance is the case of compounds that are excessively toxic to microorganisms (*e.g.* antibiotics, antifungal compounds). It is also the case of compounds thought or known to interfere with the mammalian cell replication system (*e.g.* topoisomerase inhibitors, nucleoside analogues or inhibitors of DNA metabolism) that most likely will not be detected. Guidelines consequently recommend performing additional *in vitro* mammalian cell tests. Different cell lines are used, some of which are metabolically competent to allow detection of genotoxins needing metabolic activation. Alternatively, for non-metabolizing cell lines, microsomal or S9 activation as in the Ames test can be applied.

2.1.2.1.6. In vitro micronucleus assay. The *in vitro* micronucleus assay is considered as a routine screening test for the rapid assessment of chromosomal aberrations (Miller et al., 1998; von der Hude et al., 2000). Micronuclei are acentric chromosomal fragments or whole chromosomes left behind during mitotic cellular division, appearing in the cytoplasm of interphase cells as small additional nuclei (Bolognesi, 2003). Micronuclei induction can result from clastogens (agents that induce chromosomal breaks) or aneugens (agents that induce mainly chromosomal gain/loss) (Fenech, 2000; Kirsch-Volders et al., 2003). Cells treated with test compound are then grown in the presence of cytochalasin-B to prevent the cytoplasmic division after nuclear division, fixed, stained and scored for binucleated and micronucleated cells (Fenech, 2000; Sanchez-Lamar et al., 2002). The detection of micronuclei, manual or, as more recently described, automated (Diaz et al., 2007; Westerink et al., 2011), provides a readily measurable index of chromosome breakage and loss. Although the test is unable to provide a measure of more subtle changes, such as balanced translocations, such a level of detail is in most cases unnecessary (Fenech, 1997).

2.1.2.1.7. Unscheduled DNA synthesis (UDS) assay. The unscheduled DNA synthesis (UDS) assay measures chemical-induced DNA excision repair by detecting labeled thymidine (^{3}H-TdR) incorporation. The induction of DNA repair mechanisms is presumed to have been preceded by DNA damage, indicating the DNA damaging ability of a chemical (Lambert et al., 2005; Bakkali et al., 2008). A core limitation of the UDS assay is its inability to indicate if a xenobiotic is mutagenic; indeed, it provides no information regarding the fidelity of DNA repair and it does not identify DNA lesions handled by mechanisms other than excision repair (Lambert et al., 2005).

2.1.2.1.8. Sister chromatid exchanges (SCE) assay. Various cytomolecular protocols have been used to perform the sister chromatid exchanges (SCE) assay (Djelic et al., 2006; Kaya and Topaktas, 2007; Bakkali et al., 2008; Hseu et al., 2008). This method relies on the differential staining of sister chromatids during replication to visualize reciprocal genetic exchanges between them. Such an exchange arises when, during DNA replication, two sister chromatids break and rejoin with one another (Wilson and Thompson, 2007); this natural mechanism is increased by exposure to genotoxic agents capable of inducing DNA damage (Djelic et al., 2006).

2.1.2.1.9. Mouse lymphoma assay (MLA). This assay exploits mouse lymphoma cells (L5178Y $tk^{+/-}$ 3.7.2C), heterozygous at the thymidine kinase locus (*Tk1*) on chromosome 11. Inactivation of the tk^+ allele ($Tk1^b$) induces trifluorothymidine resistance, allowing easy selection of $tk^{-/-}$ mutants (Clive et al., 1987; Nohynek et al., 2004; Isbrucker et al., 2006). MLA can detect a variety of mutations, including point mutations and small mutations within *Tk1*, losses of $Tk1^b$ or larger deletions including $Tk1^b$. In contrast to the *in vitro* micronucleus test, MLA also detects translocations (Liechty et al., 1998). Most of the substances that are positive in this mammalian gene-mutation test also induce clastogenic effects (Kirkland et al., 2011).

2.1.2.1.10. In vitro comet assay (single-cell gel electrophoresis assay). In this well-established, highly sensitive, rapid, and simple genotoxicity test, isolated cells embedded in agarose are lysed, washed to remove membranes and proteins, briefly electrophoresed, stained and examined under epifluorescence microscopy; strand breaks, coming from either strand breakage or excision repair, result in DNA extending towards the anode in a structure resembling a "comet" (Singh et al., 1988; Speit et al., 2009; Berthelot-Ricou et al., 2011). Depending on experimental conditions, the migrating DNA (comet tail or derived parameters) reflects the amount of single- or double-strand breaks, alkali-labile sites, including incomplete excision repair sites, but also of DNA–DNA and DNA–protein cross-links (Duez et al., 2003; Speit et al., 2005; Speit and Henderson, 2005; Santos et al., 2009; Verschaeve et al., 2010). A broad spectrum of DNA damage can then be detected either by visual classification of comet morphologies ("*visual scoring*") (Ramos et al., 2001; Cavalcanti et al., 2010) or from morphological parameters obtained by image analysis and integration of intensity profiles using in-house or commercially available systems. There are only few limitations of the comet assay with regard to its application and interpretation. Short-lived primary DNA lesions such as single strand breaks, which may undergo rapid DNA repair, could be missed when using inadequate sampling times. Another limitation is that indirect mechanisms related to cytotoxicity (*e.g.* DNA fragmentation in apoptosis) can lead to positive effects (Speit et al., 2005).

2.1.2.1.11. γH2AX assay. The phosphorylation of the carboxyl-tail of a specific variant of the H2A histone protein, H2AX, at Serine 139, to become γH2AX, occurs at sites flanking DNA double-strand breaks (DSBs). The measurement of γH2AX by a labeled monoclonal antibody and microscopy (determination of foci number) or flow cytometry (Muslimovic et al., 2008; Smart et al., 2011) allows detection of potential genotoxic activity (Watters et al., 2009; Smart et al., 2011). The γH2AX assay was found capable of detecting DNA damage at levels 100-fold below the detection limit of the alkaline comet assay (Verschaeve et al., 2010).

2.1.2.2. Advantages and limitations of in vitro genotoxicity assays. It is recognized that *in vitro* genotoxicity assays are extremely useful. Their set-ups are small and use minimal amounts of test substances, allowing low costs, high numbers of replicates, miniaturization and automation; they have generally been validated to detect an impressive number of genotoxic agents and mechanisms. However, these simple cellular models are often thought to be a too reductionist approach. The main limitations of *in vitro* models include the artificial and non-physiological conditions in which the cells are maintained which do not reflect the body temperature, the blood electrolyte concentrations, the extracellular matrix or the extent of cell–cell interactions within tissues. Most cell systems represent only one cell type, often cancerous in origin, with uncertain DNA damage signalization and repair status and possibly further degenerated during maintenance culture. Moreover, culture media are not always homeostatic during experiments (Hartung, 2011).

2.1.3. In vivo methods for genotoxicity assessment

In order to overcome some limitations of the *in vitro* investigations, *in vivo* methods have been also developed, not to replace them, but to complete their information on a whole organism. Despite the mainstream willingness to substitute animal experimentation with *in vitro* models, animal studies remain the core component of toxicity assessment of drugs and plant-based medicinal products. Number of test animals, gender, suitable controls and dose and time parameters are important components of these experiments and are generally specified in the relevant guidelines (Hartung, 2011). At various periods after the treatment, blood

samples are collected by venipuncture (usually from the tail), the animals are sacrificed and organs (liver, kidney, femurs, tibias . . .) are removed for the analysis.

2.1.3.1. In vivo micronucleus assay. Blood sample and bone marrow are collected by venipuncture and removed from the femur, respectively; they are then smeared, stained and scored as described for the *in vitro* assay. As a measure of toxicity of test compounds on bone marrow, the polychromatic erythrocytes: normochromatic erythrocytes ratio is scored; the incidences of micronuclei are also calculated to highlight clastogenic properties (Chen et al., 2001; da Silva et al., 2002; Nohynek et al., 2004). The frequency of micronucleated reticulocytes can also be determined (Leopardi et al., 2005).

2.1.3.2. In vivo unscheduled DNA synthesis (UDS) assay. The *in vivo* UDS is generally evaluated in the hepatocytes of treated animals following the same detection systems as its corresponding *in vitro* model.

2.1.3.3. Mouse spot test. The "mouse spot test", a rapid screening test to detect gene mutations and recombinations in mice somatic cells (Lambert et al., 2005), is based on the observation that genotoxic compounds can induce color spots on the coat of mice exposed *in utero*. The color spots arise when mouse melanoblasts, heterozygous for several recessive coat color mutations, lose a dominant allele through a gene mutation, chromosomal aberration or reciprocal recombination, allowing the recessive gene to be expressed (Lambert et al., 2005; Wahnschaffe et al., 2005).

2.1.3.4. Transgenic rodent (TGR) mutation assay. Transgenic animals carry multiple copies of chromosomally integrated plasmid and phage shuttle vectors that harbor reporter genes to detect, quantify and sequence mutations *in vivo*. The frequency of mutations occurring in the animal is scored by recovering the shuttle vector and analyzing the phenotype of the reporter gene in a bacterial host; molecular analysis of the gene can provide further mechanistic information (Lambert et al., 2005). Some deletions and insertion mutations may however not be detected in phage-based TGR. The test does not involve a large number of animals and a major advantage is that mutations can be evaluated in any tissue; the protocol is reproducible but requires well-trained experts, is not yet automated and the assay cost is superior to most of the other genotoxicity assays (Lambert et al., 2005). Some cell lines have been derived from the *in vivo* TGR models and are similarly used *in vitro* (Ryu et al., 1999; McDiarmid et al., 2002).

2.1.3.5. In vivo comet assay. After treatment and sacrifice of animals, blood lymphocytes and/or cells, dissociated from organs by mincing a small piece into very fine fragments, are treated as per the same protocols as *in vitro* studies (Chiu et al., 2000; da Costa Lopes et al., 2000; Cavalcanti et al., 2010). The *in vivo* comet assay detects low levels of DNA damage, requiring small numbers of cells per sample (Brendler-Schwaab et al., 2005).

2.1.3.6. Somatic mutation and recombination test (SMART). In view of minimizing the number of higher organisms used in toxicological research, a somatic mutations and recombination test (SMART) in the wings of *Drosophila melanogaster* ("*wing-spot test*") has been developed. This test, based on the loss of heterozygosity for two recessive markers (Idaomar et al., 2002; Carmona et al., 2011b), is a tool to evaluate gene mutations, chromosome aberrations and rearrangements related to mitotic recombination (Munerato et al., 2005). Recently, the comet assay has been adapted to be used *in vivo* in *Drosophila melanogaster* (Carmona et al., 2011a,b; Sharma et al., 2011).

2.2. "Omics" technologies

The term "omic" is derived from the Latin suffix "ome" meaning *mass* or *many*. "Omics" studies involve a high number of measurements per endpoint to acquire comprehensive, integrated understanding of biology and to simultaneously identify the

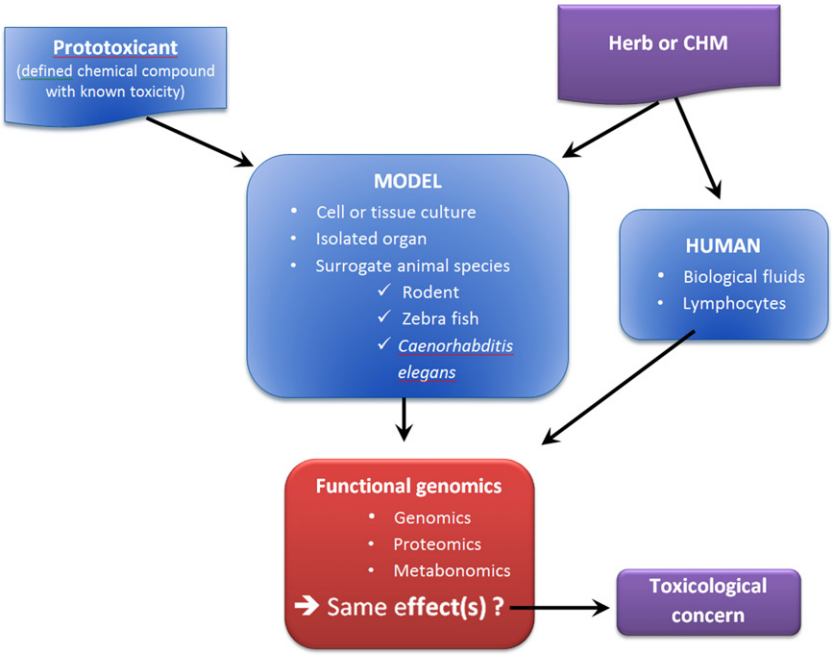

Fig. 2. Testing strategy for "omics" methods. An *in vitro* or *in vivo* model is exposed to a chemically defined toxic with known properties ("prototoxicant"); the response of the model is evaluated by an "omics" method and possible/likely biomarkers of exposure are examined. When the same model is exposed to an unknown compounds mixture or to an herbal extract, a similar pattern of response indicates probable toxicological concern. For traditional medicinal herbs or drugs that are classically used in humans, experimenters additionally may have access to patients body fluids (urine, blood) to investigate for eventual toxicity biomarkers, an important avenue for research but also for patient's monitoring.

different factors (*e.g.*, genes, RNA, proteins and metabolites) rather than each of those individually (Lay et al., 2006). "Toxicogenomics" (Borner et al., 2011) aim to study the interaction between the structure and activity of the genome and the adverse biological effects of exogenous agents (Bishop et al., 2001). This discipline is based on the concept that the toxic effects of xenobiotics on biological systems are generally reflected at cellular level by their impact on the expression of genes (transcriptomics) and proteins (proteomics) and on the production of small metabolite (metabonomics) (Fig. 2) (Aardema and MacGregor, 2002; Marchant, 2002; Heijne et al., 2005; Marques et al., 2011; Borner et al., 2011).

2.2.1. Transcriptomics

Transcriptomics analyze the expression level of genes by measuring the transcriptome, the genome-wide mRNA expression (Davies, 2010; Wilson et al., in press). Transcriptomics uses high density and/or high-throughput methods of assessing mRNA expression.

2.2.1.1. Microarrays and qrt-PCR. Microarrays ("DNA microarrays", "DNA arrays", "DNA chips", "biochips", "gene chips"), the most common approach used for gene expression profiling (Davies, 2010), are generated by immobilizing a high number of oligonucleotides on an extremely small surface (up to 200,000 spots/cm^2). Based on the target sequences, significant changes of mRNA can be estimated for thousands of genes (Eisenbrand et al., 2002; Oberemm et al., 2005; Ulrich-Merzenich et al., 2007b). Specialized sub-sets of gene expression changes and quantitative real-time reverse transcriptase-polymerase chain reactions (qrt-PCR)-based approaches that focus on specific genes have also been developed. The latter is a highly recommended confirmatory tool for quantifying gene expression with improvements in sensitivity and specificity (Wilson et al., in press).

2.2.1.2. Open systems. Other technologies such as serial analysis of gene expression (SAGE) (Velculescu et al., 1995), massively parallel signature sequencing (MPSS) (Brenner et al., 2000) and total gene expression analysis (TOGA) (Sutcliffe et al., 2000) are also successfully used to detect changes in transcriptomes. In contrast to microarray technology (which can only measure transcript abundances with pre-selected, known probe sequence), these approaches are "open systems" and thus suited for gene discovery; they offer linear gene expression quantification over a wide dynamic range (Kussmann et al., 2006).

2.2.1.3. Specific genes targeting. Published microarrays genomics studies have been quite inconclusive for genotoxicity prediction; they nevertheless pointed to some genes, *GADD45a* (Hastwell et al., 2006), *p53R2* (Ohno et al., 2005), *Ephx1*, *Btg2*, *Cbr3* and *Perp* (Hendriks et al., 2011) of which a robust induction of expression was noted for a series of genotoxins with apparently high sensitivity and specificity. There is a considerable interest in genes involved in tissue development, cell death, cell-to-cell signaling, cell cycle and cellular growth, proliferation, DNA damage signaling and DNA repair (Ellinger-Ziegelbauer et al., 2009; Jordan et al., 2010).

2.2.1.3.1. Response to p53 activation. The p53 tumor-suppressor protein plays an important role by regulating the expression of a series of genes that promote genomic stability, DNA repair, cell cycle arrest and induction of apoptosis in the response to DNA damage (Levine, 1997; Nakamura, 2004). These genes include *p53R2* (subunit of ribonucleotide reductase), *CDKN1A* (cyclin dependent kinase inhibitor 1A) and *GADD45a* (Growth arrest and DNA damage) (Corn and El-Deiry, 2007; Lu et al., 2009).

- *p53R2* is activated by γ-ray, UV-irradiation and several genotoxic compounds (Tanaka et al., 2000; Guittet et al., 2001; Xue et al., 2003). A luciferase reporter plasmid dependent on three tandem repeat sequences of the p53-binding site derived from the *p53R2* gene has been developed for genotoxicity testing (Ohno et al., 2005). This high-throughput assay is available for wild-type p53 human cell lines, is easy to conduct, requires only a small number of test samples and gives few false-positive data (Ohno et al., 2008).
- *GADD45a* plays an important role in cell cycle control, DNA repair mechanisms and signal transduction (Zhan, 2005). Other cellular signaling pathways, including BRCA1, c-MYC and NF-κB are implicated in *GADD45a* induction (Harkin et al., 1999; Barsyte-Lovejoy et al., 2004; Zheng et al., 2005). *GADD45a*-dependent reporter assays, based on green fluorescent protein (GreenScreen HC) or luciferase (BlueScreen HC), are commercially available for testing genotoxicity response (Hastwell et al., 2006; Adler et al., 2011).

2.2.1.3.2. Other pathways. Genes involved in cell cycle (*CDKN1A*, *GADD45*, *Cyclin E*), apoptosis (*BAX, BCL-XL*), DNA repair (*XPC*, *DDB2*, *GADD45*) and various physiological processes (*FOS/JUN*, *MDM2*, *FRA-1 IL-8*, *HSP70*), which are found to be altered by ionizing radiation could be predictive biomarkers of genotoxicity (Amundson et al., 2003; Snyder and Morgan, 2004).

2.2.1.4. Limitations of transcriptomics. Changes in genes expression levels may predict major changes in the proteins profiles in cells, tissues or organisms but there are cases where a functional protein is not produced despite gene expression; and so, changes in the transcriptome do not necessarily reflect a change in the profile of "end-products" (Davies, 2010).

2.2.2. Proteomics

Proteomics is the study of a broad spectrum of proteins within a cell or tissue, including proteins expression, structural status, functional states and their interactions with other cellular components (Kussmann et al., 2006; Wilson et al., in press). Key technologies rely on two-dimensional gel electrophoresis coupled to mass spectrometry (Merrick and Madenspacher, 2005; Ulrich-Merzenich et al., 2007b), on antibody microarrays and on LC–MS–MS of proteins fragments, a technique known as "*shotgun proteomics*" (Wolters et al., 2001) and its specific platform variations, called ICAT (isotope coding affinity tags) and MuDPIT (multi-dimensional protein identification technology) (Yates, 2000). Affinity chromatography, fluorescence resonance energy transfer and surface plasmon resonance are also used to identify protein–protein or protein–DNA interactions. X-ray tomography is used to determine the location of proteins or protein complexes in labeled cells (Ulrich-Merzenich et al., 2007b).

Techniques are available but a large database of proteomics 'fingerprint' is still needed for compounds of known toxicity; once available for a series of known carcinogens, it will be possible to identify changes in biochemical pathways and to assess the toxicity of unknown compounds. The products of the genes identified so far in transcriptomics studies are probably promising candidates as proteomics markers.

2.2.3. Metabonomics

Toxicometabonomics[1] concern the analysis, either in organs, blood or urine, of metabolites and metabolic pathways modifications that follow a toxic insult (Ulrich-Merzenich et al., 2007b).

[1] *Metabolomics* refer to the study of LMW molecules within cells, whereas *metabonomics* refer to a more systemic and complex change in tissues and body fluids (Ekins et al., 2005).

This implies the quantitative and qualitative study of a wide range of low molecular weight (LMW) molecules produced as the net result of cellular functions (Lindon et al., 2004; van Ravenzwaay et al., 2007; Youns et al., 2010) by nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared and near-infrared spectroscopy or mass spectrometry (MS). The latter technique generally requires pre-separation of the metabolic components by gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) (Tang and Wang, 2006; Lindon and Nicholson, 2008; Arita, 2009). So far metabonomics have been scarcely applied to genotoxicity studies; major applications concern the urinary profiling of damaged bases excreted upon DNA excision repair and the search for activated metabolites of precarcinogens (Kirkland et al., 2005).

2.3. Applications to herbal medicines

As genotoxicity testing aims to yield information on all types of damage, including gene mutations, structural chromosome aberrations (clastogenicity) and numerical chromosome aberrations (aneugenicity), standard test batteries have been developed (Kirkland et al., 2005) which include the assessment, with and without metabolic activation, of (i) mutagenicity in a bacterial reverse mutation test (Ames test); and (ii) genotoxicity in mammalian cells *in vitro* and/or *in vivo* (ICH, 2008). These tests have been developed for single chemicals and applying them to herbs has been quite a challenge. The current EU guidelines for herbal products (EMEA, 2007) define the Ames test as the primary endpoint that, if negative, accepts the drug as probably "non-genotoxic". This is not entirely satisfying however and there are still heavy debates on the topic (EMEA, 2008); indeed (i) the Ames test does not detect every genotoxic insult; and (ii) some common compounds, including flavonoids, yield very positive Ames tests but are not carcinogens. Given the number of herbal products on the market and relatively low budgets available for research, there are still relatively few herbs for which safety assessment according to the current guidelines has been done. Nonetheless, some herbal products and their secondary metabolites were assessed for genotoxicity by various techniques. The Ames test has been widely used to assess the mutagenicity of herbal products, including for example extracts from *Calendula officinalis* L., *Echinodorus macrophyllus* (Kunth) Micheli, *Mouriri pusa* Gardner, *Phyllantus orbicularis* Kunth, *Punica granatum* L., *Parthenium hysterophorus* L. and a green tea catechins preparation (Ramos et al., 1998, 2001; Sanchez-Lamar et al., 2002; Santos et al., 2002; Nohynek et al., 2004). Among these extracts, *Punica granatum* whole fruit extract and the enriched fractions of flavonoids and tannins from *Mouriri pusa* were found to give a positive genotoxic test. The genotoxicity of Copaiba oil (*Copaifera langsdorffii* Desf.) was demonstrated by *in vitro* micronucleus and comet assays (Rao et al., 2006). The clinical toxicity and chemical identity of Guan MuTong was critically assessed with LC–MS confirmation (Cheung et al., 2006). Metabonomics techniques have already been used in the toxicity studies of Guan Mutong (*Aristolochia manshuriensis* Kom.) (Zhao et al., 2006a) and its toxic component aristolochic acid (Jia et al., 2006); aristolochic acid I was suggested to possess genotoxic potency also by QSAR modeling (Hashimoto et al., 1999; Arvidson et al., 2008; Chen et al., 2011b). In the *in vitro* comet assay, artesunate, a semisynthetic derivative from artemisinin (*Artemisia annua L.*) induced DNA breakage in a dose-dependent manner (Efferth et al., 2008). The transgenic rodent mutation assay has been used to assess the genotoxicity of phytochemicals such as riddelliine (pyrrolizidine alkaloid) (Ulrich-Merzenich et al., 2007a).

There is certainly a need for the development of validated methods to rapidly pinpoint indicators of genotoxicity that yield warning signals and indicate which drugs need further assessment through a complete test battery.

2.4. Applications to mushrooms

Whereas many mushrooms were shown to be non-genotoxic, a number of investigations reported the mutagenic effects of mushroom extracts (Sugimura, 2000). An example is agaritine from *Agaricus bisporus* (J.E. Lange) Imbach, which is mutagenic, but not genotoxic (Pool-Zobel et al., 1990). The carcinogenic, mutagenic and teratogenic activity of 11 hydrazine analogues and diazonium ions derived from 22 mushroom species have been described (Toth, 1991). Remarkably, many of these mushrooms are considered to be edible. In several cases, even protective effects of mushrooms towards other genotoxic compounds have been observed. For example, the culinary-medicinal Royal Sun Agaricus (*Agaricus brasiliensis* S. Wasser et al.) revealed protective properties towards methyl methane sulfonate (MMS) induced mutagenicity and genotoxity in *Drosophila melanogaster* (Savic et al., 2011); the *Agaricus blazei* Murrill mushroom protected from cyclophosphamide-induced genotoxicity (Delmanto et al., 2001).

Despite reports in the literature on lacking genotoxicity potential of mushrooms, the potential hazard should not be underestimated. Mutagenicity and genotoxicity are frequently linked to each other; it cannot be ruled out that a lack of genotoxic data is due to technical reasons and that, with the advent of novel and advanced techniques, the genotoxicity of more mushroom species will be unraveled.

3. Part B: methods for teratogenicity assessment

The term "*teratogens*" refers to toxins that can lead to fetal death or developmental abnormality; teratogenicity supposes a capacity of a chemical to cross the placental barrier in sufficient concentration to be active at a specific time in gestation (Wilson, 1977; Keeler, 1984). The baseline birth defects rate is about 6% (Christianson et al., 2006) but varies considerably (2–8%) with respect to type of defect, time, place, and other demographic, genetic, and environmental factors (NRC, 2000; Levy et al., 2011); it is not known if this baseline rate is a normal frequency of errors in the highly complex developmental process or the consequence of as yet unidentified environmental or dietary factors. Between 1 and 3% of all birth defects are suggested to be attributable to chemicals and drugs, but the figure is a rough estimation (Stummann et al., 2008). Major identified teratogenic mechanisms rely on folate antagonism, neural crest cell disruption, endocrine disruption, oxidative stress, vascular disruption and specific receptor- or enzyme-mediated teratogenesis (Van et al., 2010). Although the literature details copious lists of herbs that are supposedly contraindicated in pregnancy (Lather et al., 2011), most herbal foods and medicines have never been assessed and may contribute to the supposedly baseline birth defect rate. Table 1 compares the major methods developed for teratogenicity assessment.

3.1. Conventional methods

The Organization for Economic Co-operation and Development (OECD, 2012) and the European Centre for the Validation of Alternative Methods (ECVAM, 2012) have largely investigated the validation of teratogenicity tests; the FP6 European project Reprotec has investigated possible strategies to cover the entire mammalian reproductive cycle, resulting in a series of published works that should be consulted for more details (Reprotec, 2012).

Table 1
Comparison of methods for teratogenicity assessment.

Test	Principle	Specificity/Sensitivity	Examples of tested herbs or natural compounds
***In vitro* methods for teratogenicity assessment**			
Methods based on mammalian stem cells and embryos			
Embryonic stem cell tests (ESC)	Determination and evaluation of three endpoints (cytotoxicity assays on both ESCs and 3T3 fibroblasts; ECS differentiation inhibition assay)	Good reproducibility; overall accuracy of 78% (Genschow et al., 2004) Validation data published (Seiler and Spielmann, 2011)	Epimedium sp (the flavonol icarriin stimulates ESC differentiation in cardiomyocytes) (Zhu et al., 2005) *Boehmeria nivea* (L.) Gaud. (non embryotoxic) (Tian et al., 2011a,b)
Mammalian micromass assay (MM)	Cells from the rat limb bud (day 14 of gestation) are tested for differentiation into chondrocytes. Evaluation with a chondrocyte specific dye	Overall accuracy of 70% (Genschow et al., 2002)	*Foeniculum vulgare* L. (no teratogenicity) (Ostad et al., 2004)
Mammalian whole embryo culture test (WEC)	Embryos are isolated on day 10 of gestation and cultured for 48 h; morphological, developmental, functional and growth parameters are measured	Overall accuracy of 80% (Genschow et al., 2002)	Dihydroartemisinin primarily affects primitive red blood cells, causing subsequent tissue damage and dysmorphogenesis (Longo et al., 2006)
Methods based on embryos of lower order species			
Frog embryo teratogenesis assay – *Xenopus* (FETAX)	During the first 96 h *Xenopus* embryos development parallels many of the major processes of human organogenesis (Measurement of mortality, malformation, and growth inhibition)	Overall accuracy in predicting teratogenic potential has been claimed to be 79–83% (Spielmann, 2005) The test is considered not sufficiently validated or optimized for regulatory applications (ICCVAM, 2000)	Teratogenicity shown for a glycoalkaloid from *Solanum tuberosum* (α-chaconine) (Friedman et al., 1991) and artemisinin (Efferth et al., 2008; Longo et al., 2008)
Fish embryo toxicity test (FET)	Zebrafish embryos are observed for 48 h for lethal and sublethal endpoints	75% success rate in identifying nonteratogenic compounds; 100% success rate in identifying teratogenic compounds (Ton et al., 2006) The FET has been standardized at the international level in 2007 (ISO 15088)	Flavonoids (Jones et al., 1964), delta-9-tetrahydrocannabinol (Thomas, 1975) and arecoline (Chang et al., 2004)
***In vivo* methods for teratogenicity assessment**			
Guidelines (OECD, 2012)	Prenatal developmental toxicity study Two generation reproduction toxicity study Reproductive/developmental toxicity screening test Repeated dose toxicity study combined with the reproductive/developmental toxicity screening test	OECD test guideline TG 414 OECD test guideline TG 416 OECD test guideline TG 421 OECD test guideline TG 422	
Mice	Post-natal growth and viability	Correct classification of 83% of tested chemicals (Chernoff and Kavlock, 1982; Kavlock et al., 1987)	Foetal and maternal adverse effects: *Psoralea corylifolia* L., *Ligusticum chuanxiong* Hort., *Scutellaria baicalensis* Georgi (Tian, 2009) No adverse effect: *Boehmeria nivea* (L.) Gaud. ( ) Tests of combinations indicate protective interactions (Tian, 2009) *Panax ginseng* C.A. Meyer caused fetal gross malformation (El-Ashmaoui et al., 2003)

3.1.1. Characterization of compounds bearing structural alerts for teratogenic effects

Epidemiological and experimental studies have shown that some medicinal plants (Lather et al., 2011) and their metabolites exert teratogenic effects, notably alkaloids based on the structures (i) *indolizidine* (swainsonine induced vascular resistance, vasoconstriction and generalized stunting in the fetus of sheep and cattle (Lather et al., 2011)); (ii) *pyrrolizidine* (teratogens and abortifacients (Prakash et al., 1999a)); (iii) *piperidine* (coniine, γ-coniceine, anatabine and perhaps anabaseine caused congenital contracture-type skeletal malformations and cleft palates, restricted fetal movement, arthrogrypotic limb deformities in calves); and (iv) *triterpene* (mammalian ingestion of jervane, solanidane and spirosolane steroidal alkaloids produced craniofacial congenital malformations in offspring upon administration during the primitive streak/neural plate developmental phase) (Gaffield and Keeler, 1994); cyclopamine, veratramine, veratrosine, and germine exerted teratogenic effects (Schep et al., 2006; Molyneux et al., 2007; Cong et al., 2007, 2008). Ginsenoside Rb1 (triterpenic saponine), extracted from Ginseng (a commonly used worldwide herbal medicine) exerts direct teratogenic effects on rat embryos (Chan et al., 2003).

3.1.1.1. Analytical methods. The detection of such compounds in plant-based medicinal products could predict potential teratogenicity. The analytical methods used to identify structural alerts are similar as for genotoxins and include spectrophotometry, TLC, GC/MS and LC/MS–MS (Fu et al., 2002b, 2007; Napoli et al., 2010).

3.1.1.2. In silico methods. The tools used are the same as for genotoxicity assessment, with similar limitations to their applicability to complex mixtures such as herbal extracts. These include QSAR and expert systems; the previously mentioned TOPKAT and DEREK systems have long been used to analyze the teratogenic effects of xenobiotics (Dearden, 2003).

3.1.2. In vitro methods for teratogenicity assessment

3.1.2.1. Methods based on mammalian stem cells and embryos.

3.1.2.1.1. Embryonic stem cell tests (ESC tests or EST). The potential of embryonic stem cells (ESC) to differentiate into all cell types of the mammalian organism (*pluripotency*) provides an important tool to assess adverse effects on their differentiation, effects that might be relevant for *in vivo* embryotoxicity. Taking into account that general cytotoxicity is a critical parameter for identifying embryotoxicants (Stummann et al., 2008), ESC tests are based on three endpoints, *i.e.* differentiation inhibition of ESC and cytotoxicity (often by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay) of both ESC and fibroblasts (Balls and Hellsten, 2002; Stummann et al., 2008). ESC tests differ in their readouts but also in the differentiated target cells (zur Nieden et al., 2004; Peters et al., 2008); neural cells (Stummann et al., 2009; Theunissen et al., 2010), cardiomyocytes (Buesen et al., 2009), skeletal cells (zur Nieden et al., 2004, 2010; Stummann et al., 2009) and endothelial cells (Fukushima et al., 2010) are most often investigated. The *in vitro* differentiation into cardiomyocytes has been extensively used due to the easy visual identification of contracting cells in differentiated ESC cultures (van Dartel et al., 2009). Briefly, ESCs are cultivated in hanging drops in cell culture medium without LIF (leukemia inhibitory factor) to induce the formation of cell multicellular aggregates ("*embryoid bodies*"); these are further cultured in suspension until day 3, plated on day 5 and assessed by light microscopy on day 10 for the number of embryoid bodies containing beating areas, normalizing *versus* a negative control (differentiation level in control must be over 87.5%) (Stummann et al., 2008). Human ESCs (derived from the inner cell mass of blastocysts) are generally used (Thomson et al., 1998); being more specific to the human response than cells from surrogate species (*e.g.* rat, mouse) they may generate more reliable *in vitro* prediction endpoints (West et al., 2010). Nevertheless the use of non-human embryonic stem cells has been approved by the European Centre for the Validation of Alternative Methods (ECVAM) (Genschow et al., 2002). The limitations of ESC tests are the relatively long culture duration, the subjective and laborious endpoint scoring and the so far limited characterization of its applicability domain and predictability (Marx-Stoelting et al., 2009).

3.1.2.1.2. Mammalian micromass test (MM test). This test is based on the ability of a xenobiotic to inhibit differentiation of embryonic tissue cells in the absence of cell death; the test is supposed to reflect an *in vivo* teratogenic potential (Hansen and Abbott, 2009). A limb bud or the cephalic tissue of mid-organogenesis rat embryo (Adler et al., 2011) is dissociated into a single cells suspension. These cells, placed into culture in small volumes at high density, replicate, migrate, re-aggregate, and differentiate into a specific cell type, forming numerous small foci of differentiating cells without additional stimulation (Flint, 1993; Adler et al., 2011). The cell viability and differentiation are measured by staining with neutral red and a stain specific of chondrocytes or neurons, respectively (Brown et al., 1995). The test based on limb buds is an attractive teratogenic model as it reproduces cartilage histogenesis, a fundamental step in skeleton morphogenesis, and the protocol has been validated by ECVAM (Genschow et al., 2002).

3.1.2.1.3. Mammalian whole embryo culture test (WEC tests). For the post-implantation whole embryo culture (WEC) test, mammalian (rat or mouse) embryos are maintained in culture for short periods throughout the phase from fertilization to the end of organogenesis. This allows probing major aspects of organogenesis, including heart development, closure of the neural tube, development of ear and eye, brachial bars and limb buds; disturbance during this period may lead to general retardation of growth and development or to specific malformations in one or several organs (Brown et al., 1995; Spielmann, 2005). Drug-metabolizing enzymes can be incorporated and concentrations of tested compounds and metabolites can easily be monitored in the culture medium and in embryonic tissues (Bechter et al., 1991). Mouse or rat embryos with 2–5 somites are explanted from the uterus, cultured (Piersma et al., 2004; Paniagua-Castro et al., 2008; de Jong et al., 2011) and exposed to various concentrations of the tested compounds. The morphology of the embryos is carefully assessed and a series of endpoints are scored, including the yolk sac diameter, crown-rump length and head length. Total embryonic protein increases logarithmically with embryonic age and is considered to be the most suitable measure of embryonic growth (Brown and Fabro, 1981; Paniagua-Castro et al., 2008; Piersma et al., 2008). The protocol has been validated by ECVAM (Genschow et al., 2002).

3.1.2.1.4. Comparison of tests on mammalian material. Studies of 20 compounds in a test panel reported that the WEC test shows the best concordance between *in vivo* classification and *in vitro* test results with 80% correct classifications *versus* 78% for the ESC test and 70% for the mammalian micromass test (Genschow et al., 2002). The WEC model mirrors the complexity of the embryogenesis closely, although it is laborious, animal consuming and limited to a specific window of embryonic development (Stummann et al., 2008); it allows the objective quantification of both growth and differentiation, and consequently differences between general toxicity and teratogenic effects can be seen (Hewitt et al., 2005). The MM test is much faster, but includes only differentiation of chondrocytes as toxicological endpoint (Stummann et al., 2008). The ESC test is presently the only *in vitro* test based on a cell line that has undergone a formal validation trial (Bremer et al., 2005; Stummann et al., 2008). However, all tests require the sacrifice of pregnant mammals for harvesting early embryos (Selderslaghs et al., 2009).

3.1.2.2. Embryos of lower order species test. According to regulatory guidelines, developing drugs for administration to pregnant women must be tested for teratogenic potential in a rodent and non-rodent mammalian species (Van den Bulck et al., 2011). However, low vertebrate animal models (anuran amphibian, zebrafish, chick) are increasingly proposed as alternatives to mammals to assess teratogenicity of xenobiotics. One of the reasons is that embryogenesis is highly conserved across amphibians and mammals, with similarities at the phylotypic stage at both morphological and molecular levels (Di Renzo et al., 2011). Furthermore, use of lower order species is in line with the current trend of applying the 3Rs principle (refinement, reduction, replacement) in reducing the number of animals necessary for an evaluation (Van den Bulck et al., 2011).

3.1.2.2.1. The frog embryo teratogenesis assay-Xenopus (FETAX). *Xenopus laevis* is one of the most used models for vertebrate experimental embryology due to its aquatic development, large eggs, easily manipulated embryos, low cost, reliability and reproducibility (Richards and Cole, 2006). It has been suggested as an alternative screening model for developmental toxicity purposes (Mouche et al., 2011). *Xenopus tropicalis*, highly related to *Xenopus laevis*, presents the advantages of a smaller size and shorter life cycle (Shi et al., 2010). Breeding is induced by subcutaneous injection

of human chorionic gonadotrophin in the dorsal lymph sac of both males and females. Adults are removed on the second morning after the injection, the embryos are harvested without removing their jelly coats, cultured and exposed to chemicals. At the end of the test, embryos are evaluated for mortality (absence of heartbeat) and the surviving embryos are anaesthetized and fixed with formalin. Examination under a dissecting microscope allows measurement of body length and assessment of malformations number and types (Shi et al., 2010; Di Renzo et al., 2011). This test has been evaluated by the U.S. Interagency Coordinating Committee on the validation of alternative methods who however concluded that FETAX was not sufficiently validated or optimized for regulatory applications (ICCVAM). The assay is limited by the aqueous solubility of test substances; its overall accuracy in predicting teratogenic potential has been claimed to be 79–83% (Selderslaghs et al., 2009).

3.1.2.2.2. Fish embryo toxicity test (FET). The vast majority of information to support the use and interpretation of the FET has been generated on the zebrafish, *Danio rerio* (Lammer et al., 2009; Selderslaghs et al., 2009; Van den Bulck et al., 2011). Less robust data sets exist for other species, particularly those of interest to Asia (*Oryzias latipes*) and North America (*Pimephales promelas*) (Braunbeck et al., 2005).

The zebrafish, a small freshwater fish (3 cm) offers several advantages for teratogenicity assessment including economic husbandry requirements, high fecundity and rapid *ex utero* development (Hill et al., 2005; Avanesian et al., 2009). The eggs remain transparent from fertilization up to and beyond pharyngulation when the tissues become dense and pigmentation is initiated. This allows observation of the main morphological changes during earlier developmental stages. Moreover, zebrafish embryos that are malformed, lack organs, or display organ dysfunction can usually survive well beyond the time at which those organs normally start to function in healthy individuals (Hill et al., 2005). Zebrafish embryos can activate proteratogenic/procarcinogenic substances without any addition of exogenous metabolic activation systems as they present phase I enzyme activities at very early stages of development (Weigt et al., 2011). Endpoints used for assessing the effects of tested compound include embryo mortality, yolk sac edema, tail malformation, changes in hatching rates and deficiencies in gastrulation, somite formation, head formation, heartbeat and pigmentation (Oliveira et al., 2009; Van den Bulck et al., 2011). Zebrafish embryo toxicity test presents a 75% success rate in identifying nonteratogenic compounds and a 100% success rate in identifying teratogenic compounds (Ton et al., 2006).

3.1.2.2.3. Chick embryo toxicity test. Avian embryos have rarely been used for embryotoxicity testing. The results obtained on chicks were disappointing as this test cannot distinguish general toxicity from specific developmental effects (Jelinek et al., 1985; Brown et al., 1995).

3.1.2.3. Utility of in vitro methods. Although *in vitro* models provide valuable teratogenicity data, they cover all of the aspects of prenatal development; they also lack a placenta, which offers some protection for the developing organism in mammals, and so cannot determine the real fetal exposure (Avanesian et al., 2009; Van den Bulck et al., 2011). None of these *in vitro* methods can currently be regarded as a full replacement for existing *in vivo* studies; however they can be considered as a pre-screen strategy to prioritize xenobiotics for *in vivo* assessment, reducing and optimizing animal use in teratogenicity evaluation.

3.1.3. In vivo methods for teratogenicity assessment

In vivo teratogenicity assessments are generally based on studies in rats, mice, rabbits, and even in monkeys. Pregnant females receive the tested compounds and throughout the experimental period are weighed and examined for signs of toxicity. At different time points, females are euthanized and a series of parameters in the uterus (including fetus), ovaries and placenta are examined, including number of *corpora lutea*, number of implantation sites, number of early and late resorption, number of live and dead fetuses, sex of the fetuses, number of malformed fetuses and types of external abnormalities (exencephaly, cleft palate, abdominal hernia, polydactly, open eyelid, *etc.*), weight of ovaries, weight of uterus containing placentas and fetuses, weight of empty uterus, weight of fetuses, weight of the placentas, gross evaluation of the placentas. Half of the fetuses are examined for soft tissue abnormalities and the remaining half for skeletal abnormalities (Favero et al., 2005; Tian et al., 2005; Wangikar et al., 2005; Griffiths et al., 2007). Cynomolgus monkeys (*Macaca fascicularis*) have been sometimes used in the teratogenicity assessment of xenobiotics (Hendrickx et al., 2000; Ema et al., 2007). Indeed, earlier studies demonstrated that embryonic development in this non-human primate very closely parallels that of human embryos (Hummler et al., 1990). The principle of teratogenicity assessment is identical except terminal cesarean sectioning is performed under anesthesia to recover fetuses (Ema et al., 2007).

The use of more than one species for testing is widely regarded as necessary because it increases chance to detect agents that may be teratogenic for humans. Typical guidelines recommend using two species, mostly rat and rabbit. The former is used because toxicity data are commonly obtained with this species; the latter because it has somewhat different placentation and pregnancy physiology than rodents, but also for historical reasons. Rabbits, but not rats, showed limb-reduction and deformities when they received thalidomide (Barlow et al., 2002).

3.2. Current "omics" approaches for predicting teratogenicity

Since toxicological testing with animals is expensive and time consuming and raises serious ethical issues, there is an urgent need to develop alternative or complementary models to support the 3Rs principle in animal testing. Reproductive medicine is now entering the exciting era of the "omics" (Scott and Treff, 2010); the use of these techniques in teratology is still in its infancy but their advent has added further impetus to the development of alternatives to *in vivo* toxicity testing (Bhogal et al., 2005; Luijten et al., 2010). At least 17 signaling pathways have been described during fetal development with their specific periods of activity; six of these are known to be crucial for early development in most animals, while four are more used in late development (NRC, 2000; Stummann et al., 2008). Study of these pathways important steps by "omics" methods in animal or cell models may give important clues to teratogenic agents and allow developing high-throughput tests for teratogenicity alert signals.

3.2.1. Transcriptomics

The incorporation of transcriptomics in established tests is expected to provide more informative and improved endpoints as compared with morphology, allowing early detection of differentiation modulation (Kultima et al., 2010; van Dartel et al., 2010; van Dartel and Piersma, 2011).

3.2.1.1. Application to embryonic stem cell tests (ESC tests). The very first studies that evaluated gene expression regulation as a consequence of xenobiotics exposure in the ESC test were based on the selection of well-known developmental markers (*Myh6* and *Myh7*) and showed that endpoint objectivation could be obtained through gene expression analysis (Bigot et al., 1999). In order to better understand the molecular mechanisms of teratogenesis, embryonic zebrafish cells were exposed to xenobiotics, and changes in

transcript expression were analyzed by microarrays (Fent and Sumpter, 2011).

3.2.1.2. Application to mammalian whole embryo culture tests (WEC tests). Late occurring malformations (*e.g.* cleft palate) cannot be detected morphologically in this test due to the limited period of observation (48 h), but gene expression changes occurring during this period may be early predictors for malformations that become morphologically visible only later in embryonic/fetal genesis. Transcriptomics have successfully employed for monitoring early responses in the *Hoxb1* gene expression in whole embryonic culture after exposure to the embryotoxicant retinoic acid (RA) (Morriss-Kay et al., 1991; Zhang et al., 2003; Menegola et al., 2004). Afterwards, the expression levels of 14 known RA-responsive genes could be measured in whole embryo by real-time quantitative PCR; these genes (*A2m*, *Afp*, *Bmp4*, *Cdkn1a*, *Cdx2*, *Dbccr1*, *Egr2*, *Hoxa1*, *Otx1*, *Pmp22*, *Pms1*, *Shh*, *Shox2* and *Otx2*) encode factors, whose regulation can stimulate aberrant tissue differentiation and morphogenesis (Rogers et al., 1997; Luijten et al., 2010).

3.2.1.3. Application to in vivo tests. In addition to prediction, transcriptomics studies can also help to understand the molecular mechanisms underlying teratogenicity. For example, analysis of the embryonic messenger RNA following administration of thalidomide to pregnant *Cynomolgus* monkeys has now identified many key pathways implicated in thalidomide embryopathy (Simoniello et al., 2011).

3.2.2. Proteomics

The applications of proteomics in teratogenicity testing seem very limited (Hansen and Abbott, 2009). To the best of our knowledge, no studies have been published so far.

3.2.3. Metabonomics

Profiling human embryonic stem cells for their secreted metabolites has been proposed as an alternative testing platform for assessing embryonic/fetal toxicity of compounds (Cezar et al., 2007; West et al., 2010). Metabonomics were notably able to detect differences in response to valproate, revealing novel biochemical pathways of injury that were in agreement with data from previous *in vivo* valproate studies (West et al., 2010).

3.3. Applications to herbal medicines

To the best of our knowledge, teratogenicity of herbal medicines has not been assessed with the help of "omics" technologies. Conventional *in vitro* and *in vivo* methods have been used to evaluate teratogenicity potency of herbal products. *Boehmeria nivea* aqueous extract at 32 g/kg/day did not cause significant embryotoxicity or maternal toxicity in mice, although it might cause cytotoxicity in cultured embryonic stem cells (ESC test) at a higher dose (Huang et al., 2006; Tian et al., 2011a,b). An evaluation of the teratogenicity of *Foeniculum vulgare* L. essential oil on the rat embryo limb bud culture (mammalian micromass test) showed no evidence of teratogenicity (Ostad et al., 2004). Artemisinin was found positive for teratogenicity on a *Xenopus* assay (FETAX) (Efferth et al., 2008; Longo et al., 2008); its derivative dihydroartemisinin has been shown in a rat WEC model to primarily affect primitive red blood cells, causing subsequent tissue damage and dysmorphogenesis (Longo et al., 2006). The Zebrafish embryo toxicity test has already been used to evaluate the teratogenicity of several natural products consumed by humans, including various flavonoids (Jones et al., 1964), delta-9-tetrahydrocannabinol, the major psychoactive constituent of marijuana (Thomas, 1975) and arecoline, the major alkaloid in betel nuts (Chang et al., 2004). *In vivo* treatment of pregnant mice with ginseng (*Panax ginseng* C.A. Meyer) caused fetal gross malformation especially when taken for an extended time during pregnancy (El-Ashmaoui et al., 2003).

3.4. Applications to mushrooms

It is known that ribosome-inactivating proteins (RIP) of plants can hamper embryonic development in mice. Recently, a similar effect has been shown for two mushroom RIPs, (i) lyophyllin from *Lyophyllum shimeji* (Kawam.) Hongo that also provokes embryonic abnormalities in mice (cranial neural tube, forelimb buds, branchial arches and body axis); at high concentrations, forebrain blisters appeared within the cranial mesenchyme (Chan et al., 2010); and (ii) hypsin from *Hypsizigus marmoreus* Bunashimeji. In contrast, another RIP, velutin from *Flammulina velutipes* (Curt.:Fr.) Sing. did not exert any adverse influence on mouse development, indicating that teratogenicity is not a general feature of all RIPs (Ng et al., 2010).

4. Part C: methods for nephrotoxicity assessment

Nephrotoxicity defined as a renal disease or dysfunction, is often caused by drugs, chemicals, industrial or environmental toxic agents. Factors contributing to the nephrotoxicity of herbal products may be related to an intrinsic toxicity of plant constituents and ingredients or secondary metabolites, to a contamination of the product itself by other nephrotoxic agents, to misidentification of medicinal herbs or overdose (Zhao et al., 2006b; De Smet, 2007; Lai et al., 2009). Drug-induced renal dysfunction is frequent in clinical practice. Kidneys are particularly vulnerable to drug toxicity because they are highly vascularized and play a major role in metabolism and elimination of toxicants (Karie et al., 2010). In this section, we will focus on applicable methods in assessing drug renal toxicity and discuss advantages and disadvantages of each method. *In silico* methods have also been applied to nephrotoxicity studies (Wolfgang and Johnson, 2002; Ursem et al., 2009) and some structural alerts are known such as aristolochic acids (Fang et al., 2011), ochratoxin A (Sieber et al., 2009) or the diterpen triptolide (Xia et al., 2009).

4.1. Review of applicable methods to test and evaluate herbal renal toxicity

4.1.1. In vivo animal models

Animal models represent the "gold standard" (de Broe and Porter, 2008) of toxicity assessment and are recommended by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) for investigating toxicity of new drugs. Acute, sub-acute and chronic toxicities are classically assessed in two animal species (one rodent and one non-rodent). However, these models are expensive and time consuming; therefore it is not practical to use them for screening the nephrotoxicity of a large number of samples. Moreover, extensive use of animals for toxicity assessment is not in line with the current trend of applying the 3Rs principle (Van den Bulck et al., 2011).

In brief, animals are treated with the tested compounds, and then biomarkers of nephrotoxicity are measured in urine or blood samples and kidneys are harvested for histological analyses.

However, utilization of classical markers of kidney injury (proteinuria, plasma creatinine and blood urea nitrogen (BUN)) in nephrotoxicological studies is limited. Indeed, because of the great ability of the kidneys to compensate renal mass loss and to recover after acute insult, the sensitivity of serum creatinine and BUN is very poor. It has been observed that a reduction of renal functionality occurs only after approximately two thirds of renal biomass has been lost. In addition, these markers lack specificity. Indeed,

the serum level of creatinine, a breakdown product of muscle tissue, depends on age, gender, muscle mass, and weight. It has also been reported that gastrointestinal bleeding or enhanced protein catabolism and other pathologic conditions can lead to an increase in serum BUN without any negative impact on the kidneys.

Recently, new biomarkers have been described and are considered as promising candidates to detect acute or chronic tubulotoxicity (Hoffmann et al., 2010b; Marrer and Dieterle, 2010) and are summarized in Table 2. Several studies have compared these markers with plasma creatinine and BUN and have demonstrated their superiority (more sensitive and more specific). These markers have therefore been accepted by FDA and EMA for the detection of acute kidney injury in the context of non clinical drug development. However, if these biomarkers seem to be promising, data regarding screening toxicological studies are limited, as only few studies have compared these markers with the gold standard (histopathological analyses) (Hoffmann et al., 2010b; Tonomura et al., 2010). Therefore, from our point of view, biomarkers should not be used alone to assess nephrotoxicity of TCM.

4.1.2. Isolated perfused kidney

Isolated perfused kidney from rat and mouse (de Broe and Porter, 2008) is ideal for pathophysiologic studies (such as renal vascular, tubular and glomerular functions). It can be also used to evaluate chemicals for their potential nephrotoxicity (Gandolfi and Brendel, 1990). Since the isolated perfused kidney is an intact organ and maintains structural integrity, it can reproduce the *in vivo* toxicity for very acute toxicants (Shanley et al., 1986). However, this system represents a difficult technique, suffers from limited viability and is time consuming.

Table 2
Experimental support for urinary biomarker candidates as indicators of kidney injury. (PT: proximal tubule; DT: distal tubule; AKI: acute kidney injury; CKD: chronic kidney disease.)

Marker	Comments	Presumed marker of	Experimental support
Calbindin	Vitamin D-dependent calcium binding protein expressed in the distal tubule/collecting duct: mediates uptake of Ca^{2+} from urine	DT damage	–
Clusterin	Secreted glycoprotein expressed in a wide range of tissues; Extracellular chaperone implicated in cell adhesion, tissue remodeling, membrane re-cycling, cell cycle regulation, DNA repair, and apoptosis	PT + DT damage	Increased following renal ischemia, unilateral urethral obstruction or in response to various nephrotoxins, *e.g.* increased mRNA expression in kidneys of rats treated with gentamicin, ochratoxin A, sevoflurane, cisplatin, vancomycin, bacitracin
Cystatin C	Extracellular cysteine proteinase inhibitor expressed and produced by most nucleated cells; Freely filtered from blood through the glomerulus, then reabsorbed and degraded by proximal tubule cells	PT damage	Cystatin C is considered as a better predictor than serum creatinine for glomerular function in CKD A 50% increase in cystatin C can predict AKI 48 h before the rise in serum creatinine and before creatinine clearance reduction Increased in diabetic rats and in response to subtotal or unilateral nephrectomy; Elevated during chromium nephropathy
GST-α	Cytosolic phase-II biotransformation enzyme involved in detoxification of a wide range of xenobiotics	PT damage	Increased urinary excretion in response to cadmium, fluoride, aristolochic acid; altered mRNA expression in response to a range of nephrotoxicants
KIM-1	Kidney injury molecule 1 Transmembrane protein expressed by tubule epithelial cells in response to injury Acts as a phosphatidylserine receptor and confers phagocytic capacity to clear cell debris	PT damage	Elevated in response to gentamicin, mercury, chromium, cadmium; Increased mRNA expression in kidneys of rats treated with gentamicin, ochratoxin A, sevoflurane, cisplatin, vancomycin, bacitracin
NGAL/lipocalin-2	Neutrophil gelatinase-associated lipocalin Acute-phase protein initially found in activated neutrophils but also expressed by a wide range of tissues in response to injury Participates in host innate immune defence by binding bacterial siderophores to limit bacterial iron acquisition; Glomerular filtration and reabsorption by proximal tubule cells	T damage	Increased in response to gentamicin nephrotoxicity increased mRNA expression in kidneys of rats treated with gentamicin, ochratoxin A, cisplatin, vancomycin, bacitracin
β2-Microglobulin	Protein found on the surfaces of all nucleated cells, shed into blood; Glomerular filtration and reabsorption by proximal tubule cells	PT damage	Increased urinary excretion in response to ochratoxin A, depleted uranium, ifosfamide, chlorotrifluoroethylene, 1,1-dichloro-2,2-difluoroethylene
Osteopontin	Secreted phosphoprotein, extracellular structural protein which interacts with multiple cell surface receptors; Role in immune function, chemotoxis, bone remodeling and apoptosis	PT + DT damage	Increased mRNA expression in kidneys of rats treated with gentamicin, ochratoxin A, sevoflurane, cisplatin, vancomycin, bacitracin
Timp-1	Tissue inhibitor of matrix metalloproteinase 1 Regulates extracellular matrix synthesis and degradation; Promotes tubulointerstitial fibrosis through inhibition of proteolytic matrix metalloproteinases; Exacerbates inflammation and renal scaring	PT damage	Increase in rodent model of gentamicin nephrotoxicity; Increased mRNA expression in kidneys of rats treated with gentamicin, ochratoxin A, sevoflurane, cisplatin, vancomycin, bacitracin
VEGF	Vascular endothelial growth factor Induced endothelial cell proliferation and angiogenesis, promotes cell migration, enhances vascular permeability	T damage	Increased expression in response to cyclosporin A and tacrolimus mediated nephrotoxicity

Adapted from Hoffmann et al. (2010b).

4.1.3. Renal cell culture models

Renal cell culture models (de Broe and Porter, 2008) have been used for a long time. Two models of renal cell culture are available: cell lines and primary cell cultures. Cell lines offer several advantages (easier to culture, unlimited life span, no isolation procedure and better stability) but are probably more distant from *in vivo* situation than primary cells. Several renal cell lines reproducing different renal cell types are available: cell lines with characteristics of the proximal tubule such as normal rat kidney cells (NRK52E), Lewis lung carcinoma porcine kidney (LLC-PK1), opossum kidney (OK) or human kidney-2 (HK-2 cells), generated by transduction of human primary proximal tubular cells with human papilloma virus (HPV); cell lines with characteristics of the distal tubule or the collecting duct such as Madin Darby canine kidney cells (MDCK) or Madin Darby bovine kidney cells (MDBK). Primary renal proximal tubular epithelial cells (RPTEC) share more similarities to *in vivo* cells. However, the isolation procedure and the limited access to these cells, particularly those from human source, hamper their utilization. Moreover, primary cell lines have a limited life span and their phenotype can change rapidly due to different culture conditions (no standard culture condition is available in the literature). Renal cell culture models offer a better way to understand biochemical and cellular mechanisms of cytotoxicity, are cheaper and less time consuming than animal experimentation. However, the nephrotoxic potency of a xenobiotic will/may depend on the nephron segments and the cell types: the epithelial cells from the proximal tubule are usual targets of toxic injury.

These methods do not allow studies related to absorption, distribution, metabolism and elimination (ADME). *In vitro* toxicity studies on metabolites are indeed unfeasible as metabolic pathways are not previously described.

4.1.4. Renal slice models

Contrasting with hepatotoxicity studies, only few renal slice models were used to study nephrotoxicity related to iodinated contrast compounds, cisplatin, sevoflurane (Catania et al., 2001; Vickers and Fisher, 2004; Vickers et al., 2004; Harmon et al., 2009). Selective injury has been observed following *in vitro* nephrotoxicant exposure to precision-cut renal slices (Ruegg, 1994). Renal cortical slices are generally prepared from kidneys of untreated rats and incubated with tested compound or vehicle for 2 h. At the end of the 2 h incubation, tissue gluconeogenesis capacity (pyruvate-stimulated gluconeogenesis) and lactate dehydrogenase (LDH) release are determined as measures of cellular function and cytotoxicity (Hong et al., 2002). This model provides interaction between various cells within the nephron and has proven to be a useful *in vitro* system for biotransformation and toxicity studies (Gandolfi et al., 1995; Minigh and Valentovic, 2003); however, it is not useful to assess chronic renal toxicity.

4.1.5. "Omics" technologies

"Omics" technologies have the potential for the development of molecular markers hopefully allowing for detection of early changes in signal transduction, regulation and biochemistry with high sensitivity and specificity.

4.1.5.1. Transcriptomics. Several studies have analyzed transcriptome changes following acute kidney injury in toxic kidney injury models. In their study Ozaki et al. found that hundreds of genes were found to be deregulated in a model of gentamicin acute kidney injury (Ozaki et al., 2010). The gene with the most prominent upregulation was the kidney injury molecule-1 (Kim-1) (also known as HAVCR1 gene). In another study, Rokushima et al. analyzed transcriptome profile following cephalosporin intoxication and found hundreds of genes to be deregulated (Rokushima et al., 2008). Among these, *Kim-1* was the most quantitatively upregulated. In addition, several studies have also demonstrated renal expression changes of genes encoding acute kidney injury biomarkers in response to injury induced by a variety of nephrotoxicants: Kim-1, metallopeptidase inhibitor 1 (TIMP1), clusterin, osteopontin, and neutrophil gelatinase-associated lipocalin/lipocalin 2 (NGAL) (Ichimura et al., 2004; Rached et al., 2008; Wang et al., 2008; Zhou et al., 2008; Kondo et al., 2009).

4.1.5.2. Proteomics. "Proteomics" technologies were used for qualitative and quantitative assessment of urinary protein modulation associated with toxicants exposure in rats. Samples are analyzed using two complementary analysis techniques: (i) bi-dimensional (2-DE) electrophoresis followed by mass fingerprinting (MALDI-TOF) identification; (ii) analysis of protein gels (SDS–PAGE) separation followed by (LC–MS/MS) and the use of spectral counting as a semi-quantitative tool to identify proteins that change in abundance (Mueller et al., 2008).

4.1.5.3. Metabonomics. Urinary ^{1}H NMR spectral-based metabolomics is a new approach for determining changes in endogenous metabolites in animals exposed to nephrotoxic agents (Kim et al., 2010; Zhang et al., 2012). For example, urinary ^{1}H NMR spectroscopy revealed apparent differential clustering of metabolites between the control and $HgCl_2$ (nephrotoxic agent) treatment groups as evidenced by principal component analysis (PCA) and partial least square (PLS)-discriminant analysis (DA). In $HgCl_2$-treated rats, the concentrations of endogenous urinary metabolites of glucose, acetate, alanine, lactate, succinate and ethanol were significantly increased, whereas the concentrations of 2-oxoglutarate, allantoin, citrate, formate, taurine, and hippurate were significantly decreased (Kim et al., 2010). ^{1}H NMR urinalysis could be used to predict or screen nephrotoxic potency of herbal medicinal products.

4.2. Comparisons of screening methods for herbal kidney toxicity

No study has directly compared these different methods of kidney toxicity screening. To date, the animal model is still the gold standard. In addition, the recent development of acute kidney injury biomarkers has strongly enhanced the sensitivity of early renal lesions detection. In a recent publication, Hoffman et al. evaluated and compared novel kidney biomarkers in preclinical toxicity studies (Hoffmann et al., 2010a). Interestingly, they demonstrated that Kim-1 (AUC = 0.99) and clusterin (AUC = 0.93) were sensitive and early expressed, even before histologically proven lesions in several animals. However, the toxicants tested in this study were all specific for the proximal tubule and none of them was specific for any other part of the nephron. In another study published by Rouse et al. (2011), several kidney injury biomarkers were evaluated in a gentamicin rat model. Overall, the quickest and largest response to gentamicin-induced injury concerned Kim-1 biomarker.

In addition to the classical new kidney injury biomarkers, several urinary markers have been identified with 'omics' methods. However, they were rarely compared to other toxicological screening approaches and they were not prospectively validated. A first review published in 2005 (Gibbs, 2005) evaluated the relative sensitivity and specificity of proteomics and metabonomics techniques compared with traditional techniques for assessing xenobiotic induced nephrotoxicity. The authors concluded that there was no consistent evidence that the novel methodologies were any more sensitive than the traditional methods for assessing nephrotoxicity. Another study (Ebbels et al., 2007) recently published by the COMET group (consortium for metabolic toxicology) was performed to build a model for toxicity prediction using a set of 80 toxics. Unfortunately, the sensitivity for kidney toxicity was only 41% (with a specificity of 100%). To conclude, these techniques are certainly

helpful to detect new biomarkers of renal injury but, considering the lack of validation, they should be regarded as complementary tools to detect renal injury in a screening program (Devarajan, 2008; Beger et al., 2010; Rouse et al., 2011).

4.3. Applications of screening methods to herbal medicines

Almost all the above described methods have been used to assess nephrotoxicity of several TCM products. However, they were not applied in screening purposes and no prospective validation is available up to now.

To our knowledge, renal cells culture models have been mainly used in aristolochic acids models. A series of aristolochic acid derivatives isolated from *Aristolochia spp* were analyzed for their nephrotoxic potential using the neutral red dye exclusion assay in cultures of renal epithelial cells (Balachandran et al., 2005). In addition, a recent study has investigated renal toxicity of 47 herbs traditionally used for kidney and urinary disorders in two cell lines models (Wojcikowski et al., 2009).

Renal slice models have also been used in investigating AA and tripterygium toxicity (Dan et al., 2008; Dickman et al., 2011).

Besides nephrotoxicological studies on cisplatine, ciclosporine, $NiCl_2$ and indomethacin, metabolomics and proteomics have been also used to investigate TCM nephrotoxicoly, *e.g. Aristolochia manshuriensis* Kom. (Chen et al., 2006; Zhang et al., 2006; Chan et al., 2008; Lin et al., 2010), ochratoxin A (Sieber et al., 2009), morning glory seed (dried mature seeds of *Pharbitis nil* (L.) Choisy or *Pharbitis purpurea* (L.) Voigt (Ma et al., 2010)), *Cimicifuga triterpenoid* (He et al., 2012), cinnabar and realgar (Wei et al., 2008, 2009) and *Tripterygium wilfordii* Hook (Xia et al., 2009).

4.4. Applications to mushrooms

Amanita phalloides (Vaill. ex Fr.) link is a well-known example for hepatotoxicity (Vetter, 1998); it is less known but equally relevant that α-amanitin also exerts nephrotoxicity (Vogel et al., 1979). *Cortinarius orellanus* Fr., *Cortinarius speciosissimus* Kühner & Romagnesi and other species of this genus contain a bipyridine, orellanine, responsible for lethal tubular necrosis (Schumacher and Hoiland, 1983; Tebbett and Caddy, 1984). As orellanine and the nephrotoxic bipyridinium herbicides methylviologen (paraquat) and diquat have a similar chemical structure, a similar toxicity mechanism has been controversially discussed for orellanine (Richard et al., 1995); it has been shown that oxygen radicals can be generated from the iron complex of orellanine (Richard et al., 1995; Cantin-Esnault et al., 1998) and that antioxidant defense enzymes are downregulated by orellanine (Nilsson et al., 2008). These few examples show that some musrooms bear a potential for nephrotoxicity; knowledge about a global epidemiology of mushroom poisonings has to be collected to prevent and treat new emerging mushroom poisonings (Diaz, 2005).

Active hexose correlated compound (AHCC), a mushroom extract rich in α-1,4 linked glucans, used in Japan as a dietary supplement to boost immune function (Fujii et al., 2011) did not show any evidence of kidney dysfunction in Sprague-Dawley rats. The mushroom β-glucan, a polymer of β-(1,3/1,6)-glucan, has been claimed for its health benefits and no subchronic toxicity was found in Sprague-Dawley rats (Chen et al., 2011a).

4.5. General conclusions on nephrotoxicity

Several methodological approaches have been developed to assess kidney toxicity of drugs. This reflects the need for complementary methods to achieve a valuable nephrotoxicity screening. In addition, the safety evaluation needs to be focused on the material effectively taken by a subject. Indeed, it should be recognized that there may be significant differences in pharmacokinetic behavior, such as the rate or extent of absorption, and pharmacological/toxic potency between a whole botanical and the equivalent amount of an isolated active principle. In the large majority of cases, there are no data available regarding metabolism and ADME study for a given herbal medicine product. Despite several limitations, animal models remain the gold standard to assess drugs nephrotoxicity. In the future, animal models combined to 'omics' methods could be useful to assess TCM nephrotoxicity.

5. Part D: opportunities, challenges and outlook of the "omics" technologies in genotoxicity, teratogenicity, and nephrotoxicity evaluation

5.1. Opportunities and challenges

Global analysis of genome-wide mRNA expression, protein and metabolite patterns offers the potential: (i) to predict the toxicity by establishing groups of chemicals causing the same type of transcriptomics–proteomics–metabonomics profile in a biological system, *i.e.* toxic fingerprinting; (ii) to discover the groups of genes or proteins that are effective biomarkers for a given toxicity; (iii) to elucidate the molecular mechanism of action of a xenobiotic; and (iv) to predict the synergistic or inhibitory effects of compounds in mixtures (Stierum et al., 2005; Xu et al., 2009; Villeneuve and Garcia-Reyero, 2011).

Changes in transcriptomics, proteomics and/or metabonomics profiles may serve as early, sensitive indicators of a potential toxicity and are thought to precede toxic outcomes (Aardema and MacGregor, 2002). In particular, gene expression data (transcriptomics) are thought to be more sensitive than traditional toxicological endpoints (Searfoss et al., 2005). Toxicogenomics can also help to bridge traditional *in vitro* and *in vivo* toxicity assessment (Wilson et al., in press), and could be a very effective tool in studying the safety of herbal medicines by expanding the predictivity of potential toxicity in earlier stages of product development (Amir-Aslani, 2008; Blomme et al., 2009). Most literature on "omics" technologies focuses on the pharmacological and toxicological screening of new potential drugs (Knasmuller et al., 2008; Ovesna et al., 2008; Chen and Cheng, 2009), but "omics" certainly have their place in the toxicity assessment of plant-based medicinal products, especially Chinese medicinal plants. Indeed, the integrative approach of "omics" is in line with the holistic concept and practices of TCM (Cho, 2007).

Despite their potential, there are a number of general challenges to "omics" technologies applied to toxicology. These technologies are not standardized yet for all laboratories (Aardema and MacGregor, 2002). So far, inter-laboratory evaluations of genomic fingerprints have demonstrated the need for caution in interpreting the data; regulatory agencies are likely to face future challenges with transcriptomics datasets coming from different laboratories, models, strains and different durations of exposure. A wise use of "data-rich omics" outputs will take time and validation, well-trained experts and advanced capabilities for data analysis (Fielden et al., 2008).

5.2. Outlook

In this post-genome era, a plethora of structural alerts and *in silico*, *in vitro* and *in vivo* methods could be applied to predict the genotoxicity, teratogenicity and nephrotoxicity of herbal medicinal products. The rapid progress in the power of bioinformatics creates a great opportunity to employ "omics" technologies to create a sound basis for better hazard identification, for increased

understanding of underlying mechanisms and for more relevant genotoxicity assessment. However, these technologies are not standardized yet; this will be the main challenge for regulatory agencies to overcome in establishing uniform and transparent methods to interpret and apply available "omics".

Considering the aforementioned issues, it is critical that toxicologists in industry, regulatory agencies and academic institutions develop rigorous "omics" methods to reach a consensus about the reliability and interpretation of endpoints. It will also be important to regulate the puzzle of conventional methods of genotoxicity assessments and their relation with new "omics" technologies. Furthermore, investigations must be undertaken to discover novel structural alerts of toxicity from herbal medicinal products which will certainly facilitate an analytical assessment of traditional medicinal plants toxicity at early stages of testing.

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