CHIMIA 49 (1995) Nr. 10 (Oktober)

MEDICINAL CHEMISTRY

Chimia 49 (1995) 361–364

ISSN 0009–4293

In Vitro Test Systems for Identifying Potential Chemical Carcinogens

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Abstract. The development of *in vitro* test systems for evaluating the toxic, mutagenic, and carcinogenic potential of substances has led to a reduction of animal experiments besides of being better suited for many differentiated mechanistic investigations. In a pre-screening approach, substances which exhibit the aforementioned characteristics *in vitro* may be excluded from further studies *in vivo*. On the other hand, negative results obtained with *in vitro* test systems cannot be extrapolated to the animal or to the human situation with sufficient certainty. Thus, if a substance has proven to be nontoxic and non-mutagenic *in vitro* there is still the need to set up an animal experiment. In this review we report on suborganismic systems which have proven to be useful tools for evaluating the mutagenic and carcinogenic potential of chemicals which are relevant to human health.

1. General Considerations on Chemical Carcinogenesis

In the Western World neoplastic diseases rank on place two of causes of death surpassed only by cardiovascular diseases. The etiology of cancer is diverse, including endogenous factors, viruses, physical and chemical stimuli. The contribution of these factors to neoplastic transformation is not exclusive but rather can complement each other.

Chemical carcinogens occur as environmental pollutants or as occupational chemicals and may also unintentionally emerge from the efforts to design new drugs. Structurally they are as diverse as is their mechanism of function which finally leads to or at least contributes to neoplastic desease. The spectrum reaches from very simple inorganic compounds such as asbestos and certain chromium salts to more complex structures such as aflatoxins, nitrosamines and polycyclic aromatic hydrocarbons (PAH). One of the most critical events which precede neoplastic transformation is the ability of certain substances or their metabolites to form adducts with the bases of DNA, thereby inducing mutations in critical genes, *e.g.* oncogenes and tumor suppressor genes. For instance, epidemiological and experimental evidence has revealed a variety of human tumors to be associated with mutational hot spots in the p53 tumor suppressor gene [1]. These tumor-associated mutations mostly occur in higly conserved domains of the protein, leading to functional inactivation of the protein, to transdominant negative effects on wild type p53 or to as yet more or less undefined gains of function contributing to cellular transformation. In particular geographic regions with a high incidence of hepatocellular carcinoma a specific G to T transversion in codon 249 is frequently found in such tumors [2][3]. This transversion leads to an arginine to serine exchange in the polypeptide chain and is supposed to be due to chronic exposure to the endemic hepatocarcinogen Aflatoxin B1. This correlation has first been demonstrated in epidemiological studies and the causative role of the relevance of this specific mutation has been substantiated experimentally by incubating HepG2 hepatoma cells with Aflatoxin B1 which led to adduct formation and finally to a miscoding G to T transversion in codon 249 [4].

The first irreversible step in the process finally leading to cancer such as a mutation following adduct formation is called initiation and is followed by promotion, *i.e.*, gain of a selective growth advantage of the respective cell and by progression, which means tumor formation and acquirement of invasive (*i.e.* malignant) growth. Whereas some chemicals can directly bind to DNA others need to be metabolically activated. The finding that numerous xenobiotics acquire mutagenic and carcinogenic potential only upon metabolic activation represents a hallmark in cancer research [5]. *Scheme 1* shows, as an example, a simplified overview of the metabolism of benzo[*a*]pyrene, a typical representative of the class of polycyclic aromatic hydrocarbons. This compound, a product of incomplete combustion, easily enters the cell due to its lipophilicity and is subjected to several enzymatic reactions primarily intended to render this molecule hydrophilic for urinary and biliary excretion. The enzymes which are involved in the metabolic conversion are divided into phase I, functionalizing and phase II, conjugating enzymes. The first reaction is the epoxidation at the 4,5-, 7,8-, or 9,10-position of the benzo[*a*]pyrene molecule. This reaction is catalyzed in a stereoselective manner by a member of the phase I enzyme family of cytochromes P450 (CYP). The 9,10-epoxide can spontaneously rearrange into the 9-phenol and then be conjugated to form glucuronides or sulfates. The 4,5-epoxide can directly bind to DNA but is also efficiently detoxified by conjugation to glutathione. An alternative pathway of the 4,5-oxide consists in opening of the epoxide ring, a reaction

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catalyzed by the enzyme microsomal epoxide hydrolase (mEH), to yield the benzo[*a*]pyrene-4,5-dihydrodiol which can be conjugated to form glucuronides or sulfates. The formation of the 7,8-epoxide represents a prelude for the formation of the most mutagenic and carcinogenic metabolite of benzo[*a*]pyrene. After introduction of the epoxide function at the 7,8-postion, the microsomal epoxide hydrolase opens the ring to yield the benzo[*a*]pyrene-7,8-dihydrodiol which again is a good substrate for CYP introducing an epoxide at position 9,10. This metabolite is, due to the location of the epoxide moiety in the bay region (the angular region present also in phenanthrene as the most simple case), especially reactive (preferential assistence of the heterolytic cleavage of the C–O bond due to resonance stabilization of the carbocation) and it is a poor substrate for the enzymes catalyzing its further metabolism (for a review see [6]). In line with this it represents the most active ultimate carcinogen of benzo[*a*]pyrene. This metabolite reacts with the DNA to form adducts, primarily *via* the exocyclic N(2) position of guanine, the N(6) of adenine and N(4) of cytosine [7][8].

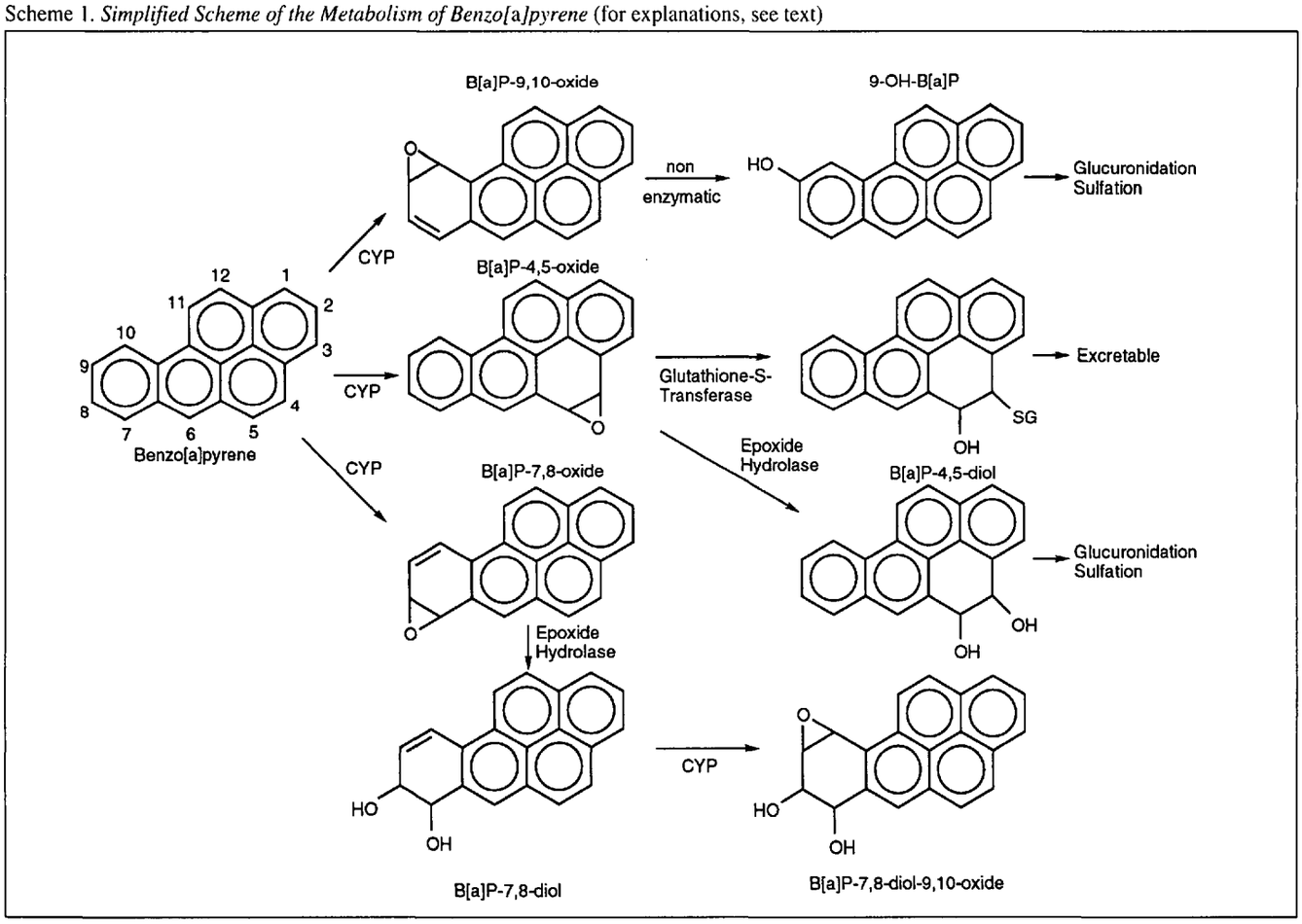
From the example of the complex metabolism of benzo[*a*]pyrene, it is clear that the formation of potentially carcinogenic metabolites is dependent on the individual metabolizing enzymes involved and on their relative expression levels. The knowledge of the enzyme composition both, qualitative and quantitative, of a given human individual thus could allow to make predictions about the metabolic profile and thus mutagenic potential of a test compound, if it is known by which enzymes it is metabolized.

2. *In Vitro* Test Systems

Whether a new substance is to be commercialized by the chemical and pharmaceutical industry or occupational exposure to it occurs during an industrial process, information on its acute toxicity and mutagenic and carcinogenic potential is needed. There is no way of totally avoiding animal experiments in order to obtain toxicological data which can be extrapolated to the human situation. Nevertheless, in an effort to reduce such animal experiments several 'alternative methods' have been developed during the last decades. In its simplest form the substances under investigation are incubated *in vitro* with preparations of potentially metabolizing enzymes and the metabolites are analyzed by biochemical and/or chromatographic methods. However, this approach does not utilize a biological endpoint and does not define a genotoxic potential.

In order to test substances for their mutagenic potential, a bacterial test system, the *Ames* test, has been developed in which an indicator strain of *Salmonella typhimurium* is incubated with a substance under investigation together with an exogenously added metabolically activating system, the S9 fraction [9]. The latter is a subcellular preparation of a rat liver homogenate which is obtaind as a supernatant after centrifugation at 9000 g (hence the name S9). It contains the microsomal and cytosolic enzymes, *i.e.*, most phase I and II enzymes but the assay conditions are adjusted in order to favor CYP mediated catalysis. The bacteria are dependent on exogenous histidine (they are said to be auxotrophic for histidine), because they have the genes which code for enzymes

Scheme 1. *Simplified Scheme of the Metabolism of Benzo[a]pyrene* (for explanations, see text)



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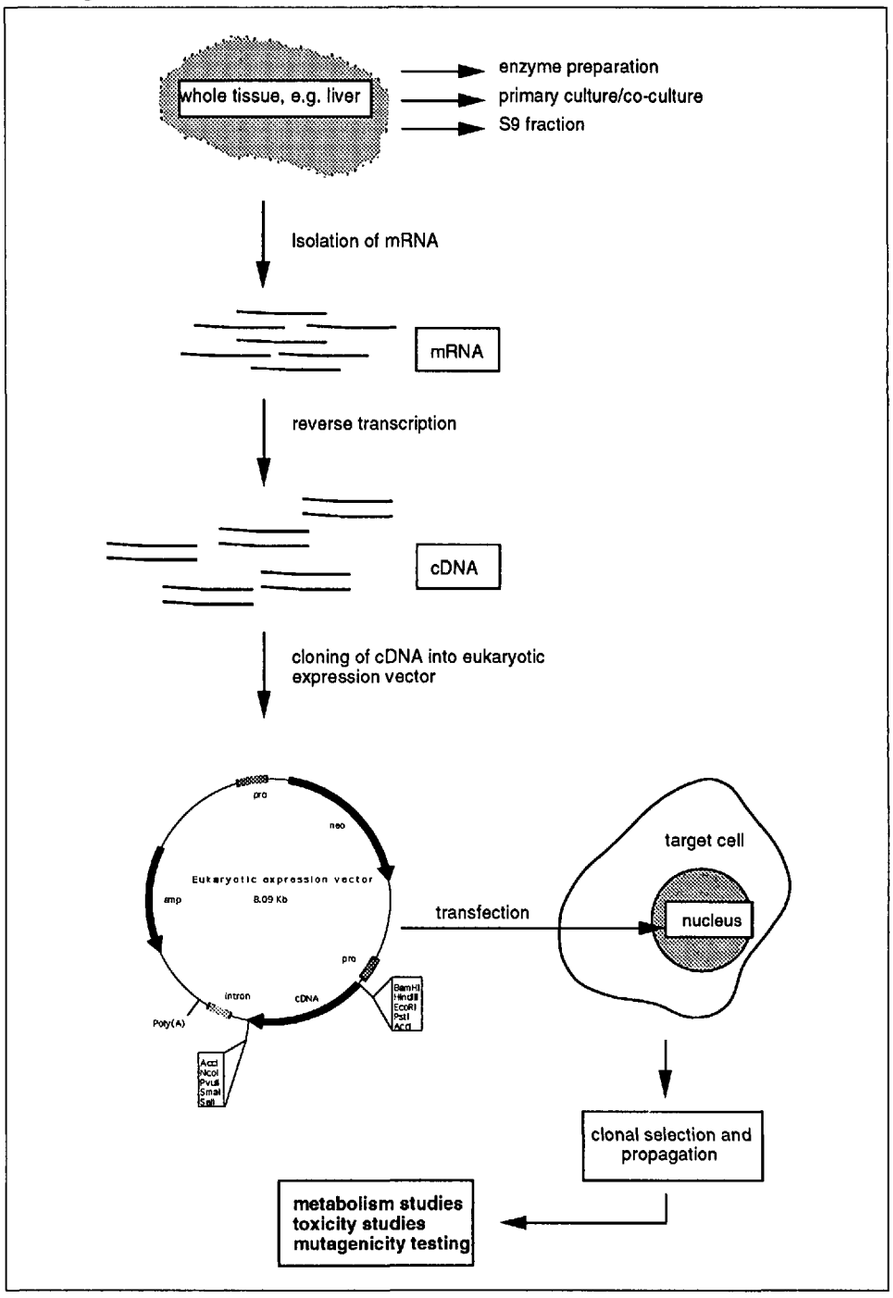
necessary for the synthesis of this amino acid mutated. Thus, if the respective substance is activated by the S9 enzymes to a mutagenic metabolite and if it permeates through the bacterial cell wall and plasma membrane, it will induce many mutations, amongst others the one which reverts the auxotrophic phenotype to a prototrophic one. The number (per unit tested compound) of bacterial colonies growing on a histidine-deficient culture medium is proportional to the mutagenic potential of the substance.

As the genome of bacteria is not organized into structures such as eukaryotic chromosomes only gene mutations can be detected with this assay. The demonstration of clastogenic effects, *i.e.*, structural chromosomal aberrations due to strand breaks, and genome mutations, *i.e.*, numerical chromosomal abberations, is only possible with eukaryotic cells.

Eukaryotic test systems take advantage of the ability of freshly isolated cells to be cultured *in vitro*. This has been extensively done with hepatocytes from various experimental animals as this cell type is characterized by a high metabolic competence. The expression of individual xenobiotic metabolizing enzymes can be selectively enhanced by pretreating the animals from which the hepatocytes will be isolated with specific inducers. For instance, intraperitoneal injection of phenobarbital leads to the induction of CYP 2B1 and mEH whereas treatment with isoniazid leads to the induction of CYP 2E1. Unfortunately, these primary cultures are not propagatable and they loose their metabolic potential within a short period of time. It could be shown that the level of CYP is reduced to *ca.* 50% within 24 h and to 7% within one week of culture [10]. In addition, the content of the different CYP isoforms decreases to a different extent, making interpretations of metabolism studies difficult. Loss of metabolic functions is a general problem when dealing with cultured cells [11]. A further drawback of this system is that the toxicological endpoint in terms of mutations cannot be detected as primary hepatocytes do not proliferate to an extent which could allow the manifestation of mutations. However, it is possible to stabilize the 'metabolic phenotype' by coculturing primary hepatocytes with non-parenchymal liver epithelial cells [10][12]. In this situation many CYP activities are maintained for *ca.* ten days although a selective loss of some CYPs is observed. But still, at the low-to-nonexistent proliferation rate of primary hepatocytes in culture, the detection of gene mutations is not achieved, although they have been demonstrated to be useful for the detection of micronuclei and chromosomal abberrations induced by several compounds [13][14]. In order to circumvent the latter problem, cells which are suitable for the detection of a gene toxicological endpoint (requirements see below) are co-cultered with freshly isolated hepatocytes of induced animals [15][16] or with the microsomal fraction of the same [15][17]. In this model the metabolic activation of the compound occurs outside the target cells, as is the case with the *Ames* test. Thereby false negative

Scheme 2. *A Typical Strategy for Generating Transgenic Cell Lines.* Total mRNA of a whole tissue is isolated and then reverse transcribed into cDNA. This cDNA is inserted into an eukaryotic expression vector which contains sequences allowing replication of the plasmid in bacteria and expression in eukaryotic cells. neo: cDNA of the neomycin resistance gene, conferring resistance towards G-418. cDNA: any sequence to be expressed, *e.g.* cDNA coding for a xenobiotic metabolizing enzyme. pro: promoter, initiates transcription of the cDNA. poly(a): polyadenylation signal, leads to the addition of a poly(A) tail, a typical post-transcriptional modification of eukaryotic mRNAs. intron: the inclusion of a non-coding intervening sequence, provoking a splicing event, again a post-transcriptional modification of eukaryotic mRNAs, enhancing the efficiency of mRNA generation. amp: ampicillin resistance gene allowing selection and propagation of the plasmid in bacteria in order to obtain μg to mg quantities of the plasmid to be transfected. Boxed are the restriction endonuclease cleavage sites representing the multiple cloning site into which any sequence can be inserted without disturbing the other functions of the vector.



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results may be obtained if the metabolite does not enter the target cells.

A target cell should ideally fulfill the following criteria: propagatable, stable karyotype, low number of chromosomes, short doubling time. The requirement for propagatability has already been discussed. The need for a stable karyotype and a manageable number of chromosomes refers to the fact that genotoxic effects often are ascertained from the occurrence of chromosomal aberrations, sister chromatid exchange, strand breaks, unscheduled DNA synthesis, *etc.* Short doubling times will lead to a rapid manifestation of the genotoxic lesion. In addition, metabolic competence and the potential to demonstrate the toxicological endpoint in the same cell would be highly advantageous. Several such systems have been described, for example human hepatoma cells [18], long-term cultured rat hepatocytic cell lines [19] and established epithelial liver cells from Chinese hamster [20]. However, the expression pattern of xenobiotic metabolizing enzymes in these cells is on one hand incomplete and on the other hand ill-defined so that it is difficult to investigate the contribution of individual enzymes in the generation of toxicological relevant metabolites.

A way out of this dilemma is to establish cells which posess all the aforementioned criteria with respect to the detection of the gene toxicological endpoint, are easy to handle and ideally established, *i.e.*, permanently propagatable and providing them with the genetic information for xenobiotic metabolizing enzymes which normally are not expressed by these cells. This is achieved by transfection, a gene transfer method by which a cDNA is introduced into cells rendering them proficient for the respective protein (*Scheme 2*). A cDNA is the result of *in vitro* reverse transcription of mRNA isolated from a given tissue and thus consists mainly of the amino acid coding sequence of the respective gene. This cDNA is then cloned into an eukaryotic expression vector, *i.e.*, a plasmid which contains upstream signal sequences such as an eukaryotic promoter (see *Scheme 2*). In order to select for those cells which have internalized and stably integrated the vector into their genome a selection marker either on the same or a different plasmid is included in the transfection experiment. Commonly used selection markers are cDNAs coding for resistance towards antibiotics, *e.g.* the gentamycin derivative G-418, puromycin or hygromycin, or towards antimetabolites, *e.g.* histidinol. The choice of the recipient cell depends mainly on the specific question. For instance, V79 cells, well established in mutagenicity studies, are deficient for CYP. BHK cells, also frequently utilized, express mEH to a scarcely detectable level. Both cell types are kept in culture as established cell lines and are well characterized concerning their metabolic potential. We generated transgenic V79 cells by transfecting several CYP cDNAs [21][22] and transgenic BHK cells by transfecting the mEH cDNA [23][24]. All the cell lines generated exhibited the expected metabolizing functions as ascertained by the incubation with standard substrates. In addition, as they are continuously proliferating they allowed to determine the toxicological endpoint in terms of mutation frequency. Thus, these cell lines are valuable tools for answering the following questions: *a*) Is a substance at all a substrate for the respective enzymes and *b*) if this is the case, do the metabolites have mutagenic potential?

3. Conclusion

Although *in vitro* test systems do not obviate the need for animal experiments in the efforts to identifying chemical carcinogens they lead to their reduction besides of being better suited for many differentiated mechanistic investigations. This is in agreement with the demand formulated already more than 30 years ago as the 'concept of the three 'R's': replace, reduce, refine animal experiments.

The test systems described in this contribution all have advantages and disadvantages. With a whole battery of transgenic cell lines at hand, each expressing a different xenobiotic metabolizing enzyme or a varying combination of the same with a constant genetic background, it would be possible to screen substances for their metabolic pathways and for their mutagenic and thus carcinogenic potential. Besides the advantages of such transgenic cell lines for studying the 'toxicological contribution' of specific enzymes towards the metabolism of certain compounds or as tools to investigate mechanistic aspects of enzyme function, they represent a simplification of the complex orchestra concerned with the metabolism of foreign compounds. Nevertheless, this strategy together with further innovative developments in the field of transgenes, *e.g.* the generation of cell lines with inducible metabolic functions, and in concert with other *in vitro* approaches will continue to contribute to the reduction of animal experiments and to new insights into how chemicals become carcinogenic.

Received: August 23, 1995

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