

# *In vitro* Screening of Plant Extracts and Phytopharmaceuticals: Novel Approaches for the Elucidation of Active Compounds and Their Mechanisms

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Received: August 19, 1999; Accepted: October 16, 1999

**Abstract:** The advantages of cell culture systems for the screening of active compounds of plant extracts and phytopharmaceuticals are discussed and exemplified for hepatocytes and nerve cells. Recent developments and new experimental techniques allow easy access even to complex specific functions of these cells and render it possible to draw conclusions about molecular mechanisms. These *in vitro* approaches, therefore, may contribute essentially to the reduction of animal use in such studies. Furthermore, they may give full insight into not only the therapeutic potential but also the possible dangers of phytopharmaceuticals and may eventually lead to hints on new therapeutic fields that can be further explored.

**Key words:** Cell culture, hepatocytes, multi-electrode arrays, neurons, phytopharmaceuticals, reduction of animal testing, screening.

## Introduction

Elucidation of active components of plant extracts as well as (phyto-)pharmaceuticals and of their mechanisms of action is a major challenge for pharmaceutical biology, pharmacology and biochemistry. In the past, model systems with either high complexity (animals, organ cultures) or low molecular organization (subcellular fractions, organ and cell homogenates) predominated this field (Fig. 1). The last decade, however, has seen an enormous trend towards isolated cellular systems, primary cells in culture and cell lines. These systems provide the desirable complexity of structurally and functionally intact cells combined with excellent experimental accessibility. Thus, they not only fill the gap in the spectrum of model systems (Fig. 1) favoring the extrapolation of experimental results, but also offer the unique possibility to elucidate interactions with vital cellular functions such as metabolism, intercellular communication, signal transduction, growth and death that were formerly difficult to address. In particular, the combination of different *in vitro* assay systems may not only enhance the capacity to screen for active compounds, but may also lead to better conclusions about possible mechanisms and therapeutic effects (Fig. 2). Ultimately, the results

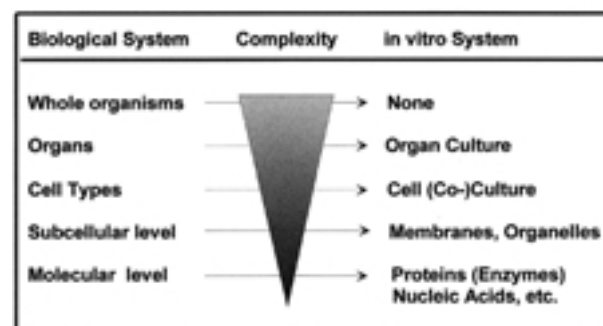
of these screens may provide first hints on new therapeutic fields for the respective extracts or drugs that have not been recognized before. Their potential can then be further tested both experimentally (in disease models) and clinically.

No doubt, the development of cellular screening systems currently is in an expansive state and the literature is growing exponentially. Since it is beyond the scope of this review to present an exhausting overview and because of space limitation, the focus will be on two examples, namely hepatocytes and neuronal cells, which can illustrate the design and potential of such studies.

## Hepatocyte Cultures as Tools for Studying Liver Functions

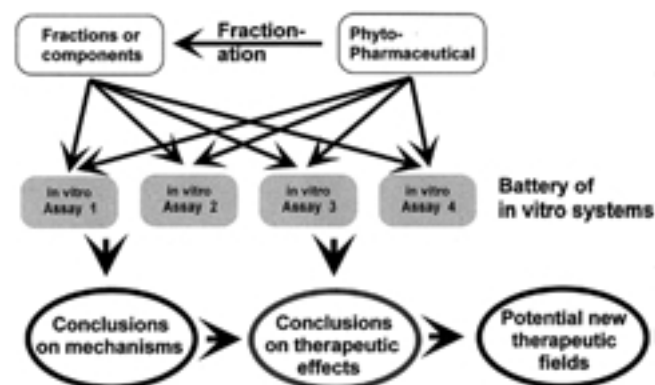
Primary cultures of hepatocytes from various species including man have been shown to mimic liver functions in many respects [cf., reviews published in Cell Biol. Toxicol. Vol. 13 (4–5), 1997]. Only the maintenance of biotransformation capacity, i.e., cytochrome P450 enzymes and conjugating enzymes, may often not be sufficient, but can be considerably improved by altering the culture conditions as, for instance, by cocultivation with liver-derived cell lines (1–3) or by using sandwich cultures (4) and perfusion (3), (5). Table 1 provides an overview on different functions that are influenced by natural products as well as on the respective methods for analysis.

Simple interactions of plant-derived materials with cell metabolism and biosynthetic functions can easily be detected by measuring the final product or the incorporation of a radiola-



**Fig. 1** Correspondence between the complexity of biological systems and different *in vitro* systems.

belled precursor. This approach was successfully applied to the screening for compounds that inhibit cholesterol and/or fatty acid biosynthesis (6–9). Likewise, interference with bile acid synthesis or carbohydrate and energy metabolism was



**Fig. 2** Advanced strategy for *in vitro* screening and evaluation of efficacy of phytopharmaceuticals and their constituents. Most suitably, a battery of complementary *in vitro* assays is used which may be composed of different cellular systems or a combination of cellular and non-cellular assays. These allow the evaluation of the influence of plant extracts or constituents on different cellular functions and the elucidation of mechanisms of action. This approach may lead to direct conclusions about therapeutic effects and their molecular basis. In addition to the experimental support for already known beneficial effects of phytopharmaceuticals, it is possible that new interactions with cellular functions are detected that provide hints on alternative therapeutic fields.

assessed (10). In general, it is helpful to measure also the influence of the materials directly on (key) enzymes of the metabolic pathways, in order to distinguish direct from indirect effects exerted through modulation of regulatory mechanisms (see below) (7), (9), (11). Furthermore, the induction as well as suppression of gene expression for enzymes and other proteins can be easily studied by various techniques (Table 1). Often, phytopharmaceuticals and drugs modulate the levels of enzymes involved in the antioxidative protection of the cells (12), (13) and of drug metabolizing enzymes (14), (15).

Interactions of drugs with cellular transport phenomena are especially interesting in liver cells, because of their involvement in the uptake and secretion of cholephilic compounds. With respect to uptake, the culture model has been used extensively in the past [for reviews, see (16), (17)]. The methods applied are usually uptake of radiolabelled compounds combined with inhibition by specific inhibitors. Transfilter well techniques (18) are especially suited for such measurements. However, the culture approach also allows for the evaluation of secretory events into preexisting (19) or newly formed bile canaliculi (16), (20). Such measurements, mainly performed with fluorescent compounds in combination with computerized image analysis (16), (19), (20), may be complemented by structural investigations using electron microscopy (21), (22). This may render it possible to study phenomena that are seen in extra- and intrahepatic cholestasis *in vivo* (21) and to study the influence of phytopharmaceuticals on this pathology (22).

Furthermore, interactions with elements of the cytoskeleton can be studied and easily monitored by fluorescence micro-

**Table 1** Hepatocyte cultures: *in vitro* assay systems.

Function	Methods	References
Biosynthesis and Metabolism:	HPLC, Scintillation counting	
carbohydrates	incorporation of labelled precursors	10
fatty acids	incorporation of acetate	8
cholesterol	incorporation of acetate, sterol pattern	6, 7, 9
bile acids	labelled cholesterol	
proteins, enzymes	labelled amino acids, Western blots, Northern blots, enzyme assays	12–15
Cellular Transport:	Uptake studies, Image analysis	
uptake of compounds	labelled compounds	17, 18
biliary secretion	fluorescent compounds	16, 20
Stress and Toxicology:	Various methods:	
metabolism of xenobiotics	HPLC, GC-MS	45, 23
oxidative stress	radicals, GSH levels, ROS	32–34
lipid peroxidation	MDA-production	9, 36
cytotoxicity	MTT-assay, LDH-leakage	27, 30
autophagy	microscopy, sequestration	24
DNA damage	unscheduled DNA synthesis	81
Signal transduction:	Various methods:	
phosphorylation	antibodies, incorporation of <sup>32</sup> P	44, 46, 47
protein adaptors	antibodies, Western blots	
transcription factors	Western blots, transient transfection assays	41
Growth and Death:	Various methods:	
cell proliferation	thymidine incorporation, BrdU-incorporation	49, 52
necrosis	LDH-leakage, propidium iodide	25, 26
apoptosis	annexin V/propidium iodide, electron microscopy, DNA strains	53, 54

scopy or other microscopical techniques (23). This approach has also been used in combination with other techniques for demonstrating the influence of several flavonoids on hepatocyte autophagy and endocytosis (24).

Experiments on toxicology are traditionally among the most intensively performed studies (25). A variety of methods is available to assess cytotoxicity of compounds including dye uptake (26), leakage of lactate dehydrogenase (6), (26), (27), and reduction of MTT (9), (27). Metabolism of xenobiotics may be hampered by the above-mentioned problems with the cytochrome P450 system (29). Nevertheless, the interest in biotransformation is rather high, since components of phytopharmaceuticals may not only be the subjects of this process, leading to inactivation (sometimes activation) and excretion (26), (29), (30), but may in some cases severely alter the metabolic capacity for other drugs (14), (15), (30). Usually metabolites are detected by HPLC or GC-MS. In this respect, the perfusion system may be of considerable advantage, because of the steady-state determination of intermediate metabolites and kinetic constants (31).

Oxidative stress and lipid peroxidation represent another field highly relevant for natural products (32–34). Many constituents of plant extracts such as flavonoids and carotenoids are potent antioxidants (35–37), but may occasionally also exert pro-oxidant actions in simple *in vitro* assays (38). The cellular system may distinguish whether such pro-oxidant effects are of biological importance. Furthermore, it is particularly suited to elucidate the mechanisms by which oxidative stress is prevented and its risk reduced (39), (40). Radical scavenging and/or metal chelating activities can be studied as can the potencies to enhance the antioxidative capacity of the cells including induction of respective enzyme activities (12), (41).

An aspect brought to attention in recent years is the fact that many constituents of plant extracts interact with signal transduction. It is already well known that plant constituents, depending on their structure, occasionally act as agonists or antagonists at the level of hormone receptors (42), (43). However, it became increasingly obvious that there are many possibilities for interactions with steps of the signal transduction pathway downstream of the receptors that could lead to the modification (generation or inhibition) of the signal. Since many signal transduction pathways include the action of one or more protein kinases, these represent interesting targets for such compounds (44), (45). Through such interactions many different cellular functions may be affected, and it seems likely that this represents a major mechanism by which phytopharmaceuticals exert their effects in higher organisms. Examples include antiproliferative actions of flavonoids exerted at tyrosine kinases (46), (47), regulation of apolipoprotein B metabolism by flavanones (48), selective inhibition of NF- $\kappa$ B activation by silymarin (49), interference with the phosphorylation/dephosphorylation of HMGCoA reductase by AMP-dependent kinase (9), (11), and interactions with cytoskeletal elements through protein kinase C (Gebhardt, R., unpublished results).

Interference of plant-derived compounds with cell growth and death may be relevant for repair processes such as in partial hepatectomy and even more so for development of fibrosis, (viral) hepatitis, and cancer. Of course, depending on the

type of pathology both stimulation or inhibition of proliferation and of apoptosis, the tissue-compatible type of cell death, may be desirable. Accordingly, screening for antiproliferative activities relevant for anticancer drugs was mainly done with hepatoma cells (15), (50). However, such screening is possible also with primary hepatocytes after stimulation by growth factors (51) and may be more appropriate to the situation in diseases other than cancer. Usually, incorporation of radiolabelled thymidine into DNA is measured (51), but determination of incorporation of BrdU and possibly immunocytochemical detection provides a good alternative (52). In many cases, particularly for flavonoids, more specific information concerning the mechanism was obtained. For instance, it was found that different steps of the cell cycle may be blocked (46), (47). Studies on apoptosis have come into focus more recently. Various techniques are used for the detection of apoptosis (53). However, it is of particular importance that the morphological criteria can be easily checked by (electron) microscopical observation in cell culture systems, since (programmed) cell death can occur in many variations (54) not necessarily expressing features like DNA laddering.

Of considerable interest are possibilities of co-cultivation of hepatocytes with other cell types (30), (55). There, important phenomena relevant to intercellular communication or to the involvement of different cell types in pathogenic mechanisms can be studied (55). Probably the best and most important example for such interactions not reviewed in detail herein is the modulation of the immune system by constituents of phytopharmaceuticals (56). However, also in the case of the liver, (abnormal) communication between different cell types may be the cause or consequence of many diseases. In culture, it is possible to dissect this communication and to elucidate at which level phytopharmaceuticals may interfere. Such strategies, if properly applied for screening, may lead to the discovery of new compounds with a high therapeutic potential. Since most liver diseases cannot be adequately and efficiently treated with currently applied therapies, *in vitro* screening may help us to improve this unsatisfactory situation (57).

### Neuronal Cell Culture as Tools for Studying Brain Functions

Cultured neurons offer excellent possibilities for studying neurotrophic effects of phytopharmaceuticals and drugs (Table 2). Besides neurons, also astrocytes may be a primary target for modulating brain function. Although the use of astrocytes was more common in the past, because they are not so difficult to isolate and culture than neurons, emphasis herein will be mainly on neurons because of space restrictions.

As listed in Table 2, normal neuronal cell cultures are suitable for analysis of general nerve functions and metabolism. Similar techniques as reviewed for hepatocytes were used to demonstrate the influence of various plant-derived extracts and compounds on basic neuronal features such as metabolism, cellular transport, ion homeostasis, survival, and proliferation (see references given in Table 2). There are, however, specific functions of neurons that need special attention and more sophisticated experimental systems. Such functions are coupled to activation of these cells and include interactions of neurotransmitters with cellular receptors, uptake of neurotransmitters, and particularly spike generation and synaptic activity. In the past, adequate experimental systems to ap-

**Table 2** Studies on the effects of plant-derived materials on cultured neurons.

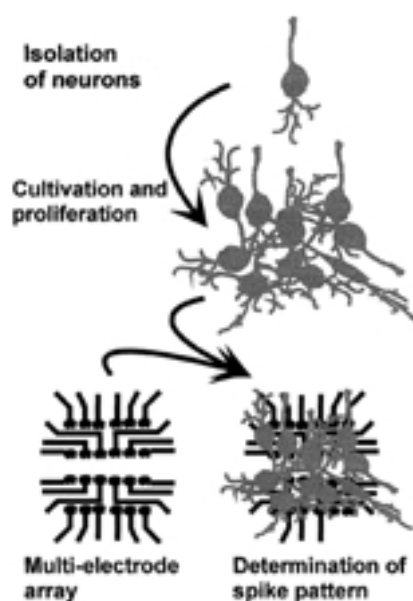
Function	Plant-derived material	References
Metabolism, Ion homeostasis and Activation:		
Ca <sup>2+</sup> elevation	baicalin, baicalein	66
Ca <sup>2+</sup> mobilization	sho-saiko-to, other extracts, various	68 69
neuronal activation	<i>Panax ginseng</i> extracts	72
excited state processes	hypericin	74
Cellular Transport:		
Inhibition of P-glycoprotein (blood brain barrier model)	verapamil (synthetic drug)	
serotonin uptake (astrocytes)	hypericum	64
Stress and Toxicology:		
nitric oxide synthase expression	epigallocatechin gallate	58
nitric oxide production	various dietary compounds	67
cytotoxicity	ricin, volkensin, repin	59 75
excitotoxic injury	2-deoxy-D-glucose glutamate receptor antagonists, various	60 71 69
Survival, Proliferation and Apoptosis:		
protection against seizure activity	kinase inhibitors	70
cell survival and differentiation	inhibitors of cyclin-dependent kinases	62
neurite outgrowth	malonylginsenoside Rb1	73
G2-M arrest	apigenin	61
staurosporin-induced apoptosis	<i>Ginkgo biloba</i> extract	63

proach these functions were rare. However, the recent development of multi-electrode arrays (76–78) for determination of spike generation and propagation in brain slices or in neurons cultured on these arrays (Fig. 3) has considerably improved this situation. The suitability of multi-electrode arrays for screening purposes was already demonstrated with normal drugs (77), (79), (80) and has revealed a great perspective. Another feature, excitotoxicity, is also coupled to neuron activation. There, high concentrations of excitatory neurotransmitters such as glutamate lead to cell death by apoptosis (82). In the past, single cell recording of the electrophysiological activity of the neurons was applied in such studies (83). Again, the use of multi-electrode arrays may revolutionize mechanistic studies on excitotoxicity which might play an important role in neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, trauma, and many others. Although phytopharmaceuticals have not yet been tested using multi-electrode arrays, it can be expected that this new and sophisticated experimental system may considerably advance studies on neurotrophic and neuroprotective effects of plant-derived materials.

#### General Aspects of the Screening of Plant Materials for Effects on Cultured Cells

Plant materials under consideration for efficacy testing are usually composed of complex mixtures of different compounds with different solubility in aqueous culture media. Furthermore, inert additives may also be included. These properties render it necessary to search for appropriate incubation conditions. Some examples relevant for mixtures or

compounds with different solubilities are provided in Table 3. Of course, direct contact of insoluble material with the cultured cells has to be avoided as have higher concentrations of solvents in the culture medium (e.g., exceeding 2% DMSO).



**Fig. 3** Schematic illustration of the use of multi-electrode arrays for determination of neuronal spike generation and propagation. Neurons are isolated from brain tissue and cultured on multi-electrode arrays coated with appropriate substrates (77), (78). Alternatively, organ cultures of brain slices can be used (76). The multi-electrode arrays allow the simultaneous recording of spike generation by different neurons on many (e.g., 64) different channels after

amplification. Using computerized analysis of the spike pattern by so-called neuronal networks it is possible to determine the influence of drugs on spontaneous or induced spike generation and propagation in a semiquantitative manner.

**Table 3** Techniques for the incubation of cultured cells with plant derived materials.

Material	Solubility in water	Handling/Solubilization	Incubation technique
powdered material	low	adsorption to filter paper dispersion in culture medium	floating filter technique bottom chamber of transfilter well cultures
	medium	extraction with solvents (DMSO, alcohols, hexane, etc.)	normal incubation; special solvent controls necessary: solvent alone; solvent added after extraction with water
	high	extraction with water or culture medium	normal incubation
liquid material	low	adsorption on filter paper	floating filter technique
	medium	addition of or extraction with solvents	floating filter technique after evaporation of solvent; normal incubation including solvent controls
	high	dilution with culture medium	normal incubation

This may sometimes severely limit the testing of water-insoluble material. In many cases the so-called floating filter technique may provide a (partial) solution, since excess solvent can be evaporated. If solvents are unavoidable, appropriate control incubations are of greatest importance and should include not only addition of solvent alone to the culture medium, but also to an extract obtained by another extraction procedure.

### Conclusions and Perspectives

As demonstrated for hepatocytes and neuronal cells, cell culture systems provide valuable *in vitro* approaches for the screening of plant extracts, phytopharmaceuticals and drugs as well as for the elucidation of possible mechanisms of action on a molecular level. It is hoped that such systems may reduce animal testing considerably. However, it should be emphasized that the results do not necessarily reflect all actions that may occur in the whole organism, either in animals or in man. For instance, bioavailability remains a critical factor that must be determined in the intact organism. It may depend on different routes of administration as well as on interactions with other constituents or extract formulations. Furthermore, systemic and other complex effects may be responsible for beneficial or adverse effects not detectable by these *in vitro* approaches. Thus, careful clinical investigations must accompany all *in vitro* studies, particularly if high-dosed extracts are considered. Despite these limitations, however, the *in vitro* studies with intact cells may considerably advance our knowledge about the beneficial effects of phytopharmaceuticals and, in some cases, may even provide the only source by which such detailed information can be obtained.

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