Nephrotoxicity Testing *In Vitro*

The Report and Recommendations of ECVAM Workshop 10[1,2]

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Preface

This is the report of the tenth of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM's main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become better informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best ways forward (1).

The workshop on Nephrotoxicity Testing *In Vitro* was held in Angera, Italy, on 16–20

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[1]The European Centre for the Validation of Alternative Methods. [2]This document represents the agreed report of the participants as individual scientists.

May 1994, under the co-chairmanship of Gabrielle Hawksworth, Peter Bach and Fred Nagelkerke. The participants comprised scientists working in both academia and industry, and included representatives from five of the six groups which participated in the recent European Commission (DGXII)-funded BRIDGE (Biotechnology Research for Innovation, Development and Growth in Europe, 1990–1994) nephrotoxicity project. This report describes the systems currently employed for nephrotoxicity testing, the methods used for the determination of various endpoints, and the applications of these methods in industry; it also outlines future developments in the field. As a result of the discussions which took place at the workshop, a number of recommendations have been made concerning the use of *in vitro* systems for nephrotoxicity testing, which are given at the end of this report.

Systems in Current Use

The *in vitro* systems currently employed for nephrotoxicity studies were reviewed during the workshop; the advantages and disadvantages of each system for undertaking toxicological studies are summarised below.

Isolated perfused kidney

The isolated, cell-free, perfused (rat) kidney (IP[R]K) is the most appropriate system for studying potentially nephrotoxic xenobiotics when tubulo-vascular integrity is required (2). This system is not influenced by extrarenal regulatory systems (for example, nervous, hormonal and blood-borne factors). The IPK enables precise control of the concentrations of compounds which are being studied. However, it is not a system to be used for routine studies.

The disadvantage, compared with studies of the kidney *in situ*, is that renal function is maintained for only a relatively short period (up to four hours), although the limited lifetime of this preparation can be improved considerably by adding oxyphoretic compounds to the perfusate (3). The marked decrease in concentrating ability leads to an elevated fractional excretion of water. The perfusion system also leads to higher renal vascular flow (RPF) and lower glomerular filtration rates (GFR), as well as to decreased filtration fractions (FF) and fractional sodium excretion (F_{ENa}), relative to the *in vivo* situation.

Table I: Conditions for using the isolated perfused rat kidney

Parameter	Value
Perfusion pressure (P_A)	80–110mmHg
Perfusate flow (RPF)	20–35ml/minute
Fractional sodium excretion (F_{ENa})	< 7%
Glomerular filtration rate (GFR)	> 0.5ml/g kidney

Three different modes of perfusion are available: a) single pass perfusion; b) recirculation of the perfusate (which minimises the costs incurred due to adding albumin, etc. to the perfusate); and c) recirculation and dialysis of the perfusate. Each of these requires optimal oxygenation of the perfusate. The methodology has been described by Bowman (4), Maack (5) and Bekersky (6). Minimum criteria which should be met when using the IPRK have been outlined in Table I. Experiments should be carried out using sterile materials and solutions, since this will result in longer term viability of the preparation. It should be noted that data obtained using the IPK may differ from those obtained from kidney perfusion studies *in situ*.

Isolated perfused nephron

The isolated perfused nephron is not suitable for routine nephrotoxicity studies. However, investigations conducted with the non-perfused nephron have provided valuable data on enzyme localisation and receptor distribution, which can be used for confirming the site of origin of isolated cell systems (7).

Renal cortical slices

Renal cortical slices have been used extensively for the study of renal transport and toxicity, typically for no more than a two-hour period (8–11). More recently, the use of precision-cut renal cortical slices has become fashionable. They are easy to produce, and differences in the preparation of slices between investigators are minimised by

Table II: Advantages and disadvantages of renal cortical slices

Advantages	Disadvantages
Simple technique	Limited lifetime
Multicellular system	Morphological, functional and biochemical heterogeneity
No enzymatic tissue digestion	Lack of reproducibility of nephron segments
Three-dimensional structure	Lumens may be collapsed (hence only passive transport of *p*-amino-hippuric acid)
Cell–cell contacts maintained	Surface of slice represents damaged area
Site-specific effects can be studied	
Several functional parameters can be assessed	
Renal-specific parameters are maintained	
Rapid and simple species comparisons can be undertaken	

using commercially available tissue slicers. Precision-cut renal cortical slices have been used for periods of up to 24 hours (12–14). The advantages and disadvantages of renal cortical slices are summarised in Table II.

Although this system still needs to be optimised, it shows considerable promise. The following aspects need to be studied further: optimisation of the culture conditions (for example, medium, temperature and pO_2); preparation time; slice thickness and regional distribution (cortical slices should comprise all of the nephrons, from the surface to the outer medulla); and definition of suitable criteria for assessing viability and biotransformation capacity, and for conducting species and sex comparisons. Successful cryopreservation of renal slices would enable more efficient use of human tissue and permit the transfer of material between research groups (15).

This relatively simple technique offers the opportunity to study both renal function and toxicity in different regions of the kidney (i.e. cortex, medulla or papilla), in an *in vitro* system that exhibits many of the characteristics seen *in vivo*.

Isolated glomeruli and proximal tubular fragments

Freshly isolated fragments of renal tissue have been used for assessing the acute effects of chemicals on glomeruli and proximal tubules, including comparisons of superficial and deep glomeruli. The isolation is based on forcing small blocks of tissue (either untreated or subjected previously to enzymatic digestion), in oxygenated buffer, through a series of stainless steel sieves. The glomeruli are resistant to shearing forces and are harvested on a 63μm (rat) or a 106μm (human) sieve. The proximal tubules are collected on a 50μm (rat) or a 75μm (human) sieve. The purity of the fragments is high (> 90% for both glomeruli and proximal tubules), and can be improved by subjecting the preparation to Percoll isopycnic density gradient centrifugation (16). Alternatively, iron oxide particles can be infused into the intact kidney. These lodge in the glomeruli, which facilitates the collection of higher purity glomeruli by magnetic separation (17), and also results in the production of an improved preparation of proximal tubules due to the exclusion of glomeruli. Renal fragments can also be prepared by microdissection.

The glomeruli or tubular fragments can be used to measure a whole range of non-specific, but sensitive, endpoints (see later), as well as a number of processes specific to the cell type of interest. For glomeruli, this would include metabolism, and the synthesis of proteoglycan, collagen and fibronectin; with proximal tubules, metabolism and transport studies can be undertaken. Studies have been undertaken with renal fragments isolated from rat, rabbit, pig, dog and human kidneys. To isolate the fragments, the same basic technique as for isolating cells from these fragments is used (see the following section).

The advantage of using renal fragments is that they retain the architecture of the

anatomical regions of the kidney from which they are derived. They offer a simple, rapid, inexpensive and flexible method for measuring various endpoints in glomeruli and proximal tubules, both of which can be isolated from the same kidney. Renal fragments can also be used to assess alterations in biochemical functions at various times after injury, and may provide useful information about mechanisms of toxicity (18). The limitations of the method are that the surfaces of the renal fragments represent damaged areas, and thus the fragments are only viable for four–six hours at the most; also, the lumen of the tubule may be collapsed, which would preclude their use for luminal transport studies. The isolation of fragments from kidneys with well-developed fibrous networks (i.e. from older animals or humans) may be less successful, unless enzymatic digestion is used during the isolation procedure. However, it is possible to extend the lifetime of these preparations by using sterile conditions and optimised medium, and by the addition of antioxidants during the isolation procedure.

Isolated proximal and distal tubular cells

Cells are isolated by two-stage collagenase perfusion, as originally described by Ormstadt *et al.* (19). The animal is anaesthetised and, after laparotomy, the aorta is cannulated and the kidneys are perfused with a buffer solution containing collagenase. After excision of the kidneys, the capsule is removed and the tissue is dispersed in buffer. The suspension is then filtered through nylon gauze or Endecott sieves, and the filtrate is subjected to Percoll density gradient centrifugation. Rat proximal tubular cells band at a density of 1.02–1.05g/ml; rat distal tubular cells band at a density of 1.08–1.12g/ml. Alternatively, for the isolation of cells from human or rat kidneys, cortical tissue can be minced coarsely prior to two-stage collagenase perfusion and Percoll density gradient centrifugation (20, 21). It is recommended that about 10g human kidney cortex, or two–four rat kidneys, are used. The yield of cells from rat kidney is lower using the latter method, but the procedure is simpler and the functional capacity of the cells is not compromised. Identification of the cells is carried out by determining their enzyme distribution, receptor responsiveness and transport capabilities (22). Tubular cells can also be isolated without the use of digestive enzymes, for example, by free flow electrophoresis (23).

These preparations have been used extensively for acute nephrotoxicity studies. The cells retain most of the characteristics seen *in vivo*. They can, therefore, be used to study mechanisms of toxicity at the cellular level, and to design strategies for reducing/protecting against cell toxicity (i.e. for cytoprotection). Relevant *in vitro–in vivo* extrapolations can also be undertaken, and comparisons can be made between species. However, cell suspensions have a limited lifetime (up to six hours), and primary cell cultures are needed for studies over a longer period. The use of primary cell cultures also enables the effects of lower, and possibly more relevant, concentrations of chemicals to be investigated.

Primary cell cultures

A number of different procedures are used to obtain primary cell cultures. Microdissection techniques have been used to establish explant cultures for all nephron segments. The origin of the cell type is well defined, but microdissection usually results in a low yield, and growth factors and serum-containing medium are needed. The proximal tubular cells obtained are responsive to parathyroid hormone, but not to arginine, vasopressin or calcitonin. They show low activities of alkaline phosphatase, Y-glutamyltranspeptidase and Na^+/K^+-ATPase. The technique can be used to isolate human proximal convoluted tubular cells and proximal straight tubular cells separately (24).

Two immunologically based techniques have been described. Immunodissection has been used for isolating rat and human proximal tubular cells, using a monoclonal antibody against microvillus membrane proteins of rat renal cortical cells. The cells show sodium-dependent glucose transport and Na^+/H^+ exchange. The second immunological method involves flow cytometry, and has been used to isolate human proximal and distal tubular cells (from the same sample) by employing different monoclonal antibodies (25). The main advantage of this technique is the very high purity of the cell preparation obtained, although the yield is low and it is a time-consuming procedure, taking about 20 hours. Nevertheless, it is anticipated that,

with the availability of more antibodies and with further technical developments, the full potential of this technique should be realised.

Higher cell yield is obtained using enzymatic digestion or mechanical dissociation followed by density gradient centrifugation (see the previous section). This procedure can be used to obtain isolated cells or three or four cell fragments, which have a higher seeding efficiency than single cells (16). It results in cell preparations which are enriched in proximal tubular enzymes (for example, alkaline phosphatase) relative to distal tubular enzymes (for example, hexokinase). The method can incorporate glomerular separation by using iron oxides, but this is not essential (17). Mechanical dissociation of cortical tissue has mainly been used to isolate rabbit and rat proximal tubular cells (26). This isolation technique, which was originally described by Brendel & Meezan (27), yields preparations with higher intracellular ATP levels, respiration rates and transport activities than those for preparations isolated by proteolytic digestion and isopycnic density gradient centrifugation (28). Care should be taken to ensure oxygen saturation of the solutions during the isolation procedure. Good seeding efficiencies are obtained on uncoated plastic. Growth initiation can be improved by short, mild treatment with collagenase (5 minutes; 0.125mg/ml). When using small-well plates, it is important to check the seeding efficiency and cell growth. The proliferation of rabbit proximal tubular cells in primary culture decreases as the size of the wells decreases. Seeding densities in the range $1 \times 10^5 - 2 \times 10^5$ cells/cm^2 are used for human and rat proximal tubular cells.

The procedure used for isolating proximal tubular cells may be crucially important for subsequent cell growth requirements. Constant hyperoxygenation of cells should be employed to obtain optimal cell seeding efficiency. The most widely used culture medium is 1:1 Dulbecco's modified Eagle's medium/Ham's F12. For optimal attachment after Percoll separation, fetal bovine serum, collagen-coated plastic, or both of these, are needed. The medium usually contains hydrocortisone/dexamethasone and insulin. Following mechanical dissociation, the cells have much less stringent growth requirements, and the use of fetal calf serum (FCS) for cell attachment should be avoided because of its detrimental effects on cell differentiation. With rabbit proximal tubular cells, the use of glucose-free and insulin-free medium may improve cell differentiation, and the maintenance of carbohydrate metabolism (by preventing loss of the gluconeogenic pathway), brush border enzyme activities and cellular architecture.

The medium should contain only low concentrations of antibiotics (25μg/ml for the first 48 hours) and no antifungals. When using porous membranes, culture of cells on Type IV collagen is the most appropriate method for maintaining both organic anion and cation transport (29). Culture on porous membranes is essential for studying vectorial transport and results in better maintenance of the brush border in proximal tubular cells. It also enables measurement of the confluency and intactness of the renal epithelial cell barrier. Coating the porous membranes with "Matrigel" or other substrata results in better differentiation, and the cellular morphology more closely resembles that observed *in vivo*. It has been shown that the culture of Madin-Darby canine kidney (MDCK) cells on Type I collagen results in dedifferentiation, with the induction of a fusiform shape and the loss of apical–basal polarity (30). More data are needed on the maintenance of enzyme activities, such as those associated with glutathione-*S*-transferases and cysteine conjugate C-S lyases, which are strongly implicated in the nephrotoxicity of several xenobiotics. Proximal tubular cell cultures from rats, rabbits, dogs and humans have been described, but there are no data on cultures derived from either marmosets or mini-pigs. In addition, better methods are needed for the culture of rat proximal tubular cells. It is difficult to obtain confluent cultures of rat proximal tubular cells in the absence of serum and growth factors, but these additives are detrimental to cell differentiation.

Isolated human proximal tubular cells can be cryopreserved in FCS containing 10% dimethyl sulphoxide, and they show good recovery and viability post-thawing. However, rabbit proximal tubular cells do not recover and grow in culture when cryopreserved using similar conditions.

Separation by Percoll density gradient centrifugation can also be used to obtain a cell population enriched with distal tubular cells

(see the previous section), but these cells have not been purified to the same extent as proximal tubular cells (25). The criteria employed for assessing distal tubular function are the transport characteristics of the cells.

Cells from the medullary thick ascending loop of Henlé (mTALH) have been separated by using centrifugal elutriation or immunological methods; they are characterised by the presence of the Na^+/K^+-cotransporter (31).

Human mesangial and epithelial cells from the glomerulus are isolated following dissociation of the tissue and collection of the glomeruli on a 106μm sieve. The glomeruli are cultured, and epithelial cells can be seen growing out of the glomeruli after 7–10 days. After 2–3 weeks, the mesangial cells overgrow the glomerular epithelial cells (32). The disadvantage of this method is that the cultured cells cannot be compared with freshly isolated cells to ensure that dedifferentiation has not occurred. Morphological and immunohistochemical, rather than functional, markers are used for identification purposes. These cells have not been widely used for toxicity studies, although the effect of cadmium on proteoglycan sulphation and excretion by cultured rat mesangial cells has been investigated (33).

Medullary interstitial cells have been isolated from rats, rabbits and dogs and, more recently, from humans. For species other than man, cultures have been established from cells derived from subcutaneous autotransplants or isogeneic transplants, or by selective growth of cells in hypertonic medium (34). Human medullary interstitial cells have been isolated from primary explants of papillae by selective overgrowth of the interstitial cells. They are characterised morphologically and histochemically, and by the determination of prostaglandin E_2 production and angiotensin II responsiveness (35). These cells need to be further characterised and compared with readily available cell lines, such as 3T3 cells, to assess their usefulness for toxicity studies.

Renal cell lines

Cell lines derived from the kidneys of various species are commercially available (Table III). It should be noted that differences in cell lines obtained from the same source have been observed. The cell lines used most in toxicological studies are those which exhibit *in vitro* some of the properties of the proximal tubular epithelium (PTE) *in vivo*, such as LLC-PK_1, LLC-RK_1 and OK cells. In culture, these cells grow to form a confluent monolayer, and they exhibit some degree of differentiation (for example, they show the morphological asymmetry of PTE cells which is observed *in vivo*). They have been shown to exhibit apical–basolateral polarisation, with brush border microvilli and basolateral infoldings being present. The expression of brush border microvilli is greater with LLC-PK_1 cells than with the LLC-RK_1 and OK cell lines, and is similar to that observed in the PTE *in vivo*. OK cells can be used to study parathyroid hormone-dependent sodium and phosphate transport. The expression of differentiated functions specific to the PTE varies with the particular cell line, and has been reviewed in detail by Gstraunthaler (36). The level of expression may differ according to the passage number, and with the composition of the culture medium, in particular in the presence of FCS or in hormonally defined medium (37).

Transepithelial transport may be evaluated if the cells are grown on porous membranes. Unfortunately, the permanent cell lines which have been characterised with respect to their transport properties lack an organic anion transporter; the only exception to this is the OK cell line, which has been shown to accumulate *p*-aminohippurate.

The main advantages of renal cell lines are their ease of handling, the speed with which experiments can be conducted, and the fact that laboratory animals are not needed to obtain tissues. However, none of the cell lines available show complete expression of those functions expected of proximal tubular cells. They often lack both Phase I and Phase II metabolising enzyme systems, and receptor-linked signalling systems. Another major disadvantage is the lack of useful cell lines which are representative of the different parts of the nephron, and which have been derived from the key species used in toxicological studies, such as the rat and dog, and from primates and humans. More information is required on the metabolic capabilities of existing cell lines, since biotransformation is often an important determinant of the mechanism of chemical-induced nephrotoxicity.

Table III: Some readily available renal epithelial cell lines

Cell line	Species of origin	Nephronal origin	Catalogue No.
LLC-PK_1	Pig	Proximal tubule	CRL 1392[a]
LLC-RK_1	Rabbit	Proximal tubule	CCL 106[a]
OK	Opossum	Proximal tubule	CRL 6551[a]
JTC-12	Monkey	Proximal tubule	RCB 456[b]
MDCK	Dog	Distal tubule/collecting duct	CCL 34[a]
MDBK	Cow	Cortex	CCL 22[a]

[a] *American Tissue Culture Collection*

[b] *Japanese Tissue Culture Association*

Hybrid cells

Fusion of primary cells with established cell lines derived from the same region of the nephron may yield new cell lines which better retain some *in vivo* characteristics. Classically, mouse B lymphocytes can be immortalised by fusion with a murine myeloma cell line; the hybrid cells retain the expression of antibody (38). However, with other cell types, the hybrid cells do not always show an immortal phenotype, nor is expression of differentiated phenotypes always observed. Furthermore, hybrid cells show chromosomal segregation and therefore do not have a stable phenotype.

Immortalised cells

The approach to cell immortalisation is to introduce oncogenes, for example, SV40 T antigen, *myc* or adenovirus E1A, into cells by gene transfer, in order to overcome cellular growth mechanisms. These oncogenes act by binding to normal cellular proteins which are encoded by tumour suppressor genes, thereby permitting progression through the cell cycle. One approach is to introduce the gene into the embryo and then to isolate cell lines from the transgenic animal. This works well for the mouse, but is not so useful for other species. Alternatively, the gene can be added to primary cultures of cells, for example, by calcium phosphate precipitation. The oncogene can be expressed constitutively or can be placed under the control of an inducible promoter (for example, heavy metal induction is used for the metallothionein promoter). The latter approach provides the opportunity to switch between cell growth and expression of the differentiated function (39, 40).

Immortalised cell lines have been isolated from the proximal tubule from transgenic mice (41), and by the transfection of rabbit mTALH (42, 43), rabbit proximal tubule (43), rat kidney epithelium (44), and human embryonic kidney (45, 46) cells. Cell lines have also been isolated by SV40 infection (39, 40). Many of these express tissue-specific characteristics, such as the expression of Tamm-Horsfall glycoprotein and ion transport systems. They have not, to date, been used for toxicity studies.

Future developments should include the characterisation of existing cell lines with respect to their xenobiotic metabolising activities, and the isolation of more cell lines which express the oncogene in a regulatable manner. Isolation of cell lines from different species would enable comparative data on interspecies differences in metabolism to be generated.

Genetically engineered cells

Cell lines which have been transfected with cloned genes, for example, with genes for transport protein receptors or xenobiotic metabolising enzymes, provide a powerful tool for studying mechanisms of action, since it is possible to investigate a particular system in isolation (47). They are less likely to be useful for *in vitro* toxicology studies, because not all of the systems involved in bioactivation or detoxification are present.

Subcellular fractions

Fractionated organelles and membranes prepared from the kidneys in general, or from defined cell types, can be used for specific cell-free investigations. These *in vitro* systems, which are usually prepared by differential centrifugation, include nuclei, mitochondria (48), lysosomes (49) and membrane vesicles (50). The purity of the preparations is confirmed by marker enzyme assays, and by transmission and scanning electron microscopy. Lysosomal membrane integrity is evaluated by measuring enzyme latency (49), and mitochondrial function is determined by respiratory control assessment (48). Brush border and basolateral vesicles can be prepared from the same proximal tubular cells, and they can be used to study the membrane-specific transport of xenobiotics and the effects of chemicals on the transport of endogenous compounds.

Databases

The use of databases for predicting the potential nephrotoxic effects of chemicals is an area which needs to be developed, since it could reduce the duplication of studies and decrease the use of laboratory animals. The major problems with this approach are the reliability of, and the differing conditions used for, many of the *in vivo* and *in vitro* studies which have been reported in the literature. It is recommended that databases should incorporate data on the toxic response patterns of known nephrotoxins. Information submitted by industry (unattributable, if possible) would be very valuable, particularly where *in vivo* nephrotoxicity data are available. Otherwise it may be necessary to conduct *in vivo* studies to generate these data.

Endpoints of Toxicity

Morphological

Light microscopy is widely used for assessing cellular changes. The perfused kidney, renal tissue slices and tissue/cellular fragments can be fixed and processed by routine histopathology techniques, and can be assessed subjectively or quantitatively. The use of histochemical and immunohistochemical techniques can provide additional information which is relevant to both screening and mechanistic studies. The endpoints that are commonly used include lethal (necrosis) and sub-lethal cell injury, and the loss or accumulation of those cellular structures, macromolecules and/or activities which can be detected histochemically.

The use of light microscopy is especially appropriate for assessing cells in culture by phase-contrast or fluorescence microscopy. These techniques are most commonly used to monitor, qualitatively, cell growth and dynamic changes; in combination with video microscopy, these changes can be quantified. Useful endpoints include cell counting, determination of the differentiated structure (domes), and the maintenance of specific functions. The use of confocal microscopy and appropriate fluorescent probes (for example, to study gap junctions) provides quantitative information on the distribution and dynamics of subcellular structures, such as lysosomes, the cytoskeleton and membrane proteins. Time-lapse video microscopy, image analysis and spectroscopic measurements at the cellular level (spectrofluorimetry, single photon counting, and confocal microscopy) have been used to facilitate documentation of the sequence of cellular changes (such as alterations in ion distribution, volume and intracellular transport processes) which occur following exposure to chemicals, and also during any subsequent recovery of the cells (51). Subtle subcellular changes can be assessed qualitatively by electron microscopy.

Microscopy thus forms the bridge to *in vivo* toxicology, especially for the more complex *in vitro* systems. The application of new morphological techniques combines assessment of both biochemical and functional data at a cellular level. The disadvantage is that many of the techniques alter or kill the cells, so that these data represent single, discontinuous endpoints. There is also the risk of introducing bias if subjective assessments are made. It is recommended that quantitative image analysis should be used more widely, especially in combination with histochemical methods.

Functional

Various functional endpoints, and their applicabilities in the *in vitro* systems described earlier, are summarised in Table IV.

Table IV: Functional endpoints used in *in vitro* nephrotoxicity assays

Endpoints	Applicability	Comments
Cell viability		
Dye exclusion/retention Enzyme/ion leakage	Renal cortical slices Renal fragments Isolated cells (in suspension) Primary cell cultures Cell lines	Applicable in intact cell systems and slices (for example K^+ content) Enzyme leakage provides information on regiospecificity NB: MTT assay is not specific for mitochondrial damage
Synthesis of macromolecules		
Protein DNA/RNA Matrix elements	Renal cortical slices Renal fragments Isolated cells Primary cell cultures Cell lines May be useful in glomerular/ interstitial cell cultures	Check uptake of precursors
Rate of proliferation		
(Clonogenic assays)	Cell lines	Total protein measurements (usually reflect cell number)
Carrier-mediated transport		
Glucose Organic ions Inorganic ions Low molecular weight proteins	Systems containing proximal tubule cells Isolated tubules (transepithelial transport) Cultures on porous membranes	Can be quantified by uptake or transport Uptake applicable to all transepithelial systems Check effects of specific inhibitors Check paracellular transport Not for routine use Not widely used
Endocytosis of labelled proteins or carbohydrates		
Cultured cells of proximal tubular origin		Usually use radiolabel or horse radish peroxidase Easy *in vivo/in vitro* comparison
Barrier function	All epithelial systems, intact epithelia or confluent cultures on porous membranes	Assessment of diffusion of extracellular markers, or electrophysiological measurements

Biochemical

The scheme outlined in Figure 1 illustrates how a chemical may interact with a renal cell to result in cellular injury and/or repair.

Studies with the IPK can provide valuable information on the intrarenal handling of xenobiotics and their metabolites, and they may also provide information on selected endpoints, such as interactions with macromolecules, and effects on ATP synthesis and glutathione depletion (Figure 1). The IPK is the only system in which the integrated function of the whole organ can be studied, but it is not applicable for routine studies.

Well-defined *in vitro* renal cell systems are ideal for studying the potential interactions of a chemical with cellular structures and functions. Initial studies should explore the time-response and dose-response relationships; further work should then be conducted at relevant, sublethal, doses. The concentrations used should be relevant to those delivered to the kidney *in vivo*. It is possible to elucidate the mechanisms of toxicity of chemicals by studying the biochemical cascade of events outlined in Figure 1, and to correlate the early effects of the interaction of a chemical (for example, the formation of protein adducts) with late endpoints relevant to observed changes in cell function. Flow cytometry is a promising new approach in which a number of biochemical parameters, including second messenger systems and $[Ca]_i$ and $[pH]_i$, can be studied simultaneously with cell cycle events and DNA cleavage. Mechanisms of cell death, including apoptosis and/or necrosis, can be studied in real-time fashion following the exposure of cells to toxic compounds (52).

The major problems with the *in vitro* systems used currently are their limited, or undefined, capacities for xenobiotic biotransformation. If renal cell lines are to be applied more widely, such as for routine testing purposes, the biotransformation capacities of the cells used should be evaluated and, for specific purposes, cells expressing certain key metabolising enzymes could be constructed using gene technology.

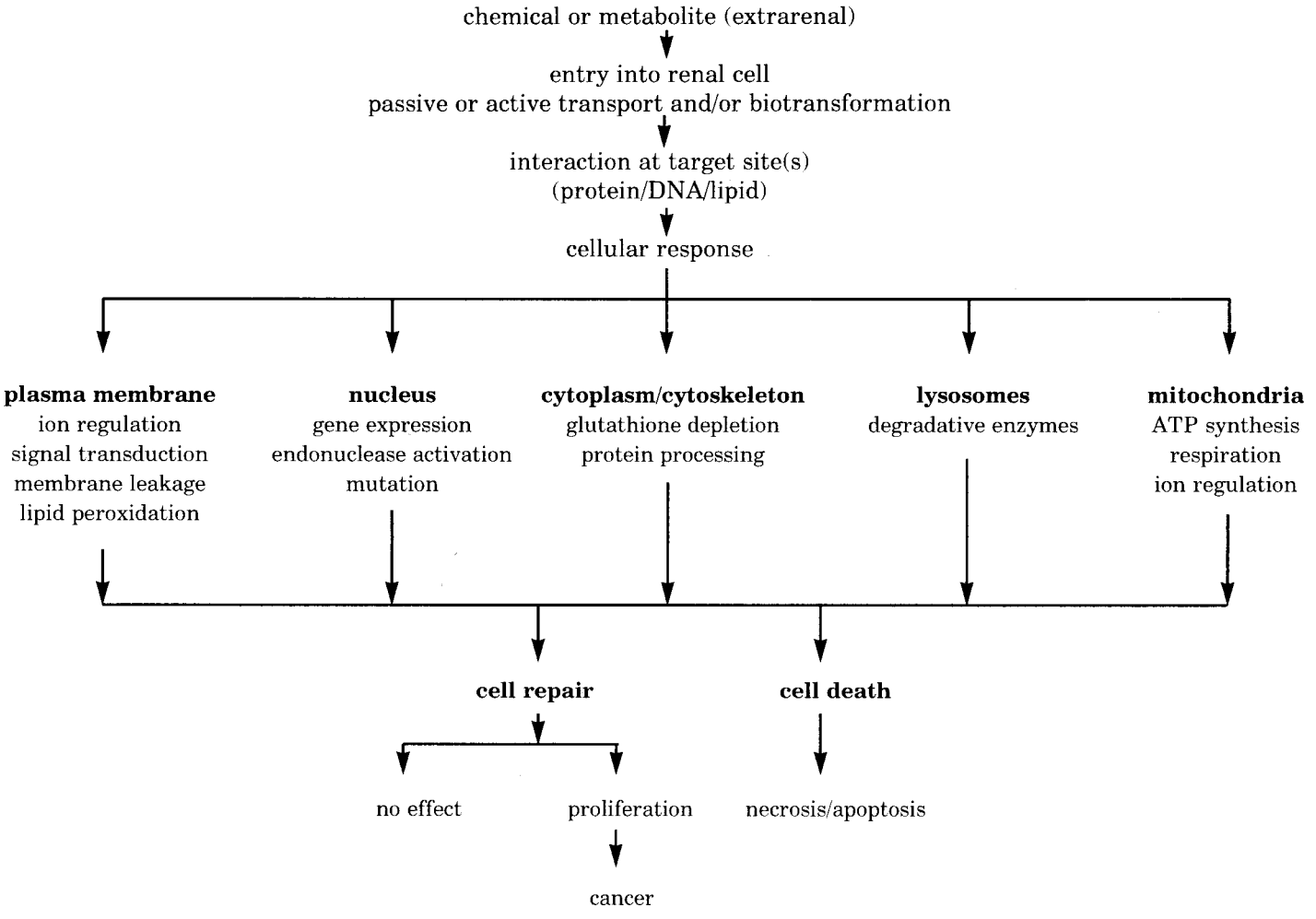
Applications

Risk assessment procedures are applied to provide the best estimate of the expected adverse effects of chemicals in exposed populations. These adverse effects depend on the possible toxic effects of the chemical itself (i.e. the hazard) and the extent of human exposure. Toxic effects of chemicals are influenced by their toxicokinetics and toxicodynamics. Toxicokinetic parameters describe the uptake, distribution, biotransformation and excretion of the chemical, and thus define the concentration of the chemical at the target site. Toxicodynamic parameters describe the interactions of the chemical with cellular macromolecules, which result in the toxic effects observed. Risk assessment is usually performed (at least in Europe) by collecting all available data on the adverse effects of a chemical in humans, experimental animals and simpler biological systems, data on toxicokinetics in animals and, if possible, in humans, and information relating to probable exposure levels.

The major potential for *in vitro* techniques to assist in the hazard identification process for nephrotoxic compounds relates to their use for elucidating mechanisms of chemical-induced acute renal damage, and in identifying certain early endpoints known to be important in chronic processes, such as in carcinogenicity. In combination with other studies on biotransformation, *in vitro* systems which retain the architecture of the intact kidney may also provide information on selected aspects of toxicokinetics, such as on renal handling and intrarenal bioactivation.

The major limitations to using the currently available *in vitro* systems for risk assessment purposes are the limited/undefined biotransformation capabilities of these systems and, due to their dissociation from the intact organism and *in vivo* toxicokinetics, the poor predictivity of these systems for the dose-response curves expected in animals. Moreover, nephrotoxicity due to the production of metabolites in organs other than the kidney, or due to the accumulation of a xenobiotic because of a particular route of administration (for example, the hepatic first pass effect may be important following exposure via the oral route), may not be detected by the exclusive application of *in vitro* systems for nephrotoxicity testing. Further limitations are the inability of the current *in vitro* systems to predict complex degenerative processes which result in organ damage. Future developments, including the co-culture of renal cells with non-renal systems,

Figure 1: Biochemical endpoints in *in vitro* nephrotoxicity assays



such as hepatocytes or cells of the immune system, may help to overcome some of these problems.

In vitro techniques can therefore be introduced into the hazard identification strategy as major tools for elucidating the mechanisms of action of nephrotoxic chemicals. Moreover, *in vitro* systems can be used to screen series of chemical analogues, in order to identify those compounds which have the lowest nephrotoxic potentials, when the toxicokinetics of at least one of the compounds is known or can be estimated by substrate structure-activity relationships.

The specific needs of industry include the availability of metabolically competent systems for nephrotoxicity studies, derived from rodent and non-rodent species, and from humans. These systems should be capable of distinguishing organ-specific and site-specific effects. Co-culture of renal systems wth extrarenal metabolising systems (for example, by adding inserts of renal cell cultures to tissue culture plates seeded with hepatocytes) is also needed. For primary screening, precision-cut renal slices, including those from human kidney, could be used to undertake rapid interspecies comparisons of metabolic profiles and chemical-induced toxicity. These slices can be used for up to 24 hours and they enable the investigation of site-specific toxicity. The technique requires less expertise than cell culture, and could now be subjected to prevalidation. Slices, renal fragments, and isolated (in suspension) and cultured renal cells can be used for secondary screening and mechanistic studies. The availability of immortalised cell lines from a range of species would be advantageous. The actual *in vitro* systems employed will obviously be determined on a case-by-case basis, depending on the specific question being addressed.

Future Developments

It is likely that future technical developments will include the production of: a) immortalised cell lines, derived from different renal cell types, which maintain all of the cell-specific functions found *in vivo*; b) heterologous expression systems for xenobiotic metabolising enzymes, which express, in particular, key human enzyme activities; and c) more elaborate cell culture systems, such as continuous flow cultures and co-cultures of renal cells with extrarenal cells. The co-culture of freshly isolated cells and/or permanent cell lines with vascular cells or cells of the immune system could lead to the development of *in vitro* systems which more closely resemble the situation *in vivo*. A new method for primary cell culture, incorporating the continuous superfusion of medium, appears to be promising, since it enables the maintenance of primary cultures of different tissues for prolonged periods of time without the loss of cell-specific functions (53). The latter two approaches may be more successful than cell immortalisation in achieving the maintenance of cell-specific functions. The establishment of databases containing *in vitro* and *in vivo* nephrotoxicity data suitable for use for comparative purposes is important for the future validation of *in vitro* systems for nephrotoxicity testing.

Summary and Recommendations

The state-of-the-art with respect to nephrotoxicity testing *in vitro* can be summarised as follows:

1. *In vitro* systems can be used to study the relative toxicities of a series of compounds.
2. *In vitro* systems are useful for mechanistic studies.
3. With optimised phenotypic expression, cell culture systems can be used over a longer period of time than freshly isolated preparations, such as cell suspensions or tissue slices. In theory, this enables compounds to be tested at more realistic concentrations, which may be more relevant for the prediction of subchronic effects *in vivo*. However, more attention needs to be focused on the refinement of cell culture systems, in order to model toxicity which is manifest *in vivo* only after chronic administration.
4. The use of existing renal cell lines is limited by their incomplete characterisation, and by the absence of some renal cell-specific transport processes and biotransformation systems.

The following recommendations are made:

1. There is a dearth of information in the

public domain on correlations between *in vivo* and *in vitro* data. A database, drawing on both published and unpublished information from proprietary sources, and preserving confidentiality where necessary, should be established and maintained. Wherever possible, dose-response data from rodent and non-rodent species, and humans, should be included. The need to establish such a database should be addressed by ECVAM.

2. For screening purposes, the most relevant and sensitive endpoints for the assessment of toxicity should be determined. These will probably vary for different *in vitro* systems. Using representative nephrotoxins, known to act by different mechanisms, the cascade of toxic events should be elucidated and the early nephrotoxic changes identified. These early changes must be relevant to the final manifestation of toxicity, if they are to serve as indices of nephrotoxicity. Barrier function tests performed using simple and relatively inexpensive electrophysiological equipment may be useful screens with cells grown on microporous supports.

3. Most of the *in vivo* nephrotoxicity data available are derived from the rat, but it is difficult to obtain confluent cultures of rat proximal tubular cells. Thus, the optimisation of culture techniques for these cells is required.

4. The most appropriate non-rodent species for the development of *in vitro* systems should be determined. Most of the data on primary proximal tubular cell cultures are from the rabbit, a species rarely used for toxicity testing *in vivo*. Tissue culture systems from the dog, mini-pig and marmoset should be considered.

5. The availability of metabolically competent systems is of great importance. The metabolic capabilities of *in vitro* systems used for studying nephrotoxicity should be evaluated and compared. Strategies should be developed to preserve or restore metabolising enzyme activities *in vitro* relative to those *in vivo*. It is also essential that *in vitro* systems exhibit the same cell-specific transport systems that are present *in vivo*.

6. There is a considerable amount of literature on the use of primary cultures of proximal tubular cells for pharmacological and toxicological studies. Improved *in vitro* systems are needed for other cell types, such as distal tubular, glomerular, collecting duct and medullary interstitial cells. Comparative toxicity studies should be undertaken with primary cell cultures and available cell lines, including those derived from humans.

7. Co-cultures of renal cells/tissue slices with extrarenal metabolising cells are needed, so that the toxic effects of compounds activated in other organs, particularly in the liver, and then excreted by the kidney, can be investigated. Co-culture systems incorporating cells from the immune system are also required, since some nephrotoxic effects may, either directly or indirectly, involve these cells.

8. There is a key requirement for *in vitro* systems which are able to predict chemical-induced nephrotoxic effects which occur *in vivo* only following chronic administration. Thus, efforts should be directed toward the development of stable *in vitro* systems which are capable of detecting chronic and sub-chronic effects of chemicals.

9. Precision-cut cortical and medullary/papillary slices should be evaluated for their usefulness in determining site-specific and species-specific toxicity. The applicability of previously cryopreserved human kidney slices for biotransformation and nephrotoxicity studies should be assessed.

10. Two alternative strategies for optimising *in vitro* cell culture systems should be considered: a) stable immortalised cell lines, derived from the principal laboratory test species and from humans, should be developed and characterised with respect to their metabolic competence and transport properties; and b) existing renal cell culture systems should be characterised and manipulated to optimise them relative to the functions and properties expressed by the cells *in vivo*. The difficulties inherent in the production of suitable immortalised cell lines suggest that progress will be slow and, therefore, both strategies should be pursued concurrently.

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