# Toxicity testing: the search for an *in vitro* alternative to animal testing

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**ABSTRACT**

Prior to introduction to the clinic, pharmaceuticals must undergo rigorous toxicity testing to ensure their safety. Traditionally, this has been achieved using *in vivo* animal models. However, besides ethical reasons, there is a continual drive to reduce the number of animals used for this purpose due to concerns such as the lack of concordance seen between animal models and toxic effects in humans. Adequate testing to ensure any toxic metabolites are detected can be further complicated if the agent is administered in a prodrug form, requiring a source of cytochrome P450 enzymes for metabolism. A number of sources of metabolic enzymes have been utilised in *in vitro* models, including cell lines, primary human tissue and liver extracts such as S9. This review examines current and new *in vitro* models for toxicity testing, including a new model developed within the authors' laboratory utilising HepG2 liver spheroids within a co-culture system to examine the effects of chemotherapeutic agents on other cell types.

Key words: Animal testing alternatives.
Cytochrome P-450 enzyme system.
Drug toxicity.
Prodrugs.

## Introduction

Prior to introduction to the clinical setting, new pharmaceuticals must undergo rigorous toxicity testing during development to ensure their safety. Various toxicity tests are conducted depending on the length of potential drug usage in humans. Acute oral toxicity tests involve the administration of a single large dose to two different mammalian species and the short-term effects in organs and tissues are observed over several weeks to determine specific toxic effects and the mode of toxicity.[1]

Subchronic and chronic toxicity tests are performed for pharmaceuticals intended to be used in humans for periods of less than or greater than three months, respectively. Therefore, daily administration of the agent in two mammalian species enables longer-term toxicity and pathology to be evaluated, including, for example, the occurrence of neurological and haematological abnormalities. In addition, if an agent is intended for use over periods greater than six months, carcinogenicity studies and mutagenicity studies may be necessary, as well as teratogenicity evaluation, if appropriate.[2]

While current toxicity testing plays an extremely important role in drug development, it is not without room for improvement, with major disadvantages including cost, false-positive/false-negative results, and large numbers of animals used in the process. As knowledge and technology advance, there is a continual drive to improve the efficacy of such testing and a move towards a greater number of *in vitro* alternatives.

## Traditional animal testing

Traditionally, drug safety testing has been achieved through *in vitro* tests and testing involving large numbers of animals, and is an extremely costly and time-consuming process. Indeed, a large backlog of chemicals on the Western market are still awaiting testing in order to characterise toxicity properly. A recent study by Ukelis *et al*.[3] estimated that only 5000 of 100,000 available chemicals have undergone appropriate testing.

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The use of animals in drug safety testing is, however, a sensitive area and subject to an ever-increasing demand to reduce the numbers used. This was first suggested by Russell and Burch[4] as the 3Rs principle, to reduce, refine and replace use of laboratory animals.

Indeed, according to the Home Office Statistics of Scientific Procedures on Living Animals,[5] toxicology testing and safety and efficacy evaluation accounted for 416,400 procedures in 2007, 39% lower than in 1995. However, although widely publicised, toxicology studies account for only 13% of animal testing, with large numbers of animals also used in areas such as genetic studies, production of biological materials and pharmaceutical research.

In addition to reducing the numbers of animals used in individual trials, the need for improved communication to reduce unnecessary duplication of tests was recognised, and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) subsequently was formed in 1990 with the aim of minimising animal testing and duplication of human trials without compromising safety. Greater similarity between animal testing guidelines in different countries is also necessary. Current EU guidelines for acute oral toxicity testing require tests to be conducted in two or more species, preferably rodents. However, in Japan, two

species are required (including one non-rodent), and three species are necessary in the USA, one of which must be non-rodent.[3]

## Progress of animal testing

In recent years, progress has been made with toxicity testing *in vivo* in terms of reducing the numbers of animals used and also in the extent of tests used. Traditionally, the LD50 assay has been used, which is a measure of the dose required to result in death in 50% of the animals in the study. An alternative to this assay was first suggested by the British Toxicology Society in 1984 and was based on administering a series of fixed doses and relying on the observation of clear signs of toxicity, rather than the endpoint of the assay being death. This was introduced as a test guideline in 1992 and was found to use fewer animals and cause less suffering than the LD50 test. The LD50 test for acute oral toxicity has since been abolished following an OECD Joint Meeting in 2001.[1]

A recent initiative between 18 European countries has also reviewed the use of acute toxicity tests in pharmaceutical development. The analysis indicates that acute toxicity test results in practice are not used to determine doses to be administered in phase I clinical trials, nor are they useful in terminating production of drugs in development. Conclusions reached by the group determined that acute toxicity tests are no longer often the first test carried out, and generally they are less useful than data generated from other tests routinely incorporated into toxicity testing.[6]

## Concordance of animal data with human toxicity

Besides the ethical reasons for reducing animal testing, lack of predictability of human toxicity from animal trials has been cited as a concern. While generally animal trials can give good indications as to human toxicity, many studies have reported a lack of concordance, termed interspecies uncertainty.[7] False-positive results from animal trials may result in withdrawal of a drug from trials or the use of subclinical doses. Conversely, possibly a worse scenario involves false-negative results, which may lead to unexpected human toxicity.

This was illustrated dramatically in the TGN 1412 phase I clinical trial in 2006. TGN 1412 is a monoclonal antibody thought to have an anti-inflammatory effect via activation of T-regulatory cells, and have potential use in the treatment of leukaemia and autoimmune diseases. However, the opposite effect was seen in human trials, with the production of massive systemic inflammatory responses.[8] A subsequent investigation concluded that these serious adverse effects were not predicted in humans following apparently adequate preclinical animal tests.[9]

While TGN 1412 is a particularly dramatic example, further lack of concordance between human and animal trials has been reported previously in other studies. A report conducted in 2000 by Olson *et al*. examined data from 12 pharmaceutical companies on 150 compounds (including 221 reported human toxicity events) and aimed to understand better the concordance between human toxicity and that observed in laboratory animals.[10]

Concordance overall was found to be 71% when using rodent and non-rodent test species. However, using non-rodents (primarily dogs and some monkeys) alone reduced concordance to 63%, and to just 43% when using rodents only (primarily rats, with some studies in mice), possibly highlighting potential issues where guidelines do not enforce the use of non-rodent species in combination with rodents, as is the case, for example, in the EU.[3]

Extrapolating safety tests on animals to set human doses is often achieved by taking a dose associated with a particular toxicity and dividing by a series of uncertainty factors. This is designed to take into account species differences, and the correlation of toxicity generally rising with increasing body weight.[7] In the USA, for example, interspecies uncertainty is generally assigned a ratio of 10:1 (to allow for humans potentially being up to 10 times more sensitive to the agent than test animals). However, a recent study by Price *et al*.[7] investigated the validity of this ratio in terms of antineoplastic agents and concluded that it may be inappropriate for a number of drugs.

When comparing human and mouse toxicity ratios, in particular, the mean ratio in 54 agents was 20:1. In contrast, human:dog toxicity ratios were most closely correlated, with a mean value of 3.5:1 – well within the value of 10 typically used in safety tests. Additionally, the report illustrates that human toxicity data are derived from individuals with cancer, who are often also elderly and tend to possess

**Table 1.** Important anticancer agents metabolised by the cytochrome P450 enzyme system.

| Agent | Activating enzymes | Deactivating enzymes | References |
|---|---|---|---|
| Cyclophosphamide | 2B6, 2C19 | 3A4, 2A6 | 11, 12 |
| Dacarbazine | | 1A1, 1A2, 2E1 | 11 |
| Etoposide | | 3A4, 1A2, 2E1 | 11 |
| Flutamide | 1A2 | | 12 |
| Paclitaxel | | 2C8, 3A | 11 |
| Tamoxifen | 2D6, | 3A4, 3A5, 2B6, 2C9, 2C19 | 12 |
| Tegafur (prodrug of 5-flurouracil) | 2A6, 2C9 | | 12 |
| Vincristine | | 3A | 11 |

**Agents are used in the treatment of the following tumours:** sarcoma, breast, ovarian, leukaemia (cyclophosphamide), metastatic melanoma (dacarbazine), lymphoma, osteosarcoma, testicular, small cell lung (etoposide), prostate (flutamide), ovarian, breast, non-small cell lung (paclitaxel), breast (tamoxifen), metastatic colorectal (tegafur), lymphoma (vincristine).

diminished DNA repair capabilities, and may therefore be compromised in terms of health and resistance to toxicity compared with the general population.

## Toxicity testing of prodrugs

Safety testing of clinical agents is increasingly difficult if the drug in question requires metabolism to the active form (e.g., by cytochrome P450 enzymes). Inadequate testing of such prodrugs may result in poor prediction of toxic metabolites, both active and inactive. While only a limited number of pharmaceuticals are administered in a prodrug form, the vast majority of drugs still require the presence of P450 enzymes to aid detoxification and elimination from the body (a small number of examples of which are given in Table 1). Lack of concordance with animal models, or poor sources of P450 enzymes in *in vitro* models, may prevent adequate evaluation of drugs in terms of the extent and kinetics of their clearance metabolism and any toxic by-products.

*In vivo*, enormous variation may be seen due to species differences as well as within species due to the highly polymorphic nature of the cytochrome P450 enzymes.[13] Dietary and environmental factors can also significantly affect P450 enzymes; for example, CYP1A2 is significantly higher in smokers than non-smokers, and only two weeks of a high protein/low carbohydrate diet can significantly increase levels of CYP1A1.[14] In addition, gene copy number variation (CNV) where large amounts of DNA (>1 kb) are duplicated or deleted can also greatly affect gene expression and inter-individual variation, a phenomenon known to be involved in P450 genetic variability.[15]

Consequently, only 30–60% of common drug therapy is effective, at least in part due to genetic variation between individuals.[16] Providing a source of enzymes such as P450 in an *in vitro* test system and allowing for large inter-individual variability present challenges to drug safety testing in the laboratory.

Traditionally, cytochrome P450 enzymes have been sourced from preparations such as S9 liver extracts, where, following processing, a specific liver homogenate fraction is a rich source of P450 enzymes.[17] Sources include rat liver, where agents such as phenobarbital are often administered to increase protein and activity levels.[18] However, phenobarbital itself has been reported to be mutagenic,[19] and species differences occur within the P450 system. S9 preparations are also available from human liver sources, removing the challenge of species variation; however, cost and availability of such preparations may limit their use in *in vitro* safety testing.

Lack of inducibility may also limit use of S9 preparations as a representative P450 system, as S9 is a protein extract only and cannot be induced to up-regulate gene expression. *In vivo*, however, some agents, including the commonly used chemotherapeutic agent cyclophosphamide, are able to up-regulate the gene expression of relevant P450 enzymes, thereby inducing their own metabolism in subsequent exposures.[20]

Primary liver tissue as a source of enzymes for *in vitro* testing is a highly attractive option; however, difficulties with ethics, cost and obtaining sufficient quantities greatly limit use. In addition, primary tissue in culture has a low proliferative rate, which in toxicity studies may be a disadvantage as proliferation can be used as a sensitive marker of adverse events such as hepatotoxicity.[21]

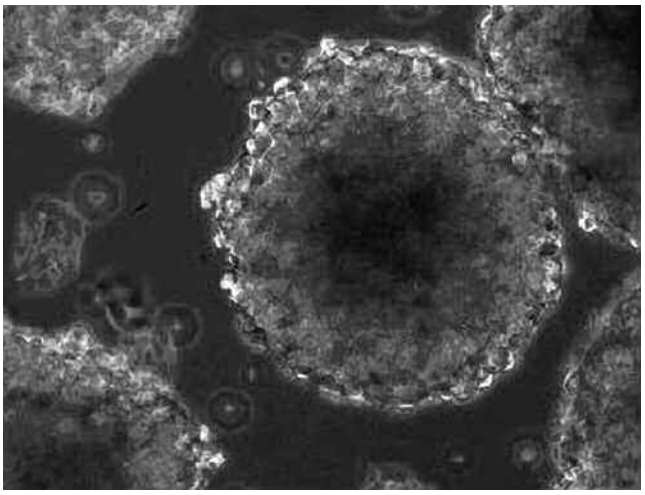

**Fig. 1.** Mature HepG2 liver spheroids after six days in culture. Following monolayer culture until confluency, HepG2 cells can be cultured on a gyrotatory shaker to enable the formation of spheroids (original magnification x10).

Many hepatocyte cell lines have also been used, with one of the best characterised being HepG2. Originally, the HepG2 cell line was established from a liver biopsy of hepatocellular carcinoma from a 15-year-old Caucasian male by culturing tumour minces on feeder layers of irradiated mouse cell layers and serially passaging over several months.[22] Morphologically, HepG2 cells resemble liver parenchymal cells and are reported to have the biosynthetic capabilities of normal liver parenchymal cells.[23] However, differences have been reported between sources of HepG2,[24] culture conditions and passage number.[25] Additionally, the presence of one genotype only (i.e., does not allow for polymorphism or inter-individual variation), which has been suggested to lead to low or even absent levels of P450s, may be a limiting factor; however, other reports conclude HepG2 to be a suitable model for biotransformation studies.[26]

More recently, new hepatocyte cell lines have shown promise in drug toxicity testing. These include HBG BC2, which has levels of some important metabolic enzymes (e.g., CYP3A4) considerably higher than HepG2.[27]

Advantages of utilising cell lines such as HepG2 include availability, low cost, lack of ethical issues and inducibility.[26] However, by definition, cell lines are immortalised and therefore may not be truly representative.

## New approaches to *in vitro* testing models

Traditionally, *in vitro* metabolism studies have involved cells cultured into monolayers. However, while cells can be easily established in monolayer conditions, the interactions of cells with their surrounding environment can greatly affect shape, cell function and gene expression.[28] Comparisons of cells grown in two-dimensional or three-dimensional (3D) cultures have revealed differences between the two systems in terms of mechanisms such as cell-to-cell adhesion and resistance to drug-induced apoptosis.[29] Within the human

body, cells form 3D structures, therefore it is likely that 3D *in vitro* culture models will be more representative of the *in vivo* environment.

Recently, a method has been developed in the authors' laboratory to enable culture of HepG2 cells into 3D spheroids (Fig. 1). These spheroids are able to survive significantly longer in culture than can monolayer cells and are more representative of human liver in terms of liver-specific functions such as P450 enzyme activity and albumin synthesis.[29,30]

Co-culture of these liver spheroids with other cell types such as mesenchymal stem cells (MSC) has enabled chemotherapeutic damage from prodrugs such as cyclophosphamide (CY) to be studied, with results obtained resembling the damage seen in patients who have received chemotherapeutic treatment *in vivo* (Fig. 2).

Several morphological changes can be seen in MSC following exposure to CY (Fig. 2). Untreated MSC form a confluent monolayer, with cells exhibiting a uniform fibroblast-like appearance. Following CY treatment, there is some loss of this morphology, with many cells forming irregular shapes with long filaments extending across the culture flask surface. Mesenchymal stem cells also generally show an increase in cell size, a decrease in cell numbers and reduced proliferative capacity (Fig. 3).

It can be seen in Figure 3 that MSC proliferative capacity is reduced following *in vitro* treatment with CY. Independently, CY has some effect on reducing MSC expansion. It is considered to be largely inactive until metabolised. In an aqueous solution, however, some spontaneous breakdown of CY occurs over extended periods.[31] In the presence of a source of P450 enzymes required for metabolism (e.g., S9 liver extract), an increasingly significant reduction in MSC expansion can be observed ($P<0.001$). However, S9 itself appears to affect MSC considerably ($P<0.05$) and there is some evidence that it is toxic to cells, particularly over prolonged exposure periods.

Indeed, Ku *et al*.[32] recommend that incubations with S9 extract should be limited to two to four hours, due to its instability and toxicity, making it unsuitable for *in vitro* metabolism studies such as modelling damage from chemotherapy where treatment can typically involve administering agents over two consecutive days prior to a stem cell transplant.[33] Conversely, HepG2 spheroids appear to be non-toxic when co-cultured with MSC and do not significantly reduce the proliferative capacity of MSC. However, when cultured with MSC in the presence of CY, the expansion of MSC is reduced ($P<0.01$).

Following a shorter incubation time of three hours, the contribution of S9 itself to the decrease in MSC expansion over longer periods can be appreciated by comparing with 48-hour incubation results (Fig. 3). At three hours, the role of HepG2 spheroids in actively metabolising CY can also be seen more clearly, with cells in the presence of CY alone showing no change from untreated cells, whereas, following the addition of spheroids, MSC exhibited a significant reduction in expansion ($P<0.05$).

In addition, HepG2 spheroids are known to perform liver-specific functions such as the production of albumin, to which metabolites of CY may bind, as would occur *in vivo*. Therefore, while metabolising CY, spheroids may also provide beneficial factors for the MSC in the culture environment. Therefore, HepG2 spheroids may provide a more suitable source of P450 enzymes for *in vitro* metabolism studies, removing the issues of toxicity experienced with other sources such as S9 and offering a model more representative of the *in vivo* situation.

Similarly, a novel *in vitro* system utilising normal primary human cells from five different organs, together with a breast cancer cell line, was developed by Li *et al*.[34] to allow study of the effects of an agent on more than one cell type simultaneously. The model uses a 'well within a well' system in which six cell types can be cultured within separate wells in specialised media, but all six wells are contained within one larger well, allowing it to be flooded with an agent able to access all internal wells. Consequently, the effect of the agent on several organ systems can be studied, as well as interactions between the different systems, resulting in a much more realistic model of the *in vivo* situation. To enable metabolism of the agent and the effect of any metabolites to be studied, one of the cell types incorporated can be hepatocytes; however, these would need to be cultured as a monolayer, which is less representative of human liver than is spheroid culture.[30]

Developments in detection of subtle indicators of toxicity may also enable progress in *in vitro* toxicity testing. Many traditional *in vitro* tests rely on detecting parameters involved in lethal toxicity events (e.g., apoptosis or necrosis) and often lack sensitivity. A study by O'Brien *et al*.[21] investigated the possibility of using new technology to detect earlier subtle sublethal indicators of toxicity *in vitro*. The High Content Screening (HCS) assay involves automated quantitative epifluorescence microscopy to monitor live cells *in vitro* in real time, examining parameters such as plasma membrane permeability, nuclear size, cellular mitochondrial membrane potential, concentration of

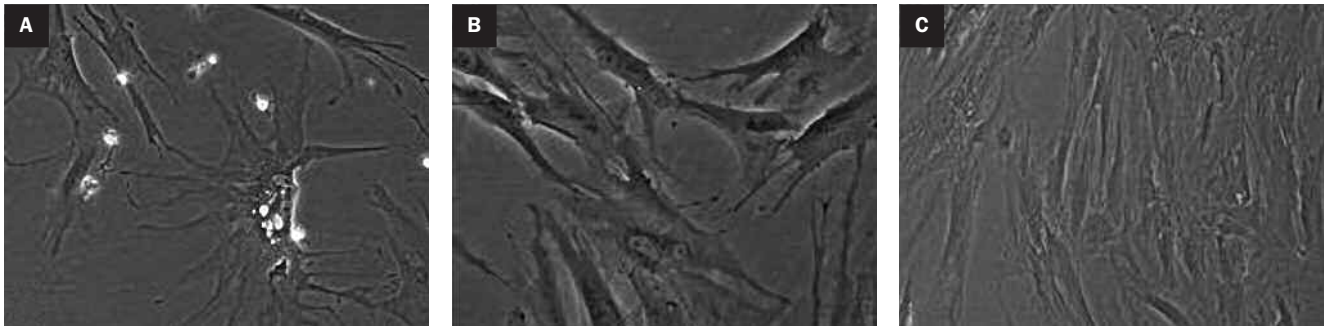


**Fig. 2.** Comparison of *in vitro* modelling of cyclophosphamide treatment with effects seen in patients who have received *in vivo* therapy. Mesenchymal stem cells (MSC) from a patient previously treated with CY and fludarabine *in vivo* (A), MSC exposed to CY *in vitro* in the presence of HepG2 liver spheroids (B), control untreated MSC (C). (Images A and C, original magnification x10; image B, original magnification x20)

intracellular free calcium, and cell number. Cell number was the first parameter to be affected in 56% of drugs that tested positive for hepatotoxicity. Altered nuclear size was found to be the most precise, with changes caused by 70% of hepatotoxic drugs. In many cases, this was seen as a significant decrease in nuclear size (up to 50%); however, a small increase was seen with some drugs, and even a 50% increase following acetaminophen exposure.[21]

The assay was found to have greater than 90% concordance with human toxicity and offers the potential for high-throughput screening. Although the assay failed to detect some toxicities, it does demonstrate the need for, and benefits of, continued research into *in vitro* toxicity testing methods as new technologies are developed and improved.

In addition to methods for toxicity testing, arguments for adapting the level of testing depending on the setting in which the pharmaceutical will be used can also be put forward. For example, in patients with a terminal illness, it may be appropriate to minimise safety testing as the risks of toxicity events, particularly longer-term, are greatly reduced and may not outweigh possible clinical benefits.

## Conclusions

Much progress has been made in both *in vitro* and *in vivo* toxicity testing. Following the 3Rs principle, numbers of animals involved in testing have been greatly reduced, and, as increased knowledge of toxicity has been gained, methods of testing have been improved. Additionally, developments in technology are currently enabling the introduction of increasingly specific and sensitive *in vitro* alternatives.

Currently, there is still a place for animal testing within the toxicity setting, as it has a well-documented history and provides the opportunity to study the entire organism. However, many alternative *in vitro* methods are now available and in development, and, while not currently a complete replacement for animal testing, can be used prior to, and in some cases to complement, existing techniques. With growing developments in knowledge and technology, *in vitro* tests should become more predictive of the *in vivo* situation and should be used wherever possible. However, care must be taken to consider possible limitations of the models used.

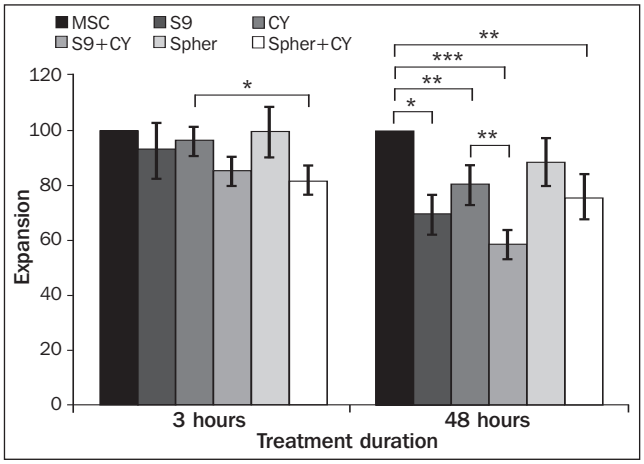


**Fig. 3.** Initial expansion of MSC following 48-h or 3-h CY exposure *in vitro*. Bone marrow-derived MSC were exposed to 0.5 mmol/L CY in the presence of HepG2 liver spheroids or S9 liver extract. Two weeks after treatment, cells were trypsinised, cell counts performed and expansion calculated as a percentage of MSC control ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). Data presented as mean+SE (3 h, $n=5$; 48 h, $n=7$).

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