On 26 November 2009, a groundbreaking international conference was held at the prestigious Royal Society in London. It was hosted by the Safer Medicines Trust, a charity which aims to improve human health by emphasising the need for human-relevant research. The conference succeeded in drawing together representatives from the pharmaceutical industry and biotechnology companies, as well as academics and regulators, to learn about and exchange ideas on the latest techniques in drug safety testing.

Unlike other initiatives addressing the use of more human-focused technologies in drug safety testing, this meeting was not looking at the potential for future developments, or making the case for increased funding to create more reliable, human biology-based methods. Rather, it looked at how we can use today’s existing technologies to address the US Food and Drug Administration’s (FDA) charge that ‘…researchers are trying to bring 21st century medical innovations to market using 20th century tools to evaluate them’.1

Another key objective was to raise awareness of, and support for, the Safer Medicines Trust’s proposal for a direct comparison of the efficacy of animal tests in predicting drug effects on humans with the ability of a battery of human biology-based tests to do so. This would involve taking a number of drugs for which both animal and human data are available, and running them through a set of human biology-based assays. By comparing the three data sets, it should then be possible to determine whether the use of human biology-based tests would constitute a better strategy for drug safety screening.

The clinical trial disaster at Northwick Park hospital, and the withdrawal of Vioxx® from the market, highlighted the urgent need for improvements in how new medicines are tested. About 92% of all new drugs that make it to clinical trials fail—an unsustainable figure.2 The safety of even the few that pass is not assured, however: adverse drug reactions are estimated to account for 197,000 deaths per year in the EU, at a cost to society of €79 billion.3

Although animal tests for drug safety have been required since 1968, as a consequence of the thalidomide tragedy, they have never been validated in the way that any test designed to replace them must be. In the light of their many demonstrated failures to predict safety (including Vioxx®, TGN1412 and HRT, which raises women’s risk of heart disease, contrary to the reduction in risk predicted by animal studies), and in view of the explosion of human biology-based technologies now available, we believe that it is time to ask whether animal tests are the best way of assessing the safety of new medicines. The Safety of Medicines (Evaluation) Bill 2009 was launched by a cross-party group of MPs in January 2009, to answer precisely that question. The Bill would require, for the first time, a direct comparison of the effectiveness of animal tests for drug safety with a battery of human biology-based tests, as mentioned above.

We do not think that any one technology should replace the currently required animal tests. The central concept is that the new technologies should be evaluated as a synergistic set of tests, each contributing an important piece of the overall puzzle. If the comparison identifies strengths which cannot be matched by the current animal testing regime, the implementation of updated regulations requiring these tests, rather than those currentlyfavoured, would confer great benefits on patients.

In a report commissioned by the US Environmental Protection Agency, the US National Academy of Sciences recognised the urgent need for a ‘paradigm shift’ in environmental chemical testing, which currently relies largely on animal tests. The report acknowledged that a move away from animal tests toward faster, cheaper and more-accurate methods (of the sort developed for medical testing) would be resisted, due to ‘deeply ingrained’ practices in toxicology assessment. However, they stated that such a move will be required over the coming decade, to ensure that the backlog of chemicals for which few reliable data are available can be evaluated.4 FRAME has long been campaigning for precisely such a move with respect to the EU REACH system.

Dr Bob Coleman, co-founder of Pharmagene (now Asterand) — the world’s first human tissue company, consultant to the pharmaceutical industry and science advisor to the Safer Medicines Trust, gave the first talk of the day. He emphasised that animal tests had always been unreliable, and suggested that the increasing demands of modern drug devel-
opment meant that ever more-sophisticated safety testing methods were required, and 'we should focus on the target, i.e. human, species'. Dr Coleman then introduced our idea for a comparison of animal tests with human biology-based tests.

Dr Paul Newbold, from AstraZeneca, discussed various advanced assays, including the use of human tissues and mathematical modelling, employed at the company to make crucial decisions about which drugs should make it to clinical trials. He outlined the difficulties of extrapolating from preclinical data to humans, and went on to explore ways of designing clinical trials to ensure that relevant populations were included. Future clinical trials may make more use of stratified populations, with a view to tailoring treatments to sub-groups of patients with particular disease phenotypes.

Professor Chris Hillier, Glasgow Caledonian University, and co-founder of the human tissue company, Biopta, expanded on another theme brought up by Dr Coleman, that of the pressing need for improved infrastructure to ensure that tissues donated by consenting patients or their relatives are collected and distributed to researchers who need them to investigate diseases and try out new drugs. Professor Hillier went through some of the myriad of tests developed by Biopta, which use biopsies from various organs to look at a range of physiological responses to new drugs. It may seem counter-intuitive to expect whole system properties such as blood pressure to be captured in tiny slivers of tissue, yet this is only one property of many that can successfully be assessed in this way.

Dr Greg Baxter, Cornell University, Ithaca, NY, USA, explained how human tissue samples, arranged in Hurel™, a microfluidic device replicating the dimensions of the human body on a tiny scale, can provide further whole-system information about the effects of new drugs.

Professor Johannes Doehmer, BioProof, Munich, Germany, followed, with very convincing arguments for the absolute necessity of examining metabolism in a strictly human context, and explained how this can now be achieved rapidly and inexpensively by using cell cultures.

Also on the theme of applying deceptively simple cell culture solutions to drug safety problems, Dr Quin Wills, SimuGen, London, described his company's assays, which combine cell cultures, genetic analysis and advanced mathematical models to not only predict which drugs will be toxic, but also to suggest ways of manipulating those molecules to reduce this likelihood.

Professor Zvia Agur, Optimata, Ramat-Gan, Israel, also works in the field of computer modelling and specialises in virtual patient technology. She discussed one of Optimata's programmes for modelling dose responses down to the level of individual patients, furthering the goal of personalised medicine.

Professor Russell Higbee, VaxDesign, Orlando, FL, USA, presented VaxDesign's advanced in vitro immune system. Produced from donated blood, this 'clinical trial in a test tube' can be used to assess vaccine immunogenicity, amongst other things. Such innovations are clearly required, for example, in the face of unremitting failure in the field of HIV vaccine development.

Dr Katya Tsaioun, Apiredica, Watertown, MA, USA, elaborated on the use of cell cultures in novel ways, in her presentation on how to make the best use of ADME (Absorption, Distribution, Metabolism, Excretion) assays as early as possible in drug development. This is particularly pertinent, given the extremely low success rate of drugs entering the clinical trial phase, as the later in the drug development process a drug fails, the more resources have been wasted — costs which are passed on to healthcare providers when a drug eventually makes it to market.

In the last session, the discussion moved to consider technologies which enable drugs to be tested as safely as possible, and at a much earlier stage, in human volunteers. Professor Markus Müller, Medical University of Vienna, Austria, impressed the audience with the uses and achievements of microdialysis. For more than a decade, this technique has been used to test the responses of individual organs to drugs at therapeutic doses, but in an extremely localised way. The theme of Dr Mark Seymour, Xceleron, York, was that 'the best model for humans is human.' He went on to introduce Xceleron's microdosing technology, which uses a combination of ultra-sensitive analysis and PET scanning to follow the ADME of a drug at extremely low, safe doses. Despite the low doses administered, he was able to reassure the audience that the results obtained have proven very amenable to extrapolation to therapeutic dosage.

We were delighted that Dr Ian Gibson MP, then Chair of the All Party Parliamentary Cancer Group, and a member of the Select Committee on Innovation, Universities, Science and Skills and of the All Party Parliamentary Patient Safety Group, among others, took time out from his parliamentary duties to address the audience on the subject of the Bill that he co-sponsored. Dr Gibson noted that the latest technologies could positively impact healthcare in two ways: by improving drug safety through improved test accuracy, but also by bringing down the cost of drug development, and hence of drugs themselves. The cost factor is a particularly important consideration, given rising healthcare costs and an ageing population. He also emphasised that 'we want to make sure the regulations move with the times', and expressed the opinion that interest in the techniques being discussed would continue to grow rapidly.

We hope that the conference and these Proceedings will help to inform the decisions of
those who are responsible for getting new drugs to market as safely as possible, as well as the regulators tasked with judging whether a new treatment has been proven suitable for use in patients. We were delighted with the level of discussion stimulated, and the collaborations forged between the attendees at the meeting.

We hope that ATLA readers will find these Proceedings useful, and would warmly welcome any feedback: we can be contacted at Margaret@safermedicines.org or Kathy@safermedicines.org. The speakers’ presentations are available at: www.drugtestingconference.com.

We are very grateful to the speakers for presenting their work at the Conference, and for generously contributing papers to these Proceedings that constitute an important means of furthering awareness of the 21st century technologies now at our disposal for improved drug safety testing. We wish to thank FRAME for its generous financial support in making this publication possible.

References


Introduction

It is generally agreed that there is a problem in the ability of the Pharma Industry to introduce effective and safe new medicines to the market (1, 2). Despite ever-increasing R&D expenditure, a more thorough understanding of the molecular basis of disease, and the introduction and application of new technologies, new drugs continue to fail in terms of their clinical efficacy and/or safety profiles. The industry has now been in a state of continuous upheaval for more than a decade, ever restructuring itself in an attempt to find the answer to its productivity problems, apparently without any significantly positive outcome. It is clear that something needs to be done if the Pharma industry is to continue to exist as an entity responsible for the development of new and effective medicines for human disease.

Drug Discovery at Glaxo

I started my career in the Pharma Industry in 1965, when I joined Allen & Hanbury’s, part of the Glaxo Group, as laboratory technician. My arrival there coincided with the discovery and development of salbutamol (marketed as Ventolin), the drug which opened the way to something of a ‘Golden Age’ for Glaxo. The company’s successes were the direct result of the leadership and inspiration of Dr (now Sir) David Jack. It was Sir David, inspired by the example of his fellow Scot, Sir James Black, who instigated a move away from the largely empirical approach to drug discovery and development that had prevailed in the industry up to that time, by taking a more structured and more rational line, based in the main on an understanding of the targets at which drugs act (3). This approach led to the successful introduction, not only of Ventolin, but also of a whole series of important and well known drugs for a range of indications, including ranitidine (Zantac), beclomethasone (Becotide), labetalol (Trandate), cefuroxime (Ceftazidine), ondansetron (Zofran), sumatriptan (Imigran), fluticasone (Flixotide), and salmeterol (Serevent).

Despite the rationality of his approach, and the success that ensued, in one aspect Sir David could be regarded as having been lucky in achieving this prodigious output. Virtually all of the pre-clinical...
work that resulted in the discovery and development of these drugs was conducted on laboratory animals. And it proved successful. Salbutamol and salmeterol, for example, are bronchodilators, and are as effective in relaxing airways smooth muscle in guinea-pigs as they are in humans (4). Ranitidine inhibits gastric acid secretion in the rat as effectively as it does in man (5). In addition, in terms of their safety profiles, both these classes of drug proved to be relatively benign in both experimental animals and human subjects (6, 7).

**Shortcomings of Animal-based Approaches**

So the question arises, ‘If the animal-based approach worked so well then, why is it proving to be so much less productive today?’ — ‘What has changed?’ The answer, of course, is that nothing has changed, it is simply that experimental animals have always been unreliable in their predictive power for human efficacy and safety, providing useful information on some drug candidates, but not on others. Despite the impressive output from the Glaxo stable during the latter third of the 20th century, many other exciting programmes that were progressing at the same time came to nothing. Such failure was due, at least in part, to the failure of experiments on animals to predict outcomes in human patients. It may well be that the drug mechanisms for which animals can provide some useful prediction of potential efficacy and safety in man, have already been exploited, so the proportion for which they are less useful, or even frankly misleading, is increasing. There may, of course, be additional issues. For example, it may well be that the very ingenuity of experimental scientists in mimicking human diseases in animals blinds them to the fact that underlying the induced disorder is a non-human physiology. There is a wealth of evidence, in a wide range of physiological systems, that even closely related species can use quite different biochemical pathways to achieve the same biological end (8–12), and even within species, choice of strain can have a profound influence (9, 10, 13, 14).

Although the unreliability of tests in non-human species for predicting efficacy and safety in man is increasingly well accepted, it seems to have had limited impact on the enthusiasm of scientists for their use. Despite the wealth of information testifying to unreliability of the mouse and the sheep to accurately indicate which drugs are likely to be effective in human asthma (15, 16), researchers into respiratory medicines continue to use them (17, 18). To a degree, the mapping of the human and mouse genomes may be to blame; it seems that all the lessons painfully learned as to the unreliability of mice as human surrogates in drug discovery and development generally were forgotten as soon as we discovered the level of concordance between the human and murine genomes (19), despite the fact that similarity in gene sequence does not necessarily reflect similarity in expression, and therefore in function (20).

**Human-based Approaches to Drug Discovery and Development**

So, if animals can no longer be relied upon, what alternatives are there? I suggest that the answer, at least in principle, is startlingly simple: ‘We should focus on the target species, i.e. humans’. There are many ways in which human biology can be accessed and exploited, not only through clinical studies on human volunteers and patients, whether these involve full clinical trials or highly focused micro-dosing studies, but also via studies on human cells, tissues and even organs *in vitro*. *In vitro* approaches are now available in abundance, and while these may involve studying the effects of novel agents on single cells or tissues in isolation, this is by no means an absolute limitation, and technologies are now becoming available for studying the effects of drugs on integrated systems *in vitro*. Alternatively, we can use humans indirectly by constructing *in silico* models based on the ever-increasing amount of human data available. There are, of course, systems that are too complex and/or intractable to lend themselves to a relatively simple *in vitro* approach, and where *in vivo* studies in non-human species seem the only way forward. In such cases, comparative studies in animal and human cells and tissues *in vitro* can prove invaluable for the identification of the most relevant animal species to employ in any particular disease modelling exercise.

A concern commonly expressed when discussing such approaches, is that they are too expensive and low-throughput to be useful to the Industry. I think that it is worth bearing in mind that there is nothing more expensive in drug discovery and development than getting the wrong answer. Also, while throughput may currently be a problem, this may merely represent a challenge to human ingenuity. There also seems to be an underlying concern that making any change is risky, and this appears to be particularly true in this case, where there is no guarantee that novel human-focused approaches will be any better than the animal-based approaches in current use.

It is for this reason that the Safer Medicines Trust and the Safer Medicines Campaign (21) are proposing the setting up of a study to answer this question. It is their intention that a range of drugs with established clinical profiles should be submitted to a battery of alternative human-focused
approaches, in order to establish directly their value in identifying the efficacy and safety profiles of the selected compounds, both in absolute terms, and also in comparison with the ‘classical’ methods that were originally employed during their development. Should the human-focused approaches prove more effective in profiling the strengths and weaknesses of the selected compounds, it will provide compelling evidence for a new, more powerful direction in tomorrow’s drug development.

**Who Should be Responsible?**

The question arises, ‘Who should be driving and, of course, funding such a human-focused approach?’ In view of the fact that, in the UK, the National Health Service (NHS) needs improved medicines to enhance its provision of effective healthcare, and it is the Pharma Industry’s role to provide those medicines, it seems logical that these two bodies should be responsible for providing the necessary resources. However, in reality, while some Pharma companies are indeed beginning to wake up to the necessity to move in this direction, some more enthusiastically than others, the main drivers have for some time been academics, charitable research groups and small biotech companies. These enthusiasts are all motivated by a keen desire to employ new, more reliable approaches to increase the human-focus of the R&D process, and thereby to enhance its ability to introduce safer, more effective drugs to the market. Unfortunately, they are all limited by funding constraints, relying on academic grants, venture capital funding, and some support from major Pharma companies, usually through the provision of services.

**How to Proceed?**

Of course, such human-focused approaches require human participants, as clinical volunteers or as donors of cells, tissue and organs, and the question arises ‘How are these to be found?’ Fortunately, humans are not scarce, and in general, they are willing to contribute, both in terms of volunteering to be clinical ‘guinea-pigs’ and in the donation of their organs, tissues and cells following surgery or post mortem. It is unclear why, to date, this resource has been so poorly tapped, particularly for the donation of viable tissues for direct pharmacological and toxicological evaluation.

Over 20 years ago, my colleague, Gordon Baxter, and I founded Pharmagene, the world’s first company to attempt drug discovery and development work solely through the use of voluntarily donated human tissues and cells. At this time, the acquisition of the necessary human biomaterials was difficult, there being no clear legal or ethical guidelines through which to work, or indeed, much in the way of the necessary infrastructure in the NHS to enable such acquisition from would-be donors (22). This situation came to a head with the Alder Hey scandal, but what this did was to give the public a voice in stating that they did not object to donating material for research, simply that they did want to be asked. It also led to legislation in the form of the Human Tissue Act 2004 (23) and the work of the Human Tissue Authority (24), which between them provided a legal, ethical and practical framework for the acquisition and use of human organs, tissues and cells (25). Despite this, in 2008, the acquisition of viable biomaterials for research purposes remains a considerable bottleneck.

Of course, the situation is not limited to the research community, and the Prime Minister, Gordon Brown, along with the Chief Medical Officer, Sir Liam Donaldson, and the British Medical Association have recently been reported as proposing the implementation of a policy of ‘presumed consent’ to improve the availability of human organs for transplant (26). However, consent may well not be the issue, but rather the lack of recovery teams at the sites of would-be donation (27). Without these, no amount of presumed consent will have any marked effect on the availability of transplantable organs. I would suggest that UK Transplant, the Pharma Industry and politicians need to work together, to make possible a system through which human biomaterials can be acquired, and then made available to assist in all aspects of human healthcare.

To conclude, the current paradigm for the delivery of novel, safe and effective medicines is not working, and an alternative, human-focused, approach has been suggested. It must be put to the test, and the fact that it requires human participation should not be regarded as a problem; it simply requires imagination and commitment from the relevant stakeholders.

**References**

Could Fresh Human Tissues Play a Key Role in Drug Development?

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Summary — Biopta was founded in 2002, to provide human tissue-based drug development and testing services to the pharmaceutical industry. Although animal tissues are readily available and are relatively inexpensive, they frequently fail to faithfully predict the results seen in the clinic. Human tissues can provide integrated responses to test drugs in a manner more representative than individual cell types or cell lines alone, and more-directly relevant to the species of interest — Homo sapiens. In order to expand the use of human tissues, however, an improved infrastructure for the collection and distribution of fresh, functional tissues is highly desirable. Moreover, where there is the potential to obtain tissue from various locations, it becomes possible to test tissue that is specific to the site of drug activity. This is important, as differences may occur between the same tissue types in different locations in the body. The detection of adverse effects is greatly helped by knowledge of how existing drugs behave in the human body. These drugs can act as reference compounds, so that new compounds can then be compared, by using standard concentration–response type studies, in a huge variety of tissues, and their effects extrapolated from what is known of the reference compounds.

Key words: angiogenesis, biopsy, cardiac, HRT, human tissue, neural, respiratory, vascular, Vioxx®.

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Introduction

Biopta was founded in 2002, following in the footsteps of Pharmagene, the first company in the world to really focus on human tissue for drug discovery and development. The premise underlying Biopta was to commercialise the wide range of human tissue assays that were being developed and performed by academic units at Glasgow Caledonian University.

Animal tissue is the method of choice for most academic and commercial users, because it is convenient, inexpensive and readily obtained. However, Biopta’s experience, particularly within the commercial sphere, is that it is generally a poor predictor of the human response, in some cases bearing no resemblance whatsoever to human-derived data. It is therefore not surprising that there has been much speculation about whether or not there is a correlation between an over-reliance on animal data and the current problems that the pharmaceutical industry is suffering, including late-stage failures, post-marketing withdrawals, and a decline in drug discovery pipelines.

Novel technologies that focus on human tissue-derived data, such as those developed by Biopta and others, are able to significantly assist in the lead selection process. Human tissues are ideally suited for use at this stage, as the responses obtained can help researchers to decide which of a group of leads is most likely to be successful in the clinic.

It is important to understand what is meant by human tissue. In this context, human tissue does not refer to cell lines, individual cell types, primary or secondary cells, but groups of different cell types together as a working tissue, e.g. an isolated blood vessel or airway. The interaction between different cell populations is critically important to the overall behaviour of the tissue and its response to drugs under test; therefore, an intact section of human cardiac muscle, whilst not as valuable as a whole organ, is clearly a more relevant test system than an isolated cardiomyocyte. Although data derived from isolated tissues cannot reveal everything about the whole organism, they can be much more useful than data from separate cell lines. Thus, Biopta can expose a tissue to a candidate drug and then measure the integrated response of...
all the cells working together, in order to find out whether the drug behaves as intended in human tissue, which is the ultimate goal. Biopta can perform a range of assays to assess efficacy and potency, and there is a growing range of different tissue types available. However, perhaps the most rapidly growing application of human tissues is in the field of safety pharmacology.

In the early days, Biopta focused on vascular and respiratory assays, and these remain a particular strength. Isolated blood vessels can be obtained after many types of surgery, but with respect to drug development and discovery, the smallest of these are of the greatest interest. Tiny arteries, between 150µm and 300µm in diameter, are the primary regulator of blood flow to all organs (1). These are ubiquitous, and can be obtained in sufficient numbers from very small biopsies of tissue, from a range of organs including the skin, kidneys, mucosa, heart and skeletal muscle (2–4).

The ability to use residual surgical tissues from various locations means that test blood vessels can often be used that are specific to the site of drug action. For example, Biopta is developing assays on very small blood vessels from neural tissue, for use in studies concerning the blood–brain barrier.

Major blood vessels are often the focus of investigational studies. However, the blood vessels in which Biopta is primarily interested are only about the diameter of a human hair. They are the major determinants of peripheral vascular resistance, and hence have a critical role in regulating blood pressure. In addition, specialised techniques are required to study such small blood vessels, and in order to achieve a sufficiently high-throughput to make human tissue tests a routine part of drug development, Biopta has developed expertise in miniaturised pharmacological techniques. These assays usually require blood vessels to be assessed as ring preparations of a few millimetres in length, by means of either ‘strain-gauge’ or ‘pressure/flow’ methodology. The strain-gauge system involves two metal wires, placed through the lumen of the vascular section, that are fixed to a micrometer and a transducer, which is able to measure changes in force as the walls of the vessel pull on the force transducer following drug application. The tissues are submerged in physiological solution, and can remain functional for up to 24 hours (for some responses), if kept at 37°C and oxygenated. The technique is robust and reliable; however, important differences exist between this assay and the in vivo scenario. The isolation of the artery involves dissection of the nerves, so central neural influence is abolished. A measure of sympathetic drive can be assessed, however, by using electrical field-stimulation, where the blood vessel is placed between two platinum or silver plates, through which a small voltage is applied. The resulting electrical field stimulates the sympathetic nerves on the blood vessel to release their catecholamine stores, thus producing a measurable vasoconstriction response.

An example of the value of such a vascular assay comes from tests of hormone replacement therapy (HRT), which has an effect on the cardiovascular health of post-menopausal diabetic women. Both women with diabetes and post-menopausal women have independently increased cardiovascular risks. These factors act synergistically to create a greater risk in post-menopausal women with diabetes. Small biopsies of skin or residual skin and subcutaneous tissue from surgery are donated by patients; in this study biopsies were taken before and after the patients were given HRT (5). Tissue can also be obtained from age-matched subjects as controls. Our data showed that, in controls, a normal response is measured to the agonist acetylcholine, which essentially is a marker of vascular health. Prior to HRT, post-menopausal women with diabetes showed an impaired response to acetylcholine, as expected from information in the literature. However, results from the second post–HRT period, in the same patients, showed that it had markedly improved.

This example demonstrates the power of this technology, i.e. multiple biopsy data can be derived from the same patients or residual surgical tissues from groups of patients known to be taking the drug of interest. This type of study can even be extended to perform further longitudinal studies over, for example, a year or more. This may help to identify small chronic changes that are invisible in short-term clinical trials, but are revealed only after a drug has been marketed, as was recently seen with Vioxx® and other drugs. However, this type of study is time-consuming, and simpler, more-rapid tests using tissue residual to surgery are more commonplace, where no invasive biopsies are required, yet the current medication and medical status of the patient is known and patients can still be “recruited” to the project by the use of defined exclusion/inclusion criteria.

An alternative technique to the wire system is the perfusion/pressure system, in which the vascular tube is cannulated at each end, which allows fluid to be perfused through the vessel. In this case, the transducers do not measure force, but pressure, flow and changes in diameter or vascular permeability.

The choice of whether to choose a strain gauge test or a pressure/perfusion system depends on the level of sensitivity required. The pressure/perfusion systems offer up to ten times more sensitivity than the strain gauge, but for higher-throughput, the strain gauge test is preferable.

The strength of this approach in discovery biology is evidenced by many achievements, including: the identification of the vascular endothelium as a
major modulator of vascular function (6), and the discovery of the nitric oxide pathway (7), the endothelin pathway (8), vascular endothelial growth factor (9), and endothelium-derived hyperpolarising factor (10).

One of the major applications of these assays is the prediction of off-target effects that may lead to hypertension or hypotension during clinical use. Blood vessels sourced from within the same tissue produce similar responses, but there can be differences in receptor populations and functional pathways in blood vessels from different tissues. This means that a number of vascular beds should ideally be screened to predict blood pressure changes. The skin, gut and skeletal muscle represent over 90% of the body's circulatory system, so the screening of small arteries from these areas constitutes a fairly comprehensive screen. Skin is the most easily accessible, and is also easy to use. Up to twenty small arteries can be obtained from a single skin sample obtained from cosmetic procedures, or in some instances, from biopsies, and both 'control' (healthy) and patient groups can be studied. These often allow direct in vitro 'clinical' trial-type comparisons to be made during the preclinical or clinical phases.

Vascular permeability is also of interest. In vivo measures of permeability currently involve injecting animals with a dye, then killing them and looking at the tissues to see how the dye has escaped from the vascular compartment. However, this can be done by using isolated blood vessels in vitro: the dye is injected through the lumen, then the leakage of the dye out of the cannulated vessel because of changes in endothelial tight junctions, can be imaged. This can be analysed and quantified both before and after the application of a drug, so a measure of how a compound alters vascular permeability can be obtained.

Angiogenesis, the process of new blood vessel formation, is involved in a number of disease processes, including tumour growth, gastrointestinal disorders, and macular degeneration (11, 12). In cancer, therapeutic strategies are being developed for anti-angiogenic purposes, to prevent blood flow to a growing tumour; and for pro-angiogenic purposes, to enhance the availability of cytotoxic drugs to tumour cells (13). Tumour tissue is readily available from surgery, although it is highly sought after by researchers.

There are two types of assay for angiogenesis. One involves the rapid and automated immuno-histochemical identification of vascular endothelial cells in tissue slices, by using antibodies against von Willebrand factor, a specific marker of vascular endothelial cells. This methodology involves classical fixation, labelling, and imaging, but can now be carried out in a plate-based format that significantly increases throughput (14).

A different assay can predict the responses of tissue to angiogenic drugs following the manipulation of growth promoting factors, including VEGF, FGF-2, PDGF, and TGF-beta. These assays involve the use of thin, 2mm sections of explant tissue, which are placed into a 96-well culture plate. Each segment is then tested with different concentrations of an anti-angiogenic test compound, with both negative and positive controls. The organotypic cultures are then examined for endothelial 'sprouting' at various time-points, for up to two weeks, by using light microscopy (15). This type of study is well-suited to target selection and optimisation studies.

Respiratory Tissue

Lung tissue is regularly removed in surgery, but much of it is required by pathologists for diagnosis. At this stage, it frequently undergoes processes which render it useless for live tissue studies, which limits its availability for that purpose. Working with the respiratory system is also technically difficult. It is a low pressure system with very fragile tissue, so special skills are required. The techniques used in blood vessel analysis are therefore altered and specifically designed for use with lung tissue.

Lung bronchial tissue has contractile properties, and can be tested as prepared strips or rings for measurement of force generation, by using a modified strain-gauge technique. The assays used provide insight into bronchoconstriction or dilation effects (16), in much the same way that the observation of changes in arterial tone elucidates likely blood pressure changes. Isolated bronchial rings, dissected within 24 hours of surgery, retain the ability to respond to nerve stimulation (17). They can generate an IgE-mediated sensitisation of the responses to bronchoconstrictors (18), which makes them a useful in vitro model for the study of allergies in patients with powerful allergic responses compared to control subjects. Initially, acetylcholine is applied, in order to measure the basic contraction response. Subsequently, one preparation is incubated in blood serum from a non-allergic individual, whilst another is incubated in serum from a patient with a hyper-immune response, before challenge with, say, house dust mites, resulting in constriction of the tissue incubated with serum from the asthma sufferer. Drugs can then be tested in an attempt to modulate this response.

Although lung assays provide useful screens for compounds designed to produce changes in airway tone, Biopta is often asked to do this kind of study for companies with no particular interest in respiratory physiology, but who need to know that there are no respiratory issues associated with their drug delivery methodology.
Cardiac Tissue

The most readily-available source of cardiac tissue is the atrial appendage, which is often removed when a patient is connected to a perfusion machine during bypass surgery (19); however, ventricular muscle, which is of greater interest for safety pharmacology studies is also available through heart tissues not suitable for transplant. Cardiac tissue requires specialised dissection and handling techniques for the maintenance of its unique electrical properties, and needs to be used either extremely rapidly following removal from the patient, or to be placed in an ‘arresting’ solution to reduce metabolic activity. The trabeculae (round muscular columns that exist throughout the cardiac tissue), papillary muscles or strips from within the ventricles can be studied for their contractile and electrophysiological properties. They have similar inotropic (force of contraction), lusitropic (rate of relaxation) and chronotropic (rhythm) responses to drugs as the whole heart, except with the obvious loss of central nervous system control. An example data trace from a trabeculae study might include contraction/relaxation over time or the response to parasympathetic neurotransmitters or catecholamines. Currently, only hERG testing is regulated and required by the FDA for cardiac drug safety relating to prolongation of QT intervals; however, it could be argued that these other data, especially from ventricular muscle, also provide valuable information.

Human tissue assays are not restricted to contractile tissues, or tissue that can generate a force. Valuable information can also be obtained from tissue sheets from skin, or respiratory, gastrointestinal, and some glandular tissues. These types of assays may involve the addition of a compound to one surface of the tissue sheet, and the measurement of a response at the other surface, such as the production of cytokines or other local hormones. These measurements may also include measures of absorption, secretion, or electrochemical changes.

Transmembrane Studies

An abundance of skin tissue is now available through cosmetic surgery, as well as reconstituted skin preparations made by companies such as MatTek and L’Oréal. In tests on full-thickness skin, a diffusion cell (most commonly, a Franz cell) is used to create a sealed system, whereby drugs can be applied to a suspended portion of tissue, with the active compound passing through the epidermis and dermis. However, this technique is limited by the use of dead skin, which has led to modified versions that use functional full-thickness skin in an organ culture system. These modified techniques permit the simultaneous measurement of functional changes such as cytokine and MMP (matrix metalloproteinase) production (20). Biopta uses this technique to model the inflammatory changes found in atopic dermatitis and psoriasis and by achieving a throughput of 20–30 “biopsies” per skin sample, means that, for the first time, human tissue can be viewed as a secondary screening tool.

The Ussing chamber, a 40-year-old methodology, is currently proving an extremely useful basis for in vitro drug testing with human tissue (21). Skin or gastrointestinal mucosa obtained from resection, because of GI tumours or inflammatory bowel disease, are most frequently studied. Two chambers are used together, separated by a small insert in which the test tissue is placed. Electrodes for voltage and current are used to monitor the potential gradient between the two chambers, which provides important information on possible side-effects produced by the transepithelial transport of drugs.

Neural Tissue

The development of new assays concerning neural tissues is one of the focuses of research and development for drug testing companies, although there is still a long way to go. It is difficult (but not impossible) to access human neural tissue which is removed at surgery, for example, in the treatment of epilepsy or cancer. However, there are currently only a few good validated functional tissue assays that are relevant to the main challenges, such as Alzheimer’s Disease, Parkinson’s Disease, psychosis and depression. Assays are available for the study of brain slices (22, 23), and electrophysiology can be performed on neurons (22), by using stimulation electrodes similar to those employed in the field stimulation approach, but this time involving point-to-point stimulation and measurement of radiolabelled neurotransmitters.

Despite the vulnerability of human neural tissue to time-dependent failure, studies on neuronal behaviour in the hippocampus, hypothalamus and cortex have been successful. Whereas a number of assays are in use in academic institutions, they are generally not commercially useful. However, Stopps et al. have recently made advances in this area, by developing a system that allows electrophysiological recordings from multiple brain slices (23). The slices can remain viable long enough (over three hours) for investigations on synaptic plasticity, although, up to now, this work has been conducted on brain tissue from mice (23).

Meeting Future Needs

Access to fresh human tissue can be difficult, and the cost, time and logistical problems that exist
when using fresh tissue have not yet been overcome. The provision of surplus tissue for drug discovery is correctly of secondary importance, and there may be a conflict between a pathologist's responsibilities and a researcher's need to place tissue in a physiological storage solution, thereby limiting the availability of fresh tissue for researchers and tissue suppliers. However, the major limitations on tissue availability are logistical, rather than reflecting a lack of sufficient tissue for the pathologist or a reluctance on the part of would-be donors; in fact, we estimate that less than 2% of surgical residual tissues that are suitable for research are made available to researchers despite over 95% of patients being willing to donate their residual surgical tissue to research (24).

Human tissue tests do present unique challenges. For many tissues, particularly those with the highest metabolic activity rates, such as the brain and heart, there is only a short experimental “window” during which they can be used. Improved transport and storage networks are therefore required, to ensure a ready supply of fresh tissue, in a state in which it can be exploited for functional assays. Studies to prolong the “shelf-life” of organs and tissues, by using various transplant solutions, artificial blood, etc. are under way, but little progress has been made so far.

The recent increase in numbers of tissue banks, although primarily focused on creating banks of frozen or fixed tissues, may soon provide a more-structured system for the supply of fresh tissues. A number of commercial companies offer to supply processed fixed or frozen human tissue. Lung and vascular tissues have been frozen, and upon thawing have retained many of their functional responses (25). However, those seeking fresh human tissue for their research need to either create a bespoke tissue supply network or to outsource the experimental work to a contract research organisation with a developed tissue network.

The Role of Regulations

EU legislation provides guidance on the standards for quality and safety surrounding all aspects of human tissue procurement and use, except for blood (which is currently covered by other legislation). In the UK, the Human Tissue Authority (HTA) and the Human Fertilisation and Embryology Authority (HFEA) are responsible for implementing the EU Directive. The use of post-mortem tissue is the focus of the UK’s Human Tissue Act 2004, which, although still broadly covering surgical samples from living donors, delegates the responsibility for surgical tissue control to the National Health Service and individual biobanks. In the UK, patients are asked to consent to the use of their tissues for specific research purposes, as outlined in the document accompanying the consent form. In Scotland, where separate regulations operate, the broader term “authorisation” is used, which implies that the donor (or their relatives) authorises the researchers to use their tissue for research as they see fit, potentially allowing for greater flexibility. We hope that the nation-wide regulation of human tissue donation will eventually lead to greater public trust in the process of tissue collection and distribution for research, and will ultimately make it easier for patients to donate tissues for research purposes.

References


**Hurel™ — an In Vivo-surgeon Assay Platform for Cell-based Studies**

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**Summary** — Accurate prediction of the human response to potential pharmaceuticals is difficult, often unreliable, and invariably expensive. Traditional *in vitro* cell culture assays are of limited value, because they do not accurately mimic the complex environment to which a drug candidate is subjected within the human body. While *in vivo* animal studies can account for the complex inter-cellular and inter-tissue effects not observable from *in vitro* assays, animal studies are expensive, labour intensive, time consuming, and unpopular. In addition, there is considerable concern as to whether animal studies can predict human risk sufficiently precisely, because, first, there is no known mechanistic basis for extrapolation from high to low doses, and second, cross-species extrapolation has frequently been found to be problematic with respect to toxicity and pharmacokinetic characteristics. To address these limitations, an interactive, cell-based microfluidic biochip called a Hurel™ was developed. The Hurel system consists of living cells segregated into interconnected “tissue” or “organ” compartments. The organ compartments are connected by a re-circulating culture medium that acts as a “blood surrogate”. The fluidics are designed so that the primary elements of the circulatory system, and more importantly, the interactions of the organ systems, are accurately mimicked. Drug candidates are exposed to a more-realistic animal or human physiological environment, thus providing a higher and more accurate informational content than can the traditional *in vitro* assays. By affording dynamic assessment of potential toxicity, metabolism, and bioavailability, the device’s capabilities hold the potential to markedly improve the prioritisation of drug leads prior to animal studies.

**Key words:** cell culture analogue, *in vitro*, microfluidic biochip, pharmacokinetic modelling.

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**Introduction**

Traditional methods of predicting human response to potential therapeutics involve the use of surrogates — either *in vitro* cell culture assays or *in vivo* animal models. *In vitro* cell-based assays are often of limited predictive relevance, because they do not mimic with sufficient realism the complex environment to which a drug candidate is subjected within a living organism. *In vivo* animal studies can account for complex inter-cellular and inter-tissue effects not observable from *in vitro* tests, but animal studies also have their limitations. Alternatives to animal studies and *in vitro* cell culture models include mathematical or computational models. Mechanistic information can be combined with physiological information, such as blood flow, circulatory distribution and organ volumes, to create physiologically-based pharmacokinetic (PBPK) models (1). A PBPK model mathematically simulates the absorption, distribution, metabolism, and elimination (ADME) processes of living systems, providing a method to link mechanistic data obtained with *in vitro* cell cultures with system-wide toxicological and pharmacological information. However, a realistic PBPK model often requires parameters that are difficult to estimate, particularly those associated with the kinetics of metabolism. Further, a prime limitation on a PBPK model is that all the relevant mechanisms, whether direct or indirect, must be anticipated and included in the model. Often, secondary effects are not explicitly included.

A cell culture analogue (CCA) system is a physical replica of the PBPK model (2–7). Mammalian cells are cultured in different compartments, to represent organs or tissues, which in turn are interconnected by a re-circulating cell culture medium that acts as a blood surrogate. Design parameters such as compartment residence times and flow distribution are based on the corresponding PBPK model. By using representative cell types in the compartments, one can obtain system-wide rate parameters that can then be used to refine the PBPK model. In addition, because mam-
malian cells from different species of origin can be applied to the CCA, this system provides a potential means of studying the cross-species extrapolation of toxicological and pharmacological profiles. The accuracy of the PBPK parameters obtained from the CCA depends very much on the authenticity of the tissue constructs. Therefore, it is important that the CCA compartments provide a physiologically realistic environment. Non-physiological conditions, in parameters such as liquid-to-cell ratio, organ residence time and shear stresses, could potentially give rise to less than authentic metabolic and physiological functionalities from the cells. Also, a more realistic environment may be obtained, if the characteristic length scale in a physiological system is duplicated. Since biological length scales are of the order of 10µm, microfabrication techniques are ideal for creating features with similar dimensions. This advantage has been recognised previously. For example, a microphysiometer is a microfabricated device that detects functional responses from cells by measuring the change of extracellular pH (8). This device has been extended to detect receptor-mediated events, as well as to probe toxicological and pharmacological profiles (9). However, the response of the microphysiometer is still based on a single cell type, which cannot model the secondary effects of xenobiotic metabolism addressed by the CCA. Drawing on the strengths of a CCA system in capturing secondary effects, and possibly a more physiologically-realistic environment in the microscale, a microfluidic cell culture analogue device was developed, (known as Hurel™).

The Hurel microfluidic circuit comprises an arrangement of separate, but fluidically interconnected, “organ” or “tissue” compartments (10). Each compartment contains a culture of living cells drawn from, or engineered to mimic the primary functions of, and thereby to represent, the respective organ or tissue of a living animal. Microfluidic channels between the compartments allow a culture medium that serves as a “blood surrogate” to re-circulate as in a living system. Drug candidates of interest are added to the culture medium and allowed to re-circulate through the device. They then distribute to, and interact with, the cells in the organ compartments, much as they would in the human body. The effects of drug compounds and their metabolites on the cell type within each respective organ compartment are detected by measuring or monitoring key physiological events, such as cell death, cell proliferation, differentiation, immune response, or perturbations in metabolism or signal transduction pathways. The cell types employed can be adherent or non-adherent, and can be derived from either standard cell culture lines or primary tissues.

The physical features of a Hurel embody parametric values derived from a PBPK model (10). The geometry of the chambers and channels and fluid flow rate of the device are fashioned to replicate the values for drug residence time, circulatory transit time, organ cell density, tissue size, shear stress, and certain other physiological parameters found in the living animal, so as to mimic the fluid-mediated interactions of the organ systems represented in the microfluidic circuit.

One embodiment of the Hurel device, depicted in Figure 1, consists of four organ compartments: a “liver” compartment to represent the organ primarily responsible for xenobiotic metabolism, a “lung” compartment representing a target tissue, a “fat” compartment to provide a site for bio-accumulation of hydrophobic compounds, and an “other tissues” compartment to assist in mimicking the circulatory pattern in non-metabolising, non-accumulating tissues. Alternative device geometries can be configured to mimic various animal species, additional organs, or particular experimental applications, such as drug absorption or bioavailability.

**Predictive Toxicology Applications**

Sin et al. (11) have described the design, fabrication, and operation of a simple three-chamber Hurel system (lung-liver-other tissue). The device was fabricated from silicone substrate, by using standard microfabrication techniques of photolithography and etching. The resulting chip is enclosed between two acrylic pieces. In addition, a fluorescence-based oxygen sensor was integrated into the system, to investigate the adequacy of oxygen transfer in the system operating with cells. The results obtained indicate that the oxygen exchange rate is sufficiently rapid to fully meet the need of cells cultured in the Hurel device.

Naphthalene was chosen as a model toxicant, and the pathway of naphthalene biotransformation has been described previously (12). Briefly, naphthalene is metabolised by the cytochrome P450 mono-oxygenase (CYP-450) system into naphthalene epoxide, which can undergo several competing reactions: conjugation to glutathione (GSH), binding to protein, non-enzymatic rearrangement into naphthol, or enzymatic conversion to dihydrodiol. Both naphthol and dihydrodiol are enzymatically converted to naphthalenedioli, which is subsequently oxidised to naphthoquinone through redox cycling, generating reactive oxygen species (ROS). These reactive oxygen species may cause severe oxidative stress within the cells, leading to cell death. Naphthoquinone can conjugate with cellular GSH, which reduces ROS formation, and these metabolites can be secreted. In addition, quinone can form adducts with proteins or DNA, leading to cellular damage. It has been observed in mice that metabolites generated in the liver can circulate to the lung, causing cell death there.
Hurel™ — an *in vivo*- surrogate assay platform for cell-based studies

Hurel™ chips containing human hepatocytes (HepG2/C3A) in the liver compartment and rat lung epithelial cells (L2) in the lung compartment, were exposed to naphthalene (50 µg/ml, in medium supplemented with serum). Cell viability was determined by Calcein-AM, and GSH levels were determined by using monochlorobimane (12). The cells remained viable throughout the experiment, and GSH levels, in both naphthalene-treated L2 and HepG2/C3A cells, gradually decreased to about 60% of that in control cells, at 6 hours. To determine whether HepG2/C3A cells in the liver compartment were responsible for GSH depletion in the L2 cells, Hurel devices were operated with and without cells cultured in the liver compartment. After a 6-hour exposure to naphthalene, there was no intracellular GSH depletion in the L2 cells in the absence of liver cells. This result shows that, by itself, naphthalene had no effect on GSH depletion in the L2 cells. To determine whether the HepG2/C3A cells were affected by circulating naphthalene metabolites, a system was used that contained no cells in the lung compartment. The level of GSH in HepG2/C3A cells was reduced to about half of that of control cells, which is consistent with the finding that naphthalene metabolites do affect the GSH level in HepG2/C3A cells. A key feature of the Hurel device is the dynamic co-culture system that permits the different roles of metabolising cells (HepG2/C3A), and non-metabolising cells (L2), in naphthalene toxicity to be addressed. These results demonstrate that reactive metabolites produced by HepG2/C3A cells in the liver compartment circulate to the lung compartment and decrease GSH level in L2 cells in a time-dependent manner. The Hurel system clearly demonstrated that naphthalene itself is not cytotoxic, and that the presence of the CYP-450 system is necessary for naphthalene-induced GSH depletion, which is consistent with the known mechanism of naphthalene toxicity.

Understanding the dynamic process of bioaccumulation is very important in predicting the responses

Figure 1: A schematic representation of the Hurel™ microfluidic biochip

In this embodiment, the Hurel biochip consists of four organ compartments: a "liver" compartment to represent the organ primarily responsible for xenobiotic metabolism, a "lung" compartment representing a target tissue, a "fat" compartment to provide a site for bio-accumulation of hydrophobic compounds, and an "other tissues" compartment to assist in mimicking the circulatory pattern in non-metabolising, non-accumulating tissues.
of humans and animals to exposure to chemicals or pharmaceuticals. Bioaccumulation has become a critical consideration in the regulation of chemicals, and is important in understanding the pharmacokinetic and toxicity characteristic of the drug candidates (13). Lipophilic compounds can accumulate in the lipid component of the adipose tissue in humans and animals, faster than they are metabolised or excreted, which increases the concentration of those particular compounds in the body over time. To address this issue, Viravaidya et al. (12, 14) constructed a Hurel device that incorporates a fat tissue compartment to provide a bioaccumulation site for hydrophobic compounds. This three-cell system allows all the bioaccumulation, distribution, metabolism, and toxicity studies to be performed with the same device.

Hurel chips containing differentiated 3T3-L1 mouse adipocytes in the fat compartment were used to test for the bioaccumulation of naphthalene (12). To study the effects of the addition of fat tissue on naphthalene toxicity, a three-cell Hurel system (with differentiated 3T3-L1 adipocytes) was challenged with naphthalene-saturated medium, in a similar manner to a two-cell system (without 3T3-L1 adipocytes), and the GSH levels in L2 and HEPG2/C3A cells upon exposure to naphthalene were compared. The intracellular GSH level of L2 cells in the Hurel system without 3T3-L1 adipocytes decreased more than that of L2 cells in the system with 3T3-L1 adipocytes. In the liver chamber, the GSH of HEPG2/C3A cells in the system with 3T3-L1 adipocytes remained high through the experiment, while the GSH level in HEPG2/C3A cells in the system without 3T3-L1 adipocytes decreased significantly compared to that of the controls. Thus, the presence of 3T3-L1 adipocytes in the Hurel system reduced the level of response to naphthalene, which suggests that the fat compartment bioaccumulates the toxic metabolites of naphthalene (12).

**Pro-drug Activation**

For initial validation studies on pro-drug activation, three-compartment Hurel chips were fabricated on 22mm × 22mm, coverslip-sized, silicon chips that were engineered to receive cultures of human cell types. These devices are comprised of a liver compartment, a target tissues compartment, and an other tissues compartment. The liver compartment was seeded with the human hepatocyte cell line, HepG2/C3A, the target tissues compartment was seeded with the human colon cancer cell line, HCT116, and the other tissues compartment contained no cells. Once the cells had been seeded into their respective compartments, the chips were sealed inside acrylic chip housings. The top of the housings contained fluid interconnects to provide cell culture medium to the chips. Stainless steel tubes were connected to micro-bore peristaltic pump

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**Figure 2:** The Hurel™ device used to demonstrate the concentration-dependent cytotoxic effect of tegafur on a human colon cancer cell line

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Total cells</th>
<th>Dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero (control)</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>12.5 µg/ml</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>25.0 µg/ml</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>50.0 µg/ml</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Hurel chips were cultured with the human liver cell line HepG2/C3A in the liver compartment and the human colon cancer cell line HCT116 in the target tissues compartment. Tegafur was added to the re-circulating culture medium and pumped through the Hurel devices at various concentrations for 24 hours. Following drug exposure, the chips were treated with the membrane-permeable nucleic acid dye Hoechst 33342, and the membrane-impermeable nucleic acid dye ethidium homodimer. The fluorescence of the Hoechst dye is seen in all cells, alive or dead, whereas the ethidium only identifies dead cells.
tubing and inserted into a small hole in the top of a micro-centrifuge tube containing culture medium, with or without test compound. The pump tubing was connected to the peristaltic pump, primed with the culture medium, and connected to the inlet and outlet ports of the chip housing, thus completing the fluidic circuit. The entire instrument was placed in a CO₂ incubator at 37°C.

The Hurel system was tested by using the cancer chemotherapeutic pro-drug, tegafur (5-fluoro-1-[2-tetrahydrofuryl]-2,4-pyrimidinedione; 16). Tegafur is inactive and requires metabolic activation by CYP-P450 enzymes present in the liver to generate the active metabolite, 5´-fluorouracil (5-FU). Like most cancer chemotherapeutic agents, 5-FU induces marked apoptosis in sensitive cells, through the generation of ROS.

Hurel chips were cultured with the human liver cell line, HepG2/C3A, in the liver compartment and the human colon cancer cell line, HCT116, in the target tissues compartment. Tegafur was added to the re-circulating culture medium and pumped through Hurel devices at various concentrations for 24 hours. Following drug exposure, the chips were treated with the membrane-permeable nucleic acid dye, Hoechst 33342, and the membrane-impermeable nucleic acid dye, ethidium homodimer. With these stains, all the cells appear fluorescent blue, but dead cells are marked by the fluorescent red ethidium homodimer (Figure 2). In a comparative test, HCT116 cells were cultured on glass coverslips placed in 35mm culture dishes, and various concentrations of tegafur were added to the culture medium.

With the Hurel system, tegafur was found to be cytotoxic to HCT116 cells in a concentration-dependent fashion. However, tegafur was ineffective when tested by using the traditional, static cell culture

**Figure 3:** The Hurel™ device permits metabolite generation while traditional homogeneous static assays do not

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a) **Hurel™** (24 hours). Cells of the human liver cell line HepG2/C3A were cultured in the liver compartment and cells of the human colon cancer cell line HCT116 were cultured in the target tissue compartment of Hurel chips. Both the pro-drug tegafur and its active metabolite 5´-fluorouracil (5-FU), caused concentration-dependent cytotoxicity of the HCT116 cells.

b) **Static cell culture** (48 hours). In traditional, static, cultures of HCT116 cells tegafur was not cytotoxic. The cytotoxic effect of 5-FU was observed after 48 hours of exposure in the static cultures, compared to 24 hours in the Hurel device.

● = tegafur; ○ = 5-FU.
assay (Figure 3). In addition, although the active metabolite, 5-FU, triggered cell death in the traditional assay, cytotoxicity was not observed until after 48 hours of exposure. In comparison, when the Hurel system was used, cytotoxicity was observed after 24 hours of exposure to either 5-FU or tegafur.

In a separate study, to demonstrate that the Hurel liver compartment was responsible for the bio-activation of tegafur, Hurel chips were seeded with HCT116 cells only (i.e. while cells were cultured in the target cell compartment as before, no cells were cultured in the liver compartment). Either tegafur or 5-FU was added to the re-circulating culture medium for 24 hours, and the cell chip was then treated as described above. These experiments showed that, in the absence of a functional liver compartment, tegafur had no effect on the HCT116 cells, whereas the active metabolite, 5-FU, caused significant cell death (Figure 4). To demonstrate that the circulating medium of the Hurel device provides a further advantage over traditional static cell culture assays, HepG2-C3A cells were incubated in the presence of tegafur or 5-FU for 24 hours. The cell culture medium from this culture was then applied to a static culture of HCT116 cells and allowed to incubate for up to 48 hours. The conditioned medium from either the tegafur culture or the 5-FU culture was not effective at inducing cytotoxicity in the HCT116 cells (Figure 4). These results suggest that, although the HepG2-C3A cells were effective at metabolising the tegafur and 5-FU, the 24 hour incubation time most likely resulted in the formation of inactive metabolites. The results of these experiments confirm that tegafur is metabolised to an active drug in the Hurel liver compartment, whence it circulates to another organ compartment and is cytotoxic to the target cancer cell.

The Hurel system was also tested by using the nonsteroidal anti-inflammatory drug, sulindac. In addition to its anti-inflammatory properties, sulindac has been shown to induce apoptosis in a variety of epithelial cancer cells, and has shown cancer chemopreventive activity in animal model systems, as well as in early-phase chemoprevention clinical trials (16). Sulindac is itself inactive, but following ingestion the parent compound (sulindac sulphoxide), is converted into sulindac sulphide, which appears to be the primary cyclooxygenase (COX)-inhibiting metabolite. The second major metabolite, sulindac sulphone, has also been shown to inhibit carcinogenesis through COX-independent pathways (Figure 5).

Hurel chips were cultured with the human liver cell line, HepG2/C3A, in the liver compartment and the human colon cancer cell line, HT29, in the target tissues compartment. Sulindac or the two active metabolites, sulindac sulphoxide and sulindac sulphone, were added to the recirculating culture medium and pumped through Hurel devices at various concentrations, for 24 hours. The chips were then removed from the device, the cells were fixed and apoptosis was determined by using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (ApopTag™). Sulindac and its two active metabolites induced a concentration-dependent increase in apoptosis of the HT29 cells in Hurel devices containing an active liver compartment (Figure 5a). In the absence of an active liver compartment, the parent drug sulindac was not effective, but the two active metabolites were effective, at inducing apoptosis of the HT29 cells (Figure 5b). These results further demonstrate that an inactive pro-drug can be metabolised to an active drug in the Hurel liver compartment, and can circulate to, and interact with, another organ compartment, where an effect (apoptosis) can be observed.

Conclusions

Embodied on a microfluidic biochip, the Hurel, multi-compartmental and fluid-circulatory attrib-
utes permit the simulation of the inter-organ or inter-tissue interactions found in a living animal. Traditional, static cell-based assays do not possess this capability. For this reason, assays with the Hurel may provide more and more-accurate information relevant to a wider range of applications (e.g. information on drug absorption, distribution, bioaccumulation, metabolism, efficacy and toxicity) than is obtainable from static in vitro assays. The Hurel should therefore be understood, not simply as a technological substitute for the static, cell-based assay, but as a more broadly-applicable “in vivo-surrogate” assay platform that, in various circumstances, may precede, supplant, or complement in vivo tests. The Hurel has been successfully tested using a variety of assays (immunohistochemical, immunofluorescent, and others). It is anticipated that the Hurel will be adaptable to many different experimental applications, and will be compatible with virtually any type of assay used in traditional, static in vitro formats. Moreover, the Hurel devices can be designed for “plug-and-play” compatibility with plate readers and other standard laboratory instruments, they can be manufactured and operated on a 96-well, and smaller, scale, and their use can be automated for increased throughput and reproducibility.

By affording dynamic assessment of potential toxicity, metabolism, and bioavailability, the Hurel has the potential to markedly improve the prioritisation of drug leads prior to the preclinical (animal) testing phase. Earlier prioritisation will reduce the number of animals needed for toxicological testing, increase throughput in the preclinical phase, decrease the time and cost of preclinical studies, and increase the efficiency of clinical trials.

References


An Immunologic Model for Rapid Vaccine Assessment — A Clinical Trial in a Test Tube


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Summary — While the duration and size of human clinical trials may be difficult to reduce, there are several parameters in pre-clinical vaccine development that may be possible to further optimise. By increasing the accuracy of the models used for pre-clinical vaccine testing, it should be possible to increase the probability that any particular vaccine candidate will be successful in human trials. In addition, an improved model will allow the collection of increasingly more-informative data in pre-clinical tests, thus aiding the rational design and formulation of candidates entered into clinical evaluation. An acceleration and increase in sophistication of pre-clinical vaccine development will thus require the advent of more physiologically-accurate models of the human immune system, coupled with substantial advances in the mechanistic understanding of vaccine efficacy, achieved by using this model. We believe the best viable option available is to use human cells and/or tissues in a functional in vitro model of human physiology. Not only will this more accurately model human diseases, it will also eliminate any ethical, moral and scientific issues involved with use of live humans and animals. An in vitro model, termed “MIMIC” (Modular Immune In vitro Construct), was designed and developed to reflect the human immune system in a well-based format. The MIMIC® System is a laboratory-based methodology that replicates the human immune system response. It is highly automated, and can be used to simulate a clinical trial for a diverse population, without putting human subjects at risk. The MIMIC System uses the circulating immune cells of individual donors to recapitulate each individual human immune response by maintaining the autonomy of the donor. Thus, an in vitro test system has been created that is functionally equivalent to the donor’s own immune system and is designed to respond in a similar manner to the in vivo response.

Key words: clinical trial, drug testing, functional assays, high-throughput, immune response, infectious disease, in vitro, vaccine.

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MIMIC® System Technology Overview

The MIMIC System is based on the multidimensional interrogation of leucocytes. It can simulate a clinical trial, including the effects of immunotherapy on human population subgroups, where responses can be clustered into groups that capture genetic diversity and other important population characteristics, such as HLA haplotypes, age, autoimmune status, and gender. We hope that this dataset can guide the design of rapid and incisive adaptive clinical trials, as well as overcome limiting and misleading animal studies in predicting the immunogenic potential of non-homologous proteins and many vaccine candidates.

The MIMIC System is comprised of four different steps: 1) leucocyte collection and preservation, 2) the Peripheral Tissue Equivalent (PTE), 3) the Lymphoid Tissue Equivalent (LTE), and 4) functional assays for assessing the in vitro immune response (Figure 1).

Step one begins with the collection of donor leucocytes by apheresis at a local blood bank. The processing of the leucocytes typically begins within an hour after collection, and the entire process to cryopreservation takes less than 4 hours. From a single apheresis donation, approximately 10 billion leuco-
cytes are obtained and processed for cryostorage by standard methods, whereby the donor’s cells may be used at a later date. This approach allows multiple experimental iterations, such as different compounds, doses or combinations, including enough of each to obtain statistically meaningful data. Because of the nature of the studies and the ability to cryopreserve cells, all the relevant controls, such as no treatment, drug alone and pathogen alone, can be run on the same “surrogate human” at the same time. This is in striking contrast to what can be done with non-human primates (NHP) or in human clinical trials. An additional advantage over both NHP studies and human clinical trials is that the experiments can be repeated on the same “individual”, as a portion of the primary cells can be frozen and stored for future use.

The second step is to simulate a peripheral tissue. For this, we developed innate immune responses in the Peripheral Tissue Equivalent (PTE) module. The
PTE module allows for a broad assessment of responses, from toxicity to proinflammatory immuno-reactogenicity (testing vaccines, antigens, adjuvants, biologics, therapeutics and chemicals), to antigen processing, vascular leakage and leucocyte infiltration, maturation and extravasation. This module is a high-throughput, fully-automated, flexible and reproducible, 3-dimensional tissue-engineered construct, mimicking peripheral microvasculature and recapitulating peripheral circulatory and fast innate responses. The PTE also links to the adaptive arm of the immune system, allowing self-differentiation of extravasating monocytes into potent antigen-presenting migratory dendritic cells (DCs).

The third step is to simulate adaptive immune responses in the Lymphoid Tissue Equivalent (LTE) module. The LTE is essentially an artificial lymph node, where antigen-presenting cells from the immune system, the body’s “sentinel” cells, start working with the immune system’s T-cells and B-cells. Specifically, the LTE is designed to reflect the spatio-temporal kinetics in a lymph node, e.g., DC–T-cell interactions, antigen–B-cell interactions, T-cell and B-cell interactions, Th1 or Th2 polarisation bias, antigen-specific antibody production or cytotoxic T-cells, can all be assessed from this in vitro module.

The fourth step is to assess the immunocytes and biomolecules from the previous modules in a functional assay, such as microneutralisation assays, haemagglutination inhibition, adherence inhibition, CTL responses, or disease modelling. Having all of these modules operating in a robotics platform provides a high-throughput, reproducible platform where multiple drug/vaccine candidates can be tested on multiple donors at the same time, without subjecting the actual individual to a potentially dangerous substance.

Figure 2: The three populations of cells which arise in the Peripheral Tissue Equivalent (PTE) module

Three populations of cells arise following the application of peripheral blood mononuclear cells (PBMCs) to the PTE module. Sub-population 1 comprises immature CD14
+ dendritic cell precursors while sub-population 2 comprises immature dendritic cells (CD14
-), and sub-population 3 comprises mature dendritic cells. A fourth population is more macrophage-like and is retained in the collagen matrix. DC = dendritic cells, APCs = antigen presenting cells.

The PTE Module

The PTE module has been well characterised, and is a unique method of generating autologous dendritic cells by using a 3-dimensional tissue engineered construct (1–3). Simply put, the PTE module is a high-throughput module that spontaneously and autonomously generates dendritic cells (DCs), the principal antigen processing cells (APCs) of the immune system. The PTE is comprised of a human endothelial monolayer grown to confluence over a 3-dimensional extracellular matrix (Figure 1), onto which purified peripheral blood mononuclear cells (PBMCs) are placed. The immature monocyte population of cells extravasates across the endothelial monolayer, migrating into the matrix, where they spontaneously and autonomously differentiate into migratory DCs with different maturation states (Figure 2). The DCs then spontaneously reverse transmigrate across the endothelial layer — a process that reflects APCs crossing the lymphatics. Upon reverse transmigration across the endothelium, the APCs are then collected. At any point along their journey through the PTE, cultures can be stimulated with antigen/adjuvant/drug compounds of choice.

Figure 3: A comparison of the cytokine response to immunomodulators in the MIMIC System and the industry-standard PBMC assay

A comparison of the MIMIC System PTE module and the industry-standard PMBC assay was made using the same individual donor’s blood cells. Immunomodulators, representing different classes, were applied to the PTE and the PMBC assay and the cytokine response was plotted on a log scale. PTE = Peripheral Tissue Equivalent; PBMC = Peripheral Blood Mononuclear Cell; IL= interleukin; poly I:C = polyinosinic : polycytidilic acid; CpG = unmethylated synthetic cytosine-phosphate-guanosine oligodeoxy nucleotides; LPS = lipopolysaccharide. Gardiquimod™ is an imidazoquinoline compound developed by InvivoGen.

A) Poly I:C
B) CpG 2006
C) Gardiquimod
D) LPS

= IL-1α; = IL-1β; = IL-6; = IL-8; = IL-10; = TNFα.
The monocyte extravasation and the DC development kinetics match in vivo physiology. An important aspect of the PTE is that the migratory DCs remain largely immature in the absence of an external stimulus. We have found that these immature DCs can acquire and process antigen when properly stimulated by adjuvants, maturing into potent DCs capable of initiating antigen-specific immune responses in LTE co-cultures. These DCs have shown the capacity to induce antigen-specific lympho-proliferation, cell-mediated cytotoxicity and T-helper cytokine production.

We have found that PTE-derived DCs are very similar to in vivo DCs. The PTE has shown that naturally extravasated monocytes constitutively and autonomously differentiate into either migratory DCs, or resident macrophages, in absence of stimulation, and this normally takes between 1–3 days; similar kinetics have been reported in vivo for both humans and animals (4–8). The transendothelial migration of blood monocytes promotes differentiation into potent antigen-presenting DCs in humans and animals (9–11), as observed in the PTE; extravasation of leucocytes is increased via endothelium activation in both the PTE and in vivo (12). Crossing the endothelium in the abluminal-to-luminal direction (reverse transmigration) in the PTE resembles the in vivo entrance of DCs into the lymphatics (13).

Similarly, in vivo, skin DCs will be one of the first cell types to engage a pathogen or foreign material, such as a vaccine or a topicaly applied chemical. The DCs produced by the PTE have been extensively characterised, and were found to be very similar in the expression of numerous surface markers to those of human dermal explants (14). Additionally, three subpopulations of DCs are also characteristic of PTE-generated DCs: immature DC precursors (CD14+), immature DCs (CD14−), and mature DCs (CD14−, HLA-DR+, CD86−, CD83+), along with a fourth population of cells that differentiate to a more macrophage-like phenotype and do not reverse transmigrate back across the endothelial monolayer (Figure 2).

The PTE has been found to largely recapitulate innate immune responses, when tested with vaccines, adjuvants, biologics, immunopotentiators, immunosuppressants and various pathogens. The PBMC assay is the accepted industry-wide standard for studying immune reactions (15). The MIMIC System has been found to produce a more physiologically relevant response than the PBMC assay (Figure 3) for various adjuvants and immunomodulators.

To evaluate the immunopotency of Toll-like receptor (TLR) agonists in the in vitro lymphatic PTE module, we measured TLR-induced cytokine production. Overall, TLR agonists induced higher levels of cytokines in the PTE module than in

![Figure 4: The cytokine response to vaccines: A comparison of the MIMIC™ System PTE module and the industry-standard method](image-url)

**Figure 4:** The cytokine response to vaccines: A comparison of the MIMIC™ System PTE module and the industry-standard method

- **a)** PTE-derived DCs; **b)** monocyte-derived DCs.

Dendritic cells (DCs) created in the MIMIC System PTE module and monocyte-derived dendritic cells were exposed to three vaccines and the production of three inflammatory cytokines (IL-6, IL-8 and MCP-1) was measured. PTE = peripheral tissue equivalent; HBV = hepatitis B virus vaccine; IL = interleukin; MCP = monocyte chemotactic protein; DTaP = diphtheria, tetanus toxoid and acellular pertussis vaccine; Fluzone® = a killed, trivalent influenza vaccine.
conventional PBMC cultures (Figure 3). For example, polyinosinic:polycytidylic acid (Poly I:C) and unmethylated synthetic cytosine-phosphate-guanosine oligodeoxy nucleotides (CpG 2006), both induced the proinflammatory cytokines IL-1 α/β in the PTE module, but neither of these cytokines were observed in PBMC cultures. Poly I:C also triggered the production of TNFα only in the PTE culture (Figures 3a and 3b). Moreover, Poly I:C and CpG 2006 treatments elicited approximately 100–1000 fold greater levels of IL-6 and IL-8 in the PTE module than in PBMC cultures. Although Gardiquimod and LPS dramatically induced IL-1 α/β, IL-6, IL-8, IL-10, and TNFα, both in the PTE module and in the PBMC cultures, the PTE module produced approximately 3–6 fold more IL-1 α/β and IL-10, and 10–50 fold more IL-6 and IL-8, than PBMC cultures (Figures 3c and 3d). Hence, the PTE module was found to be more sensitive than conventional PBMC cultures in response to TLR stimulation (Ma et al. Assessing the immunopotency of Toll-like receptor agonists in an in vitro tissue engineered immunological model. Manuscript in preparation).

When stimulated by vaccines, the PTE module mimics the in vivo state by producing cytokines known to be involved with inflammatory processes. Figure 4 shows the general innate reactogenicity of the commercially available vaccines, DTaP (Sanofi Pasteur, Inc.), Fluzone® (Sanofi Pasteur, Inc.) and Recombivax® (Merck & Co., Inc.), assessed by detection of a panel of proinflammatory cytokines in the PTE module. As can be seen, the reactogenicity is highest for DTaP probably as a result of a combination of bacterial components from diphtheria and acellular pertussis, and the presence of alum adjuvant. Fluzone shows moderate reactogenicity, that may be linked to a residue of egg albumin and the inherent stimulatory capacity of influenza virus proteins; and finally, the purified HBsAg sub-unit vaccine of Recombivax has less potency. Interestingly, several inoculations of the Recombivax vaccine are normally required in vivo, to elicit protective immunity.

The LTE Module

The Lymphoid Tissue Equivalent (LTE) module has been designed to largely recapitulate human adaptive immune responses in the lymphoid tissues of the body. Although there are many cellular

Figure 5: A comparison of the specific immune response to tetanus vaccine, in vivo, and in vitro in the MIMIC System

13 volunteers were vaccinated with a commercial tetanus vaccine. Blood samples from each individual were taken before and after vaccination.

a) The levels of tetanus-specific antibodies in the individuals’ sera were determined by enzyme-linked immunosorbent assay (ELISA), pre and post-vaccination.

b) Peripheral blood mononuclear cells, purified from both the pre and post-vaccination blood samples of the individual donors, were simultaneously evaluated in the MIMIC System. The number of tetanus-specific antibody secreting cells, after 7 and 12 days of in vitro culture, were determined by enzyme-linked immunosorbent spot (ELISpot) assay.

= pre vaccination; = post vaccination.
types within any given lymph node, the MIMIC system incorporates two-, three- and four-way interactions of the key immune cells (DCs, follicular dendritic cells, B-cells and T-cells). The application of these cells to the LTE is in sequential order, to mimic these immunologically-relevant responses, similar to what is known to occur in vivo (16). VaxDesign follows fundamental design observations, whereby the right cells (CD4+ T-cells, B-cells, DCs, and follicular DCs) are placed together at the right time and in the right order, in an automatable, scalable, reproducible system to get the appropriate response (Moser, J.M., Sassano, E.R., Leistritz, D.C., Eatrides, J.M., Gaucher, D., Filali-Mouhim, A., Phogat, S., Koff, W., Sékaly, R.P., Haddad, E.K. & Drake, D.R. [2009]. Dendritic cell-based assay for the in vitro priming of naïve human CD4+ T cells. Manuscript submitted).

As one example to validate the potential of this approach, we monitored the in vitro-generated tetanus toxoid (TT)-specific antibody levels in a cohort of donors before and after receiving tetanus vaccination. Purified CD4 T-cell and B-cell populations were combined with autologous tetanus vaccine-pulsed dendritic cells, to generate specific antibody. Enumeration of the TT-specific IgG antibody-secreting cells by enzyme-linked immunosorbent spot (ELISpot) assays displayed a significant increase in the magnitude of this population after vaccination. The relative magnitudes of the in vitro–generated TT-specific antibody response before and after vaccination, largely recapitulated the TT-specific IgG serum titre profiles measured in the same individuals, as shown in Figure 5 (17).

These findings provide evidence that the MIMIC System can be a rapid and representative in vitro method for measuring vaccine immunogenicity via induction of the memory B-cell response. In-house studies have clearly demonstrated that the use of purified lymphocyte populations and autologous DCs is more sensitive than bulk PBMC assays at generating both T-cell and B-cell immune responses (unpublished data).

Similar results have been obtained with other commercial vaccines, such as those for recall antigens (e.g. hepatitis B virus and influenza viruses),

Figure 6: Antibodies produced by the MIMIC System show effective neutralisation activity

1:32
1:16
1:8
1:4

HAI titre

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Individual human donors’ leucocytes were placed in the MIMIC System and stimulated with a commercial live-attenuated influenza vaccine. To test whether the antibodies produced by the B-cells in the MIMIC System are effective in neutralising the influenza virus they were tested, at serial dilution, in a standardised haemagglutination assay (HA). In this assay influenza virus particles cause agglutination of red blood cells (RBCs). Antibodies specific to influenza surface proteins will bind to the viral particles and will inhibit the haemagglutination. The greater the dilution of antibody-containing material, that still causes inhibition of agglutination, the greater the concentration of specific antibody. HAI = haemagglutination inhibition assay.
and primary response antigens, such as yellow fever virus.

**Functional Assays**

Functional assays are at the heart of the MIMIC System and determine whether the responses observed in previous modules of the MIMIC System are going to be effective against the original challenge material. For example, are the antibodies produced by B-cells effective at neutralising the original virus, as observed in the case for influenza (Figure 6), or do T-cell antigen-specific responses lead to an increase in cytokine production, cytolytic activity or overall proliferation? Cytotoxic T-cell assays examined CD107a and interferon-gamma (IFN-γ), both markers for cell killing.

Since the MIMIC System can be re-stimulated *in vitro* many times, this is similar to the prime-boost scenarios used *in vivo*. This unique strategy allows for the possible application and testing of different primary and secondary stimulating antigen combinations to be delivered, all *in vitro*. CD8⁺ T-cell responses have been observed in the MIMIC System, for both recall antigens (live-attenuated influenza vaccine) and for naïve antigens (live-attenuated yellow fever vaccine).

Disease modelling can also be performed with the MIMIC System. Tuberculosis (TB) is classified as one of the most devastating granulomatous diseases world-wide (18). The MIMIC System has been able to successfully recapitulate granuloma formation *in vitro*, and to drive *Mycobacterium tuberculosis* to latency. Histological analysis of the PTE module seeded with *M. tuberculosis* and PBMCs revealed spontaneous granuloma formation. Haematoxylin and eosin staining of sectioned PTE modules seeded with *M. tuberculosis* and PBMCs, clearly showed initial stages of granuloma formation in culture (Figure 7). In this disease module, new antibiotics can be tested to determine whether they are effective on latent TB and could lead to new therapeutic regimens. (Pawar et al. *An in vitro model of human tuberculosis granuloma and Mycobacterium tuberculosis latency*. Manuscript in preparation).

**Automation**

The MIMIC system has been streamlined and automated from beginning to end, by using a unique, reliable and robotic system to construct and test each component of this *in vitro* cell-based technology. Automation allows for precise fluid handling, consistency between wells and tests,

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**Figure 7:** A cross-section of a MIMIC System PTE module seeded with *Mycobacterium tuberculosis*  

A MIMIC PTE module was seeded with *M. tuberculosis* and PBMCs. Examination of cross-sections, stained with haematoxylin and eosin, clearly showed the initial stages of granuloma formation. Macrophages, T-cells and B-cells, NK cells and fibroblasts are among the cells that aggregate to form the granuloma, with lymphocytes surrounding the infected macrophages. The MIMIC System has been able to recapitulate in vivo granuloma formation. This represents a novel disease model in which new antibiotics can be tested for efficacy against latent tuberculosis. PBMCs = peripheral blood mononuclear cells.
from one donor’s cells to another, and provides a rapid platform to accomplish a very high-throughput system in the most cost-efficient manner currently available.

Conclusions

The MIMIC System is a high-throughput, automated, in vitro modular technology, which is capable of examining individual human donor immune cell responses to many different compounds, such as vaccines, adjuvants, proteins, chemicals and drugs. Innate immune responses primarily are observed in the PTE module, with the capacity to mimic multiple mucosal surface types, as well as different antigen delivery sites. LTE responses recapitulate in vivo adaptive immune response with the right cells, at the appropriate time, and under appropriate conditions, to permit the production of effective antibody production and/or T-cell responses to vaccines, biologics, biologicals, or pathogens. Functional assays test these antibodies or T-cells for performance against the stimulating antigen. Many of these involve cytokine production, increased titres in vitro, viral neutralisation, or cytotoxic T-cell assays. The MIMIC System allows testing for a variety of demographic groupings, such as for HLA typing, gender or age biases, and geographic regional differences.

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References

The Use and Value of In Vitro Technologies in Metabolism Studies

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Summary — The detailed investigation of the metabolism of drugs is one of the key issues in drug development. Several in vitro metabolism assays have been developed over the last two decades, to replace time-consuming and expensive animal studies. These have the potential to speed up drug development and increase drug safety, as they can be used to improve the prediction of the effects of drugs on humans. The key factors to be identified in metabolism are: a) the enzymes involved, and b) the metabolites produced by these enzymes. Cytochromes P450 (CYP-450s) are the key enzymes in drug metabolism. Cloning the genes encoding the CYP-450s, and the genetic engineering of suitable cells for heterologous expression, have provided new cell lines for studies on drug metabolism in vitro, under highly defined conditions. The V79 cell line, derived from Chinese hamster lung fibroblasts, was found to be suitable for heterologous expression, as these cells themselves do not express CYP-450s, thus providing a clean background for genetically engineering for the stable expression of any cloned CYP-450. In this way, V79 cell lines were created which specifically express CYP-450s from human, mouse, rat, and fish. These recombinant V79 cells have been applied in several drug metabolism and toxicity studies.

Key words: cytochrome P450, heterologous expression, in vitro metabolism, V79 Chinese hamster cells.

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Introduction

The development of procedures to permit the genetic engineering of mammalian cells for the stable expression of drug metabolising enzymes began in 1985, and has resulted in highly specific tools for use in investigating drug metabolism (1, 2). In this way, a variety of Chinese hamster V79 cells have been constructed for the stable expression of individual cytochromes P450 (CYP-450s) from human, mouse, rat, and fish. These new cell lines have been applied in several metabolism studies (3–5). V79 cells were particularly useful for this approach, as they do not express any endogenous CYP-450s, thus expressing only the CYP-450 which is encoded by foreign cDNA stably integrated into the V79 genome after transfer with a suitable expression plasmid vector. Here, as an example, the construction and application of the V79 cells expressing CYP-450 2D6 is presented (6). Although the abundance of CYP-450 2D6 in the liver is low in comparison to other CYP-450s, there is a 30% chance that a drug will be metabolised by CYP-450 2D6. In addition, the gene encoding CYP-450 2D6 is highly variable among humans, with more than 40 DNA sequence variations, resulting in a wide diversity of slow-metabolising and fast-metabolising individuals. As many as five of these genetic variations encode active enzymes. These CYP-450 2D6 genes were cloned and individually expressed in V79 cells, in order to study the variant-dependent metabolism of drugs, e.g. the hydroxylation of bufuralol, which is CYP-450 2D6 specific (6).

Materials and Methods

Construction of V79 cells for stable expression of CYP-450 2D6

The cDNAs encoding CYP-450s 2D6 *1, *2, *9, *10 and *17 variants were cloned and inserted into the expression plasmid vector, pSV450, and transferred as recombinant expression vector pSV450h2D6 into V79 cells, as described previously (6).

Characterisation of V79 cells expressing CYP-450 2D6 variants

Protein-expressing cells were selected and characterised for CYP-450 2D6 content and activity (6).
Figure 1: Making CYP-450 genes work in cells

The cDNA sequences encoding the human CYP-450 2D6 variants, *1, *2, *9, *10 and *17, were cloned and inserted into a plasmid expression vector. Chinese hamster V79 cells were transfected with the vectors and expression was confirmed by immunostaining the cells with a CYP-450 2D6 antibody.
Application of V79h2D6 cells in drug metabolism

Variant-dependent metabolism was studied by bufuralol and tamoxifen hydroxylation. Hydroxylated bufuralol was detected fluorimetrically after HPLC chromatography, and hydroxylated tamoxifen was detected by HPLC/ESI-MSD, as described previously (6).

Results

All five CYP-450 2D6 genes were cloned and verified by DNA sequencing, before insertion into the expression vector, yielding the recombinant expression vector pSV450h2D6. The recombinant gene was inserted into the genomic DNA of the recipient V79 cells. Protein expression was confirmed by immunohistochemical staining with a CYP-450 2D6-specific antibody (Figure 1).

Variant-specific kinetics of bufuralol-hydroxylation were observed, with *10 being the least active variant, and *1 the most active variant (Figure 2).

Discussion and Conclusions

The cloning and expression of the CYP-450 2D6 variants, and the subsequent characterisation of variant-specific bufuralol hydroxylation, provide just one example of what can be studied with the recombinant V79 cell lines. This study could be potentially extended to include the other CYP-450 enzymes. In general, these cell lines represent analytical in vitro tools for the investigation of drug metabolism. This includes the identification of the metabolically-competent CYP-450, the identification of the metabolites that are formed, and the determination of the kinetics of the reaction. These would be difficult to investigate with animal studies, due to the complexity of the in vivo situation.

Via the cloning and expression of human genes encoding the CYP-450s, the recombinant V79 cells can be employed to give data with immediate relevance to the human exposure situation. This avoids problems associated with transferring and extrapolating results from animal studies to humans. Thus, there is the potential for educated knowledge to be available on drug metabolism, long before clinical trials are performed, which would help to structure and speed up this very last step in drug development, with a much higher safety margin than if it just relied on animal data.

Last, but not least, it is worthwhile to note that there is no animal model available which provides insight into the genetic variant-dependent metabolism of humans, which is of increasing importance in order to avoid serious adverse consequences due to overdosing or underdosing. This permits the consideration of what is nowadays termed “individualised medicine”.

Figure 2: The enzymatic activities of the five variants of CYP-450 2D6

The activity of the five variants of CYP-450 2D6 were determined by their ability to catalyse the hydroxylation of bufuralol.
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Toxicogenomics in Drug Discovery and Development — Making an Impact

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Summary — As a branch of pharmacogenomics aimed at predicting drug safety concerns, toxicogenomics drew much excitement with the emergence of technologies such as gene expression microarrays. A few years down the line, the evidence is scant that current approaches to toxicogenomics are really making an impact in areas such as preclinical toxicology. It has been argued that there needs to be a re-focus of application toward high-throughput approaches which combine the best of tissue and genomic modelling. This commentary gives a brief introduction to in vitro toxicogenomics, drawn from the perspectives of the specialist toxicogenomics company, SimuGen.

Key words: cell culture, drug safety, in vitro, pharmacogenomics, toxicogenomics, toxicology.

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Introduction

At this meeting on Speed and Safety in Drug Discovery (1), academics, service providers and stakeholders were invited to present their views on in silico, in vitro, ‘omic’ and clinical trial trends, with the aim of improving the prediction of human drug safety. Much of the discussion centred on speeding up decisions and the reduction of drug development attrition rates — the ‘fail early’ paradigm highlighted in the US Food and Drug Administration’s (FDA’s) 2004 white paper on critical path challenges (2). Five years have passed since this FDA publication and the launch of the ‘Critical Path Initiative’ (3), which calls for some retrospection on how far we have come with ‘omic’ tools in drug safety.

Over the last few years, ‘toxicogenomics’ has stepped up as a term for ‘omic’ methods used to better understand and predict unwanted chemical and physical effects in organisms and ecosystems. In reality, its industrial application has largely been limited to the use of transcriptomic (gene expression) methods in animal trials, for the better understanding of small molecule toxicity in humans. With the current low levels of drug innovation, patents ending on a generation of blockbuster drugs, the notable development attrition, and market withdrawals of pharmaceuticals due to safety concerns, toxicogenomics has been touted as a ‘better, cheaper and faster’ option. In reality, the publication evidence does not support the claim that toxicogenomics makes decisions any faster, any less complicated or any more cost-effective. A study by Foster et al. (4) suggests that current advantages might be limited to overlaying traditional pre-clinical assessment with some mechanistic insights. Gene expression methods are no longer as immature as they were just a few years ago; whilst they still prove a technical challenge, it is these authors’ opinion that much of the problem lies with how we have made use of them.

Toxicogenomics as a Tool

Being a fairly young, fast evolving, collection of sciences, ‘omics’ is sometimes driven by the latest technologies and what questions the technologies can help answer, rather than drawing from the questions that need be answered, and answering them in a way that best facilitates a decision. This remains a particular problem with large R & D pipelines, where different questions are asked of data that might not be appropriate for answering all of the questions as satisfactorily as possible. To the medicinal chemist, or other team members involved in drug discovery/development toxicology, a toxicogenomics tool would need to answer three questions in real-time: What toxicity?, How toxic?, and What could be done to make the hit/lead less toxic?
Today, there is no shortage of commercially-available gene expression microarrays for use in trying to answer these questions, including specialist microarrays and/or analysis software. For example, a market leader, Affymetrix Inc. (5), offers two products focused on drug metabolism and safety. Their DMET™ product focuses on pharmacogenetic markers for drug metabolism studies, featuring markers in FDA-validated genes and defined by pharmaADME (6). An analysis suite, ToxFX™, combining gene expression arrays from Affymetrix with the analysis software and a database from Entelos (7), is also available for preclinical work, based on signatures and pathways of toxicological interest in the rat genome. Using this type of gene expression data, two major analysis paradigms exist (Figure 1). The first would be to treat gene expression results as an exercise in pattern recognition. If a novel compound exhibits an expression pattern very similar to that of another, a) Toxicity by association: traditional pattern recognition may cluster compounds 1 and 2 together with the ‘signature’ of other necrotic drugs in a database of compound-related gene expression data, but not provide any further information to allow confidence in this association. The figure shows a Principal Component Analysis with three variables. ○ = known non-necrotic drugs; ● = known necrotic drugs; 1 and 2 are drug candidates of unknown toxicity.

b) Understanding toxicity with bioinformatics: An example of four genes reacting to two compounds is shown. Black boxes represent genes being down-regulated (decreased expression), while the white boxes represent genes being up-regulated (increased expression), in two converging gene pathways (P1 and P2) associated with toxicity. The arrows denote known positive effects on downstream genes, while the T lines denote known inhibitory effects on downstream genes. The example here demonstrates that compound 1 inhibits P1 enough to allow increased expression of the gene common to the two pathways. Compound 2 has the reverse effect on P1 and the common gene, but the same effect on P2. Such results might be academically useful to begin understanding varying toxic mechanisms, but have proven to be of very little use as toxicity assays for routine industry use.

Figure 1: The traditional toxicogenomic methods used when assessing compounds for toxic effects

a)

b)
well-defined, compound in a database, then they might be assumed to cause similar toxicities (Figure 1a). Large databases, such as DrugMatrix® (7), have been used, in part, for this purpose. A second, increasingly popular, approach is to use bioinformatics-driven methods to mechanistically study molecular pathways thought to be central to toxic endpoints (Figure 1b). The first approach — practiced in the extreme — ignores biology, the second relies on a very poor understanding of biology. Both have traditionally ignored, or paid little attention to, the explicit modelling of dose–toxicity relationships; which is central to deciding acceptable toxicity.

As suggested by Foster et al. (4), current methods may ultimately prove to be useful in pre-clinical work, but still do not address the ‘fail early’ needs of industry. It is our opinion that the most significant drug safety impact can be made by applying toxicogenomics to high-throughput in vitro assays, so that toxicity can be considered alongside early stage ADME. It is an opinion similarly expressed by some in industry (8), and will be the focus for the remainder of this commentary, with particular reference to liver toxicity. The approach advocated by SimuGen (9) is to slot toxicogenomics in with early stage human in vitro assays, to answer the questions of What toxicity? How toxic? and What could be done about it?, at a stage when considering other predicted compound properties. With SimuGen’s approach, compounds are added over multiple concentrations to human cell cultures, and the gene expression trends are used in dose–response models for particular relevant clinical toxic endpoints that have been validated against compounds of known human toxicity (Figure 2). In doing so, in vitro molecular events are mapped directly onto human toxic pathology. With the testing of a chemical series, rather than applying traditional pattern analysis to raw gene expression data, the parameters from the dose–response models can be used, and the results linked to compound structures, to identify any structure–toxicity relationships. This approach differs from many current in vitro toxicogenomic studies that measure gene expression changes in cells exposed to single, highly cytotoxic doses of compounds — arguably, far beyond a level from which to extrapolate normal biology.

The Use of In Vitro Models

In vitro studies reduce the reliance on preclinical testing (10), and outperform preclinical testing as high-throughput, more-reproducible models. It has been routinely argued that primary human hepatocytes represent the gold standard model for human in vitro studies on xenobiotic metabolism and liver toxicity (10). However, such arguments sometimes fail to take into account the basic sensitivity/specificity concerns of creating a good model: a model that is less biased from reality, but with a poor signal-to-noise ratio because of technical performance, does not make for a good model. Primary hepatocytes are associated with a number of limitations, such as scarce or unpredictable availability, limited growth activity and life-span, culture instability (early and unpredictable phenotypic alterations), and wide variations in functional activities from one hepatocyte population to another that needn’t reflect sample genetic differences (10).

Cell lines derived from hepatocellular carcinomas, as an alternative, are easier to grow and store. SimuGen’s approach enables the modelling of in vitro molecular events, to predict at which relative doses these events would become clinically significant in humans. The horizontal dotted line on the graph represents the threshold at which toxicity is established.

![Figure 2: Combining toxicogenomics with dose–response modelling — the SimuGen approach](image)

By combining gene expression trends with in vitro assays to determine the extent of toxicity, over a wide range of compound concentrations, SimuGen is able to map the dose-response gene expression to the dose-response effect in humans. The figure uses zone 3 human liver necrosis as an example to show dose–response curves for two compounds, with compound 2 exhibiting higher toxic potency than compound 1. By using compounds with well-described human safety concerns to validate the model, SimuGen’s approach enables the modelling of in vitro molecular events, to predict at which relative doses these events would become clinically significant in humans. The horizontal dotted line on the graph represents the threshold at which toxicity is established.
maintain, which makes them useful for in vitro studies. The HepG2 cell line, for example, has been widely used to assess xenobiotic metabolism and toxicity. It is more stable than human primary hepatocytes, but has its limitations: the cells contain little Phase I drug metabolising activity, and do not mimic the regulation of gene expression observed in normal hepatocytes (10). A newer cell line, HepaRG®, exhibits liver parenchymal differentiation, greater metabolic competence for Phase I and II enzyme activities, greater culture stability than primary human hepatocytes, and can be cryopreserved without marked cell loss (11). HepaRG is proving increasingly popular as a scalable monoculture model, and is currently being assessed as a toxicogenomics tool in a project between SimuGen and the Centre for Proteomic and Genomic Research (9, 12).

The Future of In Vitro Toxicogenomics

With a focus on improving cell methods and genomic modelling, in vitro studies open up large possibilities for exploring mechanistic and predictive toxicology. Tissue engineering is pushing medium-throughput to high-throughput methods beyond current simple, single layer cultures, to complex co-cultures and 3-D cultures. For example, a ‘lab-on-a-chip’ specialist, the Hurel Corporation (13), are researching microfluidic circuits to permit the testing of multiple tissues together. The Griffith Lab at the Massachusetts Institute of Technology are also developing 3-D ‘liver-on-a-chip’ bioreactors, each only a few centimetres long (14). These comprise liver cells growing on silicon scaffolds, forming channels that resemble the capillary bed for the constant perfusion of nutrients. There are many such examples, with bodies such as the European Centre for the Validation of Alternative Methods (ECVAM), established to validate and list these newer culture approaches (15). Beyond tissue engineering, toward genomic engineering, in vitro gene expression can also be manipulated in a number of ways, to create functional variation more in line with human population variation, or to better understand toxicity mechanistically. Using RNA interference (RNAi), Dai et al. (16) have established an approach to suggest the causal sufficiency order network for liver hypertrophy in the rodent, based on the expression profiles in response to the small interfering RNAs against the gene for the PPARα receptor.

In vitro methods will also open up opportunities for the more sophisticated ‘omic’ study of toxic perturbation, such as drug–drug interactions and multiple dosing for chronic effects at the molecular level. Multiple drug regimens are often administered to a single patient, and drug–drug interactions occur during these regimens, resulting in adverse effects that may become life threatening. Rifampicin is a well known example, which is commonly used in clinical studies as a prototypical inducer of drug-metabolising enzymes, to test for effects with other drugs (17). Multi-dose, chronic studies in cell cultures are still complicated by the fact that many cell models become unstable when cultured for long periods of time. However, the HepaRG cell line again proves promising, by demonstrating relatively stable functional activities, weeks after confluence.

In summary, the field of toxicogenomics is likely to have a significant impact on drug discovery, if the fields of functional genomic and tissue culture modelling are brought together more successfully. It is with this goal that SimuGen models in vitro dose-response molecular events, mapping them directly to human toxic endpoints, rather than applying traditional pattern recognition or bioinformatics. This provides decision-focused, reliable computation models, bringing toxicogenomics into a high-throughput ADME toxicology pipeline.

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Use of Virtual Patient Populations for Rescuing Discontinued Drug Candidates and for Reducing the Number of Patients in Clinical Trials

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Summary — The decreasing cost-efficiency of drug development threatens to result in a severe shortage of innovative drugs, which may seriously compromise patient healthcare. This risk underlines the urgency to change the paradigm in clinical research. Here, we examine a novel concept of conducting virtual clinical trials for efficiently screening drug candidates, and for evaluating their prospects of being brought to the market successfully. The virtual clinical trials are carried out by using virtual patients (denoted Optimata Virtual Patients — OVPs). The OVP, a set of mathematical algorithms that describe the main pathological and physiological dynamic processes affected by the administered drug, has been shown to accurately predict docetaxel efficacy and safety in individual breast cancer patients. We report a test case in which virtual clinical trials have been conducted by using OVP populations for rescuing a discontinued oncology compound, ISIS-5132 (ISIS Pharmaceuticals Inc.). Our in silico study suggested that ISIS-5132 may be efficacious in combination with another drug, sunitinib malate (Sutent®, Pfizer Inc.), for the treatment of prostate cancer. The recommended combined treatment is predicted to result in a higher five-year Progression-Free Survival than monotherapy with either drug alone.

Key words: discontinued compounds, in silico, mathematical model, oncology, virtual clinical study.

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Introduction

Drug development is a challenging, costly and time-consuming process. Drugs that fail in clinical trials, often due to low efficacy and/or high toxicity, are shelved or altogether discontinued (1). Since the pipelines for new compounds seem to be increasingly exhausted, it becomes mandatory that the pharmaceutical industry abandons its customary work paradigm, and examines novel ways for efficiently bringing molecules to the market. Scientific tools which have been in use in other fields of research and industry should be looked at for streamlining clinical research.

The concept of the Optimata Virtual Patient (OVP) has been developed and used to suggest improved individualised treatment schedules in oncology. This is achieved by using computer simulations of sets of mathematical models, which allow for drug–patient dynamic interactions. These models represent the integration of the pharmacokinetics (PK) and pharmacodynamics (PD) of the drug or drugs with the pathological and the related physiological processes, to give one general framework (2, 3). This methodology enables the prediction, not only of the short-term efficacy and toxicity of drugs, as conventional pharmacokinetics/pharmacodynamics (PK/PD) models do, but also of their long-term effects.

Recently, this method has been employed for identifying an improved treatment for a mesenchymal chondrosarcoma (MCS) patient. To this end, growth curves and gene expression analysis of xenografts, derived from the patient’s lung metastasis, served to adjust the OVP to the specific MCS progression. The PK and PD of several drugs were modelled. The xenografted animals were treated by various monotherapy and combination therapy schedules, and the MCS-xenograft model was computer-simulated under the same treatment scenarios. The mathematical model for xenograft growth was then up-scaled to retrieve the MCS patient’s tumour progression under different treatment schedules. An average accuracy of 87.1% was obtained, when comparing model predictions to the observed tumour growth inhibition in the xenografted animals. The simulation results suggested that a regimen of bevacizumab given intra...
venously in combination with once-weekly docetaxel, would be more efficacious in the MCS patient than all the other simulated schedules. Weekly docetaxel in the patient resulted in stable metastatic disease, and relief of pancytopenia due to tumour infiltration (4).

Here, we suggest the use of populations of OVPs for conducting virtual clinical trials, in order to rescue prematurely shelved or discontinued compounds. An OVP population represents the distributions, in selected real patient populations, of the crucial parameters of the physiological and pathological interactive processes. To illustrate this approach, we will use a case study of a discontinued drug, ISIS-5132 (ISIS Pharmaceuticals Inc., Carlsbad, CA, USA).

ISIS-5132 is an antisense oligonucleotide targeted against the c-ras-1 kinase oncogene (5, 6). The c-ras-1 kinase is the direct downstream mediator of the ras protein, the oncogene downstream of which is associated with more than 30% of human solid tumour types, including: lung, colon, and pancreatic cancers (7). ISIS-5132 has been tested in Phase I trials in melanoma, pancreas, colorectal, breast, brain, small cell lung, and non-small cell lung cancers (8, 9). Phase II studies were conducted in ovarian, prostate, small cell lung, and non-small cell lung cancers (10–13). However, these Phase II trials failed to show significant antitumour activity. The compound has also been tested preclinically in xenograft models of breast, small cell lung, non-small cell lung, prostate, and colorectal cancers (14).

Below, we briefly describe the disease modelling work, the creation of the OVP populations, and their use in redirecting the discontinued drug ISIS-5132 to a more promising avenue of development.

Constructing the Disease Model

The OVP vascular tumour growth model has been described in detail elsewhere (3, 15, 16). To adjust the generic vascular tumour growth model to reflect "real" patient populations, one would be required to embed in the model, parameters characterising the untreated tumour growth dynamics of the relevant indication. However, since measurements of untreated cancer patients are not available, synthetic curves, mimicking the growth of such untreated tumours, can be constructed. These curves are based on the initial tumour size measurements and doubling times of untreated cancer patients found in the literature (1, 17, 18). Accordingly, the size of the untreated tumour at a given moment can then be calculated by using the exponential growth model, as described by Usuda et al. (18).

Briefly, the size of an untreated tumour on day $t$ can be calculated by the following formula:

$$V_t = V_0 \times 2^{t/VDT}$$

where $t$ represents the time of measurement (in days), $V_0$ represents the initial tumour size (in number of cells) and VDT represents the tumour-volume doubling time. The synthetic curves that are generated by this process serve as an input for estimating the cancer OVP's average vascular tumour parameters, $F_{ave}$.

Constructing OVP Populations

In order to predict the effect of a treatment on a population, an OVP population must be generated. For each OVP, the values of the model parameters are set. The individuals belonging to this population share most of the model parameters. However, several parameters are individually selected from a predefined distribution. The parameters and their values are selected, based on studies that indicate that they can have a prognostic value and that most of them are readily measurable in the laboratory (19–21).

Pharmacokinetics and Pharmacodynamics

PK profiles were modelled for the drugs analysed here, based on the literature information, which suggested a linear compartmental model based on the concentrations of the drugs in the plasma (13, 22–25). In addition, PD models for both drugs were estimated, based on in vitro and in vivo data (5, 14, 26, 27). The PK/PD effects were then allometrically scaled to human PK/PD (28, 29). A combined PK/PD model was created, assuming an additive PD relationship with no PK interaction between the two drugs.

Drug Rescue Case Study — Combining a Chemotherapeutic and an Anti-angiogenic Drug

The aim of this in silico study was to rescue ISIS-5132 for the treatment of prostate cancer. Prostate cancer was selected for this demonstrative investigation, based on the availability of pre-clinical pharmacological data for the discontinued drug candidate. We ran virtual clinical trials of ISIS-5132, as briefly described below.

Essentially, we carried out virtual “Phase II” clinical trials by using a "synthetic" prostate cancer patient population. In the first stage of this study, many drug candidates were screened and analysed (results not shown). Here, we present the virtual clinical trial results of the combination of ISIS-5132 and sunitinib malate, which proved superior to other treatment combinations involv-
ing ISIS-5132. Sunitinib malate is approved by the US Food and Drug Administration (FDA) for the treatment of advanced renal cell carcinoma (RCC) and gastrointestinal stromal cancer (GIST).

Model parameters, including drug PK and PD parameters, were evaluated, based on experimental data reported in the literature. The parameter estimation process was performed separately for each of the drugs, as well as for the combination of the two drugs. A concentration–effect function was created to assess the effects of ISIS-5132 on tumour cell proliferation. Similarly, the effects of sunitinib malate on tumour cell proliferation, on the number of pericytes, and on the formation of new vessels, were estimated, based on the known drug action mechanism.

A Prostate Cancer OVP Population was created and simulated under numerous possible regimens of sunitinib malate/ISIS-5132 combinations (Table 1). Each of these simulated regimens was evaluated in terms of the Progression-Free Survival (PFS). Since the doubling time in untreated prostate tumours is estimated as one year and above (30), we examined the efficacy of the studied drug combinations, two and five years after the commencement of treatment.

The results are presented by using Kaplan Meier Curve survival analysis, which describes the probability of PFS at a given moment post-treatment (Figure 1). For the purpose of this study, tumour progression is defined as an increase by a factor of 1.75 from the baseline tumour volume.

The results of the simulations are summarised graphically in Figures 1 and 2. These simulations suggest an approximately 70% five-year PFS for the best selected regimen, under the accepted dose-limiting toxicity (DLT). This result is to be compared to a predicted 40% PFS in patients treated with sunitinib malate alone, or to a 25% PFS in patients treated with ISIS-5132 alone. As seen from these figures, the most effective regimen is ISIS-5132 (6mg/kg by intravenous infusion; every third day) + sunitinib malate (50mg orally; daily; 4 weeks on/2 weeks off). Note that for simplifying the demonstration, toxicity was accounted for by considering doses below the DLT observed in Phase I. The possibility that the studied combination will create new toxic effects was neglected in the current study, possibly accounting for the visibly over-estimated PFS. However, even if somewhat over-estimated, these results demonstrate the relative improvement of the ISIS-5132/sunitinib malate combination, thus providing an opportunity to rescue ISIS-5132 by administering it to prostate cancer patients in combination with sunitinib malate.

**Discussion**

Much attention has been devoted over the past few decades to the development of new oncology compounds. Despite novel discovery technologies, such as structure-based drug design, combinatorial chemistry, high-throughput screening, and genomics, and despite the enormous amount of money spent in the pharmaceutical industry on clinical research, there still exists a serious gap in productivity. In this study, we present a new concept that may help to bridge this gap. We suggest the use of virtual clinical studies for identifying improved treatments for cancer patients. Here, we focus on the rescue of discontinued compounds, which have shown insufficient efficacy. We show how virtual clinical trials can be employed for identifying efficacious treatment by the combined use of such drugs with drugs on the market. The bio-mathematical modelling option permits an accurate prediction of the optimal match between any discontinued oncology compound, any registered drug and any clinical indication, and the further selection of those triplets that are predicted to arrive at an improved treatment. This is done by virtual clinical trials in OVP populations, and the simulation of a variety of regimens from the possible regimen space. This process can save both time and resources that would otherwise be spent on the de novo development of new, not necessarily better, drugs.

In the study reported here, we have used the OVP approach for integrating a large amount of biological and physiological information obtained

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Route of administration</th>
<th>Dose</th>
<th>Regimen*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunitinib malate</td>
<td>Oral</td>
<td>25, 37.5 or 50 mg</td>
<td>Daily: 1 week on/1 week off Daily: 4 weeks on/2 weeks off</td>
</tr>
<tr>
<td>ISIS-5132</td>
<td>Intravenous infusion</td>
<td>2–10 mg/kg</td>
<td>Every 3 days</td>
</tr>
</tbody>
</table>

*The duration of treatments was approximately 6 months
from laboratory experiments involving the various compounds examined in combination. The experimental data enabled us to adjust our PK, tumour growth, and PD models, and to evaluate the parameters of all these drugs. Once the models retrieved the observed experimental results, and were further evaluated in independent simulation experiments, their predictive powers could be used for illuminating as yet undiscovered treatment options.

Our suggested method for rescuing prematurely shelved, or discontinued, drugs by virtual clinical trials, has been illustrated by discussing the case-study of a discontinued drug, ISIS-5132. Our virtual clinical trials have shown that by combining ISIS-5132 with a licensed drug, sunitinib malate, the treatment of prostate cancer could be improved, with more patients reaching PFS at five years, as compared to either ISIS-5132 or sunitinib malate monotherapy.

In view of the poor success of current oncology drugs and, at the same time, the necessity that the pharmaceutical industry remains at the forefront of medical research and continues to help patients, new measures for reducing the number of participants in the clinical development of new compounds must be urgently sought. We believe that the option of conducting virtual clinical trials is an important solution, not only for rationalising drug development, but, most importantly, for easing the practical and ethical bottleneck of patient recruitment.
Different treatment regimens: In all four examples ISIS 5132 was given as an i.v. infusion every three days and sunitinib malate was given orally each day on a 4 week on/2 week off schedule.

- = ISIS 5132 2mg/kg + sunitinib malate 50mg.
- - = ISIS 5132 6mg/kg + sunitinib malate 50mg.
- - - = ISIS 5132 6mg/kg + sunitinib malate 25mg.
- - - - = ISIS 5132 6mg/kg + sunitinib malate 37.5mg.

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De-Risking Drug Discovery with ADDME — Avoiding Drug Development Mistakes Early

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Summary — The advent of early Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) screening has increased the elimination rate of weak drug candidates early in the drug-discovery process, and decreased the proportion of compounds failing in clinical trials for ADMET reasons. This paper reviews the history of ADMET screening and why it has become so important in drug discovery and development. Assays that have been developed in response to specific needs, and improvements in technology that result in higher throughput and greater accuracy of prediction of human mechanisms of toxicity, are discussed. The paper concludes with the authors’ forecast of new models that will better predict human efficacy and toxicity.

Key words: ADME, ADMET, DMPK, drugability, drug discovery, pharmacokinetic studies, PK, toxicity, toxicology.

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Introduction

The goal of this paper is to briefly review the advances that have been made in the science of predicting human absorption, distribution, metabolism, excretion, and toxicity (ADMET) from in vitro assays; to review the currently available assays and those in the process of being validated; and to present a few examples of how in vitro ADMET programmes are used to advance drug-development programmes at the lead-optimisation and preclinical candidate selection stages.

Drug attrition late in clinical development, or after marketing, is a serious economic problem in the pharmaceutical industry (1). The cost of getting a drug to market is approaching $1 billion, and the cost of advancing a compound to Phase I trials can reach up to $100 million, according to the Tufts Center for the Study of Drug Development (2). It was also estimated that each day a drug is in the development stage costs $37,000 in direct out-of-pocket costs, and represents opportunity costs of $1.1 million in lost revenue (2). Given these huge expenditures, substantial savings can accrue from early recognition of problems that would make a compound unlikely to succeed in development (3).

The costs associated with withdrawing a drug from the market are even higher. Consider, for example, the case of terfenadine, which is a potent hERG ligand, and which is metabolised by cytochrome P-450 (CYP)3A4. Terfenadine was frequently co-administered with ketoconazole or erythromycin (4), both of which are CYP3A4 inhibitors. The consequent overload resulted in increases in plasma terfenadine to levels that caused cardiac toxicity (5). This toxicity caused terfenadine to be withdrawn from the market (6) at an estimated cost of $6 billion. Another example is the broad-spectrum antibiotic, trovafloxacin, which was introduced in 1997 and soon became Pfizer’s top seller. In vivo, it was metabolically activated and formed a highly reactive metabolite, resulting in severe drug-induced hepatotoxicity (7). Trovafloxacin was black-labelled in 1998 (8), potentially costing Pfizer $8.5 billion in lawsuits (9). With the ADMET assays now available, the liabilities associated with these drugs could have been recognised early in preclinical development.

The purpose of preclinical ADMET is to eliminate weak candidates. This allows drug-development resources to be focused on fewer, but more-likely-to-succeed, drug candidates. In 1993, 40% of drugs failed in clinical trials because of pharmacokinetic (PK) and bioavailability problems (10). Since then, major technological advances have occurred in molecular biology and screening, which allow major aspects of ADMET to be assessed much earlier, at the lead-optimisation stage. By the late 1990s, the pharmaceutical industry as a whole recognised the value of early

This paper was originally presented at the Speed and Safety in Drug Discovery discussion meeting, hosted by Safer Medicines Trust, and held on 26 November 2008 at the Royal Society, London (www.drugtestingconference.com).
ADMET assessment, and began to employ it routinely. The results were striking. ADME and drug metabolism pharmacokinetics (DMPK) reasons for failure fell from 40% to 11% (3). Now, lack of efficacy and human toxicity are the major reasons for failure (11).

The terms “drugability” and “drug-likeness” were coined by Dr Christopher Lipinski, who also proposed what has come to be known as “Lipinski’s Rule of 5”, due to the frequent appearance of “5” in the rules (12). The Rule of 5 has come to be a compass rose for the drug discovery industry (13). It stipulates that small-molecule drug candidates must have:

— a molecular weight less than 500g/mol
— a partition coefficient (logP — a measure of hydrophobicity) less than 5
— no more than 5 hydrogen bond donors
— no more than 10 hydrogen bond acceptors

A compound with fewer than three of these properties is unlikely to become an orally active drug. There are exceptions to Lipinski’s Rule of 5 that have become marketed drugs, such as drugs that are taken up by transporter proteins, natural compounds, oligonucleotides, and proteins.

The drug-discovery industry is experiencing dramatic structural changes. It is no longer just the domain of traditional pharmaceutical companies. Now, venture-capital funded start-ups, governments, non-profit organisations, and academic institutions are significant participants in the search for new drug targets, pathways, and molecules. Thus, it is becoming increasingly important to ensure that the money of investors, donors, and taxpayers is used efficiently, so that new drugs for unmet medical needs can be delivered to the public. ADMET profiling has been proven to weed out poor drug candidates and to speed discovery and development.

The Evolving Science of ADMET

Regulatory authorities have relied upon in vivo testing to predict the behavior of new molecules in the human body since the 1950s. Bioavailability, tissue distribution, pharmacokinetics, metabolism, and toxicity are assessed in one rodent and one non-rodent species before a drug may be tested in human safety clinical trials (Phase I).

Biodistribution is assessed by using radioactively labelled compounds later in development, because it is expensive both in terms of the synthesis of sufficient amounts of radioactively labelled compound and the performance of animal experiments.

The pharmacodynamic (PD) effectiveness of test compounds is normally first assessed through in vitro models, such as receptor binding, followed by confirmation through in vivo efficacy models in mice or rats. The predictive ability of these models depends on the therapeutic area and the model itself. Infectious disease models are considered to have the best predictive ability, whereas central nervous system (CNS) and oncology animal models are generally the least predictive of human efficacy. In vivo PK studies in a variety of animal models are routinely used for lead optimisation, to assess drug metabolism and absorption. Understanding the PK/PD relationship is crucial in developing understanding of the mechanism of action and metabolic fate of the molecule, which help explain efficacy results. However, there are significant differences in absorption and metabolism among species, which may cause over-prediction, or under-prediction, of absorption or metabolic degradation of the new chemical entities (NCEs).

Toxicity and safety studies are performed in models that are relevant to the mode of action and therapeutic area of the NCE. In vivo toxicity assays are required for an Investigational New Drug Application (IND) to the US Food and Drug Administration (FDA), but these have substantial predictive weaknesses. In a retrospective study of 150 compounds from 12 large pharmaceutical companies, the combined animal toxicity study with rodents and non-rodents, accurately predicted only 50% of the human hepatotoxicity. Worse, at this poor level of accuracy, large numbers of compounds that would have been safe in humans would have shown toxicity in animals, causing the compounds to be removed from development without going forward into human trials (14). The failure to predict the toxicity of the other 50% was attributed to “idiosyncratic human hepatotoxicity that cannot be detected by conventional animal toxicity studies.”

It has long been widely known that mechanisms for toxicity are frequently quite different between species; yet, animal testing remains the “gold standard.” The FDA and other regulatory agencies are in the process of evaluating alternatives to animal testing, with the aim of developing truly predictive toxicology methods and eventually eliminating in vivo testing altogether.

The ADMET Feedback Loop

As discussed above, historically, ADMET studies were focused on in vivo assays. These are, however, expensive and low-throughput, which has caused them to be delayed until later in the development process, when more resources are released for studies on the few molecules that have advanced to this stage. With the advent of in vitro high-throughput screening and miniaturisation technologies in the 1990s, early ADMET assays were developed to pre-
Ingredient in vitro animal and human results, at a level of
cost-effectiveness appropriate for the discovery
stage. This produced a major advance in the science
of ADMET, and has created a new norm that drug-
discovery programmes follow in advancing com-
ounds from hit to lead, from lead to advanced lead,
and on to nominated clinical candidates. Now, early
in the discovery phase, with the use of human
enzymes and human cells, drug-discovery pro-
grammes are able to obtain highly actionable in-
formation about the drug-likeness of their new
molecules, and early indications of known human
mechanisms of toxicities. ADMET assessments of
varying complexity are now routinely conducted on
compounds that have shown in vitro efficacy, and at
the same time as, or just prior to, demonstrating
early proof of principle in vivo.

The application of early ADMET assessments is
unique to each drug-discovery programme. It
depends on the therapeutic area, route of adminis-
tration, chemical series, and other parameters.
Correspondingly, the importance of the various
ADMET assays depends on the specifics of the drug-
discovery programmes. ADMET assays can also be
divided into those that are routine and those
reserved for more advanced profiling, with the divi-
sion being a function of cost-effectiveness and the
need for the specific information. For instance, one
does not normally need to know, during the hit-to-
lead phase, which transporters in the gastrointesti-
nal tract are involved in transporting the drug;
however, later in the development process, this
issue becomes more relevant.

In some cases, the FDA has moved to require some
of the new in vitro ADMET assays. For example, in
vitro drug–drug interaction (DDI) studies may now
be conducted, under the guidance from the FDA
dated September 2006. The guidance document
defines precisely how to conduct CYP inhibition and
induction, and P-glycoprotein interaction studies
(15).

How should a discovery team employ early
ADMET? The answer is not simple and formula-

cic: It is a process, which should involve starting
from the goal and working backward toward dis-
covey. The drug-discovery team should first
define the target product profile (TPP). Once the
TPP has been identified, then major design ele-
ments of the Phase II and III clinical trials
should be outlined, which in turn lead to ques-
tions about the product’s tolerable toxicity and
safety, which will then define the GLP toxicity
studies in animals, which in turn will lead the
team to the discovery and preclinical-develop-
ment questions to be addressed, via their specific
early ADMET programme.

In the discovery phase, at the beginning of this
exciting and risky journey, how does one put this
information into action? For example, if a compound
has high target receptor binding, and biological
activity, in cells and in relevant in vivo animal mod-
els, how can one ensure that it will become a suc-

cessful drug? A molecule needs to cross many
barriers on the way to its target. The first barrier is
seemingly simple: Solubility. A solubility screen will
provide information about the NCE’s solubility in
fluids compatible with administration to humans.
Next question: Chemical and metabolic stability.
Chemical stability in buffers, simulated gastric and
intestinal fluids, and metabolic stability in plasma,
hepatocytes or liver microsomes of different species,
can be measured to predict a compound’s stability in
the various environments it will encounter on the
way to its target in the human body.

The second step is to define some of the absorp-
tion properties of the compounds. Are they likely to
be bioavailable? Measurement of permeability
across Caco-2 cell monolayers is a good predictor of
human oral bioavailability. Measurement of bind-
ing to plasma proteins indicates the degree of
availability of the free compound in the blood. This
is critical, as only unbound drugs are able to get to
the target and exert their pharmacologic effects.
Metabolism and drug-drug interaction issues can
be detected by screening for inhibition of liver
CYPs. All these assays allow chemists and biolo-
gists to obtain actionable information early, allow-
ing them to gain understanding of Structure–
Activity Relationships (SARs) and Structure–
Property Relationships (SPRs).

The next step is required for advanced lead opti-
misation. Are drug-drug interactions involved? The
effects of drug transporters on permeability, and the
effects of drugs on transporter activity, can be mea-
sured in Caco-2 or other models. The in vitro toxic
effects on human cells can be predicted in vitro
by measuring cytotoxicity in mammalian cell lines or
primary cells. The effects of a compound on CYP
metabolism can be identified by determining the 50%
inhibitory dose (IC50) for each CYP. These relation-
ships between the NCE and metabolising enzymes
are important to understand, for several reasons:

1. The compound may affect the effective plasma
concentrations of other drugs taken concomit-
antly with the compound of interest, if they are
metabolised by the same CYPs. (See the dis-
cussion of the terfenadine case above).

2. If the parent drug is a CYP inducer, it may
increase the clearance rate of concomitantly
administered drugs, which are metabolised by
these CYPs. This may result in a decrease in
the effective plasma concentrations of these
drugs, thus decreasing their pharmacologic
effects.

3. Metabolites formed via CYP metabolism may be
responsible for undesirable side-effects, such as
organ toxicity.
4. The metabolite of a compound may actually be responsible for the compound’s efficacy, and not the parent compound itself. In fact, the metabolite may have better efficacy, safety, and pharmacokinetics profile than its parent. If so, metabolism can be exploited to produce a better drug, which will substantially change the medicinal chemistry strategy.

5. The identification of drug-metabolising enzymes involved in the major metabolic pathways of a compound helps in predicting the probable drug–drug interactions in humans. This information may also make human clinical trials, designed to detect drug-drug interaction, unnecessary, thus resulting in a substantial cost savings during development.

ADMET is a tool that supports overall programme goals. Seldom will negative results from a single ADMET assay kill a compound or a programme. Remember, the Rule of 5 requires that only three of the four conditions are met, and even then, there are exceptions. Instead, as was illustrated above, in the case of metabolism, the results are more likely to just change the direction of the medicinal chemistry.

After assessing compounds in a few simple mechanistic systems, such as plasma and liver microsomal stability screens in relevant species, one moves on to lead optimisation by using assays to identify potential liabilities. Finally, at the stage of advanced lead optimisation and development, more-complex systems are used, to provide a more-thorough understanding of a compound’s metabolic fate and absorption mechanism, and this understanding is used to drive efficient development. As ADMET roadblocks are discovered, which they inevitably will be, the loop will be repeated until a clear path is found (Figure 1).

The Impact of ADMET

Early ADMET not only provides the data necessary for selecting preclinical candidates, by permitting better-informed medicinal chemistry, it can also accelerate the timelines for IND and, subsequently, for New Drug Application (NDA) submission, which means more time on the market under patent protection and hence greater profits. For investors, this is a major parameter. Data compiled by the Tufts Center for Drug Discovery have identified that, for a typical, moderately successful proprietary drug ($350 million annual sales), each day’s delay equates to $1.1 million in lost patent-protected revenues — revenue that provides the return on investment needed to fund drug discovery (2). Further, the shorter the discovery and development timelines, the faster venture capital and angel investors can get to a liquidity event. As drug discovery takes longer to commercialise than any other form of product development, its slowness to produce returns is a major impediment for obtaining investment. Speeding up drug discovery and development will attract more investment in drug discovery research.

ADMET technologies remain a work in progress. At present, we are unable to detect all the mechanisms of human idiosyncratic toxicity. These mechanisms cause the most expensive, harmful, and disheartening form of drug attrition — post-commercialisation toxicity. Progress is being made. Many idiosyncratic drug reactions are due to the formation of short-lived reactive metabolites that bind covalently to cell proteins (16), and the extent to which a compound will generate these can now be detected before a compound reaches human patients. Other mechanisms of human toxicity can now also be detected early in discovery. Some of the assays that are available to detect them will be briefly described in the following section.

New ADMET Tools

Idiosyncratic hepatotoxicity, or drug-induced liver injury (DILI), occurs in only one out of about 10,000 patients. Thus, it is usually statistically impossible to discover it during clinical trials. Yet, in spite of its name, which literally means “rare event with undefined mechanism,” some mechanisms have now been defined. One of these is mitochondrial toxicity, and another is the formation of...
reactive metabolites. A further mechanism of human toxicity, that is not limited to the liver, but may also affect lung, spleen, and heart tissues, is phospholipidosis.

**Mitochondrial toxicity**

Mitochondrial toxicity is increasingly implicated in drug-induced idiosyncratic toxicity. Many of the drugs that have been withdrawn from the market due to organ toxicity, have been found to be mitochondrial toxicants (17). These toxicants injure mitochondria by inhibiting the respiratory complexes of the electron chain, inhibiting or uncoupling oxidative phosphorylation, inducing mitochondrial oxidative stress, or inhibiting DNA replication, transcription, or translation (18).

The toxicity testing of drug candidates is usually performed in immortalised cell lines that have been adapted for rapid growth in a reduced-oxygen atmosphere. Their metabolism is often anaerobic, by means of glycolysis, despite their having functional mitochondria and an adequate oxygen supply. On the other hand, normal cells generate ATP for energy consumption aerobically, by mitochondrial oxidative phosphorylation. The anaerobic metabolism of transformed cell lines makes them less sensitive to mitochondrial toxicants, which is why mitochondrial toxicity is systematically under-reported in toxicity testing in these cell lines (18, 19). To address this, HepG2 and NIH/3T3 cells can be grown in media in which glucose is replaced by galactose (19). This changes their metabolism, such that the respiratory substrate becomes more like that of normal cells, so they become more sensitive to mitochondrial toxicants, without reducing their sensitivity to non-mitochondrial toxicants (Figure 2).

**Formation of reactive metabolites**

Another property of compounds that can cause idiosyncratic toxicity is their ability to form reactive intermediates (20). The formation of short-lived reactive metabolites is known to be the mechanism of toxicity of some compounds, such as acetaminophen (paracetamol; 21). The formation of reactive metabolites can be identified by incubating test compounds with liver microsomes and adding glutathione to trap the reactive intermediates, which are then identified by liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS; Figure 3). The conversion of more than 10% of the test agent to reactive intermediates was evaluated, each with two HepG2-derived cell lines. The two toxic compounds were: antimycin A, which is known to be toxic to mitochondria, and imipramine, which exerts its toxicity by other means. The two cell lines were: HepG2 cells cultured in the standard manner, which makes them resistant to mitochondrial toxicants (Mito-R), and a HepG2 cell line that had been adapted to grow in a medium that changed the metabolism of the cells, restoring the sensitivity to mitochondrial toxicants (Mito-S).

**Figure 2:** Drugs that cause mitochondrial toxicity can be identified by the use of a specially adapted, mitochondrial toxicant-sensitive cell line

![Graph showing growth inhibition against antimycin A concentration](image)

![Graph showing growth inhibition against imipramine concentration](image)

*a) Mitochondrial toxicant b) Non-mitochondrial toxicant*
ates indicates that the compound may be implicated in idiosyncratic toxicity.

Phospholipidosis

Phospholipidosis is a lysosomal storage disorder. It can be caused by drugs that are cationic amphiphiles (22). The disorder is considered to be mild, and often resolves by itself, but drugs that cause phospholipidosis can also cause organ damage, which makes phospholipidosis of concern to the regulatory agencies (23). A cell-based assay for phospholipidosis has been developed, which involves the accumulation of a fluorescent phospholipid, resulting in an increase of fluorescence in the lysosomes of cells that have been treated with drugs that cause phospholipidosis (Figure 4). If phospholipidosis is absent, the phospholipid is degraded and the fluorescence does not increase. Many of these drugs are also cytotoxic, so increases in fluorescence are usually normalised in terms of cell number (Figure 4).

Genotoxicity

The genotoxicity of drugs is an important cause for concern to the regulatory authorities. The FDA recommends a number of in vitro and in vivo tests for measuring the mutagenic potential of chemical compounds, including the Ames test in Salmonella typhimurium (24). GreenScreen GC, a new, high-throughput assay that links the regulation of the human GADD45a gene to the production of Green Fluorescent Protein (GFP) has become available. The assay relies on the DNA damage-induced up-regulation of the RAD54 gene in yeast, which is measured by using a promoter–GFP fusion reporter (25). The test is more specific and sensitive for genotoxicity than those currently recommended by the FDA, such as the Ames and mouse lymphoma tests.

Figure 3: The formation of reactive metabolites

Acetaminophen was incubated with microsomes and glutathione in the presence and absence of NADPH (nicotinamide adenine dinucleotide phosphate). The presence of the acetaminophen reaction product was revealed by LC/MS/MS.

a) In the presence of NADPH, an adduct of glutathione with acetaminophen was formed, confirming the production of a reactive metabolite; b) when NADPH was absent, no adduct was formed.
Great progress in the field of ADMET profiling has been made in the last 15 years. This progress has decreased the proportion of drug candidates that fail in clinical trials for ADME reasons, making it a bright spot in an otherwise dismal picture of declining productivity in drug discovery. Now, the principal barrier is the toxicity portion of ADMET. The prediction of human-specific toxicology must be improved.

Cell-based assays, involving the use of established cell lines and co-cultures, have been used to determine toxicity to various organs, but many of these cell lines have lost some of the physiological activities present in the equivalent normal cells. HepG2 cells, for instance, have greatly reduced levels of metabolic enzymes. Primary human hepatocytes can be used, but, not only are they expensive, they also suffer from high donor-to-donor variability, and they maintain their characteristics for only a short time. Three-dimensional models have been developed for cell-based therapies, including micropatterned co-cultures of human liver cells that maintain the phenotypic functions of the human liver for several weeks (26), which should provide more-accurate information about toxicity when used in ADMET screening. This approach could be extended to other organ-specific cells, leading to development of integrated tissue models in the so-called “human on a chip” (27). The potential of stem cells to differentiate into cell lines of many different lineages can be exploited, to develop human and animal stem-cell-derived models for major organ systems (28).

High content screening (HCS) has been used for early cytotoxicity measurement since 2003, and holds great promise. It is based on automated epi-fluorescence microscopy and the image analysis of cells in a microtitre plate format. By using four fluorescent dyes of different colours, it is possible to

**Current Challenges and Future Directions**

Drug-induced phospholipidosis (PLD) is determined by measuring the accumulation of a fluorescent phospholipid in cells treated with increasing drug concentrations. Fluorescence is measured, normalised to cell number, and expressed relative to an untreated control. Fluorescence is increased in cells treated with compounds that are known to cause PLD (chlorpromazine, tamoxifen, amiodarone), but it is not increased in cells treated with a compound that is known not to cause PLD (acetaminophen).

**Figure 4: Drug-induced phospholipidosis in HepG2 cells**

- = chlorpromazine; ○ = tamoxifen; ■ = amiodarone; □ = acetaminophen.
analyse multiple parameters at the single-cell level, including morphological and biochemical parameters that indicate pre-lethal cytotoxic effects, and represent different mechanisms of toxicity (29). This method has been optimised for hepatocytes, and is more predictive of hepatotoxicity than the currently-available methods. In the future, HCS could be applied to cells of other organs.

Molecular profiling is another alternative. It is defined as any combination, or individual application, of mRNA expression, proteomic, toxicogenomic, or metabolomic measurements that characterise the state of a tissue (30). This approach has been applied in an attempt to develop the profiles or signatures of certain toxicities. Molecular profiles, in conjunction with agents that specifically perturb cellular systems, have been used to identify patterns of change in gene expression and other parameters at sub-toxic drug concentrations that might be predictive of hepatotoxicity, including idiosyncratic hepatotoxicity (31). In the future, larger data sets, high-throughput gene disruptions, and more-diverse profiling data will lead to more-detailed knowledge of disease pathways, which will facilitate making target choices and constructing detailed models of cellular systems for use in ADMET screening to identify toxic compounds early in the discovery process. The combination of in silico, in vitro, and in vivo methods and models into multiple content data bases, data mining, predictive modelling algorithms, visualisation tools, and high-throughput data-analysis solutions can be integrated to predict systems ADMET properties. Such models are starting to be built, and will be widely available in a decade or so (32). The use of these tools will lead to a greater understanding of the interactions of drugs with their targets, and will permit the prediction of their toxicities.

Hence, in the foreseeable future we can look forward, not only to a decrease in late-stage development failures and withdrawals of marketed drugs, but also to shorter timelines from discovery to market, and reduced development costs through the reduction of late-stage failures.

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Monitoring Tissue Drug Levels by Clinical Microdialysis

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Summary — The in vivo assessment of drug distribution has long been treated as a “forgotten relative” of pharmacokinetics, mainly due to a lack of appropriate methodology. Research was long restricted to the measurement of drug concentrations from biological specimens that are relatively easy to obtain, or to indirect modelling. However, data obtained by these approaches have resulted in considerable confusion about drug distribution and target site delivery, as their interpretation was flawed by several misconceptions, such as the lack of physiological input to pharmacokinetic models, the erroneous view that a tissue is a uniform matrix, and the notion that the entire drug fraction present in various tissue spaces exerts pharmacological activity. Today, drug distribution to the well defined tissue compartment — “interstitial space fluid”, the biophase for many drugs — can be measured relatively cheaply, minimally invasively, and reproducibly, via microdialysis.

Key words: in vivo drug distribution, microdialysis,

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Introduction

The rationale for tissue distribution measurements was highlighted long ago by Paul Ehrlich, who pointed out that a drug has to reach its target site to become pharmacologically active. However, a number of misconceptions have developed about the tissue distribution process, most notably, the idea that a blood–target side equilibrium, achieved by instantaneous diffusion, can be taken for granted (1). A number of arguments can be listed against this view, such as the presence of active transporters, but also the influence of other mechanisms, such as pH partition and the Gibbs-Donnan effect. These mechanisms lead to a lack of equilibrium, even under steady state conditions. Until recently, there was a perceived inability to measure tissue distribution accurately, except for one-point-in-time measurements from homogenised tissues — a concept which has been gleaned from the idea of the Nernst partition coefficient. The problematic assumption of this approach is that a tissue is a homogenous matrix, which is clearly not the case, since “tissue” contains a number of sub-compartments, including cells and the interstitial space.

The Principle of Microdialysis

Most regulatory agencies demand distribution studies, and the US Food and Drug Administration (FDA) has expressed an interest in viewing drugs and their actions directly at the level of the drug target, rather than indirectly via plasma concentrations. One tool in a potential critical-path toolbox is microdialysis (2, 3). Microdialysis is a probe technology, where a little probe is inserted by a guide cannula into the tissue of interest. The probe is perfused by means of a pump at a very low flow rate (1–5µl per minute). The perfusate is pumped to the tip of the probe, where a semi-permeable membrane permits diffusion of diffusible compounds out of the interstitial space into the perfusate. This is then called a “tissue dialysate”, which is pumped out of the tissue and subjected to chemical analysis.

Microdialysis permits measurement of the interstitial, unbound, pharmacologically active, drug concentration in various organs. To date, PubMed has reported almost 12,000 publications on the application of microdialysis in various settings, and a number of reviews on the regulatory perspectives of this technology are also available.
able, which can be used in humans in almost every organ, e.g. the brain or lung. Tissue chemistry has been monitored in the amygdala of patients suffering from epilepsy, indicating that dopamine plays a role in cognition (4). An interesting area of CNS research has been the measurement of beta-amyloid concentrations in the brain of Alzheimer’s Disease patients (5). It is now also possible to study small proteins, up to a size of approximately 5kDa.

A substantial amount has been published on microdialysis drug research. The method is particularly relevant for the study of anti-infective agents, because the interstitial space is the site of the bacterial infection and is therefore the actual target site (6–11). Highly protein-bound drugs, such as cefixime, attain relatively low tissue concentrations due to protein binding (9). Thus, high protein binding impedes drug penetration into the interstitial space, whereas, for drugs with relatively low binding to protein, e.g. cefpodoxime, the plasma–tissue concentration difference is less marked (9). In healthy subjects, it has been shown that unbound plasma concentration reflects tissue concentration. However, the situation is different in patients, e.g. intensive care unit patients, where it has been shown that peripheral target concentrations are much lower, compared to those in healthy controls (10). In pathological states, the development of penetration barriers was observed, e.g. due to the application of vasoconstrictors. In septic patients, a lag-time to equilibration can be observed. This might become meaningful, if a drug should act rapidly. However, concentration measurements alone might not be meaningful without relating them to pharmacodynamics. A concept, which has been studied extensively, is to measure tissue concentrations in vivo by microdialysis, and to use the values to mimic the respective drug concentration versus time profiles in vitro on bacterial cultures, to measure the effect on bacterial killing (11). Such experiments have shown that, by applying plasma values, drug effects might be overestimated. Such experiments have also provided evidence that, by changing the formulation and employing tissue concentration measurements, improved kill curves can be achieved, which might provide guidance in the dose selection process.

Similar microdialysis work has been done on solid tumours (12–14). This has suggested pharmacokinetic explanations for non-responsiveness to chemotherapy due to the presence of diffusion barriers, e.g. in breast cancer patients (12, 13). Also in cancer research, in vivo PK/in vitro PD concepts can be employed. For 5-fluorouracil, it was shown that pharmacokinetics explains pharmacodynamics, i.e. the higher the concentration at the target site in the bio-phase surrounding the cells, the more pronounced is the killing effect on cancer cells (12), whereas for methotrexate no such correlation seems to exist (13). These experiments provide valuable information about drug action and rate-limiting steps in cancer therapy, indicating that, for methotrexate, intracellular events are more important for the overall drug effect than the drug load at the interstitial target.

Another interesting field for microdialysis is transdermal research (15–17). Until recently, no tool was available for measuring target site concentration in tissues underneath the application of a topical formulation, such as creams, gels or ointments. By employing microdialysis in the skin, the effects of various interventions on transdermal penetration have been studied. Tape stripping of the skin has resulted in log-fold increases in topical concentrations (16). Recently, the use of microdialysis for dermato–pharmacokinetic evaluations and bioequivalence studies has been described (17).

Future research questions which might be tackled by microdialysis are the measurement of biomarkers in tissues, topical bioequivalence, and the optimal dosing of anti-infectives. Although microdialysis is a promising research tool for drugs acting via cell surface receptors, other research areas, such as the determination of intracellular distribution, do not qualify for microdialysis measurements. Here, imaging tools such as positron emission tomography (PET) might be more suitable (18, 19). A combination of imaging and microdialysis, an approach which also allows the modelling of intracellular concentrations, might be the best strategy to use in studies on tissue pharmacokinetics and pharmacodynamics in humans (19).

In conclusion, clinical microdialysis is a mature, safe, reproducible, and ethically acceptable tool, which can be employed for tissue concentration measurements in most human organs (20).

References


The Best Model for Humans is Human — How to Accelerate Early Drug Development Safely

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Summary — Traditionally, the choice of which candidate compounds to take forward into development has been based on pre-clinical data. However, lack of predictivity of the human clinical situation in the models used has led to poor decision-making, and the later in the development process that such mistakes are realised, the more costly and time-consuming it is to correct them. Furthermore, compounds that may have made perfectly good drugs, have been dropped due to poor pharmacokinetics in animal models. Accelerator mass spectrometry (AMS) is an ultra-sensitive detection technique that can be used to quantify carbon-14. By administering very small amounts of 14C-labelled compounds, AMS can be used to obtain human clinical data very early in the drug development process. Such studies: a) can be helpful in understanding human pharmacokinetics using microdosing; b) can provide early human metabolism information, to validate the choice of animal species used in pre-clinical safety testing and identify unique or disproportionate human metabolites during Phase 1; and c) can provide fundamental human pharmacokinetic data, including absolute bioavailability, by facilitating a scientifically optimal and cost-effective study design. The provision of these clinical insights at the earliest possible opportunity can lead to improved decision-making, and therefore can reduce the time and cost involved in the drug development process.

Key words: absolute bioavailability, accelerator mass spectrometry, intravenous pharmacokinetics, metabolites in safety testing, microdosing.

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Introduction

Accelerator mass spectrometry (AMS) was originally devised as a technique for measuring 14C, in order to carry out the radio-carbon dating of archaeological artefacts. The first biological application of the technique was reported from the Lawrence Livermore National Laboratory, California, USA, in 1989. Xceleron has pioneered the use of AMS in the biomedical field since 1997 (1).

AMS is a tracer technique and, as such, requires the synthesis of a compound labelled with a rare isotope. That isotope does not have to be 14C, because AMS does not measure radioactivity. However, inevitably, because drug developers are used to working with this isotope, virtually all the biomedical work carried out by using this technique does involve 14C-labelled compounds. In common with any other tracer technique, the assumption is made that the labelled drug behaves exactly like its non-labelled analogue. Essentially, any experiment that can be carried out by using conventional radioactivity measurements can be carried out by using AMS. However, the unique sensitivity of AMS gives a number of advantages over conventional techniques. In particular, because it can be used with very low doses of radioactivity, it is possible to conduct AMS-based studies in humans at a much earlier stage in drug development. The aim is to provide relevant human clinical data as early as possible, so that better decisions can be made during the development phase for a potential drug. In this way, it is possible to select better candidates to take forward, thus reducing the money spent on candidates that will ultimately prove unsuitable and reducing the overall time (and cost) to market for successful drugs.

Methods

A scintillation counter measures the decay of the radiolabelled isotope, and it takes about a billion 14C atoms to get one disintegration per minute. On the other hand, AMS physically separates the isotopes 12C, 13C and 14C, and counts individual
atoms. Just 1000 $^{14}$C atoms are required to make a valid AMS measurement. In order to make the AMS measurement, the biological sample is first converted into carbon. This involves a three day manual process of combustion, to form carbon dioxide, cryogenic transfer of the CO$_2$, then reduction of the CO$_2$ to graphite. The graphite is then ionised; $^{12}$C, $^{13}$C and $^{14}$C ions are accelerated at high energy and counted in individual detectors; the resulting data are expressed as isotope ratios ($^{13}$C:$^{12}$C and $^{14}$C:$^{12}$C); the total amount of carbon in the sample is then used to calculate the amount of $^{14}$C in the original sample. AMS has many potential applications in the biomedical field. However, this paper will focus on three specific areas: microdosing (also known as Phase 0), META-ID™ and absolute bioavailability studies.

**Microdosing**

In microdosing studies, a sub-therapeutic dose (1/100 of the pharmacological dose, if known, up to a maximum of 100µg) of a compound is administered, labelled with a tracer amount (typically 200nCi) of $^{14}$C. Because of the very low doses involved, minimal pre-clinical safety data are required, and, because of the very low radioactive dose, in regulatory terms, such studies are considered non-radioactive, and specific authority to administer radioactivity to humans is not required. However, it should be borne in mind that microdosing does not provide efficacy or safety data, because, by definition, the administered dose is below the level where any pharmacological effects are expected. What microdosing does provide is human pharmacokinetic (PK) data at the earliest opportunity. Inevitably, this leads to the question, “Is a microdose predictive of a pharmacological dose?”, i.e. can dose-linear PK be assumed over several orders of magnitude? In order to try to answer this question, the Consortium for Resourcing and Evaluating AMS Microdosing (CREAM) trial was carried out (2). In this study, the microdose PKs of five compounds (four marketed drugs and one compound that had been rejected during development, because of inadequate PK characteristics) chosen to represent situations in which prediction of human pharmacokinetics from non-clinical data might be considered problematic, were compared to the PKs at the therapeutic dose. In a recent review by Lappin, the results from CREAM and other published data were found to indicate that microdosing was predictive of the therapeutic dose in 15 out of 18 cases where the data were available (3).

Overall, Xceleron has experience of microdosing more than 50 compounds. In many cases comparative data at therapeutic dose levels are not available. However, where they are, the microdose data are predictive in more than 80% of cases. This “success rate” compares very favourably with the correlation between animal and human data, which, in many cases, is poor (4, 5).

The microdosing approach is often used comparatively — for example when a number of potential leads have been identified by using in silico and in vitro screens and/or in vivo animal data. In a recent example of this type of study, three candidates were identified for which the in vitro and in vivo pre-clinical metabolism data conflicted. Each compound was administered to a separate group of four healthy male volunteers. Each subject received a single oral microdose (100µg; 200nCi), followed, after a one week washout, by a single intravenous microdose (100µg; 200nCi). Plasma levels of total $^{14}$C and of parent compound were measured following each dose, by using AMS techniques. The oral bioavailabilities of two of the candidates were very low (plasma concentrations following oral dosing were below the lower limit of quantification of the assay), whilst that of the third compound (compound B) was significantly higher at 8% (Figure 1). Thus, the human microdose data supported the nomination of the third compound for full scale development, whereas, had the in vitro and pre-clinical data been used without the microdose data, that compound would have been the least favoured candidate.

**META-ID™**

In February 2008, the US Food & Drug Administration issued Guidance for Industry on Safety Testing of Drug Metabolites (the so-called MIST guidelines [6]). The guidance indicated that any metabolite with a systemic exposure greater than 10% of that of the parent compound can potentially raise safety concerns, if such metabolites are present in humans at levels disproportionately higher than in the species used to generate the pre-clinical safety data.

Subsequently, there has been much interest in the possibility of using AMS to produce information on human metabolism at as early a stage in the drug development process as possible. Because AMS requires such low levels of radioactivity, it is possible to add a microtracer amount (typically 200nCi) to an existing human Phase 1 study (e.g. a single or multiple ascending dose study), such that, in addition to the measuring of plasma levels of unchanged drug by using, for example, liquid chromatography-mass spectrometry (LC–MS), by measuring total $^{14}$C levels using AMS, information on circulating levels of total drug-related material can be obtained. The difference between the two reflects the overall extent of metabolism. If this
The best model for humans is human exceeding the 10% of parent limit, metabolite profiling can be undertaken, by using high performance liquid chromatography (HPLC)–AMS, in order to determine the number and relative amounts of metabolite(s) present. Typically, profiling will initially be carried out on an AUC plasma pool (see the example shown in Figure 2), so that the size of each peak is proportional to exposure and it is easy to identify any peaks representing more than the 10% of parent threshold. The human profiles obtained can be compared directly with animal data, and, if required, metabolite(s) associated with specific peaks of radioactivity can be identified by using standard techniques such as LC–MS and/or NMR.

If urine and faeces are collected, it is also possible to obtain information on routes and rates of excretion and mass balance. Metabolite profiles can also be obtained from excreta. In fact, this kind of study could be used to completely replace a separate regulatory human absorption–metabolism–excretion study.

Mean plasma concentrations of compound B, determined by HPLC–AMS analysis, following oral or intravenous administration of a microdose (100µg; 200nCi) of the 14C-labelled compound to healthy male volunteers (n = 4; error bars = 1SD).

• = oral; ○ = intravenous.

Absolute Bioavailability Studies

The conventional approach to determining absolute bioavailability is to administer an oral dose to a group of volunteers, collect blood samples and measure concentrations of unchanged compound in these using, for example, LC–MS. After a washout period, an intravenous (i.v.) dose is administered to the same volunteers and samples are collected and analysed in the same way. Using AMS, it is possible to determine absolute bioavailability using a study design with a single study period. The oral, non-radiolabelled dose, is given at the therapeutic dose, in exactly the same way as for the conventional study. However, the i.v. dose is given as a microdose (typically 10µg) incorporating a microtracer amount of 14C-labelled compound (typically 200nCi), either at the same time as the oral dose or, more usually, as a short infusion that finishes at the expected Tmax for the oral route. The concentration of the administered compound arising from the oral dose is measured using the
standard LC–MS methodology and, using another aliquot of the same samples, concentrations of the administered compound arising from the i.v. dose are measured using an HPLC–AMS assay. Total $^{14}$C (arising from the i.v. dose) can also be determined in the same samples. Because the levels arising from the i.v. microdose are very low relative to those from the oral dose, they do not affect the concentrations measured by LC–MS.

There are a number of advantages to using the AMS approach. Because only a microdose is administered via the i.v. route, no i.v. toxicology studies are required: the oral safety data already in place by Phase 1 study are deemed to provide sufficient cover for the exposures generated by the i.v. microdose. Although the study involves the administration of radioactivity to humans, the microtracer amounts involved do not require regulatory approval, so no dosimetry studies are necessary. The small amount of material dosed intravenously also minimises any i.v. formulation issues: it is generally possible to get enough material to dissolve for a simple physiological buffer to be used as the dosing vehicle. There are also time and cost savings, because there is only one dosing period and one set of samples to collect. Scientifically, the study design is optimal. There are no temporal effects to consider, since the subjects are dosed both orally and intravenously at the same time. The possible impact of dose-dependent kinetics is obviated, since the body does not discriminate between compounds arising from the two dose routes and therefore handles them both in exactly the same way.

Although information about the bioavailability of a drug is required for product registration, there is no regulatory requirement to determine the absolute bioavailability or to define i.v. PK, so, given the time and cost associated with generating i.v. safety data and developing a suitable i.v. formulation, companies often decide not to include a human i.v. study in their development strategy. However, as well as absolute bioavailability, the

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**Figure 2**: HPLC–AMS radio-chromatogram of an extract of a plasma pool, obtained following a single oral dose of a drug candidate, showing the detection and quantification of metabolites

An extract of AUC plasma pool, comprising samples from 0–96 hours pooled in proportion to the time interval each represents, following a single oral administration of $^{14}$C-labelled compound D to healthy male human volunteers was analysed by HPLC–AMS (1.1dpm injected onto column).
study design described above provides human PK in a cost-effective way. Knowledge of the fundamental properties of a compound, such as clearance and volume of distribution, can be invaluable in interpreting data obtained from other studies, leading to more-effective decision making.

**Conclusions**

Traditionally, decisions on which candidate drugs to take forward into development have been based on pre-clinical information, including *in silico*, *in vitro* and *in vivo* animal data. However, the poor predictivity of these models can lead to wrong decisions, and the later these mistakes are realised, the higher can be the cost of putting them right. AMS can be used in a number of ways to provide *in vivo* human data at an early stage, and these data can facilitate better, more-effective decision-making. None of the approaches discussed in this paper is, in itself, a panacea. However, it seems self-evident that the best model for the human is the human, and AMS can provide human clinical data at the earliest possible opportunity. As such, it is a valuable tool in the drug developer’s toolbox, and is increasingly being used both for problem solving and as a routine element of early pharmaceutical development.

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