

High-Throughput Genotoxicity Testing

With over 25% of compounds with a genotoxic liability reaching preclinical development, there is an urgent need for more accurate genotoxicity testing; a novel assay fulfils this need by combining high sensitivity with an extremely low false positive rate – enabling the all-important non-genotoxic compounds to become the focus of drug discovery research.

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The registration of all pharmaceuticals requires a comprehensive safety assessment of their potential to cause direct or indirect genotoxic damage. This article reviews current methods employed for regulatory genotoxicity testing and new techniques developed for early high throughput screening. A novel *in vitro* mammalian assay is described which detects all common mechanistic classes of genotoxins, and delivers both high specificity and sensitivity to address the urgent need for more accurate genotoxicity testing.

REGULATORY TESTING

Genotoxicity assessment of drugs is a mandatory element of preclinical safety testing and comprises a battery of complementary regulatory tests, as no single test to date has been capable of detecting all relevant genotoxic agents. The battery includes a test for gene mutation in bacteria (commonly the Ames bacterial reverse mutation test), paired with an *in vitro* mammalian test for chromosomal damage or gene mutation, and an *in vivo* rodent assay for chromosomal damage. A chromosome aberration assay is often used to evaluate *in vitro* cytogenetic (IVC) chromosomal damage, or alternatively an *in vitro* mouse lymphoma thymidine kinase (tk) assay (MLA) is used to detect mutations and chromosomal breakage. Additional genotoxic agents are detected by the *in vivo* rodent micronucleus test (MNT) for chromosomal damage, using rodent bone marrow or peripheral blood cells.

The International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) has an Emerging Issues Subcommittee on the Relevance and Follow-up of Positive Results in *In Vitro* Genetic Toxicology Testing (IVGT). One of its objectives is to improve the scientific basis of the interpretation of results from *in vitro* genetic toxicology tests for purposes of more accurate human risk assessment. The critical issue in this respect is that although most carcinogens test

positive in the *in vitro* genotoxicity tests, this is accompanied by an extremely high rate of non-carcinogens testing falsely positive.

RE-EVALUATION OF THE STANDARD BATTERY

The battery of *in vitro* genotoxicity tests to discriminate between rodent carcinogens and non-carcinogens has recently been re-evaluated using a wide range of published data (1). The results clearly demonstrate the strengths and weaknesses of the current regulatory test batteries for identifying genotoxins. Although 93% of the carcinogens evaluated (515/554) had corresponding positive results in at least one of the three tests, this success in detecting carcinogens (sensitivity) contrasted with a poor ability to identify non-carcinogens (specificity). More than 80% of the 183 compounds testing negative in both male and female rat and mice cancer studies had positive *in vitro* genotoxicity data. When analysed test-by-test, the specificity of the Ames test was fairly predictive, but all mammalian cell tests included in the assessment had poor specificity, and therefore a high rate of compounds falsely classified as potential carcinogens (see Figure 1).

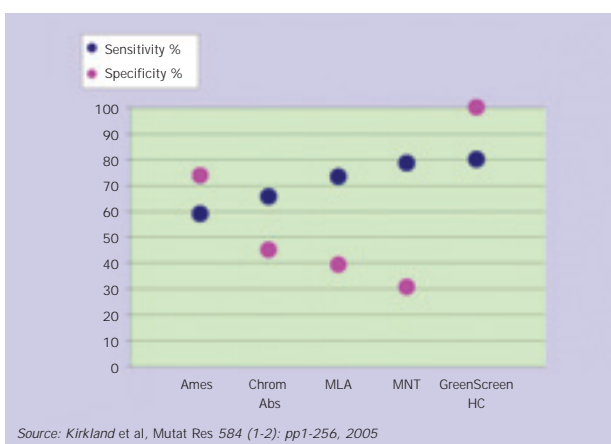


Figure 1: Existing *in vitro* genotoxicity tests show good sensitivity but poor specificity

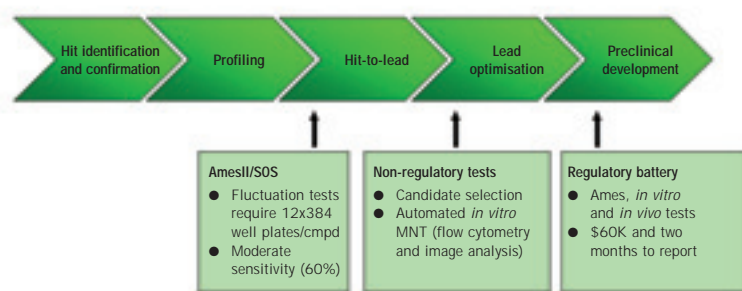


Figure 2:
Current tests for genotoxicity at different stages of the drug discovery process

As it is generally considered that data obtained *in vitro* is a demonstration of the intrinsic genotoxic properties of the test compounds, other data such as negative results from *in vivo* tests are needed to help determine the biological significance of the *in vitro* positive result. The European Centre for the Validation of Alternative Methods has stated that there is “a need to reduce false positive results with *in vitro* genotoxicity testing and avoid unnecessary follow-up animal tests”. Significant amounts of time and money are spent distinguishing unique true positives from unique false positives. As well as each requiring multi-gram quantities of valuable drug candidate, the regulatory tests can take two to three months to issue completed reports and cost approximately \$60,000 per compound to perform. Combined with the high numbers of compounds emerging from primary screens, these factors produce a toxicity-testing bottleneck in the drug development process. The regulatory tests are therefore not performed until late in the preclinical stage – too late to influence the hit and lead identification processes, and at a point where a positive result can be very costly to manage in terms of both time and resources.

THE NEED FOR EARLY SCREENING

The identification of problems, such as a genotoxicity liability, early in screening represents the single largest cost-saving opportunity in the pharmaceutical industry, as studies performed in animals are too slow to be used for real-time feedback in a drug discovery campaign. There have been limited attempts to introduce early genotoxicity screening at the hit-to-lead stage prior to application of the regulatory tests. These include high-throughput fluctuation tests (such as Ames II) that require many microplates per compound (12 x 384 wells) but predict GLP Ames results very effectively; and bacterial SOS reporter assays (such as Vitotox and SOS UmuC) that are simpler and less compound hungry, but not such effective Ames test predictors.

There are also microplate versions of the regulatory mouse lymphoma assay (MLA) which measures the induction of gene mutations, and emerging flow and imaging cytometric methods for the micronucleus test

(MNT) which measures damage to chromosomes. The analysis of micronuclei has traditionally been performed by microscopic inspection, a labour-intensive method that can hinder rapid and efficient testing. With increasing use of the *in vitro* MNT as an alternative to the more costly and time-consuming *in vitro* chromosome aberration assay, a number of automated systems utilising either flow cytometry or image analysis techniques have been developed, but a definitive approach has yet to be agreed. These methods have been tested at the lead optimisation stage, although they cannot be regarded as high-throughput screens as their throughput is limited to approximately 1,000 compounds per year per system (see Figure 2).

In silico approaches and the increasing knowledge-bank of chemical features associated with genotoxicity are routinely used at an early stage, and these are useful in highlighting chemistries that might have genotoxic liability, to allow priority setting for *in vitro* tests. Despite these tools, it is still common to see over 25% of compounds with a genotoxic liability reach preclinical development. A major issue is the prokaryotic nature of the more commonly used bacterial early screening tests; this means several mechanistic classes of genotoxicity are missed because of the different targets in eukaryotic cells. The aspirational gold standard is therefore a rodent or human cell-based screen that can be applied in parallel with eADME toxicity screens.

NOVEL *IN VITRO* MAMMALIAN SCREEN

A novel *in vitro* mammalian test using human cells, GreenScreen HC (Gentronix, UK), has been developed for application both in preclinical development prior to the regulatory battery and also early in drug discovery during profiling, hit-to-lead and lead optimisation. The test requires only a small amount of compound (approximately 1mg) and uses a simple microplate assay format for easy integration with standard automation equipment, making it the first effective *in vitro* mammalian assay suitable for high-throughput screening.

The over-sensitivity of existing *in vitro* mammalian assays may partly be explained by a deficiency – in many of the cell lines – of p53, a key protein known as “the guardian of the genome”. In these cells, the inability to respond correctly to DNA damage leads to increased cytotoxicity of genotoxins, and aberrant genotoxic damage at high concentrations of non-genotoxic cytotoxins. The new assay uses a human p53-competent cell line (TK6) and exploits p53- and genome damage-dependent expression of GFP (Green Fluorescent Protein) using elements of the human GADD45 α (Growth Arrest and DNA Damage) gene. By linking the regulation of the GADD45 α gene to the production of

Figure 3: Mechanistic classes of genotoxins identified by GreenScreen HC

Mechanistic classes of genotoxins	Example compounds
Direct acting genotoxins	4-Nitroquinoline-N-Oxide Ethyl methanesulfonate Methyl nitrosourea
Aneugens	Colchicine Nocodazole Paclitaxel
Nucleotide synthesis inhibitors	5-Fluorouracil Pyrimethamine Zidovudine
Topoisomerase inhibitors	Camothecin Etoposide Doxorubicin
Reactive oxygen species	Bleomycin sulfate Hydrogen peroxide

GFP, cells become increasingly fluorescent as the damage occurs in the presence of genotoxic agents.

A study of 75 well-characterised genotoxic and non-genotoxic compounds with diverse mechanisms of DNA damage (including aneugens, which cause changes in chromosome numbers per cell) was undertaken to test the hypothesis that this human cell-based assay responds positively to all classes of genotoxic damage (2). Genotoxic compounds were divided into groups according to their mechanism of action (see Figure 3). The screen gave negative results for all 41 non-genotoxins studied; notably, these included 11 compounds with cytotoxicity-associated 'false positives' in the regulatory *in vitro* mammalian cell assays, representing potentially valuable compounds at risk of being discarded inappropriately. The results showed that when compared with the regulatory tests or cancer studies, the sensitivity was as high as any of the established *in vitro* tests for genotoxicity, but critically not at the expense of specificity – that is, there were no false positives.

Further validation trial data also demonstrated exceptionally high specificity and an extremely low false positive rate, as well as high sensitivity to detect all mechanistic classes of genotoxins. The assay effectively distinguishes the genuinely hazardous from the misleading positives generated in other tests. The timely detection of compounds with genotoxic liabilities means that fewer enter preclinical development – minimising delays to clinical trials, as well as costly mechanistic studies and the unnecessary use of animal testing.

THE VALUE OF EARLY SCREENING

Early screening provides early alerts to medicinal chemists and, through the elimination of genotoxicity positive compounds, improves the quality of those progressing into development. The 96-well plate assay described is simple to set up, tests four compounds simultaneously per plate over nine serial dilutions, and

the results are available in 48 hours using a microplate reader and intuitive software. Based on a manual protocol, half of a full time equivalent (FTE) can assay 40 compounds a day, three days a week – that is, over 6,000 compounds per year; this compares very favourably with existing methods, and this throughput can be significantly increased with the use of robotic liquid handling systems.

The GreenScreen HC assay can be applied at different stages of the drug discovery process, at each of which the investment per compound increases exponentially with time. During profiling and conversion of hits to leads, where the investment per compound has reached around \$2-3K, the assay can be used cost-effectively to identify class-dependent liabilities and the best series for lead optimisation. The cumulative investment increases to approximately \$50K per compound at lead optimisation, where the assay can be used to select candidates. At the preclinical stage, it can prioritise compounds for regulatory testing and resolve data conflicts between true and false positives, but by this point the estimated cost per compound of late failure is over \$10M. Mechanistic studies additionally cost several \$100K and delay the initiation of clinical trials that, for a 'first-in-class' candidate, can reach \$1M per day in lost sales.

In conclusion, it can be seen that the GreenScreen HC assay fulfils the urgent need for more accurate genotoxicity tests, combining high sensitivity with an extremely low false positive rate. The first *in vitro* mammalian cell assay suitable for early screening, it provides the required throughput, cost and economy of compound, in a format compatible with existing cell culture and screening automation equipment, enabling the all important non-genotoxic compounds to become the focus of drug discovery research.

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