

Reducing late stage attrition with early high-throughput genotoxicity screening

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There is a clear requirement for an accurate genotoxicity screen that can be applied early in the drug discovery process. A screen of this type should have many attributes: high sensitivity to correctly identify genotoxins; high specificity to avoid incorrect calls on non-genotoxins; and suitability for high-throughput use. This article describes such a recently developed screen (GreenScreen HC) and the context in which it can be applied in the discovery process. It is believed that this screen can have a similar impact on reducing genotoxicity attrition as the introduction of eADME has had on reducing failures due to adverse preclinical metabolism and pharmacokinetics over the last decade.

Genotoxicity is a significant issue in early drug development - at least 35% of compounds entering preclinical evaluation will have a potential genotoxicity liability. Many of these compounds will fail, at significant cost, and require the accelerated evaluation of backup and reserve compounds. Those compounds with equivocal genotoxicity data that do continue in development demand further rigorous and expensive genotoxicity tests, usually in animals, to ensure their safety before entering human trials. In addition, it is now accepted that the regulatory in vitro genotoxicity tests, whilst identifying the majority of problem compounds correctly, over-call dramatically i.e. erroneously identify 'clean' compounds as genotoxins.

The introduction of early high-throughput ADME screening (along with improved animal models) has played a major role in reducing late stage compound attrition due to unfavourable metabolism and pharmacokinetics, with failure rates down to approximately 10% (from 40% ten years ago). A typical research site may profile up to 20,000 compounds per annum in a range of ADME screens - amounting to 500+ compounds per discovery project after hit confirmation and during hit-to-lead and lead optimisation. Figure 1 depicts a schematic overview of the discovery process with estimated times, costs and attrition rates for each stage.

Problems with existing genotoxicity screens

There have been limited attempts to introduce early genotoxicity screening prior to the application of the regulatory tests (as described later in this article). These include high-throughput fluctuation tests (such as Ames II) which require many microplates per compound but predict GLP Ames results very effectively; and bacterial SOS reporter assays (such as Vitotox and SOS UmuC) which 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 micronu-

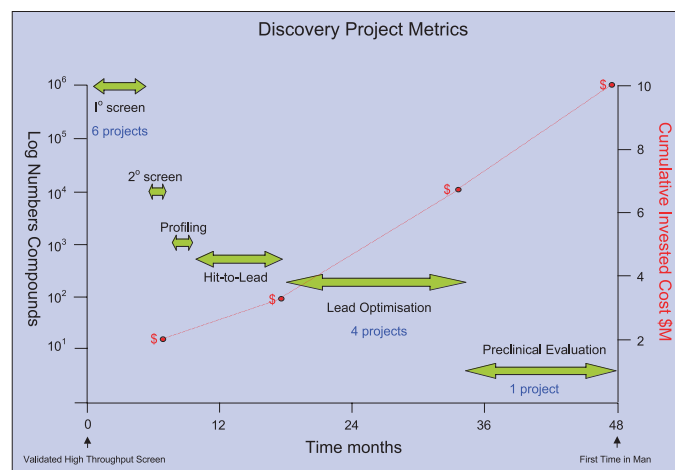


Figure 1. Schematic overview of the stages and associated costs of the drug discovery process.

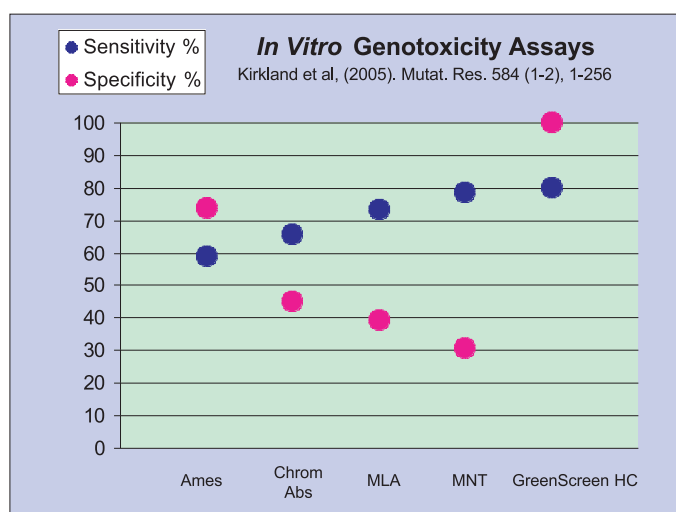


Figure 2. Based upon the data of Kirkland et al [1], existing in vitro genotoxicity tests show good sensitivity but poor specificity. In contrast, GreenScreen HC identifies the majority of genotoxins without making incorrect calls on non-genotoxins.

cleus test (MNT), which measures damage to chromosomes, though none of these can be regarded as high-throughput screens. 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 35% or more genotoxic compounds reach preclinical development. One of the main issues is that the prokaryotic nature of the more commonly used early screening tests, which use bacteria, means several mechanistic classes of genotoxicity are missed because of the different targets in eukaryotic cells. The aspirational gold standard is a rodent or human cell based screen that can be applied in parallel with eADME screens.

Issues with regulatory genotoxicity tests

Genotoxicity assessment of drugs is a mandatory element of preclinical safety testing and comprises a battery of regulatory tests. These include a bacterial assay (e.g. the Ames test); an in vitro mammalian cell test (e.g. MLA or chromosome aberration assessment); and an in vivo rodent assay (e.g. MNT using bone marrow or peripheral blood cells). Common features to all these assays are that they are time-consuming, expensive to

perform and require multi-gram quantities of precious drug candidate per assay. In total these regulatory tests cost approximately \$60,000 per compound and can take 2-3 months to the issue of completed reports. All these factors contribute to the toxicity-testing bottleneck and dictate that such tests are performed in the lead up to the first studies in human volunteers - too late to influence the hit and lead identification process, and at a stage where a positive result can be very costly to manage in both time and resources.

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. Figure 2 depicts the relative specificities and sensitivities of the in vitro tests, highlighting the issue of poor specificity.

Late stage genotoxicity failure costs

There are several ways to quantify the cost of a compound failing due to genotoxicity, and every pharmaceutical company will have their own metrics and costing/value model.

A simple approach is to take the costs of the various stages of drug discovery from HTS through to preclinical evaluation and divide by the number of compounds evaluated per stage, accounting for the accepted attrition rate between stages. There are large variations in these numbers depending upon how a company approaches discovery, the therapeutic area and the target class, but Figure 1 summarises accepted averages. For illustrative purposes, it is possible to attribute a 'value per



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compound' to each stage of the discovery process. Expressed in US dollars per compound, for HTS it is single digit \$, rising up to \$10,000 for lead optimisation and up to \$3M for preclinical evaluation. This is a notional cost of failure per compound per stage, i.e. what has been spent to date on progressing each compound through the end of that stage.

Mechanistic Classes of Genotoxins	Example Compounds
Direct Acting Genotoxins	4-Nitroquinoline-N-Oxide Ethyl methanesulphonate Methyl nitrosourea
Aneugens	Colchicine Nocodazole Paclitaxel
Nucleotide Synthesis Inhibitors	5-Fluorouracil Pyrimethamine Zidovudine
Topoisomerase Inhibitors	Camptothecin Etoposide Doxorubicin
Reactive Oxygen Species	Bleomycin sulphate Hydrogen peroxide

Figure 3. Mechanistic classes of genotoxins identified by GreenScreen HC.

The true cost is even higher

A review of the preclinical development process further illustrates the costs of late stage positive genotoxicity results failure. A compound that is negative in a regulatory Ames test will go through a genotox screening process that costs approximately \$60,000 including in vitro and in vivo tests. At this stage a positive result in the in vitro mammalian test may not be sufficient reason to abandon the compound. For example, a compound with an antineoplastic indication might be an effective medicine through a genotoxic mechanism. Alternatively there might be a plausible mechanistic explanation for the mammalian cell test to be positive. If such a compound is progressed, the in vivo MNT has to be carried out and in addition a second in vivo assay using a different tissue is also required. A proportion of these compounds will indeed be carcinogens, for which the positive in vitro result was a correct identification of a potential hazard. Mechanistic studies and additional ani-

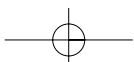
mal testing are costly in themselves, but the consequent delay in progressing the compound to market can cost tens of millions of dollars per month in lost sales. In parallel with these genotoxicity tests many other development functions are active, including repeat dose toxicology, safety pharmacology, pharmacokinetics/pharmacodynamics, chemical development and formulation. The repeat dose toxicology (starting with 14 day rat and non-rodent studies) will cost >\$100,000 and it is quite possible that 28 day studies will be in process when a compound is identified as a genotoxin. Taking into account all these functions, the total cost that will be lost due to genotoxicity failure could be many million dollars per compound.

It is therefore clear that significant savings can be made by use of a genotoxicity screen that is both sensitive and specific, and is suitable for use in the stages of drug discovery prior to preclinical evaluation, i.e. hit profiling, hit-to-lead and lead optimisation.

A new genotoxicity screen for use in early discovery

A new human cell genotoxicity screen (GreenScreen HC) has been developed that links the regulation of the human GADD45a (Growth Arrest and DNA Damage) gene to the production of Green Fluorescent Protein (GFP)[2]. Cells become increasingly fluorescent as the damage occurs, indicating the presence of genotoxic agents. Developed as a simple microplate assay, GreenScreen HC requires only a small amount of compound (<1mg), and is easily integrated with standard automation equipment - making it the first effective in vitro mammalian assay suitable for high-throughput screening.

One of the reasons for the oversensitivity of existing in vitro mammalian assays is that the cell lines are often deficient in a key protein, p53, known as "the guardian of the genome". In such 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 using elements of the human GADD45a gene.



Validation of GreenScreen HC

Seventy five well-characterised genotoxic and non-genotoxic compounds with diverse mechanisms of DNA damage (including aneugens, which cause changes in chromosome numbers per cell) were studied to test the hypothesis that the 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 for an overview of mechanistic classes of genotoxins]. All classes were represented by at least three examples and positive results were all obtained at concentrations below 10 mM, the highest concentration required by the guidelines issued by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Of these compounds only 3 actually required a test concentration higher than 100mM.

GreenScreen HC gave positive results for 31/34 genotoxic carcinogens; 10/10 direct acting agents, 4/4 topoisomerase II inhibitors, and 9/10 aneugens were positive, (with the negative compound being thiabendazole, for which the initial positive in vivo MNT data has not been reproduced by other laboratories). 7/8 nucleotide synthesis inhibitors were positive, (the negative compound was didanosine, which has given equivocal results in other tests). Apart from methyl viologen dichloride, 2/3 reactive oxygen species were positive.

The screen gave negative results for all 41 non-genotoxins studied so far. Importantly this included nine compounds with cytotoxicity-associated 'false positives' in accepted in vitro mammalian cell assays for genotoxicity. These compounds represent potentially valuable compounds at risk of being discarded inappropriately. From the study of 75 compounds, 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 was not at the expense of specificity, which stood at 100%, as there were no false positives in this dataset - see Figure 2 for a comparison with the regulatory in vitro tests. The next stage in development is to extend the assay to include the 'promutagenic' compounds, which only become genotoxic following metabolism, principally by the liver. Preliminary tests in which liver extracts ('S9') were used to treat three com-

monly used promutagen controls gave the correct positive results.

Conclusions

Increased knowledge of the DNA damage response in mammalian cells as provided by the new GADD45a screen will inevitably lead to a better understanding of genotoxic hazard. A purely in vitro prediction of cancer hazard from exposure to genotoxins is close, but at the moment this is at the expense of falsely identifying some non-carcinogens as potential carcinogens. The GADD45a screen provides a combination of high specificity with high sensitivity and at the very least should allow effective prioritisation of compounds early in the discovery process, and should minimise the number of hazardous compounds needlessly proceeding to animal tests or needless delays in the development of useful drugs.

References

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