Genotoxicity Evaluation of Inks

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Abstract

An important part of the assessment of any new ink for safety in use is the evaluation for potential genotoxicity, as a screen for chemicals which might possess carcinogenic or mutagenic properties. Although there are many tests to examine for genotoxicity, the last few years has seen the emergence of clearer guidelines for assay conduct and most importantly, for a strategy to allow the interpretation of genotoxicity data in the context of hazard to man. The strategy is based on the understanding that *in vitro* genotoxicity data provide an assessment of *intrinsic* genotoxicity of a chemical but that *in vivo* genotoxicity data provide an assessment of any activity that is *expressed* in the whole animal. Data from the *in vivo* assays carry more weight in the extrapolation to man. The use and relevance of this strategy for chemical classes commonly used in inks is discussed.

Introduction

Genotoxicity tests have a key role in contributing to the toxicological assessment of whether a chemical has the potential to cause somatic or germ cell effects in animals, i.e. the potential to induce cancer or heritable mutation as end points. These end points are clearly of significant concern, and an evaluation of a new chemical for inkjet, or other ink/dye application, is almost certain to include an assessment of genotoxicity. This may be for internal company prioritisation/selection of candidate materials, for hazard assessment for employee/customer safety assessment, or for submission to regulatory bodies for notification. It is clearly necessary to have a testing strategy which ensures that the number of tests conducted and the nature and order of the tests is such as to allow a meaningful evaluation of the genotoxicity of a material without the risk of drawing false positive or false negative conclusions.

Discussion

The core principle underlying genotoxicity tests is whether the chemical or a metabolite interacts with, and damages, the DNA of a test organism. There is a large number of such test systems available, varying markedly in complexity and covering a range of cell types and genetic end points. Until recently there has in some quarters been an apparent comparability in the way assays as disparate as the Ames test (an *in vitro* bacterial assay) and the mouse specific locus assay (involving hundreds of mice) can be considered to give an assessment of the genotoxic (gene mutation), properties of a chemical[1]. Such an equivalence would render testing strategies valueless. However, the suggestion by Bridges[2], and the promotion by Ashby[3] of a stepwise approach to genotoxicity testing has allowed the development of both effective and efficient hierarchical testing strategies to assess the likely genotoxicity of a chemical to the whole animal. This approach, together with the results of international trials designed to assess the value and reliability of the plethora of available genotoxicity assays for their ability to detect carcinogens and discriminate non-carcinogens[4-7], has led to the current focused testing strategies employing a limited number of validated assays interpreted in a coherent and stepwise manner.

The core principle of the current strategies generally accepted by genetic toxicologists is that an initial assessment is made using *in vitro* assays (using for example bacteria or isolated mammalian cells), which are designed to be sensitive and to detect any *intrinsic* genotoxic activity. If clear evidence of genotoxicity is seen in one or more of these assays, an assessment is made using *in* vivo assays in order to determine whether this intrinsic genotoxic activity is *expressed* in the whole animal. This *in vivo* assessment of chemicals found to be genotoxic *in vitro is* essential if a meaningful evaluation of their genotoxic hazard to animals (and ultimately man) is to be made. The *in vitro* tests selected are, by definition as *primary* screens, designed to be oversensitive. They involve the incubation of cells in a test tube or on a plate with large or toxic doses of chemical, with no barriers (absorption/distribution) or excretion processes as are available in the whole animal. As a result, there are a large number of false positives *in vitro,* i.e. chemicals which show genotoxicity *in vitro* but which are not genotoxic/carcinogenic *in vivo*[8]. Such positive findings *in vitro* do not pose a problem if considered in the light of an overall testing strategy involving further evaluation of the compound (i.e. *in vivo* testing), but they do if decisions are based on the positive *in vitro* data alone.

Since the *in* vivo assays are designed to assess the relevance of the *in vitro* results to the whole animal, it is recommended that the route of exposure used should be one relevant for potential human exposure. To do otherwise undermines the unique role of the animal studies in this testing strategy. They must be allowed to superimpose the effects of absorption, distribution, metabolism, excretion together with cellular processes such as DNA repair, onto the established intrinsic activity of the chemical. This then provides a toxicologically relevant assessment of any genotoxic effects in animals.

For such a strategy, the available data indicate that a thorough evaluation *in vitro* can be achieved by using the Ames test together with an *in vitro* cytogenetic assay in mammalian cells, and a thorough evaluation *in vivo* by again using two assays, such as the mouse bone marrow micronucleus assay and the rat liver unscheduled DNA synthesis assay (Figure 1). These assays provide an effective screen for the detection of genotoxic carcinogens[9].
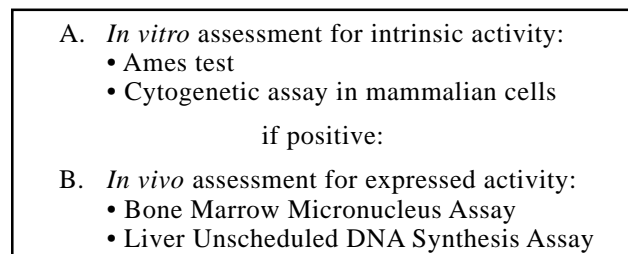

A. *In vitro* assessment for intrinsic activity:
  • Ames test
  • Cytogenetic assay in mammalian cells

  if positive:

B. *In vivo* assessment for expressed activity:
  • Bone Marrow Micronucleus Assay
  • Liver Unscheduled DNA Synthesis Assay

Figure 1.

These principles are reflected in recent genotoxicity testing guideline updates both within[10,11] and outside Europe[12].

In such a strategy, once clear genotoxic activity has been determined *in vitro,* further *in vitro* assays are of limited value. What is required is the evaluation *in vivo*. The use of chemical structure-activity considerations can be invaluable in aiding the interpretation of any activity seen in these assays[8,13,14]. The structure-activity considerations derive from the underlying principle that genotoxic chemicals bind covalently to DNA, i.e. they have (or are metabolised to have) an electrophilic centre which binds covalently to one or more of the nucleophilic sites on DNA. An analysis of chemical substructures against the relevant genotoxic and carcinogenicity data for a wide range of chemical structures has led to a good basis of structure-activity relationships for many classes of chemicals. This applies both to an assessment of the likely active groups on a base structure (e.g. amino group on a phenyl ring) through to the likely mitigating effects of further substitution (e.g. amino group on a phenyl ring which is ortho-substituted with a sulphonic acid group[15]). In addition to assessing likely activity, a knowledge of structure-activity relationships can be used also to assess whether the profile of activity both across assays and within an assay, is consistent with the chemical structure of the material under test, and this can be an aid to assessing whether a chemical is genotoxic or whether attention should be focussed onto either impurities or artifacts. For example, a simple aromatic amine structure based on 4-aminobiphenyl that gives an Ames positive response in strain TA98 (+S9) has produced a response consistent with chemical structure and therefore will not benefit from further *in vitro* evaluation—it is clearly genotoxic *in vitro;* the evaluation should move immediately to assess any effects in the whole animal. There is no value in conducting further *in vitro* assays simply because they are present in a test strategy.

Although the example here was for a simple aromatic amine based on 4-aminobiphenyl, the same fundamental principles can be applied to any chemical class found in inks. Thus, for example, in the case of anthraquinone structures, it is not the anthraquinone nucleus *per se* that is genotoxic, but rather it is the nature and pattern of substituents on the anthraquinone moiety that will determine whether such a compound shows no genotoxic activity, genotoxic activity *in vitro* alone, or genotoxic activity both *in vitro* and *in vivo*[13]. Furthermore, the substituents may not govern the observed genotoxicity by virtue of chemical reactivity alone. The solubility of a chemical may be critical *in* vivo such that certain pigments, although apparently structurally alerting for possible genotoxicity, do not prove carcinogenic to animals[8]. In such cases the insolubility almost certainly prevents exposure of the tissues to a significant amount of material. The same features of chemical reactivity and physico-chemical characteristics (e.g. solubility) act to determine the genotoxic activity of other classes of inks such as azo materials or phthalocyanine materials.

From the above it is clear that the *in vitro* assays are designed to assess the *intrinsic* activity of a chemical to isolated cells. As such, they provide valuable information on the *hazard* of a chemical i.e. the ability to cause an effect, but no indication of the potency of any response in animals. Thus it cannot be concluded that a

chemical producing a strong response in an *in vitro* assay (e.g. an Ames test) will be a potent carcinogen to animals; it may of course even prove to have no carcinogenic activity at all[8].

The above strategy involves an assessment *in vitro* followed if necessary by *in vivo* investigations conducted in somatic cells and is sufficient to define the genotoxicity of the test material *in vivo*. If the material is negative in the *in vivo* somatic cell assays, this can be considered to indicate a lack of genotoxic activity in both somatic and germ cell tissues. The justification for this is that in a number of reviews of the available data[16-18] it has been shown that those chemicals that can reliably be regarded as germ cell mutagens in rodents are detected in *in* vivo somatic cell assays. In fact there are a number of somatic cell mutagens that are not germ cell mutagens; thus germ cell mutagens are a subset of somatic cell mutagens. Based on these observations therefore, a chemical identified as non-genotoxic in *in vivo* somatic cell assays will not show genotoxic activity in germ cells and further evaluation is unnecessary.

The above testing strategy, which is based on experience with established carcinogens and germ cell mutagens therefore allows the detection of both somatic cell and germ cell genotoxins in a process using the most efficient use of resources. For such a strategy to operate effectively, common sense and good scientific practice must be applied at each stage, bearing in mind the overall aim of the endeavour. For example, testing in the *in vitro* assays should be to sufficient protocols to provide a thorough investigation[9], yet not to excessive stringency (e.g. marked cytotoxicity, where significant pH changes are induced or where the test material is at grossly precipitating concentrations). Such situations may lead to the generation of equivocal or positive responses which are of little or no relevance biologically and yet may be viewed as warranting follow-up *in vivo* studies by a rigid application of the testing strategy. This clearly may not be relevant. In a similar vein, any extension of testing outside of the core strategy should be based on established precedents that it will allow an increased detection of genotoxins. For example, the value of conducting an *in vitro* mammalian cell gene mutation assay following clearly negative Ames test and *in vitro* cytogenetic assays is not clear.

Such a testing strategy must, of course, be applied on a case by case basis and with good scientific principles employed. If this is done, then the evaluation of inks and related imaging materials for possible genotoxic activity can be carried out in a scientific and efficient manner.

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