March 1998

Significance of the bacterial reverse mutation test as predictor of rodent and human carcinogenicity

ETAD Information Notice No. 7

ACKNOWLEDGEMENT

Although this document represents a collective effort, particular thanks are due to
Dr. U. Mentzel, Ciba SC, for its initial drafting and subsequent revision.

This document is currently available also on our Internet homepage
http://www.etad.com

SIGNIFICANCE OF THE BACTERIAL REVERSE MUTATION TEST AS PREDICTOR OF RODENT AND HUMAN CARCINOGENICITY

1. Summary

The bacterial reverse mutation test is a useful tool as an initial *in vitro* screen for potential *in vivo* genotoxic activity. Positive results indicate the possibility of mutagenic activity but have no definitive status since there are numerous reasons why the activity may not be expressed *in vivo*. The test does not provide direct information on carcinogenic potential in mammals.
The bacterial reverse mutation test is one of several tests which can be employed in the first step of hazard identification during the process of carcinogenic risk assessment.

2. Introduction

A large number of *in vitro* and *in vivo* mutagenicity tests are presently available for the investigation of genotoxicity. These tests detect the two main categories of mutations, gene mutation and chromosomal aberration.
The bacterial reverse mutation test is an *in vitro* mutagenicity test, commonly used as initial screen for genotoxic activity and more particularly for point mutation inducing activity.
This type of short-term mutagenicity test was developed to identify chemical carcinogens in the environment. After two decades of development and validation, the bacterial reverse mutation test is well established in routine testing schemes, but the opinion of its utility in carcinogenicity prediction and risk assessment has undergone a re-assessment.
Aim of the present document is to shed light on the obvious discrepancy between usefulness in mutagenicity testing on the one hand and its limited predictive power for rodent and human carcinogenicity on the other hand.

3. Definition of mutagenicity, genotoxicity and carcinogenesis

In order to characterise the role of the bacterial reverse mutation test as predictor of carcinogenicity, it is important to distinguish between mutagenicity, genotoxicity and carcinogenesis.
Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of genetic material of cells or organisms. These changes, "mutations", may involve a single gene or gene segment, a block of genes, or whole chromosomes.
Genotoxicity is a broader term and refers to potentially harmful effects on genetic material, which may be mediated directly or indirectly, and which are not necessarily associated with mutagenicity.
It is generally accepted that carcinogenesis is a multiphase process, and a mutational event may represent not more than one of these steps. The process of carcinogenesis is strongly

influenced by a number of variables such as age, diet, hormonal balance, and in the case of bioassays by animal species and strain. Carcinogenesis could be defined as the development of cancer starting from the very first phase, called the initiation phase, followed by the promotion phase and ending in the final phase of the disease, the progression phase.
Specifically, a mutagenic or genotoxic event is accepted as a critical stage in the initiation phase of carcinogenesis.

4. Test procedure: Bacterial reverse mutation test

The bacterial reverse mutation test (specifically the Salmonella/microsome assay otherwise termed the Ames test) has become the most extensively used *in vitro* short-term test in the screening for mutagenicity. Reversion assays involve bacteria which are already mutant at a locus whose phenotypic effects are easily detected. The assay determines the frequency at which exposure to the test-chemical reverses or suppresses the effect of the pre-existing mutation. The genetic target presented to the test-chemical is therefore small, specific and selective. Several bacterial strains or a single strain with multiple markers, are necessary to overcome the effects of mutagen specificity. The reversion of bacteria from growth-dependence on a particular amino acid to growth in the absence of that amino acid is the most widely used marker in reverse-mutation assays. In order to make bacteria more sensitive to mutation by chemical and physical agents, several additional traits have been introduced: these include various DNA-repair deficiencies, increased permeability of the bacterial wall to hydrophobic chemicals as well as the development of strains with mutations located on a multicopy plasmid.
Since test systems using micro-organisms *in vitro* do not, or insufficiently, possess enzyme systems responsible for metabolic processes, *in vivo* liver-enzyme extracts (the so-called S9 fraction) have to be added to the test-system. By the use of the S9 fraction it is possible to assess mutagenic activity in bacteria of the parent compound as well as the activity of possible metabolites.

5. Validation of the bacterial reverse mutation test as predictor of carcinogenicity

Before a test procedure can be used for a human health risk assessment, it must be insured that it accurately detects effects that are recognized as being detrimental to human health. The necessary process is called test validation.
Validation is the determination of the ability of one endpoint to predict the occurrence of an indirectly or distantly related, but different endpoint.
The introduction of the bacterial reverse mutation test led to the hope of using this type of *in vitro* mutagenicity test for the detection of carcinogens. There was even hope that such tests

would eventually become surrogates for the rodent cancer bioassay, which represents the fundamental basis for the human carcinogenic risk assessment.

The predictive power of the bacterial reverse mutation test for identifying carcinogens has been often evaluated through comparisons with results of carcinogenicity assays in rodents. Especially recent validation studies demonstrated a relatively low degree of correlation, further highlighting the fact that mechanistic differences exist between both endpoints, mutagenicity in bacteria and carcinogenicity in rodents. An analysis of data for 301 chemicals tested for carcinogenicity in mice and rats revealed that the bacterial reverse mutation tests detected 56 percent of the carcinogens in the entire data set and 70 percent of those that were carcinogenic in both mice and rats.

Reasons for this inadequacy can be ascribed generally to the specific nature of the endpoints detected (cancer and gene mutation), differences in metabolic conditions or differences in bioavailability as well as repair mechanisms *in vivo* and detoxification of metabolites.

As already mentioned the rodent bioassay represents the key animal study for the human carcinogenic risk assessment. Therefore, in order to get a deeper understanding of the limitations for the use of the reversal bacterial mutation test in human carcinogenic risk assessment, the sources of discordance between both tests have to be discussed in more detail.

6. Sources of discordance between the reversal bacterial mutation test and the rodent cancer bioassay

There are two major sources of discordance between the bacterial reverse mutation test and the cancer bioassay.

First, the bacterial reverse mutation test cannot detect agents that can induce cancer by non-genotoxic mechanisms.

Second, the *in vitro* bacterial reverse mutation test has a much higher sensitivity compared to *in vivo* tests including the cancer bioassay and thus, tends to overpredict *in vivo* activity.

It follows that a discussion about the predictive capacity of the bacterial reverse mutation test for carcinogenicity always deals with the question about the differences between *in vitro* exposure and *in vivo* exposure. There are several important differences and each of these factors can limit the degree to which the predictions of both tests agree.

In particular the following points have to be considered.

6.1 Dose

In bacterial reverse mutation tests the dose is only limited by the chemical's solubility in the treatment medium and by its cytotoxicity to the cultured cells. In contrast, in the rodent cancer bioassay, the dose to which the target is exposed is dependent on uptake, distribution,

metabolism and excretion. The dose might be further limited by the lethality of the test chemical to the entire animal.

The high dose treatment leads to two major consequences. (1) High doses increase the chance of detecting an effect with *in vitro* tests and thereby maximise their sensitivity relative to the cancer test. (2) The use of high doses may allow small amounts of impurities to reach sufficient concentrations to induce mutagenic effects. However, these concentrations may not be sufficient to induce effects *in vivo*.

6.2 Tissue-specific effects - Uptake - Distribution

Some proven bacterial mutagens are principally not active in *in vivo* mammalian tests. The reason is a very low bioavailability in mammals. Chemical and physical parameters are decisive factors in terms of gastro-intestinal, dermal or inhalative absorption, aspects which do not arise in *in vitro* mutagenicity tests.

On the other hand a carcinogenic event is often observable only in one particular organ. The reasons might be an unequal distribution of the chemical throughout the body or a tissue-specific metabolic event leading to a DNA reactive form of the chemical. In contrast, the bacterial reverse mutation test is not able to mimic this particular situation in mammalian organs.

6.3 Metabolism

Many chemicals require metabolic activation to convert them into sufficiently reactive entities for DNA interaction. *In vivo* there exists a broad range of activation and detoxification systems, located not only in the liver but also in other tissues of the living animal. The amount of a chemical converted into an active metabolite depends on the concentration of activating and deactivating enzymes.

The exogenous metabolic activation system used in bacterial reverse mutation tests (liver S9 fraction) does not represent the complicated enzyme network in mammalian tissue.

It must be borne in mind that not all chemicals are converted in the liver, for instance conversion can also occur in the lungs, the skin or the kidney. There are considerable differences between various animal species and humans as far as metabolic transformation in different organs is concerned.

During the production of S9 fractions the ratio of activating and deactivating enzymes may be significantly altered leading to the most aberrant metabolic activation conditions. Usually the activating enzymes of the P-450 complex are retained, but the deactivating enzymes, present in the cytosolic fraction, may be preferentially discarded.

6.4 General pharmacokinetics

The presence of activated metabolites at the critical target site represents another important difference between bacterial reverse mutation tests and *in vivo* tests. The direct addition of the chemical to the target cells and the use of media with reduced serum content prevents for instance an effective protein binding which might be able to reduce the dose delivered to the target site *in vivo*. *In vivo*, the probability that an active metabolite will succeed in a critical reaction with DNA will depend on the stability of the metabolite, the distance it has to travel and the availability of appropriate active trapping groups other than DNA.

6.5 Lesion fixation and expression

The end result mutation may be regarded as a competition between DNA replication manifesting a mutation and DNA repair which removes for instance adducts and restores DNA to its normal condition. As above mentioned most of the employed test strains in bacterial reverse mutation tests lack DNA repair systems and may thus be extremely sensitive to genotoxic agents and tend to overpredict the results under *in vivo* conditions. In recent years it has become more and more obvious that cell proliferation plays a pivotal role in carcinogenicity and in mutagenicity. Cellular proliferation has two effects on the process of lesion fixation: it increases the opportunity for DNA adducts to occur, possibly because of increased turnover and because the DNA is less well protected by chromosomal proteins and chromatin structure against electrophilic attack, and it reduces time during which DNA repair can occur.

Since bacteria show a very high rate of cellular proliferation, there is a real need to consider this additional point in comparing bacterial reverse mutation tests and *in vivo* test systems in relation to their apparent sensitivity.

7. Human relevance

Most of the above mentioned points also apply to the human situation. In addition, the development of cancer in humans is even more complicated than the experimental development of cancer in rodents under high dose treatment with complete carcinogens. Humans are exposed to various complex mixtures of initiating and promoting agents. This situation is absolutely not comparable to the high dose treatment of bacteria *in vitro*.

8. Conclusion

Although the introduction of the bacterial reverse mutation test represents a major step in the development of mutagenicity testing, it is generally acknowledged that one single assay does not constitute an adequate reflection of mutagenic alterations occurring in mammalian cells *in vivo*.

Our current understanding of carcinogenesis indicates that it is a complex multiple-step process where genotoxic effects are important contributors in the initiation phase of carcinogenesis. Since the bacterial reverse mutation test is a test for the induction of point mutations in bacteria, rather than a test for the complex multiple step process of carcinogenesis, it would not be logical to expect a close correlation with the rodent cancer bioassay.

In order to establish a meaningful genetic and carcinogenic risk assessment positive results in the bacterial reverse mutation test should be evaluated in connection with further *in vitro* mutagenicity tests using mammalian cells. When this genotoxic potential has been demonstrated *in vitro*, it is necessary to determine whether and under what circumstances that activity will be expressed *in vivo*, if accurate predictions of carcinogenic potential are to be made.

This step by step approach ensures that the bacterial reverse mutation test can be used as an extremely sensitive first screen for bacterial mutagenicity and thus contribute together with further tests to the development of meaningful genetic and carcinogenic risk assessments.