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Collaborative study on fifteen compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies

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ABSTRACT

A collaborative trial was conducted to evaluate the possibility of integrating the rat-liver Comet assay into repeat-dose toxicity studies. Fourteen laboratories from Europe, Japan and the USA tested fifteen chemicals. Two chemicals had been previously shown to induce micronuclei in an acute protocol, but were found negative in a 4-week Micronucleus (MN) Assay (benzo[*a*]pyrene and 1,2-dimethylhydrazine; Hamada et al., 2001); four genotoxic rat-liver carcinogens that were negative in the MN assay in bone marrow or blood (2,6-dinitrotoluene, dimethylnitrosamine, 1,2-dibromomethane, and 2-amino-3-methylimidazo[4,5-f]quinoline); three compounds used in the ongoing JaCVAM (Japanese Center for the Validation of Alternative Methods) validation study of the acute liver Comet assay (2,4-diaminotoluene, 2,6-diaminotoluene and acrylamide); three pharmaceutical-like compounds (chlordiazepoxide, pyrimethamine and gemifloxacin), and three non-genotoxic rodent liver carcinogens (methapyrilene, clofibrate and phenobarbital).

Male rats received oral administrations of the test compounds, daily for two or four weeks. The top dose was meant to be the highest dose producing clinical signs or histopathological effects without causing mortality, i.e. the 28-day maximum tolerated dose. The liver Comet assay was performed according to published recommendations and following the protocol for the ongoing JaCVAM validation trial.

Laboratories provided liver Comet assay data obtained at the end of the long-term (2- or 4-week) studies together with an evaluation of liver histology. Most of the test compounds were also investigated in the liver Comet assay after short-term (1–3 daily) administration to compare the sensitivity of the two study designs. MN analyses were conducted in bone marrow or peripheral blood for most of the compounds to determine whether the liver Comet assay could complement the MN assay for the detection of genotoxins after long-term treatment.

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Most of the liver genotoxins were positive and the three non-genotoxic carcinogens gave negative result in the liver Comet assay after long-term administration. There was a high concordance between short- and long-term Comet assay results. Most compounds when tested up to the maximum tolerated dose were correctly detected in both short- and long-term studies. Discrepant results were obtained with 2,6 diaminotoluene (negative in the short-term, but positive in the long-term study), phenobarbital (positive in the short-term, but negative in the long-term study) and gemifloxacin (positive in the short-term, but negative in the long-term study). The overall results indicate that the liver Comet assay can be integrated within repeat-dose toxicity studies and efficiently complements the MN assay in detecting genotoxins. Practical aspects of integrating genotoxicity endpoints into repeat-dose studies were evaluated, e.g. by investigating the effect of blood sampling, as typically performed during toxicity studies, on the Comet and MN assays. The bleeding protocols used here did not affect the conclusions of the Comet assay or of the MN assays in blood and bone marrow. Although bleeding generally increased reticulocyte frequencies, the sensitivity of the response in the MN assay was not altered. These findings indicate that all animals in a toxicity study (main-study animals as well as toxicokinetic (TK) satellite animals) could be used for evaluating genotoxicity. However, possible logistical issues with scheduling of the necropsies and the need to conduct electrophoresis promptly after tissue sampling suggest that the use of TK animals could be simpler. The data so far do not indicate that liver proliferation or toxicity confound the results of the liver Comet assay. As was also true for other genotoxicity assays, criteria for evaluation of Comet assay results and statistical analyses differed among laboratories. Whereas comprehensive advice on statistical analysis is available in the literature, agreement is needed on applying consistent criteria.

1. Introduction

The *in vivo* Comet assay (single-cell gel electrophoresis assay) is increasingly used in regulatory genotoxicity testing for the evaluation of DNA damage and repair in various tissues of mice and rats. Test guidelines for pharmaceuticals recommend that for follow-up testing to evaluate positive results in genotoxicity test *in vitro*, *in vivo* assays should be done in two tissues [1]. In practice this is usually achieved by performing an erythrocyte chromosome-damage test and a genotoxicity assay in liver, because some genotoxic carcinogens are known to be positive in the liver but not in the bone-marrow assays. Current regulatory guidance considers the rodent Comet assay as a useful follow-up test in case of positive results in *in vitro* genotoxicity assays [2,3]. It is suggested as a second *in vivo* test to be conducted in the liver (e.g. as an alternative to the *in vivo* liver UDS test [4]) in addition to, or as part of the standard test battery [2]. It is also recognised as a useful tool for the evaluation of genotoxicity in organs/cell types that cannot easily be evaluated with other standard tests, e.g., in skin and stomach [5]. Protocols for conducting the *in vivo* Comet assay were developed by different expert panels, e.g., at the 2nd and 4th International Workshops on Genotoxicity Testing [6,7] and the 4th International Comet assay Workshop [8]. Moreover, an international validation study on the rodent Comet assay coordinated by the Japanese Centre for the Validation of Alternative Methods (JaCVAM) is currently ongoing, using a short-term protocol (1–3 daily administrations).

There is a current shift in paradigm towards integration of genotoxicity endpoints in repeat-dose toxicity studies, e.g., the 28-day rat-toxicity study. Recently proposed guidance on the requirements for genotoxicity testing of pharmaceuticals [2] and chemicals [5] encourage such an integration of genotoxicity tests into repeat-dose toxicity studies, whenever possible and scientifically justified. Besides the obvious contribution to the reduction of animal use in genetic toxicology [9], an integrated measurement of genotoxicity endpoints also offers the possibility for an improved genotoxicity risk assessment, since such data will be evaluated in conjunction with routine toxicological information obtained in the repeat-dose toxicity study, such as haematology, clinical chemistry, histopathology and exposure data.

In addition to the experience with repeat-dose testing in mice (e.g., [10,11]) several published studies have confirmed the feasibility of integrating the MN assay into repeat-dose toxicity studies in rats [12–18]. Investigations into the sensitivity of the long-term *vs* short-term MN assays, however, also indicated that the detection of a few genotoxins may be impaired because of the lower dosages typically employed in a long-term study as compared with an acute/short-term study [17].

To evaluate whether the liver Comet assay is suitable for integration into repeat-dose toxicity studies in the rat, a collaborative study was performed, involving 14 laboratories from Europe, Japan and the USA. Selected liver-genotoxins and non-genotoxic liver carcinogens were investigated in the liver Comet assay after repeated dosing of male rats over two or four weeks. The sensitivity of the Comet assay to detect chemicals known to be genotoxic in the liver at doses that are tolerated without mortality over 28 days (28-day maximum tolerated dose) was compared with Comet assay results obtained after short-term (acute or three-day) dosing. MN measurements were also performed in most of the studies to assess whether the liver Comet assay complements the MN assay in detecting genotoxic compounds. The specificity of the liver Comet assay, i.e. its potential to give false positive results as a consequence of cytotoxicity or liver hypertrophy/hyperplasia was investigated by testing known non-genotoxic liver carcinogens at doses that induced significant changes in the liver.

Among the fifteen compounds evaluated in this study were two compounds (benzo[*a*]pyrene (B[*a*]P) and 1,2-dimethylhydrazine(1,2-DMH)) that had been shown to induce micronuclei in an acute protocol but not in a 4-week MN assay (Hamada et al., 2001); four rat-liver carcinogens negative in the MN assay in bone marrow or blood (2,6-dinitrotoluene (2,6-DNT), dimethylnitrosamine (DMN), 1,2-dibromomethane (1,2-DBE), and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ); three compounds currently evaluated in the ongoing JaCVAM validation study of the acute Liver Comet assay (2,4-diaminotoluene (2,4-DAT), 2,6-diaminotoluene (2,6-DAT), and acrylamide (ACR); three pharmaceutical-like compounds (chlordiazepoxide (CDZ), pyrimethamine (PYR) and gemifloxacin (GF)), and three non-genotoxic rodent carcinogens (methapyrilene (MP), clofibrate (CFB) and phenobarbital (PHE)).

Finally, practical aspects of integrating the liver Comet assay and the MN assay into repeat-dose toxicity studies were assessed. For example, the potential effect of blood sampling, as typically performed for toxicokinetic (TK) measurements or for routine haematological and clinical chemistry investigations, on genotoxicity results (i.e. DNA strand-breaks measured in Comet assay and MN induction) was evaluated.