MEDIUM-TERM BIOASSAYS
AS ALTERNATIVE CARCINOGENICITY TEST

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ABSTRACT — A medium-term liver bioassay system for rapid detection of carcinogenic agents using male F344 rats has been developed, in order to bridge the gap between long-term carcinogenicity tests and short-term screening assays. The system is fundamentally based on the two-stage hypothesis of carcinogenesis: initiation with diethylnitrosamine (200 mg/kg bw, ip) is followed by test chemical administration during the second, in combination with 2/3 partial hepatectomy. It requires only 8 weeks for animal experimental treatment and a further few weeks for quantitative analysis of immunohistochemically-demonstrated glutathione S-transferase placental form positive hepatic foci. A total of 291 chemicals/substances have already been analyzed in this laboratory and the efficacy of the system for hepatocarcinogens has thereby been well established. This bioassay is particularly useful for dose-response and chemical mixture studies, usually requiring large-scale experiments and also for evaluation of chemopreventive agents. Another bioassay, a medium-term multi-organ bioassay system, using 5 different chemical carcinogens, diethylnitrosamine (DEN), N-methyl-nitrosourea (MNU), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), 1,2-dimethylhydrazine (DMH) and 2,2'-dihydroxy-di-n-propyl-nitrosamine (DHPN), has also been established for rapid detection of not only hepatocarcinogens, but also other organ-target carcinogens. Rats were initially treated with a single ip administration of 100 mg/kg DEN, 4 ip administrations of 20 mg/kg MNU, 4 sc doses of 40 mg/kg DMH for 2 weeks and then 0.1% DHPN for 2 weeks. Test chemicals are administered after the carcinogens exposure. Animals were sacrificed at the end of week 36, and major organs were examined histologically. Carcinogenic activities of test chemicals were compared between the test chemical treated group and carcinogen exposures group (control group). It is increasingly becoming regarded that these bioassays are useful methods and are appropriate alternative tests systems for carcinogenicity risk assessment. Therefore, the International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use\(^*\) has proposed that these two bioassays can be used as "additional tests for carcinogenic activity in vivo."

INTRODUCTION

Two year long-term in vivo testing using rodents has been used for detection of carcinogenic potential of test compounds. However, it is time-consuming and expensive. Therefore, a large number of short-term in vitro assays have been developed [1]. However, their validity is limited by substantial rates of false negative and false positive determinations [2]. To overcome the disadvantages of in vitro short-term screening tests and long-term in vivo bioassays, medium-term in vivo screening assay systems have been developed [3]. Solt and Farber described rapid induction of enzyme altered liver foci in rats [4]. Subsequently, the preneoplastic nature of hepatic altered foci and the usefulness of these lesions as early indicators for hepatocarcinogenicity have been well established and the validity of foci for detection of carcinogenic agents has been emphasized [5].

In our laboratory, attention has been focused on development of in vivo medium-term bioassay systems using the rat liver [6, 7].

MEDIUM-TERM LIVER BIOASSAY PROTOCOL

Male F344 male rats are divided into three groups;
Group 1 is given a single intraperitoneal injection of DEN (200 mg/kg) dissolved in 0.9% NaCl to initiate hepatocarcinogenesis and after a 2 week recovery period, receives test compound in the basal diet, drinking water, or by repeated intraperitoneal, subcutaneous or intravenous injections. The rats are subjected to partial hepatectomy (PH) at week 3. Group 2 is given DEN and PH in the same manner as for group 1 without administration of any test compound. Group 3 rats are injected with 0.9% NaCl instead of DEN solution and then subjected to administration of test compound and PH. All animals are sacrificed at week 8, and four slices of each liver are fixed in ice-cold acetone for immunohistochemical demonstration of glutathione S-transferase placental from (GST-P) positive foci.

Numbers and areas of GST-P positive foci of more than 0.2 mm in diameter are measured using a color video image processor, and the results are assessed by comparing the values between group 1 (DEN-test compounds) and group 2 (DEN alone). Group 3 serves to assay the potential of the test chemicals themselves to induce GST-P positive foci without prior DEN exposure. Statistical analysis of differences between means is carried out using the Student’s t-test or Welch's t-test after application of the preliminary F-test for equal variance, and scoring of carcinogenicity, promotion or inhibition is made on the basis of differences in P-values between groups 1 and 2: positive = increase at P < 0.05 in either the number or the area of foci.

**Results for 291 Chemicals**

Using this system, we have examined a total of 291 substances in a variety of categories. Test substances were classified as hepatocarcinogens, carcinogens other than hepatocarcinogens, non-carcinogens and chemicals whose carcinogenicity had not previously been evaluated. The compounds were also divided into 3 groups according to their reported mutagenicity in the Ames’ test; mutagenic compounds, non-mutagenic compounds and unknowns. Our results, compared with reported Salmonella/microsome and long-term carcinogenicity test findings, are summarized in Table I. Detailed results for 206 compounds [8] and for 277 compounds [9] have already been published.

It is especially noteworthy that 57 of 63 (90%) hepatocarcinogens gave positive results, irrespective of their mutagenicity, leaving only 6 false negatives; 30 out of 31 (97%) mutagenic, and 26 out of 31 (84%) non-mutagenic compounds gave positive results. 4,4’-Diaminodiphenylmethane, 4 carcinogenic peroxisome proliferators and tamoxifen did not enhance GST-P positive foci development. With regard to the carcinogenicity of non-mutagenic peroxisome proliferators, it is important to remember that they depress GST-P expression and the lesions associated with their hepatocarcinogenicity are not positive for this marker enzyme [10]. In these cases, hyperplastic hepatic nodules, which can be detected histopathologically, are used as markers.

Twenty-nine out of 47 compounds (62%) negative in the Ames’ test and evaluated to be carcinogenic

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**Table 1. Results for 291 Compounds in the Medium-term Liver Bioassay.**

<table>
<thead>
<tr>
<th>Carcinogenicity</th>
<th>Ames/test</th>
<th>Positive/Examined (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>30/31 (97%)</td>
<td>26/31 (84%)</td>
</tr>
<tr>
<td>Other than liver</td>
<td>7/25 (28)</td>
<td>3/16 (19)</td>
</tr>
<tr>
<td>Not carcinogenic</td>
<td>0/6 (0)</td>
<td>2/38 (5)</td>
</tr>
<tr>
<td>Unknown</td>
<td>4/14 (29)</td>
<td>29/83 (35)</td>
</tr>
<tr>
<td>Total</td>
<td>41/76 (54)</td>
<td>60/168 (36)</td>
</tr>
</tbody>
</table>

a: Negative

4,4’-Diaminodiphenylmethane (DDPM)

b: Negative

Clofibrate
Di(2-ethylhexyl) adipate (mouse)
Di(2-ethylhexyl) phthalate
Trichloroacetic acid (mouse)
Tamoxifen

c: Positive

Malathion
Vinlozolin

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Vol. 23 Suppl. II
(non-genotoxic carcinogens) prove positive in the present system. These include estrogenic hormonal agents, and chlorinated pesticides, hepatotoxic agents, hepatic microsomal enzyme inducers. The present system, thus, is very reliable for rapid detection of hepatocarcinogens.

Among 42 carcinogens targeting organs other than the liver, 10 compounds gave positive results (24%) and 2 pesticides, malathion and vicilozolin [11], in the total of 45 compounds reported to be non-carcinogenic demonstrated positivity (4%). Since the present system is using preneoplastic lesions as an indicator of carcinogenicity, the results for these 12 chemicals, as well as for 47 out of 141 chemicals of unknown carcinogenicity, suggest that they are weak hepatocarcinogens or liver tumor promoters.

The present system has also provided information concerning inhibitory potential of test compounds. Some are carcinogenic in one or more organs, whereas others are possible chemopreventive agents for which no carcinogenicity has been observed. Furthermore, this system can be used for analyzing mixture carcinogenicities, especially for low dose mixture by plenty number (more than 20) of pesticides, those can not be analyzed by conventional long term carcinogenicity assay.

Medium-term multi-organ bioassay protocol

For detecting carcinogenic potential in whole body, as well as liver, a medium-term multi-organ bioassay system has also been developed in our laboratory. For wide spectrum initiation, 5 different chemical carcinogens are used; DEN, MNU, BBN, DMH, DHPN. Rats were initially treated with a single ip administration of 100 mg/kg DEN, 4 ip administrations of 20 mg/kg MNU, 4 sc doses of 40 mg/kg DMH for 2 weeks and then 0.1% DHPN for 2 weeks. Test chemicals are administered after the carcinogens exposure. Animals were sacrificed at the end of week 24 or 36, and major organs were examined histologically. Carcinogenic activities of test chemicals were compared between the test chemical treated group and carcinoma exposures group (control group).

DISCUSSION

It is proposed that the present experimental protocol, which requires far fewer animals and shorter duration than conventional long-term carcinogenicity tests has advantages for rapid screening of the large number of environmental chemicals which may possess hazard potential for induction of liver cancer in man. A total of 291 chemicals have been analyzed in this laboratory and the efficacy of the system for hepatocarcinogens has thereby been well established. The system is now regarded as an appropriate alternative bioassay system for risk assessment of carcinogenicity and has found practical application with many investigators. The system is also useful for studies which require large experiments and for investigation of chemopreventive agents.

Since the two-step liver assay model is based on the induction of preneoplastic hepatocyte lesions, it primarily provides information on whether a test compound is carcinogenic to the liver. We have also developed whole body system models using wide spectrum initiation by sequential treatment with five potent carcinogens having different target organs. These multiple organ initiator models (medium-term multi-organ models) has indicated that most carcinogens can be detected by increased development of lesions in target organs within 24 or 36 weeks [12].

In conclusion, the present rapid bioassay system in the rat liver is a useful tool for risk assessment. A judicious blend of liver and multi-organ medium-term bioassay systems appears to be the best approach for detection of chemicals of potential hazard to man.

REFERENCES


