Effect of buprenorphine on genotoxicity evaluation of chemicals by the rat liver micronucleus test with partial hepatectomy

Satoru Itoh, Mayumi Nagata, Chiharu Hattori and Wataru Takasaki

Medicinal Safety Research Laboratories, Daiichi Sankyo Co., Ltd., 1-16-13, Kitakasai, Edogawa-ku, Tokyo 134-8630, Japan

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ABSTRACT — In the view of animal welfare considerations, we investigated the suitability of modifying the rat liver micronucleus test with partial hepatectomy to include administration of an analgesic drug to minimize pain and distress as much as possible. The effects of the analgesic, buprenorphine, on the genotoxicity evaluation of structural chromosome aberration inducers (cyclophosphamide, diethylnitrosamine and 1,2-dimethylhydrazine) and numerical chromosome aberration inducers (colchicine and carbenazid) were examined. The genotoxicants were given orally to 8-week-old male F344 rats a day before or after partial hepatectomy and hepatocytes were isolated 4 days after the partial hepatectomy. Buprenorphine was injected subcutaneously twice a day with at least a 6-hr interval for 2 days from just after partial hepatectomy. As results, buprenorphine caused neither change in clinical signs (except for one animal death) nor increase in the incidence of micronucleated hepatocytes of vehicle treated animals. In the case of concomitant treatment of buprenorphine and a genotoxicant, one out of 8 animals died in each group given buprenorphine with cyclophosphamide, carbendazim or colchicine (lower dose level only). Slight changes in clinical signs were noted in the group given buprenorphine with cyclophosphamide or carbendazim. A statistically significant increase in the incidence of micronucleated hepatocytes was obtained in concomitant treatment of buprenorphine and genotoxicant compared with genotoxicant alone for 1,2-dimethylhydrazine, colchicine and carbendazim. It is concluded that use of buprenorphine as an analgesic drug to minimize pain and distress for rats that are given partial hepatectomy is not appropriate under the present experimental conditions, because it could enhance the general toxicity and genotoxicity of the test chemical.

Key words: Liver, Micronucleus test, Partial hepatectomy, Analgesic drug, Buprenorphine

INTRODUCTION

The liver micronucleus test in animals with partial hepatectomy is a useful method to detect in vivo clastogens and aneugens, especially when the active metabolites of the genotoxicant are unstable and do not reach the bone marrow due to their short lifespan such as diethylnitrosamine (Tates et al., 1980). We have already reported that structural chromosome aberration inducers have to be given before, and that numerical chromosome aberration inducers have to be given after, partial hepatectomy to detect their genotoxicity in the liver of rats (Itoh et al., 2012). In recent years, animal welfare considerations have become an important and unavoidable issue globally in examinations using experimental animals. In this point of view, use of the partial hepatectomy model might become a major concern and actually the possibility to prohibit use of this technique in some countries has been pointed out (Uno et al., 2014).

In the present study, we investigated the suitability of modifying the rat liver micronucleus test with partial hepatectomy to include administration of an analgesic drug to minimize pain and distress as much as possible. Buprenorphine was used as the analgesic drug and the effect of buprenorphine on the genotoxicity evaluation of known genotoxicants was examined in male rats. The structural chromosome aberration inducers diethylnitrosamine and 1,2-dimethylhydrazine, and the numerical
chromosome aberration inducers colchicine and carbenzadizim, which were all used in the previous study (Itoh et al., 2012), were used in this study. Cyclophosphamide was also used as a structural chromosome aberration inducer for the following reason. Increase or decrease in the toxicity of cyclophosphamide would be expected due to an alteration of chemical metabolism, because cyclophosphamide is metabolized by cytochrome P450 3A4 (Brade et al., 1985; Boddy and Murray, 2000) as is buprenorphine (Kobayashi et al., 1998).

**MATERIALS AND METHODS**

**Chemicals**

Diethylnitrosamine (CAS no. 55-18-5) and 1,2-dimethylhydrazine dihydrochloride (CAS no. 306-37-6) were purchased from Tokyo Chemical Industry, Co., Ltd., (Tokyo, Japan). Colchicine (CAS no. 64-86-8) and cyclophosphamide monohydrate (CAS no. 6055-19-2) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Carbendazim (CAS no. 10605-21-7) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Buprenorphine (CAS no. 52485-79-7) was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Diethylnitrosamine, 1,2-dimethylhydrazine and colchicine were dissolved in water for injection and carbendazim was suspended in 0.5% methylcellulose. Cyclophosphamide and buprenorphine were dissolved and diluted with physiological saline, respectively.

**Animals and treatments**

Seven-week-old male F344/DuCrIclrj rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan); housed in cages with bedding; maintained in a room with a 12 hr light/dark cycle, temperature of 23 ± 3°C and relative humidity of 30-70%; and used at 8 weeks of age. Certified pellet food (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were provided ad libitum. The dose level of each genotoxicant was selected based on the results of a preliminary test for cyclophosphamide or previous study for the others (Itoh et al., 2012). In the preliminary test, cyclophosphamide was administered to 3 animals per group one day before partial hepatectomy. The general toxicity and the frequency of micronucleated hepatocytes (MNH) were observed according to the procedures described after this. A dose of 100 mg/kg, which was maximally tolerable and apparently induced MNH, was selected for the main study. Each genotoxicant was administered once by oral gavage at a dose volume of 10 mL/kg to 4 animals per group. In the case of the analgesic buprenorphine, the 50% effective dose (ED50) in rats after subcutaneous injection is reported to be 0.014 mg/kg (Hiyama et al., 1982); therefore, 15 μg/kg and 30 μg/kg (twice the ED50) at a dose volume of 0.25 mL/kg were set as the dose levels for this study. The Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. recommends using buprenorphine at 0.01-0.1 mg/kg by subcutaneous injection for surgery in rodents.

Treatment schedules are illustrated in Fig. 1. The genotoxicant or vehicle was given either before or after partial hepatectomy. In the case of dosing before partial hepatectomy for diethylnitrosamine, 1,2-dimethylhydrazine and cyclophosphamide, the genotoxicant was given on Day 1, partial hepatectomy was performed on Day 2, and the regenerated liver was removed 4 days after partial hepatectomy (i.e., on Day 6). For dosing after partial hepatectomy for colchicine and carbendazim, in contrast, the genotoxicant was given one day before or after partial hepatectomy (PH) and the hepatocytes were isolated 4 days after partial hepatectomy. Buprenorphine (Bup) or physiological saline was administered twice a day with at least a 6-hr interval for 2 days from just after partial hepatectomy in both regimens. The day of genotoxicant dosing is defined as Day 1.

**Fig. 1.** Treatment schedules. The genotoxicant or vehicle was administered to rats one day before or after partial hepatectomy (PH) and the hepatocytes were isolated 4 days after partial hepatectomy. Buprenorphine (Bup) or physiological saline was administered twice a day with at least a 6-hr interval for 2 days from just after partial hepatectomy in both regimens. The day of genotoxicant dosing is defined as Day 1.
partial hepatectomy was performed on Day -1, the genotoxicant was given on Day 1 and the regenerated liver was removed on Day 4. Buprenorphine or physiological saline was administered twice a day with at least a 6-hr interval for 2 days (total of 4 injections) from just after partial hepatectomy in both regimens. This study was conducted in compliance with the following law and guidelines: "Law Concerning the Protection and Control of Animals", Japanese Law No. 105, October 1, 1973, revised on June 22, 2005; "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain", Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006 and "Guidelines for Animal Experimentation", the Japanese Association for Laboratory Animal Science, May 22, 1987; and according to the methods approved by the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

Partial hepatectomy
The animal was anesthetized with isoflurane (Mylan Seiyaku Ltd., Tokyo, Japan). Laparotomy of about 2 cm in length was made just under the xiphoid process. The liver was pushed out through the incision; the roots of the medial and lateral left lobes, and of the medial right lobe of the liver were ligated with surgical silk; and then the lobes were removed, the remaining liver was pushed back inside and the incision was sutured.

Clinical signs and body weight
The animals were observed for their clinical signs more than once a day throughout the experiments excluding Days 4 and 5 in the regimen of dosing before partial hepatectomy or Days 2 and 3 in the regimen of dosing after partial hepatectomy. The animals were weighed before administration, partial hepatectomy and hepatocyte isolation. Body weights of the animals were measured with an electronic balance.

Collection of hepatocytes
The regenerated liver was removed 4 days after partial hepatectomy. The animal was sacrificed by exsanguination from the abdominal aorta under isoflurane anesthesia. The liver was excised and weighed with an electronic balance. The hepatocytes were isolated by a simplified method for liver perfusion (Igarashi and Shimada, 1997). Briefly, liver perfusion medium (Invitrogen Corporation, Carlsbad, CA, USA) prewarmed to 40°C was perfused through the liver via the vena cava from a stomach tube connected to a 10-mL syringe. Subsequently, a solution of 125 units/mL collagenase (Type IV, Sigma-Aldrich Corporation) was perfused through the liver. The liver was cut into small pieces with scissors, and shaken well in 20 mL of 10% FBS-MEM to separate the hepatocytes. The suspension was filtered through metal meshes and a 70-μm mesh strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and then centrifuged at 50 x g for 2 min. The sediment was resuspended in 10 mL of 10 vol% neutral buffered formalin and centrifuged at 50 x g for 2 min, and then this procedure was repeated two more times for each sample.

Observation of hepatocytes
The sediment was again resuspended in 10 vol% neutral buffered formalin. Equal volumes of liver cell suspension and acridine orange-DAPI solution were mixed well, and one drop was placed on a slide glass just before observation. The specimens were observed under a fluorescence microscope (BX60F5 and BX53, Olympus, Tokyo, Japan). Two thousand hepatocytes excluding metaphase cells and nuclear fragment cells from each animal were observed manually for the MNH count.

Statistical analyses
The difference in the incidence of MNH between the concomitant treatment of buprenorphine and genotoxicant versus genotoxicant alone was analyzed by two-tailed Fisher’s exact test with significance level at 5% using EXSUS Ver. 7.6 (CAC EXICARE Corporation, Tokyo, Japan).

RESULTS
Clinical signs and body weights
The results obtained from clinical signs in rats treated with buprenorphine and genotoxicant are shown in Table 1. The treatment with buprenorphine and vehicle (physiological saline, water for injection or 0.5% methylcellulose), without genotoxicant, caused no change in clinical signs except for death of one animal on the day after partial hepatectomy.

Slight changes in clinical signs (smudge of perinasal area and eye region, decrease in locomotor activity and emaciation) were observed in the groups given concomitant treatment with buprenorphine and cyclophosphamide, and one animal showed smudge of perinasal area in the group given buprenorphine and carbendazim. It was also noted that one out of 8 animals (results for the two dose levels of buprenorphine combined) died in each group given concomitant treatment of buprenorphine with cyclophosphamide, carbendazim or colchicine (lower dose level only).

No statistically significant change in body weight was
Micronucleated hepatocyte induction by structural chromosome aberration inducers

The results obtained from evaluation of the effect of buprenorphine on the induction of MNH by cyclophosphamide, diethylnitrosamine and 1,2-dimethylhydrazine are shown in Fig. 2A, 2B and 2C, respectively. The incidences of MNH at both dose levels of buprenorphine with vehicle (physiological saline or water for injection) were comparable to those obtained in the groups treated with vehicle alone (without buprenorphine) and to the values after vehicle treatment obtained in the previous study (Itoh et al., 2012). Neither dose level of buprenorphine affected the induction of MNH by cyclophosphamide or diethylnitrosamine (Fig. 2A and 2B, respectively), whereas, at both levels of buprenorphine, statistically significant increases in the incidence of MNH were observed in the combined treatment compared with 1,2-dimethylhydrazine alone (Fig. 2C).

**DISCUSSION**

The liver micronucleus test with partial hepatectomy is a useful method to detect in vivo clastogens and aneugens, especially when the active metabolites of the genotoxicant are unstable and do not reach the bone marrow due to their short lifespan (Tates et al., 1980). We have evaluated structural and numerical chromosome aberration inducers by the liver micronucleus test with partial hepatectomy and reported that this method is useful for detecting in vivo chromosome aberration inducers, and is especially effective for detecting numerical chromosome aberration inducers (Itoh et al., 2012). On the other hand, the partial hepatectomy might become a major concern obtained in any group (data not shown).

**Micronucleated hepatocyte induction by structural chromosome aberration inducers**

The results obtained from evaluation of the effect of buprenorphine on the induction of MNH by cyclophosphamide, diethylnitrosamine and 1,2-dimethylhydrazine are shown in Fig. 2A, 2B and 2C, respectively. The incidences of MNH at both dose levels of buprenorphine with vehicle (0.5% methylcellulose or water for injection) were comparable to those obtained in the groups treated with vehicle alone (without buprenorphine) and to the values after vehicle treatment obtained in the previous study (Itoh et al., 2012). The incidence of MNH in the concomitant treatment of carbendazim and either of the two dose levels of buprenorphine was statistically significantly higher than that in carbendazim treatment alone (Fig. 3A). Similarly, statistically significant enhancement of MNH induction was noted in the concomitant treatment with colchicine and the higher dose level of buprenorphine (Fig. 3B).

**Table 1. Clinical signs in rats treated with buprenorphine and structural or numerical chromosome aberration inducers.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Buprenorphine (μg/kg/injection)</th>
<th>0</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structural chromosome aberration inducers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diethylnitrosamine</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1,2-Dimethylhydrazine</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>DE (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>-</td>
<td>DE (4 or 5), SN, SE, SN*, SE*, AD*, EM*</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Numerical chromosome aberration inducers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbendazim</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>-</td>
<td>SN</td>
<td>DE (1)</td>
<td></td>
</tr>
<tr>
<td>Colchocine</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
<td>DE (4)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*: These clinical signs were observed in one animal. Four animals per group were used.
-: No abnormality, SN: Smudge of perinasal area, SE: Smudge of eye region, AD: Decrease in locomotor activity, EM: Emaciation, DE: Death

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in the view of animal welfare considerations, and thus we investigated the suitability of modifying the rat liver micronucleus test with partial hepatectomy to include administration of an analgesic drug to minimize pain and distress as much as possible.

The effect of the analgesic drug, buprenorphine, on the genotoxicity evaluation of structural and numerical chromosome aberration inducers was examined in male rats. The vertical bar represents S.D. One animal died in the cyclophosphamide and 15 μg/kg of buprenorphine treatment group (A), or the water for injection and 30 μg/kg of buprenorphine treatment group (C). Statistical analyses were performed on the incidence of MNH between concomitant treatment of buprenorphine and structural chromosome aberration inducer versus genotoxicant alone by two-tailed Fisher’s exact test with significance level at 5% (*).

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Fig. 2. Incidence of micronucleated hepatocytes (MNH) in concomitant treatment of buprenorphine and structural chromosome aberration inducers: (A) cyclophosphamide (CP); (B) diethylaminoethylcarbamate (DENA); (C) 1,2-dimethylhydrazine (1,2-DMH). Mean of 3 or 4 animals. The vertical bar represents S.D. One animal died in the cyclophosphamide and 15 μg/kg of buprenorphine treatment group (A), or the water for injection and 30 μg/kg of buprenorphine treatment group (C). Statistical analyses were performed on the incidence of MNH between concomitant treatment of buprenorphine and structural chromosome aberration inducer versus genotoxicant alone by two-tailed Fisher’s exact test with significance level at 5% (*).

Fig. 3. Incidence of micronucleated hepatocytes (MNH) in concomitant treatment of buprenorphine and numerical chromosome aberration inducers: (A) carbendazim (CBZ); (B) colchicine. Mean of 3 or 4 animals. The vertical bar represents S.D. One animal died in the carbendazim and 30 μg/kg of buprenorphine treatment group (A), or the colchicine and 15 μg/kg of buprenorphine treatment group (B). Statistical analyses were performed on the incidence of MNH between concomitant treatment of buprenorphine and numerical chromosome aberration inducer versus genotoxicant alone by two-tailed Fisher’s exact test with significance level at 5% (*).
ment with cyclophosphamide or diethylnitrosamine and buprenorphine. It has been reported that cyclophosphamide is metabolized by cytochrome P450 3A4 (Brade et al., 1985; Boddy and Murray, 2000) as is buprenorphine (Kobayashi et al., 1998). Therefore, an increase or decrease in the induction of MNH by cyclophosphamide was expected due to an alteration in the metabolism of the two compounds, but no change in MNH incidence was observed. On the other hand, slight changes in clinical signs and one animal death were noted in the buprenorphine and cyclophosphamide treated group whereas no change in clinical signs and no death was obtained in the treatment with cyclophosphamide alone. Those changes would indicate that an alteration of chemical metabolism had occurred after treatment with buprenorphine and cyclophosphamide, although no effect on the induction of MNH was obtained. No change was obtained in clinical signs, body weight or MNH induction by diethylnitrosamine.

In contrast, enhancement of general toxicity and genotoxicity occurred in concomitant treatment of buprenorphine and numerical chromosome aberration inducers. One animal death was observed in each of the groups treated with buprenorphine and carbendazim or colchicine whereas no death was observed in the treatment with genotoxicant alone, and a statistically significant increase in the incidence of MNH compared with carbendazim or colchicine alone was also observed in those groups. In the case of dosing after partial hepatectomy, an essential regimen for detecting MNH induction by numerical chromosome aberration inducers (Itoh et al., 2012), the administration of the genotoxicant and the third injection of buprenorphine occurred at almost the same time (Fig. 1), so interaction between the two chemicals might have contribute to the enhancement of toxicity. This concern has less weight for dosing before partial hepatectomy, a standard regime for detecting MNH induction by structural chromosome aberration inducers (Itoh et al., 2012), because buprenorphine was injected 24 hr after the genotoxicant treatment in this regimen (Fig. 1).

When taking together all results obtained in the present study, concomitant treatment of buprenorphine and a genotoxicant resulted in the enhancement of general toxicity or genotoxicity in four of five chemicals. The reasons for the enhancement of those toxicities are not clear, but buprenorphine alone or concomitant treatment of buprenorphine and a genotoxicant could act as a stress on recovery from partial hepatectomy and result in the enhancement of general toxicity. We have observed that some stresses, such as decreased body temperature during surgery or lengthy surgery, exert an influence on mortality after partial hepatectomy. This speculation is supported by the fact that no changes in clinical signs and no deaths were observed in the groups without buprenorphine treatment in this study. In the case of the enhancement of general toxicity, lower dose levels of the test chemical compared with those used with the test chemical alone could be used, and consequently exposure would be reduced, the level of exposure might not be sufficient for evaluation of the test chemical, in some cases. In other cases, the enhancement of genotoxicity could possibly lead to a false positive result for the test chemical. Therefore, the enhancement of general toxicity and genotoxicity are not appropriate conditions for the evaluation of the potential of the test chemical to induce MNH.

In conclusion, we judged that use of buprenorphine as an analgesic drug to minimize pain and distress for rats that are given partial hepatectomy is not appropriate under the present experimental conditions, because it could enhance the general toxicity and genotoxicity of the test chemical.

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Conflict of interest—The authors declare that there is no conflict of interest.

REFERENCES


