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# Liver initiation activity of norfloxacin but not nalidixic acid, pipemidic acid, and ciprofloxacin on in vivo short-term liver initiation assay in rats

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**Abstract**

This study aimed to examine the in vivo initiation activity of the quinolone antimicrobials—nalidixic acid (NA), pipemidic acid (PPA), ciprofloxacin (CPFX), and norfloxacin (NFLX)—by using an in vivo short-term liver initiation assay. Rats were subjected to a two-thirds partial hepatectomy on day 0 and 12 h after completion of this procedure were treated once orally with each quinolone or vehicle. Subsequently, they were fed a basal diet for 14 days and a diet containing 0.015% of 2-acetylaminofluorene for the following 10 days. On day 19, a single oral dose of carbon tetrachloride at 0.8 mL/kg body weight was administered. On day 34, they were sacrificed under ether anesthesia, and liver slices were fixed in 10% neutral buffered formalin for immunohistochemical examination of glutathione *S*-transferase placental form (GST-P) positive foci. Administration of NFLX resulted in a significant increase in the mean number and area of GST-P positive foci; however, administration of the three other quinolones did not produce any increase. These results suggest that only NFLX has an initiation activity in rats under the conditions used in the present study.


*Keywords:* Quinolone; Initiation activity; Nalidixic acid; Pipemidic acid; Ciprofloxacin; Norfloxacin

## 1. Introduction

Quinolone antimicrobials, which have been widely used for clinical and veterinary therapy, are classified into old quinolones and new quinolones. The pharmacological effects of quinolones include inhibition of DNA gyrase (bacterial topoisomerase II) or topoisomerase IV; they are also known to have slight inhibitory effects on eukaryotic topoisomerase II (Akasaka et al., 1998), which is responsible for the DNA double strand breakage/reunion reaction (Liu and Wang, 1999). It has been demonstrated that topoisomerase II inhibitors interfere with the breakage/reunion reaction of topoisomerase II, resulting in DNA single strand breaks (SSBs) and double strand breaks (Fukuda et al., 1996). In view of these data on DNA breakage by topoisomerase II inhibitors, some quinolones may have genotoxic and carcinogenic potentials. Some previously published studies have discussed their genotoxicity and carcinogenicity in eukary-

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otes. Nalidixic acid (NA) (Fort, 1992), pipemidic acid (PPA) (Maura and Pino, 1988), oxolinic acid (OA) (Fort, 1992), ofloxacin (OFLX) (Fort, 1992; Albertini et al., 1995), ciprofloxacin (CPFX) (Fort, 1992; Mukherjee et al., 1993; Albertini et al., 1995), norfloxacin (NFLX) (Pino et al., 1991; Fort, 1992), levofloxacin (Shimada et al., 1992), and lomefloxacin (Singh et al., 2003) were reported to produce positive results in some genotoxicity studies in eukaryotes. In addition, NA (Morrissey et al., 1991) and OA (Yamada et al., 1994) were reported to have carcinogenic potential in rodents. However, since these drugs have a short dosing period in human clinical therapy, submission of data regarding carcinogenicity studies is not always required for their governmental approval. Meanwhile, attention has recently been focused on the possibility of chronic exposure of humans to quinolones by the consumption of meat produced from domestic animals treated with these drugs to prevent various bacterial infections.

Flumequine (FL), an old quinolone used for veterinary treatment of various bacterial infections (Greenwood, 1978), was found to induce hepatocellular tumors in a conventional 18-month carcinogenicity study in mice. The 48th Food and Agriculture Organization (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) in 1997 concluded that FL was a non-genotoxic carcinogen with only promoting activity and established the acceptable daily intake (ADI) of FL as 0–30 μg/kg body weight (bw) (WHO, 1998). However, Kashida et al. reported that FL was not only a hepatic tumor promoter but also a hepatic tumor initiator based on an in vivo alkaline single-cell gel electrophoresis (comet) assay and two-stage hepatocarcinogenesis study in mice (Kashida et al., 2002). Therefore, the 60th JECFA in 2003 was unable to rule out the possibility that FL is a genotoxic carcinogen and withdrew the ADI (WHO, 2003). Additionally, the 62nd JECFA in 2004 reestablished the ADI of FL as 0–30 μg/kg bw based on data indicating a negative result in an additional study of unscheduled DNA synthesis (WHO, 2004). However, it is still unclear whether FL is a genotoxic carcinogen.

As described above, some quinolones have been reported to show DNA alteration in eukaryotes. However, many new quinolones have been widely used for clinical therapy. Old quinolones such as FL, which are used for veterinary treatment rather than human clinical therapy, tend to affect humans via food of animal origin; therefore, it is important to ascertain the possibility of DNA alteration not only by new quinolones but also by old quinolones. We previously investigated the genotoxic potential of four old quinolones, namely, NA, PPA, OA, and piromidic acid (PA), and four new quinolones, namely, enoxacin (ENX), OFLX, CPFX, and NFLX, by using the in vitro comet assay, and reported that CPFX and NFLX induced SSBs (Itoh et al., 2006). In addition, we performed the in vitro micronucleus (MN) test with four quinolones (NA, PPA, CPFX, and NFLX) and reported that NFLX-induced SSBs resulted in chromosome aberrations (Itoh et al., 2006).

Glutathione *S*-transferase placental form (GST-P) positive liver cell focus is generally regarded as a hepatocellular preneoplastic lesion in rats. A medium-term liver initiation assay based on the induction of GST-P positive foci resulting from gene mutations in rats has been developed to identify in vivo genotoxic carcinogens as an alternative to long-term carcinogenicity studies (Tsuda et al., 1980; Sakai et al., 2000, 2001, 2002a,b). In addition, it has been shown that genotoxic carcinogens, which target or do not normally target the liver, enhanced the induction of GST-P positive foci in this in vivo liver initiation assay (Sakai et al., 2002b). Hence, it is generally recognized that genotoxic carcinogens can be detected by this initiation assay, regardless of the target organs, in long-term carcinogenicity studies in rats and mice. Therefore, this initiation assay appears to be a useful method to clarify whether chromosome aberrations and/or gene mutations are fixed as a result of DNA damage induced by quinolones in the enhanced GST-P foci. In the present study, we performed the initiation assay to investigate the liver initiation activity of two old quinolones (NA and PPA) and two new quinolones (CPFX and NFLX) and focused on whether the in vitro genotoxicity data of quinolones was linked to their in vivo genotoxicity and carcinogenicity.

## 2. Materials and methods

### 2.1. Animals

Male F344 rats (Charles River Japan, Atsugi, Japan), housed individually in stainless steel cages, were maintained under constant conditions (12:12 h light/dark cycle, 40–70% humidity at 20–26 °C) on a CA-1 diet (Japan Clea Co., Tokyo, Japan) and tap water ad libitum. They were acclimatized for 1 week before the start of the experiments, at which time they were 7-weeks old.

### 2.2. Chemicals

We purchased NA and carbon tetrachloride ($CCl_4$) from Wako Pure Chemicals Industries Ltd. (Osaka, Japan); PPA and NFLX from Sigma–Aldrich Co. Ltd. (St. Louis, MO); CPFX from MP Biomedicals Inc. (Irvine, CA); and 2-acetylaminofluorene (2-AAF) from Tokyo Kasei Co. (Tokyo, Japan).

NA, PPA, and NFLX were dissolved in corn oil (Nakalai Tesque, Kyoto, Japan), and CPFX was dissolved in water for injection (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan). In order to determine the appropriate dose of the four quinolones, we preliminarily administered a single oral dose of NA at 1000 and 500 mg/kg bw, PPA at 5000 and 2500 mg/kg bw, CPFX at 5000 and 2500 mg/kg bw, and NFLX at 4000 and 2000 mg/kg bw to partially hepatectomized rats 12 h after the completion of two-thirds partial hepatectomy. This was because the $LD_{50}$ of NA was reported to be 1160 mg/kg bw; PPA, >5000 mg/kg bw; CPFX, >5000 mg/kg bw; and NFLX, >4000 mg/kg bw in intact male rats by the each interview form. It was found that almost all the partially hepatectomized rats administered with NA at 1000 mg/kg bw and NFLX at 4000 mg/kg bw died within a few days of the administration (data not shown). Therefore, the dose of the four quinolones in the present study was set at 750, 325, and 187.5 mg/kg bw for NA; 5000, 2500, and 1250 mg/kg bw for PPA; 5000, 2500, and 1250 mg/kg bw for CPFX; and 3000, 1500, and 750 mg/kg bw for NFLX.

### 2.3. Liver initiation assay

The in vivo short-term liver initiation assay was performed by using a slightly modified method of Sakai et al. (2002b).

Rats were subjected to a two-thirds partial hepatectomy on day 0. The partially hepatectomized rats were subdivided into four groups of eight rats each (except for the PPA low dose group that consisted of seven rats) in each assay to examine each of the four quinolones. The rats were treated once orally each with vehicle and low, intermediate, and high doses of each quinolone 12 h after the completion of the two-thirds partial hepatectomy. Dosing volumes of the vehicle and suspensions of each quinolone were calculated at 10 mL/kg bw.

Subsequently, the rats were fed with a basal diet for 14 days followed by a diet containing 0.015% of 2-AAF (Japan Clea Co., Tokyo, Japan) for the following 10 days. On day 19, $CCl_4$ at 0.8 mL/kg bw was orally administered once to the rats. On day 34, the rats were sacrificed under ether anesthesia, and slices of all liver lobes were fixed in 10% neutral buffered formalin.

For immunohistochemical analysis, tissues of the fixed liver lobes were embedded in paraffin, sectioned, and stained with an immunohistochemical stain using an antibody GST-P, a marker of preneoplastic lesions in the rat liver, by the avidin–biotin complex method. With a computer-assisted image analyzer (Win Roof; Mitani Corp., Japan), we measured the number and area of GST-P positive foci (>0.1 mm in diameter) and the total area of each liver section examined in each animal and then calculated the mean number and area of GST-P positive foci per unit area of all the liver sections examined per animal.

### 2.4. Statistical methods

The data were expressed as mean ± S.D. and statistically analyzed according to the below-mentioned multi comparison test between the vehicle control group and the quinolone treated groups in each assay. The data were tested by Bartlett's method for homogeneity of variance. When the variances were homogeneous, Dunnett's method was used. When the variances were heterogeneous, a Dunnett-type method using a rank order was used. *P*-values lower than 0.05 were considered statistically significant in all analyses.

## 3. Results

During the period of the liver initiation assay, four of the eight rats in the NA high dose group and one of

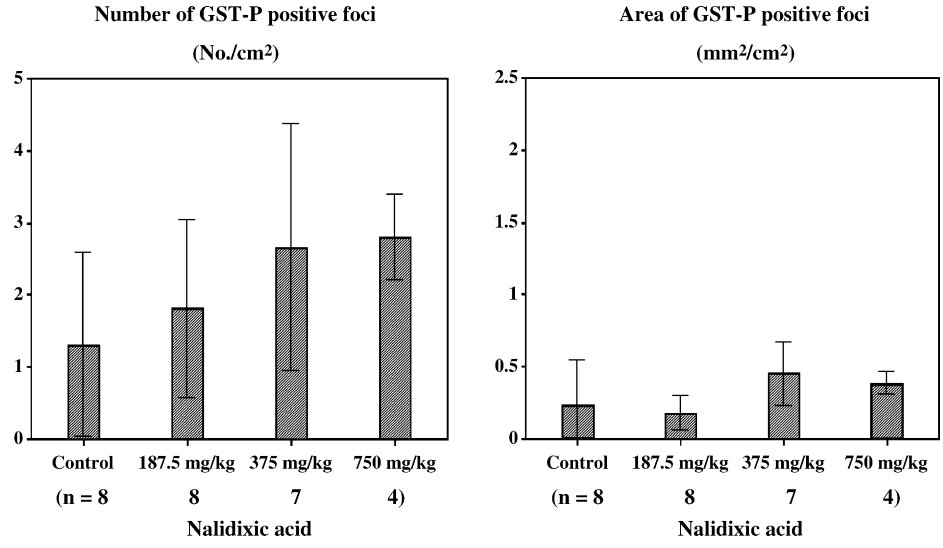


Fig. 1. Number and area of GST-P positive liver cell foci (>0.1 mm in diameter) in partially hepatectomized rats treated with vehicle or nalidixic acid. Data are expressed as the mean ± S.D. of each group.

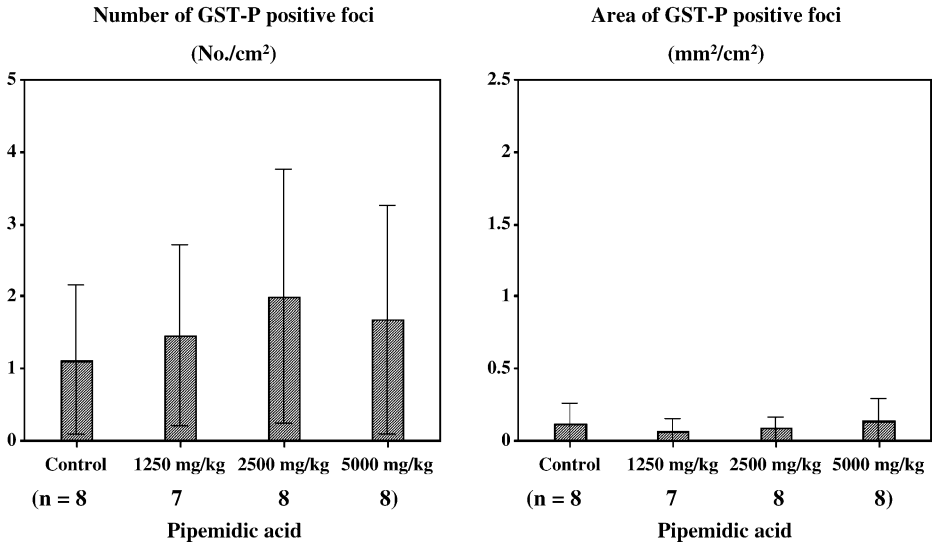


Fig. 2. Number and area of GST-P positive liver cell foci (>0.1 mm in diameter) in partially hepatectomized rats treated with vehicle or pipemidic acid. Data are expressed as the mean ± S.D. of each group.

the eight rats in the NA intermediate dose group died within 2 days of NA administration. Meanwhile, two of the eight rats in the NFLX intermediate dose group died 23 days after NFLX administration, and one of the eight rats in the NFLX low dose group died 32 days after NFLX administration. Data obtained from liver initiation assay are shown in Figs. 1–4.

As shown in Figs. 1–3, no significant differences were observed in the mean number or area of GST-P positive foci between each of the vehicle control groups and the NA-, PPA-, or CPFX-treated groups.

As shown in Fig. 4, the mean number and area of GST-P positive foci significantly increased in the NFLX intermediate and high dose groups. We measured the number and area of GST-P positive foci in another section from the same tissues embedded in paraffin to ascertain the reproducibility of the results. This second trial yielded similar results (data not shown). Therefore, the reproducibility of the results was ascertained. The mean area of GST-P positive foci in the NFLX low dose group was higher than that in the NFLX high and intermediate dose groups, but the differ-

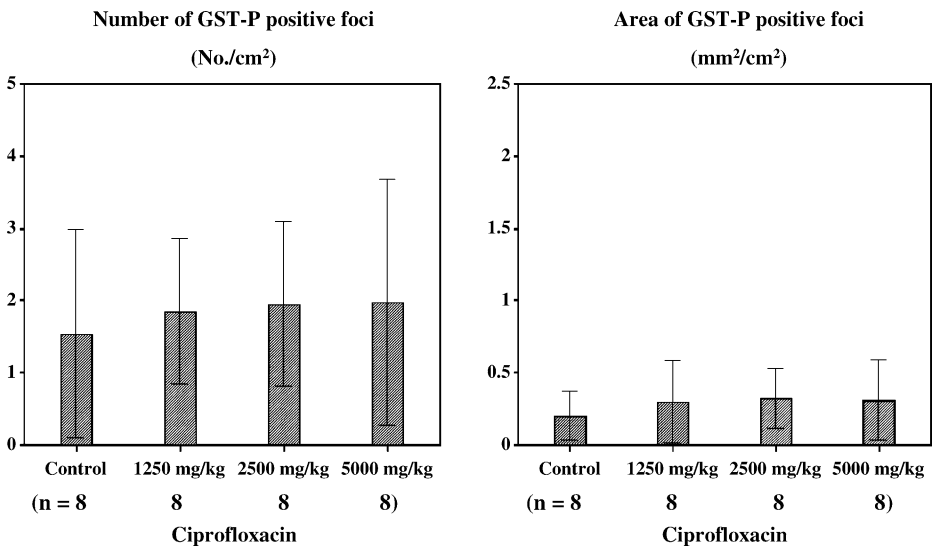


Fig. 3. Number and area of GST-P positive liver cell foci (>0.1 mm in diameter) in partially hepatectomized rats treated with vehicle or ciprofloxacin. Data are expressed as the mean ± S.D. of each group.

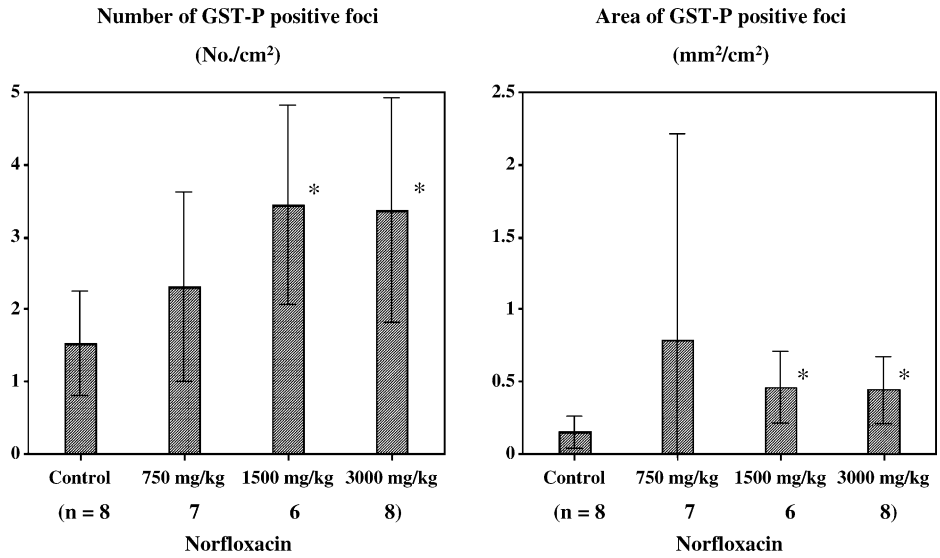


Fig. 4. Number and area of GST-P positive liver cell foci (>0.1 mm in diameter) in partially hepatectomized rats treated with vehicle or norfloxacin. Data are expressed as the mean ± S.D. of each group. *, significantly different from control group at $P < 0.05$.

ence from the control value was not statistically significant.

## 4. Discussion

In the present study, NFLX significantly increased the mean number and area of GST-P positive foci, suggesting that NFLX has a liver initiation activity in rats. We previously investigated the genotoxic potential of OA, PA, ENX, and OFLX as well as the four quinolones by the in vitro comet assay using WTK-1 cells (Itoh et al., 2006). As a result, NFLX and CPFX were found to be positive both at pH 12.1 and at pH > 13, but six other quinolones were negative. These results suggested that CPFX and NFLX induced SSBs. In addition, in the same study, we performed the in vitro MN test with the four quinolones (NA, PPA, CPFX, and NFLX) by using WTK-1 cells. It was found that NFLX was positive, but three other quinolones were negative, suggesting that NFLX-induced SSBs resulted in chromosome aberrations (Itoh et al., 2006). Furthermore, Pino et al. reported that NFLX caused DNA strand breaks in fetal tissues of rats in vivo (Pino et al., 1991). It is generally considered that DNA damage is closely related to the initiation activity as the first step in the two-stage hypothesis of carcinogenesis (Kashida et al., 2002). Therefore, we believe that the genotoxic potential of NFLX, which was detected by the in vitro comet assay and MN test, caused DNA damage and tumor initiation in the liver of the partially hepatectomized rats by a single oral dose administration.

The mean area of GST-P positive foci in the NFLX low dose group was higher than that in the NFLX high and intermediate dose groups, although the difference from the control value was not statistically significant. This contradictory result for dose dependency was due to the occurrence of a very large area of GST-P positive foci in the liver of a rat in the NFLX low dose group. On the contrary, the number of GST-P positive foci in the liver of the rat was almost equal to that of the other rats in the NFLX low dose group. Thus, the mean number of GST-P positive foci in the NFLX low dose group was lower than that in the NFLX high and intermediate dose groups. In general, increase in the number of foci is considered to be related to the initiating activity of chemicals and increase in the area of foci to their promoting activity (Sakai et al., 2000). Therefore, the contradictory result for dose dependency in the mean area of GST-P positive foci in the NFLX-treated groups was considered to be due to the individual difference in response to the promotion process.

Although NA was reported to produce positive results in the genotoxicity studies in eukaryotes (Fort, 1992), we previously reported that NA was negative in the comet assay and MN test using WTK-1 cells (Itoh et al., 2006). In the present study, NA did not produce any increase in the mean number or area of GST-P positive foci. Therefore, it can be concluded that NA does not have any initiation activity in rats under the conditions used in the present study. Within 2 days of administration of NA, four of the eight rats in the NA-high dose group and one of the eight rats in the NA-intermediate dose group

died. We considered that these deaths were caused by NA after the completion of two-thirds partial hepatectomy. It has been reported that partial hepatectomy causes severe damage to the body, especially to the liver (Tsuda et al., 1980; Okamura et al., 2002). Thus, it is likely that the partially-hepatectomized rats died of exacerbation of liver damage caused by NA. Therefore, although it is possible that different results may occur under other conditions, we believed that the doses of NA, which were determined for the present study, were enough to examine the initiation activity. Morrissey et al. reported that NA induced preputial gland tumors in male rats and clitoral gland tumors in female rats (Morrissey et al., 1991). Based on the present study, we consider that NA does not have initiation activity and NA is a non-genotoxic carcinogen.

Although Maura and Pino reported that PPA produced DNA alkali-labile sites in granuloma tissue cells in the rat granuloma pouch assay (Maura and Pino, 1988), we previously reported that PPA was negative in the comet assay and MN test using WTK-1 cells (Itoh et al., 2006). In the present study, PPA did not produce any increase in the mean number or area of GST-P positive foci. In addition, we previously reported that CPFX induced SSBs in WTK-1 cells (Itoh et al., 2006). Furthermore, CPFX was reported to produce positive results in some genotoxicity studies in eukaryotes (Fort, 1992; Mukherjee et al., 1993; Albertini et al., 1995). However, in our previous study, CPFX was negative in the MN test using WTK-1 cells (Itoh et al., 2006). In the present study, no increase in the mean number or area of GST-P positive foci was observed in the CPFX treatment. In addition, Herbold et al. suggested that CPFX cannot reach concentrations at which topoisomerase II is inhibited under in vivo conditions (Herbold et al., 2001). Therefore, it can be concluded that PPA and CPFX have no initiation activity in rats under the conditions used in the present study. The high dose of PPA and CPFX in the present study was set at 5000 mg/kg bw, which is usually adopted as the maximum dose for repeated dose toxicity studies. Therefore, although it is possible that different results may occur under other conditions, we believed that the doses of PPA and CPFX, which were determined for the present study, were enough to examine the initiation activity. Based on the present study, we consider that neither PPA nor CPFX has initiation activity.

In the present in vivo short-term liver initiation assay, we slightly modified the method of Sakai et al. (2002b). In a preliminary study, we performed the assay according to the method of Sakai et al. (2002b), and many rats, including controls, died after being given the diet containing 0.015% 2-AAF for 14 days (data not shown). Because the rats died 29 days after partial hepatectomy and administration of test compounds or thereafter, we concluded that the deaths were caused mainly due to the strong toxicity of 2-AAF and $CCl_4$. Therefore, we shortened the feeding period of the rats on the diet containing 0.015% 2-AAF to 10 days and changed the timing for administration of $CCl_4$ to 19 days. Nevertheless, three rats in the NFLX intermediate or low dose groups died 23 days after partial hepatectomy and administration of NFLX or thereafter. Because all rats in the NFLX high dose group survived the study period, we concluded that the deaths were mainly caused not due to the administration of NFLX, but due to the strong toxicity of 2-AAF and $CCl_4$. In a positive control study, we injected 1,2-dimethylhydrazine (DMH) dissolved in water for injection at 4 mg/kg bw in four rats under the same conditions as in the present study. It was found that DMH obviously increased the mean number and area of GST-P positive foci (the mean number: $35.62 \pm 7.57$ No./cm$^2$; the mean area: $11.00 \pm 6.12$ mm$^2$/cm$^2$). Thus, we confirmed that the 10-day feeding period of the diet containing 0.015% 2-AAF was sufficient for the promotion process in the in vivo short-term liver initiation assay. Furthermore, the mean number and area of GST-P positive foci were compared among the four control groups by Student's *t*-test or Aspin–Welch's *t*-test after *F*-test; however, no significant differences were observed. Therefore, we believed that the present study conditions were suitable for the in vivo short-term liver initiation assay.

Based on the present study, it can be concluded that the genotoxic potential of NFLX, which was detected by the in vitro comet assay and MN test, caused DNA damage and initiation in the liver of the partially hepatectomized rats by a single dose oral administration. NFLX has been used for clinical therapy, including pediatrics and veterinary therapy. This finding suggests that patients treated with NFLX and consumers ingesting food of animal origin that is contaminated with NFLX used for veterinary treatment are at a risk of exposure to such genotoxic carcinogens. However, it remains unclear whether the hepatocellular preneoplastic lesions induced by NFLX will result in hepatocellular adenomas or carcinomas. To clarify whether the initiating potentials of NFLX induce hepatocellular neoplastic lesions, further studies are in progress in our laboratory.

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