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Mechanistic Study on Flumequine Hepatocarcinogenicity Focusing on DNA Damage in Mice

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In order to elucidate the tumor-initiating potential of flumequine (FL) in the liver, male C3H mice were given dietary administration of 4000 ppm FL throughout the study or for 2 weeks at the initiation stage, and then received 2 intraperitoneal injections of D-galactosamine (Gal) at weeks 2 and 5, with or without 500 ppm phenobarbital (PB) in their drinking water for 13 weeks to provide tumor-promoting effects. Hepatocellular foci were observed in 2 out of 8 and 6 out of 7 animals in the FL/PB + Gal and FL/FL + Gal groups, respectively. In addition, in an alkaline single-cell gel electrophoresis (comet) assay that was performed using adult, infant, or partial hepatectomized male ddY mice to evaluate the potential of FL at 500 mg/kg or less, to act as a DNA damaging agent. FL induced dose-dependent DNA damage in the stomach, colon, and urinary bladder of adult mice at 3 h but not at 24 h after its administration. Similarly, DNA damage was noted in the regenerating liver and the livers of infant mice at the 3 h time point. Furthermore, in *in vitro* assays that were conducted to investigate the potential of FL to inhibit eukaryotic topoisomerase II, which is responsible for the double-strand DNA breakage reaction as well as bacterial gyrase, inhibitory effects of FL on topoisomerase II were high relative to the influence on bacterial gyrase. The results of our studies thus strongly suggest that FL has initiating potential in the livers of mice that is attributable to its induction of DNA strand breaks.

Key Words: **flumequine; carcinogenicity study; DNA damage; comet assay; DNA gyrase; topoisomerase II.**

Flumequine (FL), a quinolone-antibacterial agent, has been used for veterinary treatment of infections (Greenwood, 1978). Although it was found to elicit hepatocellular tumors in a conventional 18-month carcinogenicity study in mice, the Food and Agriculture Organization (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) considered that the carcinogenic effect was due to a non-genotoxic mechanism resulting from a necrosis-regeneration response of hepatocytes (WHO, 1997). However, Yoshida *et al*. reported FL to not only enhance development of altered foci and adenomas in the livers of CD-1 mice given a diet containing 4000 ppm FL for 30 weeks, after initiation with diethylnitrosamine, but also to induce small numbers of hepatocellular foci at the same dose without any initiation treatment (Yoshida *et al*., 1999). In addition, we have previously demonstrated elevated incidences of hepatocellular foci and increases in their cell proliferation in animals receiving FL with or without DMN-initiation treatment in heterozygous p53-deficient CBA mice that are sensitive to genotoxic carcinogens (Takizawa *et al*., 2001). Based on the results of these studies, FL was suspected to have both initiation and promotion effects on the liver. In the present study, we have performed a medium-term 2-stage carcinogenicity study in mice to further test for tumor initiating potential, comet assays to evaluate the potential of FL to act as a DNA damaging agent, and an *in vitro* DNA gyrase/topoisomerase II inhibitory assay to investigate the potential of FL to inhibit eukaryotic topoisomerase II.

MATERIALS AND METHODS

Thirteen-week, 2-stage hepatocarcinogenicity study in male C3H mice. The sample of FL, a fine white crystalline powder, was a kind gift from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Phenobarbital (PB) and D-galactosamine (Gal) were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Four-week-old male C3H mice from Japan SLC, Inc. (Shizuoka, Japan) were acclimated for about 3 weeks and then divided into 6 groups (5 or 8 animals each) with comparable ranges of body weights. Animals were housed 4 or 5 per cage in an air-conditioned animal room with a 12-h light-dark cycle and allowed free access to powdered diet and tap water.

The study period consisted of a 2-week initiation phase and a 13-week promotion phase. Mice in Groups 1–3 were fed basal diet (BD, Oriental Yeast, Tokyo, Japan) throughout the study period. Groups 4 and 5 received a diet containing FL at 4000 ppm for 2 weeks, followed by BD for 13 weeks, and Group 6 was fed a diet containing the same dose of FL throughout the study period. Groups 2–6 were intraperitoneally injected with Gal at 300 mg/kg during weeks 2 and 5. In addition, Groups 3 and 5 were exposed to 500 ppm of PB in their drinking water. The treatments with both Gal and PB were employed to provide tumor promotion effects within a relatively short experimental period. The animals were observed daily and weighed once a week throughout the study.

At the end of the experiment, all survivors were killed by exsanguination

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under ether anesthesia, and were necropsied. The livers were weighed and fixed in 10% phosphate-buffered formalin. The left lobe was embedded in paraffin, sectioned for staining with hematoxylin and eosin (H&E), and examined under a light microscope.

Quantitative data were analyzed using the F-test followed by a *t*-test to detect any significant differences between treatment and corresponding control groups. Incidences of hepatocellular foci were analyzed with Fisher's exact test. A *p*-value less than 0.05 was considered statistically significant.

Comet assay in CHL/IU cells. The Chinese hamster lung cell line CHL/IU was routinely maintained in monolayer culture in Dulbecco's modified MEM medium supplemented with 10% fetal bovine serum at 37°C under a 5% CO_2 atmosphere. Exponentially growing cells were treated with FL dissolved in DMSO (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 1 h. The dose range was chosen in order to obtain both damaged and highly damaged cells as observed in a dose range-finding study. Following FL treatment, cells were embedded in GP42 agarose (Nakalai Tesque, Kyoto, Japan) dissolved in saline at 1% (Miyamae *et al*., 1997). Cell number and cell viability (Trypan blue exclusion method) were determined for each dose. Slides were placed in a chilled lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, pH 10) and kept at 0°C in the dark for >60 min, then in chilled alkaline solution (300 mM NaOH and 1 mM Na_2EDTA, pH 13) for 20 min in the dark at 0°C. Electrophoresis was conducted at 0°C in the dark for 20 min at 25 V (0.96 V/cm) and approximately 250 mA. The slides were then neutralized and stained with 50 μl of 20 μg/ml ethidium bromide.

The length of the whole comet was measured for 50 nuclei for each dose, and differences between the means in treated and control plates were compared with the Dunnett test after 1-way ANOVA. A *p*-value less than 0.05 was considered statistically significant.

Comet assay in mice. Infant and young-adult male ddY mice were obtained from Japan SLC, Inc. at 4 and 7 weeks of age, respectively, and were used after 1 week of acclimatization. Groups were treated once orally with FL at <500 mg/kg. Adult mice were sacrificed at 3 and 24 h after treatment, and 8 organs, the stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow, were removed. Infant mice were sacrificed 3 and 24 h after treatment, and the livers were excised. In another study, the genotoxicity of FL was studied in the regenerating liver of adult mice. For this purpose, male mice at 8 weeks-of-age were anesthetized with ether and 3 major lobes of the liver, left lateral lobe, left medial lobe, and right lateral lobe, were removed. Four days after the hepatectomy, mice were subjected to oral administration of FL once. They were sacrificed 3 h after FL-treatment and regenerated livers were sampled. Slides for the comet assay were prepared at each set time, according to our methods established for multiple mouse organs (Sasaki *et al*., 2000). Slides prepared from nuclei isolated by homogenization were placed in a chilled lysing solution as described above, then in chilled alkaline solution (300 mM NaOH and 1 mM Na_2EDTA, pH 13) for 10 min in the dark at 0°C. Electrophoresis was conducted at 0°C in the dark for 15 min at 25 V (0.96 V/cm) and approximately 250 mA. The slides were neutralized and thereafter stained with 50 μl of 20 μg/ml ethidium bromide.

We calculated migration as the difference between the whole comet length and the diameter of the head for each. Mean migration of the 50 nuclei from each organ was calculated for each individual animal and differences between the averages for 4 treated animals and untreated controls were compared with the Dunnett test after 1-way ANOVA. A *p*-value less than 0.05 was considered statistically significant.

In vitro DNA gyrase/topoisomerase II inhibitory assay. As control compounds, 3 quinolones, enrofloxacin (Bayer AG, Leverkusen, Germany), nalidixic acid (Sigma, St. Louis, MO) and levofloxacin (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) were employed. Purified subunits A and B of DNA gyrase and relaxed pBR322 plasmid DNA were kindly supplied by Daiichi Pharmaceutical Co., Ltd. Human topoisomerase II and Topo II Assay Kit were purchased from TopoGEN, Inc. (Columbus, OH) and kinetoplast DNA (kDNA) was obtained from Nippon Gene (Toyama, Japan). Inhibitory activities of quinolones against DNA gyrase and topoisomerase II were assayed electrophoretically as described previously (Akasaka *et al*., 1998). For the supercoiling assay of DNA gyrase, 20 μl of reaction mixture containing subunits A and B (1 U of each), 0.2 μg of relaxed pBR322 plasmid DNA, and various concentrations of quinolones were incubated for 1h in buffer containing 20 mM Tris–HCl (pH 7.5), 20 mM KCl, 4 mM $MgCl_2$, 1 mM spermidine-HCl, 1 mM ATP, 1 mM dithiothreitol and 20 μg/ml of bovine serum albumin at 37°C. After stopping the reaction by adding sarcosyl with gel-loading buffer, supercoiled and relaxed pBR322 were separated by agarose gel electrophoresis. For the decatenation assay of topoisomerase II, 20 μl of reaction mixture containing 0.4 μg of kinetoplast DNA (kDNA, Nippon Gene, Toyama, Japan) with 1 U of topoisomerase II and serial dilutions of each antibacterial agent were incubated for 5 min at 37°C in buffer containing 20 mM Tris–HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothreitol, and 30 μg/ml of bovine serum albumin. After stopping the reaction by adding sarcosyl with gel-loading buffer, catenated and decatenated kDNAs were separated by agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed using UV light (302 nm) with a Gel Print 200i/VGA (Bio Image Co., Ann Arbor, MI), and the brightness of bands was traced with an Image Analyzer (Bio Image Co.). Each band was quantified and the amount of DNA treated with each concentration of quinolone was measured to determine the 50% inhibitory concentration against DNA gyrase and topoisomerase II.

RESULTS

Thirteen-week 2-stage hepatocarcinogenicity study in male C3H mice. During the treatment, all the FL-treated groups (Groups 4–6) showed significant lowering of body weight, as compared to the corresponding controls, but recovery was evident after switching to BD (Groups 4 and 5). Absolute liver weights were significantly elevated in Groups 3, 5, and 6, as compared to the corresponding control groups, reflecting long-term treatment with PB or FL. Relative liver weights were higher in all treated groups (Groups 2–6) as compared to the corresponding controls. On histological examination, hepatocellular foci were observed in 2 out of 8 and 6 out of 7 mice in Groups 5 and 6, respectively (Table 1). All animals from Group 6 showed centrilobular hepatocellular fatty degeneration. Centrilobular hepatocellular hypertrophy was consistently found in mice in Groups 3 and 5.

Comet assay. Since gross precipitation was observed at 1250 μg/ml, CHL cells were treated with FL at 625 μg/ml or lower. Immediately after the 1-h treatment with FL, a dose-dependent and significant increase in the mean tail length was observed (Fig. 1). At that time, cell viability ranged from 80 to 90%. In the comet assay experiments, no death, morbidity, or clinical signs were observed after any treatment. Necropsy and histopathological examination of tissue sections stained by hematoxylin-eosin revealed no treatment effect on any organ examined. Induction of comets having well separated head and tail, showing apoptosis, was not observed after any treatment. Thus, any DNA damage observed was not likely to be due to general cytotoxicity (necrosis) and apoptosis. In adult mice, FL induced dose-related DNA damage in the stomach, colon, and urinary bladder, 1 and 3 h but not 24 h after its administration (Fig. 2). FL did not yield significant DNA migration in the kidney, lung, brain, and bone marrow (data not shown). DNA damage in the liver was sporadic. On the other hand, FL

TABLE 1
Incidence of Hepatocellular Lesions of Male C3H Mice Given 500 or 0 ppm Phenobarbital for 13 Weeks with or without 2-Week Flumequine Treatment

Group	No. mice examined	Centrilobular fatty degeneration	Centrilobular hypertrophy	Hepatocellular foci
1. BD/BD	4	0	0	0
2. BD/BD + Gal	8	0	0	0
3. BD/PB + Gal	8	0	$8^{a,b}$	0
4. FL / BD + Gal	8	0	0	0
5. FL / PB + Gal	8	0	$8^{a,b,c}$	2
6. FL / FL + Gal	7	$7^{a,b,c}$	0	$6^{a,b,c}$

Note. BD, basal diet, Gal, D-galactosamine, PB, phenobarbital, FL, flumequine.
[a] $p < 0.05$ (vs. Group 1).
[b] $p < 0.05$ (vs. Group 2).
[c] $p < 0.05$ (vs. Group 4).

induced dose-related DNA damage in the regenerating liver of adult mice and the livers of infant mice 3 h after its administration (Fig. 3). Since necropsy and histopathological examination revealed no treatment-related effects in any organ examined, any DNA damage observed was not likely to be due to general cytotoxicity.

Inhibitory effects against DNA gyrase and topoisomerase II. With FL, the ratio of the 50% inhibitory concentration for topoisomerase II versus DNA gyrase was obviously lower than that of enrofloxacin or levofloxacin, although it was slightly higher than that of nalidixic acid (Table 2).

DISCUSSION

In the present study, in mice given a diet containing 4000 ppm FL for 2 weeks followed by PB treatment for 13 weeks, hepatocellular foci were observed in 2 of 8 animals. So far, there has been no unequivocal demonstration that hepatocellular foci are induced in animals given FL during a 2-week initiation phase followed by hepatic tumor promoters, although Yoshida *et al*. (1999) and Takizawa *et al*. (2001) have already suggested the possibility that FL has tumor initiating activity. Therefore, our results strongly suggest that FL can act as a tumor initiator.

It is generally considered that initiation is closely related to adverse effects on DNA. The main reason that FL has been considered a promoter but not an initiator is its lack of genotoxicity in mutagenicity studies, including assays for gene mutation in bacteria and mammalian cells *in vitro* and for chromosomal aberrations in mammalian cells *in vivo* (WHO, 1997). In the present study, we revaluated its genotoxicity using the comet assay to measure DNA breakage in CHL/IU cells and showed FL to induce dose-dependent damage. In the liver of adult mice, only sporadic genotoxic effects were observed although FL targets the liver with regard to its carcinogenicity. In the regenerating liver of adult mice and the liver of infant mice, FL induced dose-dependent DNA damage; the most prominent effects were exerted in the regenerating liver. In B6C3F1 male mice, the frequency of hepatocytes in growth-

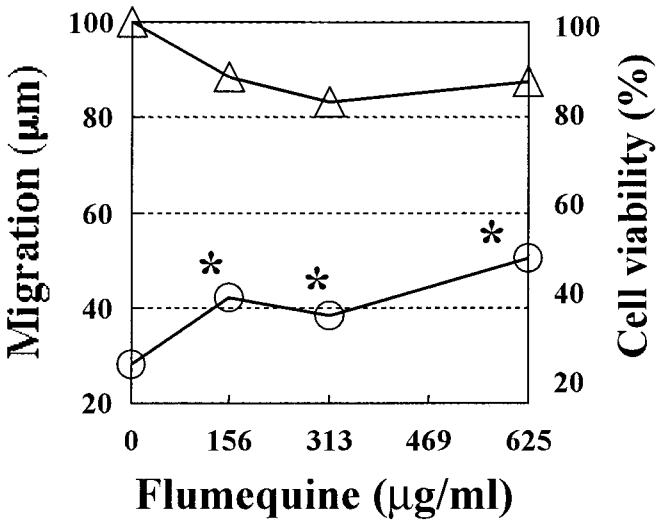

FIG. 1. Migration of nuclear DNA from CHL cells treated with flumequine. CHL cells were treated with FL for 1 h. Circles, mean migration (μm) of 50 cells; triangles, cell viability (%); *$p < 0.05$.

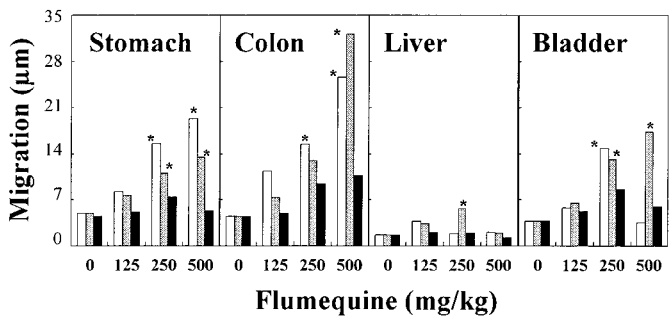

FIG. 2. Migration of nuclear DNA from organs of adult mice treated with flumequine. Mice were sacrificed 1 h (white bar), 3 h (gray bar), and 24 h (black bar) after FL dosing; *$p < 0.05$.

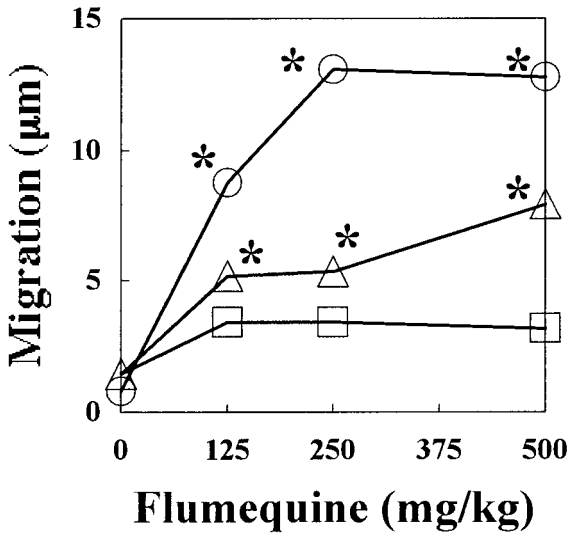

FIG. 3. Migration of nuclear DNA from the liver of infant mice and regenerating liver of adult mice treated with flumequine. Circles, regenerating liver of adult mice sacrificed 3 h after FL treatment; triangles, liver of infant mice sacrificed 3 h after FL treatment; squares, liver of infant mice sacrificed 24 h after FL treatment; $*p < 0.05$.

phase is high (about 1%) at 5 weeks of age and constantly low (below 0.25%) after 6 weeks of age (Miyagawa *et al*., 1995). From our results, FL would be expected to induce dose-dependent DNA damage in the liver in growth-phase, in line with the dose-dependent present findings for 3 organs with relatively high mitotic activities in adult mice: the stomach, colon, and urinary bladder.

Concerning the mechanism of DNA breakage induced by FL, Yoshida *et al*. (1999) demonstrated that staining of 8-hydroxy-2′-deoxyguanosine (8-OHdG) was increased in the mouse liver treated with FL. They therefore concluded that oxidative stress might be a crucial factor for FL carcinogenicity. As another possible mechanism, inhibitory effects on DNA topoisomerases may be important. Though quinolone antibacterial agents work by inhibiting DNA gyrase (bacterial topoisomerase II) or topoisomerase IV, they are also known to have slight inhibitory effects on eukaryotic topoisomerase II, which is responsible for the double-strand DNA breakage/reunion reaction (Wang *et al*., 2001), due to similarities in the biochemical mechanisms and amino acid sequences between these enzymes (Lynn *et al*., 1986). Accordingly, information on the relative selectivity toward bacterial target enzymes versus eukaryotic topoisomerase II is of significance, and a great deal of effort has been made to improve the specificity for gyrase or topoisomerase IV versus topoisomerase II (Lawrence, 2001). Both FL and nalidixic acid belong to the first generation of quinolones and their selectivity regarding bacterial gyrase is extremely low when compared to those of new quinolones such as enrofloxacin and levofloxacin, as confirmed in the present study. Actually, it has been demonstrated that topoisomerase-II inhibitors impair the DNA strand rejoining function of the enzyme, resulting in DNA single- and double-strand breaks (Snyder, 2000). The comet assay can detect DNA damage caused directly by faulty repair or through alkali-labile lesions (Fairbairn *et al*., 1995), and it has been already reported that it can detect the induction of DNA strand breaks by topoisomerase II inhibitors (Godard *et al*., 1999). Thus etoposide, a typical example, has been shown to induce DNA strand breaks in dividing cells by the comet assay *in vitro* and *in vivo* (Godard, 1999). In the *in vitro* study, DNA damage was observed in CHO cells exposed for 1 h. In rats at 4 to 10 weeks of age, etoposide intraperitoneally injected at 5 or 50 mg/kg yielded DNA damage in the liver, intestine, thymus, and bone marrow but not in the kidney. Since the ages of the rats whose livers were damaged was not cited in their report, any growth-dependence is not clear. In the mucosa of gastrointestinal organs, however, positive results with etoposide in comet assay coincide well with those for FL. Therefore, the positive results of comet assay in the liver in the growth-phase and in different mucosa are consistent.

If flumequine's genotoxicity is essentially limited to dividing cells, the observed lack of effects in the bone marrow, a tissue with significant mitotic activity, is puzzling. In fact, the highest incidence of DNA fragmentation with etoposide was observed in the bone marrow 1 h after its injection (Godard *et al*., 1999). One possible explanation for the lack of DNA-damage induction by FL might be that FL and/or its metabolites do not reach to the bone marrow. Since there are no reliable data on kinetics of this compound in mice, an answer to this question remains for further studies.

How do the genotoxic effects shown by the *in vivo* comet assay relate to rodent hepatic carcinogenicity? In carcinogenicity studies, including that reported here, adult animals are generally employed. As shown by Miyagawa *et al*. (1995), the frequency of hepatocytes in growth-phase is lower than 0.25% in mice after 6 weeks of age. One technical disadvantage of the comet assay is that it cannot evaluate large numbers of cells and fails to detect damaged cells if present at only very low frequency, in spite of its generally accepted high sensitivity (Tice *et al*., 1999). Therefore, the failure to detect clear dose-dependent induction of DNA damage in the livers of adult mice might reflect the small number of growth-phase hepatocytes. In

TABLE 2
Inhibitory Effects of Quinolones against Topoisomerase II (topoII) and Gyrase

	50% Inhibitory concentration (μg/ml)		Selectivity
Quinolone	Gyrase	Topo II	Topo II/gyrase
Flumequine	1764	3.92	447
Enrofloxacin	2281	0.29	7861
Nalidixic acid	2226	17.26	129
Levofloxacin	2526	0.30	8425

addition, since FL has been shown to induce cell proliferation in hepatocytes of heterozygous p53-deficient mice initiated with DMN (Takizawa *et al*., 2002), it cannot be ruled out that FL enhances fixation of spontaneous and FL-induced DNA damage of these cells due to increased cell proliferation, and might result in its hepatocarcinogenicity.

FL has generally been considered to be a non-genotoxic carcinogen with only promoting activity (WHO, 1997). However, recent data in the literature and the results of the present study provide evidence that FL was not only a hepatic tumor promoter but also a hepatic tumor initiator. In addition, our results suggest that the initiating activity is due to its induction of DNA strand breaks. Accordingly, more extensive safety assessment of this compound is warranted.

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