Abatement by naringin of lomefloxacin-induced genomic instability in mice

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Lomefloxacin is a difluorinated quinolone antibacterial drug. It is widely used against infectious diseases including meningitis, those of the genitourinary and upper respiratory tracts, and skin infections. Lomefloxacin, like other fluoroquinolones, is mutagenic and the formation of reactive oxygen species appears to be responsible for this genomic instability. The anti-mutagenic effects of naringin, a grapefruit flavonone, against lomefloxacin-induced genomic instability in vivo were evaluated in mouse bone marrow cells by chromosomal aberration and micronucleus (MN) assays. Naringin was neither genotoxic nor cytotoxic in mice at doses equivalent to 5 or 50 mg/kg. Pretreatment of mice with naringin significantly reduced lomefloxacin-induced chromosomal aberrations and the MN formation in bone marrow. The protective effect of naringin was found to be stronger at the higher dose, indicating the dose-dependent effect of naringin. Lomefloxacin induced marked biochemical alterations characteristic of oxidative stress, including enhanced lipid peroxidation and reduction in the reduced glutathione level. Prior administration of naringin ahead of lomefloxacin challenge ameliorated these biochemical markers. It is concluded that naringin has a protective role in the abatement of lomefloxacin-induced genomic instability that resides, at least in part, in its anti-radical effects. Thus, naringin might be a good alternative to reduce genotoxic risks associated with lomefloxacin therapy.

Introduction

Many mutagens have been identified and are known to be potentially deleterious to human health. Two general strategies have been suggested to cope with this problem: (i) to reduce the exposure of an individual to known mutagens as much as possible and (ii) to take advantage of inhibitors of mutagenesis with the final purpose of their eventual application as anti-mutagenic agents (1). The latter field has been widely explored in bacteria and cultured mammalian cells (25,26), chromosomal aberration or micronucleus (MN) induction in cultured mammalian cells (25,27,28) and DNA strand breaks in in vitro and in vivo comet assays (25,29–31), causing genomic instability that may lead to mutagenesis or carcinogenesis. In photocarcinogenicity studies using Skh-1 hairless mice with ultraviolet-A irradiation, lomefloxacin, a difluorinated quinolone, has been reported to cause cystic squamous cell carcinoma in the majority of animals treated (32,33).

An in vivo genotoxicity study has shown that lomefloxacin induced a statistically significant reduction in mitotic index and an increase in chromosomal aberrations and per cent abnormal metaphase in mouse bone marrow (34). Moreover, in an in vivo study carried out by Itoh et al. (35), in which single doses of 25 or 50 mg/kg lomefloxacin were given by oral intubations followed by light irradiation and the mice were killed on days 2, 3, 4, 5 or 8 after treatment, lomefloxacin at either dose caused significant increases in MN frequency, which peaked on day 4. Nevertheless, no significant increase in MN frequency was observed after treatment without light irradiation at these doses. Lomefloxacin was previously tested in our laboratory with the mouse bone marrow MN assay (S.M. Attia, unpublished data); the MN frequency was significantly increased in poly-chromatic erythrocytes (PCEs) following dosing with 320 mg/kg at both 24 and 48 h. Together, these results demonstrate that lomefloxacin has genotoxic activity.

The cause of genotoxicity induced by quinolones is thought to be the formation of reactive oxygen species, such as superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen (36–40). These reactive oxygen species have been associated with cancer, aging and many other degenerative diseases. Therefore, it is advisable to avoid or reduce use of quinolones or to propose alternative DNA-damage reduction methods such as chelating agents, antioxidants and radical scavengers that may help in reducing the mutagenic effects of these drugs.
methods (41). In order to reduce the genotoxic damage caused by exposure to free radicals due to chemical compounds, therapeutic drugs, air pollutants and metabolic procedures, the use of some anti-mutagens found in the normal diet has been studied as a possible DNA-damage reduction method (41,42). On the other hand, flavonoids, such as lomefloxacin, are extremely useful therapeutic drugs, whose use cannot be avoided, despite them being reactive oxygen species generators. Consumption of a diet rich in antioxidants, which are required as micronutrients in the human diet, is perhaps a good alternative to improve health and reduce the risks associated with reactive oxygen species exposure (43).

Flavonoid-induced oxidative damage may be inhibited by antioxidant compounds. In several short-term mutagenicity test systems, Krziková et al. (44) inhibited ofloxacin mutagenesis of Escherichia coli TA102 induced by norfloxacin is inhibited by β-carotene or α-tocopherol (44–46). It is noteworthy that neither of these compounds altered the minimum inhibitory concentration for norfloxacin. Meanwhile, the use of vitamins is strongly advised with prescribed antibiotics, to reduce DNA damage, as long as they do not reduce the bactericidal potency of the antibiotics (43). This may be because the DNA gyrase inhibition of norfloxacin is not dependent on reactive oxygen species generation for its mutagenic effect, which can be inhibited by antioxidants (44–46).

The free radical scavenging and anti-mutagenic properties of naringin prompted the investigation of whether naringin can ameliorate lomefloxacin-induced genomic instability in mice bone marrow cells. In the current study, the scoring of chromosomal aberrations, MN formation and mitotic activity were undertaken as markers of cytogenotoxicity. Serum glutathione and lipid peroxidation were measured as markers of oxidative damage.

Materials and methods

Animals
Adult male white Swiss albino mice weighing 20–25 g (10–12 weeks old) were obtained from the Egyptian Organization for Biological Products and Vaccines (VACSERA, Giza, Egypt). The animals were maintained under standard conditions of humidity, temperature (25 ± 2°C) and light (12 h light/12 h dark). They were fed with a standard pellet mouse diet (El-Nasr Co., Cairo, Egypt) and had free access to water. The animal experiments were conducted according to the regulations of the Committee on Bioethics of the College of Pharmacy, Al-Azhar University, Cairo, Egypt. The total number of animals was 60 treated mice and 10 vehicle control mice.

Drugs and chemicals
Naringin, lomefloxacin and cyclophosphamide (Sigma-Aldrich, St Louis, MO, USA) were dissolved in phosphate-buffered saline immediately before use. Naringin was administered at the dose levels of 5 and 50 mg/kg. The anti-mutagenic doses for naringin were chosen by reference to earlier studies. Naringin has been described to be present in grapefruit juice at concentrations ranging from 101 to 866 mg/litre (47). Upon conversion of animal dose to the equivalent human dose [human dose (mg/kg) = mouse dose (mg/kg) × (3/37)], a dose of 50 mg/kg naringin in mice corresponded to 4.05 mg/kg in humans. Accordingly, for an mutagenic doses for naringin were chosen by reference to earlier studies. Naringin prompted the investigation of whether naringin can ameliorate lomefloxacin-induced genomic instability in mice bone marrow cells. In the current study, the scoring of chromosomal aberrations, MN formation and mitotic activity were undertaken as markers of cytogenotoxicity. Serum glutathione and lipid peroxidation were measured as markers of oxidative damage.

Experimental protocol
The animals were randomly divided into seven groups consisting of 10 mice each. Three groups were administered lomefloxacin [320 mg/kg, intraperitoneally (i.p.),] two groups received a single i.p. injection of naringin at a dose of either 5 or 50 mg/kg body weight 1 h prior to lomefloxacin administration. A vehicle-treated control group and naringin (5 and 50 mg/kg) groups were also included. The experiment included a positive control group administered cyclophosphamide at the dose of 40 mg/kg. The animals were killed by cervical dislocation at 24 h after lomefloxacin treatment.

Chromosome analysis
Five mice in each group were separated and injected i.p. with colchicine at 4 mg/kg body weight, 90 min before sacrifice. The slides were prepared essentially as per the modified method of Adler (48). In brief, both femurs were dissected and bone marrow was flushed from the femoral cavity with foetal calf serum. The cells were dispersed by gentle pipetting and collected by centrifugation at 1100 rpm; the harvested bone marrow cells were incubated in 10 ml of 0.075M KCl for 20 min at 37°C. At the end of the incubation period, 1 ml of 1× saline’s fixative (cold 30% glacial acetic acid–methanol, 1:3, v/v) was added to each tube and then centrifuged for 10 min at 1100 rpm. The supernatant was discarded and 10 ml of fresh fixative was gently pipetted onto the cells without disturbing the pellet. Two to three changes of fixative were required before the preparation of slides. Finally, the cells were suspended in a small volume of fixative and burst open on a clean slide to release chromosomes. The slides were air dried and stained with 5% Giemsa and coded before observation. All slides were scored under 1000× magnification using a Nikon microscope. One hundred well-spread metaphase plates per mouse (500 metaphases for each group) were scored for both structural and numerical aberrations (polyploidy) in bone marrow cells. Cells were classified according to the most severe damage which had occurred and were placed in one of five categories: cells with gaps only, cells with breaks, acentric fragments, centric rings and polyploidy. Gaps were defined as achromatic lesions in one or both chromatids not exceeding the width of a chromatid, and breaks as discontinuities greater than the width of a chromatid, irrespective of whether or not the distal fragment was disconnected. In the tabulated data, the column headed ‘abnormal metaphases’ gives the mean of damaged cells in the total population of cells analysed. Damaged cells include those with one or more breaks, fragments, rings or polyploidy, but not those with gaps. Cells with gaps were also not included in the percentage of total aberrations. From the same slides, 1000 cells from each animal were taken into consideration for the mitotic index study. The mitotic activity of bone marrow was evaluated by calculating the number of dividing cells in a population of 1000 cells.

The MN assay
The remaining five animals from each group were employed for the bone marrow MN test. The animals were sacrificed and bone marrow collected in tubes containing 3 ml of foetal calf serum and centrifuged for 10 min at 1100 rpm. An aliquot of bone marrow cells was transferred into test tubes and cells were resuspended in a small volume of foetal calf serum for smear preparation. Two smears of bone marrow were prepared from each mouse. After air-drying, the smears were stained by May-Grünwald/Giemsa (48). With this method, PCEs stain grey-blue and normochromic erythrocytes (NCE) stain orange, while nuclear material shows a dark purple colour which is an indicative of MN. All glass slides were coded before observation. From each animal, 1000 PCEs were examined for micronucleated polychromatophilic erythrocytes (MNPCe)s under 1000× magnification using a Nikon microscope. In addition, the number of PCEs among 1000 NCE per animal was recorded to evaluate bone marrow toxicity; PCE/NCE ratio was calculated as %PCE = [PCE/(PCE + NCE)] × 100.

Determination of serum lipid peroxidation and reduced glutathione levels
To study the effect of naringin on the oxidative damage induced by lomefloxacin treatment, peripheral blood samples from the same groups used for the MN assay were collected from the heart for estimation of serum lipid peroxidation and reduced glutathione (GSH). All blood samples were centrifuged at 3000 rpm at 4°C for 10 min to obtain the serum. The serum samples were placed on ice for immediate use. GSH was assayed with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) according to the protocol described by Tietze (49) with slight modifications. Briefly, 300 μl of fresh serum was deproteinized by addition of 300 μl of 5% perchloric acid and the mixture was centrifuged at 3000 rpm for 5 min. In total, 200 μl supernatant, 1750 μl 0.1 M phosphate buffer (pH 7.4) and 50 μl DTNB (4 mg/ml in 0.1 M phosphate buffer) were incubated at 37°C for 5 min. The absorbance of the resultant yellow colour was measured spectrophotometrically after 5 min at 412 nm against a blank. The concentration of serum GSH (expressed as micrograms per millilitre) was calculated from a standard curve that was obtained from freshly prepared standard solution of GSH.

The extent of lipid peroxidation in the serum was assayed by measuring one of the products of this process, the thiobarbituric acid-reactive substances (TBARS) by the method of Gervais and Saltman (50) with some modifications. Briefly, the fresh serum (0.25 ml) was added to 0.1 ml of 8.1% sodium dodecyl...
The antimutagenic effects of naringin

The results of the conventional MN test are presented in Figure 1. The frequency of MNPE with cyclophosphamide was significantly higher than in the solvent control group. At a dose of 40 mg/kg cyclophosphamide, the frequency of MNPE was 1.56% compared to 0.32% in the solvent control [P < 0.01 (Mann–Whitney U-test)]. The frequency of MN in both negative and positive control is consistent with other published studies and confirmed the sensitivity of the experimental protocol followed in the detection of genotoxic effects. Similarly, lomefloxacin at a dose of 320 mg/kg significantly increased the frequency of MNPE from 0.32% in the control to 0.84% (P < 0.01). Naringin treatment did not exhibit a significant difference in the frequency of MNPE compared to the solvent control at either dose tested. With regard to the animals treated with naringin plus lomefloxacin, a weak protection was observed with 5 mg/kg of naringin. However, this protection was not statistically significant in comparison to the lomefloxacin alone (P > 0.05). With 50 mg/kg, however, naringin produced a clear significant inhibitory effect on the MNPE induced by lomefloxacin alone (0.42% compared with the lomefloxacin value of 0.84%, P < 0.05) and not statistically significant in comparison to the solvent control (Kruskal–Wallis test followed by Dunn’s multiple comparisons test).

Effects of naringin and/or lomefloxacin on the mitotic activity at interphase

The results for the ratio of PCE:NCE are presented in Figure 2. The positive control cyclophosphamide significantly decreased the per cent PCE from 48.4% in the control to 42.8% [P < 0.05 (Mann–Whitney U-test)] indicating a reduction in erythroblast proliferation most likely by mitotic arrest. Naringin did not modify the ratio in comparison with the one observed in the control group. Lomefloxacin treatment caused a significant

Table I. Distribution of the different types of chromosomal aberrations, percentage of total aberrations, abnormal metaphases and mitotic index in bone marrow of mice after treatment with cyclophosphamide (CP), naringin (Nar) and/or lomefloxacin (Lomo)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chromosomal aberrations</th>
<th>Percentage of total aberrations (mean ± SD)</th>
<th>Abnormal metaphases (mean ± SD)</th>
<th>Mitotic index (mean ± SD)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>G</td>
<td>B</td>
<td>F</td>
<td>R</td>
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<tr>
<td>Control</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>1</td>
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<tr>
<td>CP 40 mg/kg</td>
<td>14</td>
<td>53</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Nar 5 mg/kg</td>
<td>3</td>
<td>4</td>
<td>1</td>
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<td>10</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Lomo and Nar 50 mg/kg</td>
<td>4</td>
<td>7</td>
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</table>

One hundred metaphases were scored for chromosomal aberrations per mouse, for a total of 500 metaphases per treatment. G = gaps, B = breaks, F = fragments, R = rings, P = polyploidy and SD = standard deviation. Cells with gaps were not included in the total aberrations or abnormal metaphases. The mitotic activity per animal was evaluated by calculating the number of dividing cells in a population of 1000 cells. **P < 0.01 versus control and *P < 0.05 versus lomefloxacin alone (Kruskal–Wallis test followed by Dunn’s multiple comparisons test). *P < 0.05 and **P < 0.01 versus control (Mann–Whitney U-test).
decrease in the per cent PCE from 48.4% in the control to 44.0% \( P < 0.05 \) (Mann–Whitney U-test). The combination of 50 mg/kg naringin with lomefloxacin produced a response which was close to that observed with the solvent control.

**Effect of naringin on lomefloxacin-induced reactive oxygen species production**

The effect of naringin on lomefloxacin-induced oxidative stress in mice was assessed by measuring serum GSH and MDA levels. The serum level of GSH in the solvent control is consistent with other published studies. Serum GSH level did not show significant variation in naringin-treated animals compared to the solvent control. The GSH level observed in lomefloxacin-treated animals was significantly decreased compared to the solvent control \( (P < 0.01) \). Animals pre-treated with naringin showed a significant increase in GSH level over the lomefloxacin-treated group and the maximum increase in GSH was observed in animals pre-treated with naringin 50 mg/kg (Figure 3). In the control group, the value of 1.2 nmol/ml MDA is relatively low; however, it is in agreement with the 2.54 nmol/ml of serum MDA in the control mice reported by Alesa *et al.* (51). The treatment with naringin failed to induce any significant changes in the serum levels of MDA at both doses. A significant rise in serum MDA level was observed in the lomefloxacin group \( (P < 0.01) \). Pre-treatment with naringin was found to significantly \( (P < 0.01) \) decrease the MDA concentrations only at the higher dose (50 mg/kg) relative to the values obtained after treatment with lomefloxacin alone (Figure 4).

**Discussion**

It has been reported that naringin has various pharmacological and therapeutic actions. In spite of its potent efficacies, naringin shows little toxicity or mitogenicity in mice. More specifically, naringin exhibits no toxicity when administered orally at 1000 mg/kg, which corresponds to a human dose of 81 mg/kg body weight of naringin (13). Since naringin is a safe and readily available antioxidant, one needs to examine its interaction with drugs. The current study demonstrates that naringin was neither genotoxic nor cytotoxic at the doses...
tested. Moreover, it is able to protect mouse bone marrow cells against the lomefloxacin-induced genomic instability and produce a decline in cell proliferation as observed by the reduction in chromosomal aberrations, MN frequencies and increase in mitotic activities, respectively. An in vivo genotoxicity study has shown that lomefloxacin induced statistically significant increase in chromosomal aberrations, per cent abnormal metaphase and reduction in mitotic index in mouse bone marrow after treatment with lomefloxacin (34). In agreement with this report, the present study showed that exposure to lomefloxacin caused significant increase in the total chromosomal aberrations and abnormal metaphases. The treatment of mice with naringin before exposure to lomefloxacin resulted in significant reduction in both the total number of chromosomal aberrations and abnormal metaphases induced by lomefloxacin. A similar effect has been also reported for radiation-induced chromosomal aberrations in the mice bone marrow. Irradiated mice pre-treated with naringin had significantly fewer chromosomal aberrations in bone marrow than those pre-treated with double-distilled water (7).

Lomefloxacin was previously tested in our laboratory with the mouse bone marrow MN test. At 24 and 48 h after treatment with 320 mg/kg, the MN showed significant increase in PCE. Furthermore, lomefloxacin caused a significant depression of erythroblast proliferation indicating an inhibition of erythroblast proliferation most likely by mitotic arrest. The current study showed a significant inhibitory effect of naringin on the MN produced by lomefloxacin. However, in regard to this response, it is worthwhile to emphasize the observed dose-dependent effect of naringin, with the stronger inhibitory effect produced with 50 mg/kg; this suggests the possibility of using an even higher amount of the chemical with good results. The genotoxicity protection was also directly correlated with mitotic activity as more bone marrow protection was noted with animals pre-treated with 50 mg/kg pre-treated animals when mitotic activity was examined at interphase stage (PCE:NCE ratio). Alvarez-González et al. (10) observed a reduction in ifosfamide-induced MN and mitotic activity in mouse bone marrow cells pre-treated with naringin. Similar anti-mutagenic effects have been also reported for radiation-induced MN in the mouse bone marrow and bleomycin-induced MN in V79 cells (5,8).

The exact mechanism by which naringin protected against lomefloxacin-induced genomic instability in the form of chromosomal damage and MN is not well understood. One possible explanation is that simultaneous treatment with naringin would allow interception of free radicals generated by lomefloxacin before they reach DNA. In the present work, in order to evaluate whether the observed anti-mutagenic effect was due to an enhancement of the scavenger of free radicals generated by lomefloxacin, oxidative stress markers such as lipid peroxidation and GSH were measured after the animals were treated with lomefloxacin, compared with the simultaneous treatment with naringin and the solvent control animals. The results demonstrate that naringin pre-treatment reduced the lomefloxacin-induced lipid peroxidation and prevented the reduction in GSH significantly. The increased GSH level suggests that protection by naringin may be mediated through the modulation of cellular antioxidant levels. These observations confirm earlier studies in which naringin was reported to scavenge free radicals and lipid peroxides (14–20,52).

It is well known that quinolones produce superoxide anion, hydrogen peroxide, hydroxy radical and singlet oxygen, which cause damage to cellular genome and also the cell membrane leading to lipid peroxidation (37–40). MDA, the product of lipid peroxidation also interacts with DNA causing strand breaks that in turn develop into chromosomal breaks. These chromosome breaks may appear as MN in the daughter cell after the first cell division. Earlier studies have shown that naringin scavenged peroxyl, superoxide and hydroxyl radicals in a concentration-dependent manner. Moreover, naringin has been reported to elevate glutathione peroxidase, glutathione reductase, glutathione, superoxide dismutase and catalase and to reduce lipid peroxidation and iron-induced toxicity (6,7,20,52). Scavenging of these free radicals by naringin seems to be an important mechanism against the lomefloxacin-induced genomic instability.

It is, however, worth noting that combination therapy with antioxidants and quinolone provided protection against the quinolone’s genotoxicity without interfering with its bactericidal efficacy (44–46). These results might be explained if it is considered that the bactericidal effect of this antibiotic group is due to inhibition of DNA gyrase, which is the result of the covalent link of the 3-carboxyl and 4-carbonyl groups with the -NH2 terminal group of the bacterial DNA gyrase, and not by free radical production, which can be inhibited by antioxidants (44). Moreover, as mentioned in the Introduction, naringin also possesses antimicrobial and anti-fungal effects (3,53,54). Other studies have reported that antioxidants are able to reduce the development of antibiotic resistance among pathogenic bacteria, because oxygen-free radicals generated by antibiotic metabolism may also be a major cause of antibiotic resistance development (55). Therefore, the simultaneous use of antioxidants with quinolone therapy is advisable to reduce the genotoxic risk of reactive oxygen species generated by quinolone.

In conclusion, prior administration of naringin ahead of lomefloxacin challenge to male mice ameliorated all the cytogenetic parameters altered by lomefloxacin. Apart from the regulatory role of naringin on MDA production observed in the current work, the anti-mutagenic effects of the flavonoid could possibly reside for the most part on its antioxidant effects. Thus, naringin might be a good alternative to reduce genotoxic risks associated with lomefloxacin therapy.

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References


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