Effect of dihydrokainate on the capacity of repair of DNA damage and apoptosis induced by doxorubicin

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The intention of the current study was to investigate the effect of non-toxic doses of dihydrokainate on the capacity of repair of DNA damage and apoptosis induced by doxorubicin in mouse bone-marrow cells. The scoring of micronuclei and olive tail moment was undertaken in the current study as markers of DNA damage and repair. Apoptosis was analysed by the occurrence of a hypodiploid DNA peak. Oxidative stress markers such as bone-marrow reactive oxygen species, lipid peroxidation, reduced and oxidised glutathione were assessed as a possible mechanism underlying this amelioration. In addition, the influence of dihydrokainate on doxorubicin-induced topoisomerase II inhibition was examined. Dihydrokainate was neither genotoxic nor apoptogenic at doses equivalent to 10 or 20 mg/kg/day for 7 days. Pre-treatment of mice with dihydrokainate significantly enhances the repair of doxorubicin-induced DNA damage and reduced doxorubicin-induced apoptosis depending on dose. Doxorubicin induced marked biochemical alterations characteristic of oxidative stress, including increased reactive oxygen species, enhanced lipid peroxidation and reduction in the reduced/oxidised glutathione ratio. Prior administration of dihydrokainate ahead of doxorubicin challenge ameliorated these oxidative stress markers. Importantly, dihydrokainate treatment had no antagonising effect on doxorubicin-induced topoisomerase II inhibition. Conclusively, this study provides for the first time that dihydrokainate enhances the repair of doxorubicin-induced genomic damage in normal cells using dihydrokainate without diminishing doxorubicin’s anti-topoisomerase II activity. Thus, improvement of doxorubicin’s therapeutic index may be achieved by using dihydrokainate.

Introduction

For several years, researchers have been constantly trying to find new ways to enhance the ability of the anticancer drugs to work and to reduce their side effects. Among these trials, biochemical modulation has been studied and applied in clinical therapy. In biochemical modulation, the pharmacodynamics of an antitumour agent is modulated by combination with another drug in order to enhance the antitumour activity or to reduce the side effects, and the efficacy of chemotherapy is thereby enhanced (1). The enhancement of antitumour activity was observed on biochemical modulation (2,3); however, the side effects of antitumour agents increased at the same time. Thus, improvement of the therapeutic index may not be achieved. Therefore, the development of more effective modulators is warranted.

Previously, dihydrokainate (DHK), a component of green tea, was shown to enhance the antitumour activity of doxorubicin (DOX) against Ehrlich ascites carcinoma (EAC) cells both in vivo and in vitro (4). It was considered that DHK prevented the efflux of DOX by inhibiting the glutamate transporter, which increases the concentration of DOX in the tumour cell. On the contrary, DHK tended to reduce the DOX concentration in normal tissues. That means that DHK selectively enhances toxicity only where it is needed (4). This makes DHK different from the drugs presently being used to overcome ‘multi-drug resistance’. It is worth noting that the subtypes of the glutamate transporter expressed in the tumour probably differ from those in normal cells; therefore, the efficacy of DHK may be distinct in the tumour and normal tissues.

DOX is an anthracycline antibiotic that has been used for a long time in therapy of an array of human malignancies either alone or combined with other cytotoxic agents (5). The clinical usefulness of DOX, however, has been hampered due to the development of acquired drug resistance by the cancer cells and various side effects in the host such as cardiotoxicity, genotoxicity and carcinogenicity, preventing the use of high doses of DOX to take full advantage of the therapeutic efficacy (6–8). The primary mechanism of tumour cytotoxicity appears to be the inhibition of topoisomerase II (9). DOX stabilises the topoisomerase II–DNA complexes, preventing the rapid turnover of the protein-cross-linked DNA strand breaks, and interferes with processes that require changes in DNA topology, such as DNA replication, repair and transcription. Other mechanisms of DOX include DNA intercalation, nuclear helicases inhibition and free radical formation (5). As a consequence of these multiple effects, single- and double-strand breaks are introduced into the DNA leading to genomic damage.

The genotoxic potential of DOX has become of great interest because of its serious effects on the DNA of non-tumour cells, leading to secondary malignancies. DOX has been reported to be a genotoxic agent of somatic and germ cells, capable of inducing genomic damage in humans and animals (6–8, 10, 11). Moreover, the precise mechanisms of these toxicities are subtle and efficient treatment to decrease these devastating complications of DOX treatment is not available. The concept of providing protection against genomic damage in non-tumour tissues will represent a promising approach of preventing the unavoidable toxicity from cytotoxic chemotherapy; this will allow the safe use of increased drug doses for the benefit of future cancer patients.

Considering the widespread use of DOX in clinical oncology and the ability of DHK to enhance the antitumour activity of DOX (4) prompted us to investigate whether the non-toxic doses...
of DHK in combination with DOX can enhance DNA repair and ameliorate DOX-induced DNA damage and apoptosis in mice non-tumour cells. The unrepaired DNA damage was assessed by the bone-marrow micronucleus test and the kinetics of repair of DNA damage was assessed by comet assay. Apoptosis was analysed by the occurrence of a hypodiploid DNA peak in mouse bone-marrow cells. In addition, the biochemical alterations characteristic of oxidative stress induced by DOX, such as reactive oxygen species (ROS), lipid peroxidation, reduced glutathione (GSH) and oxidised glutathione (GSSG) have been conducted. Further, the *in vitro* catalytic activity of topoisomerase II was evaluated in the presence of DHK and/or DOX.

**Material and methods**

**Animals**

Adult male Swiss albino mice aged 10–14 weeks and weighing 25–30g were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. The animals were maintained in an air-conditioned animal house at a temperature of 25–28ºC, relative humidity at ~50% and photocycle of 12:12h light and dark periods. The animals were provided with Purina rodent’s chow pellets (Grain Silos and Flour Mills Organization, Riyadh, Saudi Arabia) and water *ad libitum*. All experiments were carried out according to the Guidelines of the Animal Care and Use Committee at King Saud University.

**Experimental protocol**

Mice were divided into eight groups and each group consisted of five mice in each experimental set up as follows: Group 1: mice served as a control group and were treated daily with saline only for 7 consecutive days; Groups 2 and 3: mice were treated orally with DHK (Sigma-Aldrich, St Louis, MO, USA); Groups 4 and 5: mice were injected intraperitoneally with a single dose of 12mg/kg DOX (Sigma-Aldrich, St Louis, MO, USA); Groups 5 and 6: mice were treated with DHK at 10 and 20mg/kg, respectively, once a day, for 7 consecutive days and 12mg/kg of DOX was administrated on the day 7, 1 h after regular DHK administration. On conversion of animal dose to the equivalent human dose [human dose (mg/kg) = mouse dose (mg/kg)/3 (3/37)], a dose of 20mg/kg/day of DHK in mice corresponded to 1.62mg/kg/day in humans. Accordingly, for an average person weighing 60kg, 97.2mg/day DHK would be needed. Although not reasonably achievable through drinking green tea, this concentration may be provided by taking a daily DHK supplement. The dose of DOX was selected on the basis of its effectiveness in inducing bone-marrow genotoxicity (10, 11) and the selected dose is within the dose range used for human chemotherapy. After drug administration, the animals were maintained with food and water *ad libitum* until being sacrificed.

**Measurement of unrepaired DNA damage**

Mice were sacrificed at 24 h after DOX treatment and femur bone-marrow cells were collected in tubes containing fetal calf serum. Bone-marrow smears were prepared and stained with May–Gruenwald/Giemsa solutions as described previously (12,13). From each animal, 2000 polychromatic erythrocytes (PCE) were examined for the presence of micronuclei (MN) using a Nikon microscope. In addition, the number of PCE among 1000 normochromatic erythrocytes (NCE) per animal was recorded to evaluate bone-marrow suppression and mitotic activity was calculated as %PCE = [PCE/(PCE + NCE)] × 100.

**Kinetics of repair of DNA damage**

The kinetics of repair of DNA damage in animals treated with DOX and/or DHK were evaluated by sacrificing the exposed mice at 12, 24 or 48h after the exposure to DOX. At specific time, bone-marrow cells from one femur were collected and the comet assay was performed as previously described (14). The slides were stained with ethidium bromide and then studied using a fluorescent microscope equipped with appropriate filter (Figure 1). Images from 100 cells per animal were randomly selected for analysis using TriTek CometScore version 1.5 software and the olive tail moment ([Tail mean × Head mean] × Tail % DNA/100) was calculated.

**Detection of apoptosis**

To study the effect of DHK on the DOX-induced apoptosis in mouse bone-marrow cells, the remaining femora from the same animals used for the comet assay were used for estimation of apoptosis. Apoptotic cells were quantified with propidium iodide according to the method described by Nicoletti et al. (15) with some modifications as previously described (16). The stained cells were then analysed by flow cytometry using a FACSCalibur cytometer (BD Biosciences). Cells containing hypodiploid DNA were considered apoptotic. The percentage of apoptotic cells was calculated using a computer system CellQuest (BD Biosciences).

**Measurement of oxidative stress markers**

To study the effect of DHK on oxidative damage induced by DOX, animals were killed by cervical dislocation at 24 h after DOX and/or DHK treatment to estimate bone-marrow ROS, lipid peroxidation, GSH and GSSG levels. Intracellular peroxide-dependent oxidation of 2’,7’-dichlorodihydrofluorescein diacetate was measured as an end point for oxidative stress, as previously demonstrated (17). Malondialdehyde (MDA) generated by lipid peroxidation was quantified according to the method of Ohkawa et al. (18), based on thiobarbituric acid reactivity. The MDA concentration in the samples was calculated from the standard curve using 1.1,3,3 tetramethoxypropane. GSSG was assayed with 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) according to the protocol described by Ellman (19). GSSG was assayed with DTNB, glutathione reductase and NADPH as described previously (20). The concentrations of GSH and GSSG were calculated from standard curves that were obtained from freshly prepared standard solutions of GSH and GSSG, respectively. The value obtained for GSH was divided by the GSSG value to get the GSH/GSSG ratio.

**Topoisomerase II inhibition assay**

To determine if DHK have an antagonising effect on DOX-induced topoisomerase II inhibition, topoisomerase II*-t* activity was measured by inhibition of decatamation of kinetoplast DNA (kDNA) by 500nM DOX and/or 100 µM of DHK using a TopoGen (Columbus, OH, USA) assay kit as described previously (21). Gels were photographed and the intensity of DNA bands was quantified by the ImageJ® image processing program.
Results and discussion

The intention of the current study was to investigate the effect of non-toxic doses of DHK on the capacity of repair of DNA damage and apoptosis induced by DOX in mouse bone-marrow cells. The current study demonstrated that DHK was neither genotoxic nor apoptogenic in mouse bone-marrow cells with the doses tested. Moreover, it is able to enhance the repair of DOX-induced DNA damage in mouse bone-marrow cells. The apoptogenic effect of DOX was also reduced by DHK pre-treatment. These effects, however, are dose dependent, having been detected clearly at the higher dose of DHK. It has been reported that the micronucleus assay has been widely used to measure unrepaired DNA damage (22). As seen in Table I, DOX treatment caused a significant increase in the frequency of MNPCE. Moreover, the mitotic activity was significantly decreased after treatment with DOX compared with the solvent control group. These results are in agreement with previous published data reported in other in vivo studies (10,11,14). This genetic damage, particularly in the population of undifferentiated cells that constitutes bone marrow, is dangerous because it can lead to mutations and DNA rearrangements. If such cells survive and proliferate, the risk of secondary acute myeloid leukemia and other drug-related cancers can increase. Prior administration of DHK ahead of DOX challenge ameliorated MN formations and clearly suggests the protective role of DHK on DOX’s genotoxic potentials. Moreover, the reduction of mitotic activity induced by DOX was found to be restored by DHK pre-treatment (Table I). The improvement in mitotic activity of bone-marrow cells of animals pre-treated with DHK in DOX toxicity may focus attention on the beneficial effect of DHK to overcome one of the most serious problems in cancer chemotherapy, which is the bone-marrow suppression and related immunosuppression.

In addition to DNA damage, DNA repair and susceptibility to apoptosis are factors with an emerging role in cancer. Therefore, it was interesting to study the impact of DHK on these processes in normal cells. In order to investigate the impact of DHK on the capacity of repair of DNA damage induced by DOX, the kinetics of repair of DNA damage in animals treated with DOX and/or DHK were evaluated by sacrificing groups of exposed mice at 12, 24 or 48 h after the exposure to DOX. At specific times, bone-marrow cells were collected and comet assay was performed. As shown in Figure 2, DOX caused significant increases in olive tail moment at all sampling times with the highest value at 24 h sampling time. Moreover, the frequency of apoptotic cells observed in bone-marrow cells increased steadily and reached a peak level at 48 h after DOX treatment (Figure 3). Animals pre-treated with DHK showed an increased repair capacity, indicating that DHK enhances the repair of DOX-induced DNA damage (Figure 2). Moreover, the current study showed that DHK markedly decreased the level of apoptotic cells induced by DOX to near to that in the control groups (Figure 3).

DOX has been shown to be metabolically activated to a free radical state and interacts with molecular oxygen to generate radicals. DHK enhances the repair of DOX-induced DNA damage and apoptosis

Table I. Frequencies of MNPCE and mitotic activity (% PCE) in bone marrow of mice after treatment with the indicated doses of DOX and/or DHK

<table>
<thead>
<tr>
<th>Treatment group (mg/kg)</th>
<th>% MNPCE (mean ± SD)</th>
<th>% PCE (mean ± SD)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.30±0.07</td>
<td>49.0±2.23</td>
</tr>
<tr>
<td>DHK (10)</td>
<td>0.32±0.08</td>
<td>48.6±1.51</td>
</tr>
<tr>
<td>DHK (20)</td>
<td>0.28±0.08</td>
<td>49.4±1.34</td>
</tr>
<tr>
<td>DOX (12)</td>
<td>2.34±0.20**</td>
<td>39.2±4.43**</td>
</tr>
<tr>
<td>DOX (12) + DHK (10)</td>
<td>1.26±0.28b</td>
<td>46.0±3.53b</td>
</tr>
<tr>
<td>DOX (12) + DHK (20)</td>
<td>0.60±0.23c</td>
<td>48.8±1.30c</td>
</tr>
</tbody>
</table>

MNPCE, micronucleated polychromatic erythrocytes; DOX, doxorubicin; DHK, dihydrokainate.

Numbers of PCE were counted in microscopic fields, which contained 1000 normochromatic erythrocytes (NCE); % PCE were calculated as % PCE = [PCE/(PCE + NCE)] × 100.

*P < 0.05 and **P < 0.01 compared with the corresponding DOX alone (Mann–Whitney U-test).

**P < 0.01 compared with the solvent control (Kruskal–Wallis test followed by Dunn’s multiple comparisons test).

Fig. 2. Influence of DHK on the kinetics of the repair of DNA damage induced by DOX. *P < 0.05 and **P < 0.01 compared with the corresponding solvent control (Kruskal–Wallis test followed by Dunn’s multiple comparisons test). $P < 0.05$ and $P < 0.01$ compared with the corresponding DOX alone (Mann–Whitney U-test).

Fig. 3. Percentage of apoptotic cells in bone marrow of mice 12, 24 or 48 h after treatment with DOX and/or DHK. % Apoptotic cells denote the percentage of cells with hypodiploid DNA content. **P < 0.01 compared with the corresponding solvent control (Kruskal–Wallis test followed by Dunn’s multiple comparisons test). *P < 0.05 and **P < 0.01 compared with the corresponding DOX alone (Mann–Whitney U-test).
Superoxide radicals (23). Superoxide radicals can deplete antioxidant cellular sulphydryl compounds and react with hydrogen peroxide to form highly reactive hydroxyl radicals through the iron-catalysed Haber–Weiss reaction (24). Secondary derived hydroxyl radicals can cause protein and DNA damage and initiate lipid peroxidation, ultimately inducing tissue damage and secondary malignancies (25). To determine whether the observed protective effects of DHK were due to an enhancement of the scavenger of free radicals generated by DOX, oxidative stress markers such as bone-marrow ROS, lipid peroxidation, GSH and GSSG levels were measured after the animals were treated with DOX and/or DHK (Table II). The present study showed that DOX treatment caused significant increases in ROS and lipid peroxidation levels and DHK pre-treatment reduced the DOX-induced ROS and lipid peroxidation significantly. The current study also demonstrated that DOX induces a decrease in the GSH/GSSG levels in the mouse bone-marrow cells. This can induce genomic damage through the failure of the antioxidant defence mechanisms because antioxidants such as GSH are able to protect normal cells from the hazardous effects of free radicals. The elevated GSH and GSH/GSSG levels in DHK pre-treated animals suggest that protection by DHK may be mediated through the modulation of cellular antioxidant levels. These observations confirm the earlier study in which the glutamate transporter inhibitor theanine reduces the adverse reactions of doxorubicin by changing the glutathione level (26). In the liver, the glutamate transporter inhibitors can be easily taken up and immediately metabolised to glutamate, and then converted to GSH (26). This increment in GSH concentration may result in reduced ROS and lipid peroxidation levels induced by DOX in normal cells. Consequently DHK protects bone-marrow cells from DOX toxicity.

A crucial consideration of co-administration of DOX and DHK is how it will possibly affect the anticancer treatment efficacy; there are, however, important differences between these two processes, which suggest that a reduction in side effects does not necessarily go hand in hand with a reduction in the antitumour effects. To determine whether DHK antagonises DOX-induced topoisomerase II inhibition, the activity of topoisomerase II-α was measured by inhibition of decatenation of kDNA by DHK and/or DOX. Figure 4 shows the fully catenated kDNA (Lane 1) cannot enter the gel due to its large size; however, following incubation with purified topoisomerase II-α (Lanes 3–6), two decatenation products are seen: nicked monomer kDNA (upper) and covalently closed circular DNA (lower; relaxed). As shown in Lanes 3 and 4, the solvent control and DHK (100 µM) do not affect decatenation of kDNA, whereas 500 nM DOX block the reaction (Lane 5). Importantly, the addition of DHK could not reverse DOX-induced topoisomerase II inhibition (Lane 6). Consequently, DHK should not diminish the antitumour activity of DOX through its ability to prevent the formation of free radical intermediates.

![Table II](https://academic.oup.com/mutage/article-abstract/28/3/257/1293471)

<table>
<thead>
<tr>
<th>Treatment group (mg/kg)</th>
<th>ROS (%) of control</th>
<th>MDA (µmol/g protein)</th>
<th>GSH (µmol/g protein)</th>
<th>GSSG (µmol/g protein)</th>
<th>GSH/GSSG ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>99.41 ± 7.0</td>
<td>0.68 ± 0.19</td>
<td>15.6 ± 2.0</td>
<td>4.2 ± 1.0</td>
<td>3.9 ± 1.0</td>
</tr>
<tr>
<td>DHK (10)</td>
<td>103.4 ± 4.7</td>
<td>0.62 ± 0.19</td>
<td>14.4 ± 2.1</td>
<td>3.8 ± 0.4</td>
<td>3.8 ± 0.8</td>
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<tr>
<td>DHK (20)</td>
<td>98.61 ± 9.7</td>
<td>0.90 ± 0.25</td>
<td>16.2 ± 3.4</td>
<td>3.8 ± 0.8</td>
<td>4.5 ± 1.7</td>
</tr>
<tr>
<td>DOX (12)</td>
<td>175.3 ± 17**</td>
<td>2.10 ± 0.4**</td>
<td>9.61 ± 1.9**</td>
<td>7.4 ± 2.7*</td>
<td>1.3 ± 0.3*</td>
</tr>
<tr>
<td>DOX (12) + DHK (10)</td>
<td>121.4 ± 22.8**</td>
<td>1.10 ± 0.39**</td>
<td>12.8 ± 2.2**</td>
<td>5.0 ± 1.2</td>
<td>2.6 ± 0.7**</td>
</tr>
<tr>
<td>DOX (12) + DHK (20)</td>
<td>109.4 ± 9.5*</td>
<td>0.92 ± 0.28*</td>
<td>13.6 ± 2.6*</td>
<td>4.8 ± 1.6</td>
<td>3.1 ± 1.2*</td>
</tr>
</tbody>
</table>

DOX, doxorubicin; DHK, dihydrokainate; ROS, reactive oxygen species; MDA, malondialdehyde; GSH, reduced glutathione; GSSG = oxidised glutathione.

*P < 0.01 and **P < 0.05 compared with DOX alone (Student t-test).
*P < 0.05 and **P < 0.01 compared with the solvent control (one-way ANOVA followed by Tukey–Kramer multiple comparisons test).

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**Fig. 4.** DHK did not reverse the inhibitory effects of DOX on kDNA decatenation by human topoisomerase II α. Lane 1 is a kDNA marker (kDNA without topoisomerase II α); Lane 2 is a linear kDNA marker produced by digestion of kDNA with Xho I; Lanes 3–6: kDNA, topoisomerase II α and drugs. Lane 3: solvent control; Lane 4: DHK (100 µM); Lane 5: DOX (500nM); Lane 6 DHK + DOX. This gel is representative of at least three independent experiments.
Conclusions

In summary, DHK enhances the repair of DOX-induced DNA damage. The improvement in mitotic activity of bone-marrow cells of animals pre-treated with DHK in DOX toxicity may focus attention on the beneficial effect of DHK to overcome one of the most serious problems in cancer chemotherapy, which is the bone-marrow suppression and related immunosuppression. The DNA damaging effects of DOX might be, at least in part, mediated by an oxidative stress mechanism that may be prevented by DHK. DOX has a direct inhibitory effect on topoisomerase II, an important component of its antitumour activity, and this will be unchanged by DHK pre-treatment. As mentioned in the Introduction, DHK enhances the antitumour activity of DOX against EAC cells both in vivo and in vitro. (4) Another study has also reported that DHK is able to increase the uptake of DOX by M5076 ovarian sarcoma cells and significantly inhibited the DOX efflux from those cells (27). However, further studies are essential to investigate whether DHK disturbs the cytotoxic and genotoxic effects of DOX on several cancer cell lines of distinct origins. Nevertheless, at present oncologists should consider the additional beneficial effect of DHK (which could possibly enhance the repair of DOX-induced genomic damage in normal cells), which seems especially important for patients receiving DOX.

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