Genotoxic risk of quinocetone and its possible mechanism in in vitro studies†

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Quinoxalines possessing the quinoxaline-1,4-dioxide (QdNOs) basic structure are used for their antibacterial action, although their mechanism of genotoxicity is not clear. After comparing the sensitivity of V79 cells and HepG2 cells to quinocetone (QCT) and other QdNOs, it was found that HepG2 cells are more sensitive. The results show that QCT induces the generation of $\text{O}_2^-$ and $\text{OH}^\cdot$ during metabolism. Free radicals could then attack guanine and induce 8-hydroxy-deoxyguanine (8-OHdG) generation, causing DNA strand breakage, the inhibition of topoisomerase II (topo II) activity, and alter PCNA, Gadd45 and topo II gene expression. QCT also caused mutations in the mtDNA genes COX1, COX3 and ATP6, which might affect the function of the mitochondrial respiratory chain and increase the production of reactive oxygen species (ROS). Nuclear extracts from HepG2 cells treated with QCT had markedly reduced topo II activity, as judged by the inability to convert pBR322 DNA from the catenated to the decatened form by producing stable DNA–topo II complexes. This study suggests that QCT electrostatically bound to DNA in a groove, affecting the dissociation of topo II from DNA and impacting DNA replication. Taken together, these data reveal that DNA damage induced by QCT resulted from $\text{O}_2^-$ and $\text{OH}^\cdot$ generated in the metabolism process. This data throws new light onto the genotoxicity of quinoxalines.

1 Introduction

Quinoxaline-1,4-dioxides (QdNOs) are widely used as antibacterial drugs1 and possess broad bioactivity.2 Carbadox (CBX) and olaquindox (OLA) have been banned by the European Commission because of their potential to induce cancer via genetic aberrations and mutations.3 Mequindox (MEQ) and quinocetone (QCT) are new members of the QdNO family and there have been only a few reports about their potential genotoxicity.4–6

In previous studies, the genotoxicity of QdNOs was found to be closely related to the production of reactive oxygen species (ROS). MEQ genotoxicity is attributable, in part, to its role as a potent inducer of DNA damage via ROS.7 QCT has toxic effects on HepG2 cells and results in the induction of mitochondria-dependent and mitochondria-independent pathways of apoptosis.8,9 Previous research has also shown that ROS plays an important role in DNA damage induced by QCT10,11 and OLA.12,13 QCT increases the generation of ROS in the liver and kidney, and decreases superoxide dismutase (SOD) and catalase (CAT) activity.14 However, the source of ROS and the relationship between DNA damage and ROS induced by QdNOs is still far from clear. Furthermore, there are some other factors that could cause DNA damage induced by quinoxalines, such as topoisomerase inhibition and DNA adducts, which should also be considered.15

ROS are mainly composed superoxide anion radicals ($\text{O}_2^-$), hydroxyl radicals ($\text{OH}^\cdot$), and hydrogen peroxide ($\text{H}_2\text{O}_2$). These compounds attack DNA, carbohydrates and proteins, and cause DNA double strand breaks, affect enzyme activity and lead to many kinds of toxic reactions.16,17 ROS mainly originate in the mitochondria and maintain normal life activity.18–21 If mitochondrial DNA is damaged, ROS are generated excessively.22 Equally, certain exogenous chemicals might induce the redox cycle following metabolism in cells, with the subsequent production of electrons that could be transferred to molecular oxygen, producing superoxide.23 $\text{O}_2^-$ can be converted to $\text{OH}^\cdot$ by SOD, and is the most toxic free radical.24 Previous research has implied that the toxicity of quinoxalines is related to the $\text{N}$-oxide group reduction and the generation of ROS.2 Whether this supposition is correct
requires further analysis. Moreover, the source of the ROS generated by QdNOs and the exact species of ROS also remain unclear.

Topoisomerase II (topo II) plays an important role in DNA replication and repair. It changes DNA topology during the DNA replication process and keeps the replication fork moving forward. Topomerase α and β are responsible for unwinding DNA in two ways, i.e., double strand breaks (DSBs) and single strand breaks (SSBs). There are many topoisomerase inhibitor drugs which inhibit topoisomerase activity and cause irreversible DNA damage, including adriamycin and etoposide. These compounds block the religation stage, and thereby generate frank DSBs. Drugs that stabilize topo II with DNA DSBs are termed topo II poisons. Tirapazamine (TPZ), one of the QdNOs, has anticancer activity because it is a tumor-specific topo II poison.

There are some interactions between DNA and drugs, such as non-covalent binding (groove, embedded or electrostatic) and covalent binding. No matter what the interaction between DNA and drugs, these cause DNA damage that cannot be easily repaired. Genes, such as POLB, PCNA, topo II, Gadd45, DNA-PK, RPA3, OGG1, RFC, CDC6, RAD50 and BRCA1 play a role in DNA replication and repair. When physical or chemical factors affect their expression, this leads to DNA damage and impacts on DNA replication.

QdNOs have variable mutagenic toxicity. 3-Methyl-quinoxaline-2-carboxylic acid (MQCA), as the residue of OLA also causes DNA strand breaks. The genotoxic sensitivity of mammalian cells to quinoxalines is not consistent. Therefore, investigations into genotoxic metabolites and screening for the most sensitive cells and most toxic quinoxaline compounds is necessary (Fig. 1). Quinoxalines have similar genotoxicity as they cause DNA strand breaks; therefore DNA strand break was chosen as an indicator of toxicity. HepG2 cells and V79 cells are more commonly used in toxicology research studies than human normal liver cells (L02 cells) and animal primary cells, respectively. Furthermore, because the mutagenicity of QdNOs under lower oxygen conditions is stronger than under aerobic conditions, HepG2 cells seemed to be more suitable than L02 cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and single cell gel electrophoresis (SCGE) were used to determine the most genotoxic quinoxaline compound and the most sensitive cells (HepG2 or V79 cells).

QCT was found to be the most genotoxic compound. It was hypothesized that QCT is metabolized and generates O2‘− and OH’. A dihydroethidium, 7′-dihydro-8-oxodeoxyguanosine (8-OH-dG) ELISA kit and the SCGE method were used to detect O2‘− and OH’, respectively. Because the structure of QCT is similar to that of TPZ, it was thought that QCT might be a topo II poison, as is TPZ. Therefore, the nuclei from mammalian cells treated with QCT were extracted to detect topoisomerase activity. Considering the fact that OLA and CBX can interact with plasmids and induce mutations, it was hypothesized that QCT would interact with DNA and cause DNA strand breaks and mutations. Moreover, the gene expression of DNA replication and repair enzymes was assessed to investigate the relationship between DNA damage and the inhibition of gene expression. These results shed new light on the mechanism of genotoxicity of QdNOs, which will help the

![Fig. 1](https://academic.oup.com/toxres/article/5/2/446/5568657) The chemical structures of QdNOs and their metabolites.
efficient use of currently available drugs, and to push the development of novel compounds with more potential and fewer harmful effects.

2 Materials and methods

2.1. Chemicals and reagents
Olaquindox (OLA, 99%), mequindox (MEQ, 99.8%) and quinoctone (QCT, 99%) were purchased from Zhongmu Pharmaceutical Co. Ltd (Wuxue, PR China). Carbadox (CBX, 98%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cyadox (CYA, 99.8%) was obtained from the Institute of Veterinary Pharmaceuticals, Huazhong Agricultural University (Wuhan, PR China). All the metabolites (purity, 99%) were obtained from the Department of Veterinary Pharmacology and Toxicology, China Agricultural University (Beijing, PR China). All five compounds and their metabolites were dissolved in dimethyl sulfoxide (DMSO, Amresco, USA) and then diluted in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Logan, USA) at the desired concentrations. Collagenase (type IV, 268 U mg\(^{-1}\)) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Gibco-BRH (Gibco, Grand Island, NY, USA). Methylthiazolotetrazolium, phenylmethanesulfonyl fluoride (PMSF), dihydroethidium, 2',7'-dichlorodihydrofluorescein, dimethyl sulfoxide, dAMP, dTMP, dCMP and dGMP were purchased from Sigma (St. Louis, USA). A human 8-hydroxy-deoxyguanine (8-OHdG) ELISA kit was obtained from CUSABIO (Wuhan, PR China). Human topo II and human topo II α polyclonal antibodies were provided by Topogen Inc. (Columbus, OH, USA). pBR322 DNA was obtained from the Beijing Huaxia Ocean Science and Technology Co., Ltd (Beijing, PR China). All other chemicals and reagents were of high analytical grade.

2.2. Cell culture
HepG2 cells and V79 cells were purchased from the Shanghai Institute for Biological Sciences, Chinese Academy Cell Resource Center (Shanghai, PR China). HepG2 cells and V79 cells were cultured in DMEM and RM1640 supplemented with 10% fetal bovine serum (FBS), respectively. Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂.

2.3. Cell viability
HepG2 cells and V79 cells (5 × 10⁴ mL⁻¹) were seeded in 96-well flat-bottomed plates and allowed to adhere for 12 h. Cells were treated with quinoxalines and their metabolites at 5, 10, 20, 40, 80 and 160 µM for 0.5, 1, 2, 4 and 8 h, respectively. These compounds were easily dissolved in DMSO, and they were diluted with cell culture medium to the concentration indicated with a final DMSO concentration of ≤0.1% (v/v). The cells were treated with 0.1% DMSO as a control. Thereafter, the cells were treated with a final concentration of 0.5 mg mL⁻¹ MTT and incubated at 37 °C for 4 h. The purple formazan crystals were dissolved in 150 µL of DMSO. Then, the optical density (OD) was measured using a Microquant plate reader (Bio-Tek Instruments) at 570 nm. Cell viability in response to treatment with drugs was calculated as: Cell Viability = (OD of sample well − OD of control well)/(OD of control well − OD of blank well).

2.4. DNA strand break analysis using the SCGE assay
DNA strand breaks were detected using a protocol for the alkaline comet assay described by Singh and Bhat. DNA microliters of the cell suspension (approximately 10⁶ cells) was mixed with 130 µL of 0.8% low melting point (LMP) agarose melted in PBS in Eppendorf tubes at 38 °C. The slides, with coverslips, were removed, then were immersed in a cold, freshly prepared lysis solution [2.5 mol L⁻¹ NaCl, 100 m mol L⁻¹ EDTA, 10 mol L⁻¹ Tris, 1% sodium N-lauroylsarcosine (SLS) with pH 10, 1% Triton-100 and 10% DMSO added right before use] for 4 h in the refrigerator. After lysis was completed, the slides were rinsed with distilled water and then were placed in a horizontal gel electrophoresis box containing fresh, chilled electrophoresis buffer to a level 0.25 cm above the slides. The slides were left for 20 min to let the DNA fully unwind so that alkali-labile damage could be expressed. Electrophoresis was conducted at 4 °C for 20 min at 25 V and 300 mA. Slides were drained and neutralized with three changes of neutralization buffer (0.4 M Tris, pH 7.5), each time for 5 min to remove the detergent and alkali. The slides were removed from the neutralization solution, rinsed gently, and then stained with 40 µL of 20 µg mL⁻¹ ethidium bromide. Slides were observed at a magnification of 400× using a fluorescent microscope (Olympus, CK40) equipped with a BP546/10 excitation filter and a 590 nm barrier filter. On each replicate slide, 100 cells were scored (200 cells total for each concentration) using a comet image analysis system (CASPP). Data on tail length, tail moment and the DNA content of comet tail were recorded. Only cells with a defined head were scored, and dead cells were excluded.

2.5. The integration analysis of DNA with QCT
The integration analysis of DNA with QCT was performed as described previously. Calf thymus DNA (ct-DNA) (12.5, 25, 50, 100 and 200 µM) was treated with 40 µM of QCT for 4 h. The integration was detected by UV-visible absorption spectra (Beijing Purkinje General Instrument Co., Ltd, PR China). The value of absorption peak was observed by UV scanning from 200 nm to 400 nm.

After 4 h incubation with 12.5 mg mL⁻¹ of dAMP, dTMP, dCMP and dGMP with 40 µM of QCT, respectively, the reaction products were detected using HPLC (Shimadzu Corporation, Kyoto, Japan). A Waters Symmetry C-18 column (5 µm, 4.6 × 250 mm) was used for the detection of the samples. The mobile phase consisted of A (methanol) and B (0.1% formic acid – 0.032% ammonium formate aqueous solution) with gradient elution. From 0 to 40 min, the mobile phase was A (650%) and B (94–50%), and from 40–50 min, the mobile phase was A (50–6%) and B (50–94%). The flow rate was 0.7 mL min⁻¹. The column was maintained at 46 °C, and the
injection volume was 30 µL. No endogenous or extraneous peaks were observed interfering with the separation.

2.6. Generation of ROS analysis using the fluorescence probe assay

ROS generation was measured with the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay described by Eruslanov and Kusmartsev (2010). Following exposure to the drug (5, 10, 20, 30 and 40 µM) for 0.5, 1, 2, 3 and 4 h, the cells were trypsinized and washed with ice-cold PBS. Then, 1 mL of PBS containing 20 µM DCFH-DA was added, and the cells were incubated for 30 min at 37 °C. The fluorescence emission from DCF was analyzed using a fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA) with excitation and emission spectra set at 480 and 530 nm, respectively.

2.7. Generation of O$_2^-$ using a fluorogenic probe assay

O$_2^-$ was measured using the dihydroethidium (DHE) assay as described by Peshavariya with some modifications. O$_2^-$ generation resulted from the incubation of QCT with xanthine oxidoreductase (XOR). After exposure to the drug (10, 20, 40 and 160 µM) for 4 h, followed by treatment with XOR (50 µM) at 37 °C for 30 min, the OD values were determined at 580 nm.

After the cells were exposed to 0, 5, 10, 20, 30 and 40 µM of QCT or Q6 or SOD (3.25 U µL$^{-1}$) for 4 h, the culture medium was removed and PBS was added. DHE (1 µM) was added to the cell culture well and incubated for 30 min. Then, cells were collected and centrifuged at 1500g for 5 min three times. Cells were resuspended in PBS and added to 96-well plates. Changes in fluorescence were monitored with a multiwell plate reader for 10 min at 37 °C. Data are expressed as the net increase in fluorescence.

2.8. LC/MS-ITTOF analysis of the metabolites of QCT

HepG2 cells were incubated with 20 µM QCT at 37 °C for 4 h. QCT and its metabolites in the cell or supernatant samples were detected using hybrid IT/TOF mass spectrometry coupled to a high-performance liquid chromatography system (LC/MS-ITTOF) (Shimadzu Corp., Kyoto, Japan). The liquid chromatography system was equipped with a solvent delivery pump (LC-20AD), an autosampler (SIL-20AC), a DGU-20A$_1$ degasser, a photodiode array detector (SPD-M20A), a communication base module (CBM-20A) and a column oven (CTO-20AC).

The cells were collected and centrifuged at 1500g for 10 min. After adding 200 µL of PBS, cells were lysed using a CV18 ultrasonic cell disruption device from Nanjing Xinchen Biological Technology Co., Ltd (Nanjing, PR China). The lysed product was centrifuged at 10 000g for 15 min after adding 200 µL of methanol. The supernatant was collected, and 10 mL of acetonitrile was added and it was vortex mixed for 5 min. After vigorous shaking, followed by centrifugation at 10 000g for 15 min, the supernatant was dried under N$_2$ in a 35 °C water bath. The residue was reconstituted in 5 mL of distilled water. The total supernatant was applied to a methanol and water pre-washed HLB 3cc cartridge (Waters Corporation, Milford, Mass US). The samples were then sequentially washed with 3.0 mL of water and 5% methanol in water. The cell extracts were eluted into plastic tubes with 5 mL of methanol. The eluate was evaporated to dryness under nitrogen at 35 °C and the samples were reconstituted in 500 µL of a methanol: water (40:60 v/v) solution and passed through a 0.22 µm filter membrane for LC/MS-ITTOF. HPLC separation was performed as described above except an isocratic solvent mixture composed of 75% water, 25% acetonitrile, and 0.1% phosphoric acid was used at a flow rate of 0.2 mL min$^{-1}$. Positive ion electrospray was used for ionization and collision-induced dissociation (CID) using argon gas. Other instrument settings included a capillary voltage of 4.5 kV, a capillary temperature of 200 °C and a column temperature of 40 °C. The separation was performed on a Zorbax eclipse XDB-C18 column (150 mm × 2.1 mm, 3.5 µm) using gradient elution consisting of mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile). The gradients were 5% B-20% B (0–16 min), 35% B (25 min), 60% B (30 min), 100% B (35–37 min), 5% B (37.1 min), 5% B (45 min). The injection volume was 10 µL. The flow rate was 0.2 mL min$^{-1}$, and the wavelength used was 306 nm.

2.9. Effect of QCT metabolism on DNA damage

A xanthine/xanthine oxidase (XXOR) enzyme system as one-electron reducing agent for the activation of QCT was used to investigate the effect of the metabolism of QCT on DNA damage under aerobic and low oxygen conditions (Tables S1 and S2†). In this assay, DNA strand scission was readily measured by observing the conversion of supercoiled (form I) plasmid DNA to the open circular form (form II) resulting from nicking the DNA backbone. Assays were prepared in an inert atmosphere glove bag and the solutions were freeze–pump–thaw degassed or purged with inert gas to remove molecular oxygen. The final concentrations of DNA, xanthine, XOR, QCT, TPZ and SOD were 0.05 µg µL$^{-1}$, 100 µM, 8.75 U mL$^{-1}$, 160 µM, 500 µM and 3.25 U µL$^{-1}$, respectively. After incubation at 37 °C for 2 h, the whole content was observed in a 1% agarose gel stained with ethidium bromide after electrophoresis for 1 h.

2.10. Topo II activity analysis

Nuclear extracts were prepared as described with some modifications. Briefly, untreated HepG2 cells were pelleted and lysed in 1.0 mL of nuclear buffer A [1 mmol L$^{-1}$ KH$_2$SO$_4$, 150 mmol L$^{-1}$ NaCl, 5 mmol L$^{-1}$ MgCl$_2$, 1 mmol L$^{-1}$ EDTA, 0.1 mmol L$^{-1}$ PMSF, 0.1 mmol L$^{-1}$ DTT, and 10% glycerol (v/v)]. After initial lysis, the cells were rinsed with nuclear buffer A and spun at 460g for 10 min. Pelleted cells were resuspended in 1.0 mL of nuclear buffer A and 9.0 mL of nuclear buffer B (nuclear buffer A containing 0.3% Triton X-100). Samples were gently rotated for 10 min and spun at 460g for 10 min. After centrifugation, the supernatants were removed and centrifuged again at 12 000g for 15 min. The supernatant was obtained and the protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, PR China).

Topo II activity was assayed as described. Reactions contained 0.1 µg pBR322DNA, 50 mmol L$^{-1}$ Tris-HCl, 120 mmol L$^{-1}$
KCl, 10 mmol L\(^{-1}\) MgCl\(_2\), 0.5 mmol L\(^{-1}\) of DTT, ATP, and 1 µL 2 U µL\(^{-1}\) topo I and topo II. The reactions were incubated for 30 min at 37 °C and terminated with 1 µL proteinase K and 2 µL 10% SDS. Samples were extracted once with an equal volume of chloroform:isoamyl alcohol (24:1). Following brief centrifugation in a microfuge, the blue upper layer was loaded directly onto an agarose gel. The decatenation products were analyzed on 1% agarose gels run either without or with 0.5 µg ethidium bromide as specified. Electrophoretic analyses of kDNA were performed using standard gel electrophoresis units.

The trapped in agarose DNA immunostaining (TARDIS) assay was performed as described by Willmore et al. with some modifications. Slides were stained with anti-topo II rabbit polyclonal antibody (1:100; TopoGEN, TG2010-1) in PBS containing 0.1% Tween 20 and 1% BSA for 1 h at room temperature. Slides were then stained with FITC-conjugated goat antirabbit IgG antibody in PBS containing 0.1% Tween 20 and 1% BSA for 1 h at room temperature and treated with DAPI and Hoechst33258 for 5 min and visualized using a fluorescent microscope (Olympus, CK40).

### 2.11. Mutation of mtDNA analysis using sequence analysis

mtDNA was isolated as described earlier. The samples were digested with proteinase K and ethanol precipitated. The quality of the DNA was checked by PCR for β globin as an internal control. The DNA was used to amplify the entire region of the mitochondrial genome. The mtDNA was amplified using the forward and reverse primers shown in Table 1.

Briefly, 50 ng of extracted DNA was amplified in a 25 µL final reaction volume under the following conditions: 1 × DNA polymerase buffer [16 mmol L\(^{-1}\) of (NH\(_4\))\(_2\)SO\(_4\), 67 mmol L\(^{-1}\) of tris-HCl (pH 8.8), 0.1% polysorbate], 1.5 mmol L\(^{-1}\) of MgCl\(_2\), 500 mmol L\(^{-1}\) of each primer, and 1 U of Super Taq. PCR conditions were as follows: 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s; and a final extension step at 72 °C for 5 min. Next, the total PCR products were purified and sent to Wuhan Anygene Biotechnology Corporation Limited (Wuhan, PR China) to analyze the sequence.

### 2.12. Expression of mRNA assay using RT-PCR

Total cellular RNA was isolated from the cells with a few minor modifications. The purity of the RNA sample was defined by the A\(_{260}/A_{180}\) ratio. One microgram of RNA was reverse transcribed to cDNA with the ReverTra Ace™ First Strand cDNA Synthesis Kit (Promega, Madison, WI, USA). cDNA was amplified by qRT-PCR (BioRad, Hercules, CA) using SYBR Premix Ex Taq RT-PCR kit (Takara, Code BKA701, China). Each 25 µL reaction mixture consisted of 12.5 µL SYBR Premix Ex Taq, 0.5 µL of each primer (10 µM), 2 µL of cDNA, and 9.5 µL RNAs-free dH\(_{2}\)O. Cycling conditions were as follows: step 1, 30 s at 95 °C; step 2, 45 cycles at 95 °C for 5 s, 60 °C for 30 s; step 3, dissociation stage. The endpoint used was real-time PCR quantification. Relative quantification of gene expression was calculated using the 2\(^{-\Delta\Delta C_{T}}\) data analysis method, as previously described and normalized to GAPDH in each sample. Primers used in this study are provided in Table 2.

### Table 1 Primer sequence of mtDNA

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### 3 Results

#### 3.1. The effects of drugs on cell viability

Dose and time-dependent increases in cytotoxicity occurred when HepG2 cells and V79 cells were exposed to the drugs (data not show). It was found that cell viabilities induced by 40 µM QCT, CBX, OLA and MEQ for 4 h were 76.40 ± 4.50%, 83.50 ± 2.60%, 80.20 ± 5.40% and 84.50 ± 6.60%, respectively, indicating that QCT presented greater cytotoxicity than the other parent drugs. Furthermore, the MTT results also show that the parent drugs had more toxicity than their metabolites. Similar results were observed in V79 cells after treatment with QdNOs and their metabolites. Doses of 10–160 µM for QdNOs and their metabolites were selected for future studies based on the MTT results.

#### 3.2. Appraisal of the most sensitive cells and the genotoxicity of quinoxaline compounds by the SCGE assay

The total number of DNA strand breaks in HepG2 cells and V79 cells were analyzed by the comet assay. Experiments were performed on QdNOs and their metabolites at 40 µM for 4 h. The data demonstrate that CBX, OLA, MEQ, QCT and their metabolites all induce DNA strand breaks. The results are summarized in Fig. 2. Tail-DNA% and Olive Tail Moment (OTM) were used as the indices of DNA strand breaks. The OTM of HepG2 cells and V79 cells was 14.66 ± 3.12% and 9.82 ± 3.08% for OTM of HepG2 cells and V79 cells was 14.66 ± 3.12% and 9.82 ± 3.08% for OLA, respectively, with 25.00 ± 3.44% and 12.40 ± 4.82% for QCT, 22.50 ± 2.68% and 18.85 ± 3.08% for OLQ, respectively, indicating that QCT presented greater cytotoxicity than the other parent drugs.
40 μM exposure for 4 h. The results suggest that HepG2 cells were more sensitive to the quinoline compounds than V79 cells. The ability of the parent drugs to induce DNA strand breaks was greater than their metabolites. QCT showed the most potential to induce DNA strand breaks. Therefore, QCT was chosen as the most genotoxic compound among the QdNOs and their metabolites.

DNA strand break induction by QCT was assessed in a dose and time-effect relationship. Three time points and five concentrations of QCT were selected to investigate the ability to induce DNA strand breaks.

### 3.3. The interaction of DNA with QCT

The absorbance values of DNA and QCT were 260 and 317 nm, respectively. Following the increased concentration of ct-DNA, the absorbance value of QCT increased and a hyperchromic effect occurred. The maximum UV absorption wavelength of ct-DNA was offset by 3 nm in the shortwave direction, indicating that QCT interacted with ct-DNA in a non-intercalative mode. The interaction of dAMP, dTMP, dCMP and dGMP with QCT was detected. The results show that QCT could not combine with them, and therefore no covalent interaction occurred between QCT and DNA (Fig. 3).

### 3.4. Generation of ROS, O$_2^{-}$ and metabolites induced by QCT

The results show that QCT induced intracellular ROS generation in a time- and dose-dependent fashion (Fig. 4A). The amount of O$_2^{-}$ generated by HepG2 cells occurred in a dose-dependent manner (Fig. 4B). The results show that QCT could induce intracellular O$_2^{-}$ generation in a dose-dependent fashion with the catalysis of XOR, suggesting that XOR plays an important role in the production of O$_2^{-}$ (Fig. 4C). Fig. 4D shows that QCT induced HepG2 cells to generate O$_2^{-}$, and SOD significantly eliminated the free radical.

Additionally, the results confirm that OH$^-$, one of the most highly reactive ROS, was generated by HepG2 cells treated with QCT. Some studies have suggested that OH$^-$ can attack DNA and generate 8-OhdG. In the present study, 8-OhdG levels increased in a dose-dependent manner after treatment with QCT at various concentrations for 4 h (Fig. 4E). Compared with the control, Q6 did not induce the generation of O$_2^{-}$, while QCT treatment resulted in a significant increase in O$_2^{-}$ production, suggesting that N-O groups play a critical role in the generation of O$_2^{-}$ (Fig. 4F). It was also found that N4-deoxy quinocetone (Q3), N1-deoxy quinocetone (Q4) and Q6 was generated by HepG2 cells, indicating that the reduction of the N-O group might be the reason for the generation of ROS and O$_2^{-}$ (Fig. 4G and H).

### 3.5. The effect of the metabolism process of QCT on DNA damage

It was found that QCT, in conjunction with the X/XOR system, caused direct single-strand breaks in DNA (Fig. 5). In the absence of QCT or X/XOR, there was no significant effect on plasmid DNA, indicating that X/XOR plays an important role in the DNA damage induced by QCT. The DNA damage induced by QCT was similar to the positive control TPZ, suggesting that the metabolism of QCT with X/XOR resulted in DNA damage. Without the presence of QCT, both DMSO and SOD showed no significant effect on DNA integrity. DMSO, a scavenger of OH$^-$, led to a significant decrease in DNA damage induced by QCT, indicating that OH$^-$ was the free radical attacking DNA. While SOD, a scavenger of O$_2^{-}$, contributed to DNA damage induced by QCT. It was presumed that, during the process, SOD contributed to converting O$_2^{-}$ to OH$^-$, which has a highly toxic effect on DNA. Additionally, it was found that plasmid DNA breakage was more obvious under low oxygen conditions than that under aerobic conditions.

### 3.6. The relationship between topo II activity inhibition and DNA strand breaks induced by QCT

Topoisomerase activity analysis was performed in vitro. Topo I and II and pBR322 DNA were treated with various concentrations of QCT to determine whether topo II could support the decatenation of pBR322 DNA. It was found that QCT markedly reduced topo II activity as judged by the inability to convert pBR322 DNA from the catenated form to the decatenated form, whereas the activity of topo I was not changed.

In the TARDIS assay, topo II, covalently attached to DNA, was detected by staining cells with anti-topo II antibodies and secondary antibodies conjugated to FITC. In untreated HepG2 cells, little staining for anti-topo II was present, but significant
staining for topo II was observed when the cells were treated with QCT (Fig. 6).

3.7. Mutation of mitochondrial DNA
Mitochondrial DNA was extracted using the high-concentration-salt precipitation method. HepG2 cells were treated with QCT at a concentration of 40 μM, for 4 h. Then, HepG2 cells were cleaved and the mitochondrial DNA was extracted. Sequence analysis showed mutations to the ATP6, COX1 and COX3 genes (Table 3).

3.8. Influence of gene expression of DNA replication and repair
HepG2 cells were treated with QCT at different concentrations for 4 h. The results show that QCT decreased the levels of expression of many genes, but these were not significantly changed after exposure at 10 μM and 20 μM. When cells were exposed to 20 μM QCT for 4 h, the expression of PCNA and topo II decreased significantly by over two-fold and a two-fold increase in Gadd45 expression was found compared with the...
The interaction of ct-DNA with QCT was exposed at 37 °C, for 4 h. From a to g: 200 μM DNA + 40 μM QCT, 100 μM DNA + 40 μM QCT, 50 μM DNA + 40 μM QCT, 200 μM DNA, 25 μM DNA + 40 μM QCT, 12.5 μM DNA + 40 μM QCT, and 40 μM QCT; (B) QCT; (C) dAMP; (D) dTMP; (E) dCMP; (F) dGMP; (G) dAMP + QCT; (H) dTMP + QCT; (I) dCMP + QCT; (J) dGMP + QCT.

Fig. 3  Ultraviolet absorption spectroscopy of ct-DNA upon the addition of QCT (A), and HPLC diagram of dinucleotide (12.5 mg mL$^{-1}$) with 40 μM of QCT (B, C, D, E, F, G, H, I and J). (A) The interaction of ct-DNA with QCT was exposed at 37 °C, for 4 h.
control group, this dose was then chosen to investigate the
time-effect relationship. The results show that the expression 
of PCNA, topo II and Gadd45 exhibited a time-effect relation-
ship. In addition, SOD significantly weakened the influence of 
QCT on gene expression (Fig. 7).

4 Discussion
A number of studies have clearly shown that QdNOs are 
potentially genotoxic agents, but little is known about their '
genotoxic mechanism. In the present study, it was found that
QCT was the most genotoxic agent among the quinoxalines by SCGE analysis. It was first identified that $O_2^{•-}$ and OH$^-$ were generated during the process of N-oxide group reduction of QCT by XOR in the cytoplasm. Furthermore, in the present study, it was found that quinoxalines could also interact with DNA, and inhibited the dissociation of DNA-topo II complexes, significantly changing gene expression related to DNA repair and causing DNA strand breaks (Fig. 8).

The mutagenic and antibacterial activity of prototype QdNOs and their metabolites was enhanced under anaerobic conditions. In the present study, these QdNOs derivatives had their genotoxicity in normal cells (V79) and cancer cells (HepG2) compared, and it was found that HepG2 cells were more sensitive to QdNOs. HepG2 is a kind of cancer with low oxygen conditions, which might be one of the important reasons that HepG2 cells were more sensitive to the hypoxia-activated compounds, such as QdNOs. In addition, some QdNOs (e.g., TPZ, DCQ) have been used as anticancer and hypoxia-selective drugs in humans. The results suggest that QCT presented the hypoxia-selective DNA cleavage, indicating its potential anticancer activity.

Some studies have suggested that the potential of genotoxicity of QdNOs is closely related to the N-oxide group reduction. The N-oxide reduction progress of QdNOs might lead to the formation of ROS and oxidative stress. In the present study, the genotoxicity potential of prototype drugs was significantly higher than that of the metabolites, identifying the important role of the N-oxide group in the genotoxicity of QdNOs. However, in the present study, it was also found that some N-oxide group reduction metabolites (e.g. Q6) could induce DNA strand breaks, suggesting that the genotoxic mechanism of Q6 might be different from that of QCT. It was previously reported that Q6 (5–20 μg mL$^{-1}$) could induce cell cycle arrest at the S phase in Chang liver cells, while the same doses of QCT could not, indicating S phase arrest induced by Q6 might be one reason for its genotoxicity. However, the reason why Q6 induced S phase arrest remains unknown. Although the metabolites were less toxic than their parent drugs, residual amounts of these chemicals in animal products might pose a hazard to consumer health, and their toxic and mutagenicity mechanism should be further elucidated.

Oxidative DNA damage induced by ROS is the most important type of damage to DNA. Usually, the generation of 8-OHdG and ROS are considered to indicate oxidative DNA damage. In a previous study, OLA was reported to induce oxidative DNA damage. However, the genetic mechanism of QCT is far from clear. In the present study, it was observed that the generation of ROS occurred in a dose and time-dependent relationship. In the ROS generation and SCGE assay, ROS was found to play an important role in DNA strand breaks in HepG2 cells induced by QCT. As the primary species of ROS, $O_2^{•-}$ was detected for the first time when cells were treated with QCT. ROS are produced excessively in animals and humans when XOR and catecholamine increase, or by chemical substances generated during metabolism. A number of reports have suggested that the toxicity of quinoxalines is related to N-oxide group reduction. XOR, cytochrome p450 and aldehyde oxidase have been suggested to be the major metabolic enzymes of quinoxalines, they are located in the cytoplasm, endoplasmic reticulum and mitochondria, respectively. XOR has been found to be responsible for N-oxide group reduction. In the present study, MS analysis showed that the desoxy-quinocetone was detected in HepG2 cells treated with QCT, indicating that QCT can enter cells and induce N-oxide group reduction. At the same time, there was excessive $O_2^{•-}$ and 8-OHdG generation in the cytoplasm induced by QCT. Here, ROS generation induced by quinoxalines was identified for the first time. Furthermore, guanine in the cytoplasm was easily attacked by OH$^-$ to generate 8-OHdG. The increase in 8-OHdG in the cytoplasm implied that OH$^-$ generated by QCT plays an important role in DNA damage. It has been reported that the OGG1 gene product is responsible for removing 8-OHdG from DNA and to repair damaged DNA. In the present study, QCT significantly affected the expression of the topo II, PCNA and Gadd45 genes; the influence of gene expression was significantly decreased by SOD, indicating that ROS induced by QCT plays a key role in oxidative DNA damage.

In previous studies, it was found that c-MYC-dependent activation of the mitochondrial apoptotic pathway and tumor necrosis factor receptor (TNFR) pathway may be associated with QCT-induced toxicity. The activation of c-MYC and
TNFR pathways could result in the activation of caspase-8 that cleaves effector caspase-3 either directly or indirectly via the mitochondrial route.\(^6^4\) Therefore, it was suggested that the damage to mitochondria might play a critical role in the genotoxicity of QCT. In the present study, mutations in mitochondrial DNA have been noted in cells treated with QCT, suggesting that not only mitochondrial apoptotic pathways but also mitochondrial DNA were affected by QCT.

Mitochondrial DNA is located in close proximity of the respiratory chain, which is the main cellular source of ROS.\(^6^5\) ROS can induce oxidative base lesions in mitochondrial DNA, which might affect oxidative phosphorylation and result in further ROS production.\(^6^6\) Mutations in \(ATP6\), \(COX1\) and \(COX3\) have been noted in cells treated with QCT. \(ATP6\) is the ATP synthesis subunit 6 and participates in ATP synthesis.\(^6^7\) The mutation of \(ATP6\) might affect ATP synthesis and result in disrupted ATP generation. ATP participates in many physiological functions, such as biosynthesis, energy transfer, metabolism and respiratory functions.\(^6^8\) If the synthesis of ATP is blocked, many physiological functions are affected, such as the respiratory chain, causing electron transport disruption and leading to the generation of ROS.\(^6^9\) \(COX3\) is cytochrome c oxidase III which participates in the composition of oxidase.\(^7^0\) Mutations in the \(COX3\) gene might affect oxidase function, cause the electron transport disruption and result in electron leaks, which is the source of ROS. Thus, mutations in these genes in the mitochondrial DNA shed new light onto the mechanism of genotoxicity induced by QCT.

Topo II is highly enriched in the nuclear matrix and is responsible for resolving topological states that are encountered during replication and transcription.\(^7^1\) Here, we showed that exposure to QCT inhibited topo II activity in nuclear extracts from HepG2 cells. Similarly, we found the same results regarding the recombination skills of topoisomerase with pBR322 DNA. In the topo II catalytic cycle, enzyme binding to double-stranded DNA introduces a transient DNA DSB and passes the unbroken strand through the break. Topo II then religates the transient break and dissociates from the DNA. The topo II poison etoposide stabilizes the DNA–topo II complex and prevents religation, producing DNA DSBs.\(^7^2\)

The TARDIS assay shows that topo II is covalently bound to DNA in individual cells.\(^7^3\) The present results for the first time show that QCT can induce HepG2 cells to form DNA–topo II complexes and suggests that the inhibition of topo II activity was most likely the result of the depletion of free topo II. ROS can attack enzymes and affect their activity.\(^7^4\) QCT could induce HepG2 cells to generate excessive ROS, which might attack topoisomerase. In the present study, it was found

<table>
<thead>
<tr>
<th>Mutation site</th>
<th>Gene</th>
<th>Blank sequence</th>
<th>Mutation sequence</th>
<th>Codon changes</th>
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<tbody>
<tr>
<td>6488</td>
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<td>TAC</td>
<td>TCTCTCTCCT</td>
<td>Insertion</td>
</tr>
<tr>
<td>8849</td>
<td>ATP6</td>
<td>CCCTATGAG</td>
<td>CCCCTATTAGAG</td>
<td>Insertion</td>
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<tr>
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<td>COX3</td>
<td>CTCAGAGTAC</td>
<td>CTCAGAGTAC</td>
<td>GAG &gt; AAG</td>
</tr>
</tbody>
</table>

![Table 3](https://academic.oup.com/toxres/article-lookup/doi/10.1093/toxres/mfv099)
that the inhibition of topo II decreased by adding SOD, suggesting for the first time that ROS plays a role in the inhibition of topo II.

Genotoxic carcinogens such as polycyclic aromatic hydrocarbons (PAH) and aflatoxin B1 (AFB1) are thought to cause cancer because of their ability to form covalent bonds with
DNA bases.\textsuperscript{75,76} The structures formed through the covalent bonding of these intermediates to DNA bases are referred to as carcinogen–DNA adducts.\textsuperscript{77} Failure of the cell to repair these adducts can lead to mutations in the DNA code.\textsuperscript{78} We measured the interaction of DNA with QCT using ultraviolet absorption spectrophotometry and HPLC. The results show that non-covalent binding between DNA and QCT occurred. The non-covalent binding mode might be electrostatic or groove binding, but not insert binding. The interaction of DNA with QCT might affect the dissociation of topo II from the DNA and lead to a decrease in free topo II, which would induce DNA strand breaks. In the present study, DSBs were generated after 2 h of exposure to 20 μM QCT, and the degree of DNA strand breakage was most serious after 4 h exposure to 40 μM QCT. However, the inhibition of topo II was greater at 20 μM than at 40 μM, indicating that a high dose of QCT exerts different genotoxic mechanisms compared with those of a low dose of QCT. Further studies should be carried out to investigate how a high dose of QCT exerts a toxic effect on topo II.

In summary, it was demonstrated for the first time that QCT causes DNA strand breaks and induces DNA damage by generating O$_2^{-}$ and OH$^-$ during the metabolism process driven by XOR. After all defence systems were damaged, ROS could easily attack DNA and led to DNA strand breaks and mutations. QCT could induce the generation of topo–DNA complexes and affect DNA replication. These results provide valuable information on the mode and molecular mechanism of QCT toxicity.

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References
