

Mechanism of apoptosis induced by quinoxalone from the myxobacterium *Stigmatella eracta* WXNXJ-B in B16 mouse melanoma cell line

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The biological activities of quinoxalone, a novel small molecular substance isolated from the broth of the myxobacterium *Stigmatella eracta* WXNXJ-B, was investigated. This study was designed to determine the anti-proliferative, apoptotic property of quinoxalone, using B16 mouse melanoma cells as a model system. The results showed that quinoxalone has antitumor activity and can significantly inhibit the proliferation of B16 cells. The extent and the timing of apoptosis were strongly dependent on the dose. After treating with quinoxalone and staining with Hoechst 33342, B16 cells showed typical apoptotic morphological features such as chromatin condensation by fluorescent microscopy. DNA isolated from B16 cells cultured with quinoxalone showed a typical DNA ladder of apoptosis in agarose gel electrophoresis. Further investigation results showed that the apoptotic machinery of B16 induced by quinoxalone was associated with drop in mitochondrial membrane potential from 5.35% to 23.7%, up-regulation of Bax and down-regulation of Bcl-2 in a dose-dependent manner. And a significant increased activation of caspase-3. Our finding suggests that quinoxalone could suppress the growth of B16 cells and reduces cell survival via disturbing mitochondrial membrane potential and inducing apoptosis of tumor cells.

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ABSTRACT The biological activities of quinoxalone, a novel small molecular substance isolated from the broth of the myxobacterium *Stigmatella eracta* WXNXJ-B, was investigated. This study was designed to determine the anti-proliferative, apoptotic property of quinoxalone, using B16 mouse melanoma cells as a model system. The results showed that quinoxalone has antitumor activity and can significantly inhibit the proliferation of B16 cells. The extent and the timing of apoptosis were strongly dependent on the dose. After treating with quinoxalone and staining with Hoechst 33342, B16 cells showed typical apoptotic morphological features such as chromatin condensation by fluorescent microscopy. DNA isolated from B16 cells cultured with quinoxalone showed a typical DNA ladder of apoptosis in agarose gel electrophoresis. Further investigation results showed that the apoptotic machinery of B16 induced by quinoxalone was associated with drop in mitochondrial membrane potential from 5.35% to 23.7%, up-regulation of Bax and down-regulation of Bcl-2 in a dose-dependent manner. And a significant increased activation of caspase-3. Our finding suggests that quinoxalone could suppress the growth of B16 cells and reduces cell survival via disturbing mitochondrial membrane potential and inducing apoptosis of tumor cells.

Subjects: Toxicology, Pharmacology

Keywords: *Stigmatella eracta* WXNXJ-B, Quinoxalone, Apoptosis, Mitochondrial membrane potential, Bax, Caspase-3

31 INTRODUCTION

32 Myxobacteria are gram-negative unicellular rod shaped bacteria that move by gliding and travel
33 in swarms, containing many cells kept together by intercellular molecular signals (*Wioletta et al.*,
34 2016). They can be frequently isolated from soil, dung of herbivorous animals and other decaying
35 organic material (*Shimekets et al.*, 2006). They are unusual bacteria characterized by gliding
36 behavior and forming fruiting body, and not obtained by the routine method due to their
37 complicated life cycle (*Velicer and Vos*, 2009).

38 Myxobacteria are one of the important sources for natural microbial products besides
39 actinomycetes and fungi (*Gerth et al.*, 2003). Ambruticin, the first myxobacterial antibiotic, was
40 isolated from a strain of *Sorangium cellulosum* (*Connor et al.*, 1977). The first structure of
41 myxothiazol was reported by Gerth et al. (1980). Within the last 30 years, the myxobacteria have
42 emerged as a promising alternative source of bioactive molecules (*Johnson et al.*, 2012; *Schmitz*
43 *et al.*, 2013; *Plaza and Müller*, 2014; *Schaberle et al.*, 2014). Myxobacterial secondary
44 metabolites do not commonly produce by other microbes, such as hybrids of polyketides and
45 non-ribosomally peptides (*Diez et al.*, 2012). The metabolites exhibit many unique structural
46 features and novel modes action, making them attractive and promising sources for drug
47 development. Many compounds from myxobacteria show quite different mechanisms of action.
48 They inhibit the protein synthesis of prokaryotic and eukaryotic and stimulate potassium export
49 from gram-positive bacteria. But, the mechanism of action of most compounds has not yet been
50 elucidated (*Weissman and Muller*, 2010).

51 Epothilones from *Sorangium cellulosum* and their analogues have demonstrated antitumor

activity towards multidrug resistant tumor cells (*Altman et al., 2009; Gong et al., 2014*). These compounds target the eukaryotic cytoskeleton, interference with microtubuli in the cell disabling the assembly of functional mitotic spindles required for cell proliferation and thus resulting in apoptosis. One such analog, known as Ixabepilone is a FDA-approved chemotherapy agent for the treatment of metastasis breast cancer (*Wioletta et al., 2016*). Several other metabolites are currently being evaluated in preclinical studies (*Kim et al., 2013*). Due to their extraordinary ability to produce novel classes of secondary metabolites, myxobacteria represent a very promising source for the discovery of new lead structures and novel natural products (*Wenzel and Muller, 2009*).

Due to their potent biological activities, which results in various applications in the pharmaceutical and agrochemical industry, many research groups have tried to identify novel groups of natural product producers over the last decade. In continuing effort to find novel bioactive metabolites from myxobacteria, the researchers in our lab obtained five myxobacteria which showed strong antitumor bioactivity *in vitro* (*Guo and Tao, 2008*). Quinoxalone, isolated from the myxobacterium *Stigmatella eracta* WXNXJ-B and stored in our lab, is a novel bioactive metabolite (Figure 1). Our previous study showed that quinoxalone exhibited significant effect of anti-proliferation on the tumor cells *in vitro*, however, the mechanism of anti-proliferative has yet not been elucidated clearly (*Wang et al., 2014*). In the present report, we investigated the quinoxalone antitumor activity by evaluating its effects on the B16 mouse melanoma cell line and its possible apoptosis mechanism.

MATERIALS AND METHODS

Microorganism and culture conditions

The strain myxobacteria *Stigmatella erecta* WXNXJ-B was used in this study. Medium for slant was CY medium. Medium composition for seed and fermentation cultures was as described by Wang et al (2014). In the fermentation medium, about 20 g/L XAD-16 adsorbent resins (Rohm & Haas, USA) were added to adsorb the bioactive metabolites. *S. erecta* WXNXJ-B was grown on CY medium at 30°C for 5 days, then inoculated in seed medium for flask culture at 30°C with shaking at 150 rev/min. After 2 days, the seed broth was transferred to fermentation medium and fermented at 30°C with shaking at 150 rev/min for 7 days.

Preparation of quinoxalone

After fermentation, the XAD-16 adsorbent resins were separated. The resins were extracted with methanol for 6 h. The extract was concentrated at 45°C and further purified by partition between water and chloroform. The chloroform extract was isolated by chromatography using a Sephadex LH-20 column, with two gradients of 80% and 90% methanol at flow rate 2 mL/min. The fraction eluted with 90% methanol was purified using a preparative RP-HPLC using a Sephax C₁₈ column (5 µm ×10 mm×150 mm, with mobile phase 80% methanol at flow rate 3 mL/min). Quinoxalone was obtained.

Cell lines

B16 mouse melanoma cell line, CT-26 murine colon carcinoma cell line MDA-MB231 and MCF-7 human breast cancer cell line, HepG2 human liver hepatocellular cell line were provided by college of Medicine and Pharmaceutics, Jiangnan University, China. All cells were cultured in

RPMI-1640 medium (Gibco, USA) with 10% inactivated fetal bovine serum (Gibco, USA), streptomycin (100 µg /mL) and penicillin (100 U/mL) at 37°C in a 5% CO₂ incubator. Epothilone B and Paclitaxel were purchased from Sigma-Aldrich Co.

Evaluation of quinoxalone in vitro (MTT assay)

B16, CT-26, HepG2, DMA-MB231 and MCF-7 cells were used to evaluate the antitumor effects of quinoxalone. Cells were harvested, counted, diluted and seeded into 96-well plates at a density of approximately 7000 cells/well. After incubating for 24 h, 200 µL medium with different concentration quinoxalone which was dissolved into dimethylsulfoxide (DMSO) was added into per well. To avoid the influence of DMSO, medium containing 0.5% DMSO was used as a control. Incubation was carried out for another 48 h. The cell viability was assessed by MTT (colorimetric 3-[4,5--2-Yl]-2,5-diphenyl tetrazolium bromide) assay. Twenty microlitre of MTT solution (5 mg/mL) was added into each well and incubated at 37°C for additional 4 h. The formazan product was dissolved by adding 200 µL DMSO and shaken for 5 min. Then, the absorption was measured at 570 nm with a microplate reader. The inhabitation rate was calculated as follows: inhabitation rate= $(1 - \text{OD}_{\text{treated}} / \text{OD}_{\text{control}}) \times 100\%$. Data were obtained from six repeat experiments.

Fluorescence microscope observation of B16 cells

To observe the change in nuclear structure, B16 cells were plated onto glass cover slips in 6-well plates and treated with 5 and 10 µg/mL quinoxalone for 48 h. Then, cells were washed twice with PBS, fixed with 1% glutaraldehyde, stained with Hoechst 33342 (Sigma, USA) for 15 min at room temperature. Nuclear morphology was examined by fluorescence microscope (Olympus, Tokyo, Japan).

115 DNA fragmentation assay

116 B16 cells were treated with quinoxalone at concentrations of 0, 2.5, 5 and 10 µg/mL for 48 h.
 117 Following with centrifugation at 600 g for 5 min, the harvested cells were lysed in a DNA
 118 extraction buffer(containing 20 mmol/L EDTA, 100 mmol/L Tris, 0.8%(w/v) SDS) and incubated
 119 at 37 °C for 30min. After centrifugation at 10000 g for 10min, 10 µL Rnase A (500 U/mL) was
 120 added into the lysate of cells at 50 °C for 90 min, followed by treatment with 10 µL proteinase K
 121 (500 µg/mL) at 50°C for 90 min. The supernatant was extracted using phenol: chloroform:
 122 isoamyl: alcohol (25:24:1) and centrifugated at 12000 g for 10 min. The supernatant was
 123 precipitated with ice-cold ethanol for 24h. The precipitated DNA was dissolved in TE buffer (10
 124 mM Tris/HCl, 1 mM EDTA) and electrophoresis containing 1% agarose gel and 0.5 µg/mL
 125 ethidium bromide was then performed. DNA ladders were visualized after staining with
 126 bromophenol blue.

127 Measurement of mitochondrial transmembrane potential

128 The inner mitochondrial transmembrane potential of B16 cells was analyzed using a FACScan
 129 flow cytometer according to the reported method (*Sun, et al., 2006*). B16 cells were treated with
 130 different concentrations of quinoxalone for 48 h. After trypsinization, B16 cells were washed
 131 twice with PBS, and then the concentration of cell suspension was adjusted to 1×10^7 cells/mL.
 132 One hundred microliter Rhodamine 123 solutions (Rh123, 20 µg/mL) was added to the harvested
 133 cells and incubated at 37°C in the dark for 30min. Then, the cells were washed with PBS again
 134 and stained with propidium iodide solution (PI, 100 µg/mL), rinsed with PBS twice, and checked
 135 immediately with flow cytometer. All data were collected and analyzed with Cellfit Analysis
 136 Software.

The analysis of expressions levels of Bcl-2, Bax and P53

After treating with different concentrations of quinoxalone (0, 5 and 10 $\mu\text{g/mL}$), the expressions levels of Bcl-2, Bax and P53 proteins in B16 cells were checked by flow cytometer (*Aggarwal and Gupta, 1998*). B16 cells were collected, washed and sequentially fixed with 2% paraformaldehyde for 10 min. The cells were treated with 75% ethanol for 1h at 4°C. After washing, the cells were respectively incubated with anti-Bcl-2, anti-Bax and anti-53 (Beyotime Biotechnology Inc., China) for 30 min at 37°C. The cells were incubated with FITC-conjugated goat anti-mouse IgG (Beyotime Biotechnology Inc., China) for 30 min at 37°C. Then, the cells were washed twice with PBS, and checked with FACScan flow cytometer. The percentage of positive cells expressing fluorescence intensity of Bcl-2, Bax and P53 was measured by mean fluorescence channel number.

Caspase-3 activity

Caspase-3 is an important molecular in the regulation of apoptosis. Activity of caspase-3 was detected by using a Caspase-3 colorimetric assay kit (Biovision Inc., USA) according to the manufacturer's protocol. B16 cells were incubated for different time (12, 24 and 48 h) in the absence (control) or presence of various concentrations of quinoxalone (5, 10 $\mu\text{g/mL}$). The cells were collected, washed twice, resuspended in 50 μL of chilled cell lysis buffer and incubated on ice for 10min. The lysate was centrifuged at 10000 g at 4°C for 10 min. Then, 50 μL of reaction buffer (containing 10 mM DTT) was added to each sample. After incubation at 37°C for 1.5 h with 5 μL DEVD-pNA substrate (200 μM final concentration), the absorbance was measured at the wavelength of 405 nm in a microtiter plate reader. Results were expressed as the fold relative to control in absorbance.

159 **Statistical analysis**

160 Data were represented as mean \pm SD. Statistical differences were determined by Student's t-test.

161 Samples with P values of $p < 0.05$ were considered statistically different.

162 RESULTS

163 Antitumor evaluation of quinoxalone on different tumor cells

164 In this study, B16, DMA-MB231, MCF-7, HepG2 and CT-26 cell lines were used to
 165 evaluate the antitumor bioactivity of quinoxalone in vitro. The five cell lines were treated by
 166 different concentration quinoxalone for 48 h. Paclitaxel and Epothilone B were the positive
 167 controls. As shown in Figure 2, quinoxalone showed strong cytotoxicity to HepG2, B16, MCF-7,
 168 CT-26 and DMA-MB231 tumor cell lines, which the value of IC₅₀ were 2.42, 2.2, 6.73, 2.05 and
 169 3.04 µg/mL, respectively. CT-26 cell was more sensitive to quinoxalone than the others.
 170 Comparing with the positive controls, the cytotoxicity of quinoxalone was similar with Paclitaxel.
 171 The results suggested that quinoxalone showed broad spectrum activity to tumor cells.

172 Effect of quinoxalone on the morphology of B16 cells

173 In order to check the influence of quinoxalone on B16 cells, the cells were treated with 0, 5, 10
 174 µg/mL quinoxalone for 48h. As we can see from Figure 3, it could significantly inhibit the
 175 growth of B16 cells. When the dose were 10 and 20 µg/mL, the inhibit rate was about 60% and
 176 80%, respectively, and, some cells turned round and floated comparing with the control. The
 177 nuclear morphology of B16 cells treated with quinoxalone for 48 h was observed under a
 178 fluorescence microscope by Hoechst 33342 staining (Figure 4). The nuclei of the treated B16
 179 cells have nuclear shrinkage and condensed chromatin.

180 Effect of quinoxalone on DNA of B16 cells

181 Externalization of phosphatidylserine and cleavage of DNA, the hallmarks of apoptosis, were
 182 also recently found in alternative types of programmed cell death (*Jakopec et al., 2006*). To
 183 determine whether the processes were induced because of quinoxalone, B16 cells were treated
 184 with different concentration of quinoxalone (0, 2.5, 5, 10 and 20 µg/mL) for 48h. As shown in
 185 Figure 5, the results of agarose gel electrophoresis showed that DNA fragmentation about 180–
 186 200 bp called “DNA ladders” were detected. The concentration of DNA ladders increased

187 following the concentration of quinoxalone. This observation suggested that quinoxalone could
188 induce apoptosis of B16 cells in the concentration dependent manner.

189 **Effect of quinoxalone on mitochondrial transmembrane potential in B16 cells**

190 Mitochondria is an important organelle in cell. It is very sensitive to around environment and
191 plays an important role in the propagation of apoptosis. The mitochondrial transmembrane
192 potential decreases in apoptosis cell because the permeability of mitochondrial membrane
193 happens to change (*Zhang and Huang, 2006*). In this study, B16 cells were used to check the
194 effect of quinoxalone on the mitochondrial membrane potential and plasma membrane integrity
195 with PI and Rh123 double-staining method. As we can see from Figure 6, after treatment with 0,
196 2.5, 5 and 10 $\mu\text{g/mL}$ quinoxalone for 48 h, the percentages of were PI negative and strongly
197 stained by Rh123(Rh123⁺PI⁻, normal cells) decreased insignificantly from 93.0% to 65.9%. The
198 PI negative and low-staining by Rh123 (Rh123⁻PI⁻, early apoptosis cells) group increased
199 markedly from 5.35% to 23.7% in a dose-dependent manner. But, there is no significantly
200 difference between the experimental groups and the control in the cell death group of Rh123⁻PI⁺
201 and Rh123⁺PI⁺. These results indicated that quinoxalone target the mitochondria in treatment-
202 induced apoptosis in B16 cells.

203 **Effect of quinoxalone on expression of relative gene-proteins p53, bcl-2 and bax in B16 cell**

204 Many factors influence the process of apoptosis, including of p53, bax and bcl-2 genes. In these
205 genes, bcl-2 and p53 are anti-apoptosis genes. But, bax is promoting-apoptosis gene(*Kenji et*
206 *al.,2003*). Based on the apoptosis analysis on cell arrest, p53, bax and bcl-2 genes were checked
207 after treated with different concentrations of quinoxalone, and the expressions levels of P53, Bax
208 and Bcl-2 proteins were analyzed by flow cytometry. As shown in Figure 7, quinoxalone
209 significantly increased the expression level of Bax protein, but significantly decreased the
210 expression of Bcl-2 protein. P53 protein expression level changed slightly after treating with
211 quinoxalone. The results suggested that quinoxalone can efficiently induce B16 cells apoptosis,
212 which is correlated with up-regulating bax expression and down-regulating bcl-2 expression.

213 **Effect of quinoxalone on caspase-3 activity in B16 cell**

214 Caspase-3 plays a critical role in apoptosis and its activity has been suggested as an index of
 215 apoptosis (*Cohen, 1997*). To examine the role of caspases in the apoptosis induced by
 216 quinoxalone, its activation was measured using fluorometric detection. As shown in Figure 8,
 217 caspase-3 activity assay showed an enhancement of enzymic activity at all experimental time,
 218 and reached a peak after 48 h of exposure to quinoxalone. Caspase-3 activity increased following
 219 the enhancement of quinoxalone concentration (from 5 $\mu\text{g/mL}$ to 10 $\mu\text{g/mL}$). The results
 220 suggested that quinoxalone activated caspase-3 in a time and dose dependent manner.

221 DISCUSSION

222 Tumor is a diseases state characterized by proliferation disorder and apoptosis obstacle. Its key
 223 characteristics are uncontrolled cellular growth and proliferation. So, the efficient methods to treat
 224 tumor are to inhibit cell proliferation and induce apoptosis. Apoptosis is a regulated process
 225 characterized by cell shrinkage, nuclear disintegration, selective degradation of DNA, and
 226 formation of apoptotic bodies with a relatively intact plasma membrane (*Cui et al., 2007*). Many
 227 natural products inhibit the proliferation of some tumor cell via the apoptosis, for example, a
 228 flavone nitroderivative caused murine mammary adenocarcinoma cells death by apoptosis
 229 (*Mariano et al., 2009*). The ability to induce cell apoptosis is an important property of the
 230 candidate anti-cancer drugs.

231 Cytotoxicity determining, a common method to evaluate the biology activity of nature
 232 products, is helpful to confirm whether nature products have potential anti-tumor properties
 233 (*Bruna et al., 1999; Kim et al., 2005*). In the previous study, we reported that quinoxalone, a
 234 novel compound isolated from the broth of myxobacteria *Stigmatella erecta* WXNXJ-B, showed
 235 significant cytotoxic effect and the proliferation inhibition on different tumor cells. The
 236 elucidation of the type of cell death induced by quinoxalone and the role of apoptosis/necrosis is
 237 very important for understanding the bioactivity of quinoxalone. With the purpose of
 238 investigating the importance of programmed cell death in the cytotoxicity of quinoxalone, we
 239 used some different methods, which enabled us to know the mode of cell death and the process of
 240 apoptosis induced by quinoxalone. B16 cells were examined for biochemical hallmarks of
 241 apoptosis such as morphological changes, DNA fragmentation, sub-G1 cell population (apoptotic
 242 cell), mitochondrial transmembrane potential, expression of relative gene-proteins (p53, bcl-2 and
 243 bax), and caspase-3 activity.

244 In this study, we have found that B16, CT-26, HepG2, DMA-MB231 and MCF-7
 245 cells exhibited markedly different sensitivity to quinoxalone. The cytotoxicity
 246 of quinoxalone was similar to that of taxal. Using agarose gel electrophoresis,
 247 flow cytometry and fluorescence microscopy, we have demonstrated that

quinoxalone can cause B16 cells apoptosis. DNA fragmentation is very typical characteristic of the apoptotic process, with generation of a series of multiplets of a 180-200 bp subunit. In present study, typical DNA ladder of apoptosis in B16 cells after treated with quinoxalone was detected at every concentration in B16 after treatment, which further indicated that quinoxalone could induce the apoptosis of B16 cells.

Mitochondrial membrane potential plays an essential role in mediating apoptosis (*Desagher and Martinou, 2000; Guo and Tao, 2008*). In our research, the change of mitochondrial membrane potential was investigated with double-staining experiment (PI and Rh123) by flow cytometry. The experiments showed that the mitochondrial membrane potential decreased following the increase of concentration of quinoxalone. When the cells were treated with quinoxalone at the concentration of 10 $\mu\text{g/mL}$, we observed that the percentages of PI⁺Rh123⁺ decreased from 93.6% to 65.9%, the percentages of PI⁺Rh123⁺ increased from 5.35% to 28.7%. The results suggested that the mitochondrial apoptotic death-signal pathway plays a critical role in quinoxalone -induced apoptosis in B16 cells.

Apoptosis is a tightly regulated process and its mechanisms involve in mainly two signaling pathways, including cell death receptor pathway and mitochondrial pathway (*Reed, 2001*). Apoptosis is a cell death process that plays a critical role in mammalian development and tissue homeostasis. It has now become clear that apoptosis is also the mechanism of tumor cell death in response to a variety of chemotherapeutic agents. The Bcl-2 family of proteins plays a key role in the regulation of apoptosis. Some members of this family, including Bax, Bak, Bid, and Bik, function as proapoptotic factors, and others, including Bcl-2, Bcl-xL, Mcl-1 and A1, function as antiapoptotic proteins (*Marc and Mark, 2015*). In this study, quinoxalone increased the expression level of Bax protein, but significantly decreased the expression of Bcl-2 protein in B16 cells.

P53, an anti-oncogene, is related to cancer development and progression by its regulation of the tumor cell cycle when DNA is damaged or stressed. P53 functions primarily as a transcription factor, which exerts its downstream functions by activating or repressing a large number of genes that mainly initiate one of three programs of cell cycle arrest, DNA repair or apoptosis (*Shu et al., 2007*). In this study, we found that P53 protein expression level changed

277 slightly after treating with quinoxalone.

278 Apoptosis is caused by activation of intracellular proteases, known as caspases, which are
 279 responsible directly or indirectly for the morphological and biochemical events that characterize
 280 the apoptotic cell. Related references revealed that caspase-3, is essential for DNA fragmentation,
 281 the morphological change associated with apoptosis, and its activation represents a key and
 282 irreversible point in the development of apoptosis (*Janicke et al., 1998; Eva et al., 2014*). To
 283 study further insight into the quinoxalone bioactivity, we checked the activity of caspase-3 in B16
 284 cells. Results showed that quinoxalone enhanced caspase-3 enzymic activity in a time and
 285 concentration dependent manner. This result suggested that the mechanism of quinoxalone-
 286 induced apoptosis in B16 cells probably involved caspase-3 activation.

287 CONCLUSION

288 In this study, we have confirmed that quinoxalone has potent anti-tumor bioactivity, arrest cell
 289 cycle and induce apoptosis in B16 cells. The induction of apoptosis was associated with the
 290 increase of mitochondrial transmembrane potential, Bax and caspase-3 expression level, the
 291 decrease of Bcl-2 expression level. Nevertheless, further studies are needed to clarify the cellular
 292 signaling process which quinoxalone induces apoptosis in B16 cell. This makes quinoxalone
 293 interesting for further investigations as a potential anti-cancer drug.

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296 **Competing Interests**

297 The authors declare there are no competing interests.

298 **Author Contributions**

299 Dahong Wang analyzed the data and wrote the paper.

300 Lanlan Wei performed the experiments, prepared figures.

301 Shuaiying Zhang performed the experiments.

302 **Data Availability**

303 The following information was supplied regarding data availability:

304 The raw data in the study has been supplied as a Supplemental File.

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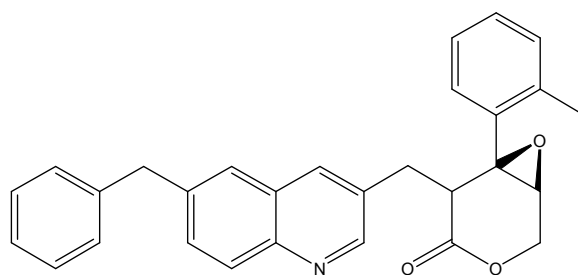
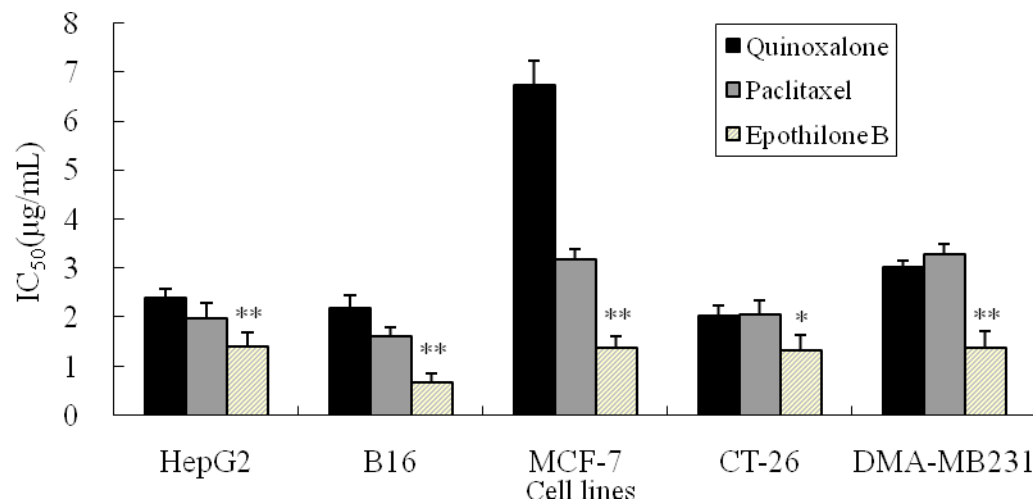


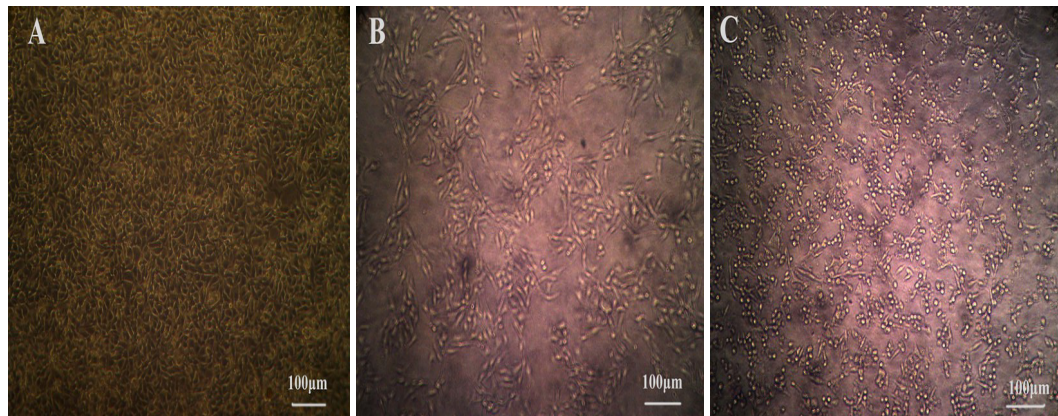
Figure1 The structure of quinxalone



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404 Figure 2 Cytotoxicity of quinoxalone to different tumor cell lines. B16, CT-26, HepG2, MCF-7
 405 and DMA-MB231 cells were incubated for 24 h and were treated with different concentration of
 406 quinoxalone for another 48 h. Taxol and Epothilone B were the positive controls. The
 407 inhabitation rate and IC₅₀ value were calculated. Data were obtained from three repeat
 408 experiments.

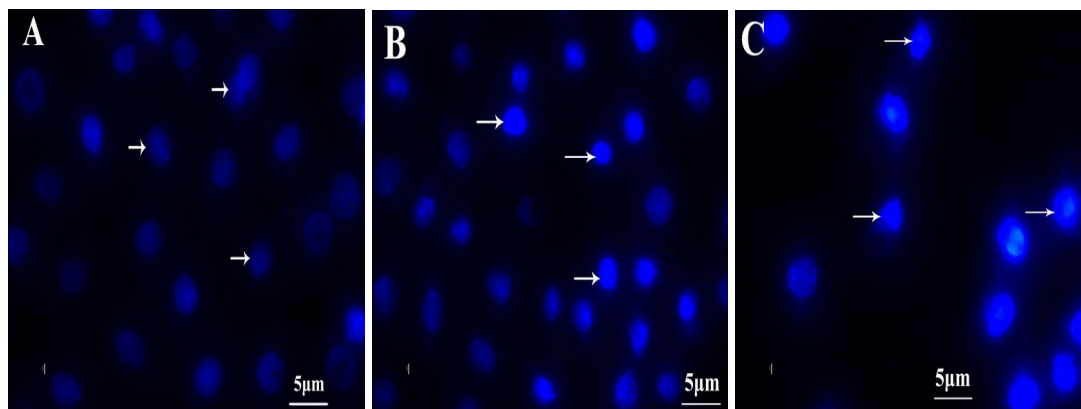
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411 Figure 3 The influence of quinoxalone on B16 cells observed by inverted microscope ($\times 100$).
 412 B16 cells were plated onto glass cover slips in 6-well plates and treated with quinoxalone (A:
 413 control, B: 5 $\mu\text{g}/\text{mL}$, C: 10 $\mu\text{g}/\text{mL}$) for 48 h. Then, cells were washed twice with PBS, fixed
 414 with 1% glutaraldehyde, stained with Hoechst 33342 for 15 min at room temperature. Nuclear
 415 morphology was examined by fluorescence microscope.

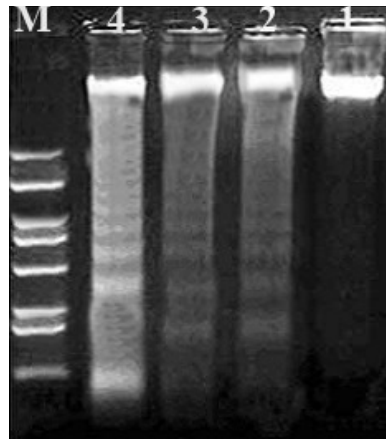
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418 Figure 4 Fluorescence micrographs of B16 cells stained with Hoechst 33342. B16 cells were
 419 treated without (A) and with quinoxalone (B: 5 µg/mL, C: 10 µg/mL) for 48 h. White arrow were
 420 the normal cells in A. White arrow were the apoptosis cells in B and C.

421



422

423 Figure 5 Effect of quinoxalone on DNA of B16 cells. B16 cells were treated with different dose
 424 of quinoxalone for 48h. Isolated DNA was analysed in agarose gel electrophoresis as described in
 425 Material and Methods. 200 bp DNA ladder marker (novoprotein, China) was used as marker (M)
 426 of DNA fragment size. Lane 1: Control; lane 2: 2.5 µg/mL, lane 3: 5 µg/mL; lane 4: 10 µg/mL;
 427 M: marker.

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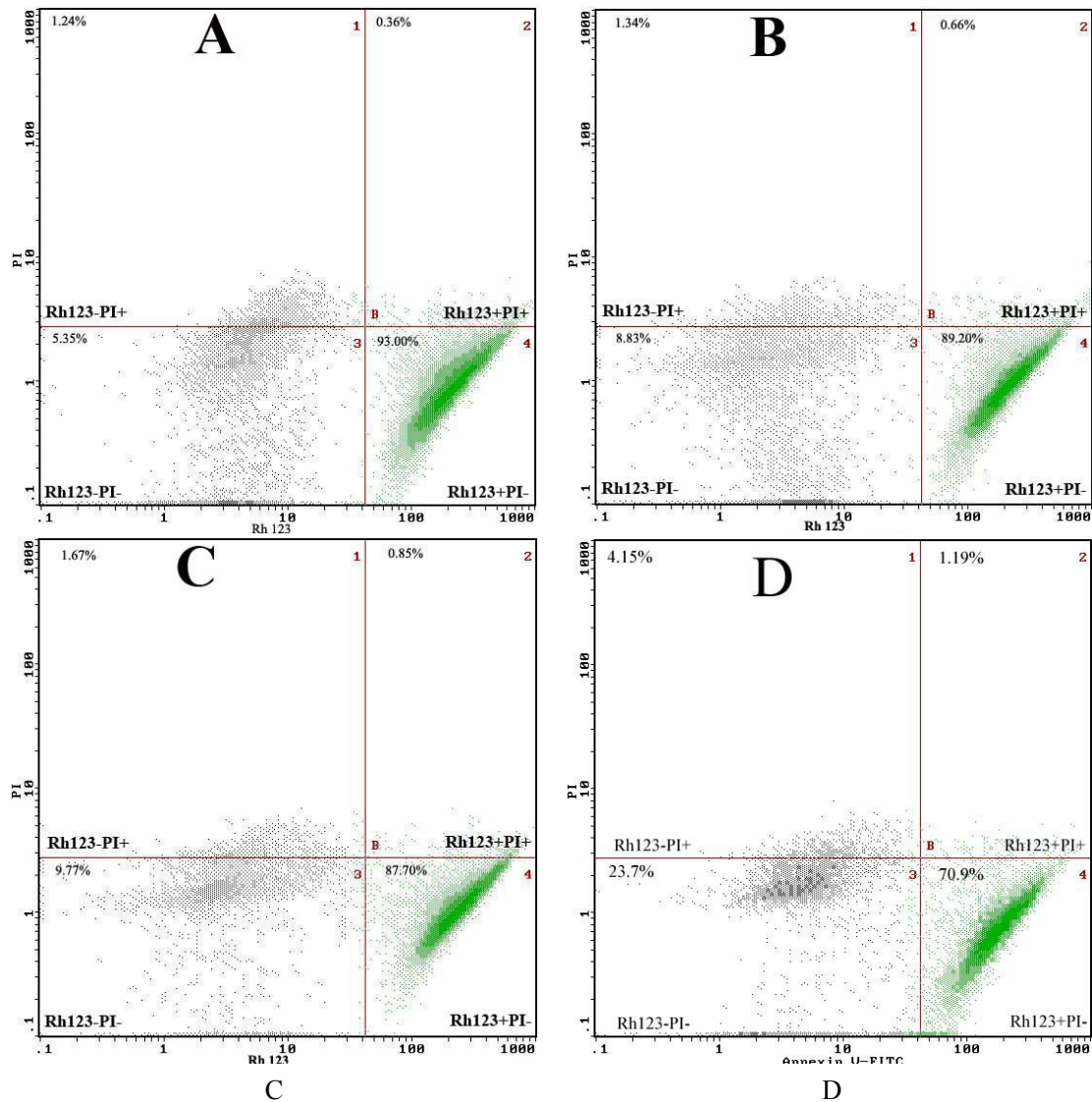
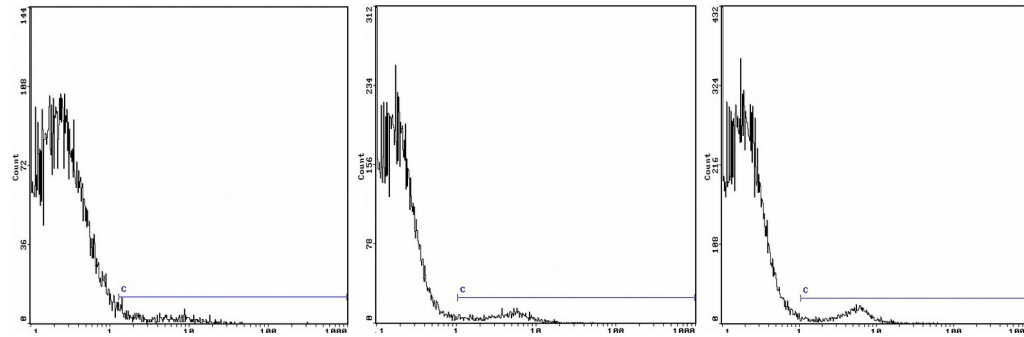
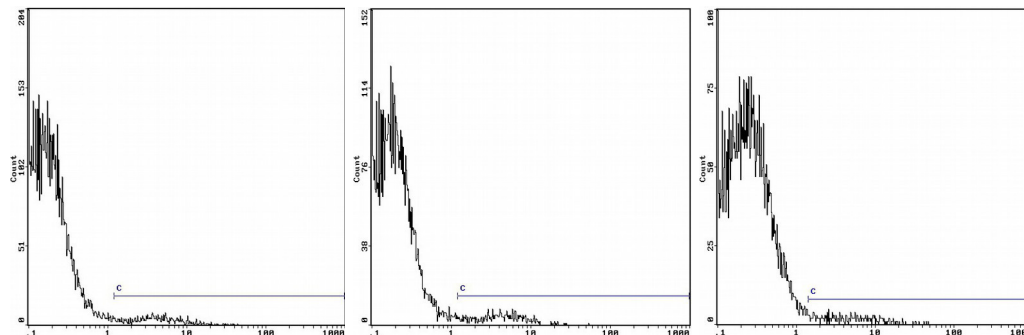


Figure 6 Effect of quinoxalone on mitochondrial transmembrane potential in B16 cells. After treatment without (A, control) and with 2.5 µg/mL (B), 5 µg/mL (C), 10 µg/mL (D) quinoxalone for 48 h, the cells were double-stained with Rhodamine-123 and PI for 30 min, respectively. The percentages of PI negative and low-staining (Rh123-PI-) group represent the apoptotic cell group.

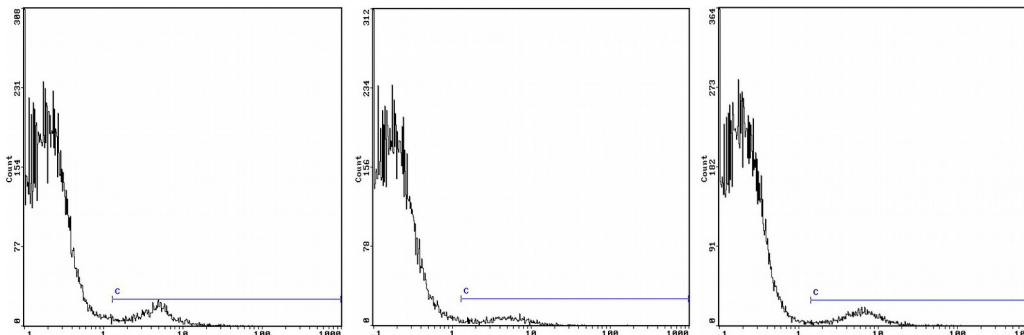
433 A



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435 B



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437 C

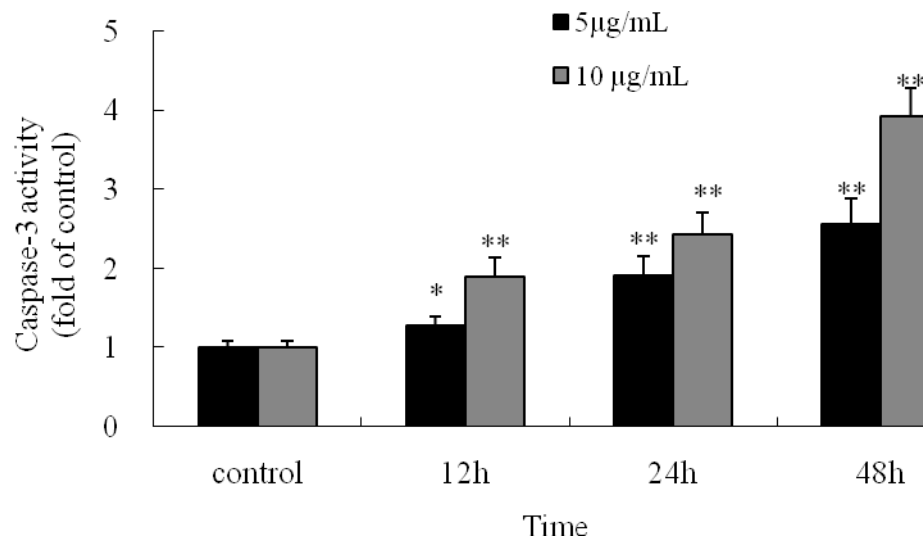


Control 5 µg/mL 10 µg/mL

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439

440 Figure 7 Effect of quinoxalone on the expression of Bax, Bcl-2 and P53 protein in B16 cells.
441 Cells were treated without (control) and with 5, 10 µg/mL quinoxalone for 48 h. After washing
442 with 75% ethanol, the cells were respectively incubated with anti-Bcl-2 antibody, anti-Bax
443 antibody and anti-53 antibody. Then, the cells were incubated with FITC-conjugated secondary
444 goat anti-mouse IgG. Bax (A), Bcl-2 (B) and p53 (C) levels were checked by flow cytometry.



445

446 Figure 8 Effect of quinoxalone on the activation of Caspase-3 in B16 cells. Cells were treated
 447 with 5 and 10 µg/mL quinoxalone for 12, 24 and 48 h. The levels of caspase-3 activities were
 448 evaluated using specific fluorogenic substrates. Data are means \pm SD of three repeat experiments.
 449 Significant differences with control were designated as * $p < 0.05$, ** $p < 0.01$.