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ORIGINAL ARTICLE

Thiocoraline mediates drug resistance in MCF-7 cells via PI3K/Akt/BCRP signaling pathway

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Abstract Thiocoraline, a depsipeptide bisintercalator with potent antitumor activity, was first isolated from marine actinomycete *Micromonospora marina*. It possesses an intense toxicity to MCF-7 cells at nanomolar concentrations in a dose-dependent manner evaluated by MTT assay and crystal violet staining. We established a human breast thiocoraline-resistant cancer subline of MCF-7/thiocoraline (MCF-7/T) to investigate the expression variation of breast cancer resistance proteins (BCRP) and its subsequent influence on drug resistance. Colony-forming assay showed that the MCF-7 cells proliferated faster than the MCF-7/T cells in vitro. Western blot analysis demonstrated that thiocoraline increased the phosphorylation of Akt. Additionally, the sensitivity of tumor cells to thiocoraline was reduced with a concurrent rise in phosphorylation level of Akt and of BCRP expression.These studies indicated that thiocoraline probably mediated the drug resistance via PI3K/Akt/BCRP signaling pathway. MK-2206 dihydrochloride, a selective phosphorylation inhibitor of Akt, significantly decreased MCF-7 cell viability under exposure to thiocoraline compared to the control. However, it was not obviously able to decrease MCF-7/T cell viability when cells were exposed to thiocoraline.

Keywords Thiocoraline · MCF-7 · Multidrug resistance · BCRP · PI3K/Akt/BCRP signaling pathway · MK-2206 dihydrochloride

Jin Jin and Yujia Zhao have contributed equally to this work.

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Introduction

Thiocoraline, a depsipeptide bisintercalator with potent antitumor activity and antibiotic activity against Gram-positive bacteria, was first isolated from the mycelial cake of a marine actinomycete strain L-13-ACM2-092, *Micromonospora marina* (Baz et al. 1997; Perez et al. 1997). It has shown antineoplastic activity against human cancer cells, such as

leukaemia, non-small cell lung cancer, colon, melanoma, breast, neuroendocrine cancer at nanomolar concentrations in vitro (Erba et al. 1999; Negri et al. 2007; Sohn et al. 2012). Furthermore, thiocoraline demonstrated a strong cytotoxic effect on human carcinoma xenografts in vivo (Faircloth et al. 1997; Wyche et al. 2014). As a DNA-binding drug, thiocoraline binds to DNA through the insertion of two planar chromophores between the bases, resulting in unwinding of duplex DNA (Boger et al. 2001). Previous research has shown that in LoVo and SW620 cells, thiocoraline directly inhibited DNA polymerase—α activity to prevent DNA elongation (Erba et al. 1999). Thiocoraline blocked cell proliferation by arresting cells in G1 phase of the cell cycle and decreased the rate of S phase progression towards G2/M phases (Erba et al. 1999). In addition, thiocoraline decreased neuroendocrine tumor markers (CgA, ASCL1) expression and mediated growth inhibition via apoptosis (Sohn et al. 2012). Recently, it was shown that thiocoraline activated the Notch pathway in MTC-TT, BON cells and changed the expression of downstream targets of the Notch pathway, resulting in a potent therapeutic effect (Tesfazghi et al. 2013; Wyche et al. 2014). However, there is few researches demonstrated the issue of potential thiocoraline resistance in vitro and in vivo. Currently it becomes a big challenge for the breast cancer chemotherapy because many chemotherapy medicines produce drug resistance.

Based on our previous research (Ying et al. 2013; Zheng et al. 2014), we studied the drug resistant mechanism of thiocoraline in breast cancer cell line MCF-7. Here we report the establishment of the human breast cancer thiocoraline-resistant subline MCF-7/T, the investigation of the expression variation of breast cancer resistance proteins, and its influence on drug resistance.

Materials and methods

Cell culture

Human breast cancer cell line MCF-7 and normal human hepatocyte cell line L-02 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biowest, Ru du Vieux Bourg, France), 100 U/mL penicillin and 100 mg/mL streptomycin (Beyotime Biotechnology, Shanghai, China) at 37 °C in a humidified 5% CO_2 (Thermo, Waltham, MA, USA) incubator.

Thiocoraline and other reagents

Thiocoraline was supplied by Dr. Williams Fenical group in Scripps Institution of Oceanography. University of California San Diego (San Diego, CA, USA). MK-2206 dihydrochloride was obtained from Topscience Guangzhou, China.

Cell viability assay

Normal cell lines and tumor cell lines were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated in the presence of the medicine at the indicated concentration and time. The rate of cell viability was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO, USA) assay at the absorbance value of 490 nm (Wang et al. 2015a, b). The rate was calculated according to the formula below: cell survival = (absorbance value of treated cells—blank)/(absorbance value of untreated cells—blank).

Crystal violet staining

MCF-7 cells were seeded in the 24-well plates at a density of 5×10^4 cells/well and incubated with the medicine at the indicated concentration for 48 h. Discarded supernatant, and then cells were stained for 15 min with 1% crystal violet solution at room temperature (Sigma, St. Louis, MO, USA).

Western blot analysis

The total protein was separated by SDS/PAGE and transferred to a PVDF membrane (Millipore Corp., Bedford, MA, USA) after cell lysis (Posch et al. 2013). Expression levels of Akt (Cell Signaling Technology, Danvers, MA, USA), p-Akt (CST,USA), BCRP (Abcam, Cambridge, UK), β-actin (Huabio, Shanghai, China) proteins were detected by enhanced chemiluminescence (Posch et al. 2013).

MCF-7/T construction

Thiocoraline-resistant subline MCF-7/T was established from the human breast cancer cell line MCF-7. MCF-7 cells were exposed to thiocoraline from 20 nmol/L increased to 540 nmol/L in 48 h. The 50% inhibitory concentration (IC_{50}) of previously selected cells was determined. Cells were incubated in the presence of thiocoraline at 0.2 IC_{50} value of concentration to maintain drug resistance. The resulting cell line was named as MCF-7/T.

Clonogenicity assay

MCF-7 cells and MCF-7/T cells were seeded in 24-well plates at a density of 300 cells/well. The colonies were grown by incubation in fresh medium for 14 days and stained with 1% crystal violet solution for 15 min at room temperature.

MCF-7/Akt1 construction

A 1443 bp Akt1 cDNA (XM_005267401.1) was inserted into the pLJM1 vector, forming a recombinant plasmid pLJM1-Akt1. Then, pLJM1-Akt1, psPAX2, pMD2.G were co-transfected into 293T cells at a mass ratio of 10:5:3 by lipofectamine (Qiagen, Germantown, MD, USA) to produce a recombinant lentivirus. The recombinant plasmid was purified by the plasmid extraction kit according to the kit's instruction (GENEray Biotech, Shanghai, China). The supernatant was recovered for 48 h after transfection, centrifuged at 2000 rpm, and filtered through a 0.45 μm microfiltration membrane to collect lentiviruses. MCF-7 cells were seeded in a 6 cm dish, and 1 ml of recovered lentivirus at a confluency of 50% was added, 12 h later 1.3 mL of lentivirus was added again to duplicate the infection well. After 24 h, the lentiviral solution was discarded and MCF-7 cells were incubated with normal medium for 48 h. The MCF-7/Akt1 cells were selected and enriched with 0.6 μg/mL puromycin.

RT-PCR and Real-Time qPCR

Total RNAs were extracted from 3×10^5 cells with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized with 2 μg total RNA by ReverTra Ace qPCR RT Kit (TOYOBO, Kyobashi, Japan). For quantitative analysis, all gene transcripts were determined by real-time PCR and two-step RT-PCR using SYBR Green (TOYOBO, Kyobashi, Japan). The transcript level of gene was calculated with the $2^{(-\Delta\Delta Ct)}$ method. GAPDH was used as an endogenous control. Primer sequences showed in Supplementary Table S1.

Statistical analysis

All the data were presented as means and S.D.s for the indicated number of independent experiments. The comparison of two experimental groups was done by the two-way Student's t test and the nonparametric Mann-Whiteney test. Statistical analysis was conducted using the GraphPad Prism statistical software version 6.0 (GraphPad Software, San Diego, CA, USA).

Results

Thiocoraline inhibits MCF-7 cells proliferation

We detected the toxicity of thiocoraline to MCF-7 cells by MTT analysis. The results showed high antitumor capacity at nanomolar concentrations in a dose-dependent manner (Fig. 1a). The IC_{50} value of thiocoraline to MCF-7 cells was 53.8 ± 2.53 nmol/L in 48 h. An intercalator doxorubicin (Sigma, USA) was used as positive control. Doxorubicin also inhibited MCF-7 cells proliferation in a dose-dependent manner at micromolar concentrations (Fig. 1b). The IC_{50} value of doxorubicin to MCF-7 cells was 3.983 ± 0.453 μmol/L in 48 h. Crystal violet staining showed similar result as that of the MTT assay (Fig. 1c). MCF-7 cells were more sensitive to thiocoraline than to doxorubincin under the same drug concentration. Thiocoraline had lower toxicity effect on the normal liver cell line L-02 as compared to the MCF-7 cells (Fig. 1d).

Thiocoraline increases phosphorylation of Akt in MCF-7 cells

To investigate the drug resistance of MCF-7 cells to thiocoraline, we established thiocoraline-resistant subline MCF-7/T by gradually increasing the concentration of thiocoraline in the medium ranged from 20

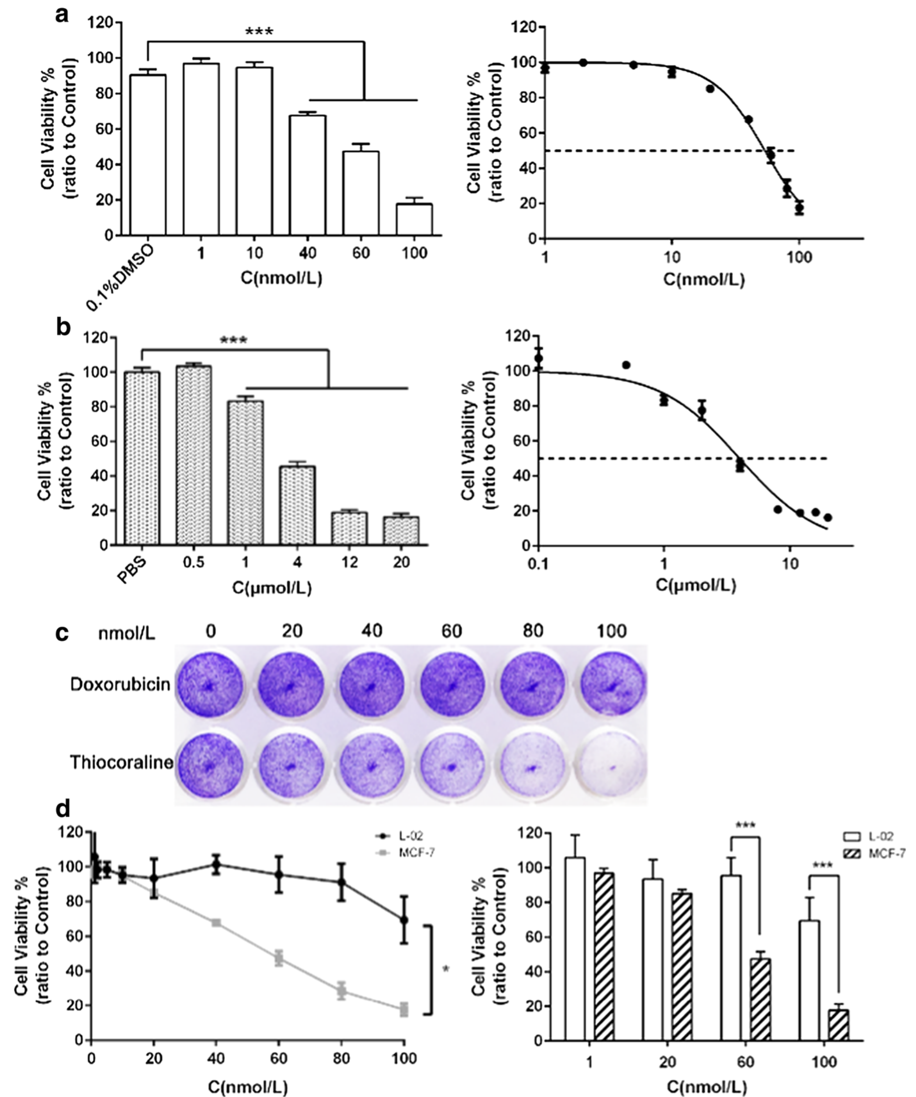

Fig. 1 The toxicity of thiocoraline to cells **a** cell viability percentages and IC_{50} value of MCF-7 after being incubated in thiocoraline for 48 h, **b** cell viability percentages and IC_{50} value of MCF-7 after being incubated in doxorubicin for 48 h, **c** crystal violet staining of MCF-7 cells after being incubated in doxorubicin/thiocoraline for 48 h, **d** Cell viability percentages of L-02 and MCF-7 after being incubated in thiocoraline for 48 h. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

to 540 nM with a gradient of 20 nM (Fig. S1). MCF-7/T cells were selected based on continuous exposure to thiocoraline in a dose escalation way. Colony-forming assay showed that MCF-7 cells proliferated faster than MCF-7/T cells (Fig. 2a).

Assay of RT-PCR and Real-Time qPCR revealed that mRNA expression levels of Akt1 and BCRP increased significantly in MCF-7/T cells but mRNA expression level of mrd1 unhanged (Fig. 2b). However, mRNA expression of Akt1 and BCRP in MCF-7 cells almost remained the same compared to the control after being incubated in thiocoraline for 48 h (Fig. 2d). These results implied that the mRNA level of Akt1 and BCRP increased due to long period of treatment with thiocoraline. Further research found that protein expression levels of Akt, p-Akt and BCRP were all up regulated in MCF-7/T cells, especially the Akt phosphorylation level (Fig. 2c). Consistent with our findings in RNA level, expression of Akt and BCRP protein remained unchanged when MCF-7 cells were incubated in different concentrations of thiocoraline (Fig. 2e). Level of phosphorylation of Akt was up regulated at the nanomolar concentrations of thiocoraline in a dose-dependent manner (Fig. 2e).

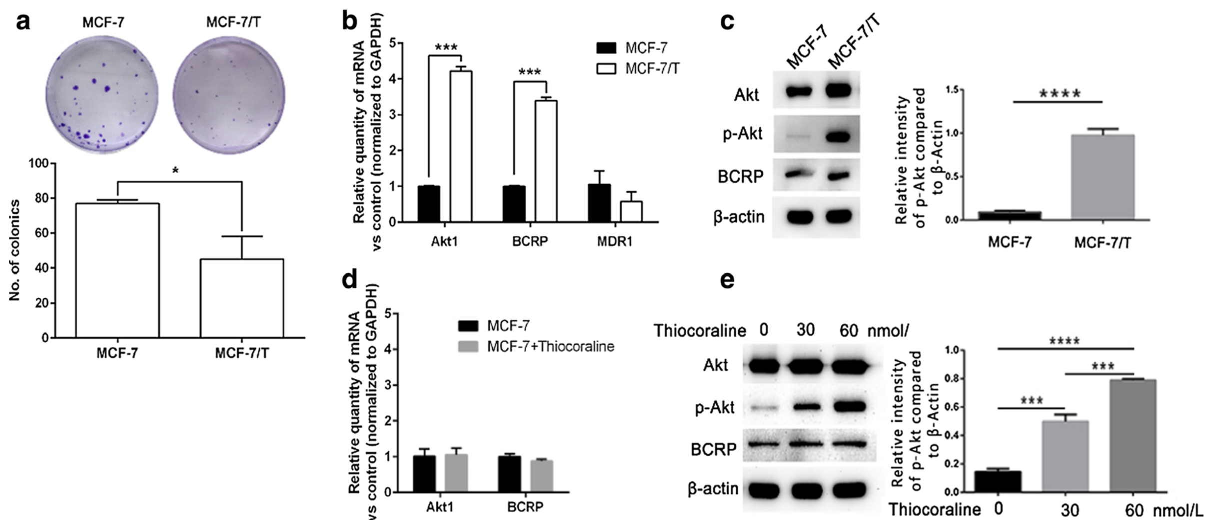

Fig. 2 Thiocoraline increased phosphorylation of Akt **a** colony-formation ability of MCF-7 cells and MCF-7/T cells were tested by Clonogenicity assay. **b** RT-PCR and Real-time qPCR assay. mRNA expressions of Akt1 and BCRP in MCF-7/T cells were all significantly increased compared to MCF-7. mRNA expression of MDR1 almost remained the same in MCF-7/T cells and MCF-7 cells. **c** The protein expressions of Akt, p-Akt and BCRP in MCF-7 and MCF-7/T cells were detected by Western blotting. β-actin was used as internal control. The expressions of Akt, p-Akt and BCRP all increased in MCF-7/T cells compared to control. **d** mRNA expressions of Akt1 and BCRP in MCF-7 cells were tested after being incubated in 30 nmol/L of thiocoraline for 48 h and almost remained unchanged. **e** The protein expressions of Akt, p-Akt and BCRP in MCF-7 were detected by Western blotting after being incubated in 0, 30,60 nmol/L thiocoraline for 48 h. The expression of P-Akt increased as the dose of drug increased (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$)

Inhibition of Akt phosphorylation increases sensitivity of MCF-7 cells to thiocoraline

We examined whether phosphorylation of Akt would affect the sensitivity of MCF-7 cells to thiocoraline. Western blot assay showed that Akt expression in 293T/Akt1 was higher than in 293T/WT (Fig. S2). It meant that recombinant pLJM1-Akt1 lentivirus was successfully packaged in 293T cells. MCF-7 cells with Akt1 overexpression (MCF-7/Akt1 cell) were constructed by incubating recombinant pLJM1-Akt1 lentivirus with MCF-7 cells. Apparently higher level of Akt phosphorylation as well as BCRP was observed in MCF-7/Akt1 cells compared to normal MCF-7 cells (Fig. 3a, Fig. S4). MTT assay revealed higher cell viability of MCF-7/Akt1 cells compared to the normal MCF-7 cells after the treatment with thiocoraline at concentrations of 1, 20, 40, 60 nmol/L, respectively (Fig. 3b).

MK-2206 dihydrochloride, a highly selective phosphorylation inhibitor of Akt1/2/3, was nontoxic to MCF-7 cells at the concentration of 0–100 nmol/L (Fig. S3a). To investigate the effect of MK-2206 on the drug sensitivity of MCF-7 cells to thiocoraline, MCF-7 cells were exposed to 65 nmol/L MK-2206 for 72 h. The result suggested that Akt phosphorylation was almost completely inhibited by MK-2206 (Fig. 3c). Meanwhile, 65 nmol/L MK-2206 pretreatment significantly decreased viability of MCF-7 cells under the exposure to thiocoraline compared to the control (Fig. 3d). It implied that the sensitivity of MCF-7 cells to thiocoraline was increased by inhibiting Akt phosphorylation.

MK-2206 dihydrochloride pretreatment increases the sensitivity of MCF-7/T cells to thiocoraline

MK-2206 also significantly blocked expression of Akt phosphorylation in MCF-7/T cells compared to the control (Fig. 4a). We further examined whether inhibition of Akt phosphorylation could improve the sensitivity of MCF-7/T after MK-2206 pretreatment. MTT assay indicated that MCF-7/T cells with 65 nmol/L MK-2206 pretreatment decreased cell viability as compared to the control when both of them were treated with the same concentration of thiocoraline (Fig. 4b).

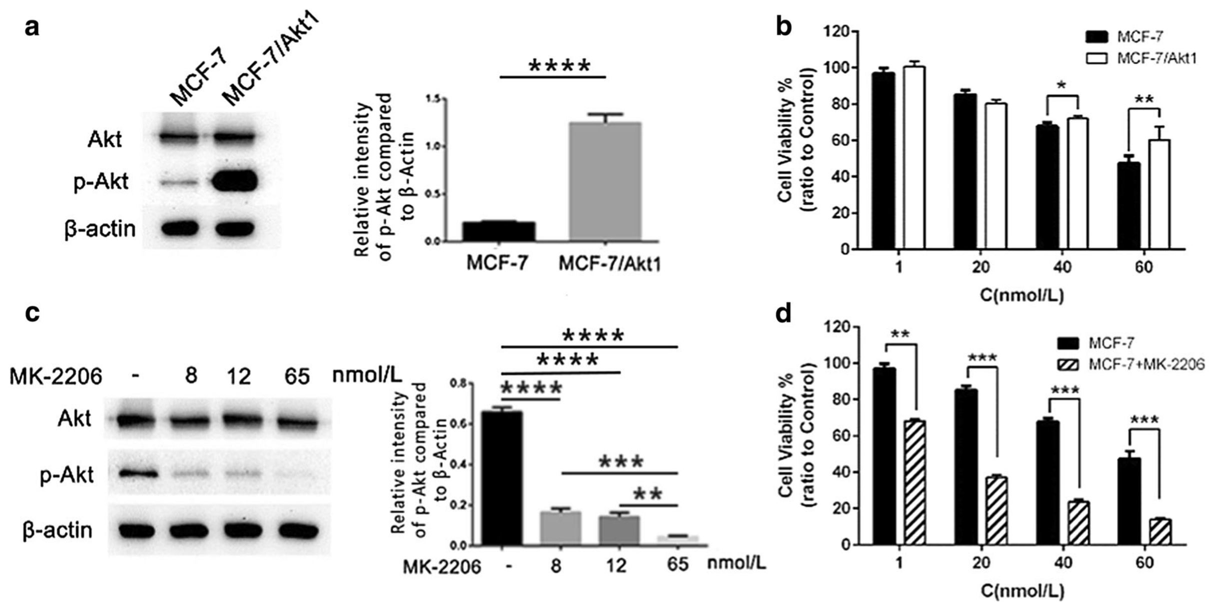

Fig. 3 The sensitivity of MCF-7 cells to thiocoraline was related to phosphorylation degree of Akt **a** p-Akt high expression in MCF-7/Akt1 cell lines was detected by Western blotting. The expression of p-Akt was significantly increased. **b** Cell viability percentages of MCF-7 and MCF-7/Akt1 were tested by MTT. MCF-7 and MCF-7/Akt1 cells were incubated in 1, 20, 40, 60 nmol/L thiocoraline for 48 h. **c** Inhibition of Akt phosphorylation in MCF-7 cells by MK-2206 dihydrochloride was detected by Western blotting. MCF-7 cells were incubated in 0, 8,12, 65 nmol/L MK-2206 dihydrochloride for 72 h. The expression of p-Akt was decreased as the dose of inhibitor increased. **d** Cell viability percentages of MCF-7 was tested by MTT, MCF-7 cells were incubated in 1, 20, 40, 60 nmol/L thiocoraline for 72 h after 65 nmol/L MK-2206 dihydrochloride pretreatment. ($*P < 0.05$, $**P < 0.01***P < 0.001$, $****P < 0.0001$)

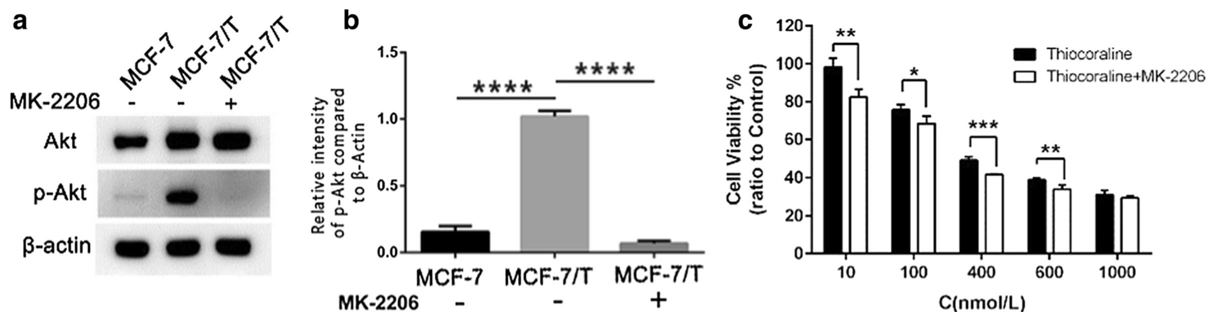

Fig. 4 Phosphorylation inhibition of Akt increased the sensitivity of MCF-7/T cells to thiocoraline **a** Akt and p-Akt expression of cells were detected by Western blotting. The expression of Akt in MCF-7/T with/without MK-2206 pretreatment all increased. Phosphorylation of Akt was significantly inhibited by 65 nmol/L MK-2206 dihydrochloride pre-treated MCF-7/T cells. **b** Cell viability percentages of MCF-7/T were tested by MTT. MCF-7/T cells were incubated in 10, 100, 400, 600, 1000 nmol/L thiocoraline for 72 h after 65 nmol/L MK-2206 dihydrochloride pretreatment. ($*P < 0.05$, $**P < 0.01***P < 0.001$, $****P < 0.0001$)

Discussion

The poor pharmacokinetics, side effects and particularly rapid emergence of drug resistance compromise the efficiency of anticancer drugs (Tandon et al. 2017). Therefore, the quest of novel and efficient agents is still an extremely critical mission. Marine organism has evolved chemical means in order to defend themselves against the complex environment (Gammone et al. 2016). Such chemical and biological adaptation generates structural diversity of compounds with various biological activities and potential

anticancer effect (Yue et al. 2017). Up to 2012, more than 22,000 natural products from marine source have been reported (Gerwick and Moore 2012). The discovery of selective and potent therapeutic activity agents from marine natural products can drive developments of novel anticancer drug in the future. Drug resistance is still a big challenge for efficient breast cancer therapy. Our study presents a new perspective in the investigation of the mechanism of drug resistance of marine derived compound—depsipeptide thicoraline.

Thiocoraline is a bisintercalators, inserting into the DNA helix to interfere with DNA replication (Camunas-Soler et al. 2015), and possesses broad-spectrum anticancer activities thanks to its property of being an effective bisintercalator (Dawson et al. 2007). Echinomycin is the first bisintercalator that showed antitumor activity in Phase I clinical trials (Harvey et al. 1985). However, the Phase II trials were uniformly disappointing, with minimal to no activity against cervical squamous cell carcinoma (Dawson et al. 2007). Both thiocoraline and echinomycin work by inhibiting the hypoxia inducible factor-1 (HIF-1) (Kong et al. 2005; Vippila et al. 2015), which bind to hypoxia response elements (HRE), resulting in the suppression of the growth of cancer cells (Wang et al. 2011). Thiocoraline is now moving close to clinical development (Dawson et al. 2007).

Thiocoraline binds to DNA with high affinity but has little or no sequence selectivity (Boger et al. 2001) and inhibits DNA polymerase α (Erba et al. 1999). To investigate the potentials of being developed into an antitumor agent, we first evaluated the toxicity aspect of thiocoraline. In the MTT assay in vitro, we showed that thiocoraline had a potent toxicity to the breast cancer cells but low toxicity to the normal liver cells.

To investigate the antitumor potency of thiocoraline, we used doxorubicin as the control drug, which is a widely used DNA-binding chemotherapeutic agent for treatment of solid tumors and leukemia. Doxorubicin acts on the DNA by causing inhibition of enzyme topoisomerase and macromolecular biosynthesis (Fornari et al. 1994; Momparler et al. 1976). The MTT assay and crystal violet staining demonstrated that the breast cancer MCF-7 cells were more than ten times sensitive to thiocoraline than to doxorubicin with an IC_{50} value of 53.8 nmol/L versus 3.983 μmol/L. These results indicate that thiocoraline might have a brighter prospect in clinical trials than doxorubicin and echinomycin.

The specific mechanisms of multidrug resistance for breast cancer still are not very clear. In tumor cell lines, multidrug resistance is often associated with cell-cycle checkpoint transformation (Kandel et al. 2001), abnormal transduction of signaling pathways (Kruh 2003), anti-apoptotic effect (Hong et al. 2010), P-glycoprotein for drug efflux from the brain (de Gooijer et al. 2018), multidrug resistance-associated protein (Nakanishi and Ross 2012), and breast cancer resistance protein (Brechbuhl et al. 2010).

We further studied the potential mechanism of the antitumor effects of thiocoraline in vitro in the thiocoraline-resistant breast cancer cell line MCF-7/T. In these cells, the mRNA and protein expression level of Akt, p-Akt and BCRP increased while their expression remained unchanged in the regular cell line. PI3K/Akt pathway plays an important role in cell physiology and associates with drug resistance in breast cancer (Guerrero-Zotano et al. 2016; Wang et al. 2015a, b). Akt, a serine/threonine kinase with three isoforms Akt1, Akt2 and Akt3, is a downstream target of PI3K (Engelman 2009). Breast cancer resistance protein (BCRP) is an ATP-binding cassette (ABC) transporter to protect cells by excreting its substrates out of cells. One report suggests that Akt-induced ABCG2 activation results from its transport to the plasma membrane (Bleau et al. 2009).

Taking this information and our data into account, we hypothesize that thiocoraline probably mediate the drug resistance in MCF-7 cells via PI3K/Akt/BCRP signaling pathway. The mechanisms of the function of BCRP in thiocoraline- resistance and the possible role of BCRP in PI3K/Akt signaling pathway remain unknown and will be studied the next.

In this study, we have demonstrated that the inhibition of phosphorylation of Akt in the drug-resistant MCF-7/T cells by pretreatment with its inhibitor MK-2206 dihydrochloride renewed the sensitivity of MCF-7/T cells to thiocoraline. Our research provides a potential possibility to combat thiocoraline resistance by combination of Akt inhibitor MK-2206.

Regards to the mechanism of BCRP, it was previously shown that PI3K inhibitors LY294002 and wortmannin induced internalization of BCRP (Takada et al. 2005). Another report showed that LY294002, but not wortmannin, reversed the BCRP-

mediated SN-38 and topo-tecan resistance (Imai et al. 2012).

Thiocoraline is currently undergoing preclinical pharmacodynamic evaluation by PharmaMar and promising to get approval to enter Phase I clinical trials (Dawson et al. 2007). Further research of the mechanisms of thiocoraline resistance in breast cancer cells involved in PI3K/Akt/BCRP signaling pathway will help the clinical studies and applications of thiocoraline in breast cancer treatment. It may provide further evidence that targeting the PI3K/Akt/BCRP pathway may be a logical strategy for overcoming resistance to breast cancer therapy.

Conclusion

In this in vitro study, thiocoraline inhibited breast cancer cells proliferation with low toxicity to normal liver cells. Gene expression and protein levels of Akt, p-Akt and BCRP were all up-regulated in thiocoraline-resistant cells. Thiocoraline also induced the increase of Akt phosphorylation, and the inhibition of Akt phosphorylation significantly reinforced the sensitivity to thiocoraline. These evidences suggest that thiocoraline probably modulates the multidrug resistance of breast cancer MCF-7 cells via PI3K/Akt/BCRP signaling pathway. We need more studies to be performed related to thiocoraline resistance via PI3K/Akt/BCRP signaling pathway.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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