Carvacrol Promotes Cisplatin-induced Apoptosis of Triple-negative Breast Cancer Through TRPM7 Channels

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Abstract

Triple-negative breast cancer (TNBC) is the most dangerous type of breast cancer. Cisplatin is a chemotherapy agent for solid tumors treatments, but it is subjected to drug resistance. A natural compound, carvacrol, showed anti-cancer potential for TNBC and can be a cisplatin sensitizer. Carvacrol inhibits the transient receptor potential melastatin-like 7 channel (TRPM7), which plays a critical role in cancer apoptosis. In this study, we proposed that carvacrol can promote cisplatin-induced apoptosis of TNBC through TRPM7 channels. We tested this hypothesis using five TNBC cell lines. Cell viability and apoptosis were determined using the MTT and Bax (Bcl-2-associated X protein) ELISA respectively. 4T1/BALB/c mice model was used to test the effect of carvacrol on cisplatin treatment. TRPM7 and Bax expression in the tumors were determined using western blotting. We also tested the effect of TRPM7 knockdown and TRPM7 inhibitor, 2-APB, on carvacrol actions in MDA-MB-231. The TRPM7 channel inhibition by carvacrol was confirmed using patch-clamp and Fura-2-based fluorescence quench assay. Results showed that carvacrol enhanced the suppression of cisplatin and cisplatin-induced apoptosis in all five TNBC cancer cell lines. Carvacrol improved cisplatin treatment in mice model by promoting cisplatin-induced apoptosis in tumors, but did not affect TRPM7 in tumors. Both knockdown and 2-APB inhibition of TRPM7 channels eliminated the effects of carvacrol and 2-APB showed a similar effect as carvacrol. Our study demonstrated that carvacrol is a cisplatin sensitizer in TNBC chemotherapy and TRPM7 channels might play a role in the synergistic effect of carvacrol on cisplatin.

1. Introduction

Breast cancer is the most common cancer type for females and also the second most common cancer type overall in the world [1]. Every year, breast cancer leads to over 2,000,000 cancer cases and results in over forty thousand clinical deaths [2]. Although breast cancer has been studied extensively and the therapy of this disease has achieved a great improvement, breast cancer is still one of the most dangerous diseases with the highest mortality and the worst prognosis [2]. Thus, further study of breast cancer is required to improve clinical breast cancer therapy.

Surgical treatment is one of the most frequently used therapies for solid breast cancer, but patients with surgical treatment also have a high risk of cancer recurrence and metastasis [3, 4]. Chemotherapies are one of the most effective therapies for breast cancer. Triple-negative breast cancer (TNBC) is the most dangerous subtype of breast cancer because patients with TNBC mostly has undesirable prognosis [5]. TNBC accounts for about 10–20% of cases among all breast cancer cases [6]. TNBC does not express three key receptors, including (1) the estrogen steroid receptor (ER), (2) progesterone steroid receptor (PR), and (3) tyrosine kinase human epidermal growth factor receptor 2 (HER2) [7]. As TNBC lack known therapeutic receptors, no therapeutic targets are available for an effective TNBC treatment. Only about half of the TNBC cases respond to common chemotherapies [8, 9]. Studies have shown that most conventional chemotherapies for TNBC treatment are subjected to drug resistance during prolonged treatment [10].

Cisplatin is one of the most frequently used chemotherapy agents in solid tumors treatments [11]. However, cisplatin is also subjected to the issue of drug resistance [12]. Clinical application of cisplatin is also found to result in many side effects and led to worse life quality for patients [13]. Therefore, it is important to search for proper chemotherapy synergists to enhance therapeutic potency and reduce the side effects of cisplatin. A natural compound, carvacrol, has been found to have anti-cancer properties [14–16]. The clinical combination used of cisplatin plus carvacrol has achieved desirable therapeutic outcomes in some cases in our hospital (data not published).

Studies have shown that carvacrol showed anti-cancer potential for breast cancer [17, 18], including some TNBC cell lines [18]. Interestingly, carvacrol was also reported to inhibit the transient receptor potential melastatin-like 7 channel (TRPM7) [19, 20]. Ion channels have been regarded to be critical in cancer development [21–24]. TRPM7, a divalent cations channel [25], is a member of melastatin-like transient receptor potential (TRPM) subfamilies [26] that are expressed in mammalian tissues [27]. TRPM7 is expressed and plays a role in multiple cancers including breast cancer [28], glioblastoma [20], and ovarian cancer [29]. TRPM7 channels have been found to be critical for apoptosis in cancer [30, 31]. In this preclinical study, we proposed that carvacrol can promote cisplatin-induced apoptosis of triple-negative breast cancer through TRPM7 channels. We hope this study can improve understanding and provide more evidence to support the use of carvacrol as a chemotherapy synergists for cisplatin in clinical TNBC treatments.

2. Methods And Materials

2.1. Reagents

Carvacrol, cisplatin, MTT Formazan (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan), protease inhibitor, and TRPM7 inhibitor 2-Aminoethyl diphenylborinate (2-APB) were provided by Sigma-Aldrich (St. Louis, MO, USA). TRPM7 primary antibody (TRPM7 Monoclonal Antibody, S74-25) and RIPA lyse buffer were provided by ThermoFisher Scientific (Waltham, MA, USA). GAPDH primary antibody (Anti-GAPDH antibody, ab8245), Bax (Bcl-2-associated X protein) primary antibody (Anti-Bax antibody, ab3191), and all the secondary antibodies were provided by Abcam (Cambridge, UK). The Lipofectamine® 2000 kit was obtained from ThermoFisher Scientific (Waltham, MA, USA). Pre-designed TRPM7 siRNAs (4390824) and Silencer™ Select Negative Control siRNA (4390846) were provided by ThermoFisher Scientific (Waltham, MA, USA). Fura-2-acetoxymethyl ester (Fura-2) was provided by Abcam (Cambridge, UK).

2.2. Cell lines and cell culture

Triple-negative breast cancer (TNBC) cell lines MDA-MB-231, MDA-MB-157, MDA-MB-453, BT-20, and MDA-MB-468 were provided by ATCC (Washington, USA). Cells were cultured in DMEM supplied with 10% FBS (Gibco, ThermoFisher Scientific) using a humidified 5% CO₂ 37°C cell culture incubator.

2.3. MTT

Cell viability was determined using the MTT assay, which was used previously [32]. Cells were cultured in the FBS-free medium for 24 h for starvation before the exposure of testing agents. At the endpoint of the assay, MTT stock solution (12mm) was added to the cells (40 μ L /well) for 4 h. Then the OD at 490 nm was measured.

2.4. ELISA

The apoptosis level was measured by testing the Bax level, which was used previously [33]. Human Bax ELISA Kit (ab199080) was provided by Abcam (Cambridge, UK). Briefly, cells were lysed in RIPA lyse buffer with protease inhibitor. 50 μ L sample was added to the wells, then Antibody Cocktail was added to the wells, followed by the incubation at room temperature for 1 hour. The plate was washed properly and the TMB solution was added to the plate. After 15 min, the stop solution was added to stop the reaction. OD at 450 nm was measured.

2.5. Western blotting

The western blotting assay was used to analyze protein levels in samples, which was described previously [34]. Briefly, samples were lysed in RIPA buffer with protease inhibitor. SDS gel electrophoresis was used to separate the proteins, followed by the transferring of the proteins onto 0.2- μ m polyvinylidene difluoride membranes. The membranes were incubated with 5% skimmed milk in TBS to block the non-specific binding. Membranes were then incubated with primary (1:2000 diluted in blocking solution, 4°C, overnight) and secondary antibodies (1:5000 diluted in blocking solution, RT, 2 h). ECL was used to visualize the proteins on the membranes.

2.6. siRNA transfection for TRPM7 knockdown

TRPM7 was knocked down by transfecting siRNA into cells. The transfection method was used previously [35]. Briefly, different concentration of TRPM7 siRNAs was mixed with negative siRNAs accordingly to ensure every transfection transfected 20 nM total siRNA. The siRNAs were transfected to cells using Lipofectamine® 2000. The expression of TRPM7 in cells was analyzed by western blotting assay.

2.7. Patch-Clamp Recording

Whole-cell currents were recorded using the patch-clamp recording method, which was described previously [36]. Briefly, the bath solution contained 140 mM NaCl, 25 mM HEPES, 1.3 mM CaCl_2 , 1.0 mM MgCl_2 , and the pH was adjusted to 7.3 using KOH, 320–335 mOsm. The electrode solution contained 140 mM CsF, 7 mM NaCl, 10 mM HEPES and 11 mM EGTA, and the pH was adjusted to 7.3 using CsOH, 300 mOsm. Capacitance and series resistance was compensated. The voltage was ramped from – 80 to + 80 mV in a second. Currents were normalized to current density by dividing whole-cell capacitance.

2.8. Fura-2-based fluorescence quench assay

The TRPM7-mediated Mn^{++} influx was measured with Fura-2-based fluorescence quench assay, which was used previously [37]. Briefly, the tested cells were plated in 96-well plates then loaded with fura-2

loading-buffer (2 mM in bath solution used in patch-clamp) for 20 min at 37 °C. The plate was washed properly and incubated with tested agents in bath solution for 5 min. 10 mM Mn⁺⁺ solution was added and the fluorescence (excitation 360 nm/ emission 510 nm) of fura-2 was measured.

2.9. Orthotopic murine breast cancer models

The orthotopic murine breast cancer model was used to mimic the development and therapy of breast cancer. The model was used in many other studies [38–40]. Briefly, 4T1 cells were injected in the mammary fat pad of female BALB/c mice to induce breast cancer. The mice were evenly allocated to four groups according to their body weight. 4 groups (n = 8) were injected intraperitoneally with the vehicle, 3 mg/kg cisplatin, 200 mg/kg carvacrol, and 3 mg/kg cisplatin + 200 mg/kg carvacrol respectively. The animals were fed until the death of the animals and the survival data of all animals was recorded. In another set of animal experiment, 4T1 cells were injected in the mammary fat pad of female mice and the mice were allocated to four groups according to their body weight. 4 groups were injected intraperitoneally with vehicle (n = 6), 3 mg/kg cisplatin (n = 7), 200 mg/kg carvacrol (n = 6), and 3 mg/kg cisplatin + 200 mg/kg carvacrol (n = 8) respectively. The body weight and tumor volume were measured every three days. All the animals were euthanized at the endpoint of the experiment (day 21) and the net tumor weight was determined. The tumors were collected for western blotting assay. Data of animals that died before the endpoint of the experiment were excluded.

2.10. Statistical analysis

Data are presented as means ± SEM. Student's t-test or one-way analysis was used to analyzed significance. A P-value of 0.05 or lower was considered significant.

3. Results

3.1. IC₅₀ of carvacrol and cisplatin inhibition toward viability of TNBC cell lines.

To determined a proper concentration for this study, we firstly measured the IC₅₀ of carvacrol and cisplatin inhibition toward viability of five TNBC cell lines we used in this study. Cell viability was determined using a common viability assay, MTT assay [41]. After 48 hours exposure, the IC₅₀ of carvacrol for all five TNBC cell lines ranged from 193 – 166 µM. MDA-MB-231 had the highest IC₅₀ and BT-20 had the lowest IC₅₀ (Fig. 1A-E). A previous study reported that carvacrol at 50–500 µM significantly inhibited the viability of multiple breast cancer cell lines and the IC₅₀ was about 500 µM which was consisted with our results. To be mentioned, the IC₅₀ of TRPM7 suppression by carvacrol was reported to be 83 µM [42]. We used carvacrol at 100 µM in the subsequent study because this concentration only slightly inhibited viability and it is enough to inhibit considerable amount of TRPM7 currents according to the previous study [42]. In addition, the IC₅₀ of cisplatin for all five TNBC cell lines were between 43.75 to 56.05 µM. MDA-MB-468 had the lowest IC₅₀ and MDA-MB-453 had the highest IC₅₀ (Fig. 1F-J). A previous

study showed that IC_{50} of cisplatin inhibiting breast cancer cells was about 50 – 30 μ M [43] which was consisted with our results. In this study, we used 40 μ M cisplatin in the subsequent study.

3.2. Carvacrol promoted cisplatin-induced cell viability suppression in TNBC cell lines

We tested whether carvacrol affected cisplatin-induced cell viability suppression in TNBC cell lines. For the control, cisplatin at 5 μ M significantly inhibited the viability of all TNBC cancer cell lines. Compared to cisplatin alone, all cell lines treated with 5 μ M cisplatin plus 100 μ M carvacrol dramatically reduced the cell viability (Fig. 2A-E). This indicated that carvacrol dramatically facilitates the suppression of cisplatin toward cell viability.

3.3. Carvacrol promoted cisplatin-induced cell apoptosis in TNBC cell lines

Since carvacrol dramatically increased the suppression of cisplatin toward cell viability, we proposed that the effect was mediated by cisplatin-induced apoptosis, thus we used ELISA to test the apoptosis biomarker Bax level. For the control, cisplatin at 5 μ M significantly induced the apoptosis of all TNBC cancer cell lines. Compared to cisplatin alone, all cell lines treated with 5 μ M cisplatin plus 100 μ M carvacrol dramatically increased the cell apoptosis (Fig. 2F-J). This indicated that carvacrol dramatically facilitates the apoptosis induction of cisplatin toward TNBC cells. We suggested that the effect of carvacrol on cisplatin might be mediated by apoptosis induction of cisplatin.

3.4. Carvacrol promoted cisplatin-mediated survival improvement of an orthotopic murine breast cancer model.

To further explore the feasibility of carvacrol as a chemotherapy synergist for cisplatin, we tested our hypothesis in an orthotopic murine breast cancer model (Fig. 3A). Firstly, we determined the effect of cisplatin plus carvacrol treatment on animal survival. Results showed that cisplatin significantly improved the survival of breast cancer-bearing animals, while carvacrol further enhanced the effect of cisplatin (Fig. 3B).

3.5. Carvacrol improved cisplatin-mediated suppression of breast cancer development in an orthotopic murine breast cancer model.

In this study, we also focused on the effect of the treatment on the tumor size and body weight. We did another set of animal experiments using the same model and observed the change of tumor size and body weight of the animals survived at day 21 (Fig. 4A). Results showed that cisplatin treatment dramatically decreased the bodyweight of the animals, while cisplatin plus carvacrol treatment only cause a slight reduction in body weight (Fig. 4B). The treatment of cisplatin also significantly suppressed the tumor growth as indicated by the tumor size and tumor volume, while cisplatin plus carvacrol

treatment further decreased the growth of the tumor (Fig. 4C-E). These results indicated that the carvacrol might improve life quality and facilitated the suppression of cisplatin toward breast tumors.

3.6. Carvacrol improved cisplatin-induced apoptosis of breast cancer in an orthotopic murine breast cancer model.

In the in vitro study, we had demonstrated that carvacrol promoted cisplatin-induced cell apoptosis in TNBC cell lines. Here we also analyzed the Bax level in tumor samples to determine the apoptosis of breast cancer cells in tumor tissues. Results showed that cisplatin significantly increased the Bax expression while the cisplatin plus carvacrol further increased Bax expression in tumors (Fig. 4FH). These results demonstrated that carvacrol promoted cisplatin-induced tumor apoptosis in an orthotopic murine breast cancer model.

3.7. TRPM7 mediated current regulation is a potential mechanism for apoptosis regulation of carvacrol.

A previous study has shown that TRPM7 mediated currents were critical for breast cancer cell viability [28]. In this study, we hypothesis that TRPM7 mediated current regulation is a mechanism for carvacrol actions. We analyzed the TRPM7 expression in tumor samples we collected in the animal study. Results showed that the treatment did not affect TRPM7 expression (Fig. 4FG). Hence, we suggested the effect of carvacrol was not mediated by the regulation of TRPM7 expression, but might be mediated by the regulation of TRPM7 currents suppression.

3.8. TRPM7 knockdown in MDA-MB-231.

To further tested whether the TRPM7 plays a role in the enhancement effect of carvacrol on cisplatin suppression, we transfected siRNA to silence TRPM7 expression in MDA-MB-231. The siRNA concentration-dependently reduced TRPM7 level. 0.5, 1, 2, 4, 10, and 20 nM siRNA reduced TRPM7 by 4%, 52%, 64%, 88%, 94%, and 99% respectively (Fig. 5AB). Thus, we used 20 nM siRNA in the subsequent study because this concentration almost completely silenced TRPM7 expression.

3.9. TRPM7 knockdown eliminated the effects of carvacrol

After the TRPM7 knockdown, the carvacrol failed to affect the viability of MDA-MB-231 and also failed to promote the suppression of cisplatin toward MDA-MB-231 viability (Fig. 5C). In terms of apoptosis, after the TRPM7 knockdown, the carvacrol failed to affect the apoptosis of MDA-MB-231 and also failed to increase cisplatin-induced apoptosis in MDA-MB-231 (Fig. 5D). These results indicated that TRPM7 was involved in the actions of carvacrol.

3.10. The inhibition of carvacrol on TRPM7 channels.

The knockdown experiment demonstrated that TRPM7 was involved in carvacrol actions, however, at this point we were not certain whether the effect was mediated by TRPM7 channels or the kinase domain of TRPM7. Therefore, we determined to used a TRPM7 inhibitor, 2-APB, to block the channels. The TRPM7 channel function was determined using both patch-clamp recording and Fura-2-based quench assay. The

patch-clamp recording measured the outward rectification as TRPM7-like currents. Results showed that 2-APB at 200 μ M reduced TRPM7-like currents in MDA-MB-231 by 75% while carvacrol at 100 μ M reduced TRPM7-like currents in MDA-MB-231 by 40% (Fig. 6AB). The Fura-2AM-based quench assay measured the Mn^{++} influx mostly mediated by TRPM7. Results showed that 2-APB at 200 μ M reduced TRPM7 mediated Mn^{++} influx in MDA-MB-231 by 80% while carvacrol at 100 μ M reduced TRPM7 mediated Mn^{++} influx by 50% (Fig. 5EF). These results confirmed that the TRPM7 was significantly inhibited by both 2-APB and carvacrol respectively.

3.11. TRPM7 inhibitor 2-APB eliminated the effect of carvacrol

We repeated the experiment with 200 2-APB. With 2-APB, the carvacrol failed to affect the viability of MDA-MB-231 and also failed to promote the suppression of cisplatin toward MDA-MB-231 viability (Fig. 6E). In terms of apoptosis, with 2-APB, the carvacrol failed to affect the apoptosis of MDA-MB-231 and also failed to increase cisplatin-induced apoptosis in MDA-MB-231 (Fig. 6F). These results indicated that the effects of carvacrol were mediated by TRPM7 channels.

3.12. TRPM7 inhibitor 2-APB showed a similar effect as carvacrol.

As we proposed carvacrol promoted the potency of cisplatin through TRPM7 channels, the blocking of TRPM7 channels by other agents should also promote the potency of cisplatin as carvacrol. As a result, we expected that 2-APB had a similar role as carvacrol. Results verified our hypothesis. Compared to cisplatin alone, 2-APB significantly decreased the viability and also increased apoptosis of MDA-MB-231. This further confirmed that TRPM7 channel function is involved in cisplatin-induced apoptosis in MDA-MB-231.

4. Discussion

Undesirable drug resistance and side effects limit the clinical application of cisplatin in TNBC patients. In our hospital, the clinical application of cisplatin combined with traditional medicine containing carvacrol resulted in favorable outcomes in some TNBC patients. The combination treatment of cisplatin plus carvacrol has shown promising therapeutic potential for TNBC. The main purpose of this study was to collect preclinical evidence to support the application of this combination treatment in clinical TNBC patients. As carvacrol can inhibit the TRPM7 channels [19, 20], we also identified TRPM7 channels as the pharmacological target involved in carvacrol action.

Although the application of cisplatin alone has resulted in significant inhibition toward cancer both in vivo and in vitro, a clinical trial has shown that it had no significant therapeutic effect in TNBC patients

[44]. The drug resistance of cisplatin might account for the insignificant in the clinical trial. Besides, cisplatin treatment also resulted in side effects such as peripheral neuropathy [45], brain damage, and cognitive impairment [46]. In our hospital, we often apply cisplatin with the prescription of traditional medicine according to patients' conditions. Many traditional medicines are used in cancer treatments [47]. A great number of studies have revealed that some compounds from traditional medicine can be useful for the therapy of many diseases [48–53]. Thus, traditional medicine is a good source for the discovery of proper synergists for cisplatin. Carvacrol was identified as one of such promising candidates.

In this study, we conducted both in vitro and in vivo studies to test the enhancing effect of carvacrol on cisplatin. We expected that carvacrol can increase the inhibition of cisplatin toward TNBC cells. We tested this hypothesis in five TNBC cell lines including MDA-MB-231, MDA-MB-157, MDA-MB-453, BT-20, and MDA-MB-468. In all five TNBC cell lines tested, carvacrol significantly enhanced the viability suppression of cisplatin. Cisplatin inhibits cancers by damaging DNA double-strand [54]. Many studies also showed that cisplatin induces apoptosis of cancer cells by multiple pathways [55–57]. We proposed that the carvacrol action is associated with its effect on apoptosis, thus we test the apoptosis factor Bax protein level in these cell lines. We found that carvacrol promoted the cisplatin-induced apoptosis of TNBC cells.

We also tested the effect of carvacrol on cisplatin treatment in an animal model. Results showed that the application of carvacrol enhanced the potency of cisplatin. Compare to the group with cisplatin alone, the tumor volume in the group with cisplatin plus carvacrol treatment was significantly lower. In addition, body weight loss results from the decrease in food intake are always concerns for clinical cisplatin treatment [58]. The bodyweight of animals in the group with cisplatin plus carvacrol treatment did not decrease as that in the group with cisplatin treatment. Thus, we suggested animals in the group with cisplatin plus carvacrol group might have a better life quality. We also determined the Bax level in tumor samples to test our hypothesis in apoptosis. Results showed that the group with cisplatin plus carvacrol showed a higher apoptosis level than that in the group with cisplatin alone. This demonstrated that carvacrol improved the cisplatin-induced apoptosis in the tumor.

Because carvacrol was first known as a non-specific inhibitor for TRPM7 [19, 20], we proposed that TRPM7 might be involved in the pharmacological action of carvacrol. We determined TRPM7 expression in tumor samples and did not found any significant difference in TRPM7 expression. We suggested that carvacrol would not affect the expression of TRPM7 but would regulate breast cancer by its inhibition toward TRPM7 channels. We conducted a silencing experiment to test this hypothesis. TRPM7 was knocked down in MDA-MB-231. Results showed that the knockdown of TRPM7 eliminated the enhancing effect of carvacrol, indicating that TRPM7 is an essential target on the carvacrol pharmacological pathway. However, the knockdown of TRPM7 not only removed the TRPM7 channel but also remove the kinase domain of TRPM7. The elimination might result in the removal of the kinase domain rather than the inhibition of the channels. Although the function of the kinase domain of TRPM7 is still largely unknown, it was supposed to play a critical role in cells [25]. To further confirm the TRPM7 channels made a difference in this action, we used a TRPM7 inhibitor, 2-APB. Results showed that blocking of

TRPM7 by 2-APB also eliminated the effect of carvacrol, and 2-APB showed a similar effect on cisplatin suppression. These results demonstrated that TRPM7 channels are involved in the enhancing effect of carvacrol on cisplatin. However, this study had several limitations. Firstly, 2-APB is not specific to TRPM7, we can not rule out its effect on other channels; secondly, although we confirmed that TRPM7 channel function was critical in this process, we are not sure whether the kinase domain also play any roles in this process; thirdly, the exact regulatory pathway connecting TRPM7 and apoptosis is still unclear.

The major clinical treatment for solid breast tumors was surgery which might involve anesthetics. Recent studies have shown that anesthetics might affect breast cancer treatments. Interestingly, a local anesthetic lidocaine has been found to inhibit TRPM7 [59]. Therefore, the understanding of carvacrol's pharmacological actions on TRPM7 might optimize the use of carvacrol as a chemotherapy synergist for cisplatin.

The main conclusion of this study was that carvacrol has promising potential as a cisplatin sensitizer in TNBC chemotherapy and that TRPM7 channels might play a critical role in the synergistic effect of carvacrol on cisplatin.

Declarations

Ethics approval

The animal study was consented by the Ethics Committee of Qingdao Hiser hospital.

Consent for publication

All authors had given consent for publication of this paper.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

There is no conflict of interest.

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Authors' contributions

Jing Li had made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. Haiyan Ye, Jun Wang, Junli Qin, and Lixia Chen have been involved in drafting the manuscript or revising it critically for important intellectual content.

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Figures

Figure 1

IC50 of Carvacrol and cisplatin inhibition toward viability of TNBC cell lines. The cells were exposed to tested drugs for 48 hours and the viability was determined using MTT assay. The IC50 was analyzed and presented in the figures.

Figure 2

Effect of carvacrol and cisplatin on cell viability and apoptosis of TNBC cell lines. A-E. Effect of carvacrol and cisplatin on cell viability and apoptosis of TNBC cell lines. The cells were exposed to test drugs for 48 hours and the viability was determined using MTT assay. F-J. Effect of carvacrol and cisplatin on cell apoptosis of TNBC cell lines. The cells were exposed to test drugs for 48 hours and the apoptosis was

determined using Bax ELISA. Statistical significance was analyzed using a Student's unpaired t-test (NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

(A)

(B)

Figure 3

Effect of carvacrol on cisplatin-mediated survival improvement of an orthotopic murine breast cancer model. (A) Schematic diagram of the experimental design. Briefly, 4T1 cells were injected in the mammary fat pad of female mice to induce breast cancer. The mice were allocated to four groups

according to their body weight. 4 groups (n=8) were injected intraperitoneally with the vehicle, 3 mg/kg cisplatin, 200 mg/kg carvacrol, and 3 mg/kg cisplatin+200 mg/kg carvacrol respectively. The animals were fed until the death of the animals and the survival data of all animals was recorded. (B) Survival data of the animals. (* = $P < 0.05$, ** = $P < 0.01$).

Figure 4

Effect of carvacrol on cisplatin-mediated suppression of breast cancer development in an orthotopic murine breast cancer model. (A) Schematic diagram of the experimental design. Briefly, 4T1 cells were injected in the mammary fat pad of female mice to induce breast cancer. The mice were allocated to four groups according to their body weight. 4 groups were injected intraperitoneally with vehicle (n=6), 3 mg/kg cisplatin (n=7), 200 mg/kg carvacrol (n=6), and 3 mg/kg cisplatin+200 mg/kg carvacrol (n=8) respectively. The body weight and tumor volume were measured every three days. All the animals were euthanized at the endpoint of the experiment (day 21) and the net tumor weight was measured. Data of animals that died before the endpoint of the experiment were excluded. (B) Body weight. (C) Tumor volume. (D) Image of tumors. (E) Tumor weight. (F) representative image of western blotting analysis. The tumor samples were used to extract proteins followed by western blotting assay with TRPM7 and Bax antibodies. (G) Normalized TRPM7 protein expression in tumor samples. (H) Normalized Bax expression in tumor samples. Statistical significance at the endpoint was analyzed using a Student's unpaired t-test (NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Figure 5

TRPM7 knockdown in MDA-MB-231. (A) A representative image of western blotting assay in TRPM7 knockdown experiment. Different concentration of TRPM7 siRNA (mix with negative siRNA) was transfected into MDA-MB-231. After 48 hours, the cells were harvested and Western blotting was used to analyzed the TRPM7 expression in cells. (B) TRPM7 expression after the transfection. (C) Effect of carvacrol and cisplatin on cell viability of TRPM7 knockdown MDA-MB-231. The cell viability was

determined using MTT assay. (D) Effect of carvacrol and cisplatin on cell apoptosis of TRPM7 knockdown MDA-MB-231. The cell apoptosis was determined using Bax ELISA. Statistical significance was analyzed using a Student's unpaired t-test (NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Figure 6

Effect of TRPM7 inhibitor 2-APB on carvacrol/cisplatin suppression. (A) Representative TRPM7-like currents in MDA-MB-231. Patch-clamp was used to measure the TRPM7-like currents in transfected MDA-MB-231. (B) TRPM7-like current density in MDA-MB-231. (C) TRPM7-mediated fluorescence quenching data in MDA-MB-231. Fura-2AM-based quench assay was used to measure TRPM7-mediated influx in MDA-MB-231. (D) Fluorescence quench amount at the last 5 s in Fura-2AM-based quench assay. (E) Effect of 2-APB on carvacrol/cisplatin viability suppression. (F) Effect of 2-APB on carvacrol/cisplatin apoptosis suppression. (G) Effect of 2-APB on cisplatin viability suppression. (H) Effect of 2-APB on cisplatin apoptosis suppression. The cell viability and apoptosis were determined using MTT and Bax ELISA respectively. Statistical significance was analyzed using a Student's unpaired t-test (NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).