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Artesunate inhibits cell proliferation, migration, and invasion of thyroid cancer by regulating the PI3K/AKT/FKHR pathway

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1 **Artesunate inhibits cell proliferation, migration, and invasion of**
2 **thyroid cancer by regulating the PI3K/AKT/FKHR pathway**

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4 **Running title: Artesunate inhibits thyroid cancer**

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6 Zhiwei Xu¹, Xiaojian Liu², Daoping Zhuang^{3, *}

7
8 ¹Department of Nail and Breast Surgery, Tongxiang First People’s Hospital, Zhejiang
9 314500, China; ²Department of General Surgery, Tongxiang First People’s Hospital,
10 Zhejiang 314500, China; ³Department of Neurology, Tongxiang First People’s
11 Hospital, Zhejiang 314500, China

12
13 ***Corresponding author**

14 Tel +86 137666735959
15 Street address: 1918, Jiaochang East Road, Tongxiang City

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Abstract

This study characterized the effects of artesunate on thyroid cancer and partially identified its related molecular mechanism. We determined the effect of artesunate on the proliferation of thyroid cancer cells using the MTT assay, cell colony formation experiments, and western blotting, and used flow cytometry to detect the apoptosis of cancer cells. Using a wound-healing assay, Transwell chamber experiments, and western blotting, we determined the effect of artesunate on cancer cell migration. By co-cultivating artesunate with the PI3K agonist, 740Y-P, we also partially identified the molecular mechanism. Artesunate significantly inhibited the growth, proliferation, migration, and invasion of thyroid cancer cells, and promoted the apoptosis of cancer cells. Using co-cultivation with a PI3K agonist, we found that the inhibitory effect of artesunate on cancer cells was mainly due to suppressing the PI3K/AKT/FKHR signaling pathway. By inhibiting the PI3K/AKT/FKHR signaling pathway, artesunate induced apoptosis of thyroid cancer cells and inhibited their proliferation and migration.

Keywords: Artesunate; Thyroid; PI3K; AKT; FKHR

36 **Introduction**

37 Thyroid carcinoma is the most common malignant tumor in the endocrine system
38 and in head implant tumors. Its main pathological types are follicular thyroid tumor
39 and papillary thyroid cancer, accounting for approximately 95% of all thyroid cancer
40 cases (Pellegriti et al. 2013). In the past 30 years, except for the limitations of disease
41 diagnoses and technology in Africa, the detection of thyroid cancer has been
42 increasing in most parts of the world (James et al. 2018) . This was largely due to the
43 use of high resolution imaging techniques to detect papillary thyroid cancer (Davies
44 and Welch 2006). Follicular thyroid tumors and papillary thyroid cancer are well-
45 differentiated thyroid cancers. If these two types of cancers can be diagnosed at an
46 early stage, patients usually have good prognoses. The overall disease-specific
47 survival (> 90%) can reach more than 10 years (Hay et al. 2002; Maia and Zantut-
48 Wittmann 2012; Paricharttanakul et al. 2016). When these two types of cancers
49 become metastatic thyroid cancer, surgery cannot be performed and radioactive iodine
50 therapy is also ineffective, so the survival of these patients is very low (Kapiteijn et al.
51 2012). Undifferentiated or poorly differentiated anaplastic thyroid cancers can
52 metastasize in up to 50% of patients (Schlumberger 2012), which is life-threatening
53 and often fatal to patients, but fortunately it is relatively rare (Liu et al. 2016).

54 The PI3K/Akt signaling pathway is highly mutated and activated in thyroid
55 cancer, which can further phosphorylate FKHR at multiple sites (Nozhat and Hedayati
56 2016). The phosphorylation of FKHR causes its accumulation in the cytoplasm and
57 the inhibition of transcriptional functions, which in turn causes cell growth and
58 proliferation (Duan et al. 2018; Nozhat et al. 2018). Thus, targeting the PI3K/AKT/
59 FKHR signaling pathway can suppress the progression of thyroid cancer.

60 Radiotherapy and chemotherapy are the main anti-tumor treatments.
61 Radiotherapy includes radioiodine therapy (Konishi et al. 2021), and chemotherapy
62 includes multi-kinase inhibitors such as sorafenib (Ishihara et al. 2019) and
63 Lenvatinib (Yeung and Cohen 2015). However, the side effects and multi-drug

resistance of these treatments often seriously affect the treatment efficacy and prognoses of patients. It is therefore of great importance to identify anti-tumor drugs with low toxicity and resistance to tolerance. Artesunate, a classic anti-malarial drug, has the characteristics of high efficacy, low toxicity, and resistance to tolerance (Benakis et al. 1997; Klayman 1985). With increasing interest in this drug, many other pharmacological effects of artesunate have been reported. It can effectively treat rheumatoid arthritis (Mirshafiey et al. 2006) and is also an anti-parasitic agent (Li et al. 1996). It also has a wide range of pharmacological activities as an anti-cancer agent. The drug activity of artesunate mainly involves the active metabolite, dihydroartemisinin (DHA). After oral administration, artesunate undergoes intestinal acid hydrolysis to DHA, while some artesunate enters the liver directly through the portal vein, where it is converted to DHA through liver metabolism (Magenta et al. 2014). Artesunate is cytotoxic to tumor cells, and its active component, which has an internal peroxide bridge, generates carbon-centered free radicals and causes oxidative stress during cell lysis (Feng et al. 2011). Previous studies have reported that artesunate and its metabolites have cytotoxic effects on human lymphocytic leukemia cells, human cervical cancer cells, human nasopharyngeal carcinoma cells, and human breast cancer cells (Li et al. 2017; Qian et al. 2018; Singh and Lai 2001; Zhang et al. 2016).

However, there are few reports on the effects of artesunate on thyroid cancer. In 2004, Rinner et al. extracted related compounds from plants of the genera *Stemona* (Stemonaceae), *Aglaia* (Meliaceae), and *Artemisia* (Asteraceae) to study the curative effects of natural plant products on medullary thyroid cancer (Rinner et al. 2004). Researchers discovered that artesunate had an anti-proliferative effect on thyroid cancer cells. However, due to the large number of compounds being studied and limitations of the experimental technology at the time, they did not characterize the effects of artesunate on other aspects of thyroid cancer cells, such as migration and colony forming ability. In the present study, we therefore characterized the effects of artesunate on thyroid cancer cells, and identified part of the molecular mechanism, which provided the basis for additional research efforts for the development of

clinical treatments for thyroid cancer.

Materials and methods

Cell culture and incubation. The thyroid cancer cell line, BCPAP (BNCC338685), was purchased from the Beijing Beina Chuanglian Biotechnology Research Institute (Beijing, China). The cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) which contained 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA). The cells were co-cultured with different concentrations of artesunate (10 μ M, 25 μ M, 50 μ M) or control (DMSO) in an incubator containing 5% CO₂ at 37°C. For signaling pathway analysis, the cells were also co-cultured with the pathway agonist, 740Y-P (15 μ M).

MTT assay. The cells were seeded into 96-well plates at a density of 1×10^4 cells/well using 200 μ L RPMI 1640 medium containing 10% FBS, and with or without artesunate and/or 740Y-P, and incubated at 37°C for 24 h. Before lysis with 150 μ L dimethyl sulfoxide, cells were incubated with 500 μ g/mL MTT reagent for 4 h at 37°C in the dark, and then the absorbance was measured using a multi-mode microplate reader (BioTek, Winooski, VT, USA) at 560 nm.

Colony formation assay. A total of 1×10^3 cells/mL in soft agar with RPMI 1640 medium containing 0.9% methylcellulose and 20% FBS, were incubated 5–10 days at 37°C in 5% CO₂. Colony Area Image J software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the number of colonies in each well. This plugin provided information about the percentage of intensity, not only considering the area covered by the colony, but also considering the direct relationship between the staining intensity and the number of cells in the colony (Guzmán et al. 2014).

Wound-healing assay. Cells treated with or without artesunate and 740Y-P were added into 6-well plates at the density of 1×10^5 cells/well. The cells were cultured with RPMI-1640, which contained 10% FBS at 37°C, until the confluency reached 90%. The monolayer was then scratched across the entire diameter of each well using a sterile pipette tip. An inverted microscope (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize images at 0 h and 48 h, and the wound closure of the cells was analyzed using ImageJ software.

Cell apoptosis. Cell apoptosis was detected using an Annexin V-FITC/PI kit (Sigma-Aldrich, St. Louis, MO, USA) and analyzed by flow cytometry (Thermo Fisher Scientific). (1) The concentration of detected cells was 1×10^6 cells/mL, and 200 µL of the cell suspension was centrifuged at 1,000 rpm at 4°C for 5 min. (2) The pellet was then washed twice with precooled phosphate-buffered saline (PBS), and centrifuged at 4°C at 1,000 rpm for 5 min. (3) The cells were mixed in 100 µL of binding buffer, and 2 µL of Annexin-V-FITC (20 µg/mL) was added, then gently mixed and incubated at 4°C for 15 min in the dark. (4) The sample was transferred to a flow detection tube, 400 µL PBS and 1 µL propidium iodide (50 µg/mL) was added, and then analyzed by flow cytometry after 2 min.

Transwell chamber experiments. Cells with a density of 1×10^5 cells/well, which were treated with or without artesunate and 740Y-P, were seeded into the upper chambers of Transwell plates with 200 µL of FBS-free medium. The lower chamber contained 500 µL of medium with 10% FBS, and the cells were incubated at 37°C for 24 h. The cells in the lower chamber were then fixed with 4% paraformaldehyde for 20 min. After removing the paraformaldehyde, the cells were stained with 0.5% Crystal Violet solution for 30 min and washed with distilled water to remove unbound dye. Photographs of invading cells were taken using an optical inverted microscope and counted and analyzed using ImageJ software.

Western blot analysis. The samples were lysed in 1% phenylmethylsulfonyl fluoride

containing total cell protein extraction reagents and using a cell ultrasonic disruptor, and then centrifuged at 14,000 rpm for 15 min at 4°C. The concentration of the protein samples was measured using the BCA kit method, and 30 µg of the sample protein were then placed in a constant temperature incubator at 95°C for 5 min to denature the proteins. Protein samples (30 µg) were resolved by SDS-PAGE using 8% to 15% gels and transferred to a polyvinylidene difluoride membrane (GE Healthcare, Chicago, IL, USA). The membranes were then blocked with 5% bovine serum albumin (BD Biosciences, San Jose, CA, USA) and were incubated with primary antibodies overnight at 4°C. Primary antibodies against the following were used: Bax (#2774), c-caspase-3 (#9661), E-cadherin (#3195), vimentin (#5741), SNAIL (#3879), p-PI3K (#4228), PI3K (#4257), p-AKT (#4060), AKT (#9272), p-FKHR (#9461), FKHR (#97635), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #5174), all from Cell Signaling Technology (Danvers, MA, USA). The membranes were washed three times with PBST for 5 min, and incubated with corresponding secondary antibodies for 1.5 h. The bands were visualized using a Pierce ECL chemiluminescence imaging system (Pierce, Rockford, IL, USA). The intensities of bands were analyzed using ImageJ software, and the levels of each protein were measured relative to that of the internal GAPDH control.

Statistical analysis. All data are expressed as the mean ± standard deviation. Prism 5.0 (GraphPad, San Diego, CA, USA) was used for statistical analyses. Dunnett's multiple comparisons were evaluated by the analysis of variance one-way test. The intragroup comparisons were evaluated using a paired Student's t test. All experiments were repeated at least three times. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The influences of artesunate on the proliferation of thyroid cancer cells

The chemical structure of artesunate is shown in Figure 1A. First, we investigated the effects of artesunate on different human thyroid cell lines, which showed that the proliferation of thyroid cancer cells (SW579, BCPAP, and TPC-1) was significantly higher than in the immortalized thyroid cell lines (Nthy-ori3-1) (Figure 1B). Similarly, the colony formation experiments also showed that the ability of colony formation in thyroid cancer cells far exceeded that of the immortalized thyroid cell line (Figure 1C). Artesunate was diluted into three samples with different concentrations, namely ART-L (low concentration, 10 μ M), ART-M (medium concentration, 25 μ M), and ART-H (high concentration, 50 μ M). We then determined the effects of artesunate on the survival and growth of BCPAP cells. The MTT method was also used to detect the survival status of cancer cells. The results showed that artesunate significantly reduced the survival of BCPAP cells in a concentration-dependent manner (Figure 1D). In the colony formation experiments artesunate also significantly inhibited the colony formation of cancer cells in a concentration-dependent manner (Figure 1E).

To further clarify the effect of artesunate on the growth of SW579 cells, SW579 cells under different treatments were analyzed using the MTT and colony formation assays (Figure 1F and G), which indicated that thyroid cancer cells treated with various doses of artesunate showed inhibited growth. Moreover, flow cytometry was used to detect apoptosis of BCPAP and SW579 cells. Artesunate induced the apoptosis of BCPAP and SW579 cells, and the percentage of apoptotic cells increased as the concentration of artesunate increased (Figure 1H and I). We also used western blots to detect changes in the expression levels of apoptosis-related proteins, and found that Bax and c-caspase-3 were significantly upregulated both in the BCPAP and SW579 cell lines (Figure 1J and K). Statistical analyses showed that changes in protein expressions were positively correlated with the concentration of artesunate (Figure 1J and K). These results indicated that artesunate inhibited the proliferation of thyroid cancer cells in a concentration-dependent manner.

207

208 *Artesunate inhibited the migration and invasion of thyroid cancer cells.*

209 To further characterize the effects of artesunate on BCPAP cells, wound-healing
210 assays were used to show that artesunate significantly inhibited the migration ability
211 of cancer cells (Figure 2A). Likewise, the Transwell experiments showed that
212 artesunate inhibited the invasion ability of cancer cells (Figure 2B). We further tested
213 the expression changes of tumor metastasis-related proteins, including epithelial-
214 mesenchymal transition (EMT)-related proteins (E-cadherin, vimentin, and Snail).
215 The results showed that as the concentration of artesunate increased the expression
216 level of E-cadherin was significantly downregulated, while vimentin and snail showed
217 the opposite results (Figure 2C), which indicated that the EMT process of cancer cells
218 was inhibited through changes in specific protein levels. Together, the results
219 indicated that artesunate inhibited the proliferation and invasion of thyroid cancer
220 cells in a concentration-dependent manner.

221

222 *Artesunate prevented the effects of 740Y-P (a PI3K agonist) on the PI3K/AKT/FKHR*
223 *pathway of thyroid cancer cells.*

224 We next characterized the molecular mechanism responsible for artesunate
225 effects on thyroid cancer cells. The results of western blotting showed that
226 phosphorylations of PI3K, AKT, and FKHR were significantly downregulated in the
227 presence of artesunate(Figure 3), in a concentration-dependent manner. Considering
228 that the normal growth state of cells should be protected with an appropriate
229 concentration of artesunate, the medium concentration of artesunate (25 μ M) was
230 used in further trials.

231 To further confirm the effect of artesunate on the abovementioned signaling
232 pathways, we used the PI3K agonist, 740Y-P. The agonist activates PI3K by

upregulating the phosphorylation level of PI3K, thereby activating the downstream proteins of the PI3K pathway (AKT and FKHR; Figure 4, third column). When both 740-P and the medium concentration of artesunate were added to BCPAP cells, the phosphorylation level of PI3K was reversed, and the phosphorylation levels of the downstream proteins, AKT and FKHR, were also reversed in the presence of artesunate (Figure 4, fourth column). Together, these results indicated that treatment with artesunate reversed the effects of the PI3K agonist on PI3K, AKT, and FKHR, and thereby acted on the PI3K/AKT/FKHR pathway in thyroid cancer cells.

Artesunate inhibited the effects of 740Y-P (a PI3K agonist) on proliferation of thyroid cancer cells.

The cell proliferation test (MTT method) showed that artesunate inhibited the survival and growth of BCPAP cells, while the PI3K agonist, 740Y-P, significantly promoted the growth of cancer cells. When the cells were co-cultured with artesunate and 740Y-P, the promoting effect of 740Y-P on cancer cells was inhibited by artesunate. Using the colony formation assay, we also found that 740Y-P significantly enhanced the colony formation ability of cancer cells, which was inhibited by artesunate (Figure 5A, B).

As shown using flow cytometry, 740Y-P reduced the apoptosis of cancer cells, but after co-culture with artesunate, the level of apoptosis of cancer cells was increased (Figure 5C), indicating that the anti-apoptotic activity of 740Y-P on cells was inhibited by artesunate. Using western blotting, we demonstrated that the expression levels of apoptosis-related proteins, Bax and c-caspase-3, were significantly downregulated when cells were treated with 740Y-P. The expression levels of the two proteins reverted back to control levels after co-culturing cells with artesunate (Figure 5D), which confirmed that the anti-apoptotic effect of 740Y-P was reversed by artesunate. Together, these results indicated that artesunate inhibited the ability of 740Y-P to promote the proliferation and anti-apoptosis of thyroid cancer

261 cells.

262

263 *Artesunate inhibited the effects of 740Y-P (a PI3K agonist) on migration and invasion*
264 *of thyroid cancer cells.*

265 Using the wound-healing assay, we found that 740Y-P significantly improved the
266 migration of cancer cells, but when cells were co-cultured with artesunate, the wound-
267 healing ability of the cells was decreased (Figure 6A). In addition, the Transwell
268 chamber experiments showed that artesunate significantly inhibited the ability of
269 740Y-P to promote cancer cell invasion (Figure 6B).

270 In the western blot analyses of tumor metastasis-related proteins, the protein
271 expression of E-cadherin was inhibited by 740Y-P, while those of vimentin and Snail
272 were upregulated (Figure 6C, third column). After adding artesunate, the expression
273 level of E-cadherin was restored, while vimentin and Snail levels were decreased
274 (Figure 6C, fourth column), which meant that the EMT process and invasion of cells
275 induced by 740Y-P were inhibited by artesunate. Overall, these results indicated that
276 artesunate effectively inhibited the effects of 740Y-P on the invasion and metastasis of
277 thyroid cancer cells.

278

279 Discussion

280 With increasing incidence of thyroid cancer, it is important to identify or develop
281 a new drug or compound with low toxicity, low side effects, and minimal tolerance.
282 After reviewing past studies, we decided to study the effects of artesunate, which is a
283 derivative of artemisinin discovered by Tu, and which is also a classic anti-malarial
284 drug (Klayman 1985). With extensive study of the role and mechanism of action of
285 artesunate, previous studies have shown that artesunate and its metabolites have
286 numerous biological activities, which are effective as anti-infection, anti-

angiogenesis, anti-immune regulation, anti-sepsis, and anti-tumor agents (Deeken et al. 2018; Meng et al. 2018; Raffetin et al. 2018; Zhang et al. 2018¹). Artesunate has the characteristics of high efficacy, low toxicity, and low side effects on the human body in anti-malarial studies. Artesunate also has effective inhibitory activity on many types of cancers, although the current research on the effect of artesunate on thyroid cancer is still preliminary. We therefore focused on its role in thyroid cancer and identified relevant signaling pathways to identify its mechanisms of anticancer action.

We found that artesunate significantly inhibited the proliferation of thyroid cancer cells at the cellular level, which is consistent with past reports (Rinner et al. 2004). Using additional experimental techniques, we found that artesunate inhibited the colony-forming ability and promoted the apoptosis of cancer cells. Invasiveness is an important aspect of cancer that has great destructive power to the body, so we characterized several aspects of invasiveness at the cellular level and found that artesunate significantly inhibited the migration and invasion of thyroid cancer cells.

We then identified a potential molecular mechanism and searched for the signaling pathways involved in these processes. There are many signaling pathways related to tumors, such as the JAK/STAT (Sansone and Bromberg 2012) and Ras/MAPK pathways (Doebele 2019). By analyzing past reports, we found that among various signaling pathways, the Akt signaling pathway mediated by PI3K is often important. The PI3K (phosphoinositide 3-kinase)/AKT (protein kinase B) signaling pathway is expressed in different types of cancer and is the target of many anti-cancer drugs. It mainly controls the pathological proliferation and invasion, survival, and anti-apoptotic properties of cancer cells (King et al. 2015; Toulany and Rodemann 2015). By targeting the PI3K signaling pathway, we identified this pathway as involved in a downstream signaling cascade to inhibit tumor growth. In the treatment of cancer, targeting this signaling pathway has been previously studied (Wang et al. 2020). As a protein kinase, AKT can phosphorylate various substrates, either resulting in activation or inhibition of targets, and can affect the physiological state of cells (Peltier et al. 2007; Song et al. 2005).

FKHR, also known as FoxO1, belongs to the FoxO transcription factor family,

together with FoxO3 and FoxO4. It is also a downstream component of the PI3K/AKT pathway. It can be phosphorylated by AKT and plays an important role in cell proliferation, apoptosis, and carcinogenesis (Zaballos and Santisteban 2013). We first determined whether artesunate affected this signaling pathway. Using western blots, we showed that artesunate significantly inhibited the PI3K/AKT/FKHR signaling pathway protein expressions. To confirm this, we also used the PI3K agonist, 740Y-P in the presence of artesunate. The results showed that artesunate not only participated in the regulation of the signal pathway, but also significantly reversed the activation by 740Y-P, the small molecule agonist. Furthermore, in experiments addressing the phenotype of thyroid cancer cells, we also confirmed that artesunate inhibited the ability of 740Y-P to promote the proliferation, anti-apoptosis, migration, and invasion of cancer cells, indicating that artesunate suppressed the growth of cancer cells by targeting this signaling pathway.

There are additional studies to be conducted. We have shown the suppressive effect of artesunate on thyroid cancer cells at the cellular level. However, if artesunate can be used to intervene after the tumor is established, the above results must be verified *in vivo*. This would make the experimental conclusions more relevant to clinical situations. We found that artesunate was involved in the regulation of the PI3K/AKT/FKHR signaling pathway. However, FKHR is one of the targets of PI3K/AKT involved in a variety of cellular processes. Whether there are other substrates, the signal molecules downstream of FKHR, and how they bind to DNA to regulate transcription are still unclear. Wei et al. found that artesunate may cause cell cycle arrest by downregulating the expressions of CDK (cyclin-dependent kinases), cyclin D1, and cyclin B1, thereby inhibiting the proliferation of glioma cells (Wei et al. 2020). Piao et al. showed that CDK1 in cells can affect the nuclear export and activity of FKHR through serine phosphorylation, thereby affecting the occurrence and development of gliomas (Piao et al. 2019). However, whether such a signal network exists in thyroid cancer remains to be studied.

In summary, we have confirmed at the cellular level that artesunate blocked the survival and invasion of thyroid cancer cells by inhibiting the PI3K/AKT/FKHR

signaling pathways, thus effectively playing an anti-cancer role, and providing direction for the treatment of thyroid cancer and the elucidation of the mechanism of artesunate.

Declarations

Acknowledgments Not applicable.

Conflicts of interest The Authors declare that there is no conflict of interest.

Contributors' statement ZWX was responsible for drafting the manuscript, and the concept and study design; XJL was responsible for laboratory experiments, data collection, data analysis, and interpretation; DPZ provided critical input and critical review of the manuscript. All authors have read and approved the final article.

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492 **Figure legends**

493 **Figure 1. Influences of artesunate on the proliferation of thyroid cancer cells. (A)**

494 The chemical formula of artesunate. (B) The MTT assay used to determine the
 495 proliferation of different thyroid cell lines. (C) Colony formation experimental results
 496 of different thyroid cell lines. (D) The MTT assay results to determine the
 497 proliferation of BCPAP cells. (E) Colony formation experiments of BCPAP cells. (F)
 498 The MTT assay results were used to determine the proliferation of SW579 cells. (G)
 499 The results of colony formation experiments of BCPAP cells. (H) Flow cytometry
 500 involving annexin V/propidium iodide labeling to determine BCPAP cell apoptosis
 501 when treated with artesunate. (I) Flow cytometry involving annexin V/propidium
 502 iodide labeling to determine SW579 cell apoptosis when treated with artesunate. (J)
 503 Western blots analyzed the expressions of Bax and c-caspase-3 in BCPAP cells when
 504 treated with artesunate. (K) Western blots analyzed the expressions of Bax and c-
 505 caspase-3 in SW579 cells when treated with artesunate. * $P < 0.05$ vs. the control
 506 group.

508 **Figure 2. Artesunate inhibits migration and invasion in thyroid cancer cells. (A)**

509 The wound-healing assay to determine the migration of BCPAP cells when treated
 510 with artesunate at 0 h and 48 h. * $P < 0.05$ vs. the control group. (B) Transwell
 511 chamber experiments to determine the cell invasive ability when treated with
 512 artesunate. * $P < 0.05$ vs. the control group. (C) Western blot analysis of the expression
 513 of epithelial-mesenchymal transition-related proteins E-cadherin, vimentin, and Snail
 514 in BCPAP cells when treated with artesunate. Glyceraldehyde 3-phosphate
 515 dehydrogenase as the loading control. * $P < 0.05$ vs. the control group.

517 **Figure 3. Artesunate inhibits the PI3K/AKT/FKHR pathway in thyroid cancer**

518 **cells.** Western blot analyses of the expression of phosphorylated PI3K and PI3K,
 519 phosphorylated AKT and AKT, and phosphorylated FKHR and FKHR in BCPAP cells

when treated with artesunate. Glyceraldehyde 3-phosphate dehydrogenase as a loading control. *P < 0.05 vs. the control group.

Figure 4. Artesunate inhibits the effects of 740Y-P on the PI3K/AKT/FKHR pathway in thyroid cancer cells. Western blots were used to analyze the expressions of phosphorylated PI3K and PI3K, phosphorylated AKT and AKT, and phosphorylated FKHR and FKHR in BCPAP cells when treated with artesunate and 740Y-P. Glyceraldehyde 3-phosphate dehydrogenase was the loading control. 740Y-P is a PI3K agonist. *P < 0.05 vs. the control group; #P < 0.05 vs. the artesunate + 740Y-P group.

Figure 5. Artesunate inhibits the effects of 740Y-P (a PI3K agonist) on proliferation in thyroid cancer cells. (A) The MTT assay experiment to determine the proliferation of BCPAP cells when treated with artesunate and 740Y-P. *P < 0.05 vs. the control group; #P < 0.05 vs. the artesunate + 740Y-P group. (B) The colony formation experiment used to determine the colony forming ability of BCPAP cells when treated with artesunate and 740Y-P. *P < 0.05 vs. the control group; #P < 0.05 vs. the artesunate + 740Y-P group. (C) Flow cytometry with annexin V/propidium iodide double-labeling to determine cell apoptosis. When treated with artesunate and 740Y-P, *P < 0.05 vs. the control group; #P < 0.05 vs. the artesunate + 740Y-P group. (D) Western blotting analyses of the expressions of Bax and c-caspase-3 in BCPAP cells when treated with artesunate and 740Y-P. Glyceraldehyde 3-phosphate dehydrogenase as a loading control. *P < 0.05 vs. the control group; #P < 0.05 vs. the artesunate + 740Y-P group.

Figure 6. Artesunate inhibits the effects of 740Y-P (a PI3K agonist) on migration and invasion in thyroid cancer cells. (A) The wound-healing assay was used to determine the migration of BCPAP cells when treated with artesunate and 740Y-P at 0 h and 48 h. *P < 0.05 vs. the control group; #P < 0.05 vs. the artesunate + 740Y-P group. (B) The Transwell chamber experiment was used to determine cell invasive

550 ability when treated with artesunate and 740Y-P. *P < 0.05 vs. the control group; #P <
551 0.05 vs. the artesunate + 740Y-P group. (C) Western blot analyses of the expression of
552 epithelial-mesenchymal transition-related proteins, E-cadherin, vimentin, and Snail in
553 BCPAP cells when treated with artesunate and 740Y-P. Glyceraldehyde 3-phosphate
554 dehydrogenase was used as a loading control. *P < 0.05 vs. the control group; #P <
555 0.05 vs. the artesunate + 740Y-P group.
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