Published in final edited form as:

Eur J Pharmacol. 2011 May 11; 658(2-3): 114–122. doi:10.1016/j.ejphar.2011.02.005.

An anticancer agent icaritin induces sustained activation of the extracellular signal regulated kinase (ERK) pathway and inhibits growth of breast cancer cells

YuMing Guo¹, XinTian Zhang, Jun Meng², and Zhao-Yi Wang

Department of Medical Microbiology and Immunology, Creighton University Medical School, 2500 California Plaza, Omaha, NE, USA

Abstract

Icaritin, a prenylflavonoid derivative from *Epimedium Genus*, regulates many cellular processes. However, the function and the underlying mechanisms of icaritin in breast cancer cell growth have not been well established. Here, we report that icaritin strongly inhibited growth of breast cancer MDA-MB-453 and MCF7 cells. At concentrations of 2–3 μM, icaritin induced cell cycle arrest at the G₂/M phase accompanied by a down-regulation of the expression levels of the G₂/M regulatory proteins such as cyclinB, cdc2 and cdc25C. Icaritin at concentrations of 4–5 μM, however, induced apoptotic cell death characterized by accumulation of the annexin V- and propidium iodide-positive cells, cleavage of poly ADP-ribose polymerase (PARP) and down-regulation of the Bcl-2 expression. In addition, icaritin also induced a sustained phosphorylation of extracellular signal regulated kinase (ERK) in these breast cancer cells. U0126, a specific ERK activation inhibitor, abrogated icaritin-induced G₂/M cell cycle arrest and cell apoptosis. Icaritin more potently inhibited growth of the breast cancer stem/progenitor cells compared to anti-estrogen tamoxifen. Our results indicate that icaritin is a potent growth inhibitor for breast cancer cells and provide a rational for preclinical and clinical evaluation of icaritin for breast cancer therapy.

Index words

Icaritin; ERK; Cell Cycle Arrest; Apoptosis; Breast Cancer Stem Cells

1. Introduction

It has been well established that estrogen is involved in the pathogenesis of breast carcinoma and development of breast cancers that express the receptors for estrogen. Approximately 70% of breast cancer patients are positive for estrogen receptor-alpha and these patients are suitable for hormonal therapy that blocks estrogen stimulation of breast cancer cells. The anti-estrogen tamoxifen has been used for hormonal therapy in both early and advanced breast cancer for the past three decades (Lewis and Jordan., 2005). Almost all patients with

Correspondence to: Zhao-Yi Wang, Department of Medical Microbiology and Immunology, Creighton University Medical School, Criss III, Room 352, 2500 California Plaza, Omaha, NE 68178, USA. Fax: +14022803543; zywang@creighton.edu.

¹Current address: Beijing Shenogen Biomedical Co., Ltd. Suit A205, 5 Shangdi Kaituo Road, Haidian, Beijing 100085, P. R. China.

²Current address: School of Food and Bioengineering, Zhengzhou University of Light Industry, Zhengzhou 450002, P. R. China.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

tumors positive for estrogen receptor- α in Western countries have been treated with this drug either as adjuvant treatment following surgery or as first-line treatment for advanced disease. However, about 50% of patients with advanced disease do not respond to first-line treatment with tamoxifen. In addition, almost all patients with metastatic disease and about 40% of the patients that receive tamoxifen as adjuvant therapy experience tumor relapse (Normanno et al., 2005). These *de novo* and acquired resistances to tamoxifen obliterate the efficacy of this treatment. Thus, novel therapeutic approaches are urgently needed for treatment of advanced breast cancer.

Icaritin is a prenylflavonoid derivative from *Epimedium Genus* that has long been used in Chinese traditional medicine. Icaritin has many pharmacological and biological activities such as neuroprotective effects (Wang et al., 2007), stimulation of neuronal and cardiac differentiation (Wang et al., 2009; Wo et al., 2008; Zhu and Lou, 2005), prevention of steroid-associated osteonecrosis (Zhang et al., 2009), enhancement of osteoblastic and suppression of osteoclastic differentiation and activity (Huang et al., 2007a), and growth inhibition of human prostate carcinoma PC-3 cell (Huang et al., 2007b). It has been reported that icaritin exhibited estrogen-like activities such as growth stimulation of estrogen receptor-positive breast cancer MCF7 cells at nanomolar (nM) concentrations (Wang and Lou, 2004). Thus, it is possible that icaritin, like tamoxifen, possesses both estrogen agonist and antagonist activities in breast cancer cells.

The extracellular signal-regulated kinase ERK1/2 [p42/p44 mitogen-activated protein kinase (MAPK)] is one of the main transducers of extracellular signals linking the stimulation of the membrane-based receptors to changes of cellular function (Ramos, 2008; Robinson and Cobb, 1997; Roux and Blenis, 2004; Stanciu et al., 2000). Previous studies demonstrated that transient activation of ERK plays a pivotal role in cell proliferation and that sustained ERK activation induces cell cycle arrest and differentiation (Adachi et al., 2002; Kim et al., 2008; Traverse et al., 1992). Recently, transient or sustained phosphorylation and nuclear accumulation of the MAPK/ERK were found to contribute to the anti- or pro-apoptotic effects of estrogens observed in osteoblasts and osteoclasts (Chen et al., 2005). Thus, the transient or sustained activation of ERK signaling may be involved in the agonist/antagonist activities of selective estrogen receptor modulators documented before.

In the present study, we demonstrated that icaritin exhibited potent growth inhibitory activity in breast cancer cells at micromolar (μ M) concentrations. Icaritin induced sustained-activation of the ERK signaling, cell cycle arrest and cell apoptosis. Unlike the well-known anti-estrogen tamoxifen, icaritin also inhibited growth of breast cancer stem/progenitor cells.

2. Materials and Methods

2.1 Chemicals and Reagents

Icaritin was obtained from Shanghai Yousi Biotechnology Co., Ltd (Shanghai, China). The MAP kinase kinase (MEK) 1/2 inhibitor U0126 was purchased from Tocris Bioscience (Ellisville, MO, USA). Anti-phospho-p44/42 ERK (Thr202/Tyr204, 197G2) mouse monoclonal antibody (mAb) and anti-p44/42 ERK (137F5) rabbit mAb were purchased from Cell Signaling Technology (Boston, MA, USA). Anti-Bcl-2, poly (ADP-ribose) polymerase (PARP), cyclin B, cdc2 and cdc25C antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Cell culture

MDA-MB-453 and MCF7 were purchased from ATCC (Manassas, VA). MDA-MB-453 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 1% antibiotic-antimycotic from

Invitrogen (Carlsbad, CA, USA). Before experiments, cells were maintained in phenol red-free media with 2.5% charcoal-stripped FBS (Thermo Scientific Hyclone, Logan, UT, USA) for 24 h and then sub-cultured in the phenol red-free medium for another 24 h. All cells were maintained at 37°C and 5% CO₂ in a humidified incubator. The ALDEFLUOR kit (StemCell Technologies) was used to enrich the cell population with high aldehyde dehydrogenases (ALDH) enzymatic activity according to manufacturer's instructions using a FACSCalibur flow cytometer (BD-Biosciences, San Jose, CA, USA).

2.3 Cell growth assay

Cells in the phenol red-free medium were seeded into 35 mm dishes at 5×10^4 cells/dish. After 24 h, the indicated concentrations of vehicle dimethyl sulfoxide (DMSO), icaritin or tamoxifen were added and incubated for 72 h. Cells were trypsinized and counted using the ADAM automatic cell counter (Digital Bio, Korea). Three dishes were used for each concentration point. For the assay of ALDH-high and-low cells, cells were seeded in the 24 wells plates at 1×10^4 cells/well.

2.4 Western blot assay

Cells were washed with cold phosphate buffered saline (PBS) twice and lysed with the RIPA buffer containing 1% proteinase inhibitor cocktail solution and 1% phosphatase inhibitor cocktail solution (Sigma). The cell lysates were boiled for 5 minutes in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with appropriate primary antibodies and visualized with the corresponding secondary antibodies and the ECL kit (Thermo Scientific, Rockford, IL, USA).

2.5 Cell cycle and cell death analysis

Cells at ~ 50% confluence were harvested and 1 ml cold 70% ethanol was added to the cell pellet while vortexing. Ethanol fixed cells were treated with 100 µg/ml RNaseA and 50 µg/ml propidium iodide (PI) in PBS at room temperature for 30 min. Flow cytometry analysis of cell cycle distribution was performed using a FACSCalibur flow cytometer (BD-Biosciences).

Cell death was detected using the annexin V-FITC apoptosis kit (Invitrogen) according to the manufacturer's instruction. Data acquisition was performed with the CellQuest software and analyzed with the ModFit software.

2.6 Statistical analysis

Data were summarized as the means $\pm$ standard deviation (S.D.) using GraphPad InStat software program. Statistical analysis was performed using paired-samples t-test, or ANOVA followed by the Student-Newman-Keuls testing and the significance was accepted for *P* values less than 0.05.

3. Results

3.1 Icaritin exhibits growth inhibitory activity in breast cancer cells

Previously, it has been reported that icaritin (Fig. 1A) has estrogen-like stimulatory activities in growth of estrogen receptor positive breast cancer MCF7 cells (Wang and Lou, 2004). Since estrogens usually have biphasic activities in breast cancer cells; i.e. stimulate cell proliferation at low concentrations while inhibit cell growth at higher concentrations, we decided to test whether icaritin also has inhibitory activities in the growth of breast cancer

cells at high concentrations. For this purpose, we used estrogen receptor positive breast cancer MCF7 cells and estrogen receptor negative breast cancer MDA-MB-453 cells to perform cell growth assay. Cells were incubated with different concentrations of icaritin for 72 h. At concentrations $> 1\mu\text{M}$, icaritin inhibited proliferation of both MDA-MB-453 and MCF7 cells more potently than the classical anti-estrogen tamoxifen (Fig. 1B & C). However, when cells treated with icaritin and tamoxifen together, an additive effect was observed in both cell lines (Fig. 1D & F). We also observed icaritin stimulated proliferation of estrogen receptor positive MCF7 cells at concentrations $< 1\mu\text{M}$ while had only slight stimulatory activity in estrogen receptor negative breast cancer MDA-MB-453 cells (data not shown), consistent with the previous report that icaritin exhibited stimulatory activity in the growth of MCF7 cells at nM range (Wang and Lou, 2004).

3.2 Icaritin induces the G₂/M arrest in breast cancer cells

To probe the mechanisms underlying the icaritin inhibitory activity, we first studied its effect on the cell cycle progression. Cell populations in the G₀/G₁, S and G₂/M phases of the cell cycle were determined with propidium iodide (PI) staining followed by flow cytometry. Icaritin increased the population of MDA-MB-453 cells in the G₂/M phase accompanied with a reduced population of the G₀/G₁ phase; $13.4\% \pm 1.8$, $23.8\% \pm 3.5$, $33.4\% \pm 5.7$, for vehicle (DMSO), $2\mu\text{M}$ and $3\mu\text{M}$ icaritin, respectively (Fig. 2A & B). In MCF7 cells, icaritin treatment also increased population in the G₂/M phase while a reduction of the S phase cells; $11.7\% \pm 0.2$, $18.6\% \pm 3.3$, $25.0\% \pm 0.9$, for DMSO, $2\mu\text{M}$ and $3\mu\text{M}$ icaritin treatment, respectively (Fig. 2C & D).

We also examined the effect of icaritin on the expression of the proteins critical for the G₂/M transition including cyclin B, cdc2 and cdc25C (Hartwell and Kastan, 1994; Molinari, 2000). Western blot analysis showed that icaritin treatment down-regulated the expression levels of cyclin B, cdc2 in a dose-dependent manner (Fig. 3A & B). In addition, the expression levels of cdc25C were decreased in the cells treated with $3\mu\text{M}$ icaritin at different time points (Fig. 3C). Altogether, these results demonstrated that icaritin treatment arrested breast cancer cells at the G₂/M phase of the cell cycle.

3.3 Icaritin induces cell death in breast cancer cells

During our experiments, we also noticed that there were floating cells in breast cancer cells treated with $3\mu\text{M}$ icaritin. We decided to determine whether icaritin at higher concentrations also induces cell apoptosis. MDA-MB-453 cells were treated with different concentrations of icaritin for 48 hours, and the annexin V-FITC and propidium iodide (PI) fluorescence assays were performed to examine the early stage apoptotic cells (annexin-positive/PI-negative), the late stage apoptotic cells (annexin-positive/PI-positive), and necrotic cells (annexin-positive/PI-positive). In MDA-MB-453 cells treated with 3, 4 and $5\mu\text{M}$ of icaritin, $13.2\% \pm 0.4$, $31.6\% \pm 13.4$ and $62.5\% \pm 8.3$ of cells were induced to apoptotic or necrotic cell death as shown as increased cell populations in groups of annexin V-positive/PI-negative, both annexin V-/PI-positive and annex V-negative/PI-positive (Fig. 4A & B). In icaritin-treated MCF7 cells, however, a different pattern of cell death was observed (Fig. 4C & D). Although cell populations in groups of annexin V-positive/PI-negative as well as both annexin V-/PI-positive were increased (apoptotic cell death), cells in the annex V-negative/PI-positive group (necrotic cell death) were also dramatically increased, suggesting icaritin may induce both apoptotic and necrotic cell death in MCF7 cells.

We also studied the effect of icaritin on the expression of anti-apoptosis protein Bcl-2. Western blot analysis revealed that Bcl-2 protein expression was down-regulated by icaritin in a dose-dependent manner in both MDA-MB-453 and MCF7 cells (Fig. 5A).

The intact PARP molecule (116 kDa) cleavage by caspases is characteristic for cell apoptosis (Lewis et al., 2005; Patel et al., 1996). Western blot analysis demonstrated that icaritin treatment induced PARP cleavage and generated the p85 fragment in both cell lines (Fig. 5B). Taken together, our results demonstrated that icaritin at higher concentrations induces apoptotic and necrotic cell death in breast cancer cells.

3.4 Icaritin induces sustained ERK activation in breast cancer cells

Although activation of the MAPK/ERK is generally considered to play a role in cell proliferation and survival (Marshall, 1995), accumulating evidence indicated that the ERK also transmits pro-apoptotic signals in several cell types (Recchia et al., 2004; Stanciu et al., 2000). To probe the mechanism underlying icaritin's inhibitory function in breast cancer cells, we investigated whether icaritin induces ERK activation in these cells. MDA-MB-453 and MCF7 cells were treated with icaritin for different time periods and activation of the ERK was examined with Western blot analysis using a phospho-specific antibody for the ERK1/2. Icaritin treatment induced ERK phosphorylation that lasted 24 hours (Fig. 6A), indicating that icaritin induced sustained ERK activation. However, we failed to consistently observe enhanced levels of phosphorylation of the other members of the MAPK family such as p38 and JNK (data not shown).

To examine if the sustained ERK activation is involved in icaritin-induced cell cycle arrest, cells were treated with icaritin alone or together with the MEK inhibitor U0126 and their effects on cell cycle were examined using PI staining followed by flow cytometry. In both cell lines, U0126 effectively arrested cells in the G1 phase of the cell cycle in the presence or absence of icaritin and abrogated the G2/M phase arrest induced by icaritin (Fig. 6B), suggesting sustained ERK activation is involved in the icaritin-induced G2/M arrest in breast cancer cells.

In addition, we also found that icaritin induced ERK activation that lasted 48 hours (Fig. 7A), suggesting that icaritin induced sustained ERK activation may be also involved in icaritin-induced cell death. To test this, MDA-MB-453 cells were treated with icaritin alone or together with the MEK inhibitor U0126 and their effects on cell death was examined using annexin V-FITC and PI staining followed by flow-cytometry. Cell death was effectively inhibited in cells treated with the MEK inhibitor U0126 [3.5 μ M: 6.8% $\pm$ 0.8 (+U0126) versus 15.0% $\pm$ 2.1, (-U0126) 5 μ M: 17.9 $\pm$ 2.1 (+U0126) versus 50.3% $\pm$ 3.9(-U0126) (Fig. 7B). A similar result was also observed in MCF7 cells (Fig. 7C). In addition, Western blot analysis demonstrated that the MEK inhibitor U0126 fully inhibited icaritin-induced sustained ERK activation in MDA-MB-453 and MCF7 cells (Fig. 7D). Thus, our data strongly indicated that sustained ERK activation is a major mechanism underlying icaritin-induced cell death in breast cancer cells.

3.5 Icaritin inhibits the proliferation of breast cancer stem/progenitor cells

Accumulating evidence demonstrated that many types of cancer, including breast cancer, are initiated from cancer stem/progenitor cells (Liu et al., 2005; Charafe-Jauffret et al., 2009). Breast cancer stem/progenitor cells are involved in resistance to chemo-and radiation-therapies (Fillmore and Kuperwasser, 2008; Shafee et al., 2008). We decided to test the possibility of icaritin's inhibitory effects on breast cancer stem/progenitor cells. To this aim, we used aldehyde dehydrogenase (ALDH) activity as a biomarker to enrich breast cancer stem/progenitor cells (ALDH-high cells) from MDA-MB-453 and MCF7 cells with the ALDEFLUOR kit and flow cytometry. ALDH expression and its activity have been used as markers to enrich breast cancer stem/progenitor cells (Ginestier et al., 2007). These ALDH-high and ALDH-low cells were treated with the different concentrations of tamoxifen or icaritin. ALDH-high positive cells from the MDA-MB-453 and MCF7 cells were more

resistant to tamoxifen treatment, compared to ALDH-low cells (Fig. 8A & C). Icaritin, however, effectively inhibited the growth of both ALDH-high and-low breast cancer cells (Fig. 8B & D). These results indicated that the ALDH-high cells, i.e. breast cancer stem/progenitor cell, are resistant to the widely used anti-estrogen tamoxifen and the novel anticancer agent, icaritin acts as a potent inhibitor on both breast cancer stem/progenitor cells and non-stem/progenitor cells.

4. Discussion

Anti-estrogen tamoxifen has been used widely to treat the patients with ER-positive breast tumors either as adjuvant therapy following surgery or as first-line treatment for advanced disease since 1971 (Cole et al., 1971). Tamoxifen was also approved as a chemopreventive agent for high-risk population, women who have a familial history of breast cancer. Although tamoxifen is effective as an adjuvant and chemopreventive agent, there are still a great proportion of patients who develop breast cancer or relapse breast cancer after tamoxifen treatment, and the emergence of advanced breast cancer is often not preventable (Muss, 1992). Thus, tamoxifen treatment is usually temporary and relapse of disease is eventually inevitable.

In this study, we found that tamoxifen inhibited growth of both estrogen receptor-positive and-negative breast cancer cells at micromolar concentrations, consistent with the previous reports that tamoxifen induced growth arrest and cell apoptosis in estrogen receptor-negative breast cancer cells *in vitro* (Mandlekar and Kong, 2001; Perry et al., 1995). Thus, induction of cell apoptotic death is one of the mechanisms involved in anti-tumor activity of tamoxifen. Tamoxifen has been used in treatment of estrogen receptor-negative tumors such as hepatoma, glioma and melanoma. However, the therapeutic efficacy of tamoxifen has been obtained at doses 4- to 8- fold higher than those used for estrogen receptor-positive tumors (reviewed in Mandlekar and Kong, 2001), indicating that tamoxifen at higher concentrations acts in a non-estrogen receptor-mediated way to induce growth arrest and apoptotic cell death in estrogen receptor-negative breast cancer cells.

In this study, we investigated the potential of icaritin, a prenylflavonoid derivative from Chinese herb *Epimedium Genus*, to inhibit growth of breast cancer cells. Previously, it was reported that icaritin exhibits estrogen-like activity in estrogen receptor-positive breast cancer MCF7 cells at sub-micromolar concentrations (Wang and Lou, 2004). At micromolar range, however, icaritin inhibited growth of prostate cancer PC3 cells (Huang et al., 2007b). These results indicated that icaritin has both agonist and antagonist activities depending on concentrations and may function as an estrogen receptor modulator to regulate cell growth. Here, we showed that icaritin potently inhibited growth of both estrogen receptor-positive and -negative breast cancer cells through induction of sustained ERK activation mediated G₂/M arrest of the cell cycle and cell death.

Eukaryotic cell cycle progression involves sequential activation of Cdks, which are controlled by a complex of proteins, including the cyclins. A complex formed by the association of Cdk1 (also called as p34cdc2) and cyclinB1 plays a major role during entering into mitosis (Hartwell and Kastan, 1994). Cdc25C regulates the cdc2-cyclinB1 complex and is believed to be the rate-limiting step in the G₂/M transition (Xiao et al., 2003). Here, we found that icaritin treatment arrested breast cancer cells mainly at the G₂/M phase of the cell cycle, which was accompanied with down-regulation of the expression levels of the proteins pivotal for the G₂/M transition. Previously, Huang et al. reported that icaritin treatment induced G₁ arrest in the prostate cancer PC-3 cell line (Huang et al., 2007b). The exact mechanism underlying this difference is not clear. One possibility is that different cell lines were used. In addition, their study also employed much higher

concentrations of icaritin (10–50 μ M), which may also provide an explanation to the difference we observed.

In this study, we observed that icaritin induced a different pattern of cell death in MDA-MB-453 and MCF7 cells; icaritin treatment mainly increased annexin V-positive (apoptotic) cells in MDA-MB-435 cell line while annexin V-negative/PI positive (necrotic) cells in MCF7 cell line. These experiments were repeated more than three times and similar results were consistently obtained. The exact mechanism underlying this difference is currently not clear. It was reported that as an important caspase in apoptotic cell death, caspase-3 (CPP32/Yama/apopain) is critical for phosphatidylserine externalization in the early stage of apoptosis (Mandal D et al., 2002). Thus, it is possible that as a caspase-3-deficient cell line (Kurokawa et al., 1999), MCF7 cells may exhibit an annexin V/PI staining pattern different from that of MDA-MB-435 after icaritin treatment. It is also possible that icaritin may mainly choose necrosis relevant pathway to induce cell death in MCF7 cells.

ERK is a member of MAPK family that plays an essential role in many cellular events such as cell growth, differentiation, survival and apoptosis through phosphorylation of specific serines/threonines of substrates (Chang and Karin, 2001; Plataniias, 2003). Previously, it has been reported that persistent or sustained ERK activation contributes to cell death in several systems (Ramos, 2008; Stanciu et al., 2000). Here, we found that icaritin induced sustained ERK activation in both estrogen receptor-positive and -negative breast cancer cells, which contributes to icaritin-induced cell cycle arrest and cell death. In this study, we did not observe that icaritin induced activation of the other members of the MAPK family, p38 and JNK. Recently, It was reported that icaritin induced cell apoptosis in the hepatoma HepG2 cell line and icaritin activated JNK1 but not ERK1/2 and p38 (He et al., 2010), suggesting that icaritin may function through different signaling pathway in different cell context.

Accumulating evidence indicated that many types of cancer, including breast cancer, originate from and are maintained by a small population of cancer stem/progenitor cells. These cancer stem/progenitor cells are resistant to most therapeutic approaches currently used. In this study, we enriched breast cancer stem/progenitor cells using the fluorescent ALDEFLUOR assay that was used to successfully isolate breast cancer stem cells from specimens of breast tumors and established breast cancer cell lines (Ginestier et al., 2007). These ALDH-high breast cancer cells exhibit stem/progenitor cell properties and display increased metastatic potentials (Ginestier et al., 2007). Here, we showed that ALDH-high breast cancer stem/progenitor cells were less sensitive to anti-estrogen tamoxifen compared to ALDH-low cells, suggesting that breast cancer stem/progenitor cells are more resistant to tamoxifen compared to non-stem/progenitor cells and tamoxifen may only inhibits growth of non-stem/progenitor cells. Our finding is consistent with the previous reports that cancer stem/progenitor cells are resistant to many current cancer therapies including chemo- and radiation-therapy (Fillmore and Kuperwasser, 2008; Hambardzumyan et al., 2006; Shafee et al., 2008). This suggests that many cancer therapies including hormone therapy, while killing the bulk of tumor cells, may eventually fail since they do not eradicate cancer stem/progenitor cells that survive to regenerate new tumors.

In this study, we found that unlike tamoxifen, icaritin inhibited growth of ALDH-high breast cancer stem/progenitor cells and ALDH-low differentiated breast cancer cells with the preferential targeting of breast cancer stem/progenitor cells. Here, we also found that a combination of tamoxifen and icaritin functioned more effectively to inhibit growth of breast cancer cells compared to either agent alone. Our results thus suggested that therapies that are directed against both differentiated breast cancer cells and breast cancer stem/progenitor cells may provide advantages to treat this deadly disease.

5. Conclusion

In summary, we have shown icaritin effectively inhibited growth of both estrogen receptor-positive and -negative breast cancer cells, which was manifested with addition of anti-estrogen tamoxifen. We have also shown that icaritin was able to induce G₂/M arrest of the cell cycle and cell death in these cells. Unlike tamoxifen, Icaritin preferentially targets breast cancer stem/progenitor cells. Furthermore, our study identified the sustained activation of the MAPK/ERK pathway by icaritin as one of the mechanisms for its effects. Thus, our findings provide a strong rationale for preclinical and clinical evaluation of icaritin alone or in combination with tamoxifen for breast cancer therapy.

Acknowledgments

We thank Perry Greg for his technical support for flow cytometry. This work was supported by the NIH grant DK070016 to Z.Y. Wang and the Nebraska Tobacco Settlement Biomedical Research Program Awards (LB-595 and LB692) to Z.Y. Wang.

References

- Adachi T, Kar S, Wang M, Carr BI. Transient and sustained ERK phosphorylation and nuclear translocation in growth control. *J Cell Physiol.* 2002; 192:151–159. [PubMed: 12115721]
- Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature.* 2001; 410:37–40. [PubMed: 11242034]
- Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, Hur MH, Diebel ME, Monville F, Dutcher J, Brown M, Viens P, Xerri L, Bertucci F, Stassi G, Dontu G, Birnbaum D, Wicha MS. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res.* 2009; 69:1302–1313. [PubMed: 19190339]
- Chen JR, Plotkin LI, Aguirre JI, Han L, Jilka RL, Kousteni S, Bellido T, Manolagas SC. Transient versus sustained phosphorylation and nuclear accumulation of ERKs underlie anti-versus pro-apoptotic effects of estrogens. *J Biol Chem.* 2005; 280:4632–4638. [PubMed: 15557324]
- Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer: an early clinical appraisal of ICI46474. *Br J Cancer.* 1971; 25:270–275. [PubMed: 5115829]
- Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res.* 2008; 10:R25. [PubMed: 18366788]
- Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell.* 2007; 1:555–567. [PubMed: 18371393]
- Hambardzumyan D, Squatrito M, Holland EC. Radiation resistance and stem-like cells in brain tumors. *Cancer Cell.* 2006; 10:454–456. [PubMed: 17157785]
- Hartwell LH, Kastan MB. Cell cycle control and cancer. *Science.* 1994; 266:1821–1828. [PubMed: 7997877]
- He J, Wang Y, Duan F, Jiang H, Chen MF, Tang SY. Icaritin induces apoptosis of HepG2 cells via the JNK1 signaling pathway independent of the estrogen receptor. *Planta Med.* 2010; 76:1834–1839. [PubMed: 20560114]
- Huang J, Yuan L, Wang X, Zhang TL, Wang K. Icaritin and its glycosides enhance osteoblastic, but suppress osteoclastic, differentiation and activity in vitro. *Life Sci.* 2007a; 81:832–840. [PubMed: 17764702]
- Huang X, Zhu D, Lou Y. A novel anticancer agent, icaritin, induced cell growth inhibition, G1 arrest and mitochondrial transmembrane potential drop in human prostate carcinoma PC-3 cells. *Eur J Pharmacol.* 2007b; 564:26–36. [PubMed: 17382317]
- Kim BW, Lee ER, Min HM, Jeong HS, Ahn JY, Kim JH, Choi HY, Choi H, Kim EY, Park SP, Cho SG. Sustained ERK activation is involved in the kaempferol-induced apoptosis of breast cancer

- cells and is more evident under 3-D culture condition. *Cancer Biol Ther.* 2008; 7:1080–1089. [PubMed: 18443432]
- Kurokawa H, Nishio K, Fukumoto H, Tomonari A, Suzuki T, Saijo N. Alteration of caspase-3 (CPP32/Yama/apopain) in wild-type MCF-7 breast cancer cells. *Oncology Reports.* 1999; 6:33–37. [PubMed: 9864397]
- Lewis JS, Jordan VC. Selective estrogen receptor modulators (SERMs): mechanisms of anticarcinogenesis and drug resistance. *Mutat Res.* 2005; 591:247–263. [PubMed: 16083919]
- Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. *Breast Cancer Res.* 2005; 7:86–95. [PubMed: 15987436]
- Mandal D, Moitra PK, Saha S, Basu J. Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes. *FEBS Lett.* 2002; 513:184–188. [PubMed: 11904147]
- Mandlekar S, Kong AN. Mechanisms of tamoxifen-induced apoptosis. *Apoptosis.* 2001; 6:469–477. [PubMed: 11595837]
- Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell.* 1995; 80:179–185. [PubMed: 7834738]
- Molinari M. Cell cycle checkpoints and their inactivation in human cancer. *Cell Prolif.* 2000; 33:261–274. [PubMed: 11063129]
- Muss HB. Endocrine therapy for advanced breast cancer: a review. *Breast Cancer Res Treat.* 1992; 21:15–26. [PubMed: 1382723]
- Normanno N, Di Maio M, De Maio E, De Luca A, de Matteis A, Giordano A, Perrone F. Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. *Endocr Relat Cancer.* 2005; 12:721–747. [PubMed: 16322319]
- Patel T, Gores GJ, Kaufmann SH. The role of proteases during apoptosis. *FASEB J.* 1996; 10:587–597. [PubMed: 8621058]
- Perry RR, Kang Y, Greaves B. Effects of tamoxifen on growth and apoptosis of estrogen-dependent and -independent human breast cancer cells. *Ann Surg Oncol.* 1995; 2:238–245. [PubMed: 7641021]
- Platanias LC. Map kinase signaling pathways and hematologic malignancies. *Blood.* 2003; 101:4667–4679. [PubMed: 12623839]
- Ramos JW. The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. *Int J Biochem Cell Biol.* 2008; 40:2707–2719. [PubMed: 18562239]
- Recchia I, Rucci N, Funari A, Migliaccio S, Taranta A, Longo M, Kneissel M, Susa M, Fabbro D, Teti A. Reduction of c-Src activity by substituted 5,7-diphenyl-pyrrolo[2,3-d]-pyrimidines induces osteoclast apoptosis in vivo and in vitro. Involvement of ERK1/2 pathway. *Bone.* 2004; 34:65–79. [PubMed: 14751564]
- Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol.* 1997; 9:180–186. [PubMed: 9069255]
- Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev.* 2004; 68:320–344. [PubMed: 15187187]
- Shafee N, Smith CR, Wei S, Kim Y, Mills GB, Hortobagyi GN, Stanbridge EJ, Lee EY. Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. *Cancer Res.* 2008; 68:3243–3250. [PubMed: 18451150]
- Stanciu M, Wang Y, Kentor R, Burke N, Watkins S, Kress G, Reynolds I, Klann E, Angiolieri MR, Johnson JW, DeFranco DB. Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *J Biol Chem.* 2000; 275:12200–12206. [PubMed: 10766856]
- Traverse S, Gomez N, Paterson H, Marshall C, Cohen P. Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem J.* 1992; 288 (Pt 2):351–355. [PubMed: 1334404]
- Wang Z, Zhang X, Wang H, Qi L, Lou Y. Neuroprotective effects of icaritin against beta amyloid-induced neurotoxicity in primary cultured rat neuronal cells via estrogen-dependent pathway. *Neuroscience.* 2007; 145:911–922. [PubMed: 17321691]

- Wang ZQ, Lou YJ. Proliferation-stimulating effects of icaritin and desmethylicaritin in MCF-7 cells. *Eur J Pharmacol.* 2004; 504:147–153. [PubMed: 15541416]
- Wo YB, Zhu DY, Hu Y, Wang ZQ, Liu J, Lou YJ. Reactive oxygen species involved in prenylflavonoids, icariin and icaritin, initiating cardiac differentiation of mouse embryonic stem cells. *J Cell Biochem.* 2008; 103:1536–1550. [PubMed: 17985362]
- Xiao D, Srivastava SK, Lew KL, Zeng Y, Hersherberger P, Johnson CS, Trump DL, Singh SV. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis. *Carcinogenesis.* 2003; 24:891–897. [PubMed: 12771033]
- Zhang G, Qin L, Sheng H, Wang XL, Wang YX, Yeung DK, Griffith JF, Yao XS, Xie XH, Li ZR, Lee KM, Leung KS. A novel semisynthesized small molecule icaritin reduces incidence of steroid-associated osteonecrosis with inhibition of both thrombosis and lipid-deposition in a dose-dependent manner. *Bone.* 2009; 44:345–356. [PubMed: 19015051]
- Zhu DY, Lou YJ. Inducible effects of icariin, icaritin, and desmethylicaritin on directional differentiation of embryonic stem cells into cardiomyocytes in vitro. *Acta Pharmacol Sin.* 2005; 26:477–485. [PubMed: 15780198]

Figure 1. Icaritin inhibits growth of breast cancer cells

(A). The chemical structure of icaritin. (B & C). Effects of icaritin or tamoxifen on the growth of MDA-MB-453 and MCF7 cells. Cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with vehicle, DMSO (0) and 1, 2, 3, 4, 5 μM of Icaritin or tamoxifen for 3 days before cells were trypsinized and counted. (D & F). Effects of icaritin or tamoxifen, icaritin and tamoxifen together on the growth of MDA-MB-453 and MCF7 cells. Cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0) and indicated concentrations of icaritin or tamoxifen alone or icaritin+tamoxifen (1:1). Three dishes were used for each concentration in all of these experiments and all experiments were repeated at least three times. Each point represents mean $\pm$ S.D. of three independent experiments and data are expressed as percent of control cells treated with vehicle (DMSO).

Figure 2. Icaritin induces G₂/M arrest in breast cancer cells

(A). MDA-MB-453 cells were treated with DMSO (vehicle) or the indicated concentrations of icaritin for 48 h. Cells were assayed with PI staining and flow cytometric analysis. All experiments were repeated three times and data from representative experiments are shown. (B). The percentages of G₂/M distribution for each group of MDA-MB-453 cells were shown with means $\pm$ S.D. (C). MCF7 cells treated with vehicle and different concentrations of icaritin were assayed with PI staining and flow cytometric analysis. (D). The percentages of G₂/M distribution for each group of MCF7 cells were shown with means $\pm$ S.D.

Figure 3. Icaritin treatment down-regulates expression levels of the G₂/M regulators (A & B). Breast cancer cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0), or indicated concentrations of icaritin for indicated time periods. Cell lysates were subjected to Western blot analysis with antibodies for cyclin B (A) and cdc2 (B). (C). Cells were treated with DMSO (–) and 3 μ M icaritin (+). Cell lysates were subjected to Western blot analysis with antibodies for cdc25C. The membranes were stripped and re-probed with β -actin antibody to ensure equal loading.

Figure 4. Icaritin induces cell death in breast cancer cells

(A). MDA-MB-453 cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0) or 3, 4, 5 μ M icaritin for 48 h. Cells were collected and analyzed with flow cytometric analysis after annexin V and PI staining. The experiment was repeated three times and the results from representative experiments were shown. (B). Percentages of total non-viable (dead) cells including cells with annexin V positive/PI negative, annexin V positive/PI positive and annexin V negative/PI positive were shown as means $\pm$ S.D. (C&D). MCF7 cells were treated as described in (A). The experiment was repeated three times and percentages of total non-viable (dead) cells were shown as means $\pm$ S.D.

Figure 5. Icaritin treatment down-regulates the intracellular level of Bcl-2 and induces PARP cleavage

(A). MDA-MB-453 and MCF7 cells were treated with DMSO (0) or 3, 4, 5 μM icaritin for 48 h. Cell lysates were subjected to Western blot analysis with a Bcl-2 antibody. The membranes were stripped and re-probed with a β -actin antibody to ensure equal loading. (B). MDA-MB-453 and MCF7 cells with DMSO (–) or 5 μM icaritin (+) for the indicated time periods. Western blot analysis was performed with a PARP antibody. The membranes were stripped and re-probed with a β -actin antibody.

Figure 6. Sustained ERK activation is involved in icaritin-induced G2/M arrest of breast cancer cells

(A). MDA-MB-453 and MCF7 cells were treated with 3 μ M icaritin for different time periods. Cell lysates were subjected to Western blot analysis to assess ERK phosphorylation. (B & C). MDA-MB-453 and MCF7 cells were treated with DMSO, U0126 (10 μ M), icaritin (3 μ M) and U0126 + icaritin, respectively for 48 h. Cells were assayed with PI staining and flow cytometric analysis. All experiments were repeated three times and data from representative experiments are shown.

A **B**

Figure 7. Sustained ERK activation is involved in icaritin-induced apoptosis of breast cancer cells

(A). MDA-MB-453 and MCF7 cells were treated with DMSO (–) or 5 μM icaritin (+) for 1, 24, 36, 48 hours. Cell lysates were subjected to Western blot analysis to assess ERK phosphorylation. (B). MDA-MB-453 cells were treated with DMSO (0), 3.5 and 5 μM icaritin, respectively for 48 hours. Cells were pre-treated with or without 10 μM U0126 for 1 hour before icaritin treatment. Cells were stained with Annexin V and PI before flow cytometry analysis. The data were representatives of three separate experiments and total non-viable (dead) cells were shown as means ± S.D. * P<0.01. (C). MCF7 cells were treated and assayed as described in (B) and non-viable (dead) cells were calculated; * P<0.01. (D). Cells were pre-treated with or without 10 μM U0126 before treatment with DMSO (–) or 5 μM icaritin (+) for 24, 36, 48 hours, respectively. Western blot analysis was performed to assess the levels of ERK phosphorylation.

Figure 8. Icaritin inhibits growth of breast cancer stem/progenitor cells

(A & B). ALDH-high MDA-MB-453 and ALDH-low MDA-MB-453 cells were sorted out with flow cytometry after stained with the fluorescent ALDEFLUOR kit. The sorted cells were treated with DMSO (0), the indicated concentrations of icaritin or tamoxifen for three days. Cells were trypsinized and counted. The data were expressed as the percentages of DMSO treated cells; * $P < 0.01$. (C & D). MCF7 cells were treated and analyzed as describe for MDA-MB-453 cells in (A & B) and the data were expressed as the percentages of DMSO treated cells; * $P < 0.01$.