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ORIGINAL ARTICLE

The effects of hypericin on ADAMTS and p53 gene expression in MCF-7 breast cancer cells

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Summary

Purpose: *The purpose of this study was to determine the effects of hypericin on MCF-7 (Michigan Cancer Foundation-7) breast cancer cells, as it is known to exert an antitumor effect on the expression and regulation of ADAMTS1, 3, 10 and the p53 gene in breast cancer cells.*

Methods: *MFC-7 cells were cultured and subjected separately to various doses (1, 5 and 7.5 μg /mL) hypericin. After 24 hrs, RNA was isolated and transcribed into cDNA. Expression analysis was performed by real time (RT)-PCR and cell survival was determined by the XTT assay.*

Results: *While the expression of ADAMTS1 in MFC-7 cells decreased to 0.04-fold after exposure to 1 μg /mL hypericin, the expression increased by 5.6- and 36-fold with 5 and 7.5 μg/mL, respectively. Furthermore, ADAMTS3 expression in MCF7 cells increased 3.9-fold with the use of 5 μg /mL of hypericin. These concentrations of hypericin did not lead to significant changes in the expression of ADAMTS10 and the p53 gene. Viability of cancer cells as evaluated by the XTT assay showed that hypericin concentration of 7.5 μg/mL led to increased apoptosis of cancer cells.*

Conclusion: *The increase in ADAMTS1 expression may prevent metastasis or facilitate the development of an adjuvant factor with tumor-suppressive effects. Hypericin may therefore exert its antitumor and apoptotic effects in MFC-7 cells via ADAMTS1 and ADAMTS3.*

Key words: *ADAMTS, breast cancer, hypericin, p53 gene*

Introduction

Breast cancer is the most common and leading cause of cancer mortality in females [1]. Metalloproteases belonging to the a disintegrin-like and metalloprotease with thrombospondin type I motif (ADAMTS) family have been widely implicated in tissue remodeling events manifested in breast cancer development, growth and progression [2].

ADAMTS1 is a member of the ADAMTS family of metalloproteases. This secreted protease participates in several biological processes, such as inflammation and angiogenesis, besides the suggested roles for ADAMTS1 in tumor invasion and metastasis [3]. Studies have suggested that it initially inhibits angiogenesis in cancer, therefore acting as an anticancer agent by blocking vascular epithelial growth factor (VEGF) [3,4]. The expression of ADAMTS1, which has both antiangiogenic and aggrecanase activities, is decreased in breast cancer [3-5].

ADAMTS3 and 10 are also members of the ADAMTS family, whose expression is reduced in breast cancer tissues [5]. ADAMTS3 exhibits collagen N-proteinase properties and degrades the propeptides at the N-extremity of procollagen, transforming it into collagen [6]. A recent study reported that ADAMTS10 may play a role in microfibril biogenesis [7]. The roles of ADAMTS3 and ADAMTS10 in human breast cancer are not fully understood and, therefore, require further

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investigation.

p53 has important effects on breast cancer development due to its role as a tumor suppressor [8]. p53 expression varied according to different histological types of breast cancer [9]. Current breast cancer therapies have considerable side effects. As some tumor cells can acquire resistance to chemotherapeutics, there is a continuing need to develop better targeted anticancer treatments [10].

One approach is to determine the anticancer effects of medicinal plant extracts. *Hypericum perforatum* has been demonstrated to exert effects on cancer cell motility, invasion, proliferation, and apoptosis [11].

However, information regarding the mechanisms underlying these effects is lacking. In this study, our objective was to determine whether hypericin extracted from *Hypericum perforatum* exerts antitumor activity by regulating the expression of ADAMTS1, 3, 10 and the p53 gene in MCF-7 cells. Our results will determine whether hypericin can act as an alternative treatment or adjuvant agent for the treatment of breast cancer.

Methods

Chemicals and reagents

Hypericin powder (Sigma, St Louis, MO, USA) (1 mg) was dissolved in 2-mL DMSO (dimethyl-sulphoxide), and made up to 10 mL with distilled non-ionized water. The solution was stored in an opaque glass bottle at - 20 °C. All other chemicals used were obtained from either Sigma or Thermo Scientific (Waltham, MA, USA).

Cell culture

ER-positive breast cancer MCF-7 cells were obtained from the American Type Culture Collection (ATCC). The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed every 2 days. Sixth-passage cells were used in all experiments.

Hypericin stimulation

All cells were initially incubated in 2 mL of medium containing 10% FBS. After 72 hrs, the medium was changed to serum-free DMEM, and the cells were incubated for another 24 hrs. The cells were then exposed to 1, 5 or 7.5 μg/mL hypericin in DMSO or phosphate-buffered saline containing 0.1% bovine serum albumin as a control (N=6 each), according to a protocol described previously [12].

Table 1. The forward and reverse primers used in the real-time PCR analyses of the ADAMTS1, 3, 10, p53 and β-Actin genes

ADAMTS1	Forward	5'TGTGATCCGAGCAGAAGCTG3'
	Reverse	5'TGTAGGCACTGCAAGGAGAC3'
ADAMTS3	Forward	5'ACTGCACCAAAACCTGTGGA 3'
	Reverse	5'CCGACTCAGGCTTTTCACCA 3'
ADAMTS10	Forward	5'CTTCCTGCTGAACCTGACCC 3'
	Reverse	5'GGTACTCTTCCTCGTCTGCC 3'
p53 gene	Forward	5'TGACTGTACCACCATCCACTA3'
	Reverse	5'AAACACGCACCTCAAAGC 3'
β-Actin gene	Forward	5'TTCCTGGGCATGGAGTCCT3'
	Reverse	5'AGGAGGAGCAATGATCTTGATC3'

Total RNA isolation and cDNA construction

Total RNA was extracted using TRIzol (Ambion Life Technologies/ Invitrogen, Carlsbad, CA, USA) according to methods described previously [12]. One microgram of RNA was reverse-transcribed using Reverse Transcriptase (Thermo Scientific) with oligo (dT) primers according to the manufacturer's instructions (Table 1). Mouse β-actin was amplified as a control for the PCR reaction. Samples lacking reverse transcriptase were amplified to control for the presence of genomic DNA contamination.

RT- PCR

RT-PCR was performed on cDNA samples obtained using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) as described previously [12]. The PCR mixture consisted of forward and reverse primers and cDNA of samples and SYBR Green PCR Master Mix, which included DNA polymerase, SYBR Green I dye, dNTPs, PCR buffer, in a total volume of 50 μl/mL. Amplification of β-Actin, a housekeeping gene, was used to normalize the efficiency of cDNA synthesis and the amount of RNA applied (Figure 1). The PCR was performed with an initial denaturation at 95 °C for 5 min, followed by amplification for 40 cycles for β-Actin and ADAMTS10, and 45 cycles for ADAMTS1, ADAMTS3 and p53, each cycle consisting of denaturation at 95 °C for 30 sec, annealing at 58 °C for β-Actin and ADAMTS1, 3 and 10, and at 60 °C for p53, polymerization elongation at 72 °C for 1 min and a final polymerization elongation at 72 °C for 5 min.

XTT cell viability assay

MCF-7 cells were cultured at 1×10^4 cells per well in 96-well culture plates. The cells were exposed for 4, 6, 8 and 24 hrs to 0.2, 0.5, 1, 5, 7.5, 10 and 20 μg/mL hypericin, respectively. Cells not exposed to hypericin were used as controls, and the assay was repeated three times per hypericin concentration.

The effects of hypericin on cell viability were evaluated using a Cell Proliferation Kit II (XTT)

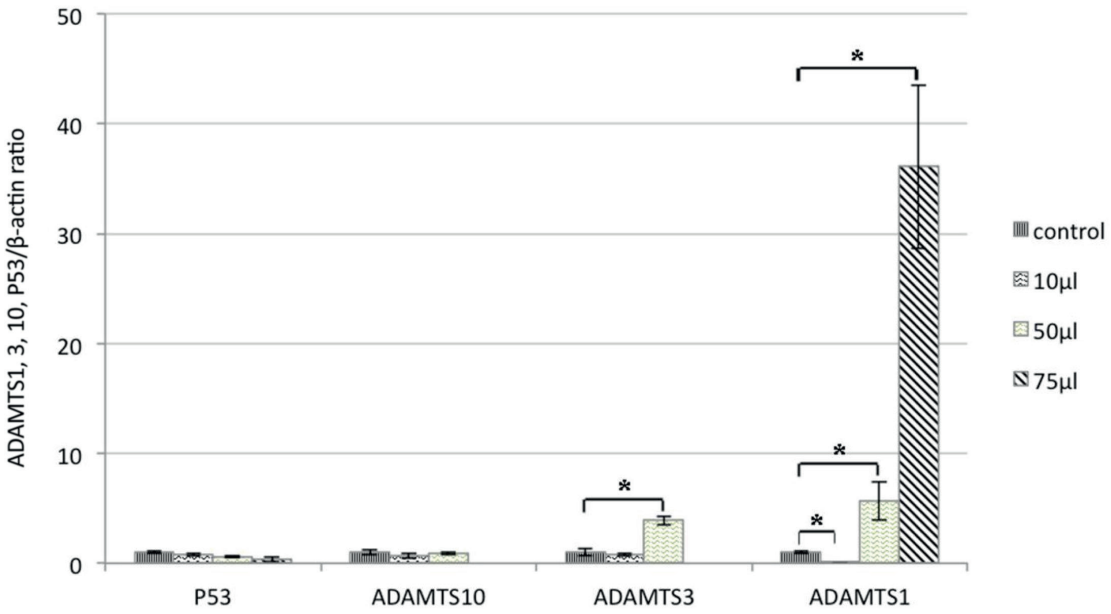

Figure 1. The effects on ADAMTS1, 3, 10 / β-actin ratio and p53/β-actin ratio after hypericin induction of MCF-7 cells. All means marked with *($p<0.05$) are significantly different from the control values.

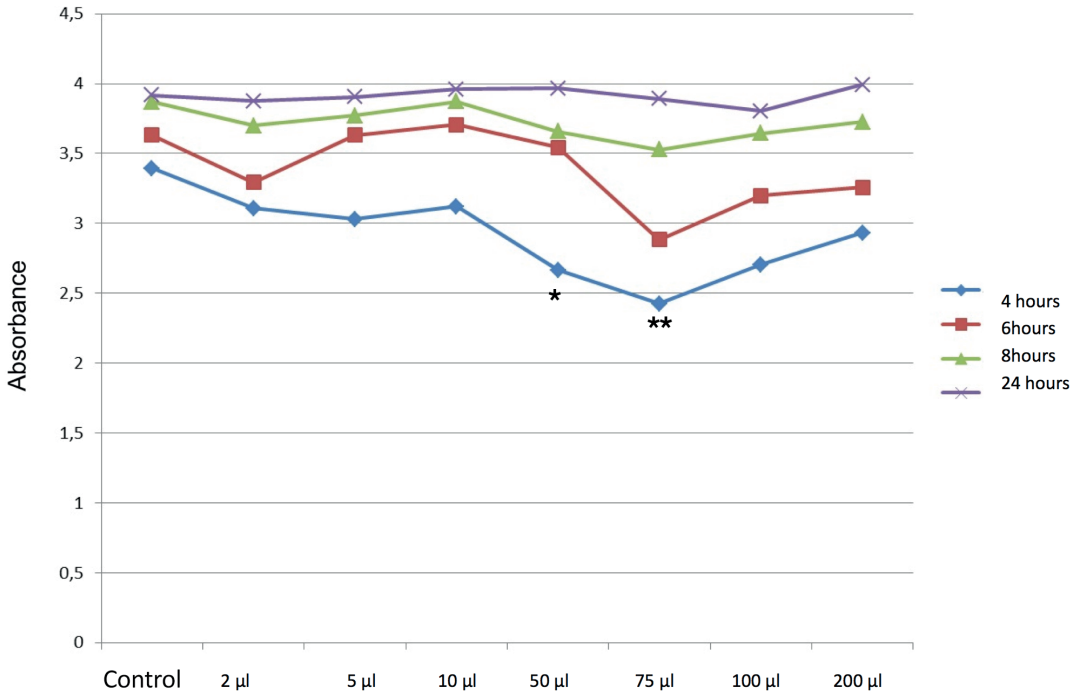

Figure 2. The cytotoxic effects of hypericin as determined by XTT assay. Each point represents the mean ± SEM of three independent experiments, each of which consisted of three replicates per treatment group. All means with *($p<0.05$) and **($p<0.005$) are significantly different from the control values.

(Roche, Penzberg, Germany). After culturing, XTT solution (2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxyanilide salt) was added and cells were cultured for a further 4 hrs. Absorbance at 490 nm was then measured using an ELx800 Absorbance Microplate Reader (BioTek, Winooski, VT, USA). The absorbance values relative to the control were calculated, and the cytotoxicity was determined.

Statistics

Comparison of the data between groups was carried out using the Student's t-test. Statistical signifi-

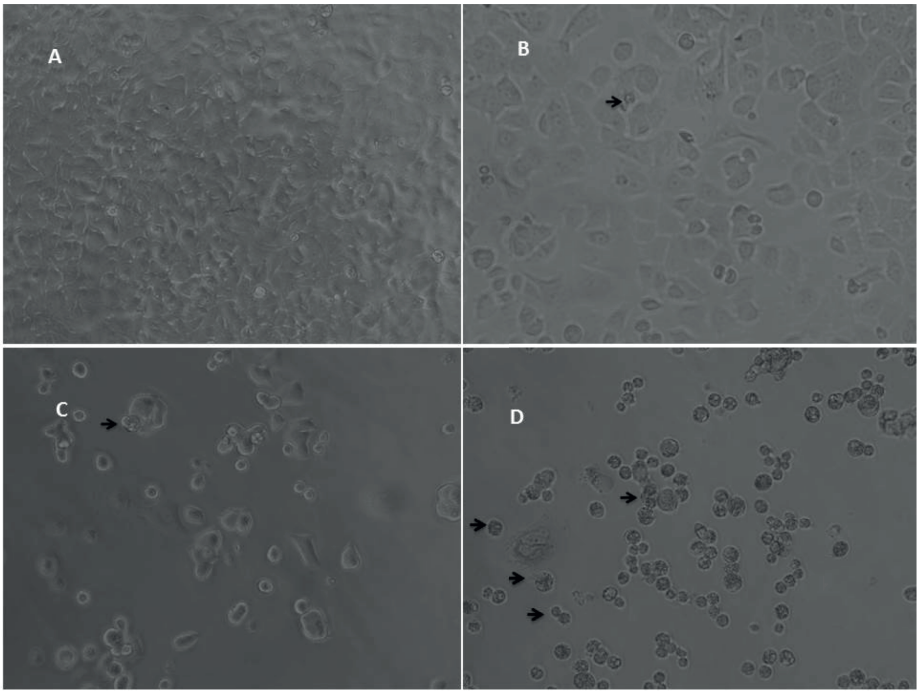

Figure 3. Morphological changes of MCF-7 cells treated with 2, 5 and 7.5 μg/mL hypericin for 24 hrs as viewed under an inverted phase-contrast microscope (200 ×). **A:** Control cells (without hypericin treatment). **B:** Cells treated with 2 μg/mL hypericin for 24 hrs. The arrow shows the nuclei of apoptotic cells and reduced cell density. **C:** Cells treated with 5 μg/mL hypericin for 24 hrs. The arrow shows small cell clusters and reduced cell density. Some cells became rounded and partially detached. **D:** Cells treated with 7.5 μg/mL hypericin for 24 hrs. The arrows indicate apoptotic bodies and extremely dense chromatin, characteristic of apoptosis.

cance was set at $p<0.05$. For all calculations the SPSS 11 software was used.

Results

The expression levels of ADAMTS 1, 3 and 10 and p53 RNA were determined in MCF-7 breast cancer cell lines. There was a significant difference ($p=0.04$) between the control cells and the stimulated cells in terms of the ADAMTS1/β-Actin ratio. The effect of hypericin on the ADAMTS1/β-Actin ratio was more pronounced at 7.5 μg/mL, followed by 5 μg/mL. While expression of ADAMTS1 in MFC-7 cells decreased 0.04-fold after exposure to 1 μg/mL hypericin, the expression increased by 5.6- and 36-fold at 5 μg/mL and 7.5 μg/mL respectively. Furthermore, ADAMTS3 expression in MCF7 cells increased 3.9-fold with the use of 5 μg/mL of hypericin. These concentrations of hypericin did not lead to significant changes in the expression of ADAMTS10 and the p53 gene (Figure 1). XTT test showed that hypericin concentration of 7.5 μg/mL led to increased apoptosis of cancer cells. The XTT results showed that hypericin had cytotoxic effects when used at concentrations of 5 and 7.5 μg/mL with p-values of 0.04 and <0.005, respectively (Figure 2).

Hypericin caused various morphological changes in MCF-7 cells, depending on the concentration administered (Figure 3).

Discussion

In this study, when MCF-7 cells were exposed to hypericin for 24 hrs, ADAMTS1 expression increased significantly but this didn't apply for ADAMTS3 and 10, and p53. We have shown that use of hypericin at ≥50 μg/mL leads to rapid death of cancer cells.

Recent studies have shown the importance of ADAMTS1 activity in the development and progression of breast cancer [2,3]. A study on the expression profiles of the ADAMTS genes of human neoplastic and non-neoplastic breast tissue showed that the expression of seven ADAMTS genes (ADAMTS1,3,5,8,9,10 and 18) decreased in carcinoma cases [5]. In head and neck, prostate, non-small-cell lung carcinomas and hepatocellular cancers, the ADAMTS1 mRNA expression levels were lower compared to healthy control groups [13-15]. Despite the reduced ADAMTS1 expression at the beginning of carcinogenesis, it increased during cancer progression and metastasis. In rat models of breast cancer, the increase in

ADAMTS expression led to tumor growth and a reduction in longevity [2]. This suggests that the increase in ADAMTS1 expression, as seen in our study, may lead to an increase in aggrecanase activity in breast cancer cells exposed to hypericin, making the environment more favorable for cancer cell proliferation, migration and progression.

However, ADAMTS1 plays an active role in the inhibition of angiogenesis [1-3]. Angiogenesis is known to play a critical role in cancer tissue invasion and metastasis [2]. The increase in ADAMTS1 expression seen in our study may repress tumor tissue vascularization via an antiangiogenic effect. Thus, by altering the nutrition available to breast cancer tissues, it may act as an apoptosis-facilitating factor. In another recent study, the increase in ADAMTS1 expression in normal cells prevented cell migration and invasion by repressing VEGF. However, in the same study, an increase in ADAMTS1 expression in tumor cells inhibited VEGF repression and thus facilitated metastasis [3]. According to these results, the development of breast cancer may vary according to whether the aggrecanase or antiangiogenic effect of ADAMTS1 expression dominant. The decrease in ADAMTS expression in primary cancers represents a pro tumorigenic effect [3]. For this reason, assays of gene expression representative of normal breast tissue, rather than for overexpression, may present a tumor suppressor effect. ADAMTS3 plays a crucial role during the wound healing process [6]. Therefore, the increase in ADAMTS3 expression seen in our study may act as an apoptosis-facilitating factor. The effects of hypericin on cell viability were evaluated using the XTT method. The XTT method is simple, accurate, and yields reproducible results for assessing cell viability. The use of XTT in colorimetric proliferation assays offers significant advantages over 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), resulting from reduced assay time and sample handling while offering equivalent sensitivity [16]. The cells were exposed to 0.2, 0.5, 1, 5, 7.5, 10 and 20 μg/mL hypericin in DMSO according to a protocol that was previously described by Takabashi et al. [17]. In their study both compounds specifically inhibited the protein kinase C with IC50 values 1.7 μg/ml and 15 μg/ml, respectively, and showed antiproliferative activity against mammalian cells [17]. In our study, ≥5 μg/mL hypericin led to rapid death of cancer cells, suggesting that hypericin may inhibit cell proliferation by means of its tumor suppressor and cytotoxic effects, which are mediated via ADAMTS1.

Many antiangiogenic agents are tested in clinical trials to determine their effects in human cancers. It has been determined that, at low levels, hypericin has an antiproliferative effect, and at high doses it has an apoptosis-inducing effect, and it may block mitosis in the cell [18]. We have shown that in addition to these effects, hypericin may also have a dose-dependent antiangiogenic effect on tumor tissues, mediated via ADAMTS1 and ADAMTS3. In a previous study on the relationship between breast cancer cells and ADAMTS9, we reported that hypericin alters ADAMTS9 expression in a dose-dependent manner [12]. Hypericin may exert its antitumor effects via ADAMTS1, 3 and ADAMTS9, based on the apoptotic and extracellular effects of ADAMTS9 and the antiangiogenic effects of ADAMTS1. While hypericin increased ADAMTS1 gene expression, the expression levels of ADAMTS 10, and p53 were unaffected. Therefore, these genes may act via different pathways.

The most important limitation of our study is that we focused only on mRNA expression levels. However, we will perform protein analysis of the proteins of interest, for example, western blotting and MiRNAs in a following study. The second limitation of our study is that we used a single type of breast cancer cell line. It would be better if different breast cancer cell lines with different hormone receptor properties were used and compared in a similar study.

Further molecular studies of the preventive and therapeutic effects of hypericin in breast cancer are required to confirm our results.

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