Selective anticancer activity of hirsutine against HER2-positive breast cancer cells by inducing DNA damage

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Abstract. Hirsutine is one of the major alkaloids isolated from plants of the *Uncaria* genus and is known for its cardioprotective, anti-hypertensive and anti-arrhythmic activities. We recently reported that hirsutine is an anti-metastatic phytochemical by targeting NF-κB activation in a murine breast cancer model. In the present study, we further examined the clinical utility of hirsutine against human breast cancer. Among six distinct human breast cancer cell lines, hirsutine showed strong cytotoxicity against HER2-positive/p53-mutated MDA-MB-453 and BT474 cell lines. Conversely, HER2-negative/p53 wild-type MCF-7 and ZR-75-1 cell lines showed resistance against hirsutine-induced cytotoxicity. Hirsutine induced apoptotic cell death in the MDA-MB-453 cells, but not in the MCF-7 cells, through activation of caspases. Furthermore, hirsutine induced the DNA damage response in the MDA-MB-453 cells, but not in the MCF-7 cells, as highlighted by the upregulation of γH2AX expression. Along with the induction of the DNA damage response, the suppression of HER2, NF-κB and Akt pathways and the activation of the p38 MAPK pathway in the MDA-MB-453 cells were observed. Considering that there was no difference between MDA-MB-453 and MCF-7 cells in regards to irinotecan-induced DNA damage response, our present results indicate the selective anticancer activity of hirsutine in HER2-positive breast cancer by inducing a DNA damage response.

Introduction

Breast cancer is the most commonly diagnosed cancer among women and is second only to lung cancer in terms of cancer-related mortality (1). Despite advances in breast cancer research and therapy, the mortality rate remains high (2); therefore, the development of new agents for breast cancer therapy is clinically important.

Phytochemicals are a promising source for the development of novel cancer therapeutics. Due to their potential effectiveness and low toxicity profiles (3), phytochemicals generally have been successful in clinical drug development to treat many diseases (4,5). Hirsutine is one of the major alkaloids found in plants of the *Uncaria* genus and is known for its cardioprotective (6), anti-hypertensive and anti-arrhythmic activities (7). Recently, we identified hirsutine as an anti-metastatic phytochemical by targeting NF-κB activation in a murine breast cancer model. We demonstrated the significant anti-metastatic activity of hirsutine both *in vitro* and *in vivo* (8).

In the present study, we further explored the clinical utility of hirsutine against breast cancer by testing the effect of hirsutine against six human breast cancer cell lines. Notably, hirsutine showed selective anticancer activity against human breast cancer cell lines with HER2-expressing and p53-mutated phenotypes. We further identified that the induction of DNA damage response can be a primary target of hirsutine, leading to apoptotic cell death of breast cancer cells.

Materials and methods

Reagents. Hirsutine and a Cell Counting kit were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Muse Annexin V and Dead Cell Assay kits were purchased from EMD Millipore Corporation (Merck Millipore, Billerica, MA, USA). The reagent for the Caspase-Glo3/7 Assay was obtained from Promega (Madison, WI, USA). Irinotecan was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cells. All cell lines used in the present study were obtained from the American Type Culture Collection (ATCC). Human breast cancer cells ZR-75-1 and BT474 were maintained in RPMI-1640 medium containing 10% bovine serum (Nissui, Tokyo, Japan). MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-453 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine serum (Nissui). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell viability assay. For detection of the neoplastic activity of cells grown in 96-well plates, the human breast cancer cells

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in exponential growth were placed at a final concentration of 2×10^4 cells/well in a 96-well plate. After a 3-h incubation, the cells were treated with hirsutine (6.25, 12.5, 25 and 50 μM) or with the vehicle (vehicle control, 0.5% DMSO) for 24 h. After treatment, 10 μl of WST-1 reagent was added. The microplate was incubated for another 2 h in a humidified atmosphere (37°C, 5% CO_2) to allow the formation of formazan dye and to get a higher sensitivity. The absorbance was measured in a microplate reader at 450/620 nm. Cell viability was determined from the absorbance of soluble formazan dye generated by the living cells.

Detection of apoptosis. Cells were grown in 24-well plates and treated with 50 μM of hirsutine for 15 h before assaying using Muse™ Cell Analyzer (Merck Millipore). Floating and adherent cells were collected after the treatments. The cells were washed twice with PBS and resuspended in PBS. The apoptosis profiling and apoptotic cell counts were obtained with Muse Annexin V and Dead Cell Assay kit. The assay was conducted in triplicate and in accordance with the manufacturer's instructions.

Measurement of caspase activity. For measurements of the activities of caspase-3 and caspase-7, the Caspase-Glo® 3/7 Assay was carried out according to the manufacturer's instructions. This kit is based on the cleavage of the amino acid sequence DEVD of a luminogenic substrate by caspase-3 and caspase-7, which results in a luminescent signal. Human breast cancer cells in exponential growth were placed at a final concentration of 1×10^4 cells/well in 90 μl in a white-walled multi-well plate. After 3 h of incubation, the cells were treated with hirsutine (25 and 50 μM) or with the vehicle (vehicle control, 0.5% DMSO) for another 3 h. Then 100 μl of Caspase-Glo® 3/7 reagent was added, and the plates were incubated for another 30 min. Caspase-3 and -7 activities were recorded in a Glomax Multi-Detection system.

Western blot analysis. Cells were exposed to 50 μM hirsutine for 0, 1, 3 and 6 h. Treated cells were collected, washed with PBS, and lysed in lysis buffer [25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylemethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 10 mg/ml aprotinin, and 10 mg/ml leupeptin]. The cell lysates were separated by 5-10% SDS-PAGE and transferred to PVDF membranes using a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8) and 20% (v/v) methanol]. After blocking with Block Ace for 4 h at room temperature, the membranes were incubated overnight with the primary antibodies, and then for 60 min with the secondary antibodies. Primary antibodies were used at a dilution of 1:1,000. The secondary antibodies were used at a dilution of 1:2,000 and visualized with an enhanced chemiluminescence system (Amersham Biosciences, Amersham, UK).

The following antibodies from Cell Signaling Technology (Danvers, MA, USA) were used for western blot analysis according to the manufacturer's recommendations: anti-phospho-Akt (Ser473, #9271L), anti-phospho-p65 (Ser536, #3033S); anti-caspase-3 (#9662S), anti-phospho-p38 (p-p38) (Thr-180/Tyr-182, #4511), anti-phospho-histone H2AX
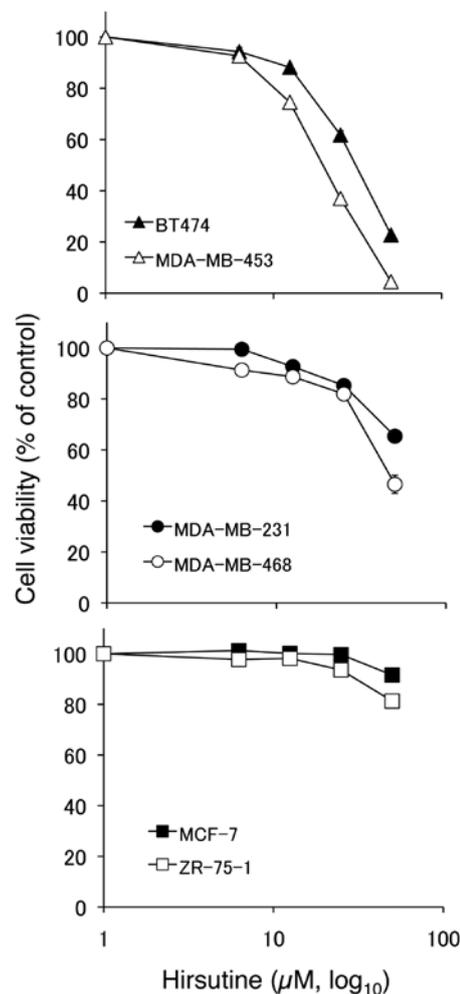

Figure 1. Selective cytotoxic effect of hirsutine on human breast cancer cell lines. Human breast cancer cells in exponential growth were placed at a final concentration of 2×10^4 cells/well in a 96-well plate and incubated for 3 h. After incubation, the cells were treated with hirsutine (6.25, 12.5, 25 and 50 μM) or with the vehicle (vehicle control, 0.5% DMSO) for 24 h. After treatment, 10 μl of WST-1 reagent was added. The microplate was incubated for another 2 h to allow the formation of formazan dye and to obtain a higher sensitivity. The absorbance was measured in a microplate reader at 450/620 nm. Cell viability was determined from the absorbance of soluble formazan dye generated by the living cells.

(Ser139, #9947). Anti-actin (sc-1615, #G1312), anti-caspase-9 (sc-7885, #B0807), anti-ErbB2 (sc-284, #L0706), anti-p65 (sc-109G, #J1003), anti-Akt1 (sc-1618, #131705) and anti-p38α (sc-535, #K1010) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis. All the data are expressed as mean ± SD of at least 3 independent experiments and were analyzed for statistical significance using the Student t-test. P-values <0.05 were considered statistically significant.

Results

Selective cytotoxicity of hirsutine against human breast cancer cells. We recently found hirsutine as an anti-metastatic phytochemical by targeting NF-κB activation in a murine breast cancer model. To further extend the utility of hirsutine, we examined the anticancer effect of hirsutine against human
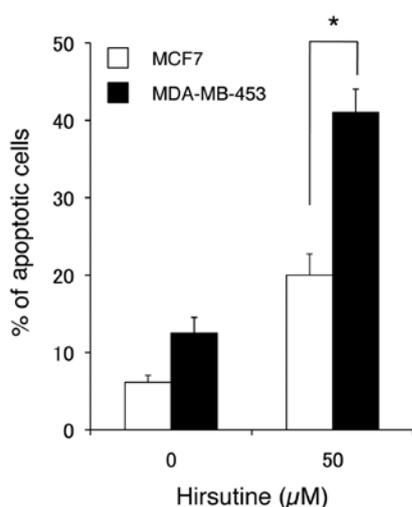
Figure 2. Effect of hirsutine on apoptotic cell death in the MDA-MB-453 and MCF-7 cells. MDA-MB-453 and MCF-7 cells were grown in 24-well plates and treated with 50 μM of hirsutine for 15 h before assaying using the Muse™ Cell Analyzer. Floating and adherent cells were collected after the treatments. Cells were washed twice with PBS and re-suspended in PBS. The apoptosis profiling and apoptotic cell counts were obtained with the Muse Annexin V and Dead Cell Assay kit. The assay was conducted in triplicate and in accordance to the manufacturer's instructions. *P<0.05.
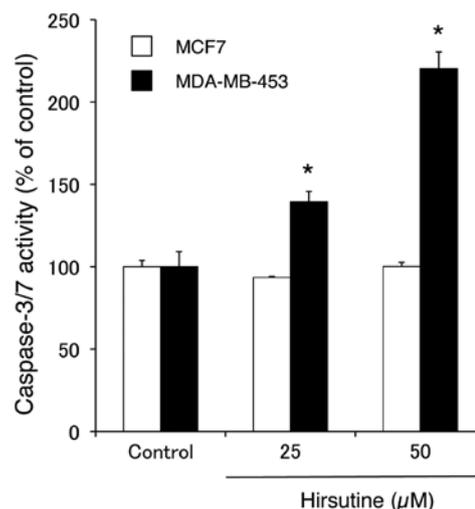

Figure 3. Effect of hirsutine on activities of caspases in the MDA-MB-453 and MCF-7 cells. The human breast cancer cells in exponential growth were placed at a final concentration of 1×10^4 cells/well in 90 μl in a white-walled multi-well plate. After 3 h of incubation, the cells were treated with hirsutine (25 and 50 μM) or with the vehicle (vehicle control, 0.5% DMSO) for another 3 h. Then 100 μl of Caspase-Glo® 3/7 reagent was added, and the plates were incubated for another 30 min. Caspase-3 and -7 activities were recorded in a GloMax Multi Detection system. *P<0.05.

breast cancer cell lines with distinct molecular background. Among the 6 tested human breast cancer cell lines, hirsutine showed strong cytotoxicity against the MDA-MB-453 and BT474 cell lines that have HER2-positive and p53-mutated characteristics (Fig. 1, top panel). While triple-negative breast cancer cell lines MDA-MB-231 and MDA-MB-468 showed a moderate response (Fig. 1, middle panel), HER2-negative and p53 wild-type MCF-7 and ZR-75-1 cells showed significant resistance against hirsutine-induced cytotoxicity (Fig. 1, bottom panel).

Induction of breast cancer cell apoptosis by hirsutine. To examine whether the cytotoxic effect of hirsutine is a result of the induction of apoptosis, both hirsutine sensitive (MDA-MB-453) and resistant (MCF-7) cells were subjected to Annexin V staining 15 h after treatment with hirsutine. In concert with the hirsutine-induced cytotoxicity (Fig. 1), hirsutine significantly induced apoptosis in the MDA-MB-453 cells, but in the MCF-7 cells (Fig. 2). Such induction of apoptosis by hirsutine was related to the activation of caspases, as noted in the elevated caspase-3/7 activity in the MDA-MB-453 cells, but not in the MCF-7 cells, 3 h after treatment with hirsutine (Fig. 3). This demonstrated that caspases played an important role in hirsutine-induced apoptosis in the MDA-MB-453 cells. These results clearly show that the induction of apoptotic cell death can be a potential mechanism for the selective anticancer effect of hirsutine against human breast cancer cells.

Hirsutine induces a DNA damage response in breast cancer cells. To further explore the possible mechanism of hirsutine to induce apoptosis in breast cancer cells, we assessed its effect on the MAPK, HER2, NF-κB, and Akt signaling pathways, as well as the DNA damage response. As shown in Fig. 4, hirsutine significantly suppressed the HER2, NF-κB, and Akt
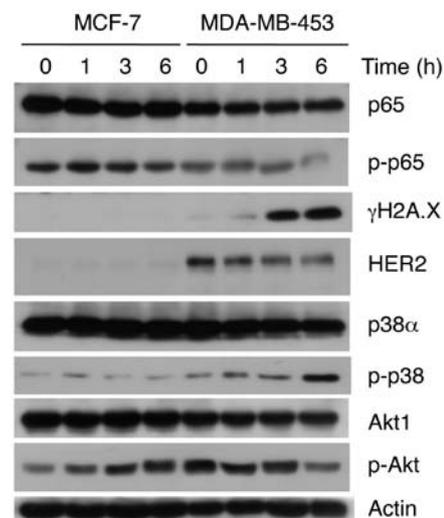

Figure 4. Effects of hirsutine on the DNA damage response in the MDA-MB-453 and MCF-7 cells. Cells were exposed to 50 μM hirsutine for 0, 1, 3 and 6 h. Cell lysates were collected and subjected to western blot analysis to detect the expression of related proteins. Results are representative of at least 3 independent experiments showing similar results.

signaling pathways in the MDA-MB-453 cells. Meanwhile, the activation of the p38 MAPK stress pathway and a DNA damage response, as noted in the expression of p-p38 and γH2AX, respectively, were observed upon hirsutine treatment in the MDA-MB-453 cells. Conversely, hirsutine did not show any significant effect on those molecular pathways or DNA damage response in the MCF-7 cells. In order to determine the selectivity of hirsutine to induce the DNA damage response in HER2-positive and p53-mutant breast cancer cells, we used irinotecan as a typical DNA damage-inducing agent to assess its effects on the MCF-7 and MDA-MB-453 cells. As shown
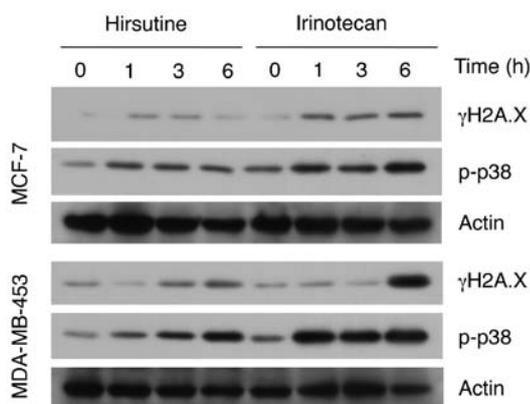
Figure 5. Selective activation of the DNA damage response by hirsutine in the MDA-MB-453 cells. Cells were exposed to hirsutine (50 μM) and irinotecan (400 μM) for 0, 1, 3 and 6 h. Cell lysates were collected and subjected to western blot analysis to detect the expression of p-p38 and γH2AX proteins in the MDA-MB-453 and MCF-7 cells. Results are representative of at least three independent experiments showing similar results.

in Fig. 5, irinotecan significantly upregulated γH2AX and p-p38 expression in both the MCF-7 and MDA-MB-453 cells, in contrast to hirsutine. Collectively, these results suggest the selective activity of hirsutine to induce DNA damage response in HER2-positive and the p53-mutated human breast cancer cells.

Discussion

In the present study, we investigated the clinical utility of hirsutine, an indol alkaloid phytochemical compound present in plants of the *Uncaria* genus, as an anticancer agent for human breast cancer cells. We found that the HER2-positive/ p53-mutated MDA-MB-453 and BT474 cell lines were relatively sensitive to hirsutine-induced cytotoxicity among the 6 tested human breast cancer cell lines (Fig. 1). To exert its cytotoxic activity against breast cancer cells, hirsutine induced apoptosis along with the activation of caspases (Figs. 2 and 3). Importantly, the expression of γH2AX, a hallmark of DNA damage response, was significantly upregulated after treatment of hirsutine along with the p38/MAPK stress signaling pathways (Figs. 4 and 5) implying that a DNA damage response was involved in the hirsutine-induced apoptosis.

Apoptosis is a programmed cell death occurring in multicellular organisms (9). In general, drug-induced apoptosis is one major mechanism of action for treating cancer, and various signaling pathways are involved in the process (10). Among these, a DNA damage response is one of the molecular events leading to apoptosis, and indeed many anticancer agents induce a DNA damage response (11-15). The mechanism involved in DNA damage-induced apoptotic cell death is known to closely correlate with the DNA repair response (16-18). The phosphorylation of the Ser-139 residue of the histone variant H2AX, forming γH2AX, is known as an early cellular response associated with the induction of DNA double-strand breaks. Therefore, induction of γH2AX has been considered as a hallmark of a DNA damage response (19-22). We originally identified hirsutine to inhibit NF-κB activation (8), and the NF-κB pathway is known to play

an important role in cancer progression (23-30). The NF-κB pathway can be activated as part of the DNA damage response by orchestrating cellular survival pathways (31,32). Indeed, we observed the downregulation of p-p65 in the MDA-MB-453 cells, but not in the MCF-7 cells, in response to hirsutine treatment along with the inhibition of Akt activity (Fig. 4), which has been widely recognized as an important determinant for DNA damage-induced apoptosis (33,34) and double-strand break repair (35-37). Considering the upregulation of p-Akt in MCF-7 cells following hirsutine treatment along with resistance to its cytotoxicity, the NF-κB/Akt pathway may be involved in the selective resistance of MCF-7 cells against hirsutine.

In addition to the NF-κB/Akt pathway, p38 MAPK activity is also known to play an important role in the DNA damage response induced by genotoxic stress with DNA-damaging chemotherapeutic agents through the activation of p53 (38). Inhibition of the p38 MAPK pathway reduced γH2AX expression (39), diminished the apoptotic fraction of cells and increased cell survival following exposure to chemotherapeutic agents (15,38,40), thus suggesting a role for p38 activation in the apoptotic response against genotoxic stress. Consistently, we observed the upregulation of p-p38 upon hirsutine treatment in the MDA-MB-453 cells, but not in the MCF-7 cells. In the context of selective cytotoxicity, hirsutine treatment downregulated the expression of HER2 in the MDA-MB-453 cells (Fig. 4), which has been known to be involved in the repair response of DNA damage induced by chemotherapeutic agents (41,42). Importantly, we obtained similar results of the DNA damage response in HER2-positive BT474 cells (data not shown). Furthermore, by using the anticancer drug irinotecan (CPT-11), which is a topoisomerase I inhibitor (43-47), we did not observe a selective induction of DNA damage response between the MDA-MB-453 and MCF-7 cells (Fig. 5). Considering the differential status of HER2 expression and/ or p53 mutation between the MDA-MB-453 and MCF-7 cells (48-50), HER2 expression and/or p53 status is presumably involved in determining the cellular response to hirsutine treatment. Although further study is clearly required to determine the exact mechanisms of action by which hirsutine targets cancer cells, our present study clearly demonstrated the selective anticancer activity of hirsutine against HER2-positive breast cancer cells by inducing DNA damage, implying its clinical utility as a novel anticancer drug.

Acknowledgements

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