Girinimbine Inhibits the Proliferation, Migration, and Invasion of Human Breast Cancer Cells via Induction of Apoptosis, Suppression of Cell Migration and Invasion and Inhibition of MEK/ERK and STAT3 Signaling Pathways

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Background: The currently used anticancer drugs against breast cancer possess serious side effects, have limited efficacy, and often lead to recurrence of the malignancy. The main aim of the current research was to investigate the anticancer potential of girinimbine, a naturally occurring carbazole alkaloid, against estrogen receptor (ER)-negative breast cancer cells (MDA-MB-453) along with its effects on cell migration and invasion, apoptosis, and the MEK/ERK/STAT3 pathway.

Material/Methods: MTT assay was used to evaluate antiproliferative effects of girinimbine while as clonogenic assay was used to study cell colony formation. Transwell migration and invasion assays were performed to study the effects on cell migration and invasion. Fluorescence microscopy using acridine orange/ethidium bromide was used to study apoptotic effects of girinimbine, which was quantified by annexin V-FITC assay. Effects of girinimbine on the MEK/ERK and STAT3 signaling pathways were evaluated by western blot assay.

Results: Results showed that girinimbine exhibited dose-dependent as well as time-dependent antiproliferative effects in MDA-MB-453 cells; in addition it strongly inhibited cell colony potency of these cancerous cells. Girinimbine could inhibit both cancer cell migration as well as invasion. Girinimbine induced potent chromatin condensation and nuclear fragmentation. The percentage of both early and late apoptotic cells increased significantly after girinimbine exposure. The anticancer effects of girinimbine were shown to be mediated via inhibition of the MEK/ERK as well as the STAT3 signaling pathways.

Conclusions: In conclusion, it may be proposed that girinimbine could be a promising anticancer agent against breast cancer provided further in-depth studies are carried out.

MeSH Keywords: Apoptosis • Cell Migration Inhibition • Flow Cytometry • Inflammatory Breast Neoplasms

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Background

Plants contain a wide variety of phytochemicals including flavonoids, terpenoids, amino acids, sterols, glycosides and proteins. Plants are also reported to have different carbazole alkaloids, which have various biological activities. Carbazole alkaloids activities include anti-inflammatory, antioxidantive, as well as anticancer activity [1]. Carbazole alkaloids also show cytotoxicity activity against different human cancer cell lines (including breast cancer) [2]. *Murraya koenigii* is a major source of carbazole alkaloids, isolated from its different parts. *M. koenigii*, also known as curry tree, grows under tropical or sub-tropical conditions and belongs to the family Rutaceae; it is native to India. It is a small tree (12 to 18 feet tall) with a trunk having a 16-inch diameter. Its leaves are highly aromatic and are pinnate with 2 to 4 cm leaflets. The berries of this plant are edible and have a sweet flavor. *M. koenigii* is a pharmacologically active plant and shows different biological activities including anticancer activity. The anticancer activity of *M. koenigii* was mainly reported to be due to its free radical scavenging and apoptosis inducing property [3,4]. Yet the effects on protein activity by the plant in intrinsic as well as extrinsic pathways (apoptosis) are not fully studied. Different carbazole alkaloids have been isolated from *M. koenigii*, but girinimbine was the first [5–7]. The girinimbine molecule has shown anti-angiogenic, anti-inflammatory, immunomodulatory, antioxidant, and antitumor activity [8–10]. Girinimbine has also been reported as an antiproliferative agent against Hepg2 mediated via cell cycle arrest and apoptosis [11,12]. It targets PI3K (phosphatidylinositol-3-kinase)/Akt, mTOR (the mammalian target of rapamycin) and Wnt/β-catenin signaling pathways in human ovarian cancer [13].

Breast cancer is regarded as the most frequent kind of cancer in women and is also the second leading cause of cancer-related deaths in the females in United States. During the last 10 to 20 years, there has been a significant improvement in the prognosis of patients with breast cancer mainly due to modifications in lifestyle, prompt detection, and advanced therapies. But the use of conventional chemotherapy has various serious side-effects, thus conventional chemotherapy has serious limitations. In some patients with advanced stage breast carcinoma, there is the growing problem of drug resistance to these chemotherapeutic agents [14,15]. When breast cancer is diagnosed, there is a very high chance of the presence of distinct metastatic lesions [16–19]. Due to distant metastasis and poor prognosis, breast cancer has generated a remarkable interest in the search for novel anticancer agents [20]. Currently, more attention is being paid towards those agents which induce anticancer effects via apoptosis, in an attempt to potentially reduce dangerous side effects of therapy. Natural products have a great potential to act against different human ailments with very few or no side-effects. Presently, the main focus of research is on natural products. The current study of girinimbine was undertaken in an attempt to unveil its anti-breast cancer activity, along with examining its impact on breast cancer cell migration and invasion, induction of apoptosis and inhibition of the MEK/ERK and STAT3 signaling pathways.

Material and Methods

Cell viability percentage was assessed via MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay; purchased from Sigma Chemical Co., St. Louis, MO, USA). Breast cancer MDA-MB-453 cells (obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) were cultured to 80% confluency at a density of 1×10⁴ cells in each well in a 96-well plate. Cells were allowed to attach in a CO₂ (5%) incubator at 37°C. Afterwards, MDA-MB-453 cells were subjected to girinimbine (Sigma Chemical Co., St. Louis, MO, USA) exposure at varying doses of 0, 8, 16, 32, and 64 μM for 24 hours and 48 hours. Treated cells were then supplemented with 5 mg/ml of MTT stock solution in phosphate-buffered saline (PBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) exposure at varying doses of 0, 8, 16, 32, and 64 μM for 4 hours. Thereafter, 200 μL DMSO (Sigma Chemical Co., St. Louis, MO, USA) was added to each well and supernatants were completely discarded followed by gently mixing. The color then developed was monitored at 550 nm through a Multiwell Microplate Reader (Thermo Scientific, USA).

Clonogenic assessment

MDA-MB-453 BC cells were exposed to girinimbine with different doses 0, 8, 32, and 64 μM for 4 hours. Dilution of the cell colonies was performed in agar solution (0.3%) followed by redisposing into 35 mm-cultural dishes. The density of cells in each plate was 3000. When agar solidifies, girinimbine treated cells were then incubated for 12 days. Afterwards, cell colonies were numbered in 2-mm grid culture dishes (Corning) (<50 cells).

Determination of cell migratory and cell invasive potency

Transwell migration assay was executed to analyze cell migratory potency of girinimbine treated MDA-MB-453 BC cells. Briefly, target cells were loaded on upper chambers containing RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a density of 4×10⁴ cells each well. Lower chambers of Transwell were loaded only with medium comprising 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Only the upper chambers were subjected to girinimbine exposure at varying doses: 0, 8, 32, and 64 μM for at least 24 hours. Treatment with girinimbine was followed by incubation at 37°C for 6 hours. Clearance of upper chambers was performed with a cotton swab to eliminate the cells which did not migrate. Formazin and crystal violet with 4% and
0.1%, respectively, were used for staining of migratory cells for 15 minutes each section was then photographed with 200x magnification via inverted microscope (Olympus Corporation, Tokyo, Japan). Similarly, cell invasive ability of MDA-MB-453 breast cancer cells was determined except for cell invasion determination using Transwell assay (coated with Matrigel).

Apoptosis analysis

Breast cancer MDA-MB-453 cells were plated in 96-well plate for determination of apoptosis and each well contained 2×10^5 cells. Cells were treated with altering girinimbine doses 0, 8, 32, and 64 μM with incubation for 24 hours. Prior to washing with PBS, cells were stained with acridine orange/ethidium bromide (AO/EBTR: 10 μM each) (Beyotime Institute of Biotechnology, Haimen, China) solution for 15 minutes; treated cells were then fixed in 10% formaldehyde (Sigma Chemical Co., St. Louis, MO, USA) and subjected to apoptosis assessment under fluorescence microscope (Olympus Corporation, Tokyo, Japan). For annexin V/PI assay, girinimbine treated cells were stained with annexin V/PI (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and apoptosis quantification was performed through flow cytometry (BD, Biosciences, USA). Each experiment was carried out in triplicates to register different events. Data were analyzed using Flowjo software (version 7.6.5; Tree Star, Inc., Ashland, OR, USA).

Western blotting assay

MDA-MB-543 cells were harvested at 80% of confluence followed girinimbine treatment at varying concentrations of 0, 8, 32, and 64 μM. Treated cells were lysed using lysis buffer (20 mM Tris-HCl (pH 7.5) 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na₃VO₄, 1 µg/mL leupeptin) (Beyotime Institute of Biotechnology) and within each lysate protein content was quantified by bicinchoninic acid (BCA) assay Kit (Beyotime, China). Proteins (45 μg/well) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed loading on nitrocellulose membranes (equal distribution). The primary antibodies included anti-Bax (1: 1,000; Abcam, Cambridge, MA, USA), anti-Bcl-2 (1: 1,000), anti-Erk and anti-phosphorylated (p-)Erk (1: 500, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Akt (1: 500) and anti-phosphorylated (p-)Akt (1: 500; Santa Cruz Biotechnology, Inc., USA), anti-STAT3 (1: 500; Santa Cruz Biotechnology, Inc., USA), anti-β-actin (1: 1,000; Santa Cruz Biotechnology, Inc., USA). After washing with tris-buffered saline plus Tween (TBST) 3 times, goat anti-rabbit IgG (1: 10 000, Santa Cruz Biotechnology, Inc.) conjugated with horseradish peroxidase was incubated for 1 hour at room temperature. Finally, the various protein bands were observed under the enhanced ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences, UK).

Statistical analysis

All the results were represented as mean and SEM (standard error of the mean). The numbers presenting P<0.05 were taken as statistically significant. Treated and control groups were compared for differences through one-way ANOVA.

Results

Cell proliferation rate of MDA-MB-543 cells after girinimbine exposure

Cell viability of MDA-MB-543 breast cancer cells after exposure to girinimbine (Figure 1) was monitored by performing MTT assay. Results showed potential time-dependent as well as dose-dependent inhibition in the cellular viability of target cells by girinimbine. Cell viability at 0, 8, 16, 32, and 64 μM of girinimbine doses was nearly 95%, 85%, 60%, 25%, and 10% after 24 hours of exposure. After 48 hours of treatment with girinimbine, the viability observed was 97%, 77%, 42%, 10%, and 5% respectively (Figure 2). Thus, the results after 24 hours and 48 hours of girinimbine treated revealed time- as
Figure 3. (A) Clonogenic survival rate reduction after girinimbine treatment of MDA-MB-543 cells. The cells were stained with crystal violet (0.5% w/v) and then photographed and the cell number was counted. (B) Graphical representation of the colony formation inhibition induced by girinimbine treatment. Individual experiments were repeated three times. Values represent mean±standard error of the mean of triplicate measurements, * P<0.05.

Figure 4. (A) Results representing cell migration potency of MDA-MB-543 cells after girinimbine treatment at indicated doses. After treatment, cells were stained with crystal violet and formalin and then photographed with an inverted microscope to determine the number of migrated cells. (B) Graphical representation (quantitative estimation) of the migration inhibitory effects induced by girinimbine treatment. Values represent mean±standard error of the mean of triplicate measurements, * P<0.05.
Figure 5. (A) Results representing cell migration potency of MDA-MB-543 cells after girinimbine treatment at indicated doses. Individual experiments were repeated three times. After treatment, cells were stained with crystal violet and formalin and then photographed with an inverted microscope to determine the number of invaded cells. (B) Graphical representation (quantitative estimation) of the invasion inhibitory effects induced by girinimbine treatment. Values represent mean±standard error of the mean of triplicate measurements, * P<0.05.

Figure 6. Acridine orange/ethidium bromide staining representing apoptotic MDA-MB-543 breast cancer cells after girinimbine exposure. Individual experiments were repeated three times. Yellow and red fluorescence indicate cells which have undergone apoptosis. Green cells represent viable cells.
well as dose-dependent, viability inhibition of MDA-MB-543 breast cancer cells.

**MDA-MB-543 cell colony inhibition by girinimbine**

Cell colonies were analyzed by executing clonogenic assay in 35 mm-culture dishes. Results presented remarkable dose-dependent inhibition in the colony forming potency of MDA-MB-543 breast cancer cells. Blue stains in the culture dishes represent cell colonies and these stains can be seen declining with increasing girinimbine doses (Figure 3A, 3B).

**Girinimbine suppressed cell migratory and cell invasive ability of MDA-MB-543 cells**

Transwell chambers assay was performed to check cell invasive as well as cell migratory potential of MDA-MB-543 cells. In the case of cell invasion Transwell chambers were coated with Matrigel. Results showed remarkable dose-dependent declination of cell invasion and cell migration. At lower girinimbine doses the cell migration only reduced by a small margin but on increasing girinimbine concentration it reduced significantly and effectively (Figure 4A, 4B). The same was observed in case of cell invasion that the number of invasive cells reduced as the girinimbine doses increased (Figure 5A, 5B).

**Girinimbine induced apoptotic cell death in MDA-MB-543 cells**

Girinimbine suppressed cell viability in MDA-MB-543 cells and it was checked weather the viability inhibitive effects are apoptosis mediated. For that AO/ETBR staining assay was performed. Results showed that untreated cells were observed with light green fluorescence and on increasing the girinimbine

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**Figure 7.** Annexin V/PI assay results revealing the dose-dependent increase in the percentage of apoptotic cells. The numbers presenting *P*<0.05 were taken as statistically significant.
doses apoptosis induction can be easily seen due to the yellow and red fluorescent cells which increased with dose (Figure 6). Further, quantification of apoptosis was performed by annexin V/PI assay and it showed that the apoptotic cell percentage enhanced from 2.1% at control to 56.2% at 64 μM of girinimbine concentration, increasing both early and late apoptotic cell percentage (Figure 7). In addition to this, western blotting analysis was executed to monitor apoptosis regulatory proteins. It showed dose reliant downregulation of BCL-2 and up-regulation of BAX proteins (Figure 8). Thus, the results from DAPI staining, annexin V/PI assay and western blotting analysis suggested that the antiproliferative effects of girinimbine are apoptosis mediated.

**Girinimbine targeted the MEK/ERK and STAT3 signaling pathways in MDA-MB-543 cells**

To analyze the MEK/ERK and STAT3 signaling pathways after girinimbine treatment to MDA-MB-543 cells, western blotting assay was performed. Results exposed that the proteins associated to MEK/ERK and STAT3 signaling pathways were significantly altered on changing girinimbine concentrations (0, 8, 16, 32, and 64 μM). The expressions of p-MEK, MEK, p-ERK and p-STAT-3 reduced and ERK increased with increasing girinimbine doses. The expressions of STAT-3 almost remained constant with all girinimbine doses (Figure 9).

**Discussion**

Different studies have shown that natural products affect specific extrinsic and intrinsic pathways in apoptosis. These pathways were testified against various human cell lines including MDA-MB-543 cells. Two types of proteins regulate apoptosis in a cancer cell that is proapoptotic (BAX) and antiapoptotic (BCL-2). Targeting STAT-3 pathway in a cancer cell by natural products is reported to be associated with declination in BCL-2 expression and enhancement in BAX expressions [21,22]. Increased BAX and decreased BCL-2 expressions evidence the apoptotic cell death in a target cancer cell. Breast cancer is a dangerous malignancy prevailing in women globally and its incidences are increasing at alarming rate. To tackle breast cancer novel treatment strategies are required. There are huge challenges in cancer chemotherapy, as the cancer cells acquire drug resistance. Breast cancer cells also acquire multi-drug resistance which leads to the failure or limitation of conventional chemotherapy. As such, there is a pressing need for novel anticancer drugs which render cancer cells to undergo cell suicide. This kind of cell death is referred to as apoptotic cell death. Natural products have been known for a long time to induce cellular apoptosis in cancer cells and as such have the potential to be developed as promising anticancer agents. The main advantage of using an apoptotic anticancer agent is to eliminate the serious side-effects which are mostly seen in conventional chemotherapy [23–25]. The present study was designed to examine the anticancer effects of girinimbine on the MDA-MB-543 breast cancer cell line. Results indicated potential time-dependent as well as dose-dependent inhibition in cell proliferation. Girinimbine could also inhibit cancer cell colony formation tendency. Furthermore, we carried out various bioassays in order to determine the anticancer mode of...
action of this molecule. Transwell cell migration and cell invasion assays showed that the antiproliferative effects of girinimbine could be due to its ability to curb cell migration and invasion, which might be a promising feature of this molecule as it would thus not allow breast cancer cells to migrate and invade other nearby tissues/cells. Various apoptotic assays showed that this molecule has the tendency to induce programmed cell death which can lead to breast cancer cell suicide leading to its antiproliferative effects. Girinimbine led to the increase of both early and late apoptotic cells. Girinimbine mode of action was further shown to be mediated via decreasing Bcl-2 expression and increasing Bax expression. Finally, it was shown that the girinimbine molecule could also inhibit MEK/ERK and STAT3 signaling pathways. These pathways are very crucial and control cell growth at the basic biochemical levels [26]. In the published literature, there are reports of apoptosis induction by molecules which are similar to girinimbine (being a carbazole alkaloid) [27] and as such complement the current findings.

Conclusions

The findings of the present study indicate promising anticancer and apoptosis inducing properties of girinimbine. The current findings also suggest that girinimbine could also inhibit cancer cell migration and invasion along with targeting key cancer pathways namely the MEK/ERK and STAT3 signaling pathways.

Conflict of interest

None.

References:

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