Evaluation of the anticancer activity of dipyridamole and Imatinib mesylate compounds against breast cancer cell line and related biochemical and genetic changes

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Abstract

Imatinib mesylate and dipyridamole were antineoplastic targeted chemotherapeutic agents, and it is here evaluating the anticancer of imatinib mesylate and dipyridamole compounds against MDA-MB231 breast cancer cell line in vitro and the related cell cycle and gene profile. MDA-MB231 cells showed more sensitivity to imatinib mesylate than to dipyridamole, with an IC50 value of 348 g/mL versus 494 g/mL for imatinib mesylate and dipyridamole, respectively (P-value < 0.05). The up/downregulation of Bax and Bcl-2 and metastasis contributing gene (MMP-1) assured the anticancer activity. Also, the apoptotic potential of dipyridamole and imatinib mesylate was verified by arresting cells in the G2/M phase and increasing the percentage of the apoptotic cells in the pre-G1 phase. The antioxidant levels were drug dependent, as they were significantly higher (P<0.05) in cells treated with imatinib than in cells treated with Dipyridamole.

Conclusion: Imatinib mesylate growth-inhibitory impact on breast cell lines, either alone or in combination with dipyridamole, may be mediated through up/downregulation of the Bax, Bcl-2, and MMP-1 genes. Imatinib is a promising treatment for breast cancer patients who require targeted therapy.

Keywords: Dipyridamole; imatinib mesylate; Breast cancer; apoptotic genes; cell cycle; antioxidants

Introduction

Breast cancer is a distinct illness that is considered a common malignancy in women worldwide, accounting for around 30% of cancer diagnoses and ranking as the second leading cause for mortality in many countries ((Spaeth et al., 2018; Siegel et al., 2018)). In 2015, 2.5 million (2.2–2.5 million) new cases of breast cancer and 523 000 (492 000–543 000) cancer-related deaths were estimated globally (Fitzmaurice et al., 2017). Even though researchers worldwide have developed several new treatments, breast cancer has the highest infection and mortality rates among women (Bray et al., 2018).

Dipyridamole (DIP) is a thromboxane synthase inhibitor that is clinically used as an antithrombotic agent and a vasodilator agent. DIP has several uses in the endothelium of the vasculature, and it has antiplatelet effects, antioxidant, anti-inflammatory
properties, and inhibition of proliferation (Thomé et al., 2019). DIP possesses potential properties for antitumor activity and enhances the sensitivity of several antitumor agents such as 5-fluorouracil, cisplatin, and methotrexate (Ge et al., 2016). DIP can enhance the sensitivity of vinblastine, doxorubicin in cancer cell lines (Zhou et al., 2020). It has the activity to inhibit the aggressive growth of triple-negative breast cancer cell lines and reduce cellular proliferation (Ge et al., 2016).

Imatinib (IM) was the first inhibitor of tyrosine kinase used for treatments of cancer and BCR-ABL tyrosine kinase that causes chronic myeloid leukaemia (CML). It inhibits platelet-derived growth factor receptor (PDGFR), which controls major cellular events (Rosenzweig et al., 2018). Imatinib has an additive effect on cell growth inhibition when combined with chemotherapy treatment such as vinorelbine and cisplatin compared to a single treatment (Weigel et al., 2009). IM can reduce the cell growth in breast cancer by the block of activated PDGFR ß (Kadivar et al., 2017).

Materials and Methods

Cell culture

For modelling late-stage breast cancer, MDA-MB-231 cell lines are frequently employed. This cell line is negative for ER, PR, and E-cadherin. The International Center for Advanced Research (ICTAR-Egypt) provided the MDA-MB231 human breast cancer cell line. Culturing breast cell line was done in RPMI-1640 (Sigma Aldrich-USA) media supplemented with 100 U/ml penicillin, 25mM sodium bicarbonate, 100g/ml streptomycin (Sigma Aldrich- USA), and 20mM HEPES (Sigma Aldrich -USA). At 37°C (Jouan-France), the incubation of the cells was done in 5% carbon dioxide (CO2) and 10% foetal bovine serum (FBS) provided by (GIBCO - USA). Trypsin was applied to the cell line for five minutes for cell dissociation post removal of the exhausted medium. Dissociated cells were maintained in tissue culture flasks or 96 well tissue culture plates.

Cell viability Cytotoxicity by using MTT assay.

In 96 well micro-titer plates (TPP-Swiss) at (5×10^3 cells/well), MDA-MB231 cells were seeded. The exhausted medium was decanted. A fresh medium was added to whole wells. Dipyridamole and imatinib were prepared at a definite concentration, added to the 1st column, and processed for two-fold serial dilutions. Untreated cells were included as a negative control. Treated plates were incubated for 24h at 37°C. Phosphate buffer saline (PBS) was used to wash away detached cells (Sigma Aldrich- USA). MTT as 0.5mg/ ml was added as 50μL / well for 3-5h. Developed purple MTT-formazan crystals were dissolved utilizing dimethylsulfoxide (DMSO) (0.05 ml) (Sigma Aldrich- USA). Using an ELISA plate reader, treated plates were read at 570 nm (ELX-800, Biotek-USA).

For calculating the percent of viability, the following equation was used:

Viability % = Mean OD of Test DilutionX100
Mean OD of Neg. Control

Using MasterPlex-2010 software, the IC50 of each test compound was determined

Expression of Apoptosis-Related Genes

The RNase kit (Qiagen-USA) was used to extract RNA from dipyridamole and imatinib mesylate-treated and untreated breast cancer cells, following the instructions of the manufacturer (Mitupatum et al., 2016). Beckman dual spectrophotometer was used to determine the RNA concentration. Real-time PCR (RT-PCR) was used to assess the expression level of apoptosis-related genes. Bax (F: 5’-ATG GAC GGG TCC GGG GAG CA-3’and R: 5’-CCC AGT TGA AGT TGC CGT CA-3’), Bel-2 (F: 5’-CCTGTG GAT GAC TGA GTA CC-3’ and R: 5’-GAGACA GCC AGG AGA AAT CA-3’), Cyc (F:5’- AGTTGTTCCAGTGCCACACCG-3’ and R: 5’TTCCTTCCCCAGAATGATCCTTT-3’) and MMP-1 (F: 5’ CTGGCCACAACTGCCAATG-3’ and R: 5’CTGTCCTGAACAGCCAGTACTTA -3’), ß-actin as housekeeping gene.
F: 5’-GTGACATCCACACCCAGAGG-3’ and R: 5’-ACAGGATGTCAAAACTGCC-3’

Using a high-capacity cDNA reverse transcriptase kit (Applied Biosystem-USA), extracted RNA (10 ng) from each sample was utilized to synthesize cDNA. Amplification of the obtained cDNA was done utilizing Syber Green I PCR Master Kit (Fermentas-Lithuania) on a step one instrument (Applied Biosystems-USA) as follows: 1 cycle at 95°C for 10 minutes for initial denaturation and activation enzyme, followed by 40 cycles at 95°C for 15 seconds, at 55°C for 20 seconds for annealing, and at 72°C for 30 seconds for an extension. The change of the target genes expression was assessed using the relative standard curve method (Δ Ct) to the mean critical threshold (CT) of the β-actin housekeeping gene.

Flow cytometry analysis.

Cell Cycle Analysis

Through centrifugation at 1500rpm/min, MDA-MB231 cells were collected, washed twice with PBS, and underwent overnight fixation at +4°C in 70% ethyl alcohol. In a dark environment, cells were pelleted and resuspended in 500 μl of 1X Binding Buffer containing 5 μl PI 50 mg/ml propidium iodide, 5 μl Annexin V-FITC, 0.1 mg/ml RNase, and 0.1 percent (v/v) Triton X-100. At 37°C and after 30 minutes, the analysis of the cells was done using a flow cytometer (Becton-Dickinson, San Jose, CA, USA) coupled with a 488 nm argon-ion laser. The cell cycle and the sub-G1 group were detected and studied.

Antioxidant profile

Before pipetting, reagents were kept reaching room temperature and were well mixed with a gentle swirl. We assessed the number of strips required for one experiment and eliminated any excess strips from the microtiter plate. The standards working were added to the first two columns in duplicate: side by side (each well contains 100 μL). The remaining wells were filled with the samples (100 μL for each well). Each plate was sealed with the sealer included in the kits and incubated for 90 minutes at 37°C. Without washing the wells, the liquid was drained, and 100 μL of biotinylated detection antibody-specific working solution was dispensed into each well. Plates were covered with plate sealers as previously. Gently were mixed up and incubated for 1 hour at 37°C. The solutions were aspirated or decanted from each well, approximately 350 μL of wash buffer were added to each well. Plates were soaked for one to two minutes, the wash solution was decanted, and the plates were inverted and blotted against clean paper towels. This wash step was repeated three times. Each well received 100 μL of HRP conjugate working solution. Plate sealers were applied to the plates, and they underwent incubation for 30 minutes at 37°C. The solution was decanted from each well five times. Each well received 90 μL of substrate solution. Plates were sealed with plate sealers, incubated at 37°C for 30 minutes, protected from light, and each well-received 50 μl of stop solution, which was well mixed. With a microplate reader set to 450 nm, the optical density of each well was determined.

Results:

Cytotoxicity (MTT assay)

Cytotoxicity was evaluated and represented as the survival proportion in comparison to untreated control cells. The MTT test was used to determine the cytotoxicity of dipyridamole and imatinib mesylate on the MDA-MB231 cell line. The mean viability percentage of imatinib mesylate and Dipyridamole treated MDA-MB231 was relatively decreased to increased concentration (5 to 10000 µg/mL). The percentage of viability following imatinib and dipyridamole treatment was not significantly different (P>0.05). Both viability and the IC50 value determined using Materplex-2010 software were cell type-dependent as the IC50 values recorded were 348 μg/mL and 494 μg/mL, respectively [Fig. 1].

Apoptotic gene

DIP recorded revealed that the application of test anticancer drugs demonstrated a significant increase in the pro-apoptotic genes (Cyc and Bax) expression
when cells were treated with imatinib and dipyridamole, in comparison to the values in untreated cell control (P<0.0001). The gene up-regulation was significantly elevated (P<0.001- p<0.00001) in imatinib-treated cells in comparison to dipyridamole-treated cells. Concurrently using drugs mix showed non-significant up-regulation of Bax and Cyc in imatinib treated cells (P >0.05). A significantly elevated up-regulation was found when cells were treated with drugs mixed with Dipyridamole (P<0.05).

The anti-apoptotic genes (MMP1 and Bcl-2) were significantly (P<0.0001) downregulated in imatinib, and dipyridamole treated cells compared to untreated control cells [Fig.2]

**Cell cycle analysis**

Regarding the flow cytometry-derived cell cycle profile, data recorded revealed an insignificant accumulation of cellular DNA during the Go-G and S phases for Dipyridamole and Imatinib treated cells (34.69%; 27.21%) and (28.42%; 25.71%) respectively compared with DNA accumulation values of cell untreated cell control (53.76%) (P >0.05) [Fig 3]. Also, after treatment with both drugs during the G2-M phase, cellular DNA accumulation was significantly increased (P<0.05). Table (1) showed that DNA accumulation was significantly higher in Imatinib-treated cells than in dipyridamole-treated cells (P<0.05). A significant increase was demonstrated as regards the total number of apoptotic cells detected post Imatinib treatment of MDA-MB231 cells compared to Dipyridamole therapy (P<0.05). In MDA-MB231 cells treated with imatinib, the early and late apoptosis detected showed a significant elevation (P<0.05) than the Dipyridamole-treated cells (6.31%; 3.82%) and (11.35% - 6.12%), respectively in comparison to the untreated negative cell control (1.61%; 0.43%). Finally, the necrosis % was significantly increased (P<0.05) (1.82%; 1.76%) in cells treated with both drugs compared with that of control (0.37%). The necrosis percentage of Imatinib-treated cells showed a significant difference than in Dipyridamole treated one (P>0.05); table (2).

**Antioxidant profile/ biochemical analysis**

**Determination of GSH, MDA, and ROS**

Under the effect of DIP and IM, there was a significantly decreased GSH level post-MDA MB 231 cell treatment in comparison to the value in the untreated cell control. GSH levels were drug dependent as IM showed a significantly decreased value of GSH compared with its level in DIP treated MDA-MB231 cell line (P<0.05). Finally, ROS could be detected and significantly elevated (P<0.05) post cell treatment with Imatinib and Dipyridamole in comparison to the level in the untreated cell control. ROS levels were drug-dependent [Fig.4].
Fig. 1. Evaluation of anti-proliferation effect of dipyridamole and imatinib mesylate relevant concentrations (5 to 10000 µg/mL) against MDA-MB231 breast cancer cell line.

Table 1. Cell cycle analysis of MDA-MB231 cell line under the effect of DIP and IM treatments.

<table>
<thead>
<tr>
<th></th>
<th>DNA content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1%</td>
</tr>
<tr>
<td>DIP</td>
<td>34.69</td>
</tr>
<tr>
<td>IM</td>
<td>27.21</td>
</tr>
<tr>
<td>Control</td>
<td>53.76</td>
</tr>
</tbody>
</table>

Table 2. Apoptosis/necrosis analysis of MDA-MB231 cell line under the effect of DIP and IM treatments

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Early</th>
<th>Late</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIP</td>
<td>11.76</td>
<td>3.82</td>
<td>6.12</td>
<td>1.82</td>
</tr>
<tr>
<td>IM</td>
<td>19.42</td>
<td>6.31</td>
<td>11.35</td>
<td>1.76</td>
</tr>
<tr>
<td>Control</td>
<td>2.41</td>
<td>1.61</td>
<td>0.43</td>
<td>0.37</td>
</tr>
</tbody>
</table>
Fig 2. Evaluation of pro and anti-apoptotic genes regulation under the effect of DIP and IM in a sole and combined form treated MDA-MB$_{231}$ compared to its level in untreated cell control.
Fig. 3 Evaluation of MDA-MB231 DNA accumulation post cell treatment with Dipyridamole and Imatinib mesylate using flow cytometry: A and B Untreated MDA-MB231 cell line, treated with a half-maximal inhibitory concentration of Dipyridamole for (C and D) and (E and F) for Imatinib mesylate respectively,
Fig. 4. Evaluation of antioxidant profile concerning GSH, MDA and ROS under the effect of Dipyridamole and Imatinib mesylate in a sole form 24-hours post-treatment.
Discussion

The main regimens of breast cancer treatment are chemotherapy, surgery, and radiotherapy, target therapy, and immunotherapy (mRNA Expression). While researchers worldwide have been creating novel treatment regimens, the most recent global cancer data revealed that breast cancer has the greatest morbidity and mortality rates among females worldwide (Bray et al., 2018). In the present study, antiproliferative and cytotoxic activities were investigated \textit{in vitro}, using DIP and IM, indicating a concentration-dependent dose-response curve. Other studies were complying with (Li et al., 2019) recording DIP, and IM IC\textsubscript{50} values of treated MC7 cell lines were 183.9 and 468.6 \textmu mol/L, respectively, and IC\textsubscript{50} values for the HCT116 cell line were (196.36 and 351.35\textmu mol/L, respectively. Cell viability was significantly decreased following sole application of DIP and DIP-IM mix; these results are similar to previous reports on the cytotoxicity of DIP (El-Sisi et al., 2020) using two breasts (MCF7, T47D) and two lungs (A549, COR L23) tumour cell lines, as well as IM cytotoxicity using human breast cancer cells (MDA MB 231, MCF 7) (Smith et al., 2000), and human pancreatic carcinoid cell line BON- (Weigel et al., 2010).

Our studies showed DIP increased the cytotoxicity of MDA-MB 231 and agreement with (Yao et al., 2007) recording that the combination of Cisplatin and dipyridamole and dipyridamole increase the cytotoxicity in the HEp-2 cell line. Our findings were in line with the MTT test, which demonstrated a significantly increased cytotoxicity across a wide range of Imatinib mesylate concentrations. The most intensive approach for therapy and prognosis has been the determination of gene expression during tumour progression (Rodrigues et al., 2004).

The current study demonstrated that imatinib causes the induction of an intrinsic signalling pathway for programmed cell death via up-regulation of Bax gene expression and down-regulation of Bcl-2. It has been demonstrated that imatinib mesylate induces apoptosis in a diversity of human solid tumour cell types, including ovarian, thyroid (Shandiz et al., 2016), and breast cancer, and has been shown to regulate Bax, Bad, Bcl-2, and Bcl-xl gene expression (de Groot et al., 2006; Jacquel et al., 2003).

Several authors used the same cell line (MDA-MB231) and same drugs, but the target difference was the mechanism of action/pathway and genetic profile (Bax and Bcl-2 and metastasis contributing gene (MMP-1)) and biochemical markers (antioxidant profile) to prove the anticancer activity. Dipyridamole was investigated for potential clinical use in Xenograft mice carrying triple-negative breast cancer 4T1-Luc or MDA-MB-231T cells for primary tumour size (68%; \( P = 0.0182 \)), metastasis formation (48%; \( P = 0.03 \)). Dipyridamole reduced activate \( \beta \)-catenin by (39%; \( P < 0.0001 \)), phospho-p65 (68%; \( P < 0.0001 \)), phospho-ERK1/2 (25 %; \( P = 0.01 \)), and doubled \( \text{IkB}\alpha \) expression (\( P = 0.0019 \)) (Spano et al., 2013).

Imatinib mesylate was found to compete with adenosine triphosphate and to inhibit the platelet-derived growth factor (PDGF) receptor, as well as certain tyrosine kinases (Bcr-Abl kinase, c-kit receptor kinases activation \( \alpha, \beta \)) (Smith et al., 2000; Ross and Hughes., 2004). Similar experiments showed that anti-apoptotic genes such as Bcl-xl, Bcl-2, and Mcl-1 could be down-regulated by imatinib (Rosenberg & Mathew., 2013), and other studies revealed that imatinib showed anti-apoptotic effects on some cancer cells, including melanoma and prostate cancer via interfering with caspase activation (Fernandes et al., 2011; Truong et al., 2003).

Imatinib mesylate antiproliferative effects on ZR751 and MDAMB231 breast cell lines might be due to a decrease in platelet-derived growth factor-BB, platelet-derived growth factor receptor-\( \beta \), c-Kit, and stem cell factor expression, thus suppressing the corresponding ligand PDGF-BB (Kadivar et al., 2018). In our studies, imatinib and dipyridamole.
enhanced the overexpression of CYC. In other studies, imatinib overexpressed platelet-derived growth factor receptor PDGFR-α and PDGFR-β (Rappa et al., 2011). The expression of MMP-1 is characteristic for many types of malignant tumours, including breast cancer, and some studies suggest that MMP1 has a synergistic effect on breast cancer (Mace et al., 2002).

In the present study, Dipyridamole induced an intrinsic signalling pathway for programmed cell death in breast cancer MDA-MB231 cell line by up-regulating Bax gene expression and down-regulating Bcl-2. This was followed (Li et al., 2019). Another study showed mild down-regulation of Bcl2 and extremely significant up regular of Bax in solid Ehrlich carcinoma-bearing mice. In our studies, dipyridamole made overexpression for CYC gene in MDA-MB231 cell line as in another studies dipyridamole increased intracellular c-AMP and inhibited Erk1/2 activation in of human peritoneal mesothelial cell proliferation (Padala et al., 2017; Choudhary et al., 2014). Some studies suggested that DIM possesses beneficial properties, either direct or indirect effects, such as downstream effects on cell signalling proliferation inhibition, anti-inflammatory and antioxidant properties (Sureechatchaiyan et al., 2018).

Our results indicated that DIP and IM were indispensable for cell cycle progression in breast cancer MDA-MB231 cell line and knocking down DIP and IM arrested cell cycle at G2/M phase. In the MDA-MB453 human breast cancer cell line, kaempferol arrested the cell cycle in the G2/M stage by down-regulating CDK1 (Chakrabarti & Freedman, 2008). In previous studies proved that DIP was found to reduce cell proliferation and inhibit growth in mice implanted with highly aggressive triple-negative breast cancer MCF10A cell lines (Akram et al., 2017). In contrast, in human cancer cells, dipyridamole induced G1 arrest that involved down-regulating several cell cycle key regulators in another study (Choudhary et al., 2012). The intensive technique for therapy and prognosis has been

monitoring gene expression throughout tumour growth (Rodrigues et al., 2004). The Bcl-2 family gene product, as well as other anti-apoptotic members (BCL-w, Mcl-1, A1, Bcl-2, and Bcl-xl), act as important regulators of apoptotic pathways (Lin et al., 2001). Bcl-2 prevents the cytochrome c release from the mitochondria induced by Bax proteins, therefore suppressing the apoptotic cascade (Zeviar et al., 2014).

In the present study, cells treated with DIP and IM showed a significantly decreased GSH level post-MDA-MB 231 breast cancer cell line compared with cell control, that agreed with another study reported by (He et al., 2004) demonstrated that the reduction in GSH concentrations might be attributed to increased consumption of GSH. In the removal of xenobiotic and peroxides and/or decreased GSH synthesis. Several investigators showed that GSH levels in the blood plasma of breast cancer patients were lower than those in control participants (Yeh et al., 2006).

In our study, MDA change under the effect of Imatinib and dipyridamole had slight and highly significantly elevated compared to its values in untreated cell control, and as in another study reported by (Lamari et al., 2008), breast cancer tissue demonstrated much lower catalase levels than the normal tissue, which was consistent with catalase role in fully detoxifying H2O2 to H2O. On the contrary, studies reported an increase in MDA plasma levels in breast cancer (Tas et al., 2005; Gönenç et al., 2006; Pande et al., 2011). Also, other studies showed that in comparison to the control group, breast cancer patients had significantly higher MDA levels (Lamari et al., 2008; Kilic et al., 2014; Ray & Husain, 2002; Elsayed et al., 2019).

Finally, ROS level was evaluated post-treatment with the IC50 concentrations for dipyridamole and imatinib mesylate compared to control and IM, and our results agreed with other studies that observed overproduction of ROS in breast cancer due to enhanced lipid peroxidation and higher production of
nitric oxide (NO). Antioxidants can offset the anticancer medications' apoptotic characteristics by decreasing the impact of reactive oxygen species generated by the therapeutic agents (Elsayed et al., 2019).

Preclinical data comparing imatinib alone or combined with dipyridamole show that imatinib was significantly more potent than dipyridamole. The preclinical data can help clinical oncologists to go through phase II studies for using imatinib in breast cancer patients.

**Conclusion**

This research evaluated the efficacy of the therapeutic potential of imatinib alone or combined with dipyridamole in breast cancer patients by up/downregulation of Bax, Bcl-2, and MMP-1 genes.

**References**


