In Silico and In Vitro Synergistic Anticancer Effects of The Metformin-Lapatinib Combination By The Regulation of Apoptotic Biomarkers Expression In SKBR3 Cells

Davood Neamati

Ahvaz Jundishapur University of Medical Sciences: Ahvaz Jondishapour University of Medical Sciences

Azam Khedri

Ahvaz Jundishapur University of Medical Sciences: Ahvaz Jondishapour University of Medical Sciences

Mohammad Aberomand

Ahvaz Jundishapur University of Medical Sciences: Ahvaz Jondishapour University of Medical Sciences

Ali-Asghar Hemmati

Ahvaz Jundishapur University of Medical Sciences: Ahvaz Jondishapour University of Medical Sciences

Ghorban Mohammadzadeh (✉ mohammadzadeh@ajums.ac.ir)

Ahvaz Jundishapur University of Medical Sciences <https://orcid.org/0000-0001-7921-4770>

Research Article

Keywords: Metformin, Lapatinib, SKBR3 Cell Line, Combination Index, Breast Cancer, Molecular Docking

Posted Date: February 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-162903/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

We examined the effect of Metformin (MET) on Lapatinib (LAP)-induced apoptosis in SK-BR3 cells. The cell viability and cell death were measured by MTT assay and Annexin V-FITC/PI

staining, respectively. The mRNA expression of Bax, Bcl2, and P21 was determined by real-time PCR. The activity of caspase 3 and 9 was measured using an ELISA kit. The MET-LAP combination index (CI) was calculated using CompuSyn software. The molecular docking studies of LAP with Akt, AMPK, and HER2 were performed using Auto Dock 4.2.5.1. The strongest synergistic apoptotic death [combination index (CI) = 0.51] was obvious in MET 100 mM plus LAP 100 nM. The MET-LAP combination causes a more powerful apoptotic death (46%) compared to LAP 100 nM individually. The combination could significantly increase the expression of Bax and P21, as well as the activity of caspase 3 and 9, but decreased the expression of Bcl2. Moreover, the combination could significantly increase the level of AMPK in LAP 100 nM plus MET 30 mM and decreased the level of Akt in LAP 100 nM plus MET 40 mM. Molecular docking studies showed that LAP interacts with the active site amino acids of Akt, AMPK, and HER2 by hydrogen and hydrophobic interactions. MET-LAP combination induces more extensive apoptotic death than either drug alone in SKBR3 cells. MET-LAP combination may be considered as a valuable therapeutic intervention in breast cancer patients. However, further in-vivo studies are needed to assess the MET-LAP combination's practical value for induction of apoptosis and an anti-cancer drug.

Introduction

Breast cancer represents one of the most common malignancies in women that have the highest rate of mortality in women worldwide, recently [1]. Approximately, twenty to twenty-five percent of breast cancer patients are HER2 positive that shows aggressive phenotype, and poor outcomes [2]. Trastuzumab, a recombinant humanized antibody is the existing and certified treatment of HER2 positive breast cancer, which can inhibit the extracellular domain of this type of tyrosine kinase receptor [3]. However, hereditarily and acquired resistance to this therapy develops within one year [4]. Thereafter, the use of therapeutics with tyrosine kinase inhibition (TKI) activity, including LAP, neratinib, and gefitinib has been more developed [5]. Though, a number of proofs about acquired resistance to these medications through modulation of their different downstream signaling proteins have been documented [6]. Therefore, a new strategy for breast cancer chemotherapy is a combination of chemotherapy that uses a natural compound with a synthetic drug. Nowadays, chemical prevention strategies by focusing on the use of natural chemopreventive compounds have been more attractive. In such therapeutic strategies, the drug used is better known to have low toxicity, desired efficacy, and can be used orally and cost-effective [7].

LAP, a dual synthetic reversible tyrosine kinase inhibitor, which selectively directs and prevents the HER2 and EGFR activity, has recently been used in clinical trials. It is usually utilized with the chemotherapeutic agent including capecitabine in breast cancer patients which previously treated with different anti-cancer drugs [8, 9]. Also, it has been indicated that LAP by blocking the adenosine monophosphate-activated protein kinase (AMPK) activity and AKT in HER2-positive cells ultimately resulted in the destruction of

cancer cells. It was also seen that the drug could sensitize the above-mentioned cell group to radiation therapy and tamoxifen [10].

MET (MET) (N, N-dimethyl biguanide), an insulin sensitizer, has long been used as one of the common drugs in type 2 diabetes mellitus [11, 12]. Several meta-analyses significantly established that MET could reduce the prevalence of several cancers including breast, colorectal, and hepatocarcinoma, and cancer-related death [13–16]. Additionally, MET has been indicated could inhibit the proliferation and induce apoptotic death in triple-negative breast cancer cell lines [17]. Although LAP and MET have been shown to exhibit an anti-cancer effect, the MET-LAP combination may have a more effective action against breast cancer. To the best of our knowledge, this combination has not been tested, therefore, in the current study; we assessed the effect of MET and LAP either individually or in combination on the canonical p53 target such as Bax, Bcl2, and P21 expression accompanied by measurement of Akt, and AMPK level and caspase 3 and 9 activity in SKBR3 cells.

Materials And Methods

Chemicals and Reagents

MET and LAP with more than 99% of purity, thiazolyl blue tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co (St. Louis, MO, USA). Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from Beyotime Institute of Biotechnology (Nanjing, Jiangsu, China). Dulbecco's Modified Eagle Medium (DMEM) and FBS were purchased from Gibco (Carlsbad, CA, USA). AMPK, AKT, and β -actin monoclonal antibodies and goat anti-rabbit IgG (labeled with HRP) were obtained from Cell Signaling (Danvers, MA, USA).

Cell Culture, cytotoxic MTT assay, and combination index determination

SKBR3 cells originated from human patients with breast carcinoma were purchased from the Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in DMEM media supplemented with 10% heat-inactivated FBS and 1% antibiotics (Pen/Strep) at 37 °C in a 5% CO₂ enriched atmosphere. Approximately 20×10^3 cells/well treated with different concentrations of MET or LAP for 24, 48, and 72 h, respectively. After treatment, 100 μ l MTT (0.50 mg/ml in PBS) was added and incubated for 4.5 h at 37 °C. Finally, the formazan crystals were dissolved in 100 μ l pure DMSO, and the absorbance was measured at 570 nm by a microplate reader (BioTek® ELx800, USA). All experiments were performed triplicate and cell viability was determined as a percentage of the control group. For calculating the fifty percent inhibition (IC₅₀) of each drug the Graph Pad Prism program (Graph Pad Software Inc., USA) was used. In order to determine the presence or absence of synergism between MET and LAP, the combination index (CI) was calculated using CompuSyn software based on the method used by Chou et al [18]. In this method, synergistic was defined as a CI < 1.0, antagonistic as a CI > 1.0, and additivity as CI values equal to 1.0.

Protein extraction and Western Blot Analysis

Approximately 5×10^5 cells/well were seeded into 6 well plates, and then incubated with different concentrations of LAP and MET at 37 °C. The collected cells were lysed in ice-cold radioimmunoprecipitation assay buffer (RIPA) contain proteases inhibitor cocktail. Total protein concentration was quantified with bicinchoninic acid (BCA) protein quantitation kit (Abcam, Cambridge, UK). After electrophoresis of proteins on the SDS-PAGE, the blots were electro transferred to the PVDF membrane and blocking was performed with BSA 5% in PBS for 1 hour. The blots were then incubated with primary anti-AKT (4691s; 1:1000 dilution), and anti-AMPK (2532s; 1:1000 dilution) antibody, and re-incubated for β -actin (4970L; 1:1000) as a loading control. After washing, the membrane was incubated in goat anti-rabbit peroxidase-conjugated antibody (7074s; 1/2000 dilution). Finally, the blots visualized with Bio-Rad ChemiDoc™ (Hercules, CA, USA), and densitometry was performed using ImageJ software.

Apoptosis assay

Apoptosis was measured using annexin V-FITC apoptosis kit (Sigma Aldrich., USA), and analyzed by flow cytometry. The cells (5×10^5 cells/well) were seeded into a 24-well plate, after treatment with MET, LAP individually and in combination, the cells were trypsinized and then pelleted by centrifugation. The pellet was washed in PBS and then in binding buffer. Then, the cells were pelleted again and resuspended in binding buffer containing Annexin V and PI and were incubated for 10 minutes in the dark. Finally, the percent of apoptotic cells, including the early apoptotic (Annexin V⁺/PI⁻) and late apoptotic/necrotic (Annexin V⁺/PI⁺) cells were analyzed using a flow cytometry system.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The cells treated with different concentrations of MET, and LAP individually and in combination, then collected and washed with PBS. Total RNA was extracted using RNeasy plus mini kit (Qiagen, Germany) according to the manufacturer's instructions. The quantity and quality of extracted RNA were assessed using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA), and electrophoresis on 1.0% agarose, respectively. To gain cDNA equal amounts of total RNA (1 μ g) was subjected to reverse transcription-PCR using Quantitate Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The primers used are listed as follows: Bax- F 5'-GGGTGGTTGGGTGAGACTC-3', Bax-R 5'-AGACACGTAAGGAAAACGCATTA-3; Bcl2-F 5'-TCGCCCTGTGGATGACTGA-3', Bcl2-R 5'-CAGAGACAGCCAGGAGAAATCA-3', and p21-F 5'-GCTCGGCTCTTCACCAAG-3', P21-R 5'-GTCACTGTCTTGTACCCTTGTG-3'. The relative expression of target genes was calculated using the comparative- Ct method ($\Delta\Delta$ Ct method) and were normalized relative to the expression of the actin housekeeping gene.

The measurement of caspase 3 and 9 activity

In order to determine the caspase 3 and 9 activity, after treatment of the cells with MET, LAP individually, and in combination, the cells were lysed, and the activity of caspases was measured using a colorimetric enzyme-linked immunosorbent assay (ELISA) kits based on manufacturer's instructions.

Molecular docking studies

The *in silico* automated docking studies were performed using AutoDock version 4.2.5.1 and AutoDock Tools (ADT) version 1.5.6 [19]. Gasteiger charges were added to each atom, and the non-polar hydrogen atoms were merged. Water and co-crystal ligand molecules were eliminated from the PDBs (Protein Data Bank). Then the receptors were converted to PDBQT (Protein Data Bank, Partial Charge (Q), and Atom Type (T) using ADT. In all experiments, the Lamarckian genetic algorithm (LGA) search method was applied to establish the best pose of each ligand in the active site of the receptors. Docking studies were accomplished by Intel®Core i5 CPU (2.53 GHz) with Windows-7 operating system. The docking study of compounds was carried out within the Akt (PDB ID: 4GV1), AMPK (PDB ID: 6C9H), and HER2 (PDB ID: 3PP0) active site, which was obtained from the protein data bank. The target was prepared by adding polar hydrogens and merging non-polar hydrogens. Kollman united atom charge, and atom type parameters were added. The dimensions of the grids were therefore $90 \times 90 \times 90 \text{ \AA}$, with a spacing of $0.475 \text{ \AA}$ between the grid points. A grid Centre with $x = 11.63$, $y = 10.18$, and $z = 16.5$ was applied. The parameters were set as follows: population size of 150, a maximum number of generations of 27,000, and a maximum number of 25,000,000 energy evaluations. The Lamarckian genetic search algorithm was applied, and docking runs were set to 100 for each ligand. The results were classified and evaluated based on docking energy. Using Chimera 1.13 software [20] and online web-based Plip software [21] the interaction of the protein-ligand was evaluated. Finally, the resulted docked ligand-receptor complexes were evaluated in terms of energy, hydrophobic interaction, and hydrogen bonding to find the best binding mode of the potent inhibitors.

Statistical Analysis

In the current study, the quantitative data are presented as mean $\pm$ SEM (standard error of the mean) of three independent experiments. The MTT assay experiments were performed triplicate and the results were reported as the percentage of viability. The comparisons of mean between groups were made with one-way analysis of variance (ANOVA) followed by post hoc test using Graph Pad Prism (Graph Pad Software, USA). Statistical significance was accepted at the level of $p < 0.05$.

Results

Synergistic cytotoxic effect of LAP- MET combination on the SKBR3 cells

After 24 h treatment with 20 mM of MET, a significant inhibitory effect on the cell viability was observed in comparison with controls ($p < 0.05$). After 72 h treatment with 40 and 50 mM of MET, its toxic effect was comparable with treatment at 24, and 48 h. (Fig. 1). The IC_{50} value was decreased to 65 and 25 mM when the cells incubated for 48, and 72 h, respectively, in comparison to 50 mM at 24 h (Fig. 1). Also, after 24 h treatment with 100 nM of LAP, a significant inhibitory effect was observed in comparison with controls ($p < 0.05$). After 72 h treatment with 100 nM of LAP, its toxic effect on the cell viability was comparable with treatment at 24, and 48 h. (Fig. 1). The IC_{50} value was decreased to 500 and 100 nM when the cells incubated for 48, and 72 h, respectively, in comparison to 800 nM at 24 h (Fig. 1). To determine whether MET could enhance the efficacy of LAP against breast cancer cells, we assessed the cytotoxic effect of the MET-LAP combination on the SKBR3 cells and then analyzed for a possible synergistic, additive, or antagonistic manner. As presented in Fig. 1, all combinations of MET with LAP had more cytotoxic effects compared to LAP individually. A combination of 100 mM of MET with 100 nM of LAP for 48 h causes 95% inhibition in comparison to LAP individually which shows 15% inhibition. An increased inhibition was also observed in the other MET-LAP combinations ranging from 10, 20, 30, 40, 50, 70, 100 mM of MET with 100 nM of LAP with 100 nM of LAP (Fig. 1). Afterward, we assessed for a possible synergism combination between MET and LAP using CompuSyn software. As presented in Table 1, all concentration of MET–LAP combination showed a synergistic effect in terms of cell viability inhibition. The strongest synergistic effects ($CI = 0.51$), however, were obvious at 100 mM concentration of MET plus 100 nM of LAP.

Table 1
MET-LAP combination index (CI) determination

	MET (10 mM)	MET (20 mM)	MET (30 mM)	MET (40 mM)	MET (50 mM)	MET (70 mM)	MET (100 mM)
LAP (100 nM)	0.89	0.82	0.79	0.73	0.72	0.59	0.51
The SKBR3 cells were given 100 nM of LAP and different concentrations of MET, and, then the combination index (CI) was analyzed by CompuSyn software. $CI < 1$, $= 1$, and > 1 shows synergistic, additive, and antagonistic effects, respectively. MET = metformin; LAP = Lapatinib							

Synergistic Apoptotic Effect Of Met-lap Combination

Annexin V-FITC/PI double staining, a quantitative apoptotic death assay was performed to verify whether the MET-LAP combination can induce apoptosis synergistically and also leading to increased apoptotic death. Our results showed that MET and LAP either individually or in combination had a dose-dependent apoptotic effect on the SKBR3 cells. The total apoptotic population was significantly increased in the

cells treated with different concentrations of MET (12.3%, 18.2%, 27%, and 29.8 %, respectively) (Fig. 2) and treated with different concentrations of LAP (18.1%, 28.1%, 33.3%, respectively) compared to controls (Fig. 3). In the cells co-treated with the least concentration (100 nM) of LAP and different concentrations of MET at 48 hours, the apoptotic death was significantly increased (10.8%,22.1%,25.3%, and 46%, respectively) compared to LAP individually (Fig. 4). These results suggested the MET-LAP combination was significantly ($P < 0.001$) increased apoptotic death in comparison to LAP individually.

Synergistic Effect of MET-LAP combination on the mRNA expression of apoptotic biomarkers

Our results indicated that the mRNA expression of pro-apoptotic protein Bax and cell-cycle inhibitory protein p21 was significantly increased (7.0-fold and 9.0-fold, respectively) in MET 100 mM plus LAP 100 nM (Figs. 5A and B). Whereas, the mRNA expression of anti-apoptotic protein Bcl2 was significantly decreased by 5.0-fold in similar concentrations (Fig. 5C). Changes in the mRNA expression of these apoptotic biomarkers were consistent with data obtained from cellular apoptosis evaluation. The caspase 3, and caspase 9 activity were also measured to further verify the apoptotic effect of MET-LAP combination. The results obtained from the activity of both caspases confirmed the results obtained from Annexin V/PI and mRNA expression of Bax (Fig. 6).

Synergistic effect of MET-LAP combination on the AMPK and Akt Expression

To determine the effects of MET and LAP either individually or in combination on the level of AMPK and Akt expression, the SKBR3 cells were treated for 48 h. Our results showed that MET and LAP individually in various concentrations had no significant effect on the protein level of Akt, and AMPK expression (Fig. 7). However, after 48 h co-treatment with MET-LAP combination the protein level of Akt was significantly decreased, and AMPK expression was significantly increased in comparison to both of them individually (Fig. 8).

Molecular Docking Properties

LAP showed a binding energy value of $-11.31 \text{ kcal.mol}^{-1}$ with the docked Akt and interacted with 15 active site amino acids of Akt, namely Val164, Ala177, Lys179, Leu181, Glu191, Glu198, Thr211, Met227, Glu228, Ala230, Met281, Thr291, Asp292, Gly294, Leu295 (Table 2), by forming five hydrogen bonds (Fig. 9). LAP showed a binding energy value of $-7.54 \text{ Kcal.mol}^{-1}$ with the docked AMPK and interacted with 7 active site amino acids of AMPK namely Arg49, Gln50, Arg53, Thr87, Ser138, Glu139, and Gln154 (Table 2) by forming four hydrogen bonds (Fig. 10). LAP showed a binding energy value of $-11.66 \text{ Kcal.mol}^{-1}$ with the docked HER2 and interacted with 11 active site amino acids of HER2 namely Leu718, Phe723, Val726, Ala743, Lys745, Thr790, Leu792, Met793, Leu844, Asp855, and Phe856 (Table 2) by forming five hydrogen bonds (Fig. 11). To validate the docking procedures, co-crystal ligand inside the PDB file of 4GV1, 6C9H, and 3PP0 were extracted and re-docked with its target. The RMSD values for both targets were below 2 Å.

Table 2

Interaction energy values and the important active site amino acids of the Akt and AMPK responsible for interactions with LAP and MET

Receptor	Ligand	Lowest docking Energy (Kcal/mol)	Estimated Ki (nM)	Interacting residues
Akt	Lapatinib	-11.31	5.09	Val164, Ala177, Lys179, Leu181, Glu191, Glu198, Thr211, Met227, Glu228, Ala230, Met281, Thr291, Asp292, Gly294, Leu295,
	Metformin	-5.93	44910	Glu191, Asp274, Asp292, Gly294, Leu295
AMPK	Lapatinib	-7.54	2990	Arg49, Gln50, Arg53, Thr87, Ser138, Glu139, Gln154
	Metformin	-5.64	73330	Arg83, Asp136, Ser138, Glu139
Her2	Lapatinib	-11.66	2.85	Leu718, Phe723, Val726, Ala743, Lys745, Thr790, Leu792, Met793, Leu844, Asp855, Phe856
	Metformin	-4.74	336230	Met766, Leu777, Thr854, Asp855, Phe856

Discussion

Changes in HER2 function are a common molecular defect in breast malignancy and commonly associated with an aggressive phenotype and poor outcomes [2, 3]. Clinically, two main problems may be occurring after using HER2 inhibitors in breast tumors with HER2 overexpressing. First, the effect of HER2 inhibitors is limited by the development of hereditary and acquired resistance that usually occurs within one year after starting the therapy [4]. Second, since the activity of HER2 has a vital role in heart function and development, thus, the risk of cardiomyopathy should be considered [22, 23]. Though, proposed that simultaneous HER2 inhibition and AMPK activation, may demonstrate very success in cancer treatment, and ameliorate cardiac side effects [24]. Moreover, several studies indicated that combination chemotherapy had a high ability to reduce the growth of carcinoma cells, prevent angiogenesis, induction of apoptosis, and triggers an immune response [8–10]. Recently, combination therapy of chemotherapeutic agents and phytochemicals is more attention as a successful alternative therapy to increase therapeutic efficacy and decreases the systemic toxic effects of these chemotherapeutic agents. Although, various *in-vitro* and *in-vivo* combination studies between MET and chemotherapeutic agents were performed to analyze the effects of such combination on the inhibition of cell proliferation, cell cycle regulation, and apoptotic death. However, to the best of our knowledge, the combination of MET and LAP has not been examined, therefore, in the current study; we assessed the effect of MET and LAP either individually or in combination on the cell viability and apoptotic death in SKBR3 cells.

We found that LAP and MET individually and in combination had a significant toxic effect on the SKBR3 cells in a time and dose-dependent manner. Analysis of flow cytometry data showed that the MET-LAP

combination had a more apoptotic death compared to both of them individually. Also, the combination could significantly increase the mRNA expression of pro-apoptotic protein Bax, a cell-cycle inhibitory protein, p21, and caspase 3 and 9 activity. Whereas, the mRNA expression of anti-apoptotic protein Bcl2 was significantly decreased. Moreover, the combination could significantly increase the AMPK and decrease the Akt expression. Our results are in accordance with the results from preceding studies which confirmed the inhibitory effect of MET [17, 25, 26] and LAP [10, 27, 28] on the breast cancer cell lines. Moreover, they found a combination of LAP and MET could considerably reduce the growth of all cell types compared to the treatments with them individually.

Clinically, MET is commonly safe, worldwide available and it is significantly low-cost. Although, it is the first-choice oral therapy as an anti-diabetic drug, several in vitro and in vivo studies have shown that MET has anti-cancer activity in several malignancies, such as pancreatic, prostate, colorectal, and breast cancers [14–17]. Several mechanisms have been suggested to explain such anti-cancer activities of MET, for example, activation of AMPK and inhibition of mTOR [15, 29]. It is well established that Akt has a key role in the proliferation of carcinoma cells and apoptotic death. Because, the Akt is downstream of several coordinate tumor growth factors including IGF- I receptor [20, 31]. Thus, our finding that the MET-LAP combination reduced the Akt expression in SKBR3 cells may represent an important effect of this drug for controlling carcinoma cell proliferation.

A case-control study by Jiralerspong et al. [32] indicated that diabetic patients with breast cancer treated with neoadjuvant chemotherapy taking also MET had a higher pathologic response compared with patients not taking. In a study by Oliveira-Ferraro et al. [33], they found MET can inhibit the expression of M phase-related genes in the breast cancer cells by activating the AMPK pathway, consequently, the breast cancer cell cycle was arrested in the G2 phase. A study conducted by Sahra et al. [14] indicated, although, MET could inhibit the growth of the prostate cancer cells, however, could not induce apoptotic death. Other studies showed that MET can induce apoptotic death in breast, colon, and endometrial carcinoma cells [34–36]. Zhuang and Miskimins [36] found that MET individually, or in combination with tamoxifen could increase apoptotic death in SKBR3 cells via caspase and PARP activity. Also, they showed that MET could increase cell apoptosis via the activation a caspase-mediated pathway. These results are consistent with our result that found MET could increase the caspase 3 and 9 activity in combination with LAP.

LAP, a dual synthetic reversible inhibitor of tyrosine kinase activity, has potent anti-tumor activity against HER2-overexpressing breast cancer [9–12], but the exact mechanism of its anti-tumor action has not been completely understood. Akt/PKB is a key serine/threonine kinase that regulates several cellular signaling pathways including cell proliferation, viability, size, and invasion, together with glucose metabolism and angiogenesis [17]. Our results indicated that although MET and LAP individually could not affect the expression of AMPK and Akt. However, the MET-LAP combination could increase and decrease the expression of AMPK and Akt, respectively. Previous studies reported that MET could inhibit signal transduction through ERK and Akt, both of which advocate cancer development in several malignancies such as breast cancer [37, 38]. The alteration of Akt function is usually dis-regulated in

various types of malignancy and has a vital role in tumor cell invasion, proliferation, mobility, and survivance [38]. The regulation of apoptosis by Akt may be mediated through phosphorylation of several pro-apoptotic proteins including Bax and caspase 9 [39]. Our results indicated that MET and LAP individually and in combination could significantly increase the expression of Bax and caspase 3 and 9 activity in SKBR3 cells. It is speculated that these effects may be mediated by the reduction of protein level of Akt. Moreover, Akt can induce phosphorylation and inactivation of the Forkhead Box (FOXO), which is implicated in the expression of pro-apoptotic genes. On the other hand, Akt can phosphorylate and inactivate I κ B kinase (IKK), a positive modulator of NF- κ B that induce several anti-apoptotic gene expressions [40]. Also, phosphorylation of Mdm2 by Akt resulted in its activation and induces its movement in the nucleus, where it promotes the degradation of p53 [41, 42]. Otherwise, Akt by induction of several transcription factors phosphorylation, can decrease p53 mRNA expression [43]. Our results indicated that MET and LAP individually and in combination could significantly increase the expression of a cell-cycle inhibitory protein, p21, which is a target of P53. It is suggested that this effect may be due to the reduction of the inhibitory effect of Akt on the P53 expression.

Since inhibition of HER2 tyrosine kinase activity is associated with blocking of downstream signaling pathway of Akt and AMPK in HER2-overexpressing breast carcinoma cells [44], thus, to gain more insight into the LAP-induced apoptotic death, its molecular docking studies were performed on the Akt, AMPK, and HER2 targets. Results of the molecular docking verified the inhibitory effect of LAP on the HER2. LAP showed an interaction energy value (ΔG_{bind}) of -11.31, -7.54, and - 11.66 kcal.mol⁻¹ in binding to Akt, AMPK, and HER2, respectively. However, it showed stronger binding energy in binding to HER2 in comparison to Neratinib (-9.13 kcal.mol⁻¹), as HER2 tyrosine kinases inhibitors. We confirmed that the main amino acids inside the active site of the HER2, which is responsible for essential interactions with LAP are Leu718, Phe723, Val726, Ala743, Lys745, Thr790, Leu792, Met793, Leu844, Asp855, and Phe856. Immunoblot analysis showed that MET-LAP combination could decrease and increase the expression level of Akt, and AMPK. Which is in consistent with the molecular docking results, which showed that the inhibitory effect of LAP in binding to HER2 can down-regulate and up-regulate the Akt and AMPK, respectively. Molecular docking of LAP in binding to Akt, and AMPK was also conducted to find out the binding modes, binding sites, and the best binding orientation of LAP to Akt and AMPK. Even though the binding of LAP to Akt, and AMPK cannot exactly explain the mechanism of down-regulation, and up-regulation of Akt, and AMPK, respectively.

Conclusion

Our results indicated that the MET-LAP combination has a synergistically cytotoxic effect on the cell viability and apoptotic death of SKBR3 cells by the regulation of apoptotic biomarkers including Bax, Bcl2 expression, and caspase 3 and 9 activity. These effects may be associated with an increased and decreased expression of AMPK and Akt, respectively. Also, based on the molecular docking results, LAP binds to the active sites amino acids of Akt, AMPK, and HER2 through conventional hydrogen and hydrophobic interactions. Finally, our results confirmed the previous studies that indicate MET sensitizes

cancer cells to clinically use therapeutic anticancer agents and supply credible proof for the therapeutic potential of this compound for the treatment of breast patients with overexpression of HER2 receptors.

Declarations

Acknowledgments This paper is issued from the thesis of Davood Neamati and financial support was provided by Hyperlipidemia Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (grant no. HLRC-9601).

Author contributions GM and AK proposed the project and designed the experiments. Experiments were performed by DN, AK, MA, and AAH. The results were analyzed by DN, AK, and GM. The manuscript was prepared by GM and AK. All authors have read and approved the final manuscript for publication in the journal.

Data availability Yes, can be supplied upon the requirement

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest

Research involving human and/or animal rights This project does not contain any studies with human participants or animals performed by any of the authors.

References

1. Ghoncheh M, Pournamdar Z, Salehiniya H (2016) Incidence and Mortality and Epidemiology of Breast Cancer in the World. *Asian Pac J Cancer Prev* 17(S3):43–46
2. Dawood S, Broglio K, Buzdar AU, Hortobagyi GN, Giordano SH (2010) Prognosis of women with metastatic breast cancer by HER2 status and trastuzumab treatment: an institutional-based review. *J Clin Oncol* 28:92–98
3. Brower V (2013) Cardiotoxicity Debated for Anthracyclines and Trastuzumab in Breast Cancer. *J Natl Cancer Inst* 105:835–836
4. Chen FL, Xia W, Spector NL (2008) Acquired resistance to small molecule ErbB2 tyrosine kinase inhibitors. *Clin Cancer Res* 14:6730–6734
5. Agrawal A, Gutteridge E, Gee JM, Nicholson RI, Robertson JF (2005) Overview of tyrosine kinase inhibitors in clinical breast cancer. *Endocr Relat Cancer* 12(Suppl 1):S135–S144
6. Sierra JR, Cepero V, Giordano S (2010) Molecular mechanisms of acquired resistance to tyrosine kinase targeted therapy. *Mol Cancer* 9:75
7. Mitra S, Dash R (2018) Natural Products for the Management and Prevention of Breast Cancer. *Evid Based Complement Alternat Med* 2018:8324696. doi:10.1155/2018/8324696

8. Blackwell KL, Burstein HJ, Storniolo AM, Rugo H, Sledge G, Koehler M et al (2010) Randomized study of LAP individually or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. *J Clin Oncol* 28:1124–1130
9. Tanizaki J, Okamoto I, Fumita S, Okamoto W, Nishio K, Nakagawa K (2011) Roles of BIM induction and survivin downregulation in LAP-induced apoptosis in breast cancer cells with HER2 amplification. *Oncogene* 30:4097–4106
10. Nahta R, Yuan L, Du Y, Esteva F (2007) LAP induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling. *Molecular. Cancer Therapeutics* 6:667–674
11. Komninou D, Ayonote AI, Richie JP, Rigas B (2003) Insulin Resistance and Its Contribution to Colon Carcinogenesis. *Exp Biol Med* 228:396–405
12. Baron JA, Weiderpass E, Newcomb PA, Stampfer M, Titus-Ernstoff L, Egan KM et al (2011) Metabolic disorders and breast cancer risk (United States). *Cancer Cause Control* 12:875–880
13. Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F, Viollet B, Thompson CB (2007) Systemic treatment with the antidiabetic drug MET selectively impairs p53-deficient tumor cell growth. *Cancer Res* 67:6745–6752
14. Sahra B, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P et al (2008) The antidiabetic drug MET exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene* 27:3576–3586
15. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M (2006) MET is an AMP kinase-dependent growth inhibitor for breast cancer cells. *Cancer Res* 66:10269–10273
16. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N (2007) MET inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. *Cancer. Res* 67:10804–10812. 2007
17. Shi P, Liu W, Wang H, Li F, Zhang H, Wu Y et al (2017) MET suppresses triple-negative breast cancer stem cells by targeting KLF5 for degradation. *Cell Discovery* 3:17010. doi:10.1038/celldisc.2017.10
18. Chou TC (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 58:621–681
19. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS et al (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* 30(16):2785–2791
20. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC et al (2004) UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* 25(13):1605–1612
21. Salentin S, Schreiber S, Haupt VJ, Adasme MF, Schroeder M (2015) PLIP: fully automated protein–ligand interaction profiler. *Nucleic acids research* 43(W1):W443–W447
22. Force T, Krause DS, Van Etten RA (2007) Molecular mechanisms of cardiotoxicity of tyrosine kinase inhibition. *Nat Rev Cancer* 7:332–344

23. Yarden Y, Smith B, Lyass L et al (2007) Activation of AMP-activated protein kinase by human EGF receptor 2/EGF receptor tyrosine kinase inhibitor protects cardiac cells. *Proc. Natl. Acad. Sci. USA* 104: 10607–10612
24. Shell SA, Lyass L, Trusk PB et al (2008) Activation of AMPK is necessary for killing cancer cells and sparing cardiac cells. *Cell Cycle* 7:1769–1775
25. Cantrell LA, Zhou C, Mendivil A, Malloy KM, Gehrig PA et al (2010) MET is a potent inhibitor of endometrial cancer cell proliferation— implications for a novel treatment strategy. *Gynecol Oncol* 116:92–98
26. Zhuang Y, Miskimins WK (2011) MET induces both caspase-dependent and poly (ADP-ribose) polymerase-dependent cell death in breast cancer cells. *Mol Cancer Res* 9:603–615
27. Gril B, Palmieri D, Mason J, Herring JM, Vega-Valle E, Feigenbaum L et al (2008) Effect of LAP on the Outgrowth of Metastatic Breast Cancer Cells to the Brain. *J Natl Cancer Inst* 100:1092–1103
28. Guan M, Tong Y, Guan M, Liu X, Wang M, Niu R (2018) LAP Inhibits Breast Cancer Cell Proliferation by Influencing PKM2 Expression. *Technol Cancer Res Treat* 17:1–12
29. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J et al (2001) Role of AMP-activated proteinkinase in mechanism of MET action. *J Clin Invest* 108:1167–1174
30. Faivre S, Kroemer G, Raymond E (2006) Current development of mTOR inhibitors as anticancer agents. *Nat Rev Drug Discov* 5:671–688
31. Guertin DA, Sabatini DM (2005) An expanding role for mTOR in cancer. *Trends Mol Med* 11:353–361
32. Jiralerspong S, Palla SL, Giordano SH, Meric-Bernstam F, Liedtke C, Barnett C et al (2009) MET and pathologic complete responses ton neoadjuvant chemotherapy in diabetic patients with breast cancer. *J Clin Oncol* 27:3297–3302
33. Oliveira-Ferraros C, Vazquez-Martin A, Menendez JA (2009) Genome-wide inhibitory impact of the AMPK activator MET on kinesins, tubulins, histones, auroras and pololike kinases M-phase cell cycle genes in human breast cancer cells. *Cell Cycle* 8:1633–1636
34. Berstein LM, Yue W, Wang JP, Santen RJ (2010) Isolated and combined action of tamoxifen and MET in wild-type, tamoxifen-resistant, and estrogen deprived SKBR3cells. *Breast. Cancer Res Treat* 128:109–117
35. Topcul M, Cetin I (2015) Effects of MET on cell kinetic parameters of SKBR3breast cancer cells in vitro. *Asian Pac J Cancer Prev* 16:2351–2354
36. Wang Y, Wei J, Li L, Fan C, Sun Y (2015) Combined use of MET and everolimus is synergistic in the treatment of breast cancer cells. *Oncol Res* 22:193–201
37. Mundi PS, Sachdev J, McCourt C, Kalinsky K (2016) AKT in cancer: new molecular insights and advances in drug development. *Br J Clin Pharmacol* 82:943–956
38. Steelman LS, Navolanic P, Chappell WH, Abrams SL, Wong EW, Martelli AM et al (2011) Involvement of AKT and mTOR in chemotherapeutic- and hormonal-based drug resistance and response to radiation in breast cancer cells. *Cell Cycle* 10:3003–3015

39. Downward J (2004) PI 3-kinase, AKT and cell survival. *Semin Cell Dev Biol* 15(2):177–182
40. PomMier Y, Sordet O, Antony S, Hayward RL, Kohn KW (2004) Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. *Oncogene* 23:2934–2949
41. Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC (2001) HER-2/neu induces p53 ubiquitination via AKT-mediated MDM2 phosphorylation. *Nat Cell Biol* 3:973–982
42. Mayo LD, Donner DB (2001) A phosphatidylinositol 3-kinase/AKT pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc. Natl. Acad. Sci. U S A* 98:11598–11603
43. Park S, Kim D, Dan HC, Chen H, Testa JR, Cheng JQ (2012) Identification of an AKT interaction protein, PHF20/TZP, that transcriptionally regulates p53. *J Biol Chem* 287:11151–11163
44. Rusnak DW, Lackey K, Affleck K, Wood ER, Aligood KJ, Rhodes N et al (2001) The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines in vitro and in vivo. *Mol Cancer Ther* 1:85–94

Figures

Figure 1

In vitro cytotoxic effect of MET, LAP, and MET-LAP combination on the SKBR3 cells. The cells were given different concentrations of MET, LAP, and MET-LAP combination for 24, 48, and 72 h, respectively. The MTT assay was used to determine cell viability. Each experiment was achieved triplicate and data expressed as means $\pm$ SEM. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to control.

Figure 2

Flow cytometry analysis of MET-induced apoptotic death in SKBR3 cells. The cells were given different concentrations of MET for 48 hours and, then the percent of apoptotic death was measured. Data are expressed as means $\pm$ SEM of three independent experiments. ***p < 0.001 in comparison to control

Figure 3

Flow cytometry analysis of LAP-induced apoptotic death in SKBR3 cells. The cells were given different concentrations of LAP for 48 hours, and, then the percent of apoptotic death was measured. Data are expressed as means $\pm$ SEM of three independent experiments. *** $p < 0.001$ in comparison to control

lapatinib and metformin combination (48h)

Figure 4

Flow cytometry analysis of MET-LAP combination-induced apoptotic death in SKBR3 cells. The cells were given MET-APT combination with different concentrations for 48 hours, and, then the percent of apoptotic death was measured. Lane 1 shows controls; Lane 2 shows the cells gave LAP alone; lanes 3,4,5 show the cells gave LAP - MET combination. Data are expressed as means $\pm$ SEM of three independent experiments. ***p < 0.001 in comparison to control.

Figure 5

The effect of MET-LAP combination on the expression of Bax, P21, and Bcl2 in SKBR3 cells. The cells were given 100 nM of LAP in combination with different concentrations of MET for 24 hours, and then the expression of Bax, P21, and Bcl2 was analyzed by qRT-PCR. Data are expressed as means $\pm$ SEM of three independent experiments. **P < 0.01, ***p < 0.001 in comparison to control.

Figure 6

The effect of MET-LAP combination on the caspase 3 and 9 activity in SKBR3 cells. The cells were given 100 nM of LAP in combination with different concentrations of MET for 24 hours, and, then the activity of caspases 3 and 9 was analyzed with a colorimetric enzyme-linked immunosorbent assay (ELISA) kits. Data are expressed as means $\pm$ SEM of three independent experiments. **P < 0.01, ***p < 0.001 in comparison to control.

Figure 7

The effect of MET and LAP on the expression of AMPK and Akt in SKBR3 cells. The cells were given different concentrations of MET and LAP for 48 hours, and, then the expression of AMPK, Akt, and β -actin was analyzed by Western blotting. Data are expressed as means $\pm$ SEM of three independent experiments. *P < 0.05, **p < 0.01 in comparison to control.

Figure 8

The effect of MET-LAP combination on the AMPK and Akt expression in SKBR3 cells. The cells were given MET- LAP combination for 48 hours and then the expression of AMPK, AKT, and β -actin was analyzed by Western blotting. Data are expressed as means $\pm$ SEM of three independent experiments. *P < 0.05, **p < 0.01, ***p < 0.001 in comparison to control.

Figure 9

A schematic representation (2D and 3D) of the interaction between LAP and the active site amino acids of Akt

Figure 10

A schematic representation (2D and 3D) of the interaction between LAP and the active site amino acids of AMPK

Figure 11

A schematic representation (2D and 3D) of the interaction between LAP and the active site amino acids of HER2