EV Targeting MCF-7 Breast Cancer Cell Lines Inhibit Both mTOR and HIF-1A: Molecular Docking Study

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Abstract

Background and Objective: The mortality dramatically increase in breast cancer are caused by inadequate of the benefits of different types of therapies. mTOR is an atypical serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family. The aim of this study to investigation of ability of EV to inhibiting both mTOR and HIF-1A through MCF-7 BC cell lines targeting and docking study by specific computational program.

Method: In vitro cytotoxicity study of different doses of EV were quantitatively measured by employing on MCF-7 cell lines. Docking study was done by computing 3D programs (in silico), the information in protein data bank (PDB) Zinc15docking and phyre2 protein homology tools.

Results: There was another suggestion for inhibition of HIF-1A protein (PDB reference code 4AJY) by incubation of MCF-7 cells with 5 mg/day of EV that is may be binding of EV as analogue in other sites differs from active site and may be stimulate the hydroxylation and acetylation of the protein and enhanced normal degradation pathway.

Conclusion: This study suggesting the mechanism of inhibition of HIF-1A by EV in addition to inhibition of mTOR pathway.

Keywords: *Breast Cancer, mTOR, HIF-1A, Everolimus, Docking.*

Introduction

Mammalian target of rapamycin (mTOR) is a protein kinase that regulates proteinsynthesis and cell growth in response to growth factors, nutrients, energy levels, andstress[1]. mTOR is an atypical serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinasefamily and interacts with several proteins to form two distinct complexes named mTORcomplex 1 (mTORC1) and 2 (mTORC2)[2]. The mTOR signaling pathway plays an essential role in cell growthand proliferation by coordinating anabolic processes with oxygen,energy and nutrient availability, as well as extracellular cues. Onefundamental characteristic of cancer cells resides in their ability tosustain chronic proliferation in the absence of growth-promoting signals. This proliferative advantage is achieved, at least in part, bygenetic events that cause aberrant activation of mTORC1 signaling[3]. Indeed, mTORC1 lies downstream of the Ras/MAPK and PI3K/Akt signaling pathways, where gain-of-function mutations inRas, Raf, PI3K and Akt oncogenes, and loss-of-function mutationsin the tumor suppressors neurofibromatosis-related protein-1 (NF-1), phosphatase and tensin homolog (PTEN) are found inup to 80% of human cancers[4]. Themajority of catalytic mTOR inhibitors is currently in phase I clinicaltrial,

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and these compounds are being tested as single agents or incombination with other chemotherapeutic agents. Currently, these compounds are being tested against several types of cancer, includingbreast cancer, endometrial cancer, non-Hodgkin lymphoma and advanced stages of solid tumors [5]. The HIF-1A subunit has two transactivation domains (TAD): NH2-terminal (N-TAD) and COOH-terminal (C-TAD). These two domains are responsible for HIF-1A transcriptional activity [6]. C- TAD interacts with co-activators such as CBP/p300 to modulate gene transcription of HIF-1A under hypoxia. N-TAD is responsible for stabilizing HIF-1A against degradation [7]. Moreover, all HIF-1A subunits are distinct from HIF-1B in that they all have an oxygen- dependent degradation domain (ODDD) over lapping N-TAD in their structures . This ODDD domain is important in mediating O2 regulation stability [8]. Given that cells and organs need to adapt to changes inoxygen supply, it would not be surprising to find that asignificant variety of the HIF-1 target genes are regulated ina tissue-specific manner. To date, there are more than 100HIF-1A downstream genes identified with varying functions(Table 1.2). HIF-1A activates the expression of these genes bybinding to HRE located in theirenhancer and promoter regions[9]. Everolimus (RAD001(40-O-(2-hydroxyethyl)-rapamycin))(molecular weight, 958.2 g/mol) is an orally active derivative of rapamycin that inhibits the Ser/Thr kinase, mTOR[10]. The aim of this study to investigation of ability of EV to inhibiting both mTOR and HIF-1A through MCF-7 BC cell lines targeting and docking study by specific computational program.

Materials and Method

***In vitro* cytotoxicity effect of EV on MCF-7 BC cell lines.**

1. In vitro cytotoxicity study of different doses of EV were quantitatively measured by employing on MCF-7 cell lines.
2. MCF-7cells were cultivated in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/ streptomycin, non-essential amino acids (0.1 mM), insulin (10 ug/mL) and sodium pyruvate (1 mM) at 37 Cº in incubator with 5% CO^2.
3. The cells were seeded in 96-well plate for 1 day, 2 days, and 3 days and then the medium was changed with various concentrations of EV(1,10,20, 40,100 mg/dl).
4. In vitro cell viability was determined using the MTT assay as described in following:

MTT protocol (1 day):

a. The cells(1×10^4 cells/well) were seeds in 96-well plate with culture medium in final volume of 100 µl/ well.

b. The plate was incubated at 37C° (5% CO_2 incubator) for 1 day hr.

c. Cells were then treated with various conc. of EV for one day at 37C° in a 5% CO2 incubator.

d. One hundred of fresh medium were added.

e. Ten µl of MTT reagent were add to each wells of plate.

f. The plate was incubated at 37°C in a 5% CO2 incubator for 4 hr.

g. The medium was carefully removed from wells

h. Two hundredµl of DMSO was add to wells and mixed to dissolving all crystals and incubated for 30 min.

i. The absorbance has been reads at 540 nm.

j. Untreated cells represent the control cells (not exposed to EV).

Percentage of viability and cytotoxicity **Cell viability (%) = (Abs_{540} Treated cells/Abs_{540} Control cells) × 100, Cytotoxicity (%)= 100- Cell viability%**

Results and Discussion

Cytotoxicity study: Figure 1 showing the MCF-7 cell viability % between EV at given concentrations and control during 1, 2,and 3 days of period of incubation:

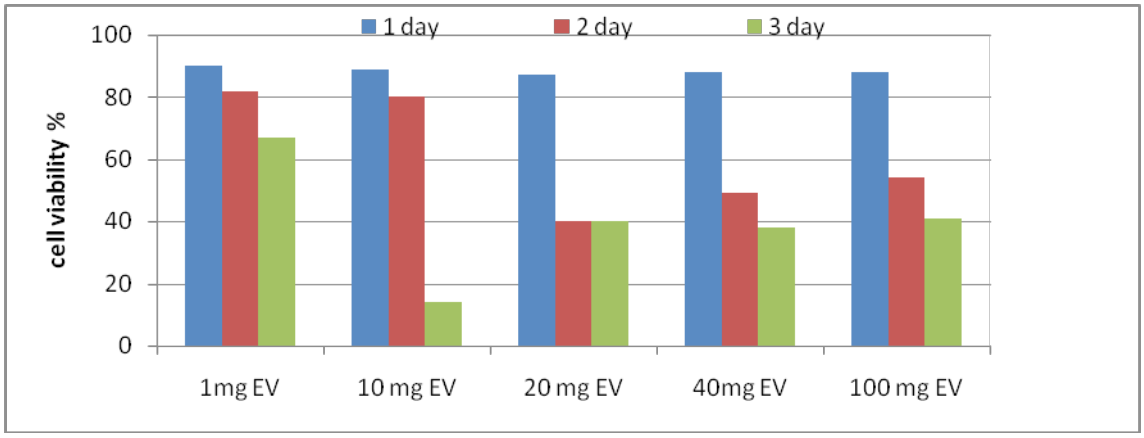

Figure (1): Viability of MCF-7 BC cell lines cultured with EVin different conc. at 1, 2, and 3 days incubation time, the values are mean (n=3) ± SD.

MTT assay is one of the common assays used to study cell viability and proliferation. It depends on the ability of viable cells to reduce the yellow MTT dye to insoluble purple formazan crystals. Here, MTT assays were used to study the anti-proliferative activity of EV on MCF-7 cell lines. The results of present study showing that no statistical differences in cell viability % when incubation of EV free with MCF-7 cell lines at one and second day of period of incubation (92±5, 82±4) (p-value>0.05), but the results show significant differences between one and third day of incubation time (91±2, 65±3) (p-value<0.05). The results also showing highly statistically differences in cytotoxicity% when incubation 10 mg of EV with MCF-7 BC cell lines at three day of incubation (10±1) (p-value< 0.001). The chemical structure of EV has more than one hydroxyl groups and this may be facilitated of binding with HIF-1A leading to inhibitory effects. It was reported by *Greenberger L et al*., (2008) that inhibition of HIF-1A by EZN-2698 attenuates HIF-1A protein level and tumor progression in various in vitro (human prostate and glioblastoma cell lines) and in vivo studies[11]. *Georgina N et al*., (2015) were reported anthracyclinesare the potent well-known chemotherapeutic agents, alsoactasHIF-1A inhibitors by preventing to binding with DNA [12].Another novel class of molecularly targeted anticancer agents consists of inhibitors of heat shock protein 90 (HSP90), such as geldanamycin, 17-allylaminogeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin, which target HIF-1A forproteasomal degradation[13]. A number of HIF-1A inhibitors have been synthesized or discovered for treating cancer, particularly for advanced and refractory solid tumors. They inhibit the expression and/or functions of HIF-1A through direct and indirect mechanisms.

Molecular docking study

By application molecular docking study and Ligand-based drug design (protein-ligand interaction) of online computing 3D programs (in silico) (https://www.rcsb.org), the information in protein data bank (PDB) (https://www.pdb.org/),https://pubchem.ncbi.nlm.nih.gov,Zinc15.docking and phyre2 protein homology tools there was another suggestion for inhibition of HIF-1A protein (PDB reference code 4AJY) that is may be due to binding of EV as analogue in other sites differs from active site and may be stimulate the hydroxylation and acetylation of the protein and enhanced normal degradation pathway or may be formation the HIF-1A-EV complex that preventing of translocation of HIF-1A into nucleus to binding with HIF-1B and in turn inhibition the controlling of HIF1A-HIF-1B dimer on gene expression of target genes, as showing in figure 1:

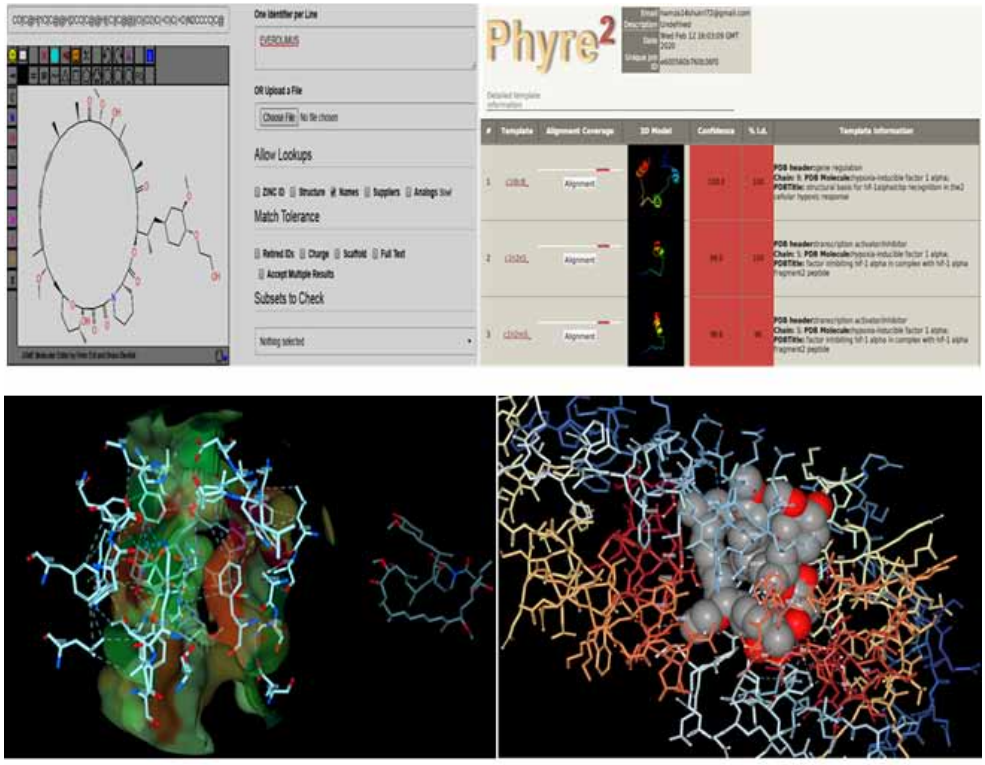

Figure (1): A-Zinc15. docking website for drawing EV structure B-Phyre2 protein homology tool results

The amino acids sequence of HIF-1A protein is shown in figure 2:

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        10         20         30         40         50
MEGAGGANDK KKISSERRKE KSRDAARSRR SKESEVFYEL AHQLPLPHNV
        60         70         80         90        100
SSHLDKASVM RLTISYLRVR KLLDAGDLDI EDDMKAQMNC FYLKALDGFV
       110        120        130        140        150
MVLTDDGDMI YISDNVNKYM GLTQFELTGH SVFDFTHPCD HEEMREMLTH
       160        170        180        190        200
RNGLVKKGKE QNTQRSFFLR MKCTLTSRGR TMNIKSATWK VLHCTGHIHV
       210        220        230        240        250
YDTNSNQPQC GYKKPPMTCL VLICEPIPHP SNIEIPLDSK TFLSRHSLDM
       260        270        280        290        300
KFSYCDERIT ELMGYEPEEL LGRSIYEYYH ALDSDHLTKT HHDMFTKGQV
       310        320        330        340        350
TTGQYRMLAK RGGYVWVETQ ATVIYNTKNS QPQCIVCVNY VVSGIIQHDL
       360        370        380        390        400
IFSLQQTECV LKPVESSDMK MTQLFTKVES EDTSSLFDKL KKEPDALTLL
       410        420        430        440        450
APAAGDTIIS LDFGSNDTET DDQQLEEVPL YNDVMLPSPN EKLQNINLAM
       460        470        480        490        500
SPLPTAETPK PLRSSADPAL NQEVALKLEP NPESLELSFT MPQIQDQTPS
       510        520        530        540        550
PSDGSTRQSS PEPNSPSEYC FYVDSDMVNE FKLELVEKLF AEDTEAKNPF
       560        570        580        590        600
STQDTDLDLE MLAPYIPMDD DFQLRSFDQL SPLESSSASP ESASPQSTVT
       610        620        630        640        650
VFQQTQIQEP TANATTTTAT TDELKTVTKD RMEDIKILIA SPSPTHIHKE
       660        670        680        690        700
TTSATSSPYR DTQSRTASPN RAGKGVIEQT EKSHPRSPNV LSVALSQRTT
       710        720        730        740        750
VPEEELNPKI LALQNAQRKR KMEHDGSLFQ AVGIGTLLQQ PDDHAATTSL
       760        770        780        790        800
SWKRVKGCKS SEQNGMEQKT IILIPSDLAC RLLGQSMDES GLPQLTSYDC
       810        820
EVNAPIQGSR NLLQGEELLR ALDQVN
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Figure (2): Amino acids sequence of HIF-1A protein

To date, no study have reported that HIF-1A are inhibit by EV. This study reported for the first time about the interactions between EV loaded NPs and HIF-1A. It can be concluded that EV loaded NPs had the potential of inhibiting HIF-1A activity. The molecular protein-ligand docking study suggestion that five amino acids participate in this interaction, the residues were (Asn 670, Phe 540, Val 599, Tyr 522, and Lys 629) participating in hydrogen bonding while the residues (Thr 611, His 646, Lys 719, Trp 752) (4 bonds) forming van-der Waalsinteractions between protein (HIF-1A) and ligand (EV) with high binding energy -12.2334 kcal/mol. Active binding sites of EV and HIF-1A protein depending on type of interactions is shown in table-1:

Many of the novel anticancer drugs that target specific pathways have been shown to have anti-angiogenic effects that appear to be inhibition of HIF-1A activity, as shown in table-2:

Table(1): Active binding sites of EV and HIF-1A protein depending on type of interactions

Target protein (HIF1-A)	Active binding sites	Binding affinity energy
Hydrogen bonding	Asn 670, Phe 540, Val 599, Tyr 522, and Lys 629	-12.2334 kcal/mol
Van-der Waals interactions	Thr 611, His 646, Lys 719, Trp 752	

Table (2): Selected drugs that inhibit HIF-1A activity

Drug	Effect	Reference
Doxorubicin	Decreased HIF-1A DNA binding	[14]
Cetuximab	Decreased HIF-1A synthesis	[15]
Digoxin	Decreased HIF-1A synthesis	[16]
Trichostatin A	increased HIF-1A degradation	[17]
Allylaminogeldanamycin.	Decreased HIF-1A transactivation	[18]
Taxotere	Decreased HIF-1A synthesis	[19]
Bortezomib	Decreased HIF-1A transactivation	[20]
EZN-2968 Topotecan	Inhibit HIF-1A mRNA expression Inhibit HIF-1A mRNA translation	[21] [22]
Everolims	Inhibit HIF-1A mRNA expression	This study

In present study, when HIF-1A mRNA expression decreasing by incubation with EV (10 mg)and in results declines the levels of GLUT-1,CD44 and VEGF (the results not appear in this paper)[23], and this means that the transcription process may be inhibited and all events after this may be not occurs. This events are due to blocking of proteins those responsible on transcription of HIF-1A mRNA such as transcription factors by EV and decrease levels of HIF-1A and in turn prevent translocation it to nucleus and don't binding with HIF-1B on its response elements on DNA. Other explanation is may be due to prevent the signals such as growth factors to binding on its receptors on cell membrane by blocking this receptors and this need further investigations.

Cellular targets of biological signals in development for BC through hypoxia events. EV free can be inhibit of mTOR, EV loaded NPs can be inhibit of both mTOR and HIF-1A mRNA and protein and in turn controlling on gene expression of target genes through prevent the accumulation, translocation, and binding of HIF-1A with nucleus protein (HIF-1B). The suggested schematic diagram can be summarize all the findings elucidated by the current study, as shown in figure 3:

Figure (3): Cellular targets of biological signals in development for BC through hypoxia events: EV inhibit both mTOR and HIF-1A.

Conclusion

Suggesting the mechanism of inhibition of HIF-1A by EV in addition to inhibition of mTOR pathway

Data Availability: The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: None

Funding: Self-funding

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