β Arrestin inhibition induces autophagy, apoptosis, G0/G1 cell cycle arrest in agonist activated V2R receptor in breast cancer cells

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Abstract

Non-visual arrestins (β-arrestins) are endocytic proteins that mediate agonist activated GPCRs internalization and signaling pathways in an independent manner. The involvement of β-arrestins in cancer invasion and metastasis is increasingly reported. So, it is hypothesized that inhibition of β-arrestins may diminish the survival chances of cancer cells. This study aimed to evaluate the in vitro impact of inhibiting β-arrestins on the autophagic and/or apoptotic responsiveness of breast cancer cells.

We used Barbadin to selectively inhibit β-Arr/AP2 interaction in AVP stimulated V2R receptor of triple negative breast cancer cells (MDA MB-231). Autophagy was assessed by the microtubule-associated protein 1 light chain 3-II (LC3II), apoptosis was measured by Annexin-V/PI staining and cell cycle distribution was investigated based upon the DNA content using flow cytometry. Barbadin reduced cell viability to 69.1% and increased the autophagy marker LC3 II and its autophagic effect disappeared in cells transiently starved in Earle's balanced salt solution (EBSS). Also, Barbadin mildly enhanced the expression of P62 mRNA and arrested 63.7% of cells in G0/G1 phase. In parallel, the drug induced apoptosis in 29.9% of cells (by AV/PI) and 27.8% of cells were trapped in sub-G1 phase. The apoptotic effect of Barbadin was enhanced when autophagy was inhibited by the PI3K inhibitor (Wortmannin).

Conclusively, the data demonstrate the dual autophagic and apoptotic effects of β-βArr/AP2 inhibition in triple negative breast cancer cells. These observations nominate β-Arrs as selective targets in breast cancer treatment.

Introduction

Autophagy is regarded as protective mechanism when cells experience unfavorable conditions. This paradigm may be altered to cytotoxic event, where excessive autophagy is implicated in autophagic (Type II) cell death [1]. Diminishing the survival chances of cancer cells may require inhibiting the autophagy mediated cytoprotective to enhance their responsiveness to chemotherapy, thereby promoting cell death either alone or in association with apoptosis [2]. The autophagy/apoptosis relation is complex and involves several intermediary molecules and signaling pathways. Both mechanisms may act independently, in parallel, or may influence one another [3]. Arrestins are a family of intracellular proteins that include visual arrestins (Arr1 and Arr4) and non-visual arrestins (β-Arr1 β-Arr2) [4]. Both β-Arr1&2 are widely expressed in most mammalian cell types, known with their regulatory role in GPCRs desensitization [5], and subsequent receptor-mediated signal termination. Liganded GPCRs are internalized through a mechanism involving arrestin-dependent binding to clathrin-coated pits [6]. Subsequent studies have introduced β-Arrs as a part of the multi-protein complex (receptorsome) involved in targeting receptor kinase complex to clathrin-coated pits and receptor internalization [7]. Moreover, β-Arrs mediate other pathways distinct from the G protein-dependent activation. These pathways are extremely diverse, derive variable cellular responses through different GPCRs, or independent of G protein activation [8]. These events determine life-or-death decisions in the cell. The role of β-Arrs in cancer invasion and metastasis is reported through various signaling pathways including
mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK), Wnt/β-catenin, and phosphoinositide-3 kinase (PI3K)/Akt [9]. Other reports have proposed the direct implication of β-Arrs in diverse types of cancer phenotypes and their participation in different stages of oncogenesis including initiation, promotion and progression [10]. Breast cancer is the leading cause of cancer related mortality among females, where the triple negative breast cancer (TNBC) constitutes about 20% of cases worldwide [11]. There are accumulating evidences indicating the implication of β-Arr in breast cancer phenotype and metastasis. In triple negative breast cancer cells (MDA MB-231), β-Arrs contributed in PAR-2 mediated migration via ERK1/2 MAP kinase activation [12] and involved in cellular proliferation and migration via AMPK activation [13]. Also, they contributed in GPR161-mediated proliferation and cell migration via IQGAP1 dependent mechanism [14] and associated with resistance through regulating the expression of multidrug resistance gene (MDR1) [15]. These findings and others have nominated β-Arrs as a potential therapeutic target against breast cancer and other cancers. The consequences of blocking the interaction between β-Arrs and the adaptor protein (AP2) of clathrin are not adequately investigated. Thus, this work was designed to explore the autophagic and apoptosis effects of the inhibition of β-Arr/AP2 interaction in hormonally stimulated breast cancer cells.

**Materials And Methods**

**Key Reagents:**

Barbadin (3-amino-5-(4-benzylphenyl)-3H,4H-thieno[2,3-d]pyrimidin-4-one) (Cat No. B118250), Arginine Vasopressin acetic acid salt (AVP) (Cat No. V991535) and Wortmannin (Cat No. W499400), were purchased from Toronto Research Chemicals, Toronto, Ontario, Canada). Trichostatin A (TSA) and dimethyl sulfoxide (DMSO) were from Sigma Chemicals, USA. Earle's balanced salt solution (EBSS) and other cell culture reagents (DMEM 4.5 g/L glucose with L glutamine, penicillin/streptomycin, fetal bovine serum (FBS) and Trypsin/EDTA) were from Lonza Pharma & Biotech.

**Cell culture and treatment**

MDA MB-231 cells [16] was generously provided by Department of Cancer Biology, NCI, Cairo University. Cells were cultured in Dulbecco's modified Eagle's Minimal Essential Medium (DMEM) supplemented with 10% heat inactivated FBS, 1% Penicillin/Streptomycin in a humidified atmosphere of 95% air and 5% CO$_2$ at 37 °C. Initially, cells were seeded with a low cell density then subcultured with particular densities in 100 mm, 6 wells, or 96 wells plates according to the experimental settings.

**Cell treatments**

Autophagy was induced by glucose oxygen deprivation, where cells were starved for 4 hours in EBSS, which does not contain nutrients nor growth factors, at 37 °C. To inhibit autophagy, 100 nM of the PI3K/mTOR inhibitor (Wortmannin) was included in the culture media for 24 h. Blocking the interaction between β-Arr1 and AP2 adaptin protein, cells were treated with 100 µM Barbadin for 30 min then
stimulated with AVP for extra 45 min. Inhibition of histoneacetylases (HDACs) was performed by incubating cells in media containing 0.25 µM TSA dissolved in DMSO for 24 h.

**Autophagy and Apoptosis assessments**

LC3 II was determined by fluorescent antibody labeling of the microtubule associated protein using Rabbit anti-Homo sapiens (Human) MAP1LC3B Polyclonal antibody (MAP1LC3B Antibody, FITC conjugated), (CUSABIO, USA). Apoptosis assay was performed using Annexin V-FITC kit (Miltenyi Biotech, Auburn, CA, USA) following the manufacturer's instructions. Briefly, subconfluent treated cells were detached from the plates and centrifuged at 1000 rpm for 5 min. Cell pellet was resuspended in 1 ml PBS and then incubated with 0.25 µg/ml Annxin V in 1X binding buffer for 15 min, followed by two washes with Wash Buffer. Cells were resuspended again in binding buffer containing 0.5 µg/ml Propidium Iodide (PI) then subjected to flow cytometer (BC Novus). The data was recovered and analyzed by Kaluza software.

**Cell cycle analysis**

Cells were treated as previously outlined. After treatment periods adherent cells were collected and washed twice with PBS. Cells were permeabilized with 70% ethanol with PBS (v/v) and stored at 4 °C for at least 2 h. After incubation, cells were washed with PBS and stained with a solution of PBS containing PI (50 µg/ml), Triton X-100 and RNase A for 30 min at room temperature in a dark place. Cells suspension was filtered then analyzed on Accuri C6 flow cytometer (Becton Dickinson, Sunnyvale, CA, USA).

Expression of P62 and β-arrestin 1

The expression of genes (β-Arr1 and P62) was assessed at mRNA levels using Qiagen Rotor-Gene Q PCR Cycler 5 Plex. Total RNA was recovered using GeneJET RNA purification kit, (ThermoFisher Scientific, USA) and 200 ng was used for cDNA synthesis using SensiFAST™ cDNA Synthesis Kit (Bioline Inc, USA) following the manufacturer's protocol. For quantitation, 50 ng/µl (2 µl) of cDNA was used as a template in cycling reactions (20 µl) containing 50 nmol/µl (2 µl) of gene-specific primers (Table 1). The reaction mix included 10 QIAGEN SYBR green, raised to 20 µl with water then subjected to a thermal cycling program consisted of a single step of denaturation followed by 45 cycles (each consisted of single denaturation at 94 °C for 5 sec, annealing at 62 °C, 55 and 62 and 65 °C (for P62, and β-Arr 1, and GAPDH, respectively) and an extension step at 72 °C for 20 sec. Reactions were terminated with a single step at 99 °C to produce a melt curve. In parallel, the expression of GAPDH was used as an internal control to determine the fold expression changes of the targeted genes. The critical threshold (Ct) of target genes was normalized with quantities (Ct) of GAPDH by using the $2^{-\Delta\Delta Ct}$. 
**Table 1**  
Sequence of primers used in the expression analysis of epigenetic and drug metabolism related genes.

| Primer Sequence | Primer Sequence | Primer Sequence | Primer Sequence |
|-----------------|-----------------|-----------------|-----------------|
| **Beclin1**     | For: 5’-AGCTGCCGTTATACTGTTCG-3’  | Rev: 5’ACTGCCTCCTGTGCTTTCAATCTT3’ |                 |
| **B arrestin1** | For: 5’TTCGCAAGGACCTGTGTTG-3’, | Rev: 5’-GGAGGGATCTCAAAGGTGAAG - 3’ |                 |
| **P62**         | For: ACAGATGCCAGAATCCGAAG       | Rev: ATCTGGGAGAGGAAGTTCAAT        |                 |
| **GAPDH**       | For: GGCGCTGAGTACGCTGAGGT       | Rev: AGTTGGTGGTCAGGAGGCATT        |                 |
|                 | For: Forward (sense)            | Rev: Reverse (antisense)          |                 |

**Statistical data analysis**

SPSS13.0 software package was used to analyze the data. All cell culture work was performed in triplicates and the average values were determined. Apoptosis was measured and displayed in histograms and as a percent of the control and represented as the mean of 3 runs ± SD (standard deviation). P values less than 0.5 were considered to indicate significant differences. ClustVis was used to cluster the effect TSA versus Barbadin and Wortmannin.

**Results**

We first determined the level of the autophagy marker when cells were transiently starved by the autophagy inducing media (EBSS), treated with the autophagy inhibitor (Wort) or both. Autophagy was assessed by fluorescent antibody labeling of the microtubule associated protein, LC3 II, and compared to its basal level of the untreated cells, grown normal media. Also, the mRNA expression of P62 was determined. Then we compared these conditions with the corresponding effect observed in agonist (AVP) activated cells treated with Barbadin as β-Arr inhibitor. Cells starved in EBSS for 4 h demonstrated higher expression of LC3II protein (66.7 ± 2.2) compared to the basal level (30.03 ± 0.95) seen in cells grown in normal nutrients and growth factors (P < 0.001). The PI3K inhibitor (Wort), in contrast, significantly reduced LC3II protein to 13.2 ± 0.55 (P < 0.05). In cells transiently grown in EBSS followed by Wort, the LC3II was 54.2 ± 2.61 indicating the irreversible effect of starvation induced autophagy even when PI3K was inhibited (Fig. 1). To inhibit the interaction between β-Arr1 with the adaptor protein AP2, Barbadin was used in treatment of agonists (AVP) stimulated cells, where it increased LC3II to 51.27 ± 0.11 compared to Barbadin untreated and AVP unsensitised cells (P < 0.001). Barbadin/AVP treatment,
however, did not induce additive autophagic effect in pre-starved cells (LC3II: 55.8 ± 2.25, P > 0.05). Moreover, no synergistic autophagic effect was observed in cells treated with both Barbadin and Wort. (LC3II: 49.7 ± 1.3) (Fig. 1).

We next examined whether these autophagic effects were associated with apoptosis. Cells which were starved with EBSS, treated with Wort or Barbadin/AVP exhibited morphological characteristics of apoptosis including shrinkage, rounding and detachment (Fig. 2A). The percent of cell viability was reduced when cells grown in EBSS, cells treated with Wort, Barbadin/AVP or both (Wort + Barbadin/AVP) to 71.3%, 75.4%, 69.1% and 67.1%, respectively. Meanwhile, apoptosis was developed in 28%, 22 %, 29.9% and 32.7%, of cells respectively (Fig. 2B&C). As in apoptotic cells, the low molecular weight internucleosomal DNA fragments are extracted during cell staining in aqueous solution, so apoptotic cells are identified on DNA content frequency histograms as sub-G1 fraction as a hallmark of apoptosis. As Fig. 3 shows, the sub-G1 fractions developed in cells starved with EBSS, treated with Wort or Barbadin/AVP were 39.5%, 27.8 % and 31.5%, respectively. Also, the percent of cells arrested in G0/G1 phase was 54.2%, 77.6% and 79.4%, respectively. All the changes observed in sub-G1 and arrested cells were significant compared to the normally grown untreated cells.

The mRNA levels of some relevant genes were investigated using reverse transcription PCR. Downregulation was the hallmark of β-Arr1 and none of the drugs were able to modulate its expression profile. P62, in contrast, which is used as autophagy marker at its protein level, demonstrated varying mRNA expression levels, where it was upregulated (about 2 fold increase) in cells grown in EBSS, cells treated with Wort or in agonist activated cells treated with Barbadin. Also, combined treatment with Wort and Barb/AVP exerted a synergistic effect in P62 regulation and Beclin 1 (Fig. 4). As histone deacetylase 6 (HDAC6) is involved in β-Arr mediated tubulin acetylation/deacetylation that affects cell migration, we thought to monitor the corresponding autophagic/apoptotic potential of HDAC inhibition compared to that of Barbadin. Cells treated with TSA (HDACs inhibitor) showed apoptotic cell morphological appearance, developed autophagy (LC3 II: 48.2 ± 1.5), apoptosis (27.8% of cells) and arrested 63.7% of cells in G0/G1. Moreover, TSA did not affect the expression of β-Arr1 but rather downregulated P62 compared to Barbadin (Fig. 5).

Discussion

In this work, Barbadin was used to functionally hinder the interaction between β-Arr1 and β2-adaptin subunit of the clathrin adaptin protein-2 (AP2). This does not interfere with the formation of the membrane receptor/β-Arr complex [17]. The hormonal agonist (AVP) we used, is known to ligand with V2R receptor and stimulates ERK1/2 pathway (independently of heterotrimeric G protein signaling). Also, ERK1/2 stimulation involves β-Arr [18]. Thus, it was anticipated that treatment of cells with Barbadin may block V2R-stimulated ERK1/2 activation and prohibits the intracellular accumulation of cAMP and downstream signaling. MDA MB-231 cells are highly metastatic TNBC cells [19], expresses V2R receptor [20] and non-visual arrestins (β-arr 1&2). We have employed this cellular, in vitro, model to reveal the direct
impact of β-Arr inhibition on cellular events including autophagy, apoptosis and cell cycle progression and the subsequent anticancer potential of Barbadin.

Growing cells in starvation conditions (in EBSS) is well established protocol to induce autophagy [21]. Limited starvation (4 h) was sufficient to induce autophagy, as indicated by 2-fold increase in the microtubule-associated protein light chain 3 (LC3II) compared to its basal level in cells grown in nutrients rich condition. Starvation-induced autophagy was associated with apoptosis in 28% of cells dually stained with AV/PI. The displacement of Bcl-2 from Beclin-1 and Bax, may be the driving force that triggered both autophagy and apoptosis [22]. In similar work, starved MDA MB-231 cells developed autophagy through the AMBRA1/mTOR pathway leading to an increase of LC3II, increase of autophagosomes but decrease of p62 protein [23]. The PI3K inhibitor (Wort) is commonly used as an autophagy inhibitor, based on its inhibitory effect on class III PI3K activity, which is known to be essential for induction of autophagy. The LC3II in Wort treated cells was significantly reduced. Unexpectedly, both cytotoxic mechanisms were associated with mild increase in the expression of P62 mRNA. Although P62 protein was not assessed in this work, the relative overexpression of its coding gene may help to replenish selective degradation of P62 protein during the initial stages of autophagy [24]. This may occur during the first few hours of starvation and before the turnover of P62 protein. In this process, P62 protein acts as an intracellular receptor for ubiquitinated proteins, where the P62-ubiquitinated complex is merged with LC3II, and the new complex is sequestered in the autophagosomes [25]. Also, the increased P62 mRNA, we observed, may be attributed to the increased response to the proteasome inhibitor (PSI) or prostate-derived Ets factor (PDEF) [26].

An altered scenario was observed when cells were treated with Wort, where autophagy was inhibited, as indicated by the significant decrease of LC3 II compared to its basal level. Additionally, Wort developed apoptosis in 22% of cells. Wort-mediated autophagy inhibition may occur through inhibiting the conversion of PIP2 into PIP3 and phosphorylation of AKT leading to the inhibition of PI3k/AKT pathway [27]. Cells transiently exposed to harsh conditions followed by Wort have maintained their high LC3II protein and developed more apoptosis (31% of cells) indicating the irreversibility of starvation-induced autophagy. In similar studies, concomitant exposure of mouse embryonic fibroblasts (MEF) cells to Wort or 3-methyladenine during starvation, led to the suppression of starvation-induced autophagy [28]. Cell cycle analysis confirmed the association between autophagy and apoptosis, where about 39% of cells were trapped in Sub-G1 phase, and more cells (54%) were arrested in G0/G1 phase, the observation previously reported in both TNBC (MDA MB-231) [27] and the less aggressive luminal A cells (MCF-7) [29].

Nonvisual β-Arrs, on the other hand, play a key role in mediating cellular cytoprotective events through the activation of GPCRs [30]. They participate in cancer invasion and metastasis [9]. Herein, Barbadin mediated β-Arr inhibition, in cells grown in normal condition, was associated with autophagy and developed apoptosis as similar as starved cells (EBSS-incubation). The inhibition of β-Arr/AP2 interaction may provide more chance for β-Arr to act as a caspase substrate, where its cleaved fragments participate in the core mechanism of apoptosis and assist other product of caspase activity in releasing the mitochondrial Cytochrommone [31]. Also, Barbadin autophagic/apoptotic effect was associated in G0/G1
arrest. These observations are supported by some previous reports in which β-Arr1 depletion, markedly induced neuronal apoptosis/necrosis in vivo and in vitro [32]. Also, siRNA-mediated silencing of β-Arr1 &2 reduced ERK1/2 activation and MDA MB-231 cells metastasis [12]. In addition, β-Arr2-associated type III transforming growth factor-β receptor negatively mediated the migration and invasion of MDA-MB-231 breast cancer cells via NF-κB signaling [33]. Our observations indicated that Barbadin did not exert additional autophagic effect after cells were desensitized with EBSS, most probably due to the internalization of their GPCRs. This was evidenced by the insignificant differences in LC3II protein, P62 mRNA and apoptosis in Barbadin treated cells with or without starvation. Moreover, the autophagic role of Barbadin did not involve PI3K signaling, where cells cotreated with Barbadin and Wort did not show significant changes in the magnitude of autophagy. More importantly, the data predicts the dependence of Barbadin-induced apoptosis on autophagy, where cotreatment of cells with Wort and Barbadin resulted in higher proportion of cells in Sub-G1 peak (31.9%) and higher percent of apoptotic cells (32.7%) (Fig 2) The intervening role of P62 seems to be cell type and/or autophagy phase dependent. P62, it is multifunction and involved in other pathways including the UPS [34]. The increased synthesis of P62 could be triggered through the effect of some transcription factors, such as Nrf2, where P62 is phosphorylated to participate in autophagy flux [35]. This hypothesis is supported by the decreased expression of P62 in Wort treated cells. Other expression data revealed low expression of β-Arr1 mRNA is consistent with clinical studies in which β-Arr 1 was inversely correlated with the histological grade of breast cancer and positively associated with TNBC patient survival, suggestive of a tumor-suppressive function of β-Arrs1 in breast cancer patients [13]. Silencing of β-Arr1 increased the migration potential of MDA MB-468 and MDA MB-231 cells [36]. The autophagy/apoptosis effect of Barbadin is exerted through the hindrance of the covalent interaction between β-Arr and AP2 β-adaptin subunit, where Barbadin is superimposed with the β-Arr1 C-terminus peptide where Phe-388, Phe-391 and Arg-395 are the three key residues for β-Arr binding [17]. This may exclude possible direct effect of Barbadin on β-Arr1 expression.

In parallel, histone deacetylases (HDACs) 1, 6 and 8 are deeply involved in invasion of breast cancer [37]. HDAC6, in particular, plays a critical role in the ubiquitinated aggregate formation and autophagosome–lysosome fusion. Although this role is mediated by P62, other studies revealed the involvement of HDAC6 in β-Arr mediated tubulin acetylation/deacetylation that affects cell migration. Thus, we thought to monitor the autophagic/apoptotic potential of HDAC inhibition compared that induced by β-Arr inhibitor. Treatment of cells with HDACI (TSA), resulted in regression of cells viability, induction of autophagy, massive necrosis and cell cycle arrest in G0/G1 phase. These observations are largely consistent with previous reports that nominated TSA as anticancer drug through the induction of both autophagy [38] and apoptosis [39]. These findings, however, predict that TSA may adopt different anticancer mechanism as indicated by its necrotic effect and the downregulation of P62 mRNA. Correlation based clustering revealed that TSA effects did not tightly cluster with Barb/AVP or Wort effects (Fig 5). This added to its well reported epigenetic mechanism in acetylating genes and transcription factors.

Conclusion
Although the mechanisms underlying these events need further exploration, the outcomes revealed the potential and beneficial therapeutic role of β-Arr inhibition in breast cancer management. The study suggests autophagic and apoptotic effects of β-arrestin inhibition by Barbadin in a dependent manner and the association of both mechanisms with cell cycle arrest in G0/G1 phase. This approach promotes selective targeting the intracellular endocytic proteins as therapeutic targets in advanced stages of breast cancer.

**Declarations**

**Disclosure statement**

The authors report no conflicts of interest.

**Authors’ contributions:**

Conceptualization: MH and TD; Methodology and investigations: MA, MK, TD and MH; Editing the original draft and revision: MH, MK and TD; Project administration, MH; Funding acquisition, MH, TD, MK.

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Figures
β-arrestin inhibition induced apoptosis in MDA MB-231 cells in association with autophagy. Phase-contrast photomicrographs taken after starving cells for 4 h, treatment with the autophagy inhibitor (Wort), or β-arrestin inhibitor (Barbadin/AVP) revealed apoptotic morphological changes including cell shrinkage, rounding and detachment. magnification ×400 (A). Annexin V-FITC and PI staining was used to determine the percentage of viable, dead, early and late apoptotic fractions by flow cytometry (B). Cell
viability and apoptosis was assessed in following starving cells in EBSS, treatment with Wort, EBSS+Wort, Barbadin/AVP, EBSS+Barbadin/AVP or Barbadin/AVP+Wort. Cell viability significantly decreased in cells incubated in EBSS or treated with Wort, EBSS+Wort, or Barbadin/AVP. Also, treatments resulted in variable degrees of apoptosis. Bar represent the mean (±SD) of 3 independent experiments (C). (*): significant difference between total apoptosis of the corresponding data versus DMSO treated cells.

Figure 5
Comparison the effect of HDACs inhibition versus inhibiting the interaction between β-arr/AP2 adaptin protein on cells viability, apoptosis (Sub G1 fraction), autophagy-related proteins (LC3II), and the expression levels of β-Arr1 and P62 mRNA in MDA MB-231 cells. Cells were cultured in presence TSA for 24 h, collected and then used to assess the level of LCII and cell cycle by flow cytometry as explained in Materials and Methods. Also, the mRNA was isolated, reverse transcribed and the levels of β-arr1 and the autophagy marker P62 were determined by reverse transcription PCR. TSA acts as similar as Barbadin in inducing apoptosis cell morphology (panel A), autophagy and cell cycle arrest in G0/G1 phase. TSA, however, downregulated the expression of P62 mRNA (panel B). Panel C represents heatmap of the correlation-based clustering the individual or the combined effect of Barb/AVP, Wort and TSA. Both rows and columns are clustered using correlation distance and average linkage. TSA did not tightly cluster with Barb/AVP or Wort effect indicting its varying anticancer mechanism.