



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 LC3II 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 - β Arr/AP2 inhibition in triple-negative breast cancer cells. These observations nominate β -Arrestins as selective targets in breast cancer treatment.

Keywords Autophagy · Apoptosis · B-Arrestin · Breast cancer · Barbadin · Wortmannin

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 and 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 -Arrestins 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 -Arrestins mediate other pathways distinct from the G protein-dependent

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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 -Arrestin 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 -Arrestin 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 -Arrestin in breast cancer phenotype and metastasis. In triple-negative breast cancer cells (MDA MB-231), β -Arrestin contributed in PAR-2 mediated migration via ERK1/2 MAP kinase activation [12] and involved in cellular proliferation and migration via AMPK activation [7]. Also, they contributed in GPR161-mediated proliferation and cell migration via IQGAP1-dependent mechanism [13] and associated with resistance through regulating the expression of multidrug resistance gene (MDR1) [14]. These findings and others have nominated β -Arrestin as a potential therapeutic target against breast cancer and other cancers. The consequences of blocking the interaction between β -Arrestin 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 -Arrestin/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 [15] were 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% CO₂ at 37 °C. Initially, cells were seeded with a low cell density then subcultured with particular densities in 100 mm, 6-well, or 96-well plates according to the experimental settings.

Cell treatments

Autophagy was induced by glucose oxygen deprivation, where cells were starved for 4 h 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 -Arrestin1 and AP2 adaptor protein, cells were treated with 100 μ M Barbadin for 30 min then stimulated with AVP for extra 45 min. Inhibition of histone deacetylases (HDACs) was performed by incubating cells in media containing 0.25 μ M TSA dissolved in DMSO for 24 h.

Autophagy and apoptosis assessments

LC3II 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 Biotec, 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 μ g/ml Annexin-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 μ g/ml Propidium Iodide (PI) then subjected to flow cytometer (BC Novus). The data were 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 μ g/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 by RT PCR

Real-time PCR was used to evaluate the expression of genes (β -Arr1 and P62) was assessed at mRNA levels using Qiagen Rotor-Gene Q PCR Cyclor 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 real-time PCR quantitation, 50 ng/ μ l (2 μ l) of cDNA was used as a template in thermal cycling reactions (20 μ l) containing 50 nmol/ μ l (2 μ l) of gene-specific primers (Table 1). The ready-to-use master mix included the fluorescent dye QIAGEN SYBR green 1 and HotStar Taq DNA polymerase raised to 20 μ l 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 s, annealing at 62 °C, 55 °C and 62 °C and 65 °C (for P62, and β -Arr 1, and GAPDH, respectively) and an extension step at 72 °C for 20 s. Reactions were terminated with a single step at 99 °C to produce a melt curve that represents the change in fluorescence observed when dsDNA with incorporated dye molecules dissociates, or “melts” into single-stranded DNA ssDNA as the temperature of the reaction is raised. In parallel, the expression of GAPDH gene 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 using the $2^{-\Delta\Delta Ct}$.

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 \pm 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, LC3II, 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 unsensitized 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 and 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

Table 1 Sequence of primers used in the expression analysis of epigenetic and drug metabolism-related genes

Primer	Sequence
Beclin1	For: 5'-AGCTGCCGTTATACTGTTCTG-3' Rev: 5'ACTGCCTCCTGTGTCTTCAATCTT-3'
B-arrestin1	For: 5'TTCGCAAGGACCTGTTTGT-3', Rev: 5'-GGAGGGATCTCAAAGGTGAAAG-3'
P62	For: 5'-ACAGATGCCAGAATCCGAAG-3' Rev: 5'-ATCTGGGAGAGGGACTCAAT-3'
GAPDH	For: 5'-GGCGCTGAGTACGTCGTGGAGT-3' Rev: 5'-AGTTGGTGGTGCAGGAGGCATT-3'

For Forward (sense), Rev Reverse (antisense)

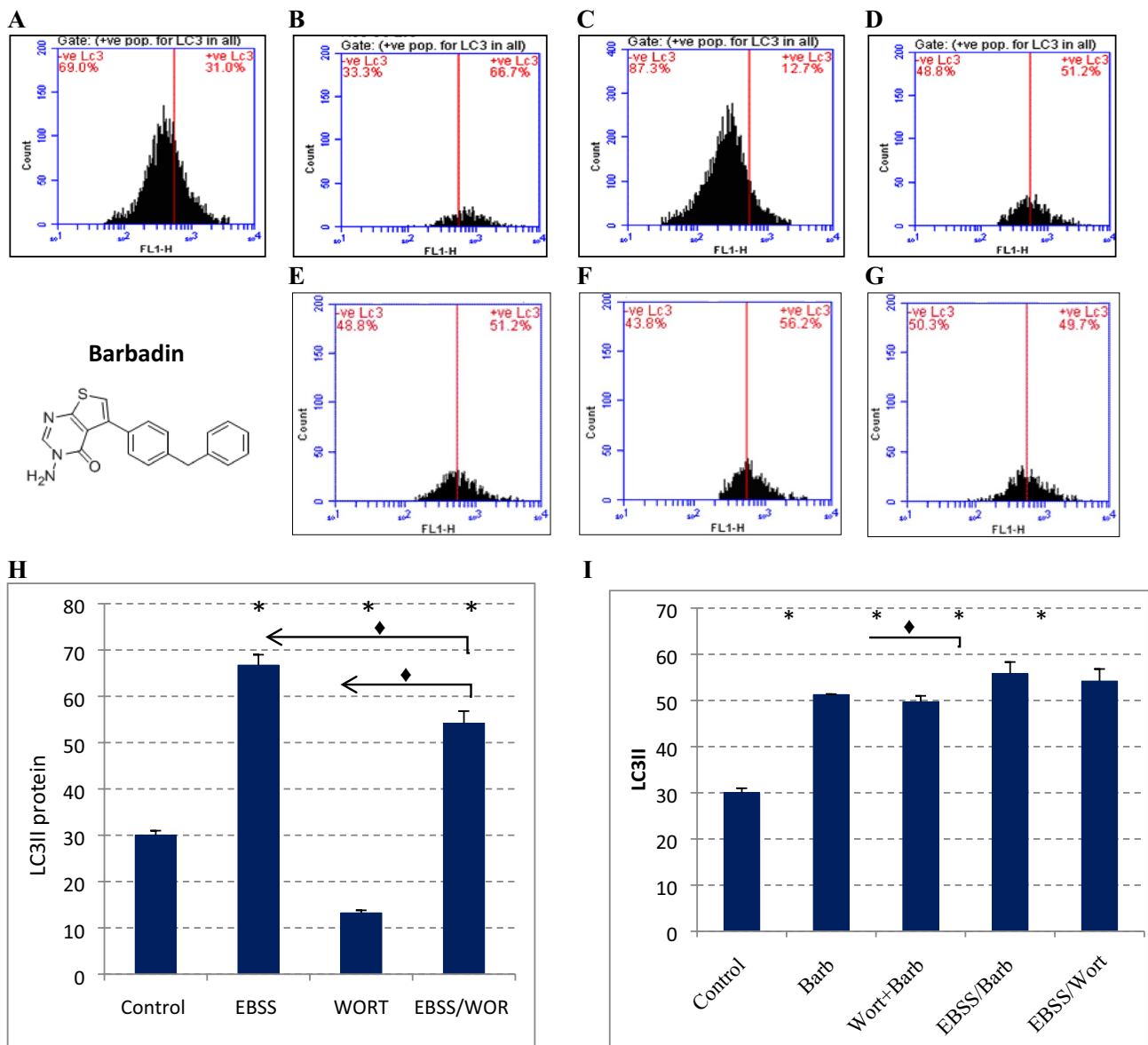


Fig. 1 β -arrestin inhibition induces PI3K-independent autophagy in triple-negative breast cancer cells. MDA-MB-231 were seeded with an initial cell density 4×10^4 grown in nutrient-rich conditions and left untreated (a), or starved in EBSS (b), treated with Wort (c), starved for 4 h and then treated with Wort (d), sensitized with AVP then treated with Barbadin (e), sensitized with AVP then treated with Barbadin and Wort (f), or pre-starved with EBSS, sensitized with AVP then treated with Barbadin (g). After treatment periods, cells were collected and the expression of autophagy protein LC3II was determined by flow cytometry. The autophagy was significantly induced

by EBSS and inhibited by Wort. Cells pre-starved with EBSS, flowed by Wort treatment, mildly reduced the expression of LC3II but did not restore its basal level. Also, inhibition of the interaction between β -Arr and AP2-stimulated autophagy, independently of PI3K inhibition (in presence or absence of Wort). Also, Pre-starving cells did not affect the autophagic effect of β -arrestin inhibition. Data are presented as mean \pm SD (h & i). (*): refers to significant ($P < 0.001$) difference compared to the untreated cells and (\blacklozenge) refers to significant ($P < 0.01$) difference between the indicated cells

and arrested cells were significant compared to the normally grown untreated cells.

The mRNA levels of three relevant genes were investigated using reverse transcription real-time PCR. Down-regulation was the hallmark of β -Arr1 and none of the drugs used were able to modulate its expression level.

P62, in contrast, which is used as autophagy marker at its protein level, demonstrated varying mRNA expression levels, where it was upregulated (about twofold increase) in cells grown in EBSS, cells treated with Wort or in agonist-activated and treated with Barbadin. Also, combined treatment with Wort and Barb/AVP exerted a synergistic

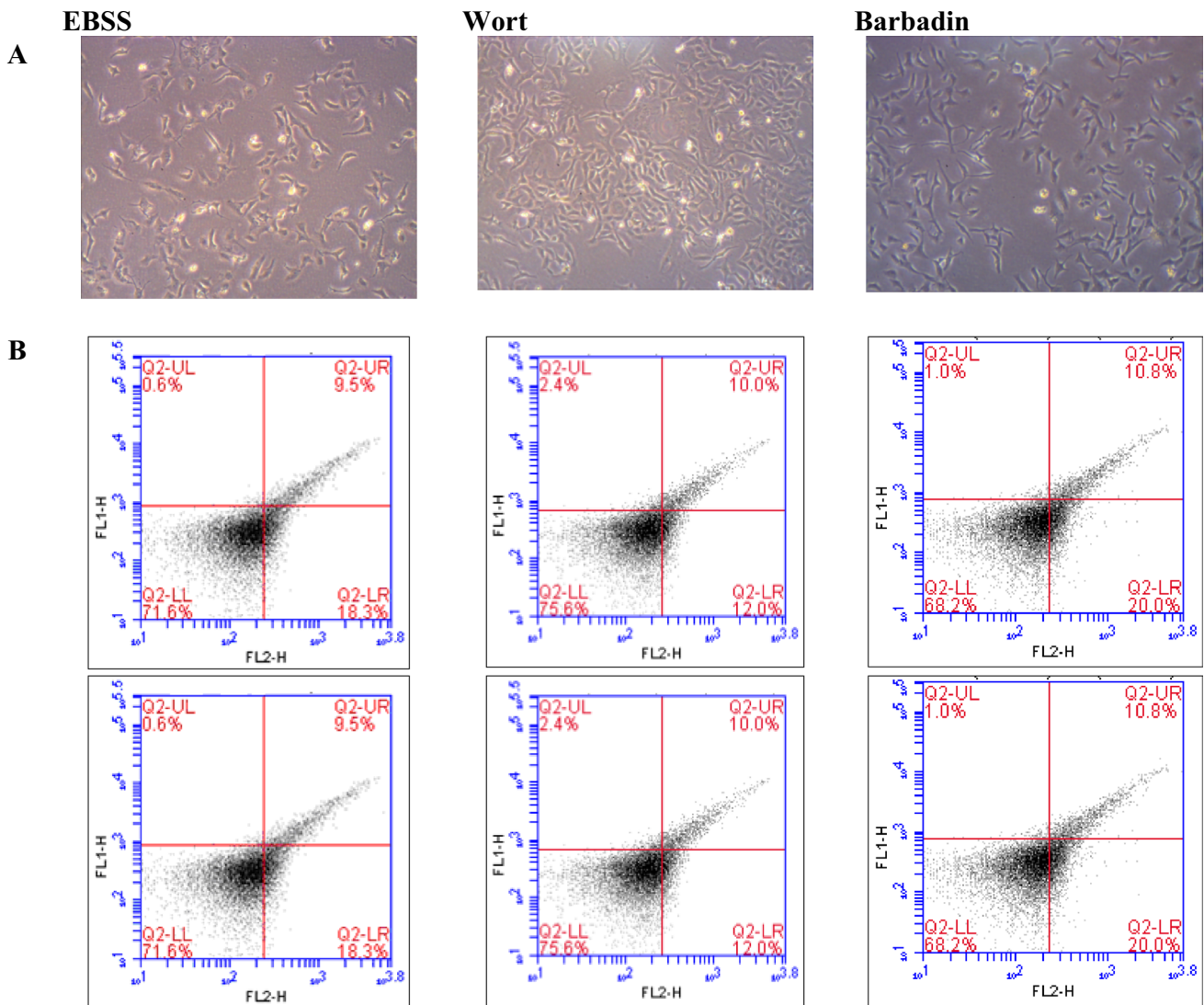


Fig. 2 β -arrestin inhibition induced apoptosis in MDA MB-231 cells in association with autophagy. Phase-contrast photomicrographs taken after starving cells for 4 h, treatment with the autophagy inhibitor (Wort), or β -arrestin inhibitor (Barbadin/AVP) revealed apoptotic morphological changes including cell shrinkage, rounding and detachment. magnification $\times 400$ (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 apop-

tosis 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 (\pm SD) of 3 independent experiments (c). (*): significant difference between total apoptosis of the corresponding data versus DMSO-treated cells

effect on P62 and Beclin1 (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 (LC3II: 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 β 2-adaptin subunit of the clathrin adaptin protein-2 (AP2). This does not interfere with the formation of the membrane receptor/ β -Arr complex [16]. 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 [17]. Thus, it was anticipated that treatment of cells with Barbadin may block

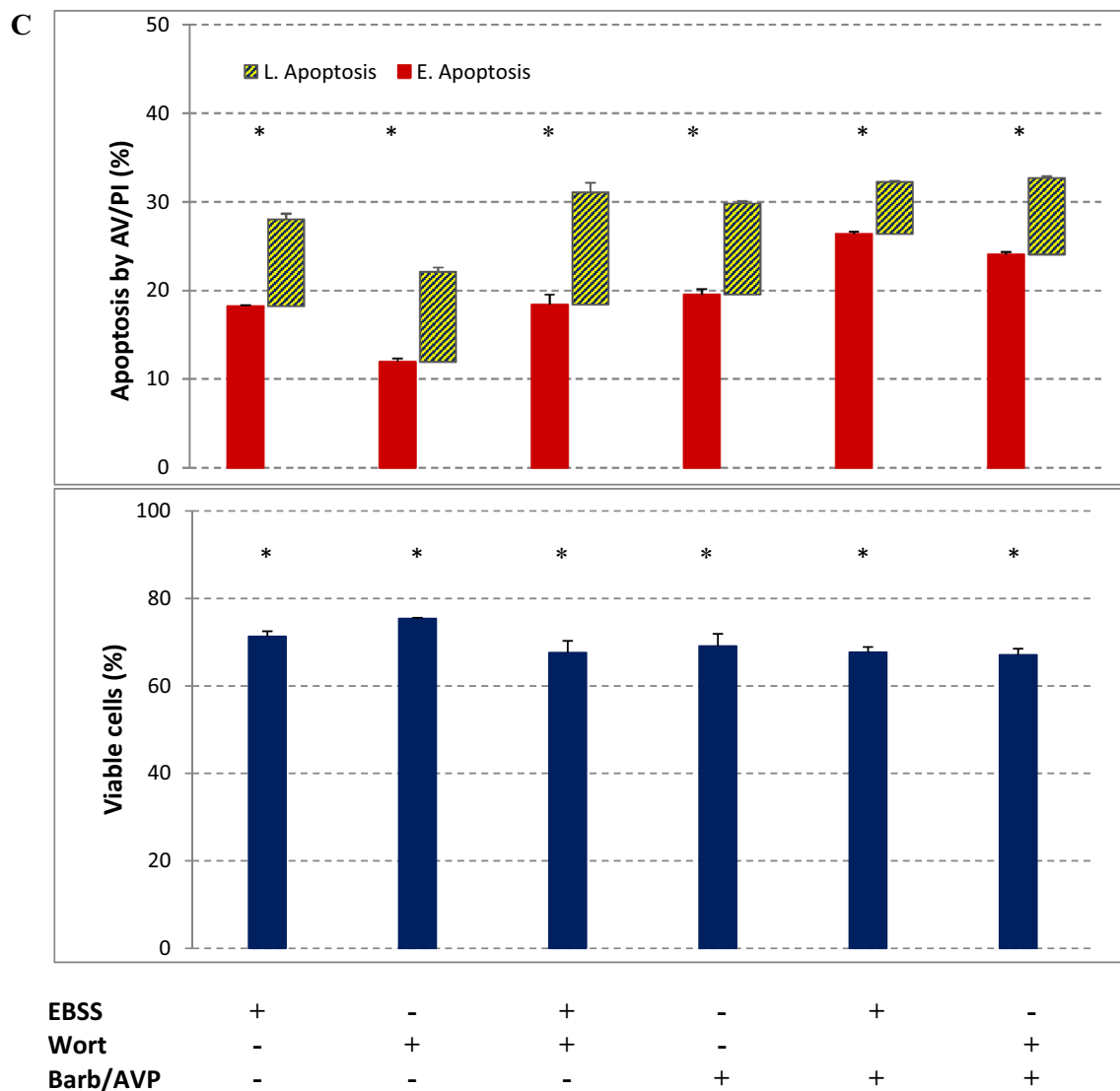


Fig. 2 (continued)

V2R-stimulated ERK1/2 activation and prohibits the intracellular accumulation of cAMP and downstream signaling. MDA MB-231 cells are highly metastatic TNBC cells [18], expresses V2R receptor [19] 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 [20]. Limited starvation (4 h) was sufficient to induce autophagy, as indicated by twofold increase in the microtubule-associated protein light chain 3 (LC3II) compared to its basal level in cells grown in nutrient-rich condition. Starvation-induced autophagy was associated with apoptosis in 28% of cells dually stained with AV/PI. The displacement of Bcl-2 from

Beclin1 and Bax, may be the driving force that triggered both autophagy and apoptosis [21]. 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 [22]. 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 [23]. This may occur during the first few hours of starvation and before the turnover of P62

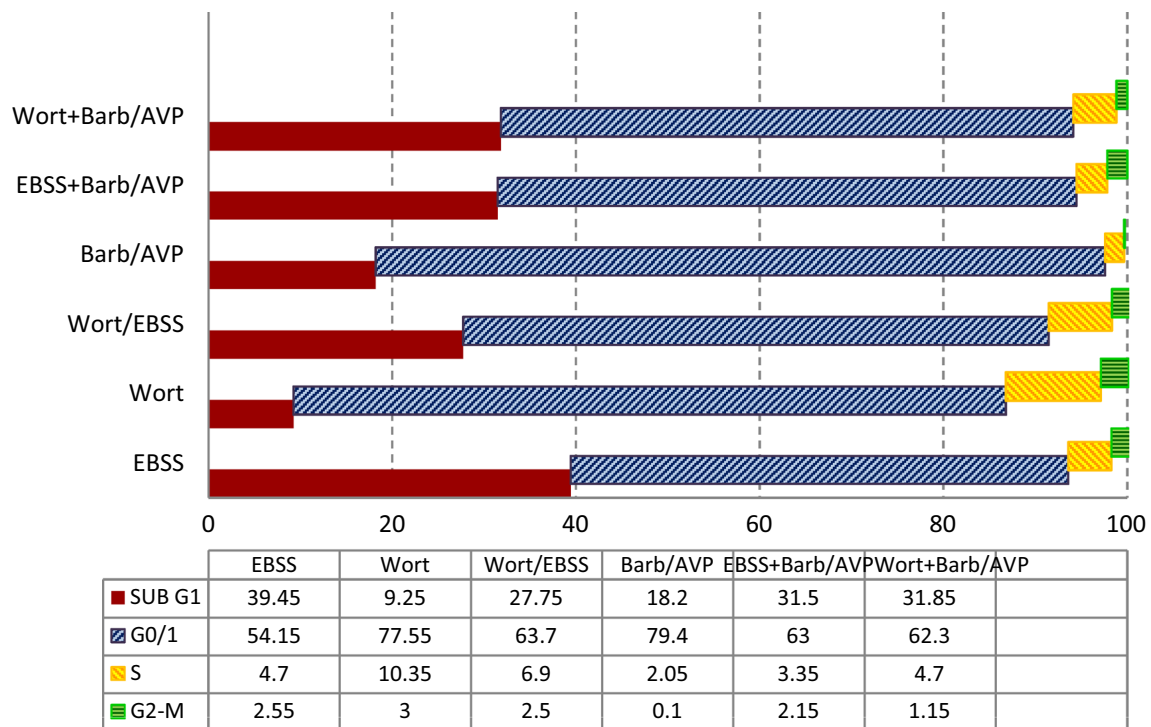


Fig. 3 β -arrestin and autophagy inhibition, independently arrested breast cancer cells in G0/1 phase. MDA MB-231 cells were incubated in EBSS, treated with agonist mediated β -Arr inhibitor (Barbadin/AVP) or the autophagy inhibitor (Wort). At the end of treatment

periods, cells were collected and stained with PI and then cell cycle distribution analysis was performed by flow cytometry. Cells were arrested in G0/G1 phase and significant apoptotic fractions of cells were trapped in the sub-G1 phase

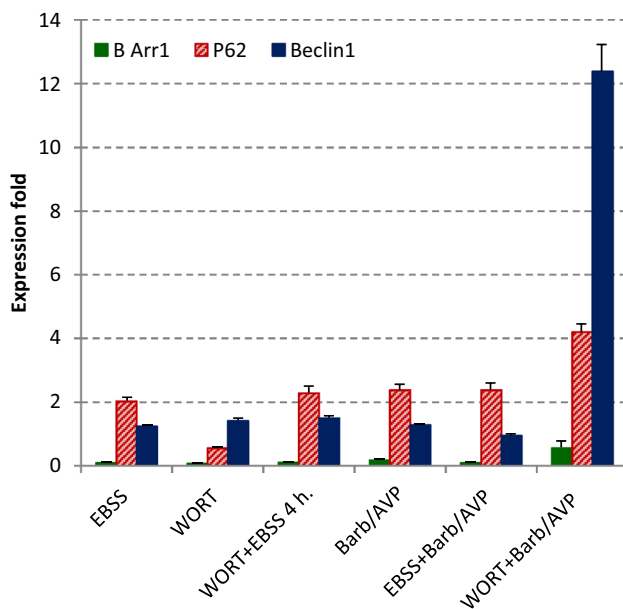
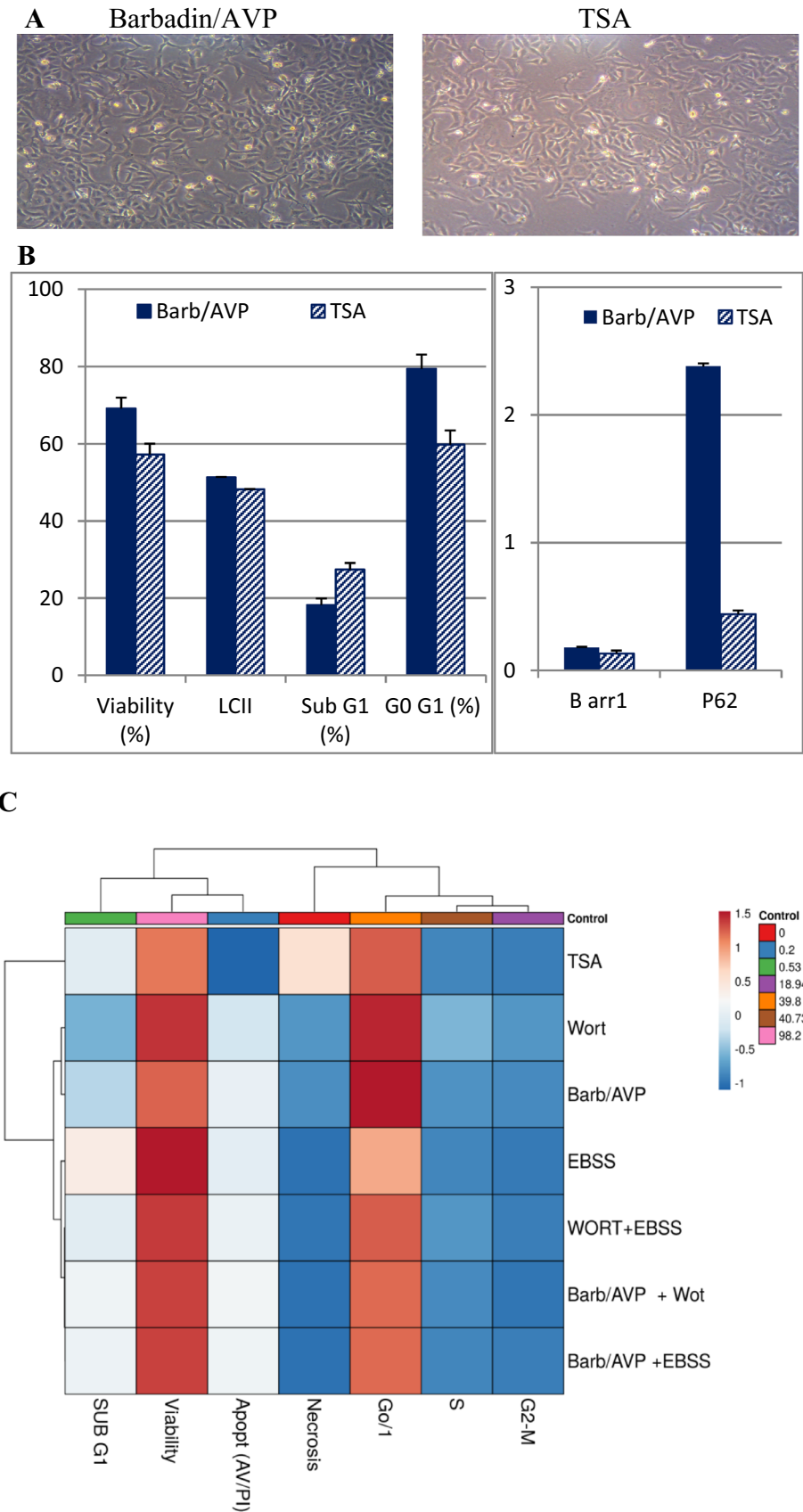


Fig. 4 Expression of β -Arr1, P62 and Beclin1 in MDA MB-231 cells. Cells were grown in starving media (EBSS), or treated with Barbadin after sensitization with AVP and/or the autophagy inhibitor Wort. After cell collection, mRNA was isolated and reverse transcribed into cDNA, which was used to quantitate the expression level of B Arr1, P62 and Beclin. β -Arr1 was downregulated, whereas P62 upregulated by Barbadin

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 [24]. 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) [25].

An altered scenario was observed when cells were treated with Wort, where autophagy was inhibited, as indicated by the significant decrease of LC3II 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 [26]. 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 [27]. 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

Fig. 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 indicating its varying anticancer mechanism



reported in both TNBC (MDA MB-231) [26] and the less aggressive luminal A cells (MCF-7) [28].

Non-visual β -Arrestins, on the other hand, play a key role in mediating cellular cytoprotective events through the activation of GPCRs [29]. 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 Cytochrome [30]. Also, Barbadin autophagic/apoptotic effect was associated in G0/G1 arrest. These observations are supported by some previous findings in which β -Arr1 depletion, markedly induced neuronal apoptosis/necrosis *in vivo* and *in vitro* [31]. 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- κ B signaling [32]. 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 effect of Barbadin did not involve PI3K signaling, where cells cotreated with Barbadin and Wort did not show significant change in the magnitude of autophagy. More importantly, the data predict 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 [33]. 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 [34]. 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 -Arrestin1 in breast cancer patients [7]. Silencing of β -Arr1 increased the migration potential of MDA MB-468 and MDA MB-231 cells [35]. The autophagy/apoptosis effect of Barbadin is

exerted through the hindrance of the covalent interaction between β -Arr and AP2 β -adapitin 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 [16]. 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 [36]. 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 [37] and apoptosis [38]. 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 more 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.

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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.

Declarations

Conflict of interest The authors report no conflicts of interest.

References

- Tsujimoto Y, Shimizu S. Another way to die: autophagic programmed cell death. *Cell Death Differ*. 2005;12(2):1528–34.
- Gewirtz DA. The four faces of autophagy: implications for cancer therapy. *Can Res*. 2014;74(3):647–51.
- Eisenberg-Lerner A, Bialik S, Simon H-U, Kimchi A. Life and death partners: apoptosis, autophagy and the cross-talk between them. *Cell Death Differ*. 2009;16(7):966–75.
- Gurevich VV, Gurevich EV. The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacol Ther*. 2006;110(3):465–502.
- Kang Y, Zhou XE, Gao X, He Y, Liu W, Ishchenko A, Barty A, White TA, Yefanov O, Han GW. Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature*. 2015;523(7562):561–7.
- Goodman OB, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL. β -Arrestin acts as a clathrin adaptor in endocytosis of the β 2-adrenergic receptor. *Nature*. 1996;383(6599):447–50.
- Luttrell L, Ferguson S, Daaka Y, Miller W, Maudsley S, Della Rocca G, Lin F-T, Kawakatsu H, Owada K, Luttrell D. β -Arrestin-dependent formation of β 2 adrenergic receptor-Src protein kinase complexes. *Science*. 1999;283(5402):655–61.
- Jean-Charles PY, Kaur S, Shenoy SK. GPCR signaling via β -arrestin-dependent mechanisms. *J Cardiovasc Pharmacol*. 2017;70(3):142.
- Song Q, Ji Q, Li Q. The role and mechanism of β -arrestins in cancer invasion and metastasis. *Int J Mol Med*. 2018;41(2):631–9.
- Shukla AK, Dwivedi-Agnihotri H. Structure and function of β -arrestins, their emerging role in breast cancer, and potential opportunities for therapeutic manipulation. *Adv Cancer Res Elsevier*. 2020;145:139–56.
- Wahba HA, El-Hadaad HA. Current approaches in treatment of triple-negative breast cancer. *Cancer Biol Med*. 2015;12(2):106.
- Ge L, Shenoy SK, Lefkowitz RJ, DeFea K. Constitutive protease-activated receptor-2-mediated migration of MDA MB-231 breast cancer cells requires both β -arrestin-1 and -2. *J Biol Chem*. 2004;279(53):55419–24.
- Feigin ME, Xue B, Hammell MC, Muthuswamy SK. G-protein-coupled receptor GPR161 is overexpressed in breast cancer and is a promoter of cell proliferation and invasion. *Proc Natl Acad Sci*. 2014;111(11):4191–6.
- Jing X, Zhang H, Hu J, Su P, Zhang W, Jia M, Cheng H, Li W, Zhou G. β -arrestin 2 is associated with multidrug resistance in breast cancer cells through regulating MDR1 gene expression. *Int J Clin Exp Pathol*. 2015;8(2):1354.
- Cailleau R, Young R, Olive M, Reeves W Jr. Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst*. 1974;53(3):661–74.
- Beautrais A, Paradis JS, Zimmerman B, Giubilaro J, Nikolajev L, Armando S, Kobayashi H, Yamani L, Namkung Y, Heydenreich FM. A new inhibitor of the β -arrestin/AP2 endocytic complex reveals interplay between GPCR internalization and signalling. *Nat Commun*. 2017;8(1):1–16.
- Charest PG, Oligny-Longpré G, Bonin H, Azzi M, Bouvier M. The V2 vasopressin receptor stimulates ERK1/2 activity independently of heterotrimeric G protein signalling. *Cell Signal*. 2007;19(1):32–41.
- Liu WJ, Ye L, Huang WF, Guo LJ, Xu ZG, Wu HL, Yang C, Liu HF. p62 links the autophagy pathway and the ubiquitin-proteasome system upon ubiquitinated protein degradation. *Cell Mol Biol Lett*. 2016;21(1):29.
- Garona J, Pifano M, Orlando UD, Pastrian MB, Iannucci NB, Ortega HH, Podesta EJ, Gomez DE, Ripoll GV, Alonso DF. The novel desmopressin analogue [V4Q5] dDAVP inhibits angiogenesis, tumour growth and metastases in vasopressin type 2 receptor-expressing breast cancer models. *Int J Oncol*. 2015;46(6):2335–45.
- Cui J, Lu K, Shi Y, Chen B, Tan S-H, Gong Z, Shen H-M. Integrated and comparative miRNA analysis of starvation-induced autophagy in mouse embryonic fibroblasts. *Gene*. 2015;571(2):194–204.
- Chiu C-F, Chin H-K, Huang W-J, Bai L-Y, Huang H-Y, Weng J-R. Induction of Apoptosis and Autophagy in Breast Cancer Cells by a Novel HDAC8 Inhibitor. *Biomolecules*. 2019;9(12):824.
- Zhu W, Qu H, Xu K, Jia B, Li H, Du Y, Liu G, Wei HJ, Zhao HY. Differences in the starvation-induced autophagy response in MDA-MB-231 and MCF-7 breast cancer cells. *Anim Cells Syst (Seoul)*. 2017;21(3):190–8.
- Ichimura Y, Komatsu M. Selective degradation of p62 by autophagy. *Paper Present Sem Immunopathol*. 2010;32(4):431–6.
- Komatsu M, Ichimura Y. Physiological significance of selective degradation of p62 by autophagy. *FEBS Lett*. 2010;584(7):1374–8.
- Thompson HGR, Harris JW, Wold BJ, Lin F, Brody JP. p62 overexpression in breast tumors and regulation by prostate-derived Ets factor in breast cancer cells. *Oncogene*. 2003;22(15):2322–33.
- Li J, Li F, Wang H, Wang X, Jiang Y, Li D. Wortmannin reduces metastasis and angiogenesis of human breast cancer cells via nuclear factor- κ B-dependent matrix metalloproteinase-9 and interleukin-8 pathways. *J Int Med Res*. 2012;40(3):867–76.
- Wu Y-T, Tan H-L, Shui G, Bauvy C, Huang Q, Wenk MR, Ong C-N, Codogno P, Shen H-M. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. *J Biol Chem*. 2010;285(14):10850–61.
- Akter R, Hossain MZ, Kleve MG, Gealt MA. Wortmannin induces MCF-7 breast cancer cell death via the apoptotic pathway, involving chromatin condensation, generation of reactive oxygen species, and membrane blebbing. *Breast Cancer Targets Therapy*. 2012;4:103.
- Ahn S, Kim J, Hara MR, Ren X-R, Lefkowitz RJ. β -Arrestin-2 mediates anti-apoptotic signaling through regulation of BAD phosphorylation. *J Biol Chem*. 2009;284(13):8855–65.
- Kook S, Gurevich VV, Gurevich EV. Arrestins in apoptosis. *Arrestins-Pharmacology and Therapeutic Potential*: Springer; 2014. p. 309–39.
- Wang P, Xu T-Y, Wei K, Guan Y-F, Wang X, Xu H, Su D-F, Pei G, Miao C-Y. ARRB1/ β -arrestin-1 mediates neuroprotection through coordination of BECN1-dependent autophagy in cerebral ischemia. *Autophagy*. 2014;10(9):1535–48.
- Seo J, Tsakem EL, Breitman M, Gurevich VV. Identification of arrestin-3-specific residues necessary for JNK3 kinase activation. *J Biol Chem*. 2011;286(32):27894–901.
- Shin WH, Park JH, Chung KC. The central regulator p62 between ubiquitin proteasome system and autophagy and its role in the mitophagy and Parkinson's disease. *BMB Rep*. 2020;53(1):56.
- Liu Y-L, Chou C-K, Kim M, Vasisht R, Kuo Y-A, Ang P, Liu C, Perillo EP, Chen Y-A, Blocher K. Assessing metastatic potential of breast cancer cells based on EGFR dynamics. *Sci Rep*. 2019;9(1):1–13.
- Lundgren K, Tobin NP, Lehn S, Stål O, Rydén L, Jirström K, Landberg G. Stromal expression of β -arrestin-1 predicts clinical outcome and tamoxifen response in breast cancer. *J Mol Diagn*. 2011;13(3):340–51.
- Park SY, Jun J, Jeong KJ, Heo HJ, Sohn JS, Lee HY, Park CG, Kang J. Histone deacetylases 1, 6 and 8 are critical for invasion in breast cancer. *Oncol Rep*. 2011;25(6):1677–81.
- Bai Y, Chen Y, Chen X, Jiang J, Wang X, Wang L, Wang J, Zhang J, Gao L. Trichostatin A activates FOXO1 and induces autophagy in osteosarcoma. *Arch Med Sci AMS*. 2019;15(1):204.

38. Alao JP, Lam EW, Ali S, Buluwela L, Bordogna W, Lockey P, Varshochi R, Stavropoulou AV, Coombes RC, Vigushin DM. Histone deacetylase inhibitor trichostatin A represses estrogen receptor α -dependent transcription and promotes proteasomal degradation of cyclin D1 in human breast carcinoma cell lines. *Clin Cancer Res.* 2004;10(23):8094–104.

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