Prestimulation of breast cancer cells with arginine vasopressin accentuates the anticancer effect induced by Dynamin 2 inhibitor

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Research Article

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Abstract

Purpose: Breast cancer cells abnormally express arginine vasopressin hormone (AVP) and its receptors. Clathrin-mediated endocytosis (CME) involves a machinery of intracellular endocytic proteins, including large Dynamins. The impact of Dynamin inhibition on the survivability and metastasis of triple-negative breast cancer cells, stimulated by AVP, was not well addressed.

Methods: To explore this, Dynamin 2 was selectively inhibited by dynasore (DYN) in MDA MB-231 cells, which was prestimulated with AVP, or cotreated with, a PI3K/AKT/mTOR inhibitor.

Results: Dynasore induced apoptosis in 19.2±1.5% of cells, whereas in cells transiently prestimulated with AVP or co-treated with Wortmannin (Wort), apoptosis has increased to 28.0±1.4% and 35.4±1.5%, respectively. This was associated with an increase in the expression of the autophagy indicators (LC3II protein and Beclin-1 mRNA), whereas, Wort reduced both markers. Moreover, 85.1%, 76.3% and 74.8% of cells were arrested in G0/G1 phase, when they were exposed to AVP, DYN or both, respectively. The Phosphorylated Akt (pAkt) decreased in DYN-treated cells in presence of AVP, Wort or both. Besides, DYN enhanced the expression of Bax and Caspase-3 genes, downregulated the multidrug resistance gene (MDR1), and reduced cell's invasion.

Conclusion: These results suggest the antineoplastic, anti-metastasis effects of Dynamin inhibition in triple negative breast cancer cells, in which V2R receptor was stimulated with AVP. Mechanistically, the drug repressed AKT/PI3K pathway, upregulated the apoptosis related genes and enhanced cells responsive to chemotherapy as similar as Wort. These events may nominate dynamin as anticancer target in breast cancer.

Introduction

Dynamins (Dyns) are small family of large (≈100 kDa) intracellular proteins with an intrinsic GTPase activity localized in their N-terminal domain. Compared to small GTPases (G proteins), they demonstrate higher GTP-hydrolysis rate associated with increasing polymerization, during dynamin-dependent endocytic pathways [1, 2]. In this process, Dynamin is recruited and assembled into oligomeric spiral around the necks of budding vesicles, where the GTPase hydrolyzing effect mediates the constriction and the subsequent vesicles fission. Also, Dynamins are involved in some clathrin-independent and dynamin-dependent mechanisms [3, 4]. Additionally, they play an integral role in the completion of cells mitosis and cytokinesis [5]. Although Dyn inhibition is useful strategy to explore their role in cellular events, particularly receptor-mediated endocytosis and mitochondria severing, some reports have evidenced their participation in the tumorigenesis and enhancing tumor invasion and metastasis. Abnormally expressed or mutated Dynamin, for example, was reported in many cancers, including acute myeloid leukemia, colon adenocarcinomas and non-small cell lung cancer (NSCLC) [6, 7, 8]. Moreover, dysregulations of dynamins, were reported in progressive prostate cancer, pancreatic tumor, hepatocellular carcinoma (HCC), glioblastomas, breast cancer, bladder cancer, cervical cancer and T-cell acute lymphoblastic

leukemia [9–14]. Similarly, the dysregulation of the dynamin 3 was implicated in HCC development [15]. These accumulating evidences may nominate Dynamins as anticancer targets, where many small-molecule inhibitors, like Dynasore (DYN), its hydroxyl analogs (Dyngo™) [16] and Dynole 34 – 2 [17, 18], were introduced as potent dynamin GTPases inhibitors. Dynasore, in particular, selectively inhibits the GTPase activity of Dyn1, Dyn2 and Drp-1[19]. Also, other studies have reported that it suppressed cell proliferation, migration and enhanced the antitumor efficacy of cisplatin in osteosarcoma via STAT3 pathway [20], reduced the metastasis of cervical cancer [12] (Lee et al., 2016) and induced apoptosis in NSCLC cells [21]. Recently, we reported that the inhibition of early endocytic events of CME, through inhibiting the interaction between β-arrestin and AP2 adaptor protein induced both apoptosis and autophagy in invasive breast cancer [22].

In another context, as vasopressin receptor (V2R) is ectopically expressed in several cancers, including breast cancer, it makes the cells responsive to the endogenously expressed arginine-vasopressin hormone (AVP), where it acts as an autocrine growth factor [23]. Accordingly, agonist activation of V2R will initiate CME and subsequent intracellular events including the recruitment, activation of Dyn-related GTPase activity. In this regard, it is not known how the inhibition of Dyn2 will modulate AVP-stimulated breast cancer cells survival, proliferation and migration. In parallel, the mechanochemical enzymatic function of Dyn2 involves phosphatidylinositol 4,5-bisphosphate (PIP2), where the interaction between PIP2 and the AP2 adaptor protein is considered a main regulatory step in CME. However, the mutual effects between Dyn inhibition and the transition of PIP2 to PIP3, during the initial steps of PI3K/Akt/mTOR pathways, is not addressed yet. Thus, this work was designed to investigate the antiproliferative effect of Dyn2 inhibition in invasive breast cancer cells prestimulated with exogenous AVP or cells in which PIP2 to PIP3 transition was selectively inhibited by wortmannin. Also, the effect of Dyn2 inhibition on apoptosis, autophagy, cell migration and the expression of apoptosis some related genes (Bax and Caspase-3), endogenous AVP, Beclin-1 and the multi-drug resistance gene (MDR1) expression will be explored at the mRNA level.

Materials And Methods

Key reagents

Dynasore (3-Hydroxy-[(3,4-dihydroxyphenyl) methylene] hydrazide 2-naphthalenecarbo-xylic acid; Cat No., D826508), Arginine Vasopressin acetic acid salt (AVP) (Cat No., V991535) and wortmannin, (Cat No. W499400) were purchased from Toronto Research Chemicals, Canada). Cell culture reagents (Dulbecco's modified Eagle's Minimal Essential Medium (DMEM) with L-glutamine, penicillin/streptomycin, fetal bovine serum (FBS) and Trypsin/EDTA) were from Lonza Pharma & Biotech, Basel, Switzerland. Total and P-Akt monoclonal antibodies were from Cell Signaling Technologies (Ma, USA).

Cell culture and treatment

MDA MB-231 cells were purchased from VACSERA, Cairo, Egypt. Cells were seeded and maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% Penicillin/Streptomycin. Cells were

incubated in 95% humidified air and 5% CO_2 at 37°C. Initially, cells were seeded with low cell density and then subcultured with particular densities in T75 tissue culture flasks, 6-well plates, or 96-well plates according to the experimental settings. Dynasore (130 μM dissolved in DMSO) was used to inhibit Dyn2. The V2R receptor was agonist-stimulated by treatment of cells with 10 nM AVP for 30 min and PI3K/Akt/mTOR signaling was inhibited by 100 nM Wortmannin for 24 h.

Viability assay

Cell metabolic activity was determined using a MTT assay. Briefly, cells were cultured at a density of 2×10^4 cells/well in 96-well plates. After overnight incubation, for cells attachment, DMEM media was replaced with fresh media containing different concentrations of DYN and incubated at 37°C in 5% CO_2 for 24 h. Cells were then labeled with 20 μl of MTT solution (5 mg/mL in PBS) per well, followed by 5 min of shaking, after which they were incubated in the dark for 4 h. The medium was then removed, dimethyl sulfoxide (DMSO) was added to dissolve the formazan, and then the absorbance was measured at 546 nM.

Apoptosis and autophagy assessments

Apoptosis assay was performed using Annexin-V FITC kit (Miltenyi Biotec, CA, USA) following the manufacturer's guidelines. Briefly, subconfluent treated cells were detached from the flasks by trypsinization and then centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in 1 ml PBS and incubated with 0.25 μg/ml Annexin-V in 1X binding buffer for 15 min, followed by two washes with Wash Buffer. Cells were resuspended again in a binding buffer containing 0.5 μg/ml Propidium Iodide (PI) and then subjected to flow cytometer (BC, Novus). The data were analyzed by Kaluza software. In parallel, autophagy flux was determined by measuring the abundance of LC3II protein by fluorescent antibody labeling of the microtubule-associated protein using Rabbit anti-Homo sapiens MAP1LC3B Polyclonal antibody (MAP1LC3B Antibody, FITC conjugated) (CUSABIO, USA).

Cell cycle analysis

After treatments with DYN, Wort, AVP or DMSO, cells were harvested, washed twice with PBS then fixed with 70% ethanol (in PBS, v/v). After incubation, at 4°C for at least 2 h, cells were washed with PBS and stained with PBS containing PI (50 μg/ml, Triton X-100 and RNaseA) for 30 min at room temperature in a dark place. Cells suspension was filtered then analyzed by Accuri C6 flow cytometer (Becton Dickinson, Sunnyvale, CA, USA).

Akt and pAkt expression

The ready Prep™ protein extraction kit (Bio-Rad Inc., Catalog No., 163–2086) was used to extract total cells protein according to the manufacturer's instructions. Bradford Protein Assay Kit (Bio Basic Inc., Markham Ontario, Canada) was used to determine protein concentration following the manufacturer's instructions. For blotting, 20 μg protein was mixed with an equal volume of 2x Laemmli sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue and 0.125M Tris HCl, pH 6.8), where the mixture was boiled at 95°C for 5 min before loading on the gel. The blot was run, followed by

membrane blocking at room temperature for 1 h. Primary antibodies of total and phosphorylated Akt were diluted in TBST and incubated overnight with each antibody at 4°C. The blot was rinsed 3–5 times for 5 min with TBST and then incubated with the HRP-conjugated secondary antibody (Goat anti-rabbit lgG- HRP-1mg Goat mab-Novus Biologicals) for 1 h at room temperature. After another wash with TBST, the chemiluminescent substrate (Clarity TM Western ECL substrate Bio-Rad cat#170–5060) was applied and the signals were captured using a CCD camera-based imager. Image analysis software was used to read total-Akt and pAkt band intensities.

Cells invasion assay

MDA MB-231 cells were cultured in 6 well plates and left to grow up to 70% confluence, after which they were treated with DYN, Wort, AVP or DMSO. By the end of the treatment period, cells were trypsinized, washed with PBS and resuspended in serum-free DMEM. Inserts (8 µm pore size, BD Biosciences, St Louis, USA) were mounted onto the top of 6 well plate, where 100 µl serum free medium and 200 µl of treated cells ($2.5X10^5$ cell/ml) in serum-free medium were added to the upper chamber. In the lower chamber, 750 µl of 15% serum-containing medium was added, and then the treated cells containing inserts were place onto the lower champers. The plates were kept at 37 ^{O}C for about 18 h, after which the media were decanted and the inserts were washed twice with PBS. Cells were fixed with 3.7% formaldehyde (in PBS) for 2 min at room temperature. After decanting the formaldehyde, cells were washed twice with PBS and permeabilized with 100% methanol for 20 min at room temperature. Methanol was decanted and cells were washed twice with PBS, stained with 300 µl Giemsa stain, covered with tin foil, and then incubated for 15 min at room temperature. The stain was removed and cells were washed twice with PBS. Non migrated cells were scraped off with a cotton swab, and then the membrane was photographed under the light microscope, where the average number of migrated (transmitted) cells was counted using Image J software.

RNA isolation, cDNA synthesis and expression analysis

Quantitative real-time PCR was used to determine the fold expression of Beclin-1, MDR,1 ProAVP, Bax, and Caspase3genes at mRNA levels using Qiagen Rotor-Gene QPCR Cycler 5 Plex. Initially, total RNA was purified using GeneJET RNA purification kit, (ThermoFisher Scientific, USA). After quantitation and quality assessment, 200 ng RNA was used as a template for cDNA synthesis, using SensiFAST™ cDNA Synthesis Kit (Bioline Inc, USA) following the manufacturer's protocol. For real-time PCR quantitation, 50 ng/µl (2 µl) of cDNA was used as a template in 20 µl thermal cycling reactions containing 50 nmol/µl (2 µl) of the genes-specific primers (Table 1), the ready-to-use master mix of fluorescent dye QIAGEN SYBR green 1 and HotStar*Taq*DNA polymerase. Reactions were subjected to a thermal cycling program consisted of a single denaturation step followed by 45cycles (each consisted of a denaturation step at 94°C for 5 s, annealing at 62°C, 55°C and 62°C and 58°C, 56.8°C and 57.9°C (for Beclin-1, MDR1, Pro-AVP, Bax, and Caspase3, respectively) and an extension step at 72°C for 20 sec. Reactions were terminated with a single step at 99°C to produce melt curves that represent the changes in fluorescence observed when dsDNA with incorporated dye dissociates, or "melts" into single-stranded DNA (ssDNA) as the reaction temperature is raised. In parallel, the expression of the GAPDH gene was used as an internal control to

determine the relative fold expression of the targeted genes. The critical threshold (C_t) of target genes was normalized with quantities (Ct) of GAPDH using the $2^{-\Delta\Delta Ct}$.

Table 1
Sequence of primers used in the expression analysis of resistance genes, autophagy and apoptotic genes.

Gene		Sequence (5'-3')
MDR1	For	5'-CCCATCATTGCAATAGCAGG-3'
	Rev	5'-GTTCAAACTTCTGCTCCTGA-3'
Pro-AVP	For	5'CTTCTCCT CCGCGTGCTA-3
	Rev	5'CGTCCAG CTGC GGCGTTGCT-3'
Beclin1	For	5'-AGCTGCCGTTATACTGTTCTG-3'
	Rev	5'ACTGCCTCCTGTGTCTTCAATCTT-3'
BAX	For	5'-AAGCTGAGCGAGTGTCTC-3'
	Rev	5'-TCCCGCCACAAAGATGGT-3'
Caspase3	For	5'-TTTGTTTGTGTGCTTCTGAGCC-3'
	Rev	5'-ATTCTGTTGCCACCTTTCGG-3'
For Forward (sense), Rev Reverse (antisense)		

Statistical data analysis and graphing

Data analysis was performed using the SPSS13.0 software package (IBM, Chicago, IL, USA). All cell culture work was performed in triplicates. Apoptosis and the autophagy markers were measured and displayed in histograms as a percent of the control and represented as the mean of 3 runs ± standard deviation. Par graphs were constructed by Microsoft excel. *P* values less than 0.5 indicate significant differences. ClustVis was used to cluster the effect of DYN, combined with AVP-mediated stimulation or PI3K/Akt/mTOR pathway inhibition, based upon correlation distances and variable values were represented by squares with different color intensities.

Results

Initially, the changes in the metabolic activity of cells were assessed by MTT assay. Dynasore treatment decreased the MDA MB-231 cells viability in a concentration dependent manner, with IC_{50} value 8.5 µM. Also, Dynamin inhibition, in absence or presence of AVP or Wort, resulted in apoptotic morphological

changes including cells shrinking, rounding and detachment. Representative images of treated cells and the associated changes in cell viability are shown in Fig. 1. The percent of viable cells was decreased to 93.8 ± 1.7%, 76.5 ± 1.3%, 69.8.1 ± 1.09%, 671.7 ± 0.9%, and 62.6 ± 0.85%, in cells treated with AVP, DYN, DYN + AVP, Wort and DYN + Wort, respectively. Also, Annexin-VFITC/PI staining and flow cytometry analysis revealed that DYN alone induced apoptosis in 18.3 ± 1.5% and cell death in 5.3% of cells. More apoptosis (29.7 ± 1.4%) was observed when cells were prestimulated with exogenous AVP, before their exposure to DYN. Also, co-treatment of cells with DYN and Wort led to more apoptosis (35.3 ± 1.45%). Except in AVP stimulated cells, all the observed apoptotic effects of other treatments were significantly ($P < 0.001$) higher compared to DMSO- treated cells. Also, combined treatment with DYN + Wort induced more apoptosis compared to DYN + AVP ($P < 0.001$) (Fig. 2).

During Annexin-V FITC staining for apoptosis assessment, the low molecular weight internucleosomal DNA fragments are extracted in aqueous solution. Accordingly, apoptotic cells are identified on DNA content frequency histograms as sub-G1 fraction. As Fig. 3 shows, the sub-G1 fractions developed in DYN-treated cells was 19.2%. Also, Dynamin inhibition induced cell cycle arrest in G0/G1 phase in 76.3% of cells and 74.8% in cells prestimulated with AVP, before DYN treatment. Also, treatment of cells with exogenous AVP alone led to significant cell cycle arrest in G0/G1 phase (85.1%). Wort alone failed to induce cell cycle arrest, where 28.6% of cells were still dividing (in S-Phase fraction). Also, treatment of cells with DYN in combination with Wort did not induce cell cycle arrest, where 36.4% of cells were still dividing (Fig. 3). Next, to investigate whether Dynamin inhibition is associated with autophagy, fluorescent monoclonal antibody labeling of the microtubule-associated protein, LC3II was estimated and compared it to its basal level in DMSO-treated cells. The levels of LC3II, when cells were individually exposed to DYN, AVP or Wort were 50.7 ± 1.2%, 37.0 ± 1.0% and 20.8 ± 0.638. Compared to the basal level (31.0%), AVP did not significantly change the LC3II expression, whereas DYN and Wort induced a significant increase and decrease, respectively ($P < 0.001$). Also, in cells transiently treated with PV followed by DYN, (DYN + AVP) or (DYN + Wort), the LC3II levels were significantly increased to 58.2 ± 1.1% and 42.4 ± 0.9%, respectively indicating the independent effect of DYN in both cases (Fig. 4).

To investigate dynamin-independent effect of Dynasore and its involvement in PI3K/Akt/mTOR pathway modulation, cells were incubated with DYN in presence or absence of AVP or Wort (that selectively inhibits PI3K/Akt/mTOR pathway at PIP2-PIP3 transition reaction). The immunoblotting results demonstrated that DYN alone or in cells prestimulated with AVP, significantly decreased the level phosphorylated AKT (pAKT) compared to its corresponding level in DMSO-treated cells (Fig. 5). Also, DYN inhibition demonstrated a synergistic effect with Wort, where a lower pAKT expression was observed in cells co-treated with DYN and Wort. The least level of pAKT was obtained in cells treated with DYN in presence of both AVP and Wort.

Expression analysis at the mRNA level included the expression of 2 apoptosis related genes (Bax and Caspase-3), Beclin1, endogenous AVP (Pro-AVP) and the multidrug resistance gene (MDR1). The results indicated that Dynasore led to the upregulation of both Bax and Caspase-3, especially in cells prestimulated with AVP, whereas the expression of MDR1 was mildly downregulated (Fig. 6A) Pro-AVP

mRNA was expressed in MDA MB-231 cells and its expression was enhanced in cells treated with DYN (Fig. 6B). Also, DYN enhanced the expression of Beclin1, especially in cells prestimulated with AVP. Wort in contrast reduced Beclin1. To monitor the impact of Dynamin inhibition on cells invasion, transwell assay was performed to cells treated with DYN, prestimulated with AVP or cotreated with Wort. As Fig. 7 shows, Dynamin inhibition significantly reduced cells migration.

Discussion

CME is orchestrated with a set of scaffolding and adaptor proteins, including high molecular weight dynamins. Although this mechanism is utilized mainly to internalize liganded- membrane receptors, recent evidences demonstrated that some viruses, including SARS-CoV2, entry is established through their attachment with certain membrane receptors, like angiotensin-converting enzyme 2 (ACE2) receptors [24], followed by CME-mediated internalization. Accordingly, CME and the related proteins were extensively targeted by several small molecules to inhibit CME-mediated viral infection. However, the crosstalk between inhibitors of these proteins and non-endocytic targets is not well explored. Previously, we demonstrated that the inhibition of the interaction between β-arrestin, an endocytic accessory protein, and AP2 adaptor protein, decreased the viability of TNBC cells [22]. In the present study, Dyn2, (integral endocytic proteins with an intrinsic GTPase activity) was inhibited by 3-hydroxynaphthalene-2-carboxylic acid-(3,4-dihydroxybenzylidene)-hydrazide (commonly known as Dynasore) to explore the associated side effects on TNBC cells viability and their metastasis, especially when cells were prestimulated with exogenous AVP hormone. Compared to other large dynamins inhibitors (like Dynole 34 – 2 and Mdivi-1), Dynasore (DYN) selectively inhibits both isoforms (Dyn 1 & Dyn 2) in addition to the mitochondrial dynamin-related protein (Drp-1). More importantly, DYN does not affect small GTPases like Ras, Rho, Rab families [25, 26]. The involvement of Dyn2 in insulin secretion and plasma glucose homeostasis [27] may explain the progressive reduction of cells metabolic activity when Dyn2 was inhibited, as indicated by the MTT assay.

Dynasore was able to induce apoptosis in 18.3% of cells, increase the expression of the apoptotic markers Bax and caspase-3 and downregulated the activation of AKT. Although the inhibition of large GTPase activity represents its main mode of action [18], some studies revealed that DYN cytotoxic effect is mediated through blocking cytokinesis and/or induction of caspase-mediated apoptosis following cytokinesis failure [17]. Also, the direct involvement of Drp-1 in mitochondrial fission, suggests the mitochondrial dysfunction mediated cell death. In similar manner, Wort exerted a synergistic apoptotic effect with DYN. This is attributed to Bak activation and the subsequent mitochondrial damage [28]. Although breast cancer cells express AVP gene, more apoptosis was observed in cells prestimulated with exogenous AVP. Dyn 2 plays an important role in the completion of the last stages of mitosis [29], where it is intensely localized at the mitotic spindle via its proline-rich domain (PRD) [30]. Although the Dyn2 GTPase activity is not involved in the microtubule polymerization, the obtained results suggest that DYN may induce cytokinesis failure and inhibit proliferation of TNBC breast cancer, where cells were arrested in G0/G1 phase in absence or presence of AVP. Such effect was previously observed in other cancers including cervical and lung cancers [31]. In agreement with previous reports [32], Wort alone failed to

induced cell cycle arrest, where 28.6% of cells were still dividing (S phase). Moreover, cells cotreated with DYN combined with Wort did not show cell cycle arrest, where 36.4% of cells were still dividing. AVP in contrast was able to accumulate cells in G0/G1 phase, due to the cumulative effects of both the endogenously expressed (Pro-AVP) and the exogenous AVP and their direct inhibitory effect on cyclin D1 expression [33].

In parallel, the involvement of Dynamin in regulation of autophagy was previously reported, where mutations in Dyn 2, led to the impairment of autophagy in mice [34]. The observed increase of the autophagic marker (LC3II) in Dynasore-treated cells may be explained by the inhibition of Drp-1. Moreover, the increase of LC3II protein was associated 1.8-fold increase in the expression of Beclin1 in Dyn-inhibited cells and more expression (2.5-fold) in presence of AVP, the observation previously reported in senescent Endothelial Cells. In contrast, Wort was associated with the downregulation of both markers, the finding previously reported in brain [36] and colorectal cancer [37]. In a similar manner, the auophagic effect of AVP was evidenced through 1.6 and 2.5-fold increment in LC3II and Beclin 1. Moreover the enhance AVP-mediated responsiveness of cells could be attributed to V2R activation [38] and the associated downstream signaling. Although AVP did not induce observable apoptotic changes (about 6%), it led to cell cycle arrest possibly due to the inhibition of cyclin D1 [33]. The obtained data excludes mitogenic effect of AVP, however it enhanced the apoptotic effect of Dynasore in agreement with reports, that suggested the antiproliferative of AVP-mediated activation of V2R in renal collecting duct [39]. AVP binding with V1R, for example, enhanced cellular proliferation, whereas its binding to V2R was associated with antiproliferative effects. Moreover, the downstream signaling effect of AVP varies when it liganded with V1ar, V1br or V2R, that distributed among different cell types [40]. The contradicting effects of AVP may predict its apoptotic or anti-apoptotic effect that varies according to the cell type and the type of VR receptor, it binds with [41].

To compare the antineoplastic effect of DYN, Wort was utilized, where it inhibits cell proliferation via the inhibition of PI3K/AKT/mTOR signaling [32]. DYN treatment was associated with an observable decrease pAkt. This may be explained by RagA-Raptor binding that reduces the recruitment of mTORC1 to the lysosome and the subsequent reduction of Akt activation [42]. A similar regression in AKT activation was observed in cells prestimulated with AVP, where AVP-mediated activation of the V2R receptor, leads to the recruitment and oligomerzation of Dyn2, around the necks of nascent AVP/V2R containing vesicles [43]. During this process, Dyn2 is anchored to membrane PIP_2 (through ithe pleckstrin-homology domain PH domain, shown in Fig. 1) and to the negatively charged lipids through its positive residues. Similar to Wort [44], the depletion of PIP2 may explain the synergistic effect of AVP with DYN in down-regulating AKT pathway.

The potential of cell migration and invasion gives breast cancer the ability of local, regional and systemic metastasis, that convey to poor prognosis and death. Previous reports predicted the implication of large dynamins in facilitating cell migration and invasion [45]. Also, other studies have indicated that metastasis of breast cancer cells may involve GTP binding and/or hydrolysis by dynamin [46]. The obtained data showed that Dynamin inhibition led to a reduction of cells invasion. As PI3K/Akt signaling

is a common pathway regulating cell proliferation, migration, and invasion, the observed down-regulation of the cell's invasiveness, may be attributed to the associated DYN-mediated PI3K/Akt inhibition.

Although the study did not involve genome or proteome-wide analysis, we thought to mine all observations (13 variables) performed in cells treated with either DYN or Wort using a simple clustering tool. Correlation based clustering using ClustVis demonstrated that DYN and AVP/DYN effects were tightly clustered. Similarly, Wort and combination of Wort/DYN were tightly clustered (Fig. 8) indicating the corresponding anticancer effects of Dynamin inhibition and PI3K/Akt/mTOR inhibition. The overall scenario depicts that the apoptotic and the autophagic effect of Dynamin inhibition by Dynasore. This may represent a beneficial role in limiting the growth rate of invasive breast cancer, especially when cells are prestimulated with AVP. These events are supported with other observations including, limitation of metastasis. Also there is a relative improvement in cell's sensitivity to chemotherapy, as indicated by the downregulation of the drug resistance gene that encodes the multidrug efflux protein P-glycoprotein (PGY1).

Summary

In summary, to our knowledge, this work represents the first study that monitored the effect of Dyn 2 inhibition on breast cancer cells. The study design aimed to investigate the DYN effect after prestimulating cells with AVP and compare it with corresponding effect of Wort. The obtained data documented the antiproliferative effect of DYN, where it induced apoptosis, autophagy and cell cycle arrest in MDA MB 231 cells. These observations were enhanced by AVP, which stimulates the abnormally expressed V2R receptor. The antiproliferative effects of DYN was associated with a decreased activation of AKT pathway, increased expression of both Bax & Caspase-3, subsidizing cells invasion potentials and increased their drug responsiveness. Also, the drug synergistically enhanced the apoptotic effect of Wort (as PI3K/mTOR inhibitor). Collectively, these observations nominate Dyn2 as a promising antineoplastic target in breast cancer.

Abbreviations

CME: Clathrin mediated endocytosis, AVP: arginine vasopressin, TNBC: Trible negative breast cancer,

Declarations

Authors' contributions:

Conceptualization: **MH**; Methodology and investigations: **SS, TD, SL** and **MH**; Original draft preparation **MH;** Review and editing, **MH, SL, SS** and **TD**. All authors read and approved the final manuscript and its revised version.

Competing interest

The authors declare that they have no competing interests

Financial interest

All authors declare that they have no financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

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Figures

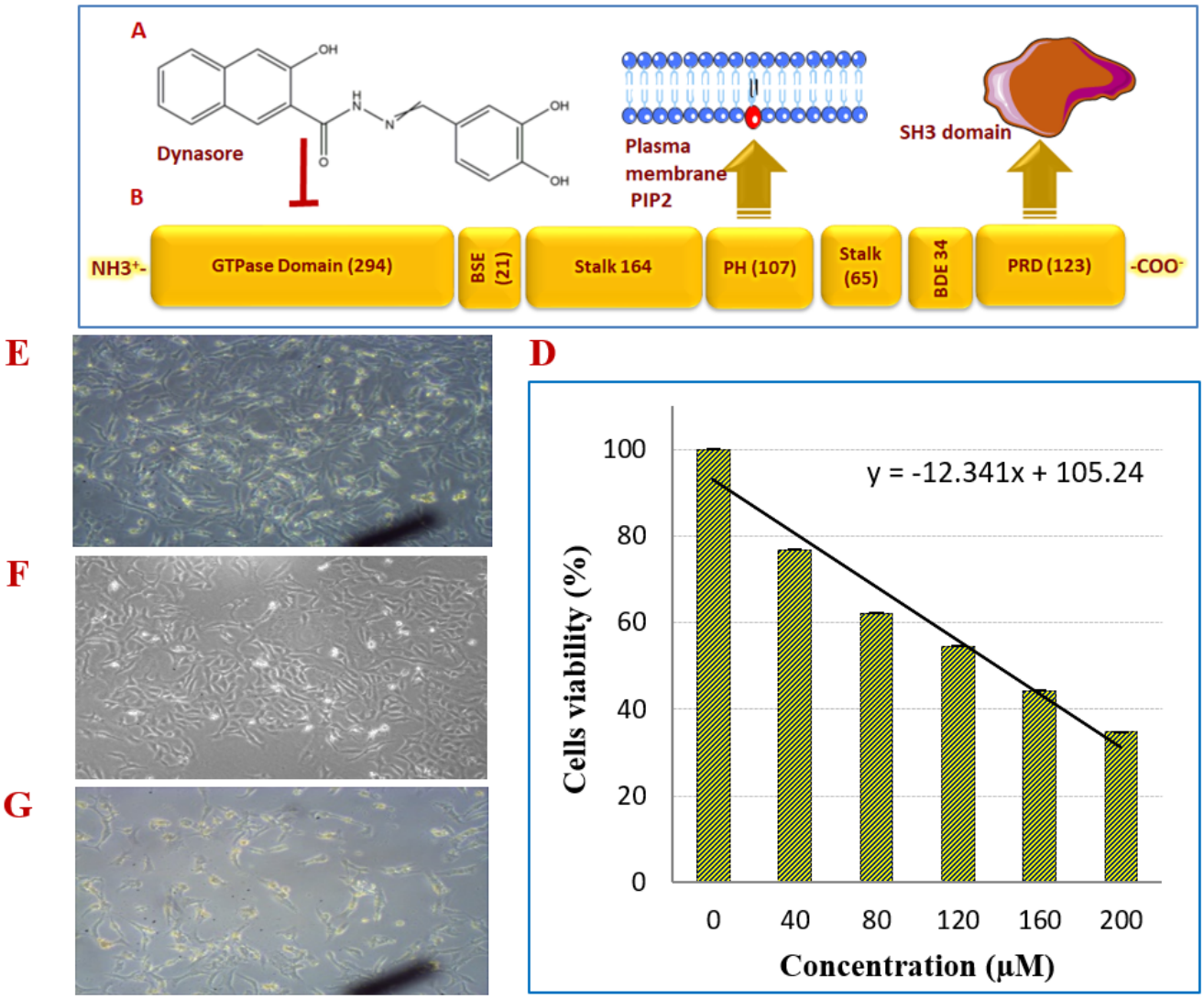

Figure 1

Cytotoxic effect of Dymanin inhibition. Dynasore (A), a selective inhibitor of Dynamin 2 activity localized at its GTPase domain (B), induced a concentration dependent cytotoxic effect in triple negative breast cancer cells. MDA MB-231 cells were incubated with varying concentrations of a Dynasore (DYN) for 24 hours and cells metabolic activity was determined by MTT assay. Data are expressed as means ±SD of multiple experimental replicates (*n*=5) (D).E-G are representative phase contrast photomicrographs of cells treated with, DYN or combinations of DYN with AVP or the PI3K inhibitor (Wort), respectively. Dynamin inhibition resulted in cells morphological abnormalities including shrinking, rounding and detachment. Representative images from three independent experiments are shown (magnification of 400X).

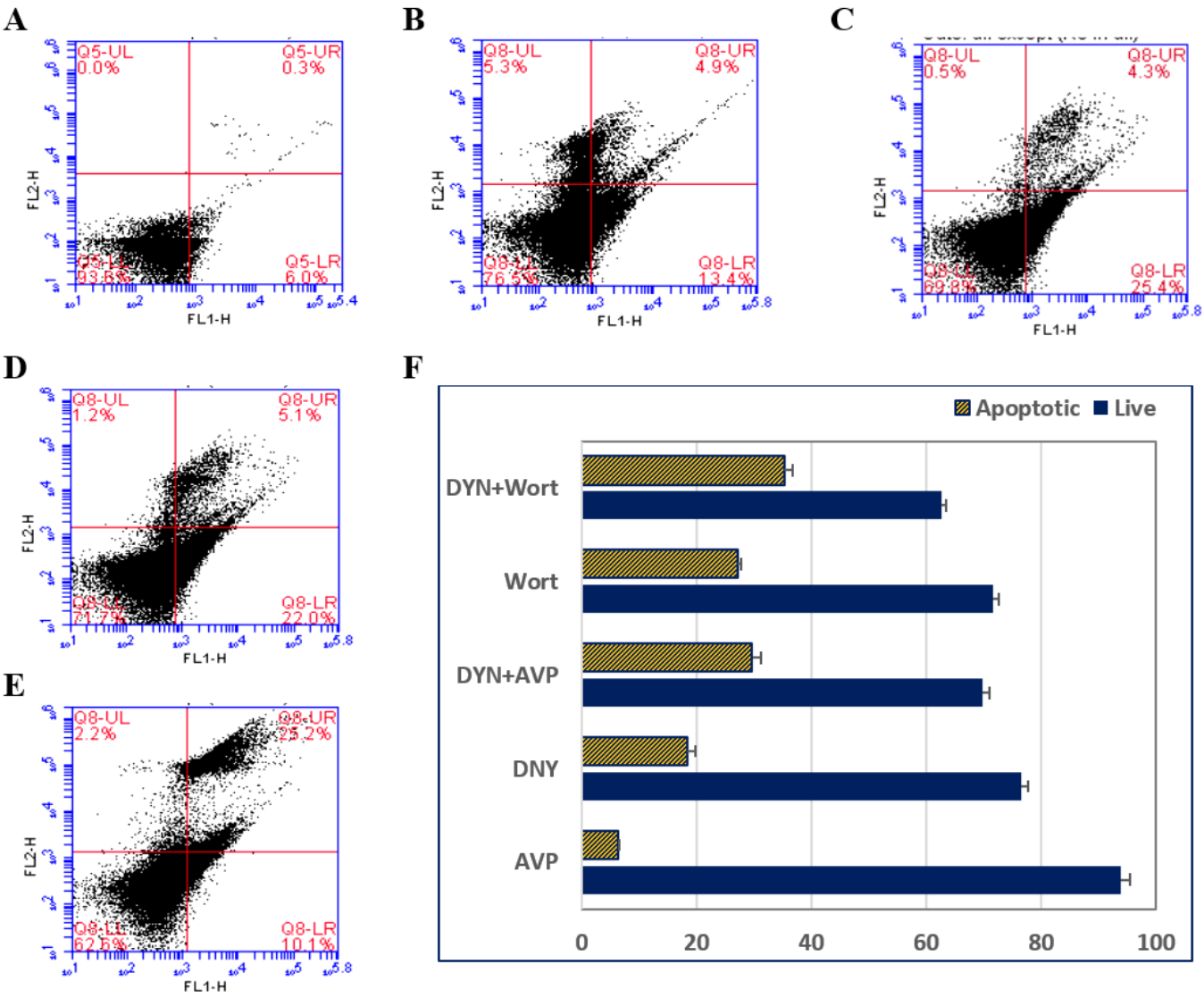

Figure 2

Apoptotic effect of dynamin inhibition in invasive breast cancer cells. Plots (A) through (E) show Annexin V-FITC and PI-stained cells treated with AVP (A), DYN (B), DYN after cell stimulation with AVP, Wort (D) or combination of DYN with Wort (E). In each scatter plot, the lower left quadrant, the upper left quadrant, the lower right quadrant and upper right quadrants represent the percent of viable cells, dead cells, early apoptotic cells and late apoptotic cells, respectively, Significant apoptosis was observed in DYN treated cells and more apoptosis developed in presence of AVP or Wort combined with DYN treatment (F). Results are presented as mean ± SD as a percent of the control.

(***) and (**) refer to P<0.001 and 0.01 indicating significant apoptosis relative to the untreated cells.

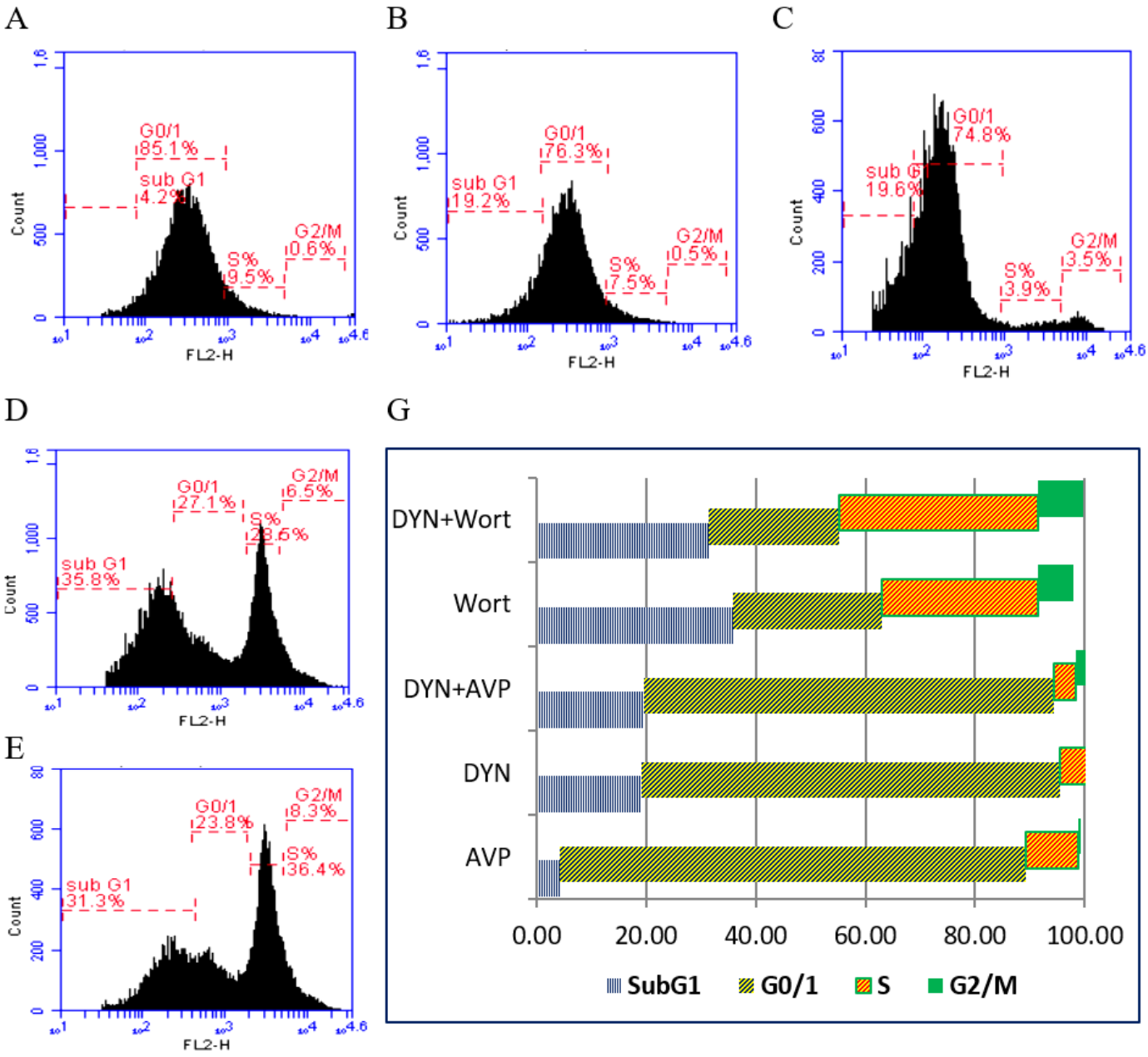

Figure 3

Dynamin inhibition induces cell cycle arrest in G0/G1 phase in breast cancer cells. MDA MB 231were treated with AVP (A), DYN (B), prestimulated with AVP then treated with DYN, Wort, or a combination of DYN and Wort. After cell fixation, they were PI-stained and analyzed for cell cycle by flow cytometry. A-E are representative scatter plot of cell cycle analysis and (F) is bar graph of cell fractions distributed in different phases. DYN and AVP arrested cells in G0/G1 phase.

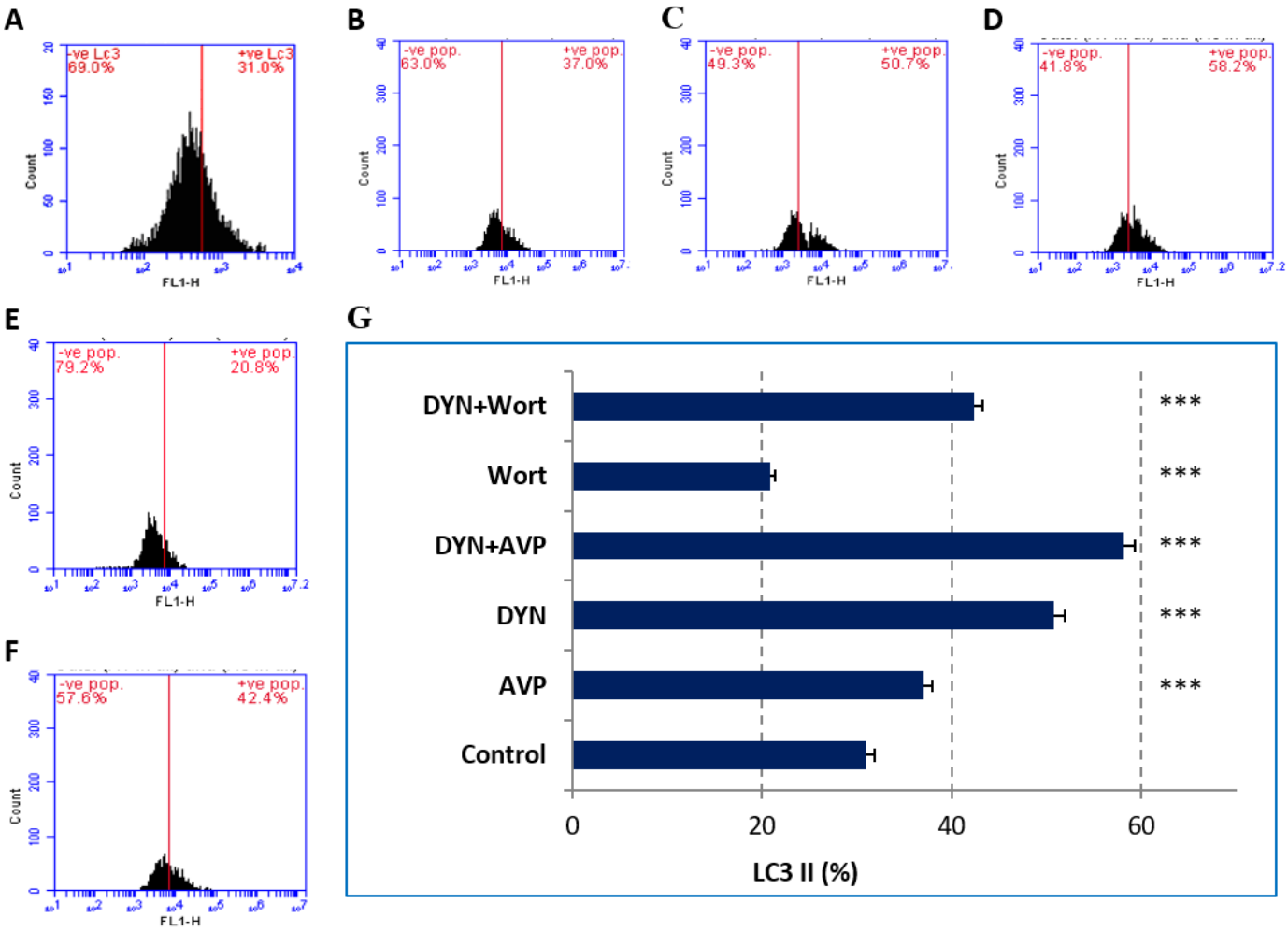

Figure 4

Dynamin inhibition promotes cytotoxic PI3K-independent autophagy in triple-negative breast cancer cells. MDA-MB-231 were seeded with an initial cell density $4X10^4$ grown in nutrient-rich conditions and left untreated (**A**), transiently treated with AVP (B), DYN (C), DYN after cells stimulation with AVP (D), Wort (**E**) or DYN in combination with Wort (**F**). Following treatments, the expression of autophagy marker (LC3II protein) was determined by flow cytometry. The autophagy was significantly induced in Dynamin inhibited cells and reduced in Wort treated cells. Dyn inhibition induced significant increase in LC3II even in cells in which PI3K was inhibited by Wort. Data are presented as mean ± SD (G). (***): refers to significant ($P < 0.001$) differences between the indicated group compared to the untreated cells.

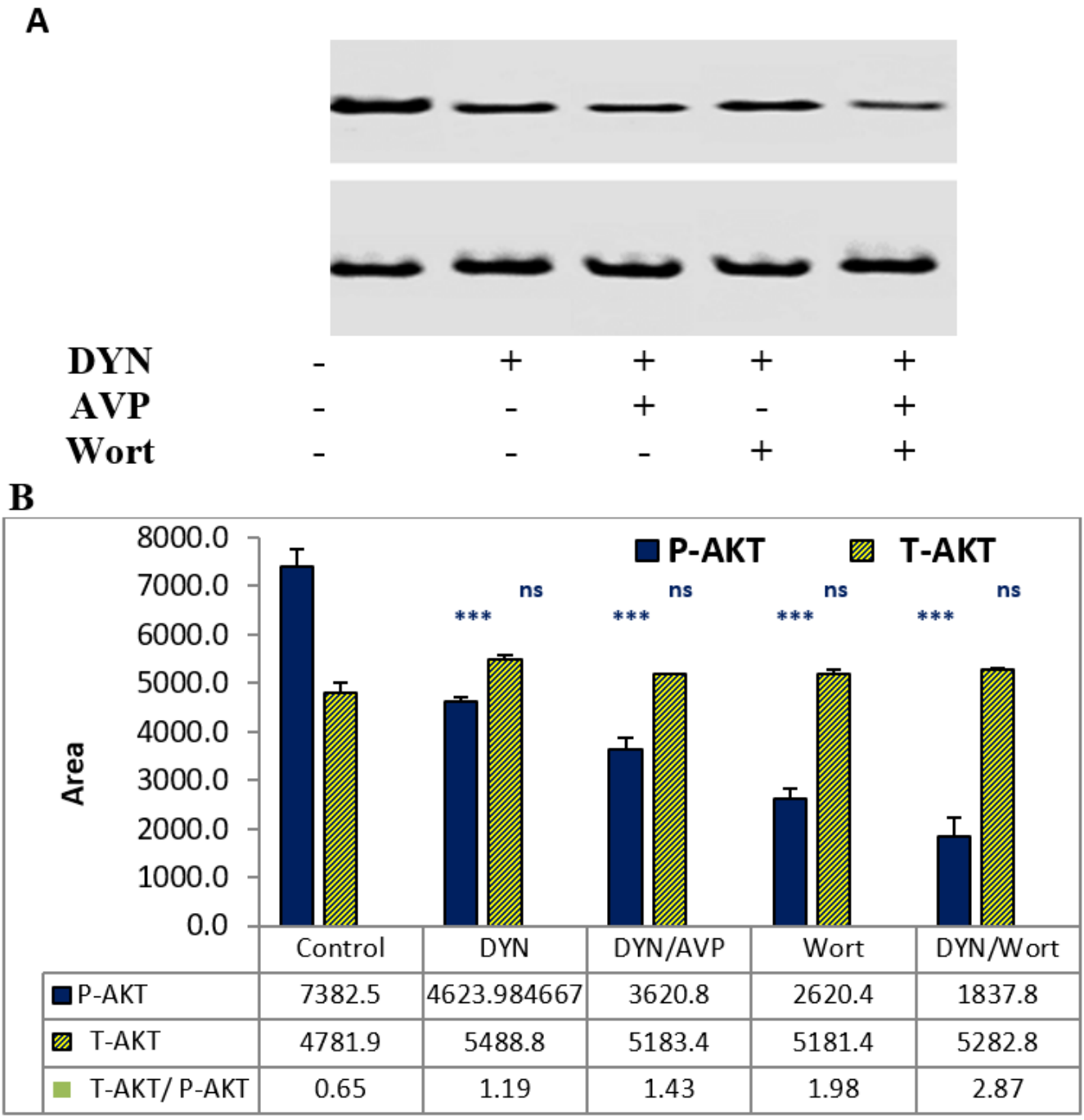

Figure 5

Expression of total and phosphorylated Akt (P-Akt) at (Ser473) in breast cancer cells stimulated with AVP or PI3/AKT inhibitor in combination with DYNs inhibitor by Western blot analysis. Top panel: MDA MB-231 cells in which V2R receptor was prestimulated with AVP or PI3K pathway was inhibited by Wort in combination with the dynamins inhibitor (DYN). Cell lysate was analyzed using Akt or P-Akt monoclonal antibodies. Bottom: band intensities of the quantification of P-Akt (Ser 473) and the ratio T-Akt/P-Akt are the means ± SD of data from independent experiments. (***) P<0.001 determined by one-way ANOVA followed by the Student-Newman-Keuls posthoc test, ns: non-significant difference.

D: Dynasore; Wort: Wortmannin, AVP: arginine vasopressin.

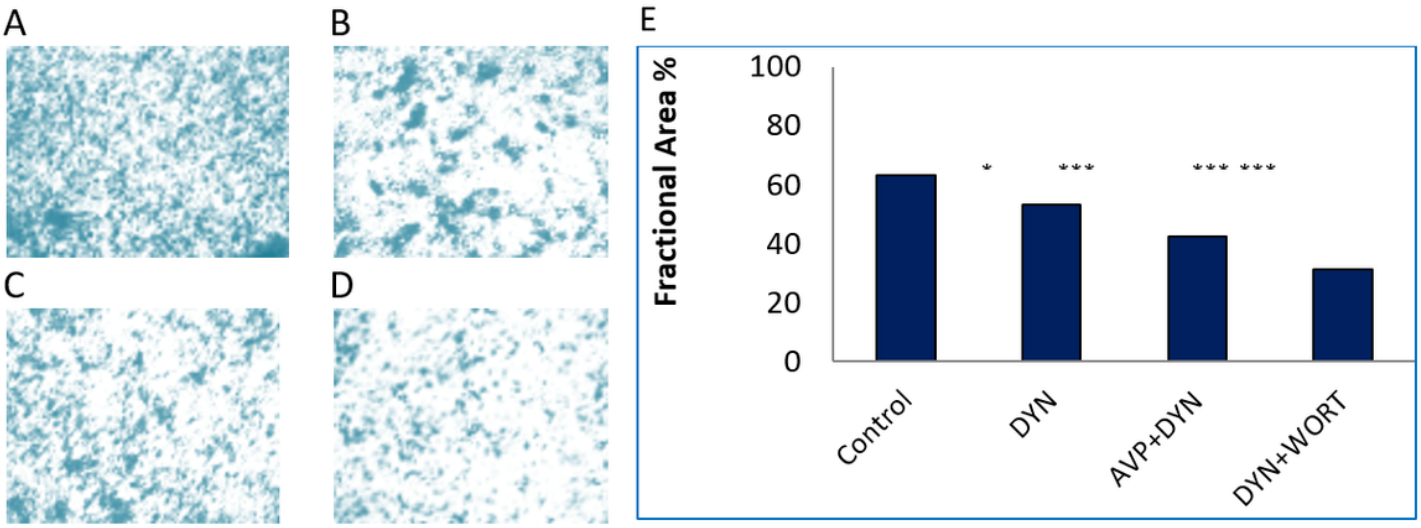

Figure 6

Effects of Dynamin inhibition on AKT and p-AKT expression of in breast cancer cells. Dynamin inhibition in presence of AVP or Wort (A). The decreased expression of phosphorylated Akt is observed in cells treated with DYN alone, AVP+DYN, or DYN+Wort interdicts the inhibitory role of DYN on PI3K pathway as similar as Wort effect as a selective PI3K inhibitor (B).

*** P,0.001, *ns*: not significant

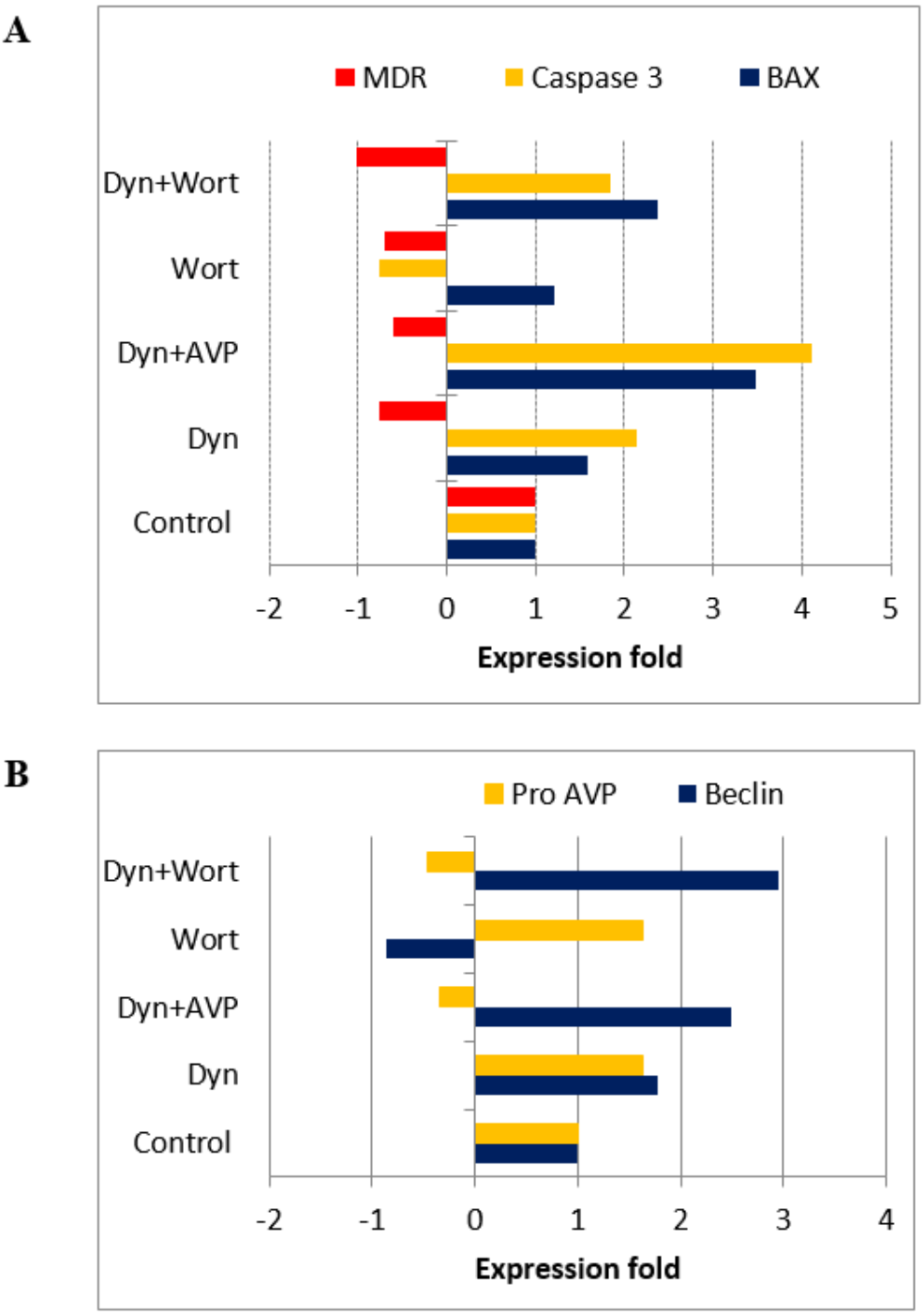

Figure 7

Relative expression of Bax, Caspase-3, MDR1 (A), Pro-AVP and Beclin1 (B) in dynamin inhibited breast cancer cells. MDA MB-231 cells were treated with DYN alone, after their prestimulation with AVP, or cotreated with Wort. Cells mRNA was isolated, reverse transcribed, and the cDNA was used as a template in qRT-PCR to determine the relative expression. Dynamin inhibition was associated with upregulation of apoptosis and autophagy related genes and downregulation of drug resistance gene.

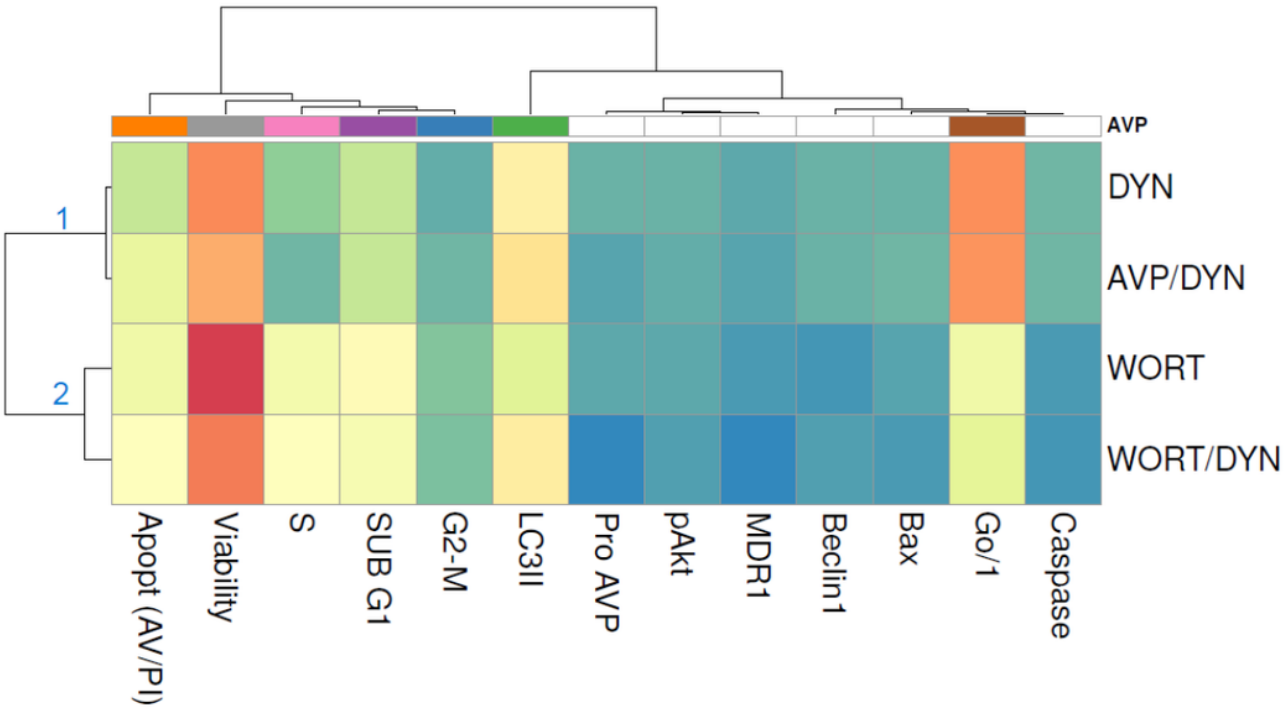

Figure 8

Heatmap of the correlation-based clustering of the effect Dynamin inhibition, with Dynasore, or the combined effect of Dynasore with either AVP or Wort. Rows are centered; unit variance scaling is applied to rows Both rows (representing drugs treatments) and columns (representing the estimated variables) are clustered based on correlation distance and average linkage. Variation in variable values are indicated by the corresponding squares with different color intensities. Two distinct clusters were obtained, where DYN/AVP+DYN cluster (1) and Wort/Wort+DYN (2) are shown indicating the similar antineoplastic effects of Dynasore and Wort