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Research Article

GOLD NANOPARTICLE-ENHANCED 5-FU POTENTIATES CHEMOTHERAPY SENSITIVITY OF BREAST CANCER CELLS METASTASIS AND INVASIVENESS BY DOWN REGULATING THE EXPRESSION OF MENIN AND ETV7, AND PI3K/AKT/mTOR SIGNALING PATHWAYS

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Abstract

Breast cancer progression, especially in the case of metastasized phenotypes has been a significant challenge clinically due to its aggressive nature, poor prognosis, and resistance to therapy. The key molecular drivers such as Menin and ETV7 have been implicated in promoting metastasis to enhance breast cancer cell proliferation, survival, and drug resistance. 5-fluorouracil (5-FU) is a widely used chemotherapeutic agent, thus BEPANT-6 an improved 5-FU formulation gold nanoparticle was designed specifically to down regulate survival proteins and was used to target Menin and ETV7 expressions, and other interactable molecules involved in the progression of BC cells. BEPANT-6 gold nanoparticles (BEPANT-6) could enhance the therapeuticeffect on BC cells (MDA-MB-231 and MCF-7) through targeting and down regulating Menin and ETV7 oncogenicity, PI3K/AKT/mTOR signaling pathways transduction and the regulation potential to reduce heterogeneity which is among the key factorsthat underpins therapy resistance making the BC cell's sensitivity to treatment, andcontrols metastasis potential to spread. The mitigations of these key molecules by BEPANT-6 reduced BC cells' invasiveness and metastatic ability, thus improving patient outcomes with advanced BC. Therefore, the BEPANT-6 could potentiate treatment strategy against BC cell proliferation, survival, and invasiveness which could play an important role in BC cell sensitivity to treatment.

Keywords: BEPANT-6 gold nanoparticle, 5-FU, Menin, ETV7, and PI3K/AKT/mTOR.

INTRODUCTION

Breast cancer (BC) has become the second most commonly diagnosed type of cancer worldwide and the number one most widespread cancer in women, recording up to 2.3 million new cases and 665,684 in 2022 globally (1). It is a heterogeneous disease that is classified into different subtypes with percentages of occurrence such as human epidermal growth factor receptors (HER2, 10-20%), triple-negative breast cancer (TNBC,10-20%), luminal A and B (50-60%), and normal-like BCs (2–4). TNBCs are particularly difficult to respond to treatment compared to other subtypes (4). BC can be caused by various factors: hormonal imbalance, BRCA 1 and 2 mutation, age, diet, etc. (2,5). Annually, approximately 5-10% of therapy-resistant BC (TRBC) phenotypes are diagnosed with primary metastatic BC (pMBC) and distant metastases, and patients that degenerate to metastasized TRBC (mTRBC) remain a menace to cure. However, treatment with palliative intentions, depending on the BC subtypes has a median overall survival (OS) within 3-4 years (6,7). Previously studies have shown that metastasized cancer cells migration to other organs is the leading cause of cancer deaths (4,8,9). Therefore, increased survival outcomes and the patient's overall quality of life have been pivotal to clinicians and drug developers, and monotherapies: molecular-targeted therapy, chemotherapy, immunotherapy, hormonal therapy, and irradiation therapy were seen as the mainstay to treat metastasized BC (mBC) cells (10). However, there were successes with the use of monotherapies but somepatients eventually relapsed, most are patients with advanced BC and the metastasized phenotypes, especially the TNBC subtypes (11–13). Thus, there is an urgent and unmet need to develop more effective and targeted treatment(s) for mBC cells, anda combination of these therapies has proven more effective. The FDA has approved somecombination therapies for treating pMBC, mTRBC, and non-mTRBC (nmTRBC) (12–16). However, there are some incidences of side effects from combination therapies due to multiple mechanisms that contribute to the emergence of multidrug resistance (MDR)(17,18). Studies have seen chemotherapy as the most efficient choice for BC treatment (19, 20). Despite the arrays of therapies for mBC, there is an urgent demand forimproved chemotherapy agents that can effectively treat TRBC progression (21–25). In addition, nano-drug delivery systems of chemotherapy agents are seen as the mainstayto improve the cellular heterogeneous distribution, and therapy target of the therapeuticagents for a prolonged period to achieve safe drug delivery and increase therapeutic efficacy (21–24). Previous studies have shown that 5-FU is widely known as a chemotherapy agent that can inhibit thymidylate synthesis (TS), distorting DNA synthesis, and repair used as an anticancer treatment for numerous cancers, e.g. colorectal, breast, and prostate (26–29). Although, 5-FU has several therapeutic advantages, there are noticeable limitations in its clinical application, due to its poor bioavailability, rapid degradation, and non-specific toxicity, leading to the development of therapy resistance of the cancer cells (26–29).

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While continuous administration of 5-FU to improve therapeutic efficiency on breast andprostate cancers, revealed incidences of side effects, such as mucositis, diarrhea, vomiting,nausea, and stomatitis. Therefore, to reduce system toxicity, the dose, and the therapy duration, demand the use of nano-vehicles, as targeted drug delivery systems to effectively deliver 5-FU in a pharmacologically adjustable dose to increase its therapeuticeffect on BC cells (30–32). In addition, improving the NP's surface to be conjugated to target molecules such as cytokines, growth factors, folic acid, peptides, and antibodies, thus increasing the target on cancer cells has shown positive outcomes (33–35). Meanwhile, the formulation of 5- FU into a nano-vehicle has been seen as an effective option for reversing multidrug resistance and has been shown to increase chemotherapy drugs' sensitivity to treat cancer cells without the incidence of adverse therapeutic side effects (30,32,36,37).

In addition, 5-FU as a chemotherapy agent remains one of the central components of BC treatment in adjuvant settings and in the treatment of mBC (29,30,32), which is seen as the main cause of cancer-related lethality(17). Moreover, survival signaling pathways play important roles in the invasion, metastasis, EMT, and autophagy in BC and TRBC cells,leading to aggressiveness of BC progression (38,39). Furthermore, P13K/AKT/mTOR (PAM) transduction signaling pathway is known as a master regulator due to the extensive cross-talk with other cancer cell signaling networks that is prevalently over activated in human cancers and PAM hyper activation is mostly underpin in treatment resistance in cancer cell migration, and the pathway is controlled by the regulatory proteins' phosphorylation and dephosphorylation via modulating the level of protein expression (40–42). The upregulation of the survival protein expression level in BC cells contributes to cell growth, survival, proliferation, and angiogenesis (39,39). Hence, therapeutic targeting of the signaling molecules and the components of these survival pathways are seen as therapeutic options to mitigate and downregulate the transcription and expression of the survival proteins in BC cells could be an interesting therapeutic target for BC progression (4,16,38,39,40).

Previous findings have shown a considerable link between multiple endocrine neoplasia type 1 (MEN 1) and breast carcinogenesis. Moreover, the protein Menin is encoded by theMEN 1 gene bound to and activates the estrogen receptor (ER) to upregulate the level of Menin expression to enhance the growth stimulus of BC cells (43,44). Menin is predominantly a scaffold protein that mechanistically crosstalk, and interacts with numerous partner signaling regulators to regulate gene transcription and interplay with multiple signaling pathways such as PAM in breast carcinogenesis (43,45). While ETS variant transcription factor 7 (ETV7) also known as TEL2 is an oncoprotein from the E26 transforming-specific (ETS) family that is upregulated and over expressed inall subtypes of BC. ETV7 also crosstalk with the PAM signaling pathways and the increasedtranscriptional activity accelerates the onset of tumor and promotes tumor penetrance (46). ETV7 is associated with the BC-causing mechanisms that promote tumorigenic transformation, oncogenic progression, and genetic regulation that are involved in the development of chemo- and radio-resistance, and the increased cancer cell proliferation,differentiation, and stemness in BC progression (46–48).

Importantly, Menin and ETV7 co-regulation of survival genes and crosstalk with PAM signaling pathway transduction increases the transcription of metastasis-associated genes to promote cancer cell survival and metastasis, thus the down regulation of Menin and ETV7 expression and interaction with partner signaling regulators and pathways, andthe inhibition of the transcriptional activity respectively, could mitigate the transduction of PAM signaling pathway to upregulation of the synthesis of survival proteins expressionresponsible for BC proliferation and invasiveness(36,42,43,47,49,50). Therefore, to improve the therapeutic targeting of TRBC cells with 5-FU, we synthesizeda 5-FU derivative conjugated to gold nanoparticle known as BEPANT-6 gold nanoparticles (BEPANT-6), formulated as a target drug delivery nanoparticle agent (21,24,36). It has faster and increased penetration into the cancer cells with a relatively higher therapeutic activity. BEPANT-6 has a pharmacokinetically adjusted dose of 5-FU and functions as a chemotherapy agent to target and inhibit BC cells and the therapy-resistant phenotypes to cancer therapy, to improve the mBC cells' (mTRBC) sensitivity to treatments (4, 16, 33, 38). For this study, BEPANT-6 was tested on breast cancer models MDA-MB-231(highly metastatic) and MCF-7 (non-metastatic) cells *in vitro*, in *vivo*, and *ex vivo* analyses. It was found that BEPANT-6 had a more significant effect in mitigating mBC cells' sensitivity to treatment than 5-FU. The results confirmed that continuous exposure to a pharmacokinetic dose of 5-FU by a nano vehicle-targeted delivery system prevents cell growth in TRBC and induces sensitivity to cancer treatment.

RESULTS

BEPANT-6 enhanced heterogenous distribution promotes the disruption of BCcell division and induces apoptotic death

The internalization and impact of BEPANT-6 on BC cells (MDA-MB-231 and MCF-7) division and cell death were examined. The cells were treated with BEPANT-6 on MDA- MB-231 and MCF-7 cells respectively. We observed an increased presence of BEPANT-6 in both the cytosol and nucleus of the BC cells (Fig. 1A) and a significant rate of cells in the G0-G1 phase on both BC cell lines with an increased fraction of 71.4% (Fig. 1B, ***: $p < 0.001$) in MDA-MB-231 cells and 68.5% (Fig. 1C, ***: $p < 0.001$) in MCF-7 cells. This indicates that the increased internalization of BEPANT-6 and its heterogeneous distribution enhanced the supply of 5-FU to induce BC cell cycle arrest more in the G0-G1 phase when compared with the other phase (Fig. 1A-C).

The heterogeneous distribution of BEPANT-6 and its ability to disrupt BC cell division at the G0-G1 phase hindering the BC cell transition to the S phase promotes a significant inducement of apoptotic cells that was observed on both BC cell lines after BEPANT-6 treatment with increased fractions of apoptotic MDA-MB-231 cells of 65.3% (Fig. 1D,**: $p < 0.01$) and MCF-7 cells of 68.1% (Fig. 1E, ***: $p < 0.001$) compared to the non-treated controls cells. In addition, western blotting analysis revealed significant induction of cleaved PARP and cleaved caspase-3, BAX, and p21, and significant inhibition of Bcl-2 expressions on both BC cell lines (Fig. 1F). Overall, the results show BEPANT-6 increasedheterogeneous distribution induces MDA-MB-231 and

MCF-7 cells cycle arrest at the G1-phase with an increased proportion of cells trapped in the phase by the down regulation of cell cycle checkpoints and Bcl-2 expression via the cyclin-dependent kinases (CDKs) that are involved in transition to the S-phase and activate the upregulation of pro-apoptotic proteins such as BAX and p21 that reinforce elevated G1-phase arrest of BC cellsleading to apoptosis. However, 5-FU and its derivatives such as BEPANT-6 have known mechanisms to mitigate survival proteins and pathways to trigger apoptotic cell death through both the intrinsic (mitochondria) and extrinsic pathways. The apoptotic pathways are activated primarily via the pro-apoptotic factors to initiate and induce caspase-apoptotic cascade (i.e. mitochondrial dysfunction, caspase activation, and Fas ligand upregulation), most importantly the activation of caspase-3 that cleavage cellular components and apoptosis to execute cell death and to suppress BC cell growth and metastasis (20, 27, 29, 31, 36).

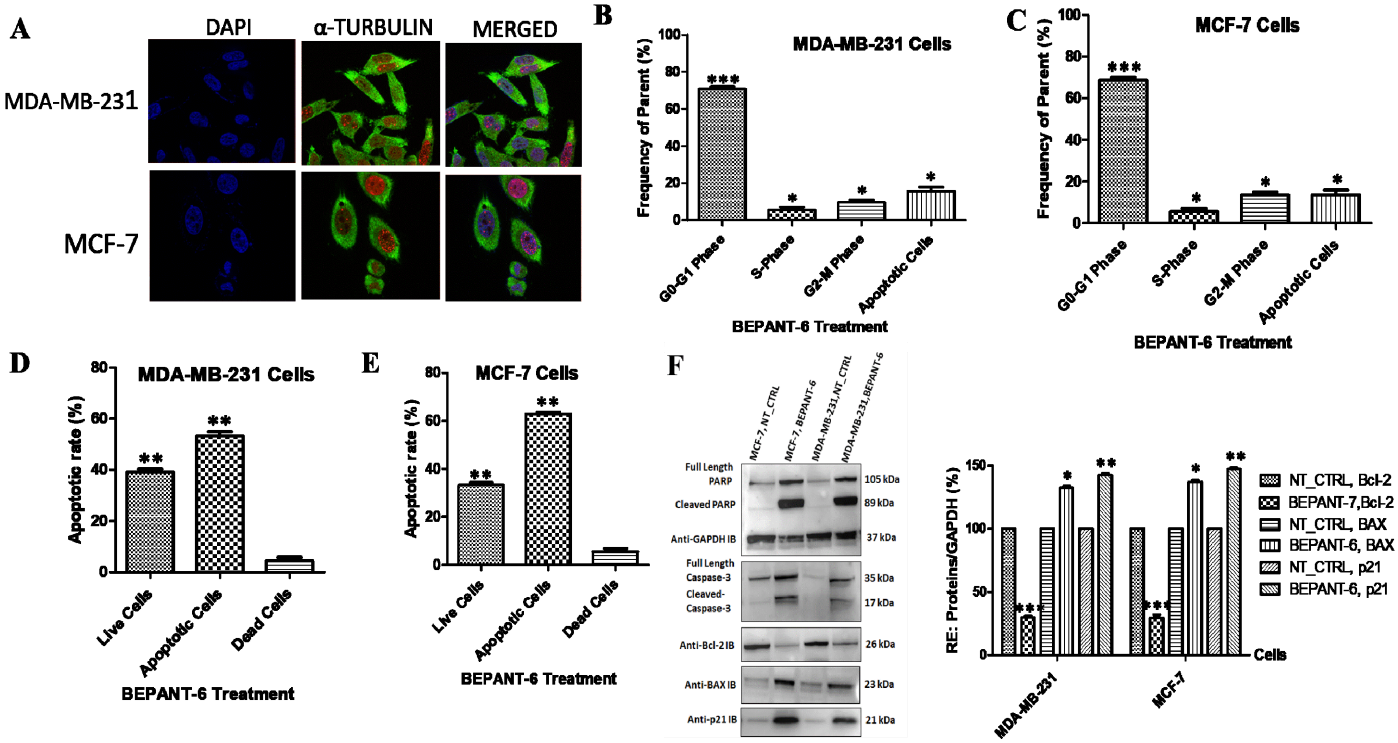

Fig. 1 BEPANT-6 enhanced heterogeneous distribution promotes the disruption of BC cell division and induces apoptotic death. (A) BEPANT-6 was significantly distributed heterogeneously in the BC cell lines (MDA-MB-231 and MCF-7). **(B-C)**BEPANT-6 significantly arrested BC cells in the G_0-G_1 phase with increased fractions of 71.4% and 68.5% in MDA-MB-231 and MCF-7 respectively. **(D-E)**BEPANT-6 significantly induced the increased fractions of 65.3% and 68.1% apoptotic MDA-MB-231 and MCF-7 cells respectively. **(F)**Graphical representation of BEPANT-6 inhibiting anti-apoptotic Bcl-2 and inducing pro-apoptotic cleavage PARP, cleavage caspase-3, BAX, and p21 expressions. Experimental analysis was in triplicates and statistical values were represented*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

BEPANT-6 shows a better antiproliferative effect and reduced invasiveness onBC cells at lower concentrations than 5-FU

To elucidate the comparison of BEPANT-6 and 5-FU antiproliferative effects and to reduce invasiveness on cancer cells, we performed a cell invasion assay on BC cell lines (MDA- MB-231 and MCF-7). The cells were treated with BEPANT-6 and 5-FU concentrations for48 h. We observed the treatment response of the MDA-MB-231 and MCF-7 cells to both chemotherapies. MDA-MB-231 and MCF-7 cell invasion was reduced by 78% and 71% with BEPANT-6 treatment compared to 15% and 10% with 5-FU treatment respectively (**Fig. 2A).** The BEPANT-6 displayed a better therapeutic enhanced anti-invasive effect when compared to the original 5-FU chemotherapy in both BC cell lines invasiveness (***: $p < 0.001$). To further elucidate the REPANT-6 effect to mitigate BC cell proliferation at lower concentrations than 5-FU, we performed an MTT assay on BC cell lines (MDA-MB- 231 and MCF-7)**.** The cells were treated with BEPANT-6 concentrations (from 1 to 20 µM) and 5-FU concentrations (from 1 to 200 µM) for 48 h, and we observed a dose-dependent response to the treatments**.** The BC cell lines (MDA-MB-231 and MCF-7) viability was reduced by 42% and 39% when treated at the lowest doses at the concentration (1µM) of BEPANT-6 respectively, and 5-FU treatment at the concentration (1µM) do not affect cell viability (Fig. 2B-D). Also, a dose-dependent analysis was performed on the BC cell lines treated with BEPANT-6 and 5-FU, and the results showed IC/EC50 values for MDA-MB-231were 2.2µM and 52.2µM, while MCF-7 IC/EC50 values were 2.4µM and 57.8µM respectively (Fig. 2E-I).

Overall, the results showed a significant effect of BEPANT-6 on the BC cell lines (MDA-MB-231 and MCF-7) viability and proliferation from considerably low concentrations compared to 5-FU (Fig. 2A, ***: $p < 0.001$). Therefore, the results reveal that BEPANT-6 could overcome the biological challenges of 5-FU, reduce systemictoxicity and drug resistance, and have an enhanced drug delivery to increase its therapeutic capability to effectively mitigate BC cell lines proliferation and aggressiveness, especially in MDA-MB-231 that is highly metastatic triple-negative BC (TNBC) cell line, and MCF-7 is a luminal BC cell line correlates with previous studies (21, 29, 31, 51).

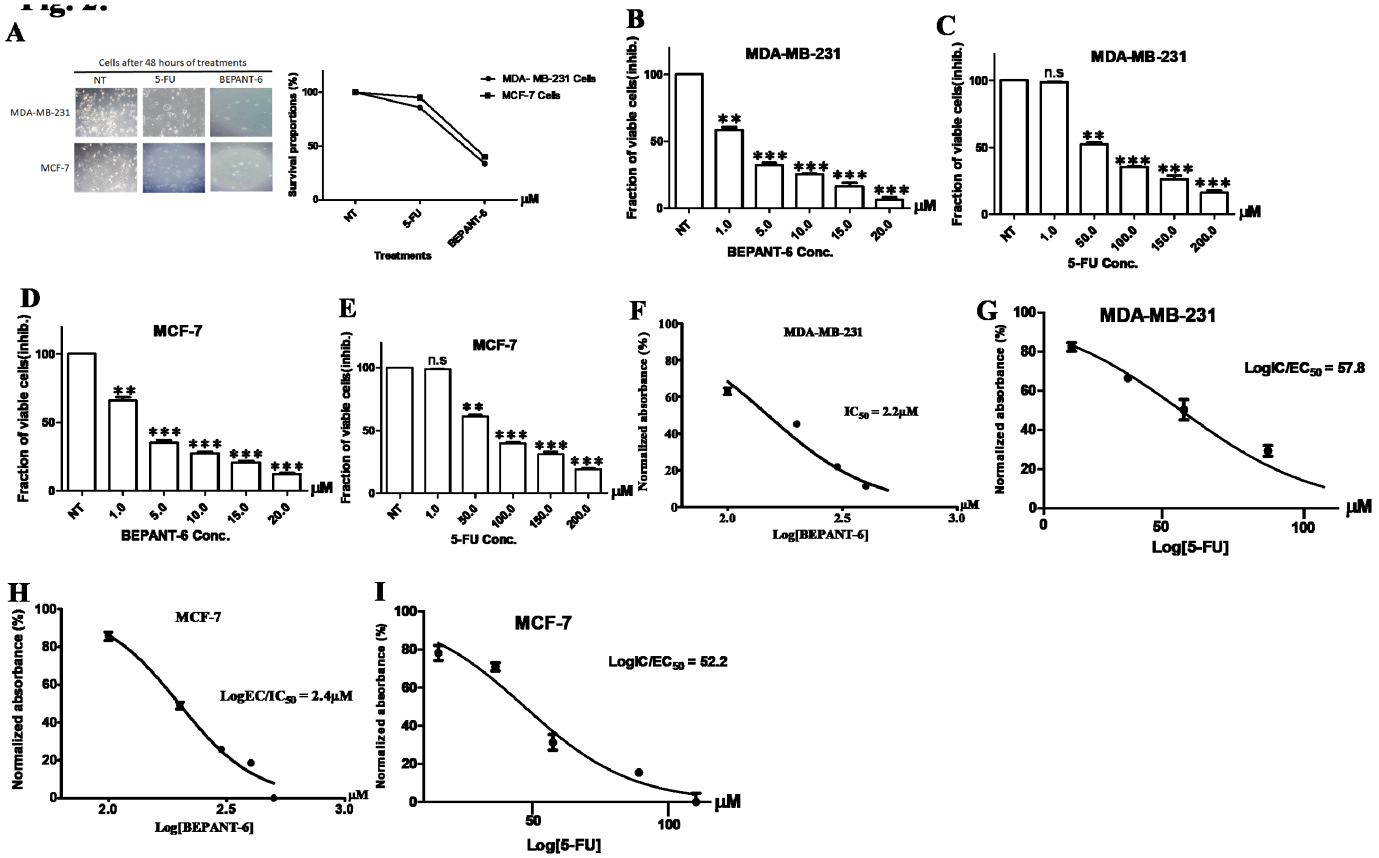

Fig 2: BEPANT-6 nanoparticle effectively mitigates BC cell proliferation. (A) The micrograph view and graphical representation of BEPANT-6 and 5-FU therapeutic effect on BC cell lines (MDA-MB-231 and MCF-7)invasion for 48h show BEPANT-6 have a significantly enhanced effect on the cell's invasiveness than 5-FU(***: p <0.001). **(B-E)** Graphical representation of BEPANT-6 nanoparticle and 5-FU therapeutic effect in increasing concentrations (1 to 20µM), and (1 to 200µM) on MDA-MB-231 and MCF-7cells viability for 48h, show BEPANT-6 have increased significant effect on the BC cells at lower concentrations than 5-FU. **(E-I)** Calculated log. of BEPANT-6 and 5-FU IC_{50} values on MDA-MB-231 and MCF-7 cells were 2.2µM and 2.4µM, and 52.2µM, and 57.8µM respectively. Experimental analysis was in triplicates and statistical values were represented *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

BEPANT-6 treatment down regulates the expressions of Menin and ETV7oncogenic involvement in the survival of BC cells

To decipher the molecular mechanism that underpins the action of BEPANT-6, we examined the effect on Menin and ETV7 levels of expression and molecular factors known to be associated with increased invasiveness and metastasis of BC cells. They play crucial roles in regulating survival gene transcription linked with cancer stemness and progression and promote oncogenic pathways through the mechanism of cellular resistance to apoptosis induced by anticancer treatments such as chemotherapy (43, 47, 48, 52). Thus, in interrogating the protein levels of Menin and ETV7 expression andtheir interaction in BC cell lines MDA-MB-231 and MCF-7 after BEPANT-6 treatment usingwestern blot (WB) and immunoprecipitation (IP) analysis. Moreso the level of mRNA expression in the BC cell lines was analyzed using the RT-PCR method. In addition, using WB analysis determines the effect of BEPANT-6 on the protein expression levels of PI3K,pAKT, and mTOR (i.e., PAM signaling pathways) involved in BC cellular survival.

The concentrations of MDA-MB-231 (2.2µM) and MCF-7 (2.4µM) for BEPANT-6 treatmentand at the concentrations of 52.2µM and 57.8µM for 5-FU treatment of MDA-MB-231 andMCF-7 cells respectively were used to analyze the therapeutic effect on Menin and ETV7 expression using western blot method. We observed a significant inhibition and suppression of Menin and ETV7 expressions when treated *in vitro* with BEPANT-6 and 5-FU compared with the non-treated controls in MDA-MB-231 and MCF-7 cells (Fig. 3A). Furthermore, BEPANT-6 treatments show a better inhibition effect on Menin and ETV7 than 5-FU treatments with 30-40% (Fig. 3A, *: $p < 0.05$**)**. Therefore, the significant difference in the inhibitions of BEPANT-6 compared to the 5-FU, suggests that BEPANT-6 has an increased inhibitory effect and possibly higher molecular half-life on the BC cells. Thus BEPANT-6 have shown an enhanced therapeutic efficiency on the BC cell lines in lower concentrations while minimizing side effects and could overcome the biological challenges such as poor bioavailability, rapid degradation, systemic cytotoxicity, and drug resistance that are commonly linked with 5-FU (24,53). Moreover, results for the interactions between Menin and ETV7 were significantly disrupted by BEPANT-6 on both BC cell lines (Fig. 3B-C, ***: $p < 0.001$**)**, while the resultsof BEPANT-6 on mRNA expressions showed a significant decrease of Menin and ETV7 levels of mRNA expressions in MDA-MB-231 and MCF-7 cells with 57.3% and 59.7%, and 70.5% and 67.9% reductions respectively compared with the non-treated controls (Fig. 3D-E,**:$p<0.01$,***:$p<0.001$). Additionally, the results show a significant effect of BEPANT-6 on mTOR, PI3K, and pAKT levels of expression on MDA-MB-231 and MCF-7 cells (Fig. 3F, **: $p<0.01$, ***: $p<0.001$). Therefore, the results suggest that BEPANT-6 nanoparticles could target, disrupt, and mitigate Menin and ETV7 transcriptional and oncogenic activity, downregulate the transduction of the regulation proteins PI3K, pAKT and mTOR phosphorylation, and drug resistance mechanisms, thus inducing

apoptosis and reducing BC cells proliferation, invasiveness and metastatic potential (31,32,43,49,52,54). Overall, Menin and ETV7 interaction and expression create a synergistic effect and transcriptional amplification of genes that enhance the invasiveness of BC cells and BEPANT-6 disrupt Menin and ETV7 interactions and expression, dephosphorylate the regulation proteins to control the PAM signaling pathway, and decrease the levels of mRNA expression impair the transcription of gene expression involved in chemoresistance, metastasis, and cell survival (54-59).

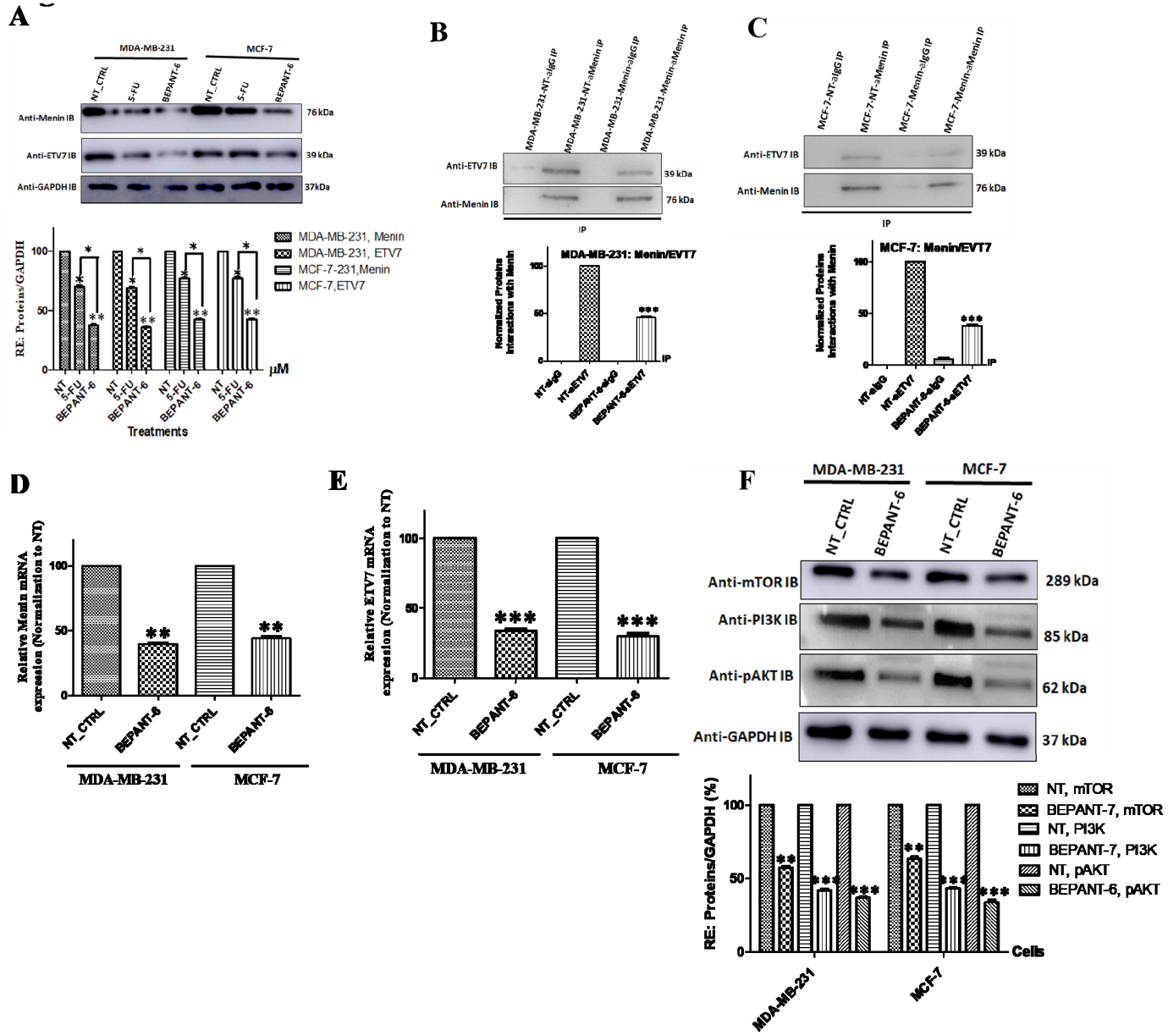

Fig. 3: BEPANT-6 treatment downregulates the expressions of Menin and ETV7 oncogenic involvement in the survival of BC cells. (A) BEPANT-6 and 5-FU inhibitory impact on Menin and ETV7 expressions. Using western blot analysis, graphical relative protein quantification was normalized with GAPDH on MDA-MB-231 and MCF-7 cells, showing BEPANT-6 superior significant effect on target proteins Menin and ETV7 expressions. **(B-C)** BEPANT-6 disruption of Menin and ETV7 interactions in MDA-MB-231 and MCF-7 cells using immunoprecipitation (IP) analysis. Graphical representations of results show significant disruption of Menin and ETV7 interactions. **(D-E)** The use of RT-PCR analysis on RNA levels of expression of Menin and ETV7 in MDA-MB-231 and MCF-7 cells. The graphical representation of the results revealed significant impairment of Menin and ETV7 expressions normalized with GAPDH in the BC cell lines (MDA-MB-231 and MCF-7).**(F)** BEPANT-6 nanoparticles decrease the transduction of PI3K, pAKT, and mTOR expression levels in MDA-MB-231 and MCF-7 cells. Using western blot analysis and graphical representation reveals protein quantification normalized with GAPDH. Experimental analysis wasdone in triplicates and statistical values represented *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

BEPANT-6 delay metastasized breast tumor progression with better efficacy than ordinary 5-FU in preclinical mouse models

To determine the therapeutic effect of BEPANT-6 treatments on breast xenograft tumor growth, 6-weeks-old female Bal-C Nude mice were inoculated with highly metastasized MDA-MB-231 cells, a triple-negative breast cancer cell line to form a tumor growth ($100mm^3$). The mice were 9/group and treated with BEPANT-6 and 5-FU (2mg/kg) with a corresponding not-treated control for 5-weeks, and observed without treatments fromweeks 6 to 8, to determine tumor regeneration. The *in vivo* treatments of BEPANT-6 and 5-FU on the mice demonstrate a significant delay in tumor growth after week 3 oftreatment compared to the control.

The results show that BEPANT-6 therapeutic effect ontumor growth is significantly more potent by 45% than 5-FU. The xenograft measurement (cm) shows a reduction of size in 5-FU and a significant decrease in tumor size, with no regeneration of tumor growth (weeks 6 to 8) in BEPANT-6 treatments (Fig. 4A *P<0.05; ***P<0.001). In the *ex vivo* Ki-67 proliferation analysis on the xenografts, the micrograph results show a significant reduction of proliferating cells in both BEPANT-6 and 5-FU treatments compared to the controls (Fig. 4B). The result showed that BEPANT-6 treatment has substantially reduced proliferating cells than 5-FU, which suggests that BEPANT-6 treatment has more significance in inducing apoptosis. These results further potentiate BEPANT-6 an improved 5-FU delivery chemotherapy agent with enhanced therapeutic efficiency to inhibit BC cell proliferation and induce apoptotic deathto mitigate TRBC growth (4, 21, 29, 31, 35, 51).

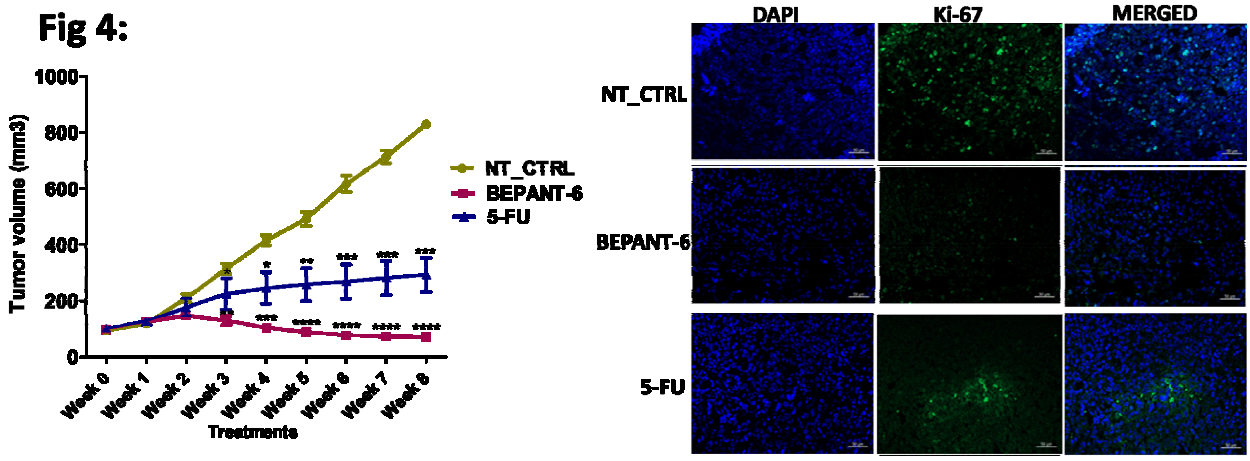

Fig. 4: BEPANT-6 nanoparticles delay metastasized breast tumor progression with better efficacy than ordinary 5-FU in preclinical mouse models (A) *In vivo* experiment, MDA-MB-231 cells inoculated on 6-weeks-old male Bal-C nude mice and after two weeks of inoculation with tumor growth of 100mm^3, mice were treated with BEPANT-6 and 5-FU twice a week for four weeks on nine (9) mice for each condition and controls. The tumor growth and xenograft size were compared with the controls. Calculated statistical representation of tumor volume per week for 5- weeks and the regeneration observation in weeks 6 to 8 after both treatments. There was a significant delay in tumor growth in both BEPANT-6 and 5-FU treatment compared to the controls. BEPANT-6 treatment significantly delayed tumor growth than 5-FU treatments**.** Also, there was no regeneration of tumor growth in BEPANT-6. **(B)***Ex vivo* analysis of Ki 67 proliferation testing, DAPI was used to stain the nucleus of the cells that represent the bluish color, and Ki 67 binds to the proliferation cell's nucleus represented with the greenish color. The results show a significant reduction of proliferating cells in BEPANT-6 and 5-FU treatments compared to the control, which signifies there is induction of tumor apoptotic cells in the BEPANT-6 and 5-FU treatments compared to the control. The BEPANT-6 treatments are more effective than the 5-FU treatment.

DISCUSSION

Breast cancer treatment remains a clinical challenge, especially the aggressive and metastatic cases due to the cancer cell's ability to evade cell death and unchecked proliferation. A classic chemotherapeutic agent 5-FU with the potential to mitigate BC progression can disrupt DNA synthesis via thymidylate synthase (TS) inhibition leading to cell cycle arrest and inducement of apoptosis. However, 5-FU poor biological potency limits its therapeutic efficacy, and thus needs improvement to overcome these challenges.The nanoparticle-enhanced intracellular delivery of 5-FU such as BEPANT-6 will increasethe therapeutic efficiency in suppressing and downregulating oncogenic drivers, which promotes BC cell proliferation and survival for managing progression and metastasized BC to be sensitive to treatment (21,51,53).

However, previous findings have shown oncogenic drivers Menin and ETV7 play a criticalrole in BC progression. Menin and ETV7 are transcriptional regulators integral to BC cells'progression and survival. The transcriptional regulation functions of these genes are pivotal in the cancer cells maintaining proliferation, thus Menin and ETV7 that are transcriptional factors influence the increased expression of oncogenes and anti- apoptotic proteins such as Bcl-2, and downregulate pro-apoptotic proteins/factors such as BAX to promote cell cycle progression via supporting resistance to apoptosis and influence genes to drive G1-phase transition of the cell cycle to enhance cellular survival pathways and effectively prevent BC cells to chemotherapeutic-induce cell death (42, 55, 56, 57).

Menin and ETV7 proteins are associated with cellular survival pathways, (such as transduction of PAM signaling pathways) that enhance tumor proliferation, aggressiveness, invasiveness, and therapy resistance. Therefore, the alteration of the cellular machinery of Menin and ETV7 can potentially distort cell cycle progression and induce apoptosis of cancer cells like MDA-MB-231 and MCF-7 cell lines, leading to decreased cell/tumor viability and increased BC cell death (42,45,48,50,55-58). The heterogeneous distribution of BEPANT-6 in the BC cells enhanced the disruption of the intrinsic and extrinsic pathways, which promotes the target and inhibition of Menin and ETV7 expressions to downregulate the cyclin-dependent kinases (CDKs) and cyclins levels to prevent the BC cells especially the MDA-MB-231 cells from progressing from G1-phase to S-phase, where DNA replication occurs, thus increasing the proportion of BC cellcycle arrest in the G1-phase**,** which also leads to enhanced BC cells sensitivity to chemotherapy agents such as BEPANT-6 , and upregulate apoptotic proteins-like BAX andp21 and activity to induce apoptotic cell death (42, 52, 55-58).

The results suggest BEPANT-6 could enhance the cytotoxic effect on BC cells that rely on Menin and ETV7 oncogenicity for survival, targeting the expression of Menin and ETV7 transcriptional and regulation to reduce drug resistance making the BC cell's sensitivity to treatment, and control metastasis of BC cells potential to spread, thus improving patient outcomes (31, 52, 58, 59). The results shown indicate BEPANT-6 that is formulated with an adjustable pharmaceutical dose of 5-FU and specific ligands or antibodies to improve pharmacokinetics (i.e. the circulation extension time and enhanced permeability and retention effect), targeted delivery, and control release to enhance its accumulation in tumor tissues to target receptors that are overexpressed, reduce the off-target effects, and increase the local concentration in tumor cells for consistent delivery and therapeutic efficiency to mitigate cancer cells proliferation and progression of BC cells invasiveness (29, 53, 56-59). Overall, Menin and ETV7 are implicated in promoting oncogenic pathways by the processes of histone methylation interactions, cellular apoptotic resistance, and chemotherapy, influencing cancer stemness and epithelial-mesenchymal transition (EMT), and supporting invasiveness and metastasis of cancer cells. Additionally, Menin has a transcriptional co-activator role that may affect ETV7 transcriptional activity, thus Menin and ETV7 interaction has been suggested to regulate key pathways that promote BC progression. Moreover, the disruption of Menin and ETV7 interaction distorts the chromatin environment and accessibility of target genes to promote BC cell invasiveness(55, 56, 57, 59). BEPANT-6 down regulation of Menin and ETV7 expressions and their abilityto co-regulate genes could degrade and impair the transcription of metastasis-associatedgenes that support metastasis leading to the reduction of matrix metalloproteinases (MMPs) expression and the inhibition of angiogenesis that allows cancer cells to invade surrounding tissues (55-59). Therefore, BEPANT-6 targeting and down regulating Menin and ETV7 expression, disrupting the transduction of PAM's signaling pathway is a promising approach to mitigate BC cell and the metastatic phenotype progressions (Fig.5).

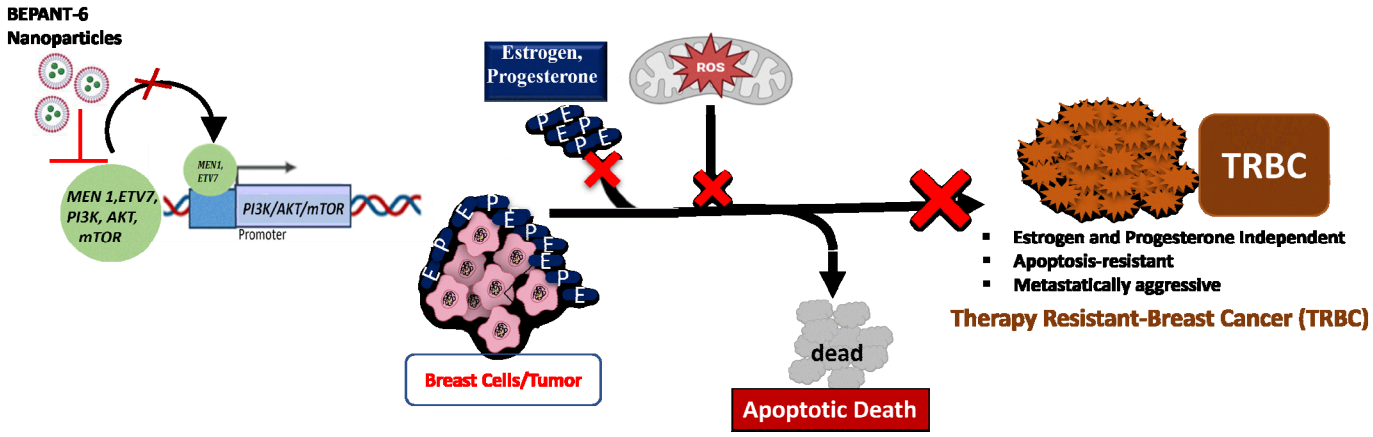

Fig.5: Schematic representation showing BEPANT-6 therapeutic efficiency to downregulate the increased expressions and impact of Menin and ETV7 transgenetic (transcriptional) regulation via altering the hyper activation of PAM transduction signaling pathway tocross-talk with other BC cell signaling networks to underpins the development of treatment resistance phenotypes and promoting BC cell survival, growth, and progression. Hence, the therapeutic efficiency of BEPANT-6 could inhibit the expressions of Menin, ETV7, and PAM, and impair elevated levels of estrogen and progesterone supplyand mitochondrial functions for cellular survival and progression in BC. The BEPANT-6inhibitions and therapeutic efficacy promote the disruption of cell division and induce apoptosis to mitigate metastasis and therapy resistance to potentiate the sensitivity of BC cells to chemotherapeutic treatment.

Conclusion

BEPANT-6 superior efficacy over 5-FU enhanced drug delivery and overcome resistance mechanisms to mitigate and downregulate the molecular action that underpins Menin, ETV7, and PAM's oncogenic involvement in BC cells therapy resistance, metastasis, and invasiveness, represents a significant advancement in targeted cancer therapy. Therefore, BEPANT-6 offers a new therapeutic intention that could potentiate an effective treatment sensitivity of BC therapy-resistant, aggressive, and metastasized phenotypes, as a promising chemotherapy agent to improve patient outcomes.

MATERIALS AND METHODS

Synthesis of BEPANT-6 Gold nanoparticle

BEPANT-6 is derived from the phosphonium group and is formulated with a pharmacology adjustment dose of 5-FU synthesized with gold nanoparticles to form BEPANT-6 gold nanoparticles. The stock of BEPANT-6 gold nanoparticle concentration of10mM was dissolved and diluted in phosphate-buffered saline (PBS) to the working concentrations used for the experiments.

Cell Lines and Cell Culture

The androgen-independent breast cancer cell line MCF-7, androgen-dependent breast cancer cell line MDA-MB-231 were purchased from the American Type Culture Collection(ATCC, Manassas, VA, USA) and maintained in Nitrogen oxide (NO2). Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI 1640) from (Life Technology, Inc., Saint Aubin, France) supplemented with 10% fetal bovine serum (FBS) from (Invitrogen, Paisley, UK) were used to cultivate MCF-7 and MDA-MB-231 cell lines at 37 ◦ C in 5% CO2. Trypsin-EDTA for trypsinization was purchased from

Gibco (Gaithersburg, MD, USA), and harvested cells were washed with 1% phosphate-buffered saline (PBS) purchased from WELGENE, Inc. (Gyeongsan-si, Gyeongsangbuk-do, Korea).

Treatment of BC cell lines MDA-MB-231 and MCF-7 with BEPANT-6

BC (MDA-MB-231 and MCF-7) cells were seeded into different culture-well plates according to the experiments to be carried out. After 24 h, the cells were treated with BEPANT-6, according to our previous experiment (35). The effects of the treatment with BEPANT-6 were analyzed after 48 h compared with the control(s).

Inhibition of BC cell viability and proliferation Assay

BC cell viability and proliferation were performed according to our previous protocol (35)

Immunofluorescence Imaging

To observe, BEPANT-6 nanoparticles heterogeneous distribution on the BC cell lines (MDA-MB-231 and MCF-7 cells (1 x 10^6)) were seeded in flat bottom Petri-dishes containing cover glasses covered. After 24 h, the cells were treated with BEPANT-6 for 48h. After incubation, the cells were washed with phosphate-buffer saline 1X (PBS1X) and fixed with formaldehyde 4% (Thermo Fisher Scientific, Strasbourg, France) for 15-30' RT.The Cells are washed with PBS 1X twice and then permeabilized in PBS 1X containing 0.1% triton X-100 and blocked in PB 1X containing 1% bovine serum albumin (BSA) (Thermo Fisher Scientific, Strasbourg, France). The cells were incubated with anti-α- tubulin primary antibody (1:500 dilution in 1% BSA-PBS, Sigma-Aldrich Inc) in combination with anti-mouse secondary antibody conjugated with Alexa Fluor 488 (1:1000 dilution in 1% BSA-PBS, Thermo Fisher Scientific, Strasbourg, France). The BC cells nuclei are counterstained with µg/ml dilutions of 4',6-diamidino-2-phenylindole (DAPI) solution (Thermo Fisher Scientific, Strasbourg, France) at RT for at least 10'. The cover slides containing the cells were mounted on glass slides using Prolong Gold anti- fade reagent (Life Technologies, Villebon-sur-Yvette, France) and were kept away from light rays to dry in RT. Zeiss 510 META fluorescence confocal microscope plan 40×/1.4 (Carl Zeiss, Paris, France) was used to capture images.

Cell Cycle and Apoptosis Analysis

The cytostatic effects and induction of apoptosis of the BEPANT-6on BC (MDA-MB-231 and MCF-7) cells were performed using the Phosphate H-3 and PI staining method for cell cycle assay and the APC Annexin V/PI staining method for apoptotic assay, respectively. Both analyses were read with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and Cell Quest (BD Biosciences, San Jose, CA, USA) as previously performed (35).

Western Blot and Immunoprecipitation Analysis

The therapeutic effect of BEPANT-6 on the target proteins' expression levels and interactions on BC cell lines (MDA-MB-231 and MCF-7) was performed using western blot (WB) and immunoprecipitation (IP) analytic methods (35). The following primary antibodies were used: rabbit Menin (anti-*MEN1*) antibody (Assay Designs, Villeurbanne, France, 1/10000), rabbit ETV7 (anti-ETV7) antibody (Cell Signaling Technology, 1/1000, Massachusetts, USA), rabbit antibody Caspase-3 (Cell Signaling Technology, 1/1000, Massachusetts, USA), rabbit Poly(ADP-ribose) polymerase (anti-PARP) antibody (Cell Signaling Technology, 1/1000, Massachusetts, USA), rabbit Bcl-2 antibody (anti-Bcl-2) (Cell Signaling Technology, 1/1000, Massachusetts, USA), rabbit BAX antibody (anti-BAX) (Cell Signaling Technology, 1/1000, Massachusetts, USA),rabbit pAKT antibody (anti-pAKT) (Cell Signaling Technology, 1/4000, Massachusetts, USA), rabbit mTOR antibody (anti-mTOR) (Santa Cruz, 1/2000, Heideberg, Germany), rabbit p21 (anti-p21) antibody (Cell Signaling Technology, 1/1000, Massachusetts, USA), rabbit PI3K antibody (anti- PI3K) (Cell Signaling Technology, 1/2000, Massachusetts, USA), mouse anti- glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibody (Santa Cruz Biotechnology, Heidelberg, Germany 1/5000.) as an internal control, anti-rabbit IgG HRPconjugate antibody (Santa Cruz Biotechnology, Heidelberg, Germany, 1/5000), and anti- rabbit True blot IgG HRP conjugate antibody (eBiosciences, 1/1000, Villebon-sur-Yvette,France), rabbit anti-IgG (ThermoFisher Scientific, USA), True blot anti-rabbit Ig IP beads(eBiosciences, Paris, France), and protein sample buffer (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's protocols. Re-blot Plus Mild Solution (Millipore,Molsheim, France) was used for membrane stripping for 10 minutes at RT.

Messenger RNA (mRNA) Quantitative Real-time RT-PCR Analysis

Total RNA was extracted from harvested MDA-MB-231 and MCF-7 cells treated with BEPANT-6 nanoparticles for 72 h using previous protocols (35).

Tumor Evaluation with *in vivo* and *ex vivo* experimental analysis

For the *in vivo* study, MDA-MB-231 cells were inoculated subcutaneously into 6-week-oldBAL C Nude mice (NOD SCID) through a 27-gauge needle under halothane anesthesia. Themice were treated with 2mg/kg of BEPANT-6 and 5-FU for 5-weeks and further observed for regeneration for weeks 6 to 8. The tumor growth and Ki-67 proliferation analysis to determine the level of proliferating cells, using previous protocols (35).

Statistical Analysis

Data shown in the figures are statistically analyzed using Graph Pad Prism v 6.0 software,Excel, Image J, and R-programming, and either representative experiments or the mean +/− SEM (standard error of the mean) represents results. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, and ****: $p < 0.0001$were considered significant.

Interest Declaration

All authors declared no conflict of interest

Contributions of Authors

J.O. conceived and wrote this project; All authors were involved in analyzing theexperiments; Authors proofread and reviewed the manuscript before submission.

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