Autophagy inhibition synergistically enhances anti-cancer efficacy of RAMBA, VN/12-1 in SKBR-3 cells and tumor xenografts

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Running Title: Efficacy modulation of RAMBAs by autophagy inhibition.

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Njar VCO holds an ownership interest in the RAMBAs patents and technologies thereof. The other authors declare no potential conflict of interest.

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Abstract

VN/12-1 is a novel retinoic acid metabolism blocking agent (RAMBA) discovered in our laboratory. The purpose of the study was to elucidate the molecular mechanism of VN/12-1’s anticancer activity in breast cancer cell lines and in tumor xenografts. We investigated the effects of VN/12-1 on induction of autophagy and apoptosis in SKBR-3 cells. Further, we also examined the impact of pharmacological and genomic inhibition of autophagy on VN/12-1’s anti-cancer activity. Finally, the anti-tumor activity of VN/12-1 was evaluated as a single agent and in combination with autophagy inhibitor chloroquine (CHL) in an SKBR-3 mouse xenograft model. Short exposure of low dose (< 10 μM) of VN/12-1 induced endoplasmic reticulum stress (ERS), autophagy and inhibits G1-S phase transition and caused a protective response. However, higher dose of VN/12-1 initiates apoptosis \textit{in vitro}. Inhibition of autophagy using either pharmacological inhibitors or RNA interference of Beclin-1 enhanced anti-cancer activity induced by VN/12-1 in SKBR-3 cells by triggering apoptosis. Importantly, VN/12-1 (5 mg/kg twice weekly) and the combination of VN/12-1 (5 mg/kg twice weekly) + chloroquine (50 mg/kg twice weekly) significantly suppressed established SKBR-3 tumor growth by 81.4% (p < 0.001 vs. control) and 96.2% (p < 0.001 vs. control), respectively. Our novel findings suggest that VN/12-1 may be useful as a single agent or in combination with autophagy inhibitors for treating human breast cancers. Our data provides a strong rationale for clinical evaluation of VN/12-1 as single agent or in combination with autophagy inhibitors.
Introduction

Breast cancer is the most common neoplasia in women. Despite significant advances in treatment, breast cancer remains incurable due to emergence of alternative pathways adopted by cancer cells to overcome the effects of anti-cancer therapy. To overcome this issue, a rational approach would be to concomitantly target clinically relevant cellular abnormalities with combination therapy or to use a potent multi-targeted agent. Mounting evidence indicates that the anti-tumor effects of all-trans retinoic acid (ATRA) are attributed to its ability to interfere with multiple facets of oncogenic signaling pathways (1, 2). Moreover, ATRA proved to be an effective anticancer agent to treat hematological cancers (3). However, several factors compromise the widespread clinical use of ATRA. They include low in vitro anticancer potency, limited bioavailability and unfavorable pharmacokinetic behaviors due to rapid metabolism by CYP26 enzymes (4, 5). Consequently, the structural modification of ATRA to develop novel retinoic acid metabolism blocking agents (RAMBAs) with improved potency and metabolic stability has been the focus of our group for many years. Our RAMBAs are considered to be atypical, because in addition to being potent inhibitors of ATRA metabolism, they also possess potent intrinsic multiple anti-cancer activities.

4-(±)-(1H-imidazol-1-yl)-(E)-retinoic acid (VN/14-1) and its corresponding methyl ester, VN/12-1 (Figure 1A and Supplementary Figure S1) are amongst our lead RAMBAs (6). Previously, we had reported that flow cytometric analysis of breast cancer cells treated with our RAMBAs revealed significant growth arrest with only weak apoptosis (7), a phenomenon also reported for a variety of retinoids (8). In an effort to search for strategies that could enhance cancer apoptosis mediated by RAMBAs, we looked for possible pro-survival pathways that may be activated in response to RAMBAs. VN/12-1 does not bind to or transactivate retinoic acid.
receptors (RARs) (7) and its exact molecular target (other than CYP26 inhibition) is yet to be identified. Herein, we report the induction of pronounced autophagy by VN/12-1-treated SKBR-3 cells and tumors.

Autophagy is an intracellular process in which proteins and cytoplasmic organelles are degraded (9). It has been implicated in various physiological processes such as survival in stress, response to starvation and pathogenesis (10, 11) and a mechanism of cell protection against drug-induced apoptosis (12-14). It is now generally accepted (15) that the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (ER) results in cellular stress that initiates a specialized response, designated ER stress response (ERS). In most cases, induction of cellular ERS subsequently leads to activation of autophagy (16, 17).

While the estrogen receptor-alpha (ER-α) proficient breast tumors respond to several therapies, ER-α deficient tumors are less sensitive partly due to activation of growth factor signaling pathways such as human epidermal growth factor receptor-2 (Her-2) (18). Therefore, there is an urgent need for development of effective therapy against this type of breast cancer. In this study, we primarily focused on an ER-α deficient cell line which overexpresses Her-2 (SKBR-3 cells). We note that SKBR-3 xenograft model is one of the less commonly investigated model because of reported difficulty of SKBR-3 cells to form tumors in nude mice (19). However, a few research groups have previously successfully used this xenograft model in SCID mice (20, 21).

The current study evaluated the potency and anti-tumor activity of VN/12-1, as a single agent and in combination chloroquine (CHL) (Figure 1A) (15), an autophagy inhibitor that blocks lysosome acidification and autophagosome degradation. We show that VN/12-1 has dual effects on the cancer cells based on the dose. Low dose (< 10 μM) of VN/12-1 induces
-autophagy, ERS and cell cycle arrest as an immediate protective response in SKBR-3 cells and xenograft tumors. Importantly, we demonstrate that inhibition of autophagy using either pharmacological inhibitors (e.g. CHL) or RNA interference of essential autophagy gene Beclin-1 potentiates apoptotic cell death induced by VN/12-1. Notably, VN/12-1 produced impressive tumor inhibitory effects when used alone or in combination with CHL (p < 0.001).

Methods and Materials

Cell culture, cell growth inhibition, western blot, Annexin FITC, electron and immunofluorescence microscopy, siRNA, statistical analysis, in vivo studies See Supplementary Information Materials and Methods

Chemicals and reagents: ATRA and 4-hydroxytamoxifen (Figure S1) were purchased from LKT Laboratories Inc. (St Paul, MN, USA), while VN/12-1, other RAMBAs, letrozole (Figure S1) (22) and 3-methyl adenine (3-MA) (23) were synthesized in our laboratory. Chloroquine (15) was purchased from Sigma Aldrich.

Cell growth inhibition (MTT colorimetric assay): MTT (Sigma Aldrich) assay was performed as previously described (24).

Cell cycle analysis: Cell cycle analysis was performed using method a previously described method (24).

In vivo tumor growth

All animal studies were performed according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University. Female SCID mice were obtained from the National Cancer Institute (Fredrick, MD). The compounds used in this study and their doses are as shown in Figure 5A. See supplementary section for details.
Results

**VN/12-1 inhibits the growth of Breast Cancer Cell Lines**

To determine if VN/12-1 exerts a growth inhibitory effect, MCF10A, MCF-7, SKBR-3 and MDA-MB-231 cells were treated with VN/12-1 and cell viability was analyzed using MTT assay (Figure 1B; Supplementary Table S1). VN/12-1 did not induce any significant growth inhibitory effects in immortalized non-cancerous MCF10A cell line. However, it was quite potent against the cancer cell lines with IC$_{50}$ in the low µM range. Clinically used anti-cancer drugs ATRA, 4-hydroxytamoxifen (4-OHT) and letrozole also effectively inhibited the growth of MCF-7 cells (Supplementary Table S1). However, their potencies were significantly less than that of VN/12-1 against SKBR-3 and MDA-MB-231 cells.

**Short exposure of VN/12-1 to breast cancer cells does not reduce cell viability**

Cancer cells have fundamental differences in their metabolism and sensitivity to preconditioning, autophagy and apoptosis, so the dosage and duration of treatment with the therapeutic agents is important. Typically, short incubation time of treatment induces a protective mechanism by way of autophagy (25). To test this, MCF-7, SKBR-3 and MDA-MB-231 cells were treated with 10 µM VN/12-1 and the compound was washed away at various time points (see Figure 1C). The cells were then maintained in regular growth media until the endpoint of the MTT assay. As shown in Figure 1C, continuous exposure to VN/12-1 for at least 24-36 hours was needed to reduce the cell viability by ~20% for all the cells tested (p < 0.05). This indicates that the induction of growth inhibitory effect of VN/12-1 is time dependent. Collectively, these data also suggest that the cells treated with VN/12-1 for a shorter time (< 36 hours) manage to counter the growth inhibitory effect of VN/12-1, possibly, by a protective mechanism.
**VN/12-1 induces endoplasmic reticulum stress (ERS)**

Many cancer therapies including retinoids are known to induce ERS in various cancers (26-28). The transduction of ERS is mediated by the binding of the chaperone BiP (binding immunoglobulin protein) to the misfolded proteins and its concomitant dissociation from PERK (protein kinase RNA-like endoplasmic reticulum kinase) (29). This dissociation results in its activation and subsequent initiation of a cascade of downstream signals (e.g. attenuation of protein translation by inhibition phosphorylation of eIF2) that ultimately aim to preserve cellular homeostasis facilitating cell survival. Prolonged ERS however, beyond levels at which cellular homeostasis can be maintained, becomes proapoptotic by upregulating CHOP (CCAAT-enhancer-binding protein homologous protein) and IRE1-α (17, 30). To test the role of ERS, western blot analysis was performed on SKBR-3 lysates treated with VN/12-1 for 6 and 24 hours. As shown in **Figures 1D (a) and 1D (b)**, there was an upregulation of eIF2α phosphorylation (Ser51), and BiP in response to VN/12-1 within 6 hours which was further enhanced in 24 hours. PERK phosphorylation remained unchanged (data not shown). These data show that induction of ERS is a concentration-dependent early event (i.e. after 6 hours) following VN/12-1 treatment.

**VN/12-1 induces autophagy and inhibits G1-S phase transition**

Autophagy represents a failed attempt to adapt to stress and survival (31). ER stress can also trigger autophagy (16, 17). The observation that VN/12-1 mediated induction of ER stress and modest anti-cancer activity following shorter treatment of VN/12-1 led us to hypothesize that SKBR-3 cells undergo a protective response, possibly autophagy, at lower µM concentrations of VN/12-1 or upon short exposure.
To better understand the morphologic changes induced by VN/12-1, transmission electron microscopy was performed on SKBR-3 cells treated with vehicle control (Figure 2A) or VN/12-1 (10 μM) for 24 hours (Figures 2B (a), 2B (b) or 2B (c)). There was an abundance of autophagosomes in the images from the treatment group. VN/12-1 treatment caused nucleolar disruption, cytoplasmic vacuolization (Figure 2B (a)), distortion of mitochondrial shape and dilated ER (an evidence of ERS) (Figure 2B (b)). Numerous autophagosomes in various stages of maturation containing cellular organelles were also noted (Figure 2B (c)). Another efficient way to confirm autophagy is by measuring lapidated form of LC3 (LC3B) which is formed from the LC3 a microtubule associated protein (32). On the fluorescence microscopy, the presence of autophagosomes is indicated by punctate pattern of LC3B staining unlike uniform staining in the absence of autophagy (32). On fluorescence microscopy, 20 μM treatment of VN/12-1 for 24 hours increased the number and size of punctate LC3B aggregates (Figure 2C (b)) compared to control (Figure 2C (a)). Collectively, these studies showed that VN/12-1 can induce morphologic features of autophagy in SKBR-3 cells.

In order to confirm the upregulation of biochemical markers, immunoblots were performed to probe various autophagy markers. CHL inhibits the fusion of autophagosomes with lysosomes and thus leads to accumulation of lapidated form of LCB (LC3B) (33). Following 24 hours incubation, the cells treated with VN/12-1 demonstrated a dose-dependent upregulation of LC3B (Figure 3A (a)). Nevertheless, there is a need to further discriminate between 2 physiologically distinct scenarios—increased autophagic flux without impairment in autophagic turnover versus impaired clearance of autophagosomes (31). For these studies, we next exposed the cells to VN/12-1 in the presence of CHL (a weak lysosome stabilizing base). Co-treatment with CHL resulted in enhancement of LC3B band (Figure 3A (b)). Beclin-1 is an important
autophagy related protein and its upregulation correlates with induction of autophagy (34). Both, dose dependent (Figure 3A (a)) and time dependent (Supplementary Figure S2A) Beclin-1 upregulation in western blots further supported our premise of autophagy induction by VN/12-1.

Under various conditions, an inverse relationship is found between the percentage autophagy and the degree of phosphorylation of p70S6 kinase and Akt, key proteins in Akt-mTOR pathway (35-37). In our studies, a dose-dependent downregulation of phosphorylation of p70S6K (Thr389) and Akt (S473) following VN/12-1 treatment was evident which correlates well with induction of autophagy (Figure 3A (a)). Following the addition of CHL to VN/12-1, there was an abolishment of reversible ERS marker, BiP and induction of the irreversible ERS markers- IRE1-α and CHOP (Figure 3B). This indicates that the addition of CHL to VN/12-1 pushes the cells from reversible ERS to irreversible ERS which is a well-known trigger for apoptosis (29). Thus, our data suggest that VN/12-1 induced autophagy due to enhanced induction rather than decreased clearance. Upregulation of LC3B and abolishment of AKT phosphorylation and p70S6K phosphorylation were also evident in MCF-7 cells (Supplementary Figure S2B) suggesting that VN/12-1 mediated autophagy is not cell line dependent. Basal level of autophagy as indicated by LC3B formation was evident in the untreated MCF-7 cells.

Since autophagy represents a phase of dormancy or arrest, we expected VN/12-1 to have negative effect on cell cycle proteins such as cyclin D1. Although CHL alone did not have any major effect on cyclin D1 expression, VN/12-1 (10 μM) alone or in combination with CHL completely abolished the expression of cyclin D1 (Figure 3B). Analysis of cell cycle distribution by flow cytometry revealed that VN/12-1 treatment resulted in a significantly higher % increase in G0/G1 population after 24 hours (Supplementary Figure S2C).
Pharmacological inhibition of autophagy enhances the growth inhibitory activity of VN/12-1

To assess whether VN/12-1-induced autophagy contributed to survival response of the cells, we measured VN/12-1-induced reduction in cell viability in the presence or absence of IC_{20} concentrations of known inhibitors of autophagy: chloroquine (CHL) (15), 3-methyladenine (3-MA) (33, 38). When combined with VN/12-1 both the autophagy inhibitors enhanced the anticancer potency of VN/12-1 (p < 0.01) (Figure 3C (a) and 3C (b)). We also found that VN/12-1, combined with either CHL (Supplementary Figure 2D) or 3-MA (Supplementary FigureS2E), resulted in synergistic effects, as assessed by isobologram analysis using the median-effect principle of Chou and Talalay (39). These data suggest that VN/12-1 and autophagy inhibitors elaborate unique individual effects that are complementary when these compounds are used together.

In order to quantitatively evaluate the effect of the combination of VN/12-1 with CHL, the dose reduction index (DRI) (40) and combination index (CI) were calculated from the data (Supplementary Table S2) using Calcusyn software program at 50-95% inhibition levels of cell viability. DRI values indicate that the synergic combination can result in 2-8 fold reduction of the VN/12-1 dose and 3-9 fold reduction of CHL dose in order to achieve 50-95% cell growth inhibition respectively compared to the dose if they were used as single agent. The values of the CI suggest that the combination of VN/12-1 and CHL was synergistic at 50-95% of growth inhibition. Collectively, these results confirm that autophagy is a protective cellular response that lowers VN/12-1’s anti-cancer potency and its inhibition by pharmacological means enhances the growth inhibition by VN/12-1.
Genomic silencing of Beclin-1 enhances the growth inhibitory activity of VN/12-1

To determine if autophagy inhibition by way of silencing an important autophagy gene—Beclin-1 enhances the anti-cancer activity of VN/12-1, si-RNA technology was used. For Beclin-1, the si-RNA sequence that showed 90-100 % knockdown of the protein was selected for the studies (Supplementary Figure S2F). As expected, SKBR-3 cells treated with VN/12-1 alone showed a decrease in viability by the MTT assay when compared to the vehicle treated (no si-RNA) and scrambled sequence si-RNA (scrambled-si-RNA) controls (p < 0.05) (Figure 3D). Cells treated with scrambled-siRNA plus VN/12-1 showed no change in activity compared to VN/12-1 alone (p>0.05). In the absence of VN/12-1, siRNA targeting Beclin-1 exerted little effects on SKBR-3 cell viability (p>0.05), but the combination of VN/12-1 with si-RNA for Beclin-1 resulted in a dramatic reduction in cell viability (p< 0.0005) (Figure 3D).

VN/12-1 induces caspase-dependent apoptosis

Literature evidence suggests that the compounds that are autophagy inducers at smaller dose can induce apoptosis at higher dose (9). In many situations, autophagy accompanies rather than causes apoptosis. Immunoblots were done to determine whether VN/12-1 has apoptotic activity. The hallmarks of apoptosis, PARP cleavage and an upregulation of pro-apoptotic protein Bad were evident when VN/12-1 was combined with CHL (Figure 4A). 20 µM VN/12-1 as a single agent initiated apoptosis in cells as shown by Annexin/ PI staining. However, addition of CHL to VN/12-1 significantly enhanced the percentage of apoptotic cells (p < 0.001) (Figure 4B). Pan caspase inhibitor z-vad-fmk was used to determine if VN/12-1 mediated apoptosis was caspase dependent. Pre-treatment with 30 µM z-vad-fmk reversed the growth inhibitory effects of VN/12-1 and its combination with CHL in MTT assay (p < 0.01) (Figure 4C).
**Preliminary toxicology and pharmacokinetics (PK) of VN/12-1 in SCID mice**

Prior to assessment of the *in vivo* anti-tumor efficacy of VN/12-1, we conducted preliminary toxicity and PK studies. Preliminary toxicity studies in SCID mice showed that VN/12-1 was not toxic as a single agent or in combination with CHL in the subcutaneous (s.c.) doses 2.5 and 5 mg/kg twice a week. In addition, preliminary pharmacokinetics following administration (s.c) of 20 mg/kg of VN/12-1 showed that the peak plasma level after was 41.38 μg/ml and the mean t1/2 was 6 hours. Details of these studies will be reported in a future manuscript.

**VN/12-1 inhibits the growth of SKBR-3 xenografts**

We evaluated VN/12-1’s ability to inhibit the growth of SKBR-3 xenografts when given via s.c. administration. Mice were divided into 8 groups as detailed in the methods section. As shown in (Figure 5A (a) and 5A (b)). Geometric means of starting tumor sizes were comparable across the 8 groups and ranged from 208 to 266 mm³ (p = 0.224). Supplementary Table S3 summarizes the estimated tumor growth parameters (daily tumor growth rate and tumor doubling time), while Supplementary Table S4 shows the p-values for all the pairwise comparisons among the 8 treatment groups. Daily tumor growth was not significantly different between ATRA alone and VN/12-1 2.5 mg/kg alone (3.9% vs. 3.6%, p = 0.551), but was significantly slower with VN/12-1 5.0 mg/kg (2.2%, p = 0.001 vs. both ATRA and VN/12-1 2.5 mg/kg) (Supplementary Table S3).

The combination of VN/12-1 (either dose) with CHL slowed tumor growth compared to treatment with VN/12-1 alone (1.9% vs. 3.6% for the low dose, 1.2% vs. 2.2% for the high dose, p = 0.001 for both). The combination of VN/12-1 at 5 mg/kg with CHL was better than the combination of VN/12-1 (2.5 mg/kg) with CHL (1.2% vs. 1.9%, p = 0.086). The combination of
VN/12-1 (at either dose) with CHL was better than the combination of ATRA with CHL (p = 0.001 for both comparisons).

Representative pictures of tumors from each group are shown in Figure 5B. Figures 5C (a) and 5C (b) show the average tumor weights and the body weights of the mice in different groups respectively. As shown in Figure 5C (a), tumors in treatment groups involving the combination of VN/12-1 (either dose, 2.5 mg/kg and 5 mg/kg twice a week) and CHL had statistically significant reduction of tumor weights (p < 0.05 and p < 0.01 respectively). However, there was no significant change in body weights of mice in any group.

In summary, the growth of tumors were significantly inhibited by all treatments (Figures 5A (a) and 5A (b)), but treatments with VN/12-1 (5 mg/kg) alone and its combination with CHL were the most effective, with impressive inhibitory values of 81.4% (p < 0.001 vs. control) and 96.2% (p < 0.001 vs. control), respectively without causing any toxicity.

**Effect of VN/12-1 treatment on SKBR-3 tumor protein expressions**

Immunoblot analysis of the tumor lysates confirmed the activation of autophagic pathway as there was a rise in LC3B expression in the VN/12-1 treatment groups but not in the ATRA group. As in the *in vitro* results, addition of CHL enhanced VN/12-1 upregulation of LC3B. PARP cleavage and upregulation of pro-apoptotic Bad was noted in all the treated groups (except CHL group), more so in VN/12-1 and CHL combination groups (Figure 5D). Thus, these data identify apoptosis induction as a major mechanism underlying the ability of CHL to potentiate the anti-tumor effects of VN/12-1. There was a marked upregulation of CHOP in the groups treated with VN/12-1 alone or with CHL. This further supports *in vitro* findings of activation of ERS by VN/12-1. These data support our proposed mechanism that autophagy is the initial response of the cells to VN/12-1 treatment and inhibition of VN/12-1-induced autophagy by
CHL leads the cells to apoptotic pathway. Cyclin D1 was down-regulated in all the treatment groups except CHL (Figure 5D). Collectively, these in vivo tumor suppressive responses exhibited by VN/12-1 or VN/12-1 plus CHL strongly correlates to mechanisms identified in vitro. The overall mechanism of action of VN/12-1 and its combination with CHL in SKBR-3 human breast cancer cells is summarized in Figure 6.

**Discussion**

Because of the heterogeneous nature of breast cancer (41), combination therapies or multi-target agents are required for effective therapy. VN/12-1 has dual action on SKBR-3 cells based on the dose and duration of treatment. Shorter exposure of smaller dose induced protective effect by autophagy. VN/12-1 initiated apoptotic activity at doses higher than 20 μM in SKBR-3 cells in vitro. However, such high plasma VN/12-1 levels were not achievable in vivo without producing toxic effects in the mice. For these reasons, alternative approach that combines VN/12-1 with a second agent exhibiting a different mechanism of action was required to further improve VN/12-1’s potency and reduce its dose and yet achieve apoptosis. Importantly, immortalized breast MCF10A cells were much less sensitive to VN/12-1. This selectivity offers VN/12-1 a clear advantage over many other compounds that are in development as potential new breast cancer therapies.

Perhaps the most striking aspect of our data was the effective inhibition of established SKBR-3 xenograft growth by VN/12-1 alone at doses as low as 2.5 mg/kg body weight, twice weekly, and when combined with CHL, with no apparent toxicity as there was no change in body weight of the mice. Recently, Samaddar *et al.* demonstrated that the combination of a 4-OHT with 3-MA, resulted in potent anti-cancer activity against MCF-7 breast cancer cells (42). Wu *et al* also demonstrated similar combination strategy in prostate cancer PC-3 model using src
family inhibitors in combination with autophagy inhibitors (43). Indeed, a number of clinical trials have been initiated in patients with solid and hematopoietic tumors to test the overall hypothesis that autophagy is a mechanism of therapeutic resistance (2).

Overall, following treatment of SKBR-3 cells with VN/12-1, our findings clearly demonstrate that VN/12-1 has dual effects (autophagy and apoptosis) based on the dose and duration of treatment. We also show that: (1) autophagy acts as a protective mechanism in SKBR-3 cells; (2) inhibition of autophagy can be exploited to potentiate VN/12-1-induced cell death, and (3) the combination of VN/12-1 with autophagy inhibitor(s) can reduce the dose of VN/12-1 and thus reduce its toxicity (if any). As with our other lead RAMBAs, the exact molecular targets (except CYP26) of VN/12-1 are yet to be identified. We envision that this will be achieved with ongoing studies directed at design and synthesis of appropriate biotin-RAMBAs conjugates (molecular probes that retain potency in bioassays) (44).

In summary, this study provides the first evidence that a RAMBA rapidly activates autophagy in breast cancer cells and that induction of autophagy by VN/12-1 can be exploited as a target to achieve enhanced anti-tumor efficacy. We suggest that VN/12-1 or the combination therapy of VN/12-1 and CHL is a potential strategy for the treatment of patients with breast cancer.

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References:


Figure legends:

**Figure 1: Effect of VN/12-1 on the growth of human breast cancer cells and ERS markers in vitro.** (A) Chemical structures of VN/12-1 and CHL (B) VN/12-1 inhibits the growth of MCF-7 (green), SKBR-3 (blue), MDA-MB-231 cells (red), MCF-10A (black). Curves generated from an MTT assay after 96 h exposure to VN/12-1. Points, mean of replicates from three independent experiments; bars, SE. Solid line, best-fit sigmoidal-dose response (variable slope). (C) Cells were exposed to 10 μM VN/12-1 for time points shown. After each time point, the compound was washed away and cells were maintained in normal growth medium for 96 hrs, and cell viability was measured with an MTT assay.* p < 0.05, ** p < 0.01. (D(a)-D(b)) Effect of VN/12-1 on ERS markers. Cells were treated with indicated concentrations of VN/12-1 for 6 h (D(a)) or 24 h (D(b)), whole cell lysates were tested for BiP and p-eIF2α. T-Thapsigargin (20 μM) was used as a positive control. Total eIF2α was used as loading control.

**Figure 2: VN/12-1 induces the formation of autophagosomes.** (A-B) Transmission electron microscopy images of VN/12-1-treated SKBR-3 cells. Cells were treated with ethanol (A) or 10 μM VN/12-1 (B (a), (b), (c)) for 24 h. (A and B(a)) represent comparison of nuclear and nucleolar morphology (arrow heads); (A and B(b)) represent normal versus VN/12-1-treated mitochondria (solid arrows in (A) vs arrow head in B(b)); B(a) VN/12-1-treated cell with vacuolated cytoplasm (solid arrow); B(b) VN/12-1-treated cell with dilated ER showing ER stress (solid arrow); and B(c) early (solid arrow) and late (arrow head) autophagosomes containing cellular organelles. (C) SKBR-3 were treated with ethanol (a) or 10 μM VN/12-1 (b) for 24 h. VN/12-1 induces LC3 aggregation (punctate staining—peculiar of autophagy formation as shown in figure 2C (b)).
Figure 3: VN/12-1 induces autophagy markers and cell cycle arrest in SKBR-3 cells.

(A(a) and A(b)) Effect of VN/12-1 on autophagy markers. Cells were treated with indicated concentrations of VN/12-1 and CHL for 24 h, whole cell lysates were tested for LC3B, Beclin-1, p-p70S6K and p-Akt. β-actin and total Akt, total p70S6K were used as control. (B) Cells were treated with indicated concentrations of VN/12-1 and/or CHL for 24 h. Whole cell lysates were tested for ERS markers and cyclin D1. V-VN/12-1, C-CHL. The number following V or C indicates the concentration in μM. (C) SKBR-3 cells were treated with indicated concentrations of VN/12-1 and IC20 concentrations of (C(a)) CHL, (C(b)) 3-Methyladenine (3-MA) for 96 h in an MTT assay. Columns are the mean of viable cells in three experiments; bars, SE. ** p < 0.01.

(D) Cells were transfected with si-Beclin-1 or si-Scrambled as described above. After 72 hours, they were treated with indicated concentrations of VN/12-1 and cell viability was assessed by MTT assay as described above. * P < 0.05, ** P < 0.01.

Figure 4: VN/12-1 (in combination with CHL) induces caspase dependent apoptosis (A)

SKBR-3 cells were treated with indicated concentrations of VN/12-1 or CHL for 24 h and protein expression of cleaved PARP and Bad was determined. V-VN/12-1, C-CHL. The number following V or C indicates the concentration in μM (B). The percent of late apoptotic/necrotic cells as determined by Annexin V-FITC and PI positive staining by flow cytometry. **p < 0.01. SKBR-3 cells were treated with indicated concentrations of VN/12-1 or CHL for 24 h. % cells stained with FITC/PI was calculated using flow cytometry. (C) Indicated concentrations of VN/12-1, CHL and/or 30 μM Z-vad-fmk used in an MTT assay (as described in Methods section) experiment to determine the cell viability in SKBR-3 cells. Columns are the mean of three experiments; bars, SE. * P < 0.05, ** P < 0.01.
Figure 5: VN/12-1 (alone or in combination with CHL) inhibits the growth of SKBR-3 xenografts (A) The effect of VN/12-1 (2 doses- 2.5 and 5 mg/kg twice a week), ATRA (5 mg/kg twice a week) alone or in combination with CHL (50 mg/kg twice a week) were evaluated in a SKBR-3 xenograft model in female SCID mice. Mice (n = 8) were injected subcutaneously. Tumors were measured twice a week. (A(a)) Arithmetic tumor means and (A(b)) geometric means was plotted against time. (A(b)) has logarithmic scale on y-axis. P-values are indicated separately in supplementary Table S4 (B) Representative tumors from the 8 groups (C(a)) Mean tumor weights taken upon euthanizing all mice and collecting tumors. *p < 0.05, **p < 0.01. Data are mean (± s.e.). (C(b)) mean body weights. Mice were weighed once a week for the duration of the study. (D) Western blot analysis of protein expression in SKBR-3 tumors taken from mice. Autophagy marker (LC3B), ERS marker (CHOP), Cell cycle marker (cyclin D1), Apoptosis markers (Bad, PARP cleavage) were probed.

Figure 6: Mechanisms of action of VN/12-1 in combination with CHL: VN/12-1 induces ER stress. This results in inhibition of protein translation, downregulation key cell cycle proteins such as cyclin D1 and arrest in G1-S phase cell cycle transition. Addition of CHL enhances the ER stress and switches it from reversible (protective) pathway to irreversible (apoptotic) pathway by CHOP upregulation. VN/12-1 inhibits PI3K-Akt-mTOR pathway and activates autophagy. Inhibition of VN/12-1-mediated autophagy by CHL potentiates apoptosis by activation of caspase 9 and PARP cleavage.
Figure 1A

Chloroquine

Figure 1B

![Graph showing cell viability](image)

Figure 1C

![Bar graph showing cell viability](image)

Figure 1D (a)

![Western blots showing BiP and p-eIF2α](image)

Figure 1D (b)

![Western blots showing p-eIF2α and Total eIF2α](image)
Figure 2A

Figure 2B (a)

Figure 2B (b)

Figure 2B (c)

Figure 2C (a)

Figure 2C (b)
Figure 6
Molecular Cancer Therapeutics

Autophagy inhibition synergistically enhances anti-cancer efficacy of RAMBA, VN/12-1 in SKBR-3 cells and tumor xenografts

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