Autophagy Inhibition Synergistically Enhances Anticancer Efficacy of RAMBA, VN/12-1 in SKBR-3 Cells, and Tumor Xenografts

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Abstract

VN/12-1 is a novel retinoic acid metabolism blocking agent discovered in our laboratory. The purpose of the study was to elucidate the molecular mechanism of anticancer activity of VN/12-1 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. Furthermore, we also examined the impact of pharmacologic and genomic inhibition of autophagy on anticancer activity of VN/12-1. Finally, the antitumor activity of VN/12-1 was evaluated as a single agent and in combination with autophagy inhibitor chloroquine in an SKBR-3 mouse xenograft model. Short exposure of low dose (<10 μmol/L) of VN/12-1 induced endoplasmic reticulum stress, autophagy, and inhibited G1-S phase transition and caused a protective response. However, a higher dose of VN/12-1 initiated apoptosis in vitro. Inhibition of autophagy using either pharmacologic inhibitors or RNA interference of Beclin-1 enhanced anticancer 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. Mol Cancer Ther; 11(4); 898-908. ©2012 AACR.

Introduction

Breast cancer is the most common neoplasia in women. Despite significant advances in treatment, breast cancer remains incurable because of emergence of alternative pathways adopted by cancer cells to overcome the effects of anticancer 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 multitargeted agent. Mounting evidence indicates that the antitumor 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 hematologic 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 (RAMBA) 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 anticancer activities.

4-[(4-((1H-imidazol-1-yl)-yl)-(E)-retinoic acid (VN/14-1) and its corresponding methyl ester, VN/12-1 (Fig. 1A and Supplementary Fig. S1) are among 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 prosurvival pathways that may be activated in response to RAMBAs. VN/12-1 does not bind to or transactivate retinoic acid receptors (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 physiologic 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 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 (15). In most cases, induction of cellular ER stress subsequently leads to activation of autophagy (16, 17).

Whereas the estrogen receptor-α-proficient breast tumors respond to several therapies, estrogen receptor-α–deficient tumors are less sensitive partly because of activation of growth factor signaling pathways such as human epidermal growth factor receptor-2 (Her-2; ref. 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 estrogen receptor-α–deficient cell line that 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 successfully used this xenograft model in severe-combined immunodeficient (SCID) mice previously (20, 21).

This study evaluated the potency and antitumor activity of VN/12-1, as a single agent and in combination with chloroquine (Fig. 1A; ref. 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 μmol/L) of VN/12-1 induces autophagy, ER stress, and cell-cycle arrest as an immediate protective response in SKBR-3 cells and xenograft tumors. Importantly, we show that inhibition of autophagy using either pharmacologic inhibitors (e.g., chloroquine) 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 chloroquine ($P < 0.001$).

**Materials and Methods**

For cell culture, cell cycle inhibition, Western blot, Annexin fluorescein isothiocyanate (FITC), electron and immunofluorescence microscopy, short interfering RNA (siRNA), statistical analysis, and *in vivo* studies, see Supplementary Data Materials and Methods.

**Chemicals and reagents**

ATRA and 4-hydroxytamoxifen (Supplementary Fig. S1) were purchased from LKT Laboratories Inc., whereas VN/12-1, other RAMBAs, letrozole (Supplementary Fig. S1; ref. 22), and 3-methyl adenine (3-MA; ref. 23) were synthesized in our laboratory. Chloroquine (15) was purchased from Sigma Aldrich.

**Cell growth inhibition (MTT colorimetric assay)**

MTT (Sigma Aldrich) assay was carried out as previously described (24).

**Cell-cycle analysis**

Cell-cycle analysis was done using method as previously described (24).

**In vivo tumor growth**

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

**Results**

**VN/12-1 inhibits the growth of breast cancer cell lines**

To determine whether 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 (Fig. 1B; Supplementary Table S1). VN/12-1 did not induce any significant growth inhibitory effects in immortalized noncancerous MCF10A cell line. However, it was quite potent against the cancer cell lines with $IC_{50}$ in the low μmol/L range. Clinically used anticancer drugs ATRA, 4-hydroxytamoxifen, 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 μmol/L VN/12-1, and the compound was washed away at various time points (Fig. 1C). The cells were then maintained in regular growth media until the endpoint of the MTT assay. As shown in Fig. 1C, continuous exposure to VN/12-1 for at least 24 to 36 hours was needed to reduce the cell viability by approximately 20% for all the cells tested ($P < 0.05$). This indicated that the induction of growth inhibitory effect of VN/12-1 is time dependent. Collectively, these data also suggested 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**

Many cancer therapies including retinoids are known to induce ER stress in various cancers (26–28). The transduction of ER stress 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 ER kinase; ref. 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 ER stress, however, beyond levels at which cellular homeostasis can be maintained becomes proapoptotic by upregulating CHOP (CCAAT-enhancer-binding protein homologous protein) and IRE1α (17, 30). To test the role of ER stress, Western blot analysis was carried out on SKBR-3 lysates treated with VN/12-1 for 6 and 24 hours. As shown in Figs. 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 showed that induction of ER stress is a concentration-dependent early event (i.e., after 6 hours) following VN/12-1 treatment.
Efficacy Modulation of RAMBAs by Autophagy Inhibition

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 anticancer activity following shorter treatment of VN/12-1 led us to hypothesize that SKBR-3 cells undergo a protective response, possibly autophagy, at lower μmol/L concentrations of VN/12-1 or upon short exposure.

To better understand the morphologic changes induced by VN/12-1, transmission electron microscopy was carried out on SKBR-3 cells treated with vehicle control (Fig. 2A) or VN/12-1 (10 μmol/L) for 24 hours (Fig. 2B (a), (b), and (c)). There was an abundance of autophagosomes in the images from the treatment group. VN/12-1 treatment caused nucleolar disruption, cytoplasmic vacuolization [Fig. 2B (a)], distortion of mitochondrial shape, and dilated ER [an evidence of ER stress; Fig. 2B(b)]. Numerous autophagosomes in various stages of maturation containing cellular organelles were also noted [Fig. 2B(c)]. Another efficient way to confirm autophagy is by measuring lapsed 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 μmol/L treatment of VN/12-1 for 24 hours increased the number and size of punctate LC3B aggregates [Fig. 2C(b)] compared with control [Fig. 2C(a)].

To confirm the upregulation of biochemical markers, immunoblot analysis was carried out to probe various autophagy markers. Chloroquine inhibits the fusion of autophagosomes with lysosomes and thus leads to accumulation of lapsed form of LCB (LC3B; ref. 33). Following 24 hours incubation, the cells treated with VN/12-1 showed a dose-dependent upregulation of LC3B [Fig. 3A(b)]. 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 chloroquine (a weak lysosome stabilizing base). Cotreatment with chloroquine resulted in enhancement of LC3B band [Fig. 3A(b)]. Beclin-1 is an important autophagy-related protein, and its upregulation correlates with induction of autophagy (34). Both, dose-dependent [Fig. 3A(a)] and time-dependent (Supplementary Fig. 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 [Fig. 3A(a)]. Following the addition of chloroquine to VN/12-1, there was an abolishment of reversible ER stress marker, BiP, and induction of the irreversible ER stress markers—IRE1-α and CHOP (Fig. 3B). This indicated that the addition of chloroquine to VN/12-1 pushes the cells from reversible ER stress to irreversible ER stress, which is a well-known trigger for apoptosis (29). Thus, our data suggested that VN/12-1–induced autophagy due to enhanced induction rather than decreased clearance. Uptregulation of LC3B and abolishment of AKT phosphorylation and p70S6K phosphorylation were also evident in MCF-7 cells (Supplementary Fig. 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.

Because 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 chloroquine alone did not have any major effect on cyclin D1 expression, VN/12-1 (10 μmol/L) alone or in combination with chloroquine completely abolished the expression of cyclin D1 (Fig. 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 Fig. S2C).
Pharmacologic 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 IC50 concentrations of known inhibitors of autophagy: chloroquine (15) and 3-methyladenine (3-MA; refs. 33, 38). When combined with VN/12-1 both the autophagy inhibitors enhanced the anticancer potency of VN/12-1 (Fig. 3C (a) and C (b)). We also found that VN/12-1, combined with either chloroquine (Supplementary Fig. 2D) or 3-MA (Supplementary Fig. S2E), resulted in synergistic effects, as assessed by isobologram analysis using the median effect principle of Chou and Talalay (39). These data suggested that VN/12-1 and autophagy inhibitors elaborate unique individual effects that are complementary when these compounds are used together.

To quantitatively evaluate the effect of the combination of VN/12-1 with chloroquine, the dose reduction index (DRI; ref. 40) and combination index (CI) were calculated from the data (Supplementary Table S2) using CalcuSyn software program at 50% to 95% inhibition levels of cell viability. DRI values indicate that the synergic combination can result in 2- to 8-fold reduction of the VN/12-1 dose and 3- to 9-fold reduction of chloroquine dose to achieve 50% to 95% cell growth inhibition, respectively, compared with the dose if they were used as single agent. The values of the CI suggest that the combination of VN/12-1 and chloroquine was synergistic at 50% to 95% of growth inhibition. Collectively, these results confirmed that autophagy is a protective cellular response that lowers anticancer potency of VN/12-1, and its inhibition by pharmacologic 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 whether autophagy inhibition by way of silencing an important autophagy gene, Beclin-1, enhances the anticancer activity of VN/12-1, siRNA technology was used. For Beclin-1, the siRNA sequence that showed 90% to 100% knockdown of the protein was selected for the studies (Supplementary Fig. 2F). As expected, SKBR-3 cells treated with VN/12-1 alone showed a decrease in viability by the MTT assay when compared with the vehicle treated (no siRNA) controls (Fig. 4B). Pancaspase inhibitor z-vad-fmk was used to determine whether VN/12-1–mediated apoptosis was caspase dependent. Pretreatment with 30 μmol/L z-vad-fmk reversed the growth inhibitory effects of VN/12-1 and its combination with chloroquine in MTT assay (P < 0.01; Fig. 4C).

Preliminary toxicity and pharmacokinetics of VN/12-1 in SCID mice

Before assessment of the in vivo antitumor efficacy of VN/12-1, we conducted preliminary toxicity and pharmacokinetics studies. Preliminary toxicity studies in SCID mice showed that VN/12-1 was not toxic as a single agent or in combination with chloroquine in the subcutaneous doses 2.5 and 5 mg/kg twice a week. In addition, preliminary pharmacokinetics following subcutaneous administration 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 article.

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

We evaluated the ability of VN/12-1 to inhibit the growth of SKBR-3 xenografts when given via subcutaneous administration. Mice were divided into 8 groups as detailed in the Methods section. As shown in Fig. 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), whereas 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 chloroquine slowed tumor growth compared with 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 chloroquine was better than the combination of VN/12-1 (2.5 mg/kg) with chloroquine (1.2% vs. 1.9%, P = 0.086). The combination of VN/12-1 (at either dose) with chloroquine was better than the combination of ATRA with chloroquine (P = 0.001 for both comparisons).

Representative pictures of tumors from each group are shown in Fig. 5B. Figure 5C (a) and (b) show the average tumor weights and the body weights of the mice in
different groups, respectively. As shown in Fig. 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 chloroquine 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 [Figs. 5A(a) and 5A(b)], but treatments with VN/12-1 (5 mg/kg) alone and its combination with chloroquine 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 chloroquine enhanced VN/12-1 upregulation of LC3B. PARP cleavage and upregulation of pro-apoptotic Bad was noted in all the treated groups (except chloroquine group), more so in VN/12-1 and chloroquine combination groups (Fig. 5D). Thus, these data identify apoptosis induction as a major mechanism underlying the ability of chloroquine to potentiate the antitumor effects of VN/12-1. There was a marked upregulation of CHOP in the groups treated with VN/12-1 alone or with chloroquine. This further supports in vitro findings of activation of ER stress by VN/12-1. These data supported 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 chloroquine leads the cells to apoptotic pathway. Cyclin D1 was downregulated in all the treatment groups except chloroquine (Fig. 5D). Collectively, these in vivo tumor-suppressive responses exhibited by VN/12-1 or VN/12-1 plus chloroquine strongly correlated with the mechanisms identified in vitro. The overall mechanism of action of VN/12-1 and its combination with chloroquine in SKBR-3 human breast cancer cells is summarized in Fig. 6.

Discussion

Because of the heterogeneous nature of breast cancer (41), combination therapies or multitarget 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 μmol/L 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 the potency of VN/12-1.
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 chloroquine, with no apparent toxicity as there was no change in body weight of the mice. Recently, Samaddar and colleagues showed that the combination of 4-hydroxytamoxifen with 3-MA resulted in potent anticancer activity against MCF-7 breast cancer cells (42). Wu and colleagues also showed 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 (13).

Overall, following treatment of SKBR-3 cells with VN/12-1, our findings clearly show that VN/12-1 has dual effects (autophagy and apoptosis) based on the dose and duration of treatment. We also show that (i) autophagy acts as a protective mechanism in SKBR-3 cells; (ii) inhibition of autophagy can be exploited to potentiate VN/12-1-induced cell death, and (iii) 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; ref. 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 antitumor efficacy. We suggest that VN/12-1 or the combination therapy of VN/12-1 and chloroquine is a potential strategy for the treatment of patients with breast cancer.

Disclosure of Potential Conflicts of Interest
V.C.O. Njar holds an ownership interest in the RAMBAs patents and technologies thereof. No potential conflicts of interest were disclosed by other authors.

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