

Akt2 and acid ceramidase cooperate to induce cell invasion and resistance to apoptosis

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Abbreviations: ASAH1, N-acylsphingosine amidohydrolase 1, acid ceramidase; CI, combination index; DMSO, dimethyl sulfoxide; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; PI3K, phosphoinositide-3 kinase; PTEN, phosphatase and tensin homolog; SPHK1, sphingosine kinase 1

Both Akt 2 and acid ceramidase (ASAH1) are found aberrantly overexpressed in cancer cells, but whether these two enzymes cooperate to induce malignant transformation is not known. We found that in immortalized, non-transformed cells, ectopic co-expression of Akt2 and ASAH1 is significantly more effective than expression of each gene alone at inducing cell invasion and at conferring resistance to apoptosis. Consistent with these observations, siRNA-mediated depletion of both Akt2 and ASAH1 is much more potent than depleting each alone at inhibiting cell viability/proliferation and cell invasion. Furthermore, pharmacological inhibitors of Akt (TCN or MK-2206) and ASAH1 (B13) synergize to inhibit cell viability/proliferation, and combinations of these drugs are more effective than single-agent treatments at inhibiting cell invasion. Taken together, the results suggest that these two enzymes cooperate to induce malignant transformation and warrant further preclinical studies to evaluate the potential of combining inhibitors of Akt and ASAH1 to treat cancer.

Introduction

Cancer cells ensure their survival and sustain malignant transformation by harboring several aberrantly activated signaling pathways that are complementary and inter-dependent.^{1,2} Often suppression of only one of these pathways is insufficient to induce tumor cell death and to reverse malignant transformation. Therefore, understanding the relationship among such pathways can lead to a rational exploration of drug combinations that may be more effective.^{3–8} Two frequently hyperactive pathways in cancer are those regulated by the serine/threonine kinase Akt and acid ceramidase ASAH1 (N-acylsphingosine amidohydrolase 1). Although these two enzymes regulate distinct yet overlapping pathways that are important for survival and malignant transformation (see below), it is presently unknown whether some human tumors require both pathways for oncogenesis, and whether targeting these two enzymes simultaneously would be more beneficial than targeting each alone.

Akt is an important promoter of tumor cell survival, proliferation as well as migration and invasion.^{9–11} PI3K-catalyzed formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), is required for Akt recruitment to the plasma membrane and subsequent activating phosphorylation at T308 and S473. Akt phosphorylates many substrates that mediate tumor progression.¹⁰ This has made Akt a major target for cancer drug discovery.^{12–15}

There are three mammalian Akt isoforms that may play distinct but also overlapping roles in development, normal physiology and tumorigenesis. For example, in mice, loss of Akt1 function results in smaller body size and significant growth defects.^{16,17} Mice lacking Akt2 are unable to maintain glucose homeostasis and are diabetic,¹⁸ while Akt3-knockout mice have smaller brains but are otherwise normal.¹⁹ In cancer, Akt1 is frequently found persistently phosphorylated and hyperactivated. Furthermore, Akt2 is often overexpressed in human cancer, and its forced overexpression results in increased PI3K-dependent invasion and metastasis of breast and ovarian cancer cells.²⁰ Increased Akt3 expression and loss of PTEN result in the development of melanoma, and Akt3 siRNA stimulates apoptosis and inhibits melanoma development.²¹

ASAH1 is a ubiquitously expressed enzyme that converts ceramide into sphingosine and free fatty acids.^{22–24} Ceramide is a major intracellular activator of apoptotic cell death, whereas sphingosine, after its conversion to sphingosine-1-phosphate by sphingosine-1 kinase (SPHK1), stimulates cell growth and proliferation.^{22–24} SPHK1 is known to activate Akt²⁵ and is itself subject to activating phosphorylation.²⁶ ASAH1 is upregulated in many cancers, particularly prostate cancer,²⁷ and is believed to have an important role in tumor promotion. For instance, in prostate cancer cells, stable overexpression of ASAH1 stimulates cell proliferation and cell invasion and confers resistance to apoptosis.²⁸

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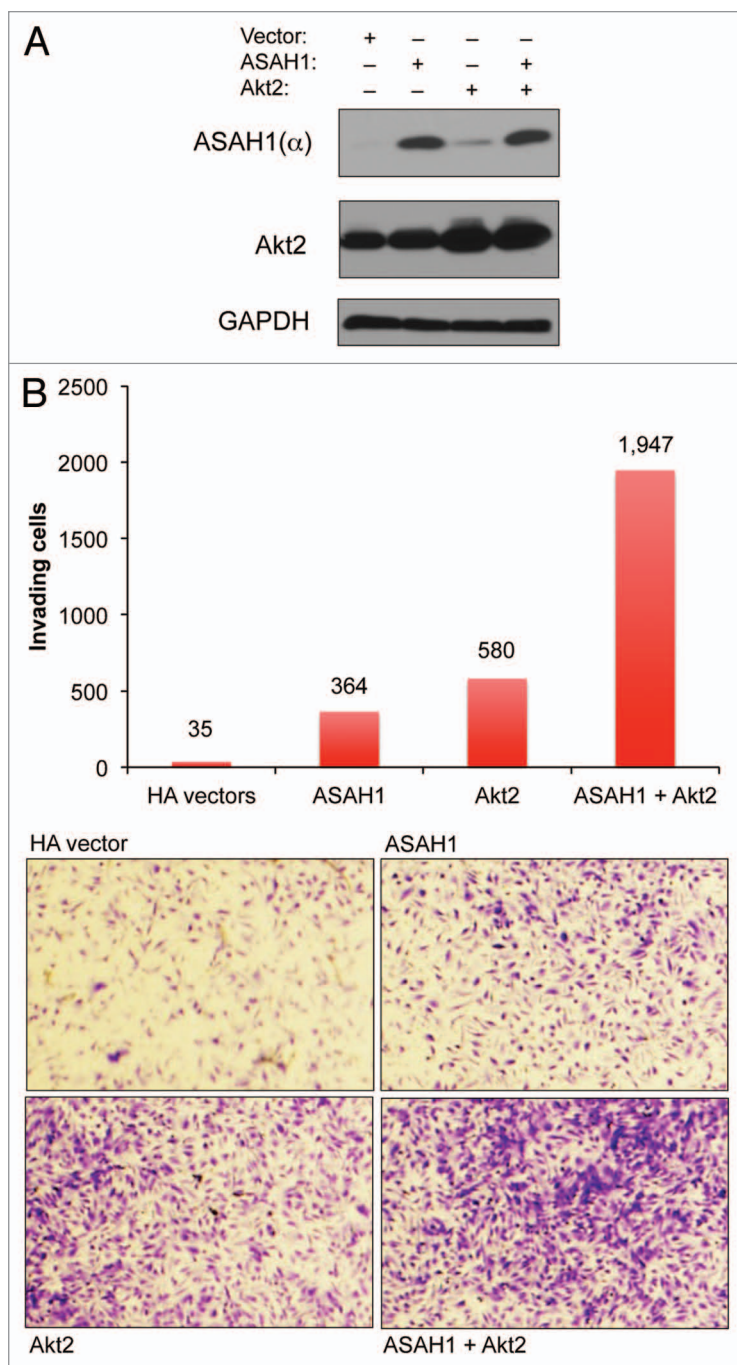


Figure 1. ASAH1 and Akt2 cooperate to stimulate cell invasion. HPNE cells were transfected with 0.4 μ g of plasmids encoding ASAH1 and/or Akt2. (A) Cells were harvested 48 h post-transfection, and the resulting lysates were analyzed for the levels of ectopically expressed proteins by western blotting. (B) Cells were harvested 48 h post-transfection, counted, and 20,000 cells were incubated in invasion chambers for another 48 h. The number of invading cells is indicated above each column. The data are representative of three independent experiments.

Therefore, ASAH1 has emerged as a promising cancer drug target (reviewed in refs. 29–31).

In this paper we have explored whether ASAH1 and Akt cooperate to induce, and whether combined inhibition of ASAH1 and

Akt blocks, malignant transformation. Thus, we show here that co-expressing ASAH1 and Akt2 is more effective than expressing each enzyme alone at inducing cell invasion and at causing resistance to apoptosis. We also show that the concomitant knockdown of both ASAH1 and Akt by siRNA is more effective at suppressing cell viability/proliferation and cell invasion. These observations were confirmed by demonstrating that pharmacological inhibitors of ASAH1 and Akt synergistically inhibit cell viability/proliferation, and that the drug combination is more effective than single drugs at inhibiting cell invasion.

Results and Discussion

Akt2 and ASAH1 together are more effective than each alone at promoting cell invasion and inducing resistance to apoptosis in immortalized non-transformed cells. Both ASAH1 and Akt2 have individually been implicated in cell invasion via distinct mechanisms,^{20,32–34} raising the question whether these two enzymes cooperate to induce cell invasion. To address this question, we transfected immortalized, non-transformed HPNE cells with ASAH1 and Akt2, either alone or together, and determined their effects on cell invasion as described in “Materials and Methods.” **Figure 1A** shows that HPNE cells express little ASAH1 and reasonable amounts of Akt2. **Figure 1B** shows that overexpression of ASAH1 in HPNE cells increased the ability of HPNE cells to invade over 10-fold, from 35 invading cells in empty vector-transfected cells to 364 invading cells in ASAH1-transfected cells. **Figure 1B** shows that overexpression of Akt2 resulted in even a greater increase in invasion from 35 to 580 (17-fold) invading cells. Overexpression of both ASAH1 and Akt2 resulted in much more than an additive effect, leading to 1,947 invading cells (55-fold increase relative to control). Therefore, the fact that ASAH1 or Akt2 stimulated invasion by 10- or 17-fold, respectively, but that together they promoted invasion by 55-fold, suggests that these two enzymes cooperate to stimulate invasion. These effects are most likely not due to enhanced proliferation rates, since overexpression of each enzyme alone increased the growth rate by 1.3-fold, and, more importantly, co-expression did not provide further growth advantage, and also only increased the growth rate by 1.3-fold (**Fig. 2A**).

Since both ASAH1 and Akt also regulate cell survival, we investigated whether co-expressing these two enzymes is more effective than expressing each alone at protecting against drug-induced apoptosis. To this end, we expressed ASAH1 and Akt2 alone or together in HPNE cells and determined their effects on doxorubicin-induced apoptosis by measuring the ability of doxorubicin to activate caspase 3. **Figure 2B** shows that in the absence of doxorubicin, cleaved caspase 3 was not detected. In contrast, treatment with doxorubicin resulted in activation of caspase 3. Furthermore, overexpression of either ASAH1 or Akt2 alone had little effect on the ability of doxorubicin to activate caspase

3. In contrast, co-expression of both ASAHI and Akt2 inhibited the ability of doxorubicin to activate caspase 3 (Fig. 2B). Similar results were also obtained by determining the effects of these two enzymes on cell death as measured by trypan blue, as described in “Materials and Methods.” Figure 2C shows that doxorubicin induced 18, 9 and 7% cell death in empty vector-, ASAHI- and Akt2-transfected cells, respectively. In contrast, Doxorubicin’s ability to induce cell death was reduced to 3% when both enzymes were concomitantly expressed. Since co-expression of ASAHI and Akt2 did not result in a growth advantage (Fig. 2A), the enhanced invasion, resistance to apoptosis and cell death following co-expression of these two enzymes was most likely not due to increased cell proliferation. Together, these data suggest that ASAHI and Akt may cooperate to induce or maintain malignant transformation, raising the question whether inhibition of both of these pathways could be beneficial to human cancer patients.

Depletion of both Akt and ASAHI together is more effective than depletion of each alone at inhibiting cell survival/proliferation and cell invasion. Figures 1 and 2 demonstrated that ASAHI and Akt2 cooperate to stimulate invasion and inhibit apoptosis. Therefore, we reasoned that some human cancer cells might be dependent on ASAHI and/or Akt for cell survival and invasion. To this end, we first assessed the requirement of Akt and ASAHI alone or together on the survival/proliferation of human cancer cells by depleting Akt alone, ASAHI alone or both in 13 human cancer cell lines of different lineages. Of the three Akt isoforms, we focused on Akt1 and Akt2, because Akt3’s role in cancer is not as well established as that of the other two isoforms. Following transfection with 20 nM siRNA targeting each enzyme, cells were incubated for 48, 72 and 96 h, and their viability/proliferation was examined by MTT assay as described under “Materials and Methods.” Figure S1 shows that transfection with the siRNAs was effective in depleting the corresponding proteins in all 13 cell lines. Figure 3A shows that, 96 h post-transfection with the indicated siRNAs, the survival/proliferation of 7 out of the 13 cell lines (RXF-393, MCF-7, PANC-1, 786-O, AsPC-1, U-87-MG and MDA-MB-231) was not significantly affected by depletion of Akt1/2 and ASAHI, either alone or together, suggesting that 54% of the human cancer cell lines tested do not depend on Akt1/2 and/or ASAHI for survival/proliferation. In contrast, three cell lines (23%, PC-3, MDA-MB-468 and DU-145) depended on combined, but not individual, Akt1/2 and ASAHI functions for survival/proliferation. Similar effects were seen 48, 72 and 96 h post-transfection with siRNAs (Fig. 3B; Fig. S2). Figure 3A also shows that one cell line (LnCaP) was dependent on either Akt1/2 or ASAHI individually. Others, such as SW-1990 and SkOV-3 were dependent on either Akt1/2 or ASAHI alone, respectively. These results suggested that 6 out of 13 human cancer cell lines tested

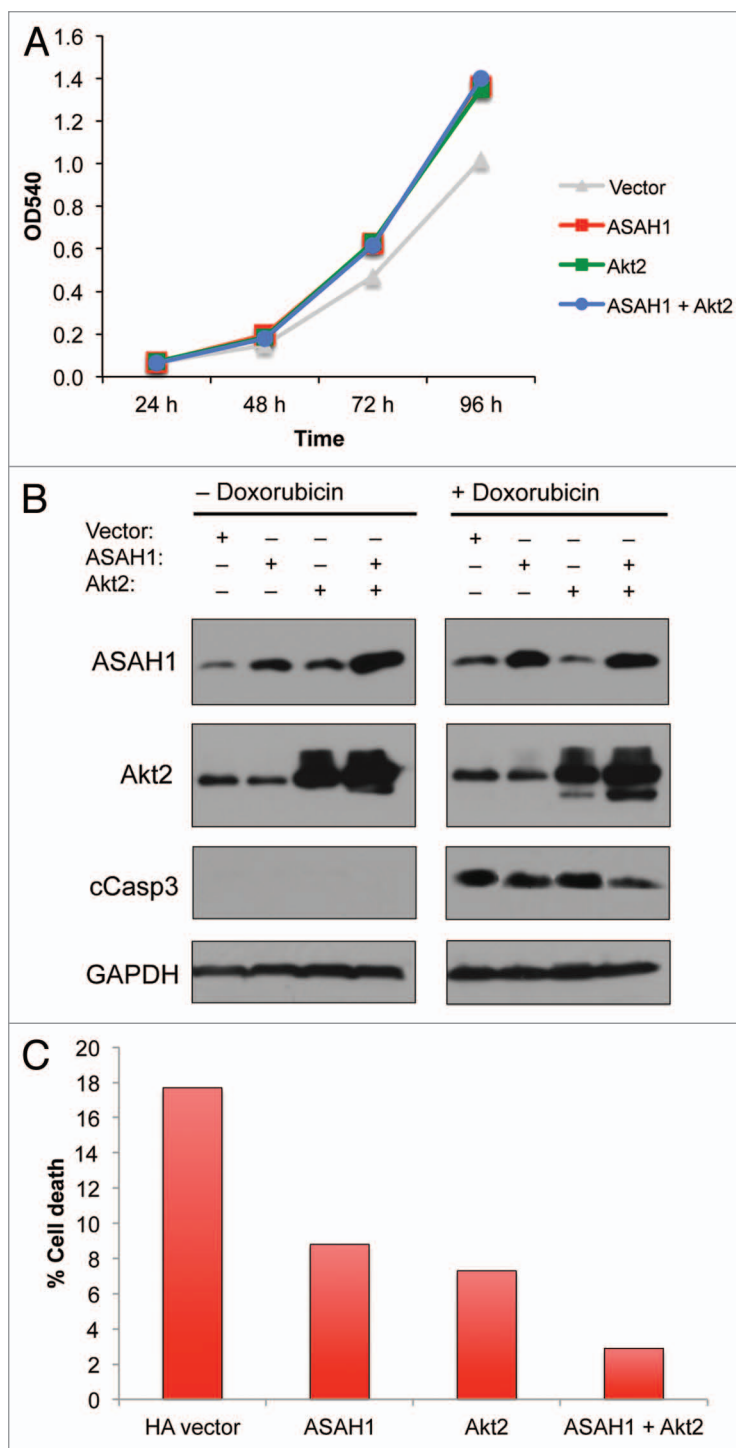


Figure 2. ASAHI and Akt2 cooperate to evade drug-induced apoptosis, but not to stimulate cell proliferation. HPNE cells were transfected with 0.4 μ g of plasmids encoding ASAHI and/or Akt2. (A) Cell proliferation/viability: cells were harvested 48 h post-transfection, counted and seeded at 1,000 cells per well in 96-well plates. Cell proliferation/viability was assessed by MTT assay for up to 96 h. (B) Apoptosis: western blots are showing the overexpression of ASAHI and Akt2 and the extent of caspase-3 cleavage following exposure to doxorubicin for 24 h. (C) Cell death: 48 h post-transfection, cells were exposed to 1.0 μ M doxorubicin for 24 h, cells were harvested, and the amount of cell death was quantified by determining the number of cells incorporating trypan blue. The data are representative of two independent experiments.

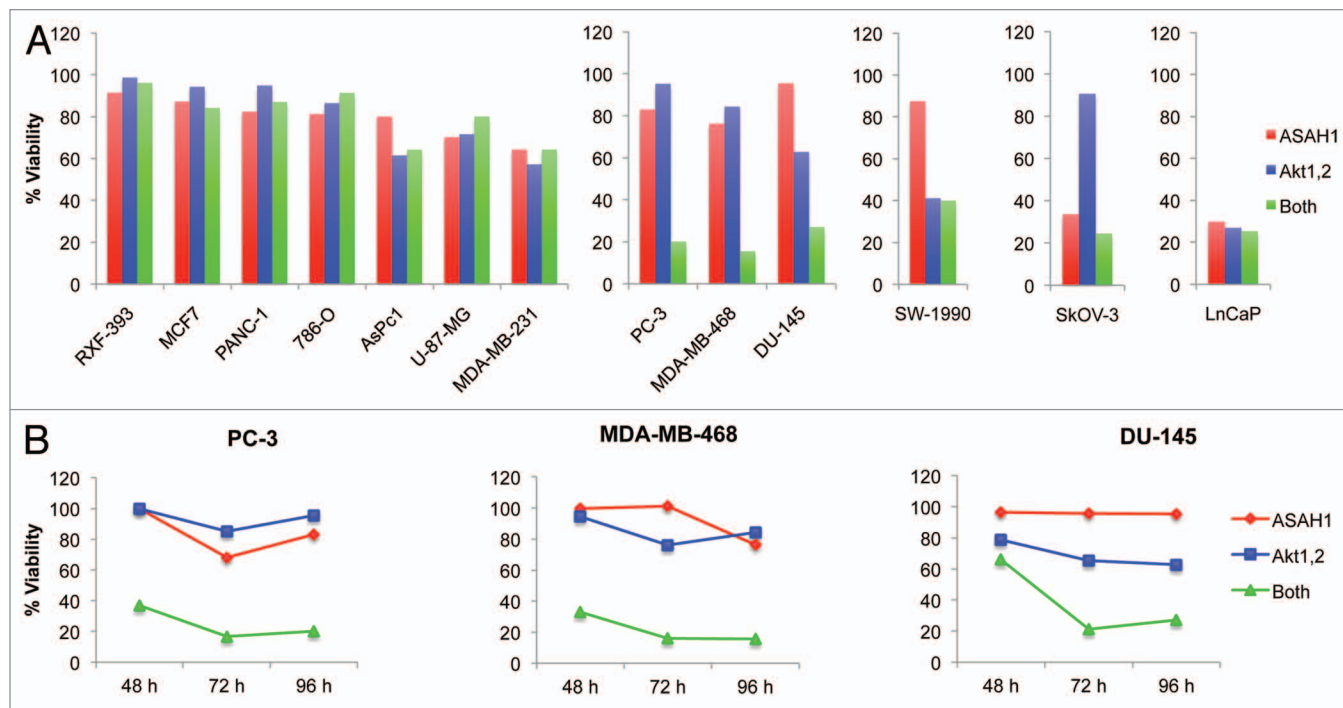


Figure 3. Combined silencing of Akt and acid ceramidase is synthetically lethal in a subset of human cancer cells. Thirteen human cancer cell lines were transfected with 20 nM siRNA targeting Akt1/Akt2 and ASAH1, either alone or in combination for 48, 72 or 96 h. Cell proliferation/viability was then determined by MTT assay. Cell viability was calculated as percent of cells treated with the appropriate concentration of non-targeting siRNA. The graphs show representative data of at least two independent experiments. (A) Ninety-six hours post-transfection, seven cell lines did not depend on either ASAH1 or Akt1/2, and three cell lines depend on Akt1/2 or ASAH1 or either for proliferation/viability. (B) Three out of 13 cell lines tested are significantly affected by combined, but not individual, depletion of ASAH1 and Akt1/2. The knockdown efficiency associated with these experiments and the proliferation/viability data for all three time points are shown in **Figures S1 and S2**, respectively. The data are representative of at least two independent experiments.

(PC-3, MDA-MB-468, DU-145, LnCaP, SW-1990, SkOV-3) depend on Akt1/2 and/or ASAH1 for survival/proliferation. In three out of these six cell lines (PC-3, MDA-MB-468, DU-145), loss of either Akt1/2 or ASAH1 had only a minor to moderate effect, whereas the combined loss of both resulted in dramatic loss of viability, perhaps suggesting a synthetic lethal interaction. These data suggest that the effects of ASAH1 or Akt depletion on cell viability are highly cell type-dependent. For Akt, this is consistent with studies by Koseoglu et al.³⁵ who showed that MDA-MB-468, BT-474 and LnCaP cells, but not U-87-MG or PC-3 cells, become apoptotic upon knockdown of Akt1/2/3. With the exception of MDA-MB-468 cells, our results confirmed these data. This discrepancy may be due to the fact that these authors depleted in MDA-MB-468 cells all three, rather than just two, Akt isoforms.

Figure 1 demonstrated that ASAH1 and Akt2 cooperate to stimulate invasion. Therefore, we next evaluated the requirements of Akt and ASAH1 alone and together on cell invasion as described under "Materials and Methods." As shown in **Figure 4A**, in MDA-MB-468 cells, siRNA to ASAH1 inhibited invasion by 13% relative to NT siRNA, whereas siRNA to Akt2 inhibited by 45%. Combined depletion of ASAH1 and Akt2 resulted in 73% inhibition of invasion. Similar results were obtained with PC3 cells where the inhibition of invasion was 44, 64 and 87% for ASAH1, Akt2 and ASAH1 + Akt2,

respectively (**Fig. 4A**). **Figure 4B** shows that the inhibition of invasion was associated with very efficient knockdown of both enzymes. Together with the results from **Figure 1**, the data suggest that ASAH1 and Akt2 cooperate to promote invasion, and that inhibition of both enzymes may be required to suppress invasion.

The Akt inhibitors TCN or MK2206 and the ASAH1 inhibitor B13 act synergistically to inhibit proliferation/survival and suppress cell invasion. The results from **Figures 3 and 4** demonstrated that depletion of ASAH1 and Akt together is more effective at suppressing cell survival/proliferation and invasion in some human cancer cells and prompted us to determine whether combining Akt and ASAH1 pharmacological inhibitors is more effective than single-agent treatment. First, we determined the effect of combining the Akt inhibitors TCN³⁶ or MK-2206³⁷ with the ASAH1 inhibitor B13³⁸ on cell viability/proliferation. We investigated the effects of these two drugs employing eight different dose combinations at a fixed ratio of 1:1. TCN, MK-2206 and B13 inhibited cell survival/proliferation in a concentration-dependent manner in both MDA-MB-468 and PC3 cells (data not shown). Using Calcsyn software and the Chou and Talalay method, we then determined whether these two drug combinations have synergistic effects.^{39,40} **Figure 5** and **Table S1** show that in MDA-MB-468 and PC-3 cells, 8 out of 8 combination indices

(CIs) were below 1, which indicates synergistic interaction between these compounds. For example, in MDA-MB-468 and PC3 cells, the average CIs for the eight TCN/B13 combinations were 0.20 and 0.42, respectively, indicating a highly synergistic interaction. Similarly, the combinations of MK2206 and B13 were also synergistic with average CIs of 0.55 and 0.37 for MDA-MB-468 and PC3 cells, respectively. Therefore, using two structurally distinct Akt inhibitors, TCN and MK-2206, we demonstrated that inhibition of Akt and ASAH1 is significantly more effective than inhibition of each alone to suppress cell survival/proliferation. These results obtained with pharmacological inhibitors corroborate the results of our genetic depletion approach.

Next, we studied the effects of these Akt and ASAH1 pharmacological inhibitors on cell invasion. To this end, we treated MDA-MB-468 cells with TCN (0.3, 1.0 and 3.0 μM), MK2206 (0.3, 1.0 and 3.0 μM) or B13 (1.0, 3.0 and 10 μM), either alone or in combination (TCN/B13 and MK2206/B13). **Figure 6** shows that in MDA-MB-468 cells all the TCN/B13 and MK2206/B13 combinations (0.1/3.0, 1.0/3.0 and 3.0/10 μM) were much more effective than the single-agent treatments, with the highest combination (3.0/10 μM) resulting in complete inhibition of invasion. Similar results were obtained with PC3 cells, where the combination of either TCN/B13 or MK2206/B13 was much more effective at inhibiting invasion than treatment with each drug alone. These results are consistent with the siRNA data of **Figure 4**, where depletion of both ASAH1 and Akt2 was more effective than the single depletion at inhibiting invasion.

Thus, in this paper we demonstrate that ASAH1 and Akt cooperate to induce malignant transformation, and that their combined depletion is more effective in suppressing malignant transformation. Furthermore, a synergy between agents targeting these two proteins was demonstrated. These important findings may be due to the fact that ASAH1 and Akt affect distinct pathways that regulate proliferation, cell death and invasion. Indeed, Akt stimulates cell survival and proliferation by acting on a number of proteins (Forkhead, IKK, Mdm2, TSC1/2) that are apparently not affected by ASAH1.⁹⁻¹¹ Furthermore, Akt was shown to promote invasiveness by upregulating the expression of IGF-1 receptor, β 1-integrin and MMP-9, respectively,^{20,33,34} whereas ASAH1 was shown to promote proliferation and survival by affecting retinoblastoma protein (RB) phosphorylation⁴¹ and Bax levels,⁴² and to stimulate invasion by upregulating cathepsin B.³² In addition, there may be crosstalk between ASAH1 and Akt-regulated pathways. For example, ASAH1 increases cellular sphingosine-1 phosphate, which was previously shown to activate Akt.²⁵ Furthermore, a major role of ceramide is to activate the protein phosphatases PP2A and PP1,⁴³⁻⁴⁶ and the upregulation of ASAH1 often seen in cancer would decrease ceramide and, therefore, PP1/PP2A activities. PP2A is known to act as a tumor suppressor via dephosphorylating T308 in Akt,^{47,48} RalA,⁴⁹ SPHK1,⁵⁰ but also anti-apoptotic Bcl-2⁵¹ and pro-apoptotic Bax.⁵² Similarly, PP1 plays anti-proliferative and

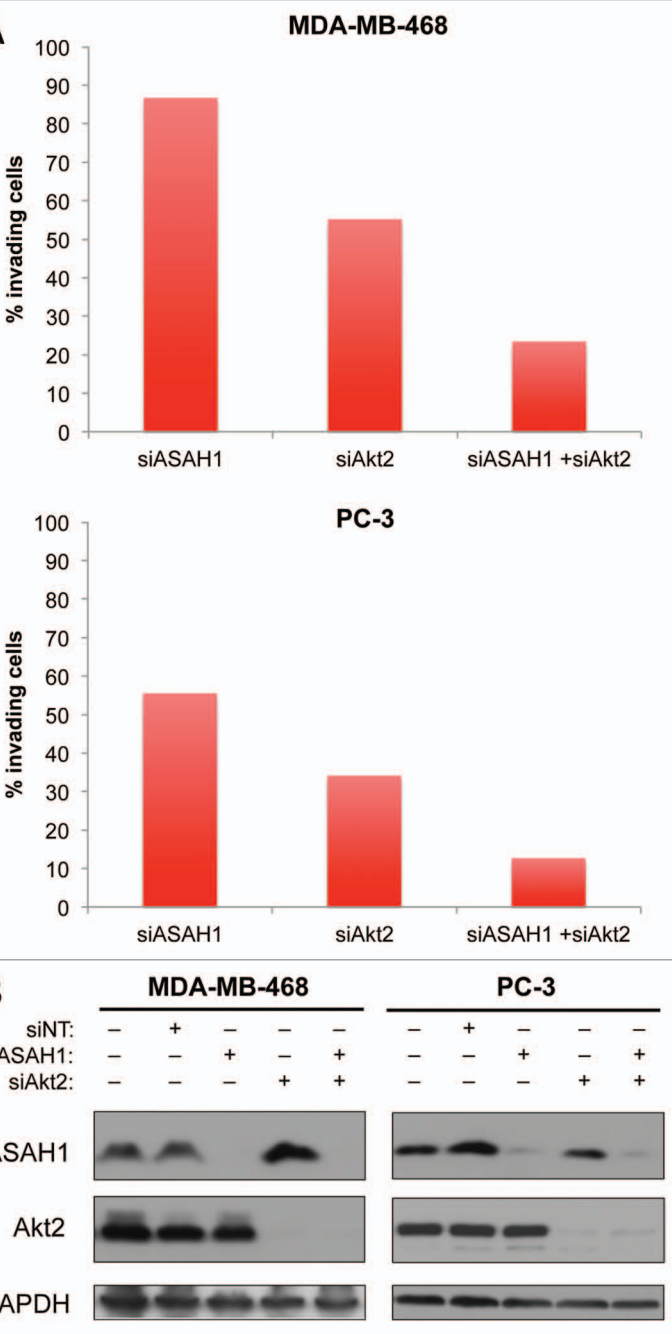


Figure 4. Depletion of ASAH1 and Akt2 is more potent at inhibiting cell invasion than depletion of ASAH1 or Akt2 alone. MDA-MB-468 or PC-3 cells were transfected with 12.5 nM siASA1 and/or 12.5 nM siAkt2. (A) Cells were harvested 48 h post-transfection and used in invasion assays. The graphs show invading cells (in % of NT siRNA). (B) Western blots show the knock-down efficiencies of siASA1 and siAkt2 associated with this experiment. The data are representative of three independent experiments.

pro-apoptotic roles via dephosphorylating RB,⁵³⁻⁵⁵ Bad,⁵⁶ Bax,⁵⁷ caspase-9⁵⁸ and T450 in Akt.⁵⁹ Therefore, inhibition of both Akt and ASAH1 would be predicted to result in maximal dephosphorylation of key regulators of the malignant phenotype.

In summary, our results suggest a novel strategy for cancer therapy that would exploit the fact that certain cancer cells

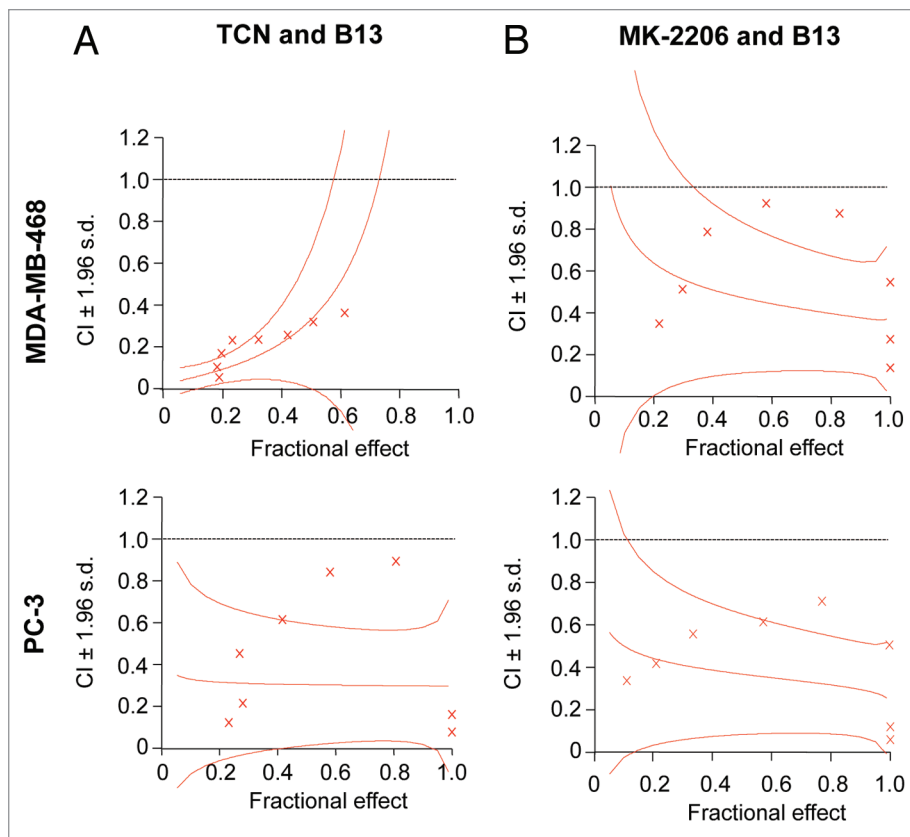


Figure 5. Pharmacological inhibitors of Akt and ASAHI act synergistically to inhibit cell viability/proliferation. Human cancer cells were treated in 96-well plates with either TCN and/or B13 (A) or MK-2206 and/or B13 (B) for 24 h. The concentrations of the drugs used in the combination treatments were at a ratio of 1:1. The viability of cells was then determined by MTT assay with six replicates per condition, and the data was processed using CalcuSyn software for combination index (CI) analysis to determine synergy (defined as CI values below 1) as described in "Materials and Methods." The graphs display CI values vs. the fraction affected (F_0) by each combination treatment (effect). The effect ranges from 0 (no inhibition) to 1 (complete inhibition). The data are representative of two independent experiments.

depend on both Akt and ASAHI function for cell survival. Further investigations are needed to delineate the mechanism(s) by which the combined inhibition of these two enzymes is beneficial in a subset of cancer cells.

Materials and Methods

Cells and reagents. The following cells were used in this study and purchased from the American Type Culture Collection: breast cancer cell lines MCF-7, MDA-MB-468 and MDA-MB-231, brain cancer cell line U-87-MG, kidney cancer cell lines 786-O and RXF-393, ovarian cancer cell line SkOV-3, pancreatic cancer cell lines AsPc-1, PANC-1 and SW-1990, prostate cancer cell lines DU-145, LnCaP and PC-3 and the immortalized pancreatic duct epithelial cells HPNE. TCN was obtained from the National Cancer Institute, and MK-2206 was purchased from Chemietek (#CT-MK2206). The ASAHI inhibitor B13 was from Cayman Chemical (#10006305).

Antibodies were purchased from Cell Signaling (Akt1, #2938; Akt2, #3063; cleaved caspase-3, #9664), BD Biosciences

(ASAHI α subunit, #612302), Covance (GAPDH, #MMS-580S) and Sigma-Aldrich (vinculin, #V9131). Peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch (goat anti-mouse IgG, #115-035-003 and goat anti-rabbit IgG, #111-035-003).

siGENOME SMARTpool siRNA reagents targeting Akt1 (#M-003000-03-0005), Akt2 (#M-003001-02-0005) and ASAHI (#M-049456-01-0005) as well as the non-targeting siRNA #2 (#D-001206-14-05) were purchased from Dharmacon.

RNAi-mediated silencing. Cells were seeded in either 6-well or 96-well plates and allowed to adhere overnight. The next day, cells were transfected with a mixture of siRNA and transfection reagent lipofectamine RNAiMAX (Invitrogen, #13778150) in OptiMEM (Invitrogen, #31985) at a ratio of 0.1 μ l per pmol of siRNA.

Ectopic expression of Akt2 and ASAHI. HA-ASAHI was amplified from a cDNA clone of acid ceramidase 1 (Invitrogen, Clone ID 3923451) using PCR with HA-pcDNA3 as a vector as described for RhoB by us.⁶⁰ The forward and reverse primers used for PCR were ATGCCGGGCC GGAGTTGCGT CGCCTTAGT and TCACCAACCT ATACAAGGGT CAGGGCAGTC CCGC, respectively. The PCR product was digested with *Bam*H1 and *Xho*I, cloned into HA-pcDNA3 vector and further confirmed by sequencing. The pLXSN-HA-Akt2 construct was provided by Dr Jin Cheng. Cells were seeded in 6-well plates using media free of antibiotics, and the next day the growth media was replaced by 2 ml OptiMEM, to which was added a mixture of Lipofectamine 2000 (Invitrogen, #11668019) and plasmid constructs in 0.5 ml OptiMEM. Six h later, the OptiMEM was replaced by 2 ml complete growth media.

MTT cell viability assay. Cells were seeded in 96-well plates and allowed to adhere overnight. Following transfection or drug treatment for the times indicated in the figure legends, $\frac{1}{2}$ volume of MTT reagent (Calbiochem, #475989) (3 mg/ml filtered PBS) was added to each well and incubated for ≥ 3 h at 37°C. The supernatant-containing medium and MTT reagent was then aspirated and replaced with 100 μ l DMSO. The plates were placed on an orbital shaker for 5 min, and the absorbance at 540 nm was determined with a μ QUANT multiplate reader (Bio-Tek Instruments).

Cell invasion assay. Invasion assays were performed using BioCoat Matrigel invasion chambers (BD Biosciences, #354480). For studies involving siRNA or recombinant DNA, cells were

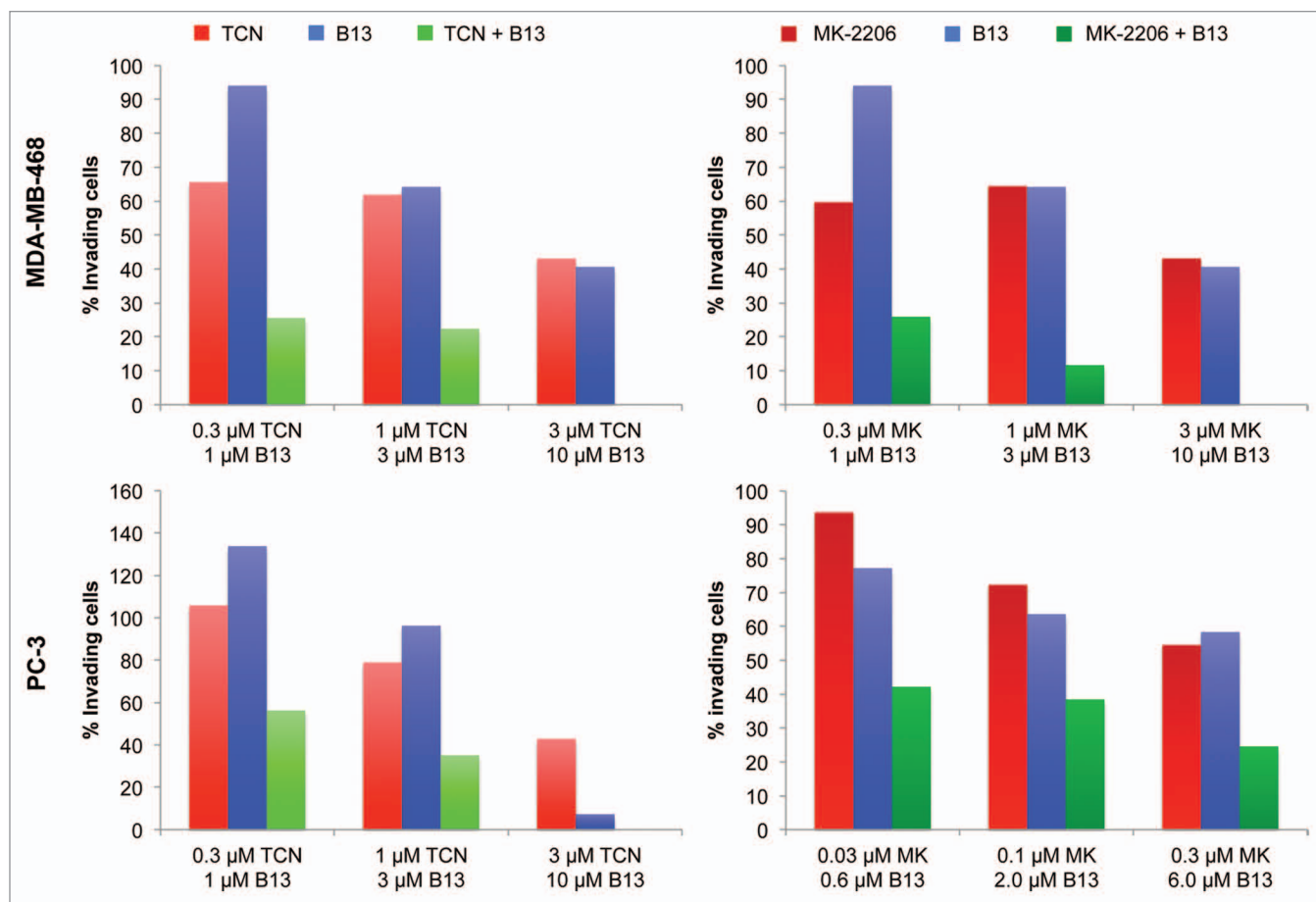


Figure 6. Pharmacological inhibition of ASAH1 and Akt is more effective at inhibiting cell invasion than single-agent treatment. MDA-MB-468 and PC-3 cells were mixed with the indicated drug concentrations and then replated to determine the extent of cell invasion as described in "Materials and Methods." The data are representative of three independent experiments.

transfected with the indicated concentrations of ASAH1 or Akt2 siRNAs/cDNAs and allowed to grow for 48 h on 6-well plates. The cells were then trypsinized, resuspended in serum-free medium and counted by the trypan-blue exclusion method. An equal number of live cells were then plated in the inserts at the indicated concentrations. The bottom chamber contained 10% FBS as the "chemoattractant." After 48 h at 37°C, the inserts were treated as described in the manufacturer's instructions, and invading cells were fixed in methanol and stained with 0.2% crystal violet. After an overnight drying period, the inserts were photographed with an Olympus Stereomicroscope, and invading cells were counted using ImagePro 6.2 software.

Synergy analysis. CalcuSyn (Biosoft), a software based on the Chou-Talalay combination index (CI) method,^{39,40} was used to determine the effects of the drug combinations. In the CI method, which is based on the median-effect equation, a derivation from the mass-action law, Drug1 is combined with Drug2 at a constant ratio $(IC_{50}^{\text{Drug1}})/(IC_{50}^{\text{Drug2}})$, and their effects on proliferation are determined by MTT assay. The resulting data, along with the data obtained from single drug treatments, were entered

into CalcuSyn to determine a CI value for each combination point, which quantitatively defines additivity (CI = 1), synergy (CI < 1) and antagonism (CI > 1). The resulting values were used to construct a plot of CI values over a range of fractions affected (F_a -CI plot).^{39,40}

Various methods. Estimation of protein concentration and western blotting was performed by using standard laboratory procedures as described by us previously.³⁶

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/cc/article/25043

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