Validation of a Novel Statistical Model for Assessing the Synergy of Combined-Agent Cancer Chemoprevention

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Abstract
Lung cancer is the leading cause of cancer death, developing over prolonged periods through genetic and epigenetic changes induced and exacerbated by tobacco exposure. Many epigenetic changes, including DNA methylation and histone methylation and acetylation, are reversible. The use of agents that can modulate these aberrations is a potentially effective approach to cancer chemoprevention. Combined epigenetic-targeting agents have gained interest for their potential to increase efficacy and lower toxicity. The present study applied recently developed statistical methods to validate the combined effects of the demethylating agent 5-aza-2-deoxycytidine (5-AZA-CdR, or AZA, or decitabine) and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA or vorinostat). This validation compared AZA alone with SAHA alone and with their combinations (at later or earlier time points and in varying doses) for inhibiting the growth of cell lines of an in vitro lung carcinogenesis system. This system comprises isogenic premalignant and malignant cells that are immortalized (earlier premalignant), transformed (later premalignant), and tumorigenic human bronchial epithelial cells [immortalized BEAS-2B and its derivatives 1799 (immortalized), 1198 (transformed), and 1170-I (tumorigenic)]. AZA alone and SAHA alone produced a limited (<50%) inhibition of cell growth, whereas combined AZA and SAHA inhibited cell growth more than either agent alone, reaching 90% inhibition under some conditions. Results of drug interaction analyses in the $E_{\text{max}}$ model and semiparametric model supported the conclusion that drug combinations exert synergistic effects (i.e., beyond additivity in the Loewe model). The present results show the applicability of our novel statistical methodology for quantitatively assessing drug synergy across a wide range of doses of agents with complex dose-response profiles, a methodology with great potential for advancing the development of chemopreventive combinations. Cancer Prev Res; 3(8); 917–28.

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Introduction
Lung cancer is the leading cause of cancer death in the United States (1). The severe morbidity and mortality from lung cancer have not been reduced, and the overall 5-year survival rate is a discouraging $\sim$15%, which has not improved substantially (despite advances in various treatment modalities) over the past 20 years (2). For these reasons, novel approaches for preventing and treating lung cancer are urgently needed (2).

Activation of oncogenes or amplification or mutations in growth-factor signaling molecules, along with deletions, loss of expression, or diminished function of tumor-suppressor genes, are hallmarks of cancer development (3, 4). Epigenetic events are also implicated in cancer development, including the silencing of critical genes by aberrant methylation of CpG islands in gene promoter regions and by changes in chromatin structure resulting from alterations in the state of histone acetylation (5). Certain compounds that modulate DNA methylation [e.g., DNA methyltransferase inhibitors including 5-aza-2′-deoxycytidine (5-AZA-CdR, or AZA, or decitabine) and 5-azacytidine (azacitidine)] or inhibit histone deacetylation [e.g., histone deacetylase (HDAC) inhibitors including suberoylanilide hydroxamic acid (SAHA, or vorinostat), butyrate derivatives, depsipeptide, and valproic acid] can reactivate the expression of epigenetically silenced genes and induce differentiation or apoptosis of malignant cells (5). Furthermore, phase II and III clinical trials of AZA and

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phase III trials of several HDAC inhibitors have produced promising results in several neoplastic settings (6). Combinations of modulators of DNA methylation and HDAC inhibitors have been shown to exert more than additive effects or synergistic effects in certain preclinical studies with cultured tumor cell lines (7, 8).

Comprehensive experiments to establish synergistic, antagonistic, or additive interactions are very difficult to perform in vivo because the assessment of synergy requires many challenging animal experiments to measure responses to single and combined agents over different dose ranges and time periods. Therefore, many studies have used in vitro cell systems that usually are fully malignant (e.g., refs. 9, 10). Information on agent effects in premalignancy requires the availability of cells that represent distinct stages of carcinogenesis. An excellent in vitro model of human bronchial epithelial (HBE) cells has been developed in the well-characterized BEAS-2B cell line, which was derived by immortalizing normal human bronchial epithelial (HBE) cells through simian vacuolating virus 40 (SV40) T/Adeno12 virus (11). The three cell lines, 1799 (immortalized), 1198 (transformed), and 1170-I (tumorigenic), were then derived from BEAS-2B cells after subcutaneous growth as xenotransplants in nude mice. 1198 and 1170-I were isolated after in vivo exposure of BEAS-2B transplants to cigarette smoke condensates (12). The full complement of cell lines reflects a spectrum of neoplasia from early premalignancy (BEAS-2B) to midstage premalignancy (1799) to advanced premalignancy (transformed 1198) to malignancy (1170-I). This in vitro human lung carcinogenesis model offers opportunities to identify new agents or combinations of agents that affect early and/or late stages of lung carcinogenesis and are potentially relevant to human lung cancer chemoprevention and therapy. We previously used this cell system to test the efficacy of combined celecoxib and fenretindine (13).

The standard median-effect equation for analyzing a two-drug combination (14, 15) is limited in its ability to address the complex dose-response pattern of chemopreventive combinations involving various doses and schedules. This complexity is underscored by the dose-response pattern of increasing doses of single agents, which can involve a monotonic relationship (increasing response) until a plateau effect is reached, or can involve a U-shaped curve; that is, increasing activity up to a point where activity begins to decrease again with higher doses. Such nonmonotonic dose-response relationships may relate to the body’s regulatory mechanism to maintain homeostasis and can be observed with natural agents. Therefore, we used the $E_{\text{max}}$ model to address the potential plateau effect of increasing doses of DNA methylation modulators and HDAC inhibitors and extended the semiparametric model (which is a recently developed theoretical model for assessing two drugs given simultaneously) to examine the complex drug interaction contour of different doses and schedules of our two-drug combinations (16, 17). Our present study validated this novel statistical methodology for quantitating the synergy indicated by previous qualitative analyses for the interaction between AZA and SAHA in suppressing the growth of premalignant and malignant HBE cells in vitro. The significance of this work is heightened by the growing, crucial potential of cancer chemoprevention with agent combinations (18).

**Materials and Methods**

**Cells and cell culture**

A collection of isogenic cells comprising an in vitro lung carcinogenesis model that includes immortalized (BEAS-2B and 1799), transformed (1198), and tumorigenic (1170-I) human HBE cells was obtained from Dr. Klein-Szanto (Fox Chase Cancer Center, Philadelphia, PA). BEAS-2B and 1799 cells were grown in keratinocyte serum-free medium (K-SFM, Gibco, Invitrogen Corporation) containing epidermal growth factor (5 ng/mL) and bovine pituitary extract (50 μg/mL) at 37°C in a humidified atmosphere of 95% air and 5% CO2. 1198 and 1170-I cells were maintained in K-SFM with 3% fetal bovine serum (HyClone). However, in some experiments, 1198 and 1170-I cells were cultured in serum-free K-SFM supplemented with epidermal growth factor and bovine pituitary extract as above. The four cell lines have been submitted for short tandem repeat (STR) genotyping (Powerplex 1.2 to the Johns Hopkins CORE Fragment Analysis Facility, Baltimore, MD) and the results obtained on November 9, 2009, have indicated that all cell lines had an identical STR pattern as expected because all were derived from the BEAS-2B. The STR profile was as follows: AMEL: X, Y; CSF1PO: 9, 12; D13S317: 13, 13; D16S539: 12, 12; D5S818: 12, 12; D7S820: 10, 13; THO1: 7, 9, 3; TPOX: 6, 11; vWA: 17, 18. This pattern was distinct from those of all cell lines listed in the American Type Culture Collection STR Database for Human cell lines in a search done on November 16, 2009 (http://www.atcc.org/CulturesandProducts/CellBiology/STRProfileDatabase/tabid/174/Default.aspx).

**Reagents**

SAHA was purchased from the Midwest Research Institute. It was dissolved in DMSO and diluted into growth medium before cell exposure. AZA was purchased from Sigma Chemical Co. It was also dissolved in DMSO at a concentration of 10 mmol/L, and aliquots were stored at −80°C. The stock solutions were diluted to the desired concentrations with culture medium before their use, keeping the final concentration of DMSO <0.1%.

**Single agents: protocol and dose-response/growth inhibition model**

The effects of AZA and SAHA as single agents were examined following an experimental design that is described pictorially in Fig. 1 (groups B–D). The cells were harvested by trypsinization from subconfluent stock cultures and seeded in 96-well plates at a density of 3,000 per well.
24 hours before treatment (time designated as -24 hours) to allow them to attach to the surface of the dishes. BEAS-2B and 1799 immortalized cells were analyzed for single-agent effects in their optimal growth medium, which is serum-free, whereas 1198 transformed cells and 1170-I tumorigenic cells were analyzed in both their optimal growth medium, which contains 5% serum, as well as in the same serum-free medium as the immortalized cells. AZA was added to untreated cells either at time 0 hour (Fig. 1; designated as early AZA) or 72 hours later (late AZA) or at both times. SAHA was added to untreated cells only at 72 hours. Cell numbers in triplicate wells were estimated at time 144 hours by the colorimetric sulforhodamine B assay with reagents purchased from Sigma-Aldrich using an automated plate reader (model MR5000, Dynatech Laboratories, Inc.), and the values were used to calculate the percentage of growth inhibition (%GI) using equation A:

\[
\% \text{GI} = (1 - \frac{At}{Ac}) \times 100 \quad (A)
\]

where At and Ac are the optical densities in treated and control cultures within the same plate, respectively (19).

The percentage of growth inhibition versus the dose levels for early AZA, late AZA, and SAHA when used alone were plotted in Fig. 2A to F. In each figure panel, the solid line is the fitted dose–growth inhibition curve for each single agent based on the following \(E_{\text{max}}\) model (20):

\[
y = \frac{E_{\text{max}}}{1 + \left(\frac{d}{ED_{50}}\right)^m} \quad (B)
\]

In the \(E_{\text{max}}\) model (Eq. B), \(E_{\text{max}}\) is the maximum growth inhibition attributable to the single agent, \(ED_{50}\) is the dose required to produce 50% of the maximal growth inhibition (0.5\(E_{\text{max}}\)), and \(d\) is the dose level that produces growth inhibition \(y\). Note that the \(E_{\text{max}}\) model does not require 100% growth inhibition as the dose of the drug increases to infinity. It reflects the nature of many chemopreventive agents; that is, as the dose continues to increase, the effect may reach a plateau.

**Combined agents: growth inhibition and statistical method to assess synergy**

The protocols used for treating cells with combinations of AZA and SAHA are depicted in Fig. 1 (protocols E, F,
Fig. 2. Dose-response curves of treating immortalized, transformed, and tumorigenic human bronchial epithelial cells with 5-AZA-CdR or SAHA as single agents. Cells were grown without serum (A, B, C, and D) or with serum (E and F) and treated with the indicated single agents according to protocols B, C, and D (see Fig. 1), where the growth inhibition was calculated relative to the growth of cells under protocol A (no treatment).
and G). In protocol E, AZA was added to cells at time 0 hour (early AZA) and SAHA was added 72 hours later. In protocol F, AZA was added at time 0 hour (early AZA), and a combination of AZA (late AZA) and SAHA was added 72 hours later. In protocol G, a combination of AZA (late AZA) and SAHA was added at 72 hours. We did not include an early SAHA treatment followed by AZA in the protocol because demethylation is a prolonged process (requiring DNA replication and incorporation of AZA), whereas HDAC inhibition is a rapid process that occurs within hours. Protocol H was a control for consecutive treatment of cells with early AZA and late AZA without SAHA. The cells were harvested 144 hours after treatment initiation, and growth inhibition was estimated as described above. To examine the growth inhibition for different combined doses, we summarized the mean and SEM for the observed growth inhibition at each combination dose. For each fixed early AZA dose level (range 0–0.5 μmol/L without serum and range 0–2.0 μmol/L with serum), we plotted the growth inhibition versus late AZA at each of the dose levels for SAHA (0–2 μmol/L), as shown in Fig. 3A to F. These plots enabled us to examine when the maximal growth inhibitions were obtained and how the growth inhibitions vary as dose levels for early AZA, late AZA, and SAHA increase. We decided to use the Loewe additivity model (15) for this analysis because we have recently shown that this model is one of the best general reference models for evaluating drug interactions. The Loewe additivity model (Eq. C) for three drugs can be described as

\[
\frac{d_1}{D_{y,1}} + \frac{d_2}{D_{y,2}} + \frac{d_3}{D_{y,3}} = 1 \quad \text{(C)}
\]

where \((d_1, d_2, d_3)\) is the combination dose of early AZA, late AZA, and SAHA in the mixture that produces a growth inhibition \(y\) under additivity; \(D_{y,1}, D_{y,2},\) and \(D_{y,3}\) are the respective doses of early AZA, late AZA, and SAHA that result in the growth inhibition \(y\) when used alone. The Loewe additivity model can be equivalently written as

\[
d_1 + \frac{D_{y,1}}{D_{y,2}}d_2 + \frac{D_{y,1}}{D_{y,3}}d_3 = D_{y,1}
\]

where the ratio \(D_{y,1}/D_{y,2}\) is called the relative potency of drug 2 versus drug 1. Thus, the growth inhibition produced by drug 2 at dose level \(d_2\) is the same as the growth inhibition produced by drug 1 alone at dose level \(D_{y,1}d_2/D_{y,2}\). Similarly, the growth inhibition produced by drug 3 at dose level \(d_3\) is the same as the growth inhibition produced by drug 1 alone at dose level \(D_{y,1}d_3/D_{y,3}\). Without drug interaction, the growth inhibition produced by the combination dose \((d_1, d_2, d_3)\) should be the same as the growth inhibition produced by drug 1 alone at dose level \(d_1 + \frac{D_{y,1}}{D_{y,2}}d_2 + \frac{D_{y,1}}{D_{y,3}}d_3\); that is, \(D_{y,1}\). Based on the estimated dose–growth inhibition curve for each single drug and the Loewe additivity model, one may predict the additive growth inhibition at a combination dose by solving \(y\) in Eq. C. Synergy occurs when the actual growth inhibition is more than the predicted growth inhibition, whereas antagonism occurs when the actual growth inhibition is less than the predicted growth inhibition.

To obtain an overall picture of drug interactions for the combination doses of early AZA, late AZA, and SAHA, we extend the semiparametric model (17) to assess the growth inhibition beyond additivity for the three drug combinations. To this end, we first obtained the differences of the observed growth inhibitions and the predicted growth inhibition at each observed combination dose \((d_{1p}, d_{2p}, d_{3p})\) \((i = 1, ..., n)\). Based on this information, we can then estimate the function \(f(d_1, d_2, d_3)\), the growth inhibition beyond additivity, for any combination dose \((d_1, d_2, d_3)\) by using the thin plate splines (21), where \(f(d_1, d_2, d_3)\) can be expressed as

\[
f(d_1, d_2, d_3) = \gamma_0 + \gamma_1d_1 + \gamma_2d_2 + \gamma_3d_3 + \sum_{k=1}^{K} \eta(\frac{d_1}{D_{y,1}}, \frac{d_2}{D_{y,2}}, \frac{d_3}{D_{y,3}})
\]

where

\[
\eta(\frac{d_1}{D_{y,1}}, \frac{d_2}{D_{y,2}}, \frac{d_3}{D_{y,3}}) = \sqrt{\left(\frac{d_1 - k_{1k}}{k_{1k}}\right)^2 + \left(\frac{d_2 - k_{2k}}{k_{2k}}\right)^2 + \left(\frac{d_3 - k_{3k}}{k_{3k}}\right)^2}
\]

and \((k_{1k}, k_{2k}, k_{3k})\) \((k = 1, ..., K)\) are the knots for the thin plate splines, which are selected as all the different observed combination doses. We used the technique developed by Kong and Lee (17) to estimate the function \(f(d_1, d_2, d_3)\). In addition, we constructed a 95% confidence interval for \(f(d_1, d_2, d_3)\). Thus, different patterns of drug interactions can be clearly identified.

In short, the \(E_{max}\) model (20) was applied to describe the dose–growth inhibition relationship of early AZA, late AZA, or SAHA used alone. The predicted additive growth inhibition for combinations was calculated based on the Loewe additivity model and the marginal dose–growth inhibition curves. After that, the growth inhibition beyond additivity was determined by applying the semiparametric model (17) such that the patterns of drug interactions (i.e., synergy, additivity, or antagonism) over all experimental combinations can be characterized.

**Results**

**Single agents**

The dose-response curve for each single drug calculated using \(E_{max}\) model in S-PLUS is presented in Fig. 2A–F. When early AZA, late AZA, and SAHA were used alone, the estimated maximum growth inhibitions \((E_{max})\) of BEAS-2B cells were 15%, 19%, and 19%, respectively (Fig. 2A). Note that these estimated maximum growth inhibitions are obtained based on the \(E_{max}\) model (E2),
which may be larger than the maximum observed growth inhibition. The estimated maximum growth inhibitions of 1799 cells for early AZA, late AZA, and SAHA used alone were 45%, 19%, and 45%, respectively (Fig. 2B). Treatment of 1198 cells with early AZA, late AZA, and SAHA alone in medium without serum (Fig. 2C) resulted in estimated maximum growth inhibitions of 36%, 36%, and 30%, respectively. Similar results (33%, 33%, and 44%, respectively) were observed when 1198 cells were treated with these agents in medium with serum (Fig. 2E). Treatment
of 1170-I cells with early AZA, late AZA, and SAHA alone resulted in estimated maximum growth inhibitions of 47%, 16%, and 44%, respectively, in medium without serum (Fig. 2D) and 55%, 42%, and 30%, respectively, in the presence of serum (Fig. 2F).

The four cell lines exhibited dose-dependent inhibition of growth, which was variable among the different cell lines and between the two agents and growth media. For most cell lines, the growth inhibition to AZA peaked at low doses (e.g., 0.5 μmol/L) and was not improved much with increased agent dose up to 2 μmol/L. In contrast, most cell lines showed progressive increase in growth inhibition by SAHA as the dose of this agent increased up to 2 μmol/L. The presence of serum increased the sensitivity of 1198 cells to late AZA and SAHA (Fig. 2C and E), whereas serum increased the sensitivity of 1170-I cells to early AZA and late AZA (Fig. 2F).

**Combined agents**

Because, with a few exceptions, AZA alone and SAHA alone were not very potent (<50% growth inhibition), we examine the possibility that a combination of the two agents, which reverse epigenetic gene silencing by distinct mechanisms, may produce additive or synergistic effects on growth inhibition. The dose-response curves for the drug combinations administered according to protocols E to H (Fig. 1) are presented in Fig. 3A to E. When the Loewe additivity model was applied to these results for each cell line under each condition (growth with or without serum), we obtained the predicted growth inhibitions for each combination dose. We then estimated the growth inhibition beyond additivity by applying the semiparametric model as described in Materials and Methods. The differences of the observed growth inhibitions and their predicted growth inhibitions (namely, residuals) based on the pure additivity model were plotted versus the predicted growth inhibitions in panel 1s of Fig. 4A to F, and the residuals based on the semiparametric model were plotted versus the predicted growth inhibitions in panel 2s of Fig. 4A to F. When the residuals based on the pure additivity model are small and centered around zero, the result suggests that the pure additivity model fits the data well. Positive residuals from the pure additivity model indicate synergistic combinations whereas negative residuals illustrate antagonistic effects.

By examining panel 1s of all six data sets, we conclude that the additivity model could not adequately describe the observed growth inhibition data. Most residuals from the pure additivity model lie in the positive regions, suggesting synergistic effects. After applying the semiparametric method for modeling the combination data, the resulting residuals are centered around zero, as shown in panel 2s of the corresponding figures. Furthermore, Fig. 5 shows the corresponding contour plots of the residuals beyond additivity using the semiparametric model. Each panel displays the contour plot of the beyond additivity growth inhibition of SAHA and late AZA by conditioning on early AZA. More detailed descriptions on the patterns of drug interactions for each individual cell lines are described below.

**Immortalized BEAS-2B and 1799 cells treated with early AZA, late AZA, and SAHA combinations in serum-free medium.** Growth inhibition of BEAS-2B cells (Fig. 3A) and 1799 cells (Fig. 3B) increased as early AZA increased up to 0.5 μmol/L and SAHA increased up to 1.0 μmol/L. The figure also indicates that the combination of both agents even at low doses, for example, early AZA at 0.5 μmol/L, late AZA at 0.1 μmol/L, and SAHA at 1 μmol/L, can achieve more than 70% growth inhibition of BEAS-2B cells, whereas any single agent only achieved <20% growth inhibition. In 1799 cells, late AZA had no enhancing effect but the combination of early AZA at 0.5 μmol/L and SAHA at 1 μmol/L achieved nearly 80% growth inhibition compared with <45% by any single agent.

The residuals based on the Loewe additivity model versus the predicted growth inhibition are presented in Fig. 4A.1 and B.1 for BEAS-2B and 1799 cells, respectively. Many of the differences, particularly for higher predicted growth inhibitions, are above zero, suggesting that this pure additivity model does not describe the data adequately. Applying the semiparametric model to each of the data sets, residual plots in Fig. 4A.2 for BEAS-2B cells and Fig. 4B.2 for 1799 cells show that the residuals are centered around zero, indicating that the semiparametric model is a better model to describe these experimental data. We have also drawn the contour plot for the growth inhibitions beyond additivity at each fixed-dose early AZA level as shown in Fig. 5A for BEAS-2B cells and in Fig. 5B for 1799 cells. Panels 1, 2, and 3 in each figure show that the combination doses in the areas above the thick solid curves (in light blue) are antagonistic, and the combination doses in the areas below the thick dashed lines (uncolored) are additive, and the combination doses in the area under the thick dashed lines (in light pink) are antagonistic. The combination doses with early AZA at 0.5 μmol/L (panel 4) are mostly synergistic.

**Transformed 1198 cells treated with early AZA, late AZA, and SAHA or their combination in serum-free and serum-supplemented media.** We found that in 1198 cells grown in serum-free medium, growth inhibition increased as early AZA increased up to 0.5 μmol/L as well as SAHA increased up to 1 μmol/L (Fig. 3C). Further enhancement of growth inhibition was achieved when a small dose of late AZA (e.g., 0.1 μmol/L) was added, but no further inhibition was obtained by increasing the late AZA dose to 1 μmol/L (Fig. 3C). The combination of 0.5 μmol/L early AZA, 0.1 μmol/L late AZA, and 1 μmol/L SAHA achieved almost 80% growth inhibition, whereas the best single agent could only achieve <36% growth inhibition. When 1198 cells were treated in serum-supplemented medium, growth inhibition increased as early AZA increased up to 2 μmol/L, late AZA increased to 1 μmol/L, and SAHA increased up to 2 μmol/L (Fig. 3E). Increasing the early AZA doses above 0.5 μmol/L did not improve its growth-inhibitory effects in combination with 0.5, 1, and...
Fig. 4. A–F, panel 1 shows the plots of the difference between observed and predicted growth inhibition on the Y axis versus predicted growth inhibition on the X axis based on the pure additivity model, whereas panel 2 shows the plots of the difference between observed and fitted growth inhibition on the Y axis versus the predicted growth inhibition on the X axis based on the semiparametric model.
Fig. 5. Contour plots for the growth inhibitions beyond additivity of the indicated cell lines at each fixed dose level of early AZA with SAHA doses on the Y axis and the late AZA doses on the X axis. The combination doses in the light-blue areas are synergistic (Syn.); the combination doses in the uncolored areas are additive (Add.); and the combination doses in the light-pink areas are antagonistic (Ant.). For the cells grown in serum-free medium (A–D), when the dose level for early AZA is nonzero, the experimental dose range for late AZA is 0 to 1 μmol/L, and the dose range for SAHA is 0 to 1 μmol/L.
2 μmol/L SAHA. The combination doses of early AZA (0.5 μmol/L), late AZA (0.1 μmol/L), and SAHA (1 μmol/L) achieved 90% growth inhibition, whereas, under the same condition, each single agent could only achieve <44% growth inhibition (Figs. 2E and 3E).

Based on the Loewe additivity model, we obtained the predicted additive growth inhibition for each combination dose as previously described. For 1198 cells treated in the absence (Fig. 4C.1) and in the presence of serum (Fig. 4E.1), most of the residuals based on the Loewe additivity model were found to be above zero, indicating that the additive model is not adequate for describing the data. However, the semiparametric model was found to be a better model to describe the experimental data as indicated by the finding that the residuals from the semiparametric model are centered around zero in Fig. 4C.2 and E.2.

Contour plots for the growth inhibitions without and with serum beyond additivity at each fixed dose level for early AZA indicated the type of drug interactions (Fig. 5C and E, respectively). In each panel, the combination doses in the area above the thick solid curve (light-blue area) are synergistic. Figure 5C and E indicates that most of the combination doses of SAHA, late AZA, and early AZA are synergistic.

Tumorogenic 1170-I cells treated with combinations of early AZA, late AZA, and SAHA in serum-free and serum-supplemented media. Treatment of 1170-I cells with combination of agents in serum-free medium showed that the response from 0.1 to 1 μmol/L SAHA was improved by pretreatment with early AZA in the dose range from 0.05 to 0.5 μmol/L (Fig. 3D). Addition of late AZA at a low dose level of 0.1 μmol/L had a small effect on growth inhibition; however, addition of late AZA at 0.5 μmol/L or beyond did not increase growth inhibition. The combination of 0.5 μmol/L early AZA, 0.1 μmol/L late AZA, and 1 μmol/L SAHA achieved 80% growth inhibition, whereas any single agent could only achieve <47% growth inhibition (Fig. 2D).

When 1170-I cells were treated in the presence of serum, their sensitivity to the combination treatment increased relative to the serum-free treatment. Growth inhibition increases as early AZA dose increased up to 0.5 μmol/L and SAHA dose increased up to 2 μmol/L. The addition of late AZA enhanced the growth-inhibitory effects when early AZA doses were 0.05, or 0.1 μmol/L, but only slightly for early AZA dose at 0.5 μmol/L and beyond, reaching plateaus for late AZA dose at 0.5 μmol/L and beyond (Fig. 3F). The efficacy of the combination of early AZA and SAHA reached a plateau after early AZA doses >0.5 μmol/L. Figure 3F also indicates that the combination of 0.5 μmol/L early AZA and 2 μmol/L SAHA achieved 85% growth inhibition, which was much higher than any single agent could achieve.

Under the additive model, Fig. 4D.1 shows that there were some negative residuals when the predicted growth inhibition was small. Moreover, most residuals in Fig. 4D.1 (in the absence of serum) and F.1 (in the presence of serum) were positive, indicating that the additive model had failed to describe the data well. In contrast, the residual plots based on the semiparametric model (Fig. 4D.2 and F.2) show that the residuals are centered around zero, indicating that the semiparametric model describes the experimental data well. The contour plots for the growth inhibitions beyond additivity at each fixed dose level for early AZA are shown in Fig. 5D and F for cells treated in serum-free and serum-supplemented medium, respectively. In each panel in Fig. 5D, the combination doses in the light-blue areas are synergistic, the combination doses in the light-pink areas are antagonistic, and the combination doses in the uncolored areas are additive. When tumorigenic 1170-I cells were treated in serum-free medium (Fig. 5D.1), in the absence of early AZA, the concurrent combination of SAHA and late AZA are antagonistic or additive. The addition of a low dose of late AZA following early AZA enhanced the synergistic effect (Fig. 5D). In the presence of serum, most of the combination doses of early AZA, late AZA, and SAHA were synergistic except when the early AZA and SAHA are both low; for example, early AZA ≤0.05 μmol/L and SAHA ≤0.5 μmol/L (Fig. 5F).

It is noted that the dose range for late AZA is 0 to 1 μmol/L and the dose range for SAHA is 0 to 1 μmol/L for cells grown in serum-free medium (Fig. 5A–D) when the dose level for early AZA is nonzero. Although the statistical model provides estimates in the entire dose range, the extrapolations beyond the actual data range should be done with caution.

**Discussion**

Although complex, the present findings clearly indicate that combining AZA with SAHA synergistically inhibited growth in most settings (versus results of either agent alone) and that increasing SAHA doses (within the studied range) almost always increased growth inhibition. We also found in regard to the AZA-SAHA combinations that the dose-response (growth inhibition) pattern of late AZA varied between combinations with lower-concentration early AZA (<0.5 μmol/L) and higher-concentration early AZA (≥0.5 μmol/L); the concentration range of early and late AZA was 0–2 μmol/L. As evidenced by these data, early and late AZA were evaluated as two different drugs, illustrating the applicability of our model to three-drug combinations and its value for finding complex, subtle synergistic effects. Furthermore, the overall growth-inhibition results validate our novel statistical method for quantitatively assessing drug synergy (over a wide range of doses and different schedules and medium conditions) with comparisons that can be tested for significance. Although effective chemopreventively (in immortalized BEAS-2B or 1799 cells and transformed 1198 cells) or therapeutically (in malignant 1170-I cells), this method is especially important for assessing the complex dose-response relationships of chemopreventive combinations. Another advantage of the method for chemoprevention is its ability to analyze synergistic interactions between agents or doses with relatively modest effects.

Several agent combinations are more effective than single agents in treating certain cancers (9, 22–24). It is thought that future strategies will focus on synergistic drug
combinations that can enhance efficacy by targeting several alternative pathways and decrease side effects (e.g., by lowering constituent-agent doses). These fundamental principles of combination approaches are applicable to both therapy and chemoprevention and to cytotoxic and noncytotoxic agents. Epigenetic therapy targets different chromatin modifications (e.g., methylation and acetylation) to reach an effective reversal of epigenetic alterations (25). The use of HDAC inhibitors and demethylating agents in chemoprevention has received increasing attention as the role of epigenetic alteration at early stages of cancer development has been recognized (26–28). Indeed, combinations of demethylating agents (mostly decitabine) and HDAC inhibitors (phenyl butyrate, valproic acid, SAHA) have shown enhanced activity relative to single agents in various preclinical studies (7, 29, 30), and some combinations have been tested in animal models and clinical trials (31, 32). For example, short-term, low-dose AZA decreased the incidence of tobacco carcinogen–induced lung neoplasms by 30%, the HDAC inhibitor phenylbutyrate alone was ineffective, and combined AZA and phenylbutyrate reduced these neoplasms by >50% in female mice, suggesting that the combination effect was more than additive (29). In another mouse model, a combination of AZA and the HDAC inhibitor valproic acid was more effective than either agent alone in preventing the development of spontaneous medulloblastoma and rhabdomyosarcoma in Patched (Ptch) heterozygous mice (30). These studies used a single dose of each agent and thus precluded the determination of additivity or synergy for the combinations. Even previous in vitro studies, however, suggested a synergistic interaction between decitabine and HDAC inhibitors, absent any formal quantitative analysis (33) or with calculations of the combination index (CI; refs. 7, 8, 34).

We did not address the mechanisms by which AZA, SAHA, and their combination inhibited cancer cell growth because we focused this investigation on the nature of the interactions between these epigenetic modifiers. We and others have shown previously that SAHA and AZA can modulate the expression of genes involved in cell cycle progression and apoptosis induction by both the mitochondrial and death-receptor pathways (35); these effects could mediate these agents’ synergistic inhibitory effects on cell growth in the system reported here as well.

Studying the effects of 48-hour treatment with AZA or SAHA or both combined in breast carcinoma MDA-MB-231 cells, Beltran et al. (8) generated dose-effect curves and median-effect plots of cell proliferation, using a median-effect equation, $E_{\text{max}} = I$, that can be considered a special case of the $E_{\text{max}}$ model. The median-effect dose ($ED_{50}$) and slope ($m$) were calculated from the median-effect plots and introduced into the isobologram equation for the calculating the CI (14, 15). They found CI values between 0.5 and 1, indicating a synergistic effect.

Kumagai et al. (7) analyzed the in vitro effects of the combination of AZA and SAHA on pancreatic cancer cells. Their colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that treatment for 96 hours with SAHA (2.5 μmol/L) plus AZA (1 μmol/L) in medium with 10% fetal bovine serum decreased cell growth of the pancreatic cancer cell lines PANC-1 by 31% and AsPC-1 by 16% (versus control). These levels of growth inhibition were much higher than those accomplished with each agent alone at the same concentrations. They then used the CI to evaluate the nature of the interaction between the two agents (29). The CIs were 0.54 (<1.0) for PANC-1 and 0.48 (<1.0) for AsPC-1. Because both CIs were <1, the authors concluded that combined AZA and SAHA had synergistic antiproliferative effects on these pancreatic cancer cells.

A major contribution of this report is in extending and applying novel statistical methods to complex combined-agent studies. Single chemopreventive agents usually have a limited ability to inhibit growth. Chou and Talalay’s median-effect equation (14) assumes that growth inhibition reaches 100% at a certain (increased) dose, which is not always true. Hence, we applied the $E_{\text{max}}$ model to address the phenomenon of a plateau dose, past which growth inhibition does not advance toward 100%. We plotted additivity in the Loewe model and then compared this plot with that of overall activity to determine whether Loewe additivity provided an adequate fit of the data. Many combination doses produced positive residual plots, indicating drug synergy. We then applied the semiparametric model proposed by Kong and Lee (17) to model combination drug effects beyond additivity. Furthermore, many older methods require fixed ratios of doses in combinations (also known as the ray design) for calculating the CI (14, 34). In contrast, our method can be applied to any dose ratios and thus can present an overall picture of drug interactions for the entire range of experimental doses. The patterns of drug interactions based on our approach are fully determined by the experimental data without any presumed parametric models. By examining growth inhibition over all combined doses (Fig. 3) and the patterns of drug interactions (Fig. 5), we can identify low doses with highly synergistic combination effects on growth inhibition.

Although translating in vitro data to the clinic requires caution, our results have clinical implications in demonstrating that low doses of AZA (<0.5 μmol/L) and SAHA (<1 μmol/L) can effectively suppress the proliferation of immortalized lung cells and cells representing later premalignant and malignant lung epithelial lesions. Our results also indicate that early administration of AZA greatly enhances the effect of combined AZA and SAHA. Low combined doses of these agents were effective and have the potential to decrease adverse effects of the higher dose that either agent alone would need to be effective; this reduction in toxicity is an important advantage for chemoprevention, where side effects should be minimal or absent.

In conclusion, the present findings suggest that combined demethylating agents and HDAC inhibitors may be a rational approach for clinical cancer chemoprevention or therapy trials in the lung. These results also validate our novel statistical method for quantitatively assessing drug synergy. We succeeded in assessing the complex
dose-response relationship of chemopreventive combinations and in analyzing synergistic interactions between doses with relatively modest single-agent effects, and this methodology has great potential for advancing the development of cancer chemoprevention with combined agents, one of the most important directions of this field.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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Validation of a Novel Statistical Model for Assessing the Synergy of Combined-Agent Cancer Chemoprevention

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