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Melding a New 3-Dimensional Agarose Colony Assay with the E_{max} Model to Determine the Effects of Drug Combinations on Cancer Cells

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Abstract

The goal of our study was two-fold: (i) develop a robust 3D colony assay methodology to interrogate drug combinations using GelCount™ and (ii) to develop 2-drug combinations that might be useful in the clinic for the treatment of high-grade gliomas. We used three glioma cell lines (U251MG, SNB19, and LNZ308) and two adenocarcinoma cell lines (MiaPaCa and SW480) grown as colonies in a two-tiered agarose cultures. We evaluated two-drug combinations of difluoromethylomithine (DFMO), carboplatin, vorinostat (SAHA), and docetaxel. To analyze for antitumor efficacy we used GelCount™ to measure the area under the curve for tumor colony volumes ($\mu\text{m}^2 \times \text{OD}$) in each plate. The non-linear dose-response E_{max} model and the interaction index based on the Loewe additivity are applied to calculate two-drug synergy, additive, and antagonistic interactions.

For glioblastoma cell lines, (i) carboplatin followed by DFMO was synergistic or additive in 2/3 cell lines, (ii) carboplatin before SAHA was synergistic in 1 cell line, (iii) carboplatin before docetaxel was synergistic in 2/3 cell lines and partially additive in the third, (iv) SAHA before docetaxel was synergistic in 1/3 cell lines, (v) docetaxel before DFMO was additive or partially active in 3/3 cell lines, and (vi) DFMO plus SAHA was inactive regardless of order. In the MiaPaCa cell line, synergy occurred when DFMO followed carboplatin and, at short exposure times, when SAHA was combined with carboplatin (regardless of order). In the SW480 cell line synergy occurred only in short exposures for carboplatin followed by docetaxel; additive and mixed partial effects were also seen with DFMO plus carboplatin or docetaxel (regardless of order), carboplatin before DFMO, carboplatin before SAHA, and docetaxel before carboplatin.

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In conclusion, by applying the Gelcount™ automated counting and sizing of colonies and the use of $E_{\max}$ and Loewe models to define drug interactions, we can reliably define drug combination efficacy as a function of log dose and duration of drug exposure.

Keywords

Glioma; Alpha-difluoromethylornithine; Carboplatin; Docetaxel; Vorinostat

Introduction

The purpose of this article is two-fold: (i) to describe a methodology for evaluating drug combinations in 3-dimensional agarose colony growth assay using a newly developed methodology utilizing GelCount™ equipment (1) and (ii) to investigate the efficacy of individual agents and drug combinations in three-dimensional (3D) cultures of glioblastoma and adenocarcinomas cell lines. The driving force for our research is the fact that there is a dearth of drugs and effective drug combinations for the treatment of high-grade [World Health Organization (WHO) grade 3 and 4] tumors. To an extent, the treatment of glioblastoma (WHO grade 4) and other high-grade glial tumors is an unmet clinical need with median survival of approximately 12–14 months for glioblastoma and 4–6 years for anaplastic gliomas (WHO grade 3) (2, 3). In both cases the morbidity of treatment and the societal costs are among the highest of any cancer.

We recently developed a new and efficient preclinical 3D growth method that makes use of GelCount™ technology to analyze drug effects over time (1). The method we developed allows a more accurate representation of the cell growth compared to the traditional 2-D assay. The technology is non-destructive so cell growth can be measured repeatedly over time. In this manner it is more reproducible and quantitative than traditional colony assays that rely on staining and the visual recognition of colony size and number. In addition, the assay is also more robust, since it allows repetitive measurements of colony size without the need for staining and/or the inactivation of colony growth. In this paper we utilize this methodology to further assess its applicability and reliability for studying drug interactions based on measuring the integral of cell colony size over time.

For this report, we wanted to emphasize less commonly studied drugs that do not alkylate DNA. By choice we excluded the nitrosoureas and temozolomide. As a result, we studied various combinations of alpha-difluoromethylornithine (DFMO, eflornithine), carboplatin, vorinostat [suberoyl-anilide hydroxamic acid (SAHA)], and docetaxel (taxotere). DFMO is an irreversible inhibitor of ornithine decarboxylase [L-ornithine decarboxylase (ODC)] that converts ornithine to the polyamine putrescine and that has shown good antitumor activity in clinical trials (4–7). The relatively safe clinical profile of DFMO was also a good reason for trying to combine it with various other drugs for clinical application.

We chose carboplatin, a platinum-based compound that binds to DNA, since combinations of cisplatin and DFMO have shown effects ranging from antagonism (8–12) to additivity or synergism (13–15) on the growth of tumor cell lines *in vitro*.

SAHA inhibits all class I and II histone deacetylases (HDACs); induces growth arrest, differentiation, and/or apoptosis of transformed cells *in vitro* (16–20); and inhibits tumor growth *in vivo* (21–25). HDAC inhibitors target the cell cycle checkpoint and chromatin structure, which can be altered during tumorigenesis. SAHA has also shown promise in preclinical studies with glioma models (26–28), has been tested against a variety of cancers in clinical trials (29, 30), and is now under investigation for the treatment of high-grade gliomas.

Docetaxel is a microtubule-stabilizing agent that, by interfering with spindle microtubule dynamics, causes cell cycle arrest and apoptosis. The usefulness of docetaxel in gliomas has not been established (31, 32) and its usefulness is limited by development of drug resistance (33).

Materials and Methods

The methodology used in the agarose colony formation assay has been described previously (34). Portions of the sections that follow were taken from that paper but are reproduced here to aid the reader (see Cartoon 1). While the emphasis of these studies is drug combinations active against glioblastoma cell lines, we studied two adenocarcinoma cell lines in parallel to determine if the response to the glioblastoma cell lines was unique to those lines.

Cell Lines and Culture Conditions

We used three glioma cell lines (U251MG, SNB19, LNZ308) and two adenocarcinoma cell lines MiaPaCa (pancreas) and SW480 (colon). Our choice of cell lines was based partly on their levels of ODC and sensitivity to DFMO (1) (S. Panchabhai, personal communication, 2008). They were maintained in Dulbecco's modified essential/F12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere of 95% air/5% carbon dioxide at 37 °C. Cells were harvested with trypsin/ethylenediamine tetraacetic acid solution, rinsed twice with Ca²⁺- and Mg²⁺-free phosphate-buffered saline, and resuspended to yield a final concentration of 1200 cells/ml of matrix volume in a 12-well plate.

We studied DFMO, carboplatin, SAHA, and docetaxel over a 3-log dose range, both as single agents and in combination. DFMO was a gift of Patrick Woster (Wayne State University, Detroit, MI). Carboplatin was purchased from Sigma-Aldrich (St. Louis, MO). SAHA was a gift of William Bornmann (The University of Texas M. D. Anderson Cancer Center, Houston, TX). Docetaxel was purchased from The University of Texas M. D. Anderson Cancer Center Pharmacy.

DFMO and carboplatin were dissolved in pyrogen-free water or culture medium and remains stable in solution for years at concentrations to 200 mg/ml. SAHA was dissolved in dimethylsulfoxide and stored as 10 mM (2.643 mg/ml) stock solutions at –80 °C. Docetaxel was dissolved in 13% ethanol in water (using the diluent provided with the vial (sanofi-aventis, Bridgewater, NJ) and stored as stock solutions of 0.010 mg/ml at 4 °C.

3D Agarose Colony Formation Assay

Cells were grown in 12-well plates at a total of 1200 cells/well (details in *Clonogenic Assay* below). Between 3–15 days, colonies were counted using the GelCount™ technology (Oxford Optronix Inc., Oxford, United Kingdom). GelCount™ scan and image acquisition were done using Microsoft Windows-based software developed by Oxford Optronix that uses a proprietary image processing algorithm with their high-resolution scanner. Using their digital image processing and software algorithms, one can achieve accurate colony detecting and counting (1).

Two-drug Combination Assay

The first drug was added on day 5 for glioma cell lines and day 2 for adenocarcinoma cell lines. The second drug was added 48 hours later. Drug dosing always covered a 3-log dose range and doses were equally spaced, but depending on the cell line and the experiment, the DFMO dose could vary between 1 and 4416 μM , SAHA between 0.05 and 126 μM , carboplatin between 0.2 and 315 μM , and docetaxel between 0.1 and 141 μM .

After adding different dose combinations of the two drugs, we determined the time course of growth suppression of colonies in each well. Colonies were counted 3, 5, and 7 days after the second drug was added. For each drug combination experiment, the two drugs were studied separately to determine an average concentration inhibiting cell growth by 50% (IC_{50} values) of each drug alone (Table I).

Clonogenic Assay

Day 1: Making the Assay—All assays were performed in triplicate using non-tissue culture-treated 22-mm culture wells. Cells ($n = 1,200$) were suspended in a 1.0 ml volume, 70% of which was medium, 13% of which was fetal calf serum, and 17% of which was 0.5% agarose (Fisher Scientific, Austin, TX). This suspension was layered over a solidified 1.0 ml matrix, 70% of which was medium, 13% of which was fetal calf serum, and 17% of which was 0.7% agarose. An additional 0.4 ml of media was layered over this cell matrix. Matrices formed as gels in < 20 minutes.

Day 6 (Glioma Cells) or Day 2 (Adenocarcinoma Cells): Addition of First Drug

—After a 5-day equilibration period for glioma cell lines and 1 day of equilibration for adenocarcinoma cell lines, the first drug dissolved in 0.2 ml of medium was sterile filtered using a 0.2 μm mesh filter and mixed proportionally with medium to achieve threefold log doses. The 2.8 μl working volume was taken into account during calculations. Control wells received 0.2 ml of medium alone.

Day 8 (Glioma Cells) or Day 4 (Adenocarcinoma Cells): Addition of a Second Drug

—After 48 hours of exposure to the first drug, the second drug (dissolved in 0.1% DMSO) was sterile filtered and proportionally mixed with 0.2 ml of medium. The 2.8 ml working volume was taken into account when calculating the desired doses. Control wells received 0.2 ml of medium without drug.

Days 8–15 (Glioma Cells) or Days 4–11 (Adenocarcinoma Cells): Imaging and Counting—Because the growth rate of control colonies varied among cell lines, images were obtained, based on optimal colony growth, between days 4 and 15 using GelCount™. Note that for assessing drug interactions under each specific condition, the cell volume on the same day for the each of the single agents and their combinations were used in the subsequent calculations.

Analysis of the GelCount™ Data

GelCount™ scans of the 3D colonies yielded outputs to Microsoft Excel files regarding five parameters: measured diameter (d , in μm), measured optical density (OD, in units), calculated area (A , in μm^2), calculated volume (V , in μm^3), and the distance of the nearest neighbor colony (μm). The last was not used in these studies. Where

$$A = \pi d^2 / 4 \quad [1]$$

$$V = A \times \text{OD} \quad [2]$$

From these measures, we approximated the area under the colony size curve (AUC) as the sum of colony volumes in each plate:

$$\text{AUC} = V_1 + V_2 + V_3 + \dots + V_n \quad (3)$$

where V_i is the volume of each colony with i ranges from 1 to n .

Statistical Methods

The interaction index (IAI) based on the Loewe's additivity model between two drugs defines the magnitude of drug interaction as:

$$IAI = \frac{d_1}{D_{y,1}} + \frac{d_2}{D_{y,2}} \quad [4]$$

where y is the effect at the combination dose (d_1, d_2). $D_{y,1}$ and $D_{y,2}$ are the respective doses of drug 1 and drug 2 required to produce the same effect y when used alone (35). The value of IAI is estimated by the point estimator and its 95% confidence interval for which the delta method is used to calculate the variance of IAI (36). $IAI < 1$, $IAI = 1$, and $IAI > 1$ correspond to the drug interactions' being synergistic, additive, and antagonistic, respectively.

$D_{y,1}$, $D_{y,2}$, and (d_1, d_2) are estimated by modeling the dose-effect relationship for each of the three curves (drug 1 alone, drug 2 alone, and the combination of drugs 1 and 2 a constant ratio between d_1 and d_2) using the modified E_{max} model in Equation [5]:

$$y(d) = E_0 - E_{\max} + \frac{E_{\max}}{1 + (d/ED_{50})^m} \quad [5]$$

where E_0 is the base effect, corresponding to the measurement of the colony growth as defined by AUC in Equation [3] when no drug is applied; $E_{\max}$ is the maximum effect attributable to the drug; ED_{50} is the dose level producing half of $E_{\max}$; d is the dose level that produces the effect $y(d)$; and m is a slope factor (Hill coefficient), measuring the sensitivity of the effect within a given dose range for the drug. Thus, $E_0 - E_{\max}$ is the net drug effect when a very large dose of the drug is applied. Although Equation [5] allows different values of E_0 and $E_{\max}$ for different curves, to calculate the IAI value, we need to assume that all curves have the same E_0 so that the “base measure” of no drug effect is the same in all curves. This assumption was achieved by dividing all effect measures by the mean of the controls. Thus, the dose-response curve following the $E_{\max}$ model is

$$y(d) = 1 - E_{\max} + \frac{E_{\max}}{1 + (d/ED_{50})^m} \quad [6]$$

The modified $E_{\max}$ model [6] provides an adequate fit for most data. For the $E_{\max}$ model, parameter estimation is obtained by means of an iterative non-linear least squares method. Non-convergence of the parameter estimation can occur in situations where data are insufficient at either low or high dose levels. When non-convergence happens due to insufficient data at the low dose end, one intermediate point at half of the minimum dose is added with an assuming effect also half the effect at the minimum dose. Conversely, when non-convergence occurs at the high dose end, two or three points are added assuming the exponential attenuation model with the limiting $y(d)$ set at 90% of the observed minimum. In the case of repeat experiments, each data set for each experiment is analyzed separately and meta-analysis using a fixed-effect model is performed to combine the results from the repeat experiments (37).

We performed all analyses using software written in open-source R software that was derived from an earlier study (35).

Results

Reliable log dose versus AUC curves were obtained for all drugs studied. Figure 1 shows a typical plot of AUC (total volume) versus log dose for SNB19, a glioblastoma cell line treated with SAHA for 7 days. Occasionally, the highest dose used was not high enough to bring the AUC close to a plateau, causing non-convergence in the $E_{\max}$ model fitting. For some cell lines and drugs, the dose range might exceed 5 logs in order to extend the plateau or the drug (*e.g.*, DFMO) or the drug might have limited solubility at high concentrations or it could be too costly to use high molar doses. The first case was not uncommon with DFMO that would have, in some instances, required the highest dose to exceed 4 mM. Some SAHA experiments also would have required a dose of more than 100 μ M. In these cases, we

electd to add computed values to the bottom of the curve. Occasionally, this strategy was also needed for the top of the curve (at low doses) to more closely approximate the value 1 at a very low dose level when the treatment effect is minimal as shown in Equation [6]. An example of such a data fit is shown in Figure 2, which presents results for the SNB19 cell line treated with DFMO for 7 days with four points added.

Figures 3 is an example of the effects of carboplatin (5 day exposure) combined with SAHA (3 day exposure) analyzed by E_{max} plots that demonstrates that the two drugs are synergistic in SNB19 cell line at all dose combinations studied. Similarly, Figure 4 demonstrates that all dose combinations of DFMO (5 day exposure) and carboplatin (3 day exposure) are additive in MiaPaCa cell line. Figure 5 shows antagonism at all the combination doses of SAHA (7 day exposure) and docetaxel (5 day exposure) in SW480 cell line. Figure 6 shows the results of each of the three experiments repeated under the same conditions for the combination of carboplatin (7 day exposure) and DFMO (5 day exposure) in the SNB19 cell line as well as the result of the combining all three replicates using the meta-analysis technique. Using this approach, we determined the relationship between effect and IAI value for all drug combinations at different time points. The complied data for the five cell lines with all the drug combinations are summarized in Tables II–VI.

In combination studies, DFMO and carboplatin produced different degrees of synergism and additivity at different effect levels depending on the cell line and order of drug treatment. The combination of DFMO followed by carboplatin was additive to synergistic in LNZ308 cells on day 7 after the first drug was added depending on the d1:d2 ratio, with most synergy at d1:d2 = 10 (Table IV). In SW480 cells, additivity was most consistent on day 7 after the first drug was added, with d1:d2 = 28 (Table V). For MiaPaCa cells, synergy was seen on days 3, 5, and 7 after the first drug was added at the low-effect doses, with d1:d2 = 7 (Table VI). This sequence, however, was less active and sometimes antagonistic in SNB19 (Table II) and U251MG (Table III) cell lines; reversing the drug order so that carboplatin was followed by DFMO was more effective in terms of growth inhibition/cell killing, with synergy seen in SNB 19 cells on days 5, 7, and 9 and in U251MG cells on day 7 after exposure to the first drug.

The combination of SAHA and DFMO in either order was mostly antagonistic in the SNB19, U251MG, LNZ308, and SW480 cell lines. For this reason, the combination was not studied in MiaPaCa cells. Moreover, neither increasing the length of exposure nor increasing the dose improved the combination's performance. Thus, we conclude that SAHA and DFMO are purely antagonistic.

Combination studies of SAHA and carboplatin revealed variable interactions. In general, SAHA before carboplatin was synergistic in SNB19 and MiaPaCa cells, but the effect deteriorated after the 4th day of treatment. The same combination, however, proved to be antagonistic in U251MG, LNZ308, and SW480 cell lines, except for some additivity at higher Effect doses in LNZ308 on day 9. On the other hand, in SNB19 cells carboplatin before SAHA was synergistic with persistence of efficacy during days 5 and 7 but deterioration to antagonism by day 9. For MiaPaca cell lines, carboplatin before SAHA was synergistic with decreasing activity over time with synergy seen on day 3, additivity on day

5, and antagonism by day 7. In SW480 cell lines, the trend for this combination was primarily additive on days 5 and 7 and antagonistic on day 9 after the first drug was added. The combination was not completely studied in U251MG cells, but on day 7 after the first drug was added, the results suggested only additivity at the highest two effect levels.

The combination of carboplatin and docetaxel (in either order) was synergistic in SNB19 cells, while in U251MG, LNZ308, and SW480 cells the combination was additive to synergistic only when carboplatin preceded docetaxel; if the order was reversed, the two drugs were almost antagonistic at all doses. The combination was not studied in MiaPaCa cells. Thus, this was a good example of a synergistic combination that is effective in four cell lines when carboplatin is followed by docetaxel.

The combination of SAHA and docetaxel was, for the most part, antagonistic in all the cell lines except SNB19, for which it was synergistic at days 5 and 7 and additive at day 9. This effect decreased, however, at later days of exposure and when docetaxel preceded SAHA.

The combination of DFMO and docetaxel was antagonistic for the adenocarcinoma cell lines when the drugs were added in either order and showed only little additivity when administered in high doses in the SW480 cell line (Table V). The combination of docetaxel followed by DFMO produced more persistent synergy in glioma cell lines than the combination of DFMO followed by docetaxel. Overall, the combination was effective in gliomas and ineffective in adenocarcinomas.

Our reported studies have some limitations, in that we did not report combinations that were not done in triplicate, the number of cell lines studied was limited, some drug combinations were so inactive that additional study seemed pointless, and the time of the measurements were occasionally incomplete since cell lines grew slower than expected.

Discussion

The goal of our study was twofold: (i) develop a robust 3D colony assay methodology to interrogate drug combinations using GelCount™ and (ii) to develop 2-drug combinations that might be useful in the clinic for the treatment of highgrade gliomas. In addition, we obtained some potentially valuable drug interaction data for a colon and pancreatic adenocarcinoma cell line. We believe that we were successful in our first goal. We demonstrated the capabilities of an easily performed quantitative 3D assay using GelCount™ that reflects drug action on both colony size and number and, using an E_{max} statistical model, can be used to interrogate 2-drug combinations with respect to drug order, log dose, and time after drug treatment. Using this methodology, we were able to define additive and synergistic two-drug combinations of anticancer drugs against five cell lines.

Anchorage-independent growth, the cell's ability to proliferate without attachment to, or spreading onto, a substratum, is one of the hallmarks of neoplasia and a predictable *in vitro* indication of tumorigenicity. We believe that 3D colony growth rather than monolayer culture viability should be used to study drug combinations; however, until recently, economical 3D systems for such analysis were lacking. For this reason, we developed quantitative methodology using an agarose colony growth assay and the new GelCount™

technology (1). In the methodology described in this paper, we sought to be as quantitative as possible in our determination of the log dose-and time-effect of the drug(s) on colony formation. To that end, we elected to test the system using a range of five cell lines, three of which were gliomas and two of which were adenocarcinomas. While the emphasis of these studies is drug combinations active against glioblastoma cell lines, we studied two adenocarcinoma cell lines in parallel to determine if the response to the glioblastoma cell lines was unique to those lines. Drugs were added to the culture when the cells had equilibrated and started to form colonies. In this way, we ensured that we could evaluate the drugs' ability to decrease the number of colonies already formed as well as their potential to inhibit new colony formation. We also followed the effects of drug treatment on all colonies over time by repeat imaging at 2-day intervals. In these first studies, we also elected to use drugs known to be stable in aqueous media so that culture pharmacokinetic analyses would not be needed.

In biologic systems, the dose-effect curves for different drugs are mostly sigmoidal, with extreme difficulty reaching $AUC = 0$ (100% cell kill) or plateau effect. This is true for our agarose colony assay as well as for clinical trials. It is not always possible to treat agarose colonies or patients with high enough doses to reach the low or plateau effect with unequivocal certainty. Our modification of the E_{max} model allows us to improve on the estimation precision of the IC_{50} value for the individual drugs, as well as for the interaction of two drugs. This consideration becomes much more important when we deal with cytostatic drugs (e.g., DFMO) rather than cytotoxic drugs since achieving a high enough dose to produce a 0 AUC might be too expensive in terms of drug cost or may be physically unrealistic because of drug solubility. The statistical approach presented here enables us to observe the complexities of drug-drug interactions at different doses of two drugs (more than two-drug combinations could be studied as well), different exposure times, and different orders of drug administration.

Our second goal was less clearly achieved; however, we did find cell line variability in drug effects, a limited number of synergistic combinations that was coupled to order of drugs, and log dose-and time-dependence of drug exposure. In the glioblastoma cell lines we found: carboplatin followed by DFMO was generally synergistic or additive, carboplatin before SAHA was infrequently synergistic, carboplatin before docetaxel was generally synergistic or additive, SAHA before docetaxel was occasionally synergistic, docetaxel before DFMO was usually somewhat additive, and DFMO and SAHA was inactive in either order. In comparison, MiaPaCA pancreatic carcinoma cell line synergy was seen only with DFMO followed by carboplatin and, at short exposure times, when SAHA followed carboplatin or carboplatin followed SAHA. In the SW480 colon cancer cell line synergy was seen at short exposure days for carboplatin followed by docetaxel and additive and mixed effects were seen with DFMO before carboplatin, carboplatin before DFMO, carboplatin before SAHA, docetaxel before carboplatin, DFMO before docetaxel, and docetaxel before DFMO.

While the most clinically relevant DFMO drug interactions to date have been with the nitrosoureas (38–43), DFMO has been evaluated with numerous other cytotoxic chemotherapy agents with variable results. *In vitro* combination studies using cisplatin and DFMO showed either an antagonistic (812) or an additive/synergistic (13–15) effect on the

growth of tumor cell lines. Pretreatment with DFMO resulted in the depletion of intracellular polyamines and significantly decreased the cytotoxicity of cisplatin in rat 9L gliosarcoma cells and the pancreatic adenocarcinoma cell lines PANC-1 and MiaPaCA (8–11). It was believed that polyamine depletion by DFMO reduced the total number of inter-strand cross-links formed by cisplatin in 9L rat brain tumor cells *in vitro* (10, 12). On the other hand, the combination of cisplatin and DFMO was shown to be synergistic at the higher DFMO concentration (14) and produced a significant decrease in the bromodeoxyuridine labeling over either drug alone (15). Also pretreatment of U251MG human malignant brain tumor cells with polyamine analogues resulted in an increased incorporation of cisplatin into the linker region of the chromatin and enhancement of cisplatin cytotoxicity (44). Against pancreatic adenocarcinoma cells, DFMO alone was predominantly cytostatic and its effects were reversible by putrescine; when DFMO was combined with cisplatin, the effect was roughly additive (13).

In the current study, carboplatin, a platinum analog, was given either after cells had been exposed to DFMO for 48 hours or prior to DFMO exposure. In our studies, combinations in which DFMO was given prior to carboplatin had variably additive or synergistic effects against adenocarcinoma cell lines and one glioma cell line; moreover, except in the pancreatic adenocarcinoma, which needed higher dose combinations to produce synergy, additivity could occasionally be seen at lower drug doses (high Effect) in other cell lines. For the other two glioma cell lines, carboplatin prior to DFMO produced the best synergistic effect at lower doses, but when the sequence was reversed, the drugs were almost antagonistic at all doses.

The second drug to be studied with DFMO was SAHA. This HDAC inhibitor can induce programmed cell death in cancer cells via mitochondria-mediated apoptosis and caspase-independent autophagic cell death (45). SAHA also selectively alters the transcription of expressed genes in transformed cells (46, 47). Since the depletion of polyamines by DFMO alters chromatin structure, allowing easier extraction of acetylated histones (48), polyamine depletion may alter global chromatin structure in a manner that activates the spindle checkpoint to prevent tumor cells treated with HDAC inhibitors from entering an aberrant mitosis and undergoing apoptosis (48, 49). Also, a recent study showed that HDAC activity in tumors did not appear responsive to DFMO treatment (50). Our studies confirm this finding, as DFMO exposure prior to SAHA failed to suppress the colony AUC and SAHA followed by DFMO produced even more antagonism in all the cell lines. Thus, it would appear that regardless of dose sequence, the interactions between DFMO and SAHA is generally antagonistic.

However, SAHA combined with carboplatin was moderately more effective. In our studies, the combination of SAHA and carboplatin was synergistic only in SNB19 and MiaPaCa cell lines, but the effect occurred in more cell lines and a greater number of exposure days when carboplatin was followed by SAHA. The combination was antagonistic in U251MG, LNZ308, and SW480 cells. Several studies have investigated the activity of SAHA in combinations with cytotoxic agents targeting chromatin DNA (such as etoposide, camptothecin, cisplatin, doxorubicin, 5-fluorouracil, and cyclophosphamide) and have shown synergistic and additive activity in a variety of cultured human transformed cell lines

(51). It has been shown that SAHA allows for a reduction in the standard dose of carboplatin, with improvement in the overall therapeutic index (52). Based on synergy in only one of the three glioblastoma cell lines, it is doubtful that this approach would be efficacious against glioblastoma in a clinical setting.

The combination of carboplatin and docetaxel is currently of interest as a potentially relatively safe and very effective treatment for advanced cancer of the ovary, fallopian tubes, uterine cervix, metastatic breast cancer, and non-small cell lung cancer (53–56). Also, the combination is synergistic in human non-small cell lung cancer cell lines (57) and has shown activity in clinical trials for this cancer (58–60). Our studies confirmed this synergy when carboplatin was given prior to docetaxel, and the combination was very effective in the four cell lines tested. However, if docetaxel was given prior to carboplatin antagonism resulted with occasional additive effects. This finding thus stresses the importance of the order of drug addition in deciding drug combinations.

Clinically, the combination of SAHA and docetaxel has proven to be beneficial in breast, lung, bladder, and prostate cancers (61, 62). These two agents together comprise an effective way to induce terminal differentiation, cell growth arrest, and/or apoptosis of neoplastic cells when they are treated with SAHA followed by docetaxel (63); however, in our studies this combination was generally antagonistic in all the cell lines except for SNB19 over time.

The combination of docetaxel followed by DFMO was synergistic in the glioma cell lines and less effective in MiaPaCa and SW480 adenocarcinoma cell lines. This suggests that depletion of cellular polyamines may or may not interfere with cell cycle changes induced by docetaxel, depending on cell line characteristics (64).

In conclusion, by applying the Gelcount™ automated counting and sizing of colonies and the use of an E_{max} model to define drug interactions, we can reliably define additive, synergistic, and antagonistic combinations in cell lines as a function of log dose and duration of drug exposure. With the considerable growth in combinational chemotherapies for different cancers and with the premise of targeted chemotherapy, more and more relevant combinations are needed in the clinic. The reliable preclinical data that can be provided by the methodology presented in this paper may expedite this process, however, the data will imply rather than confirm an active/inactive combination, and *in vivo* experiments and, ultimately, clinical trials to fully validate the findings.

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Figure 1.
Dose effect plot for the SNB19 cell line treated with 10 doses of SAHA for 7 days.

Figure 2.
Dose effect plot for the SNB 19 cell line treated with 10 doses of DFMO for 7 days. Due to initial non-convergence, three additional points () were added to make a better fit.

Figure 3.

Assay results for the SNB19 cell line treated with carboplatin for 5 days and SAHA for 3 days. **(A)** Red dashed line indicates dose response curve of carboplatin as a single agent, green dotted line indicates dose response curve of SAHA as a single agent and bold blue line is the dose response curve of carboplatin and SAHA in combination. Three additional points (in pink) were added to the extremes to improve the fit. **(B)** Effect versus Interaction Index demonstrating synergy at all the dose combinations of the two drugs.

Figure 4.

Assay results for MiaPaCa cell line treated with DFMO for 5 days and carboplatin for 3 days. **(A)** Red dashed line indicates dose response curve of DFMO as a single agent, green dotted line indicates dose response curve of carboplatin as a single agent, and bold blue line is the dose response curve of DFMO and carboplatin in combination. Three additional points (in pink) were added to the extremes to improve the fit. **(B)** Effect versus Interaction Index demonstrating additivity at all the dose combinations of two drugs.

Figure 5.

Assay results for the SW480 cell line treated with SAHA for 7 days and docetaxel for 5 days. **(A)** Red dashed line indicates dose response curve of SAHA as a single agent, green dotted line indicates dose response curve of docetaxel as a single agent, and bold blue line is the dose response curve of SAHA and docetaxel in combination. **(B)** Effect versus Interaction Index demonstrating antagonism at all the dose combinations of two drugs.

Figure 6. Results of triplicate experiments with the SNB19 cell line treated with carboplatin for 7 days and DFMO for 5 days. The effect was synergistic in all three experiments (**A–C**). (**D**) Meta-analysis of the three experiments supporting synergy of the two-drug combination. In all cases $d1:d2 = 0.03$.

Cartoon 1.

Pictorial representation of the 3-dimension agarose culture technique and its relationship to the GelCount methodology.

Table 1

Mean IC₅₀ values for four drugs (carboplatin, DFMO, SAHA, and docetaxel) in five cell lines (LNZ308, MiaPaCa, SNB19, SW480, and U251MG) calculated at 3, 5, 7, and 9 days of drug exposure.

Cell Line	Drug	IC ₅₀ , μ M (SE)			
		Days of Drug Exposure			
		3	5	7	9
LNZ308	Carboplatin	–	9.8 (1.9)	5.0 (1.1)	3.9 (0.5)
MiaPaCa	Carboplatin	12.2 (4.5)	3.1 (0.8)	2.2 (0.6)	–
SNB19	Carboplatin	–	7 (3)	3.4 (0.6)	2.1 (0.2)
SW480	Carboplatin	–	14.7 (3.2)	7 (1.5)	5.2 (1.2)
U251MG	Carboplatin	–	32.2 (7.8)	8.4 (2.1)	5 (1.7)
LNZ308	DFMO	–	182 (34)	107 (19)	82 (30)
MiaPaCa	DFMO	105 (29)	42 (14)	70 (18)	–
SNB19	DFMO	–	456 (66)	385 (35)	363 (24)
SW480	DFMO	–	82 (30)	74 (9)	79 (13)
U251MG	DFMO	–	–	237 (38)	149 (16)
LNZ308	SAHA	–	1.0 (0.2)	1.02 (0.15)	13 (0.3)
MiaPaCa	SAHA	1.3 (0.3)	0.9 (0.2)	0.8 (0.2)	–
SNB19	SAHA	–	2.8 (0.5)	23 (0.3)	2.2 (0.3)
SW480	SAHA	–	2 (0.5)	1.6 (0.1)	1.4 (0.3)
U251MG	SAHA	–	–	2.1 (0.4)	13 (0.1)
LNZ308	Docetaxel	–	2.1 (0.2)	1.5 (0.3)	0.8 (0.2)
MiaPaCa	Docetaxel	1.5 (0.8)	0.9 (0.5)	0.1 (0.0)	–
SNB19	Docetaxel	–	10.7 (3.4)	3.4 (0.8)	2.0 (0.4)
SW480	Docetaxel	–	2.1 (0.7)	2 (0.6)	1.1 (0.3)
U251MG	Docetaxel	–	2.0 (0.8)	1.8 (0.7)	33 (1)

SE, Standard error of the mean.

Table II

Summary of the effects of the two-drug combinations in the SNB19 cell line.

										Interpretation at Effect									
D1	Expodays	D2	Expo days	D1:D2	N	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9					
DEMO	7	Carboplatin	5	28	3		++	+	--	--	--	--	--						
DEMO	9	Carboplatin	7	28	3		+	--	--	--	--	--	--						
Carboplatin	5	DEMO	3	0.03	3				++	++	++	++	++						
Carboplatin	7	DEMO	5	0.03	3				++	++	++	++	++						
Carboplatin	9	DEMO	7	0.03	3				++	++	++	++	++						
SAHA	5	Carboplatin	3	0.5	3		++	++	++	++	++	++	+						
SAHA	7	Carboplatin	5	0.5	3			+	+	+	+	--	--						
SAHA	9	Carboplatin	7	0.5	3			+	--	--	--	--	--						
Carboplatin	5	SAHA	3	2	2				++	++	++	++	++	++					
Carboplatin	7	SAHA	5	2	2				++	++	++	++	++	--					
Carboplatin	9	SAHA	7	2	2			+	+	--	--	--	--	--					
Carboplatin	7	Docetaxel	5	4	2				++	++	++	++	++	++					
Carboplatin	9	Docetaxel	7	4	2				++	++	++	++	++	++					
Docetaxel	7	Carboplatin	5	0.2	2				+	++	++	++	+	+					
DEMO	7	Docetaxel	5	70	2			+	+	--	--	--	--						
DEMO	9	Docetaxel	7	70	2			+	+	+	--	--	--						
Docetaxel	7	DEMO	5	0.006	2				++	++	++	+	--	--					
Docetaxel	9	DEMO	7	0.006	2				+	++	++	++	+	+					
SAHA	5	Docetaxel	3	2	2					++	++	++	++	++					
SAHA	7	Docetaxel	5	2	2					++	++	++	++	++					
SAHA	9	Docetaxel	7	2	2					+	+	+	+	++					
Docetaxel	7	SAHA	5	1	2				+	+	+	+	--						
Docetaxel	9	SAHA	7	1	2			+	+	--	--	--	--						
SAHA	5	DEMO	3	0.01	2				--	--	--	--	--	--					
SAHA	7	DEMO	5	0.01	2				--	--	--	--	--	--					

D1	Expodays	D2	Expo days	D1:D2	N	Interpretation at Effect									
						0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	
SAHA	9	DFMO	7	0.01	2										
SAHA	5	DFMO	3	0.02	2										
SAHA	7	DFMO	5	0.02	2										
SAHA	9	DFMO	7	0.02	2										

Expo days, Days of drug exposure; N, Number of replicate experiments; --, Antagonistic activity; +, Additive activity; ++ Synergistic activity.

Table III

Summary of the effects of the two-drug combinations in the U251MG cell line.

Interpretation at Effect														
D1	Expo days	D2	Expodays	D1:D2	N	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
DFMO	7	Carboplatin	5	7	2			--	--	--	--	--	--	
DFMO	7	Carboplatin	5	15	2			--	--	--	+	+	+	
Carboplatin	7	DFMO	5	0.03	2				++	++	++	+	+	+
Carboplatin	9	DFMO	7	0.03	2				++	+	--	--	--	--
SAHA	7	Carboplatin	5	0.5	3				++	+	--	--	--	--
SAHA	9	Carboplatin	7	0.5	3				--	--	--	--	--	--
Carboplatin	7	SAHA	5	2	2				+	+	--	--	--	--
Carboplatin	7	Docetaxel	5	2	2					++	++	++	++	++
Carboplatin	9	Docetaxel	7	2	2				++	++	++	+	--	--
Docetaxel	5	Carboplatin	3	0.5	2				--	--	--	--	--	--
Docetaxel	7	Carboplatin	5	0.5	2			--	--	--	--	--	--	--
Docetaxel	9	Carboplatin	7	0.5	2			--	--	--	--	--	--	--
DFMO	9	Docetaxel	7	70	2			++	++	++	++	+	--	--
Docetaxel	7	DFMO	5	0.01	2				+	+	+	+	+	--
Docetaxel	9	DFMO	7	0.01	2				+	+	+	+	--	--
SAHA	7	Docetaxel	5	1	2					--	--	--	--	--
SAHA	9	Docetaxel	7	1	3					--	--	--	--	--
Docetaxel	7	SAHA	5	1	1				--	--	--	--	+	+
SAHA	7	DFMO	5	0.01	2				++	+	--	--	--	--
SAHA	9	DFMO	7	0.01	2				--	--	--	--	--	--

Expo days, Days of drug exposure; N, Number of replicate experiments; --, Antagonistic activity; +, Additive activity; ++, Synergistic activity.

Table IV

Summary of the effects of the two-drug combinations in the LNZ308 cell line.

Interpretation at Effect														
D1	Expo days	D2	Expo days	D1:D2	N	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
DFMO	7	Carboplatin	5	4	15				+	+	+	+	+	
DFMO	7	Carboplatin	5	10	15				++	++	++	++	++	
DFMO	7	Carboplatin	5	28	15				++	+	+	+	--	
Carboplatin	7	DFMO	5	0.1	2					+	+	--	--	--
Carboplatin	9	DFMO	7	0.1	2					+	--	--	--	--
SAHA	5	Carboplatin	3	0.25	21		+	+	--	--	--	--	--	
SAHA	7	Carboplatin	5	0.25	21		--	--	--	--	--	--	--	
SAHA	9	Carboplatin	7	0.25	15				--	+	+	+	+	
Carboplatin	5	Docetaxel	3	2.5	8					+	--	--	--	
Carboplatin	7	Docetaxel	5	2.5	12			+	+	+	+	+	+	
Carboplatin	9	Docetaxel	7	2.5	8			++	++	++	++			
Docetaxel	5	Carboplatin	3	0.5	10			--	--	--	--	--	--	--
Docetaxel	7	Carboplatin	5	0.5	14			--	--	--	--	--	--	--
Docetaxel	9	Carboplatin	7	0.5	14			--	--	--	--	--	--	--
Docetaxel	5	DFMO	3	0.01	12			+	+	+	--	--	--	--
Docetaxel	7	DFMO	5	0.01	14			--	+	++	++	++	++	++
Docetaxel	7	SAHA	5	2	12			--	--	--	--	--	--	--
Docetaxel	9	SAHA	7	2	12			--	--	--	--	--	--	--
DFMO	5	SAHA	3	140	8				--	--	--	--		+
DFMO	7	SAHA	5	140	10			--	--	--	--	+	+	+
DFMO	9	SAHA	7	140	10			--	--	--	--	+	+	+
SAHA	7	DFMO	9	0.01	2		+	--	--	--	--	+	+	+
SAHA	9	DFMO	11	0.01	2		--	--	--	--	--	--	--	--

Expo days, Days of drug exposure; N, Number of replicate experiments; --, Antagonistic activity; +, Additive activity; ++, Synergistic activity.

Table V

Summary of the effects of the two-drug combinations in the SW480 cell line.

D1	Expodays	D2	Expo days	D1:D2	N	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
DFMO	5	Carboplatin	3	28	3	+	+	--	--	--	--	--	--	--
DFMO	7	Carboplatin	5	28	3	+	+	+	+	+	+	+	+	+
DFMO	9	Carboplatin	7	28	3	--	--	--	--	+	+	+	+	+
Carboplatin	5	DFMO	3	0.07	3	++	++	+	+	--	--	--	--	--
Carboplatin	7	DFMO	5	0.07	3	++	+	+	+	--	--	--	--	--
SAHA	5	Carboplatin	3	0.25	3	+	+	--	--	--	--	--	--	--
SAHA	7	Carboplatin	5	0.25	3	--	--	--	--	--	--	--	--	--
SAHA	9	Carboplatin	7	0.25	3	--	--	--	--	--	--	--	--	--
Carboplatin	5	SAHA	3	4	2	++	+	+	+	+	--	--	--	--
Carboplatin	7	SAHA	5	4	2	+	+	+	+	+	+	--	--	--
Carboplatin	9	SAHA	7	4	2	--	--	--	--	--	--	--	--	--
Carboplatin	7	Docetaxel	5	3.5	2	++	++	++	++	++	++	++	+	+
Carboplatin	9	Docetaxel	7	3.5	2	+	+	+	+	+	+	+	+	+
Docetaxel	5	Carboplatin	3	0.5	2	+	+	+	+	+	+	--	--	--
Docetaxel	7	Carboplatin	5	0.5	2	+	+	+	--	--	--	--	--	--
Docetaxel	9	Carboplatin	7	0.5	2	+	+	+	+	--	--	--	--	--
DFMO	7	Docetaxel	5	70	2	+	+	--	--	--	--	--	--	--
DFMO	9	Docetaxel	7	70	2	+	+	--	--	--	--	--	--	--
Docetaxel	7	DFMO	5	0.03	2	++	+	+	+	--	--	--	--	--
Docetaxel	9	DFMO	7	0.03	2	++	+	+	--	--	--	--	--	--
SAHA	7	Docetaxel	5	1	2	+	+	--	--	--	--	--	--	--
SAHA	9	Docetaxel	7	1	2	--	--	--	--	--	--	--	--	--
Docetaxel	5	SAHA	3	2	3	--	--	--	--	--	--	--	--	--
Docetaxel	7	SAHA	5	2	3	--	--	--	--	--	--	--	--	--
Docetaxel	9	SAHA	7	2	3	--	--	--	--	--	--	--	--	--

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D1	Expodays	D2	Expo days	D1:D2	N	Interpretation at Effect									
						0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	
DFMO	7	SAHA	5	70	2			--	--	--	--	--	--	--	
DFMO	9	SAHA	7	70	2		--	--	--	--	--	--	--	--	
DFMO	7	SAHA	5	31	2			--	--	--	--	--	--	--	
DFMO	9	SAHA	7	31	2		--	--	--	--	--	--	--	--	
SAHA	5	DFMO	3	0.01	3		--	--	--	--	--	--	--	--	
SAHA	7	DFMO	5	0.01	3		--	--	--	--	--	--	--	--	
SAHA	9	DFMO	7	0.01	3		--	--	--	--	--	--	--	--	

Expo days, Days of drug exposure; N, Number of replicate experiments; --, Antagonistic activity; +, Additive activity; ++, Synergistic activity.

Table VI

Summary of the effects of the two-drug combinations in the MiaPaCa cell line.

D1	Expo days	D2	Expo days	D1:D2	N	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
DFMO	3	Carboplatin	1	7	3	++	++	+	+	+	+	+	--	--
DFMO	5	Carboplatin	3	7	3	++	++	++	++	+	--	--	--	--
DFMO	7	Carboplatin	5	7	3	++	++	++	++	+	+	--	--	--
Carboplatin	3	DFMO	1	0.25	3			++	++	+	+	--	--	--
Carboplatin	5	DFMO	3	0.25	3			--	--	--	--	--	--	--
Carboplatin	7	DFMO	5	0.25	3			--	--	--	--	--	--	--
SAHA	3	Carboplatin	1	0.1	3		++	++	++	++	++	++	++	++
SAHA	5	Carboplatin	3	0.1	3		++	--	--	--	--	--	--	--
Carboplatin	3	SAHA	1	4	2		+	++	++	++	++	+	+	+
Carboplatin	5	SAHA	3	4	2		+	+	+	+	--	--	--	--
Carboplatin	7	SAHA	5	4	2		--	--	--	--	--	--	--	--
DFMO	3	Docetaxel	1	70	2		--	--	--	--	--	--	--	--
DFMO	5	Docetaxel	3	70	2		--	--	--	--	--	--	--	--
DFMO	7	Docetaxel	5	70	2		--	--	--	--	--	--	--	--
Docetaxel	3	DFMO	1	0.025	2			--	--	--	--	--	--	--
Docetaxel	5	DFMO	3	0.025	2			--	--	--	--	--	--	--
SAHA	5	Docetaxel	3	2	2			--	--	--	--	--	--	--
SAHA	7	Docetaxel	5	2	2			--	--	--	--	--	--	--

Expo days, Days of drug exposure; N, Number of replicate experiments; --, Antagonistic activity; +, Additive activity; ++, Synergistic activity.