Identification of unique synergistic drug combinations associated with downexpression of survivin in a preclinical breast cancer model system

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An in-vitro 72-h assay using median effect analysis and curve shift analysis was used to evaluate the utility of potentially clinically useful combinations of agents for synergism or antagonism. Six human breast cancer cell lines, both receptor rich and receptor poor, were studied. Panobinostat (LBH-589), a pan histone deacetylase inhibitor with a multitude of biological effects, exhibits time-dependent synergistic effects in breast cancer cell lines with docetaxel, doxorubicin, or gemcitabine in clinically relevant concentrations. Survivin expression was markedly downregulated in the presence of panobinostat with gemcitabine. Bortezomib, a proteasome inhibitor, markedly enhanced the cytotoxic effects of panobinostat combined with gemcitabine. Panobinostat did not demonstrate universal enhancement of cytotoxic drugs, and therefore, synergy was dependent on the second agent selected. No synergy was noted with anti-Her2 agents in Her2 overexpressing cell lines. Metformin combined with panobinostat demonstrated no synergy in this test system. These effects were confirmed by an apoptosis assay and caspase-3 production. A positive drug interaction was identified. The triplet of panobinostat with either doxorubicin/carboplatin or gemcitabine/carboplatin was especially potent in all cell lines. As all these agents are clinically available, further studies of the potent combinations are warranted. *Anti-Cancer Drugs* **23:272–279** © **2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

In the US, the incidence of breast cancer has decreased over the last decade but still remains a major source of morbidity and mortality [1]. In turn, the use of pharmacological treatment of this disease has slowly improved but the majority of women with relapsed or disseminated disease ultimately still die due to this disease [2]. Therefore, new approaches with therapeutic intent are needed.

One recent approach has been to combine classical cytotoxic agents with biologics or small molecules that perturb signal pathways or enhance apoptosis. This approach has been most successful in the use of trastuzumab in Her2/neu overexpressing breast cancer although drug resistance occurs [3,4]. As a consequence of 'proof of principle,' numerous agents have been developed to interfere with cellular pathways. Many of these agents are currently under clinical evaluation. In addition, recent preclinical findings suggest that many of the small molecules perturb the cell through a variety of mechanisms and are not as specific as originally thought. We were interested in small molecules that perturb several cell functions. One such group of agents is the nonselective histone deacetylase inhibitors such as panobinostat [5], which have numerous on-target and off-target effects including enhancement of endoplasmic stress in the tumor cell leading to apoptosis [6], interference with histone deacetylase 6 function, which plays a critical role in both oncogenic transformation and the ability of the transformed cell to form an intact tumor [7], inhibition of heat shock protein 90 chaperone function [8], protein degradation [9], and perturbation of aggresome function [10]. In a human colon cancer cell line (HCT116p21 +), survivin levels were also reduced in the presence of a nonselective histone deacetylase inhibitor [11]. Survivin has been implicated as a major drug resistance factor in the MDA-MB 231 breast cancer cell lines by using small inhibitory RNA interference [12] and causes primary resistance to trastuzumab in Her2 + lines [13,14]. In preclinical myeloma models, application of panobinostat with bortezomib resulted in interference with the aggresome function and with histone 6 deacetylase function, resulting in a marked increase in tumor cell death [15,16].

For several years we have used a semiautomated model in-vitro system to screen for synergism and antagonism of clinically available drugs to make drug combinations more rational [17–21]. This nonbiased screening approach is based on the finding of unexpected interactions between agents when combined [22] and the ability of multi-targeted agents to exhibit synergistic effects [23]. This

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assay does not presume that a specific pathway is responsible for anticancer effects and is thus adaptable to drugs known to cause multiple perturbations of cell function. This short in-vitro assay allows up to three agents to be studied in combination and also allows scheduling of agents to be examined. If a positive result is identified, further studies on the potential mechanism of action can be initiated. Recently, using this approach, we identified marked synergy between a pan histone deacetylase inhibitor, panobinostat, and gemcitabine in ovarian carcinoma cell lines [21]. We therefore report our findings in breast cancer cell lines with panobinostat, classical cytotoxic agents, a proteasome inhibitor, and metformin, which has assumed prominence in the clinical trials program [24,25]. Metformin had previously been demonstrated to enhance taxane cytotoxicity in a murine xenograft model [26]. Here, we demonstrate that the pan histone deacetylase inhibitor, panobinostat, does indeed reduce survivin levels in human breast cancer cell lines and that this effect is associated with enhancement of cytotoxicity with several classical therapeutic agents.

Materials and methods

The human breast cancer cell lines MCF7/wt, BT474, SK-BR-3, and HTB25 were obtained from the American Tissue Culture Collection (ATCC, Rockville, Maryland, USA). MCF7/adr (a multiple-drug-resistant line grown in the presence of doxorubicin) was obtained from the National Cancer Institute as a gift from Dr Kenneth Cowan and HCC1395 from Dr John Minna, University of Texas, Dallas. DU145 is a prostate cancer cell line obtained from ATCC used as a negative control. MCF7/wt and BT 474 express the estrogen receptor; SK-BR-3 and MCF7/adr are receptor negative and overexpress Her2/neu [27]. HTB-25 and HCC1395 are triple-negative variants [27,28]. The phenotypes of these cell lines were reassayed using a quantitative enzyme-linked immunosorbent assay (ELISA) method as discussed below as a phenotypic drift may occur in cell lines [29].

Panobinostat (LBH-589) and fluvastatin were gifts from Novartis Pharmaceuticals (East Hanover, New Jersey, USA); docetaxel was a gift from Sanofi-Aventis Corporation (Bridgewater, New Jersey, USA). GW282974X (an analogue of lapatinib) was supplied by GlaxoSmithKline (Philadelphia, Pennsylvania, USA). The following agents were obtained from Sigma-Aldrich (St Louis, Missouri, USA): carboplatin, docetaxel, doxorubicin, 5′-DFUR (metabolite of capecitabine), and paclitaxel. Bortezomib, gemcitabine, topotecan, and trastuzumab were obtained from commercial stock.

The various cell lines were grown to confluence in T-150 tissue culture flasks (Corning Glass Works, Corning, New York, USA) using RPMI 1640 media (Invitrogen, Carlsbad, California, USA) with 10% heat-inactivated fetal calf serum and 5% CO_2. Cells were then replated into 96-well dishes (Falcon 3072; Becton Dickinson & Co., Franklin Lakes, New Jersey, USA) at concentrations of 5000 cells per well and grown in the exponential phase. Drug or solvent control was then added and the cells were incubated for 72 h. No sequential studies of agents were carried out. For triplet studies containing clinically achievable levels of metformin (1.0 μmol/l), the metformin concentration was maintained constant and not varied during the determination of the dose–response curve. An identical procedure for bortezomib was followed at 0.26 μmol/l, which is the clinically achievable concentration.

Cell death was identified by MTT assay [30] and read at 595 nm on a BioRad 3550 MicroPlate Reader (BioRad, Hercules, California, USA). We had previously compared this assay with Trypan blue exclusion, with concordant results [17]. For determination of the IC_{50} (the concentration required for 50% growth inhibition), a dose–response curve of a given agent applied for 72 h was analyzed by the EZ ED_{50} program (Perrella Scientific, Conyers, California, USA) and extrapolated from the curve with all values of the means of three to four experiments. For median effect isobologram analysis [31], 10 fixed drug ratios above and below the IC_{50} of the combination were used to identify the dose–response curve as previously described [32]. Fa is defined as the fraction of cells affected and a plot of log dose versus log (Fa/1-Fa) gives parallel slopes if no biologic interaction is present (mutually exclusive) or converse if there is an interaction between the drugs (mutually nonexclusive). This plot suggested that the nonexclusive criteria should be used. The data were therefore analyzed using the nonexclusive criteria and the computer program of Chou [33]. CI (combination index) values were determined at Fa_{50} (50% effect) as the method is a linear approximation of a nonlinear process and most accurate at Fa_{50} [34]. CI values less than unity are synergistic effects, unity additive, and greater than unity antagonistic. Assays were performed in triplicate to determine SD. Results were confirmed by curve shift analysis, which normalizes concentrations using the program ACT Analysis (Optimum Therapeutics, Columbus, Ohio, USA) [35].

Apoptosis was measured using the Cell Death Detection ELISAplus kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's directions. This method detects 5-bromo-2′-deoxyuridine-labeled fragmented DNA. The resulting enrichment factor is defined as the relative fold increase in apoptosis as measured by optical density compared with the control as calculated by the formula: mU of dying or dead cells/mU of untreated cells, where mU = absorbance 405–490 nm. Quantification of total caspase-3 protein was performed using the Genscript Total caspase-3 colorimetric assay Kit (Cat # L00289). The sandwich ELISA kit (Genscript Inc., Piscataway, New Jersey, USA) was used as per the

manufacturer's instructions. The method is based on a solid-phase sandwich ELISA that detects endogenous levels of caspase-3. The Survivin ELISA kit was obtained from Calbiochem (La Jolla, California, USA; Cat # CBA048). Reactions were measured at 450 nm in a manner analogous to MTT. Human recombinant caspase-3 protein (Cat # 10-663-46608), human recombinant estrogen receptor protein (Cat # 10-786-259004), human recombinant progesterone receptor protein (Cat # 10-786-259007), and human recombinant Her2/neu protein (Cat # 10-511-248341) were all obtained from GenWay Biotech Inc. (San Diego, California, USA), and were used to generate a standard curve for protein assays using a Lowry protein assay kit (Sigma-Aldrich). Results were read at 595 nm. ER (Cat # 49296) and PR (Cat #49396) sandwich ELISA kits were obtained from Active Motif (Carlsbad, California, USA) and assays were performed as per the manufacturer's instructions. Total Her2/neu Sandwich ELISA was obtained from Cellsignal (Cat #7310; Danvers, Massachusetts, USA) according to the manufacturer's instructions.

Statistical methods

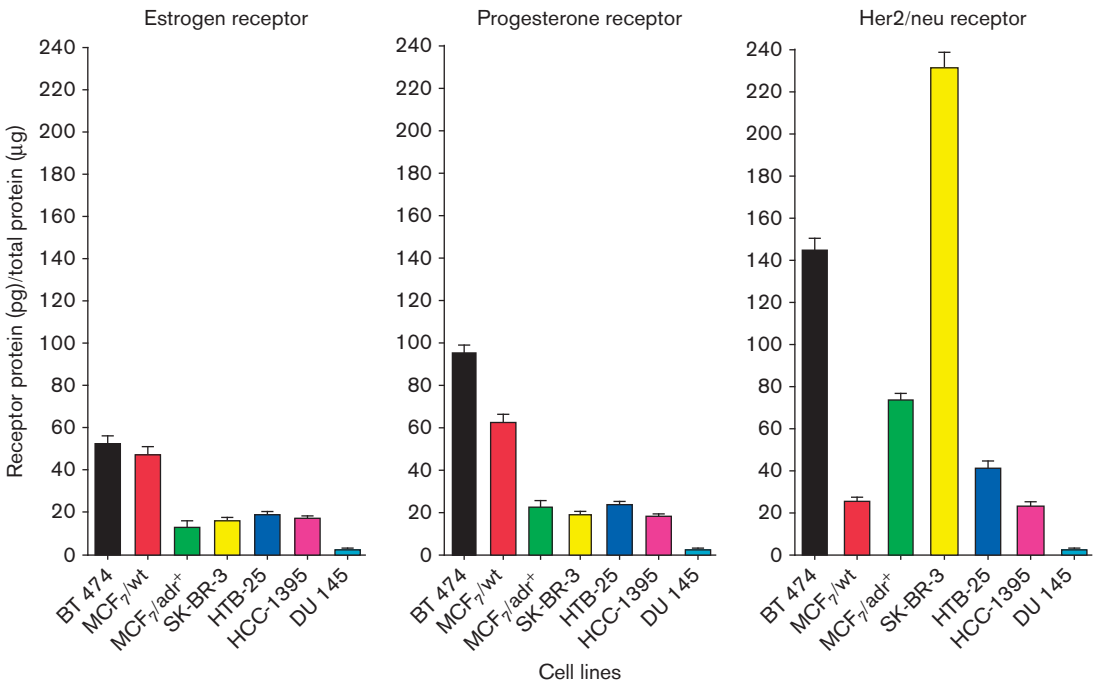
For analysis of doublet combinations, the study was a $2 \times 2 \times 4$ factorial design. Three-way analysis of variance with interaction terms was used to compare pictograms of caspase-3 and as for survivin across treatment groups and time. The three factors were panobinostat, docetaxel, and time for the caspase-3 analysis and panobinostat, gemcitabine, and time for the survivin analysis. Caspase-3 was log-transformed in order to meet the standard assumptions of Gaussian residuals and equality of variance. Summary statistics are presented on the untransformed data for ease of interpretation. SD was calculated where applicable.

Results

As cell lines can genetically drift from their original origin [29], we first rephenotyped our cell lines using a quantitative ELISA assay (Fig. 1). BT 474 and MCF_7wt are estrogen and progesterone receptor rich, whereas MCF_7/adr, SK-BR-3, HTB-25, and HCC-1395 are receptor poor. BT 474, MCF_7/adr, and SK-BR-3 have high expressions of Her2/neu. The DU 145 is a negative human prostate cancer control.

The IC_{50} of each agent in the various cell lines is shown in Table 1. All results are for a 72-h exposure of drug. The most active agent in this system is doxorubicin. Doublets of panobinostat with other agents were then tested after the 72-h incubation, with the median effect results (CI) reported at Fa_{50} (Table 2). Panobinostat generated synergistic to additive effects in the majority of cell lines with most of the agents. Of interest, panobinostat was markedly synergistic with doxorubicin or with docetaxel in

Fig. 1



Quantitative expression of phenotype using an enzyme-linked immunosorbent assay assay. DU 145 is a negative control prostate cell line. Error bars show SD.

Table 1 IC_{50} **of the various agents incubated for 72 h in the six human breast cancer cell lines**

Drug	MCF7/wt	MCF7/adr+	BT-474	SK-BR-3	HCC-1395	HTB-25
Bortezomib	0.2±0.0	0.2±0.0	0.4±0.0	0.2±0.1	0.1±0.0	0.1±0.0
Carboplatin	87.9±14.9	44.1±8.8	31.0±4.7	37.3±10.9	24.5±5.2	41.9±13.6
5′-Deoxy-5-fluorouridine	6.0±0.6	5.5±2.5	22.9±4.3	36.6±14.5	12.3±1.2	18.6±4.6
Docetaxel	14.9±2.1	3.00±0.5	33.1±19.3	8.0±0.4	22.4±5.5	44.9±7.4
Doxorubicin	0.005±0.001	49.4±7.4	0.3±0.0	1.1±0.3	2.3±0.9	0.3±0.1
Fluvastatin	34.2±5.7	5.3±1.5	23.2±7.7	57.7±7.7	75.8±4.6	33.4±2.9
Gemcitabine	2.3±0.5	2.9±1.3	7.7±1.3	1.9±0.1	63.0±8.4	13.8±6.2
GW 282974X	7.6±1.8	6.8±1.5	4.8±1.7	0.6±0.1	1.5±0.2	29.7±7.6
Paclitaxel	18.7±3.8	20.5±1.0	32.8±9.5	42.4±5.4	87.7±15.0	33.8±3.7
Panobinostat	27.9±3.2	2.1±0.9	23.2±4.7	24.4±5.0	102.9±9.0	122.1±23.3
Topotecan	28.2±7.5	12.3±0.4	15.5±1.7	26.8±2.2	34.9±4.6	45.8±2.4
Trastuzumab	4.7±1.6	1.2±0.3	3.0±0.8	7.2±1.1	0.4±0.01	3.5±0.2

Concentrations are in μmol/l with the means±SD of 3–5 determinations.

Table 2 **Combination index results at** Fa_{50} **for 72-h incubation**

Combination	MCF7/wt	MCF7/adr+	BT 474	SK-BR-3	HCC-1395	HTB-25
Mutually nonexclusive	CI-50	CI-50	CI-50	CI-50	CI-50	CI-50
Panobinostat + carboplatin	0.7±0.0	0.8±0.1	0.6±0.1	0.4±0.1	0.5±0.1	0.8±0.1
Panobinostat + 5′-DFUR	0.3±0.1	0.9±0.1	*1.0±0.0*	0.8±0.1	0.8±0.1	0.4±0.0
Panobinostat + doxorubicin	**1.6±0.2**	0.6±0.1	0.9±0.0	0.3±0.1	0.3±0.0	0.5±0.1
Panobinostat + docetaxel	0.9±0.1	0.4±0.0	0.3±0.1	0.3±0.1	**2.0±0.1**	0.5±0.1
Panobinostat + fluvastatin	**2.0±0.1**	*1.0±0.4*	*1.0±0.2*	**1.8±0.1**	**1.3±0.3**	0.7±0.2
Panobinostat + gemcitabine	0.4±0.1	0.9±0.0	**1.3±0.1**	0.6±0.1	0.4±0.1	0.8±0.1
Panobinostat + GW282974X	**1.1±0.3**	0.6±0.0	**1.7±0.1**	**1.6±0.2**	0.9±0.1	0.7±0.1
Panobinostat + paclitaxel	0.7±0.1	0.9±0.1	**1.2±0.2**	0.8±0.1	**1.2±0.1**	0.5±0.1
Panobinostat + topotecan	**1.1±0.1**	*1.0±0.2*	*1.0±0.1*	0.4±0.1	0.9±0.2	0.8±0.2
Panobinostat + trastuzumab	*1.0±0.4*	0.7±0.1	**1.6±0.2**	**1.6±0.2**	0.6±0.1	0.6±0.1

CI<1 are synergistic (shaded values), 1 additive (italics), >1 antagonistic (bold). Values are means±SD. Six human breast cancer cell lines were tested.
CI, combination index; 5′-DFUR, 5′-deoxy-5-fluorouridine.

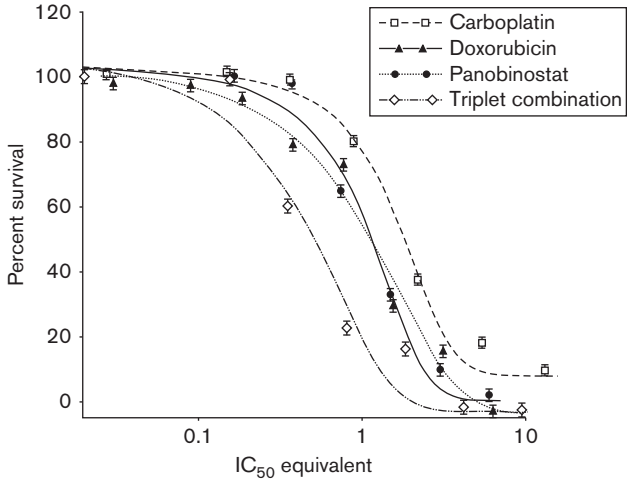
Table 3 **Combination index results at** Fa_{50} **for select triplet combination of agents**

Combination	MCF7/wt	MCF7/adr+	BT 474	SK-BR-3	HCC-1395	HTB-25
Mutually nonexclusive	CI-50	CI-50	CI-50	CI-50	CI-50	CI-50
Panobinostat + carboplatin	0.7±0.0	0.8±0.1	0.6±0.1	0.4±0.1	0.5±0.1	0.8±0.1
Panobinostat + 5′-DFUR	0.3±0.1	0.9±0.1	*1.0±0.0*	0.8±0.1	0.8±0.1	0.4±0.0
Panobinostat + doxorubicin	**1.6±0.2**	0.6±0.1	0.9±0.0	0.3±0.1	0.3±0.0	0.5±0.1
Panobinostat + docetaxel	0.9±0.1	0.4±0.0	0.3±0.1	0.3±0.1	**2.0±0.1**	0.5±0.1
Panobinostat + fluvastatin	**2.0±0.1**	*1.0±0.4*	*1.0±0.2*	**1.8±0.1**	**1.3±0.3**	0.7±0.2
Panobinostat + gemcitabine	0.4±0.1	0.9±0.0	**1.3±0.1**	0.6±0.1	0.4±0.1	0.8±0.1
Panobinostat + GW282974X	**1.1±0.3**	0.6±0.0	**1.7±0.1**	**1.6±0.2**	0.9±0.1	0.7±0.1
Panobinostat + paclitaxel	0.7±0.1	0.9±0.1	**1.2±0.2**	0.8±0.1	**1.2±0.1**	0.5±0.1
Panobinostat + topotecan	**1.1±0.1**	*1.0±0.2*	*1.0±0.1*	0.4±0.1	0.9±0.2	0.8±0.2
Panobinostat + trastuzumab	*1.0±0.4*	0.7±0.1	**1.6±0.2**	**1.6±0.2**	0.6±0.1	0.6±0.1

CI<1 is synergistic (shaded values), 1 is additive (italics), and >1 antagonistic (bold). Values are means±SD.
CI, combination index; 5′-DFUR, 5′-deoxy-5-fluorouridine.

the receptor poor cell lines. Fluvastatin, an inhibitor of prenylation, which was active in some of our previous studies [32], did not demonstrate additional benefit in this study. In the Her2/neu-rich cell lines, panobinostat did not synergize with either a tyrosine kinase Her2/neu inhibitor (GW282974X) or with trastuzumab. The p53 mutant cell line BT 474 demonstrated the greatest resistance to sensitizing effects with panobinostat. Triplet combinations were also evaluated (Table 3). In contrast to the doublets, most of the triplet combinations revealed synergistic activity with panobinostat/doxorubicin/carboplatin and panobinostat/gemcitabine/carboplatin, demonstrating the most synergism. A typical curve shift analysis confirming this synergistic cytotoxic effect is shown for SK-BR3 cells in Fig. 2. In addition, bortezomib at clinically achievable concentrations in combination with either gemcitabine or as a triplet with gemcitabine and panobinostat exhibited marked synergistic cytotoxic effects in the receptor-poor/her 2-negative HCC-1395 cell line (Fig. 3). The CI for the entire Fa of the triplet panobinostat, gemcitabine, bortezomib) is shown in Fig. 3b. Figure 3c shows the CI versus Fa curve for panobinostat, doxorubicin, and carboplatin demonstrating synergy. In contrast, the addition of metformin at clinically achievable concentrations did not enhance panobinostat doublet combinations (data not shown).

Fig. 2

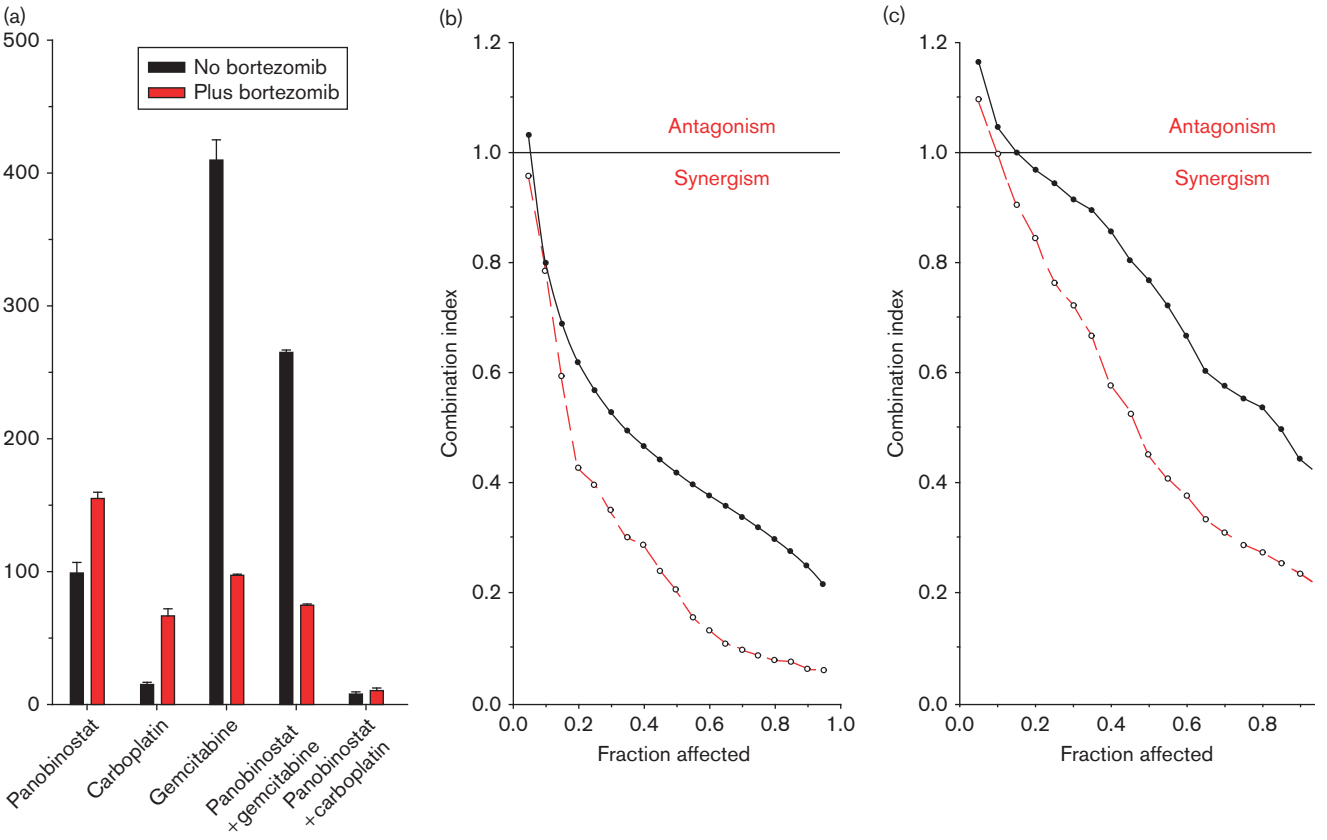


Drug concentrations are normalized with the cytotoxic effect measured at 72 h of incubation. A shift to the left indicates synergy as is shown for the triplet combination.

To determine whether or not the synergistic effects were time dependent, SK-BR-3 cells were exposed to a single agent, control (media), panobinostat, docetaxel, or panobinostat with docetaxel for 24, 48, or 72 h (Fig. 4). As measured by the ELISA apoptosis assay, the combination of panobinostat with docetaxel demonstrated marked enhancement of the cytotoxic effect and this effect was time dependent. Caspase-3 was also measured to show that this effect was due to apoptosis (Fig. 5). The expression of survivin in the presence or absence of panobinostat, gemcitabine, or the combination over time was examined as shown in Fig. 6. All drugs lowered survivin, with the doublet of gemcitabine and panobinostat demonstrating marked effects (5% of control at 3 days). This effect was rapid, occurring within the first 24 h of drug exposure.

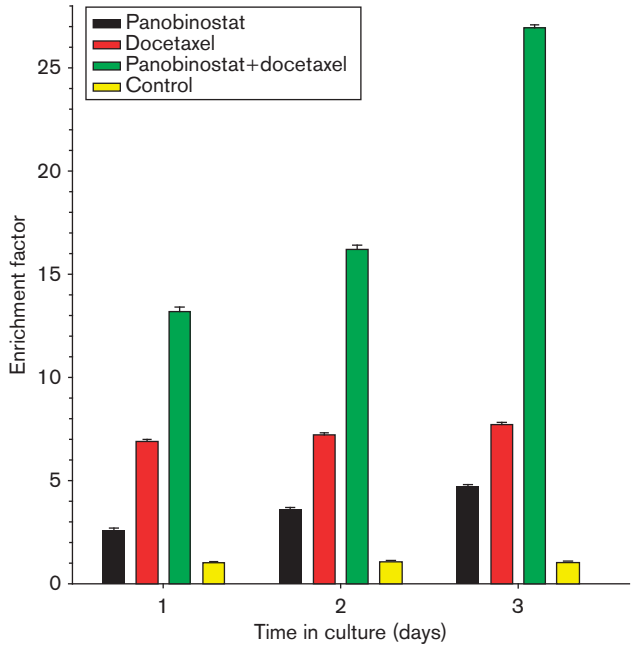
Analysis of variance demonstrated that there were indirect pharmacodynamic effects with a significant three-way interaction between panobinostat, docetaxel, and time ($P < 0.0001$). In the caspase-3 studies, a significant interaction ($P < 0.0001$) was noted by day 1

Fig. 3



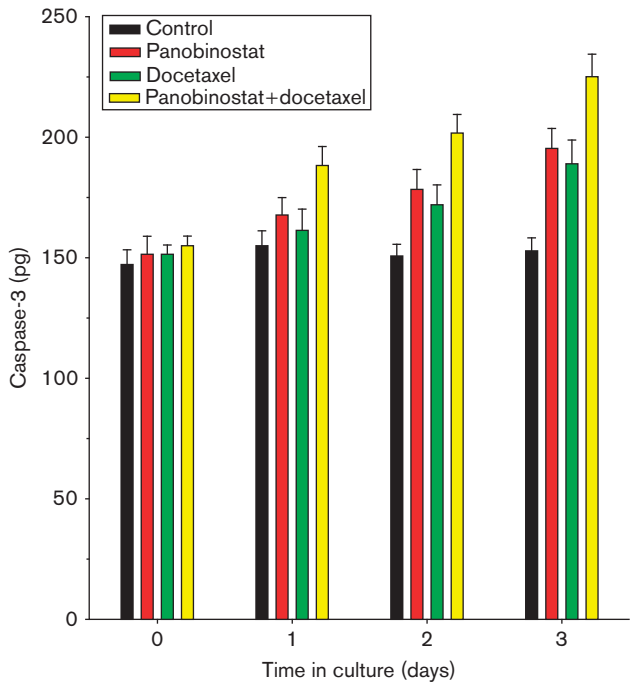
The IC_{50} of single agent gemcitabine is enhanced in the presence of bortezomib in the receptor-poor HCC-1395 breast cancer cell line (a) with marked synergy (red line) of the triplet (panobinostat, gemcitabine, bortezomid) in HCC-395 cells as demonstrated by the combination index curve (b). (c) Demonstrates synergy (red line) with panobinostat, doxorubicin, and bortezomab.

Fig. 4



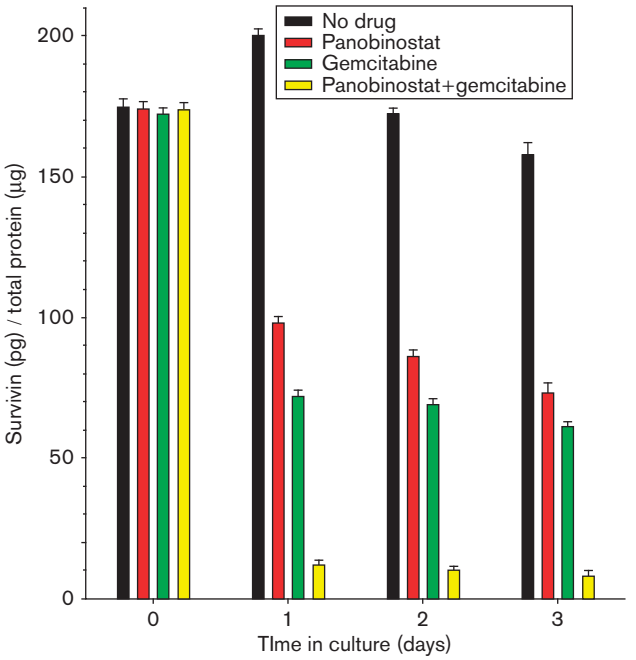
Time-dependent apoptotic effects of panobinostat with or without docetaxel *in vitro* in SK-BR-3 cells. SD is shown by the error bars. Enrichment factor is the relative fold increase in 5-bromo-2′-deoxyuridine DNA fragments compared with the control.

Fig. 5



Apoptotic effect as measured by caspase-3 formation. Error bars indicate the SD.

Fig. 6



Profound downexpression of survivin when panobinostat is combined with gemcitabine. SK-BR-3 cells were exposed to the drug for 72 h. Error bars show SD.

(supplemental Figure A), with panobinostat in the presence of docetaxel increasing caspase-3 by 14%. No interaction was identified at day 2 ($P < 0.53$) but there were significant effects by day 3 ($P < 0.0001$). Three-way analysis of panobinostat, gemcitabine, and time (supplemental Figure B) indicated a significant interaction ($P < 0.0001$). At day 1, gemcitabine decreased survivin by 62% whereas the panobinostat/gemcitabine combination decreased survivin by 87%. The effect of incubation time on drug effect was seen in a similar manner on day 2 ($P < 0.0001$) and day 3 ($P < 0.0001$), with an 86% decrease in survivin for the two-drug combination.

Conclusion

Recent progress in cellular physiology has demonstrated that cellular pathways have complex interactions that, when perturbed, exhibit redundant mechanisms for survival [36]. Hence, targeting of a specific overexpressed protein in a malignant cell may not result in an optimal outcome, except for instances such as Her2 overexpression in breast cancer or BCR-Abl expression in chronic myelogenous leukemia. In addition, small molecules may have off-target effects that may be clinically more interesting [22]. Recently, the utility of defined cell lines in drug development has been re-emphasized [37].

Using our short-term culture assay for synergism or antagonism, we have identified that the pan histone deacetylase inhibitor, panobinostat, exhibits time-dependent synergistic effects with classical agents such as docetaxel

or doxorubicin, especially in receptor-poor breast cancer cells, and has enhanced effects when combined as a triplet with either doxorubicin/carboplatin or gemcitabine/carboplatin. The effect of panobinostat involves apoptosis as measured by an ELISA assay and is time dependent, with increasing effects with longer exposure duration. Survivin, a major antiapoptotic protein, is normally increased by noxious insults such as chemotherapy [38] and was thus also studied in these experiments. Previous studies in a colon cancer cell line had demonstrated that histone deacetylase inhibition is associated with the downregulation of survivin [11], and we now demonstrate that this effect occurs in human breast cancer cells. This effect is enhanced when panobinostat is combined with gemcitabine. Furthermore, we have shown that in a receptor-poor human breast cancer cell line, the addition of bortezomib, a clinically used proteasome inhibitor, to panobinostat and gemcitabine results in a marked antitumor effect *in vitro*. A previous preclinical study using a different pan histone deacetylase inhibitor, suberoylanilide hydroxamic acid, with a different proteasome inhibitor also enhanced cell death [39] but did not explore the effects when combined with classical chemotherapeutic agents.

These findings have potential clinical importance. Bortezomib has already been combined with docetaxel in a phase I/II study of patients with breast cancer with evidence of activity [40]. Histone deacetylase inhibitors have been combined with chemotherapeutic agents in several tumors in preclinical models, with activity noted when combined with gemcitabine [41,42], but with a negative trial in humans with pancreatic cancer [43]. Other doublets and triplets, especially with a proteasome inhibitor have not been reported in patients with breast cancer. These results suggest that the type of tumor, the chemotherapeutic agent used, the histone deacetylase inhibitor chosen, and perhaps the schedule of administration may be critical for clinical outcome.

In our studies, the use of panobinostat did not enhance the cytotoxic effects of all chemotherapeutic agents tested and did show some variation with the cell line. In addition, the current experiments were performed on a model system that does not take into account distribution of drug, metabolism, and therapeutic index. However, the present results, especially in receptor-poor tumors, suggest that further investigation is warranted.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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