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Original Article

A novel method for potentiation of chemotherapy in soft tissue sarcomas with BromAc

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Abstract: Single-agent doxorubicin currently forms part of standard care for patients with sarcomas. However, efficacy is limited by the presence of dose-dependent cardiotoxicity and toxicity to renal, hepatic, and neurological systems. Therefore, there is a pressing need for novel drug regimens which can provide increased efficacy and safety. BromAc is a novel drug combination developed as a mucolytic agent which has demonstrated anticancer activity both in vitro and in vivo in several cancers. Here, we investigated the efficacy of BromAc in combination with doxorubicin for four subtypes of sarcoma. Cell proliferation, alongside western blot for a variety of cell cycle, apoptosis, and autophagy biomarkers assays was performed following treatment of cell lines in vitro at various concentrations of BromAc and doxorubicin. The impact of drug treatment on MUC1 and MUC4 levels was assessed through immunecytological methods. Drug agent synergy was assessed through the Chou-Talalay framework. BromAc treatment in combination with doxorubicin was more efficacious than single-agent doxorubicin, with synergistic effects observed. The immuno-cytological analysis demonstrated significant mucin depletion following treatment with BromAc and doxorubicin used in combination, providing a potential mechanistic underpinning for the observed anticancer effects.

Keywords: Bromelain, acetylcysteine, cancer, soft tissue sarcoma, fibrosarcoma, synovial sarcoma, epithelioid sarcoma, chemo-resistance, chemo-sensitisation

Introduction

Sarcomas are neoplasms that are defined by their origin from mesenchymal cells. They can be categorized into two general major groups with different staging and treatment approaches. The majority of cases (80%) are classified as soft tissue sarcomas (STS), with the remainder being primary bone sarcoma [1]. Soft tissue sarcomas can be further classified into subtypes through light microscopy and analysis of genetic mutations, each representing a different cell origin. Signs and symptoms of sarcomas would depend on their location and include presence of a mass, pain, and rarely a fever. Diagnosis is achieved through biopsy and histopathological analysis to determine cellular origin [2]. Furthermore, histologic grading, size, and location of the primary tumor are important factors for establishing prognosis and guiding treatment. Localised disease can be managed with surgery alone, while tumors with higher grade or stage may require adjuvant or neoadjuvant chemotherapy, surgery, and potentially radiotherapy [2, 3].

Doxorubicin is an anthracycline that is commonly used for soft tissue sarcoma. Its primary mechanism is the inhibition of topoisomerase II, through the stabilisation of DNA-doxorubicin-topoisomerase complex. This prevents the normal re-ligation of DNA, resulting in double strand breaks of DNA. Another mechanism is the generation of reactive oxygen species through a process of redox cycling involving CYP450 [4]. Single agent doxorubicin is considered as standard care in advanced soft tissue sarcoma, with combination therapies currently providing little additional benefit, and additional toxicity [5, 6]. Low to moderate response rates are achievable, around 10-30% depending on dosage, although efficacy is limited by pres-

ence of dose-dependent cardiotoxicity, as well as renal, hepatic, and neurological toxicity [7]. Reduction of doxorubicin toxicity has been attempted through continuous infusion rather than bolus administration, as well as liposomal formulations which have a longer half-life [7, 8]. However, at their core, these methods are not able to circumvent the underlying cellular mechanisms by which doxorubicin exerts its off-target toxicities, since they are no more than refinements to the pharmacokinetic profile of the drug. Furthermore, in a trial for metastatic breast cancer, the efficacy of pegylated liposomal doxorubicin was found to be no different to that of conventionally delivered doxorubicin for metastatic breast cancer [9]. Considering the low response rates in current practice, further strategies are urgently needed to both improve efficacy and reduce toxicity, in the treatment of sarcoma through doxorubicin.

One potential strategy for increasing efficacy and reducing toxicity is the combination of doxorubicin with other suitable agents. Previously, we have shown that bromelain and acetylcysteine (BromAc) inhibit the growth of soft tissue sarcoma as single agents, but more importantly in combination [10]. This drug combination was initially developed as a mucolytic agent for pseudomyxoma peritonei having completed a phase 1 trial in 20 patients [11]. The potentiation of chemotherapeutic agents with BromAc has been explored in various colorectal cancers and peritoneal mesothelioma with promising results [12, 13]. In these studies, it has been shown *in vitro* that the efficacy of cisplatin, 5-FU, paclitaxel, and vincristine was increased when used in combination with bromelain and acetylcysteine. As bromelain and acetylcysteine are two readily available natural agents which have demonstrated high safety profiles, they potentially have value in the treatment of soft tissue sarcomas, both as a standalone treatment, and combination treatment with doxorubicin. Hence in this study, we report that a novel combination of these agents with doxorubicin increases the treatment efficacy in four subtypes of soft tissue sarcomas.

Materials and methods

Cell culture

Human sarcoma cell lines HT1080, SW872, VAESBJ and SW982 were attained from the American Type Culture Collection (ATCC, USA). All cells were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. HT1080 and SW872 cells were grown in RPMI-1640 medium (Sigma-Aldrich, USA) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin (Invitrogen, USA). High-glucose DMEM supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin and 1% (v/v) L-glutamine was used for VAESBJ and SW982.

Drug preparation

Bromelain and acetylcysteine were sourced from MucPharm Pty Ltd, Australia and Doxorubicin was sourced from Sapphire, Australia. Stock solutions were created by dissolving into relevant culture media, filtered, pH adjusted if required and diluted for treatment.

Proliferation assay

Following trypsinization, cells were seeded into a 96-well plate at a density of 3,500-4,000 cells/well and allowed to establish confluency for 24 hours. Cells were then treated with either doxorubicin or a combined treatment. In combination treatment, cells were treated with bromelain and/or acetylcysteine followed immediately by doxorubicin. Following 72 hour treatment, proliferation of treated cells was measured using the sulforhodamine-B assay as detailed in a previous study [14].

Immuno-cytochemistry

Cells were seeded on cover slips at a density of 175,000-200,000 cells/well. After 24 hours of incubation, wells are treated with bromelain, acetylcysteine, doxorubicin, and their respective combinations. After 48-hours treatment, cells were fixed using 4% (v/v) paraformaldehyde before permealisation was achieved with 0.4% (v/v) Tween-20. Following protein blocking with 1% BSA, the primary antibodies against MUC-1 and MUC-4 (Abcam, Cambridge, MA, USA) were added at a concentration of 1:200 for overnight incubation at 4°C. Secondary antibody incubation (1:250) was completed using chicken anti-mouse Alexa 488 (Abcam, Cambridge, MA, USA) followed by counterstaining with Hoechst (1:200) (Thermo Fisher Scientific, California, USA). The Olympus IX71 fluorescence microscope (Olympus, Centre

Valley, PA, USA) and Zen program (Carl Zeiss, Cambridge, UK) were used to capture and overlay fluorescent images, respectively.

Western blot

Following trypsinization, 1.75-2 million HT1080 and SW872 cells were grown in 75 cm^3 flasks for 24 hours. Flasks were then treated with bromelain, acetylcysteine, doxorubicin and their combinations for 48 hours. A Radio-Immuno Precipitation Assay (RIPA) lysis buffer was added, and proteins were extracted and quantified using the BioRad protein assay (Bio-Rad, Hercules, CA, USA). Next, separation of proteins was achieved through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and proceeded to be transferred onto a polyvinyl fluoride membrane (Millipore, Billerica, MA, USA). Primary antibody incubation (1:1000) occurred overnight at 4°C (Cell Signaling, QLD, Australia) following by secondary antibody incubation with respective horseradish perioxidase-conjugated secondary antibodies (Cell Signalling, QLD, Australia). Finally, anti-GAPDH (Sigma-Aldrich, St. Louis, MO, USA) was used to quantify GAPDH as a loading control. The ImageQuant™ LAS 4000 Biomolecular imager and Image Quant software (GE Healthcare, Little Chalfont, Buckinghamshire, UK) were used for imaging analysis.

Statistical analysis

Statistical analysis was conducted on the proliferation of cells following treatment with doxorubicin, bromelain and acetylcysteine. Using Prism GraphPad 7, *P*-value significance of treated groups was evaluated against a doxorubicin control using Dunnett's test in a multiple comparison ANOVA. A *P*-value of <0.05 was considered statistically significant. The IC-50, the concentration at which 50% growth inhibition is achieved, was calculated using a non-linear regression of the log(dose)-response curve. The combination index (CI) in combination therapy was calculated using CompuSyn software (ComboSyn Incorporated, Paramus, NJ, USA) using the median-effect principle. Drug synergy is interpreted as $CI<0.9$ whereas $0.9<CI<1.1$ and $CI>1.1$ indicate additive synergy and antagonism. Finally, densitometry of western blot bands was quantified using ImageJ software with GAPDH normalisation.

Results

Different subtypes of soft tissue sarcoma displayed varied response to doxorubicin

Single agent doxorubicin was used in 72 h treatment to assess cytotoxic effects in HT1080, SW872, VAESBJ, and SW982. The results, as shown in **Figure 1**, exhibit varying sensitivities to both drugs depending on the cell line. HT1080 is the most sensitive to single agent doxorubicin, followed by SW872, VAESBJ, with SW982 being least sensitive.

Addition of bromelain and acetylcysteine to doxorubicin results in significant increases in efficacy in vitro

Single agent doxorubicin was compared to doxorubicin with bromelain, acetylcysteine in varying combinations. Results shown in **Figure 2** demonstrated that addition of bromelain and acetylcysteine together resulted in a significantly increased combined antiproliferative effect compared to single agent doxorubicin in all four cell lines studied. Addition of the specified levels of bromelain and acetylcysteine to doxorubicin resulted in levels of efficacy that were not achievable with single agent doxorubicin at the same concentrations, in all four cell lines.

Bromelain and acetylcysteine combined with doxorubicin demonstrate clear Chou-Talalay synergism

Chou-Talalay analysis was performed on the *in vitro* work described above. In HT1080 cells, the combination of doxorubicin with bromelain and acetylcysteine displayed clear synergism at all the concentrations of doxorubicin tested ($0.3<CI<0.7$) (**Figure 3**; **Tables 1**, **2**). In SW872 cells, there were mixed effects, with some synergy at higher doses of doxorubicin and antagonism at lower doses. In VAESBJ cells, treatment with doxorubicin, bromelain, and acetylcysteine together displayed clear Chou-Talalay synergy, although more mixed effects were noted when combining doxorubicin with bromelain and acetylcysteine individually. Finally, in SW982 cells, there was clear synergism at all concentrations tested when combining doxorubicin, bromelain, and acetylcysteine.

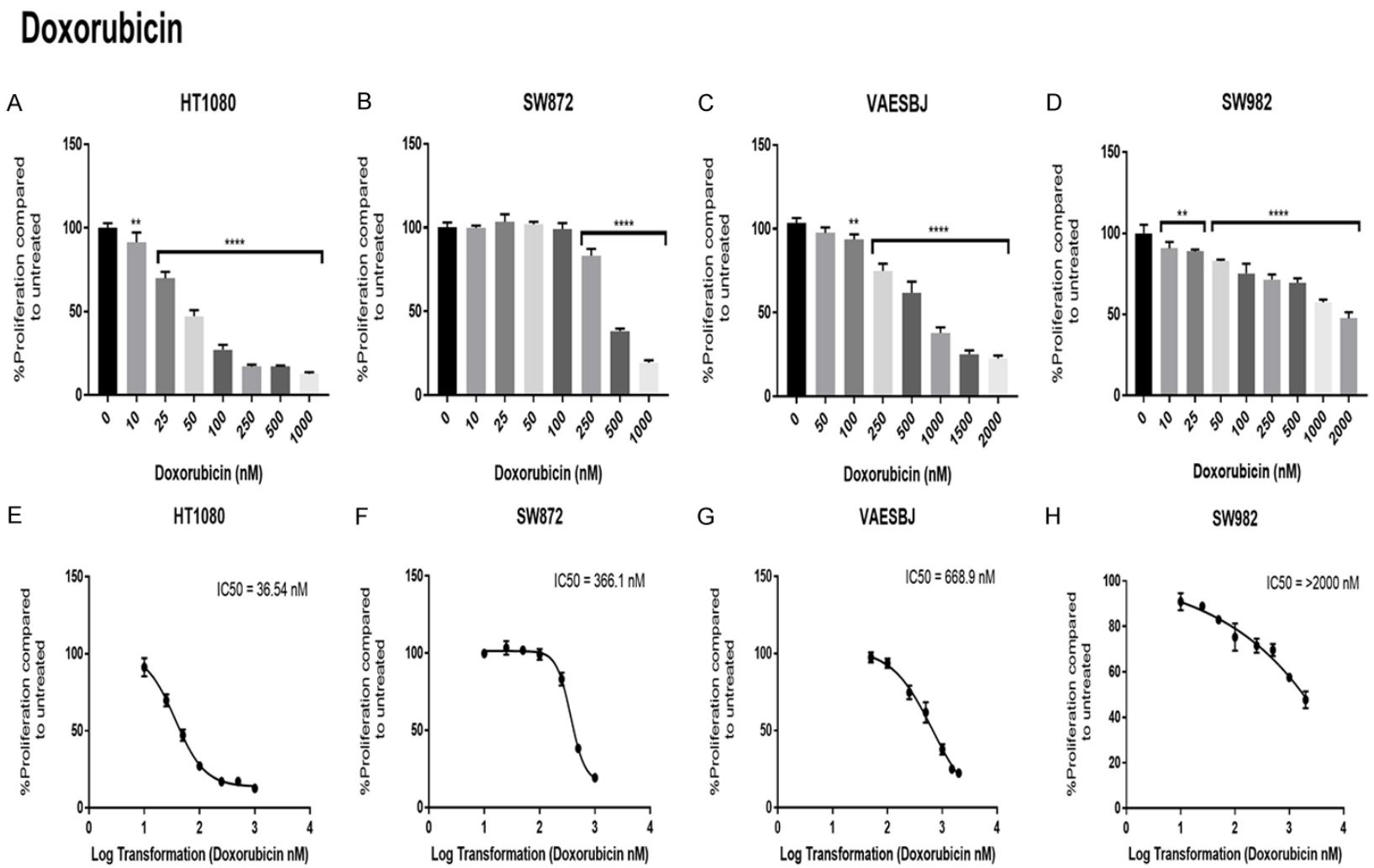

Figure 1. 72-hour treatment with doxorubicin. Top graphs represent (A) HT1080, (B) SW872, (C) VAESBJ, and (D) SW982 cell proliferation compared to an untreated control. Significance is denoted by (*) such that *P*-values are represented by P<0.05 (*), P<0.005 (**), P<0.0005 (***), and P<0.0001 (****). Bottom graphs represent dose-response curve of (E) HT1080, (F) SW872, (G) VAESBJ, and (H) SW982 cell lines with associated IC50, calculated using Prism GraphPad 7.

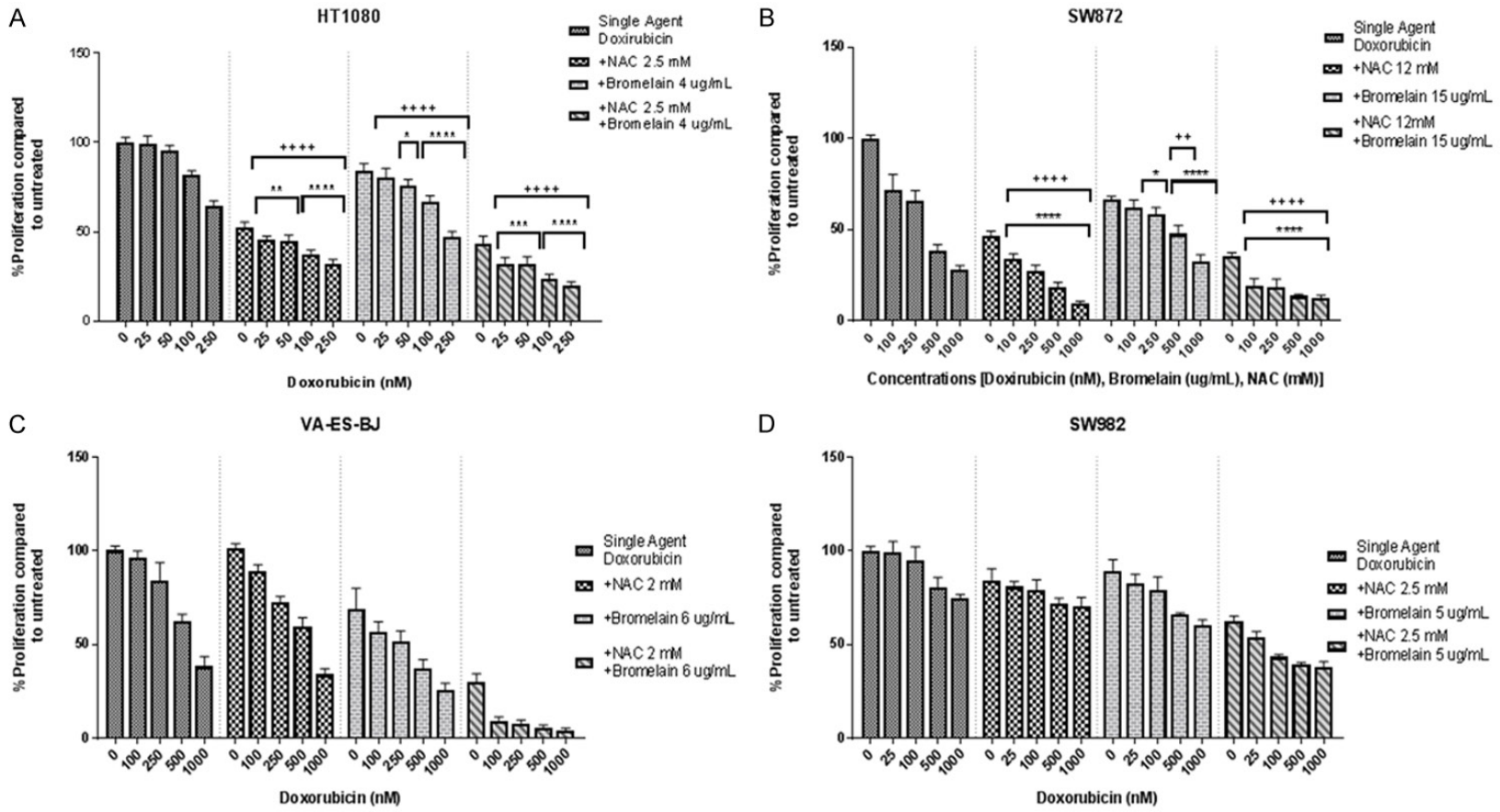

Figure 2. Efficacy of 72-hour treatment with doxorubicin alone and in combination with bromelain and acetylcysteine in (A) HT1080, (B) SW872, (C) VAESBJ, and (D) SW982 cells measured through proliferation compared to untreated controls. Data are presented as mean ± SD.

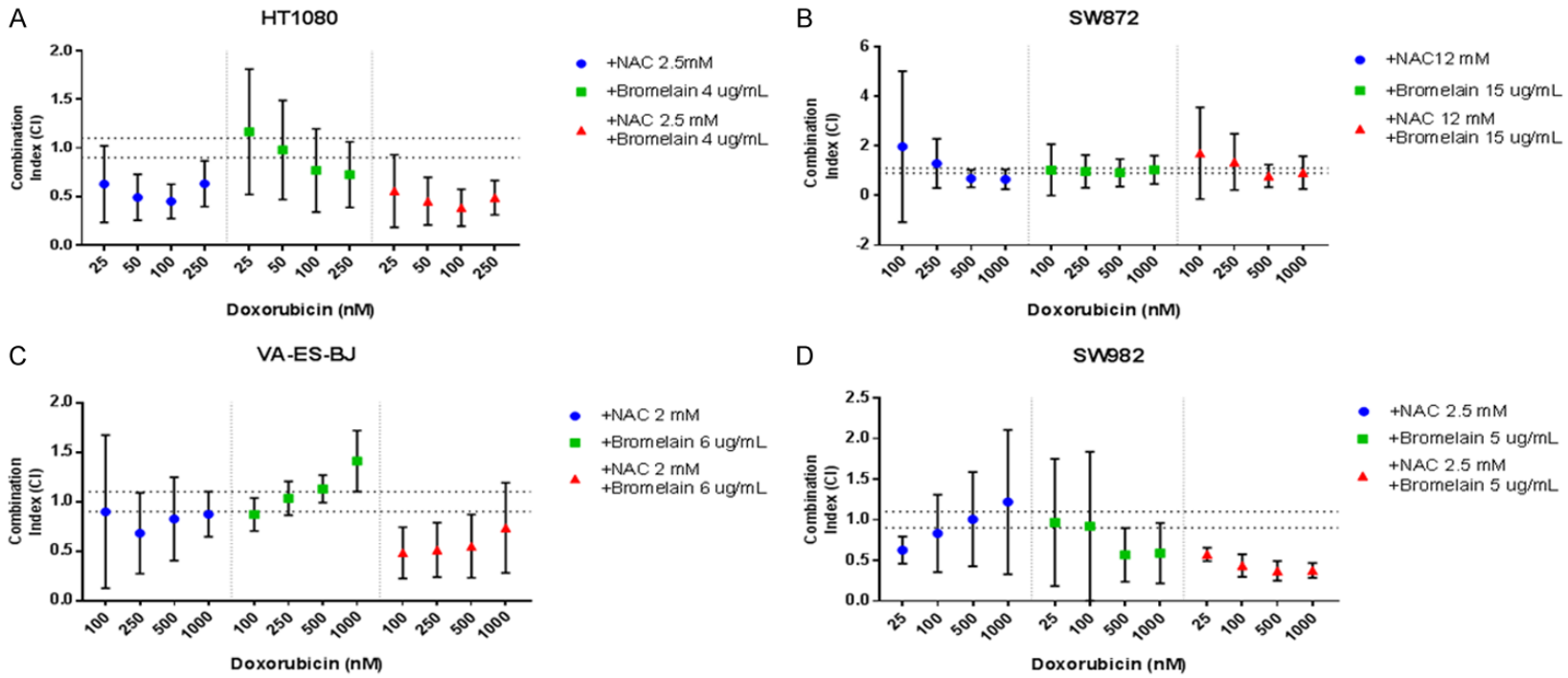

Figure 3. Combination index of doxorubicin in varying combinations with bromelain and acetylcysteine in (A) HT1080, (B) SW872, (C) VAESBJ, and (D) SW982 cell lines. The dotted lines represent thresholds for Chou-Talalay antagonism (CI>1.1) as well as synergy (CI<0.9). Additive effect corresponds to (0.9<CI<1.1). Data are presented as mean ± SD.

Table 1. Combination Index of doxorubicin in varying combinations with bromelain and acetylcysteine, displayed for various concentrations of doxorubicin

Cell line: HT1080	Combination index with doxorubicin		
Dox (nM)	Dox (nM) + NAC 2.5 mM	Dox (nM) + Br 4 ug/mL	Dox (nM) + NAC + BR
25	0.63	1.17	0.56
50	0.49	0.98	0.45
100	0.45	0.77	0.39
250	0.63	0.73	0.49
Cell line: SW872	Combination index with doxorubicin		
Dox (nM)	Dox (nM) + NAC 12 mM	Dox (nM) + Br 15 ug/mL	Dox (nM) + NAC + BR
100	1.97	1.03	1.7
250	1.29	0.97	1.35
500	0.68	0.92	0.79
1000	0.65	1.04	0.92
Cell line: VAESBJ	Combination index with doxorubicin		
Dox (nM)	Dox (nM) + NAC 2 mM	Dox (nM) + Br 6 ug/mL	Dox (nM) + NAC + BR
100	0.90	0.87	0.48
250	0.68	1.03	0.51
500	0.83	1.13	0.55
1000	0.87	1.41	0.74
Cell line: SW982	Combination index with doxorubicin		
Dox (nM)	Dox (nM) + NAC 2.5 mM	Dox (nM) + Br 5 ug/mL	Dox (nM) + NAC + BR
25	0.63	0.97	0.57
100	0.82	0.92	0.44
500	1.00	0.57	0.37
1000	1.22	0.59	0.38

Chou-Talalay antagonism is represented by CI>1.1, with synergy being CI<0.9. Additive effect corresponds to 0.9<CI<1.1. Combination indices less than 0.5 are bolded for emphasis.

Immuno-cytochemistry shows significant depletion of MUC1 and MUC4 in mucin-producing subtypes of soft tissue sarcoma

In HT1080 cells, MUC1 is moderately depleted in the bromelain, acetylcysteine, and doxorubicin combination treatment group compared to single agent doxorubicin. MUC4 expression is eliminated when bromelain and acetylcysteine are combined with doxorubicin together, whereas slight and moderate depletion of MUC4 is observed when bromelain and acetylcysteine are combined with doxorubicin individually, respectively (**Table 3**).

In SW872 cells, MUC1 is significantly depleted in the bromelain, acetylcysteine and doxorubicin combination group compared to untreated controls. MUC4 is also significantly depleted in the bromelain, acetylcysteine, and doxorubicin combination treatment group compared to both single agent doxorubicin and untreated control (**Table 3**).

Apoptosis, cell cycle progression, and autophagy were impacted in subtype dependent ways

Previously, we have investigated the mechanisms of cell death in soft tissue sarcomas with bromelain and acetylcysteine as single agents and in combination (**Figures 4**-**7**) with the conclusion that these agents predominately facilitate pathways of apoptosis and autophagy in SW872 and HT1080 cells [10]. An extension of this experiment included treatment groups consisting of doxorubicin as a single agent and in combination with bromelain or acetylcysteine, and a combination of all three agents. These blots are seen in **Figure 8**, with the densitometry analysis included in **Table 4**. Full-length blot images are provided in the Supplementary Figures 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16.

In the HT1080 cell line, treatment with doxorubicin alone similarly resulted in increased expression of caspase 8, although caspase 3 was not elevated. BcL2, BAX, and cyclin D3 were relatively unchanged, while cyclin B1 and LC3BI and LC3BII were decreased, reflecting decreased levels of autophagy. When doxorubicin was administered in combination with bromelain and acetylcysteine, notably, both cyclin B1 and cyclin D3 were downregulated significantly, potentially reflecting increased cell cycle arrest. LC3BI and LC3BII were roughly unchanged from the untreated control values, while there were limited increases in caspase 3 and 8 compared to control. Cleaved PARP was not observed in all treatment groups, reflecting limited apoptosis when HT1080 cells are exposed to the given agents.

In the SW872 cell line, treatment with doxorubicin alone resulted in slight induction of caspases 3 and 8, associated with increased expression of cyclin B1 and D3, indicating that some

Table 2. Combination index of doxorubicin in varying combinations with bromelain and acetylcysteine, displayed for various concentrations of doxorubicin

Cell line: HT1080	Combination index with doxorubicin		
Dox (nM)	Dox (nM) + NAC 2.5 mM	Dox (nM) + Br 4 ug/mL	Dox (nM) + NAC + BR
25	+++	-	+++
50	+++	±	+++
100	+++	++	+++
250	+++	++	+++
Cell line: SW872	Combination index with doxorubicin		
Dox (nM)	Dox (nM) + NAC 12 mM	Dox (nM) + Br 15 ug/mL	Dox (nM) + NAC + BR
100	---	±	---
250	--	±	--
500	+++	±	++
1000	+++	±	±
Cell line: VAESBJ	Combination index with doxorubicin		
Dox (nM)	Dox (nM) + NAC 2 mM	Dox (nM) + Br 6 ug/mL	Dox (nM) + NAC + BR
100	+	+	+++
250	+++	±	+++
500	++	-	+++
1000	+	--	++
Cell line: SW982	Combination index with doxorubicin		
Dox (nM)	Dox (nM) + NAC 2.5 mM	Dox (nM) + Br 5 ug/mL	Dox (nM) + NAC + BR
25	+++	±	+++
100	++	±	+++
500	±	+++	+++
1000	--	+++	+++

Symbols represent various ranges of combination indices. Clear synergism (+++): 0.3-0.7; Moderate synergism (++): 0.7-0.85; Slight synergism (+): 0.85-0.9; Nearly additive (±): 0.9-1.1; Slight antagonism (-): 1.1-1.2; Moderate antagonism (--): 1.2-1.45; Clear antagonism (---): 1.45-3.3.

apoptosis was induced. Some autophagy was also induced as levels of LC3B1 and LC3BII were increased. When bromelain and acetylcysteine were administrated with doxorubicin, we can infer a significant increase in apoptosis due to the increase in cleaved PARP, which is associated with increases in cyclin D3 levels. The magnitudes of these increases were highest when treatments of all three agents were combined. Increased levels of LC3BII were also observed in the combination treatment group, as well as increased BcL2 expression.

Discussion

We have investigated the effect of bromelain and acetylcysteine in combination with doxorubicin on four subtypes of sarcoma: HT1080 (fibrosarcoma), SW872 (liposarcoma), SW892 (synovial sarcoma), VAESBJ (epithelioid sarcoma). Efficacy and Chou-Talalay synergy were assessed for all four cell lines, while cytological analysis of MUC1 and MUC4 content as well as western blot analysis for markers of apoptosis, cell cycle progression, and autophagy were carried out for two cell lines: HT1080 and SW872.

All cell lines responded to single agent doxorubicin treatment in a dose dependent manner. However, there was significant variation in sensitivity between the different subtypes assessed. HT1080 had an IC50 that was less than 2% of that of the least sensitive, SW982, representing over 50 times more sensitivity to doxorubicin. Doxorubicin resistant cell lines of soft tissue sarcoma are able to be selected through sustained exposure to doxorubicin, with the remaining cells having several hundred-fold levels of resistance compared to the parent cell line [15]. Various mechanisms have been discovered to result in resistance to doxorubicin and other cytotoxic drugs. A genetic mutation (MDR1) confers doxorubicin resistance through coding for p-glycoprotein, a drug efflux pump which lowers the intracellular levels of doxorubicin [16, 17]. Furthermore, the protein Rad51 is responsible for homologous recombination DNA repair and has been found to be upregulated in a variety of cancers, specifically those that are drug resistant [18]. Overexpression of Rad51 therefore is another mechanism by which sarcoma cells can become resistant to doxorubicin treatment. Considering the observed significant variation in sensitivity between subtypes, further research into the presence of MDR1, p-glycoprotein, and Rad51 in sarcoma would be worthwhile.

In all cell lines studied, addition of bromelain and acetylcysteine to doxorubicin caused significant antiproliferative effects beyond those of doxorubicin alone. Under Chou-Talalay analysis, their combined action would be mostly classified as synergistic for most dose combi-

Table 3. Mucin depletion as assessed quantitatively through ImageJ RGB analysis

Cell Line	Treatment group	Mucin depletion	Percentage of MUCs compared to control
HT1080 MUC1	Control	-	100
	Doxorubicin	+++	44
	Dox + NAC	+++	32
	Dox + Br	++	68
	Dox + Br + NAC	++	54
HT1080 MUC4	Control	-	100
	Doxorubicin	--	128
	Dox + NAC	+	86
	Dox + Br	+++	44
	Dox + Br + NAC	+++	8
SW872 MUC1	Control	-	100
	Doxorubicin	-	100
	Dox + NAC	+++	47
	Dox + Br	+++	43
	Dox + Br + NAC	+++	46
SW872 MUC4	Control	-	100
	Doxorubicin	+	85
	Dox + NAC	++	72
	Dox + Br	++	66
	Dox + Br + NAC	+++	47

Ratio of green to red pixels were calculated and normalized to untreated control. Significant mucin depletion (<50%) denoted by +++, moderate depletion (50-75%) denoted by ++, slight depletion (75-90%) denoted by +, no depletion (90-110%) denoted by -, and slight increase (110-130%) denoted by --.

nations studied, reflecting combination indices less than 0.9 [19]. In two cell lines in particular: HT1080 and SW982, clear synergism was observed at all doses. Evidence of Chou-Talalay synergy suggests that there can be a reduction of toxicity profile through the combination of bromelain, acetylcysteine, and doxorubicin, as well as increased efficacy. A possible reason for the observed synergism is that bromelain and acetylcysteine promote increased cellular uptake and penetration of doxorubicin by disintegrating the mucinous barriers in the cytoplasm, nucleus and extracellularly. This represents a method of overcoming doxorubicin resistance, perhaps by counteracting the expression of drug efflux pumps such as p-glycoprotein.

Immuno-cytological analyses have demonstrated that bromelain and acetylcysteine in combination with doxorubicin act to significantly reduce the levels of MUC1 and MUC4 expression in sarcoma cell lines, in some cases eliminating expression of mucins. These results show consistency with the established body of literature that demonstrates the mucin-degrading properties of bromelain and acetylcysteine in combination for a wide variety of cancers both *in vitro* and *in vivo* [12, 13, 20-22], as well as in clinical studies of pseudomyxoma peritonei [11].

Mucins are aberrantly expressed in a wide variety of tumors and are thought to play an important role in cancer physiology. It has been reported in the literature that MUC1 and MUC4 are both intricately linked with various signalling pathways that have impact on cancer cell growth, proliferation, survival, and autophagy (p53, PI3K phospho-Akt/PKB, JNK, extrinsic apoptotic, etc.) [23, 24]. Therefore, depletion of mucins provides a potential mechanistic basis for the observed anticancer effect of bromelain and acetylcysteine. Further clarification as to the relative importance of mucins towards the regulation of these various pathways would provide a useful avenue for further study. Aside from direct anticancer effects, bromelain and acetylcysteine have been demonstrated to dramatically improve chemo-penetration through the depletion of mucin, representing another mode of action [22]. The current investigation validates the action of bromelain and acetylcysteine in depleting various mucins and provides further support for the anticancer effect of the drug combination, in different forms of sarcoma.

Analysis of biomarkers of cell cycle progression, apoptosis, and autophagy show variation between the two cell lines studied, suggesting that there are subtype-dependent factors that result in distinct and separate responses to treatment with doxorubicin in combination with bromelain and acetylcysteine. For HT1080, the predominant pathway responsible for anti-proliferative effects appears to be cell cycle arrest with some upregulation of apoptosis. However, for SW872, the predominant pathway is cas-

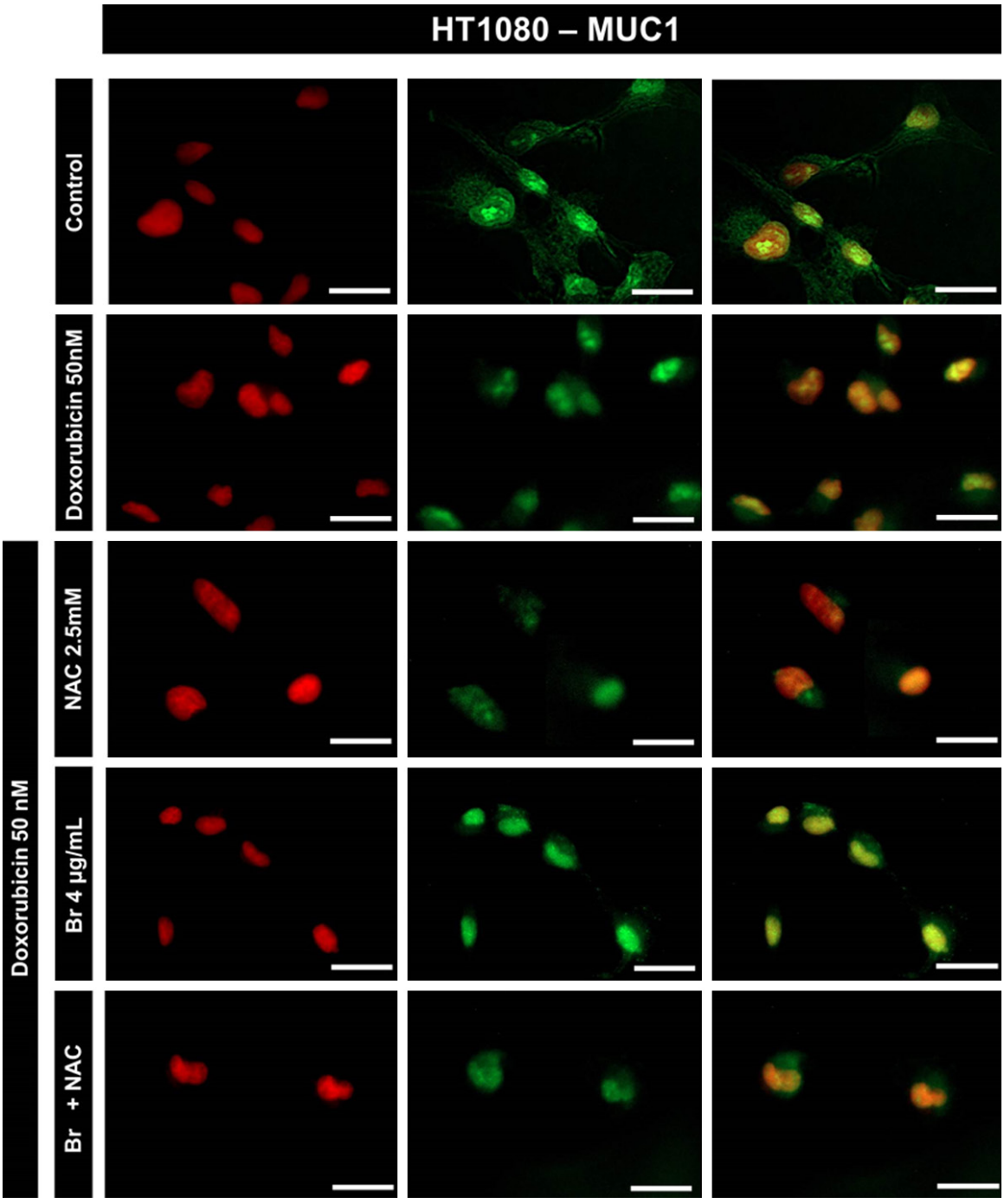

Figure 4. Immunofluorescence of soft tissue sarcoma HT1080 cells following 48 hours of treatment with bromelain, acetylcysteine, and bromelain plus acetylcysteine. Expression of MUC1 was viewed under confocal microscope with red and green fluorescence corresponding to nucleus and MUC1 staining, respectively. Scale bar: 50 µm. Final magnification =600×.

pase-mediated apoptosis, as demonstrated by the presence of cleaved PARP when bromelain and acetylcysteine are combined with doxorubicin. Autophagy is thought to increase cancer cell survival through enabling survival through conditions of high metabolic stress, limiting damage to the genome [25, 26]. Autophagy dysregulation, whether increase or decrease, has been reported to be correlated with doxorubicin resistance in a number of cell lines

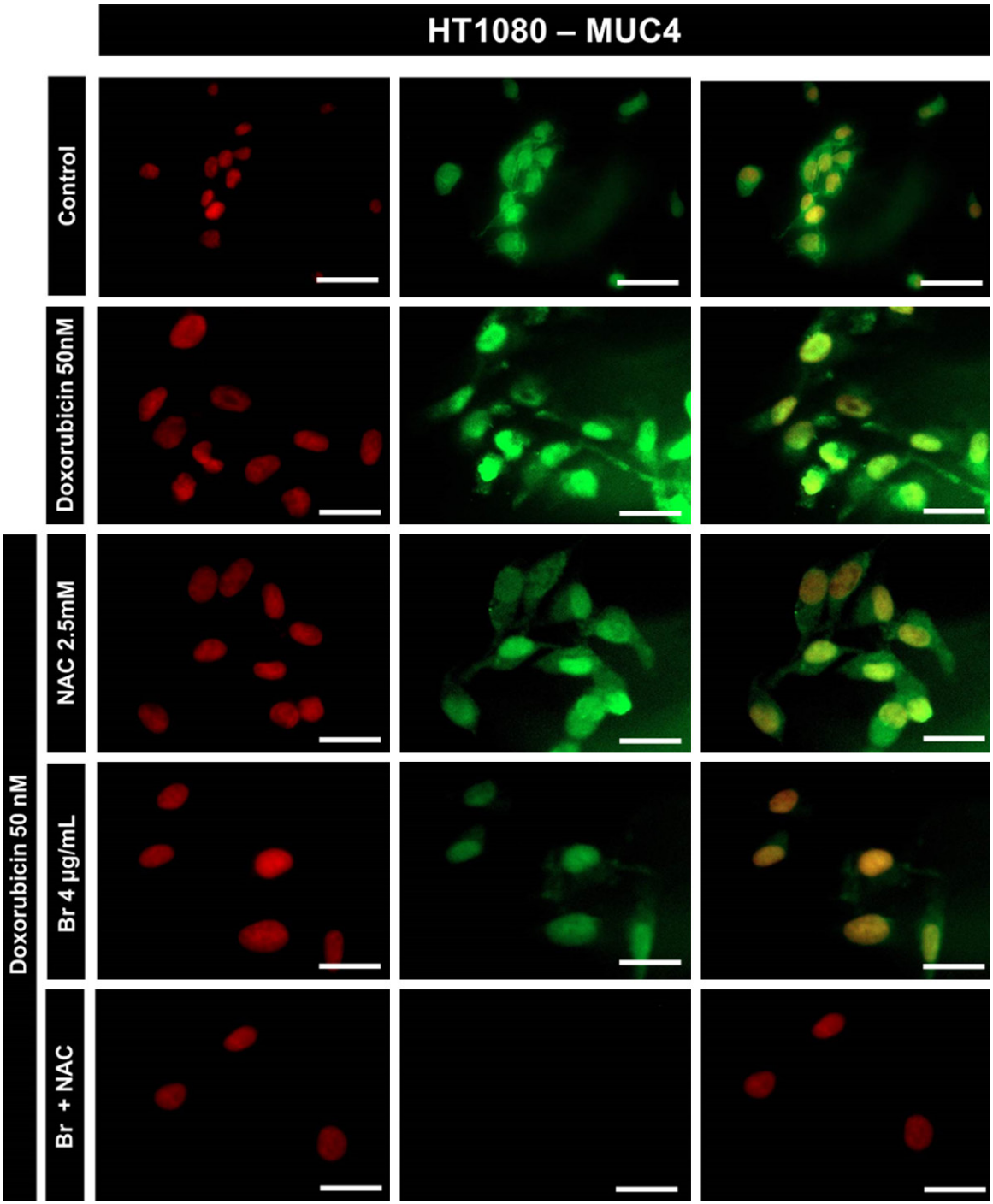

Figure 5. Immunofluorescence of soft tissue sarcoma HT1080 cells following 48 hours of treatment with bromelain, acetylcysteine, and bromelain plus acetylcysteine. Expression of MUC4 was viewed under confocal microscope with red and green fluorescence corresponding to nucleus and MUC4 staining, respectively. Scale bar: 50 µm. Final magnification =600×.

[27]. In SW872, levels of autophagy are increased following treatment with doxorubicin, bromelain and acetylcysteine, representing the tumor cell response to additional genomic and metabolic stressors from treatment. In HT1080, autophagy is not induced following the same treatment, which may partly explain the increased chemosensitivity to doxorubicin of

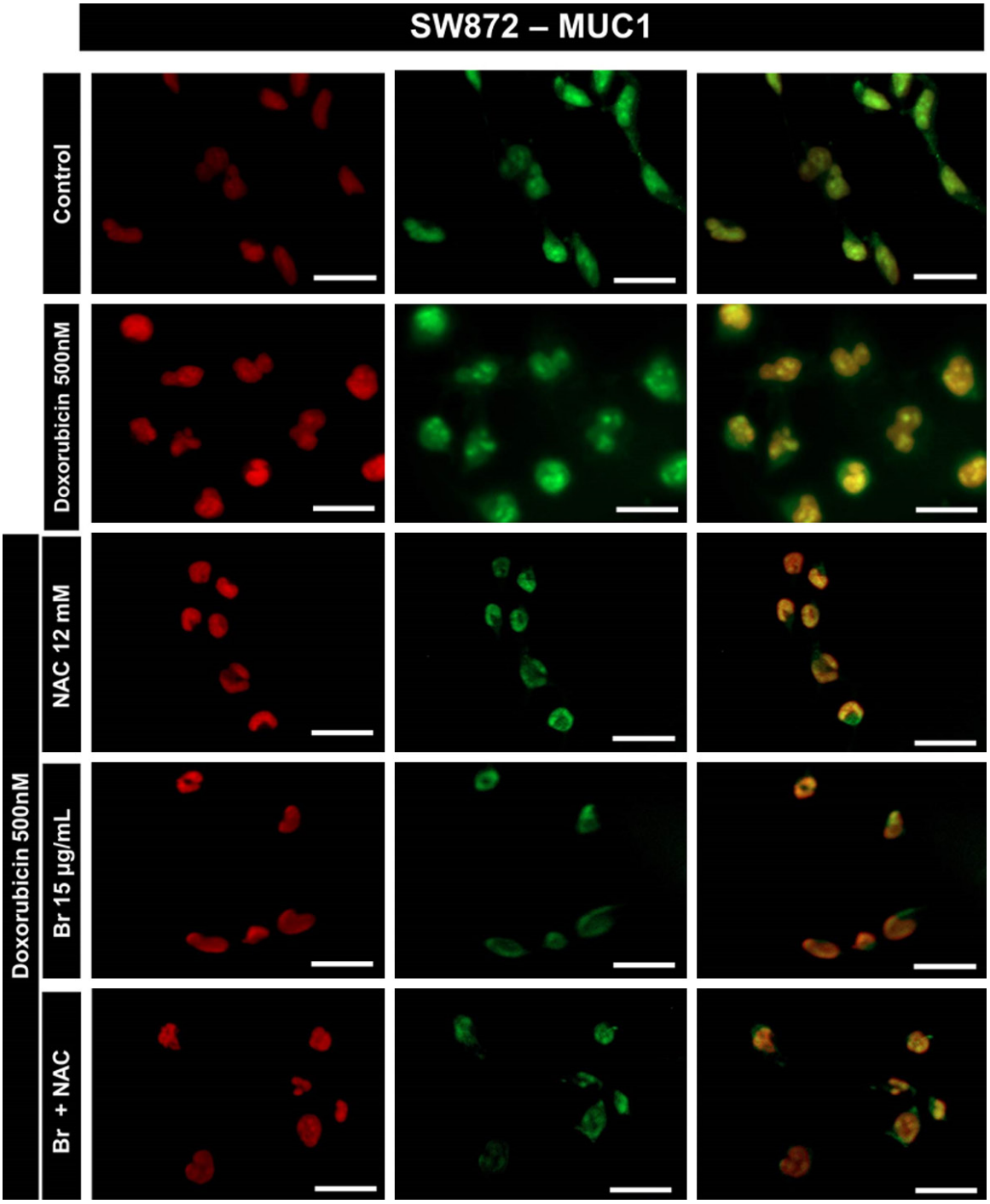

Figure 6. Immunofluorescence of soft tissue sarcoma SW872 cells following 48 hours of treatment with bromelain, acetylcysteine, and bromelain plus acetylcysteine. Expression of MUC1 was viewed under confocal microscope with red and green fluorescence corresponding to nucleus and MUC1 staining, respectively. Scale bar: 50 µm. Final magnification =600×.

HT1080 compared to SW872. Further research is needed to clarify and quantify the mechanisms behind these varied responses.

Our *in vitro* results demonstrate that significant dose reductions of doxorubicin are possible with the addition of bromelain and acetyl-

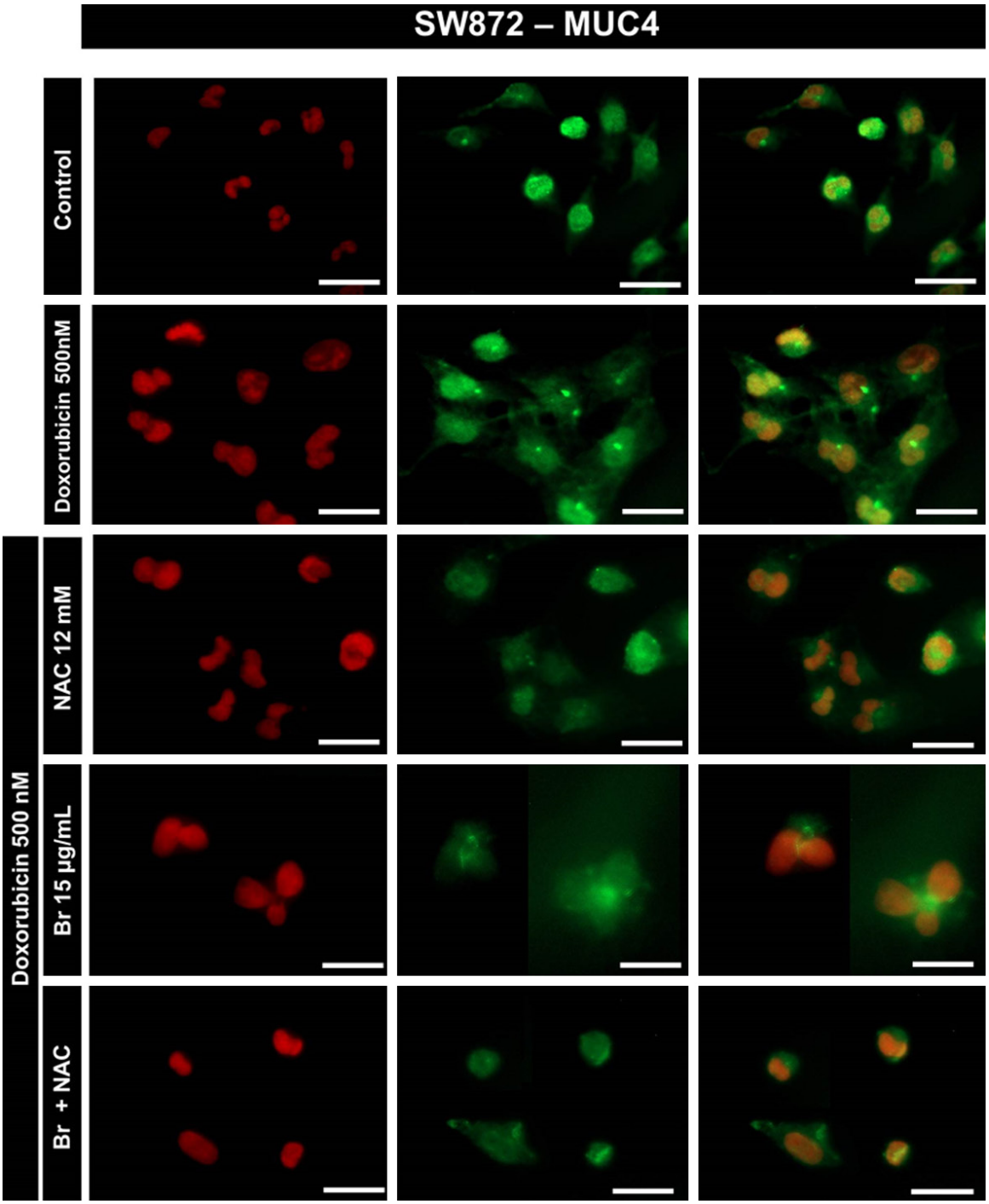

Figure 7. Immunofluorescence of soft tissue sarcoma SW872 cells following 48 hours of treatment with bromelain, acetylcysteine, and bromelain plus acetylcysteine. Expression of MUC4 was viewed under confocal microscope with red and green fluorescence corresponding to nucleus and MUC4 staining, respectively. Scale bar: 50 µm. Final magnification =600×.

cysteine, as well as significant increases in antitumor efficacy in the four cell lines studied. These results support the continued preclinical development of bromelain and acetylcysteine as an adjunctive therapy to common cytotoxic agents including doxorubicin, with potential for cytotoxic dose reductions and increased efficacy. Immuno-cytological analyses show that

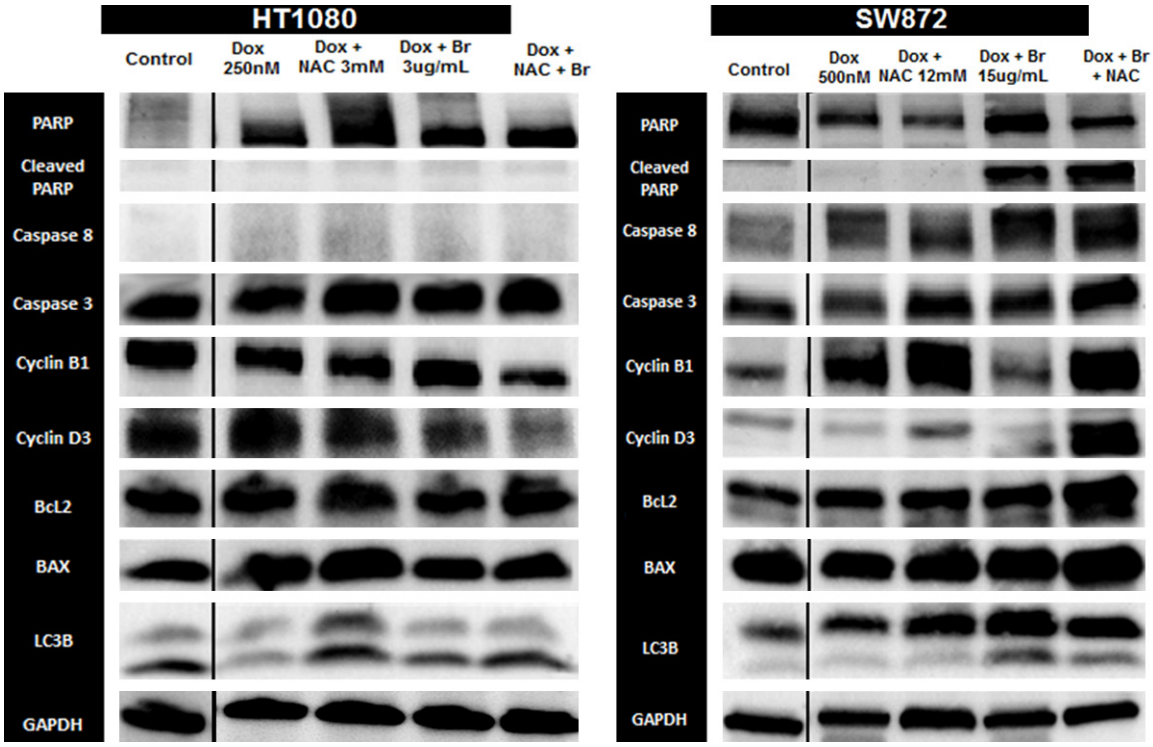

Figure 8. Western blot analysis of a panel of biomarkers for apoptosis, cell cycle progression, and autophagy in soft tissue sarcoma HT1080 and SW872 cells.

Table 4. Densitometry analysis of Western blot bands reflecting markers of apoptosis, cell cycle progression and autophagy, presented as ratios of untreated control with GADPH normalisation

HT1080				
	Dox	Dox + NAC	Dox + Br	Dox + Br + NAC
PARP	1.5	2.0	1.3	2.0
Cleaved PARP	-	-	-	-
Caspase 8	2.6	2.9	2.2	1.5
Caspase 3	0.9	1.3	1.4	1.3
Cyclin B1	0.7	0.8	0.9	0.6
Cyclin D3	1.0	0.8	0.6	0.4
BcL2	1.0	0.9	0.8	0.9
BAX	0.9	1.4	1.1	1.2
LC3BI	0.5	1.5	0.9	0.9
LC3BII	0.5	1.5	0.9	0.9
SW872				
	Dox	Dox + NAC	Dox + Br	Dox + Br + NAC
PARP	0.7	0.5	0.7	0.5
Cleaved PARP	0.5	0.6	5.9	6.5
Caspase 8	1.3	1.3	1.8	1.6
Caspase 3	1.1	1.2	1.3	1.4
Cyclin B1	2.3	2.4	1.1	2.2
Cyclin D3	1.3	3.3	2.5	8.5
BcL2	1.3	1.4	1.3	1.8
BAX	1.0	0.9	0.7	1.1
LC3BI	1.5	1.4	1.6	1.7
LC3BII	1.3	1.0	4.5	4.2

bromelain and acetylcysteine can deplete MUC1 and MUC4 in two subtypes of sarcoma, while the western blot analysis has demonstrated that bromelain and acetylcysteine in combination with doxorubicin can induce caspase-mediated apoptosis and cell cycle arrest.

Conclusion

We investigated the effects of bromelain and acetylcysteine in combination with doxorubicin for four subtypes of sarcoma. *In vitro* results showed significantly improved efficacy when the three agents were used in combination, compared to single agent doxorubicin. Chou-Talalay analysis demonstrated mostly synergistic effects at the dose combinations studied. Immuno-cytological analyses demonstrated the significant depletion of mucin in both subtypes of sarcoma tested. Western blot analysis demonstrated induction of caspase mediated apoptosis, with cell cycle arrest when bromelain and acetylcysteine were used in combination with doxorubicin. Further research would be required to better understand the mechanisms underpin-

ning the varied responses between different subtypes of sarcoma, as well as to clarify the safety and efficacy profile of the agents studied.

Disclosure of conflict of interest

DLM is the co-inventor and assignee of the licence for this study and director of the spin-off sponsor company, Mucpharm Pty Ltd. KK, AHM, JA and KP are employees of Mucpharm Pty Ltd.

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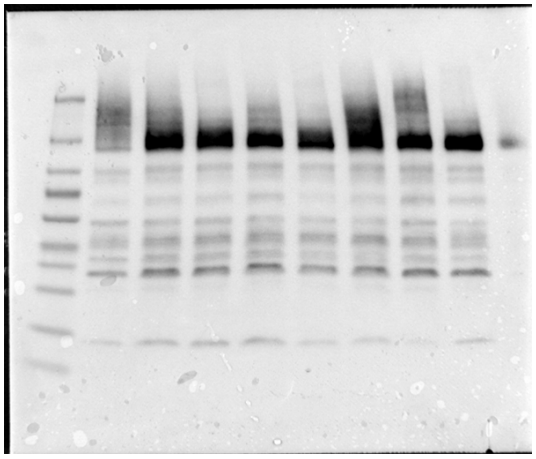
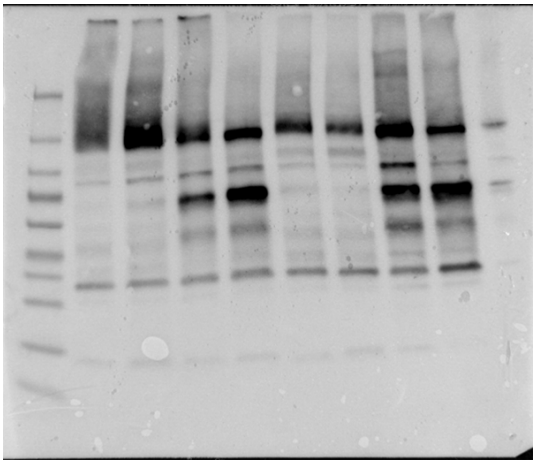
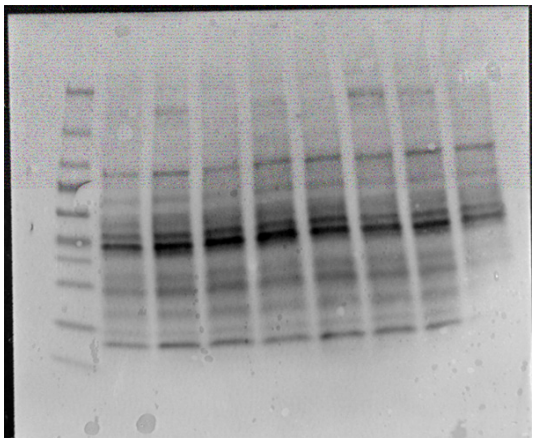


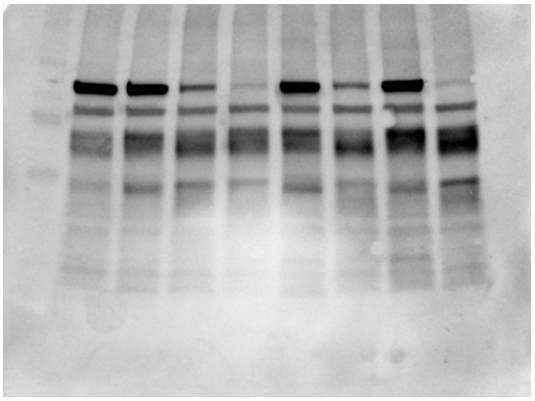
Supplementary Figure 1. Full-length blot images of HT1080 cells showing PARP and cleaved PARP.



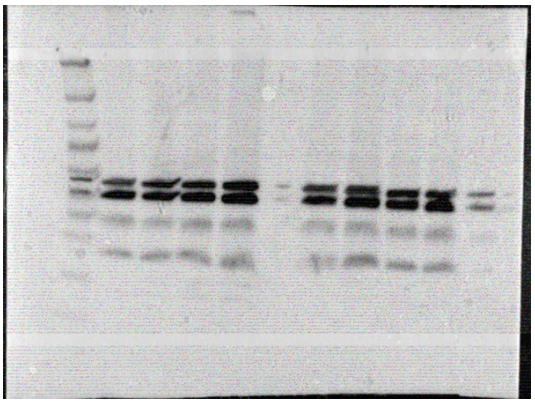
Supplementary Figure 2. Full-length blot images of SW872 cells showing PARP and cleaved PARP.



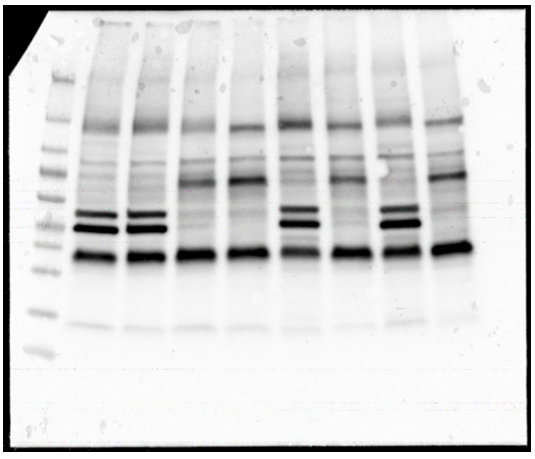
Supplementary Figure 3. Full-length blot images of HT1080 cells showing caspase 8.



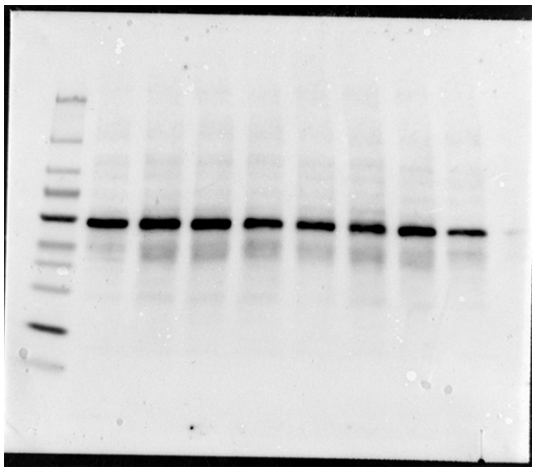
Supplementary Figure 4. Full-length blot images of SW872 cells showing caspase 8.



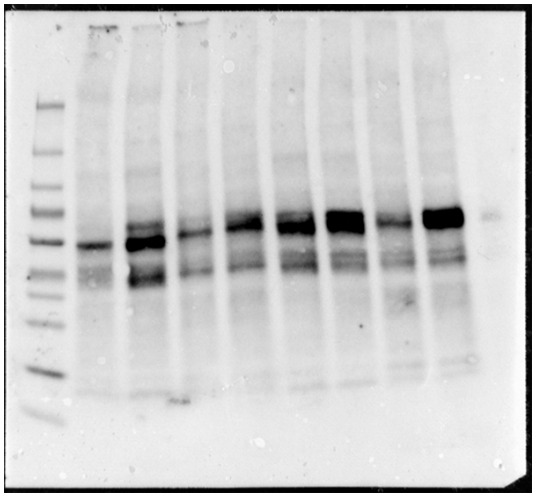
Supplementary Figure 5. Full-length blot images of HT1080 cells showing caspase 3.



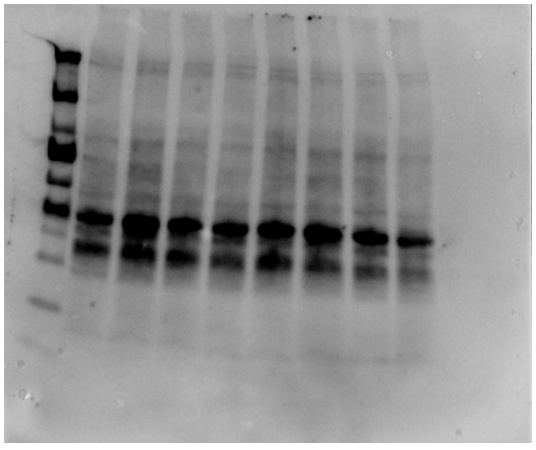
Supplementary Figure 6. Full-length blot images of SW872 cells showing caspase 3.



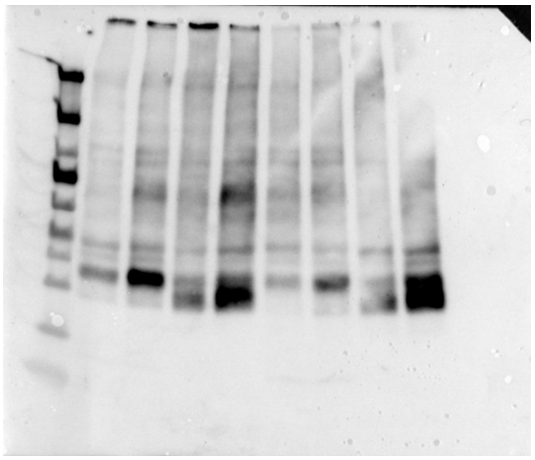
Supplementary Figure 7. Full-length blot images of HT1080 cells showing cyclin B1.



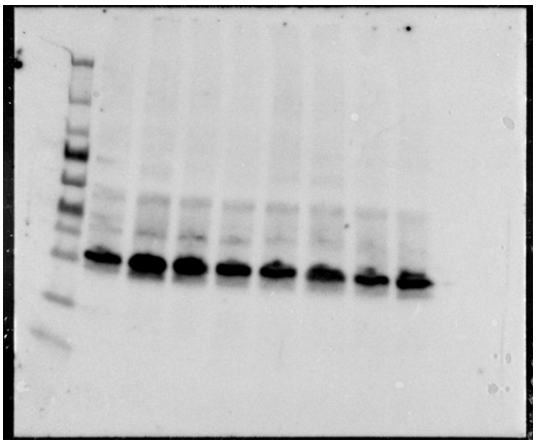
Supplementary Figure 8. Full-length blot images of SW872 cells showing cyclin B1.



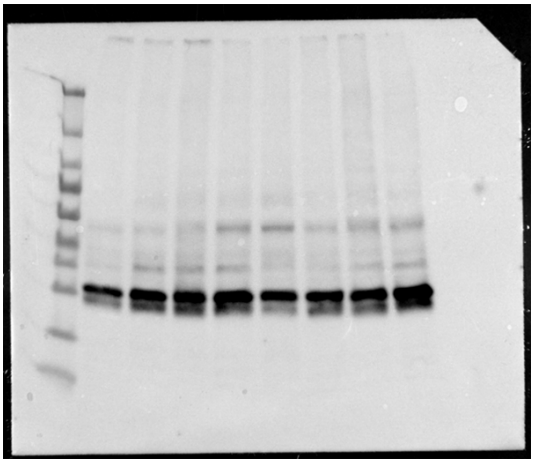
Supplementary Figure 9. Full-length blot images of HT1080 cells showing cyclin D3.



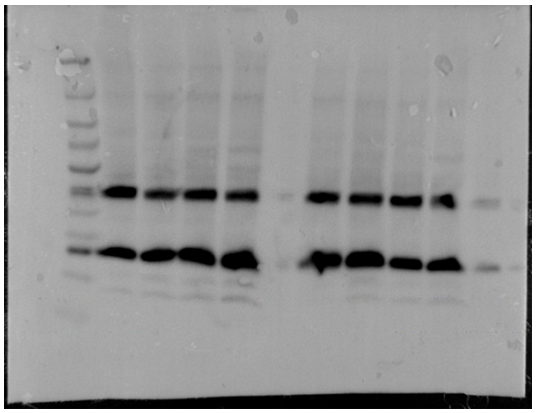
Supplementary Figure 10. Full-length blot images of SW872 cells showing cyclin D3.



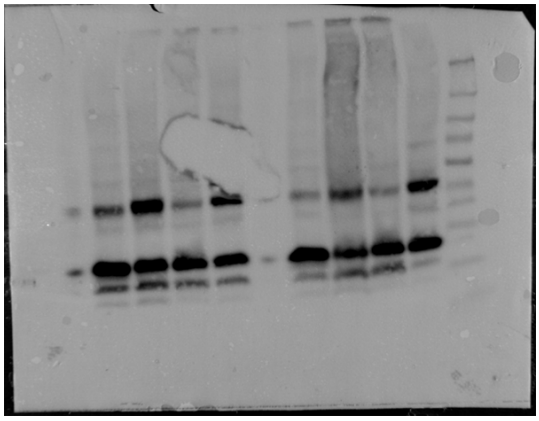
Supplementary Figure 11. Full-length blot images of HT1080 cells showing Bcl2.



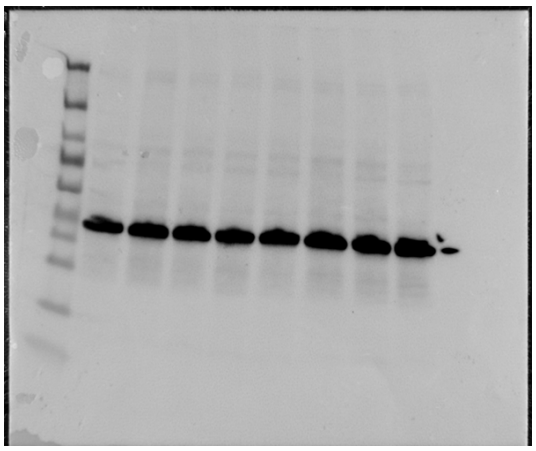
Supplementary Figure 12. Full-length blot images of SW872 cells showing Bcl2.



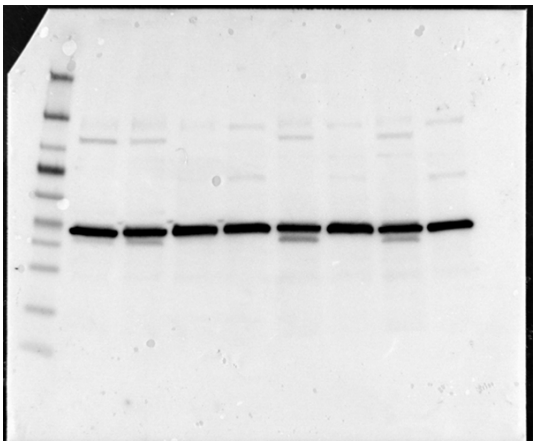
Supplementary Figure 13. Full-length blot images of HT1080 cells showing BAX.



Supplementary Figure 14. Full-length blot images of SW872 cells showing BAX.



Supplementary Figure 15. Full-length blot images of HT1080 cells showing GAPDH.



Supplementary Figure 16. Full-length blot images of SW872 cells showing GAPDH.