Int J Clin Exp Med 2019;12(12):13311-13324
www.ijcem.com /ISSN:1940-5901/IJCEM0096364

Original Article

Bromelain and N-acetylcysteine as therapeutic agents for soft tissue sarcoma

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Received April 30, 2019; Accepted October 7, 2019; Epub December 15, 2019; Published December 30, 2019

Abstract: Soft tissue sarcomas (STS) are rare mesenchymal neoplasms comprising 1% of the global burden of malignant cancers. Adjuvant chemotherapy includes doxorubicin with or without ifosfamide, however targeted therapy is limited. Since, Bromelain and N-acetylcysteine (NAC) have previously shown promising roles in cancer treatment, we tested these agents on STS. The effectiveness of treatment with Bromelain and N-acetylcysteine as single agents and combined, was tested on a panel of soft tissue sarcoma cell lines (SW872, HT1080, VA-ES-BJ, SW982). The viability of cells after 72-hour treatments was assessed using the sulforhodamine-B assay. Mucin status 48 hours post treatment was examined through periodic-acid-Schiff (PAS) staining and immunocytochemistry (ICC). Finally, cell death pathways were assessed by Western blot. Percentage of live cells after 72-hours of treatment was significantly reduced with bromelain and NAC. Efficacy was further enhanced when the two agents were combined. PAS and ICC showed a non-specific disruption of mucins/mucopolysaccharides and MUC1/MUC4, respectively. This may point to mucin depletion as the main mechanism of inhibition in treated cells. Western blot analysis indicated apoptosis as the main and autophagy as the minor pathway of cell death in non-mucin-expressing SW872 and vice versa in the mucin-expressing HT1080. From this, we can infer that the induction of cell death by bromelain and NAC have varying mechanisms in STS with differing mucin profiles. Bromelain and N-acetylcysteine as single agents, but more importantly in combination, exert significant effects on the survival of soft tissue sarcoma cells indicating that these agents can be developed into effective therapies.

Keywords: Bromelian, N-acetylcysteine, fibrosarcoma, synovial sarcoma, epithelioid sarcoma

Introduction

Sarcomas are a rare group of tumours which arise from mesenchymal and connective tissue and account for approximately 1% of the global burden of malignancies. There are two categories of sarcomas; sarcomas of the soft tissue (soft tissue sarcomas) and sarcomas of the bone (osteosarcoma, Ewing's sarcoma, chondrosarcoma) which comprise 80% and 20% of cases, respectively. Since their original classification by the World Health Organisation in 1994, there has been a continual shift in the sub-classification of these tumours, particularly in relation to clinical, histological and genetic data. In its newest classification of soft tissue tumours published in 2013, the WHO recognised over 100 histological subtypes of benign and malignant soft tissue tumours [1].

The distribution of soft tissue sarcomas are most commonly seen within the ages of 45 and 90 and cases arise equally in males and females. Generally, incidence increases with age however there is a relatively high frequency in children, adolescents and young adults. Whilst they comprise only 1% of global malignancies, they account for 10% of paediatric cases and are a significant cause of death in the under 30 s.

Treatment modality is based mainly on tumour grade. In lower grade disease, treatment includes wide local resection and radiotherapy to achieve local control with clear margins. However, in over 50% of cases, disease progression will occur presenting as distant metastasis. In high grade disease, in addition to surgical intervention, adjuvant and neoadjuvant chemotherapy are used to manage micro-metastasis and decrease tumour load, respectively [2, 3]. The most efficacious chemotherapy regime is doxorubicin and ifosfamide which has been shown to be more effective than doxorubicin alone [4].

In more recent times, popular treatment protocols have also incorporated epirubicin, MESNA and Darcarbazine with advances in immunotherapy. For patients who have failed first line therapy, second line treatment is limited. Gemcitabine and taxanes have been used, however response is limited [2]. In advanced disease and metastasis, single agent doxorubicin is commonly used despite only half of patients experiencing any clinical benefit [5]. The expected 5-year overall survival across all subtypes and stages is approximately 50% [6]. However, in advanced and metastatic disease, 5-year survival is significantly reduced to 15% and there has been little improvement over the past few decades [7].

Thus, with such diverse spectrum of subtypes, limited treatment types and poor treatment outcomes in advanced disease, there is a need for new treatments for soft tissue sarcomas, especially aimed at targeting specific subtypes [8].

Bromelain is an extract from the stems of the pineapple plant *(Ananas Comosus).* Its constituents include a mixture of proteolytic and non-proteolytic enzymes including phosphatases, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates [9]. Since its pharmaceutical release in 1956 it is a widely accepted phototherapeutic drug due to its very low toxicity, high gastrointestinal bioavailability and myriad of therapeutic benefits [10]. Due to this, its potential in medicine has garnered much attention and includes roles in burn debridement, healing of surgical traumas, prevention and minimization of cardiovascular and circulatory events, and as an anti-inflammatory and anti-mucolytic agent [11]. It has already been shown that bromelain exerts anti-proliferative effects in a variety of cancers including melanoma, sarcoma, leukaemia, lung carcinoma, colorectal cancer, and breast cancer [10-15]. In a review by Chobotova et al. the *in-vitro* and *in-vivo* anti-cancer effects of Bromelain have been related to stimulation of apoptosis, inhibition of cell survival activators and alteration of the tumour micro-environment [12].

N-acetylcysteine (NAC) is an acetylated derivative of the amino acid L-cysteine and is commonly used for treating acetaminophen, or paracetamol toxicity [13]. Furthermore since it also possesses the ability to reduce disulphide (S-S) groups to sulfhydryl (S-H) groups in mucins, it is used as a mucolytic agent in various pulmonary conditions [14]. NAC also possesses other mechanisms through which it acts as an anti-cancer agents [15, 16] and its oxido-reductive potential in generating glutathione (GSH) from its reduced form glutathione disulphide (GSSG) may also affect tumour cell survival [17]. It is known that many oncogenes possess molecular structures with disulfide linkages and thus NAC may have efficacy in disrupting vital structural linkages and thus rendering them ineffective in their tumorigenic role [18].

There have been promising studies exploring the therapeutic effects of bromelain and N-acetylcysteine as single agents, in combination with each other, and in combination with various chemotherapy agents [11, 19-29]. Bromelain has been shown to cleave glycosidic linkages in secreted mucin by hydrolysing glycosidic linkages [30] thus decreasing the integrity of the mucinous barrier in pancreatic cancer thus allowing more efficacious penetration of 5-FU [31]. Furthermore, Pillai et al., demonstrated that bromelain and NAC enhanced cytotoxicity in a synergistic manner with adjuvant chemotherapeutic agents in malignant peritoneal mesothelioma cells [32]. Similarly, Amini et al. have shown synergism between bromelain and NAC in gastrointestinal cancer cell lines [26].

Mucins are glycoproteins that are either of a secretory or transmembrane nature. They are found lining many ductal surfaces in various organs including the liver, kidney, pancreas and breast. They act as lubricants and as a barrier to exogenous molecules, however aberrant or over-expression of mucins have been implicated in a variety of cancers [33]. Mucins are glycoproteins that polymerise through glycosidic linkages (-o-) and disulfide linkages (s-s) to form either a polymer on the cellular membrane surface, or they are secreted so that they form an envelope on the cells [34]. Hence, it is conceivable how depolymerising agents such as bromelain and NAC may act on these polymeric molecules. Further, many of the other oncogenes that provide enhancement of survival through accelerated proliferation and metastasis also contain disulfide and glycosidic linkages [35] which are prone to the action of bromelain and NAC.

A common occurrence in soft tissue sarcoma is the presence of a "myxoid" matrix resulting in a tumour of gelatinous nature. Constituents of this matrix have been identified to include various mucopolysaccharides [36], however MUC1 and MUC4 have been identified in a few types of soft tissue sarcoma [37-41], there have been no studies on the role of mucins in the tumorigenicity of soft-tissue sarcomas. Both of these mucins have been linked to malignant transformation in various types of cancers including pancreatic, breast, ovarian, lung and gastrointestinal [33, 42, 43].

Materials and methods

Cell culture

The human sarcoma cell lines HT1080 (fibrosarcoma), SW872 (liposarcoma), VA-ES-BJ (epithelioid sarcoma) and SW982 (synovial sarcoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Incubation of cells occurred with 5% CO_2 at 37°C. The cell lines HT1080 and SW872 were maintained with RPMI 1640 media (Sigma, MO, USA), VA-ES-BJ and SW982 were maintained with Dulbecco's modified Eagle's medium (DMEM). All culture media received supplementation with 10% (v/v) foetal bovine serum (FBS; Wisent, Canada) and 1% (v/v) penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). DMEM was additionally supplemented with 1% (v/v) L-Glutamine as per the distributer's instructions. Routine passage of cell lines occurred at 70% confluency by washing with phosphate buffered saline (PBS) and trypsinizing for 3 minutes. Cells were separated from a trypsin-media suspension via centrifugation at 1200 rpm for 5 minutes at 21°C. Finally, with the addition of 0.06% trypan blue, cell count and viability was analysed with an automated cell counter (Thermo Fisher Scientific, California, and USA).

Preparation of Br and NAC

Bromelain and N-acetylcysteine (NAC) used in the study was acquired from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of bromelain and NAC were prepared at concentrations of 10 mg/mL and 100 mM respectively. Following pH adjustments to 7.0, solutions were filtered through a sterile cap. Drugs were stored frozen at -20°C.

Sulforhodamine B assay

Cell viability following treatment was determined through the Sulforhodamine B (SRB) assay. Following trypsinization, cells were seeded in a 96-well plate at densities of 3500-4000 cells/well and incubated for 24 hours to gain confluency. The plates were then treated for 72 hours with desired concentrations of bromelain and NAC as both single agents and in combination. Cells were then fixed with 10% (w/v) trichloroacetic acid at 4°C for 30 minutes, washed four times with water and air-dried. Sulforhodamine B dye of 0.4% (w/v)

Periodic Acid-Schiff (PAS) staining

Periodic Acid-Schiff (PAS) staining is a technique which allows for the nonspecific staining of mucins and mucopolysaccharides. PAS staining was used on the cell lines HT1080, SW872 and VA-ES-BJ to examine potential mucin depletion and disruption of the extracellular matrix. Following trypsinization, cells were seeded onto coverslips at a density of 175,000-200,000 cells/well in a 6-well plate. Confluency was established for 24 hours and cells were then treated with Bromelain and NAC for 48 hours. Upon completion, cells were fixed with 4% paraformaldehyde for 10 minutes and stained with periodic acid solution (Sigma-Aldrich, St. Louis, MO, USA) for 5 minutes. This was following by staining with Schiff's reagent (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes and finally counterstaining with hematoxylin solution (Sigma-Aldrich, St. Louis, MO, USA) for 90 seconds. The coverslips were then mounted onto slides and examined using the Leica DMLB microscope, DC200 digital imaging system (Leica Microsystems, Wetzlar, Germany).

Immunocytochemistry

Following trypsinization, cells were seeded onto sterile coverslips in a 6-well plate at a density of 175,000-200,000 cells/well and incubated for 24 hours. Plates were then treated with Bromelain and NAC as single agents and combination for 48 hours. Fixing of cells was achieved with 4% (v/v) paraformaldehyde for 10 minutes followed by permealisation with 0.4% (v/v) Tween-20 for 20 minutes. Protein blocking was conducted with 1% BSA for 1 hour. The primary antibodies MUC-1 and MUC-4 (Abcam, Cambridge, MA, USA) were added a

concentration of 1:200 and incubated for 24 hours. The secondary antibody chicken-anti-mouse Alexa 488 antibody (Abcam, Cambridge, MA, USA) was added at a concentration of 1:250 for 1 hour. Cells were then counter-stained with Hoechst (Thermo Fisher Scientific, California, and USA). For 5 minutes at a concentration of 1:200. Visualisation of cells was achieved using the Olympus IX71 laser scanning confocal microscope (Olympus, Center Valley, PA, USA). Images were taken and overlayed through the Zen program (Carl Zeiss, Cambridge, UK).

Western blotting

To investigate potential mechanisms which treatment may induce cellular death, Western blotting was used. A panel of proteins previously implicated in bromelain and NAC therapy [26] was chosen and included markers of apoptosis, cell cycle and autophagy. The following protocol was used. Following trypsinization, 1.5-2 million cells were seeded into 75 cm^2 flasks and incubated for 24 hours. Flasks were then treated with Bromelain and NAC as both single agents and in combination. After 48 hours, a lysis buffer was added for 5 minutes and cells were removed from the flask surface. Protein concentrations were calculated using the BioRad protein assay (Bio-Rad, Hercules, CA, USA). Protein separation was achieved through sodium dodecyl sulphate-polyacrylamide gel electrophoresis preceding transference onto a polyvinyl fluoride membrane (Milliposre, Billerica, MA, USA). Incubation with primary antibodies (Cell Signalling, QLD, and Australia) occurred at 4°C overnight followed by incubation with respective horseradish peroxidase-conjugated secondary antibodies (Cell Signalling, QLD, and Australia). The GAPDH protein was used to represent a loading control via the anti-GAPDH antibody (Sigma-Aldrich, St. Louis, MO, USA). Visualisation of the antigen-antibody interaction was achieved using the Image Quant LAS 4000 Biomolecular imager and Image Quant software (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Statistical analysis

The SRB data was analysed using Prism GraphPad 7 (GraphPad Software Inc., San Diego, CA, USA). Dunnett's test was used to statistically compare treated and control groups in a multiple comparison anova. A p value of < 0.05 was considered to be of statistical significance. The IC50, the concentration at which there is a 50% cell death rate was also calculated using a non-linear regression of the log (dose)-response curve. Significance represented in these figures has been calculated based on technical repeats (n = 4 or 8). The combination index (CI) of combined drug treatment was calculated using the median-effect principle on the CompuSyn software (ComboSyn Incorporated., Paramus, NJ, USA) where a $CI < 0.9$, $0.9 < CI < 1.1$, and $CI > 1.1$ indicated a synergistic, additive and antagonistic effect of the drug treatments, respectively [44]. The calculation of CI was conducted using all technical and independent repeats (n = 4 or 8, n = 3, respectively).

The densitometry of Western blots bands was measured using FIJI (Fiji Is Just ImageJ), a version of the ImageJ software [45] and normalisation was achieved using GAPDH.

Results

Bromelain and NAC as single agents exerted antiproliferative effects on all cell lines

Antiproliferative effects after 72-hour treatment with bromelain (0-100 μg/mL) and NAC (0-100 mM) was assessed using the sulforhodamine B assay. Bromelain inhibited cell proliferation in all cell lines. This result was shown to be significant with p values in HT1080 (P = 0.0004 for 0.78125 μg/mL, and < 0.0001 for all other concentrations), SW872 (P = 0.007 and 0.0004 for 1.5625 μg/mL and 3.125 μg/mL, respectively and < 0.0001 for all other concentrations), VA-ES-BJ ($P < 0.0001$ for all concentrations including and above 6.25 μg/mL) and SW982 (P = 0.0280 for 0.78125 ug/mL, and $P < 0.0001$ for all other concentrations). Similarly, NAC also significantly inhibited cell proliferation of HT1080 (P = 0.0111 for 0.78125 mM, and < 0.0001 for all other concentrations), SW872 ($P < 0.0001$ for all concentrations), VA-ES-BJ ($P < 0.0001$ for all concentrations including and above 6.25 mM) and SW982 (P = 0.0446 for 1.5625 mM, and $P < 0.0001$ for all other concentrations).

The antiproliferative effects of bromelain and NAC were found to be dose dependent for all cell lines with the IC50s as shown in **Figure 1**. For cells treated with bromelain, all cell lines

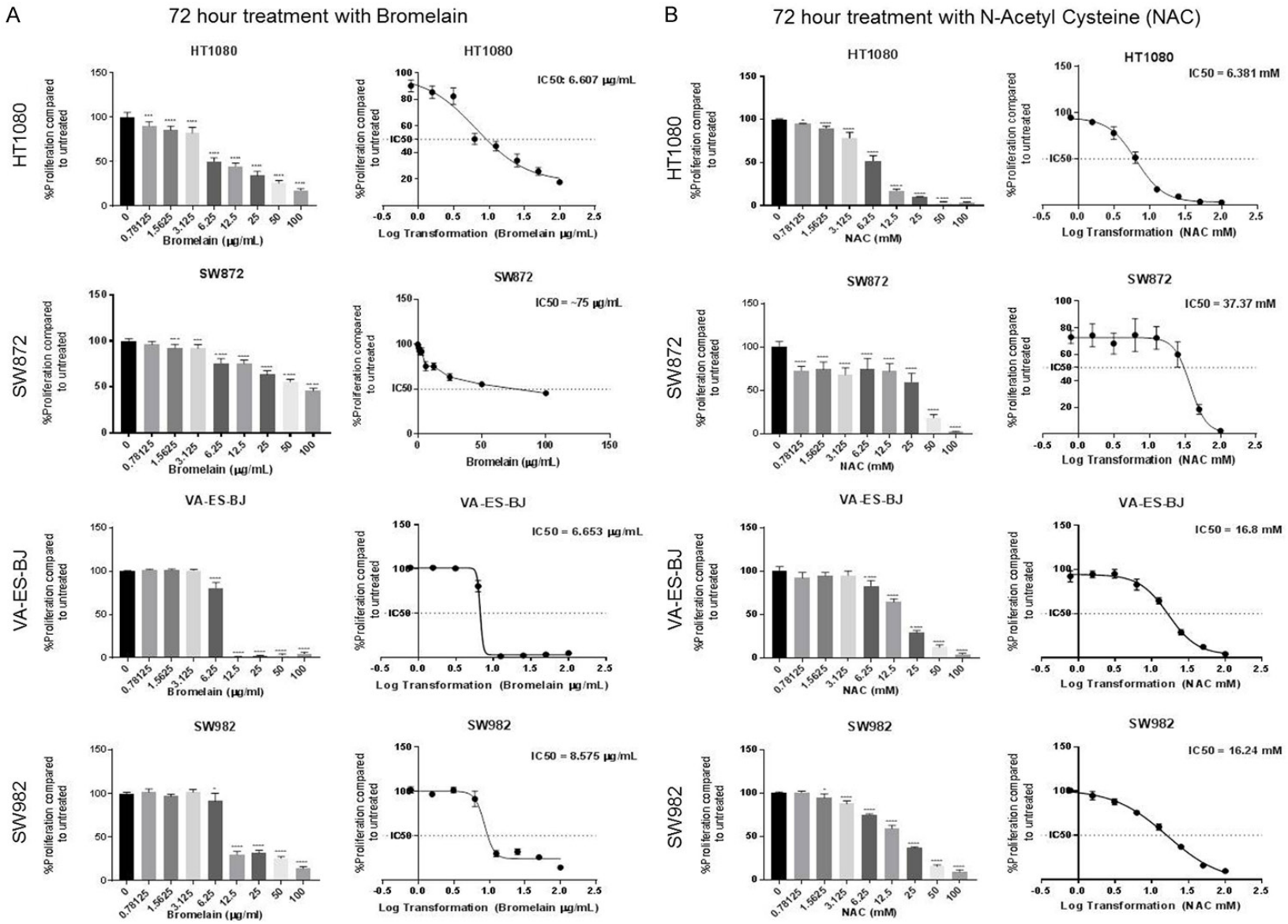

Figure 1. A, B. The results of the Sulforhodamine-B (SRB) assay of 72-hour treatment with Bromelain or N-Acetylcysteine (NAC). Left graph represents the mean proliferation of cells (n = 8) compared to untreated control. Significance is denoted by (*) P = 0.01 to 0.05, (**) P = 0.01 to 0.001, (***) P = 0.001 to 0.0001 and (****) P < 0.001. Right graph represents the log (dose)-response curve (with the exception of SW872 treated with NAC which is presented as a log-dose curve). The half maximal inhibitory concentration (IC50) was calculated using a non-linear regression using Prism Graph pad 7.

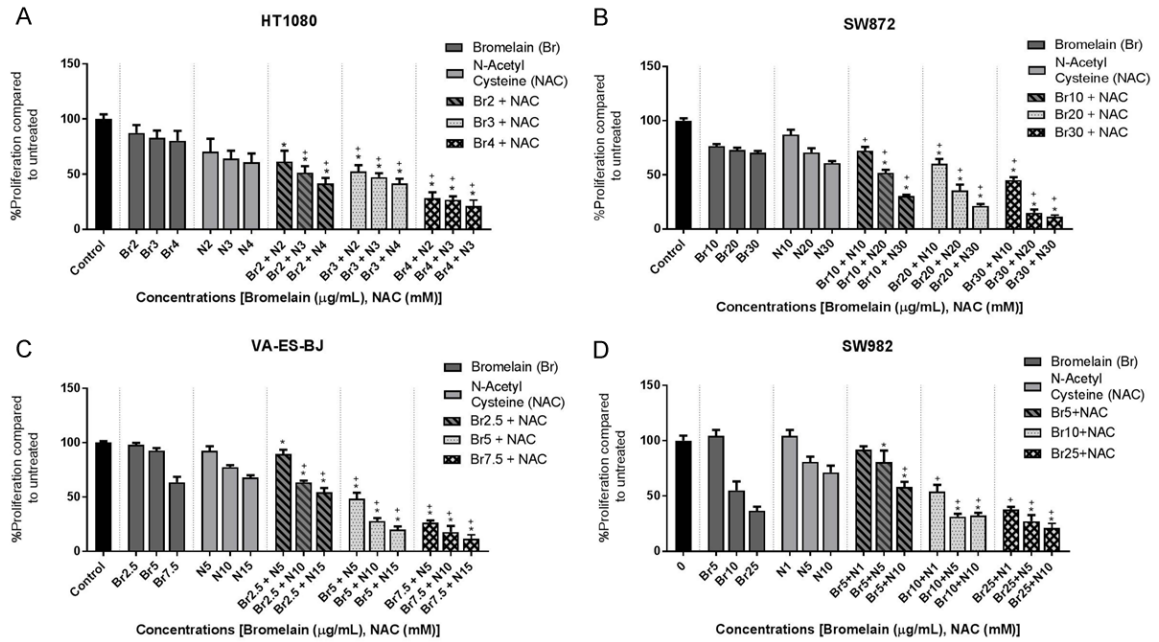

Figure 2. Graphs represent mean cell proliferation (n = 4) after 72-hour with Bromelain and NAC as both single agents and in combination. In combination therapy, additional growth inhibitory effects are seen in all four cell lines in a dose dependent fashion. The significance of combination is denoted by (*) respective to bromelain controls and (+) respective to NAC controls. A *p*-value of < 0.05 was considered a significant.

had IC50s < 10 μg/mL except SW872 which was least responsive with a significantly higher IC50 at approximately 75 μg/mL. This was also observed in NAC with the IC50s of all cell lines being < 17 mM with the exception of SW872 (IC50 = 37.37) thus making SW872 the least sensitive to both drugs. HT1080 was found to be the most responsive to both bromelain and NAC.

The growth inhibitory effects of bromelain and NAC are enhanced in combination therapy in a synergistic and dose-dependent fashion

Following single agent treatment, we studied the effects of combination therapy using three different concentrations of bromelain with three different concentrations of NAC. The results of these experiments (**Figure 2**) show that cytotoxic effects of Bromelain and NAC are amplified in combination therapy compared to single agent therapy. These inhibitory effects were demonstrated in a dose dependent manner with level of inhibition proportionate to drug dose.

Next, using the median-effect principle, the combination index (CI) was calculated for each drug combination (**Table 1**). Synergy is interpreted as CI < 0.9. A CI > 0.9 but < 1.1 is interpreted as additive effect and CI > 1.1 indicates antagonism. In HT1080 cells, drug synergy was observed in every single combination with increasing synergy as both bromelain and NAC concentrations increased. Similarly, a dose-dependent increase in synergy was seen in VA-ES-BJ cells with all combinations over Bromelain 5 ug/mL with NAC 10 mM showing increasing synergy whereas concentrations below this combination had antagonistic effects. Similarly, in SW982 synergism was present in all combinations of Bromelain 10 ug/mL and Bromelain 25 ug/mL whilst antagonism occurred at lower concentrations. The weakest drug synergy was seen with the cell line SW872 where synergy was only present in the combination of Bromelain 30 ug/mL with NAC 20 mM and NAC 20 mM. The combinations Bromelain 20 ug/mL and Bromelain 30 ug/mL with NAC 20 mM and NAC 30 mM, respectively, exerted additive effects. Drug antagonism was seen in all other concentrations. The trend of synergy over increasing concentrations of Bromelain and NAC is seen in **Figure 3** and indicates a strengthening of synergy in higher drug concentrations.

Table 1. Summary of combination index (CI) for various combinations of bromalin and NAC

HT1080			SW872			VA-ES-BJ			SW982		
Br (μg/mL)	*Nac (mM)*	*CI*	*Br (μg/mL)*	*Nac (mM)*	*CI*	*Br (μg/mL)*	*Nac (mM)*	*CI*	*Br (μg/mL)*	*Nac (mM)*	*CI*
2.0	2.0	0.90*	10	10	5.88	2.5	5.0	4.50	5.0	1.0	2.86
2.0	3.0	0.79*	10	20	6.64	2.5	10	1.47	5.0	5.0	1.40
2.0	4.0	0.86*	10	30	1.44	2.5	15	2.00	5.0	10	1.10
3.0	2.0	0.80*	20	10	2.32	5.0	5.0	1.15	10	1.0	0.52*
3.0	3.0	0.77*	20	20	2.38	5.0	10	0.66*	10	5.0	0.40*
3.0	4.0	0.70*	20	30	0.99	5.0	15	0.63*	10	10	0.61*
4.0	2.0	0.38*	30	10	0.95	7.5	5.0	0.64*	25	1.0	0.76*
4.0	3.0	0.27*	30	20	0.56*	7.5	10	0.53*	25	5.0	0.50*
4.0	4.0	0.22*	30	30	0.34*	7.5	15	0.43*	25	10	0.55*

Synergy is denoted by (*). Synergy between drugs increases as CI becomes closer to 0.

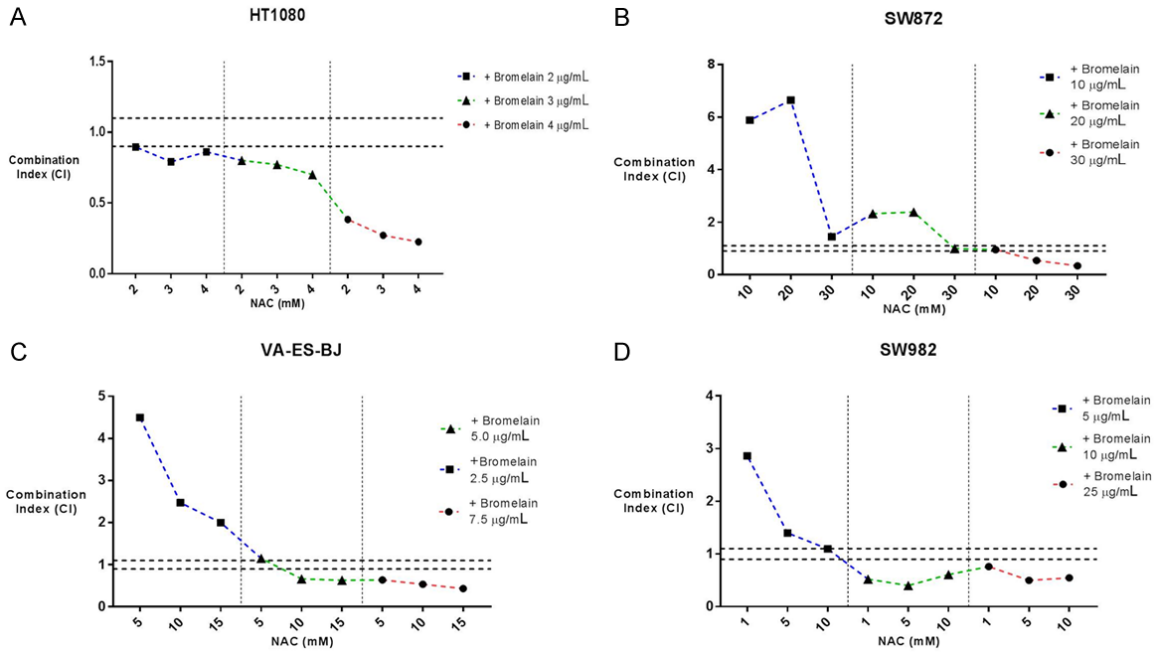

Figure 3. The combination index (CI) was calculated using the median-effect calculation and indicates the synergy, additive and antagonistic effects of multiple drugs. A CI < 0.9 indicates synergy where as 0.9 < CI < 1.1 indicates additivity and CI > 1.1 representing drug antagonism. The dashed-lines on the Y-axis represent the values CI = 0.9 and CI = 1.1. All SRB results (n = 3 independent repeats) were included in the calculation of the CI.

PAS staining indicated mucin depletion at varying degrees in cells treated with bromelain and NAC

PAS staining was conducted on untreated control cells and treated cells in the cell lines HT1080, SW872 and VA-ES-BJ (**Figure 4**). PAS staining significantly decreased in SW872 and HT1080 with the greatest reduction in intensity seen in the combined treated group. A slight decrease in amount of staining in the cytoplasm is seen in treated VA-ES-BJ cells although there is no reduction seen between the treatment groups. Furthermore, the staining reveals a change in cell morphology, especially in SW872 cells. As PAS stains the basement membrane, glycogen, some mucins [46] and other various mucopolysaccharides [47] this indicates a nonspecific disruption of such substances to a greater extent in HT1080, and SW872 but to a lesser degree in VA-ES-BJ.

Immunofluorescence of transmembrane mucins were depleted in some treated cell lines

Immunocytochemistry was used to determine mucin profiles of HT1080, SW872 and VA-ES-BJ. **Figure 5A-C** shows the immunofluorescence

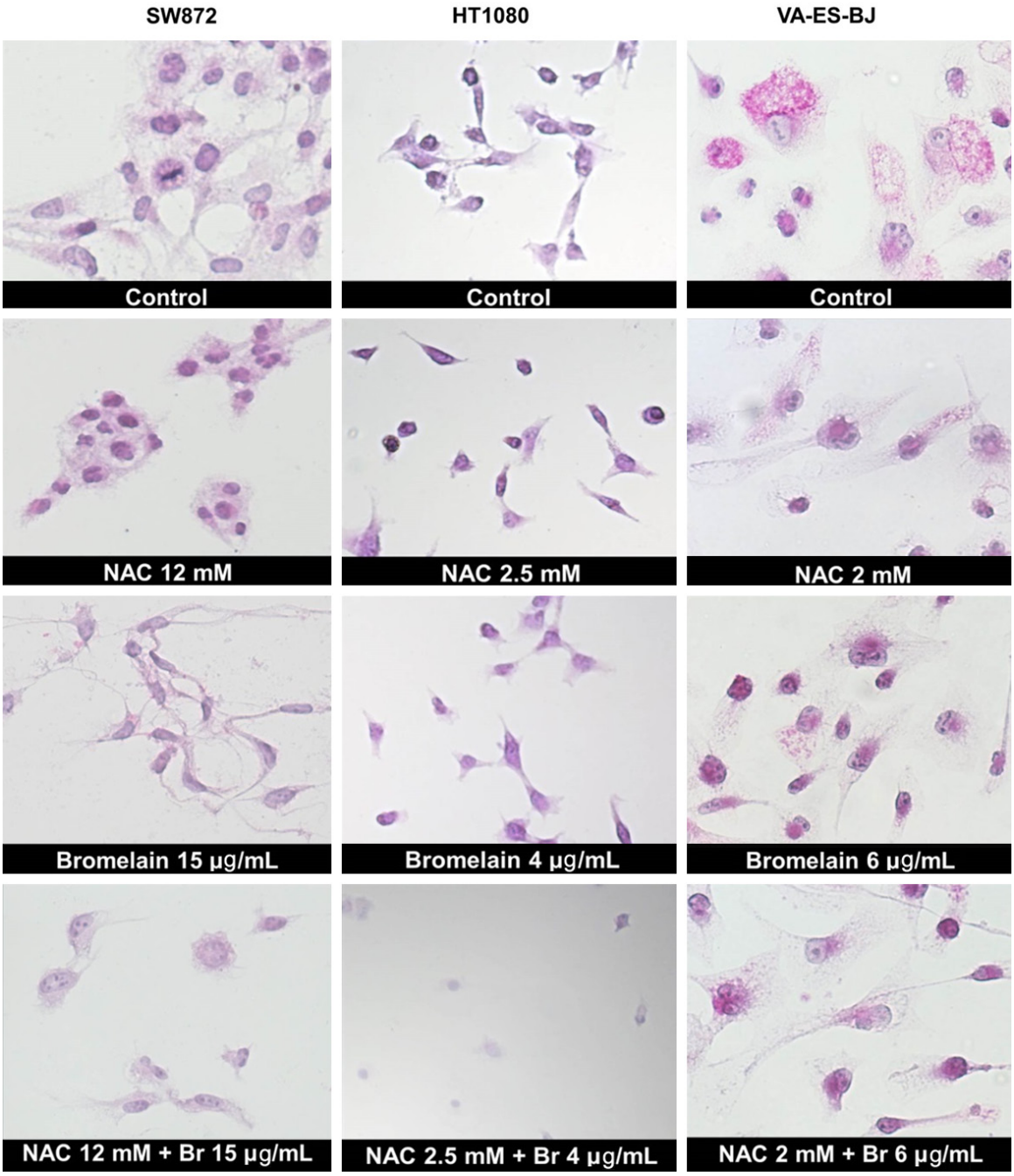

Figure 4. Periodic Acid-Schiff (PAS) staining of cells after 72-hour treatment. Positive staining of mucins and mucopolysaccharides is represented in pink and the nucleus is stained purple. Staining is decreased.

of MUC1 and MUC4 in untreated (control) and treated cells (single agent bromelain or NAC, and a combination of both). In HT1080 cells, there was a strong expression of MUC1 and MUC4 localised within the cytoplasm. Cytoplasmic expression decreased in treated cells with greatest reduction seen in MUC1 in combination treatment with bromelain and NAC. Similarly, MUC1 expression in VA-ES-BJ was also significantly decreased in cells treated with a combination of bromelain and NAC. In SW872, the fluorescence of treated cells and untreated cells did not differ.

Pathways of cell death differed between subtypes of soft tissue sarcoma

To investigate the mechanisms of inhibition and cell death, Western blot analysis was con-

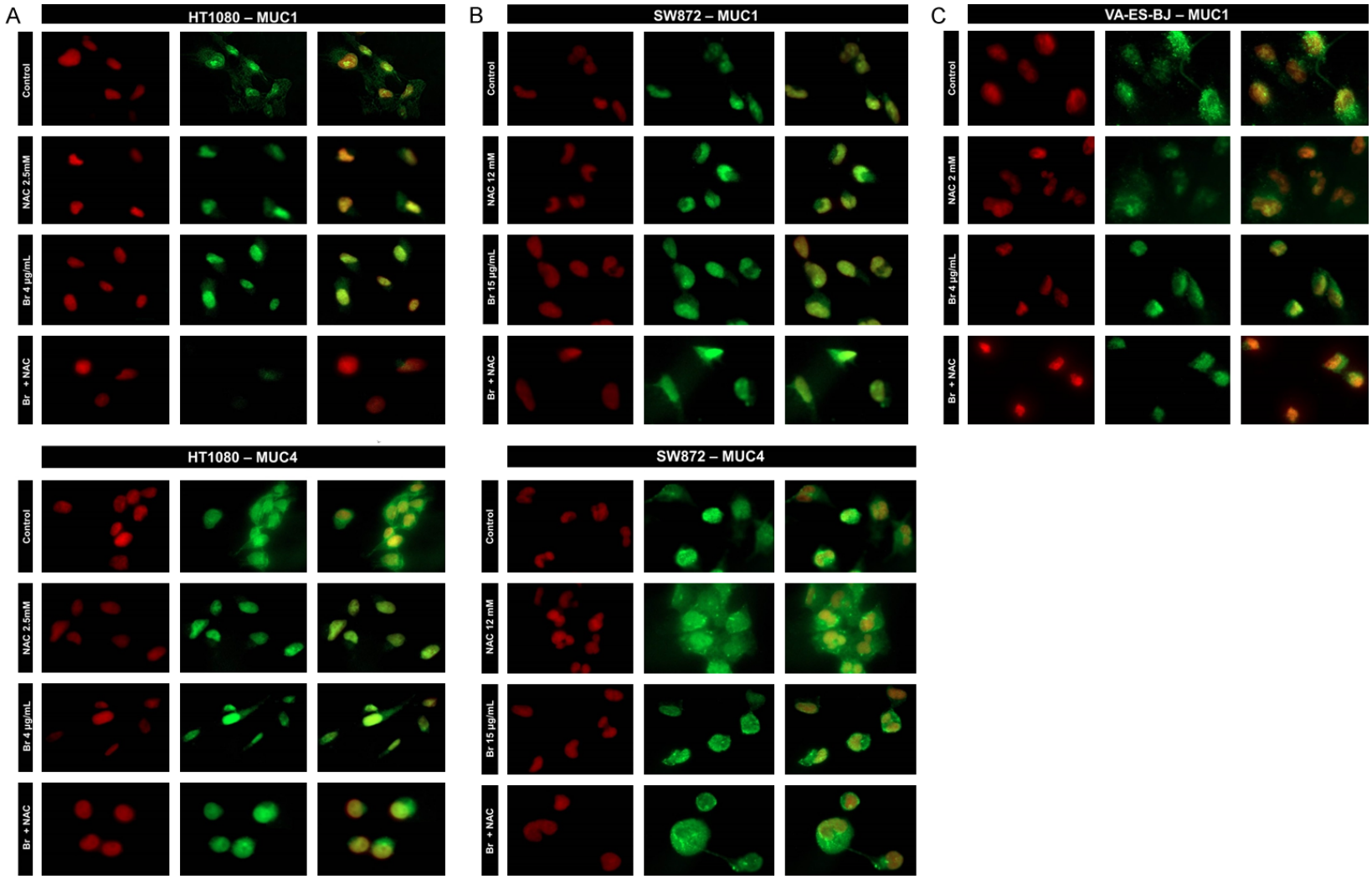

Figure 5. A-C. Immunofluorescence of soft tissue sarcoma cells following 48 hours of treatment with Bromelain, NAC and Bromelain plus NAC. Expression of MUC1 and MUC4 was viewed under a laser scanning confocal microscope with red and green fluorescence corresponding to nucleus and MUC staining, respectively. This represents pilot results (n = 1).

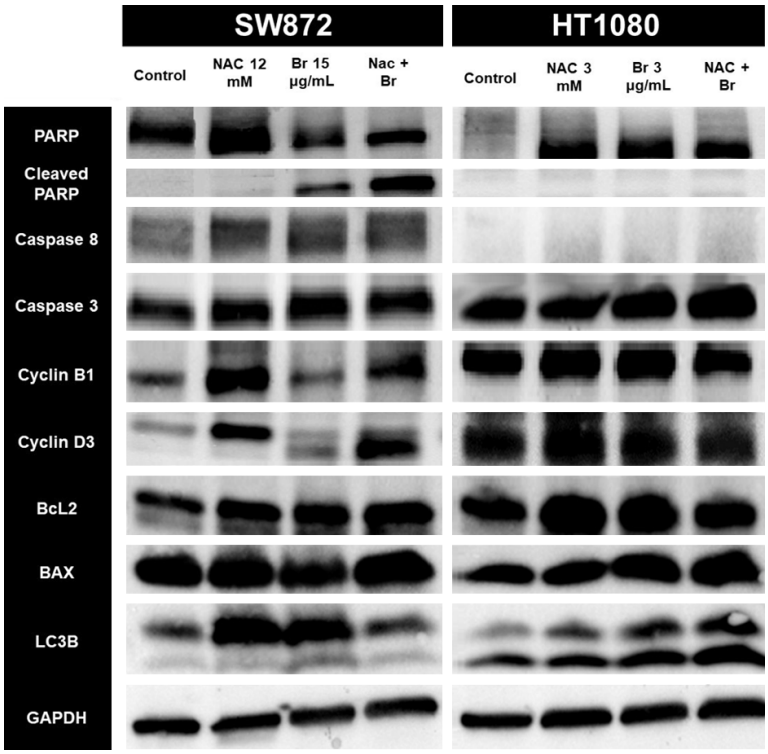

Figure 6. Western blot analysis of the expression of various proteins associated with cell cycle, apoptosis and autophagy. Analysis is conducted on a negative control, and cells treated for 48 hours with bromelain, nac and a combination of both. This represents pilot results (n = 1).

ducted on cells following 48 hours treatment with Bromelain, NAC or their combinations. Untreated cells were used as a negative control. As seen in **Figure 6**, the expression of proteins greatly varied between the fibrosarcoma HT1080 and liposarcoma SW872 cell lines. A summary of the band intensities relative to the control is presented in **Table 2**.

In SW872, there was an increase in cleaved parp in cells treated in bromelain alone, and increased even more significantly in treatment with bromelain and NAC. Caspase 8 and caspase 3 increased in treated cells compared to the control but did not vary greatly between treatment groups. The expression of cyclin B1 increased in both the NAC and combination groups. Similarly a significantly increase in cyclin D3 was seen in groups treated with NAC. BcL-2 increased in all treatment groups with a slightly greater increased in combination group. BAX decreased in single agent treatments but increased in the combination group. Finally LC3B-I (uncleaved top band) was greatly increased in single agent treatments, with slight increases in LC3BII (cleaved bottom band) in all groups. The increase of the apoptotic proteins (cleaved parp, caspase 8, caspase 3) as well as the autophagy protein (LC3B) indicates that Bromelain and NAC induce cell death via different pathways including apoptosis and autophagy.

In HT1080 cells, uncleaved PARP expression increased greatly however was negative for cleaved parp in all treatment groups. A significant change was seen in the expression of the autophagy marker LC3B, with an increase of both uncleaved (LC31) and cleaved (LC3B-II) bands. This expression increased in the bromelain group compared to NAC, and was greatest in the combination group. BAX, caspase 3, cyclin B1, cyclin D3 expression did not vary greatly between treatment groups. This indicates that in HT1080, autophagy was the primary pathway to cell death, however slight increases in caspase 3 and PARP may indicate that apoptosis is also stimulated.

Discussion

In the present study, we have investigated the effects of Bromelain and NAC in the three subtypes of sarcoma which has been shown to express mucins: fibrosarcoma (HT1080), biphasic synovial sarcoma (SW982), and epithelioid sarcoma (VA-ES-BJ). In addition, we have also included a non-mucin expressing liposarcoma (SW872) in order to compare the effects of treatment on mucin-expressing and non-mucin expressing soft tissue sarcomas.

All cell lines responded to single agent treatment with bromelain and NAC in a dose-dependent manner. The IC50s for bromelain were significantly lower in the mucin-expressing cell lines. Similarly, the IC50 for NAC was also lower in these cell lines although to a lesser extent. Furthermore, the pattern of inhibition in combi-

Table 2. Densitometry calculation of Western blot markers as compared to untreated cells

Treatment	SW872			HT1080		
	NAC 12 mM	*Br 15 µg/mL*	*Br + NAC*	*NAC 3 mM*	*Br 3 µg/mL*	*Br + NAC*
Parp	1.17	0.67	0.71	2.11	2.29	1.78
Cleaved Parp	0.67	3.72	7.21	N/A	N/A	N/A
Caspase 8	1.53	1.27	1.41	N/A	N/A	N/A
Caspase 3	1.41	1.40	1.35	1.08	1.38	1.24
Cyclin B1	2.25	1.09	1.84	0.83	0.98	0.68
Cyclin D3	4.03	2.50	6.72	1.04	0.91	0.87
BcL2	1.17	1.17	1.50	2.05	2.05	0.97
BAX	0.81	0.81	1.27	1.08	1.29	1.09
LC3BI	1.68	2.09	1.20	1.43	2.22	2.36
LC3BII	1.27	3.57	2.01	1.11	1.31	1.49

Calculations have been normalised to the loading control GAPDH.

nation therapy was predominantly synergy in these cell lines as opposed to the non-mucin expressing SW872 where synergy was only achieved at very high concentrations of the agents. The significant difference in bromelain and NAC requirement between the two types of cells (mucin expressing vs. non-mucin expressing) may indicate that the primary target of treatment was glycoproteins such as mucins. Despite this, combination treatment was still effective in SW872 which may indicate that other oncoproteins are also prone to the agents.

The results of PAS staining show a decrease in the intensity and amount of staining in HT1080, SW872 and VA-ES-BJ. This result may indicate that bromelain and NAC exert effects not only on mucins but also on other mucopolysaccharides present within the extracellular matrix. It is known that mucopolysaccharides are present within the myxoid matrix of sarcomas [36] and there was a decrease in staining intensity in SW872 cells.

Analysis of transmembrane mucin profile by immunocytochemistry showed an overall MUC1 and MUC4 fluorescence decrease in treated HT1080 and VA-ES-BJ sarcoma cells but not in SW872 indicating the absence of these two mucins in the latter case. These findings support the hypothesis that bromelain and NAC primarily target mucins resulting in their depletion. In addition, these findings also align with the current knowledge of mucins in sarcoma indicating the presence of MUC1 and MUC4 in fibrosarcomas [37-40, 48, 49] and MUC1 in epithelioid sarcomas [41]. Liposarcomas have not been reported to express mucins, despite a common presentation with "myxoid stroma" [50].

In order to compare the pathways of cell death in mucin-expressing and non-mucin expressing cell lines, Western blot analysis was conducted on HT1080 and SW872 cells. In SW872, an increase in apoptotic markers including cleaved PARP, caspase 3 and caspase 8 indicated that apoptosis was the predominant mechanism of cell death [51, 52]. Autophagy was also induced in these cells by Bromelain or NAC as single agents. Hence cell death was inducted by two separate pathways. In HT1080, although there was no elevation of caspase 8 or cleaved PARP, a noticeable elevation of caspase 3 may indicate that apoptosis was taking place. However the major pathway of cell death in this cell line was by autophagy with a dramatic increase in LC3B-II [53].

Cyclin B1 is a key molecule for G2-M phase transition during the cell cycle. It has been identified to be overexpressed in various cancers [54]. Similarly, Cyclin D3 have been identified to be overexpressed in breast cancer [55]. The increased expression of these cyclins in treatment group containing NAC may indicate that NAC facilitates cell cycle progression in SW872 although the mechanisms are unclear. Finally, the increase in BcL-2 in treated cells in both cell lines may indicate an inhibition on translocation of BAX [56], which is also increased in combined treatment in both cell lines. BAX facilitates apoptosis through mitrochondrial stress and membrane permeability [57]. Hence, this may indicate that the cells may have gained

resistance to BAX-induced apoptosis in SW872 and HT1080.

In summary, beside mucin depletion, Western blot analysis reveals that Bromelain and NAC may also work by an independent mechanism which promotes apoptosis and autophagy in cells. NAC may also have a protective role in stimulating cell cycle progression. The exact mechanisms by which Br and NAC induced apoptosis and autophagy in these cell lines needs further investigation. However, it can be envisaged that these agents may primarily be disrupting the -o- and s-s linkages found in the oncopoteins and hence disabling their vital tumorigenic role.

Remarkably, the current preliminary study indicates that bromelain and NAC as single agents or in combination may potentially be developed into therapeutic agents for the treatment of soft tissue sarcoma. Unlike many of the current chemo-agents that carry inherent adverse events owing to their high clinical dosage, Bromelain and NAC are agents with a much higher safety profile that can be regulated for either systemic or loco-regional use. Furthermore, they may also act as an add-on therapy to the current chemotherapeutic agents that are used for the treatment of sarcoma. Since, bromelain and NAC have already been shown to act synergistically with many chemotherapeutic agents for the treatment of a number of cancers (unpublished data), they may likewise act in synergy with drugs that are currently used for the treatment of sarcoma. This needs further study since some chemotherapeutic agents are not compatible with NAC as it is an antoxidant, in particular with those agents that are dependent on reactive radicals to induce cytotoxicity. Finally, the current findings need further validation in animal models before proceeding into clinical evaluation.

Disclosure of conflict of interest

Professor DL Morris is the co-inventor and assignee of the intellectual property 2017-0045 surrounding the development of bromelain and acetylcysteine in cancer, and is co-owner and director of the sponsor company, Mucpharm Pty Ltd commericalising this licence. Dr J. Akhter and Dr K. Pillai are employed by Mucpharm Pty Ltd and co-inventors of the intellectual property. Dr AH Mekkawy and Ms Samina Badar are employed by Mucpharm Pty Ltd. Ms Lillian Dong is a research student through UNSW. Professor DL Morris and the study team guarantees that the conduct of this study complies with all ethics, national and government guidelines and regulations.

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