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Original Article

Addition of bromelain and acetylcysteine to gemcitabine potentiates tumor inhibition *in vivo* in human colon cancer cell line LS174T

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Abstract: The combinations of Bromelain and Acetylcysteine (BromAc®) with cytotoxics such as Gemcitabine, 5-Fluorouracil or Oxaliplatin have shown a dramatic reduction in IC50 values in a variety of cancers, including colon cancer, suggesting the possibility of effective treatment without undesired side effects. In the current study, we investigated whether a similar effect is present *in vivo* using the colorectal cell line LS174T. Animals after acclimatization were randomized and allocated equally in the groups for the different studies (safety, dose-escalation, and efficacy). Drugs were delivered by the intraperitoneal route and animals were monitored for wellbeing. Separately, an efficacy study was conducted with intraperitoneal drug delivery after intraperitoneal tumor induction. At the termination of the experiment, tumors and other tissues were collected for evaluation. BromAc® was safe when delivered intraperitoneally in a rat model at the concentrations used. Subsequent investigations of these adjuvants in combination with Gemcitabine, Oxaliplatin, and 5-Fluorouracil in mice were also proven to be safe. Preliminary efficacy studies with Oxaliplatin and 5-Fluorouracil on tumor growth (LS174T) were negative. Gemcitabine was assessed with BromAc® showing an almost 71% tumor inhibition compared to controls. This *in vivo* study indicates that Gemcitabine at 2 mg/kg in combination with BromAc® 3 mg/300 mg/Kg was effective and safe, supporting its potential for future clinical application.

Keywords: Colon cancer, bromelain, acetylcysteine, bromac®, gemcitabine, *in vivo*

Introduction

Colon cancer was the fourth most commonly diagnosed cancer worldwide in 2018 with over 1 million cases, accounting for 6% of all cancer diagnoses [1]. Moreover, it caused over 500,000 deaths in 2018, accounting for approximately 6% of all cancer deaths [1]. Staging through TNM or Duke's classification is used in conjunction with various other factors including grading, presence of lymphatic, venous, or peritoneal spread, tumor genetic characteristics as well as tumor markers to guide prognosis and arrive at a management plan [2]. Prognosis varies considerably with TNM staging, with localized tumor corresponding to a 5-year-survival of 93%, while the presence of distant metastases results in a 5-year-survival of 8% [3]. Peritoneal dissemination is present in approximately 5-10% of all colon cancer patients and 20-50% in patients with recurrent disease in the peritoneum following primary surgery [4]. The peritoneal spread of colon cancer results in high morbidity with patients suffering from internal obstruction, ascites, cachexia, colon perforation, and even death.

Surgical resection represents the main treatment for colorectal cancer (CRC), however, not all patients are candidates for curative-intent surgery, due to factors such as high volume peritoneal spread, or the presence and severity of comorbidities preventing surgery. Patients who are initially deemed unresectable may convert to resectability following tumor regression through chemotherapy [5, 6], although evidence of peritoneal response to systemic treatments is limited. Hyperthermic intraperitoneal chemotherapy (HIPEC) together with complete

surgical removal of disease, known as peritonectomy, offers the only choice of care for long term survival of CRC with peritoneal dissemination This, however, is only effective in selected patients with a Peritoneal Cancer Index (PCI) ≤ 15 and is a major procedure [7]. Patients with significant disease burden have limited treatment options and similarly, those that recur after curative-intent surgery may not be suitable for repeat surgical procedures. Standard chemotherapeutic agents include fluoropyrimidines (5-Fluorouracil "5-FU", capecitabine), topoisomerase inhibitors (Irinotecan), platinum compounds (Oxaliplatin "OXAL"), nucleoside antimetabolites (Gemcitabine "GEM"), and biologics (Bevacizumab, Cetuximab, Panitumumab) [8-10]. These agents are often used in combination in various treatment regimes, guided by considerations of efficacy and toxicities. While this wide range of therapies has been proven to provide benefit, when considering the low survival rates of certain subgroups of patients, there is still a clear need for more effective therapies. Second-line systemic treatment options are of limited utility in peritoneal diseases.

We developed BromAc®, Bromelain in combination with Acetylcysteine, to treat a rare mucinous tumor, pseudomyxoma peritonei. Bromelain (Brom) is an enzyme extract from the fruit or the stem of the pineapple plant *(Ananas comosus)* that contains a mixture of proteases, carbohydrates, phosphatases, glycoprotein, etc. Acetylcysteine (Ac) is an antioxidant. BromAc® has been shown to have a remarkable effect of solubilizing mucinous tumor in pseudomyxoma peritonei [11] and this has been replicated in animal experiments [12, 13] and a clinical trial [14]. BromAc® has shown anticancer properties that are equivalent to GEM and other cytotoxics on several human cancer cell lines [15-19] where the effective dose of the cytotoxics can be dramatically reduced. In the case of GEM, an almost 95% reduction can be achieved [20], potentially reducing the side effects experienced by patients due to chemotherapy toxicity. Bromelain can lyse glycosidic bonds [21]. Acetylcysteine is a potent reducing agent. Hence, it can reduce disulfide bonds in proteins, disrupting their ligand bonding, and altering their structures [22]. The combination of Bromelain and Acetylcysteine has been shown to affect the tumor's biological functions as well as oncogenes which results in cytotoxicity [23]. In light of the potential advantages provided by the addition of BromAc®, we investigated the anticancer effect of OXAL, GEM, and 5-FU in colorectal tumor cells LS174T using *in vivo* models to assess the findings for future clinical utility.

Materials and methods

Cell lines

The human colon cancer cell line LS174T used in this study was originally obtained from the American Type Culture Collection and maintained according to the supplier instructions.

Drug preparation

Bromelain was purchased from Challenge Bioproducts Co, Taiwan, China. Acetylcysteine was purchased from Link Pharma, Australia. For treatment, the stock solutions were freshly made and filtered. Drugs were diluted with 0.9% NaCl according to the final treating concentrations required.

In vivo dose-escalation study

All the animal studies were conducted with ethical approval from the UNSW Animal Care and Ethics Committee. The animal dose-escalation toxicology study was designed to establish a safe dose for our prospective pre-clinical and clinical studies. Twenty-eight female Wistar rats (Animal Resources Center, WA, Australia) were randomly assigned into seven groups of four rats. Post-acclimatization, six groups of rats were treated intraperitoneally (IP) with Bromelain 3, 5, or 10 mg/kg and Ac 300, or 500 mg/kg. Control group received the vehicle (0.9% NaCl). Treatments were performed on alternate days for one week (3x in total). Animals were monitored for their health status, manifestations of toxicity, an allergic reaction, and capillary bleeding daily during the treatment week (week 1) and a fortnight post-treatment (weeks 2 and 3). After the completion of week 3, the experiment was terminated with euthanasia of all animals. Post-mortem, comparative observations of the peritoneal organs were performed and samples were collected for pathology.

In vivo safety study of combination therapy

The risks of complications of the combination therapy of BromAc® with either GEM, 5-FU, or

OXAL were not known. Besides, the signs and time course of the potential effects of these combination therapies were not defined. A pilot safety study was carried out before the commencement of a larger combination therapy efficacy study. Twenty-four, eight-week-old female BALB/c nude mice (Animal Resources Center, WA, Australia) were used in this safety study. Post-acclimatization, mice were randomly assigned to one of the four study groups (n = 6/group). Intraperitoneal treatment was continued for 4 weeks based on the treatment regimens (Supplementary Table 1). Bromelain 3 mg/kg and Acetylcysteine 300 mg/kg combinations were administered every other day for 28 days (3 times per week). Low doses of GEM (2 mg/kg), OXAL (2 mg/kg) and 5-FU (15 mg/kg) were administered once/week for 4 weeks. 0.9% NaCl was used as control and administered every other day. Regular monitoring of animals continued during the treatment period using standardized protocols. Upon completion of the treatment, animals were euthanized and internal viscera were collected.

In vivo efficacy study of combination therapy

This animal study was designed to evaluate the tumor growth inhibition of BromAc® in combination with GEM (2 or 5 mg/kg), OXAL (2 or 5 mg/kg), and 5-FU (15 or 30 mg/kg) using an *in vivo* model of colon cancer. Post-acclimatization (Day 8), Sixty-six mice (Animal Resources Center, WA, Australia) were inoculated IP with 2×10^6 log-phase growing LS174T cells in serum-free RPMI 1640. Before the commencement of the treatment, mice were randomly assigned to one of the study groups (Supplementary Table 2). IP treatments started ten days post-inoculation (Day 18). BromAc® was administered every other day (3x/week). GEM, OXAL, and 5-FU were administered once/week. 0.9% NaCl was used as control and administered every other day. Regular monitoring of animals continued during the treatment period using standardized protocols. Upon completion of the treatment (Day 32), animals were euthanized, internal viscera and tumors were excised, and tumors were weighed.

Histology and immunohistochemistry

Formalin-fixed, paraffin-embedded sections were stained using H&E standard techniques. For immunohistochemistry, BOND-III Automated IHC Stainer, Leica has been used. Sections were blocked for non-specific binding, followed by incubation with anti-human Ki67 (Cell Marque; Rabbit Monoclonal Anti-Human; Clone SP6; Cat# 275R-16; Dilution 1/200), incubated with biotinylated anti-rabbit immunoglobulins, treated with streptavidin peroxidase, and counter-stained with hematoxylin. The images were captured using a binocular light microscope with a digital camera.

Statistical analysis

Data were analyzed using GraphPad Prism version 8.0 (GraphPad Software, Inc.). All data were reported as the mean ± SEM. Qualitative variables were compared using the Student's *t*-test. Differences were considered statistically significant when $P < 0.05$. Differences in survival were tested using Kaplan-Meyer survival analysis and Dunnett's test.

Results

In vivo dose-escalation study showed no toxicity

The result from the dose-escalation study showed that IP administration of Bromelain 3, 5, or 10 mg/kg in addition to Acetylcysteine 300 or 500 mg/kg did not effect on apparent health of rats as indicated by the parameters of general wellbeing including indicators of pain and distress. Besides, there were similar trends in the increase of animal weight between control and treated groups. The results showed also that there were no apparent histological alterations by morphological assessment of tissues of the liver, kidney, spleen, intestine, and pancreas of rats between the BromAc® IP treated groups and controls using hematoxylin and eosin staining (**Figure 1**). The *in vivo* dose-escalation study showed no toxicity with 3x IP administration of Bromelain 3, 5, or 10 mg/kg in addition to Acetylcysteine 300 or 500 mg/kg in rats.

In vivo safety study of combination therapy

All animals survived until the end of the study (Day 32) except one animal from the BromAc® + 5-FU group (n = 1/6) (**Figure 2A**). This animal was culled on day 28 as it scored >3 points according to the animal safety monitoring sheet. Apart from this one animal, there

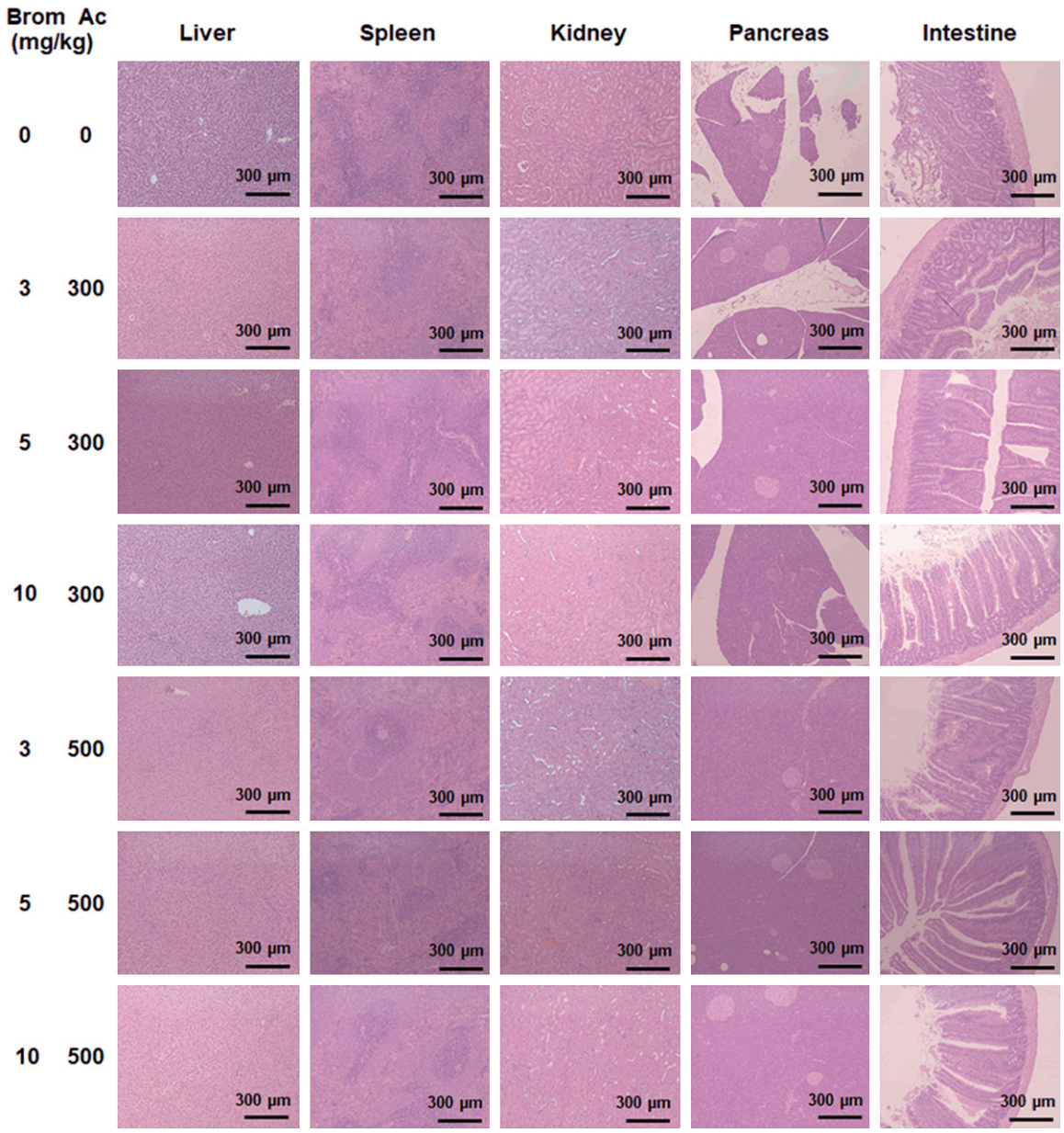

Figure 1. Histological images of liver, spleen, kidney, pancreas and intestine from control and, treated rats from the dose-escalation study. Tissues were Hematoxylin and eosin-stained (magnification, ×100; scale bar = 300 µm). The results showed no tissue toxicity after 3X IP administration with either Bromelain 3, 5, or 10 mg/kg in combination with Acetylcysteine 300 or 500 mg/kg in rats.

was no significant change in body weight through the treatment course between animals in the treatment and control groups (**Figure 2B**). There were also no drug-related toxicities in treated groups. Combination therapies did not affect the health scores of treated animals when compared to controls. After euthanasia on day 32, necropsies for abdominal organs were performed. No treatment-related gross findings including hemorrhage were observed in any of the treated animals.

A combination of Bromelain 3 mg/kg and Acetylcysteine 300 mg/kg together with low doses of GEM (2 mg/kg), OXAL (2 mg/kg), or 5-FU (15 mg/kg) were safe when administered IP in the tumor-bearing nude mice.

Combination therapy suppressed LS174T tumor growth

Initially, we used 3 animals per group to test our hypothesis (n = 3/group). Combination therapy

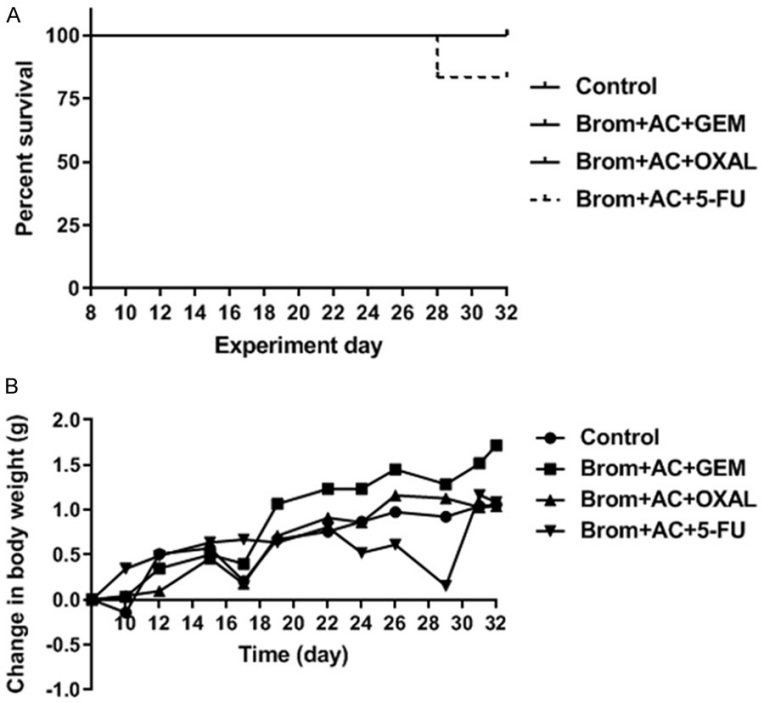

Figure 2. (A) Mean body weight fluctuation and (B) survival curve showing the safety of IP administration of Bromelain 3 mg/kg and Acetylcysteine 300 mg/kg with addition of low doses of Gemcitabine (2 mg/kg), Oxaliplatin (2 mg/kg) or 5-FU (15 mg/kg) in LS174T tumor-bearing mice.

of BromAc® in addition to either OXAL or 5-FU in this model of high tumor burden (10 days of tumor growth before the start of treatment) was not effective. There was no reduction in tumor weight in the treated group when compared to the control, hence, we discontinued our investigation using either OXAL or 5-FU. The subsequent study only involved BromAc® and GEM (n = 3/group).

All animals in the control and BromAc® in combination with either GEM 2 or 5 mg/Kg groups (Total: n = 6/group) survived until the end of the study (Day 32) (**Figure 3**). Animals that scored >3 points in the animal safety monitoring sheet were humanely culled. That is, two out of seven animals from (BromAc®) group were culled on Day 29. One animal out of seven from (GEM 2 mg/Kg) group was culled on Day 27 and another one was culled on Day 29. One out of seven animals from (GEM 5 mg/Kg) group was culled on Day 29. In contrast to the safety study where there was an increase of body weight over time, there was no significant loss in body weight in any animals in the treatment and control groups that exceeded 20% loss of body weight that need to be culled according to the animal ethics guidelines. This difference in body weight trend may be attributed to tumor growth which is supported by animals' body metabolism. After euthanasia on day 32, necropsies for abdominal organs were performed. No treatment-related abnormal gross findings were observed in any of the treated animals. Histological examination of the treated animals showed normal tissue morphology except in one animal in (GEM 5 mg/Kg) group where the liver sections in this animal showed focal necrosis (**Figure 4B**).

Tumor analysis data showed suppression of tumor weight in BromAc® in combination with either GEM 2 or 5 mg/Kg treated groups (**Figure 5A**). This finding is supported by the increase in tumor necrosis in the same groups (**Figure 5B**). Besides, a decrease was demonstrated in cell proliferation (Ki67; **Figure 5C**) in BromAc® in combination with either GEM 2 or 5 mg/Kg treated groups when compared to control, $P < 0.0001$.

A combination of Bromelain 3 mg/kg and Acetylcysteine 300 mg/kg together with GEM (2 mg/kg) was safe and effectively inhibited tumor growth when administered IP in nude mice bearing IP colon tumor. This combination showed a reduction of 71% of tumor volumes with $P \leq 0.05$ (**Table 1**). The combination index (CI) has been calculated using the reduction of tumor volumes [24]. For low dose GEM (2 mg/kg) in combination with BromAc (low dose), the CI is 0.08, whilst for high dose Gem (5 mg/kg) with BromAc, the combination index is 0.66, indicating that low dose GEM with BromAc has higher synergy.

Discussion

In vitro studies using three colon cancer cell lines (HT295M21, HT29 & LS174T) with

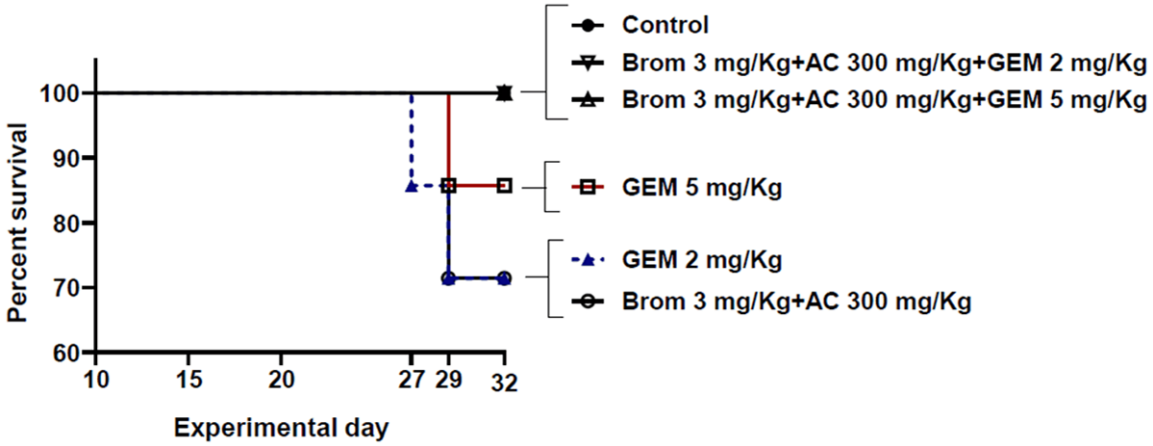

Figure 3. Kaplan-Meier survival curve of the LS174T tumor-bearing nude mice treated with either [0.9% NaCl alone], [Bromelain (3 mg/kg) + Acetylcysteine (300 mg/kg)], [Gemcitabine (2 mg/kg) alone], [Bromelain (3 mg/kg) + Acetylcysteine (300 mg/kg) + Gemcitabine (2 mg/kg)], [Gemcitabine (5 mg/kg) alone], or [Bromelain (3 mg/kg) + Acetylcysteine (300 mg/kg) + Gemcitabine (5 mg/kg)]. The survival of mice was recorded as the percentage of surviving animals on a given day.

Bromelain, Acetylcysteine, GEM, OXAL, 5-FU and their combinations (Supplementary Table 3) indicated that combination BromAc® considerably reduced the IC50 values of the chemotherapeutic agents. The potential reduction of the effective dosage of chemotherapeutic agents indicated that the side effects resulting from the present clinical dosage in use may be considerably reduced when combining these therapies in addition to enabling more frequent administration of the agents.

Dose escalation *in vivo* studies using intraperitoneal administration of Bromelain 3, 5 or 10 mg/kg with Acetylcysteine 300 or 500 mg/kg combination showed no negative effect on the animals either on physical examination or histological assessment tissue staining using hematoxylin & eosin, indicating the safety of the concentrations used. With these encouraging results, we proceeded with further safety studies using BromAc® in combination with GEM, OXAL, and 5-FU. Our results were encouraging and there were no adverse events or drug-related toxicities, indicating the safety of the concentrations used in combination (BromAc® + cytotoxics). Subsequently, we examined the efficacy of BromAc® with cytotoxics in LS174T *in vivo*. Our initial study indicated that BromAc® combinations with either OXAL or 5-FU were not effective. On the contrary, BromAc® with GEM showed considerable efficacy.

The negative results seen with OXAL or 5-FU may be related to their mode of action in the presence of BromAc®. OXAL is normally hydrolyzed to generate reactive radicals that bind to RNA strands with cross-linking resulting in interference with cellular replication [25]. As Acetylcysteine is an antioxidant, it deactivates these reactive radicals and hence nullifies their RNA intercalating effect. Further, the presence of Bromelain with its proteolytic action may also interfere with cellular transporters that are basically protein molecules [26]. Copper transporter 1 and organic cation transporters 1-3 have been implicated in the uptake of OXAL [26]. This area requires further investigation.

In the case of 5-FU (5-Fluorouracil), it is a prodrug that has to be activated by conversion into 5-fluorodeoxyuridine monophosphate (5-FdUMP), 5-fluorodeoxyuridine triphosphate (5-FdUTP) and 5-fluorouridine triphosphate (5-FUTP) of which 5-FdUMP inhibits thymidylate synthetase (TYMS) resulting in deoxynucleotide pool imbalance affecting DNA synthesis [27]. Although in the past, we were able to show that BromAc® may enable the reduction of 5-FU dosage in peritoneal mesothelioma [16] and other cell lines, the current dosage of agents used may have a critical role in either enhancing or regressing the efficacy of this molecule since the molecular ratio of 5-FU to adjuvant agents such as BromAc® is crucial for the enhancement of the cytotoxics shown in earlier work in this current study. Further, BromAc® at the concentration used may also have a negative effect on the phosphorylating enzymes

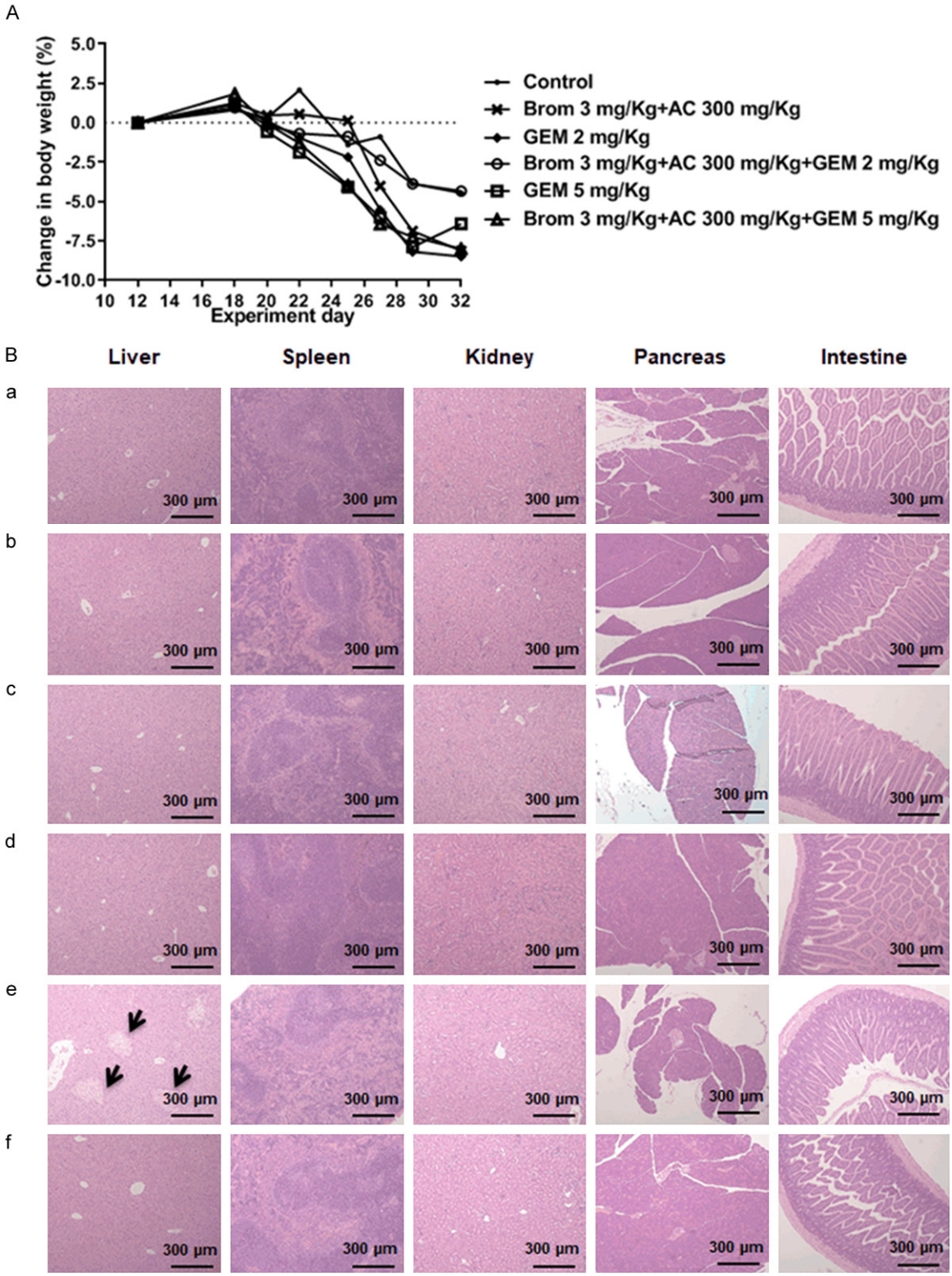

Figure 4. (A) Mean body weight fluctuations in the LS174T tumor-bearing nude mice treated with combination therapies. (B) Histological images of liver, spleen, kidney, pancreas and intestine from the LS174T tumor-bearing nude mice treated with either (a) 0.9% NaCl, (b) Bromelain (3 mg/kg) + Acetylcysteine (300 mg/kg), (c) Gemcitabine (2 mg/kg), (d) Bromelain (3 mg/kg) + Acetylcysteine (300 mg/kg) + Gemcitabine (2 mg/kg), (e) Gemcitabine (5 mg/kg). Arrows shows necrotic focal areas in one liver sample (1 out of 3) in this group, or (f) Bromelain (3 mg/kg) + Acetylcysteine (300 mg/kg) + Gemcitabine (5 mg/kg). Tissues were Hematoxylin and eosin stained (magnification, ×100; scale bar = 300 μm).

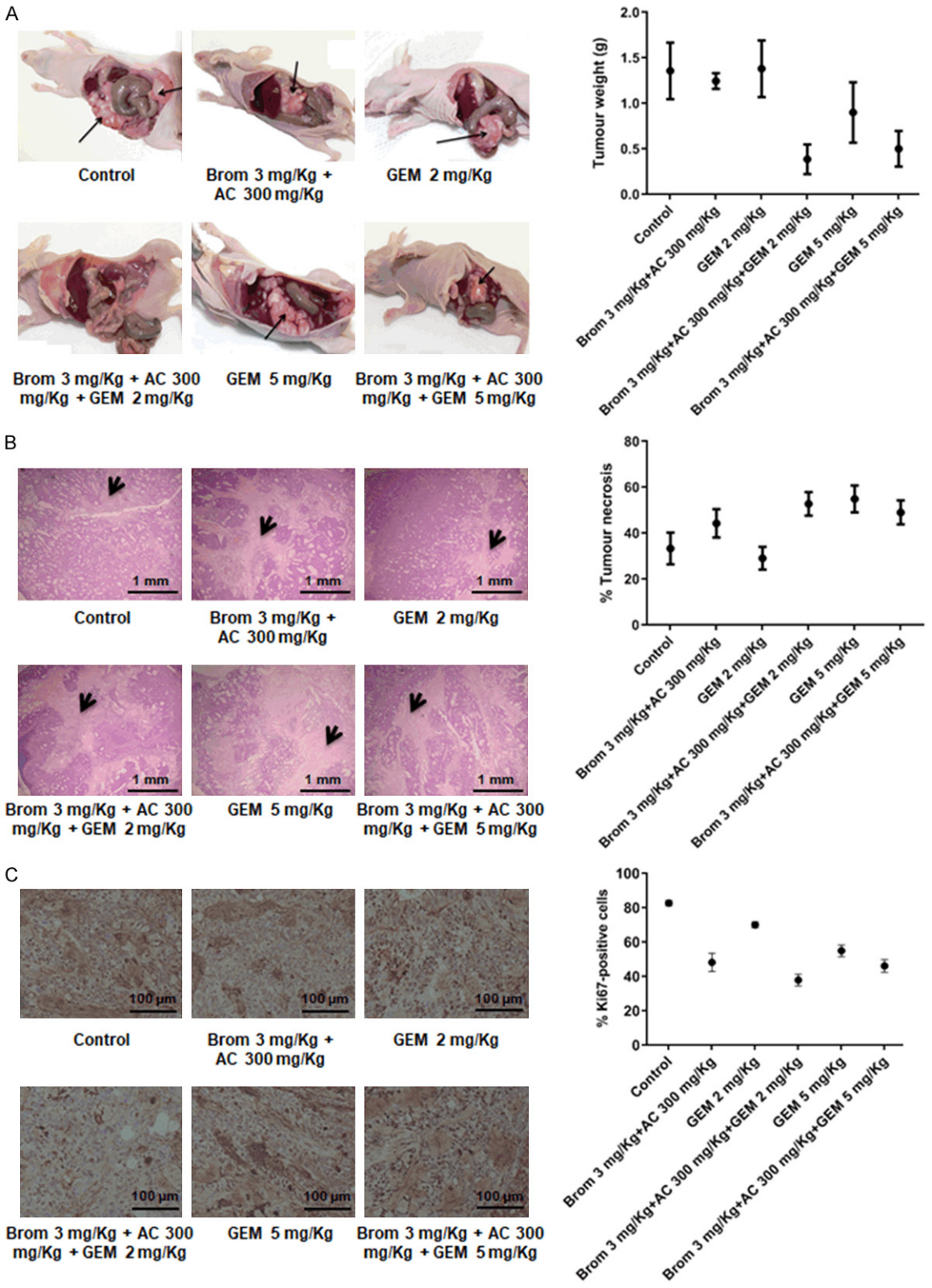

Figure 5. (A) Figures and graph show tumor size and weight, respectively, in the treated groups. The lowest weight is seen in the BR 3 mg/kg + NAC 300 mg/kg + GEM 2 mg/kg group. (B) Histological images of tumors shown in hematoxylin and eosin staining (H&E), showing drug combinations-induced necrosis (magnification, ×50; scale bar = 1 mm). Necrosis is highest in two groups - BR 3 mg/kg + NAC 300 mg/kg + GEM 2 mg/kg and GEM 5 mg/kg; (C)

Immuno-histological images of tumors samples with anti-Ki67 antibody. Sections were counterstained using hematoxylin (magnification, ×400; scale bar = 100 μm). The corner image represents a higher magnification view. The lowest expression of Ki67 is observed in the group treated with BR 3 mg/kg + 300 mg/kg + GEM 2 mg/kg which is indicative of reduced cellular replication. Quantifications of these images (A-C) are presented at the end of the rows. Data presented as mean ± SEM.

Table 1. Mean percentage reduction in tumor weight compared to control with 95% confidence intervals in the various treatment groups indicating significance ($P \leq 0.05$) in Brom 3 mg/kg + Ac 300 mg/kg + GEM 2 mg/kg with a reduction of 71% of tumor volumes that is indicative of the combination efficacy. Brom 3 mg/kg + Ac 300 mg/kg + GEM 5 mg/kg showed a reduction of 62.98% of tumor volumes with a *p* value = 0.0855. Data were analyzed using Dunnett's multiple comparisons test

Treatment Group	n	Mean Reduction in Tumor Weight vs Control (%)	95% Confidence Interval (%)	*p*-value
Brom 3 mg/Kg + AC 300 mg/Kg	6	8.186	-61.23 to 77.60	0.9975
GEM 2 mg/Kg	6	-1.806	-71.22 to 67.61	>0.9999
Brom 3 mg/Kg + AC 300 mg/Kg + GEM 2 mg/Kg	6	71.39	1.979 to 140.8	0.0421
GEM 5 mg/Kg	6	33.53	-35.88 to 102.9	0.5757
Brom 3 mg/Kg + AC 300 mg/Kg + GEM 5 mg/Kg	6	62.98	-6.434 to 132.4	0.0855

that convert 5-FU to active agents [28, 29] and this requires further studies. Cellular features that are characteristics of tumor cells may also affect the efficacy of this combination regime as we have shown in the past [16]. The combination of BromAc® at the concentration used may also affect transporters such as the ABC cassette transporters and the penetration of 5-FU may have been compromised. Only specific concentrations of BromAc® with cytotoxics show enhancement of cytotoxicity in a synergistic manner that is indicative of the specific molar ratios of drugs that are required for the optimal combination [20].

The combination of GEM (2 mg or 5 mg/kg) with Bromelain 3 mg/kg + Ac 300 mg/kg) showed no toxicity. When efficacy was examined, there was no difference between the GEM 2 mg and 5 mg/kg groups, indicating that there is a maximum effect which did not exceed 2 mg/kg in the presence of BromAc®. Further, the combination regimen had a maximum reduction of tumor weight that is indicative of synergy. By combining with BromAc®, the tumor weight was reduced from 1.3 g (GEM 2 mg/kg alone) to 0.4 g, a reduction of 71% (**Figure 5A**; **Table 1**). The combination index calculated for low dose GEM (2 mg/kg) and high dose GEM (5 mg/kg) in combination with BromAc (low dose) demonstrating that low dose GEM with BromAc has higher efficacy. Combinations such as this in cancer therapy could dramatically reduce the clinical dosage required, with a potential reduction of severe side effects. Furthermore, more frequent dosing as compared to the current regime of 4 treatments with seven days rest in between may be achieved. Frequent treatment will not only result in better tumor exposure but may also increase patient survival, as resistant cells will not have the time to recuperate and repopulate [30, 31]. BromAc® plus GEM combination showed higher tumor necrosis compared to the other treatment groups that may be indicative of treatment efficacy. Necrosis may enable tumor shrinkage [32]. Examining all the treatment groups in the initial study of efficacy (Supplementary Figure 1A, 1B), there is clear evidence that a combination of GEM 2 or 5 mg/kg with Bromelain 3 mg/kg + Acetylcysteine 300 mg/kg produces the greatest tumor regression as indicated by the tumor weight or percentage reduction of tumor weight.

The mechanism by which BromAc® enhances the cytotoxicity of GEM in LS174T cells may be speculated to be direct proteolytic action on the mucin that is present in the cells [33] as well as the disintegration of other oncoproteins present within the cells that provide replicative power and resistance to these cell lines [34, 35]. The relationship of mucin to certain important functions is well described. Mucin 1 (MUC1) induces drug resistance in pancreatic cancer cells by upregulating multi-drug resistance genes [36]. MUC4 expression is associated with a reduction hCNT1 and hCNT3 trans-

porters which are responsible for GEM drug entry [37]. MUC4 is a transmembrane glycoprotein and its expression in pancreatic cancer cell lines is associated with GEM resistance by activating anti-apoptotic pathways [38]. Another mechanism of BromAc®'s anti-tumor activity could be its effect on apoptosis. We showed previously that BromAc® is able to activate the caspase system as well as to inhibit anti-apoptotic and pro-survival processes in gastrointestinal cancer cell lines including LS174T cells [17]. These potential mechanisms need further investigation, however, we can speculate that the current therapy may offer a new treatment for colorectal cancers. Colorectal cancer cells are heterogeneous and treatment efficacy may vary [39] and therefore evaluating the response *in vitro* of tumor cells from patients may enable the tailoring of drug dosage with the combination of adjuvant agents such as BromAc®. Although individualizing treatment medicament is a slow process, the end results may be more promising for both the patient and the medical profession.

The current *in vivo* results is very promising since a combination of GEM 2 mg/kg with Bromelain 3 mg/kg and Ac 300 mg/kg show tumor volume inhibition by 71% in human colorectal cancer cell line LS174T. The molecular ratio of these agents appears to be 18:1:1840 (GEM: Bromelain: Acetylcysteine). This essentially indicates that 18 molecules of GEM are required to be combined with one molecule of Bromelain for effective tumor cytotoxicity. GEM is a nucleoside that is utilized and exhausted whilst Bromelain regenerates as it is an enzyme and hence the variation in ratio. The high molecular ratio (1840) requirement of Ac is indicative of its role as an antioxidant that affects not only the disulfide bonds that are found in oncogenes but also in the regeneration of glutathione (GSH) that are reduced to GSSG during chemotherapy [29, 40].

In conclusion, whilst the mechanism of BromAc® sensitization of colorectal and other cancer cell types to GEM has several possibilities, our data suggest that this may be achieved with safety after peritoneal administration and be capable of providing clinically meaningful growth inhibition. Further work with other cancer types and a phase I trial is planned.

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Disclosure of conflict of interest

Professor D.L. Morris is the co-inventor and assignee of the intellectual property and co-owner and director of the sponsor company, Mucpharm Pty Ltd. Dr J. Akhter and Dr K. Pillai are co-inventors of the intellectual property. Dr A.H. Mekkawy, Dr K. Pillai, Dr J. Akhter, Mr K. Ke and Ms S.J. Valle are employed by Mucpharm Pty Ltd. The University of New South Wales (NSi) has assigned the IP through license 2017-0045 to Professor D.L. Morris (inventor).

Abbreviations

5-FU, 5-Fluorouracil; Ac, Acetylcysteine; Brom, Bromelain; BromAc®, Bromelain and Acetylcysteine; CRC, Colorectal Cancer; GEM, Gemcitabine; GSH, glutathione; HIPEC, Hyperthermic intraperitoneal chemotherapy; IP, intraperitoneally; OXAL, Oxaliplatin; PCI, Peritoneal Cancer Index.

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Supplementary Table 1. The dosage regimen for the various treatment groups (n = 6/group) in the *in vivo* safety study of combination therapy

Groups	Drug 1 (Every other day)	Drug 2 (Once/week)
Group 1	0.9% NaCl (Control)	-
Group 2	Brom 3 mg/kg + Ac 300 mg/kg	GEM 2 mg/kg
Group 3	Brom 3 mg/kg + Ac 300 mg/kg	OXAL 2 mg/kg
Group 4	Brom 3 mg/kg + Ac 300 mg/kg	5-FU 15 mg/kg

Supplementary Table 2. Shows the dosage regimen for the various treatment groups

Groups	Drug 1 (Every other day)	Drug 2 (Once/week)
Group 1	0.9% NaCl (Control)	-
Group 2	Brom 3 mg/kg	-
Group 3	Ac 300 mg/kg	-
Group 4	Brom 3 mg/kg + Ac 300 mg/kg	-
Group 5	Brom 3 mg/kg + Ac 300 mg/kg	GEM 2 mg/kg
Group 6	-	GEM 2 mg/kg
Group 7	Brom 3 mg/kg + Ac 300 mg/kg	OXAL 2 mg/kg
Group 8	-	OXAL 2 mg/kg
Group 9	Brom 3 mg/kg + Ac 300 mg/kg	5-FU 15 mg/kg
Group 10	-	5-FU 15 mg/kg
Group 11	Brom 3 mg/kg + Ac 300 mg/kg	GEM 5 mg/kg
Group 12	-	GEM 5 mg/kg
Group 13	Brom 3 mg/kg + Ac 300 mg/kg	OXAL 5 mg/kg
Group 14	-	OXAL 5 mg/kg
Group 15	Brom 3 mg/kg + Ac 300 mg/kg	5-FU 30 mg/kg
Group 16	-	5-FU 30 mg/kg

Studies with Oxaliplatin and 5-FU were discontinued after initial failure to show any positive tumor regressive response.

Supplementary Table 3. Suitable combination of Bromelain (Brom) and Acetylcysteine (Ac) reduces the IC50 values considerably in all the colon tumor cell lines

A. Bromelain and Acetylcysteine IC50 values alone and in combination following 72 hour drug treatment, measured through SRB assay.

Cell Line	HT295M21	Cell Line	HT29	Cell Line	LS174T
AC IC50 (mg/mL)	0.05	AC IC50 (mg/mL)	1.7	AC IC50 (mg/mL)	4.4
Brom IC50 (µg/mL) (AC 0 mg/mL)	18	Brom IC50 (µg/mL) (AC 0 mg/mL)	6.5	Brom IC50 (µg/mL) (AC 0 mg/mL)	6.2
Brom IC50 (µg/mL) (AC 0.16 mg/mL)	13	Brom IC50 (µg/mL) (AC 0.41 mg/mL)	3.9	Brom IC50 (µg/mL) (AC 0.41 mg/mL)	5.2
Brom IC50 (µg/mL) (AC 1.1 mg/mL)	0	Brom IC50 (µg/mL) (AC 0.82 mg/mL)	2.5	Brom IC50 (µg/mL) (AC 0.82 mg/mL)	4.1
Brom IC50 (µg/mL) (AC 2.4 mg/mL)	0	Brom IC50 (µg/mL) (AC 1.2 mg/mL)	0.6	Brom IC50 (µg/mL) (AC 1.2 mg/mL)	0

B. Gemcitabine IC50 values following 72 hour drug treatment combined with Bromelain, Acetylcysteine or both, measured through SRB assay.

Cell Line	HT295M21		Cell Line	HT29		Cell Line	LS174T	
Treatment	Control: GEM IC50 (ng/mL)	Treated: GEM IC50 (ng/mL)	Treatment	Control: GEM IC50 (ng/mL)	Treated: GEM IC50 (ng/mL)	Treatment	Control: GEM IC50 (ng/mL)	Treated: GEM IC50 (ng/mL)
Brom 10 µg/mL	12	11	Brom 2.5 µg/mL	42	43	Brom 5 µg/mL	260	130
AC 0.16 mg/mL	13	4	AC 0.41 mg/mL	52	37	AC 0.82 mg/mL	70	88
Brom 10 µg/mL + AC 0.16 mg/mL	8	0	Brom 2.5 µg/mL + AC 0.41 mg/mL	34	14	Brom 5 µg/mL + AC 0.82 mg/mL	150	82

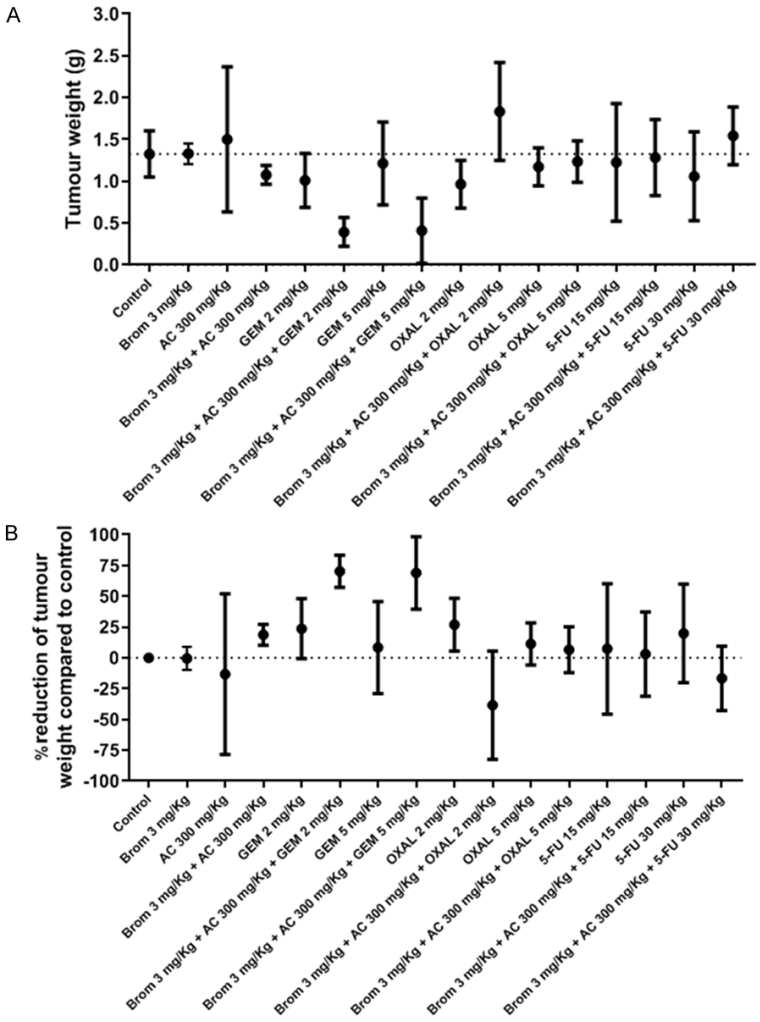
C. Oxaliplatin IC50 values following 72 hour drug treatment combined with Bromelain, Acetylcysteine or both, measured through SRB assay.

Cell Line	HT295M21			HT29			LS174T	
Treatment	Control: OXAL IC50 (µg/mL)	Treated: OXAL IC50 (µg/mL)	Treatment	Control: OXAL IC50 (µg/mL)	Treated: OXAL IC50 (µg/mL)	Treatment	Control: OXAL IC50 (µg/mL)	Treated: OXAL IC50 (µg/mL)
Brom 12 µg/mL	4	0	Brom 5 µg/mL	0.8	0.8	Brom 5 µg/mL	0	0
AC 0.16 mg/mL	3.6	4.2	AC 0.82 mg/mL	0.9	>20	AC 0.82 mg/mL	0	-
Brom 12 µg/mL + AC 0.16 mg/mL	2.4	0	Brom 5 µg/mL + AC 0.82 mg/mL	1.0	0	Brom 5 µg/mL + AC 0.82 mg/mL	2	0

D. 5-Fluorouracil IC50 values following 72 hour drug treatment combined with Bromelain, Acetylcysteine or both, measured through SRB assay.

Cell Line	HT295M21		Cell Line	HT29		Cell Line	LS174T	
Treatment	Control: 5-FU IC50 (µg/mL)	Treated: 5-FU IC50 (µg/mL)	Treatment	Control: 5-FU IC50 (µg/mL)	Treated: 5-FU IC50 (µg/mL)	Treatment	Control: 5-FU IC50 (µg/mL)	Treated: 5-FU IC50 (µg/mL)
Brom 10 µg/mL	>6.5	0	Brom 2.5 µg/mL	1.8	1.5	Brom 5 µg/mL	2.3	2.3
AC 0.16 mg/mL	>6.5	0	AC 0.41 mg/mL	1.8	1.8	AC 0.82 mg/mL	1.7	1.7
Brom 10 µg/mL + AC 0.16 mg/mL	5.3	0	Brom 2.5 µg/mL + AC 0.41 mg/mL	1.9	1.1	Brom 5 µg/mL + AC 0.82 mg/mL	2.5	0.9

A similar scenario is depicted when Bromelain and Acetylcysteine are combined with Gemcitabine (GEM), 5-Fluorouracil (5-FU) and Oxaliplatin (OXAL) that is indicative of the advantage of combinatorial effect of these agents.



Supplementary Figure 1. A shows tumor weight in the various treatment groups. Those above the dotted lines at the control group is indicative of tumor growth whilst those below shows tumor regression. Treatment with Gemcitabine 2 mg/kg in combination with Bromelain 3 mg/kg and Acetylcysteine 300 mg/kg shows the best regression whilst that with Gemcitabine 5 mg/kg with similar combinations of Bromelain and Acetylcysteine is almost at par with Gemcitabine 2 mg/kg. B shows a similar scenario in terms of % reduction of tumor weight.