

The effect of hyperthermia (42°C) on the anti-tumoral effect of bromelain, N-acetyl cysteine, chemotherapeutic agents and their combinations - an *in vitro* evaluation

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Abstract

Bromelain, N-acetyl cysteine and their combinations show cytotoxicity at 37°C. Similarly, their combinations with common chemotherapeutic agents (gemcitabine, Mitomycin C, oxaliplatin and 5-FU) show enhanced cytotoxicity. Since, hyperthermia (42°C) inhibits cellular proliferation, we set out to determine if it would further enhance the effect of these agents.

Tumour cells (pancreatic and colorectal) were grown in a 96 well plate and treated to various agents and their combinations at 37° and 42°C. The survival of cells at 72 hours was evaluated with sulphordamine assay. Colony formation assay was performed to evaluate development of resistance to these agents and finally PAS staining was carried out to determine the effect of bromelain, N-acetylcysteine (NAC), oxaliplatin and their combinations on mucin secretion in ASPC-1 (pancreatic) cancer cells.

Hyperthermia (42°C) enhanced cytotoxicity of certain agents or their combinations in some of the cell lines. It also showed the absence or a reduction of effect, with certain agents. Hyperthermia reduced colony formation in CFPAC cells and with agents (bromelain + NAC, gemcitabine, NAC + gemcitabine, Bromelain

+ NAC + Gemcitabine). A similar effect with 5FU, 5FU + NAC and 5FU + bromelain was observed. Hyperthermia reduced mucin secretion in ASPC-1 cells, with bromelain and its combinations with NAC and oxaliplatin. NAC as a single agent increased mucin with hyperthermia.

The effect of hyperthermia on cytotoxicity of bromelain, NAC and their combinations with chemotherapeutic agents varied with agents, their combinations and cell types, showing an increase, null effect or a decrease. However, hyperthermia reduced colony formation and mucin secretion.

Introduction

Hyperthermic intraperitoneal chemotherapy is routinely used for treating peritoneal surface malignancies after cytoreductive surgery. Drugs commonly used in HIPEC are cisplatin, oxaliplatin, doxorubicin, mitomycin C and 5FU at a temperature of 41-42°C.^{1,2} Recurrence has been attributed to residual tumour cells that have escaped treatment.³ Since, peritoneal surface malignancies may originate from distant tumours of different origin such as ovarian, appendiceal, colorectal, *etc.*, their response to HIPEC may also vary as these cells have different cellular characteristics.^{4,5}

Bromelain, an enzymic mixture from pineapple plant, has anti-tumoral properties in both *in vitro* and *in vivo* models^{6,7} whilst N-acetylcysteine (NAC), a commonly used antidote for paracetamol toxicity⁸ has also been shown to have anticancer properties.^{9,10} Bromelain is an effective mucolytic that targets the 1-4 glycosidic linkages in glycoproteins¹¹ whilst NAC is an antioxidant that reduces disulfide bonds.¹² Numerous cellular proteins and mucins are glycoproteins that carry both 1-4 glycosidic linkages along with the presence of disulfide bonds.^{13,14} Further, the presence of mucins (glycoproteins) in tumour cells have been correlated with chemo-resistance^{15,16} since it provides the cells with both a barrier to the transfer of drugs as well as enhancing survival through regulation of several oncoproteins.^{17,18} Hence, the cellular mechanism at both the membranous and cytoplasmic levels may be affected by these agents. Bromelain and NAC have also been shown to enhance the efficacy of a number of chemotherapeutic drugs *in vitro* and *in vivo* studies.^{19,20}

Hyperthermia (>39°C) have been shown to adversely affect the survival of mammalian cells.^{21,22} the integrity of the cellular membranes along with cellular transport proteins are affected at temperatures exceeding 39°C, whilst at the same time disrupting DNA synthesis and repair.²³ In addition, on a macroscopic level, blood circulation is enhanced in hyperthermia with accelerated

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Key words: Hyperthermia, bromelain, N-acetylcysteine, chemotherapeutic agents.

Contributions: all authors have contributed equally to the research. The script has been revised and prepared by K. Pillai and Vanessa H.L. Wan.

Conflict of interests: there is no conflict of interest to declare.

Received for publication: 29 July 2018.
Accepted for publication: 8 October 2018.

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Journal of Peritoneum (and other serosal surfaces) 2018; 3:104
doi:10.4081/joper.2018.104

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transport of cytotoxic agents.²⁴ Hence, chemotherapy with hyperthermia presents a harsh environment for cellular survival with concomitant cell death.

Our earlier *in vitro* studies at 37°C with chemotherapeutic agents such as gemcitabine, oxaliplatin, 5-FU and mitomycin C with the addition of bromelain, NAC and their combinations have shown a considerable reduction in IC₅₀ values indicating that the therapeutic dosage of these chemo-agents can be reduced whilst maintaining the therapeutic effects. (unpublished data) Essentially, this means that most of the deleterious side effects associated with current dosage can be ameliorated or reduced whilst enhancing the patient well being. Hence, the present investigation aims to determine if the IC₅₀ values of these chemo-agents can be further reduced in the presence of bromelain and NAC with hyperthermia at 42°C.

In the current study, pancreatic and colorectal cancer cell lines that are chemoresistant and invasive owing to the presence of mucin, both secretory and transmembrane, are investigated. Both CFPAC and ASPC-1 cells that are of pancreatic ductal adenocarcinoma (PDAC) origin have a wide array of mucins,²⁵⁻²⁷ whilst HT-29 and LS 174T of colorectal origin also display different types of mucins.^{28,29} Bromelain and NAC as single agents or in combination would depolymerize the glycoprotein mucin polymers and hence facilitate both the penetration of chemotherapeutic agents with the reduction of chemoresistance by down regulating the mucins. Hence, hyperthermia in conjunction with chemotherapeutics in the presence of mucin depolymerizing agents should in principal enhance cytotoxicity. Therefore, we set forth to investigate whether this paradigm exists.

Materials and Methods

Cell lines

HT-29 and CFPAC used in this study were obtained from the American Type Culture Collection. HT-29 is colorectal adenocarcinoma and CFPAC is pancreatic ductal adenocarcinoma (ATCC). The Colorectal cell line LS174T and pancreatic cell line ASPC-1 was purchased from Sigma-Aldrich. LS174T is colorectal adenocarcinoma and ASPC-1 is pancreatic adenocarcinoma (Sigma-Aldrich, USA). All cell lines were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C in RPMI-1640 medium with 10% fetal bovine serum and 1% penicillin-streptomycin.

Cell proliferation and colony formation assays

Single agent treatment

The ASPC-1, CFPAC, LS174T and HT29 were seeded in 96-well plate with 2000 cells/ well, and incubated for 24 hours. Subsequently, they were treated with N-acetylcysteine (Nac), Bromelain (Br), Gemcitabine (Gem), Mitomycin-C (MMC), Oxaliplatin (Oxali), 5-Fluorouracil (5FU) or their combinations. Control cells were included and maintained in drug-free medium in all plates. At the completion of the treatment, cell proliferation was assessed by SRB assay.³⁰

Each treatment was conducted at two temperatures *i.e.* 37 and 42°C in separate plates for a period of 72 hours (with only 2 hours at 42°C, followed by 70 hours at 37°C). Following cell fixations, cells were then washed and stained with 100 μ L of 0.4% SRB dissolved in 1% acetic acid for 20 minutes. Subsequently, unbound dye was removed by five washes with 1% acetic acid. After air drying, 100 μ L of Tris base (pH 10.5) were used to dissolve the bound SRB and read at a wavelength of 570 nm.

Combination treatment

Based on the results obtained from the single agent treatments mentioned above, desirable concentrations of Nac, Br and cytotoxic agents were selected for the combination treatment. The ASPC-1, CFPAC, LS174T, and HT29 cells were treated with escalating concentrations of Gem, MMC, Oxali and 5FU and with combinations of Nac and Br in 16 separate experiments. Untreated control groups were included in all the experiments. SRB assay was applied upon the completion of the treatment regime.

Colony formation assays

For colony formation assays, tumor cells at a density of 0.3 million were seeded in sixteen small sized flasks and allowed to attach overnight. Cells were treated with Nac, Br, Gem and their combinations accordingly at both 37 and 42°C for 72 hours (only 2 hours at 42°C, followed by 70 hours at 37°C.) Subsequently, cells were trypsinized and seeded into petri dishes at 1000 cells/plate with drug free RPMI medium, with an incubation time of 3 weeks in total with weekly change of medium. Finally, plates were washed with PBS and fixed with 100% ethanol, then stained with 0.5% solution of filtered crystal violet. Colonies were counted manually.

PAS staining

ASPC-1 cells were seeded at densities of 200,000/ wells onto sterile glass cover-slips in 6-well tissue culture plates and maintained in RPMI-1640 medium at 37°C in a humidified atmosphere with 5% CO₂ for 24 hours. After incubation, cells were treated with 2.5 mM Nac, 2.5 mM Br and 10 μ M Oxaliplatin for 48 hours, followed by three washes with ice-cold PBS and fixed in ice-cold methanol for 10 minutes. Afterwards, cells were stained with periodic acid solution for 5 minutes followed by three washes with ice-cold PBS again. Subsequently, cells were stained with Schiff's reagent for 15 minutes. The cells were rinsed with three washes of PBS. Afterwards, they were counter-stained with haematoxylin solution for 90 seconds. Followed by the final three washes with PBS, cells were mounted with glycerol gelatin and investigated using Leica DMLB microscope, DC200 digital imaging system. Staining intensity was assessed, visually.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6. Fifty percent inhibitory concentration (IC₅₀) values were calculated from concentration-response curves plotting growth percentage *versus* drug concentration using GraphPad Prism 6. Multiple t tests one per row was used to determine the statistical significance between two temperature groups. P values <0.05 were considered significant.

Results

Both colorectal cell lines: LS174T and HT29, and pancreatic cell lines: ASPC-1 and CFPAC were the reference cancer cells in this experiment. Since this experiment was to compare the cytotoxicity of the agents at both 37° and 42°C, the IC₅₀ values at the two temperatures were determined and hyperthermic efficacy index was determined.

Comparison of IC₅₀ values between the two temperatures to determine hyperthermic efficacy index (X)

The IC₅₀ values for each treatment at both the temperatures are compared to determine the efficacy of hyperthermia as follows:

$$\text{IC50 (37deg C)/ IC50 (42 deg C) = X (Hyperthermic Efficacy Index)}$$

When:

X = < 1.0 (decreased efficacy)

X = 1.0 (equal efficacy)

X = > 1.0 (enhanced efficacy)

Pancreatic cancer cells

CFPAC

Hyperthermia treatment on this cell line has shown a noticeable increase in efficacy as indicated by the X values in the following categories of treatment: BR, NAC, NAC + BR, GEM, GEM + BR, GEM + NAC + BR and OXA + NAC, whilst a very small increase in efficacy was observed in OXA + BR, OXA + BR + NAC, 5FU + BR and MMC.

With OXA and 5FU + NAC, there is a considerable reduction in efficacy (Table 1; Figure 1A-D).

ASPC-1

In ASPC-1 cells hyperthermia showed a noticeable enhancing effect in the following categories of treatment: GEM, GEM + NAC (X=5.633) - a substantial increase in efficacy with hyperthermia), OXA + NAC, OXA + NAC + BR, and a small difference with 5FU + NAC.

Considerable reduction in efficacy was found in the following categories of treatment: NAC, GEM + BR, GEM, BR + NAC and 5FU + BR + NAC (X= 0.221 - a dramatic reduction in efficacy) (Table 1, Figure 2A-D).

HT-29

With HT-29, hyperthermia produced a noticeable increase in efficacy in the following categories of treatment: BR, GEM and OXA + NAC (X=2.995) whilst very small increase in efficacy was observed with 5FU, 5FU + BR + NAC, MMC + BR and MMC + MAC.

A large reduction in efficacy was found with the addition of NAC, GEM + BR. and 5FU + NAC (Table 1, Figure 3A-D).

LS174T

Increase in efficacy was found in the following categories of treatment: BR + NAC, GEM + BR, GEM + NAC, OXA + BR, OXA + BR + NAC, 5FU.

A reduction in efficacy was found GEM + BR + NAC, OXA, 5FU + BR (X = 0.121 - a considerable reduction in efficacy), 5FU + NAC and MMC (Table 1, Figure 4A-D).

Colony formation assay

To investigate whether the combination of Nac, Br and cytotoxic drugs chosen can effectively inhibit the reproductive integrity and the ability of establishing colonies, CFPAC cells were chosen as they are actively dividing cell line. Following a 72-hour treatment at Gemcitabine (100 nM), Nac (5 mM) , Br (5 mM) as single agents and their combinations for the following 3 weeks, it was found that the combination of Gemcitabine + Nac + Br reduced colony formation of CFPAC cells and the result was more significant at 42°C (Figure 5A and B).

Following a 72-hour treatment at 5FU (1000 nM), Nac (5mM) , Br (5mM) as single agents and their combinations for the following 3 weeks, it was found that the combination of 5FU + Nac + Br showed no hyperthermic effect. However, 5FU + Br reduced more colony formation at 42°C (Figure 5C and D)

Table 1. The X values for individual reagent as well as in combination with others values below 1 denotes reduced efficacy with hyperthermia (42°C).

Reagent	Ratio of IC50 (37°C/IC50 (42°C) = X		Colorectal cancer cells	
	Pancreatic cancer cells CFPAC (X)	ASPC-1 (X)	HT29 (X)	LS174T (X)
Bromelain (BR)	<i>1.300</i>	<i>1.415</i>	<i>2.00</i>	0.810
N-acetyl cysteine (NAC)	<i>1.727</i>	0.714	0.500	0.940
BR + NAC	<i>1.333</i>	1.000	1.000	<i>1.127</i>
Gemcitabine (GEM)	<i>4.927</i>	<i>1.296</i>	<i>1.152</i>	0.865
GEM + BR	<i>1.971</i>	0.638	0.487	<i>1.76</i>
GEM + NAC	<i>0.772</i>	<i>5.633</i>	0.815	<i>1.194</i>
GEM + BR + NAC	<i>1.666</i>	0.694	0.748	0.697
Oxaliplatin (OXA)	0.581	0.966	0.793	0.724
OXA + BR	<i>1.069</i>	0.987	0.924	<i>1.751</i>
OXA + NAC	<i>2.333</i>	<i>1.100</i>	<i>2.995</i>	1.0
OXA + BR + NAC	<i>1.015</i>	0.848	0.888	<i>1.8</i>
5FU	0.910	<i>1.100</i>	<i>1.049</i>	<i>1.691</i>
5FU + BR	<i>1.041</i>	0.996	0.880	0.121
5FU + NAC	0.711	<i>1.030</i>	0.647	0.648
5FU + BR + NAC	0.588	0.221	<i>1.023</i>	1.0
Mitomycin C (MMC)	<i>1.011</i>	0.861	1.0	0.737
MMC + BR	1.0	1.0	<i>1.023</i>	1.0
MMC + NAC	1.0	1.0	<i>1.011</i>	1.0
MMC + BR + NAC	1.0	1.0	0.976	0.99

X=1 denotes equal efficacy and hence no difference with hyperthermia and X>1.0 denotes increased efficacy with hyperthermia (42°C); X values in italics indicates increased inefficacy with hyperthermia.

Effect of hyperthermia on colony formation

Hyperthermia reduces colony formation as indicated by the Y values (1.428) in the first control and 1.164 in the second control (Table 2; Figures 5A-B and 4C-D).

The addition of NAC did not affect colony formation and neither did bromelain in the first case. However in the second case a Y value of 1.114 indicates a slight reduction of colony. Again the addition of

NAC + bromelain indicated a reduction of colony in the first case whilst showing a no effect (Y=0.95) in the second case.

When Gem or 5FU was added, hyperthermia definitely reduced colony formation and so did the addition of NAC to either GEM or 5FU. The addition of Bromelain to GEM showed no effect whilst colony formation was considerably reduced when added to 5FU (Y=1.94). Finally, the triple addition for GEM indicated a reduction of colony whilst the effect was null for 5FU.

Table 2. The calculated hyperthermic efficacy index (Y) for the colony formation assay.

Hyperthermic efficacy index (Y) Reagents							
Control	NAC	BR	NAC+BR	GEM(G)	G+NAC	G+BR	G+NAC+BR
1.428	1.086	1.034	1.259	1.170	1.428	0.9	1.263
Control	NAC	BR	NAC+NAC	5FU	5FU+NAC	5FU+BR	5FU+NAC+BR
1.164	1.0	1.114	0.95	1.209	1.23	1.954	1.063

Y=colony count at 37°C/colony count at 42°C; Y<0.9 reduced hyperthermic efficacy; Y=0.9-1.0 (no effect); Y>1.0 enhanced hyperthermic efficacy. NAC=N-acetylcysteine; BR=bromelain; GEM=gemcitabine; 5FU=5-fluorouracil.

Pancreatic cancer cells- CFPAC at 37 & 42 deg C.

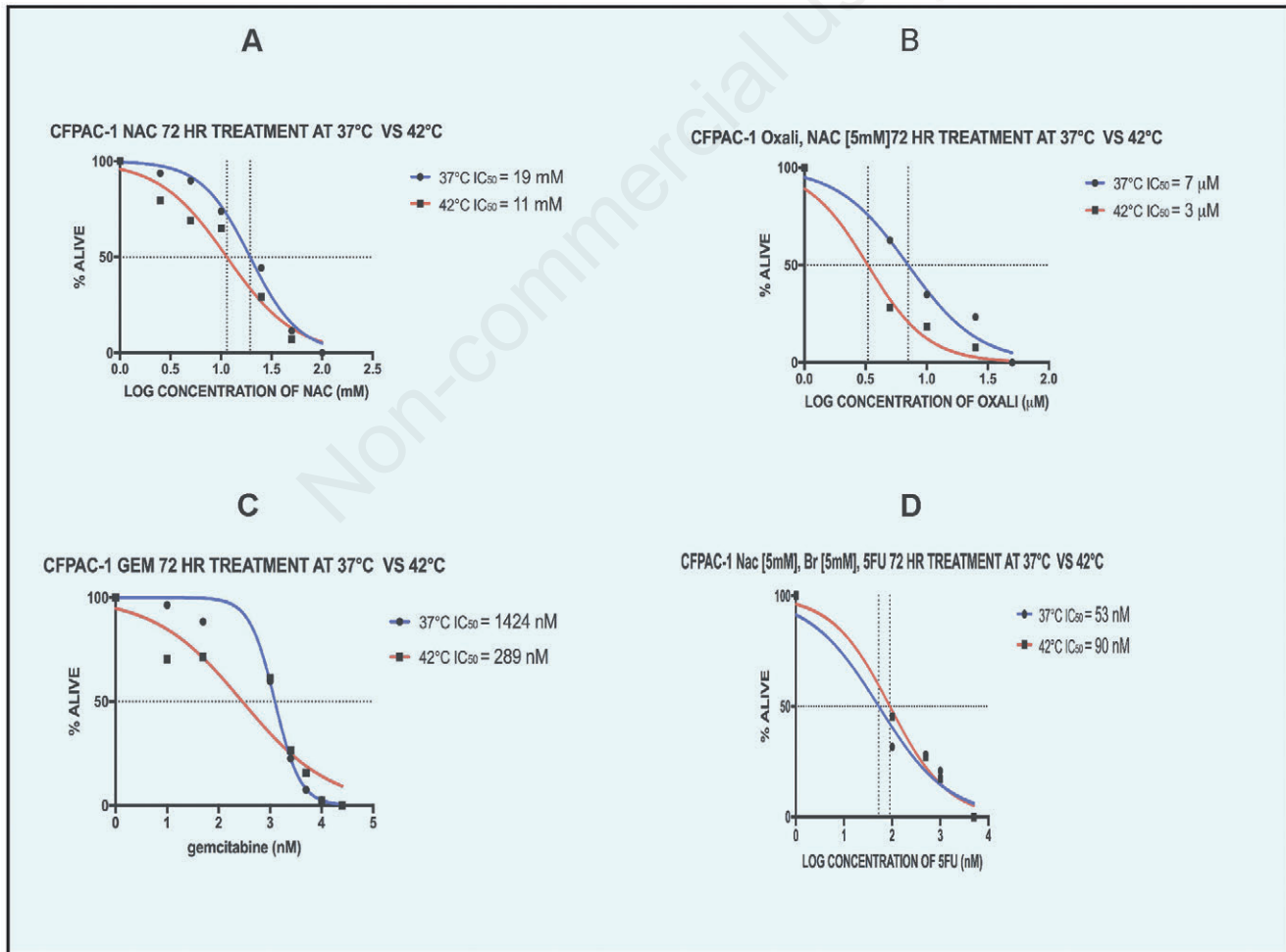


Figure 1. A-D) Shows the effect of hyperthermia on the IC50 values in CFPAC cell lines when treated with different agents. Figures 1 A-C shows enhancement of toxicity whilst D shows a reduction by hyperthermia.

PAS staining with ASPC-1 cells

In the visual grading, the highest intensity was scored with 10 points and the others were compared to this. The following score was obtained for treatment at 37° and 42°C (Figure 6, Table 3). The hyperthermic index was assessed as before with Z values.

There was a definite reduction in staining intensity by hyperther-

mia alone as indicated by the Z value of 1.430. The addition of NAC showed a reduction of efficacy, $Z=0.625$ indicating a protective role and hence an increase in mucin intensity. The addition of bromelain, oxaliplatin, oxaliplatin + NAC, Oxaliplatin + bromelain all indicated a reduction of mucin secretion as indicated by the Z value of 1.4. However, the triple combination showed no effect ($Z=1.0$).

Table 3. Hyperthermic index (Z) calculated from visual scoring. The highest intensity of staining (control) is given a score of 10 points.

No	Reagents	Visual scoring for staining intensity of mucin			Z
		(37°C)	(42°C)	% difference	
1	Control	10	7	-30 (Enhancement)	1.430
2	NAC	5	8	+60 (Reduction)	0.625
3	Bromelain (BR)	10	5	-50 (Enhancement)	2.000
4	NAC+BR	7	5	-28"	1.400
5	Oxaliplatin (OX)	7	5	-28"	1.400
6	OX+NAC	7	5	-28"	1.400
7	OX+BR	5	3	-40"	1.400
8	OX+NAC+BR	3	3	0 -	1.0

$Z > 1.0$ (enhancement); $Z = 0.9 - 1.0$ (no effect); $Z < 0.9$ (reduction of effect).

Pancreatic cancer cells – ASPC-1 at 37 & 42 deg C.

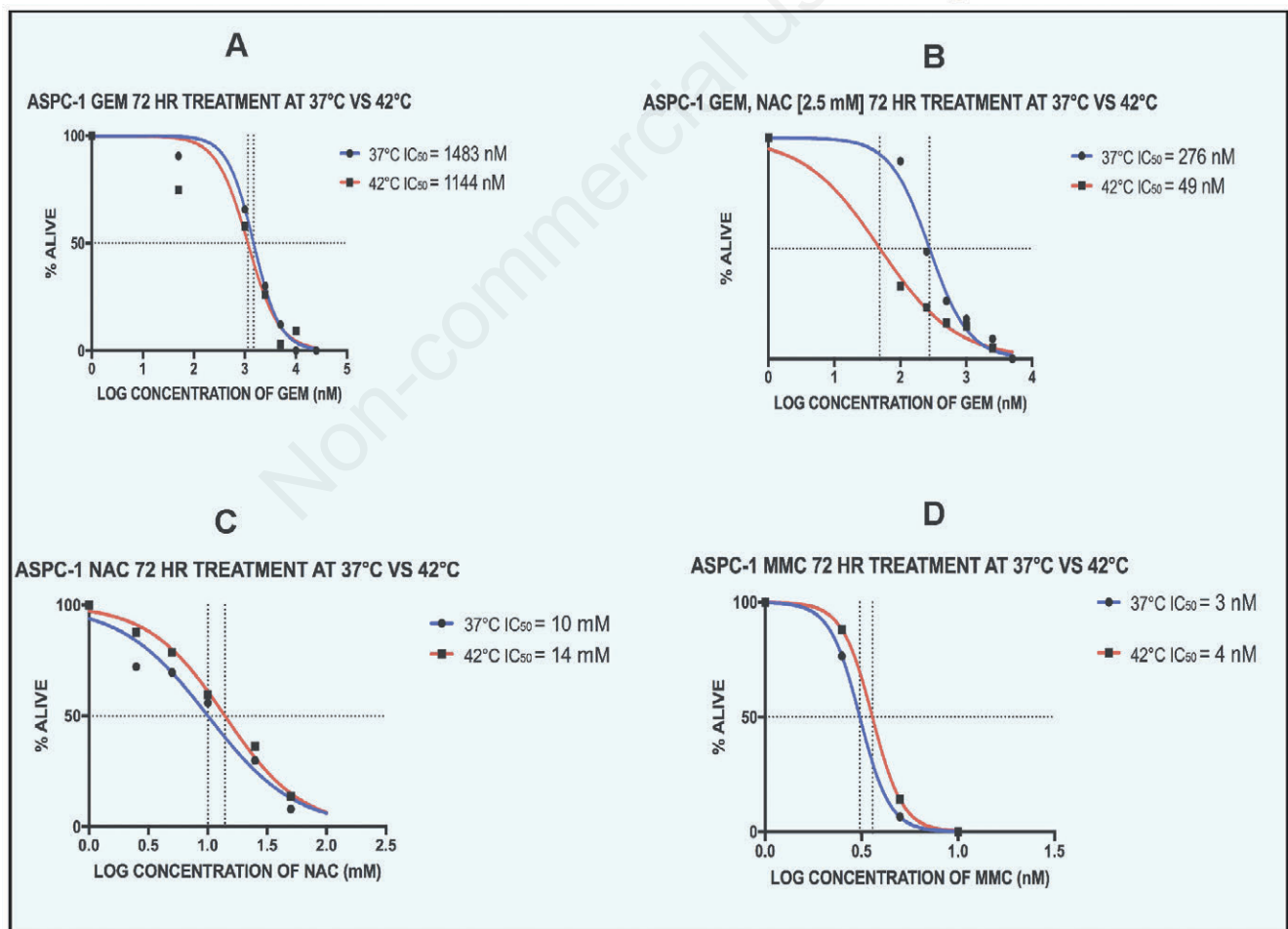


Figure 2. A-D) Shows the effect of hyperthermia on the IC50 values in ASPC1 cell lines when treated with different agents. Figures A & B shows enhancement of cytotoxicity whilst C & D shows a reduction by hyperthermia.

Discussion and Conclusions

In the present study, hyperthermia enhanced the cytotoxic effect of bromelain in pancreatic cell lines, CPPAC & ASPC-1 and in colorectal cell (HT29) whilst it had a reduced effect in LS174T cells. Based on the efficacy index (X), it was highly efficient with hyperthermia in HT29 with an efficacy index of 2.00 that may indicate that the both mucin and many other vital cellular components having 1-4 glycosidic linkages may be affected by bromelain. On the contrary, hyperthermic bromelain in LS174T was least affected and even showed a reduced hyperthermic effect indicating that bromelain in hyperthermia may be counterproductive. The predominance of either glycosidic linkages or the disulfide bonds in the polymeric mucin may dictate the reactivity of the cells to hyperthermic treatment with bromelain. This needs further investigation.

Hyperthermic NAC showed a dramatic increase in efficacy (X=1.727) in CFPAC cells, it showed a reduction in efficacy in both ASPC-1 (X=0.714) and HT29 (X=0.500) cells whilst it showed a null effect in LS174T. This may indicate that CFPAC may have mucin and other components that are predominantly

cross linked with disulfide bonds and the disintegration was enhanced with hyperthermia. On the other hand, ASPC-1 and HT29 showed a reduced effect indicating that disulfide linkages were probably minimal and hence the presence of NAC (antioxidant) may in fact provide a defence against hyperthermia with concomitant enhanced survival of the tumour cells.³¹ NAC, in the absence of substrate for reduction may in fact increase glutathione production and possibly heat shock proteins that may protect the cancer cells.^{32,33} Colorectal tumour cells (LS174T) did not respond to hyperthermia at all indicating that they are resistant to heat in the presence of NAC. Cytotoxicity was enhanced by the combination of NAC with bromelain in CFPAC and LS174T whilst there was a null effect with hyperthermia in HT29 and ASPC-1 cells. This may again indicate the heterogeneity of mucins and other cellular features within the cells.

Gemcitabine (2',2'-difluoro-2'-deoxycytidine; dFdC), is a pro-drug that requires conversion to the active triphosphate by doxycytidine kinases.³⁴ These enzymes may be thermodynamically affected by an increase in temperature in CFPAC, ASPC-1 and HT29 cells and hence enhance the effect of gemcitabine with hyperthermia. On the other hand, in LS174T, there is a slight reduction in

Colorectal cancer cells- HT29 at 37 & 42 deg C.

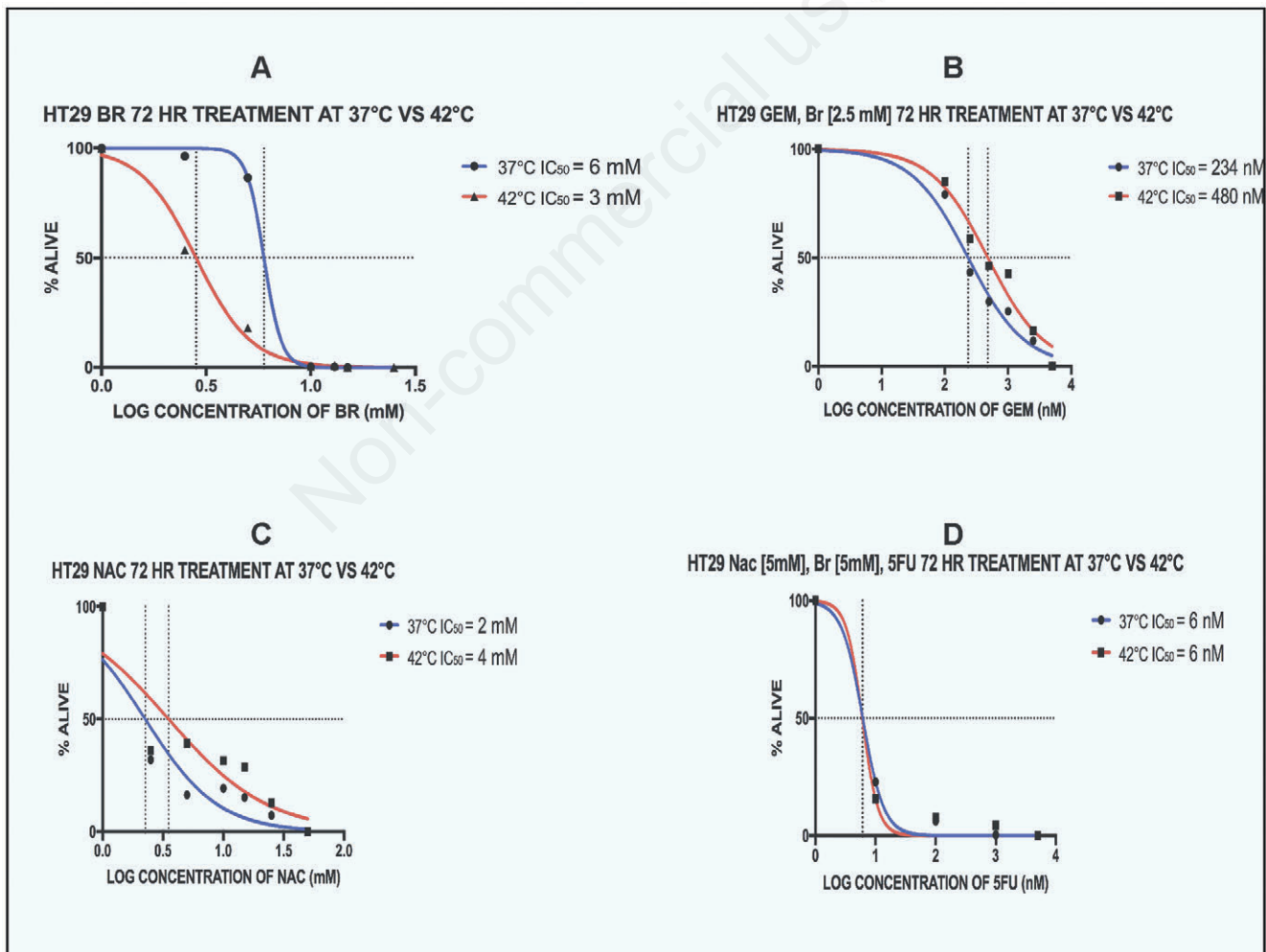


Figure 3. A-D) Shows the effect of hyperthermia on the IC50 values in HT29 cell lines when treated with different agents. Figure A shows cytotoxicity enhancement, B & C shows reduction whilst D shows a null effect by hyperthermia.

efficacy owing to temperature rise. Gemcitabine a hydrophilic molecule is normally transported through the cellular membrane by human nucleoside transporter proteins (hNTs) that are found on the cell membranes. If these are disrupted by temperature rise, then there will be a reduction of entry of gemcitabine.³⁵ The combination of bromelain and gemcitabine shows enhanced cytotoxicity due to hyperthermia in both CFPAC and LS174T cells. In the CFPAC cells, this was expected since individual agents have shown a positive effect of hyperthermia, however in the case of LS174T, it was least expected. The combination seems to overcome the negative effect of the separate agents. This paradox may be due to thermodynamic enhancement of gemcitabine cellular entry by bromelain, however further studies are necessary to decipher this observation.

The addition of NAC to gemcitabine showed a reduction in cytotoxicity with hyperthermia in CFPAC, HT29 and with an increased hyperthermic efficacy in LS174T cells and ASPC1. Antioxidants may interfere with the action of gemcitabine in CFPAC and HT29 cells by either reducing the conversion of gemcitabine into triphosphates in hyperthermia or may be affecting the entry of the molecule owing to denaturation of membrane surface cellular transport proteins.^{21,36} Remarkably, ASPC-1 cells responded very effectively with NAC with an X value of above 4.9, indi-

cating that hyperthermia is very effective in this case. Finally the triple combinations only were effective in CFPAC cells whilst it was negative for the other cells. This variation may again be due to the heterogeneity of cellular features.

Hyperthermia did not enhance the efficiency of oxaliplatin in any of the cell lines investigated, in fact, there was a reduction in efficacy in CFPAC, HT29, LS174T and with a null effect in ASPC-1 cells. With the addition of bromelain to oxaliplatin, hyperthermia showed slight enhancement of cytotoxicity in the pancreatic cell line CFPAC and with much higher efficacy in colorectal cells LS174T indicating that some molecular features such as mucin or other survival glycoproteins may be perturbed thereby enhancing cytotoxicity with hyperthermia. The addition of NAC showed hyperthermic enhancement in three cell lines (CFPAC, ASPC1 and HT29) with much higher reactivity in CFPAC and HT29 indicating the beneficial effect of an antioxidant that may be facilitating the action of oxaliplatin. The molecular mechanisms needs to be investigated, however one mode of action may be by enhancing the penetration of oxaliplatin through the cell membrane by preserving the integrity of the membrane at hyperthermia and hence thermodynamically accelerating the passage of oxaliplatin by carrier proteins.³⁷ However, the addition of bromelain to NAC + oxaliplatin (triple combinations) resulted in no hyperthermic effect in all the

Colorectal cancer cells – LS174T at 37 & 42 deg C.

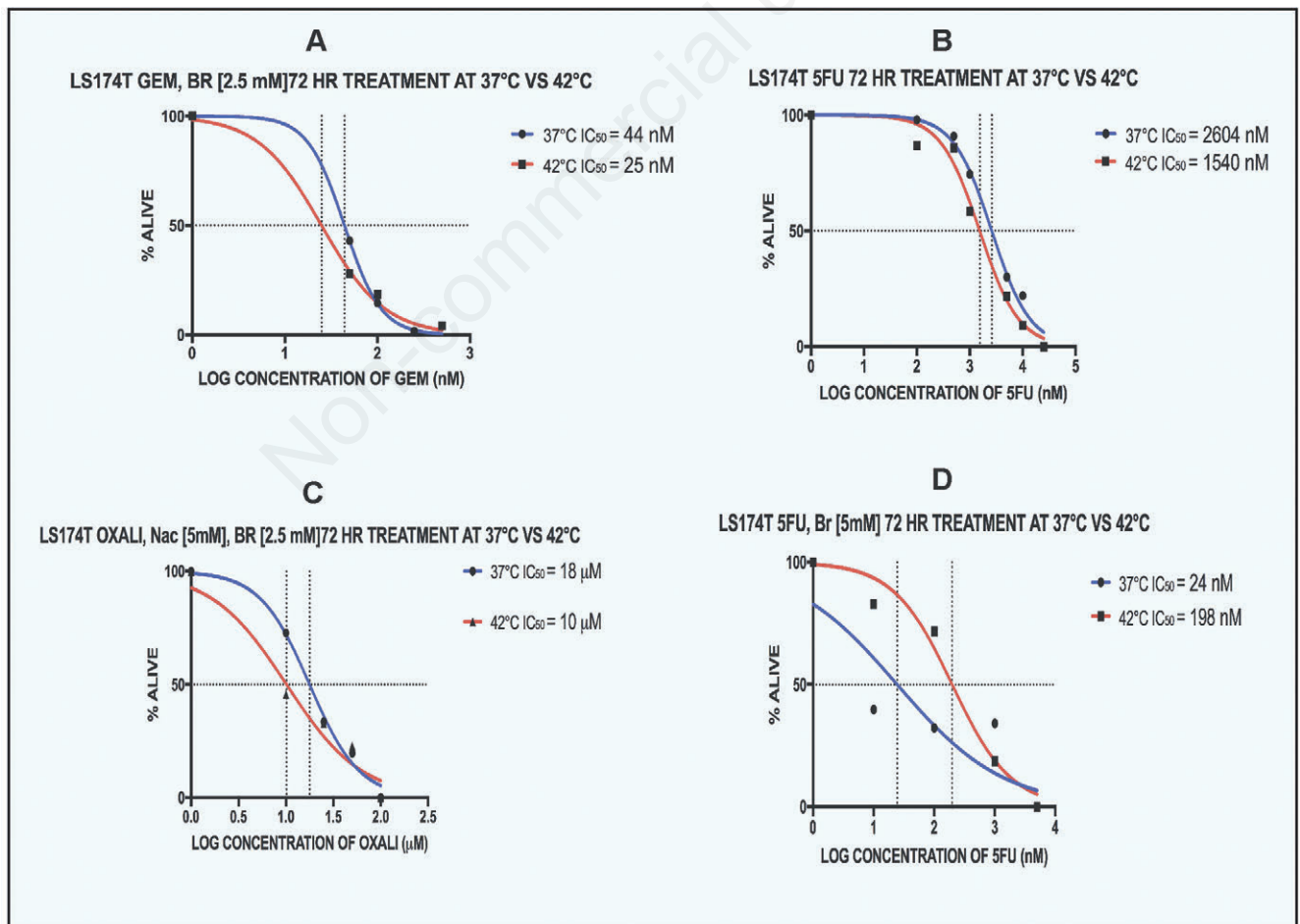
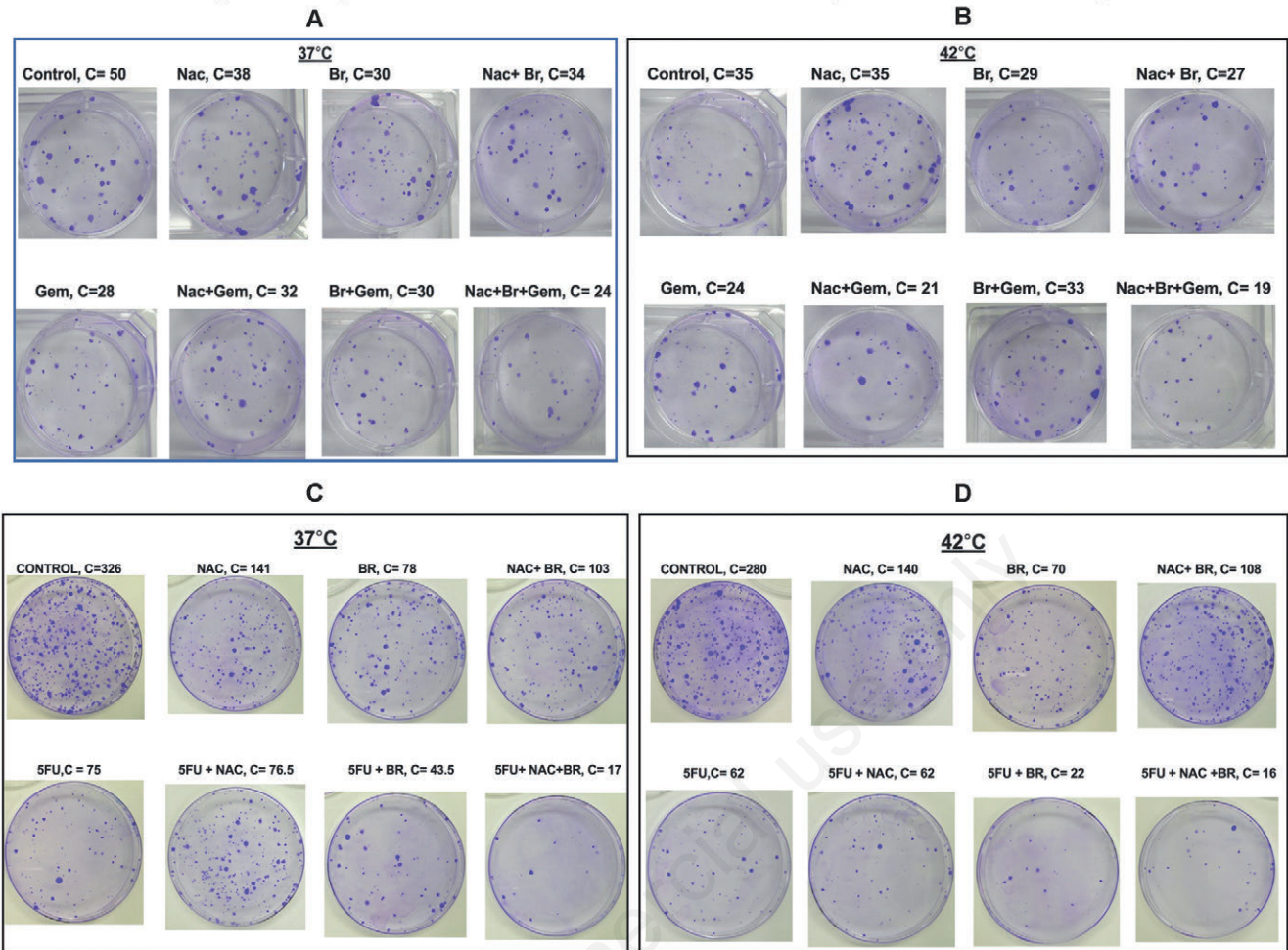


Figure 4. A-D) Shows the effect of hyperthermia on the IC₅₀ values in LS174T cell lines when treated with different agents. Figures A-C shows enhancement of cytotoxicity whilst D shows a considerable reduction by hyperthermia.

Colony count for pancreatic cancer cells CFPAC at 37 & 42 deg C treated to different agents



Figures 5. A-B) Shows the number of colony counts (C) in CFPAC cells when treated to gemcitabine (100 nM), NAC (5mM), Bromelain (5mM) and their combinations at similar concentrations at 37° and 42°C; C-D) shows the number of colony counts (C) in CFPAC cells when treated to 5-FU (1000nM), NAC (5mM), Bromelain (5mM) and their combinations at similar concentrations at 37° and 42°C.

PAS staining in pancreatic cancer cells ASPC-1 treated to different agents at 37 & 42 deg C

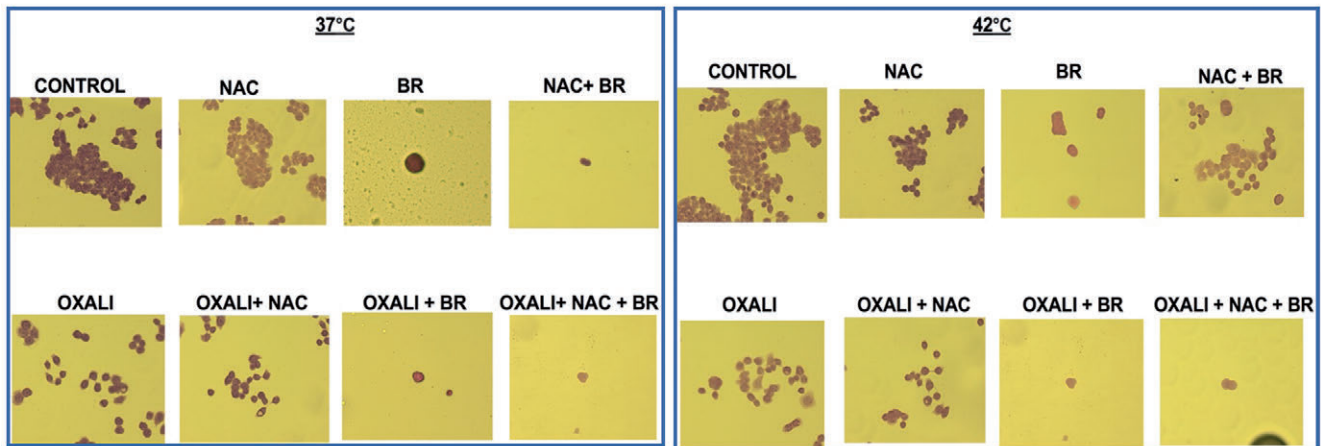


Figure 6. Shows the intensity of mucin staining in ASPC-1 cells by PAS reagent after treatment with different agents at 37° and 42°C.

cell lines except LS174T. Further studies are required to interpret this observation.

5-fluorouracil (5-FU), a uracil analogue is a widely used antimetabolite for the treatment of various cancers.³⁸ 5-FU has to be converted into one of its active metabolite *i.e.* 5-fluorodeoxyuridine monophosphate (5-FdUMP), 5-fluorodeoxyuridine triphosphate (5-FdUTP) or 5-fluorouridine triphosphate (5-FUTP).³⁹ 5-FdUMP is a suicide inhibitor of thymidylate synthase, the only de novo cellular source of deoxythymidine monophosphate (dTMP) and the depletion of dTMP leads to deoxynucleotide pool imbalance affecting DNA synthesis and repair.^{40,41}

Hyperthermia enhanced the efficacy of 5-FU in all the cell lines investigated except CFPAC cells indicating that there may have been an enhancement of intracellular penetration and subsequent conversion into active metabolite at 42°C. The enhancement was very high in LS174T ($X=1.691$). However, the addition of bromelain seems to interfere with the hyperthermic effect in all cell lines slightly except with a dramatic reduction in LS174T cells. The addition of NAC to 5FU was again counterproductive as indicated by the hyperthermic index values, there was only very slight enhancement of efficacy in ASPC-1 cells. Triple combination was like wise ineffective and in fact reduced the efficacy in CFPAC and with a dramatic reduction in efficacy in ASPC-1 ($X=0.221$). The addition of bromelain or NAC may be interfering with the enzymic conversion of 5-FU to its active metabolite.

Mitomycin C (MMC) is an antibiotic/antitumour agent that is widely used for treating a variety of cancers.^{42,43} MMC is a bio-reductive drug that needs activation into its biselectrophiles through a series of reactions initiated by the reduction of the quinone ring, thereby forming reactive intermediates with two electrophilic centres capable of interacting with DNA to form DNA-DNA adducts^{44,45} inducing cell death. Several reductases are involved in the conversion of MMC and recently GRP58, a protein with dithiol active site in its thioredoxin (Trx) like domains have been implicated in the activation of MMC.⁴⁶

Since MMC activation is an enzymatic reaction, increase in temperature may affect the reaction, particularly above 37°C. Very minute enhancement was observed with the addition of mitomycin C in CFPAC cells whilst it was reduced in both ASPC1 and LS174T cells. The addition of bromelain or NAC only showed minute enhancement of efficacy in HT29 cells and on the whole these cell lines seems to adopt a resistant profile to the treatment of the agents or their combinations with hyperthermia.

To determine the modulation of resistance by hyperthermia, the effect of individual agents and in combination with either gemcitabine or 5-FU were performed separately on pancreatic cancer cell line, CFPAC. The control showed that hyperthermia affected colony formation by reducing the number of colonies as indicated by the hyperthermic index ($Y=>1.0$) There was a null effect when NAC or bromelain was added ($Y=0$), however NAC + bromelain, gemcitabine, gemcitabine + NAC or gemcitabine + bromelain + NAC showed an increase in hyperthermic index ($Y=>1.0$) indicating a reduction of colony formation. Only the addition of bromelain to gemcitabine showed a reduction of hyperthermic index, ($Y<1.0$), indicating a protective role in hyperthermia, possibly by interfering with the penetration of gemcitabine.

In the case of 5-FU, hyperthermic effect was similar to gemcitabine but when bromelain and NAC were added together, the hyperthermic index fell below 1.0 indicating that there was an increase in colony formation. However, when 5-FU, 5-FU + NAC or 5-FU + bromelain were added, the hyperthermic index rose above 1.0 indicating a reduction of colony formation. The addition of bromelain and NAC to 5-FU showed a null effect. Hence, the effect of hyperthermia on colony formation and hence acquired

resistance was dependent on chemotherapeutic agent used with NAC and bromelain.

PAS staining to detect the effect of hyperthermia on mucin secretion treated with oxaliplatin, bromelain and NAC in pancreatic cancer cells ASPC-1 showed that heat alone reduced the secretion of mucin by about 30% but the presence of NAC prevented this effect by 60%. However, the addition of bromelain with hyperthermia reduced the secretion by 50% and likewise NAC + bromelain, oxaliplatin, oxaliplatin + NAC showed a reduction by 28%. The addition of bromelain to oxaliplatin reduced the secretion by 40% whilst the addition of the three agents had a null effect. From this observation, it appears that NAC has a protective effect against hyperthermia on mucin disintegration in ASPC-1 cells. On the other hand the mucin dissolution was enhanced by bromelain with hyperthermia. These observations seem to correlate with earlier experiments on the effect of hyperthermia on cytotoxicity in ASPC-1 cells.

Hence the present investigation suggests that hyperthermia with chemotherapy may not be beneficial for all chemotherapeutic drugs or in all types of cancers. Further when additional mucin disintegrating or chemotherapeutic agents were used either singly or in combinations, the chemotherapeutic effect in hyperthermia varied with cell types since cellular features were variable depending on cancer types and origin. Therefore, judicious use of chemotherapeutic agents or their combinations with bromelain or NAC are required for specific cancer types to achieve the positive goals of hyperthermia. However, further studies are required to determine how these agents interact with these cell lines on a molecular level in order to understand the mechanism of enhancement, stagnation or reduction in efficacy when subjected to hyperthermia.

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