Comparative study of antitumor effects of bromelain and papain in human cholangiocarcinoma cell lines

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Abstract. Cholangiocarcinoma (CC) worldwide is the most common biliary malignancy with poor prognostic value and new systemic treatments are desirable. Plant extracts like bromelain and papain, which are cysteine proteases from the fruit pineapple and papaya, are known to have antitumor activities. Therefore, in this study for the first time we investigated the anticancer effect of bromelain and papain in intra- and extrahepatic human CC cell lines. The effect of bromelain and papain on human CC cell growth, migration, invasion and epithelial plasticity was analyzed using cell proliferation, wound healing, invasion and apoptosis assay, as well as western blotting. Bromelain and papain lead to a decrease in the proliferation, invasion and migration of CC cells. Both plant extracts inhibited NF\(\kappa\)B/AMPK signalling as well as their downstream signalling proteins such as p-AKT, p-ERK, p-Stat3. Additionally, MMP9 and other epithelial-mesenchymal-transition markers were partially found to be downregulated. Apoptosis was induced after bromelain and papain treatment. Interestingly, bromelain showed an overall more effective inhibition of CC as compared to papain. siRNA mediated silencing of NF\(\kappa\)B on CC cells indicated that bromelain and papain have cytotoxic effects on human CC cell lines and bromelain and partially papain in comparison impair tumor growth by NF\(\kappa\)B/AMPK signalling. Especially bromelain can evolve as promising, potential therapeutic option that might open new insights for the treatment of human CC.

Introduction

Cholangiocarcinoma (CC) is a primary liver tumor arising from the epithelial cells lining the intra- and extrahepatic biliary tract system (1,2). The incidence and mortality rate of CC are increasing worldwide and they represent the second most common primary hepatobiliary cancer (3,4). Surgery is the only curative therapy, but CC is often diagnosed in advanced tumor stage (4,5). Currently, the combination of gemcitabine and cisplatin is the standard chemotherapy for patients undergoing first line palliative treatment (6). Many cytotoxic compounds failed to improve therapy and new antitumor treatments are urgently needed to improve survival.

Bromelain is an extract of pineapple (Ananas comosus) containing a mixture of proteases and non-protease components (7). Bromelain is sold in health food stores as a nutritional supplement to promote digestion, wound healing and as an anti-inflammatory agent (8). Oral treatment is well tolerated and there is traditional and anecdotal evidence that bromelain has different properties (7-10). Obviously, bromelain was tested in different experimental and clinical studies and it was demonstrated that this plant food has anticancer and anti-inflammatory activities (7,10-26). However, the precise molecular mechanisms are not fully understood.

Papain is a sulfhydryl protease from Carica papaya latex with a powerful digestive action superior to pancreatin, or pancreatic enzymes (27). Papain has high concentration in the fruit, stem, leaves, fruit skin and seeds. The compound has toxic effects on plant-eating bugs, supported wound healing and the juice of ripe papaya shows anti-oxidant effect (28-30). Papain is a known ingredient of herbal medicine in different countries, but there is very limited information on the molecular targets and anticancer effects. Ground-breaking studies for both compounds as anticancer therapies for CC are lacking so far. Using a model of human CC cell lines, we investigated for the first time the antitumor activity of both bromelain and papain on CC.

Materials and methods

Cell culture. Human CC cell lines (TFK-1, SZ-1) were generously provided by Nisar Malek (31). Cell lines were cultured in RPMI-1640 + Glutamax (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Biochrom, Berlin, Germany)
and 100 U/ml penicillin/streptomycin (Invitrogen) at 37°C in 5% CO₂.

**Compound preparation and in vitro treatment.** Bromelain (Sigma-Aldrich, Germany - 25 mg, 100 mM) stock solutions were prepared by dissolving them in dimethyl sulfoxide, DMSO (Applichem, Darmstadt, Germany). Papain (Sigma-Aldrich, 100 mM) stock solutions were prepared by dissolving them in distilled water. Cells were treated with DMSO or bromelain (150 and 200 µM) or papain (25, 40 and 100 µM) in different concentrations (100 mM) and were analyzed after 24, 48 and 96 h.

**Proliferation assay.** In order to measure the effect of bromelain and papain on cell proliferation, cells were plated at a concentration of 2,000 cells/ml in a 96-well plate overnight. Cells were treated with DMSO, different concentrations of bromelain (150 and 200 µM) or papain (25, 40 and 100 µM) for different time-points (1-4 days). At the respective time-points, 10 µl WST-1 reagent (Roche Diagnostics, Mannheim, Germany) was added to each well and incubated for 2 h at 37°C. The absorbance was detected at a wavelength of 492 nm with reference wavelength of 650 nm.

**Invasion assay.** Cells (2.5x10⁵ cells/ml) were seeded in serum-free media into each well of the 6-well BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences, Bedford, UK). The cells in the inserts were simultaneously treated with bromelain (150 and 200 µM) or papain (25, 40 and 100 µM) and the DMSO control. The inserts were placed into the BD Falcon TC Companon Plate containing 10% FCS and incubated for 48 h in a humidified tissue culture incubator, at 37°C, 5% CO₂ atmosphere. Then the invading cells were fixed with 100% methanol and stained with 1% toluidine blue in 1% borax. Cells were then counted under a microscope (Leica DM 5000 B, Leica, Wetzlar, Germany). The calculation of the invading cells were done according to the BD protocol where:

\[
\text{Invasion index} = \frac{\% \text{ invasion test cell}}{\% \text{ invasion control cell}}
\]

**Migration assay.** Cells were seeded in a 6-well plate and left to reach 80% confluency. Initially, cells were starved for 24 h in media containing 2% FCS. Then SZ-1 and TFK-1 were further incubated for 48 h in the starvation media containing either the control with DMSO, different concentrations of bromelain (150 and 200 µM) or papain (25, 40 and 100 µM). Afterwards a scratch was done using a white tip for each treatment. Then cells were washed with PBS and photographed using Leica DMI 6000 B microscope (Leica, Wetzlar, Germany). The images were taken of the wounded area. The migrating cells were calculated according to the following formula:

\[
\text{Migration index} = \frac{\text{Width of the wound at 0 h} - \text{Width of the wound at 24 h}}{\text{Width of the wound}} \times 100
\]

**Protein extraction and western blotting.** SZ-1 and TFK-1 cells cultured with bromelain or papain treatment for immuno-blotts were collected and rinsed with cold phosphate-buffered saline (PBS). Then harvested cells were lysed in lysis buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and protease and phosphatase inhibitor (Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany). The concentration of extracted protein was determined using DC protein assay kit (Bio-Rad, München, Germany) according to the manufacturer’s instructions. The absorption was measured at 650-750 nm using a microplate reader (Titer-trek-Berthold, Pforzheim, Germany). For immune blotting the cell lysates were loaded at a protein concentration of 30 µg per well. Gel electrophoresis (12% acrylamide gels) was performed (Bio-Rad, München, Germany). The membranes were blocked using 5% dried milk (Applichem) for 30 min at room temperature, then they were probed with primary antibodies against E-cadherin (1:1,000, Cell Signaling Technology, 24E10), N-cadherin (2:10,000; Millipore, EPR1792Y), actin (2:10,000; Sigma, AC-74), Akt (1:1,000; Cell Signaling Technology, 9131s), Rac-1 (1:000; Cell Signaling Technology, 9271s), STAT3 (1:1,000; Cell Signaling Technology, 9132), p-Stat3 (Tyr705) (1:1,000; Cell Signaling Technology, 9271s), STAT3 (1:1,000; Cell Signaling Technology, 9131s), Rac-1 (1:000; Cytoskeleton, Inc., ARCO3-A), PARP (1:1,000; Cell Signaling Technology, 9542), AMPKα (1:1,000; Cell Signaling Technology, 4685s), phospho-p44/p42 MAPK (Erk1/2) (1:1,000; Cell Signaling Technology, 9101), AMPKα (1:500, Cell Signaling Technology, 5831s), phospho-AMPKα (1:500, Cell Signaling Technology, 2535s), NFkB p65 (1:1,000; Abcam, ab16502).

**Small interfering RNA (siRNA) knockdown of NFkB.** SZ-1 and TFK-1 cells were plated at a concentration of 1x10⁵ cells/ml in a 6-well plate. After 24 h, cells were transfected with control siRNA-A (Santa Cruz Biotechnology, CA, USA) 5, 10 and 20 nM NFkB siRNA (Santa Cruz Biotechnology, sc-29410) using hiperfect transfection reagent (Qiagen, Germany) for 48 h. Trial experiments were performed with different concentrations of siRNA oligonucleotides at different time-points. The transfection efficiency was judged based on western blot analysis of NFkB.

**Apoptosis.** To determine the apoptosis, SZ-1 and TFK-1 cells were seeded (1x10⁴ cells/ml) in a 6-well plate. After 24 h, cells were transfected with control siRNA-A (Santa Cruz Biotechnology, CA, USA) 5, 10 and 20 nM NFkB siRNA (Santa Cruz Biotechnology, sc-29410) using hiperfect transfection reagent (Qiagen, Germany) for 48 h. Trial experiments were performed with different concentrations of siRNA oligonucleotides at different time-points. The transfection efficiency was judged based on western blot analysis of NFkB.

**Statistical analysis.** All the experiments were repeated 3 times. The results were analyzed using software Graphpad prism version 5.0 (GraphPad Software, San Diego, CA, USA) and SPSS Version 11.0 (SPSS, Chicago, IL, USA). The tests include one way ANOVA analysis of variance and Student's t-test.
t-test along with Bonferroni post test and paired and unpaired t-tests. Differences were considered as statistically significant when the P-value was <0.05, <0.005 and <0.001; and not significant (ns).

Results

**Bromelain and papain attenuates the viability of CC cells.** To analyze the anti-proliferative potential of bromelain and papain on TFK-1 and SZ-1 cells we determined first the IC\textsubscript{50} value for papain by constructing a dose-response growth curve and examined the effect of different concentrations of papain on CC cells. Taking into account the calculated IC\textsubscript{50}, papain was used in three concentrations: 25, 40 and 100 µM (Fig. 1A and B). For bromelain therapy the previously published IC\textsubscript{50} values were taken into account and dosage of 150 and 200 µM were used (25,26) (Fig. 1C and D). As shown (Fig. 1) both bromelain and papain treatment effectively decreases the proliferation of viable CC cells for 24, 48, 72 and 96 h. The highest anti-proliferative effect for both bromelain and papain was observed at 96 h under highest dosages (200 and 100 µM for bromelain and papain, respectively).

**Bromelain and papain treatment inhibits migration and invasion of CC cells.** We next examined the effect of bromelain (150 and 200 µM) and papain (25, 40 and 100 µM) on cell motility by wound healing assays (Fig. 2). As shown (Fig. 2A-C) and (D), bromelain inhibited significantly cell invasion in a dose-dependent manner. Approximately 90% decrease in the number of invading cells was observed compared to the control group. Papain also showed similar effective inhibition of invasion in both CC cells compared to the control (Fig. 2A, C and E).

**Bromelain and papain treatment induces apoptosis in CC cells.** In order to elucidate that the anti-proliferative effect of both compounds (bromelain and papain) the induction of apoptosis, Annexin V/PI staining was performed and the percentage of apoptotic cells was determined by FACS analysis (Fig. 4). The results clearly showed that bromelain treatment induces relatively high rate of apoptosis on TFK-1 and SZ-1 cells in comparison to SZ-1 cells (Fig. 2A-C). The highest inhibition effect was observed by papain in TFK-1 cells under 100 µM treatment. Effective (p<0.05) inhibition of wound healing was observed under 150 and 200 µM bromelain in both CC cells as compared to the DMSO controls (Fig. 2D-F). Next, we tested cell invasion using Matrigel-coated Transwell chambers under DMSO and bromelain (150 and 200 µM) and papain (25, 40 and 100 µM) (Fig. 3). As shown (Fig. 3B, D and F), bromelain inhibited significantly cell invasion in a dose-dependent manner. Approximately 90% decrease in the number of invading cells was observed compared to the control group. Papain also showed similar effective inhibition of invasion in both CC cells compared to the control (Fig. 3A, C and E).

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Bromelain effectively inhibits NFκB/AMPK pathway and common downstream effectors in CC cells in contrast to papain. NFκB/AMPK signaling has been implicated as an important pathway involved in the carcinogenesis of several tumor diseases (32). In order to gain more insight into the anticancer mechanism exerted by both bromelain and papain in CC cells, we checked the status of AMPK and NFκB both before and after treatment by immunoblotting. In both cell lines (TFK-1, SZ-1) bromelain (150 and 200 µM) effectively downregulated NFκB and other common downstream proteins such as phospho-AKT, phospho-ERK and phospho-STAT3, but the full forms remained unchanged in both cell lines for 48 and 96 h (Fig. 5). An effective upregulation of phospho-AMPK was observed under bromelain treatment for both time-points in a time- and dose-dependent manner (Fig. 5C and D). Thus, clearly indicating effective inhibition of the NFκB/AMPK pathway in CC cells by bromelain. In contrast, papain treatment (25, 40 and 100 µM) did not show effective downregulation of NFκB in either CC cell line (Fig. 5A and B). For SZ-1 cells under papain treatment full and phosphorylated forms of ERK, STAT3 remained unchanged for all tested time-points (Fig. 5B). In contrast, AKT full form remained unchanged but the phosphorylated form showed slight downregulation in SZ-1 under 100 µM of papain treatment specifically in 96 h (Fig. 5B). In TFK-1 cells papain treatment effectively downregulated phospho-STAT3 in all dosages (25, 40 and 100 µM) specifically after 96 h (Fig. 5A). Full forms of other effector proteins like AKT, ERK, STAT3 remained stable under papain treatment but the phosphorylated forms were slightly downregulated mostly under 100 µM of treatment at 96 h (Fig. 5A and B). Interestingly, NFκB and AMPK levels remained mostly unaltered under papain treatment for both CC cell lines for all time-points compared to the respective controls. Moreover, phosphorylated AMPK showed considerable upregulation in SZ-1 cells under 100 µM of...
papain treatment for both time-points (48 and 96 h) (Fig. 5B). In TFK-1 cells slight upregulation of phosphorylated AMPK was observed in a dose-dependent manner of papain treatment in comparison to the control (Fig. 5A). Thus, papain treatment partially influences, but does not inhibit NFκB/AMPK signaling in contrast to bromelain in CC cells.

We further reconfirmed the importance of NFκB status for bromelain anticancer activity in CC cells by NFκB siRNA. NFκB silencing in both TFK-1 and SZ-1 cells showed effective inhibition of all the common downstream proteins, such as AKT and ERK, as shown in Fig. 6A and B. Importantly, 200 µM of bromelain treatment achieved similar results of effective inhibition of all downstream effector proteins as that with NFκB siRNA for both tested CC cells. However, 100 µM of papain treatment did not show effective inhibition of any of the above mentioned common downstream effector proteins.

Figure 3. Bromelain and papain inhibits invasion of CC cells. SZ-1 (C-F) and TFK-1 (A, B, E and F) cells were treated for 48 h with bromelain (150 and 200 µM), papain (25, 40 and 100 µM), or their controls (DMSO, H2O). The number of cells that invaded through the membrane was determined by light microscope (x20 magnification) counterstained and invasion index was calculated as described in Materials and methods and plotted in bar graphs. Differences were statistically significant at ***P<0.001. Data are expressed as mean ± SD of triplicates.
in contrast to NFκB silencing compared to the controls for both CC cell lines (Fig. 6). Based on these results bromelain effectively impairs CC carcinogenesis via inhibition of NFκB/AMPK signaling, but not papain.

**Bromelain significantly impairs EMT in comparison to papain in CC cells.** EMT is considered a key process driving invasion and metastasis. Therefore, we examined whether bromelain or papain can attenuate EMT. CC cells were treated with either bromelain (150 and 200 µM), papain (25, 40 and 100 µM) or DMSO for 48 and 96 h. The expression of EMT markers (E-cadherin and N-cadherin) was evaluated by western blotting (Fig. 7). Bromelain treatment showed dose- and time-dependent increase in expression of the epithelial marker E-cadherin (Fig. 7B and D) and downregulation of the mesenchymal marker N-cadherin both in TFK-1 and SZ-1 cells (Fig. 7B and D). Notably, the expression of both E- and N-cadherin remained relatively unaltered under papain treatments (25, 40 and 100 µM) in both CC cell lines for 48 and 96 h (Fig. 7A and C). Our immunoblots clearly show the effective inhibition of EMT by bromelain in contrast to papain, which shows no influence on EMT in human CC cells.

![Figure 4](image-url)
Discussion

Plant derived extracts such as bromelain and papain have been used for herbal medicine and are easy to acquire. However, the exact molecular modes of actions and therapeutic effects are not fully understood. So far, no trials have been conducted to test the efficacy of bromelain and papain in human CC. We hypothesised that both compounds might impair CC carcinogenesis and investigated their efficacy in inhibiting growth and proliferation of human CC cell lines (TFK-1, SZ-1). In this study, we found that bromelain and papain have potent antitumor activity against CC. We observed four major findings: i) anticancer effect of bromelain was more effective compared to papain, ii) significant restriction of CC cells was mainly achieved via inhibition of NFκB/AMPK pathway, iii) therapy induced apoptosis, iv) EMT was only partially influenced. Importantly, in our experiments we used two human CC cell lines from different origins: TFK-1 cultured from an extrahepatic primary tumor and SZ-1 cultured from an intrahepatic primary tumor (31).

Bromelain was tested in vitro and in vivo cancer models and it was demonstrated in a skin papilloma model that bromelain application reduced tumor formation by apoptotic cell death (33). It was also reported that bromelain influences metastasis, local tumor growth, cell growth and migration (16-19). Furthermore, it is known that bromelain can down-regulate NFκB and Cox-regression in papillomas and skin cancer (34). For papain it is known that anti-angiogenic effects are accessible (35). In addition, papain has anti-inflammatory, anti-infectious and anti-diabetic characteristics (36). We found that both plant extracts significantly suppressed CC growth, influencing cell migration and invasion. Since bromelain was also previously tested for anticancer ability in other tumor models as described above we selected two widely used and established dosages (150 and 200 µM) (25,26). Our experiments on CC cells also reconfirmed these two pre-established dosages.
dosages as the most effective ones for the inhibition studies in CC cells. Interestingly, papain has not been tested as an anticancer drug, therefore, in our study we for the first time performed IC₅₀ experiments and selected three dosages that were found to be suitable for CC cell inhibition. Notably, the dosages used for papain were comparatively lower than to bromelain mainly because we picked up three dosages for papain that covered a range from low to high (25-100 µM). Notably, 100 µM of papain treatment failed to show effective downregulation of the common downstream proteins. This difference could possibly be due to the existence of some feedback loop with other cancer pathways in CC which in turn can upregulate or activate the common downstreams. Thus, reactivating the CC carcinogenesis process which was temporarily restricted by papain treatment. Papain treatment ceases or stops the proliferation of CC cells temporarily, but
fails to effectively inhibit any cancer signaling cascade or their crosstalk involved in CC carcinogenesis. On the contrary, bromelain not only inhibits proliferation of CC cells but also effectively downregulates the common downstream proteins leading to complete inhibition of CC carcinogenesis mainly via the NFκB/AMPK signalling pathway.

Our results clearly showed that the degree of effectiveness of a plant extract greatly depends on the source, as papain even in higher dosages did not show effective inhibition of AMPK NFκB signalling. The cytotoxic effect of bromelain and papain seemed to be caused by apoptotic cell death. We tried to further elucidate the mechanisms by which the cysteine proteases affect CC cells and to understand the differences between the compounds. Therefore, we investigated the expression status of NFκB and the phosphorylation status of AMPK on protein level. Bromelain treatment caused a significant decrease of NFκB and increase in AMPK phosphorylation. In contrast, papain did not alter NFκB/AMPK signalling significantly. The NFκB/AMPK signalling pathway is amongst others important for the immune system (37). Studies have demonstrated that AMPK signalling downregulates the function of NFκB (38). We tested the status of NFκB and AMPK under treatment with bromelain and papain on CC cells by selective knockdown using siRNA and compared the results with bromelain/papain single treatment on CC cells. We also further analyzed some important selective downstream effector proteins such as AKT, ERK and STAT3, known to be crucial for inhibition studies. Under treatment upregulation of phosphorylated AMPK was observed, thus, leading to the inhibition or effect of the NFκB signaling. The cytotoxic effect of bromelain and papain was confirmed by impaired immunocytotoxicity of mononuclear cells from patients with CC. Moreover, AMPK inhibition rather than papain as a potential therapy for human CC and that our results clearly showed that the degree of effectiveness of a plant extract greatly depends on the source, as papain even in higher dosages did not show effective inhibition of AMPK NFκB signalling. The cytotoxic effect of bromelain and papain seemed to be caused by apoptotic cell death. We tried to further elucidate the mechanisms by which the cysteine proteases affect CC cells and to understand the differences between the compounds. Therefore, we investigated the expression status of NFκB and the phosphorylation status of AMPK on protein level. Bromelain treatment caused a significant decrease of NFκB and increase in AMPK phosphorylation. In contrast, papain did not alter NFκB/AMPK signalling significantly. The NFκB/AMPK signalling pathway is amongst others important for the immune system (37). Studies have demonstrated that AMPK signalling downregulates the function of NFκB (38). We tested the status of NFκB and AMPK under treatment with bromelain and papain on CC cells by selective knockdown using siRNA and compared the results with bromelain/papain single treatment on CC cells.

References


