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Growth inhibitory effects of casein hydrolysates on human cancer cell lines

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The aim of this study was to investigate the effects of unhydrolysed/intact casein and eight different sodium casein hydrolysates **(a–h)** on the viability and growth of human cancer cell lines. Both human Jurkat T cells and Caco-2 cells were incubated with increasing concentrations of the test compounds (0·5–10% v/v) for 24 h. Cell viability was assessed using the MTT, lactate dehydrogenase (LDH) release and Trypan Blue assays. Cell growth was monitored using the MTT, Trypan Blue and Bromodeoxyuridine (BrdU) proliferation assays. Casein hydrolysates b, c and f had an inhibitory effect on the viability and growth of both cell lines. The casein hydrolysates did not negatively affect the membrane integrity of both Jurkat and Caco-2 cells. In Jurkat cells hydrolysates a and h had an inhibitory effect on DNA synthesis after 24 h, while in Caco-2 cells DNA synthesis was not affected. In conclusion, we found that the different casein hydrolysates had cell-specific effects which target particular functions within the cell. Overall, casein hydrolysates had no effect on membrane integrity while they had varied effects on mitochondrial activity and DNA synthesis in the different cell lines.

Keywords: Casein hydrolysates, Caco-2 cells, Jurkat cells, growth, viability.

Epidemiological evidence suggests that people who regularly consume fermented dairy foods have a reduced risk of developing certain cancers (van't Veer et al. 1989). Milk proteins, which have long been known for their nutritional and technological benefits, have also been shown to possess bioactive properties in addition to their nutritive value (Hartmann et al. 2007; Phelan et al. 2009). For instance, cytochemical studies have provided evidence that milk protein-derived peptides can affect the viability and growth of cancer cells (Mac Donald et al. 1994; Hartmann & Meisel, 2004).

Casein and other milk proteins provide a rich source of bioactive peptides that have physiological roles in the human body (Korhonen & Pihlanto, 2006). These peptides affect the major systems including the cardiovascular, immune, nervous and digestive systems (for review see Korhonen & Pihlanto, 2006). Furthermore, there is a considerable amount of scientific evidence from both in vitro and in vivo studies to suggest that bioactive peptides regulate specific physiological functions. Milk-derived peptides are inactive within the sequences of the parent protein, such as casein, but can be released during gastrointestinal digestion, fermentation and food processing (Korhonen & Pihlanto-Leppälä, 2001; FitzGerald & Meisel, 2003). Unravelling the mechanisms through which dietary factors alter the gastrointestinal environment to prevent or promote tumour formation is a major challenge and may never be completely performed using in vivo experimentation. However, in vitro models may provide clues to help us understand these mechanisms (Cameron, 1990).

Although Jing and Kitts (2004) showed that casein does not affect cellular enzymes, Laparra et al. (2008) reported that caseinophosphopeptides reduced glutathione (GSH) concentration and increased GSH-reductase activity in Caco-2 cells. More recently, we demonstrated that eight distinct casein hydrolysates may exert specific antioxidant and immunomodulatory effects on human Jurkat T cells (Phelan et al. 2009). Furthermore, studies have provided increasing evidence that food-derived bioactive peptides modulate the viability (e.g. proliferation, differentiation, and apoptosis) of different cell types such as Int-407 cells (Jing & Kitts, 2004), HL-60 cells (Hartmann & Meisel, 2004) and Jurkat T cells (Phelan et al. 2009). Therefore, the aim of the present study was to further investigate the effects of sodium caseinate and eight distinct casein hydrolysates on the viability and growth of both human

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Jurkat T cells and intestinal Caco-2 cells. Cell viability was assessed using the MTT (mitochondrial activity), lactate dehydrogenase (LDH) release (membrane integrity) and Trypan Blue (membrane integrity) assays. Cell growth was monitored using the MTT, Trypan Blue and Bromodeoxyuridine (BrdU) proliferation (DNA synthesis) assays. Hence, these assays give a broad insight into the effects of the test samples on cell membrane integrity, mitochondrial activity, and DNA synthesis of different cell lines.

Materials and Methods

Materials

Human Jurkat T cells and human adenocarcinoma Caco-2 cells were purchased from the European Collection of Animal Cell Cultures (Salisbury,Wiltshire, SP4 0JG, UK). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, PA4 9RF, Scotland). Cell culture plastics were supplied by Cruinn Diagnostics (Greiner Bio-One, Frickenhausen, Germany). All other cell culture reagents and chemicals were purchased from Sigma-Aldrich Chemical Co. (Airton Road, Tallaght, Dublin, Ireland) unless otherwise stated.

Generation of casein hydrolysates

Sodium caseinate (NaCN, 85·92% w/w protein) was provided by Arrabawn Co-op Society Ltd., Tipperary, Ireland. The protein content of the NaCN substrate was determined using the macro-Kjeldahl method (IDF, 2001), a Kjeldahl conversion factor of 6·38 was used. NaCN hydrolysates were generated using different commercially available food-grade enzyme preparations from mammalian, bacterial and plant sources. For hydrolysis with neutral proteinase preparations, aqueous solutions of NaCN ranging in concentration from 9·3 to 10·6% (w/v) protein were incubated at 50 °C at pH 7. The pH was maintained constant using a pH stat (Titrino 718, Metrohm, Herisau, Switzerland) charged with 2 M-NaOH as previously described (Flanagan & FitzGerald, 2002). In the case of hydrolysis with acid proteinase, the NaCN concentration was 5% (w/v) while the pH was maintained constant at pH 2·4 using 1 M-HCl. Enzyme inactivation was by heat treatment at 80 °C for 20 min. Hydrolysate samples were stored at −18 °C prior to subsequent analysis. The reversed-phase HPLC profile of each hydrolysate has been outlined elsewhere (Phelan et al. 2009).

Cell culture

Jurkat cells were grown in RPMI medium supplemented with 5% (v/v) FBS. Caco-2 cells were grown in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) non-essential amino acids. Both cell lines were cultured in an atmosphere of CO_2-air (5 : 95, v/v) at 37 °C and were maintained in the absence of antibiotics. The casein hydrolysates were filter sterilised (0·2 μm diameter) and diluted directly with sterile RPMI medium. Samples a, c, d, h and NaCN were diluted 1/10 prior to filtration as the concentrated samples could not pass through the filter membranes (0·2 and 0·45 μm diameter) undiluted. Control cultures comprised of cells grown in media only.

Incubation of cells with test compounds

For all experiments, Jurkat cells were seeded at a density of 2×10^5 cells/ml and Caco-2 cells were seeded at 3×10^4 cells/cm^2. Jurkat cells were supplemented with increasing concentrations (0·5–10% v/v) of NaCN and its hydrolysates (a–h) for 24 h. Caco-2 cells were allowed adhere for 24 h in complete media. After 24 h, the culture media was removed and replaced with growth medium containing 2·5% (v/v) FBS, 1% (v/v) NEAA and the test samples (0·5–10% v/v) for 24 h.

MTT assay

Measurement of mitochondrial activity can be carried out by assessing the activity of the enzyme mitochondrial reductase (Mosmann, 1983; Ciapetti et al. 1993). Mitochondrial activity was determined using the MTT assay (MTT I proliferation kit, Roche Diagnostics; West Sussex, RH15 9RY, UK). The assay is based on the conversion of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its purple formazan product (Mosmann, 1983). At the time of assay, 10 μl pre-warmed MTT labelling reagent was added to each well of a 96-well plate and then incubated for a further 4 h. Solubilising solution (100 μl) was then added to all wells and incubated overnight at 37 °C. Absorbance was measured at 570 nm, with a reference wavelength of 690 nm, using a microplate reader.

LDH Release assay

Release of LDH into the medium is an indication of compromised membrane integrity (Koh & Choi, 1987), which was measured using an in vitro LDH release assay kit (Biogenesis, Dorset, BH17 7DA, UK). At the time of assay, 100 μl media was collected from all test wells of a 96-well plate. Substrate mix (100 μl) was added to each sample and incubated at room temperature for 1 h. HCl (1 M, 50 μl) was then added to each well and the plate was centrifuged at $716\times g$ for 5 min. Absorbance was measured at 492 nm using a microplate reader. LDH release was expressed as fold difference relative to the control.

Trypan Blue Exclusion assay

This assay is based on the principle that viable cells will exclude trypan blue dye, whereas dead or dying cells

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Table 1. Viability of Caco-2 cells incubated with casein hydrolysates (a–h) and sodium caseinate for 24 h†

Values are the mean ± SE for $n=4$ independent experiments

	Cell viability (%)‡ Casein hydrolysates								
(% v/v)	a	b	c	d	e	f	g	h	NaCN
Caco-2 cells									
0·0	100·0±7·9	100·0±7·9	100·0±7·9	100·0±7·9	100·0±7·9	100·0±7·9	100·0±7·9	100·0±7·9	100·0±7·9
0·5	93·2±3·6	105·8±5·5	101·4±8·0	101·4±5·0	81·7±3·5	78·3±4·8	107·5±8·8	108·3±9·7	89·6±11·5
1·3	81·2±6·0	81·8±4·4	92·3±7·9	95·8±5·7	75·7±7·7	71·8±5·7*	103·3±5·9	111·9±6·2	92·7±9·2
2·5	82·5±6·0	4·7±6·5	82·9±6·8	103·1±9·5	68·9±2·2	72·1±5·7*	100·9±1·9	103·6±5·9	74·1±3·1
5·0	74·8±5·1	69·5±7·8*	67·3±5·6*	93·2±9·2	86·8±13·5	71·7±8·1*	103·3±11·2	97·8±7·6	82·5±4·1
10·0	70·6±3·8	70·9±8·2*	67·1±6·5*	86·4±7·4	86·8±8·3	72·9±7·8*	105·8±4·6	103·7±6·2	84·3±5·3

NaCN, sodium caseinate (which was only available at the time when using Caco-2 cells)

† Caco-2 cells (3×10^4 cells/cm^2) were supplemented with increasing concentrations of casein hydrolysates (**a–h**) and sodium caseinate for 24 h

‡ Cell viability was determined by the MTT assay and expressed as a percentage of the control

* $P<0·05$ compared with control (0% v/v): one-way ANOVA, Dunnett's test

absorb the dye and, thus, appear blue (Aras et al. 2008). At the time of assay, 1·5 ml Jurkat cells in media was centrifuged at 100 × ***g*** for 5 min. The supernatant was removed and the cell pellet was re-suspended in PBS. Trypan blue (50 μl) was mixed with 50 μl cell suspension (1 : 1) and incubated at room temperature for 3 min. The mixture was loaded onto a haemocytometer and cells were analysed using a microscope.

BrdU Cell proliferation assay

DNA synthesis was assessed using the BrdU incorporation assay (ELISA kit; Roche, DE-68305, Mannheim, Germany), which is based on the incorporation of BrdU into the newly synthesised DNA, substituting for thymidine during DNA replication (Motobu et al. 2002). Briefly, at the time of assay, 10 μl BrdU labelling solution was added to each well of a 96-well plate and the plates were incubated for a further 24 h. The cells were denatured and fixed using Fix/Denat solution for 30 min at 37 °C. Anti-BrdU-POD was then added to the wells and incubated for a further 120 min at 20 °C. Lastly, 100 μl substrate solution was added to each well and the plates were incubated at 20 °C until colour development was sufficient. Twenty-five microliters of 1 M H_2SO_4 was added as a stopping solution to each well and absorbance was measured at 450 nm, using a microplate reader, with a reference wavelength of 690 nm.

Statistical analysis

Results are presented as mean values ± SE. Statistical analysis was evaluated by one-way ANOVA followed by Dunnett's test (GraphPad Prism 4.0, GraphPad Inc, San Diego, CA, USA). The level of statistical significance was taken at $P<0·05$ or $P<0·01$.

Results

Cell Viability

MTT assay: In our previous study (Phelan et al. 2009) we mentioned that the viability of Jurkat cells decreased significantly following incubation with increasing concentrations of the casein hydrolysates (a–h), with the exception of hydrolysate e (data not shown). Caco-2 cell viability significantly ($P<0·05$) decreased in cells that were supplemented with increasing concentrations of samples b, c, and f for 24 h (Table 1), although viability did not decrease below 65%.

LDH Release assay: The presence of the casein hydrolysates did not induce LDH release from Jurkat cells (data not shown). LDH release significantly ($P<0·01$) decreased in Caco-2 cells supplemented with casein hydrolysates a–h for 24 h (Table 2).

Trypan Blue Exclusion assay: After 24 h incubation, membrane integrity, as measured by this assay, was not affected by the presence of NaCN and its hydrolysates in Jurkat cells (data not shown). There was good correlation between the Trypan Blue assay and the LDH release assay in Jurkat cells, hence membrane integrity was not assessed in Caco-2 cells using the Trypan Blue assay.

Cell growth

MTT assay: There was a significant decrease in the growth of Jurkat cells supplemented with casein hydrolysates a–d, and f for 24 h (Table 3; Phelan et al. 2009). Growth in Caco-2 cells supplemented with increasing concentrations of samples a, b, c, and f significantly decreased ($P<0·05$, $P<0·01$) after 24 h incubation (Table 3).

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Table 2. Lactate dehydrogenase (LDH) release from Caco-2 cells incubated with casein hydrolysates (a–h) and sodium caseinate for 24 h†

Values are the mean ± SE for $n=3$ independent experiments

	LDH release (fold difference)‡ Casein hydrolysates								
(% v/v)	a	b	c	d	e	f	g	h	NaCN
0·0	1·00 ± 0·06	1·00 ± 0·06	1·00 ± 0·06	1·00 ± 0·06	1·00 ± 0·06	1·00 ± 0·06	1·00 ± 0·06	1·00 ± 0·06	1·00 ± 0·06
0·5	0·77 ± 0·06	0·83 ± 0·04	0·71 ± 0·06*	0·71 ± 0·07	0·75 ± 0·07	0·81 ± 0·02	0·92 ± 0·15	0·86 ± 0·07	0·98 ± 0·09
1·3	0·78 ± 0·08	0·74 ± 0·07	0·67 ± 0·07*	0·50 ± 0·09*	0·67 ± 0·10	0·73 ± 0·05*	0·69 ± 0·10	0·78 ± 0·02	0·85 ± 0·15
2·5	0·79 ± 0·09	0·82 ± 0·12	0·54 ± 0·11*	0·64 ± 0·11	0·55 ± 0·12*	0·63 ± 0·06*	0·59 ± 0·07	0·72 ± 0·05	0·87 ± 0·19
5·0	0·64 ± 0·08*	0·65 ± 0·06*	0·62 ± 0·04*	0·46 ± 0·07*	0·60 ± 0·09*	0·64 ± 0·08*	0·57 ± 0·10*	0·70 ± 0·09	0·62 ± 0·11
10·0	0·56 ± 0·09*	0·53 ± 0·09*	0·58 ± 0·09*	0·44 ± 0·10*	0·55 ± 0·08*	0·67 ± 0·09*	0·52 ± 0·11*	0·58 ± 0·14*	1·20 ± 0·08

NaCN, sodium caseinate
† Human Caco-2 cells (3×10^4 cells/cm^2) were supplemented with increasing concentrations of casein hydrolysates (**a–h**) and sodium caseinate for 24 h
‡ Cytotoxicity was determined by the LDH release assay. LDH release is expressed as fold difference relative to control
* $P<0{\cdot}05$ compared with control (0% v/v): one-way ANOVA, Dunnett's test

Table 3. Growth of Caco-2 and Jurkat T cells supplemented with casein hydrolysates (a–h) and sodium caseinate for 24 h†

Values are the mean ± SE for $n=4$ independent experiments

	Cell growth (MTT Reduction index)‡ Casein hydrolysates								
(% v/v)	a	b	C	d	e	f	g	h	NaCN
Caco-2 cells									
0·0	0·52 ± 0·04	0·52 ± 0·04	0·52 ± 0·04	0·52 ± 0·04	0·52 ± 0·04	0·52 ± 0·04	0·52 ± 0·04	0·52 ± 0·04	0.52 ± 0·04
0·5	0·48 ± 0·03	0·54 ± 0·03	0·50 ± 0·03	0·57 ± 0·04	0·42 ± 0·02	0·40 ± 0·02*	0·59 ± 0·01	0·59 ± 0·01	0·53 ± 0·10
1·3	0·44 ± 0·04	0·46 ± 0·04	0·47 ± 0·05	0·54 ± 0·05	0·39 ± 0·04	0·37 ± 0·02**	0·56 ± 0·01	0·57 ± 0·02	0·48 ± 0·06
2·5	0·46 ± 0·03	0·43 ± 0·01	0·42 ± 0·01	0·41 ± 0·09	0·41 ± 0·04	0·37 ± 0·02**	0·56 ± 0·03	0·53 ± 0·03	0·38 ± 0·02
5·0	0·38 ± 0·03*	0·35 ± 0·03**	0·34 ± 0·02**	0·47 ± 0·02	0·36 ± 0·08	0·36 ± 0·03**	0·57 ± 0·02	0·50 ± 0·02	0·42 ± 0·02
10·0	0·36 ± 0·03**	0·36 ± 0·02**	0·34 ± 0·01**	0·43 ± 0·04	0·44 ± 0·03	0·37 ± 0·03**	0·59 ± 0·02	0·53 ± 0·02	0·43 ± 0·04
Jurkat cells§									
0·0	0·71 ± 0·03	0·62 ± 0·04	0·74 ± 0·03	0·70 ± 0·02	0·60 ± 0·05	0·63 ± 0·03	0·60 ± 0·04	0·71 ± 0·02	—
0·5	0·55 ± 0·03**	0·53 ± 0·04	0·43 ± 0·03**	0·57 ± 0·06	0·56 ± 0·06	0·47 ± 0·02**	0·56 ± 0·05	0·67 ± 0·03	—
1·3	0·48 ± 0·03**	0·47 ± 0·03*	0·39 ± 0·02**	0·50 ± 0·04*	0·56 ± 0·04	0·46 ± 0·03**	0·54 ± 0·04	0·63 ± 0·04	—
2·5	0·46 ± 0·03**	0·45 ± 0·03**	0·38 ± 0·03**	0·47 ± 0·04**	0·59 ± 0·03	0·45 ± 0·03**	0·52 ± 0·05	0·62 ± 0·04	—
5·0	0·43 ± 0·03**	0·45 ± 0·03**	0·38 ± 0·03**	0·43 ± 0·03**	0·58 ± 0·04	0·38 ± 0·03**	0·52 ± 0·06	0·60 ± 0·04	—
10·0	0·42 ± 0·03**	0·45 ± 0·04**	0·40 ± 0·03**	0·44 ± 0·04**	0·53 ± 0·05	0·36 ± 0·03**	0·53 ± 0·06	0·60 ± 0·04	—

NaCN, sodium caseinate (which was only available at the time when using Caco-2 cells)
† Cells were supplemented with increasing concentrations of samples for 24 h
§ Data that was not shown from a previous study (Phelan et al. 2009)
‡ Cell growth was determined by the MTT assay, and expressed as MTT reduction index
* $P<0{\cdot}05$, ** $P<0{\cdot}01$ compared with control: one-way ANOVA, Dunnett's test

Trypan Blue Exclusion assay: After 24 h, cell density was not affected by the presence of casein hydrolysates in Jurkat cells (data not shown). As mentioned earlier, there was good correlation between the Trypan Blue assay and the LDH release assay in Jurkat cells; hence, cell density was not assessed in Caco-2 cells using the Trypan Blue assay.

BrdU Incorporation assay (DNA synthesis): Casein and its hydrolysates exerted different effects on DNA synthesis in Jurkat cells. After 24 h supplementation, casein hydrolysates a and h, at a level of 0·5% (v/v) only, significantly ($P<0{\cdot}05$) reduced DNA synthesis to 45·6% and 55·6% (Table 4). Following 24 h treatment of Caco-2 cells with casein hydrolysates, BrdU incorporation was not affected compared with control (Table 4).

Discussion

There is evidence that milk protein-derived peptides may have a direct effect on the viability of cancer cells

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Table 4. Effects of casein hydrolysates (a–h) and sodium caseinate on DNA synthesis in Jurkat cells incubated for 24 h†

Values are the mean ± SE for $n = 4$ independent experiments

	DNA synthesis (% control)‡ Casein hydrolysates								
(% v/v)	a	b	c	d	e	f	g	h	NaCN
Caco-2 cells									
0·0	100·0 ± 8·2	100·0 ± 8·2	100·0 ± 8·2	100·0 ± 8·2	100·0 ± 8·2	100·0 ± 8·2	100·0 ± 8·2	100·0 ± 8·2	100·0 ± 8·2
0·5	101·7 ± 8·3	106·9 ± 8·8	109·6 ± 8·8	93·9 ± 12·0	97·1 ± 9·0	119·0 ± 11·5	110·4 ± 8·0	105·6 ± 9·9	104·9 ± 10·1
1·3	108·4 ± 9·5	95·6 ± 8·7	106·8 ± 8·0	105·4 ± 9·6	108·1 ± 6·0	105·5 ± 9·0	110·0 ± 4·9	115·8 ± 8·8	110·1 ± 7·9
2·5	91·4 ± 13·6	93·6 ± 8·9	89·4 ± 11·5	99·4 ± 8·7	103·5 ± 11·7	103·6 ± 14·8	96·7 ± 12·9	108·2 ± 10·1	93·1 ± 11·5
5·0	109·2 ± 13·7	107·7 ± 12·5	115·1 ± 7·6	114·6 ± 7·4	105·1 ± 12·2	104·4 ± 7·6	105·6 ± 15·4	105·4 ± 12·5	94·9 ± 16·2
10·0	108·7 ± 12·1	78·9 ± 16·4	92·5 ± 6·7	91·3 ± 8·4	106·4 ± 10·4	113·9 ± 12·7	121·3 ± 9·1	103·1 ± 9·4	101·1 ± 5·6
Jurkat cells									
0·0	100·0 ± 16·0	100·0 ± 16·0	100·0 ± 16·0	100·0 ± 16·0	100·0 ± 16·0	100·0 ± 16·0	100·0 ± 16·0	100·0 ± 16·0	100·0 ± 16·0
0·5	45·6 ± 3·1*	77·4 ± 6·1	73·0 ± 6·2	85·2 ± 19·9	92·8 ± 14·2	93·6 ± 6·5	72·9 ± 2·2	55·6 ± 0·6*	57·2 ± 2·9
1·3	74·6 ± 10·9	119·7 ± 17·2	106·1 ± 10·7	115·3 ± 9·8	104·8 ± 6·1	106·6 ± 6·5	94·1 ± 5·5	79·3 ± 8·7	101·2 ± 15·7
2·5	79·2 ± 4·5	119·7 ± 4·8	136·3 ± 8·1	125·0 ± 8·3	120·5 ± 3·6	99·6 ± 2·2	94·4 ± 8·0	80·9 ± 9·2	91·9 ± 16·7
5·0	62·1 ± 5·9	111·3 ± 11·8	109·3 ± 11·0	117·8 ± 7·2	104·4 ± 15·3	115·9 ± 2·3	73·7 ± 3·9	64·4 ± 3·8	56·8 ± 2·3
10·0	69·7 ± 9·4	76·5 ± 4·0	112·5 ± 4·4	130·3 ± 1·2	106·5 ± 9·7	85·4 ± 4·6	78·7 ± 21·1	64·6 ± 4·9	76·2 ± 7·3

NaCN, sodium caseinate

† Cells were supplemented with increasing concentrations of casein hydrolysates (**a–h**) and sodium caseinate for 24 h

‡ Cell growth was determined by the BrdU incorporation assay and expressed as a percentage of control (cells)

* $P < 0·05$ compared with control: one-way ANOVA, Dunnett's test

(Mac Donald et al. 1994; Hartmann & Meisel, 2004; Phelan et al. 2009), which offers potential for their inclusion into functional foods as these peptides are also of nutritional importance (Meisel & Schlimme, 1996).

When assessing the therapeutic potential of any novel sample in vitro, it is important to consider the cell as a whole and to study the molecular mechanisms underlying different modulating activities caused by the sample on individual components within the cell (Gülden & Seibert, 2003). To determine if casein hydrolysates influence the growth and viability of Jurkat cells and Caco-2 cells, four different assays were selected, namely the MTT, LDH release, Trypan Blue, and BrdU incorporation assays.

Both LDH release and Trypan Blue assays are based on the principle that cell death cascades alter membrane permeability (Aras et al. 2008). LDH is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity, whereas the Trypan Blue assay measures the ability of cells to exclude dye from being taken up into their cytoplasm (Aras et al. 2008). The Trypan Blue assay is very accurate; however it is very time-consuming when used as a screening assay (Durrieu et al. 2005). In the Jurkat T cell model there was good correlation between the results of the LDH release and the Trypan Blue assays, hence the Trypan Blue assay was not carried out on the Caco-2 cell model. LDH release was not negatively affected by the presence of casein hydrolysates in both the Jurkat cells and Caco-2 cells. Hence, the casein hydrolysates did not have any major effect on membrane integrity in these selected cell models.

In the MTT assay, the reduction of MTT by a mitochondrial reductase takes place in viable cells when mitochondrial reductase enzymes are active, which is used as a measure of mitochondrial activity as well as viability and growth of cells (Mosmann, 1983). The BrdU incorporation assay was selected to analyse DNA synthesis since this assay is based on the incorporation of BrdU instead of thymidine into DNA during DNA synthesis. Antibodies specific for BrdU can then be used to detect the incorporated chemical, thus indicating cells that are actively replicating their DNA (Motobu et al. 2002).

In Jurkat cells, the MTT and BrdU assays correlated well as the same trend was seen from both assays. Samples a and h tended to reduce rates of cell division in Jurkat cells. This shows that some casein samples have specific effects on BrdU incorporation during DNA synthesis. Meisel & Günther (1998) showed that bioactive sequences of casein and a lyophilized extract of Gouda cheese had both apoptotic and anti-proliferative activity in HL-60 cells at concentrations as low as 1 pmol/l. In addition, Roy et al. (1999) reported that bovine skimmed milk digested with the yeast *Saccharomyces cerevisiae* had an anti-proliferative activity toward human leukaemia HL-60 cells. On the other hand, Azuma et al. (1989) found that some human casein tryptic fragments could stimulate DNA synthesis in BALB/c3T3 cells. MacDonald et al. (1994) showed that bacterial hydrolysis of casein using commercial yogurt starter cultures yielded bioactive peptides that reduced [^{3}H]thymidine incorporation in IEC-6 cells and increased DNA synthesis in Caco-2 cells, thus suggesting

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cell-specific effects. Furthermore, Ringseis et al. (2005) showed that certain hydrolysates from casein inhibited endothelial cell (HaoEC) proliferation whereas other hydrolysates had no effect.

Recently, Ramos-Mandujano et al. (2008) showed that NaCN and its individual casein components displayed different inhibitory characteristics on the proliferation of 32Dcl3 and WEHI-3 myeloid cells. At 0·1 mg/ml, NaCN exerted the strongest growth inhibition on 32Dcl3 cells but the inhibition was not as potent on WEHI-3 cell growth. This variation in findings may be explained by the different protein concentrations and the different cell lines used in these studies (Hagiwara et al. 1995; Ramos-Mandujano et al. 2008). In addition, the types and sources of peptide can have individual effects on cells (Hagiwara et al. 1995; Meisel & Günther, 1998). In the present study, an explanation for the activity profiles of the hydrolysates may be that they relate to the different proteolytic enzyme preparations used in their manufacture (manuscript in preparation).

In conclusion, NaCN had no significant effect on the viability and growth of Caco-2 whereas its associated casein hydrolysates (a–h) had differing effects on cell viability and proliferation. Hydrolysate a had an inhibitory effect on proliferation while e had a promotive effect, which was cell-specific. The casein hydrolysates seemed to be more toxic to Jurkat cells compared with Caco-2 cells. A vast majority of tumour promoters are potent inhibitors of apoptosis (Wright et al. 1994) and therefore apoptosis-inducing peptides can be classified as probable anti-carcinogens. According to Meisel & FitzGerald (2003), effects on both cell viability and immune function may be a mechanism by which bioactive peptides exert protective effects in cancer development. Our data add to our previous findings and suggest that the eight distinct casein hydrolysates have varying effects on two different cell culture models. Further research is warranted to assess the effects of the casein hydrolysates on the cell cycle of these two cell lines (Elion, 2001).

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References

Aras MA, Hartnett KA & Aizenman E 2008 Assessment of cell viability in primary neuronal cultures. *Current Protocols in Neuroscience* **44** 1–7

Azuma N, Nagaune S, Ishino Y, Mori H, Kaminogawa S & Yamauchi K 1989 DNA-synthesis stimulating peptides from human β-casein. *Agricultural Biological Chemistry* **53** 2631–2634

Cameron L 1990 Colon carcinogenesis: modulation of progression. In *Colon Cancer Cells*, pp. 74 (Eds MP Moyer & GH Poste). New York, USA: Academic Press, Inc. New York

Ciapetti G, Cenni E, Pratelli L & Pizzoferrato A 1993 *In vitro* evaluation of cell/biomaterial interaction by MTT assay. *Biomaterials* **14** 359–364

Durrieu C, Degraeve P, Carnet-Pantiez A & Martial A 2005 Assessment of the immunomodulatory activity of cheese extracts by a complete and easy to handle *in vitro* screening methodology. *Biotechnology Letters* **27** 969–975

Elion EA 2001 The Ste5p scaffold. *Journal of Cell Science* **114** 3967–3978

FitzGerald RJ & Meisel H 2003 Milk protein hydrolysates and bioactive peptides. In *Advanced Dairy Chemistry: Proteins1A*, pp. 675–698 (Eds PF Fox & PLH McSweeney). New York: Kluwer Academic/Plenum Press

Flanagan J & FitzGerald RJ 2002 Functionality of *Bacillus* proteinase hydrolysates of sodium caseinate. *International Dairy Journal* **12** 737–748

Gülden M & Seibert H 2003 *In vitro-in vivo* extrapolation: estimation of human serum concentrations of chemicals equivalent to cytotoxic concentrations in vitro. *Toxicology* **189** 211–222

Hagiwara T, Shinoda I, Fukuwatari Y & Shimamura S 1995 Effects of lactoferrin and its peptides on proliferation of rat intestinal epithelial cell line IEC-18, in the presence of epithelial growth factor. *Bioscience, Biotechnology and Biochemistry* **59** 1875–1881

Hartmann R & Meisel H 2004 Caseinophosphopeptides and their cell modulting potential. *Biofactors* **21** 73–78

Hartmann R, Wal J-M, Bernard H & Pentzien A-K 2007 Cytotoxic and allergenic potential of bioactive proteins and peptides. *Current Pharmaceutical Design* **13** 897–920

IDF 20-2 2001 Milk: determination of nitrogen content. Part 2: Block digestion (macro) method. Brussels, Belgium: International Dairy Federation

Jing H & Kitts DD 2004 Redox-related cytotoxic responses to different casein glycation products in Caco-2 and Int-407 cells. *Journal of Agricultural and Food Chemistry* **52** 3577–3582

Koh YJ & Choi DW 1987 Quantitive determination of glutamate-mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *Journal of Neuroscience Methods* **20** 83–90

Korhonen H & Pihlanto A 2006 Bioactive peptides: production and functionality. *International Dairy Journal* **16** 975–960

Korhonen H & Pihlanto-Leppälä A 2001 Milk protein-derived bioactive peptides-novel opportunities for health promotion. *International Dairy Federation Bulletin* **363** 17–26

Laparra JM, Alegría A, Barberá R & Farré R 2008 Antioxidant effect of casein phosphopeptides compared with fruit beverages supplemented with skimmed milk against H_2O_2-induced oxidative stress in Caco-2 cells. *Food Research International* **41** 773–779

MacDonald R, Thornton WH & Marshall R 1994 A cell culture model to identify biologically active peptides generated by bacterial hydrolysis of casein. *Journal Dairy Science* **77** 1167–1175

Meisel H & FitzGerald RJ 2003 Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Current Pharmaceutical Design* **9** 1289–1295

Meisel H & Günther S 1998 Food proteins as precursors of peptides modulating human cell activity. *Nahrung* **42** 175–176

Meisel H & Schlimme E 1996 Bioactive peptides derived from milk proteins – ingredients for functional foods? *Kieler Milch Forsch* **48** 343–357

Mosmann T 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65** 55–63

Motobu M, El-Abasy M, Na KJ & Hirota Y 2002 Detection of mitogen induced lymphocyte proliferation by bromodeoxyuridine (BrdU) incorporation in the chicken. *The Journal of Vetinerary Medicine Science* **64** 377–379

Phelan M, Aherne-Bruce SA, O'Sullivan D, FitzGerald RJ & O'Brien NM 2009 Potential bioactive effects of casein hydrolysates on human cultured cells. *International Dairy Journal* **19** 279–285

Phelan M, Aherne SA, FitzGerald RJ & O'Brien NM 2009 Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. *International Dairy Journal* **19** 643–654

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Ramos-Mandujano G, Weiss-Steidera B, Meloa B, Córdovaa Y, Ledesma-Martíneza E, Bustos S, Silvestre O, Aguiñiga I, Sosa N, Martínez I, Sánchez L, García A & Santiago-Osorio E 2008 Alpha-, beta- and kappa-caseins inhibit the proliferation of the myeloid cell lines 32D cl3 and WEHI-3 and exhibit different differentiation properties. *Immunobiology* **213** 133–141

Ringseis R, Matthes B, Lehmann V, Becker K, Schöps R, Ulbrich-Hofmann R & Eder K 2005 Peptides and hydrolysates from casein and soy protein modulate the release of vasoactive substances from human aortic endothelial cells. *Biochimica et Biophysica Acta* **1721** 89–97

Roy MK, Wantanabe Y & Tamai Y 1999 Induction of apoptosis in HL-60 cells by skimmed milk digested with a proteolytic enzyme from the yeast *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering* **88** 426–432

van't Veer PJ, Dekker M, Lamers JWJ, Kok FJ, Schouten EG, Brants HAM, Sturmans F & Hermus JJ 1989 Consumption of fermented milk products and breast cancer: a case-control study in the Netherlands. *Cancer Research* **49** 4020–4023

Wright SC, Zhong J & Larrick JW 1994 Inhibition of apoptosis as a mechanism of tumor promotion. *FASEB Journal* **8** 654–660

https://doi.org/10.1017/S0022029909990471 Published online by Cambridge University Press