

Oligosaccharides from human milk induce growth arrest via G2/M by influencing growth-related cell cycle genes in intestinal epithelial cells

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Oligosaccharides are present in human milk in large amounts and in a high variety. We have previously shown that these oligosaccharides are strong inhibitors of proliferation and inducers of differentiation in intestinal cell lines. To elucidate the molecular mechanism, we investigated the influence on cell cycle events via flow cytometry and expression levels by using quantitative real-time RT-PCR. Human intestinal cells, i.e. HT-29, HIEC and Caco-2 cells, were exposed to neutral or acidic human milk oligosaccharides. Both fractions induced a concentration-dependent G2/M arrest. Cell cycle analysis for HT-29 revealed 37 % of cells in G1 and 35 % in G2/M (neutral oligosaccharides) and incubation with acidic oligosaccharides led to 42 % cells in G1 and 40 % in G2/M. In control experiments without oligosaccharides we found 71 % of cells to be in G1 and 17 % in G2/M. This G2/M arrest was associated with changes in mRNA expression of cyclin A and B. A G2/M arrest with concomitant alterations of cell cycle gene expression could also be shown for HIEC and Caco-2 cells. Analysing the expression of cyclin-dependent kinase inhibitors p21^{cip1} and p27^{kip1} and the tumour suppressor p53 we observed that the expression of p21^{cip1} was p53-independent and necessary for arresting cells in the G2/M phase, while p27^{kip1} was associated with differentiation effects. Both neutral and acidic human milk oligosaccharides were able to induce epidermal growth factor receptor, extracellular signal-regulated kinase 1/2 and p38 phosphorylation. These results suggest that oligosaccharides from human milk inhibited intestinal cell proliferation and altered cell cycle dynamics by affecting corresponding regulator genes and mitogen-activated protein kinase signalling.

Oligosaccharides: G2/M arrest: Cyclins: Cyclin-dependent kinase inhibitors: Mitogen-activated protein kinase signalling

Human milk oligosaccharides (HMO) are currently discussed as participating in the development of a normal microflora in neonates, as soluble receptors for pathogens preventing their adhesion to the epithelial cells and as modulators of inflammatory processes^(1–4). The total oligosaccharide content in human milk ranges from 5 to 15 g/l compared to about 1 g/l in mature bovine milk^(5–8). Neutral oligosaccharides comprise about 70 % of the total oligosaccharide content in human milk, whereas acidic oligosaccharides account for 30 %⁽⁹⁾. As the intestine of the breast-fed infant is continuously exposed to such high concentrations of oligosaccharides for up to several months, it is intriguing to consider whether ingested oligosaccharides may modulate neonatal intestinal development. Recent studies using the intestinal cell line Caco-2 revealed that specific neutral oligosaccharides are minimally digested, could in part be taken up and get enriched by intestinal cells^(10–12). Hence, they might have the potential to affect enterocyte function.

Renewal of the intestinal villi is a well-regulated sequential process of proliferation, differentiation and apoptosis. We have recently shown that neutral and acidic HMO were able to reduce cell proliferation and enhance alkaline phosphatase

activity particularly in undifferentiated cell lines⁽¹³⁾. However, up to now, no information is available regarding a direct effect of HMO on intestinal cell integrity and the mechanisms involved. Processes affecting cell fate are highly regulated and cell cycle genes like cyclins, cyclin-dependent kinases (CDK) and inhibitors play an important role in that context^(14,15). Dependent on their level of expression, cyclins regulate cell progression and are responsible for arresting cells at a defined state. Various molecular studies showed that a G1 arrest is associated with a decrease in cyclin D and cyclin E^(16,17). This decrease was found to be associated with changes in CDK protein levels and activity. Concomitant to these changes CDK inhibitors such as p27^{kip1} and p21^{cip1} were up-regulated and arrested cells in cell cycle phase G1 or G2^(18,19).

Although most of the *in vitro* studies on p21^{cip1} regulation have concentrated on p53-dependent and p53-independent transcriptional regulation, a series of new studies has shown that p21^{cip1} can be regulated by post-translational mechanisms. Phosphorylation of p21^{cip1} modulates not only interaction and binding with target proteins but also its stabilization⁽²⁰⁾. These phosphorylation events in cell cycle

Abbreviations: CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitors; Ct, cycle threshold; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HMO, human milk oligosaccharides; MAPK, mitogen-activated protein kinase.

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progression occur through different signal cascades such as receptor-mediated MAPK activation which could result in both G1 and G2 arrest.

Thus, the objective of the present study was to investigate how oligosaccharides induce inhibition of cell proliferation and which mechanism participates in these intestinal cell dynamics.

Materials and methods

Isolation of human oligosaccharides from human milk and human milk oligosaccharide preparation

Oligosaccharides were isolated from human milk as described previously^(7,13,21). Briefly, after centrifugation, the lipid layer was removed, and the proteins were precipitated from the aqueous phase with ice-cold ethanol. Lactose was removed by gel filtration on Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden). The total oligosaccharide fraction was further separated into an acidic fraction (acidic HMO), consisting of compounds with *N*-acetylneuraminic acid residues, and a neutral fraction (neutral HMO) without *N*-acetylneuraminic acid residues, using HPLC anion-exchange on a Resource Q column (Pharmacia Biotech) at the following conditions: 100 % eluent A (H₂O) from 0 to 7.5 min, a linear gradient to 55 % eluent B (0.6 M-NaCl) for 42.5 min, a linear gradient to 100 % eluent B for 2 min, and a constant flow with 100 % eluent B for 8 min. A flow rate of 2 ml/min was used. The eluting fractions were monitored at 214 nm. Afterwards, pooled HPLC fractions were desalted by gel filtration on Sephadex G-25 (Pharmacia Biotech).

Cell culture

The human colon cancer cell line HT-29 was obtained from the American Type Culture Collection (Rockville, MD, USA); Caco-2 cells were a gift from R. K. Kinne (Max Planck Institute of Molecular Physiology, Dortmund, Germany). The fetal intestinal colon cell line HIEC was generously donated by J. F. Beaulieu (Department of Anatomy and Cell Biology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec, Canada)⁽²²⁾. Cells were cultured in 75 cm² tissue culture flasks (Renner, Darmstadt, Germany) in RPMI 1640 (HT-29 and HIEC) or Dulbecco's modified Eagle's medium (Caco-2) supplemented with 10 % fetal calf serum, 2 mM-glutamine (Invitrogen, Karlsruhe, Germany), 100 units/ml penicillin and 100 mg/ml streptomycin (Invitrogen). The cultures were kept in a humidified atmosphere of 5 % CO₂ at 37°C. Cells were passaged at preconfluent densities using 0.05 % trypsin and 0.5 mM-EDTA (Invitrogen).

Cell cycle analysis

Intestinal cells (HT-29, Caco-2 and HIEC) were seeded at a density of 50 000 onto six-well culture flasks and allowed to adhere for 24 h. Thereafter, the medium was replaced and incubated for another 24 h in the presence or absence of the test compounds. Cells were trypsinized, pelleted by centrifugation at 500 g for 3 min, washed twice with PBS and adjusted to 1 × 10⁶ cells/ml. Cells were then fixed by suspending the cells at 4°C in ethanol for 24 h. Thereafter, the suspension

was centrifuged for 3 min at 500 g, and the pellet was washed twice in 38 mM-sodium citrate buffer (pH 7.4). After washing and centrifugation, cells were incubated with the dye solution containing 7-amino actin (1 mg/ml) and 1 unit DNase-free RNase in sodium citrate buffer. Cell cycle analysis was performed using a FACScan (Becton Dickinson, San Jose, CA, USA) and the software BD CellQuest™ Pro version 1.41 for data analysis.

RNA extraction and cDNA synthesis

Total RNA from intestinal cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instruction with slight modifications. Total RNA was quantified by measuring the absorbance at 260 nm, and the purity was checked by the 260/280 nm absorbance ratio. cDNA synthesis was carried out in a reaction volume of 50 µl containing 500 ng RNA, 50 mM-2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride (pH 8.3), 75 mM-KCl, 3 mM-MgCl₂, 10 mM-dithiothreitol, 100 ng oligodeoxythymidine 15 primer, 500 µM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP and dTTP), 10 units ribonuclease inhibitor and 200 units Moloney murine leukaemia virus RT; all reagents were obtained from Invitrogen. The samples were incubated at 37°C for 60 min, followed by an incubation at 95°C for 15 min.

Analysis of mRNA expression (real-time quantitative PCR)

mRNA expression of cell cycle genes such as cyclins (cyclin A, B, D, E), cyclin-dependent kinase inhibitors (CDKI) and p53 were determined in intestinal cells using the real-time PCR technique on a 7500 Real Time PCR System (Applied Biosystems, Darmstadt, Germany) and TaqMan® PCR master mix (Eurogentec, Köln, Germany). Gene-specific primers and probes used in the present study (Table 1) were designed using the sequences accessible in the NCBI Reference Sequence and the software Primer Express 3.0 (Applied Biosystems). Primer and probe sequences were chosen to prevent homologies to undesired genes and other coding sequences and checked by BLAST™ software. To exclude amplification from possible DNA contamination, the probes were designed to overlap exon junctions in cDNA regions derived from intron-bearing genes. All probes were labelled with the fluorescent dyes 6-carboxy-fluorescein as reporter and 6-carboxy-tetramethyl-rhodamine as quencher. Primers were purchased from MWG Biotech (Ebersberg, Germany) and probes from Eurogentec (Seraing, Belgium). A total reaction volume of 25 µl containing reaction buffer with 250 nM of each primer and 150 nM-probe and 1 µl cDNA. The PCR running conditions were: 10 min of initial denaturation at 95°C followed by forty cycles of 30 s at 95°C, 30 s at 58°C for annealing, 30 s at 60°C, and 15 s at 75°C. Samples were run in triplicates. PCR results are shown as the relative expression level of normalized samples (Δ cycle threshold (Ct)) in relation to the expression of the calibrator sample ($2^{-\Delta\Delta Ct}$), which was set at 100 %. The Ct value refers to the cycle number at which the PCR plot crosses the threshold line, ΔCt is calculated by subtracting the Ct value of the corresponding housekeeper gene GAP-DH (endogenous reference control) from the specific Ct value of the target gene. Finally,

Table 1. Primers and TaqMan® probes for real-time RT-PCR amplifications

Gene	Accession no.		Primers (5' → 3')
GAP-DH	NM_002046	FP	CCACATCGCTCAGACACCAT
		BP	GTGACCAGGCGCCAATA
		Probe	AGGTCGGAGTCAACGGATTTGG
Cyclin A	NM_001237	FP	TGCCTTTTCATTTAGCACTCTACACA
		BP	TTAAGACTTTCCAGGGTATATCCAGTC
		Probe	CGGGACAAAGCTGGCCTGAATCATTAAATA
Cyclin B	NM_031966	FP	GCTGCTGCCTGGTGAAGAG
		BP	CGCCTTATTTTCAGCATTAAATTTTC
		Probe	ATGGCGCTCCGAGTCAACCAGGAA
Cyclin E	NM_001238	FP	CGGCTCGCTCCAGGAA
		BP	TCGATTTTGGCCATTTCTTCA
		Probe	ACCGTTTTTTTGCAGGATCCA
Cyclin D	NM_053056	FP	CCGTCCATGCGGAAGATC
		BP	TGCAGGCGGCTCTTTTTTC
		Probe	ATGCTGGAGGTCTGCGAGGAACAGAAGT
p21 ^{cip1}	NM_078467	FP	CTGGAGACTCTCAGGGTCCGAA
		BP	GGCTTAGGGCTTCTCTTTGGA
		Probe	ACGGCGGCAGACCAGCATGA
p27 ^{kip1}	NM_004064	FP	CCGGTGGACCACGAAGAGT
		BP	GCTCGCCTTCCATGTCTC
		Probe	AACCCGGGACTTGGAGAAGCACTGC
p53	NM_000546	FP	TAACAGTTCTGCATGGGCGGC
		BP	AGGACAGGCACAACACGCACC
		Probe	CGGAGGCCCATCCTCACCATCATCA

BP, backward primer; FP, forward primer.

$\Delta\Delta Ct$ is obtained by subtracting the ΔCt of each experimental sample from the ΔCt of the calibrator sample.

Detection of receptor phosphorylation

Nearly (70–80%) confluent HT-29 cultures were incubated in Dulbecco's modified Eagle's medium with 10% fetal calf serum for 24 h. Subsequently, reagents were added in concentrations indicated in the legends of the figures. After incubation, cells were trypsinized, washed and lysed with extraction buffer. Protein concentration was measured using the BioRad protein assay kit (Richmond, CA, USA). The Proteome Profiler™ array, human phospho-receptor tyrosine kinase array kit to identify the phosphorylation of forty-two different receptor tyrosine kinases, was used according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA) loading 250 µg protein/membrane. Blots were developed by applying the chemiluminescence detection kit ECL-Plus (Amersham Biosciences, Arlington Heights, IL, USA). Spot intensity was then analysed with the software Biocat Software (Köln) and density is expressed as area under the curve.

Detection of mitogen-activated protein kinase phosphorylation

After incubation with oligosaccharides as indicated in the figures, cells were washed with PBS, solubilized in lysis buffer and 300 µg total protein were used for the human phospho-MAP array (R&D Systems, Heidelberg, Germany) according to the manufacturer's protocol. Briefly, arrays were incubated with whole-cell lysates overnight at 4°C and washed with the supplied washing buffer. Arrays were then incubated with anti-phosphotyrosine-horseradish peroxidase antibodies for 2 h at room temperature before incubation

with a chemiluminescent reagent and film exposure; spot intensity was then analysed applying the software Biocat Software and expressed as area under the curve.

Statistical analysis

Data were evaluated by ANOVA and statistical differences were tested by Bonferroni's test. For each variable at least three independent experiments were carried out. Results are expressed as means and their standard errors. All analyses were done using the GraphPad Software Prism 3 (San Diego, CA, USA); differences were considered significant at $P < 0.05$.

Results

Cell cycle analysis

As a consequence of growth inhibition and differentiation cells were fixed to determine their specific cell cycle phase by using flow cytometry. The cell cycle histogram plots and distribution of cell populations in different cell cycle stages for HT-29 are shown in Figs. 1 and 2. It was observed that neutral as well as acidic HMO induced a cell cycle arrest in the G2/M phase which was moderate at a concentration of 7.5 mg/ml but pronounced at 15 mg/ml. Cell cycle analysis of controls without oligosaccharide exposure revealed that 73% of the HT-29 population was in the G0/G1 phase and 16% in the G2/M phase (Figs. 1(A) and 2(A)). HT-29 cells exposed to 7.5 mg/ml neutral HMO exhibited a similar distribution of cells among the different cell cycle phases (Fig. 1(B)). The higher dose of oligosaccharides (15 mg/ml) altered the cell population in a way that 42% of the cell population was in the G0/G1 phase and 44% in the G2/M phase

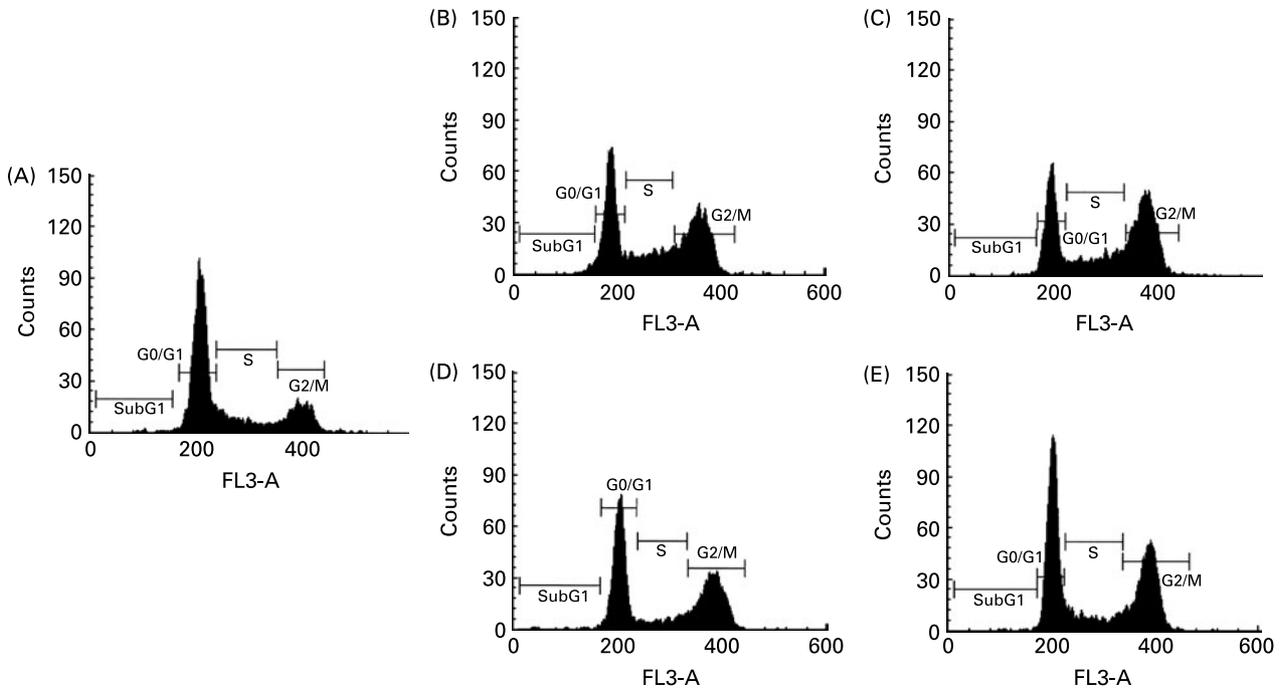


Fig. 1. Representative histograms of flow cytometric analysis of the cellular DNA content in control and oligosaccharide-treated HT-29 cells, stained with the fluorescence dye 7-aminoactinomycin. The cells were untreated (A) or treated with 7.5 mg/ml (B, D) and 15 mg/ml (C, E) neutral (B, C) or acidic (D, E) human milk oligosaccharides for 24 h. Cell cycle distribution was performed as described in the Materials and methods. FL3-A, fluorescence intensity.

(Fig. 2(A)). After a 24 h treatment with the lower dose of acidic HMO (Figs. 1(D) and 2(B)), 72% of the cells were in the G0/G1 phase. This was balanced by a lower percentage of cells in the G2/M stages (16%). Exposure of HT-29 for 24 h to the high dose of oligosaccharides resulted in a further accumulation of cells in the G2/M stage, 37% of the cell population were found to be in the G0/G1 stage and 35% accumulated in the G2/M stage (Fig. 2(B)).

In comparison to control cells, the proportion of cells in the G0/G1 phase significantly decreased in HIEC and Caco-2 cells after incubation with neutral and acidic HMO at a concentration of 15 mg/ml and thus accumulated in the G2/M phase (44 and 33% in HIEC and Caco-2 cells, respectively). The number of cells in the S phase did not change significantly

in both cell lines after incubation with oligosaccharides when compared to controls (Table 2).

Transcriptional regulation of cyclins, cyclin-dependent kinase inhibitors and tumour suppressors with quantitative real-time PCR

In order to relate cell cycle arrest to important cell cycle regulator genes such as cyclins and CDKI and tumour suppressor p53, we performed real-time PCR to quantify differences in the expression level of selected target genes. In Fig. 3, the data for the expression levels of cyclines are summarized for HT-29, HIEC and Caco-2 cells. After incubation of the cells with 15 mg/ml neutral or acidic HMO, mRNA expression

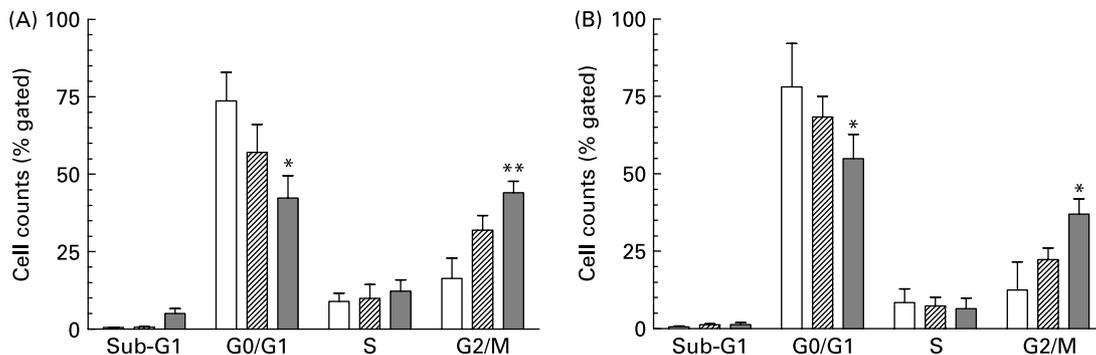


Fig. 2. Distribution of cell cycle phases based on histogram plots for HT-29 cells. The distribution of cell cycle phases is expressed as the percentage of gated cells in the G0/G1, S and G2/M phase of the cell cycle. Cells were treated with (A) neutral human milk oligosaccharides (▨, 7.5 mg/ml; ■, 15 mg/ml) or (B) acidic human milk oligosaccharides (▨, 7.5 mg/ml; ■, 15 mg/ml). Values are means with their standard errors depicted by vertical bars (*n* 2). Mean values were significantly different from those of the control group: **P* ≤ 0.05, ***P* ≤ 0.01. □, Control.

Table 2. Effects of oligosaccharides on cell cycle progression of HIEC and Caco-2 cells†
(Mean values for two independent experiments)

	Control	Neutral HMO		Acidic HMO	
		7.5 mg/ml	15 mg/ml	7.5 mg/ml	15 mg/ml
HIEC					
Sub G0/G1	0.54	1.21	6.76*	0.9	2.1
G0/G1	69.5	60.6	55.4*	59.0	49.3*
S	10.3	8.24	10.3	7.4	5.4
G2/M	18.3	24.43	29.5*	22.5	44.3**
Caco-2					
Sub G0/G1	0.73	0.33	7.5	2.5	1.9
G0/G1	63.4	63.5	53.2*	60.4	50.3*
S	8.3	8.9	9.4	7.1	5.8
G2/M	20.4	27.3	31.2*	26.5	33.2*

HMO, human milk oligosaccharides.

Mean values were significantly different from those of the control group: * $P \leq 0.05$, ** $P \leq 0.01$.† For details of procedures, see Materials and methods. Intestinal cells were seeded at a density of 10^6 cells and were allowed to attach for 24 h. Finally, they were cultured without (control) or with 7.5 or 15 mg/ml neutral and acidic HMO for 24 h. The distribution of cell cycle phases is expressed as percentage of gated cells in the G0/G1, S and G2/M phase of the cell cycle.

levels were determined using the target gene/housekeeping gene ratio by setting the control to 100%. The expression level of cyclin A, a regulator for S/G2 transition, remained unchanged in all cell lines after the treatment with neutral HMO. In contrast, after incubation with acidic oligosaccharides, a moderate but significant increase was observed in HT-29 (146 (SEM 6) %) and HIEC cells (132 (SEM 6) %), whereas the cyclin A expression level in Caco-2 cells remained unaffected (Fig. 3). The mRNA expression levels of cyclin B, which is responsible for the regulation of the G2/M cell cycle transition, were markedly increased in all cell lines after incubation with neutral or acidic oligosaccharides. After incubation with neutral HMO, the expression level of cyclin B in HT-29 was increased to 198 (SEM 12) % and to 235 (SEM 9) % after treatment with acidic HMO. Similar data were obtained for HIEC cells, i.e. both neutral and acidic HMO markedly increased cyclin B expression (221 (SEM 12) and 214 (SEM 8) %, respectively). Also, in Caco-2 cells cyclin B expression was enhanced to 150 (SEM 7) % by neutral HMO and 164 (SEM 5) % by acidic HMO compared to controls. Cyclin D and E which regulate the entry of cells into and the progression through the G1 phase of the cell cycle remained unchanged in all cell lines after oligosaccharide treatment.

We then investigated whether the block in cell cycle progression was accompanied by increased levels of CDKI such as p21^{cip1} and p27^{kip1}. It is well known that both CDKI are able to induce cell cycle arrest and/or differentiation. Treatment of the undifferentiated cell lines HT-29 and HIEC with neutral and acidic HMO resulted in an enhanced expression of p21^{cip1} and p27^{kip1} (Table 3). In HT-29 cells, 7.5 and 15 mg/ml neutral HMO enhanced expression of p21^{cip1} 3.7- and 6.6-fold, respectively; acidic HMO were able to enhance mRNA levels of p21^{cip1} 2.8- and 4.1-fold, respectively. Similar observations were made for HIEC cells. p21^{cip1} expression increased 3.2-fold (15 mg/ml neutral HMO) and 3.6-fold (15 mg/ml acidic HMO) compared to control experiments. p27^{kip1} mRNA expression was enhanced 2.8- and 4.3-fold in HT-29 and HIEC cells when exposed to neutral HMO; treatment with the same amount of acidic HMO

(15 mg/ml) increased p27^{kip1} mRNA expression 2.5- and 8.3-fold in HT-29 and HIEC cells compared to controls. In contrast to HT-29 and HIEC cells, Caco-2 cells responded only with an increased p21^{cip1} mRNA level but p27^{kip1} levels remained unchanged after supplementation with HMO. Hence, neutral as well as acidic HMO induced an increase of p21^{cip1} levels which reached a maximum of 210 (SEM 12) % (15 mg/ml neutral HMO) and 196 (SEM 10) % (15 mg/ml acidic HMO) in comparison to control cells (100%).

A well-known regulator of different checkpoints during the cell cycle is the tumour suppressor p53 which is able to regulate G1 or G2 transition. To get insight into the role of p53 in the observed G2/M arrest, we determined p53 mRNA levels in all cell lines after incubation with HMO, but no changes in p53 mRNA levels were observed in any cell line (Table 3).

Influence of signal transduction pathways

Changes in expression levels of cyclins or CDKI are a consequence of activation or inactivation of different signal cascades. Thus, we further investigated whether the effects of oligosaccharides are a result of influencing signalling pathways in the cell line HT-29, representing phenotypical undifferentiated cells. To screen the phosphorylation of several receptors and MAPK in HT-29 cells, we used the protein profiling array to detect different phosphorylation events. Results are shown in Fig. 4 for receptor phosphorylation.

Incubation with both neutral and acidic HMO induced a dose-dependent phosphorylation of the ERF receptor within 10 min indicating that oligosaccharides could interact with the epidermal growth factor receptor (EGFR). The effects of acidic oligosaccharides were more pronounced than the neutral oligosaccharide effects. Acidic HMO concentrations of 7.5 and 15 mg/ml induced an EGFR phosphorylation up to 247 (SEM 3) and 447 (SEM 34) % compared to untreated cells (100%). Neutral HMO were also able to affect EGFR phosphorylation and enhanced its phosphorylation to 168 (SEM 6) and 284 (SEM 8) % at 7.5 and 15 mg/ml, respectively. The effect of HMO was EGFR-specific because no other receptor phosphorylation was observed.

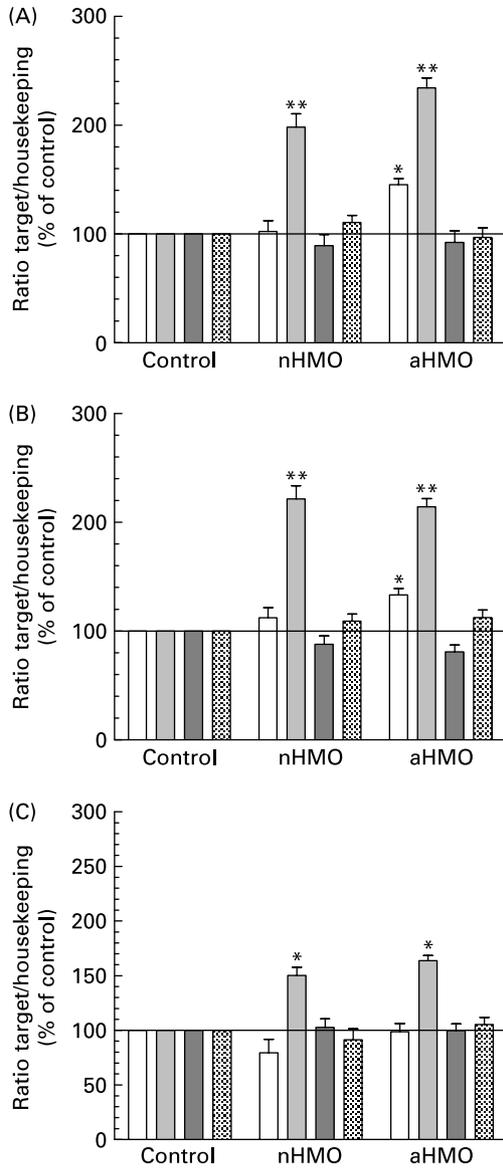


Fig. 3. Changes of mRNA expression levels of cell cycle genes in intestinal HT-29 (A), HIEC (B) and Caco-2 (C) cells with quantitative real-time PCR. Cells were treated with 15 mg/ml neutral (nHMO) or acidic human milk oligosaccharides (aHMO) after reaching a confluency of 30% over 72 h. Data were analysed as $2^{-\Delta\Delta Ct}$ (Ct, cycle threshold). Values are means of the percentage of controls with their standard errors depicted by vertical bars ($n=3$). Mean values were significantly different from those of the control group: * $P \leq 0.05$, ** $P \leq 0.01$. □, Cyclin A/GAP-DH; ■, cyclin B/GAP-DH; ▒, cyclin D/GAP-DH; ▣, cyclin E/GAP-DH.

As a consequence of receptor phosphorylation different signal pathways could be involved. To determine whether different MAPK were further activated we used the MAPK array to investigate how and to which extent oligosaccharides are able to induce downstream events from EGFR signalling. As shown in Fig. 5, the analysis of these signalling pathways revealed a role for p38 MAPK and extracellular signal-regulated kinase (ERK) 1/2 which were most significant on p38 MAPK. Phosphorylation and activation of different p38 MAPK subtypes, especially p38 α and p38 δ in HT-29 cells, was induced by acidic HMO (Fig. 5(A)) and to a lesser

extent, but still significantly, by neutral HMO (Fig. 5(B)). Furthermore, the activation of p38 α was stronger than that of p38 δ in HMO-treated cells within 30 min. In addition, PKB- β /Akt (Akt2), a growth factor-regulated protein kinase, and the downstream kinase ERK1 have emerged as critical enzymes in signal transduction pathways involved in cell proliferation and apoptosis. We have shown that both were activated by HMO at concentrations of 7.5 and 15 mg/ml.

Discussion

The intestine is a continuously renewing tissue that constantly undergoes proliferation, differentiation and apoptosis. Recently, we reported that the exposure of the intestinal cell lines HT-29, HIEC and Caco-2 to HMO significantly inhibited cell proliferation. Whereas neutral oligosaccharides exerted an anti-proliferative effect along with a significant induction of apoptosis and a slight increase in differentiation, acidic oligosaccharides inhibited intestinal cell proliferation only by an induction of alkaline phosphatase activity as a marker of differentiation⁽¹³⁾. To understand the effects of oligosaccharides on growth-associated events of intestinal cells, flow cytometric analyses were carried out. Cytometric analyses showed significant changes of the cell cycle pattern in HT-29, HIEC and Caco-2 cells following oligosaccharide treatment for 24 h (Figs. 1 and 2; Table 2). In control experiments, the major proportion of cells was arrested in the G0/G1 phase. After treatment with neutral or acidic HMO, cells accumulated in the G2/M phase of the cell cycle (44, 30 and 31 of HT-29, HIEC and Caco-2 cells, respectively, after exposure to neutral oligosaccharides; 37, 44 and 33 % of HT-29, HIEC and Caco-2 cells, respectively, after treatment with acidic HMO). This leads to the conclusion that the potent anti-proliferative effect of acidic and neutral oligosaccharides is coupled to an extensive G2/M arrest in intestinal epithelial cells. We further investigated whether the G2/M cell cycle arrest due to the exposure to oligosaccharides was associated with changes in mRNA levels for key proteins regulating the cell cycle. Progression through the cell cycle is regulated by CDK, a family of serine/threonine protein kinases which phosphorylate a variety of proteins for cell cycle control^(23,24). The cellular concentrations of cyclins vary depending on the cell cycle stage, whereas the levels of CDK remain relatively stable, but must bind to the appropriate cyclin in order to be activated. Therefore, cyclin mRNA expression levels were determined and correlated to flow cytometric events.

After 24 h of oligosaccharide treatment, there was no significant change in cyclin D and E mRNA levels suggesting that cells enter the cell cycle and proceed to G2. Previous studies in intestinal cells demonstrated that cyclin D1 is essential for the progression through the cell cycle^(25,26). The present data also indicate that additional cyclins may be responsible for transition and arrest, e.g. cyclin B1, a cell cycle regulatory protein that is generally induced at the G2/M transition and becomes deactivated as the cell exits mitosis. We found that cyclin B1 expression increased after HMO treatment (Fig. 3). This may prevent the cells from entering the G2/M stage, thus corroborating the observed cell cycle arrest at G2/M and the reduced cell proliferation when oligosaccharides were applied. This is in accordance with several other studies which had shown that blocking

Table 3. Effects of oligosaccharides on cell cycle progression of HT-29, HIEC and Caco-2 cells† (Mean values with their standard errors for three independent experiments)

	Control		Neutral HMO				Acidic HMO			
	Mean	SEM	7.5 mg/ml		15 mg/ml		7.5 mg/ml		15 mg/ml	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
HT-29										
p21 ^{cip1}	2.31	0.21	8.65	1.20***	15.25	2.64***	6.48	1.54***	9.67	1.88***
p27 ^{kip1}	1.59	0.10	3.25	0.44*	4.56	0.99*	2.66	0.41*	4.08	0.57**
p53	3.65	0.29	2.69	1.41	4.12	0.59	3.06	0.41	4.14	0.47
HIEC										
p21 ^{cip1}	3.69	0.43	6.55	0.75**	11.74	2.05**	5.98	0.51**	13.5	2.87**
p27 ^{kip1}	0.69	0.09	1.56	0.47*	2.99	0.58**	1.57	0.42*	5.73	0.39**
p53	5.69	0.41	6.87	2.56	8.74	3.68	7.08	2.54	7.41	2.62
Caco-2										
p21 ^{cip1}	3.61	0.35	5.25	1.46	7.69	1.56*	6.98	2.04*	7.09	2.10*
p27 ^{kip1}	5.24	0.52	4.56	0.62	5.08	0.41	5.88	0.85	4.80	1.08
p53	3.04	0.33	2.65	0.41	3.65	0.41	3.96	0.84	2.74	0.41

HMO, human milk oligosaccharides.

Mean values were significantly different from those of the control group: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

† For details of procedures, see Materials and methods. Intestinal cells were seeded at a density of 10^6 cells and were allowed to attach for 24 h. Finally, they were cultured without (control) or with 7.5 or 15 mg/ml neutral and acidic HMO for 24 h. Real-time PCR was carried out using cell cycle-specific primer and probes (see Table 1). Data were analysed as $2^{-\Delta\Delta Ct}$ (Ct, cycle threshold) and given as means of the percentage of controls.

cell cycle progression at G2/M was due to increasing cyclin B1 expression in different cancer cell lines⁽²⁷⁾.

In addition, the G2/M arrest seen in HT-29 and HIEC cells, but not in Caco-2 cells, was associated with a slightly increased mRNA expression level of cyclin A after incubation with acidic oligosaccharides. However, there was no response to an exposure to neutral HMO. If cyclin A were necessary for S/G2 phase transition one might speculate that the G2/M arrest in HT-29 and HIEC cells treated with acidic oligosaccharides is coupled to an increase in both cyclin A and B^(27,28).

The transition through the cell cycle is mediated not only by different time-dependent expression of cyclins but also by the activation of CDK. The interaction of cyclins with their corresponding CDK promotes further activation of target proteins responsible for cell cycle transition. On the other hand, CDKI of the CIP/KIP family are negative regulators of cell cycle progression and CDKI such as p21^{cip1} and p27^{kip1} were able

to inhibit the formation of cyclin-CDK complexes. The inactivation of the CDK-cyclin B complex by its subsequent binding to p21^{cip1} is one of the probable mechanisms of G2/M arrest. The present data demonstrate (Table 3) a significant up-regulation of p21^{cip1} mRNA expression, indicating its role in oligosaccharide-mediated G2/M arrest in all intestinal cell lines. It has recently been shown that p21^{cip1} interacts with cyclin D throughout the cell cycle, whereas interaction between p21^{cip1} and cyclin A or cyclin B occurs in the later part of the cell cycle^(29,30). Thus, the G2/M growth arrest we observed may well be a p21-dependent mechanism. Several lines of evidence suggested that p21^{cip1} and p27^{kip1} exert similar effects on cyclin-CDK complexes and cell cycle progression but other observations indicate that these CDKI have no overlapping functions and are not biologically equivalent, because of their differentially regulated expression^(31,32). In comparison to control cells HMO were able to enhance mRNA levels of p27^{kip1}

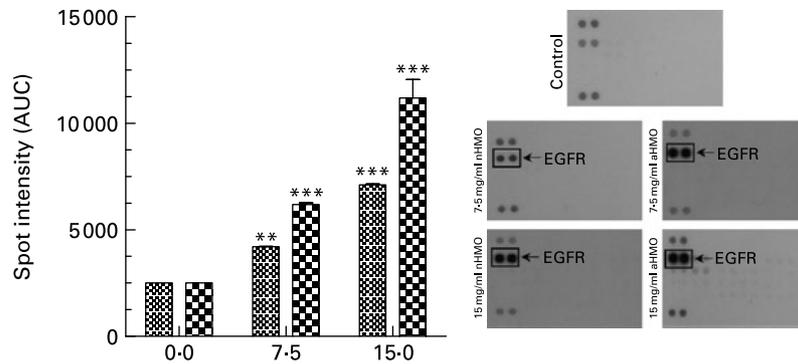


Fig. 4. Receptor tyrosine kinase antibody array analysis. Cells were treated with neutral human milk oligosaccharides (nHMO; ▨) or acidic human milk oligosaccharides (aHMO; ▩) (7.5 and 15 mg/ml) for 10 min. Lysate was prepared according to the manufacturer's instructions. Phospho-receptor tyrosine kinase array was used to detect phosphorylation of these receptor tyrosine kinases in HT-29. The signal was detected by chemiluminescence and the spot intensity is shown. Values are means of the percentage of controls with their standard errors depicted by vertical bars ($n = 3$). Mean values were significantly different from those of the control group: ** $P \leq 0.01$, *** $P \leq 0.001$. AUC, area under the curve; EGFR, epidermal growth factor receptor.

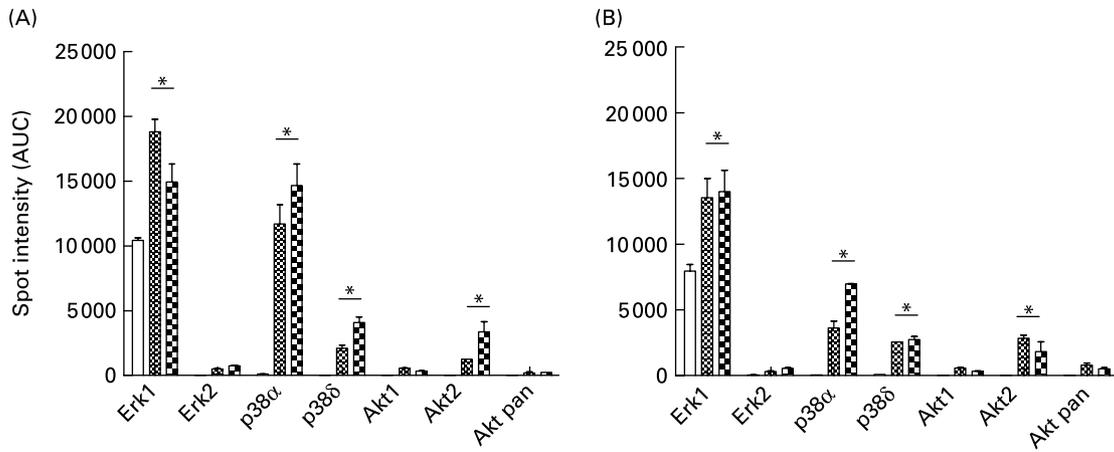


Fig. 5. Detection of phosphorylated mitogen-activated protein kinases in untreated HT-29 cells (□) and HT-29 cells treated with (A) neutral human milk oligosaccharides (▤, 7.5 mg/ml; ▥, 15 mg/ml) or (B) acidic human milk oligosaccharides (▤, 7.5 mg/ml; ▥, 15 mg/ml) after 30 min. After incubation, lysate was prepared and 300 μg lysate were used for each assay. Array signals from scanned X-ray film images were analysed using image analysis software and expressed as spot pixel density. Values are means with their standard errors depicted by vertical bars (*n* 2). Mean values were significantly different: **P* ≤ 0.05. AUC, area under the curve.

significantly in HIEC and HT-29 cells whereas in the more differentiated Caco-2 cells oligosaccharides failed to further increase p27^{kip1} mRNA levels (Table 3). The present results indicate distinct roles of these CDKI, i.e. p21^{cip1} promotes cell cycle arrest and p27^{kip1} induces cell differentiation. Cells arrested in the G2/M phase concomitant with enhanced p21^{cip1} mRNA and protein levels are often discussed in the context of a p53-dependent mechanism^(33–35). This p53-independent G2/M regulation was also seen in the present experiments; however, p53 was not changed in the intestinal cell lines tested in comparison to the control cells (Table 3).

The activation of EGFR and transmission of the extracellular signal through different signalling pathways (the ERK pathway, the stress-activated c-Jun N-terminal kinase and the p38/HOG kinase pathways) in order to provoke cell progression is a well-known and accepted process^(36,37). However, recent studies suggest that the EGFR pathway is not simply a growth-promoting signalling pathway, but phosphorylated EGFR also mediates p21^{cip1} expression and growth arrest or apoptosis via modulation of signalling pathways^(38,39). From the present study two interesting findings have emerged: firstly, we observed that oligosaccharides were able to induce EGFR phosphorylation and,

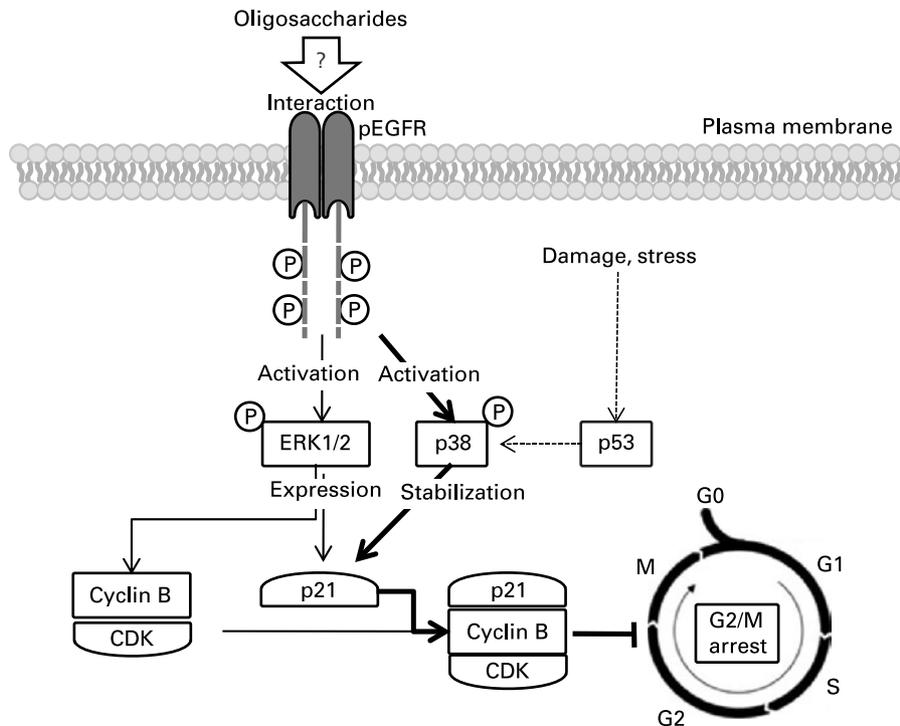


Fig. 6. Proposed mechanism for intracellular pathway-mediated G2/M arrest. CDK, cyclin-dependent kinase; EGFR, epidermal growth factor receptor.

secondly, we have confirmed that the EGFR/Ras/Raf/ERK pathway is involved. As shown in Figs. 4 and 5 neutral HMO as well as acidic HMO induce a potent phosphorylation of the EGFR within 10 min and, in addition, multiple downstream kinases such as p38 α , p38 δ , ERK1 and Akt-2/PKB were activated within 30 min. As the EGFR is a glycoprotein and interactions of oligosaccharides with glycoprotein receptors are well documented it is conceivable that oligosaccharide fractions possess a general motif inducing the effects we observed. The cross-talk between EGFR phosphorylation, p38 activation and p21^{cip1} expression is observed in a variety of *in vitro* studies. For example, the p38 MAPK pathway can activate the Sp1/Sp3 transcription factors and this seems necessary for EGFR-dependent transactivation of the p21^{cip1} promoter^(40–42).

Based upon the present observations we conclude that oligosaccharides are able to activate p21^{cip1} expression and stabilization via EGFR and p38 kinase phosphorylation. Fig. 6 summarizes a mechanism how oligosaccharides induce cell cycle arrest by possibly interacting with EGFR.

In conclusion, we identify HMO as ingredients which were able to induce growth arrest of intestinal cells by modulating EGFR signal pathways and cell cycle-associated gene expression. However, whether the ability of oligosaccharides to inhibit intestinal cell proliferation has implications for a different effect on the intestinal growth regulation in human milk-fed *v.* formula-fed infants is not known yet and requires further investigation.

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References

- Sharon N & Ofek I (2000) Safe as mother's milk: carbohydrates as future anti-adhesion drugs for bacterial diseases. *Glycoconj J* **17**, 659–664.
- Kunz C, Rudloff S, Baier W, Klein N & Strobel S (2000) Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu Rev Nutr* **20**, 699–722.
- Bode L, Kunz C, Muhly-Reinholz M, Mayer K, Seeger W & Rudloff S (2004) Inhibition of monocyte, lymphocyte, and neutrophil adhesion to endothelial cells by human milk oligosaccharides. *Thromb Haemost* **92**, 1402–1410.
- Bode L, Rudloff S, Kunz C, Strobel S & Klein N (2004) Human milk oligosaccharides reduce platelet–neutrophil complex formation leading to a decrease in neutrophil β 2 integrin expression. *J Leukocyte Biol* **76**, 1–7.
- Kobata A & Ginsburg V (1972) Oligosaccharides of human milk. 3. Isolation and characterization of a new hexasaccharide, lacto-N-hexaose. *J Biol Chem* **247**, 1525–1529.
- Kobata A, Yamashita K & Tachibana Y (1978) Oligosaccharides from human milk. *Methods Enzymol* **50**, 216–220.
- Kunz C, Rudloff S, Hintelmann A, Pohlentz G & Egge H (1996) High-pH anion-exchange chromatography with pulsed amperometric detection and molar response factors of human milk oligosaccharides. *J Chromatogr B Biomed Appl* **25**, 211–221.
- Parkkinen J & Finne J (1987) Isolation of sialyl oligosaccharides and sialyl oligosaccharide phosphates from bovine colostrum and human urine. *Methods Enzymol* **138**, 289–300.
- Kunz C & Rudloff S (2000) Oligosaccharides in human milk: structural, functional and metabolic aspects. *Annu Rev Nutr* **20**, 699–722.
- Engfer MB, Stahl B, Finke B, Sawatzki G & Daniel H (2000) Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *Am J Clin Nutr* **71**, 1589–1596.
- Gnoth MJ, Kunz C, Kinne-Saffran E & Rudloff S (2000) Human milk oligosaccharides are minimally digested *in vitro*. *J Nutr* **130**, 3014–3020.
- Gnoth MJ, Rudloff S, Kunz C & Kinne RK (2001) Investigations of the *in vitro* transport of human milk oligosaccharides by a Caco-2 monolayer using a novel high performance liquid chromatography–mass spectrometry technique. *J Biol Chem* **14**, 34363–34370.
- Kuntz S, Rudloff S & Kunz C (2008) Oligosaccharides from human milk influence growth-related characteristics of intestinally transformed and non-transformed intestinal cells. *Br J Nutr* **99**, 462–471.
- Vermeulen K, Van Bockstaele DR & Berneman ZN (2003) The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif* **36**, 131–149.
- Serrano M (2003) Proliferation: the cell cycle. *Adv Exp Med Biol* **532**, 13–17.
- Stein GH & Dulic V (1998) Molecular mechanisms for the senescent cell cycle arrest. *J Investig Dermatol Symp Proc* **3**, 14–18.
- Dai Y & Grant S (2003) Cyclin-dependent kinase inhibitors. *Curr Opin Pharmacol* **3**, 362–370.
- Mani S, Wang C, Wu K, Francis R & Pestell R (2000) Cyclin-dependent kinase inhibitors: novel anticancer agents. *Expert Opin Investig Drugs* **9**, 1849–1870.
- Baghdassarian N & French M (1996) Cyclin-dependent kinase inhibitors (CKIs) and hematological malignancies. *Hematol Cell Ther* **38**, 152–158.
- Li Y, Dowbenko D & Lasky L (2002) AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. *J Biol Chem* **17**, 277–286.
- Rudloff S, Obermeier S, Borsch CH, Pohlentz G, Hartmann R, Brösicke H, Lentze MJ & Kunz C (2006) Incorporation of orally applied ¹³C-galactose into milk lactose and oligosaccharides. *Glycobiology* **16**, 477–487.
- Perreault N & Beaulieu JF (1996) Use of the dissociating enzyme thermolysin to generate viable human normal intestinal epithelial cell cultures. *Exp Cell Res* **1**, 354–364.
- Nadella KS & Kirschner LS (2005) Disruption of protein kinase A regulation causes immortalization and dysregulation of D-type cyclins. *Cancer Res* **15**, 10307–10315.
- Ouyang W, Ma Q, Li J, Zhang D, Liu ZG, Rustgi AK & Huang C (2005) Cyclin D1 induction through I κ B kinase beta/nuclear factor-kappaB pathway is responsible for arsenite-induced increased cell cycle G1-S phase transition in human keratinocytes. *Cancer Res* **15**, 9287–9293.
- Lewis RC, Bostick RM, Xie D, Deng Z, Wargovich MJ, Fina MF, Roufail WM & Geisinger KR (2003) Polymorphism of the cyclin D1 gene, CCND1, and risk for incident sporadic colorectal adenomas. *Cancer Res* **63**, 8549–8553.

26. McKay JA, Douglas JJ, Ross VG, Curran S, Murray GI, Cassidy J & McLeod HL (2000) Cyclin D1 protein expression and gene polymorphism in colorectal cancer. *Int J Cancer* **88**, 77–81.
27. Dash BC & El-Deiry WS (2005) Phosphorylation of p21 in G2/M promotes cyclin B-Cdc2 kinase activity. *Mol Cell Biol* **25**, 3364–3387.
28. Ji P, Agrawal S, Diederichs S, *et al.* (2005) Cyclin A1, the alternative A-type cyclin, contributes to G1/S cell cycle progression in somatic cells. *Oncogene* **14**, 2739–2744.
29. Li R, Hannon GJ, Beach D & Stillman B (1996) Subcellular distribution of p21 and PCNA in normal and repair-deficient cells following DNA damage. *Curr Biol* **6**, 189–199.
30. Harper JW, Elledge SJ, Keyomarsi K, *et al.* (1995) Inhibition of cyclin-dependent kinases by p21. *Mol Biol Cell* **6**, 387–400.
31. Sherr CJ & Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* **13**, 1501–1512.
32. Blain SW, Montalvo E & Massagué J (1997) Differential interaction of the cyclin-dependent kinase (Cdk) inhibitor p27Kip1 with cyclin A-Cdk2 and cyclin D2-Cdk4. *J Biol Chem* **272**, 25863–25872.
33. Matsui TA, Sowa Y, Murata H, *et al.* (2007) The plant alkaloid cryptolepine induces p21WAF1/CIP1 and cell cycle arrest in a human osteosarcoma cell line. *Int J Oncol* **31**, 915–922.
34. Sowa Y, Murata H, Takagi K, *et al.* (2007) The plant alkaloid cryptolepine induces p21WAF1/CIP1 and cell cycle arrest in a human osteosarcoma cell line. *Int J Oncol* **31**, 915–922.
35. Aoki S, Kong D, Suna H, Sowa Y, Sakai T, Setiawan A & Kobayashi M (2006) Aaptamine, a sponge alkaloid, activates p21 promoter in a p53-independent manner. *Biochem Biophys Res Commun* **342**, 101–116.
36. Tarnawski AS & Jones MK (1998) The role of epidermal growth factor (EGF) and its receptor in mucosal protection, adaptation to injury, and ulcer healing: involvement of EGF-R signal transduction pathways. *J Clin Gastroenterol* **27**, S12–S20.
37. Sundaram MV (2006) RTK/Ras/MAPK signaling. *WormBook* **11**, 1–19.
38. Liu ZM & Huang HS (2006) As₂O₃-induced c-Src/EGFR/ERK signaling is via Sp1 binding sites to stimulate p21WAF1/CIP1 expression in human epidermoid carcinoma A431 cells. *Cell Signal* **18**, 244–255.
39. Matsumoto E, Hatanaka M, Bohgaki M & Maeda S (2006) PKC pathway and ERK/MAPK pathway are required for induction of cyclin D1 and p21Waf1 during 12-*o*-tetradecanoylphorbol 13-acetate-induced differentiation of myeloleukemia cells. *Kobe J Med Sci* **52**, 181–194.
40. Kuwahara I, Lillehoj EP, Lu W, Singh IS, Isohama Y, Miyata T & Kim KC (2006) Neutrophil elastase induces IL-8 gene transcription and protein release through p38/NF-κB activation via EGFR transactivation in a lung epithelial cell line. *Am J Physiol Lung Cell Mol Physiol* **291**, 407–416.
41. Matsuo M, Sakurai H, Ueno Y, Ohtani O & Saiki I (2006) Activation of MEK/ERK and PI3K/Akt pathways by fibronectin requires integrin αv-mediated ADAM activity in hepatocellular carcinoma: a novel functional target for gefitinib. *Cancer Sci* **97**, 155–162.
42. Xu K & Shu HK (2007) EGFR activation results in enhanced cyclooxygenase-2 expression through p38 mitogen-activated protein kinase-dependent activation of the Sp1/Sp3 transcription factors in human gliomas. *Cancer Res* **67**, 6121–6129.