Experimental evidences on the potential of prebiotic fructans to reduce the risk of colon cancer

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Inulin is extracted from the chicory root. It is a set of fructans with its monomers \( n = 2–65 \) linked by means of \( \beta(2–1) \) bonds. This linkage cannot be hydrolysed by either pancreatic or by brush border digestive enzymes in the upper intestinal tract of humans. As such the carbohydrates arrive in the colon, where they are fermented by bifidobacteria and other lactic acid producing bacteria, thus enhancing their relative populations in the gut. Recent research in experimental animal models revealed that inulin has significant anticarcinogenic properties. It acts chemopreventively by reducing the incidence of azoxymethane (AOM) — induced aberrant crypt foci and tumours in the colon. These effects may be due to the stimulation of bifidobacteria, which themselves have been shown to act as antigenotoxic in the colon and to reduce AOM-induced tumours. Also fermentation products, including the short-chain fatty acid butyrate, could contribute to the protective effects. In this case a mechanism may be the induction of apoptosis of already transformed cells. The experimental evidence from animal studies and from studies elucidating potential mechanisms strongly supports the possibility that inulin will contribute to reducing risks for colon cancer in humans. In order to obtain more insight into this possibility, human dietary intervention studies relating biomarkers of reduced risk to inulin consumption are needed.

Inulin: Oligofructose: Aberrant crypts: Chemoprevention: Anticarcinogenic food ingredients

Inulin and oligofructose could be such effective food ingredients to be included in this type of strategy. They are natural constituents of many common plant foods such as onion, garlic, tomato, banana, wheat, etc. and, as reviewed below, they have anticarcinogenic potential. Their average consumption in the normal human diet has

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; DMH, dimethylhydrazine; GSH, glutathione; GST, glutathione S-transferase; LAB, lactic acid producing bacteria.

Note: For the definition of the terms inulin and oligofructose please refer to the introductory paper (p. S139) and its footnote.

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been evaluated to amount to several grams per day (Moshfegh et al. 1999; Van Loo, 1995). Inulin is industrially obtained from chicory roots by hot water extraction, followed by refining and spray drying. It is thought that chicory inulin and its low and high molecular weight fractions owe their nutritional properties to the presence of the $\beta(2-1)$ bond, which cannot be hydrolysed by the pancreatic nor by the brush border hydrolytic enzymes of humans (Schneemann, 1999). As such inulin and oligofructose escape digestion in the upper intestinal tract and arrive almost quantitatively (>90%) in the colon (Ellgard et al. 1997). There these non-digestible oligosaccharides are completely fermented. Hereby they selectively promote the growth of certain groups of bacteria. Amongst these are the bifidobacteria and the lactobacilli, both of which produce lactic acid and are considered to be indicators of a well-balanced intestinal flora (Gibson & Wang, 1994). This modified intestinal flora and the metabolites, which it produces, interact with the surface of the intestinal tract in the human body and cause physiological beneficial effects.

Inulin and oligofructose have been subjected to extensive in vitro and in vivo (experimental models) research (Van Loo et al. 1999). At present, the relevance of most observed properties has been confirmed in human dietary intervention studies. The prebiotic effect (Gibson & Roberfroid, 1995; Roberfroid et al. 1998; Kruse et al. 1999), as well as modulation of lipid metabolism (Williams, 1999), increased calcium absorption (Coudray et al. 1997; Van den Heufel et al. 1999), modulation of the immune system in young children (Saavedra et al. 1999) and modulation of gut function (Den Hond et al. 2000) are important physiological properties that may have significant health-promoting impact in humans.

Recently an impressive variety of studies in experimental anticancer models has been performed. This review intends to bring together and discuss these data, reflect on state of art, as well as on research necessary to understand putative properties of risk reduction for cancer by these specific types of prebiotics.

Prevention of cancer and related endpoints in animal experiments

Prevention of chemically induced aberrant crypt foci and colon cancer

Several experiments with inulin and oligofructose have been performed using one of the most commonly employed animal models to determine preneoplastic lesions in the colon of rats (Bird, 1987). The carcinogenic compound used is azoxymethane (AOM), an alkylating derivative of dimethylhydrazine (DMH), that specifically targets the colon of rats, where it induces DNA damage (Pool-Zobel et al. 1996) and tumours. The highest tumour incidence is in the distal part of the colon. Rats ($n \geq 10$) are injected (e.g. subcutaneously) with two AOM doses $2 \times 15$ mg AOM/kg body weight at an interval of 1 week. The tumours appear after a period of 45–52 weeks. Intermediate endpoints induced by AOM can be detected already after 8 weeks, since it produces large quantities of preneoplastic lesions in the colon, called aberrant crypt foci (ACF). These abnormalities are due to a thickening of the wall in the pericarp of the colon crypts that can be stained and counted. Numerous aberrant crypts occur together and are visible as aberrant crypt foci (ACF). Most of these lesions, however, are eliminated by repair mechanisms. Only some of them develop into tumours, of which mainly those with high numbers of aberrant crypts per foci (multiplicity) are associated with cancer risk (Magnuson et al. 1993). The application of the model using AOM as the initiator has been developed to study chemoprevention of colon tumours (Wargovich et al. 1992; Pereira et al. 1994). An overview of the variations of this model that has been used to study inulin and oligofructose for preventive properties are presented in Table 1.

At least three studies show a reduction of crypt numbers and multiplicity, when adding inulin (10%) to the diet; Rowland et al. 1998; Coles et al. 2000). In one case (Reddy et al. 1997), it was observed that the effect of inulin is numerically more important than the effect of the oligofructose (Fig. 1). This was attributed to the lower fermentation rate of the inulin, which as a consequence may arrive in more distal parts of the colon, where the injected carcinogen (AOM) exerts its damaging activity (adapted from Reddy et al. 1997). Other authors could confirm this in a study where the whole range of chicory fructans was compared. Verghese et al. (2002a) observed an increasing protection of oligofructose < inulin < long-chain inulin < a mixture of inulin + oligofructose which is a specific mixture of short- and long-chain inulin fractions (Verghese et al. 2002a, Van Loo & Jonkers, 2001). However, there is also a report where in a similar experimental approach with oligofructose no reduction of ACF was observed (Gallaher & Khil, 1999).

In another experiment (Rowland et al. 1998), it was observed that the combination of the prebiotic inulin and the probiotic B. longum inhibit AOM-induced aberrant crypt foci in a synergistic manner. Especially the effect on the foci with multiplicity of over four crypts, which are thought to be the most relevant markers for tumour formation, may be considered of importance in this context (Fig. 2). This was the first demonstration of an effect now described as ‘symbiote’, which has been confirmed by another group (Gallaher & Khil, 1999).

A more recent study shows that the effect of inulin is dose related. By increasing the concentrations of inulin to 2.5, 5 and 10% in the diet (Verghese et al. 2002a), an increasingly more visible impact on reduction of ACF incidence is apparent. These authors, moreover, have also recently shown that the incidence of colonic tumours is reduced after life-long feeding of 10% inulin to the rat (Verghese et al. 2002b). Moreover, when offering inulin only before or only after the carcinogenic AOM injection, or by continuously administering inulin throughout the whole experiment, the effect of the prebiotic compound given either during the initiation phase (I), or during the promotion phase (P) or during the whole carcinogenic process (I + P) was investigated. It was observed that the highest impact on limiting the numbers of tumours and/or reducing the average size of the tumours was obtained...
when inulin was applied during the promotion phase, although the dietary supplementation during initiation phase also reduced the number of tumours. In this model, tumours in the small intestine also develop and these were also dramatically reduced in the inulin feeding groups. In this case the effect of supplementation during the initiation phase was about as important as during the promotion phase (Verghese et al. 2002b).

**Modulation of sporadic cancers in transgenic APC\textsuperscript{MIN} mouse model**

Inulin may also modulate the occurrence of colon tumours, which are not chemically induced. Studies were performed with a genetically predetermined model, the APC\textsuperscript{MIN} mouse. This transgenic mouse contains a nonsense mutation in the murine APC-gene and it is strongly predisposed to developing intestinal tumours at a relatively young age. It comes close to reflecting the situation of patients with familial adenomatous polyposis (FAP), or of individuals carrying the first APC mutation in somatic cells and who are then later predisposed for developing sporadic colon cancer. As is shown in Fig. 3, dietary supplementation with oligofructose (from sucrose) caused a reduction in the incidence of colonic tumours but not of small intestinal tumours (Pierre et al. 1997). The authors moreover, observed that the oligofructose-fed mice had a better-developed gut associated lymphoid tissue (GALT).

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**Table 1. Overview of the different experimental lay-outs using inulin and oligofructose based on the AOM/ACF model**

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Type of diet</th>
<th>Feeding scheme ()</th>
<th>Number of rats (n)</th>
<th>Type of rat (age)</th>
<th>AOM (mg/kg BW)</th>
<th>Biomarker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligofructose (10 %)</td>
<td>AIN-76A semi-puri-fied</td>
<td>I+P</td>
<td>12</td>
<td>male F344 (5 w)</td>
<td>15</td>
<td>ACF/multiplicity</td>
<td>Reddy et al. 1997</td>
</tr>
<tr>
<td>Inulin* (10 %)</td>
<td>CO25 high fat diet</td>
<td>P</td>
<td>15</td>
<td>male Sprague-Dawley (3–4 w)</td>
<td>12.5</td>
<td>ACF/multiplicity</td>
<td>Rowland et al. 1998, Bolognani et al. 2001</td>
</tr>
<tr>
<td>Inulin* (5 %)</td>
<td>AIN 93G</td>
<td>I</td>
<td>20 (tumors)</td>
<td>male F344 (5 w)</td>
<td>16</td>
<td>ACF/multiplicity</td>
<td>Verghese et al. 2002b</td>
</tr>
<tr>
<td>Inulin* (10 %)</td>
<td>+ B. longum (10^9)</td>
<td>I+P</td>
<td>12</td>
<td>AOM (52 w)</td>
<td>10</td>
<td>ACF/multiplicity</td>
<td>Verghese et al. in press 2002a</td>
</tr>
<tr>
<td>Inulin* (2.5; 5 and 10 %)</td>
<td>AIN 93G</td>
<td>I+P</td>
<td>12</td>
<td>ACF/multiplicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligofructose (10 %)</td>
<td>Inulin (10 %) + oligofructose (10 %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltodextrin as placebo</td>
<td></td>
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</tbody>
</table>

**Fig. 1.** Life-long feeding of inulin and oligofructose suppresses formation of aberrant crypt foci in the rat colon. Data from Reddy et al. (1997). ■ = Control, ■ = 10% oligofructose, ▲ = 10% inulin.

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I: initiation phase, rats are fed with the pre/probiotics prior to and during injection with AOM; P: progression phase, rats are fed with the pre/probiotics after injection with AOM; I + P: initiation and progression phases, rats are fed with the pre/probiotics before and after injection with AOM, throughout life; AOM: azoxymethane; ACF: aberrant crypt foci.  
* In these studies the inulin used was long-chain inulin identified as HP-Inulin.
In a similar model, Mutanen et al. (2000) compared the impact of a non-specified fraction of chicory inulin with other prebiotic food ingredients, added at higher dosage levels. Inulin was not effective at these low concentrations significantly different from the non-fibre control.

Mechanisms-directed experimental approaches

*Bifidobacteria, production and potential role as modifiers of cancer risk factors*

Bifidobacteria are present in large amounts subsequent to inulin and oligofructose consumption (Gibson et al. 1995; Kleessen et al. 1997) and these bacteria are expected to contribute to the protective effects of the prebiotics (Gibson & Wang, 1994). One important mechanism is probably the detoxification of genotoxins in the gut. This has been shown experimentally in animal models using the rat colon carcinogen 1,2-dimethylhydrazine (procarcinogen from which AOM is produced) and determining end points ranging from tumorigenesis (Singh et al. 1997) (Fig. 4), to ACF (Reddy, 1998), down to DNA damage induction (Pool-Zobel et al. 1996) (Fig. 5), all of which were markedly reduced. Owing to the complexity of cancer initiation, of cancer progression and of the exposure situation in the gut, many types of interactions between DMH metabolites and *Bifidobacteria* may be envisaged to be occurring in the gut. Notably some of our newer studies have shown that short-lived metabolite mixtures isolated from milk fermented with strains of *L. bulgaricus* and *S. thermophilus* are more effective in deactivating etiological risk factors of colon carcinogenesis than cellular components of the micro-organisms (Wollowski et al. 1999). Depending on the nature of the exposing risk factor, different mechanisms including enhanced decomposition or scavenging can be potential biochemical mechanisms involved.

*Inactivation of carcinogens by modification of toxifying and detoxifying enzymes*

Lactic acid producing bacteria (LAB), and fermented milk products containing LAB, or having bifidogenetic properties affect gut flora enzyme activities associated with colon carcinogenesis (Ling et al. 1994). These enzymes, namely azoreductase, nitroreductase, β-glucuronidase, β-glucosidase and 7α-dehydroxylase are expected to influence the carcinogenic impact of endogenous toxic and genotoxic compounds (Rowland, 1991). Examples

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**Fig. 2.** *B. longum* and inulin (given during the progression phase) synergistically suppress preneoplastic lesions in the colon of rats. Data from Rowland et al. (1998).

**Fig. 3.** Oligofructose (from sucrose) reduces the occurrence of colon tumors and develop gut associated lymphoid tissue in APC<sup>MIN</sup> mice. Data from Pierre et al. (1997). ■ = CD, ▲ = RS, ▼ = WB, ◆ = FOS.
are glucuronide metabolites of the carcinogenic food contaminants, heterocyclic amines. They need to be cleaved by β-glucuronidases before being activated to the ultimate carcinogens, delivering the electrophilic species (Turesky et al. 1991). Other examples are azoreductases that activate azo dyes, some of which were formally used as food colouring agents. Nitroaromatics are activated by nitroreductases and are environmental contaminants that may reach the gut via blood or bile; whereas 7-α-dehydroxylase may contribute to the genotoxic burden in the gut lumen by activating endogenous hormones to intermediates that are further converted to yield reactive oxygen species (ROS). Harmful and beneficial bacteria commonly found in the intestine differ in their enzyme activities (Ballongue et al. 1997). *Bifidobacteria* and *Lactobacilli* have lower activities of these xenobiotic-metabolising enzymes than *Bacteroides*, *Clostridia* and *Enterobacteriaceae*. For example, β-glucuronidase is highest in *Enterobacteria* and *Clostridia* (Morra & Boland, 1995). As a consequence of these enzymes, toxic compounds, already detoxified in the liver by conjugation, are regenerated by the release of toxic aglycones. Furthermore, products formed after hydrolysis of glucuronides can re-enter enterohepatic circulation and thus delay excretion of compounds. Therefore, although no specific evidence is available, (other than these general associative suggestions), lower activities of these enzymes are connected to lower carcinogen exposures. In contrast, an increase of β-glucosidase could potentially be regarded as an advantage for health by releasing glycosides of plant ingredients, some of which have more anti-mutagenic, antioxidative, anticarcinogenic and immune stimulatory properties than their respective glycosides.
other colonic bacteria, inulin could cause this regulation (Gibson & Wang, 1994). Bifidobacteria can have regulatory effects on the growth of other colonic bacteria, inulin could cause this regulation indirectly by leading to a selective stimulation of Bifidobacteria (Gibson & Wang, 1994).

Diet could also be important for enzyme-related detoxifying effects in the colon. Only recently, we have obtained evidence showing that resistant starch can induce the chemopreventive enzyme glutathione S-transferase \( \pi \) (GST\( \pi \)) in the colon of rats with human flora (Treptow-van Lishaut et al. 1999). In contrast, studies by other groups showed that inulin fed to rats inoculated with whole faecal flora enhanced hepatic GST-activity, but did not modulate GST in the colon (Roland et al. 1994). This interaction of gut flora with cells of the colonic mucosa or other systemically remote tissues and expression of GST and other xenobiotic metabolising enzymes has only been randomly investigated so far. In the future related studies on these aspects may reveal how inulin can be protective. Together the findings imply that there is some potential of pre- and probiotics to inactivate carcinogenic factors in the colon. However, more conclusive evidence is needed to relate these activities to a lowering of cancer risk either by modulating luminal or even colon epithelial metabolism.

**Effects of inulin and gut-products on apoptosis**

Apoptosis, first described by Kerr et al. (1972), is a physiological process of selected cell deletion. As an antagonist of cell proliferation, apoptosis contributes to keeping the cell number in tissues and organs constant, and helps to remove superfluous and damaged cells. If apoptosis is suppressed (e.g. in cells with \( p53 \)-mutations) this can result in the development of various tumours (Hollstein et al. 1991). Similarly, for example overexpression of the antiapoptotic gene \( bcl-2 \) can lead to lymphoid hyperplasia, lymphomas, and auto-aggression by self-reactive lymphocytes that are normally deleted by apoptosis. In contrast, the induction of apoptosis is essential for the therapy of neoplasm and autoimmune diseases (Thompson, 1995; Kroemer et al. 1995). During apoptosis the cells undergo various morphological and molecular changes, e.g. the formation of apoptotic blebs of the cell membrane, DNA-fragmentation in typical fragments of 180 base pairs (characteristic DNA-laddering), condensation of chromatin, or the externalization of phosphatidylserine (PS) on the extracellular side of the plasma membrane (Cohen, 1993; Kroemer et al. 1995; Hale et al. 1996; Steller, 1995). These changes can be detected in a great number of apoptosis assays.

To study the impact of inulin on apoptosis as a mechanism for anticancer properties of prebiotics, young (3–4 weeks old) male Sprague-Dawley rats (\( n = 6 \)) were fed a diet containing either inulin (5 \%) or oligofructose (5 \%) or the basal diet only for a period of 3 weeks. At the end of this period they all were gavaged with a dose of 20 mg/kg of 9,12-dimethylhydrazine (DMH) directly in the stomach. Twenty-four hours later the animals were killed. The colon was removed and cut mid-way, to obtain a proximal and distal end, and subsequently adequately embedded in paraffin wax. The apoptotic bodies in twenty good longitudinal sections of crypts were counted microscopically upon identification by means of the Apoptag kit (Appligene-Oncor, France) (Hughes & Rowland, 2001). The results clearly show that there was an effect on apoptosis. Feeding either inulin or oligofructose to the rats increased their apoptotic index (Fig. 6), which means that the prebiotic fed rats more efficiently eliminate colonic cells with defective DNA. Here the effect of inulin was numerically more important than the effect of oligofructose. It is remarkable that this is the case both in the proximal and in the distal colon (Hughes & Rowland, 2001).

**Short-chain fatty acids, products of gut fermentation**

One of the bacterial metabolites of fructan fermentation is butyrate, a short-chain fatty acid, which is one of the most physiologically relevant products of gut flora fermentation (Cummings et al. 1987). It is found in millimolar concentrations in the lumen as a consequence of microbial
Carbohydrate degradation and serves as a principle energy source for colon epithelial cells (Roediger, 1989). A hypothesis is that butyrate protects against colon cancer by inhibiting colon cell proliferation and inducing differentiation (Rehman et al. 1998; Sesink et al. 2000). Moreover, it may additionally confer protection by promoting apoptosis in colon tumour cell lines (Hague et al. 1995; Hague & Paraskeva, 1995). It is further implicated that dietary fibre may protect against colon cancer through the production of butyrate by the colonic microflora (Van Munster et al. 1994; McIntyre et al. 1993; Perrin et al. 2001). Therefore some of the properties inulin has shown in rats in vivo, including the first described mechanisms of apoptosis, could be due to butyrate.

In contrast, in vitro data with human biopsy specimens and other in vivo data in animals, show that butyrate seems to have opposite effects in non-transformed colon cells where it acts proliferate instead of antiproliferative (Lupton, 1995). For diet and cancer prevention therefore, some additional mechanisms could be caused by butyrate in non-transformed colon cells. In fact, the blocking agent activities involved in primary cancer prevention are expected to be of equal importance for overall risk reduction (Wattenberg, 1992). These activities lead to reduced exposure to genotoxic risk factors either by inhibiting their formation, by scavenging reactive intermediates or by modulating the balance of metabolizing systems in cells to favour deactivation of carcinogens (Johnson et al. 1994). In this context we have recently been able to show that pre-incubation of human and rat primary colon cells with Na-butyrate protects them from genotoxic effects induced by hydrogen peroxide (Abrahamse et al. 1999; Pool-Zobel et al. 1995). Butyrate may also alter the metabolic balance in human colon tumour cell lines by inducing glutathione S-transferase (GST) (Stein et al. 1996; Kirlin et al. 1999). GST are a family of enzymes that catalyse the conjugation of reactive chemicals with glutathione (GSH) and play a major role in protecting cells from these chemicals (Awasthi et al. 1994). GSH-conjugates are subsequently eliminated via active transport systems (Ishikawa, 1992). In human and rat colon tissue, GSTP1 is the major form of this enzyme and it is inducible by dietary factors (Peters et al. 1989; Acheson et al. 1967; van Lieshout et al. 1996; Nijhoff et al. 1995). Butyrate may also increase secretion of mucin, a barrier which can deactivate carcinogens thus protecting the epithelial cells (Kassie et al. 1999). Together the findings are subsequently the possible extensions in the line of evidence that fibre may be protective on account of butyrate production by the gut flora. In this context, an interesting recent study has shown that only fibres promoting a stable butyrate colonic ecosystem decrease the rate of aberrant crypt foci in rats (Perrin et al. 2001).

Conclusion

From the present set of results, it can be stated that there is consistent data available suggesting that the prebiotic chicory inulin and its fractions have anticarcinogenic activities, most probably through particular modification and maintenance of metabolic activity of the intestinal flora.

In rats a prebiotic effect, resulting in the proliferation of bifidobacteria (with the major metabolites lactate or acetate), as well as of other bacteria (with the metabolites butyrate or propionate and acetate), could be responsible for the observed anticancer effects in the colon of animals. The metabolites will reduce the pH of the colon lumen and direct interactions with cells of the colon epithelium may cause enhanced expression of phase II (deactivating enzymes), such as GST, or induce apoptosis to remove transformed cells, or increase mucin, a barrier which protects from attack by reactive compounds.

Since there is established evidence of a prebiotic effect in humans, these data justify further research with human volunteers using biomarkers to reveal a potential risk reduction in the gut lumen. Appropriate methods to determine the genotoxic burden in the gut lumen, or to analyse DNA damage and other parameters in cells isolated from colon biopsy specimens, are available. On the basis of so far obtained preliminary results they are probably sufficiently sensitive to reveal a significant effect of inulin or oligofructose intervention, if present (Osswald et al. 2000; Pool-Zobel & Leucht, 1997; Pool-Zobel et al. 1999). Such studies would allow evaluating the possible role of these food ingredients to reduce carcinogenic/genotoxic risk factors in the colon.

At present a human intervention study is being planned, and its outcome promises to yield more information in the potentially advantageous properties of inulin and related prebiotics in the human colon.

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