Anti-cancer function of phytic acid

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Summary
Inositol hexaphosphate (InsP$_6$ a.k.a. phytic acid or IP$_6$) is ubiquitous. In the plant kingdom it is particularly abundant in cereals and legumes; in much smaller amounts IP$_6$ and its lower phosphorylated forms (IP$_{1-5}$) are contained in most mammalian cells, where they are important in regulating vital cellular functions. Both in vivo and in vitro experiments have demonstrated striking anticancer (preventive as well as therapeutic) effects of IP$_6$. Inositol also is anti-carcinogenic, albeit to a lesser extent; it acts synergistically IP$_6$ in inhibiting cancer. In addition to reduction in cell proliferation, IP$_6$ increases differentiation of malignant cells often resulting in reversion to the normal phenotype. IP$_6$ is quickly absorbed from the rat stomach and upper intestine and distributed as inositol and IP$_1$. In vitro, it is instantaneously taken up by malignant cells undergoing variable dephosphorylation to inositol and IP$_{1-5}$, pointing towards their role in mediating the action of IP$_6$. In humans, IP$_6$ has recently been detected in urine, plasma and other biological fluids; the levels fluctuating with ingestion or deprivation of IP$_6$ or IP$_6$-rich diet. As IP$_6$ is high in high-fibre diets, these also may explain, at least in part, the epidemiological observation showing the association of ingesting high-fibre diets with a lower incidence of certain cancers. Along with safety, the reproducible efficacy of IP$_6$ and inositol in the prevention of cancer in laboratory animals warrant their inclusion in our strategies for cancer prevention and perhaps therapy in humans. Aside from the anticancer action, IP$_6$ and inositol also have numerous other health benefits. All these facts of normal physiological presence of IP$_6$ in our body the level of which fluctuates with intake, association of an IP$_6$-rich diet with low incidence of several diseases and vice versa, and finally reversal of some of these conditions, at least in part, by IP$_6$ supplementation strongly argue in favour of its inclusion as an essential nutrient or perhaps a vitamin.

Keywords
Chemoprevention, differentiation, inositol hexaphosphate, IP$_6$.

Introduction
myo-Inositol hexaphosphate (InsP$_6$ or IP$_6$) is a simple ringed carbohydrate with six phosphate groups attached to each carbon. In a pH range of 0.5–9.0, it adopts the sterically stable 1ax/5eq (one phosphate at carbon position 2 in the axial position and five phosphates in the equatorial position) and sterically hindered 5ax/1eq conformation over pH 9.5 (Barrientos & Murthy, 1996). IP$_6$ is contained in substantial amounts in cereals and legumes (0.4–6.4%), primarily existing as a form of salt with monovalent and divalent cations, e.g. Ca$^{2+}$, Mg$^{2+}$ and K$^+$ (Harland & Oberleas, 1987). Since its discovery in the mid-nineteenth century, the popularity of IP$_6$ stemmed mostly from the fact that it is the chief storage form of phosphorous for the germinating seeds. Interest in IP$_6$ in the mid-1980s was rekindled partly due to its antioxidant function as a result of its ability to chelate divalent cations. This property however, has been marred by concerns expressed during the past half-a-century about an alleged mineral deficiency that resulted from intake of foods high in IP$_6$. It was, and unfortunately to some extent, thought that IP$_6$ reduces the bioavailability of dietary minerals; hence its infamy as an anti-nutrient,
notwithstanding the reports to the contrary that even long-term intake of IP6 in food (Walker et al., 1948; Cullumbine et al., 1950) or in pure form (Henneman et al., 1958) did not cause such a deficiency in humans.

Biochemists and cell biologists on the other hand, have been interested in the phosphorylation and dephosphorylation of IP6, and how this might affect cellular functions. Lower inositol phosphates (IP1–4) are recognized as intracellular messengers. The second messenger role of inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] in bringing about a host of cellular functions including mitosis via mobilizing intracellular Ca2+ is well recognized. Its ‘cousin’ – inositol 1,3,4,5-tetakisphosphate (IP4), has also been shown to induce Ca2+ sequestration. Higher forms of IP, inositol 1,3,4,5,6-pentakisphosphate (IP5) and inositol hexakisphosphate (IP6) are also abundant and represent the bulk of IP content of mammalian cells. IP5&6 are present in virtually all mammalian cells in substantial amounts, between 10 and 100 μM (Szwergold et al., 1987), much higher than any other IP. Why should there be an intracellular abundance of these compounds which were presumed toxic by nutritionists or inert at best by biochemists (Menniti et al., 1993)? Moreover, we know very little of their purpose. Recent studies provide an increased understanding of the functional roles of IP5&6. Physiological functions of IP5 include regulation of the affinity of avian hemoglobin for oxygen, and along with IP6, it may be involved in neuronal excitation (Menniti et al., 1993). Recent demonstrations that IP5&6 are precursors of several derivatives that turnover rapidly suggest that these forms of IP are not inert or ‘metabolically lethargic’ and that they play a more dynamic role has been previously appreciated (Menniti et al., 1993). As described below, one reason why IP6 has been receiving increased attention is its anticancer action.

As IP6 undergoes dephosphorylation to IP1–5 and IP3 is central in cellular signal transduction and intracellular function, I hypothesized that IP6 exerts its anticancer function through lower inositol phosphates by entering into the intracellular IP pool (Shamsuddin, 1992; Shamsuddin et al., 1988, 1989). It was also hypothesized that the addition of inositol (Ins), a precursor of IP and also an innocuous natural carbohydrate, to IP6 may enhance the anticancer function of IP6 (Shamsuddin, 1992; Shamsuddin et al., 1989). Furthermore, because inositol phosphates are ubiquitous and are common molecules involved in signal transduction in most mammalian cell systems, I further hypothesized that the anticancer action of inositol phosphates would be observed in different cells and tissue systems (Shamsuddin, 1992; Shamsuddin et al., 1992).

Cancer chemoprevention

Because there were no prior data on the effect of IP6 on cancer, my first experiment was essentially a ‘shot in the dark’. Although experts in the field of nutrition and cancer would like such an experiment to begin by adding IP6 to the diet, conventional wisdom from pharmacology suggested that the administration of IP6 solution via drinking water would be the preferred one. Pilot studies to test the palatability of the Na-IP6 solution quickly revealed that rats only drink it up to a concentration of 5% (by the way, I tasted it prior to giving it to the rats, as it didn’t taste bad to me, I thought the rats wouldn’t mind!). However, it appeared that a solution of 1–2% Na-IP6 was the most desirable, beyond which their water intake was so reduced that higher levels would have been self-defeating. Furthermore, it is well known that IP6 readily binds with proteins and other components of the diet, rendering itself less readily available for absorption. So our experiments were designed to test the efficacies at a maximum dose of 2% in drinking water. Different species (rats and mice) and carcinogens (1,2-dimethylhydrazine, azoxymethane, dimethylbenzan-thracene) were used to examine its effectiveness across species and agents. In the colon cancer model, Na-IP6 treatment was begun 1–2 weeks prior to the beginning of carcinogen administration (pre-initiation phases) to (i) give the treatment an advantage of time and (ii) to see if treatment would directly or indirectly nullify the carcinogenic action (chemically). Six months after the beginning of the experiment, animals receiving IP6 had fewer neoplasms in their colon than the control animals which were not treated with IP6, and the tumours were approximately two-thirds smaller (Table 1). Early during this experiment I also observed that the rate of cell division in the non-tumorous colonic epithelium of IP6-treated
animals was similar to that of the normal control animals; in other words, the carcinogen-induced increase in mitotic rate was normalized, confirming my hypothesis that the anticancer action of IP6 lies in controlling cell division. Most interestingly, the mitotic rate of the animals receiving IP6 treatment but not carcinogen remained at normal rate (Shamsuddin et al., 1988, 1989; Shamsuddin & Ullah, 1989; Ullah & Shamsuddin, 1990). An interesting point of note is that adding a much higher amount of IP6 to the diet, Jariwalla et al. (1988) in a rat fibrosarcoma model have reported similar results; this points to the earlier argument I made for not using IP6 mixed in food which renders it less efficient.

Subsequently my co-workers and I showed that the inhibition of large intestinal cancer was not only dose-dependent but also related to the pH of the solution; Na-IP6 solution has a pH of approximately 11, neutralization gave better results (Ullah & Shamsuddin, 1990).

The lack of a dramatic decrease in cancer incidence implied that IP6 was not a direct antagonist to the carcinogen. Thus, coupled with the observation that the tumour size in IP6-treated animals was smaller, these facts led me to believe that IP6 might be effective even after the beginning of cancer induction (post-initiation phase). Consequently, IP6 was administered to the drinking water of rats as early as 2 weeks or as late as 5 months following carcinogen. Eight months after four doses of the carcinogen azoxymethane (8 mg kg−1), only 10% of the animals on IP6 developed colon cancer compared with 43% in the control group. Animals showed significantly lower tumour number and size even when given IP6 5 months after initiation, a time when most of the animals are expected to have cancers (Shamsuddin & Ullah, 1989) (Table 2). These findings pointed towards its possible therapeutic use (vide infra).

To test the hypothesis that the anti-tumour action of IP6 may be mediated via lower phosphorylated forms of inositol – IP, which are important in cell division, and that the addition of inositol may enhance the anti-proliferative and thus the anti-cancer action of IP6, additional experiments were conducted. It was indeed noted that inositol potentiated both the anti-proliferative and anti-neoplastic effect of IP6 in vivo. A significantly greater suppression of both cell proliferation and colorectal cancer was noted when inositol was added to IP6 (Shamsuddin et al., 1989). Similar potentiation was seen in the mammary and metastatic tumour models (Vucenik et al., 1992, 1993).

### Inositol

As regards the relationship between inositol and IP6, the phrase ‘chicken or the egg’ could best describe it. Inositol deficiency within cells in patients with diabetes is implicated in the development of various complications of diabetes, such as altered sensations (peripheral neuropathy), cataract and retinal damage, early derangements of kidney functions (diabetic nephropathy), etc. In experimentally induced diabetes mellitus, free inositol level in the peripheral nerve is reduced which correlates with a decreased motor nerve
conduction velocity. Inositol, ranging from 0.5 g twice a day to 3 g has been given to patients with diabetic neuropathy; results to date support that oral supplementation of inositol may be of benefit in the prevention and treatment of neural complications of diabetes mellitus (Holub, 1986).

Several psychiatric diseases have been treated with inositol. These include clinical depression, panic disorder and obsessive compulsive disorder. When twenty-eight depressed patients were given 12 g of inositol a day, a statistically significant overall benefit was found for inositol treatment as compared with the placebo control as early as week 4 on the Hamilton Depression Scale, the standard measure for assessing the effectiveness of an antidepressant substance (Levine, 1997). Patients with obsessive compulsive disorder were treated with 18 g of inositol or placebo per day for 6 weeks in a double-blind controlled crossover trial. Here too, inositol reduced the scored (Yale-Brown Obsessive Compulsive Scale) symptoms of obsessive compulsive disorder with good statistical significance when compared with the placebo control.

Anti-cancer action of inositol

My colleagues and I were also the first to demonstrate that myo-inositol alone or in combination with IP6 can prevent the formation and incidence of several cancers in experimental animals in soft tissue, colon, metastatic lung cancer and mammary cancers (Shamsuddin et al., 1989; Vucenik et al., 1992, 1995, 1997). Dr Hecht and Dr Wattenberg and their colleagues have shown that inositol prevents carcinogen-induced lung tumour formation in mouse models (Estensen & Wattenberg, 1993; Wattenberg & Estensen, 1996; Hecht et al., 1999).

Combined IP6 + inositol

At the beginning I alluded to my hypothesis that since IP6 undergoes dephosphorylation to IP1–5 and IP3 is central in cellular signal transduction and other functions such as cell division, IP6 could enter into the intracellular inositol phosphate pool and cause tumour suppression through lower inositol phosphates such as IP3. I had also hypothesized that inositol could also become phosphorylated to the higher inositol phosphates such as IP1–6, but most importantly IP3.

Thus addition of inositol to IP6 and administering the cocktail as therapeutic agent would allow the nascently released phosphates from IP6 to be captured by the inositol that is pretty much standing by. Therefore, delivering IP6 + inositol may cause increased availability of lower inositol phosphates, most importantly IP3 as per the following reaction:

\[ \text{IP}_6 + \text{inositol} \rightarrow 2 \text{IP}_3 \]

If indeed IP3 is involved in the transmission of signals from the growth factors in the cell’s exterior to the nucleus and cell division, too much of this signal may somehow cause a negative feedback and therefore shut down cell proliferation and growth. Furthermore, because inositol phosphates are ubiquitous and are common molecules involved in signal transduction in most mammalian cell systems, I also hypothesized that the anticancer action of inositol phosphates would be observed in different cells and tissue systems. The anticancer function of IP6 against various tumours was already discussed; the following are examples of how the combination treatment is also better than inositol or IP6 in different cancers.

Table 3 shows the results from the colon cancer study (Shamsuddin et al., 1989); similar results

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of tumour/rat</th>
<th>Tumour volume (mm³)</th>
<th>Mitotic rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOM only</td>
<td>7.1 ± 0.6</td>
<td>570 ± 110</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>AOM + IP6</td>
<td>5.2 ± 0.6</td>
<td>200 ± 60</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Significance*</td>
<td>P &lt; 0.02</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

* The reduction in the number of tumour/rat, tumour size and the rate of cell division by IP6 treatment are statistically significant at the P values given for each parameter. Note that IP6 treatment has resulted in two-thirds smaller tumours than the untreated controls.
were seen in the mammary cancer (Vucenik et al., 1992, 1995, 1997) and metastatic lung cancer (Vucenik et al., 1992) models.

It is of interest to note that the response of various cancer models to either IP<sub>6</sub> or inositol vary depending on the parameters tested. For instance in the mammary tumour model, while IP<sub>6</sub> caused a reduced incidence of tumours, the tumour size was larger than the control! But when all parameters of tumour assessments are taken the most consistent anticancer results were obtained from the combination of IP<sub>6</sub> + inositol.

Cancer therapy

The fact that colorectal cancer inhibition was observed when IP<sub>6</sub> treatment was begun as late as 5 months following initiation suggested to us that the beneficial action of IP<sub>6</sub> was not restricted to the prevention of tumour development, but perhaps treatment of existing cancers as well. That IP<sub>6</sub> normalizes cell division rate provides additional rationale for such experiments; inasmuch as cellular proliferation is integral to fully developed cancers as it is to early lesions. Thus, studies in my laboratory were extended to test the therapeutic properties of IP<sub>6</sub> in a mouse transplantable tumour model (Vucenik et al., 1992). Daily treatment of syngeneic host mice with intraperitoneal injections 0.25% Na-IP<sub>6</sub> (1 mL every other day) following subcutaneous inoculation of mouse fibrosarcoma FSA-1 cells, resulted in both a significant inhibition of tumour size and improvement of survival over the untreated controls. Intravenous injection of FSA-1 cells developed experimental lung metasta-

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Tumour prevalence</th>
<th>Tumour frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mitotic rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMH</td>
<td>63%&lt;sup&gt;†&lt;/sup&gt;</td>
<td>22/19 (1.16)</td>
<td>1.92 ± 0.17</td>
</tr>
<tr>
<td>DMH + IP&lt;sub&gt;6&lt;/sub&gt;</td>
<td>47%&lt;sup&gt;†&lt;/sup&gt;</td>
<td>13/21 (0.62)</td>
<td>1.48 ± 0.15</td>
</tr>
<tr>
<td>DMH + inositol</td>
<td>30%</td>
<td>9/20 (0.45)</td>
<td>1.01 ± 0.14</td>
</tr>
<tr>
<td>DMH + IP&lt;sub&gt;6&lt;/sub&gt; + inositol</td>
<td>25%</td>
<td>4/16 (0.25)</td>
<td>1.06 ± 0.13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Tumour frequency is represented as number of tumours (gross + microscopic cancers) per mouse. <sup>†</sup> The difference in tumour prevalence between DMH-only (carcinogen control group) and DMH + IP<sub>6</sub> + inositol is significant at P < 0.001, and between 2DMH + IP<sub>6</sub> and 3DMH + IP<sub>6</sub> + inositol at P < 0.005. Thus the combination of IP<sub>6</sub> + inositol treatment is significantly better than either one alone.

Table 3 Synergistic cancer inhibition by IP<sub>6</sub> when combined with inositol

Effect on cancer cell lines

In vitro studies of both human and rodent cancer cell lines in my laboratory demonstrate that IP<sub>6</sub> reduces cell proliferation rate in all of the cell lines tested, including MCF-7 human mammary carcinoma cells. Interestingly, unlike most other anti-cancer agents, the cells do not show an overwhelming evidence of cytotoxicity; rather, the growth of malignant cells slows and they mature and die. The reduced cell growth and enhanced differentiation of cancer cells to the point of reversion back to normal phenotype is seen in different cell lines. For instance K-562 human erythroleukaemia cells are relatively small compared with their normal counterpart, the erythrocytes; and they are devoid of haemoglobin. A striking growth inhibition can be achieved with 6–10 mmol L<sup>-1</sup> of Na-IP<sub>6</sub> and few cells remain viable after 48 h. However, at lower concentrations (e.g. 75 µmol L<sup>-1</sup>), the cells can be maintained for a longer period, albeit at reduced numbers wherein the cells become large and accumulate haemoglobin, akin to the more mature cells (Shamsuddin et al., 1992).

Reversion of malignant to normal phenotype has also been observed in the HT-29 human colon carcinoma cell line. The disaccharide tumour marker β-D-galactose-[1→3]-N-acetyl-galactosamine is expressed by malignant and premalignant cells of the colon and other epithelia, but not by the
normal cells. Following IP₆ treatment, along with a decreased rate of cell proliferation, tumour marker expression is markedly suppressed. In most cells no expression whatsoever could be seen although the cells produced the parent mucopolysaccharide (Sakamoto et al., 1993a). Thus while IP₆ suppresses the malignant phenotype, it nevertheless allows the progression of normal mucin synthesis and maturation of human colon cancer cells to structurally and behaviourally resemble normal cells.

It is to be noted that the doses or the concentration required to achieve IC₅₀ (50% inhibition of cell number) varies for the different cell lines. While the cells of haematopoietic lineage (e.g. K-562, YAC-1, HL-60, etc.) are highly sensitive to IP₆, those of the epithelial and mesenchymal lines require higher concentrations. Furthermore, whereas Ins alone has a modest anticancer effect in vivo (Shamsuddin et al., 1989; Estensen & Wattenberg, 1993; Vucenik et al., 1993) when added to IP₆ there is a synergistic effect.

Using BALB/c mouse 3T3 fibroblasts and midpoint cytotoxicity assay by neutral red method, Babich et al. (1993) compared the effects of several chemopreventive agents and demonstrated a moderate efficacy of IP₆. This modest result is not surprising because extensive experiments with human cancer cell lines in vitro in my laboratory have shown little cytotoxicity.

### Tumour abrogation

While in vitro studies using human cancer cell lines lend support to the data obtained from in vivo animal models, preparation for a human trial require further investigations. Towards that goal, we tested the efficacy of IP₆ against human rhabdomyosarcoma – a highly malignant tumour of skeletal muscle origin, common in children and young adults. We first tested in vitro cell growth inhibition which not surprisingly was striking as in all other cell lines. Consistent with in vitro observation, IP₆ also suppressed the growth of rhabdomyosarcoma cells in vivo in a xenografted nude mice model. NIH athymic male nude mice were used to determine the effect of IP₆ on the tumour forming capacity of rhabdomyosarcoma cells. Each mouse received 10⁷ viable rhabdomyosarcoma cells, injected subcutaneously into the lower dorsal region. Two days later, IP₆ (40 mg kg⁻¹ in 0.1 mL PBS) was injected around the tumour, and was continued every other day for 2 weeks (expt 1) or for 5 weeks (expt 2). When compared with controls, IP₆-treated mice produced 25-fold smaller tumours (P = 0.008), as observed after a 2-week treatment. In the second experiment, wherein the treatment period was extended to 4 weeks, a 49-fold (P = 0.001) reduction in tumour size was observed in mice treated with IP₆. Histologically no evidence of tumour cell necrosis was observed, confirming all the previous studies that IP₆ does not kill tumour cells (cytostatic, and non-cytotoxic), yet suppresses the tumour by 25–49-fold (Vucenik et al., 1998a) (Table 4).

As exciting as the results may be, this experiment only showed inhibition of tumour formation and not reduction of a preformed tumour, as the injection was begun before a visible tumour has formed. Therefore the next logical experiment was to test whether IP₆ could shrink an existing tumour. Using a similar xenotransplantation model, this time for liver cancer, we addressed this question. However, prior to this we tested the ability of IP₆ to inhibit growth of a human liver cancer cell line and then tested the effect on tumour formation and growth of human hepatocellular carcinoma HepG2 cell line in a transplanted nude mouse model (Vucenik et al., 1998b,c). A solid tumour was observed in 71% of nude mice after subcutaneous injection of HepG2 cells (1 × 10⁷/mouse) during a period of 18–41 days following transplantation, but no tumour was found in the mice which had received the same number of HepG2 cells pretreated in vitro with 5 mM IP₆ for 48 h (P < 0.03). When transplanted tumours reached 8–10 mm in diameter, an intratumoral injection of IP₆ (40 mg kg⁻¹) was given for 12 consecutive days, after which the animals

<table>
<thead>
<tr>
<th>Expt 1 (2 weeks treatment)</th>
<th>Expt 2 (5 weeks treatment)</th>
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<tr>
<td>Controls (n = 5)</td>
<td>Controls (n = 5)</td>
</tr>
<tr>
<td>818 ± 377</td>
<td>1666 ± 1122</td>
</tr>
<tr>
<td>IP₆-treated</td>
<td>IP₆-treated</td>
</tr>
<tr>
<td>33 ± 63</td>
<td>34 ± 47</td>
</tr>
<tr>
<td>Significance</td>
<td>Significance</td>
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<tr>
<td>P = 0.008</td>
<td>P = 0.001</td>
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Data presented as mean ± standard deviation.
were killed. At autopsy, the tumour weight in IP₆-treated mice was 3.4-fold less than that in control mice (0.333 ± 0.270 g vs. 1.130 ± 0.423 g, \( P = 0.016 \)).

The most intriguing finding in this experiment is that a single treatment of hepatocellular cancer cells by IP₆ resulted in the complete loss of the ability of these cells to form tumour when inoculated subcutaneously in nude mice (tumorigenicity); on the other hand untreated cells did. Moreover, then when the pre-existing liver cancers themselves were treated directly with IP₆, they regressed (Vucenik et al., 1998b,c).

**Effect on host defence mechanism**

Natural killer (NK) cells play a pivotal role in various aspects of the host’s defence system. As NK cells are important in tumour abrogation, my co-workers and I investigated whether IP₆ mediates its anti-neoplastic action via NK cells. Using YAC-1 target cells labelled with \(^{51}\text{Cr} \), the cytotoxicity of murine spleen NK cells was measured. Mice with carcinogen-induced tumours, treated \textit{in vivo} with IP₆, showed augmented NK activity over the untreated controls and NK activity correlated with tumour suppression (Baten et al., 1989). Similar enhancement of NK activity was also demonstrated when splenocytes from normal mice were treated with IP₆ \textit{in vitro} (Baten et al., 1989).

For bacterial killing by polymorphonuclear leucocytes (or neutrophils) the stimulation of respiratory burst associated with free radical generation is a critical event. Eggleton et al. (1991) demonstrated that the preincubation of human polymorphonuclear leucocytes with IP₆ results in substantially enhanced production of reactive oxygen intermediates following stimulation by phagocytic particles, or chemicals. Johnson et al. (2000) investigated the effect of IP₆ on the proliferation and viability of RAW 264.7 transformed macrophages and the role of IP₆ as a free radical scavenger. Their results suggested that IP₆ may have an excitatory effect on inflammatory cell secretions. These investigators also demonstrated a reduction of malondialdehyde (MDA) by IP₆ indicating its effectiveness as an antioxidant. Thus IP₆ may enhance host defence via these mechanisms as well.

**Mechanisms of action**

Because cancer is a major public health issue, the dramatic anti-cancer effect of IP₆ has resulted in our quest for understanding its mechanism of action. As a first step, studies in my laboratory have demonstrated that contrary to popular misconception, \(^{3}\text{H}-\text{IP₆} \) when intragastrically administered to rats is very quickly absorbed from the stomach and upper small intestine and distributed to various organs as early as 1 h following administration (Sakamoto et al., 1993b). The radioactivity isolated from the gastric epithelium at this time is associated with inositol and IP_{1-6}, and that in the plasma and urine with inositol and IP₁ indicating a very rapid metabolism of the compound. The presence of IP₆ in the gastric epithelium suggests that the intact molecule is perhaps transported inside the cell wherein it is rapidly dephosphorylated. Could IP₆ be dephosphorylated extracellularly and then be absorbed as inositol and IP_{1-5} with some rephosphorylation back to IP₆ intracellularly? This is unlikely, as there is no mucosal phytase activity in the stomach and we administered IP₆ in drinking water 2 h following fasting avoiding the action of dietary phytase. In any event, as earlier time point studies were not conducted, this study could not beyond doubt, demonstrate the transport of intact IP₆. Transport of intact IP₆ across the cell membrane is not only a normal process via various binding proteins to transport it across cell membranes (e.g. clathrin adaptor complex AP2, AP180, coatomer of COP I coat), but IP₆ may also regulate vesicle budding and fusion in the membrane. Indeed, IP₆ itself interacts with AP2 and inhibits downstream events in the cell.

Our studies of the absorption of IP₆ by malignant cells \textit{in vitro} also demonstrate that the cells almost instantaneously begin to accumulate IP₆ intracellularly, the rate of accumulation varying for the different types of cells (Vucenik & Shamsuddin, 1994). For instance, the uptake of \(^{3}\text{H}-\text{IP₆} \) by mouse YAC-1 lymphoma cell line reached a plateau as early as 10 min after incubation. The ability and the rate at which the cells metabolized IP₆ also varied; YAC-1 and K-562 cells contained only the lower IP’s whereas HT-29 human colon carcinoma cells had inositol and IP_{1-6}.

A central pathway of cancer inhibition by IP₆ is via control of cell division; and IP₆ reduces the
rate of cellular proliferation both in vivo and in vitro. Experiments in my laboratory with ³H-thymidine incorporation also demonstrated a reduction in DNA synthesis. It is possible that IP₆ may exert its control by chelating cations because the metalloproteins are important in gene regulations (O’Halloran, 1993). However, as we used a dodecasodium salt of IP₆ rather than inositol hexaphosphoric acid in our experiments, it is difficult to envision how Na₁₂-IP₆ would be able to exert any, much less substantial chelation. Insofar as the steps of carcinogenesis are concerned, our studies showed the effectiveness of IP₆ both prior to and following initiation in colon carcinogenesis models. But in a mouse skin carcinogenesis through the classical initiation → promotion model, Ishikawa et al. (1999) found that IP₆ is effective in the prevention of skin papillomas when given during the initiation phase, but not during promotion.

From the earlier discussion, one can surmise that the cellular mechanism of cancer inhibition is one of reduction of cell proliferation rate (rather normalization of mitotic rate) and induction of cellular differentiation. Further studies in my laboratory showed a suppression of DNA synthesis as measured by ³H-thymidine incorporation and down-regulation of proliferation marker PCNA by IP₆ (Yang & Shamsuddin, 1995). A marked decrease in the expression of proliferation markers indicated that IP₆ disengaged cells from active cycling. Using dual parameter flow cytometry and combined analysis of the expression of cell cycle-related proteins, El-Sherbiny et al. (2001) demonstrated that IP₆ controls the progression of the cells through the cell cycle by significantly decreasing the S-phase and arresting the cells (human colon and breast cancer cell lines) in the G₀/G₁ phase. Studies of human leukaemia cells at Professor Lambertenghi-Deliliers’ laboratory at the University of Milan demonstrate that not only does IP₆ show a dose-dependent cytotoxic effect on human leukaemia cell lines, but also the IP₆-treated leukaemia cells accumulate in G₂M phase of cell cycle, once again arresting cells in the cycle, albeit in a different phase (Lambertenghi-Deliliers et al., 2002). cDNA micro-array analysis showed an extensive down-modulation of genes involved in transcription and cell-cycle regulation (c-myc, HPTCAAX₁, FUSE, cyclin H) and an up-regulation of cell cycle inhibitors such as CKS₂, p57 and Id-2. Genes such as STAT-6 and MAPKAP, involved in important signal transduction pathways were also down regulated.

Besides the above cellular mechanism, the following biochemical pathways may also be operational for the various functions of IP₆:

Chelation of Fe³⁺ and suppression of OH formation

In the plant kingdom IP₆ functions as an antioxidant, protecting and preserving the seeds, which may remain viable for a long time. The 1,2,3 (equatorial–axial–equatorial) phosphate grouping in IP₆ has a conformation that uniquely provides a specific interaction with iron to completely inhibit its ability to catalyze hydroxyl radical formation. Protection against cancer and a multitude of other applications may be based at least in part on this antioxidant function.

The anti-oxidative function of IP₆, occurs by chelating iron by occupying all the available Fe coordination sites thus inhibiting *OH generation from the Fenton reaction and the subsequent lipid peroxidation and DNA damage. IP₆ has the unique ability to remove O₂ without generating oxy-radicals. Thus it inhibits *OH production by chelating iron in the presence of O₂, O₂⁺, or any reducing agent; can maintain the redox potential of iron by accelerating both reduction of Fe³⁺ by ascorbic acid (AH₂) and oxidation of Fe³⁺ by O₂ through following reaction:

\[
\text{Fe}^{3+} + \text{AH}_{2} \xrightarrow{\text{O}_2, \text{e}} \text{Fe}^{2+} + \text{A} + \text{H}_2\text{O}_2
\]

\[
4\text{Fe}^{2+} + \text{O}_2 + 4\text{H}^+ \rightarrow 4\text{Fe}^{3+} + 2\text{H}_2\text{O}
\]

Therefore, IP₆ could reduce the active oxygen species-mediated carcinogenesis and cell injury via its anti-oxidative function (Graf & Eaton, 1990).

Chelation of divalent cation

Mg²⁺ has been implicated in the second messenger system within the cell. Thymidine kinase, an enzyme essential for DNA synthesis and cell division, is sensitive to zinc depletion. Within 6 days of zinc depletion, there is a decrease in
thymidine kinase activity and thymidine incorporation into DNA. On the other hand the activity of ribonuclease increases with zinc deficiency (Harland & Oberlea, 1987). Inasmuch as Mg\(^{2+}\) and Zn\(^{2+}\) are essential for cell proliferation including that in tumours, deprivation of these cations may cause a decrease in tumour growth. Jariwalla et al. (1988) and Thompson & Zhang (1991) favour the theory that IP\(_6\) brings about its anticancer effect by removing these cations. Our pilot experiments using \(^{45}\)Ca in cell culture medium, done by G.-Y. Yang and myself (unpublished observation, 1993) showed that Na-IP\(_6\) did not significantly alter the availability of divalent minerals \textit{in vitro}. This is not totally unexpected considering the fact that we used salts of IP\(_4\) for all our experiments, both \textit{in vitro} and \textit{in vivo}. Thus the proposed mechanism remains inexplicable at the moment and additional experiments are needed to understand this. Furthermore, the observed rapid dephosphorylation of IP\(_6\) by both normal rats \textit{in vivo} and malignant cells \textit{in vitro} to lower IPs, particularly IP\(_{1-3}\), make the above two hypothesis less credible; they are both based on the ability of six phosphate groups to chelate divalent cations. Finally, that tumour inhibition and even abrogation by IP\(_6\) was observed long after carcinogenic stimuli suggest that other mechanisms are involved.

**Participation in intracellular inositol phosphate pool**

In support of the hypothesis that the anti-tumour action of IP\(_6\) is mediated via lower IPs, my co-workers and I have demonstrated that following IP\(_8\) treatment of K-562 human erythroleukaemia cells, there is a 41\% increase (\(P < 0.05\)) in intracellular IP\(_3\) and a 26\% (\(P < 0.02\)) decrease in IP\(_2\) (Shamsuddin et al., 1992). The fact that these measurements were done by administration of \(^{3}\)H-Ins and ion exchange chromatography of the IP and measuring the radioactivity, indicates that conversion of Ins to IP\(_{1-3}\) does take place, and that this occurs as early as within 1 h. The data from \textit{in vivo} and \textit{in vitro} experiments showing absorption of IP\(_8\) described earlier point to its rapid conversion to Ins and IP\(_{4-6}\). Thus it is clear that administration of IP\(_6\) results in alterations in the cellular inositol phosphate pool. What specifically brings about the observed biological effects are yet to be elucidated.

A pilot study demonstrated that 3 h following 0.05\% IP\(_6\) treatment, intracellular Ca\(^{2+}\) increases by 57\% (\(P < 0.02\)) (Shamsuddin et al., 1992). While the current dogma is that an increased intracellular Ca\(^{2+}\) concentration secondary to an increase in IP\(_3\) may be responsible for mitosis, our data on the other hand corresponds to a decrease in cell division. Such a paradoxical relationship between increased intracellular Ca\(^{2+}\) and biological events is not unique to our study and has also been observed by others. Further studies of time-dependent changes in intracellular Ca\(^{2+}\) concentration are warranted. Because inositol can be converted to IP\(_{1-3}\) and perhaps IP\(_{4-6}\), it is quite plausible that the observed anticancer effect of inositol given alone is perhaps owing to its conversion to metabolically active IP\(_3\)s.

**Cellular and nuclear signalling pathways**

Studies by several investigators are beginning to lead us to an understanding that IP\(_6\) may be involved in the pathways involved in cell signalling. There are other ways by which IP\(_6\) could influence the various activities within the cells. For instance, repair of double-strand breaks in DNA is essential for maintaining the stability of the genome, failure to repair may result in loss of genetic information, chromosomal translocation and even cell death. Two mechanisms for this repair have been described – homologous recombination or non-homologous end-joining. IP\(_6\) has been demonstrated to stimulate non-homologous end-joining; it has been proposed to be brought about by the binding of IP\(_6\) to the DNA-dependent protein kinase DNA-PK\(_{cs}\) (Hanakahi et al., 2000). A more recent study reported that it is not DNA-PK\(_{cs}\) (a large protein of \(~3500\) amino acids, \(M_w \sim 465\) kDa), but the DNA end binding protein Ku (consists of Ku\(~70\) – 70 kDa, and Ku\(~86\) – 83 kDa) that binds to IP\(_6\) (Ma & Lieber, 2002). Be that as it may, these studies, in spite of differences in their specific findings, clearly show a very important role of IP\(_6\) in DNA repair mechanism.

Once the assault on the cell has gone past the scope of DNA repair, the otherwise normal cell is likely to transform to a malignant (cancer) cell. Insofar as the transformation of cells from normal to malignancy is concerned, there are various
models and pathways, one of these pathways is the activation of transcription factors activating protein-1 (AP-1) and nuclear factor NFκB via phosphotidylinositol 3-kinase (PI-3 kinase). Using tumour promoter-induced cell transformation of human skin JB6 cells, Huang et al. (1997) have demonstrated that IP₆ blocks epidermal growth factor-induced PI-3 kinase and AP-1 activity. Zi et al. (2000) demonstrated similar results on DU145 human prostate cancer cells, along with a concomitant inhibition of cell growth. Upstream of these pathways lies the mitogen-activated protein kinases (MAPK) which are serine/threonine kinases that are rapidly activated upon extracellular stimulation. This family of kinases include Erks (extracellular signal-regulated kinases, JNKs (c-Jun N-terminal kinases) and p38 kinases. IP₆ inhibited the activities of Erks and JNKs, but not of the p38 kinases in human skin, prostate and breast cancer cells (Huang et al., 1997; Vucenik et al., 1999a; Zi et al., 2000; Chen et al., 2001). Thus, given the commonality shared by these three divergent cell types, the blocking of this cellular to nuclear signalling pathway appears as an important mechanism of anticancer action of IP₆.

**IP₆ controls nuclear export of mRNA**

The transcription of DNA to messenger RNA (mRNA) is carried out within the nucleus, being segregated from the cytoplasm by the nuclear envelope; the mRNA is then transported by a complex series of events through pores into the cytoplasm. York et al. (1999) demonstrated that the enzyme phospholipase C and two proteins that influence the generation of IP₆ are required for proper and efficient export of mRNA from the nucleus to the cell.

The enhancing effect of IP₆ on NK cells, already mentioned, could be an additional mode of its anti-neoplastic action. As NK-cells play an important role in host defence against neoplasia, it is quite possible that the contribution of IP₆ via boosting NK-cell cytotoxicity may be important.

**Other biological effects and applications of IP₆**

There is evidence that it plays important roles in various other conditions and holds promise for use therein.

Nearly half a century ago, Henneman et al. (1958) successfully used pure Na-IP₆ to treat the idiopathic hypercalciuria which is associated with a high frequency of kidney stones. A diet containing high IP₆ has also been used to treat hypercalciuria and kidney stones (Ohkawa et al., 1984). IP₆ has a hypocholesterolemic effect and may find potential use in the clinical management of hyperlipidaemia and diabetes (Jariwalla et al., 1990). A strong *in vitro* anticoagulant activity of Na-IP₆ has been demonstrated in the blood of various animals (Borgo, 1983). Additionally, agonist-induced platelet aggregation is markedly inhibited by IP₆ (Vucenik et al., 1999b). Moreover, administration of IP₆ efficiently protects the myocardium from ischaemic damage and reperfusion injury (Rao et al., 1991).

Finally, Otake et al. (1989) demonstrated that IP₆ inhibited the cytopathic effect of human immunodeficiency virus (HIV) and HIV-specific antigen expression in MT-4 cells; inositol hexosulphate showing an even stronger inhibition. Coupled with the facts that IP₆ enhances NK-cell activity and polymorphonuclear cell priming function, it is possible that IP₆ may have uses in the management of HIV infection and associated immunodeficiency-related problems.

**Safety**

For quite some time now, IP₆ has been blamed, albeit wrongly (as discussed below) for causing mineral, particularly Ca²⁺ deficiency, hence it had been castigated as an anti-nutrient. As Dr June Kelsay wrote in her critical review (Kelsay, 1987), unfortunately much of that stemmed from studies done on 2–6 people. In addition to the very small sample size, many of those studies suffered from poor study design; for instance, the only two subjects in the study by Walker et al. (1948) were consuming a lower than recommended amount of Ca²⁺ in the diet during the study period. The three subjects studied by Cullumbine et al. (1950) were also on a low Ca²⁺ diet who developed a negative balance during the first few weeks, but the balance reversed to a positive one after 8–9 weeks on the same diet! There were also studies (see review by Kelsay, 1987) that indicated that if mineral intakes are adequate and cereal or bran intakes are held at a moderate
level, there are no adverse effects on mineral bioavailability. Ohkawa et al. (1984) did a follow-up study of subjects ingesting 10 g rice bran (containing high IP₆) for up to 2 years and reported no decrease, significant or otherwise, in serum Ca²⁺, PO₄³⁻, Mg²⁺ and uric acid levels. Kelsay (1987) proposed that oxalic acid (rich in spinach and rhubarb) in the diet complicated the interpretation of results in studies of fruits and vegetables; there seems to be an interaction of fibre and oxalic acid resulting in increased faecal excretion of some minerals, and hence decreased mineral balances.

Direct studies of the effects of IP₆ should resolve this lingering and rather vexing argument, for administration of the isolated pure compound enjoys the benefit of non-confounding variables. Indeed those studies both in experimental animals and in humans (some done nearly half a century ago) have conclusively refuted the negative mineral balance stigma. Studies in my laboratory on experimental animals showed no significant toxic effect on body weight, serum mineral content or any pathological changes of consequence in either male F344 or female Sprague–Dawley rats for 40 weeks (Ullah & Shamsuddin, 1990; Vucenik et al., 1993). Studies by Grases et al. (1998) not only confirmed our findings, but also report that abnormal calcification is prevented in rats given IP₆.

As regards toxicity and its direct relevance to humans, of particular note is that Henneman et al. (1958) administered pure Na-IP₆ orally to thirty-five patients at a dose of 8.8 g day⁻¹ (in divided doses) for many months. Not only did they reduce the episodes of urinary stones in these patients, a long-term follow-up study of ten patients (average of 24 months) showed no untoward toxicity; the reduction in hypercalciuria and prevention of stone recurrence were rather the benefits.

**Epidemiological correlates**

There are very few, if any, well controlled epidemiological studies of the relationship of diet containing high IP₆ content and cancer incidence. However, the consistent negative correlation between those high-fibre diets rich in IP₆, and cancers of the colon, breast and perhaps prostate are interesting (Englyst et al., 1982; Yu et al., 1991; Willett, 1994). As breast cancer incidence is significantly lower in Hispanic and black women than in non-Hispanic White, Zang et al. (1994) studied the dietary pattern of these three groups and showed that beans (in Hispanics), and fruits and vegetables (in Blacks) may be responsible for the lower incidence of mammary cancer. You may recall that beans are rather rich in IP₆. Yu et al. (1991) have demonstrated that compared with Chinese in Shanghai, Americans have fourfold higher age-adjusted rates of colon cancer and a twofold higher rates of rectal cancer. The rates of prostate and postmenopausal breast cancer were 26-fold and 10-fold higher in Americans; corresponding to a 2.6-fold lower cereal consumption in the USA. A negative correlation between the incidence of breast cancer and cereal and fibre consumption was also reported by Morales & Llopis (1992).

A vitamin?!

Much of our knowledge of inositol phosphates and their metabolism comes from studies using radio-labelled inositol. Conversion of inositol to IP₆ has been demonstrated in mammalian cells in vitro (Larsson et al., 1997). However, to demonstrate such a conversion in humans in vivo is not permissible nowadays. Enter Professor Felix Grases of the University of Balearic Islands in Palma de Majorca: using a novel method of gas chromatography – mass detection analysis of high pressure liquid chromatography (HPLC) fractions, Grases et al. (2000, 2001a) were not only the first to identify IP₆ in human urine and plasma, but also that the levels fluctuate depending on the intake of IP₆ by the subjects. Healthy human volunteers become deficient in IP₆ (as demonstrated by a low plasma level of IP₆) in about 2 weeks. It takes about the same time period (2 weeks) for the plasma and urinary IP₆ levels to return to normal once the subjects are on an IP₆-rich diet; but it takes only 4 h if the subjects take the pure IP₆ as a supplement (Grases et al., 2001a). Grases et al. (2001b, 2002) were the first to detect IP₆ and its lower phosphorylated forms (IP₃ and IP₅) in mammalian cells and body fluids as they occur naturally, again demonstrating changes in the IP₆ and IP₅ levels that depended on dietary
intake of IP6. These data, taken together with the above mentioned health benefits, argue most strongly in favour of inclusion of IP6 as an essential nutrient (perhaps a vitamin) and shedding its bad name as an anti-nutrient.

Final comments

The preceding discussion has highlighted the many potential beneficial actions of IP6. The mechanism(s) of how IP6 works need to be elucidated, but it seems unlikely that all these divergent functions are mediated through a single pathway. Given the numerous health benefits, its participation in important intracellular biochemical pathways, normal physiological presence in our cells, tissues, plasma, urine, etc., the levels of which fluctuate with intake, epidemiological correlates of deficiency with disease and reversal of those conditions by adequate intake, and safety – all strongly suggest for its inclusion as an essential nutrient, or perhaps a vitamin. Meanwhile, inclusion of IP6 + inositol in our strategies for prevention and therapy of various ailments, cancer in particular is warranted. Of course, eating a healthy diet rich in IP6 would always be a prudent thing too.

References


IP₆ regresses pre-existing human liver cancer xenotransplanted in nude mice. *Anticancer Research, 18*, 4091–4096.


