Malignant H1299 tumour cells preferentially internalize iron-bound inositol hexakisphosphate

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Synopsis
In colon enterocytes and in well-differentiated colon cancer CaCo-2 cells, InsP6 (inositol hexakisphosphate) inhibits iron uptake by forming extracellular insoluble iron/InsP6 complex. In this study, we confirmed that CaCo-2 cells are not able to take up iron/InsP6 but, interestingly, found that the cells are able to internalize metal-free and Cr3+-bound InsP6. Thus, the inability of CaCo-2 cells to take up iron/InsP6 complexes seems to be due to the iron-bound state of InsP6. Since recently we demonstrated that the highly malignant bronchial carcinoma H1299 cells internalize and process InsP6, we examined whether these cells may be able to take up iron/InsP6 complexes. Indeed, we found that InsP6 dose-dependently increased uptake of iron and demonstrated that in the iron-bound state InsP6 is more effectively internalized than in the metal-free or Cr3+-bound state, indicating that H1299 cells preferentially take up iron/InsP6 complexes. Electron microscope and cell fraction assays indicate that after uptake H1299 cells mainly stored InsP6/iron in lysosomes as large aggregates, of which about 10% have been released to the cytosol. However, this InsP6-mediated iron transport had no significant effects on cell viability. This result together with our finding that the well-differentiated CaCo-2 cells did not, but the malignant H1299 cells preferentially took up iron/InsP6, may offer the possibility to selectively transport cytotoxic substances into tumour cells.

Key words: H1299 tumour cell, inositol hexakisphosphate, multiple-inositol-polyphosphate-phosphatase (MINPP1).


INTRODUCTION

In plant cells, InsP6 (inositol hexakisphosphate) mainly acts as a phosphate source and as a store of multivalent cations, because it binds Mg2+, Ca2+, Zn2+ and Fe3+ with high affinity [1]. Plant seeds express phytases during germination to get access to phosphate and multivalent cations, and mature plants use secreted phytases of rhizosphere bacteria and fungi to dephosphorylate soil InsP6 [2,3]. Also mammalian cells express a phytase, the MINPP1 (multiple-inositol-polyphosphate-phosphatase), which in lung tumour cells (H1299) is processed in the endoplasmic reticulum and subsequently secreted to the medium and transported into lysosomes [4]. The phytase activity of MINPP1 enables the cells to dephosphorylate extracellular InsP6 in the medium and, as H1299 cells are able to internalize extracellular InsP6, MINPP1 dephosphorylates InsP6 after endocytosis in lysosomes. This MINPP1-mediated dephosphorylation of InsP6 results in release of inositol, phosphate and multivalent cations, thus providing an additional source of micronutrients [4].

However, high concentrations of InsP6 mainly have negative cellular effects because physiological levels of phytases are not sufficient to dephosphorylate InsP6 concentrations ≥100 μM resulting in depletion of essential multivalent cations from the medium [5]. In enterocytes, this characteristic of InsP6 can substantially inhibit uptake of non-haem iron, because formation of Fe3+/InsP6 complexes prevents reduction of Fe3+ by Dcytb (duodenal cytochrome B) and thus transport of non-haem iron by the divalent metal transporter DMT-1 [6]. In particular plant seeds contain high concentrations of InsP6 and of Fe3+ and since

Abbreviations used: DCF, dichlorodihydrofluorescein; EM, electron microscopy; InsP6, inositol hexakisphosphate; InsPs, inositol phosphates; MDD, metal detection; MINPP1, multiple-inositol-polyphosphate-phosphatase; ROS, reactive oxygen species.

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after destruction of cells InsP₆ and Fe³⁺ are released, formation of non-soluble InsP₆ and Fe³⁺ complexes reduce the bioavailability of plant iron [7,8]. Accordingly, it has been shown that in CaCo-2 cells, which serve as cellular model for iron uptake by enterocytes, extracellular InsP₆ inhibits iron uptake (e.g. [9]). As similar as plants, H1299 cells use extracellular InsP₆ as additional source for micronutrients, in this study we examined if these cells may be able to internalize and process extracellular iron/InsP₆ complexes.

**MATERIALS AND METHODS**

**Cell culture**

The cell line H1299 was cultured in DMEM (Dulbecco’s modified Eagle’s medium); CaCo-2-cells were grown in MEM (Minimal Essential Medium). Both media were supplemented with 10% (v/v) (FCS), 4 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. For characteristics of these cells, see American Type Culture Collection (ATCC).

**Extraction of InsPs (inositol phosphates)**

InsPs were extracted from H1299 and CaCo-2 cells as described in Windhorst et al. [4]. After extraction, InsPs were analysed by MDD (metal detection) HPLC [10].

**Radioactivity measurements**

⁵⁹FeCl₃ and ⁵¹CrCl₃ in 1 M hydrochloric acid were purchased from Perkin Elmer Rodgau, Germany. Appropriate activities result in 500–2000 Bq per final probe, containing only tracer amounts of iron or chromium, were diluted with aqueous ‘cold’ FeCl₃ or CrCl₃ solutions to the desired concentrations and then lyophilized to remove HCl. In experiments, with InsP₆, this agent was pre-incubated with ⁵⁹Fe or ⁵¹Cr for 1 h at room temperature (25 °C) before adding to the respective cell culture system. ⁵⁹Fe- or ⁵¹Cr-radioactivity was measured in washed cells as well as in pooled medium and wash fractions using a sensitive large volume whole body counter.

**Analysis of iron uptake in CaCo-2- and H1299-cells in presence and absence of InsP₆**

H1299- and CaCo-2-cells (5×10⁵ cells/dish) were grown in 3.5 cm dishes at 37 °C and 5% (v/v) CO₂ in 1 ml cell culture medium for 20 h. An aliquot of 50 μl of each solution containing ⁵⁹FeCl₃ with or without InsP₆ was added to cell culture dishes to give an end concentration of 30 μM ⁵⁹FeCl₃ and 30 μM InsP₆. After cautious mixing, cells were incubated at 37 °C overnight. The media were removed and cells were washed five times with PBS.

**Dose-dependent influence of InsP₆ on iron uptake in CaCo-2- and H1299-cells**

The cell lines were grown as described above. Solutions containing ⁵⁹FeCl₃ and different amounts of InsP₆ were prepared, pre-incubated for 1 h and then added as 50 μl aliquots to the cell culture dishes (n = 3) to result a final concentration of 30 μM ⁵⁹FeCl₃ and InsP₆ (0 μM/dish); (1 μM/dish); (10 μM/dish); (30 μM/dish); (100 μM/dish). After cautious mixing, the cells were incubated overnight at 37 °C and prepared for ⁵⁹Fe-activity measurement as described above.

**Cell fractionation using differential centrifugations**

H1299 cells were treated with 30 μM ⁵⁹FeCl₃/30 μM InsP₆ or with 30 μM ⁵⁹FeCl₃ only. After incubation for 20 h, the cells were washed five times with PBS and after trypsinization, the cells were centrifuged (7 min 1.200 rev/min room temperature). The pellet was resuspended in 1 ml fractionation buffer (10 mM Tris–HCl pH 7.5, 250 mM sucrose) and the cells were homogenized in a Potter-Elvehejm homogenizer (40 times). Trypan-blue staining of homogenized cells revealed that only 20% of the cells were lysed. However, to avoid destruction of microsomes, the cells were not further homogenized. The homogenized cells were differentially centrifuged as described in [4]. ⁵⁹FeCl₃-radioactivity of P3 as well as of supernatants were measured using the HAMCO-whole body counter. The gamma radiation of the samples was measured for 10×10 s and the mean value of the activity in Bq (Becquerel) was calculated.

**Determination of iron-induced formation of ROS (reactive oxygen species)**

After cellular uptake of 5,6-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-DCF-DA, Wako) esterases remove the diacetate group leading to formation ofDCF (dichlorodihydrofluorescein). After oxidation by ROS DCF is fluorescent with excitation at 490 nm. Thus, the DCF fluorescence is linear to the concentration of cellular ROS. To measure the effect of extracellular FeCl₃ and FeCl₃/InsP₆ on formation of ROS, 1×10⁴ H1299 cells per well were seeded into black 96-well plates. After incubation for 20 h the medium was discharged, the cells were washed twice with PBS and new medium containing 10 μM DCF or, as control, medium without DCF was added to cells and incubation was continued for 1 h. Thereafter, the medium was discharged again, the cells were washed twice with PBS and new medium containing 30 μM FeCl₃, 30 μM FeCl₃/30 μM InsP₆ or 30 μM FeCl₃/100 μM InsP₆ was added and the cells were further incubated for 2 or 20 h, respectively. After these incubation times, the medium was discharged, the cells were washed twice with PBS and 120 μl PBS was added to cells. DCF fluorescence of the cells was measured in a Tecan-Reader at excitation 490 nm; emission 535 nm. The values obtained from cells that were not incubated with DCF (background) were subtracted from the values obtained from cells which were incubated with DCF.
Malignant H1299 tumour cells preferentially internalize iron-bound inositol hexakisphosphate

Figure 1  Effect of InsP<sub>6</sub> on iron uptake by CaCo-2 and H1299 cells
(A) Cells were seeded in 3.5 cm Petri dishes and grown to 70 % confluence. 59FeCl<sub>3</sub> was incubated with different InsP<sub>6</sub>-concentrations and added to the cell culture medium. As control, cells were treated with 59FeCl<sub>3</sub> only. After 20 h, the cells were washed five times with PBS and the 59FeCl<sub>3</sub> radioactivity was measured by using a HAMCO-whole body counter. The 59FeCl<sub>3</sub>-signal of cells incubated with 59FeCl<sub>3</sub> alone was set to 100 %. (B) The same experiment as described in (A) was performed. In addition 50 μM vitamin C was added to 30 μM InsP<sub>6</sub>/30 μM 59FeCl<sub>3</sub> to reduce 59FeCl<sub>3</sub> to 59FeCl<sub>2</sub>. Shown are means ± S.D. of at least three independent experiments.

Analysis of cellular ferritin concentration
Cells seeded in 24-well plates and grown to 70 % confluence were treated with 30 μM FeCl<sub>3</sub>, 30 μM FeCl<sub>3</sub>/10 μM InsP<sub>6</sub> or 30 μM FeCl<sub>3</sub>/100 μM InsP<sub>6</sub>, respectively. After 20 h of incubation, the cells were washed five times with PBS and lysed with MPER buffer (Promega). The protein concentration of these samples was analysed by the Bradford assay and the concentration of ferritin by a ferritin ELISA (Immunology Consultants Laboratory; Cat. No. E-90F) according to the manufacturer’s instructions. The ferritin concentration was calculated per mg protein.

Electron microscopy
30 μM FeCl<sub>3</sub>/30 μM InsP<sub>6</sub> or 30 μM FeCl<sub>3</sub> were incubated for 1 h at room temperature in 50 μl cell culture medium and was then added to H1299 cells cultivated in 24-well plates on 12 mm Aclar sheets (Plano). After 20 h of incubation the cells were fixed with a mixture of 4 % (v/v) paraformaldehyde and 1 % (w/v) glutaraldehyde in 0.1 M PBS at pH 7.4 for 1 h at room temperature. Thereafter they were rinsed three times in 0.1 M sodium cacodylate buffer (pH 7.2–7.4) and osmicated using 1 % (w/v) osmium tetroxide (Science Services) in cacodylate buffer. Following osmication, the cells were dehydrated using ascending ethyl alcohol concentration steps, followed by two rinses in propylene oxide. Infiltration of the embedding medium was performed by immersing the coverslips in a 1:1 mixture of propylene oxide and Epon and finally in neat Epon and hardened at 60 °C. Ultrathin sections (60 nm) were examined in a EM902 (Zeiss). Pictures were taken with a MegaViewIII digital camera (A. Tröndle,).

RESULTS
InsP<sub>6</sub> alters iron uptake by tumour cells
In order to examine potential differences in iron uptake between H1299 and CaCo-2 cells in presence and absence of InsP<sub>6</sub>, both cell lines were incubated with 30 μM 59FeCl<sub>3</sub> only as well as with 30 μM 59FeCl<sub>3</sub>, and different concentrations of InsP<sub>6</sub> (1, 10, 30 and 100 μM) for 20 h. Prior to measurement, the cells were washed five times to redissolve precipitated iron/InsP<sub>6</sub> complexes from the cell surface (see, e.g. [4]). Cellular iron uptake was examined by analysing the 59FeCl<sub>3</sub>-radioactivity of washed cells. First, we compared uptake of 59FeCl<sub>3</sub> in absence of InsP<sub>6</sub> between CaCo-2 and H1299 cells and found no significant differences between the cell lines (results not shown). To analyse the effect of InsP<sub>6</sub> on iron uptake, 59FeCl<sub>3</sub>-signals of control cells (treated with 59FeCl<sub>3</sub> only), were set to 100 % (Figure 1A). As expected, we found that InsP<sub>6</sub> inhibited iron uptake in a dose-dependent manner in CaCo-2 cells, by 25 % at low (1 μM) and by 65 % at high concentrations (100 μM) in comparison with cells incubated with 59FeCl<sub>3</sub> alone. However, in H1299 cells InsP<sub>6</sub> showed the opposite effect. In this cell line incubation with 1 μM InsP<sub>6</sub> slightly increased iron uptake (by 25 %) while addition of 100 μM increased uptake of 59FeCl<sub>3</sub> even 8-fold. Thus, in contrast to CaCo-2 cells, in H1299 cells InsP<sub>6</sub> does not inhibit but facilitates iron uptake.

Since InsP<sub>6</sub> possesses six negatively charged phosphate groups, it might bind Fe<sup>3+</sup> with higher affinity than Fe<sup>2+</sup>, and thus may preferentially bind oxidized iron. To test this assumption, 59FeCl<sub>3</sub> was incubated with vitamin C to reduce FeCl<sub>3</sub> to FeCl<sub>2</sub> and CaCo-2 and H1299 cells were incubated with 59FeCl<sub>2</sub> and with 59FeCl<sub>2</sub>/30 μM InsP<sub>6</sub>. In addition, the cells were treated with 59FeCl<sub>3</sub> and with 59FeCl<sub>3</sub>/30 μM InsP<sub>6</sub>. The 59Fe-signals
although the cells took up higher amounts of InsP6. This observation might be explained by inhibition of InsP6 dephosphorylation or it may be that dephosphorylation of InsP6 is rate limiting as only a fraction of InsP6 is hydrolysed even in absence of iron.

of cells incubated with 59FeCl3 were set to 100 %. As shown in Figure 1(B) vitamin C treatment increased iron uptake in both cell lines (by 40 to 50 %), which is in line with numerous reports describing that vitamin C facilitates uptake of iron [8]. Incubation with 30 μM InsP6/59FeCl3 again decreased uptake of iron in CaCo-2 and increased its uptake in H1299 cells. In both cell lines, addition of vitamin C to 59FeCl3/InsP6 complexes reduced the effect of InsP6 on iron uptake; in H1299 cells 1.5- and in CaCo-2 cells 2.5-fold. This finding supports our assumption that InsP6 binds Fe3+ with higher affinity than Fe2+.

Taken together our data demonstrate that InsP6 dose dependently increases uptake of iron by highly malignant H1299 lung tumour cells and decreases iron uptake by well-differentiated CaCo-2 colon carcinoma cells, whereby in both cell lines vitamin C reduces the effect of InsP6 on iron uptake.

H1299 cells preferentially internalize iron-bound InsP6

Our data that in CaCo-2 cells InsP6 inhibits iron uptake indicate that these cells are not able to take up InsP6. To examine this assumption, H1299 and CaCo-2 cells were incubated with either InsP6 alone or with 30 μM InsP6/30 μM FeCl3 for 24 h and uptake of InsP6 into cells was analysed by MDD–HPLC. This analysis revealed that in absence of iron both cell lines were able to take up InsP6 without showing significant differences of the incorporated amount (Figures 2A and 2B). Whereas addition of FeCl3 had no significant effect on InsP6 uptake by CaCo-2 cells, it increased internalization of InsP6 by H1299 cells by 79 %. After uptake of FeCl3/InsP6 by H1299 cells the concentration of Ins(1,2,4,5,6)P5 was similar as in cells treated with InsP6, although the cells took up higher amounts of InsP6. This observation might be explained by inhibition of InsP6 dephosphorylation or it may be that dephosphorylation of InsP6 is rate limiting as only a fraction of InsP6 is hydrolysed even in absence of iron.

In conclusion, our data clearly demonstrate that CaCo-2 cells are able to take up iron-free InsP6 but obviously no iron-bound InsP6.

To analyse if CaCo-2 cells in principle are not able to take up InsP6-metal complexes, we analysed internalization of 51Cr3+/InsP6 by CaCo-2 in comparison with H1299 cells. The cells were treated with 30 μM 51CrCl3+ and with 30 μM 51CrCl3+/30 μM InsP6 for 20 h and after washing the cells, the 51Cr3+ radioactivity was measured. To compare uptake of 51Cr3+/InsP6 with uptake of 59FeCl3/InsP6 the percentage of uptake was calculated and depicted in one graph (Figure 3). This comparison revealed that in absence of InsP6 CaCo-2 cells took up 0.7 % 51Cr3+ and 9 % 59FeCl3. In presence of InsP6 3 % of extracellular 51Cr3+ has been taken up, and uptake of 59FeCl3 was decreased 4.5-fold. H1299 cells took up only 0.4 % 51Cr3+, and similar as CaCo-2 cells, 10 % 59FeCl3 in absence of InsP6. In presence of InsP6, the cells took up 30 % 51Cr3+ and 75 % 59FeCl3. In summary, these results reveal that CaCo-2 cells are able to take up metal-free and also chrome-bound but not iron-bound InsP6. H1299 cells, by contrast internalized all forms of InsP6 with preference to Fe3+/InsP6.

InsP6/FeCl3 complexes accumulate in lysosomes

Metal-free InsP6 is internalized and stored in lysosomes of H1299 cells [4]. To analyse if uptake of Fe3+/InsP6 complexes occurs similar as metal-free InsP6 H1299 cells incubated with 59FeCl3 and with 59FeCl3/InsP6 were fractioned in endo/lysosomal (P3) and cytosolic fractions. The fractions were evaluated by Western-blotting using antibodies against the specific marker proteins [4]. In Figure 4(A), the 59Fe3+ radioactivity (Bq) of the endo/lysosomal (P3) fraction and of the supernatant (S/N) of cells incubated with 59FeCl3 and with 59FeCl3/InsP6 is depicted. In Figure 4(B), the 59FeCl3-signal of cells incubated with 59FeCl3 was set to 100 %. This evaluation shows that in presence of InsP6, the 59FeCl3-signal was 8.5-fold higher in P3
cells, (2) cells treated with 30 μM FeCl₃ and resemble those published by Ahlinder et al. [11,12]. H1299 cells had internalized FeCl₃/InsP₆ and stored the inositol phosphate–iron complex in lysosomes. Accordingly, also viability of cells incubated with iron only in comparison with non-treated cells, confirming our observation that H1299 cells can take up iron also in absence of InsP₆ (results not shown). In presence of 100 μM InsP₆, the ferritin level was reduced 1.3-fold in CaCo-2 cells, which is in line with our data showing that in these cells high InsP₆ concentrations inhibit iron uptake. In H1299 cells, the ferritin level was also lower (2-fold) in cells incubated with iron and 100 μM InsP₆ as compared with cells treated with iron only, although our data show that under these conditions the cells took up about 8-fold more iron than in absence of InsP₆. From this data, we conclude that in presence of InsP₆, H1299 cells store the main fraction of iron in lysosomes and transport only a small amount (about 10%) into the cytoplasm.

**InsP₆ protects H1299 cells from iron-induced formation of ROS but does not alter cell viability**

It has been shown that in vitro InsP₆ prevents iron-induced formation of ROS [14]. To examine if this is also the case in H1299 cells, formation of ROS was examined in iron- and iron/InsP₆-treated cells. The cells were treated with 30 μM FeCl₃ with 30 μM FeCl₃/10 μM InsP₆ and with FeCl₃/100 μM InsP₆ for 2 h (Figure 6A) or for 20 h (Figure 6B), respectively. Measurement of DCF-fluorescence revealed that incubation of H1299 cells with FeCl₃ for 2 h increased formation of ROS 3-fold, while incubation with FeCl₃/30 μM InsP₆ as well as incubation with FeCl₃/100 μM InsP₆ had no effect. Thus, InsP₆ seems to prevent iron-induced formation of ROS. However, after long incubation times (20 h), the FeCl₃-induced formation of ROS was vanished, indicating that during this time ROS were metabolized and free FeCl₃ had been bound to ferritin. Accordingly, also viability of cells incubated with 30 μM FeCl₃ with 30 μM FeCl₃/30 μM InsP₆ and with FeCl₃/100 μM InsP₆ was not different from that of control cells (Figure 6C).

**DISCUSSION**

In this study, we demonstrate that InsP₆ strongly promotes iron uptake by the highly malignant lung cancer cell line H1299 but inhibits iron transport by the well-differentiated colon carcinoma cell line CaCo-2. These different behaviours result from the fact that H1299 are able to internalize InsP₆/FeCl₃ complexes, whereas in CaCo-2 cells the complexes remain extracellular and inhibit uptake of free iron. Interestingly, CaCo-2 cells internalized
Figure 4 After internalization, iron/InsP₆ complexes accumulate in lysosomes of H1299 cells

H1299 cells were seeded in 15 cm Petri dishes and grown to 70% confluence. ⁵⁹FeCl₃ and InsP₆ were pre-incubated and added to the medium to an end concentration of 30 μM InsP₆/30 μM ⁵⁹FeCl₃. As control, cells were treated with 30 μM ⁵⁹FeCl₃ only. After incubation for 20 h, the cells were differently centrifuged (see Material and Methods section) and ⁵⁹FeCl₃ radioactivity of the endo/lysosomal (P₃)-fraction and the supernatant (S/N: cytosolic fraction) was analyzed. (A) Radioactivity in Bq. (B) The ⁵⁹FeCl₃-signal of cells incubated with ⁵⁹FeCl₃ was set to 100%. Shown are means ± S.D. of at least three independent experiments. (C) Cells remained non-treated or were treated with pre-incubated 30 μM InsP₆/30 μM ⁵⁹FeCl₃, with 30 μM ⁵⁹FeCl₃ and with 30 μM InsP₆. After 20 h the cells were fixed with 4% (v/v) paraformaldehyde/1% (w/v) glutaraldehyde and prepared for EM-analysis (see Material and Methods section). Shown are only cells treated with 30 μM InsP₆/30 μM ⁵⁹FeCl₃. Endo/lysosomes with dark amorphous structures, which indicate an accumulation of iron/InsP₆ aggregates, are marked with arrows.

iron-free InsP₆ and also InsP₆/Cr³⁺ complexes, showing that in principle the cells are able to take up InsP₆/metal complexes. Thus, the inability of CaCo-2 cells to internalize InsP₆/Fe³⁺ aggregates must result from the iron-bound state of InsP₆. It is well known that InsP₆ binds iron with high affinity and NMR-studies revealed that one InsP₆ molecule can bind four iron atoms by performing P–O–Fe–O–P bonds, leading to formation of large and stable Fe³⁺–InsP₆ aggregates [15,16]. Furthermore, Bretti et al. [17] demonstrated that InsP₆/Fe³⁺ complexes are more stable than InsP₆/Cr³⁺ aggregates and we detected large InsP₆/Fe³⁺ aggregates in lysosomes of InsP₆/Fe³⁺-treated H1299 cells. Based on these findings, we assume that InsP₆/Fe³⁺ aggregates are larger than InsP₆/Cr³⁺ complexes and could be taken up by H1299 but not by CaCo-2 cells. Our data that H1299 cells took up InsP₆/Fe³⁺ complexes 3-fold more effectively than InsP₆/Cr³⁺ support this assumption. However, despite this preferential uptake of InsP₆/Fe³⁺ also InsP₆/Cr³⁺ complexes were taken up more effectively by H1299 than by CaCo-2 cells. Thus, the ability of H1299 cells to take up InsP₆-metal-complexes is in general higher than the ability of CaCo-2 cells. Future experiments will elucidate the cellular mechanisms underlying this cell-specific uptake of InsP₆/Fe³⁺ complexes.

The mechanism of InsP₆-mediated iron transport in H1299 cells mainly resembles that of transferrin-mediated iron uptake,
Malignant H1299 tumour cells preferentially internalize iron-bound inositol hexakisphosphate

Figure 5 InsP₆/iron alters the ferritin levels of CaCo-2 and H1299 cells
CaCo-2 and H1299 cells were seeded in 24-well plates and grown to 70% confluence. FeCl₃ was incubated with InsP₆ and added to the cell culture medium to give end concentrations of 10 μM InsP₆/30 μM FeCl₃ and 100 μM InsP₆/30 μM FeCl₃. As control, cells were treated with 30 μM ⁵⁹FeCl₃ only. After 20 h washed cells were lysed and the ferritin concentration of the cell lysates was analyzed by a ferritin-ELISA. Microgram ferritin per mg whole cell protein was calculated and the ferritin concentration of non-treated cells (control) was set to 100%. Shown are means ± S.D. of at least three independent experiments.

because similar to the transferrin–transferrin receptor complex, the iron/InsP₆ complexes are endocytosed and processed in lysosomes. The main fraction of internalized iron/InsP₆ exists as precipitate because iron and InsP₆ in equimolar ratios are poorly soluble at pH 5 [7] and metal–InsP₆–complexes are more insensitive to dephosphorylation by MINPP1 [17]. However, a small fraction of iron must have been dissociated from InsP₆ and subsequently transported from the lysosomes into the cytosol because the cellular ferritin level of cells treated with InsP₆ and Fe³⁺ was 10-fold higher than that of non-treated cells. As at low pH InsP₆ shows a higher affinity for H⁺ than for metal atoms, Fe³⁺ might have dissociated from InsP₆ with the time of incubation and InsP₆ becomes accessible to MINPP1-mediated dephosphorylation. The Fe³⁺ ions, which are released from InsP₆ could be reduced to Fe²⁺ and transported by DMT-1 into the cytosol. However, uptake of iron/InsP₆ did not alter cell viability, which is in contrast to the effect of low concentration of metal-free InsP₆, which slightly increased proliferation of H1299 cells [4]. We assume that these differences are due to the slow and ineffective dephosphorylation of iron/InsP₆ complexes leading to release of only low concentrations of phosphate, iron and inositol. The finding that iron-bound InsP₆ does not promote viability of lung cancer cells together with our result that well-differentiated CaCo-2 cells are not able to take up iron/InsP₆ complex may offer a new application of iron/InsP₆. Coupling of cytotoxic substances to the MINPP1-sensitive 3-phosphate group of InsP₆ may enable to transport cytostatic into tumour cells and slowly release them from its carrier. Future experiments will figure out which groups of malignant tumour cells are able to internalize and process InsP₆ and might thus enable a specific transport of cytotoxic substances into malignant tumour cells.

Figure 6 Effect of InsP₆ on iron-induced formation of ROS and on cell viability
(A, B) DCF-treated cells were incubated with 30 μM FeCl₃, with 30 μM InsP₆/30 μM FeCl₃ and with 100 μM InsP₆/30 μM FeCl₃ for 2 h (A) or for 20 h (B), respectively. DCF fluorescence was measured in a Tecan-Reader. Excitation 490 nm; emission 535 nm. Shown are means ± S.D. of three independent experiments. (C) Viability of cells incubated for 20 h was measured by the MTT assay. Thereafter, the cells were treated with 30 μM FeCl₃, with 30 μM InsP₆/30 μM FeCl₃ and with 100 μM InsP₆/30 μM FeCl₃ and viability was measured after further incubation for 24 and 48 h. For normalization, ratios to control cells were calculated. Shown are means ± S.D. of three independent experiments.
AUTHOR CONTRIBUTIONS

Christina Helmis performed most of the experiments (for exceptions, see below). Christine Blechner gave technical assistance. Hongying Lin performed and evaluated the MDD-HPLC experiments. Michaela Schweizer performed and evaluated the EM analysis. Georg Mayr helped to interpret the data obtained by MDD-HPLC. Peter Nielsen and Sabine Windhorst designed the concept of the study and interpreted the data. Sabine Windhorst wrote the paper.

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REFERENCES

4 Windhorst, S., Lin, H., Blechner, C., Fanick, W., Brandt, L., Brehm, M. A. and Mayr, G. W. (2013) Tumour cells can employ extracellular Ins(1,2,3,4,5,6)P(6) and multiple inositol-polyphosphate phosphatase 1 (MINPP1) dephosphorylation to improve their proliferation. Biochem. J. 450, 115–125
9 Ma, Q., Kim, E. Y., Lindsay, E. A. and Han, O. (2011) Bioactive dietary polyphenols inhibit heme iron absorption in a dose-dependent manner in human intestinal Caco-2 cells. J. Food Sci. 76, H143–H150

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