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Effect of inositol hexaphosphate (IP_6) on human normal and leukaemic haematopoietic cells

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Summary. Inositol hexaphosphate (IP_6), a naturally polyphosphorylated carbohydrate, has been reported to have significant *in vivo* and *in vitro* anticancer activity against numerous tumours, such as colon, prostate, breast, liver and rhabdomyosarcomas. To confirm this activity in haematological malignancies and to characterize some of the mechanisms of IP_6 action, we analysed its effects on human leukaemic cell lines and fresh chronic myelogenous leukaemia (CML) progenitor cells using a combined cellular and molecular approach. IP_6 had a dose-dependent cytotoxic effect on all of the evaluated cell lines, with accumulation in the G_2M phase in two out of five cell lines tested. At the molecular level, cDNA microarray analysis after IP_6 exposure showed an extensive downmodulation of genes involved in transcription and cell cycle regulation and a coherent upregulation of cell cycle inhibitors. Furthermore, IP_6 treatment of fresh leukaemic samples of bone marrow $CD34^+$ CML progenitor cells significantly inhibited granulocyte–macrophage colony-forming unit (CFU-GM) formation ($P = 0{\cdot}0062$) in comparison to normal bone marrow specimens, which were not affected. No differentiating effect on HL60 cells was observed. Taken together, our results confirm the antiproliferative activity of IP_6 and suggest that it may have a specific antitumour effect also in chronic myeloid leukaemias, via active gene modulation.

Keywords: IP_6, haematological malignancies, cytotoxicity, CML, microarrays.

Inositol hexaphosphate (IP_6) is a polyphosphorylated carbohydrate which, together with its parental compound inositol and its lower phosphorylated forms (IP_{1-5}), is present in the great majority of vegetal and mammalian cells (Shamsuddin *et al*, 1997). All these molecules are involved in the metabolism of phospholipids and are important regulators of such vital cellular functions as cell proliferation, differentiation and signal transduction (Shamsuddin, 1999). Few data are available concerning the downstream modulation of gene expression mediated by IP_6, although it has been suggested that, after cell internalization, exogenously administrated IP_6 may be dephosphorylated to $IP_{1\text{-}5}$ and (possibly via IP_4 or IP_5) may inhibit cell growth through the activation of signal transduction pathways and transcriptional regulation (De Camilli *et al*, 1996; Odom *et al*, 2000).

Interestingly, it has been demonstrated that IP_6 has significant biological activity, inhibiting cell growth in a variety of tumoral cell lines and mammalian neoplasia, such as human colon (Sakamoto *et al*, 1993), prostate (Shamsuddin & Yang, 1995), lung (Wattenberg, 1999), breast (Shamsuddin *et al*, 1996; Shamsuddin & Vucenik, 1999), liver (Vucenik *et al*, 1998a), skin cancers (Ishikawa *et al*, 1999) and rabdomyosarcomas (Vucenik *et al*, 1998b).

As IP_6 anticancer activity has been frequently demonstrated in tumours of mesodermal origin, from which haematopoietic bone marrow precursor cells also derive, we have investigated its biological effect on normal and leukaemic human haematopoietic cells, and attempted to characterize some of the molecular pathways of IP_6 activity.

In particular, we tested the effects of pharmacological IP_6 doses on the growth and cell cycle of various human leukaemic cell lines, and of fresh normal and chronic myelogenous leukaemic human bone marrow progenitor cells. The possible specific modulation of gene expression was tested by microarray analysis of K562 cells before and after IP_6 exposure.

Our data suggest that IP_6 may have a cytotoxic effect on leukaemic progenitor cells in both cell lines and fresh

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human samples. Furthermore, the expression pattern of IP_6-treated K562 cells suggests that its effect may be mediated by the active modulation of specific genes.

MATERIALS AND METHODS

Cell cultures

Cell lines. The HL60 human promyelocytic leukaemia cell line was obtained from Interlab Cell Line Collection (ICLC, Genova, Italy). The chronic myelogenous leukaemia cell lines were K562 from ICLC, AR230 (kindly provided by Dr S. Mizutani, Tokyo, Japan) and RWLeu4 (kindly provided by Dr M.A. Santucci, Bologna, Italy); the KG1a progenitor leukaemia cell line came from the European Collection of Animal Cell Cultures (Salisbury, UK). All the cell lines were grown in Roswell Park Memorial Institute (RPMI) medium + 10% fetal calf serum (FCS) at 37°C at densities of $2–3 \times 10^5$ cells/ml.

Cytotoxicity. The effect of IP_6 (Sigma, St. Louis, MO, USA) on cell growth was evaluated using a colorimetric assay for the quantification of cell proliferation and viability based on the cleavage of the WST-1 tetrazolium salt by mitochondrial dehydrogenases in viable cells (Lambertenghi Deliliers *et al*, 1998). Briefly, 10 μl of WST-1 were added to each of the 96-well culture plates containing 100 μl of cell suspension. After 4-h incubation at 37°C in a fully humidified atmosphere, the plates were evaluated using a 1420 VICTOR multilabel counter (E. G. & G. Wallac, Turku, Finland) and data expressed as the mean percentage of three replicates normalized to the untreated control.

Cell cycle analysis. In order to evaluate the possible role of IP_6 on cell cycle phases of all the tested cell lines (AR230, KG1a, K562, RWLeu4 and HL60), 4×10^5 cells were incubated with escalating doses of IP_6 (from 0·1 to 10 mmol/l), fixed in 70% ethanol, treated with 0·5 mg/ml RNase (Sigma) in phosphate-buffered saline (PBS) plus 0·1% saponin and incubated at 37°C for 30 min before staining with 20 μg/ml propidium iodide (Sigma) for 30 min at 4°C. The cells were then analysed for DNA content using a FACSCalibur flow cytometer (Becton Dickinson, San Josè, CA, USA). The acquired histograms were analysed using CELL QUEST software (Becton Dickinson) (Delia *et al*, 1997).

Assessment of in vitro differentiation. To test the potential differentiating effect of IP_6, the morphology of the HL60 cell line, which is known to be easily driven to differentiation by the addition of several substances (such as all-trans retinoic acid or dimethyl sulphoxide), was evaluated at different doses (from 0 to 10 mmol/l) and at different times (every 72 h for 3 months). Briefly, every 72 h 5×10^4 cells were cytocentrifugated on glass slides and then stained with May–Gruenwald solution for 6 min (Reactifs RAL, Martillac, France), washed with water, stained for 2 min with Giemsa fast stain (Reactifs RAL), washed again and air dried. Cell morphology was observed using a Nikon light microscope.

Haematopoietic progenitor $CD34^+$ cell separation. $CD34^+$ haematopoietic progenitor cells were separated from five fresh leukaemic or three normal bone marrow samples (taken from donors giving their informed consent) using the MACS Miltenyi immunonomagnetic method (Servida *et al*, 1996). The bone marrow specimens were layered on a Ficoll–Paque gradient (specific gravity 1·077 g/ml; Nycomed Pharma AS, Oslo, Norway) in order to separate the low density mononuclear cells (LD-MNCs) and then washed twice in Hanks' balanced salt solution (HBSS). The adherent cells were removed by means of two 30 min pre-adherence cycles at 37°C in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum (FBS) in 25 cm^2 culture flasks. The non-adherent fraction was then resuspended in IMDM 10% FBS. To isolate the $CD34^+$ cells, the LD-MNCs were incubated for 15 min at 4°C with the QBEND10 monoclonal antibody directed against the CD34 antigen. The cells were then washed and incubated for 15 min at 4°C with immuno-magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) against QBEND10. For the flow cytometry analysis, CD34-phycoerythrin (CD34-PE) conjugated antibody (HPCA-2, Becton Dickinson, Mountain View, CA, USA) was added to the cells for 15 min at 4°C. At the end of the separation, the cells were counted and assessed for viability by means of trypan blue dye exclusion; their purity was determined by means of flow cytometry analysis. All of these steps were performed under aseptic conditions. The mean percentage of purity of the $CD34^+$ cells after immuno-magnetic separation was 90·6%.

Liquid cultures. The CD34-enriched cells were cultured in the presence of different cytokines as previously reported (Soligo *et al*, 1996). Briefly the $CD34^+$ cells from three normal bone marrow and three CML patients were cultivated in RPMI + 10% FCS and supplemented with a cocktail of cytokines, including recombinant human (rh) stem cell factor (SCF; 50 ng/ml), rh interleukin 3 (rh IL-3; 10 ng/ml), rh IL-6 (10 ng/ml) and rh granulocyte–macrophage colony-stimulating factor (rh GM-CSF; 100 ng/ml) to induce cell proliferation which otherwise would not occur. IP_6 was added at concentrations ranging from 0·1 to 10 mmol/l and cell growth and viability was measured by means of the WST-1 tetrazolium salt assay at 24, 48 and 72 h of culture (Soligo *et al*, 2001); the data were expressed as the mean percentage of three replicates of three samples for a total of nine data for each point normalized to the untreated control.

Clonogenic assay (CFU-GM). The clonogenic assays were carried out by plating 5×10^3 $CD34^+$ cells in a methylcellulose culture medium (MethoCult 4434, Stem-Cell Technologies Inc., Vancouver, Canada) containing 0·9% methylcellulose in IMDM, 30% FBS, 1% bovine serum albumin (BSA), 10^{-4} mol/l 2-mercaptoethanol, 2 mmol/l L-glutamine, 50 ng/ml rh SCF, 10 ng/ml rh GM-CSF, 10 ng/ml rh IL-3 and 3 Units/ml rh erythropoietin. The cells were pretreated for 24 h with IP_6 and then seeded in the semisolid medium. Triplicate dishes were incubated at 37°C in a 5% CO_2 fully humidified atmosphere. The aggregates of ≥ 40 and < 40 cells were, respectively, scored as colonies and clusters after 14 d of culture.

Gene expression analyses

Cell line. K562 cells were treated with IP_6 at a dose of 5 mmol/l for 24 h; the negative control consisted of untreated K562 cells. After 24 h, 20×10^6 cells were harvested, dry pelleted and snap-frozen in liquid nitrogen for microarray and reverse transcription polymerase chain reaction (RT–PCR) analyses. The IP_6 concentration and incubation time were chosen, observing induction kinetics and the extent of cell cycle alterations demonstrated by flow cytometry analysis.

Microarray analysis. Atlas 1·2II cDNA expression arrays from Clontech Laboratories (Palo Alto, CA, USA) were chosen. These arrays consist of 1176 human cDNA fragments, organized into broad functional groups. A complete list of the genes included on the membranes is available on the Clontech Web site (http://www.clontech.com). All of cDNAs printed on the arrays have been sequence-verified by the company. The microarrays were screened using radiolabelled cDNA generated from total poly(A)$^+$ RNA from K562 cells cultured in the presence or absence of 5 mmol/l IP_6 for 24 h. Differential expression was arbitrarily defined as any gene up- or downmodulated in the cell line by a ratio of more than two at any signal intensity.

RNA extraction and RT–PCR analysis. Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Gibco BRL, Paisley, UK). RNA (1 μg) was run on a 1% agarose gel (Sigma) stained with ethidium bromide (Sigma) for quality control.

For cDNA synthesis, 4 μg of total RNA were mixed with 4·6 μmol/l oligo(dT)$_{12-18}$ (Gibco BRL) and incubated at 70°C for 10 min: the reaction was stopped in ice. In a final volume of 40 μl, 8 μl of 5X buffer (Gibco BRL), 4 μl of 0·1 mol/l dithiothreitol (DTT, Gibco BRL), 400 μmol/l dNTPs (Sigma), 80 U of Ribonuclease Inhibitor (Takara, Kyoto, Japan) and 400 U of Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT, Sigma) were mixed and incubated at 37°C for 1 h. cDNA integrity was confirmed by abl and β-actin genes PCR amplification.

Table I. Primer sequence, PCR product length and PCR conditions.

Primer	Sequence (5′ - > 3′)	Primers used: product size	PCR conditions
abl FW	TTCAGCGGCCAGTAGCATCTGACTT		
abl REV	GACCCGGAGCTTTTCACCTTTAGTT	184 bp	27 cycles: 94°C × 15 s–56°C × 30 s–72°C × 30 s
β-actin FW	TCACCCACACTGTGCCCATCTACG		
β-actin REV	CAGCGGAACCGCTCATTGCCAATG	295 bp	25 cycles: 94°C × 15 s–56°C × 30 s–72°C × 30 s
canp small subunit – 1*	AATAAGGTTGTGACACGAC		
canp small subunit – 2†	GATTTGAAGGCACGGAAC	1,2: 363 bp	27 cycles: 94°C ×
canp small subunit – 3‡	GATTTGAAGGCACGGAACACTTTCATCTGAGTAGCG	1,3: 310 bp	15 s–56°C × 30 s–72°C × 30 s
c-myc – 1*	TACCCTCTCAACGACAGCAGCTCGCCCAAGTCCT		
c-myc – 2†	TCTTGACATTCTCCTCGGTGTCCGAGGACCT	1,2: 479 bp	33 cycles: 94°C ×
c-myc – 3‡	TCTTGACATTCTCCTCGGTGTCCGAGGACCTCGC-TGCGTAGTTGTGCTGATG	1,3: 377 bp	30 s–65°C × 30 s–72°C × 30 s
canp large subunit – 1*	GACTACGAGGCGCTGCGGAAC		
canp large subunit – 2†	CATCCGTTGATCTTGGCGTATG	1,2: 481 bp	52 cycles: 94°C ×
canp large subunit – 3‡	CATCCGTTGATCTTGGCGTATGCCTTGGCAGATGTCTGTGC	1,3: 231 bp	15 s–60°C × 30 s–72°C × 30 s
mcl-1 –1*	GAGGACGAGTTGTACCGGCAG		
mcl-1 –2†	GTCCTTACGAGAACGTCTGTG	1,2: 391 bp	43 cycles: 94°C ×
mcl-1 –3‡	GTCCTTACGAGAACGTCTGTGGATCATCACTCGAGACAACG	1,3: 264 bp	15 s–60°C × 30 s–72°C × 30 s
cks2 –1*	CCACAAGCAGATCTACTAC		
cks2 –2†	GGCTCATGAATCATGTAATG	1,2: 180 bp	26 cycles: 94°C ×
cks2 –3‡	GGCTCATGAATCATGTAATGGTTCTCTGGGTAACATAAC	1,3: 97 bp	15 s–56°C × 30 s–72°C × 30 s
id-2 –1*	GAAAGCCTTCAGTCCCGTG		
id-2 –2†	GCCACACAGTGCTTTGCTG	1,2: 400 bp	31 cycles: 94°C ×
id-2 –3‡	GCCACACAGTGCTTTGCTGTCTGGTGATGCAGGCTGAC	1,3: 291 bp	15 s–56°C × 30 s–72°C × 30 s

*Primer forward used in the synthesis of the target and competitor.
†Primer reverse used in the competitive reaction.
‡Primer reverse used in the synthesis of the competitor.

The following genes were studied: small and large subunits of calcium-activated neutral proteinase-encoding genes (30 kDa-canp, GenBank accession number X04106; 80 kDa-canp, GenBank accession number M23254 respectively), the c-myc oncogene (GenBank accession number V00568), the induced myeloid leukaemia cell differentiation protein-encoding gene (mcl-1, GenBank accession number L08246), DNA-binding protein inhibitor Id-2 encoding gene (GenBank accession number M97796) and cyclin-dependent kinase regulatory subunit CKS2 encoding gene (GenBank accession number X54942). The primers sequences, product size and PCR amplification conditions for each gene are listed in Table I.

For each gene, the PCR amplification linear phase was identified by progressively increasing the number of cycles. The analysis was performed in duplicate on cDNAs obtained from two different experiments in which the K562 cells were incubated with and without 5 mmol/l IP_6. β-actin and abl amplimers were used for normalization purposes.

To avoid false PCR positive results due to DNA contamination, the primers were designed across an exon–intron boundary, and 1 μg of total RNA was PCR amplified in the absence of reverse transcriptase.

Each PCR was performed in a final volume of 50 μl containing 5 μl of 10X Taq buffer (Perkin Elmer, Norwalk, CT, USA), 3 mmol/l magnesium chloride ($MgCl_2$, Perkin Elmer), 800 μmol/l dNTPs, 1·25 U of AmpliTaq Gold (Perkin Elmer), 0·4 μmol/l primer 1 and primer 2 (Gibco BRL) and 5 μl of cDNA mix (100 ng of total RNA). In the case of the canp-large subunit encoding gene, 0·8 μmol/l primer was used. The PCR product (20 μl) was run on a 2% agarose gel stained with ethidium bromide.

For competitive PCR, a shorter competitor fragment was synthetized for each gene using as template the cDNA PCR amplification product obtained with primers 1 and 2. A reverse oligonucleotidic primer, named 3, was designed combining together primer 2 sequence and a 18–21 bp sequence located 53–251 bp upstream of it (Gibco BRL). In this way a deletion occurred and a new shorter PCR fragment was generated, flanked by primer 1 and 2 sequences. After 2% low melting point (LMP-Sigma) agarose gel electrophoresis, each fragment was purified by phenol:chloroform (Sigma-Aldrich, Milan, Italy) extraction, and quantified by spectrophotometric analysis.

To carry out PCR amplification, a fixed amount of target cDNA was mixed with decreasing quantities of competitor to identify a competitor:target ratio of one (Gilliland *et al*, 1990). The exact equivalence point was extrapolated by constructing a regression line on the basis of densitometer scanning data (Image Master VDS, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

RESULTS

Cell cultures

IP_6 had a clear cytotoxic effect on all of the tested cell lines, with an IC_{50} 5 mmol/l after 72 h of culture (Figs 1A–C).

Cell cycle analysis after treatment with different concentrations of IP_6 showed abnormalities only in the K562 and the AR230 cell lines (Fig 2), in which there was an inversion of the G_2 versus G_1 phases with accumulation in the G_2M phases. This was observable at an IP_6 concentration of 5 mmol/l and progressively increased with higher drug doses.

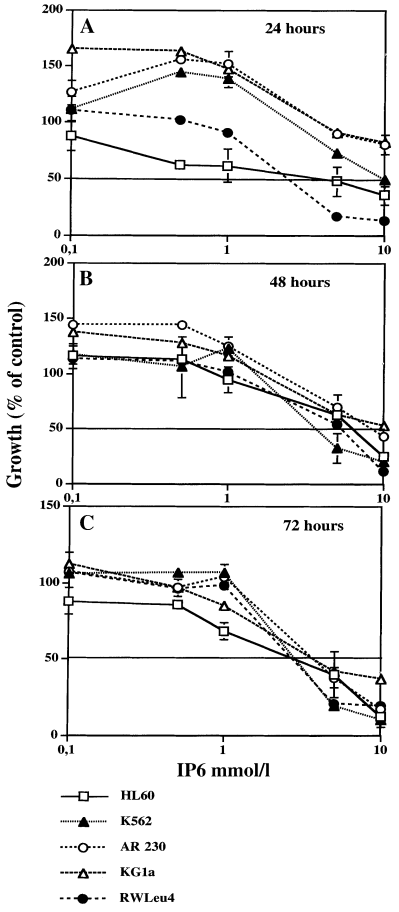

Fig 1. Dose-dependent cytotoxic response to IP_6 after 24 (A), 48 (B) and 72 (C) h treatment. The data are expressed as mean percentage ± SD of the untreated control of three experiments in triplicate; the horizontal bar represents the 50% of cell kill (IC_{50}).

No clear signs of nuclear or cytoplasmic differentiation in the HL60 cell line were seen after 3 months treatment at any of the IP_6 doses (range 0·1–10 mmol/l).

A slight cytotoxic effect (80% cell viability after treatment) was observed at all time points in the cytokine-driven liquid cultures of selected $CD34^+$ cells from fresh normal bone marrow samples only at the highest dose of 10 mmol/l (Figs 3A–C). Conversely, the $CD34^+$ cells from the CML specimens showed an IC_{50} ranging from 3 to 5 mmol/l, with the maximum result (0% cell viability) being observed at 10 mmol/l; this effect was clear after 24 h treatment (Fig 3A).

The growth of granulocyte–macrophage colony-forming units (CFU-GM) from normal $CD34^+$ cells pretreated for 24 h with different doses of IP_6 was not affected, whereas the IC_{50} of the CML samples was 1 mmol/l. When analysed using paired two-tailed Student's *t*-test, the differences

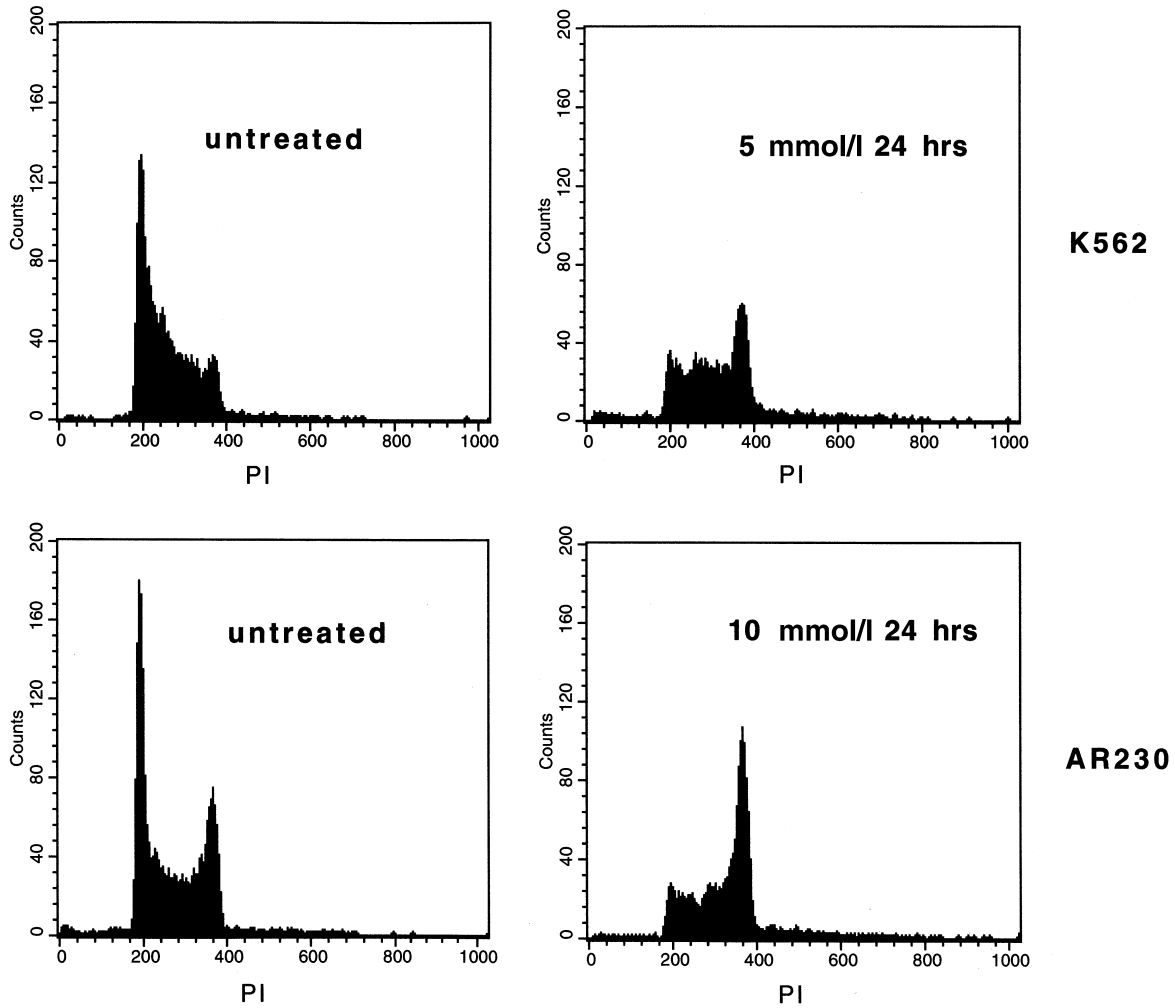

Fig 2. Cell cycle flow cytometric analysis after IP_6 treatment. The K562 and AR230 cell lines were treated, respectively, with IP_6 5 mmol/l and 10 mmol/l for 24 h, then stained with propidium iodide and analysed using flow cytometry (10 000 events per histogram; horizontal axis, linear fluorescence intensity; vertical axis, relative cell number).

between normal and CML colony formation at each concentration point were highly significant ($P = 0{\cdot}0062$) (Fig 4).

Gene expression analyses

cDNA microarray analysis revealed that 66 of the 1176 (5·61%) known genes were modulated with a more than twofold difference of expression between the IP_6 treated and the untreated K562 cells (Table II). Eight of these genes showed upregulated signals and 55 downmodulation.

To confirm microarray results, we analysed six IP_6 responsive genes (the genes encoding for small and large subunits of calcium-activated neutral proteinase, c-myc oncogene, induced myeloid leukaemia cell differentiation protein-encoding gene, DNA-binding protein inhibitor Id-2-encoding gene and cyclin-dependent kinase regulatory subunit CKS2-encoding gene) using both linear and competitive RT–PCR (see *Materials and methods*). The results obtained for all of the genes were repeatedly consistent with the microarray data (Fig 5). In the case of the large subunit of neutral proteinase, our experimental data were consistent with a threefold upmodulation compared with the 7·5-fold upmodulation shown by microarray analysis (data not shown).

For each gene, the mRNA copies number of untreated and treated cells was obtained by extrapolating the equivalence point by means of regression analysis (Fig 5).

DISCUSSION

Inositol phosphates (IPs), synthesized from the parent inositol molecule, are ubiquitously common molecules involved in the regulation of signal transduction in most mammalian cell systems. Inositol from exogenous source is absorbed through the human digestive tract and internalized in the cells by active transport mechanisms. After internalization, IP_6 is dephosphorylated to IP_{1-5} which are transported via blood plasma to distant normal and tumoral targets.

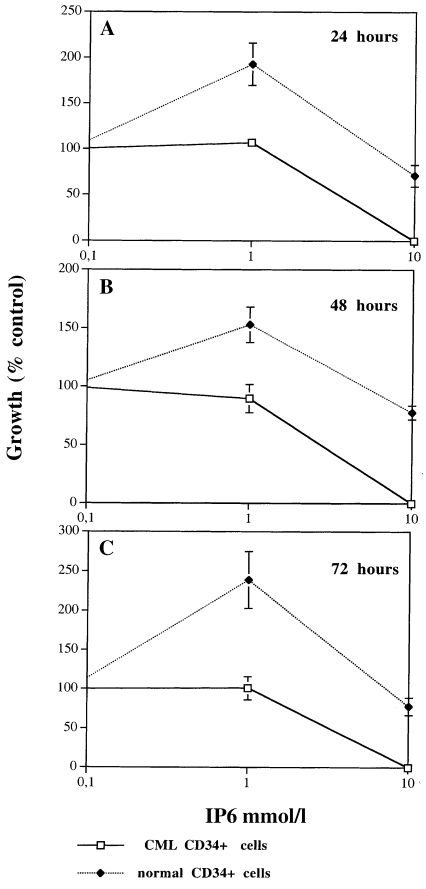

Fig 3. Effect of IP_6 on the growth of human $CD34^+$ from normal bone marrow cells and from CML samples in cytokine-driven liquid culture after 24 (A), 48 (B) and 72 (C) h treatment. The data are expressed as mean percentage ± SD of the untreated control of three experiments in triplicate.

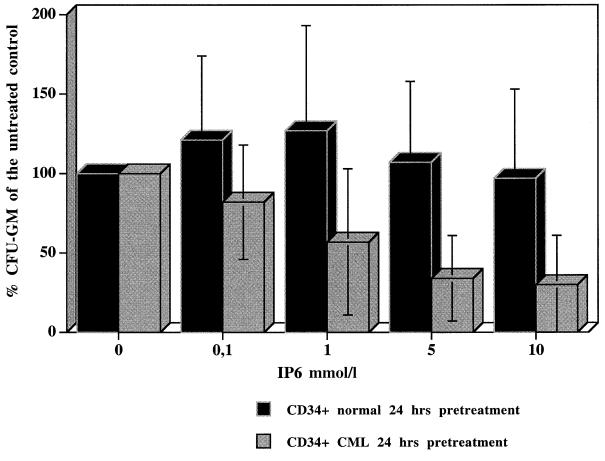

Fig 4. CFU-GM formation of three normal and five CML bone marrow $CD34^+$ purified cells after treatment with different doses of IP_6. All data are expressed as mean percentage ± SD of the untreated control.

IP_6 has been demonstrated to be instantaneously absorbed by a variety of cancer cell lines (Shamsuddin, 1999). The rate and pattern at which IP_6 is metabolized by cancer cells varies depending on the cell type (Shamsuddin, 1999). However, the mechanisms of action of IP_6 are still not completely understood. In particular, few data are available on the IP_6 effect in haematological neoplasia (Shamsuddin *et al*, 1992).

To characterize the possible biological and molecular mechanisms of action of IP_6 in these malignancies, we tested its effects on various myeloid leukaemic cell lines and fresh normal or CML-derived $CD34^+$ progenitor cells, in order to provide a pathophysiological basis for possible therapeutic applications.

Previous studies in solid tumours have suggested that IP_6 is cytostatic rather than cytotoxic (Sakamoto *et al*, 1993; Shamsuddin & Yang, 1995; Shamsuddin *et al*, 1996; Vucenik *et al*, 1998a, 1998b; Ishikawa *et al*, 1999; Shamsuddin & Vucenik, 1999; Wattenberg, 1999), but our results show that it had clear cytotoxic effects on all five of the human chronic and acute myeloid leukaemia cell lines tested.

Furthermore, after IP_6 treatment, inversion of the cell cycle with accumulation in G_2M phases were pointed out in two out of five leukaemic cell lines tested, both derived from CML patients. This was not observed in the RWLeu4 CML cell line, possibly because it is characterized by additional copies of the Philadelphia chromosome (an escape mechanism that needs further study).

As no previous data are available on IP_6 cell cycle effects in solid tumours, it was not possible to determine whether our findings are specific to haematological malignancies or represent a more general mechanism of action.

Interestingly, when we treated fresh $CD34^+$ cells from CML or normal donor bone marrow with different doses of IP_6, a specific toxic effect was observed on leukaemic progenitors from CML patients either as inhibition of the clonogenic growth (CFU-GM) or as cytotoxicity on liquid cultures, but no cytotoxic or cytostatic effect was observed on normal bone marrow progenitor cells under the same conditions.

The effect is observed at relatively high doses of IP_6. Surprisingly, at low concentrations a parodoxic growth effect is observed in $CD34^+$ normal cells, but not in $CD34^+$ CML-derived cells. The increased survival of normal $CD34^+$ cells at low IP_6 concentration may be in part due to the experimental conditions in which normal cells can be driven to grow upon cytokine stimulation. To a lesser extent, the effect is observed also in leukaemic cell lines. IP_6 appears to act specifically, as the expansion in IP_6 treated cells is as high as 2·5-fold compared with the untreated control that has been incubated with the same cytokine cocktail.

To test whether the observed cytotoxic effect may be mediated by active gene modulation, and to investigate the mechanisms underlying the reported cell cycle inversion, we used cDNA microarray analysis to study gene expression patterns after IP_6 treatment in the K562 cell line, which was chosen because IP_6 led to the most marked effect on the cell cycle and had a clear cytotoxic effect.

Table II. IP_6 up- and downregulated genes clustered by biological function.

down	up	Gene	GenBank accession number
APOPTOSIS-RELATED PROTEIN			
4·0		induced myeloid leukaemia cell differentiation protein MCL-1	L08246
4·0		tumour necrosis factor receptor 1-associated death domain protein (TRADD)	L41690
3·9		growth arrest & DNA-damage-inducible protein 153 (GADD153)	S40706; S62138
2·7		DAP kinase 1 (DAPK1)	X76104
DNA SYNTHESIS/REPAIR AND RECOMBINATION PROTEIN			
4·0		xeroderma pigmentosum group C complementing protein (p125)	D21089
6·3		replication factor C 37-kDa subunit (RFC37); RFC4	M87339
2·5		6-O-methylguanine-DNA methyltransferase (MGMT)	M29971
3·5		DNA topoisomerase II alpha (TOP2A)	J04088
4·0		serine kinase	U09564
3·0		DNA polymerase beta subunit (DPOB)	D29013
2·7		CDC21 homologue	X74794
2·1		CDC46 homologue	X74795
2·3		xeroderma pigmentosum group B complementing protein (XPB)	M31899
2·3		survival of motor neurone (hSMN)	U18423
	4·5	xeroderma pigmentosum group G complementing protein (XPG)	L20046; X69978
SIGNAL TRANSDUCTION MODULATORS AND EFFECTORS			
2·5		macMARCKS	X70326
2·7		YWHA1	L20422
2·2		protein kinase C inhibitor protein-1 (KCIP-1)	X57346
2·3		ras-related protein RAP-1B	X08004
2·4		Ral A	X15014
7·5		M-type calcium-activated neutral proteinase (CANP)	M23254
2·5		calcium-activated neutral proteinase (CANP)	X04106
2·1		MAPKAP kinase (3pK)	U09578
2·7		recoverin	S43855
TRANSCRIPTION FACTORS AND DNA BINDING PROTEINS			
2·5		signal transducer and activator of transcription 6 (STAT6)	U16031
2·3		c-myc	V00568
3·0		cAMP-response element binding protein (CREB)	M34356
5·2		early growth response protein 1 (hEGR1)	X52541; M62829
2·4		activated RNA polymerase II transcriptional coactivator p15	U12979
2·8		basic transcription factor 2 44-kDa subunit (BTF2p44)	Z30094
2·3		transcription initiation factor TFIID 31-kDa subunit	U30504
3·0		CCAAT-binding transcription factor subunit B (CBF-B)	M59079
2·5		transcriptional repressor protein yin & yang 1 (YY1)	M76541
2·5		FUSE binding protein	U05040
2·7		transcription factor LSF	U03494
3·3		PTPCAAX1 nuclear tyrosine phosphatase (PRL-1)	U48296
	2·3	basic transcription element-binding protein 2 (BTEB2)	D14520
	3·9	DNA-binding protein inhibitor Id-2	M97796
	2·8	DNA-binding protein inhibitor Id-3	X69111
PROTEIN MODULATION			
2·7		proteasome component C8	D00762
2·3		heat shock cognate 71-kDa protein	Y00371
3·6		heat shock protein 60 (HSP-60)	M34664
3·0		14·5-kDa translational inhibitor protein (p14.5)	X95384
CYTOKINES, CHEMOKINES AND GROWTH FACTORS			
2·7		vascular endothelial growth factor precursor (VEGF)	M32977; M27281
	5·0	macrophage inflammatory protein 1 beta precursor (MIP1-beta)	J04130
RECEPTORS, LIGANDS AND MEMBRANE PROTEINS			
3·0		CD40 ligand (CD40-L)	L07414
2·2		interleukin 2 receptor alpha subunit (IL2RA)	X01057; X01058; X01402
2·5		epidermal growth factor receptor	M29366; M34309
3·3		CD29 antigen	X07979
4·0		peripheral myelin protein 22 (PMP22)	D11428
2·3		major prion protein precursor (PRP)	M13667
	2·3	prostaglandin E2 receptor EP3 subtype (PGE receptor EP3 subtype)	S69200

Table II. *continued*

down	up	Gene	GenBank accession number
CELL CYCLE REGULATION			
2·6		cyclin H (CCNH)	U11791; U12685
2·6		proliferating cyclic nuclear antigen (PCNA)	M15796; J04718
	3·0	cyclin-dependent kinase regulatory subunit (CKS2)	X54942
	2·3	p57-KIP2	U22398
METABOLISM			
2·1		cathepsin C	X87212
2·5		leucocyte elastase inhibitor (LEI)	M93056
2·6		glutathione synthetase (GSH synthetase; GSH-S)	U34683
4·0		methionine aminopeptidase 2 (METAP2)	U29607
2·7		glutathione S-transferase A1 (GTH1; GSTA1)	M25627
2·9		monocarboxylate transporter 1 (MCT1)	L31801
2·2		E16 amino acid transporter	AF077866

We identified eight upregulated and 55 downregulated genes by IP_6 that were subsequently classified into nine categories according to their structures and/or functions (Table II).

On the other hand, many of the downregulated genes may be functionally important in terms of cell growth regulation. Transcription factors that promote cell proliferation, including c-myc and HPTPCAAX1 (Diamond *et al*, 1996; Thompson, 1998), were downregulated upon incubation in the presence of IP_6. Interestingly, the FUSE gene, which induces c-myc gene expression in a cell growth-dependent manner (Bazar *et al*, 1995), is also downmodulated, thus suggesting that its reduced expression levels may mediate the downregulation of c-myc.

A cluster of genes involved in DNA synthesis, repair, recombination and in the regulation of cell cycle checkpoint transitions were also downmodulated. In this regard, it is interesting to note the coherent reduction in the expression of multiple members of the transcription factor IIH multiprotein complex, namely cyclin H (a constituent of CAK complex) (Ko *et al*, 1997), xeroderma pigmentosum, complementation group C (XPC) and the 44 kDa subunit of the general transcription factor IIH (Coin *et al*, 1998), suggesting a preferential IP_6 targeting of this system in our cell model.

Interestingly, the IP_6 dependent downmodulation of cyclin H, in conjunction with the upregulation of other cell cycle regulators, such as CKS2, p57 and Id-2, may provide clues to interpreting the observed G_2 accumulation of K562 cells. In particular, CAK is responsible for cyclinB/cdc2 activation through cdc2 phosphorylation, which is necessary for the progression through the G_2 to M phase. As inhibition of the CAK-mediated cdc2 activation has been demonstrated to cause the cells to accumulate in G_2 phase (Smits & Medema, 2001), it may be hypothesized that the IP_6-mediated CAK inhibition may contribute to the G_2 phase block observed in our study. Furthermore, overexpression of CKS2, p57-KIP2 and Id-2 may be consistent with this hypothesis. In fact, even if CKS2 has been poorly studied, its homologous CKS1 is involved in the regulation of G_2/M phases and, in particular cell models, as in fission yeast and in frog egg extract, it may cause G_2 arrest and G_2/M transition delay (Pines, 1996). Furthermore, p57-KIP2 may promote the activity of cyclin D–dependent kinases, facilitating G_1 progression (Sherr & Roberts, 1999), similar to Id-2, whose expression induce G_1 to S phase progression, through pRb and pRb-related protein inhibition (Lasorella *et al*, 1996).

Further studies are necessary to elucidate this hypothesis, but our findings suggest that IP_6 may represent a novel pharmacological agent for cell cycle modulation, possibly acting through CAK transcription regulation.

Genes participating in a number of important signal transduction pathways, including STAT-6 (Takeda *et al*, 1997) and MAPKAP (Ludwig *et al*, 1996), were downmodulated. Some apoptosis agonists (DAPK1, TRADD) (Cohen *et al*, 1997; Lin *et al*, 1998) and antagonists (MCL-1, GADD153) (Kozopas *et al*, 1993; Seth *et al*, 1999) showed decreased levels of expression. As no change in the apoptosis levels were evidenced at the cell cycle analysis, the effects of the pro- and anti-apoptotic genes may reciprocally counterbalance. Other interesting genes that were downregulated, which deserve a separate comment, comprise vascular endothelial growth factor (VEGF) and calcium-activated neutral proteinases (CANPs).

The IP_6 downregulation of VEGF is particularly intriguing. This is a multifunctional cytokine with a crucial role in development of human tumour angiogenesis (Amoroso *et al*, 1997). It regulates multiple endothelial cell functions, and its biological activities are mediated through at least two receptor tyrosine kinases, Flk-1 and flt-1 (Zachary, 1998). Increased levels of VEGF have been reported in CML as well as in myeloid blasts in human leukaemia and myelodysplastic syndromes, suggesting that VEGF production may play a pathogenetic role in these haematological diseases (Aguayo *et al*, 2000; Bellamy *et al*, 2001). These observations may suggest a possible IP_6 anti-angiogenic effect through VEGF downmodulation.

Finally, calpains are calcium-dependent proteases that also play a role in oxidative stress-mediated apoptosis (Banik

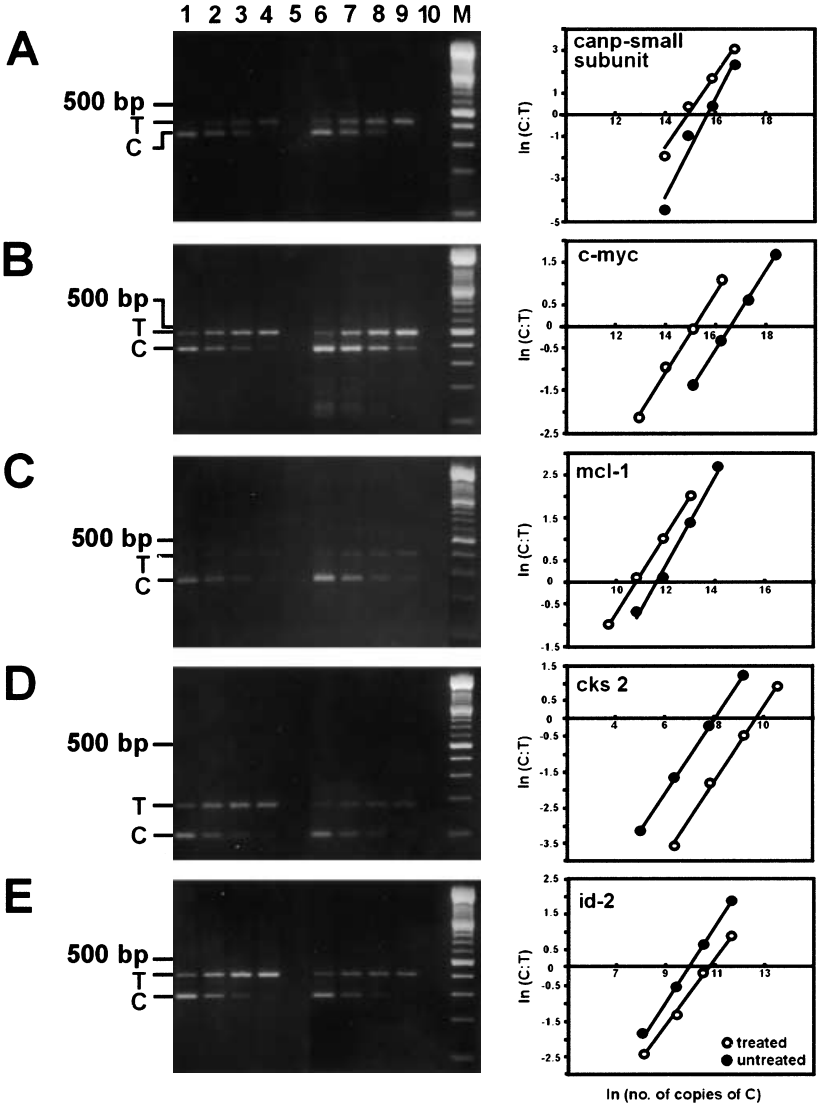

Fig 5. Competitive RT–PCR for the quantification of gene expression levels. The lower bands represent the competitor, C; and the upper bands the target cDNA, T. Lanes 1–4: competitive reaction from IP_6-treated K562 cell line. Lanes 6–9: competitive control reaction from K562 cell line. Lanes 5 and 10: negative controls represented by all reagents except DNA. M: 100 bp fragments ladder DNA molecular weight markers. (A) *canp-small subunit*. Lanes 1–4: 2×10^7, 8×10^6, $3{\cdot}2 \times 10^6$ and $1{\cdot}25 \times 10^6$ competitor copies mixed with a fixed amount of cDNA, corresponding to 20 ng of the starting RNA from IP_6-treated K562 cell line. The extrapolated number of copies/ng at the equivalence point is $1{\cdot}64 \times 10^5$ for treated K562 and $3{\cdot}45 \times 10^5$ for untreated K562 cell line, with a C:T-value of 2·1 (compared with 2·46 for microarray analysis). (B) *c-myc*. Lanes 1–4: $1{\cdot}1 \times 10^7$, $3{\cdot}7 \times 10^6$, $1{\cdot}2 \times 10^6$, 4×10^5 competitor copies were mixed with 30 ng of the starting RNA from treated K562 cell line. The extrapolated number of copies/ng at the equivalence point is $1{\cdot}21 \times 10^5$ for IP_6-treated K562 cells and $5{\cdot}6 \times 10^5$ for control cells, with a C:T ratio of 4·6 (compared with 2·29 for microarray analysis). (C) *mcl-1*. Lanes 1–4: $4{\cdot}8 \times 10^5$, $1{\cdot}6 \times 10^5$, $5{\cdot}3 \times 10^4$ and $1{\cdot}7 \times 10^4$ copies of the competitor were mixed with 40 ng of the starting RNA from treated K562 cell line. Lanes 6–9: $1{\cdot}44 \times 10^6$, $4{\cdot}8 \times 10^5$, $1{\cdot}6 \times 10^5$ and $5{\cdot}3 \times 10^4$ competitor copies were mixed with cDNA from control K562 cell line. The extrapolated number of copies/ng at the equivalence point is, respectively, $1{\cdot}3 \times 10^3$ for IP_6-treated K562 cells and 3×10^3 for control cells, with a C:T ratio of 2·3 (compared with 4 for microarray analysis). (D) *cks2*. Lanes 1–4: $1{\cdot}2 \times 10^5$, 4×10^4, $1{\cdot}3 \times 10^4$, $3{\cdot}3 \times 10^3$ competitor copies were mixed with 20 ng of the starting RNA from IP_6-treated K562 cell line. The extrapolated number of copies/ng at the equivalence point is $2{\cdot}4 \times 10^3$ for treated K562 cells and $1{\cdot}1 \times 10^3$ for control cells, with a C:T ratio of 2·18 (compared with 2·99 for microarray analysis). (E) *id-2*. Lanes 1–4: 4×10^4, 1×10^4, $2{\cdot}5 \times 10^3$ and $6{\cdot}25 \times 10^2$ competitor copies were mixed with 20 ng of the starting RNA from treated K562 cell line. Lanes 6–9: 1×10^4, $2{\cdot}5 \times 10^3$, $6{\cdot}25 \times 10^2$ and $1{\cdot}56 \times 10^2$ competitor copies were mixed with cDNA from control K562 cell line. The extrapolated number of copies/ng at the equivalence point is $8{\cdot}1 \times 10^2$ for treated K562 cells and $1{\cdot}6 \times 10^2$ for control cells, with a C:T ratio of 5·06 (compared with 3·89 for microarray analysis).

et al, 1998). In our system both the heavy (80 kDa, catalytic) and smaller (30 kDa, regulatory) subunits (Sorimachi *et al*, 1989) are downregulated. As IP_6 mediates an increase of the intracellular Ca^{++} concentration (Imajoh *et al*, 1986) that activates calpains (McCelland *et al*, 1989; Nakamura *et al*, 1989; Saido *et al*, 1994), the observed downregulation may be due to the presence of a transcriptional negative feedback aimed at protecting the cell from an excessive calpain proteolytic activity.

Taken together, our results suggest that the spectrum of neoplasia on which IP_6 has antiproliferative activity could be extended to acute and chronic myeloid leukaemias. Most interestingly, no adverse effect on the biological characteristics of normal fresh human haematopoietic progenitor cells was observed. Our molecular analysis also indicate active modulation of specific genes involved in cell cycle regulation and angiogenesis. The demonstration of a similar gene modulation in normal and CML $CD34^+$-derived cells would provide a possible pathogenetic basis for future therapeutic applications of IP_6.

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