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Molecular Mechanisms for the Antitumor Activity of Inositol Hexakisphosphate (IP_6)

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Abstract. *Background: Inositol hexakisphosphate (IP_6), a naturally occurring polyphosphorylated carbohydrate, has been reported to have significant in vivo and in vitro anticancer activity against numerous tumors. However, the molecular mechanism of the anticancer effect of IP_6 has not been fully elucidated. Materials and Methods: Using K-562 human leukemia cells we analysed the induction of the erythroid differentiation program, as well as modulation of the gene expression profile of K-562 leukemia cells treated with IP_6. Results: A single treatment with IP_6 (0.75 or 5.0 mM) resulted in a time- and dose-dependent growth inhibition of K-562 cells and also activation of the erythroid differentiation program. K-562 cells expressed a concomitant differentiation after 12 hours of exposure. Possible molecular mechanisms and key signaling pathways, as well as gene expression behind this anticancer effect were examined using oligonucleotide microarrays and quantitative real-time PCR. Treatment with IP_6 (750 μM, 5 mM) had a marked impact, resulting in early (60 min) and late (12 h) modulation of expression of about 1800 and 1200 transcripts (at $p<0.05$). Through microarray analysis, the anticancer effect of IP_6 in K-562 was found to be associated with the modulation of multiple genes involved in immunity, Wnt and IGF pathways, PI3 kinase signaling and apoptosis. Using selected subsets of genes, the microarray hits could be validated by Q-PCR. A 2-fold upregulation of the apoptosis pathway, measured using the BAX/BCL-2 ratio was observed for 12 hours. IP_6 (5 mM) induced up to 6-fold increases in differentiation measured by hemoglobin synthesis, yielding up to 70% of benzidine-positive cells at 120 hours. Conclusion: The results of this study show that IP_6 is a strong inducer of differentiation (cytostatic effect) and a moderately strong inducer of apoptosis (cytotoxic effect). Evidence has been provided to show that the growth inhibitory effects of IP_6 are mediated through the modulation of key signaling pathways.*

IP_6 (inositol hexakisphosphate, also known as phytic acid) is a polyphosphorylated carbohydrate, which is ubiquitous and found at high concentrations (0.4-6.4%) in cereals and legumes (1). Mammalian cells can either take up exogenous IP_6 through nutrition or generate it intracellularly during the metabolism of inositol polyphosphates (2). IP_6 has several roles in mammalian cells. It has been implicated in regulating growth, inflammation and neurotransmission, and is even thought to be involved in export of mRNAs from the nucleus (3, 4). IP_6 may also regulate vesicle budding and fusion with the plasma membrane (5). It might specifically bind to, and thus induce, conformational changes in a series of particular proteins, thereby altering their activity and substrate binding (6). Implication in non-homologous end-joining of double strand break repair of DNA is also described (7). Mammalian cells can take up and metabolize extracellular IP_6 (8); the rate at and the pattern with which IP_6 is metabolized by cancer cells varies with cell type.

IP_6 anticancer activity has been widely described in different tumor types, including that of the breast, colon, prostate, lung, liver and pancreas (4, 9-13). There are several explanations for the antineoplastic effect of IP_6, most importantly *via* the downstream modulation of gene expression (14): the surface of cells has receptors for IP_6; G protein coupled receptor mediates its effect through phospholipase C, which splits PIP2, a membrane component into IP_3 and DAG. IP_3 increases the intracellular Ca^{++} concentration immediately from SR as well as indirectly, with prolonged effect through Ca^{++} channels (15). IP_6 inhibits phosphatidylinositol-3 kinase (PI3K), an important signaling molecule. This action is related to the structure of IP_6 being similar to D-3-deoxy-3-fluoro-PtdIns, an inhibitor of PI3K (16). It is also a compelling hypothesis that not only intact IP_6 itself, but also its less phosphorylated derivatives (IP5, IP4, IP3), are able to interfere with cell signaling and the gene expression profile (17). IP_6 up-regulates the expression of $p21^{WAF-1/CIP1}$ in a dose dependent manner,

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which in turn causes p53-mediated cell cycle arrest (18, 19). IP_6 inhibits cell survival signaling through the Akt-NFκB pathway (20). IP_6 decreases cell adhesion and migration by suppressing the integrin receptors and their subsequent signaling pathway mediating focal adhesion (21). The anti-angiogenic effect of phytic acid has also been described (22). Furthermore, IP_6 is a known antioxidant with its ability to chelate bivalent cations consequently preventing the hydroxyl-free radical formation of iron (23). IP_6 can interfere with immune responses: it has been reported to prime human neutrophils for enhanced agonist-stimulated superoxide anion generation. This led to the proposal that the release of IP_6 from necrotic cells may augment the functional responsiveness of neutrophils at an inflammatory focus (24). This is in concordance with the finding that oral administration of IP_6 augments the immune response by enhancing the activity of natural killer (NK) cells (9, 25). However, which of these complex effects are principally due to the exact mechanism through which IP_6 exerts its anticancer effect on different tumor types is still uncertain.

Along with inhibition of cell proliferation, there is enhanced differentiation of malignant cells to a more mature phenotype, often resulting in reversion to normal (5). IP_6 exerts its differentiating effect through its dephosphorylated derivatives (IP_4, IP_5), thus contributing to the return to normal proliferative behaviour of cells that have undergone blastic transformation (26). One of the most relevant signs of differentiation is the production of markers of the more mature derivatives: for example gamma-globin for erythroid maturation and (26, 27) and lactalbumin for mammary cancer cell differentiation (28).

There is indication that IP_6 is not only cytostatic, as formerly believed (29, 30), but also cytotoxic to malignant cells (31). This is also true for the growth of the cell lines of human leukemic hematopoietic lineage, such as K-562, in a dose- and time-dependent manner. The leukaemic cell lines seem to be highly susceptible to IP_6 (31). There have been efforts to test the bulk changes of gene expression in K-562 pathways as a result of 5 mM exogenous IP_6 treatment (31). Microarrays, containing 1176 human cDNA fragments, revealed significant down-regulation of essential protooncogenes as well as up-regulation of transcripts involved in apoptosis and differentiation after 24 h treatment (31).

Using a Whole Human Genome cDNA microarray platform, representing 44,000 human transcripts, we examined the expression in pathways by which IP_6 might exert its antineoplastic effect using K-562 cell line as a model system.

Materials and Methods

Cell cultures and growth inhibition assay. K-562, a BCR-ABL positive human leukemia cell line, was grown in suspension culture in RPMI-1640 medium (GIBCO). Cells were maintained in culture at cell densities between 10^5 and 10^6 cells/ml by dilution in fresh medium every 2-3 days. Cells were treated with 0, 0.5, 1, 2.5, 5 and 10 mM inositol hexaphosphate Na salt (sodium phytate; SIGMA). In order to determine IC_{50}, $5x10^4$ log phase cells/ml were seeded in culture flasks and cell viability was monitored daily *via* the trypan-blue dye exclusion test for up to 5 days of treatment. The number of treated cells was plotted as a percentage of the control cells for each concentration and time. IC_{50} values were determined using regression curve analysis.

For individual gene expression experiments cells in log phase growth were cultured at initial seeding concentrations of $3x10^6$ cells/ml with or without IP_6.

Benzidine-staining for observing cell differentiation. K-562 leukemia cells were treated with 750 μM and 5 mM of IP_6, respectively. Benzidine staining was carried out every 24 h up to 5 days for the detection of erythroid differentiation as described elsewhere (32). To measure the capacity of the cells to undergo erythroid differentiation, the percentage of benzidine positive-staining in 1000 counted cells was determined under light microscopy.

Microarray analysis. High-purity RNA was isolated from K-562 cells treated with 750 μM IP_6 for 30 and 60 min, as well as 5 mM for 12 h, using an RNAqueous™ kit from Ambion. As a control, RNA from non-treated cells cultured for the same times was used. The integrity and concentration of the RNA was measured with an Agilent 2100 Bioanalyser. RNA was amplified and labeled with either Cy3 or Cy5 using the Agilent Low Input Amplification Kit. Amplified cRNA was purified with the RNeasy® Mini Kit from Qiagen and exact concentrations were measured with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies). 1-1 μg of differently labelled cRNAs were fragmented according to an Agilent protocol (*In situ* Hybridization Kit Plus) and hybridized to an Agilent-designed 60-mer Whole Human Genome Oligo Microarray chip representing 41K human transcripts. A complete list of the genes is available on the Agilent web site (http://www.agilent.com). Dye swaps (same samples with reverse dye-label) of each comparison was performed. A 17-h hybridization was carried out in oven at 60˚C with constant rotation, washing with SSPE and acetonitrile was performed according to the manufacturer's instructions. Fluorescence intensity was measured with an Agilent Microarray Scanner. Each feature was determined using the Agilent Feature Extraction Software (v.A.7.5.1). Data analysis was carried out with the Rosetta Luminator System analysis software (Rosetta Biosoftware) for significant up- and down-regulation of genes using ANOVA with $p<0.05$. Evaluation of the microarray data involving detection of affected pathways was carried out with PANTHER (33), the free online software of Applied Biosystems (http://www.panther.appliedbiosystems.com)

Real-time (TaqMan) PCR assay. To validate a subset of microarray hits, TaqMan PCR assays for 10 previously selected genes (*c-MYC, h-TERT, Ha-RAS, Ki-RAS, $p21^{CIP1/WAF1}$, PI3K, TP53, BCL-2, BAX, BCR-ABL*) were performed on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Q-PCR reactions were carried out in a final reaction volume of 20 μl containing TaqMan Universal Mastermix with TaqMan Gene Expression Assays for the appropriate genes and cDNA templates. The ratio change in target genes relative to the β2 microglobulin and RPLP0 as internal controls was determined using the $2^{-\Delta\Delta Ct}$ method (34). All reactions were done in quadruplicate from the cDNA templates of 3 independent reverse transcription reactions. The thermal cycling

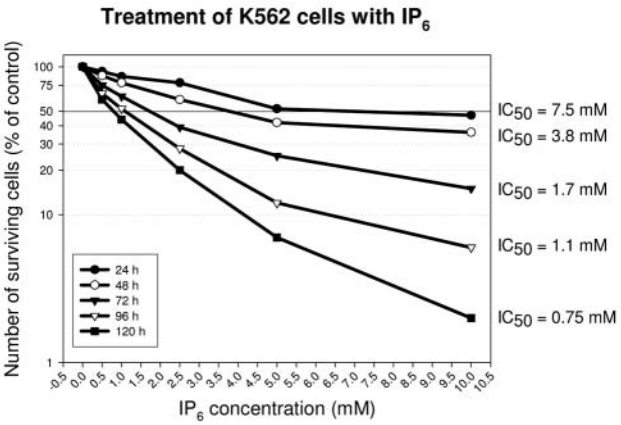

Figure 1. *Determination of IC_{50} of K-562 cells upon IP_6 treatment. The cells were treated with 0, 0.5, 1, 2.5, 5 or 10 mM IP_6 and the cell survival ratio was monitored daily up to 5 days. IC_{50} values belonging to the respective tretaments were determined using exponential regression curves. The experiments were done in triplicate, SE values <±10%.*

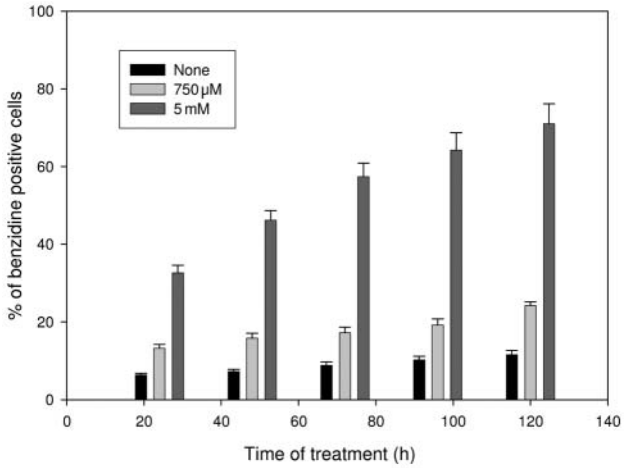

Figure 2. *Benzidine staining of K-562 cells upon IP_6-treatment. Cells were treated with 750 µM and 5 mM IP_6, respectively, and the percentage of benzidine-positive cells were counted each day for 5 days. The results are means of triplicates.*

conditions were 2 min at 50˚C and 10 min at 95˚C, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. The Sequence Detector Software 2.0 (Applied Biosystems) was used for data analysis.

Results

Antiproliferative action of IP_6 on K-562 cells. A trend for decreasing cell survival of IP_6 treated cells as compared to the untreated cells in a time- and dose-dependent manner was observed (Figure 1). IC_{50} values were determined by applying exponential regression curves. Doses corresponding to IC_{50} values at 120 h (750 µM), which confers physiological concentration, and 24-48 h (5 mM), a pharmacological concentration, were used for further treatment analysis.

Induction of erythroid differentiation. Globin production is an excellent erythroid differentiation marker in CML (26). Using specific staining of globin with benzidine (see Materials and Methods) we could follow the erythroid differentiation. After 24 h, the ratio of benzidine stained cells was elevated relative to the untreated control cells and this ratio grew larger each day. The dynamics of the increase was even more enhanced in the case of the 5 mM treatment: after 5 days the benzidine-stained cell ratio was as high as 70%, representing a 6-fold increase compared to untreated cells. The effect of the 750 µM dose was less severe, with only a two-fold increase in benzidine positive cells (Figure 2).

Evaluation of the microarray. Treatment of K-562 cells with 750 µM IP_6 for 30 or 60 min resulted in a significant change in the expression of transcripts. Thirty minutes following IP_6 treatment, non-characteristic changes in gene expressions were detected as a result of the non specific shock after administration of a concentrated chemical agent (phase of adaptation). The characteristic effect of IP_6 manifests itself after 1 h; after 12 h, a subset of genes was already apparent representing an inflammation profile and a reduction in cell proliferation. The treatment with 750 µM IP_6 for 60 min significantly affected a total 1818 transcripts: 771 of them were up regulated and 1041 down-regulated. The treatment with 5 mM IP_6 for 12 h caused a significant change in the expression of 1243 transcripts: 615 were up regulated, while 628 down regulated (data not shown). Table I summarizes the most relevant genes and pathways over- and under-represented upon IP_6 treatment. In both cases the expression of a series of histone genes was significantly reduced in agreement with a decrease in DNA-replication (data not shown). Similarly, several relevant proto-oncogenes (*MYC, CDK4, ELK1, E2F1, JUN, Ki RAS, N-RAS*) were down

Table I. *Pathways and biological processes affected as a result of IP_6 treatment.*

(750 µM/60 min)	(5 mM/12 h)
PI3 kinase pathway	PI3 kinase pathway
Insulin-IGF pathway	EGF and FGF pathway
Wnt signaling pathway	Wnt signaling pathway
TGF-beta signaling pathway	JAK/STAT signaling pathway
Oncogenes	Oncogenes
General transcription	Inflammation, immunity
Protein biosynthesis	Apoptosis
	Angiogenesis

regulated at the RNA-level at lower as well as higher doses of IP_6. Important members of the Wnt signaling pathway (*CDH13, FZD5, PLCB3*) together with other pathways mediating cell proliferative signals (insulin-IGF, PI3K pathway) were also down regulated at the transcriptional level with both treatments; suprisingly, some of them (EGF, FGF receptor signaling) seemed to be slightly activated at the higher dose (Tables II and III). Genes related to transcription (*BC006322, M62760* and *ATF4*) were down regulated at 750 μM (Table II). Significant changes in apoptotic transcripts were observed at 5 mM IP_6, however not the most common genes were up- (*NFKBIA, AMID*), and down regulated (*DAXX, ATF4, V JUN*) (Table III). Remarkable up regulation of genes for specific chemokines (*CXCL1, CXCL2, CKLFSF3, CD69*), cytokines (*IL8, IL23A, IL27RA2*) and other sequences associated with immune response (*IER3, NFATC1, IER5, PILRB*) indicates activation of immunological events, involving T-cell, as well as B-cell mediated immunity and neutrophil burst, especially at the higher IP_6 dose (5 mM for 12 h). The mRNA of a series of genes which partcipate in angiogenic events was lowered as a result of treatment with 5 mM IP_6 for 12 h (*LYN, WNT10A, PXN, DVL1*) (Table III).

We did not detect any sign towards erythroid cell differentiation at the transcriptional level. Interestingly, the expression of several differentiation markers, characteristic of dendritic cells (*HLA-DR, CD-80, CD-86, CD-83*), increased, however, with low significance ($p>0.05$) (data not shown). An increase of protein kinase-C activity was correlated with macrophage-like differentiation (4). Our results showed 1.25-fold up regulation of X07109, which is protein kinase C (PKC) type beta II. On the other hand, we detected a 2.37-fold increase of CD69, a natural killer cell activation marker, which may also be a marker of cell differentiation and inhibits tumor growth (9) (Table III).

Real-time validation. To confirm microarray results, we performed real time PCR with the most representative members detected from the pathways studied using the array (Figure 3 A-H). The results were repeatedly consistent with the microarray data. In comparison with non-treated K-562 cells, expression of the *MYC* proto-oncogene decreased. The higher dose of IP_6 resulted in a greater down regulation (Figure 3A). A similar tendency was observed for TERT transcription, where the mRNA-level of the enzyme showed gradual reduction with time and dose (Figure 3B). The expression level of small GTPases – Ha-RAS and Ki-RAS – decreased after IP_6-treatment in a dose-dependent manner (Figure 3C). The quantity of transcript of $p21^{CIP1/WAF1}$, a relevant tumor suppressor, grew with time of incubation with IP_6. This increase was much more pronounced at 5 mM IP_6 (Figure 3D). The ratio of BAX (proapoptotic) and BCL-2 (antiapoptotic) transcript clearly indicates the activation state of apoptosis (35). In our experiments, BAX-expression gradually overwhelmed that of BCL-2, however, after a culmination at 6 h a decrease in the ratio was observed with the higher IP_6-dose. The lower dose of 750 μM elicited apoptosis after a longer time ($\sim$6 h) of treatment (Figure 3E). In agreement with this, the mRNA-level of TP53, another key molecule in apoptosis, showed a mild increase upon treatment with 750 μM IP_6, especially during the early hours. The higher IP_6-dose, in contrast, brought about a decrease in TP53-transcript (Figure 3F). PI3K at a transcriptional level shows fluctuation with time, which does not seem to correlate with the IP_6-dose: its level falls for 6 h, then, when cells were incubated with 5 mM IP_6, begins to increase again (Figure 3G). Regarding the two main transcripts of the *BCR/ABL* fusion gene of K-562 cells (*b2a2, b3a2*) we observed an unequivocal decrease in the quantity of both transcripts at the higher concentration (5 mM) of IP_6 (Figure 3H).

Discussion

An antiproliferative and cytotoxic effect of IP_6 was noticed as a result of the treatment of K-562 cells compared to the untreated cells in a time- and dose-dependent manner. The most severe decline in cell survival was detected in the case of the higher, pharmacological concentration (5 mM IP_6). This is in agreement with Nickel and Belury, 1999 (36), who found that an IP_6 dose higher than 5 mM is quite cytotoxic.

Microarray experiments revealed 1,818 gene transcripts were significantly altered as a result of 60 min treatment with 750 μM IP_6 and 1,243 transcripts as a result of 12 h treatment with 5 mM IP_6. A subset of histones were down regulated in both cases. As *de novo* synthesized histones are prerequisites of nucleosomes in the newly synthesized DNA during replication, the down regulation of histones implies a significant reduction in cell proliferation. Thus, histones may act as proliferation markers in this respect, as formerly suggested (37). Sustained telomerase activity is essential for continuous proliferation of tumor cells (38). It was demonstrated that IP_6 dose-dependently represses TERT activity *via* the post-translational modification of AKT and PKCα (39). Our real-time experiments revealed that the mRNA-level of TERT decreased with IP_6 administration showing time-and dose-dependence, so a reduction of telomerase activity at the transcriptional level may also contribute to its antiproliferative action.

Microarray experiments, where a series of proto-oncogenes were down regulated while tumor suppressors and apoptotic transcripts up regulated provided further evidence that the effect of IP_6 is not only cytostatic but also cytotoxic as previously described (31) and apoptotic processes also take place. A significant decrease of expression of several major oncogenes was revealed with

Table II. *The most relevant genes significantly ($p<0.05$) affected in K-562 cells treated with 750 μM IP_6 for 60 min, clustered by pathway and biological function.*

Sequence	Accession #	Sequence description	Regulation	
			Up	Down
Oncogenes				
MYC	NM_002467	*Homo sapiens* v-myc myelocytomatosis viral oncogene homolog (avian) (MYC)		1.21
MLLT2	NM_005935	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 2		10.27
PTTG2	NM_006607	pituitary tumor-transforming 2		11.85
KRAS2	NM_033360	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog		5.15
M61108	M61108	T-cell acute lymphocytic leukemia 1		11.05
NRAS	NM_002524	neuroblastoma RAS viral (v-ras) oncogene homolog		6.2
MERTK	NM_006343	*Homo sapiens* c-mer proto-oncogene tyrosine kinase (MERTK),		1.87
E2F1	NM_005225	*Homo sapiens* E2F transcription factor 1 (E2F1)		1.49
ELK1	NM_005229	*Homo sapiens* ELK1, member of ETS oncogene family (ELK1),		2.24
VAV1	NM_005428	vav 1 oncogene	1.35	
MAFK	NM_002360	*Homo sapiens* v-maf musculoaponeurotic fibrosarcoma oncogene homolog K (avian) (MAFK)		1.88
CDKN1C	NM_000076	*Homo sapiens* cyclin-dependent kinase inhibitor 1C (p57, Kip2) (CDKN1C)		2.37
BC002646	BC002646	*Homo sapiens* v-jun sarcoma virus 17 oncogene homolog (avian)		1.70
FZD2	NM_001466	*Homo sapiens* frizzled homolog 2 (Drosophila) (FZD2)		2.48
Insulin-IGF and TGF-beta and PI3 kinase pathways				
GSK3A	NM_019884	glycogen synthase kinase 3 alpha	1.43	
FOXD1	NM_004472	forkhead box D1		9.18
FOXQ1	NM_033260	forkhead box Q1		10.78
FOXD3	NM_012183	forkhead box D3		4.07
INPPL1	NM_001567	inositol polyphosphate phosphatase-like 1		2.97
FOXC2	NM_005251	forkhead box C2 (MFH-1, mesenchyme forkhead 1)		9.13
FOXB1	NM_012182	forkhead box B1		4.99
ACVR1B	NM_004302	activin A receptor, type IB		1.72
GDF15	NM_004864	growth differentiation factor 15		1.62
RAC2	NM_002872	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)		1.59
AMH	NM_000479	anti-Mullerian hormone		4.75
RAB1B	NM_030981	RAB1B, member RAS oncogene family		1.62
RAB35	NM_006861	RAB35, member RAS oncogene family		1.64
BMP8A	NM_181809	bone morphogenetic protein 8a		15.03
GRB2	NM_002086	growth factor receptor-bound protein 2		1.55
GADD45A	NM_001924	growth arrest and DNA-damage-inducible, alpha		1.31
BCL2	NM_000657	B-cell CLL/lymphoma 2		4.54
Wnt signaling pathway				
PPP2R5C	NM_178588	protein phosphatase 2, regulatory subunit B (B56), gamma isoform		7.59
PLCB3	NM_000932	phospholipase C, beta 3 (phosphatidylinositol-specific)		9.61
HOXB7	NM_004502	homeo box B7		14.36
CTNNA3	NM_013266	catenin (cadherin-associated protein), alpha 3		1.66
CTNNB1	NM_001904	catenin (cadherin-associated protein), beta 1, 88 kDa		7.39
FZD5	NM_003468	frizzled homolog 5 (Drosophila)		8.1
NFATC1	NM_172387	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1		11.92
CDH13	NM_001257	cadherin 13, H-cadherin (heart)		8.4
PPP3R2	NM_147180	protein phosphatase 3 (formerly 2B), regulatory subunit B, 19 kDa, beta isoform (calcineurin B, type II)	2.56	
X07109	X07109	protein kinase C, beta 1		9.09
General transcription				
BC006322	BC006322	*Homo sapiens* activating transcription factor 3		2.75
M62760	M62760	*Homo sapiens* chick ovalbumin upstream promoter transcription factor II (COUP-TFII)		1.75
ATF4	NM_001675	*Homo sapiens* activating transcription factor 4 (tax-responsive enhancer element B67) (ATF4), transcript variant 1		1.39

Fold change compared to untreated cells.

Table III. *The most relevant genes significantly ($p<0.05$) affected in K-562 cells treated with 5 mM IP_6 for 12 h.*

Sequence	Accession #	Sequence Description	Up	Down
Apoptosis				
BC028013	BC028013	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	2.44	
NFKBIA	NM_020529	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3.69	
MAP3K5	NM_005923	mitogen-activated protein kinase kinase kinase 5	1.55	
X07109	X07109	protein kinase C, beta 1	1.31	
PML	NM_002675	promyelocytic leukemia	2.49	
CDKN2D	NM_001800	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	1.4	
AMID	NM_032797	apoptosis-inducing factor (AIF)-like mitochondrion-associated inducer of death	1.36	
DAXX	NM_001350	death-associated protein 6		1.47
HSPA8	NM_153201	heat shock 70 kDa protein 8		1.70
BC002646	BC002646	v-jun sarcoma virus 17 oncogene homolog (avian)		1.81
HSPA1A	NM_005345	heat shock 70 kDa protein 1A		1.58
ATF4	NM_001675	activating transcription factor 4 (tax-responsive enhancer element B67)		1.75
Inflammation, immunity				
CXCL1	NM_001511	*Homo sapiens* chemokine (C-X-C motif) ligand 1 (melanoma stimulating growth activity, alpha)	7.09	
CXCL2	NM_002089	*Homo sapiens* chemokine (C-X-C motif) ligand 2	5.1	
IER3	NM_003897	*Homo sapiens* immediate early response 3 (IER3), transcript variant short, mRNA	1.94	
IER5	NM_016545	*Homo sapiens* immediate early response 5 (IER5), mRNA	1.35	
PILRB	NM_175047	*Homo sapiens* paired immunoglobin-like type 2 receptor beta (PILRB), transcript variant 2, mRNA	1.26	
IL27RA	NM_004843	*Homo sapiens* interleukin 27 receptor, alpha (IL27RA), mRNA	1.66	
IL23A	NM_016584	*Homo sapiens* interleukin 23, alpha subunit p19 (IL23A), mRNA	1.64	
CKLFSF3	NM_144601	*Homo sapiens* chemokine-like factor super family 3 (CKLFSF3),	1.39	
JUNB	NM_002229	jun B proto-oncogene	1.37	
BC028013	BC028013	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	2.44	
NFKBIA	NM_020529	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3.69	
STAT6	NM_003153	signal transducer and activator of transcription 6, interleukin-4 induced	1.6	
IL8	NM_000584	interleukin 8	3.29	
NFATC1	NM_172387	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	1.39	
BCL3	NM_005178	B-cell CLL/lymphoma 3	2.48	
MAPK1	NM_138957	mitogen-activated protein kinase 1		1.36
CD69	NM_001781	*Homo sapiens* CD69 antigen (p60, early T-cell activation antigen)	5.61	
Angiogenesis				
WNT10A	NM_025216	wingless-type MMTV integration site family, member 10A		1.89
LYN	NM_002350	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog		1.58
BC002646	BC002646	v-jun sarcoma virus 17 oncogene homolog (avian)		1.81
PRKCZ	NM_002744	protein kinase C, zeta	1.53	
DVL1	NM_181870	dishevelled, dsh homolog 1 (Drosophila)		1.51
SPHK1	NM_021972	sphingosine kinase 1		1.3
PXN	NM_002859	paxillin		1.33
MAPK1	NM_138957	mitogen-activated protein kinase 1		1.36
EGF and FGF pathways				
STAT6	NM_003153	signal transducer and activator of transcription 6, interleukin-4 induced	1.6	
PRKCZ	NM_002744	protein kinase C, zeta	1.53	
MAP3K5	NM_005923	mitogen-activated protein kinase kinase kinase 5	1.55	
X07109	X07109	protein kinase C, beta 1	1.31	

microarray analysis. Real time confirmation of MYC, Ha-RAS and Ki-RAS expression also verified this result. The PI3K-mediated pathway and PI3K itself is inhibited by IP_6 (27). We also revealed a marked down regulation of several members of the PI3 kinase pathway on the microarray. Naturally, the inhibition of P3I kinase itself takes place at the protein level because of structural homology (16) and is not related to the transcriptional regulation of PI3K. This is

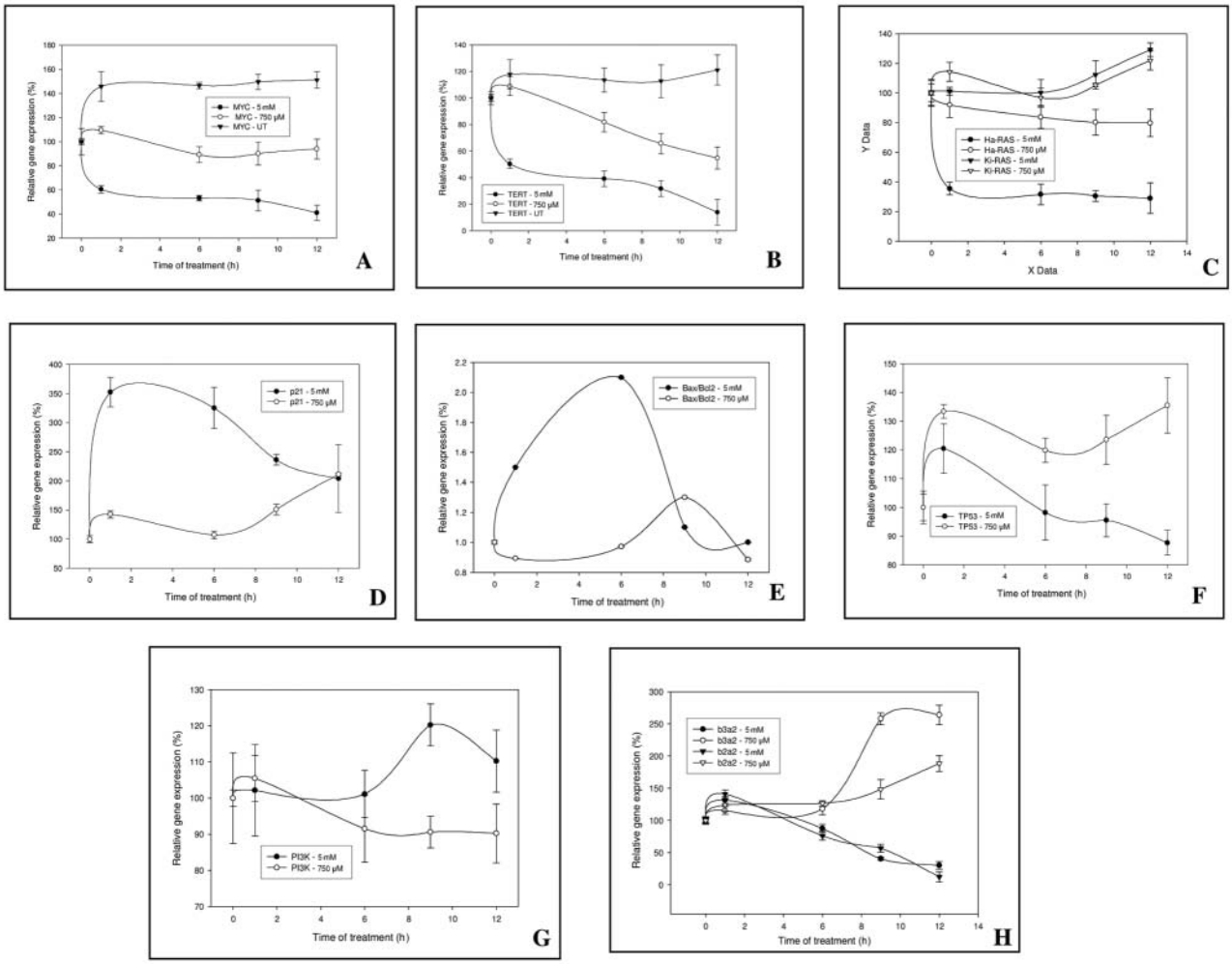

Figure 3. *Real time validation of A: c-Myc, B: h-TERT, C: Ha-RAS, Ki-RAS D: $p21^{CIP1/WAF1}$, E: BAX/BCL-2, F: TP53, G: PI3K, H: BCR-ABL b2a2 and b3a2, as a result of 750 µM and 5 mM IP_6 treatment monitored for 12 h. The gene expression values of each plot are calculated relative to the untreated values. The results are means of triplicates; SD values indicated.*

underlined with our real time analysis of PI3K, where no clear correspondence was demonstrated between IP_6 dose and PI3K mRNA.

Developing new blood vessels is essential for the growth of solid tumors. The anti angiogenic impact of IP_6 was reported (22). Our results also demonstrated up regulation of certain genes involved in this process, especially upon 5 mM treatment. The affected pathways and the pattern of down- and upregulated genes were, however, basically the same, with slight differences at the two different IP_6 doses. The general immunological activation and inflammation processes were more characteristic of the higher, 5 mM dose. In contrast, at the lower dose (750 µM, 60 min) antiproliferative effects *i.e.* suppressed Wnt and Insulin-IGF pathways and decreased expression of proto-oncogenes are characteristic. The observed mild induction of some of the proliferative pathways (EGF, FGF pathways) at the higher IP_6 dose may be due to the induction effect of the immunological events.

The increase in BAX/BCL-2 ratio is a reliable indicator of enhanced apoptotic processes (35). Our real time results showed significant elevation of BAX, as well as p53 and $p21^{WAF1}$ transcripts, which is in agreement with the findings of Weglarz *et al.* (40). It was reported, that inositol hexakisphosphate treatment resulted in activation of the apoptotic machinery in HeLa cell culture (20). Microarray results in line with real time analysis exhibited the strong dose- and time-dependence of apoptotic phenomena. Pharmacological IP_6-concentration (5 mM) resulted in a more prompt, rapidly increasing but transient apoptotic program, which culminated at around 6 h of treatment and fell to its initial level by 12 h. The physiological IP_6-concentration (750

μM), in contrast, elicited a more delayed (after 6 h of treatment) and moderate activation, which was similarly restored by 12 h. This tendency was also reflected in the microarray patterns: these showed a modest amount of apoptotic transcripts, significantly changed at both doses of IP_6.

We did not detect any sign towards erythroid cell differentiation at the transcriptional level after IP_6-treatments according to the array profile, however, real time PCR-validated mRNA of $p21^{CIP1/WAF1}$, a hallmark of erythroid differentiation (41) was elevated. On the other hand, benzidine staining unequivocally showed the increase of gamma-globin at the protein level. This contradiction may be due to the fact that 12 h-treatment is too short a time to detect the first signs of differentiation (Figure 2).

The major transcripts coding for the CML-characteristic BCR/ABL fusion protein showed a decrease as a result of treatment with 5 mM IP_6 in parallel with time. It was reported that a decrease in BCR/ABL fusion protein induces cell cycle and proliferation arrest, apoptosis and differentiation (42). This is in concordance with our findings that higher concentration (5 mM) of IP_6 reflected alterations in gene expression characteristic of apoptotic changes and differentiation.

We conclude that IP_6 treatment of the K-562 cell line elicited active modulation of specific genes involved in various biological pathways. The lower dose (750 μM for 60 min) IP_6 resulted in significant suppression in proliferative pathways, while a higher, pharmacological dose (5 mM for 6 h), called forth the most apoptotic changes. The first detectable signs of differentiation, however, started to appear after 12 h of treatment with 5 mM IP_6 and increased with time. Our study provides further evidence that IP_6 influences biological functions not only at the protein level but also profoundly alters the expression profile, modulating multiple targets of the cell signaling network.

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