Pharmacogenomics and analogues of the antitumour agent N6-isopentenyladenosine

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N6-isopentenyladenosine (i6A), a member of the cytokinin family of plant hormones, has potent *in vitro* antitumour activity in different types of human epithelial cancer cell lines. Gene expression profile analysis of i6A-treated cells revealed induction of genes (*e.g.*, PPP1R15A, DNAJB9, DDIT3, and HBP1) involved in the negative regulation of cell cycle progression and reportedly upregulated during cell cycle arrest in stress conditions. Of 6 i6A analogues synthesized, only the 1 with a saturated double bond of the isopentenyl side chain had *in vitro* antitumour activity, although weaker than that of i6A, suggesting that i6A biological activity is highly linked to its structure. *In vivo* analysis of i6A and the active analogue revealed no significant inhibition of cancer cell growth in mice by either reagent. Thus, although i6A may inhibit cell proliferation by regulating the cell cycle, further studies are needed to identify active analogues potentially useful *in vivo*.
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N6-isopentenyladenosine (i6A) is a member of the cytokinin family of plant hormones that regulate plant cell growth and differentiation[1] and is present in mammalian cells in a free form as a mononucleotide in cytoplasm[2] or tRNA-bound. As a tRNA modification, i6A contributes to reading frame maintenance,[3] and the lack of i6A in selenocysteine tRNA can affect synthesis of selenoproteins.[4]

We previously showed that i6A exerts potent *in vitro* antitumour activity on human epithelial cancer cell lines derived from different types of tumours.[5] Several i6A mechanisms of action have been hypothesized, including inhibition of cell proliferation,[5] inhibition of protein prenylation,[6] induction of apoptosis,[7,8] inhibition of DNA, RNA and/or protein synthesis,[2,9] and inhibition of nucleoside transport.[10,11] However, the precise mechanism of action of i6A in inhibiting cancer cell proliferation *in vitro* remains to be clarified.

Despite abundant evidence of the *in vitro* antitumour activity of i6A, this molecule has only a slight effect, if any, on tumour growth in rodents,[12] as well as in a pilot clinical trial.[13] The reason(s) for this discrepancy may rest in a rapid catabolism[14] or short plasma half-life of i6A, in analogy to other nucleosides.[15]

Here, we performed a gene expression profile analysis of i6A-treated cells to clarify the mechanism of action of i6A. Furthermore, we analysed 6 i6A analogues for their activity on different human cancer cell lines, and performed an *in vivo* study using 1 of these analogues compared to i6A. We also analyzed the expression levels of some genes modulated by i6A in cells treated with the analogues.

Material and methods

Cell lines

Human lung cancer (A549, NCI-H520, NCI-H596, Calu-3), breast cancer (MDA-MB-361, MCF7), hepatocellular carcinoma (HepG2), nasal septum squamous cell carcinoma (RPMI 2650), colorectal adenocarcinoma (HT-29) and ovarian adenocarcinoma (IGROV1) cell lines were used. All represent adherent epithelial

cells. Each cell line was propagated in the appropriate culture medium as recommended by American Type Culture Collection, except for MDA-MB-361, HT-29 and IGROV1 cells, which were cultured in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1.0 mM sodium pyruvate, 4.5 g/L glucose and 10% FBS.

Synthesis of i6A derivatives

I6A was purchased from Sigma-Aldrich (St. Louis, MO). Compound **1** (Fig. 1) was prepared by reaction of 6-chloropurine riboside and isopentylamine in refluxing DMF (3 hr, 150°C). Compounds **2-5** (Fig. 1) were prepared from 6-chloropurine and 1,2-isopropylidene glycerol or 1,3-isopropylidene 2-hydroxymethyl-1,4-butenediol using a Mitsunobu reaction.[16] Reaction of 6-chloropurine acyclonucleosides with isopentenylamine produced compounds **2** and **4**, whereas reaction with isopentylamine produced compounds **3** and **5**. Compound **6** (Fig. 1) was prepared according to Chladek and Smrt[17] All compounds were purified by silica gel chromatography and analyzed by 500-MHz 1H NMR to confirm assigned structures and purity. Spectra were recorded at 303 K on a Bruker AM-500 spectrometer equipped with an Aspect 3000 computer, a process control and an array processor.

RNA extraction

Total RNA was extracted using RNAfast-II RNA Isolation System (Molecular Systems, San Diego, CA) or TRIZOL® Plus RNA Purification Kit (Invitrogen, Carlsbad, CA). After DNase treatment (deoxyribonuclease I, Amplification Grade, Invitrogen) and purification (RNeasy MinElute Cleanup, Qiagen, Hilden, Germany) when necessary, RNA quality was assessed with an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA). RNA was used in microarray experiments or in kinetically monitored reverse transcriptase PCR (kRT-PCR).

Microarray gene expression analysis

Two independent microarray experiments were performed. In the first, total RNA extracted from A549 or MCF7 cells untreated or treated for 6 hr with i6A was used: for both cell lines and treatments, 4 independent replicates were analyzed. The second experiment was performed using total RNA extracted from MCF7 cells untreated or i6A-treated for 2, 6 or 24 hr. For each timepoint,
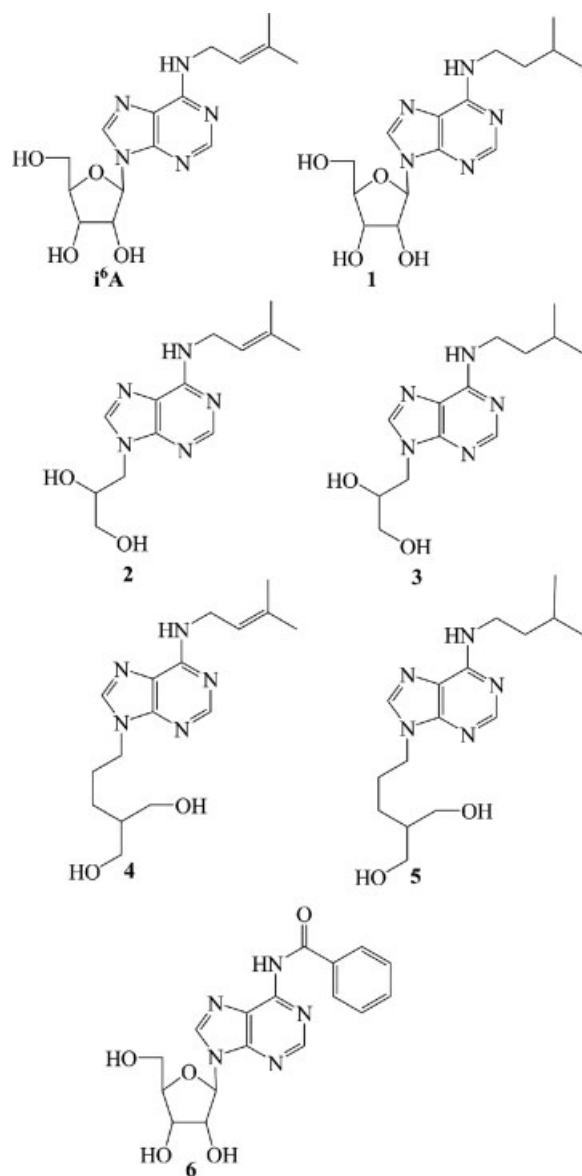
FIGURE 1 – Chemical structures of i⁶A and its analogues tested for antitumour activity.

4 replicates were performed both for i⁶A-treated and untreated cells. Biotinylated cRNA was synthesized using an RNA amplification kit (Ambion, Austin, TX) according to the manufacturers' instructions. From each sample, 1,500 ng of cRNA was hybridized for 18 hr to Human-6 v2 (first experiment, 2 technical replicates per biological replicate) or to Human-8 v3 (second experiment) Expression BeadChips (Illumina, San Diego, CA), according to the manufacturer's protocol. BeadChips were dried and scanned with an Illumina BeadArray Reader (Illumina). For differential expression analysis, genes were considered detected at $p < 0.05$.

Quantitative mRNA expression analysis

cDNAs were prepared by reverse-transcription using random hexamer primers and ThermoScript RT (Invitrogen). Amplification mixtures (final volume 25 μl) for kRT-PCR contained cDNA template diluted in RNase-free water, 12.5 μl 2X SYBR® GREEN PCR Master Mix (Applied Biosystems, Foster City, CA) and 0.3 μM PCR primers, amplifying 71–73 bp fragments specific for: DNAJB9, DDIT3, HBP1, PPP1R15A, and SESN2 genes (sequences of oligonucleotide primers are available upon request). The human hypoxanthine phosphoribosyltransferase 1 (HPRT1) gene was amplified with primers 5′-gactttgctttccttggtcagg-3′ and 5′-tccttttcaccagcaagcttg-3′ as a housekeeping control for possible differences in the amount of cDNA. Primers were designed using Primer Express 2.0 software (Applied Biosystems). All assays were run in triplicate on an ABI GeneAmp 7900 sequence detection system (Applied Biosystems). Relative expression levels were calculated using the comparative Ct method.

Clonogenic assay

Inhibition of clonogenicity was evaluated in cells seeded at 300–650 cells per well, depending on the cell line used, in 12-well plates and cultured in the appropriate medium supplemented with the compound of interest (no compound in control wells). Medium was replaced every 2 or 3 days with fresh compound-containing medium. Six replicas were performed for each concentration, compound, and cell line tested. After culture for approximately 2 weeks, cells were methanol-fixed and stained with 10% Giemsa. Clones were manually (Calu-3, IGROV1 and MDA-MB-361) or automatically (all others) counted with the Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Proliferation assay

Cell proliferation was analyzed using the AlamarBlue® Assay (Biosource, Camarillo, CA). A549 cells were plated at 1,000 cells per well in 48-well plates and cultured for 5 days in the presence of i⁶A or compound **1** at different doses (0, 1, 10 or 100 μM) and 10% AlamarBlue. Cell proliferation was monitored based on fluorescence intensity (excitation 535 nm, emission 590 nm) measured on a Tecan ULTRA multiplate reader (Tecan Group, Mannedorf/Zurich, Switzerland). Six replicas were performed for each dose and for each compound tested.

DNA synthesis assay

Inhibition of DNA synthesis was evaluated with the Cell Proliferation ELISA, bromodeoxyuridine (BrdU)-Chemiluminescent kit (Roche Applied Science, Mannheim, Germany). A549 cells plated at 15,000 cells per well in 96-well plates were treated with 0, 1, 10 or 100 μM of i⁶A or compound **1** for 18 hr. Two hours before the end of the treatment period, cells were labeled with 10 μM BrdU. Incorporation of BrdU was detected according to the manufacturer's instructions and luminescence was measured on a multiplate Tecan ULTRA reader. Eight replicas were performed for each dose for both compounds.

In vivo *tumour growth assay*

I⁶A was dissolved in 0.9% NaCl solution and 5% ethanol, aliquoted and kept at −20°C until use. A similar solution of compound **1** was prepared shortly before use. Forty female athymic Swiss nude mice (purchased from Charles River, Calco, Italy) were inoculated intraperitoneally (i.p.) with IGROV1 cells (2.5 × 10⁶ cells/mouse) and, starting on the same day, injected i.p. with i⁶A (10 mice) or compound **1** (10 mice) at a concentration of 10 mg/kg body weight for 5 days/week for 2 weeks, or injected with solvent alone (20 mice) using the same schedule. Mice were inspected daily and the day of death was recorded. The 10 mg/kg body weight dose was chosen after a preliminary experiment with higher doses. The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale Tumori of Milan. Survival analysis was performed after 25 days.

Statistical analysis

Genes differentially expressed in the 2 classes of either untreated or i⁶A-treated cells were identified using random variance t-statistics.[18] Gene expression profiles were analyzed using BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam (http://linus.nci.nih.gov/BRB-ArrayTools.html). Functional
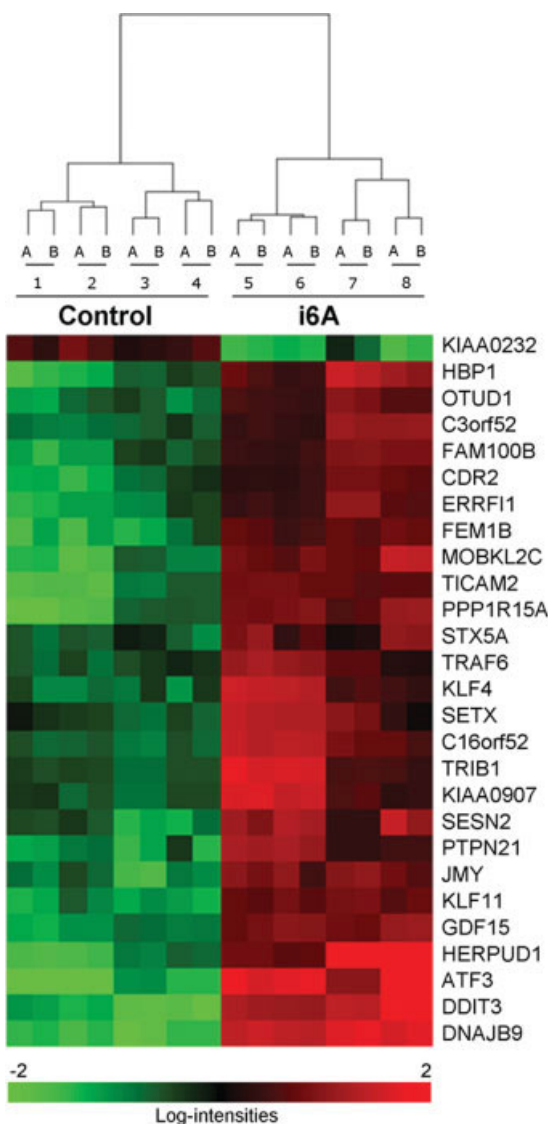
FIGURE 2 – Untreated and i[6]A-treated cells separated into 2 groups based on the gene expression profile at 6 hr after treatment. An example is shown of hierarchical clustering of differentially expressed genes, including genes whose difference in mRNA levels between untreated and i[6]A-treated A549 and MCF7 cells is significant at $p <$ 0.001 and whose expression data values have at least a 2-fold change in either direction from the gene's median value in more than 20% of samples. Gene expression levels (on the right) are indicated by the colour bar: green, low; red, high. A and B: technical replicas; 1, 2, 5 and 6: MCF7 cells; 3, 4, 7 and 8: A549 cells.

annotation in gene ontology (GO) categories was performed with the DAVID Functional Annotation Tools.[19] Differences in quantitative measures were assessed by analysis of variance. Correlations between expression levels were expressed by the Spearman's correlation coefficient. The Kaplan-Meier product-limit method and the log-rank test were used to evaluate the effect of i[6]A and compound **1** on survival of mice bearing IGROV1 tumour cells.

Results

Identification of i[6]A target genes

Gene expression profiles of A549 and MCF7 cells treated for 6 hr with 100 μM i[6]A as compared to untreated cells were obtained by microarray hybridization. Two different cell lines were used to
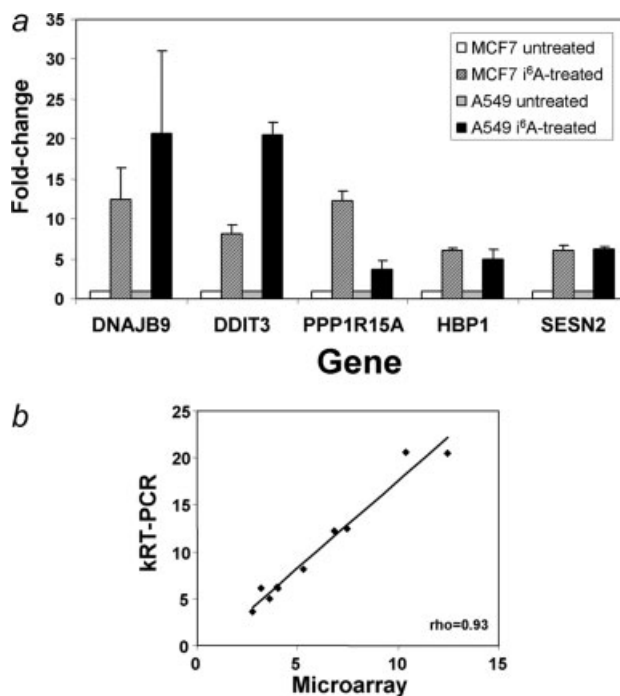

FIGURE 3 – Quantitative kRT-PCR for DNAJB9, DDIT3, PPP1R15A, HBP1 and SESN2 genes in MCF7 and A549 cells, untreated or i[6]A-treated. (*a*) Bars represent mean ± SE of fold change (i[6]A-treated/control). (*b*) Correlation analysis between microarray and kRT-PCR data.

avoid the identification of cell type-specific gene targets. Class comparison between i[6]A-treated and untreated samples of both cell lines identified 111 differentially expressed genes ($p <$ 0.001), most of which were up-regulated (70 genes $\geq$ 2-fold upregulated in treated samples), whereas only 3 were downregulated (Supporting Table 1). Figure 2 shows the genes whose expression levels were more modulated by i[6]A treatment.

Analysis of expression levels by kRT-PCR of 5 of the genes showing $\geq$3-fold up-regulation (DDIT3, DNAJB9, HBP1, PPP1R15A and SESN2) confirmed the microarray results (Fig. 3).

To analyze whether the modulation of gene expression induced by i[6]A has biological significance, gene functions were annotated and classified into functional gene ontology (GO) categories using the DAVID Functional Annotation Tool.[19] Table I lists only the biological process GO categories more significantly enriched ($p <$ 0.01) in the gene list (only the highest level, *i.e.*, 5, of GO Annotation was evaluated to obtain more specific information). The most significantly enriched GO term was "cell cycle arrest"; other significant GO categories included "negative regulation of progression through cell cycle", "cell death", "apoptosis", "protein modification process", "unfolded protein response, and "regulation of transcription" (Table I).

We performed an additional gene expression analysis, at different timepoints (2, 6 or 24 hr), of 100 μM i[6]A-treated or untreated MCF7 cells. Class comparison analysis between i[6]A-treated and untreated samples identified 16, 339 and 1,375 differentially expressed genes at 2, 6 and 24 hr, respectively ($p <$ 1.0E-06; false discovery rate (FDR)<5.0E-04). As an example, Fig. 4 shows the first 40 genes related to i[6]A treatment, adjusted by time. Overall, genes that were up-regulated at 6 hr after i[6]A treatment showed only marginally modulated mRNA levels at 2 hr after i[6]A treatment but remained up-regulated at 24 hr (Fig. 5). The 2 independent microarray experiments performed at 6 hr of i[6]A treatment showed consistent results, with a statistically significant correla-

TABLE I – GENE ONTOLOGY CATEGORIES (BIOLOGICAL PROCESS, LEVEL 5) ASSOCIATED TO THE 111 GENES SHOWING A SIGNIFICANT ($P < 0.001$) DIFFERENCE IN EXPRESSION LEVELS BETWEEN CONTROL AND i[6]A-TREATED A549 AND MCF7 CELLS

Gene ontology term	GO ID	No. of genes	p	Genes
Cell cycle arrest	0007050	6	7.61E-05	ERN1, HBP1, SESN2, PPP1R15A, DDIT3, JMY
Protein modification process	0006464	21	6.65E-04	HERPUD1, TRAF6, USP30, CBLB, TRIB1, MTMR3, PIM2, ALG2, ERN1, DUSP16, FBXL12, EIF2AK3, MED8, TRIM11, SNRK, PTPN21, WSB1, RNF44, ARIH1, MGAT1, VPRBP
Negative regulation of progression through cell cycle	0045786	7	7.72E-04	ERN1, HBP1, AXIN1, SESN2, PPP1R15A, DDIT3, JMY
Cell death	0008219	13	1.18E-03	AXIN1, TRAF6, PPP1R15A, DDIT3, PIM2, CEBPG, SETX, ERN1, FEM1B, EIF2AK3, SNRK, JMY, CEBPB
Regulation of apoptosis	0042981	10	1.87E-03	ERN1, EIF2AK3, FEM1B, TRAF6, SNRK, DDIT3, JMY, CEBPB, PIM2, CEBPG
Regulation of programmed cell death	0043067	10	2.02E-03	ERN1, EIF2AK3, FEM1B, TRAF6, SNRK, DDIT3, JMY, CEBPB, PIM2, CEBPG
Apoptosis	0006915	12	2.35E-03	ERN1, EIF2AK3, FEM1B, AXIN1, TRAF6, SNRK, PPP1R15A, DDIT3, JMY, CEBPB, PIM2, CEBPG
Unfolded protein response	0030968	3	2.36E-03	ERN1, EIF2AK3, DDIT3
Regulation of transcription, DNA-dependent	0006355	23	4.94E-03	NFIL3, DDIT3, AKNA, MEF2A, BRD1, ZNF140, KLF11, CEBPG, KLF4, HSF2, ERN1, MED8, HBP1, JMY, TSC22D1, SIN3A, CEBPB, GTF2B, ATF3, NRBF2, E2F7, ATF2, C10orf137
Regulation of transcription	0045449	24	5.16E-03	NFIL3, DDIT3, AKNA, MEF2A, BRD1, ZNF140, KLF11, CEBPG, KLF4, HSF2, ERN1, MED8, HBP1, RGMB, JMY, TSC22D1, SIN3A, CEBPB, GTF2B, ATF3, NRBF2, E2F7, ATF2, C10orf137
ER-nuclear signaling pathway	0006984	3	6.37E-03	ERN1, EIF2AK3, DDIT3
Transcription, DNA-dependent	0006351	23	6.50E-03	NFIL3, DDIT3, AKNA, MEF2A, BRD1, ZNF140, KLF11, CEBPG, KLF4, HSF2, ERN1, MED8, HBP1, JMY, TSC22D1, SIN3A, CEBPB, GTF2B, ATF3, NRBF2, E2F7, ATF2, C10orf137
RNA biosynthetic process	0032774	23	6.60E-03	NFIL3, DDIT3, AKNA, MEF2A, BRD1, ZNF140, KLF11, CEBPG, KLF4, HSF2, ERN1, MED8, HBP1, JMY, TSC22D1, SIN3A, CEBPB, GTF2B, ATF3, NRBF2, E2F7, ATF2, C10orf137
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0019219	24	6.89E-03	NFIL3, DDIT3, AKNA, MEF2A, BRD1, ZNF140, KLF11, CEBPG, KLF4, HSF2, ERN1, MED8, HBP1, RGMB, JMY, TSC22D1, SIN3A, CEBPB, GTF2B, ATF3, NRBF2, E2F7, ATF2, C10orf137
Regulation of kinase activity	0043549	6	7.83E-03	ERN1, DUSP16, MBIP, TRAF6, TRIB1, CERK

tion (rho $= 0.44$, $p < 2.2$E-16) observed between the transcript levels of the most significantly differentially expressed genes ($n = 329$).

Functional annotation in GO categories of the modulated genes at the 3 different timepoints indicated many GO terms; we reduced the redundancy of these terms by analysis using the Functional Annotation Clustering Tool and assigned names to the identified clusters according to the grouped GO terms. Supporting Table 2 lists the clusters with the higher enrichment score for each time-point and the clusters for which there was a time-dependent increase in the enrichment score. Indeed, we observed that at 2 hr after i[6]A treatment, the majority of modulated genes were those involved in transcription. This annotation cluster became more significantly enriched after 6 hr of treatment but was very poorly represented at 24 hr. By contrast, GO terms related to cell cycle and to apoptosis were not significantly enriched at the 2 hr time-point, but did increase their significance at 6 and 24 hr (Fig. 6).

Effect of i[6]A analogues on clonogenicity

Six analogues of i[6]A (Fig. 1) were synthesized (70–80% yield). ^{1}H NMR analysis established assigned structures and indicated a purity >98%. These compounds at different concentrations (0, 10 or 100 µM) were first screened on A549 cells for their ability to in-hibit clonogenicity. Only compound **1** significantly reduced the number of colonies formed after 12 days of treatment, leading to approximately 3- and approximately 5.5-fold decrease in A549 clonogenic activity at 10 and 100 µM, respectively ($p < 0.0001$; Fig. 7 and not shown). These findings contrast with the complete inhibition of A549 clonogenicity by i[6]A at the same concentrations.[5]

Compound **1** at 100 µM (the most effective dose in A549 cells) completely inhibited clonogenic activity in 6 cell lines tested (HepG2, IGROV1, MCF7, MDA-MB-361, NCI-H520 and NCI-H596), and induced approximately 5- and approximately 16-fold decrease in the number of Calu-3 and RPMI 2650 colonies, respectively ($p < 0.0001$). A slight but consistent inhibition of colony formation (less than 1.5-fold; $p < 0.0001$) was also observed in HT-29 cells (Fig. 7), the only cell line that was not completely inhibited by i[6]A treatment.[5]

Effects of i[6]A and compound 1 on in vitro *proliferation and on tumour-bearing mice*

Compound **1** inhibited both proliferation and DNA synthesis in A549 cells as measured using the AlamarBlue® assay and based on BrdU incorporation, but to a lesser extent than i[6]A. Indeed, pro-liferation at day 5 in cells treated with 10 µM i[6]A was inhibited
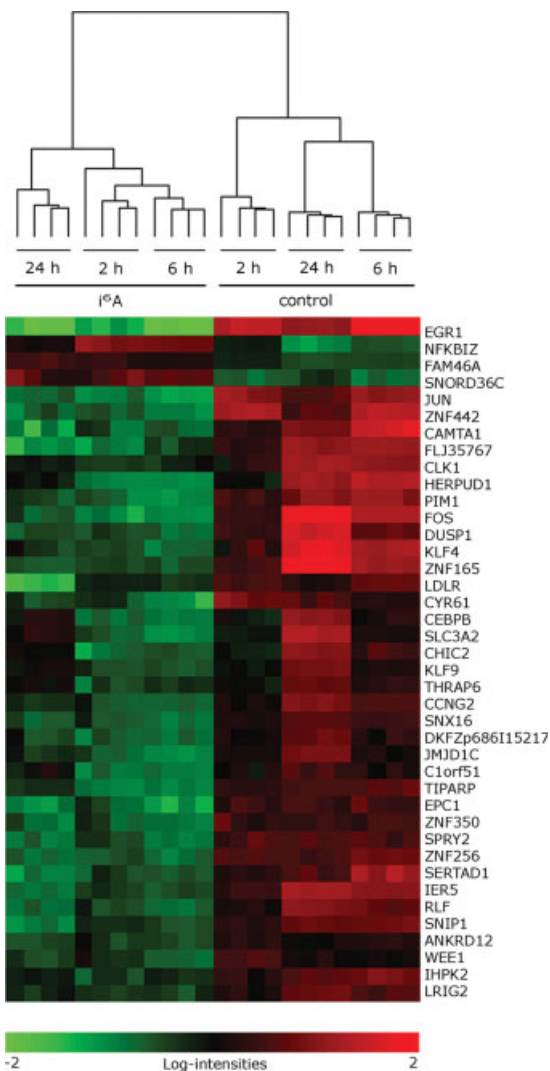
FIGURE 4 – Time-response expression profile of the first 40 more significantly differentially expressed genes ($p < 1.0E-07$) in i⁶A-treated and untreated MCF7 cells. Hierarchical clustering of differentially expressed genes is shown. Gene expression levels (on the right) are indicated by the colour bar: green, low; red, high.

FIGURE 5 – Time-course of i⁶A-induced (i⁶A/untreated ratio) up-regulation of 298 mRNA transcripts ($p < 1.0E-06$) at 6 hr after i⁶A treatment. The line within each box represents the median value of the ratio between i⁶A-treated and untreated MCF7 cells (in base 2 logarithmic units); upper and lower edges of each box represent the 75th and 25th percentile, respectively; upper and lower bars indicate the highest and lowest values analysed, respectively ($p < 2.2E-16$).

FIGURE 6 – Enrichment scores of functional annotation clusters varying with time.

approximately 3-fold but only approximately 1.5-fold in cells treated with compound **1** at the same concentration (Fig. 8*a*). Use of i⁶A and compound **1** at 100 μM reduced proliferation more than 8-fold and approximately 2.5 fold, respectively (Fig. 8*a*). The same trend upon higher doses of the chemicals was observed with the BrdU incorporation assay, with DNA synthesis maintained in only approximately 4% of cells treated with i⁶A at 100 μM but in 45% of cells treated with compound **1** at this dose, as compared to untreated cells. Treatment with 10 μM i⁶A or compound **1** reduced BrdU incorporation by approximately 30 and 20%, respectively, as compared to untreated control cells, whereas treatment with either compound at 1 μM had no significant effect (Fig. 8*b*).

Analysis of nude mice bearing i.p. IGROV1 ascites and treated i.p. daily with 10 mg/kg of i⁶A or compound **1** revealed a limited increased survival of treated mice (median survival time of 21, 20 and 18 days in i⁶A-, compound **1**-treated, or untreated mice, respectively), but the increase was not statistically significant (Fig. 9). Higher doses of i⁶A (30 mg/kg) caused lethal toxicity, with treated mice dying before the control mice, whereas higher doses (15 mg/kg) of compound **1** caused a rapid abdominal contraction
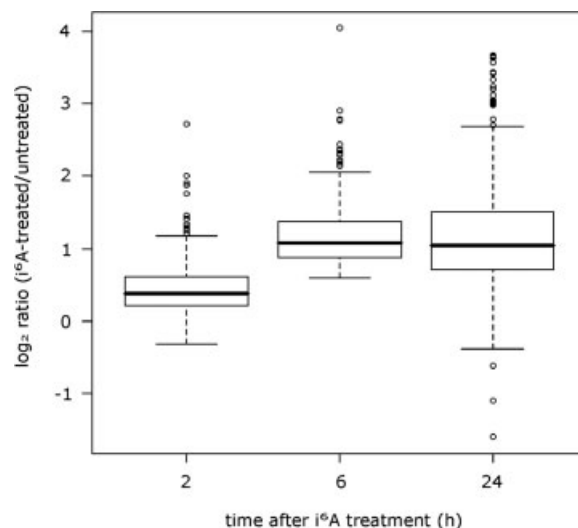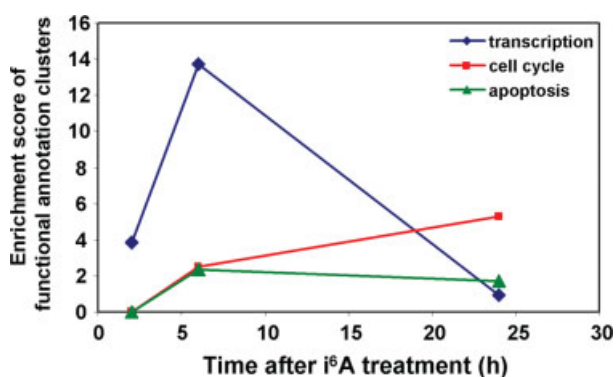
and lethargy lasting for about 30 min but had no significant affect on the median survival time.

Expression analysis of i⁶A target genes in cells treated with analogues

To test whether i⁶A target genes were also modulated by any of the i⁶A derivatives, kRT-PCR was used to measure DDIT3, DNAJB9, HBP1, PPP1R15A and SESN2 expression levels in A549 cells treated with 100 μM of each compound for 6 hr or untreated; none of the 5 genes were modulated by any of the analogues (not shown).

Discussion

Several studies reported that i⁶A has *in vitro* antitumour activity, but the mechanism of action of this molecule has not yet been identified. To this aim, we performed a gene expression profile analysis of i⁶A-treated or untreated A549 and MCF7 cells. The results of microarray analysis indicated modulation of 111 genes at 6 hr after treatment with i⁶A (Supporting Table 1).
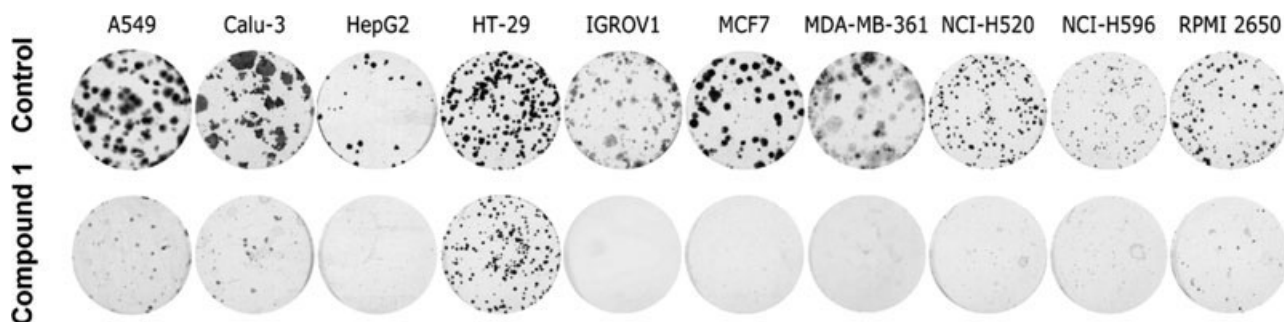
FIGURE 7 – Inhibition of clonogenicity after *in vitro* treatment of 10 human cancer cell lines of epithelial origin with compound **1** (100 μM).
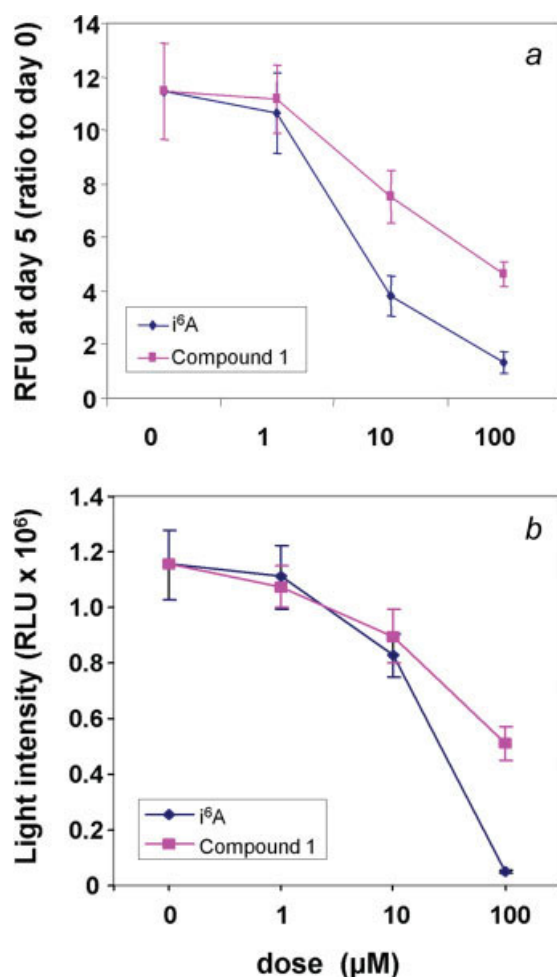

FIGURE 8 – Dose-dependent inhibition of cell proliferation in A549 cells treated with i[6]A or compound **1**. Panel A, AlamarBlue® assay at day 5 (ratio to day 0), RFU, relative fluorescence units. Panel B, BrdU uptake after 18 hr, expressed as RLU, relative luminescence units. Data are given as mean ± SE.
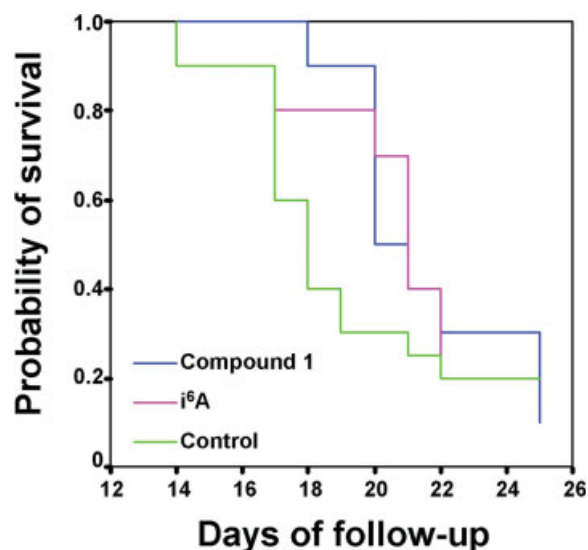

FIGURE 9 – Survival rates of nu/nu female mice inoculated i.p. with IGROV1 ascites tumour cells and treated i.p. for 5 days/week for 2 weeks with i[6]A or compound **1** at 10 mg/kg body weight or left untreated.

Functional annotation of the modulated genes indicated alterations in the regulation of transcription, consistent with previous evidence of a role for i[6]A in RNA synthesis.[2,9] The significantly enriched GO term "cell cycle arrest" is consistent with evidence that i[6]A inhibits cell proliferation and negatively regulates cell cycle progression,[5,6] while the significant association with "protein modification process" could explain a previous finding of a role for i[6]A in inhibiting protein prenylation.[6] The association with apoptosis-related GO terms is also in accord with previous studies.[5,7,8] The significant association of our gene list with the GO term "unfolded protein response" suggests that i[6]A could exert its *in vitro* biological effects by inducing cellular stress associated with an accumulation of unfolded proteins. Unfolded proteins can be correctly refolded by chaperone proteins or degraded by the ubiquitin-proteasome pathway; when levels of misfolded proteins are excessive, cells can die.[20] Maintenance of proteins in the unfolded form may require stable levels of i[6]A, possibly explaining the strong suppression of cell clonogenicity *in vitro* by i[6]A but its lack of *in vivo* effects, because i.p. injections produce peak i[6]A levels of only short duration.

Time-response expression profile analysis showed that early-response genes modulated by i[6]A treatment were those involved in transcriptional regulation. These genes could activate and/or repress other genes involved in the cell cycle and in apoptosis, as indicated by the enrichment at 24 hr after treatment of the list of genes associated to these 2 biological processes (Fig. 6).

To identify molecules with the same inhibitory effects of i[6]A on *in vitro* cell proliferation but with greater stability *in vivo*, i[6]A analogues were synthesized and tested. Initially, the isopentenyl side chain was modified or substituted to analyze whether this portion of i[6]A structure was necessary for biological activity. Thus compound **1**, in which the double bond of the isopentenyl side chain was saturated, and compound **6**, in which the isopentenyl side chain was substituted with a benzoyl group, were synthesized. The clonogenicity of A549 cells was partially inhibited by compound

1 (Fig. 7), whereas no inhibition was seen after treatment with compound **6**, suggesting that the antitumour effect of i⁶A may be conserved after subtle changes (*i.e.*, saturation) of the isopentenyl side chain, but not after its substitution with another chemical group (*i.e.*, the benzoyl group). We then synthesized 2 analogues of i⁶A (compounds **2** and **4**) and 2 analogues of compound **1** (compounds **3** and **5**) in which the unstable *in vivo* β-N-glycosidic (N-C-O) bond was substituted with a more stable (N-C-C) bond; none of these analogues inhibited clonogenicity of A549 cells. Thus, the ribose chain appears to be essential for maintenance of the inhibitory effects of i⁶A on growth of epithelial cancer cells.

Because i⁶A has a wide-spectrum inhibitory effect on different human epithelial cancer cell lines,[5] we tested whether compound **1** might inhibit the same cell lines. Although 10 μM i⁶A completely abrogated colony formation in 9 of 10 cell lines tested ([5] and not shown), 100 μM compound **1** completely inhibited clonogenicity of only 6 of the 10 cell lines (Fig. 7). Thus, besides being less effective, compound **1** appeared to have a certain cell-specificity. The lower tumour inhibitory effects of compound **1** as compared to i⁶A may reflect the minor inhibitory effects of the analogue on A549 cell proliferation and DNA synthesis (Fig. 8). Thus, inhibition of clonogenicity and of cell proliferation are correlated events in the action of these molecules.

The potential *in vivo* antitumour activity of i⁶A or compound **1** was tested in nude mice inoculated i.p. with IGROV1 cells, an *in vivo* model that can be considered the most similar to an *in vitro* assay because i.p.-injected IGROV1 cells grow in the peritoneal cavity as ascites and the i.p.-injected drug quickly comes in contact with tumour cells. However, neither compound **1** nor i⁶A affected mouse survival as compared to untreated mice (Fig. 9), suggesting that the pharmacokinetics of these chemicals do not allow them to reach time-average concentrations comparable to those *in vitro*.

To analyze whether the observed gene targets modulated by i⁶A were also involved in the response to compound **1**, and to test whether the lack of functional effects of the other analogues rested in the failure to modulate the same i⁶A gene targets, we measured the expression levels of DDIT3, DNAJB9, HBP1, PPP1R15A and SESN2 by kRT-PCR in A549 cells treated with the 6 analogues at 100 μM. No significant difference between treated and untreated cells in these gene expression levels was found for any of the 6 analogues (not shown). These results suggest that compound **1** either targets genes different from those targeted by i⁶A or that its lower antitumour effects are associated with a weaker, perhaps delayed, modulation of specific transcripts as compared to i⁶A. The unmodulated gene profile after treatment with compounds **2**–**6** is in agreement with the lack of effects of these same compounds.

Our pharmacogenomics study of i⁶A suggests that induction of cellular stress may represent a mechanism of antitumour action of i⁶A, causing cell cycle arrest and cell death. Expression data obtained in this study suggest possible i⁶A molecular targets. To date, the analogues synthesized and tested are ineffective or less effective than i⁶A, pointing to the strict requirements of molecular architecture in antitumour activity. New i⁶A derivatives are needed to identify a compound with *in vivo* antitumour activity on epithelial cancer cells.

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