Time-resolved gene expression profiling of human squamous cell carcinoma cells during the apoptosis process induced by photodynamic treatment with hypericin

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Abstract. Hypericin is used as a powerful naturally occurring photosensitizer in photodynamic therapy (PDT). Activated by visible light, it kills tumour cells and tissues via generation of reactive oxygen species (ROS). Depending on the protocol, apoptotic cell death can be achieved very effectively by hypericin-PDT. To analyze the fundamental molecular mechanisms leading to apoptosis induced by photodamage especially with regard to human skin cancer cells, we studied the alteration of the gene expression pattern in the human squamous cell carcinoma cell line A-431 at 1.5, 3, 5 and 8 h after hypericin-PDT by cDNA-macroarray technique. Radioactively labelled samples were hybridized onto macroarray filters containing PCR products of 9738 ESTs of the Incyte Human UniGEM Microarray clone set. In total, 168 genes were found to be differentially upregulated and 45 downregulated. Verification of expression changes of 45 genes of interest was performed by quantitative real-time PCR. Due to the observed significant expression changes the following can be concluded: lipoprotein receptor-mediated endocytosis could play a role in the uptake of lipophilic hypericin. Extracellular signal transduction to the cell is reduced, cell detachment facilitated, changes of the morphology, cytoskeleton and formation of apoptotic bodies occur. The promotion of $p38^{MAPK}$, ERK, JNK and Ras signalling pathways supports survival and/or apoptosis. Switches between life and death could be the strongly upregulated transcription factors *c-jun* and *FOSB* as well as the MAPK-phosphatase 1 *DUSP-1*, possibly activated via H3 histone modifications. ROS activate ER-stress pathways or adaptive response, and provoke damage protection against ROS, partly in a cell-type specific way.

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Introduction

Photodynamic tumour therapy (PDT) is applied worldwide for malignant and non-malignant lesions as well as for antimicrobial and antiviral questions. It is based on the preferential uptake and retention of a *per se* harmless dye, the photosensitizer, by target cells and tissue structures which, upon illumination with visible light of an appropriate wavelength, generates reactive oxygen species (ROS) and free radicals leading to the destruction of the target tissues and cells.

Hypericin, which is extracted from St. John's wort (*Hypericum perforatum*) is gaining rapidly in importance as photosensitizer for therapeutical use, since it is very effective in low doses, as well as fluorescence marker for diagnosis due to its bright fluorescence and high selectivity (1).

While at present the predominant clinical application of hypericin is in fluorescence diagnosis (FD) which has been carried out for bladder cancer in successful trials (2), the therapeutic application of hypericin is in its early stages, especially in tissues other than bladder. Although potentially promising from its properties (high tumour selectivity and efficiency) and proved in experiments with mice (3-5), only a few clinical data concerning the treatment of (pre)cancerous lesions of the skin exist. Hypericin was applied successfully on human skin carcinomas BCC (basal cell carcinoma) and SCC (squamous cell carcinoma) (6,7), but an extract of *Hypericum perforatum,* used in a clinical pilot study on actinic keratoses, BCC and Bowen's disease, showed only partial success (8). The results, however, are difficult to evaluate, since an extract of the whole plant may contain also inhibitory compounds.

The absence of clinical trials is mainly due to some not completely solved issues. Research is still carried out on uptake mechanisms of hypericin and its basic molecular modes of action in order to eventually optimize its clinical application (9). Therefore expression profiling after hypericin-PDT has been carried out with the bladder cancer cell line T24, showing that genes involved in metabolic processes, stress-induced cell death, autophagy, proliferation, inflammation and carcinogenesis are affected by hypericin-PDT. Validation of some relevant genes was performed at 7 h post treatment by quantitative real-time PCR (qRT-PCR). Hypericin-PDT induced T24 cell death mediated by endoplasmatic reticulum (ER)-Ca^{2+} store emptying and executed through caspase-

dependent or -independent pathways on one hand, but also activation of survival pathways via $p38^{MAPK}$ on the other hand (10). Previous studies of our group showed that hypericin-PDT on A-431 cells induced preferentially apoptosis, executed via the mitochondrial pathway activating the caspases 9, 6 and 3, but also caspase 2 of the receptor-mediated pathway, maybe in a feedback loop (11).

In order to analyse the molecular background for skin treatment and apoptosis induction by hypericin-PDT, we investigated gene expression profiles of human squamous cell carcinoma cells A-431 at different time-points (1.5, 3, 5 and 8 h) post treatment (pt = post irradiation of hypericin) to include the time window relevant for apoptotic processes. Cells were treated with doses resulting in about 80% apoptotic cell death and about 20% viable cells.

RNA was isolated from PDT-treated cells as well as of 3 controls (untreated, light only and photosensitizer only), radioactively labelled by reverse transcription with ^{33}P-dCTP and hybridized onto macroarray filters containing PCR products of 9738 ESTs of the Incyte Human UniGEM Microarray clone set. Validation of observed expression changes at the four different pt-times was carried out by qRT-PCR.

Materials and methods

Cell culture. A-431 human squamous cell carcinoma cells (ATCC: CRL-1555) and human fibroblasts WI38 (ATCC: CCL-75) were cultivated in DMEM (Dulbecco's modified Eagle's medium) with 4.5 g/l glucose supplemented with 4 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 100 mM HEPES together with 5% FCS or 10% FCS, respectively, and incubated at 37˚C in a humid 7.5% CO_2-atmosphere. No serum was added to the DMEM during photosensitizer incubations and post treatment times in order to avoid release of the lipophilic hypericin into the culture medium.

Photodynamic treatment. Cells were incubated for 16 h with 200 ng/ml hypericin and kept in darkness. All cell manipulations were performed under subdued light conditions. Irradiation was carried out with a power density of 12 mW/cm^2 using the dermatological halogen lamp 'Waldmann PDT 1200' with a cut-off filter below 600 nm. For gene expression analysis, cells were irradiated with a fluence of 0.6 J/cm^2 and harvested at 1.5, 3, 5 and 8 h pt. Untreated cells served as reference. Light-only and sensitizer-only controls were also performed.

Fluorescence microscopy. Localization studies of hypericin were performed with an inverted fluorescence microscope (Olympus IX70) coupled to a sensitive RGB camera (SPOT 2, Visitron Systems, Germany) using an excitation wavelength of 480-550 nm and an emission wavelength of >570 nm.

Apoptosis detection. Caspase 3 activity was monitored using the CaspACE Assay System (Promega, USA). Also the sub-diploid DNA content (sub-G1-peak) in cell cycle DNA-histograms, generated by a flow cytometer (FACSCalibur, Becton-Dickinson, USA) by fixing cells with 70% ethanol, removal of RNA by RNase treatment (100 μg/ml) and staining of DNA with 40 μg/ml propidium iodide, was investigated. For verification of the results apoptotic as well as viable and necrotic cells were stained with 50 μg/ml JC-1 and analyzed on a flow cytometer (FACSCalibur). As a reference apoptosis was induced by UV irradiation at 254 nm with 200 mJ.

RNA isolation and probe synthesis. A-431 cells were incubated with hypericin and irradiated according to the selected protocols. Control cells without any treatment served as a baseline sample for comparison. RNA isolation and cDNA labelling was done essentially as described by Aberger *et al* (12). Briefly, after harvesting, A-431 cells were lysed using Tri Reagent™ (MRC Inc., USA) and RNA was isolated using the provided protocol. Labelling of cDNA to high specific activity by reverse transcription was carried out by using 15 μg total RNA, 70 μCi α^{33}P-dCTP (3000 Ci/mmol, Amersham Biosciences, USA), 10 μl 3x labelling buffer (for 200:120 μl 5x SuperScript™ reverse transcriptase buffer (Invitrogen, USA), 3 μl dATP/dGTP/dTTP 100 mM each, 60 μl 0.1 M DTT, 0.8 μl 1 mM dCTP, 10.2 μl DEPC treated ddH_2O) and 2 μl SuperScript II reverse transcriptase (Invitrogen). The labelled probes were purified using GFX columns (Amersham) according to the manufacturer's protocol. Scintillation-counting was used to measure the incorporation of radioactivity and to verify that samples were labelled equally well. Incorporation of labelled triphosphate into samples was calculated from the ratio of total counts to counts after purification.

Array hybridization and data analysis. Macroarray PCR filters consisting of membranes of 22x22 cm (Hybond-N$^+$, GE Healthcare, SE) were spotted with PCR products of 9738 ESTs of the Incyte Human UniGEM Microarray clone set in duplicate on a MicroGrid II arrayer (BioRobotics, UK). After prehybridization for 2 h at 65˚C, filters were hybridized with labelled and denatured (5 min at 95˚C) cDNAs for 2 days at 65˚C in prewarmed hybridization buffer [5X Denhardt's, 5X saline-sodium citrate buffer (SSC), 1% sodiumdodecyl sulphate (SDS)]. Filters were washed each time for 20 min at 65˚C first in 2X SSC/0.1% SDS, then two times in 0.2X SSC/0.1% SDS and then in 0.1X SSC/0.1% SDS, rinsed with 2X SSC, sealed in Saran-wrap and exposed for 4 days to phosphorimager screens (Fuji, Germany). These screens were then scanned with a BAS Reader 1800 II phosphorimager (Fuji). Two arrays were hybridized for each RNA sample yielding 2 hybridization spots in duplicates for statistical analysis.

Image and data analysis. Spot intensities were calculated using the AIDA software (Raytest, Germany) and exported to Microsoft Excel (Microsoft, USA) for further calculations. The results were normalized using the total signal over all hybridizing spots.

Real-time PCR. Forty-five genes of interest from the macroarrays (expression change >2 of at least one point pt compared to untreated control) were selected for verification by real-time RT-PCR. Primers were designed mainly with Vector NTI 6 (Invitrogen) unless using previously published primers (http://medgen.ugent.be/rtprimerdb/). The design of self-constructed primers was done under the prerequisite that at least one primer for each gene should cover an exon-intron boundary to avoid amplification of genomic sequences. Primers (sequences are shown in Table I) were then ordered at Biomers (Germany).

Table I. Primer sequences.

Gene name	Accession no.		Primer sequences 5'-3'
ACTB	P60709	fwd:	TTCCTTCCTGGGCATGGAGTCC
		rev:	TGGCGTACAGGTCTTTGCGG
ADAM10	AF009615	fwd:	TCCTCCTAAACCACTTCCAGGCACTTT
		rev:	CAGTTAGCGTCTCATGTGTCCCATTTGATA
ADM	D14874	fwd:	TCTCGAAGCCCCGAAGACAGCA
		rev:	GATCTGGTGTGCCAGCTTCTGCA
ATF4	D90209	fwd:	TCCAACAACAGCAAGGAGGATGCC
		rev:	GAAGGTCATCTGGCATGGTTTCCA
CANX	M94859	fwd:	CCTGAAGAAAGCTGCTGATGGGG
		rev:	CCTCCTCCTCATCTCCCTTGTCCTT
CITED2	AF129290	fwd:	AAACGGAAGGACTGGAAATGGCA
		rev:	TTAGGGCGTTGAAGGCGTGCT
CTH	S52784	fwd:	TGGATGGGGCTAAGTACTGTTTGGC
		rev:	TGGTTTCTGGTGTAATTGCTGCCTC
CYP1B1	U03688	fwd:	ACCGCACCTCCCCGCAGCAT
		rev:	CACCTTCCAGTGCTCCGAGTAGTGGC
DKK-1	AF177394	fwd:	GCAAAAATGGAATATGTGTG
		rev:	ACCTTCTTGTCCTTTGGTGT
DNAJB1	D85429	fwd:	TTGACCATCGAAGTGAAGAAGGGG
		rev:	ATCGTCCTGCCGTCCAGAGT
DUSP1	X68277	fwd:	ACAACCACAAGGCAGACATCAGCTC
		rev:	GTTGGGAGAGATGATGCTTCGCC
ETS2	J04102	fwd:	GAGCAAGGCAAACCAGTTATACCT
		rev:	GTAGTTCATCTTGGGCTTATTTTTCCT
FAF1	AJ271408	fwd:	CTGGCGGATTTTCAGGCATGTACT
		rev:	CAGGTCCTGGTATGGTCTCACCTCC
FOSB	L49169	fwd:	TCCAGGCGGAGACAGATCAGTTG
		rev:	TCTTCGTAGGGGATCTTGCAGCC
GADD34	AK001361	fwd:	CTCCCAGCCGCCCAGACACA
		rev:	CCATCACTGGGGACAGGAGG
GAPDH	P04406	fwd:	GTGAAGGTCGGAGTCAACG
		rev:	TGAGGTCAATGAAGGGGTC
GRAP	Z35491	fwd:	GGAGATTGACACACTGATCCTGCCA
		rev:	CCATTCTTCAGGGCAGCACAGC
HIST1H3B	Z80784	fwd:	CTGGTGGGGCTGTTTGAGGACA
		rev:	GGCGAGCAAGCTGGATGTCCTT
HMGA1	M23614	fwd:	GGAGTCAGAAGGAGCCCAGCGAAGT
		rev:	GCATGGGTCACTGCTCCTCCTCC
HSP90AA1	X07270	fwd:	TCCCATTACTCTTTTTGTGGAGAAGGAACG
		rev:	CAACATCTTCAATTTCAGGTTTGTCTTCCG
HSPA13	U04735	fwd:	GATCTTAGGATCGGCTGTTTTGACTCTCC
		rev:	GGGACACTGTGATGGTCTCATTACTTGTCA

Table I. Continued.

Gene name	Accession no.		Primer sequences 5'-3'
HSPA8	Y00371	fwd:	GGAAATTGCAGAAGCCTACCTTGGG
		rev:	GCACGTTTCTTTCTGCTCCAACCTT
IL1RN	X52015	fwd:	GGAAGATGTGCCTGTCCTGTGTCAA
		rev:	TATTGGTGAGGCTGACGGGCTG
INSIG1	U96876	fwd:	CCCTACCCCAACACCTGGCATCAT
		rev:	CGGAGGAAAAGATGGTGGCGATCAC
INSR	M32831	fwd:	GCGAATGCTGCTCCTGTCCAAA
		rev:	CGAAAACCACGTTGTGCAGGTAATC
IRS2	AF073310	fwd:	GGCCACCATCGTGAAAGAGTGAAG
		rev:	ACAAGGGAAAGAGGCAGGTGACCT
ITGA3	M59911	fwd:	CCCTCTCAACCTCACTCTTTCTGACCC
		rev:	TCCACACTCGTGCCTTCACAGTCAC
ITGA6	X53586	fwd:	TCCCTGAACCTAACGGAGTCTCACAACT
		rev:	TCACATCAATGAAGGCTCGCATGAG
ITGB1	X07979	fwd:	AGTAACAATGGAGAGTGCGTCTGCG
		rev:	TGGGGTTGCACTCACACACACGAC
JUN	J04111	fwd:	CACGGCGGTAAAGACCAGAAGG
		rev:	AACTCACTTCCCAGAGCAGCGG
KRT17	Z19574	fwd:	TCTCCTCTCCAGCCCTTCTCCTGTG
		rev:	CCGCCAGCAGATCCCAGCCT
LDLR	AF217403	fwd:	TGCCTCATCCACCAATCTCTAAGCC
		rev:	CGACGCCATGAATCCCTTTCCTG
MT1A	K01383	fwd:	ATGGACCCCAACTGCTCCTG
		rev:	CACTTCTCTGATGCCCCTTTGCA
MXD1	L06895	fwd:	GGAGAGAGAAGCTGAACATG
		rev:	ATCTGCTACTGCTGTTATTC
MYC	V00568	fwd:	AGTGGAAAACCAGCAGCCTCCC
		rev:	GAGGTCATAGTTCCTGTTGGTGAAGC
NEDD9	L43821	fwd:	CCGCCTACAAAAGGGGTATATGCC
		rev:	GCCTTCCAGCTTGTCTCATGGGA
NR4A2	X75918	fwd:	TCGATTAGCATACAGGTCCAACCCA
		rev:	TGACCATAGCCAGGGCAGCAAT
PBK	AB027249	fwd:	CAGGCGGTGAGACTCTGGACTGAGA
		rev:	GCCCAAGGAGAATGAGACAAACCTC
PHLDA1	AF220656	fwd:	CCGTATCCGCATCCACATCCAC
		rev:	GAGTTGGAGGTGCTGCGGAGAA
PNPLA8	AB041261	fwd:	AGAACAACTGACCCAAAGCTCTGCA
		rev:	TCGGATTCCTCTCCCTTTCACTGG
PRE IL-1	X02851	fwd:	ACCAACCAGTGCTGCTGAAGGAGA
		rev:	GCCAAGCACACCCAGTAGTCTTGC

Table I. Continued.

Gene name	Accession no.	Primer sequences 5'-3'	
RIN2	AL136924	fwd:	AACAACTCAGGGAGGATGTATGGCG
		rev:	AAGTCGTCCACAGAGGGGATGGTCC
RND3	S82240	fwd:	GTCCTATGACCAGGGGGCAAATATGGC
		rev:	CGCTTTGTCCTTTCGTAAGTCCGTAGC
RPS3A	P61247	fwd:	CCTGGAGGAGAAGAGGAAAGAGA
		rev:	TTGAGGACCTCTGTGTATTTGTCAA
THBS1	X14787	fwd:	CGACCAGAAGGACTCTGACGGCGAT
		rev:	GGACGAGTTCTTTACCCTGATGGCG
UBC	D63791	fwd:	GATTTGGGTCGCGGTTCTT
		rev:	TGCCTTGACATTCTCGATGGT
WEE1	X62048	fwd:	CGCACACGCCCAAGAGTTTGCT
		rev:	CGACACTGTCCTGAGGAATGAAGCA
ZBTB16	AF060568	fwd:	GCTCATTCAGCGGGTGCCAA
		rev:	TCCCACACAGCAGACAGAAGACGG

Real-time PCR reactions were carried out using iTaq DNA polymerase (Bio-Rad, Germany) and labelling with SYBR Green (Molecular Probes, The Netherlands) on a Bio-Rad iCycler. Optimal PCR conditions (especially the determination of specific annealing temperatures) were established for each primer pair by checking melting curves and 2% agarose gel banding of PCR products. Relative expression changes were calculated using the Bio-Rad 'Genex' MS-Excel macro (Gene Expression Analysis for iCycler iQ® Real-Time PCR Detection System) based on the algorithms outlined by Vandesompele *et al* (13). Normalization of sample to sample variations was performed using the three house-keeping genes ß-actin, *glyceraldehyde 3-phosphate dehydrogenase* and *40S ribosomal protein S3a*. Data were collected from 3 independent PDT-treatments and RNA isolations. Each PCR reaction was run in triplicates. Dark- or light-only results were described when exceeding the threshold.

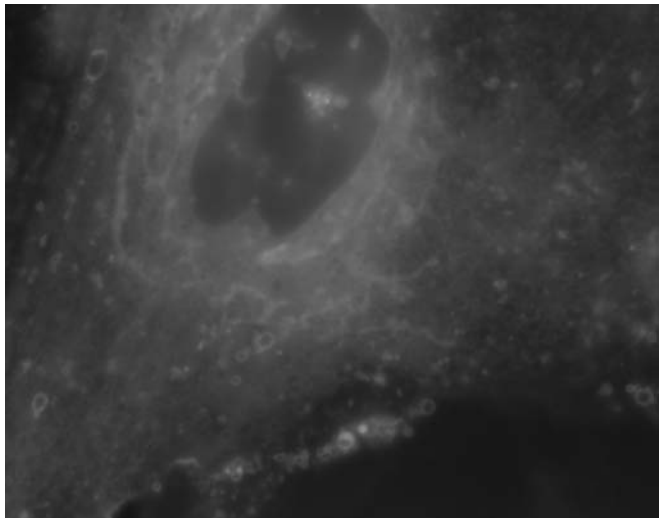

Figure 1. Localization of 200 ng/ml hypericin in WI38 cells after 16 h of incubation. Magnification x1500.

Results

A-431 cells were incubated for 16 h with 200 ng/ml hypericin and irradiated with red-light with a fluence rate of 0.6 J/cm^2 to efficiently provoke apoptotic cell death. Induction of apoptosis applying this treatment regimen was determined using three independent assays: one for measurement of DNA fragmentation via evaluation of the sub-G1 area in cell cycle histograms using flow cytometry, one for the determination of caspase 3 activation and one for analysis of mitochondrial membrane integrity via staining with the fluorescent dye JC-1. Apoptosis assays revealed an apoptosis rate of 80% and a surviving cell fraction of 20%.

The intracellular accumulation of 200 ng/ml hypericin in A-431 cells after 16 h was analysed by fluorescence microscopy. For better display of the sensitizer localisation in cell compartments fluorescence microscopy was also performed in WI38 human lung fibroblasts containing a larger cytoplasmatic area than A-431 cells. These localisation studies revealed that hypericin is accumulated mainly in the ER and Golgi apparatus (Fig. 1).

To obtain a broad overview of changes in gene expression following PDT treatment of A-431 cells based on the cDNA array results, genes showing ratios of expression changes of more than twofold compared to untreated control cells were clustered to functional classes (Table II).

In total, 168 genes were found to be significantly (>2-fold) upregulated and 45 downregulated. For validation of the cDNA array results and exact quantitative determination of gene expression changes of 45 genes of interest over the time period of 1.5-8 h pt, qRT-PCR was employed. The genes of interest were those significantly differentially

Table II. Functional classes of differentially expressed genes on the macroarrays.

Functional class	No. of genes upregulated	No. of genes downregulated
Apoptosis	55	2
Oxidative stress	25	4
Cytoskeleton and cell attachment	24	12
Proliferation and cell cycle	17	9
MAPK signalling pathway	17	5
Protein transport	10	2
Energy metabolism	8	7
G-protein pathways	6	2
RNA processing	6	2

expressed genes with the highest changes or with relation to apoptosis.

Real-time PCR verification showed the following: 4 out of the 45 genes of interest were not detectable through PCR; 8 genes did not show a significant (>2-fold) alteration, although displayed the same tendency regarding up- or downregulation as found on the cDNA arrays; 8 genes were confirmed down- and 25 upregulated.

Down-regulated genes. Verification by quantitative real-time PCR of genes of interest found downregulated on the arrays revealed the expression changes listed in Table III.

Time-resolved expression profiles of downregulated genes (Fig. 2). Thrombospondin 1 showed the strongest down-regulation with a factor of 5.8 at 8 h pt, where the other downregulated genes, except for *CYP1B1*, reach the control level or have at least a tendency to normalize their expression. All other verified downregulated genes change their expression only moderately, 2- to 3-fold, at at least one of the time points pt (Fig. 2).

Downregulation as early as 1.5 h pt can be found for all genes except *ITGA3* and *RIN2*. The respective maximum downregulation for *CYP1B1, ITGA6, ADAM10* and *CANX* is at 1.5 h, for *ITGB1* and *ITGA1* at 3 h, for *RIN2* at 5 h or for *THBS1* at 8 h pt.

Cytochrome P450 1B1 is suppressed almost equally during the whole pt time. The courses of the expression kinetics of the analysed integrins, α3, α6 and ß1 show a similar tendency, with a minimum at 1.5 or 3 h pt.

Function of downregulated genes (starting with strongest downregulation)

THBS1, thrombospondin 1: this adhesive glycoprotein is secreted by most cell types, mediates cell-to-matrix interactions, influences cellular phenotype and the structure of the extracellular matrix; it inhibits angiogenesis and migration, promotes apoptosis, and serves as integrin ß1 ligand (14-16).

CYP1B1, cytochrome P450 1B1: overexpressed in tumour cells and recognized as a biomarker of tumour phenotype, it is involved in oxidative metabolism of toxic foreign compounds and plays a central role in influencing the response of established tumours to anticancer drugs; its overexpression may lead to resistance of tumour cells to many drugs (17-19).

RIN2, Ras and Rab interactor 2: binds RAB5, a small GTPase involved in membrane trafficking in the early endocytic pathway; several Rab5-binding proteins have been identified as regulators or effectors for coordination of the docking and fusion processes of endocytic vesicles (20).

ADAM10: belongs to a gene family encoding membrane proteins with a disintegrin and metalloprotease domain and mediates cleavage of E-cadherin. ADAM10-mediated E-cadherin shedding affects epithelial cell-cell adhesion as well as cell migration. Its downregulation leads to increased adhesiveness. Its overexpression in epithelial cells increases the expression of ß-catenin and enhances cell proliferation (21).

CANX, calnexin: as a member of the calnexin family of molecular chaperones it is a calcium-binding, endoplasmic

Table III. Confirmed downregulated genes; means from 3 independent treatment series; PCR reactions were run in triplicates.

Gene symbol	Gene name	Accession no.	1.5 h pt	3 h pt	5 h pt	8 h pt
THBS1	*Thrombospondin 1*	X14787	0.41	0.39	0.56	0.17
CYP1B1	*Cytochrome P450 1B1*	U03688	0.33	0.51	0.57	0.42
RIN2	*Ras and Rab interactor 2*	AL136924	0.85	0.66	0.4	0.51
ITGB1	*Integrin β1 subunit*	X07979	0.49	0.41	0.69	0.75
ITGA6	*Integrin α6*	X53586	0.42	0.52	0.69	0.84
ADAM10	*A disintegrin and metalloprotease domain 10*	AF009615	0.42	0.44	0.68	0.79
ITGA3	*Integrin α3*	M59911	0.68	0.45	0.91	1.11
CANX	*Calnexin*	M94859	0.46	0.5	0.87	1.11

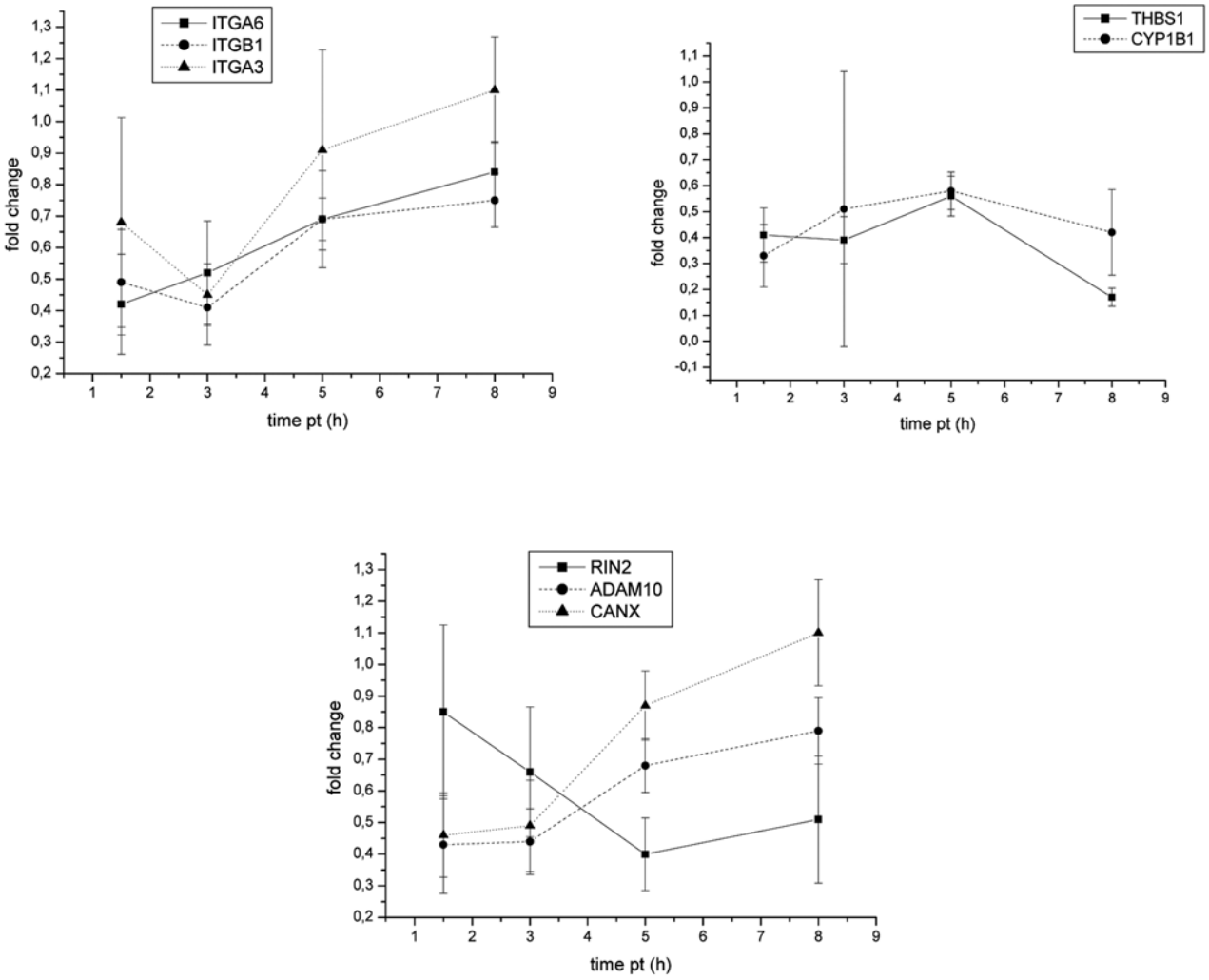

Figure 2. Kinetics of genes downregulated following hyp-PDT validated by quantitative real-time PCR. Data points represent mean values out of 3 independent PDT-treatments; each PCR reaction was run in triplicates.

reticulum (ER)-associated protein, which facilitates protein folding and assembly. It may also play a central role in the quality control of protein folding by retaining incorrectly folded protein subunits within the ER for degradation; finally it seems to suppress apoptosis independent of its chaperone functions, but dependent on its binding to Bap31 (22,23).

Integrin-family: integrins are heterodimeric integral membrane proteins composed of an α and a ß chain; they are cell surface adhesion receptors that transduce signals from extracellular matrix, promote metastasis and influences apoptosis induction via ERK and $p38^{MAPK}$ pathways. *Integrin ß1 (ITGB1), integrin α3 (ITGA3)* and *integrin α6 (ITGA6)* belong to this group (24). Thrombospondin interacts with ITGB1 (24,25), which is a multi-functional protein involved in cell-matrix adhesion, cell signalling, cellular defence, protein binding, protein heterodimerisation and receptor-mediated activity, and is highly expressed in the human body (26).

Up-regulated genes. Verification by quantitative real-time PCR of genes of interest found upregulated on the arrays revealed the expression changes listed in Table IV.

Time-resolved expression profile of upregulated genes (Fig. 3). *DUSP 1, FOSB* and *JUN, NR4A2, PPP1R15A, NEDD9, HIST1H3B, KRT17, RND3, LDLR, WEE1, INSIG1, ADM, BAG1* and *ETS2* start with a more or less pronounced elevated expression at 1.5 h pt, have their expression maximum at 3 or 5 h, respectively, and are at 8 h returning back to a level lower than at 1.5 h; in the case of the less strongly upregulated genes, to about the control level. *MXD1* follows the same course, but stays elevated at 8 h pt, similar to the level at 1.5 h. *CITED2* starts with the maximum expression at 1.5 h, and returns back to the control level at 8 h. *MT1A, DKK1, DNAJB1, MYC, PHLDA1, IRS2, PNPLA8* and *ATF4* start at or moderately above the control level at 1.5 h pt, have the maximum at 5 h and decrease again, whereas all of them are still elevated at 8 h pt.

Function of upregulated genes (starting with strongest upregulation)

DUSP1, dual specificity phosphatase 1 (MKP-1, MAPK phosphatase 1): the nuclear inducible phosphatase may be switched on by many factors such as oxidative and heat stress via the ERK (extracellular signal-regulated kinase)

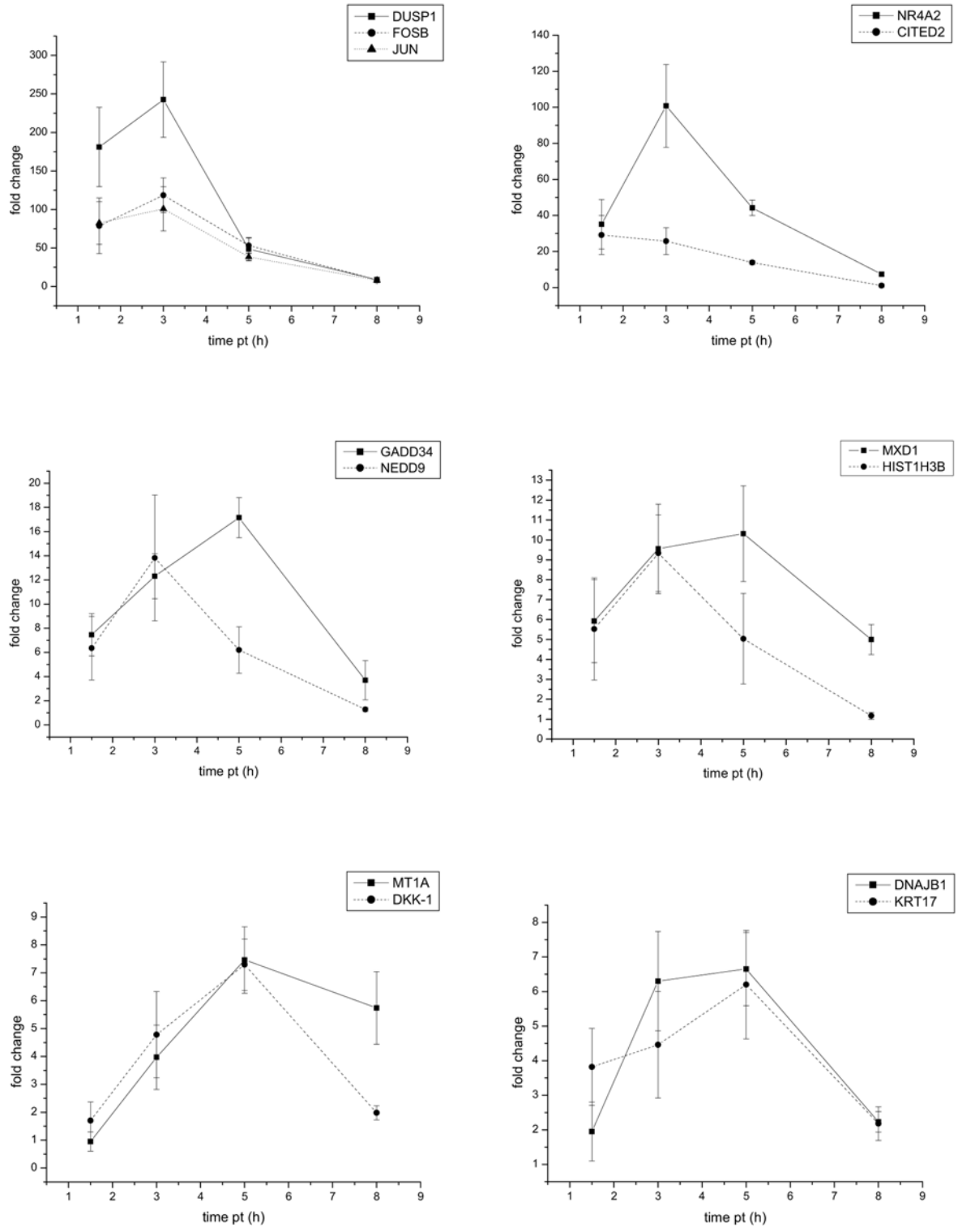

Figure 3. Part I. Kinetics of genes upregulated following hyp-PDT validated by quantitative real-time PCR. Data points represent mean values out of 3 independent PDT-treatments; each PCR reaction was run in triplicates.

or $p38^{MAPK}$ pathways being a negative regulator of ERK1/2, JNK or $p38^{MAPK}$, respectively. DUSP1 may induce apoptosis (27-30). Following exposure to stress agents various degrees of histone H3 modification at the DUSP1 chromatin occurs. It is suggested that chromatin remodelling after stress contributes to the transcriptional induction of *DUSP1* (28).

FOSB and JUN: both are immediate-early, stress response genes and proto-oncogenes. The gene products of the fos-family can dimerize with proteins of the jun-family and, together with the activating transcription factor, form the transcription factor complex AP-1, which binds to a common DNA site, the AP-1-binding site (31). AP-1 has a function

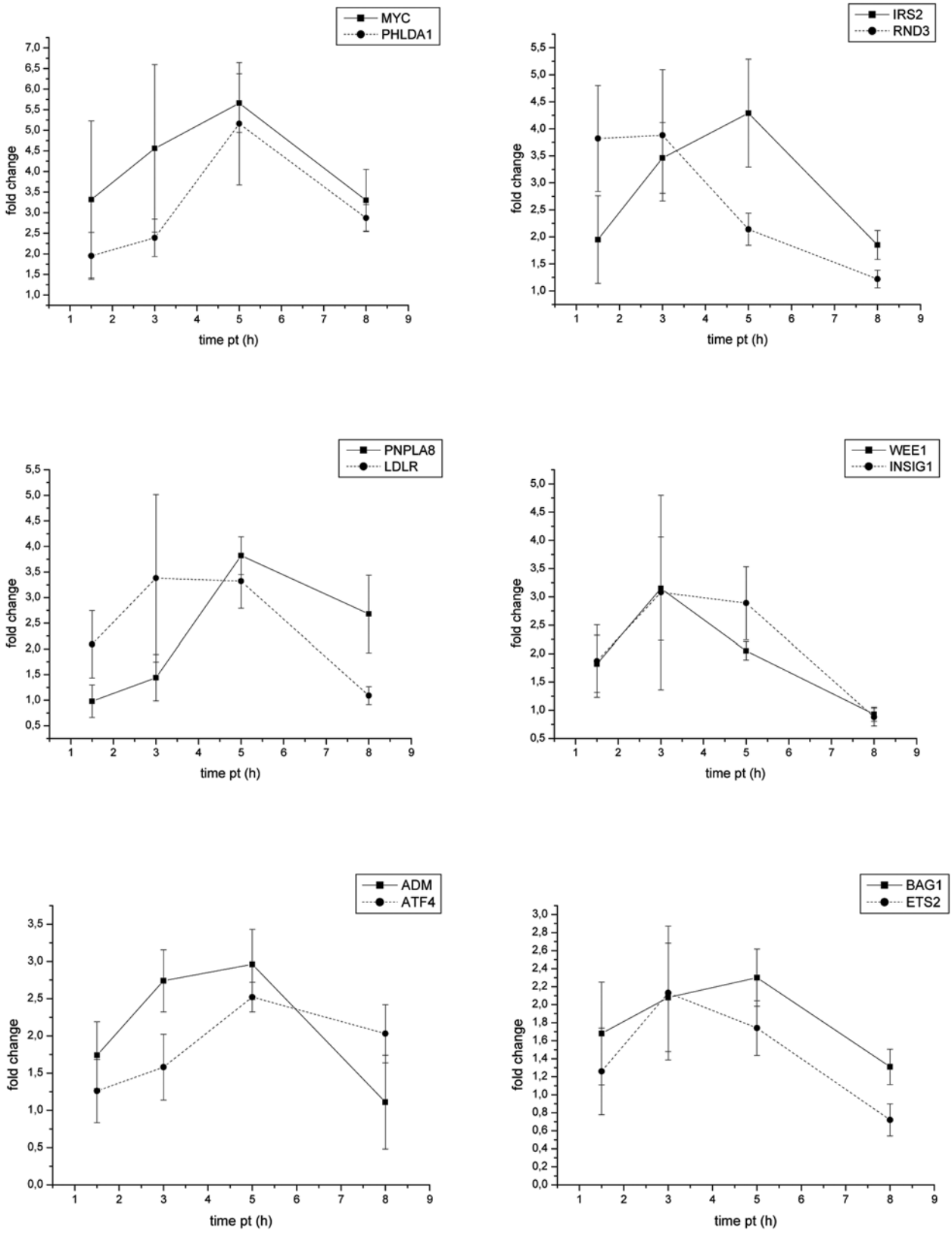

Figure 3. Part II. Kinetics of genes upregulated following hyp-PDT validated by quantitative real-time PCR. Data points represent mean values out of 3 independent PDT-treatments; each PCR reaction was run in triplicates.

in stress response, differentiation, cell proliferation and cell survival by signal transduction of growth factors in the cytoplasm to the nucleus via the MAP-kinases signalling pathway (31,32). AP-1 may modulate stress-induced apoptosis either positively or negatively, depending on the micro-environment and the cell type in which the stress stimulus is induced (32). *FOSB* is 2.46-fold upregulated by hypericin incubation alone.

Table IV. Confirmed upregulated genes; means from 3 independent treatment series; PCR reactions were run in triplicates.

Gene symbol	Gene name	Accession no.	1.5 h pt	3 h pt	5 h pt	8 h pt
DUSP1	*Dual specificity phosphatase 1 (MKP-1)*	X68277	181.02	242.56	48.5	8.91
FOSB	*FBJ murine osteosarcoma viral oncogene homolog B*	L49169	78.79	118.51	53.2	8.51
JUN	*Jun oncogene*	J04111	82.52	100.81	38.5	8.12
NR4A2	*Nuclear receptor subfamily 4, group A, member 2 (NOT)*	X75918	35.1	100.81	44.22	7.41
CITED2	*Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (MRG1, p35srj)*	AF129290	29.18	25.79	13.93	1.09
PPP1R15A	*Protein phosphatase 1, regulatory (inhibitor) subunit 15A (GADD34)*	AK001361	7.46	12.13	17.15	3.7
NEDD9	*Neural precursor cell expressed, developmentally down-regulated 9 (HEF1)*	L43821	6.35	13.82	6.21	1.28
MXD1	*MAX dimerization protein 1 (MAD1)*	L06895	5.92	9.55	10.32	5
HIST1H3B	*Histone cluster 1, H3b*	Z80784	5.53	9.33	5.04	1.17
MT1A	*Metallothionein 1A*	K01383	0.96	3.97	7.46	5.75
DKK1	*Dickkopf homolog 1*	AF177394	1.70	4.78	7.29	1.99
DNAJB1	*DnaJ (Hsp40) homolog, subfamily B, member 1*	D85429	1.95	6.3	6.65	2.23
KRT17	Keratin 17	Z19574	3.82	4.46	6.21	2.18
MYC	*v-myc myelocytomatosis viral oncogene homolog (avian)*	V00568	3.33	4.56	5.61	3.3
PHLDA1	*Pleckstrin homology-like domain, family A, member 1*	AF220656	1.95	2.39	5.16	2.87
IRS2	*Insulin receptor substrate 2*	AF073310	1.95	3.46	4.29	1.85
RND3	*Rho family GTPase 3 (RhoE)*	S82240	3.82	3.88	2.14	1.22
PNPLA8	*Patatin-like phospholipase domain containing 8 (iPLA2-gamma)*	AB041261	0.98	1.44	3.82	2.68
LDLR	*Low density lipoprotein receptor*	AF217403	2.1	3.38	3.33	1.09
WEE1	*WEE1 homolog (S. pombe)*	X62048	1.82	3.15	2.05	0.93
INSIG1	*Insulin induced gene 1*	U96876	1.87	3.08	2.9	0.88
ADM	*Adrenomedullin*	D14874	1.74	2.74	2.96	1.11
ATF4	*Activating transcription factor 4 (TAXREB67)*	D90209	1.26	1.58	2.52	2.03
BAG1	*BCL2-associated athanogene (GRAP)*	Z35491	1.68	2.08	2.3	1.31
ETS2	*v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)*	J04102	1.26	2.13	1.74	0.72

NR4A2, nuclear receptor subfamily 4, group A, member 2 (NOT): the NR4A subfamily is a transcription factor and a potential mediator of a melanocortin-1 receptor (MC1R)-coordinated DNA damage response to UV exposure in melanocytic cells. MC1R is a G-protein-coupled receptor expressed primarily in melanocytes and plays a photoprotective role in melanocytes in response to UV irradiation (33).

CITED2, cytokine inducible nuclear protein binding transcriptional coactivators p300 and CREB-binding protein (CBP) (p35srj): *CITED2* was previously known as *MRG1 (melanocyte-specific gene-related gene 1). CITED2* is inducible by varying stimuli including lipopolysaccharide, hypoxia, and cytokines such as interleukin 9 and interferon γ. It plays a major role in shear-induced down-regulation of *MMP-1* and *MMP-13* via the transforming growth factor-beta-dependent pathway (34). *CITED2* is 0.41-fold down-regulated by hypericin incubation alone.

PPP1R15A, protein phosphatase 1, regulatory (inhibitor) subunit 15A (GADD34 or FLJ10499fis): is inducible by growth arrest and DNA damage, further by oxidative stress via $p38^{MAPK}$ activation (35). GADD34 has been shown to provoke in turn growth arrest and apoptosis, and induces p53 phosphorylation and p21/WAF1 transcription. One of the functions of GADD34 is the recovery from a shutoff of protein synthesis triggered by ER stress (36).

NEDD9, neural precursor cell expressed, developmentally down-regulated 9 (human enhancer of filamentation 1 (HEF1)): belongs to the CAS protein family, localizes at focal adhesion sites, transmits various signals mainly induced by integrins, and is important for transition of 'flat' attached cells in interphase to rounded mitotic cells. NEDD9 co-ordinates invasion, apoptosis and cell cycle; its overexpression increases JNK activation and actively induces apoptosis. It serves as substrate for caspase cleavage, which leads to focal adhesion disassembly (37-39).

MXD1, MAX dimerization protein 1 (MAD1): this protein, a transcriptional repressor, competes with Myc for binding to MAX to form a sequence-specific DNA-binding complex. MAD-MAX dimers repress expression of *myc* and antagonize the function of Myc to activate transcription. It sensitizes cells to TNF-α-induced (but not Fas-induced) apoptosis, slows down proliferation and retains cells in the G1-phase (40,41). The Myc/Max/Mad network has been suggested to function as a molecular switch that regulates cell growth and differentiation by controlling a common set of genes.

HIST1H3B, histone cluster 1, H3b (H3/b): histones are basic nuclear proteins that are responsible for the nucleosome structure of the chromosomal fibre in eukaryotes. Normally, post-translational modification of histones plays an important role in many cellular processes such as gene (de)activation, mitosis, proliferation, apoptosis and differentiation. Activation of HIST1H3B induces cellular transformation through up-regulation of c-fos transcriptional activity, followed by AP-1 transactivation activity (42).

MT1A, metallothionein 1 A: as a metal ion-binding protein involved in heavy metal detoxification and scavenging free radicals MT1A is upregulated upon oxidative stress (43); its expression is regulated by AP-1 and therefore it is considered to be a downstream target of the JNK pathway (44). Additionally MT1A is involved in zinc sensitive processes (such as apoptosis) probably as a zinc donor (45). Elevation of MT1A levels protects cells against toxic levels of cadmium, heme- and iron-induced oxidation and UV light-induced apoptosis (46).

DKK1, dickkopf homolog 1: induced by Bmp-4 or strongly up-regulated by UV irradiation and several other genotoxic stimuli, DKK1 blocks Wnt/ß-catenin stress signalling leading to the activation of c-Jun *in vivo*. Normal expression of DKK1 is dependent on c-Jun; its overexpression may promote apoptosis, but also the development of several tumours (47,48).

DNAJB1, DnaJ (Hsp40) homolog, subfamily B, member 1 (HSP40): the heat-shock protein family Hsp40 plays an important role in regulation of cell proliferation, survival and apoptosis by serving as co-chaperones for HSP70s; HSP40 facilitates the ATPase activity of Hsp70, promotes the cellular protein folding and renaturation of misfolded proteins. Furthermore HSP40 may confer resistance to oxidative stress via the prevention of proteasome activity (49,50).

KRT17, keratin 17: as a major structural protein in epithelial cells responsible for the mechanical integrity it forms the cytoplasmatic network of filaments. *KRT17* is induced after disturbances in epidermal homeostasis and could serve as substrate for caspases in apoptosis (51,52). Keratin 17 can be used as a marker for squamous cell carcinoma (53).

MYC: *c-Myc* is a transcription factor for many cell cycle genes, which are active in late G1 for transition of G1 to S. It is a major apoptosis inducer by targeting the anti-apoptotic Bcl2-family, controls normal cell growth and is an immediate early growth response gene that is rapidly induced upon mitogenic stimulation of quiescent cells via the MAPK/ERK pathway (54,55). ß1 integrins regulate the expression of c-Myc through the activation of the Src family of tyrosine kinases and the MAK kinase pathway (56).

PHLDA1, pleckstrin homology-like domain, family A, member 1: it plays a role in protein translation, cell growth, cytoskeletal organization, and apoptotic cell death. It co-localizes with focal adhesion kinase, disrupts the actin cytoskeleton and thus promotes apoptosis (57). As a result of overexpression significant changes in cell morphology, decreased cell adhesion and promoted detachment-mediated apoptosis can be found (58). Persistent activation of ERK signalling by ER-stress up-regulates *PHLDA1* as a target of the ERK pathway. This event serves as a negative regulator of both ERK activation as well as matrix-detached cellular proliferation and suggests that PHLDA1 opposes ERK-mediated transformation in breast epithelial cells (59). Since *PHLDA1* was found to be down-regulated in breast cancer, it could serve as useful prognostic marker of disease outcome (60).

IRS2, insulin receptor substrate 2: a cytoplasmatic signalling molecule that mediates effects of insulin, insulin-like growth factor 1, and other cytokines by acting as a molecular adaptor between diverse receptor tyrosine kinases and downstream effectors (61); it is a negative or positive regulator of glucose transport (62,63). It cannot be excluded that ER stress has an influence on the human *IRS2* promotor via enhanced binding of SREBP-1 (Sterol regulatory element-binding protein 1) (64).

RND3, Rho family GTPase 3 (RhoE): belongs to the Rho GTPases superfamily, which regulates the organization of the actin cytoskeleton in response to extracellular growth factors. Expression of RND3 inhibits formation of actin stress fibres and integrin-based focal adhesions and induces loss of cell-substrate adhesion leading to cell rounding. It is suggested that RND3 contributes to the control of rearrangements of the actin cytoskeleton and changes in cell adhesion (65). RND3 protein levels increase upon exposure of human keratinocytes

to UVB to protect them from damage. RND3 induction counteracts UVB-induced apoptosis and may serve as a novel target for the prevention of UVB-induced photodamage regardless of the p53 status. RND3 is a pro-survival factor acting upstream of p38, JNK, p21, and cyclin D1 (66).

PNPLA8, patatin-like phospholipase domain containing 8 [intracellular membrane-associated calcium-independent phospholipase A2 gamma (iPLA2γ)]: the endogenous calcium-independent phospholipase PNPLA8 is found in ER and mitochondria (67), can be activated by ROS (68) and has an effect on the permeability transition of mitochondria and the release of intermembrane space proteins (69). It is cleaved by caspase-3 and potentiates cell death-associated arachidonic acid release (70). On the other hand, *PNPLA8* seems to be induced via MAPK pathways (71) as protection against ROS to prevent or repair oxidant-induced cell death and lipid peroxidation (67). The stimulation of PKC-ERK-p38-JNK pathways is linked with its activity (71). PNPLA8 is also involved in clearance of apoptotic cells by generating, in a caspase-3-dependent fashion, the phospholipid lyso-phosphatidylcholin (LPC) mediated attraction of monocytic cells to apoptotic cells (72).

LDLR, low density lipoprotein receptor: as a cell surface protein synthesized in the rough ER (73) it is involved in receptor-mediated endocytosis of lipoproteins, and is induced by ERK (74). $p38^{MAPK}$ inhibits LDLR expression by suppressing ERK activity (73).

WEE1, WEE1 homolog (S. pombe) (WEE1hu): it is a nuclear protein, which is a tyrosine kinase belonging to the Ser/Thr family of protein kinases. It catalyzes the inhibitory tyrosine phosphorylation of CDC2/cyclin B kinase and appears to coordinate the transition between DNA replication and mitosis by protecting the nucleus from cytoplasmically activated CDC2 kinase (75). It links the cell cycle arrest to induction of apoptosis. Overexpression of WEE-1 may suppress apoptosis (76). WEE1 is phosphorylated downstream $p42^{MAPK}$, which may either arrest or promote the G2 phase via Cdc2 (77).

INSIG1, insulin induced gene 1: as ER membrane protein it is involved in the regulation of cholesterol and fatty acid synthesis and the UPR (unfolded protein response) -ER stress response. Insulin-induced genes exert their action in the ER by binding SREBP-cleavage-activating protein (SCAP) and preventing it from escorting SREBPs to the Golgi apparatus where the SREBPs are processed to their active forms (78). Upon ER stress INSIG1 reduces SREBP activation at both the mRNA and the protein levels (79).

ADM, adrenomedullin: a vasodilatory peptide which is known to regulate cell growth, apoptosis, and migration (80). As an endogenous antioxidant it inhibits oxidative stress-induced vascular damage and counteracts oxidative stress-induced insulin resistance associated with aging (81). Markedly elevated adrenomedullin expression was found in cells stably overexpressing *c-myc*, since c-Myc transactivates the ADM gene. As a built-in feedback safety loop mechanism high c-Myc protein levels then degrade ADM mRNA (80).

ATF4, activating transcription factor 4 (TAXREB67): ATF4 transactivates the *GADD34* promotor in response to ER stress (82). Upon oxidative stress it promotes cell death and loss of glutathione (83).

BAG1, BCL2-associated athanogene (GRAP): this pro-survival molecule suppresses GADD34 mediated cellular stress response and promotes survival of cells undergoing stress (84). BAG1 interacts with BCL2 (preventing release of cytochrome C), regulating the activity of HSP70 and HSC70 (controlling formation of the apoptosome) and activating RAF-1, which induces MAP kinases. BAG1 predominantly functions to suppress apoptosis, most likely acting between the growth/survival receptors and mitochondria (85).

ETS-2 oncogene: as a transcription factor it can reverse Ras-mediated cellular transformation (86). It is induced by oxidative stress (87) and a target of the Ras/MAPK pathway (88), is proapoptotic (89) and transactivates the p53 promoter (89,90). ETS-2 overexpression results in p53-dependent apoptosis (89).

Discussion

Time-resolved expression profiles of the human squamous cell carcinoma cell line model A-431 analysed at four time-points between 1.5 and 8 h after photodynamic treatment with hypericin (200 ng/ml hypericin incubated for 16 h and irradiated with a fluence rate of 0.6 J/cm^2) using cDNA-macroarrays and qRT-PCR showed for many genes expression changes, which were most prominent at 3 and 5 h pt. Many differentially expressed genes are involved in apoptotic pathways following hypericin-PDT, which correlates with the measured effective apoptosis induction in 80% of the cells. The intracellular localization of the photosensitizer is essential for the cellular response to the treatment as it is related or even identical with the primary intracellular targets of photodynamic action. Hypericin localizes predominantly in intracellular membranes such as ER, Golgi apparatus (Fig. 1) and, under different treatment conditions, in the mitochondrial and nuclear membrane as well as in lysosomes (91). Accordingly ER stress response is an early event following hypericin-PDT leading to apoptosis, which can be executed via caspase-dependent or -independent pathways (92). A previous study of our group presented in detail the time-resolved events during the apoptosis process induced by hypericin-PDT in A-431 cells: caspase-dependent pathways are mobilized and show the highest activities of the caspases 2, 3, 6 and 9 at 5 h pt, that is when also nuclear fragmentation reaches a maximum; even ATP supply remains at a high level for 6 h in order to allow for the energy consuming apoptotic process (11).

The present study illustrates on the gene-expression level that hypericin-PDT is, depending on the applied protocol, a very efficient inducer of apoptotic cell death.

Functional gene classes with altered expression in A-431 cells. In the human squamous cell carcinoma cell model A-431 we found, based on the deployment of cDNA-arrays, significant changes in expression levels of genes involved in apoptosis induction, ROS stress response, proliferation and cell cycle, survival pathways, energy metabolism, cell attachment and cytoskeleton, protein transport, energy metabolism and RNA processing (Table II). These results are to the some extent similar to those of Buytaert *et al* (10) who detected in a bladder cancer cell model altered expression of genes, which

are involved in metabolic processes, stress-induced cell death, autophagy, proliferation, inflammation and carcinogenesis, after hypericin-PDT (10). The time-points for analysis are not directly comparable: while Buytaert *et al* (10) measured the molecular effectors and modulators of hypericin-PDT at 1 and 7 h pt via microarrays, we gained our results from four time-points pt. We found most gene expression changes to occur at 3 and 5 h pt, and at 8 h pt the tendency to return to the normal level; some of these genes did not even show significant changes at either 1.5 or 8 h pt. At 1.5 h pt only one (*CITED2*) of the qRT-PCR verified genes has reached its maximum increase.

The 33 qRT-PCR validated genes found differentially expressed in the PDT-treated skin tumour model in the present study do not include those 5 genes (*matrix metalloproteinases-1, -10, -13 precursors, HMOX1* and *AKR1C1*), which were verified in the bladder cancer cell system by Buytaert *et al* (10). The validated genes in our study can be allocated to one or more of the functional gene classes, which are coding for ROS stress response involving the ER pathway, adaptive response and antioxidant defence; for alteration of $p38^{MAPK}$, ERK1/2 or JNK survival pathways; for cell detachment, extracellular signalling, cell cycle arrest, and for cell-specific response.

These functional classes either represent the different cell states in the analysed population ranging from unaffected to dying cells or are functionally connected in one and the same cell: adaptive, antioxidant and cell-specific responses of the cells are activated as damage protection in parallel to ROS stress processing via ER and via intracellular survival pathways. Transcription factors strongly upregulated during the whole studied period may be involved in protection strategies of the cells. Changes of extracellular signalling, cell detachment processes and reduction of growth factor transmission are processed by intracellular signalling.

If all these pathways are activated in one and the same cell, this may lead to several options for the cell, be it repair or one of the cell death modes, each depending on many critical factors. The final decision between these options could be made by one or a few important crosspoints in the network (such as DUSP1, see below). The function and interaction of these genes are discussed in the following.

Extracellular signalling, cell-detachment and cytoskeleton. Integrins as receptors for extracellular matrix proteins such as fibronectin, the latter downregulated after ALA-PDT (93), mediate cell adhesion and engage in crosstalk with different growth factor receptors. Phosphorylation of these receptors may not only occur following binding of a growth ligand to the receptor but also by binding to integrins, without ligand binding. However, phosphorylation stimulates MAP kinase signalling. A similar ligand-independent mechanism was also reported for E-cadherin (cell-cell adhesion molecule) regulated EGF-receptor-mediated MAP kinase activation (94). A loss of E-cadherin expression and with that a loss of intercellular adhesion in spheroids consisting of human bladder carcinoma cells found following incubation with hypericin (95) is likely to contribute to a downregulation of the MAPK pathway, as probably does also the suppression of some integrins in the present cell line, which are *integrin ß1 (ITGB1)* (at 3 h to 0.41-fold), *integrin α3 (ITGA3)* (at 3 h to 0.45-fold) and *integrin α6 (ITGA6)* (at 1.5 h to 0.42-fold) (Table III and Fig. 2). Their significant downregulation within the first 3 h pt leads to reduced signal transduction from extracellular matrix and also to reduced cell adhesion (25) in the early phase of damage processing, all required for cell cycle stop and apoptosis. Similarly, downregulation of *integrin α2* and *ß3 precursors* were found in the bladder cancer cell model by Buytaert *et al* (10). ß1-integrins play also a role in cell detachment and apoptosis induction triggered by loss of E-cadherin following PDT with zinc (II)-phthalocyanine (96).

That thrombospondin-1 is a ligand for integrin ß1 (25) is reflected by similar expression kinetics for the pt times 1.5 to 5 h. Downregulation of *thrombospondin-1 (THBS1)* promotes cell detachment and increases cell motility mediated by calreticulin (97). The late final downregulation with a minimum expression at 8 h pt points to a longer lasting effect, which could keep the cells detached and facilitates migration during apoptosis completion (15,16). Downregulation of the *thrombospondin-1 precursor* was also found by Buytaert *et al* (10). The upregulation of the *Rho family GTPase 3 (RND3 or RhoE)* at 1.5 h (3.8-fold) and 3 h (3.9-fold) is likely to contribute to the process of cell detachment and the control of rearrangements of the actin cytoskeleton, since it inhibits integrin based focal adhesions and formation of actin stress fibres leading to cell rounding (65). RND3 antagonizes RhoA and inhibits cell cycle progression, in part by preventing translation of cyclin D1 (98). On the other hand RND3 was found to be a pro-survival factor acting upstream of p38, JNK, p21, and cyclin D1, serving as UVB protection (66).

That the overexpression of *Pleckstrin homology-like domain, family A, member 1 (PHLDA1)* between 3 and 8 h pt seems also to contribute to significant changes in cell morphology and decreased cell adhesion and is likely to be caused by ER-stress induced in the present model via the ERK pathway as a negative regulator of ERK and proliferation (59), fits the other results found in our study. The late upregulation of *PHLDA1* with a maximum at 5 h pt (5.2-fold) promotes apoptosis, either by the disruption of the actin cytoskeleton (57) or detachment of the cells (58) or both. The latter is not contradicting the present apoptosis induction by ROS, in case apoptosis signalling is boosted via a feedback loop.

However, the detachment mechanisms shown above seem to contradict the effect of downregulation of *ADAM10* at 1.5 and 3 h pt (0.42- and 0.44-fold), which results in increased adhesiveness. ADAMs (a disintegrin and metalloprotease transmembrane protein) are emerging as key modulators of cell-cell and cell-matrix interactions (99), as they have metalloprotease, integrin-binding, intracellular signalling and cell adhesion activities. In contrast to other metalloproteases, ADAMs are particularly important for cleavage-dependent activation of proteins and can bind integrins (100). The observed downregulation could be a reaction to decreased integrin expression. However, in the dying cell it would be more reasonable that ADAMs and also MMPs are upregulated following hypericin-PDT, as found in bladder cancer cells (10), unless the downregulation of *ADAM10* has another

purpose: effects such as prevention of ß-catenin expression and of proliferation (21). Loss of E-cadherin after PDT with zinc (II)-phthalocyanine triggers apoptosis via ß-catenin degradation (96). Since a similar mechanism can be assumed for hypericin-PDT, ADAM10 could play a role in this regulation.

Changes in the cell shape (cell attachment, cytoskeleton) in the course of apoptosis execution promote the formation of apoptotic bodies and assist the clearance of the bodies mainly by cells of the immune system. A special case is 'anoikis', apoptosis induced by cell detachment of anchorage-dependent cells (101). In the present study *NEDD9* or *HEF1*, localizing at focal adhesion sites, with an early starting (1.5 h pt) and longer lasting (5 h pt) upregulation (maximum at 3 h pt with a factor of 13.8) could participate in apoptosis induction as well as in its execution. Its overexpression is likely to activate JNK kinases, induce apoptosis and accelerate transition of 'flat' attached cells to rounded mitotic cells. Furthermore, it serves as substrate for caspase cleavage, which leads to focal adhesion disassembly in the course of apoptosis execution (72). The transmission of various signals by NEDD9 mainly induced by integrins is probably impaired due to integrin downregulation. Promotion of the clearance of apoptotic cells via generation, in a caspase-3-dependent fashion, of phospholipid lysophosphatidylcholin (LPC) mediating attraction of monocytic cells to apoptotic cells (72) is a function of *PNPLA8 [intracellular membrane-associated calcium-independent phospholipase A2-γ (iPLA2-γ)]*. Its late upregulation at 5 h pt (3.8-fold) and 8 h pt (2.7-fold) contributes to alterations of the cell shape as a consequence of ongoing apoptotic processes.

Due to the late downregulation of the *Ras* and *Rab interactor 2 (RIN2)* between 5 and 8 h pt docking and fusion processes of endocytic vesicles are downregulated. This may point to a function in the final apoptosis regulations (20) or in the protection against sensitizer uptake in viable cells as a late reaction. It seems very likely that lipophilic hypericin is taken up via the receptor-mediated endocytosis of lipoproteins, as was already suggested for porphyrins by Luna *et al* who found that the low density lipoprotein receptor plays a role in photosensitizer uptake (102). This interpretation is supported by the moderate upregulation of the *low density lipoprotein receptor (LDLR)* at 3 and 5 h pt (3.4- and 3.3-fold, respectively) via the ERK pathway (74). LDLR is synthesized in the rough ER (73).

Extracellular signals, cell-detachment and cell shaping processes receive or transmit their information via intracellular signalling pathways such as those of $p38^{MAPK}$ or ERK1/2 or JNK.

ROS stress affects $p38^{MAPK}$, ERK1/2, JNK and/or Ras signalling pathways and activates apoptosis. ERKs play a central role in survival by mitogenic signalling, while JNKs and $p38^{MAPK}$ are preferentially activated by environmental stresses and are actively involved in various stress responses. ERK and JNK signal to the transcription factors c-Jun and c-Fos, and $p38^{MAPK}$ to Myc and Max. The cooperation of ERK1/2 and p38 may also induce a cell cycle delay in G1 by promotion of p21(CIP1) expression (103).

The most highly upregulated gene following hypericin-PDT of our skin cell model is the nuclear inducible phosphatase *DUSP1, the dual specificity phosphatase 1 (*MKP-1*, MAPK phosphatase 1)*. Upregulation is found at all pt times, with a high starting value of 181-fold at 1.5 h and a maximum of 243-fold at 3 h which is very likely to be induced by oxidative stress transmitted either via the ERK, JNK or $p38^{MAPK}$ pathways. Being a negative regulator of ERK, JNK or $p38^{MAPK}$, DUSP1 is presumably the main switch for inactivating all these pathways, especially proliferation signalling, and inducing apoptosis. Hypericin-induced photodamage seems to specifically activate this switch. The early and long-lasting upregulation still measured at 8 h pt indicates that the presence of DUSP1 is required during the whole apoptotic program, possibly as protection against permanently incoming pro-survival signals.

It is known that DUSP1 is induced by many factors such as oxidative/heat stress (heat shock and H_2O_2) (27-30). Following exposure to stress agents various degrees of histone H3 modification at the DUSP1 chromatin may occur. It is suggested that chromatin remodelling after stress contributes to the transcriptional induction of *DUSP1* (28). We found an upregulation of *histone cluster 1, H3b (HIST1H3B or H3/b)* with the same kinetics between 1.5 and 5 h with a maximum at 3 h pt, but to a much lower degree than *DUSP1*. Histone modifications are very likely to be related to DUSP1 activation in the present case, potentially as a cause for it. Activation of HIST1H3B in turn can induce upregulation of *c-fos* transcriptional activity, followed by AP-1 transactivation activity. It can further induce many processes such as apoptosis, gene activation or deactivation (42) and this obviously still at a high level after 8 h pt. Similar results were found by Buytaert *et al* (10) with an upregulation of *HISTIH2BG* and *HIS2H2AA* after hypericin-PDT.

DUSP-1 plays also a role as link between the transcription factor E2F-1 and MAPK, mediating cellular response to oxidative stress. E2F-1 has an important function through transcriptional regulation of DUSP1 in the cell cycle but also in apoptosis as cellular response to oxidative stress (104).

Upregulation of transcription factors occurs via the ERK, JNK or $p38^{MAPK}$ pathways and is a rapid response of the cell to oxidative stress. Immediate-early or early-response genes are coding for transcription factors, which induce consequently transcription of many other genes. In our study, not all transcription factors reacted in a fast way, which suggests that they have some functions later, possibly in apoptosis execution (105).

The vast upregulation of *FOSB* and *c-jun* at all pt times with a maximum at 3 h (*FOSB*: 119-fold, *c-jun*: 101-fold) forms a high amount of the transcription factor complex AP-1 (31,32) in response to PDT stress. AP-1 is able to modulate stress-induced apoptosis positively or negatively by signal transduction of growth factors in the cytoplasm to the nucleus via the MAP-kinases signalling pathway. It is very likely that AP-1 is also an important switch between survival and apoptosis in the present cell model, monitoring the amount of ROS stress and deciding the final fate of the cell. However, under the present conditions, support of apoptosis seems to be the primary goal (49,50) and this happens very rapidly and is lasting at least up to 8 h, when apoptosis execution processes in A-431 cells are in their final stages (11).

Jun plays a prominent role in mitochondrial apoptotic pathways by the transcription of proapoptotic proteins. JNK, acting upstream of c-Jun, is involved in extrinsic and intrinsic pathways in apoptosis by stimulating the activities of proapoptotic proteins, especially those of the BH3-only family, and by inhibiting anti-apoptotic proteins such as Bcls (105). c-Jun is also a factor for the expression of DKK1. The upregulation of *DKK1 (Dickkopf-1)* at 3-5 h with the maximum value at 5 h pt (7.3-fold) in the present study is possibly dependent on the upregulation of c-Jun. In response to stress signals such as ROS, DKK1 blocks Wnt/ß-catenin signalling, a pathway which regulates e.g. cell morphology and proliferation, leading to the activation of c-Jun *in vivo*. Abrogation of the Wnt/ß-catenin signalling pathway could support a cell cycle stop. *DKK1* upregulation can be connected with apoptosis but also with tumour promotion (47,48).

The up-regulation of *FOSB* and *c-jun* transcriptional activity was already observed following PDT. While we found also a very high upregulation of *c-fos* and AP-1 after ALA-PDT inducing cell death with an approximately 50% necrotic cell fraction (93), a less pronounced upregulation of *c-jun* and *fos* after hypericin-PDT as measured in the present study, was reported by Buytaert *et al* (10). In response to Photofrin-induced ROS-mediated photodamage, AP-1, multiple protein kinase cascades such as $p38^{MAPK}$ signalling and also survival pathways inducing the prosurvival COX-2 (cyclooxygenase-2) were involved (106). Saito *et al* demonstrated that $p38^{MAPK}$ activation leads to an upregulation of COX-2 also following hypericin-PDT (20).

Additional transcription factors found with a function in hypericin-PDT are MXD1, MYC and ETS-2. The upregulation of *MAX dimerization protein 1 (MXD1 or MAD)* at all pt times with a maximum at 5 h (10.3-fold) demonstrates an early as well as a long lasting response. MXD1 competes with MYC for binding to MAX to form a sequence-specific DNA-binding complex. Higher susceptibility of cells to TNF-α-induced apoptosis, slowing down of proliferation and G1-cycle stop (40,41) is the result. *c-myc* was found to be also upregulated at all pt times with a maximum at 5 h pt (5.6-fold), which is late for an immediate-early-gene. The increased expression could indicate apoptosis induction via the MAPK/ERK pathway (54,56). Myc has several functions: it supports the progression of the cell cycle, induces apoptosis, is an angiogenic switch and decides about terminal cell differentiation (54). In contrast to the results found in the present study we observed previously a downregulation of c-myc following ALA-PDT, where cells died primarily via necrosis (93).

Oxidative stress also activates the Ras/MAPK pathway, which leads to a moderate upregulation of the transcription factor and pro-apoptotic oncogene *ETS-2* at 3 h pt (2.1-fold) *ETS-2* overexpression results in p53-dependent apoptosis (86,87,89), which is playing a minor role, if any, in the present case.

Finally, cell cycle arrest is promoted by the upregulation of the nuclear protein and tyrosine kinase *WEE1* or *WEE1hu* at 3 h pt (3.1-fold). It catalyzes the inhibitory tyrosine phosphorylation of CDC2/cyclin B kinase and protects the nucleus from cytoplasmically activated CDC2 kinase (75), arrests cells in G2 and connects the cell cycle arrest to induction of apoptosis (76). WEE1 is phosphorylated downstream of $p42^{MAPK}$ (77).

ROS affect ER-stress pathways and activate adaptive response or apoptosis. Disruption of ER homeostasis e.g,. by ROS causes accumulation of unfolded and misfolded proteins in the ER, triggering the ER stress response (107). Upon disruption of calcium homeostasis by inhibiting the ER transmembrane SERCA (sarcoplasmic/ER Ca^{2+}-ATPase) calcium pump by ER stress stimuli, several immediate-early genes, including *c-myc, c-jun, egr-1 (early growth response factor-1)*, and *fra-1 (fos-related antigen-1)*, display PERK [eIF2α (eukaryotic initiation factor-2α) kinase]-dependent expression. PERK is normally activated during the ER-stress response to protect cells from ER stress. Also activation of JNK and $p38^{MAPK}$ upon certain stimuli is PERK-dependent (108). Hypericin-PDT has the consequence that ER stress triggers cellular rescue pathways as well as apoptosis via SERCA2 photodamage and loss of ER Ca^{2+} homeostasis (92).

The upregulation of *heat shock protein 40 (DNAJB1 or HSP40)* between 3 and 5 h pt with a maximum at 5 h (6.6-fold) in the present study is likely to be a reaction to increased ER stress response mediated by the key molecular chaperone protein Grp78/BiP. The induction of Grp78 by ER stress enhances the folding capacity of the ER and promotes recovery from ER stress. ER stress may induce apoptosis unless increasing adaptive mechanisms through enhancement of ER stress-signalling pathways restore ER homeostasis and prevent apoptosis (107). Again ERK and p38 pathways are involved in upregulating *GRP78* gene transcription (109). HSP40, which binds HSP70 as a co-chaperone, is likely to be upregulated in the present study to confer resistance to oxidative stress via the prevention of proteasome activity concerning HSP70, by promoting cellular protein folding and renaturation of misfolded proteins (93). *HSP40* (*DNAJB9*) and *HSP70* were also found to be highly upregulated as an adaptive cellular response in the study of Buytaert *et al* (10).

The upregulation of the ER membrane protein *INSIG1 (insulin induced protein 1)* at 3 and 5 h pt (3.1- and 2.9-fold) is also related to the UPR-ER stress response (78). Upon ER stress INSIG1 reduces SREBP (sterol regulatory element-binding protein) activation at both, the mRNA and the protein levels (79).

Also acting in the ER stress pathway is the *activating transcription factor 4 (TAXREB67* or *ATF4)* (82), which we found moderately upregulated between 5 h (2.5-fold) and 8 h pt (2.0-fold). A similar upregulation at 7 h pt was found by Buytaert *et al* (10). ATF4 increases the expression of *GRP78* as well as of *GADD34* (the gene of *protein phosphatase 1, regulatory (inhibitor) subunit 15A or PPP1R15A or FLJ10499fis)* (83). The latter was actually found to be highly upregulated at all pt times (maximum at 5 h pt with 17.1-fold) very likely by growth arrest and oxidative stress via $p38^{MAPK}$ activation. GADD34 can induce both, apoptosis as well as recovery from a shutoff of protein synthesis induced by ER stress and was also found highly upregulated by Buytaert *et al* (10). As an antagonist, BAG1 [*BCL2-associated athanogene (GRAP)*], moderately upregulated at 5 h (2.3-fold), suppresses GADD34 mediated stress response in contrast to ATF4, and

promotes survival of cells undergoing stress (84). By interacting with BCL2 it regulates the activity of HSP70 and HSC70 and activates RAF-1, which induces MAP kinases (signalling pathway). BAG1 predominantly suppresses apoptosis, most likely acting between the growth/survival receptors and mitochondria (85) and offers protection against MAPK-signalling induced apoptosis.

It seems that increased ER stress also influences the human IRS2 promotor via enhanced binding of SREBP-1 (64) as a moderate upregulation of the insulin receptor substrate 2 (IRS2) between 3 h (3.5-fold) and 5 h pt (4.3-fold) was found. Upregulation could be additionally correlated with changes in the glucose transport (62,63) and has, however, a protective effect against apoptosis. IRS2 and INSIG1 are common targets of a transactivator of metabolic genes (110).

An opposite reaction to adaptive response and repair of misfolded proteins represents the downregulation of *calnexin (CANX)* at 1.5 h pt to 0.46-fold resulting in the prevention of protein folding and assembly in the ER; incorrectly folded protein subunits cannot be kept any longer within the ER for degradation, and apoptosis is promoted (22,23).

General and cell-type specific damage protection. Beside pro-survival signalling and adaptive response to ER stress, damage protection mechanisms are activated. For protection against oxidants such as ROS or hypoxia could serve the upregulation of *MTA1, PNPLA8 and ADM*, while the down-regulation of *CYP1B1* would counteract this.

The upregulation of the metal ion-binding protein *metallothionein-I-A (MT1A)* between 3 and 8 h pt, with a maximum at 5 h (factor 7.5), seems to serve for detoxification and scavenging free radicals (43). Regulated by increased AP-1 and as a downstream target of the JNK pathway (44) it could be involved in protection against toxic levels of heme- and iron-induced oxidation and photodamage-induced apoptosis (46). Moderately upregulated *metallothionein-1X* found by Buytaert *et al* (10) was discussed to play a role as antioxidant.

Intracellular membrane-associated calcium-independent *phospholipase A2-γ (PNPLA8 or iPLA2-γ)* is lately upregulated at 5 and 8 h pt (3.8- and 2.7-fold) via MAPK pathways and its activity is linked with the stimulation of PKC-ERK-p38-JNK pathways (71). Induced by ROS in ER and mitochondria (67-69), possibly as protection against ROS to prevent or repair oxidant-induced cell death and lipid peroxidation (67), it has an effect on the permeability transition of mitochondria and the release of intermembrane space proteins (69). It is cleaved by caspase-3 and potentiates cell death-associated arachidonic acid release (70).

Also induced by oxidative stress, *adrenomedullin (ADM)* is moderately upregulated at 5 h pt (3.0-fold). As a vasodilatory peptide and an endogenous antioxidant inhibiting oxidative stress-induced vascular damage and counteracting oxidative stress-induced insulin resistance associated with aging (80), it contributes to apoptosis and migration. Since c-myc is interrelated with ADM, an overexpression of both is very likely (81) and is found in the present study.

In contrast to this, downregulation of cytochrome *P450 1B1 (CYP1B1)*, at 1.5 h pt to 0.33-fold and at 8 h to 0.42-fold leads to reduction of antioxidant defence, which could support apoptosis. Since, antioxidant agents such as resveratrol (found in grapes) can inhibit *CYP1B1* (111), a similar mechanisms could act when irradiated hypericin is present.

Cell-specific damage protection against ROS is very likely to be provoked by the upregulation of *NOT* and *CITED2*. It can be assumed that the pronounced upregulation of the *nuclear receptor subfamily 4, group A, member 2 (NR4A2* or *NOT)* at all pt times, but mainly at 3 h pt with a factor of 101, is involved in DNA damage response as typically induced by UV exposure in melanocytes (33). This is remarkable in view of the fact that although red-light activated hypericin in our melanocytic cell model is capable of ROS production similar to UV, the photosensitizer does not enter the nucleus. Localisation can only be observed to some extent in the nuclear membranes, from where the generated singlet oxygen could diffuse to the adjacent DNA in a very restricted radius. According to this DNA is likely to be only minimally targeted by hypericin-PDT.

The high upregulation of *cytokine inducible nuclear protein binding transcriptional coactivators p300* and *CREB-binding protein (CBP) (CITED2* or *p35srj)* in A-431 cells between 1.5 and 5 h pt, but with a maximum already at 1.5 h (29-fold) is not surprising, since it is the *melanocyte-specific gene-related gene 1*. As it can be induced by hypoxia and cytokines as an early response, it is conceivable that it is also induced by ROS. It could effect the down-regulation of *MMP-1* and *MMP-13* via a transforming growth factor-ß-dependent pathway (34). This is in contrast to the strong upregulation of *MMP-1* and *MMP-13* found in the study of Buytaert *et al* (10) and could indicate that the A-431 cells are protected against ECM remodelling and the degradation of different components of the extracellular matrix by metalloproteases. Remarkably, *CITED2* is 0.41-fold down-regulated following incubation with hypericin without irradiation what could point to a protective effect of the plant constituent itself, but with the consequence that this treatment modality rather promotes cell motility.

Keratin 17 (KRT17) is a marker for squamous cell carcinoma (52). Its cell-specific upregulation in A-431 cells at 5 h pt for a factor of 6.2 could serve as relatively late response in protection against cell detachment and apoptosis. KRT17 as a major structural protein in epithelial cells is probably upregulated after disturbances in the epidermal homeostasis to increase the cytoplasmatic network of filaments and mechanical integrity and counteract the decomposition by caspases in apoptosis (50). *RND3* upregulation serves as protection of human keratinocytes against light-induced ROS such as found with UVB. It counteracts ROS-induced apoptosis and serves as a target for the prevention of ROS-induced photodamage regardless of the p53 status (66).

In can be concluded that the genes showing altered expression levels between 1.5 and 8 h following prevalent apoptosis induction by ROS generated by hypericin-PDT are functionally connected: antioxidant response, adaptive mechanisms and cell-specific damage protection against ROS, mainly by NOT and CITED2, are activated in A-431 cells as protection in parallel to ROS stress processing via ER and to signalling via survival pathways; the latter with dual functions: transmission of survival or cell death signals. It can be hypothesised that primarily survival signals are transmitted to protect the cell until one or several key points

in the network of signals detect e.g. unrepairable damage and turn on pathways finally executing apoptosis. Switches between life and death could be the massive and permanent (at least until 8 h pt) upregulation of the transcription factors *c-jun* and *FOSB* as well as of the MAPK-phosphatase 1 *DUSP-1*, possibly activated by H3 histone modifications. In the course of the apoptosis process, changes in the cell shape occur. The intracellular apoptosis signalling is accompanied by changes of extracellular signalling and cell detachment processes.

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