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Modulation of stress genes expression profile by nitric oxide-releasing aspirin in Jurkat T leukemia cells

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ABSTRACT

NO-donating aspirin (NO-ASA, *para* isomer) has been reported to exhibit strong growth inhibitory effect in Jurkat T-acute lymphoblastic leukemia (T-ALL) cells mediated in part by β-catenin degradation and caspase activation, but the mechanism(s) still remains unclear. In this study, DNA oligoarrays with 263 genes were used to examine the gene expression profiles relating to stress and drug metabolism, and characterize the stress responses at IC_{50} and subIC_{50} concentrations of *p*-NO-ASA (20 and 10 μM, respectively) in Jurkat T cells. A total of 22 genes related to heat shock response, apoptosis signaling, detoxifiers and Phase II enzymes, and regulators of cell growth were altered in expression by array analysis based on the expression fold change criteria of ≥1.5-fold or ≤0.65-fold. Real time quantitative RT-PCR confirmed that 20 μM *p*-NO-ASA strongly upregulated the mRNA levels of two heat shock genes *HSPA1A* (41.5 ± 7.01-fold) and *HSPA6* (100.4 ± 8.11-fold), and *FOS* (16.2 ± 3.2-fold), moderately upregulated *HSPH1* (1.71 ± 0.43-fold), *FMO4* (4.5 ± 1.67-fold), *CASP9* (1.77 ± 0.03-fold), *DDIT3* (5.6 ± 0.51-fold), and downregulated *NF-κB1* (0.54 ± 0.01-fold) and *CCND1* (0.69 ± 0.06-fold). Protein levels of Hsp70, the product of *HSPA1A*, and fos were increased in *p*-NO-ASA-treated Jurkat T and HT-29 colon cancer cells in a dose-dependent manner. Silencing of Hsp70 enhanced the growth inhibitory effect of *p*-NO-ASA at low concentrations. The altered gene expression patterns by NO-ASA in Jurkat T cells suggest mechanisms for carcinogen metabolism, anti-proliferative activity and possible chemoprotective activity in T-ALL.

1. Introduction

T cell acute lymphoblastic leukemia (T-ALL) is one of the primary causes of cancer-related mortality in children [1]. The Wnt/β-catenin/TCF signaling pathway is implicated in T-ALL and in a number of hematological malignancies including acute myelogenous leukemia, chronic myelogenous leukemia, multiple myeloma and B-cell chronic lymphocytic leukemia [2–4]. The protein β-catenin is abnormally expressed in Jurkat T cells, which is a T-ALL cell line, and regulates Jurkat cell proliferation and survival [2]. Aberrant activation of Wnt/β-catenin signaling and its downstream targets are also intimately linked with several other types of cancers such as colon, breast, and prostate [5–7]. For these cancer types, novel therapeutic and/or chemopreventive approaches need to be developed based on appropriate molecular targets of growth and proliferation specific to these cell types. While targeting the Wnt/β-catenin pathway is a rational design for anticancer agents, the cellular responses such as drug metabolism, detoxification of carcinogens, stress response induction may enhance or inhibit the anticancer potential of such agents. Therefore it is important to closely monitor the differential gene expression profile relating to cell death, cellular detoxification, stress and cell survival mediated by potential therapeutic agents that modulate the β-catenin pathway in these cancer cell types.

Nitric oxide-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs) consist of a traditional NSAID to which a group donating NO has been covalently attached via an aromatic or aliphatic spacer (Fig. 1A) [8]. There is evidence that NO-NSAIDs are substantially superior compared to their NSAID counterparts because these compounds combine the chemopreventive properties of traditional NSAIDs against cancer with enhanced safety, efficacy and potency [8,9]. Among the NO-NSAIDs, the *para* isomer

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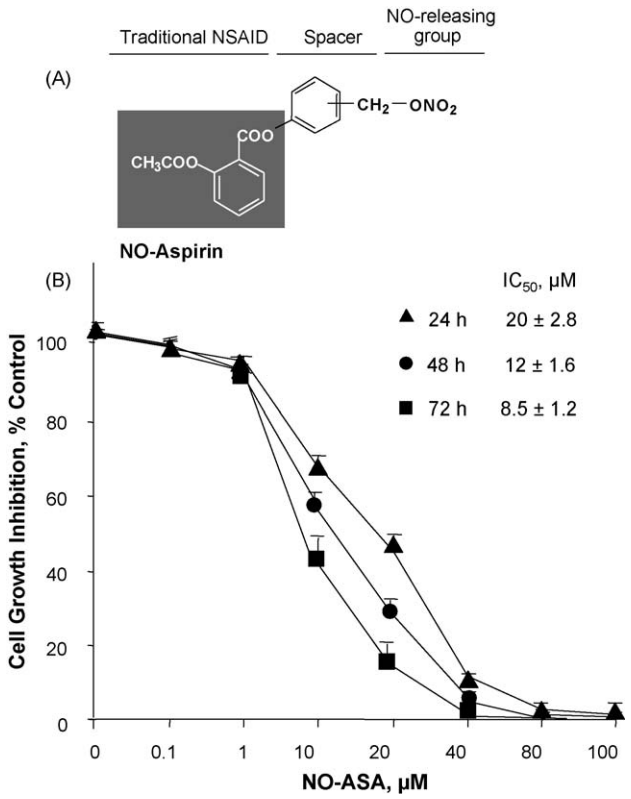

Fig. 1. (A) NO-donating aspirin and its structural components. (B) Cell growth inhibition of Jurkat T cells by *p*-NO-ASA. Cells were treated with various concentrations of *p*-NO-ASA, cell viability was determined at 24, 48, and 72 h from which IC_{50} for cell growth inhibition were determined. Results represent means ± SD of three different experiments performed in triplicate.

of NO-donating aspirin (*p*-NO-ASA) was demonstrated to be the most potent with respect to anticancer-related biological processes, such as induction of apoptosis and inhibition of cellular proliferation mainly in colon, pancreatic, and breast cancer cultured cells [10,11]. It has also exhibited a strong potential as a chemopreventive agent in various animal models [12–14]. Among the molecular targets at the mechanistic level, Wnt/β-catenin/TCF-4 pathway was found to be modulated by NO-ASA [15–17].

One of our previous studies demonstrated that NO-ASA inhibited the growth of Jurkat T leukemia cells, attributed, in part, to cleavage of β-catenin and activation of caspases as shown by treatment with inhibitors of caspase-3, -8, and -9, and partial reversal of growth inhibition by z-VAD-fmk, a pan-caspase inhibitor, suggesting that NO-ASA may have therapeutic potential in T-ALL [17]. While the caspases and the β-catenin pathway may have its implications in growth inhibition and apoptosis in Jurkat cells, other underlying mechanisms need to be explored to fully understand the strong growth inhibitory effect of NO-ASA in T-ALL. NO-ASA exerts pleotropic effects on several targets and pathways in colon and pancreatic cancer cell lines affecting the Wnt/β-catenin, NF-κB, iNOS, COX-2, MAPK, PPAR, drug metabolizing enzymes, reactive oxygen species, and pro- and anti-inflammatory cytokines (reviewed in [18]). In addition, the role of NO-donating moiety of NO-ASA on Jurkat T cell expression profile may be important since NO can regulate T cell proliferation, apoptosis and signaling activity *in vivo* and *in vitro* [19]. Therefore studies on the modulation of gene expression profiles by *p*-NO-ASA in Jurkat T leukemia cells may provide new insight into genes that may be induced or repressed. For example, differential gene expression profiles of ALL cells by NO-ASA obtained by gene array analysis can provide important mechanistic information regarding its effect on genes such as regulators of cell proliferation, cell renewal, survival, apoptosis, and chemoprotection genes coding for stress proteins and carcinogen metabolism enzymes. In addition, it may provide novel molecular targets in these cells that can be manipulated pharmacologically by NO-ASA. This is the first report investigating the differential gene expression profile of human Jurkat T leukemia cells in response to *p*-NO-ASA by gene array analysis. Our results demonstrated altered mRNA expression of several *p*-NO-ASA-regulated genes including induction of heat shock proteins. Silencing of Hsp70 was examined for potentiation or reversal of effects in response to *p*-NO-ASA. These studies provide better understanding of the therapeutic potential of *p*-NO-ASA in T-ALL.

2. Materials and methods

2.1. Cell culture and reagents

NO-ASA, *para* isomer, [2-(acetyloxy)benzoic acid 4-(nitrooxy methyl)phenyl ester]; and the *meta* isomer, [2-(acetyloxy)benzoic acid 3-(nitrooxy methyl)phenyl ester] were synthesized and purified by us according to previously published methods [20]. Stock solutions (100 mM) were made in DMSO; final DMSO concentration was adjusted to 1% in all media. The human colon adenocarcinoma cell line HT-29 (ATCC HTB38, Manassas, VA) was cultured in McCoy's 5A medium, the human colon adenocarcinoma cell line SW480 (ATCC CCL-228, Manassas, VA) was grown in RPMI 1640 medium supplemented with GlutaMAX (Invitrogen, Carlsbad, CA) and Jurkat T cells, which are a human T-acute lymphoblastic leukemia cells (ATCC TIB-152), were grown as a suspension culture in RPMI 1640 medium; all medium were supplemented with 10% fetal bovine serum and antibiotics (1% penicillin/streptomycin (v/v)) according to laboratory standard culture conditions. Jurkat cells were maintained at 10^5 cells/mL and treatment with different concentrations of *p*-NO-ASA was performed with a cell density of 0.5×10^6 cells/mL.

2.2. Cell growth inhibition assay

Dimethyl thiazol-diphenyltetrazolium bromide (MTT) was used to measure the growth inhibitory effect of NO-ASA on Jurkat cells using the MTT kit from Roche (Indianapolis, IN). This assay is based on the conversion of MTT, a soluble tetrazolium salt into insoluble formazan by mitochondrial dehydrogenase enzymes in the viable cells. Cells seeded at a density of 10,000 cells/well in a 96-well plate were incubated with various concentrations of *p*-NO-ASA and viable cells were quantified with MTT substrate according to the manufacturer's instructions. Relative cell growth was compared with the corresponding control cells. Growth inhibition was expressed as percentage of the corresponding control. All experiments were conducted using triplicate determinations per plate and each assay was repeated at least three times. The concentrations at which 50% of the cells were inhibited (IC50) were determined. These concentrations were taken as the basis for choosing the subtoxic doses for the gene expression analysis.

2.3. Arrays, RNA extraction, hybridization and data analysis

The Oligo GEArray Human Toxicology & Drug Resistance Microarray (SUPERArray, currently SABiosciences, Frederick, MD) spotted with 263 genes related to the metabolic processes of cell stress, cell toxicity, drug resistance and drug metabolism, and the Human Cancer Pathway Finder Array spotted with 96 genes on nylon membranes were used. The list of all the genes that were spotted on the oligoarrays is listed in Appendices 1 and 2. Total RNA was isolated from the cells using SUPERArray RNA

isolation and purification column. RNA was quantitated with NanoDrop spectrophotometer, and RNA quality analyzed using Agilent Bioanalyzer with RNA NanoChip 6000. The RNA samples (2 μg) were enzymatically converted into a biotinylated cRNA target probes with the TrueLabeling-AMP™ 2.0 Kit. The labeled target was hybridized to the microarrays in a standard hybridization oven. Side-by-side hybridizations were performed for two sets of treated cells and vehicle-treated cells. Microarray data were collected at Superarray Biosciences. Detection was performed with Chemiluminescent Detection Kit reagents and a Chemi-Doc imaging system (CCD camera). Microarray intensity data were extracted from the TIFF image using Agilent Feature Extraction Software 9.1.3.

The changes in gene expression in the arrays were detected using minimum value background subtraction and interquartile normalization (between the 25% and 75% quartile) of the spots. Minimum value from the lowest density spot on the array and the average value across the spot was used as the background correction value, and was subtracted from the intensity value for each spot on the array. A spot was considered "absent" if the average density of the spot was less than 1.5× of the mean value of the local backgrounds of the lower 75 percentile of all non-bleeding spots indicating a low signal to noise ratio. Furthermore, a quality control (QC) filter was used to remove questionable array features. A spot shape that deviated greatly from a circle where the ratio of area to circumference deviated by more than 20% from the value for an ideal circle were flagged and eliminated from further consideration. This approach prevented misidentification of the spot boundaries. After interquartile normalization, fold changes in expression for each gene were obtained relative to baseline control. For our studies, a cut-off value fold for determining altered gene expression for either up- or downregulation was 1.5-fold and 0.65-fold, respectively.

2.4. Quantification of mRNA levels by real time quantitative RT-PCR

Validation experiments on candidate genes that were induced or repressed were performed by real time quantitative RT-PCR analysis by reverse transcription from the RNA samples used for the gene array and for separate biological RNA samples using the SABioscience RT2 First Strand Kit. qRT-PCR was performed with SYBR Green/Fluorescein PCR Master Mix in an iCycler (Bio-Rad) in 96-well format in a volume of 24 μL per reaction. Specific primer sets for the candidate genes were purchased from SABiosciences (proprietary primers, sequence not disclosed) and the procedures were carried out at SABiosciences. Data analysis was performed via Excel using the Ct value which is the calculated cycle number where the fluorescence signal emitted was significantly above background levels. Relative gene expression was obtained by normalization to GAPDH expression first and then comparing the test sample against the control sample for the same gene. The fold increase or decrease was measured relative in triplicate to time-matched vehicle-treated controls and calculated after adjusting for GAPDH using the formula $2^{-\Delta\Delta Ct}$ where ΔCt = target gene Ct − GAPDH Ct, and ΔΔCt = ΔCt control − ΔCt treatment. Changes in gene expression were illustrated as a fold increase/decrease.

2.5. Western blot analysis

After treatment with *p*-NO-ASA or vehicle, cells were harvested and lysed in 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 0.5% IGEPAL and 10% glycerol in the presence of protease inhibitors. Proteins were fractionated by SDS-PAGE and transferred to supported nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Primary mouse monoclonal antibody against Hsp70 (HSP 70 (3A3) and c-Fos (8B5)) (Santa Cruz Biotech, CA) was used at 1:2000 dilution. Immunoreactive protein was detected using chemiluminescence substrates (Pierce Chemicals, Rockford, IL).

2.6. RNA interference and cell viability assay

siRNA against human HSPA1A (target sequence 1: TGCCATCT-TACGACTATTTCT; sequence 2: TACACTTAACTCAGGCCATTT; sequence 3: TACTGCCATCTTACGACTATT; sequence 4: TTCTA-CATGCAGAGATGAATT) and a scrambled non-target siRNA as control was ordered from SABiosciences (Frederick, MD). SW480 cells were reversed transfected in a 96-well plate at 10,000 cells/well with siRNA (10 nM) and control at the same concentration. Knockdown efficiency was confirmed by extraction of RNA from the transfected cells using SV96 system (Promega), reverse transcribing with RT^2 HT First Strand Kit (SABiosciences), and quantitative measurement by real time PCR using the comparative Ct method. Compared with the control, the HSPA1A mRNA levels were reduced by 70–80% for each of the four target sequences (data not shown). To examine the effect of silencing on NO-ASA-mediated growth inhibition, SW480 cells were transfected with siRNAs, and after 48 h the cells were treated with different concentrations of NO-ASA as indicated (10, 20, 40 and 80 μM) or with DMSO as control, for 24 h. Cell Titer-Blue Cell Viability Assay (Promega) was performed according to the manufacture's instruction to measure the combined growth inhibitory effect of HSPA1A RNA interference and NO-ASA treatment. Fluorescence signal was read with EnVision plate reader (PerkinElmer). Relative cell growth was compared with the corresponding control cells.

2.7. Biostatistical analysis

All experiments were repeated at least three times and the results are presented as the means of the three separate experiments. All data are described as (mean ± SD). Two-tailed Student's *t*-tests were used to assess differences between treated and control groups. Differences with *p*-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Low concentrations of NO-ASA inhibit the growth of Jurkat T cells

Our earlier reports have demonstrated the growth inhibiting potential of the *para*, *meta*, and *ortho* positional isomers of NO-donating aspirin in Jurkat T cells [17]. For further mechanistic studies in this investigation, the *para* isomer (*p*-NO-ASA) and its effects on cell growth was chosen primarily because it was the most potent isomer in inhibiting the growth of Jurkat cells [17]. We examined the effect of *p*-NO-ASA on Jurkat T cell growth by MTT assay for different time intervals. *p*-NO-ASA strongly inhibited cell growth in a concentration- and time-dependant manner. The IC_{50} of *p*-NO-ASA for Jurkat cell growth inhibition was determined to be 20 ± 2.8, 12 ± 1.6, and 8.5 ± 1.2 μM at 24, 48, and 72 h, respectively (Fig. 1B). At lower concentrations of *p*-NO-ASA, 1 and 10 μM, the growth inhibition was approximately 9% and 31%, respectively at 24 h. For further studies, we focused on two concentrations of NO-ASA, namely, 20 μM being the IC_{50} for cell growth inhibition and 10 μM being half the IC_{50} which may be considered a subtoxic dose.

3.2. NO-ASA modulates expression of genes related to stress response and carcinogen metabolism

The expression profile of genes altered by *p*-NO-ASA was determined for Jurkat T leukemia cells treated with 10 or 20 μM *p*-

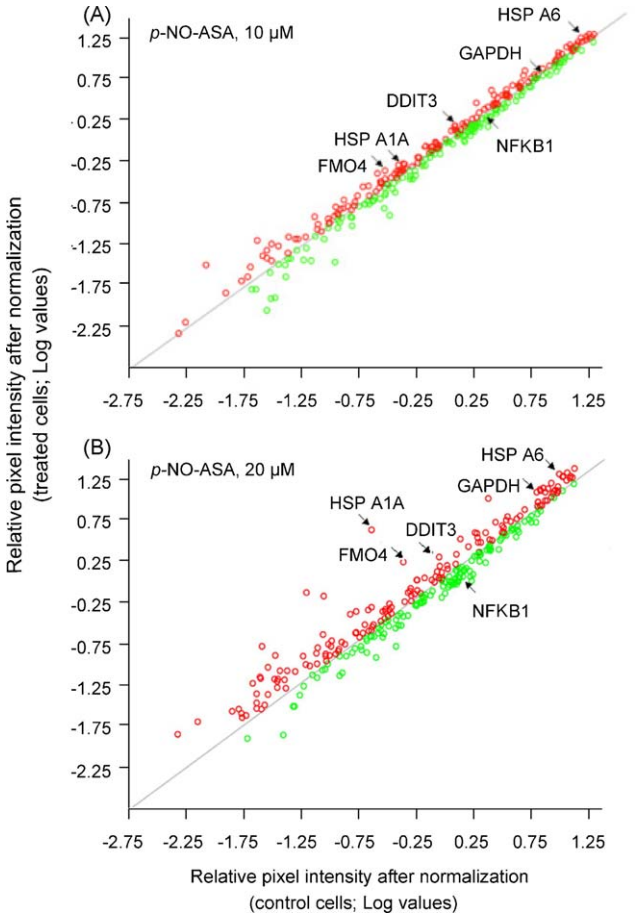

Fig. 2. Scatter plots for modulated genes in *p*-NO-ASA-treated Jurkat T cells compared with untreated cells.

NO-ASA for 24 h and compared to a vehicle-treated control. The two different concentrations were studied in order to obtain, if possible, a dose-dependent response of the gene expression. Representative scatter plots of signal intensities from one set of hybridizations belonging to the stress and drug toxicity oligoarray show the expression of normalized signals from cells treated with 10 or 20 μM *p*-NO-ASA versus the untreated cells (Fig. 2). Some genes of interest whose signal intensities were altered reproducibly are highlighted with arrows. The normalized data were grouped on the basis of major gene functions. Those genes with signal intensities that fit the criteria described in Section 2 were further analyzed, while those genes which were 'absent calls' in all three conditions, implying poorly detectable expression in this cell line, were discarded from further analysis. These absent calls are highlighted with asterisks (*) in Appendices 1 and 2. The combined analysis from the arrays revealed that *p*-NO-ASA altered the expression of several genes, which were grouped as cell growth regulators, apoptosis signaling, stress and drug metabolism in Jurkat T cells. The list of genes with expression fold change $\geq$1.5 or $\leq$0.65 treated with 10 or 20 μM *p*-NO-ASA for 24 h is shown in Table 1. *p*-NO-ASA induced the expression of 16 genes and inhibited 6 genes. Dose-dependent response for 10 or 20 μM *p*-NO-ASA was found for some genes in this list. Intriguingly, several genes that were induced several fold at 20 μM were induced less than 1.5-fold at 10 μM *p*-NO-ASA. Overall, the upregulated genes were classified mainly as follows: heat shock proteins namely *HSPA1A* (heat shock 70 kDa protein 1A or Hsp70), *HSPA6* (heat shock 70 kDa protein 6 or Hsp70B') and *HSPH1* (heat shock protein 110), and apoptosis signaling proteins which included *CASP10*, *CASP8*, *CASP9* (caspase-10, -8, and -9, respectively). Other induced genes included cell cycle regulators and DNA-damage response genes such as the DNA-damage-inducible transcript (*DDIT3*), Ataxia telangiectasia mutated (*ATM*), insulin-like growth factor binding protein 6 (*IGBP6*), and Breakpoint Cluster Region (*BCR*). Carcinogen/drug metabolism related genes were also induced; these included the detoxifiers Flavin containing monooxygenase-1 and -4 (*FMO1*, *FMO4*) and Peroxiredoxin 1 (*PRDX1*), and the Phase II enzymes and regulators such as Glutathione S-transferase omega 1 (*GSTO1*), NAD(P)H dehydrogenase:quinone oxidoreductase-1 (*NQO1*), and aryl hydrocarbon receptor (*AHR*). One gene belonging to the Phase III drug transporter gene ATP-binding cassette, subfamily G (*ABCG2*) was upregulated as well. The downregulated genes which responded dose-dependently, were cell cycle regulators, transcription factors and regulators (*CCND1*, *CDKN2D*, *NR1I3*, *NFKB1*). Of the total 22 genes altered, 5 genes were significantly altered; *HSPA1A*, *HSPA6* and *DDIT3* were induced, *RXRB* and *CDKN2D* were inhibited ($p \leq 0.05$, paired *t*-test). Among the modulated genes, *HSPA1A* was upregulated the most (11.7-fold, 20 μM *p*-NO-ASA) even though there was no major upregulation with 10 μM NO-ASA. There was no major change in the *CTNNB1* (β-catenin) mRNA expression (0.97-fold, 10 μM *p*-NO-ASA and 1.3-fold, 20 μM *p*-NO-ASA).

Several genes that were not included in the final array analysis deserve some mention. 'Absent call' genes in the treated cells including control included several genes in the Phase I enzyme category such as the *CYP*s (cytochrome P450 family); other absent calls denoted by asterisk adjacent to the gene name are given in Appendices 1 and 2. Also, expression of two genes of interest from the oxidative stress-related group, superoxide dismutase 1 and superoxide dismutase 2 (*SOD1* and *-2*) were considered borderline or unaltered, as these were insufficiently induced to just below the cut-off criteria (Appendix 1). Consistent with reports on HSP90 levels in most human cells, we observed abundant basal levels of two isoforms of HSP90, namely HSP90α (inducible) and HSP90β (constitutive) in Jurkat T cells relative to other genes in the array, and there were no changes in the fold expression for these (data not shown).

3.3. Real time quantitative RT-PCR validation of altered genes expression by NO-ASA

To confirm the gene expression differences from the array results, quantitative real time RT-PCR was performed for the following selected genes of interest using sequence-specific primers (*HSPA1A*, *HSPA6*, *HSPH1*, *FMO4*, *CASP9*, *DDIT3*, *ATM*, *NFKB*, and *CCND1*). We chose these genes based on expression levels and functional importance in the various cancer cell lines that were used to examine *p*-NO-ASA effects. In addition, validation of mRNA expression levels was performed in response to two concentrations 10 and 20 μM *p*-NO-ASA in Jurkat T cells, using the RNA that was used for the microarray hybridization and additional biological duplicate sets of treated and untreated cells. Quantitative real time RT-PCR for housekeeping gene GAPDH was also performed in these samples and was used for normalization of data. In addition, CTNNB1 (β-catenin), *SOD1* and *SOD2*, which exhibited no change in the array analysis, were examined as additional controls.

Consistent mRNA level changes were obtained compared with the array analysis. Dose-dependent modulation of the mRNA levels was observed for 5 out of the 9 genes (Fig. 3). *p*-NO-ASA significantly increased the mRNA expression of *HSPA1A* and *HSPA6* ($p \leq 0.005$, 10 and 20 μM *p*-NO-ASA) which confirmed the significant fold increases in the array analysis. It is important to note that these are both inducible HSPs and are present in very low basal levels in Jurkat T cells, which may account for high fold changes. Consistent with the array analysis, the modulations in

Table 1
Gene expression profiling of Jurkat T cells treated with *p*-NO-ASA.

Gene Id	Description	Gene name	10 μM			20 μM		
			Fold change	SD	*t*-Test *p*-Values	Fold change	SD	*t*-Test *p*-Values
Increased								
Heat shock proteins								
NM_005345	Heat shock 70 kDa protein 1A	**HSPA1A**	1.33	0.04	0.14	**11.77**	**0.98**	**0.05**
NM_002155	Heat shock 70 kDa protein 6 (HSP70B')	**HSPA6**	1.32	0.25	0.32	**3.9**	**1.71**	**0.04**
NM_006644	Heat shock 105 kDa/110 kDa protein 1	HSPH1	0.92	0.8	0.79	1.51	0.37	0.79
Apoptosis signaling								
NM_001229	Caspase-9	CASP9	1.05	0.63	0.88	1.68	0.71	0.82
NM_001230	Caspase-10	CASP10	1.23	0.82	0.90	1.96	0.85	0.49
NM_001228	Caspase-8	CASP8	2.04	0.08	0.42	2.77	0.08	0.20
DNA-damage response, cell cycle regulators, serine thr kinase								
NM_004083	DNA-damage-inducible transcript 3	**DDIT3**	1.07	0.05	0.17	**1.84**	**0.03**	**0.01**
NM_000051	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	ATM	1.04	0.17	0.88	1.69	0.28	0.19
NM_004327	Breakpoint Cluster Region	BCR	1.28	0.49	0.11	1.55	1.51	0.17
Phase I enzymes								
NM_002021	Flavin containing monooxygenase 1	FMO1	0.92	0.06	0.84	1.77	0.19	0.43
NM_002022	Flavin containing monooxygenase 4	FMO4	1.02	0.11	0.97	3.27	0.66	0.21
Phase II enzymes and regulators								
NM_001621	Aryl hydrocarbon receptor	AHR	1.12	0.52	0.76	3.03	1.2	0.38
NM_000903	NAD(P)H dehydrogenase, quinone 1	NQO1	1.50	0.03	0.50	1.93	0.09	0.10
NM_004832	Glutathione S-transferase omega 1	GSTO1	1.01	1.62	0.97	1.56	8.37	0.45
Phase III, drug transporter								
NM_004827	ATP-binding cassette, subfamily G (WHITE), member 2	ABCG2	1.20	0.09	0.64	1.99	0.28	0.40
Oxidoreductases								
NM_002574	Peroxiredoxin 1	PRDX1	1.09	2.98	0.76	1.85	15.87	0.39
Regulator of cell growth and proliferation								
NM_002178	Insulin-like growth factor binding protein 6	IGFBP6	1.4	0.13	0.31	1.57	0.07	0.16
Decreased								
Transcription factor or regulator, Negative regulator of cell growth and proliferation								
NM_004064	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CDKN1B	0.71	0.59	0.60	0.65	0.02	0.54
NM_001800	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	CDKN2D	0.70	0.08	0.01	0.54	0.09	0.01
NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B-cells	NFKB1	0.87	0.2	0.46	0.65	0.06	0.06
Nuclear receptors								
NM_005122	Nuclear receptor subfamily 1, group I, member 3	NR1I3	1.04	0.06	0.62	0.65	0.19	0.11
NM_021976	Retinoid X receptor, beta	RXRB	0.87	0.25	0.57	0.64	0.06	0.03
No change								
Oxidative stress								
NM_000454	Superoxide dismutase 1, soluble amyotrophic lateral sclerosis 1	SOD1	1.36	4.14	0.34	1.43	0.38	0.3
NM_000636	Superoxide dismutase 2, mitochondrial	SOD2	1.4	0.15	0.6	1.4	0.34	0.79
Housekeeping								
NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	0.96	4.38	0.93	1.3	1.43	0.49
NM_004048	Beta-2-microglobulin		1.12	1.78	0.58	1.48	2.22	0.15

Genes which were significantly upregulated by 20 μM *p*-NO-ASA (>1.5 fold, $p < 0.05$) are highlighted in bold.

fold expression of mRNA levels of Hsp70A or *HSPA1A* (41.5 ± 7.01-fold; increased), Hsp70B' or *HSPA6* (100.4 ± 8.11-fold; increased), *HSPH1* (1.71 ± 0.43-fold; increased), *FMO4* (4.5 ± 1.67-fold; increased), *CASP9* (1.77 ± 0.03-fold; increased), *DDIT3* (5.6 ± 0.51-fold; increased) were confirmed to be upregulated; and *NFKB1* (0.54 ± 0.01-fold; decreased) and *CCND1* (0.69 ± 0.06-fold; decreased) were confirmed to be downregulated with 20 μM *p*-NO-ASA treatment for 24 h in Jurkat T cells. Lower concentration of *p*-NO-ASA (10 μM) also induced some genes; *HSPA1A* (4.6 ± 1.01-fold), *HSPA6* (10.2 ± 2.5-fold) and *DDIT3* (1.9 ± 0.3-fold). The mRNA expression of *CTNNB1* (β-catenin) at 10 and 20 μM *p*-NO-ASA was not altered; this lends credence to our previous reports regarding inhibition of β-catenin/TCF signaling inhibition via degradation of β-catenin or disruption of binding to TCF-4. Also consistent with the array analysis, *SOD1* and *SOD2* mRNA expression did not change, whereas, contrary to the array results, we observed no changes in *ATM* mRNA levels (data not shown). An interesting result obtained was increases in *FOS* mRNA in a dose dependant manner (3.25 ± 1.2-fold, 10 μM, and 16.2 ± 3.2-fold, 20 μM *p*-NO-ASA) which was included in the validation studies because of existing evidence of Hsp70 and a concomitant c-fos induction during stress conditions [21,22].

3.4. NO-ASA increased Hsp70 protein levels

Hsp70 and c-fos protein levels were examined by immunoblot analysis in Jurkat cells. These proteins were also examined in HT-29 human colon cancer cell line since earlier studies had demonstrated growth inhibition by *p*-NO-ASA. The cells were treated with 5, 10, 20 and 50 μM *p*-NO-ASA for 18 h and compared to a vehicle-treated control. Immunoblots confirmed that Hsp70 and c-fos protein levels increased in a concentration-dependent manner in Jurkat T cells and in HT29 cells (Fig. 4).

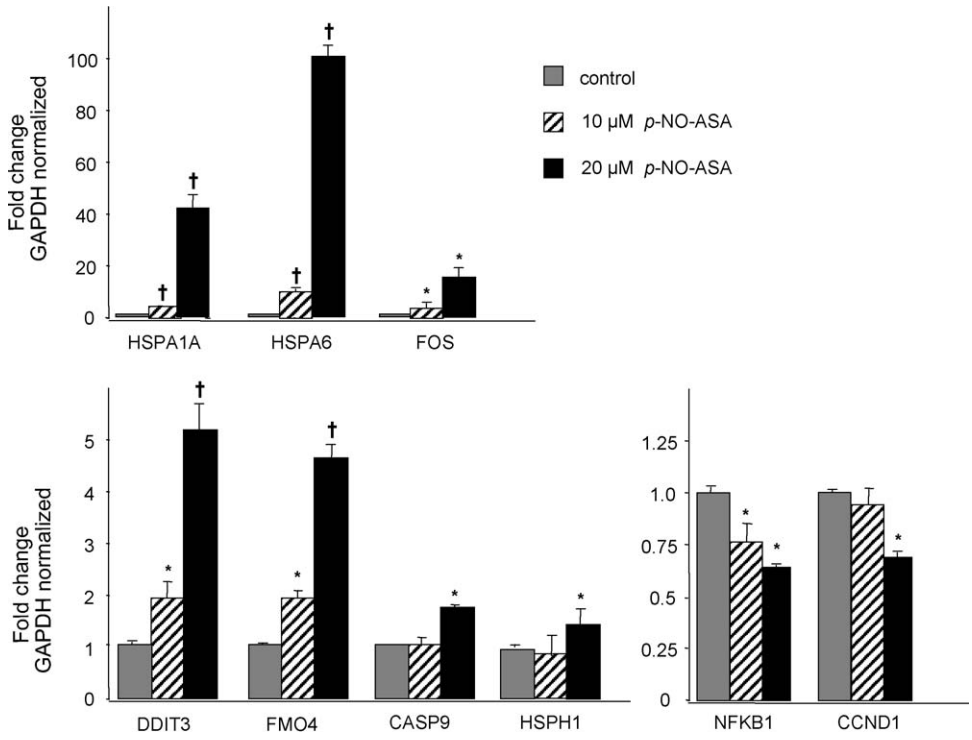

Fig. 3. Real time quantitative RT-PCR analysis of selected genes modulated by *p*-NO-ASA in Jurkat T cells. Effects of *p*-NO-ASA (10 and 20 μM) on mRNA levels are shown. Total RNA samples were isolated form Jurkat cells using gene specific primers. Fold changes in mRNA levels were obtained by comparing the treatment groups to the vehicle-control. The *GAPDH* gene was used as internal reference control. Results are mean ± SD, significant differences to the controls were calculated by Student's *t*-test, $n = 3$, $^{*}p \leq 0.05$, $^{\dagger}p \leq 0.01$.

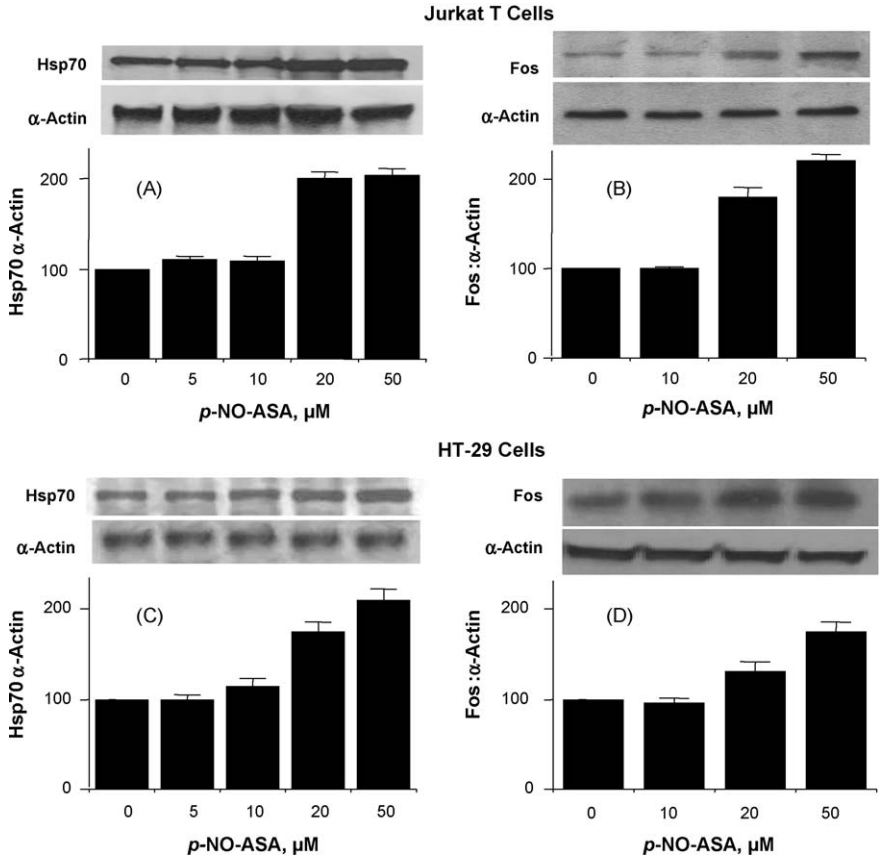

Fig. 4. *p*-NO-ASA increased the Hsp70 and Fos protein levels in Jurkat T and HT-29 colon cancer cells. Cells were treated with increasing concentrations *p*-NO-ASA for 18 h and analyzed for Hsp70 and Fos expression by immunoblot of lysates using α-actin as control. *Upper panel*: Jurkat T cells (A, Hsp70; B, Fos); *Lower panel*: HT29 cells (C, Hsp70; D, Fos). Each blot is a representative of two independent experiments. Densitometry evaluations showing mean ± range of two independent experiments are shown under each blot.

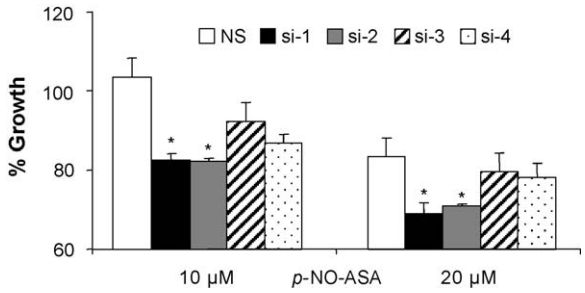

Fig. 5. Enhanced cell growth inhibition with knocking down HSPA1A together with *p*-NO-ASA treatment. *p*-NO-ASA's cell growth inhibition effect was shown to be enhanced by knocking down a Heat shock protein 70 family member HSPA1A with siRNA. This enhanced inhibition is significant as indicated by (*), $p < 0.05$ compared to the corresponding non-specific control. NS, non-specific control; siRNA targets, si1-4: (target sequence 1: TGCCATCTTACGACTATTTCT; sequence 2: TACACTTAACTCAGGCCATTT; sequence 3: TACTGCCATCTTACGACTATT; sequence 4: TTCTACATGCAGAGATGAATT).

3.5. Knocking down HSPA1A enhanced the effect of NO-ASA at low concentrations

Small interfering RNA for HSPA1A was used to determine the effect of HSPA1A (Hsp70) knockdown on the growth inhibitory response of *p*-NO-ASA. The adenocarcinoma colorectal SW480 cell line was selected for its high transfection efficiency and strong growth inhibition by *p*-NO-ASA [15]. RT-PCR in SW480 cells confirmed that the SW480 cells also strongly induced Hsp70 mRNA by *p*-NO-ASA. Validation experiments for the siRNA target sequences was performed by transfection of SW480 cells with each of the four Hsp70 siRNA and RT-PCR, which confirmed decrease of mRNA levels by 70–80% compared to non-specific control (data not shown). To examine the role of Hsp70, cells transfected with siRNA for Hsp70 were incubated for 48 h and followed by treatment with *p*-NO-ASA for 24 h. The percent cell growth inhibition by *p*-NO-ASA was enhanced compared to corresponding non-silencing control, particularly at 10 and 20 μM *p*-NO-ASA. At 10 μM *p*-NO-ASA, % growth for the non-specific control was 103 ± 9%, and for si HSP-70 was 82 ± 3%. At 20 μM *p*-NO-ASA, the % growth for the non-specific control was 83 ± 8%, and for si HSP-70 was 69 ± 4% (Fig. 5). At higher concentration of *p*-NO-ASA 40 or 80 μM, there was no enhancement or reversal of % growth with si HSP-70 compared with the non-specific control (data not shown).

4. Discussion

This study explored for the first time the differential gene expression profile of human Jurkat T leukemia cells in response to *p*-NO-ASA for genes mainly related to stress, toxicity, and some cancer pathways. This study demonstrated that expression of genes related to heat shock response, apoptosis, cell cycle, and carcinogen metabolism were altered by *p*-NO-ASA compared to a DMSO-treated control. This study presents four major considerations that merit discussion.

First, *p*-NO-ASA strongly upregulated the heat shock protein HSP70A mRNA and HSP70B' mRNA, moderately increased Hsp70A (Hsp70) protein expression, while *HSPH1* mRNA (Hsp 110/105 protein) was modestly upregulated. These members of the heat shock proteins belong to the Hsp70 family and act as molecular chaperones and a heat shock response allow the cells to regulate physiological homeostasis under stress [23–25]. Hsp70 is stress-inducible with major roles in response to stress, whereas Hsp70B' is also strictly inducible and is believed to be a secondary responder to stress [25]. Among the human hematopoietic cell types, leukemic and non-leukemic tumor cells express low levels of inducible *HSP70A* mRNA in the absence of any physical or chemical stimuli [26], which we also observed since these were 'absent calls' in the array analysis of untreated cells (Appendices 1 and 2, see footnote). So the calculated high fold induction of HSP70A and HSP70B' mRNA (40-fold and 100-fold at 20 μM *p*-NO-ASA, respectively by RT-PCR) were due to the presence of low basal levels of Hsp70 mRNA levels in Jurkat cells. It is also possible that such increase of mRNA may be caused by stimulation of the transcription, but may not necessarily lead to stabilization of the mRNA. Similar high fold induction of Hsp70 mRNA was also obtained in K562 chronic myelogenous leukemia cells (400-fold) and Raji B lymphoma cells (110-fold) after 4 h in response to heat shock treatment [26].

Second, regarding the role of HSP induction, several studies indicate that Hsp70 protein is the major stress-inducible cancer-associated antiapoptotic protein [27,28]. Targeting Hsp70 with siRNA demonstrated moderate enhancement of cell growth inhibition by *p*-NO-ASA at low concentrations, but no such effect at higher concentration, thereby indicating a low-concentration-dependent synergistic effect of *p*-NO-ASA when Hsp70 is knocked down. It is quite unlikely that Hsp70 alone can have a major effect, the role of Hsc70 (the constitutive variant) and Hsp70B' may need to be examined for a combined effect. Recent work has suggested facilitation of cell death by a combination of Hsp70 over-expression with functional inhibition of Hsp70-ATPase, probably via AKT kinase reduction [29]. However, regarding *p*-NO-ASA, such differential effects at low and high concentrations of *p*-NO-ASA have been observed by us with respect to β-catenin degradation [15]. It must be noted that *p*-NO-ASA strongly inhibits the growth of cells at very low concentrations even though Hsp70 is induced. Several studies indicate that existing high expression of HSPs is related to poor prognosis and resistance to cancer therapy (reviewed in [27,28]). Generally, HSPs are known to delay the apoptotic response [28]. However, several reports describe other roles associated with HSP due to their ability to interact with a wide range of proteins and peptides. Chemopreventive agents such as NSAIDs are known to induce a heat shock response in a variety of cell types and simultaneously induce apoptosis [30,31]. Also, there is evidence that HSP induction is associated with arrest of DNA synthesis by binding with co-chaperone Bag-1 and inhibiting Bag-1-mediated activation of Raf-1 kinase [32]. Such a mechanism has been implicated for the chemopreventive agent curcumin, which exhibits anti-proliferative activity in human leukemia K562 cells, and is associated with an increase in HSP70 mRNA and protein expression [33]. Further, there is evidence that increased Hsp70 protein expression on the tumor cell surface may lead to tumor cell sensitization for immune attacks by enhanced recognition by the natural killer (NK) cells of the immune system [34]. A series of studies demonstrated that Hsp70 stimulated the cytolytic activity of the NK cells against such tumor cells by secreting granzyme B, which is a pro-apoptotic enzyme [35,36]. A potential application was investigated where secretion of Hsp70 together with antigenic peptides improved the potency of tumor cell vaccines [37]. Therefore, at one end, HSPs are known to promote cell survival by inhibiting the apoptotic cascade that lead to cell death, and at the another end, induction of HSP70 and expression on the membrane or secretion is associated with anti-proliferative and/or immunostimulatory activity, which may be useful for tumor immunotherapy. Thus, *p*-NO-ASA-mediated induction of HSP70 in T- ALL cells may have therapeutic potential with combined immunotherapy. Adjuvant effect of NO aspirin *meta*- and *ortho* isomers (NCX4016 and NCX4060) was demonstrated in two tumor models in mice where the compounds enhanced the preventive and therapeutic effectiveness of recombinant cancer vaccines, although HSPs were not examined in this report [38].

The third consideration is regarding our own reports of modulation of various molecular targets by *p*-NO-ASA such as

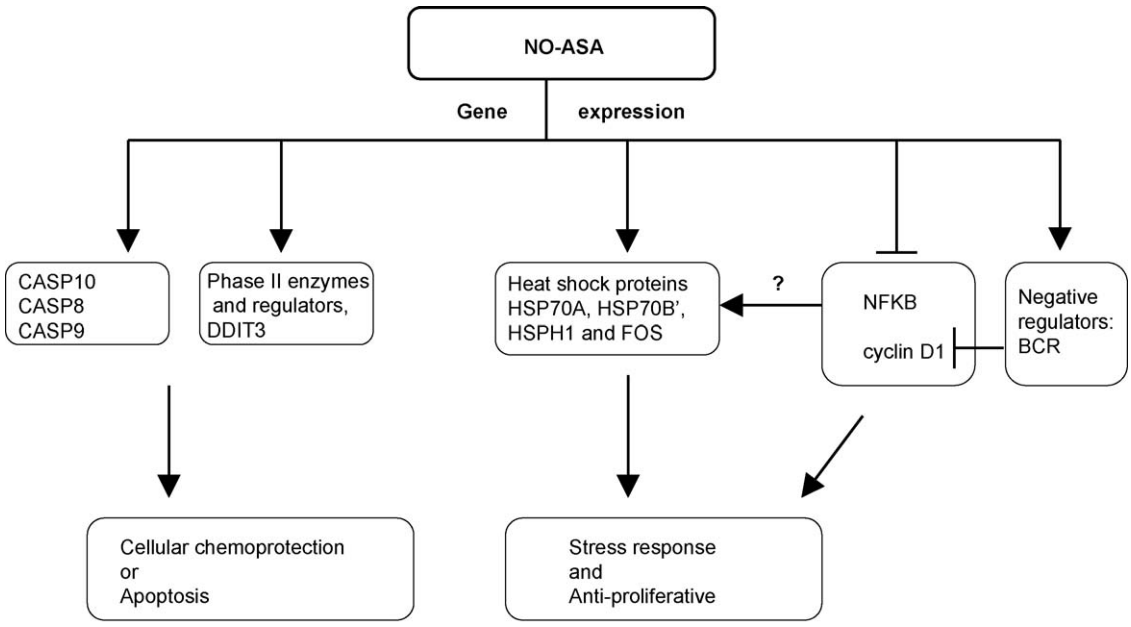

Fig. 6. Schematic diagram of a working model of the pleotropic effects of *p*-NO-ASA on the expression of molecular targets relating to stress and proliferation pathways. Based on this study, the induction of genes related to detoxification (Phase II enzymes) and stress genes (heat shock proteins) may explain, in part, its chemoprotective activity in cooperation with anti-proliferative and pro-apoptotic role of *p*-NO-ASA.

inhibition of iNOS expression, and induction of COX-2 in various cancer cell lines [39]. From several independent reports, it appears that Hsp70 induction may regulate the expression of these target molecules [39], For example, cross-regulation of iNOS and COX-2 by combined heat shock and LPS stimulation was reported [40]. HSP70 mRNA and protein were upregulated by the green fluorescent protein resulting in COX-2 induction and PGE_2 production [41]. In other studies, Ca^{2+} and PKC were shown to be involved in the regulation of Hsp70 synthesis [42]. Interestingly, this provides a possible role of Hsp70 in our recent report in MCF-7 breast cancer cells, where we described COX-2 induction by *p*-NO-ASA that was mediated in part by protein kinase C [11]. Similarly, an association between the inhibition of NF-κB activation and the induction of heat shock response was also demonstrated [43,44]. An interesting finding in our study was upregulation of the proto-oncogene *FOS*. It is noteworthy that the *FOS* gene promoter contains one copy of a specific DNA recognition sequence called heat shock element (HSE) whereas the Hsp70 promoter contains two HSEs [21]. Thus, it appears that several effects of *p*-NO-ASA may have close association with heat shock protein induction.

Phase II enzymes are involved in detoxification metabolism, considered to be an effective method to achieve chemoprotection [45]. NQO1 enzyme is a commonly used marker of Phase II enzyme induction whose increased expression is related to detoxification of harmful quinones [45]. *GST01* (Glutathione S-transferase omega-1) and *NQO1* (NAD(P)H:quinone oxidoreductase-1) (Table 1) may be relevant to the mechanism of action of *p*-NO-ASA. Consistent with the upregulation of *NQO1* observed in the array analysis, we and others have reported that NO-ASA induced *NQO1* mRNA expression in a hepatoma cell line [46] and increased NQO1enzyme activity in mouse liver adenocarcinoma cells and in *Min* ($APC^{Min/+}$) mice [47]. Further, Ahr (Table 1) which is a target of Nrf2 may result in the enhanced transcription of Phase II genes *GST01* and *NQO1* [48]. Peroxiredoxin 1 (*PRDX1*) is a scavenger of reactive oxygen species (ROS), which reduces hydrogen peroxide and alkyl hydroperoxides and thereby functions as a tumor suppressor and a detoxifier detoxification of peroxides [49]. The flavin containing monooxygenases are a family of NADPH- and oxygen-dependent enzymes that oxygenate a diverse group of xenobiotics [50]. High levels of FMO1 expression have been suggested to be important in the metabolic clearance of sulfides and tertiary amines, therefore its induction may have a chemo-protective role while the role of FMO4 is not yet clear [50]. The *BCR* gene, is a putative tumor suppressor based on its role as a negative regulator of β-catenin [51], was upregulated by *p*-NO-ASA in the array analysis (Table 1). Jurkat T cells are a BCR-Abl negative cell line which express only BCR that interacts with β-catenin and leads to downregulation of b-catenin/TCF-dependent transcription [52]. Therefore its upregulation by *p*-NO-ASA may be therapeutically important in T-ALL. In agreement with our earlier studies in colon cancer cell lines no change in the *CTNNB1* (β-catenin) gene expression, and confirmed downregulation of cyclin D1 by array analysis and qRT-PCR, agree with our earlier studies in colon cancer cell lines [15,16].

DNA-damage-inducible transcript 3 (DDIT3) or GADD153 or CHOP is a C/EBP homologous transcription factor implicated in cellular stress response and apoptosis. Curcumin-induced upregulation of *GADD153* mRNA expression was suggested to be an important molecular event leading to apoptosis [53]. Further, DDIT3 was also shown to play a key role in NO-mediated apoptosis in macrophages [54]. *ABCG2* is a member of the ABC family of drug transporters alternatively called breast cancer resistance protein (BCRP), which extrude a wide variety of anticancer drugs by altering their absorption and distribution. While low or absent levels of ABCG2 are found in several solid tumors and acute myeloid leukemia cells, its expression in placenta, liver, small intestine, colon, lung, and kidney, have suggested a role in the detoxification of xenobiotics [55]. Of the downregulated genes, RXR functions as a ligand-activated transcription factor that modulates cell differentiation, and heterodimerize with PPAR nuclear transcription factors making them ideal targets for chemoprevention [56].

Many current anti-tumor therapies seek to block NF-κB activity as a means to inhibit tumor growth or to sensitize the tumor cells to chemotherapy [57]. NF-κB mRNA expression was down-regulated by *p*-NO-ASA in this study, whereas there were no changes in NFKBIA (I-|Bα) and NFKBIB (I-|Bβ). Downregulation of NF-|B activity and cyclin D1 expression by curcumin was

demonstrated in primary adult T cell leukemia cells, which suppressed growth and induced apoptosis (reviewed in [58]). However, in our previous studies the activation of NF-κB was inhibited in human colon cancer cells without changes in protein levels of NFκB subunits [39].

A schematic depicting the possible molecular events mediated by *p*-NO-ASA in Jurkat T cells studied by gene array is presented in Fig. 6. This is the first report of modulation of a variety of genes by the *para* isomer of NO-ASA by gene array and qRT-PCR. We recognize some limitations of this study as follows. While the changes in *p*-NO-ASA-mediated gene expression are demonstrated for the single cancer cell line Jurkat T that overexpresses β-catenin, and an additional cell line HT29 was used for confirming Hsp70 and Fos induction; these findings need to be validated in other model systems. Second, the array data for genes considered 'absent calls' were not included in further analysis even though these displayed some level of altered expression mediated by *p*-NO-ASA, may need to be revisited. Expression of some of these genes could be relevant and may be investigated by an initial stimulation of expression by known mediators followed by *p*-NO-ASA treatment. For example, Interleukin 8 and Interleukin 1-α were induced by 20 μM *p*-NO-ASA 2.2-fold and 2.9-fold, respectively, even though these were considered 'absent' (Appendices 1 and 2, absent calls).

In conclusion, *p*-NO-ASA induces the stress-related *HSP70* mRNA and protein, and modulates the expression of genes related to cell proliferation, and phase II detoxifying enzymes. Hsp70 protein induced by *p*-NO-ASA is associated with increased fos. At low concentration of *p*-NO-ASA, Hsp70 seems to provide anti-growth inhibitory role, as demonstrated by knockdown studies. The effect of induced Hsp70 on other targets molecules need to be investigated. This work highlights a compact but significant study that underscores the chemopreventive and therapeutic potential of *p*-NO-ASA in T-ALL.

Acknowledgements

Supported by NYIT grant ISRC2006-2008 (NN) and National Cancer Institute contracts (KK).

Appendix A

List of genes on the Human Toxicology and Drug Resistance oligoarray (263 genes). (*) Denote absent calls after data analysis.

Gene	Description
ABCB1	ATP-binding cassette, subfamily B, member 1
ABCB4	ATP-binding cassette, subfamily B, member 4
*ABCC1**	ATP-binding cassette, subfamily C, member 1
*ABCC2**	ATP-binding cassette, subfamily C, member 2
*ABCC3**	ATP-binding cassette, subfamily C, member 3
*ABCC5**	ATP-binding cassette, subfamily C, member 5
ABCC6	ATP-binding cassette, subfamily C, member 6
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ABCG2	ATP-binding cassette, subfamily G (WHITE), member 2
ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1
ACADSB	Acyl-Coenzyme A dehydrogenase, short/branched chain
ACAT1	Acetyl-Coenzyme A acetyltransferase 1
AHR	Aryl hydrocarbon receptor
AKT1	V-akt murine thymoma viral oncogene homolog 1
AP1S1	Adaptor-related protein complex 1, sigma 1 subunit
*APC**	Adenomatosis polyposis coli
*AR**	Androgen receptor (dihydrotestosterone receptor)
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATM	Ataxia telangiectasia mutated (compl groups A, C and D)
BAG1	BCL2-associated athanogene
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BCL2L1	BCL2-like 1
BCL2L2	BCL2-like 2
BCR	Breakpoint Cluster Region
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
CALR	Calreticulin
CANX	Calnexin
*CASP1**	Caspase-1, apoptosis-related cysteine peptidase
CASP10	Caspase-10, apoptosis-related cysteine peptidase
CASP8	Caspase-8, apoptosis-related cysteine peptidase
CAT	Catalase
CCL21	Chemokine (C–C motif) ligand 21
*CCL3**	Chemokine (C–C motif) ligand 3
*CCL4**	Chemokine (C–C motif) ligand 4
CCNC	Cyclin C
CCND1	Cyclin D1
CCNE1	Cyclin E1
CCNG1	Cyclin G1
CCT2	Chaperonin containing TCP1, subunit 2 (beta)
CCT3	Chaperonin containing TCP1, subunit 3 (gamma)
CCT4	Chaperonin containing TCP1, subunit 4 (delta)
CCT5	Chaperonin containing TCP1, subunit 5 (epsilon)
CCT7	Chaperonin containing TCP1, subunit 7 (eta)
CCT8	Chaperonin containing TCP1, subunit 8 (theta)
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16, inhibits CDK4)
CDKN2D	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)
CES4	Carboxylesterase 4-like
CHAT	Choline acetyltransferase
CHEK2	CHK2 checkpoint homolog (S. pombe)
CHST1	Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1
CHST10	Carbohydrate sulfotransferase 10
CHST2	Carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2
CHST3	Carbohydrate (chondroitin 6) sulfotransferase 3
CHST4	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 4
CHST5	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 5
*CHST6**	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6
CHST7	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7
CHST8	Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 8
CLU	Clusterin (complement lysis inhibitor)
COMT	Catechol-O-methyltransferase
*CRABP1**	Cellular retinoic acid binding protein 1
CRAT	Carnitine acetyltransferase
CRYAA	Crystallin, alpha A
CRYAB	Crystallin, alpha B
*CSF2**	Colony stimulating factor 2 (granulocyte-macrophage)
CTPS	CTP synthase
*CXCL10**	Chemokine (C-X-C motif) ligand 10
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1
CYP11B2	Cytochrome P450, family 11, subfamily B, polypeptide 2
*CYP1A1**	Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2
*CYP1B1**	Cytochrome P450, family 1, subfamily B, polypeptide 1
*CYP20A1**	Cytochrome P450, family 20, subfamily A, polypeptide 1
CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1
CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1
*CYP2A6**	Cytochrome P450, family 2, subfamily A, polypeptide 6
CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6
CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8
*CYP2C9**	Cytochrome P450, family 2, subfamily C, polypeptide 9
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1
*CYP2F1**	Cytochrome P450, family 2, subfamily F, polypeptide 1
*CYP3A4**	Cytochrome P450, family 3, subfamily A, polypeptide 4
*CYP3A5**	Cytochrome P450, family 3, subfamily A, polypeptide 5
CYP4A11	Cytochrome P450, family 4, subfamily A, polypeptide 11
CYP4B1	Cytochrome P450, family 4, subfamily B, polypeptide 1
*CYP4F3**	Cytochrome P450, family 4, subfamily F, polypeptide 3
*CYP7A1**	Cytochrome P450, family 7, subfamily A, polypeptide 1
*CYP7B1**	Cytochrome P450, family 7, subfamily B, polypeptide 1
*CYP8B1**	Cytochrome P450, family 8, subfamily B, polypeptide 1
DDIT3	DNA-damage-inducible transcript 3
DHFR	Dihydrofolate reductase
CYB5R3	Cytochrome b5 reductase 3
DLAT	Dihydrolipoamide S-acetyltransferase

Appendix A (*Continued*)

Gene	Description
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1
DNAJA2	DnaJ (Hsp40) homolog, subfamily A, member 2
*DNAJA3**	DnaJ (Hsp40) homolog, subfamily A, member 3
*DNAJA4**	DnaJ (Hsp40) homolog, subfamily A, member 4
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1
DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11
DNAJB2	DnaJ (Hsp40) homolog, subfamily B, member 2
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4
DNAJB5	DnaJ (Hsp40) homolog, subfamily B, member 5
*DNAJB9**	DnaJ (Hsp40) homolog, subfamily B, member 9
*DNAJC4**	DnaJ (Hsp40) homolog, subfamily C, member 4
*DNAJC5**	DnaJ (Hsp40) homolog, subfamily C, member 5
DNAJC7	DnaJ (Hsp40) homolog, subfamily C, member 7
DNAJC8	DnaJ (Hsp40) homolog, subfamily C, member 8
DPYD	Dihydropyrimidine dehydrogenase
E2F1	E2F transcription factor 1
*EGFR**	Epidermal growth factor receptor
*EGR1**	Early growth response 1
ELK1	ELK1, member of ETS oncogene family
EPHX1	Epoxide hydrolase 1, microsomal (xenobiotic)
*EPHX2**	Epoxide hydrolase 2, cytoplasmic
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2
*ERBB3**	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3
ERBB4	V-erb-a erythroblastic leukemia viral oncogene homolog 4
ERCC1	Excision repair cross-complementing rodent repair deficiency (complementation group 1)
ERCC3	Excision repair cross-complementing rodent repair deficiency (complementation group 1)
ESR1	Estrogen receptor 1
*ESR2**	Estrogen receptor 2 (ER beta)
*FGF2**	Fibroblast growth factor 2 (basic)
FMO1	Flavin containing monooxygenase 1
FMO4	Flavin containing monooxygenase 4
FMO5	Flavin containing monooxygenase 5
GADD45A	Growth arrest and DNA-damage-inducible, alpha
GADD45B	Growth arrest and DNA-damage-inducible, beta
*GAL3ST1**	Galactose-3-O-sulfotransferase 1
GDF15	Growth differentiation factor 15
GPX1	Glutathione peroxidase 1
GPX2	Glutathione peroxidase 2 (gastrointestinal)
GSR	Glutathione reductase
GSTA3	Glutathione S-transferase A3
GSTA4	Glutathione S-transferase A4
GSTM1	Glutathione S-transferase M1
GSTM2	Glutathione S-transferase M2 (muscle)
*GSTM3**	Glutathione S-transferase M3 (brain)
GSTM5	Glutathione S-transferase M5
GSTO1	Glutathione S-transferase omega 1
GSTP1	Glutathione S-transferase pi
GSTT1	Glutathione S-transferase theta 1
GSTT2	Glutathione S-transferase theta 2
HAT1	Histone acetyltransferase 1
HIF1A	Hypoxia-inducible factor 1, alpha subunit
HMOX1	Heme oxygenase (decycling) 1
HMOX2	Heme oxygenase (decycling) 2
HNMT	Histamine N-methyltransferase
HOP	Homeodomain-only protein
HSF1	Heat shock transcription factor 1
HSPA1A	Heat shock 70 kDa protein 1A
HSPA1L	Heat shock 70 kDa protein 1-like
HSPA2	Heat shock 70 kDa protein 2
HSPA4	Heat shock 70 kDa protein 4
HSPA6	Heat shock 70 kDa protein 6 (HSP70B')
HSPA8	Heat shock 70 kDa protein 8
HSPA9B	Heat shock 70 kDa protein 9B (mortalin-2)
HSPB1	Heat shock 27 kDa protein 1
HSPB2	Heat shock 27 kDa protein 2
HSPB3	Heat shock 27 kDa protein 3
HSPCA	Heat shock 90 kDa protein 1, alpha
HSPCB	Heat shock 90 kDa protein 1, beta
HSPD1	Heat shock 60 kDa protein 1 (chaperonin)
HSPE1	Heat shock 10 kDa protein 1 (chaperonin 10)
HSPH1	Heat shock 105 kDa/110 kDa protein 1
HYOU1	Hypoxia upregulated 1
IGF1R	Insulin-like growth factor 1 receptor
IGF2R	Insulin-like growth factor 2 receptor
IGFBP6	Insulin-like growth factor binding protein 6

Appendix A (*Continued*)

Gene	Description
*IL18**	Interleukin 18 (interferon-gamma-inducing factor)
IL1A	Interleukin 1, alpha
IL1B	Interleukin 1, beta
*IL6**	Interleukin 6 (interferon, beta 2)
LTA	Lymphotoxin alpha (TNF superfamily, member 1)
*MAOA**	Monoamine oxidase A
*MAOB**	Monoamine oxidase B
MDM2	Mdm2, transformed 3T3 cell double minute 2
MET	Met proto-oncogene (hepatocyte growth factor receptor)
MGMT	O-6-methylguanine-DNA methyltransferase
MGST1	Microsomal glutathione S-transferase 1
MGST2	Microsomal glutathione S-transferase 2
MGST3	Microsomal glutathione S-transferase 3
MIF	Macrophage migration inhibitory factor
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
*MT1X**	Metallothionein 1X
*MT3**	Metallothionein 3 (growth inhibitory factor (neurotrophic))
MVP	Major vault protein
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
MYST2	MYST histone acetyltransferase 2
MYST4	MYST histone acetyltransferase (monocytic leukemia) 4
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)
NAT2	N-acetyltransferase 2 (arylamine N-acetyltransferase)
NAT5	N-acetyltransferase 5 (ARD1 homolog, S. cerevisiae)
*NAT8**	N-acetyltransferase 8 (camello like)
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer 1 (p105)
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer 2 (p49/p100)
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer inhibitor, alpha
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer inhibitor, beta
*NNMT**	Nicotinamide N-methyltransferase
*NOS2A**	Nitric oxide synthase 2A (inducible, hepatocytes)
NQO1	NAD(P)H dehydrogenase, quinone 1
NR1I2	Nuclear receptor subfamily 1, group I, member 2
NR1I3	Nuclear receptor subfamily 1, group I, member 3
NUDT1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1
PCNA	Proliferating cell nuclear antigen
PON3	Paraoxonase 3
POR	P450 (cytochrome) oxidoreductase
PPARD	Peroxisome proliferative activated receptor, delta
PPARG	Peroxisome proliferative activated receptor, gamma
*PPARGC1A**	Peroxisome proliferative activated receptor, gamma, coactivator 1, alpha
PRDX1	Peroxiredoxin 1
PRDX2	Peroxiredoxin 2
*PTGS1**	Prostaglandin-endoperoxide synthase 1
*PTGS2**	Prostaglandin-endoperoxide synthase 2
*RAD23A**	RAD23 homolog A (S. cerevisiae)
*RAD50**	RAD50 homolog (S. cerevisiae)
*RAD51**	RAD51 homolog (RecA homolog, E. coli)
RARA	Retinoic acid receptor, alpha
RARB	Retinoic acid receptor, beta
RARG	Retinoic acid receptor, gamma
RB1	Retinoblastoma 1 (including osteosarcoma)
RELB	V-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)
RXRA	Retinoid X receptor, alpha
RXRB	Retinoid X receptor, beta
*RXRG**	Retinoid X receptor, gamma
SAFB	Scaffold attachment factor B
*SERPINH1**	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1 (collagen binding protein 1)
SOD1	Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))
SOD2	Superoxide dismutase 2, mitochondrial
SRD5A2	Steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2)
ST13	Suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)
SULT1A1	Sulfotransferase family, cytosolic, 1A, member 1
*SULT1B1**	Sulfotransferase family, cytosolic, 1B, member 1
*SULT1C1**	Sulfotransferase family, cytosolic, 1C, member 1

Appendix A (*Continued*)

Gene	Description
SULT1C2	Sulfotransferase family, cytosolic, 1C, member 2
*SULT1E1**	Sulfotransferase family 1E, estrogen-preferring, member 1
*SULT2A1**	Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1
SULT2B1	Sulfotransferase family, cytosolic, 2B, member 1
*SULT4A1**	Sulfotransferase family 4A, member 1
HSPCB	Heat shock 90 kDa protein 1, beta
*TBXAS1**	Thromboxane A synthase 1 (platelet, cytochrome P450, family 5, subfamily A)
TCP1	T-complex 1
*TNF**	Tumor necrosis factor (TNF superfamily, member 2)
*TNFRSF11A**	Tumor necrosis factor receptor superfamily, member 11a, NFKB activator
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
*TNFSF10**	Tumor necrosis factor (ligand) superfamily, member 10
FASLG	Fas ligand (TNF superfamily, member 6)
TOP1	Topoisomerase (DNA) I
*TP53**	Tumor protein p53 (Li-Fraumeni syndrome)
TPMT	Thiopurine S-methyltransferase
TPST1	Tyrosylprotein sulfotransferase 1
TPST2	Tyrosylprotein sulfotransferase 2
TRA1	Tumor rejection antigen (gp96) 1
B2M	Beta-2-microglobulin
ACTB	Actin, beta
TRADD	TNFRSF1A-associated via death domain
TYMS	Thymidylate synthetase
XDH	Xanthine dehydrogenase
*XRCC2**	X-ray repair complementing defective repair in Chinese hamster cells 2

Appendix B

List of genes on Human Cancer Pathway Finder oligoarray (96 genes). (*) Denote absent calls after data analysis.

Gene	Description
AKT1	V-akt murine thymoma viral oncogene homolog 1
*ANGPT1**	Angiopoietin 1
*ANGPT2**	Angiopoietin 2
APAF1	Apoptotic peptidase activating factor
ATM	Ataxia telangiectasia mutated (compl groups A, C & D)
*BAD**	BCL2-antagonist of cell death
*BAI1**	Brain-specific angiogenesis inhibitor 1
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BCL2L1	BCL2-like 1
BIRC5	Baculoviral IAP repeat-containing 5 (survivin)
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
CASP8	Caspase-8, apoptosis-related cysteine peptidase
CASP9	Caspase-9, apoptosis-related cysteine peptidase
CCND1	Cyclin D1
CCNE1	Cyclin E1
*CD44**	CD44 antigen (Indian blood group)
CDC25A	Cell division cycle 25A
*CDH1**	Cadherin 1, type 1, E-cadherin (epithelial)
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
CFLAR	CASP8 and FADD-like apoptosis regulator
CHEK2	CHK2 checkpoint homolog (S. pombe)
COL18A1	Collagen, type XVIII, alpha 1
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa
E2F1	E2F transcription factor 1
*EGF**	Epidermal growth factor (beta-urogastrone)
EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
ETS2	V-Ets erythroblastosis virus E26 oncogene homolog 2
*FGF2**	Fibroblast growth factor 2 (basic)
*FGFR2**	Fibroblast growth factor receptor 2 (bacteria-expressed kinase)

Appendix B (*Continued*)

Gene	Description
*FLT1**	Fms-related tyrosine kinase
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
GZMA	Granzyme A (granzyme 1)
HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)
HTATIP2	HIV-1 Tat interactive protein 2, 30 kDa
*ICAM1**	Intercellular adhesion molecule 1 (CD54)
*IFNA1**	Interferon, alpha 1
*IFNB1**	Interferon, beta 1, fibroblast
*IGF1**	Insulin-like growth factor 1 (somatomedin C)
*IL8**	Interleukin 8
*ITGA1**	Integrin, alpha 1
*ITGA2**	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
*ITGA3**	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)
ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)
*ITGA5**	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
*ITGA6**	Integrin, alpha 6
*ITGAV**	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide)
*ITGB3**	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
*ITGB5**	Integrin, beta 5
JUN	V-jun sarcoma virus 17 oncogene homolog (avian)
CD82	CD82 antigen
KISS1	KiSS-1 metastasis-suppressor
MAP2K1	Mitogen-activated protein kinase kinase 1
*MAPK14**	Mitogen-activated protein kinase 14
*MCAM**	Melanoma cell adhesion molecule
*MDM2**	Mdm2, transformed 3T3 cell double minute 2
*MET**	Met proto-oncogene (hepatocyte growth factor receptor)
*MICA**	MHC class I polypeptide-related sequence A
*MMP1**	Matrix metallopeptidase 1 (interstitial collagenase)
MMP2	Matrix metallopeptidase 2
*MMP9**	Matrix metallopeptidase 9
MTA1	Metastasis associated 1
MTA2	Metastasis associated 1 family, member 2
MTSS1	Metastasis-suppressor 1
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
*NCAM1**	Neural cell adhesion molecule 1
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer 1 (p105)
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer inhibitor, alpha
NME1	Non-metastatic cells 1, protein (NM23A) expressed in
NME4	Non-metastatic cells 4, protein expressed in
PDGFA	Platelet-derived growth factor alpha polypeptide
PDGFB	Platelet-derived growth factor beta polypeptide
*PIK3CB**	Phosphoinositide-3-kinase, catalytic, beta polypeptide
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)
*PLAU**	Plasminogen activator, urokinase
PLAUR	Plasminogen activator, urokinase receptor
PNN	Pinin, desmosome associated protein
PRKDC	Protein kinase, DNA-activated, catalytic polypeptide
*PTEN**	Phosphatase and tensin homolog
RAF1	V-raf-1 murine leukemia viral oncogene homolog 1
RASA1	RAS p21 protein activator (GTPase activating protein) 1
RB1	Retinoblastoma 1 (including osteosarcoma)
S100A4	S100 calcium binding protein A4 (calcium protein)
*SERPINB2**	Serpin peptidase inhibitor, clade B, member 2
SERPINB5	Serpin peptidase inhibitor, clade B, member 5
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
SNCG	Synuclein, gamma (breast cancer-specific protein 1)
SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
SYK	Spleen tyrosine kinase
*TEK**	TEK tyrosine kinase, endothelial
*TERT**	Telomerase reverse transcriptase
*TGFB1**	Transforming growth factor, beta 1 (Camurati-Engelmann disease)
*TGFBR1**	Transforming growth factor, beta receptor I
*THBS1**	Thrombospondin 1
*THBS2**	Thrombospondin 2
TIMP1	TIMP metallopeptidase inhibitor 1
*TIMP3**	TIMP metallopeptidase inhibitor 3
TNF	Tumor necrosis factor (TNF superfamily, member 2)

Appendix B (*Continued*)

Gene	Description
TNFRSF10B	Tumor necrosis factor receptor, member 10b
TNFRSF1A	Tumor necrosis factor receptor, member 1A
TNFRSF25	Tumor necrosis factor receptor, member 25
FAS	Fas (TNF receptor superfamily, member 6)
TP53	Tumor protein p53 (Li-Fraumeni syndrome)
*TWIST1**	Twist homolog 1 (acrocephalosyndactyly 3)
*EPDR1**	Ependymin related protein 1 (zebrafish)
*VEGF**	Vascular endothelial growth factor
*ACTB**	Actin, beta

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