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# STAT1 signaling is associated with acquired crossresistance to doxorubicin and radiation in myeloma cell lines

**Mårten Fryknäs[1], Sumeer Dhar[2], Fredrik Öberg[1], Linda Rickardson[2], Maria Rydåker[2], Hanna Göransson[2], Mats Gustafsson[2], Ulf Pettersson[1], Peter Nygren[3], Rolf Larsson[2] and Anders Isaksson[2]***

[1]*Department of Genetics and Pathology, Uppsala University, Sweden*
[2]*Department of Medical Sciences, Clinical Pharmacology, Uppsala University, Sweden*
[3]*Department of Oncology, Radiation and Clinical Immunology, Uppsala University, Sweden*

**The myeloma cell line RPMI 8226/S and its doxorubicin resistant subline 8226/Dox40 were used as models to explore the potential importance of the STAT1 signaling pathway in drug and radiation resistance. The 40-fold doxorubicin resistant subline 8226/Dox40 was found to be crossresistant to single doses of 4 and 8 Gy of radiation. A genome-wide mRNA expression study comparing the 8226/Dox40 cell line to its parental line was performed to identify the underlying molecular mechanisms. Seventeen of the top 50 overexpressed genes have previously been implicated in the STAT1 signaling pathway. STAT1 was over expressed both at the mRNA and protein level. Moreover, analyses of nuclear extracts showed higher abundance of phosphorylated STAT1 (Tyr 701) in the resistant subline. Preexposure of the crossresistant cells to the STAT1 inhibiting drug fludarabine reduced expression of overexpressed genes and enhanced the effects of both doxorubicin and radiation. These results show that resistance to doxorubicin and radiation is associated with increased STAT1 signaling and can be modulated by fludarabine. The data support further development of therapies combining fludarabine and radiation.**


**Key words:** drug resistance; radiation resistance; STAT1; microarray

Failure of cancer treatment with chemo- as well as radiotherapy is often caused by resistance at the cellular level. The molecular mechanisms underlying drug resistance have to a large extent been elucidated through experiments *in vitro*, with cell lines selected for resistance. The mechanisms include efflux pumps, glutathione-based detoxification systems and DNA repair.[1] The primary mechanism for killing cells by ionizing radiation is through DNA damage. Thus, the search for molecular mechanisms that can explain induced radio resistance has largely focused on DNA repair.[2] DNA repair systems such as nucleotide excision repair and repair of double strand breaks have been implicated in resistance to both radiation and drugs.[3–5]

Signaling through the signal transducer and activator of transcription 1 (STAT1) has recently been suggested as an important mechanism for resistance to radiation.[6,7] Furthermore, we recently demonstrated that STAT1 expression correlates positively with doxorubicin resistance in a human tumor cell line panel.[8] STAT1 signaling has also been shown to mediate resistance to the platinum drug AMD473 in ovary cancer cells.[9] Understanding the underlying molecular mechanisms of cellular crossresistance is of particular interest, since it may open for therapeutic strategies to counteract cellular crossresistance and thus improve treatment.

In this work we used expression profiling and a pharmacological approach to explore the hypothesis that STAT1 signaling is one mechanism that contributes to acquired crossresistance to both drugs and radiation in myeloma cell lines.

## Material and methods

### *Cell culture*

The cell line RPMI 8226/S and its subline RPMI 8226/Dox40 (kind gifts from WS Dalton, AZ) were grown in culture medium RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (all from Sigma-Aldrich, St Louis, MO) at 37°C in humidified air containing 5% $CO_2$ with the doubling time of 24–36 hr for both RPMI 8226/S and 8226/Dox40 cell lines. The 8226/Dox40 cell line is selected for resistance to Doxorubicin and exhibits classical MDR phenotype with overexpression of ABCB1.[10] The resistant phenotype was treated once a month with doxorubicin (0.24 μg/ml).

### *Drugs and reagents*

Stock solutions of doxorubicin (Apoteket AB, Sweden; 2 mg/mL) and fludarabine (Schering AG, Germany; 50 mg/mL) were further diluted in sterile water. Calcein-AM (Molecular Probes, Eugene, OR) was dissolved in Dimethyl sulfoxide (DMSO; Merck Darmstadt, Germany) to a stock of 1 mg/mL and kept at −20°C until use.

Immunosuppressive analog SDZ PSC 833 was kind gift from Novartis, Basel, Switzerland, and dissolved in equal volumes of ethanol/phosphate-buffered saline (PBS) to a concentration of 1 mg/mL and kept at −70°C until further use. Fluorocein diacetate (Sigma-Aldrich) was dissolved in DMSO and stored at −20°C.

### *Measurement of drug activity*

The Fluorometric Microculture Cytotoxicity Assay is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescent fluorescein by cells with intact plasma membranes, described in detail previously.[11] Briefly, cells were seeded in V- shaped 96-well plates (Nunc, Roskilde, Denmark) containing doxorubicin at 5 different concentrations, obtained by 10-fold serial dilution and the maximum concentration was 100 μg/ml. Wells with medium only were used as blanks and the wells containing cells but no doxorubicin were used as controls. The cells were incubated at 37°C and 5% $CO_2$ for 72 hr. Then the plates were washed, FDA added and after 40 min of incubation, the fluorescence was measured in a Fluoroscan II (Labsystems Oy, Helsinki, Finland). The fluorescence is proportional to the number of living cells and data are presented as survival index, defined as the fluorescence of experimental wells in percent of control wells with blank values subtracted. The $IC_{50}$-value for fludarabine and doxorubicin in the two cell lines was

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Mårten Fryknäs and Sumeer Dhar contributed equally.

***Correspondence to:** Department of Medical Sciences, Uppsala University, Uppsala Academic Hospital, Entrance 61 3rd floor, SE-751 85 Uppsala, Sweden. Fax: +46-18 611 37 03.
E-mail: anders.isaksson@medsci.uu.se

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obtained from concentration–response curves constructed in Excel (Microsoft) and GraphPadPrism (GraphPad Software, CA).

*Fludarabine preexposure*

The RPMI 8226/S and RPMI 8226/Dox40 cell lines were fed fresh medium one day before the experiment. The following day cells were harvested and counted and the cell number was adjusted to $0.11 \times 10^6$ cells/ml. Ten milliliter of the cell suspension was added into each flask. Fludarabine was added to the cell suspension to yield the final concentration of 50 μg/ml. Cells unexposed to fludarabine were used as control. After 2 h of fludarabine exposure, the cells were collected and washed once with medium and resuspended to get the final cell number of $1.1 \times 10^6$ in 10 ml medium. The suspensions were made in duplicates; one sample was used for the qRT-PCR analysis, and one for testing the response to doxorubicin and radiation. For qRT-PCR analysis, the cells were washed 3 times with PBS and the pellets were frozen at −70°C.

*Calcein-AM uptake assay*

The Calcein-AM uptake assay was performed as described previously.[12] Briefly, V-shaped 96-well microtiter plates (Nunc, Roskilde, Denmark) were prepared with 20 μl of PSC 833 and fludarabine at 10 times the desired final concentration with a working concentration of 3 and 500 μg/ml for PSC 833 and fludarabine, respectively. RPMI 8226/S and 8226/Dox40 cells were harvested and the suspensions were prepared as described earlier. Cells were adjusted to $0.5 \times 10^6$ cells per ml and 180 μl of the cell suspension was seeded into the plates. Twenty microliter of 50 μg/ml Calcein-AM was added to the wells except in the row containing only PBS. The row with PBS and calcein-AM served as blank. The Calcein-AM uptake was measured for 80 min at 10 min intervals using Fluoroscan II (485 EM and 538 EX; Labsystems Oy, Helsinki, Finland). The Calcein-AM uptake was calculated as the ratio between the fluorescence from test wells and blank wells.

*Response to radiation*

The RPMI 8226/S and 8226/Dox40 cell lines were fed fresh medium 1 day before the experiment. The following day cells were harvested and counted. A cell suspension consisting of $0.11 \times 10^6$ cells/ml medium was prepared and a total of 11 ml cell suspension for each cell line in triplicate was prepared in T25 flasks. Cells were exposed to γ-radiation using a $^{137}Cs$ source at a dose of 4 or 8 Gy at a dose rate of 1.209 Gy/min. After irradiation the cells were further incubated at 37°C for 48 hr. At the end of the incubation, cells were harvested and resuspended in 1 ml medium. To measure the viability of the cells the dye trypan blue was used as previously described.[13,14] In brief, 20 μl of the cell suspension was diluted with 20 μl of trypan blue dye and mixed well. Subsequently viable cells (excluding trypan blue) were counted in a hemocytometer and the percentage of viable cells compared to untreated control was determined.

*Western blot analysis*

Whole cell extracts were prepared as described previously.[15] Nuclear and cytoplasmic extracts were prepared according to Andrews and Faller, in the presence of complete™ protease inhibitor (Boehringer Mannheim, Mannheim, Germany), 1 mM DTT, 1 mM PMSF, 0.1 mM $NaVo_3$, 10 mM NaF, 50μM $NaMoO_4$ and 1 mM $ZnCl_2$,(Sigma, St Louis, MO).[16] Five microgram of the extracts were fractionated on Novex NuPAGE (10%) precast gels using the Novex electrophoresis and blotting system (Novex, San Diego, CA). Primary antibodies used were as follows: α-pY701-Stat1 (no. 9171, Cell Signalling Technology, Beverly, MA), and α-Stat1 (sc-464) and α-actin (sc-1616) (Santa Cruz Biotechnology, Santa Cruz, CA). For detection, horseradish peroxidase HRP-linked antibodies (DAKO, Glostrup, Denmark) and ECL plus™ (Amersham) were used. The bands were quantified using a Fujix Bio-Imaging Analyzer Base 2000 (Fuji, Stockholm, Sweden).

*RNA extraction*

Total RNA was extracted from each cell line starting from $10^7$ cells, using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The purity of the RNA was ensured by measuring the optical density at 260 and 280 nm. The integrity of the RNA was controlled by capillary electrophoresis using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Only pure RNA (OD 260/280 >1.8) without any sign of ribosomal RNA degradation was used in the subsequent experiments.

*Oligo arrays*

The human oligo set (version 2, Operon) was dissolved in (30 μl) 30% DMSO to get a final concentration of 20 μM and stored at −20°C. Each 384-well plate was thawed and put on a shaker for 1 hr before printing. The oligos were printed with a Cartesian Prosys 5510A (Cartesian Technologies) on UltraGAPS (Corning Life Sciences). Sixteen pins (Stealth, Telechem) were used in a $4 \times 4$ configuration. The printing conditions were 25°C and a relative humidity of 45%. The slides were allowed to dry for 48 hr after spotting and then UV-crosslinked with 800 mJ using a UV-Stratalinker (Stratagene).

*Microarray experimental procedure and data analysis*

Samples were labeled and hybridized to arrays using Array 900™ Expression Array Detection Kit (Genisphere, Hatfield, PA), according to manufacture's protocol. Briefly, 1 μg total RNA from RPMI 8226/S and 8226/Dox40 were labeled for detection with either cy3 or cy5 dendrimers, combined and hybridized to oligoarrays. The experiment was performed in quadruplicates, with dye reversal (two in each direction). The microarrays were scanned in a GenePix 4000B scanner (Axon Instruments, Union City, CA) at wavelengths 635 and 532 nm for Cy5 and Cy3 dyes, respectively, using 5-μm resolution. Microarray images were analyzed with GenePix Pro software version 5.0 (Axon Instruments). Obvious artifacts, and spots not identified by the GenePix 5.0 software, were removed from further analysis. The data were stored and analyzed using the database system BASE,[17] which had been modified at the Linneaus Centre for Bioinformatics (LCB), Uppsala University, Sweden. Statistical analysis was performed in the analysis environment LCB Data Warehouse (https://dw.lcb.uu.se) using the R package (http://www.r-project.org) LIMMA. First systematic variation was removed by print-tip lowess normalization, and then the moderated *t*-test SAM was used to search for differentially expressed genes between the two cell lines.[18,19] The MIAME compliant data set is available to the research community through the ArrayExpress expression data repository at the EMBL (www.ebi.ac.uk/arrayexpress) using the accession number E-BASE-2 and A-BASE-2.

*Quantitative real-time PCR (qRT-PCR) validation of microarray data*

Reverse transcription of 2 μg total RNA was performed with Omniscript RT kit (Quiagen) in a volume of 20 μl using random hexamers and RNaseOUT (Invitrogen) according to the protocol of the manufacturer. TaqMan primers and probes were ordered as Assay on demand (Applied Biosystems) for 18S rRNA (product no. 4319413E), ISGF3G (Assay ID NM_006084) CXCL10 (Assay ID Hs00171042_m1), G1P2 (Assay ID Hs00192713_m1), G1P3 (Assay ID Hs00242571_m1), STAT1 (Assay ID Hs01013989_m1), IFI27 (Assay ID Hs00271467_m1) and ABCB1 (Hs00184491_m1). Amplifications were performed in 25 μl reactions using TaqMan Universal PCR MasterMix (Applied Biosystems). All PCR reactions were performed in triplicates on ABI PRISM 7000 Sequence Detector (Applied Biosystems) with the following thermocycling conditions: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The data were analyzed and converted into threshold cycle ($C_t$) values with use of the ABI Prism 7000 software system (Applied Biosystems). The $C_t$ values were then translated into relative cDNA copy numbers by comparison to $C_t$ values from serial

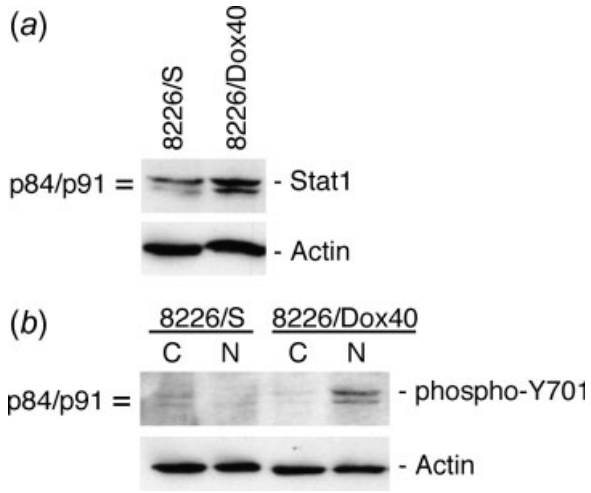


**Figure 1** – STAT1 protein expression and Tyr-701 phosphorylation. Whole cell extracts or nuclear (N) and cytoplasmic (C) extracts were prepared from RPMI 8226/S and 8226/Dox40 cells, and w.b. was performed using the indicated antibodies. (*a*) Expression of the Stat1 84 and 91 kDa species in whole cell extracts. (*b*) Cytoplasmic/Nuclear expression of phospho-tyrosine (Y701)-Stat1, a phosphorylation event required for dimerization and nuclear translocation.

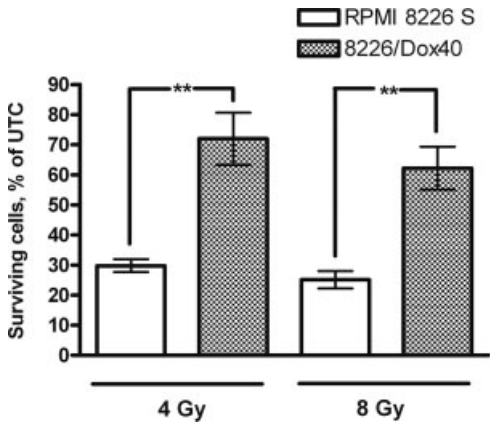


**Figure 2** – Response to radiation in RPMI 8226/S and 8226/Dox40 cells. The cell lines were exposed to 4 and 8 Gy of radiation in triplicates and the percentage of surviving cells compared to untreated controls (UTC) were assayed by trypan blue exclusion. The data were presented as mean ± SD based on triplicate measurements. The results are representative of three independent experiments. ** indicates $p < 0.01$.

dilutions. The expression levels for each gene in each sample were normalized to the expression of 18S rRNA.

## Results

### *The STAT1 expressing cell line 8226/Dox40 is resistant to both doxorubicin and radiation*

The cell line 8226/Dox40 has been selected for resistance to doxorubicin and is known to overexpress the MDR1 (ABCB1) efflux protein.[10,20] We have shown in a previous study that 8226/Dox40 expresses more STAT1 mRNA than its parental cell line RPMI 8226/S and that STAT1 expression is correlated to doxorubicin resistance.[8] The resistant cell line also showed increased expression of STAT1 protein levels, and displayed a 12-fold higher nuclear expression of Tyr-701 phosphorylated STAT1 (Fig. 1). Phosphorylation of Tyr-701 is required for dimerization and nuclear translocation, consistent with more active STAT1 in 8226/Dox40. To investigate whether STAT1 expression in the 8226/Dox40 cell line is also associated with resistance to radiation, cells were exposed to 4 and 8 Gy of radiation and cell survival was analyzed by trypan blue exclusion (Fig. 2). The percentage of surviving cells was significantly higher in the 8226/Dox40 cell line compared to the parental line for both doses of radiation ($p < 0.01$).

### *Genes involved in α/β-interferon signaling are overexpressed in the resistant cell line*

A genome-wide search for genes that in addition to STAT1 are associated with resistance to both types of exposures was conducted using oligonucleotide arrays that measured mRNA levels of over 20,000 genes. The hybridization was repeated 4 times and genes differentially expressed in the resistant cell line compared to the parental cell line were identified using Significance Analysis of Microarray data (SAM).[19] With an estimated false discovery rate of 0.73%, 50 genes were found to be more highly expressed in 8226/Dox40 (3- to 85-fold), while 60 genes were expressed to lower levels (supplementary information Table S1 available at our website; www.medsci.uu.se/klinfarm/pharmacology/supplement.htm). The top 50 overexpressed genes are listed in Table I together with their fold overexpression in the resistant cell line. The increased expression of a subset of these genes was validated using quantitative RT-PCR (Table I). One of the highly overexpressed genes (32-fold) was the efflux protein ABCB1 (MDR1) previously described to confer drug resistance in the 8226/Dox 40 cell line.[10,20] STAT1 was also found to be overexpressed (5-fold) and it is interesting to note that 16 other genes previously reported to be involved in α/β-interferon signaling are overexpressed. A majority of these genes contain interferon-stimulated response elements (ISREs) in their promoter regions. These data suggest that STAT1 is involved in the overexpression of a large fraction of the identified genes since activated STAT1, as a component of the interferon-stimulated gene factor 3 (ISGF3) transcription factor complex, is able to initiate transcription through binding to ISRE elements.[21] Real-time PCR was conducted to investigate the expression level of ISGFγ, the DNA binding subunit of the ISGF3 transcription factor complex. ISGFγ mRNA was expressed to 4-fold higher levels in 8226/dox40 compared to RPMI 8226/S (data not shown).

### *Short fludarabine exposure reduces STAT1 signaling*

To explore whether STAT1 signaling can mediate resistance to radiation and doxorubicin, we used a pharmacological approach to modulate the activity of the STAT1 signaling pathway. The anticancer drug fludarabine has previously been shown to reduce STAT1 phosphorylation (serine 727) and to downregulate STAT1 expression.[22–25]

We studied the molecular effect of a short fludarabine exposure (50 μg/ml for two hours) on STAT1 and a downstream target, the ISRE element containing gene Chemokine (C-X-C motif) ligand 10 (CXCL10), in the 8226/Dox 40 cell line. The mRNA expression was monitored for 48 hr postexposure using quantitative RT-PCR. Fludarabine exposure caused a decreased expression of both STAT1 and CXCL10 mRNA. The expression of both genes was reduced 5-fold after 2 hr and the effect of fludarabine persisted for more than 24 hr postexposure (Figs. 3*a* and 3*b*).

### *Fludarabine preexposure reduces resistance to doxorubicin*

To investigate whether fludarabine preexposure also affects resistance to doxorubicin, the myeloma cell lines were first exposed to fludarabine (50 μg/ml for 2 h), followed by 72 hr doxorubicin exposures. The $IC_{50}$ value for doxorubicin was determined for RPMI 8226/S and 8226/Dox40, either with or without fludarabine preexposure. Fludarabine preexposure made the resistant cells more sensitive to doxorubicin, while it had no sensitizing effect on the parental cell line (Figs. 4*a* and 4*b*).

**TABLE I –** THE 50 MOST UP-REGULATED GENES, RANKED BY SIGNIFICANCE ANALYSIS OF MICROARRAYS (SAM), IN THE DOXORUBICIN RESISTANT SUBLINE 8226/DOX40 COMPARED TO THE PARENTAL CELL LINE RPMI 8226/S

| Name | Symbol | Acc. | Fold-change | |
|---|---|---|---|---|
| | | | Microarray | qRT-PCR |
| **Myxovirus resistance 1, interferon-inducible protein p78**[1] | **MX1** | **NM_002462** | **14** | |
| **Chemokine (C-X-C motif) ligand 10**[1] | **CXCL10** | **NM_001565** | **47** | **79** |
| ATP-binding cassette, sub-family B (MDR/TAP), member 1 | ABCB1 | NM_000927 | 32 | |
| **Chemokine (C-X-C motif) ligand 9** | **CXCL9** | **NM_002416** | **16** | |
| **Major histocompatibility complex, class II, DM alpha** | **HLA-DMA** | **NM_006120** | **10** | |
| **Interferon, alpha-inducible protein (clone IFI-15K)**[1] | **G1P2** | **NM_005101** | **11** | **37** |
| Paralemmin | PALM | NM_002579 | 10 | |
| **Major histocompatibility complex, class II, DP alpha 1** | **HLA-DPA1** | **NM_033554** | **15** | |
| N-acetylneuraminate pyruvate lyase | NPL | NM_030769 | 6 | |
| Fc receptor-like and mucin-like 1 | FREB | NM_032738 | 6 | |
| **Interferon-induced protein with tetratricopeptide repeats 1**[1] | **IFIT1** | **NM_001548** | **16** | |
| Interleukin 8 | IL8 | NM_000584 | 10 | |
| Chromosome 2 open reading frame 23 | C2orf23 | NM_022912 | 5 | |
| Regulator of G-protein signalling 1 | RGS1 | NM_002922 | 8 | |
| **Interferon, alpha-inducible protein (clone IFI-6-16)**[1] | **G1P3** | **NM_022873** | **8** | **18** |
| **Interferon regulatory factor 7**[1] | **IRF7** | **NM_004031** | **4** | |
| S100 calcium binding protein A6 | S100A6 | NM_014624 | 4 | |
| CD9 antigen | CD9 | NM_001769 | 5 | |
| Tumor protein p53 inducible protein 5 | TP53I5 | AK026923 | 8 | |
| **CD74 antigen** | **CD74** | **NM_004355** | **5** | |
| P antigen family, member 5 | PAGE-5 | BC009230 | 5 | |
| Mesoderm specific transcript homolog | MEST | NM_002402 | 10 | |
| **Signal transducer and activator of transcription 1,91 kDa**[1] | **STAT1** | **NM_007315** | **5** | **10** |
| CD83 antigen | CD83 | NM_004233 | 5 | |
| Keratin 15 | KRT15 | NM_002275 | 4 | |
| EGF-like module containing, mucin-like, hormone receptor-like 1 | EMR1 | NM_001974 | 5 | |
| Hypothetical protein FLJ20035 | FLJ20035 | AL137464 | 6 | |
| Lipase, endothelial | LIPG | NM_006033 | 7 | |
| **Major histocompatibility complex, class II, DQ beta 1** | **HLA-DQB1** | **NM_002123** | **7** | |
| Inosine monophosphate dehydrogenase 1 | IMPDH1 | NM_000883 | 4 | |
| Homolog of mouse skeletal muscle sarcoplasmic reticulum protein | FLJ32416 | AK056978 | 6 | |
| Tight junction protein 1 | TJP1 | NM_003257 | 6 | |
| Ubiquitin D | UBD | NM_006398 | 19 | |
| **Interferon, alpha-inducible protein 27**[1] | **IFI27** | **NM_005532** | **5** | **72** |
| Transcobalamin II; macrocytic anemia | TCN2 | NM_000355 | 4 | |
| Homo sapiens growth factor-binding protein-3 precursor gene | | M35878 | 4 | |
| Galanin | GAL | M77140 | 3 | |
| Carbonic anhydrase VIII | CA8 | NM_004056 | 9 | |
| Ras association domain family 4 | RASSF4 | AK055763 | 4 | |
| Myristoylated alanine-rich protein kinase C substrate | MARCKS | NM_002356 | 4 | |
| **Purkinje cell protein 4** | **PCP4** | **NM_006198** | **3** | |
| **Major histocompatibility complex, class II, DP beta 1** | **HLA-DPB1** | **NM_002121** | **5** | |
| Cathepsin O | CTSO | NM_001334 | 4 | |
| CDNA FLJ32664 fis, clone TESTI1000088 | | AK057226 | 4 | |
| Kruppel-like factor 6 | KLF6 | NM_001300 | 4 | |
| Poly polymerase family, member 12 | ZC3HDC1 | NM_022750 | 6 | |
| **Interferon-induced protein 44-like**[1] | **IFI44L** | **NM_006820** | **8** | |
| Lymphoid-restricted membrane protein | LRMP | NM_006152 | 3 | |
| Gap junction protein, alpha 1, 43 kDa | GJA1 | NM_000165 | 7 | |

Note: Genes in bold are previously reported to be involved in the Stat 1 signaling.
[1]Promotor contains ISRE element. Acc., GenBank accession number; qRT-PCR, Quantitative reverse transcription polymerase chain reaction.

To explore whether the effect of fludarabine could be mediated by a decreased efflux of small molecules previously described as a mechanism for doxorubicin resistance in this cell line 2 experiments were performed.[10] Expression of the most highly expressed efflux pump ABCB1 was measured using quantitative RT-PCR in the two cell lines before and after fludarabine exposure. There was no significant difference in ABCB1 mRNA levels in 8226/Dox40 before and after exposure (data not shown). In addition, the functional activity of drug efflux proteins including ABCB1 was measured as internal accumulation of the fluorescent reporter compound Calcein-AM. The results show that accumulation of Calcein-AM in 8226/Dox40 is dependent on efflux pumps since accumulation can be increased by the broad-specificity inhibitor PSC 833.[26] However, there was no difference in accumulation of Calcein-AM in the 8226/Dox40 cell line after preexposure to fludarabine (Fig. 5). Thus, reduced efflux of doxorubicin and thereby increased effective cellular concentration cannot explain the increased sensitivity due to fludarabine preexposure in the 8226/Dox40 cell line.

*Fludarabine preexposure enhances sensitivity to radiation*

We also tested whether preexposure to fludarabine (50 μg/ml for 2 hr) combined with radiation could enhance the effect of radiation alone (Fig. 6). Fludarabine exposure alone had a slight effect on both the resistant and parental cell line as measured by trypan blue exclusion, but there was no significant difference between the cell lines. The cell survival after irradiation in the 8226/Dox40 cell line was significantly reduced after preexposure to fludarabine ($p < 0.01$), whereas no significant effect was observed in the parental cell line. Thus, a reduction in STAT1 signaling is associated with an increased sensitivity to both radiation and fludarabine in the 8226/Dox40 cell line.

## Discussion

The present study provides a detailed view of mRNA expression in 8226/dox40 compared to its parental cell line and detected

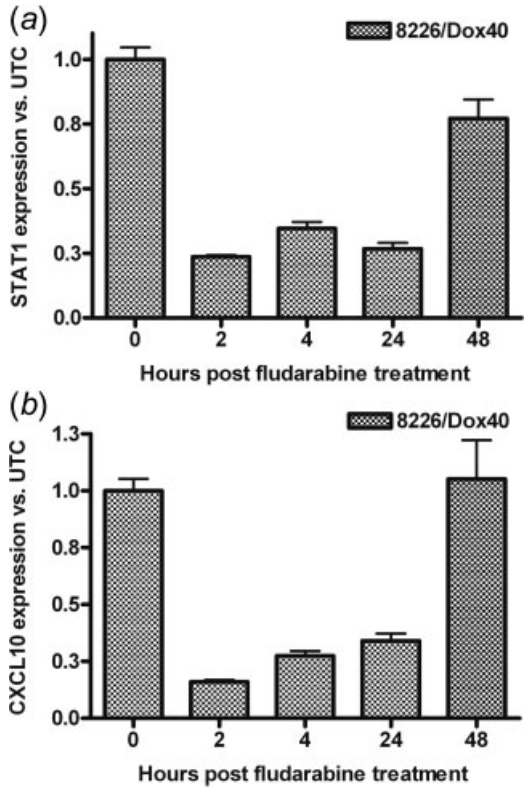


**Figure 3** – Gene expression levels after fludarabine exposure *versus* untreated control (UTC). The cell line 8226/Dox40 was exposed to fludarabine for 2 hr and mRNA levels were measured in triplicates by quantitative real-time PCR after the indicated periods of time and normalized to 18S RNA levels (mean ± SD in arbitrary units). (*a*) Reduction of STAT1 mRNA expression after exposure to fludarabine. (*b*) Reduction of *CXCL10* mRNA expression after exposure to fludarabine.

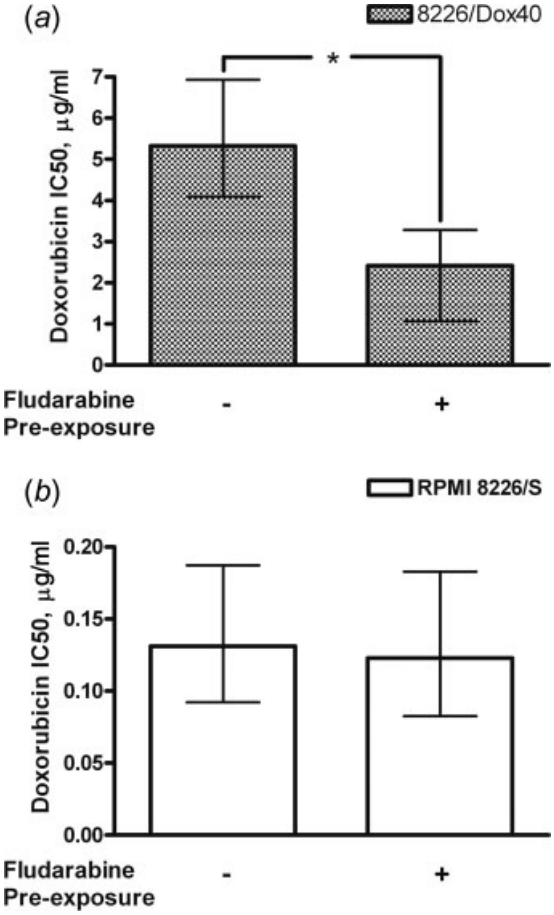


**Figure 4** – Doxorubicin $IC_{50}$ values in 8226/Dox40 and RPMI 8226/S, with or without a 2 hr preexposure with fludarabine. Error bars indicate the 95% confidence interval. Three identical experiments were performed with qualitatively similar results. * indicates $p < 0.05$.

overexpression of 17 genes in the STAT1 signaling pathway. In a previous study one of these genes, interferon-inducible 56 kDa protein (IFIT1), was identified as one of the most overexpressed genes in 8226/Dox40 compared to RPMI 8226/S.[27]

The effect of IFN-α/β (type I IFNs) on transcription has been studied extensively.[21] Briefly, Type I IFNs binds to the type 1 interferon receptor and activates STAT1, STAT2 and ISGFγ, which form the trimeric complex ISGF3. ISGF3 translocates into the nucleus where it binds to ISRE sequences and initiates transcription of interferon-stimulated genes.[21,28] We observed mRNA overexpression of both STAT1 and ISGFγ in 8226/Dox40, as well as a number of genes containing ISRE elements. STAT1 protein was over expressed and activated STAT1 (phosphorylated at Tyr-701) showed nuclear enrichment in the resistant cell line. Taken together these results suggest augmented activity of the STAT1 signaling pathway.

Fludarabine preexposure of the 8226/Dox40 cell line reduced STAT1 signaling as indicated by reduced expression of STAT1 and CXCL10. These experiments show that a clinically used drug can reduce doxorubicin resistance with about 2-fold. It should be pointed out that the 8226/Dox40 cells after fludarabine preexposure were still about 20-fold more resistant to doxorubicin than the parental cell line, presumably due to higher efflux capability (Fig. 5). Moreover, fludarabine appears to potentiate the effect of radiation in the resistant cell line but not in the parental line. Taken together these results suggest that STAT1 signaling is involved in resistance to both radiation and doxorubicin in the same cells.

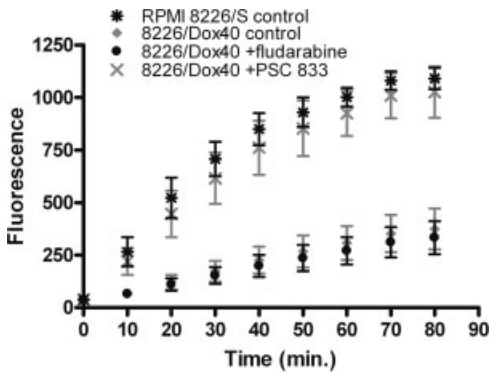


**Figure 5** – Calcein-AM uptake over time in RPMI 8226/S (*) and 8226/Dox40 (♦). Although the ABC transport inhibitor PSC 833 almost completely restored accumulation in the 8226/Dox40 cells (crosses), fludarabine had no effect (circles). The data is presented as mean ± SD from quadruplicate measurements.

Interferon exposure has previously been shown to have an anti-apoptotic effect in combination with radiation.[6,29,30] *STAT1* is one of the downstream genes of interferon signaling and has also previously been implicated in resistance to a number of stimuli. *STAT1* and many ISRE containing genes were found to be overexpressed after serial exposure to low doses of radiation in a mouse

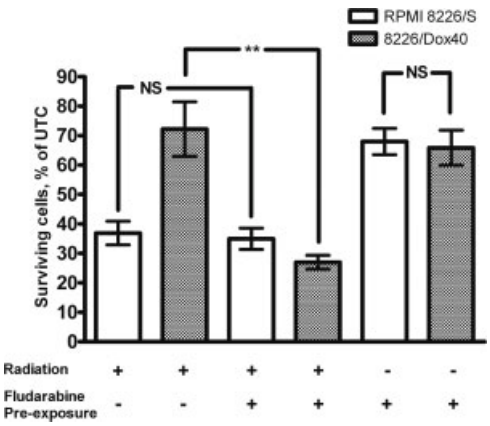


**FIGURE 6** – Sensitivity to 4 Gy of radiation without or with 2 hr pre-exposure to fludarabine in the RPMI 8226/S and 8226/Dox40 cell lines. The percentage (mean ± SD) of surviving cells after 48 hr is compared to untreated control (UTC). The 8226/Dox40 cell line cell is significantly sensitized to radiation after fludarabine exposure (two-tailed *t*-test). ** indicates $p < 0.01$. NS indicates not significant.

xenograft model with SCC-61 tumor cells, and caused radio resistance.[7] Antisense experiments reducing the mRNA level of one of the further downstream ISRE containing genes, IFITM1, led to an increased sensitivity to radiation in human RSa cells.[6] Cell lines selected for resistance to etoposide and cisplatin overexpress genes involved in STAT1 signaling.[31] ISRE containing genes were also found to be over-expressed in a tamoxifen resistant mammary carcinoma subline MaCa 3366/TAM.[32] Moreover, we previously found that STAT1 expression to be highly correlated to doxorubicin resistance in a panel of cell lines including cell lines resistant to doxorubicin, melphalan and vincristine.[8] The evidence for the functional importance of STAT1 signaling in drug resistance has recently been substantiated when STAT1 was shown to be a key component for resistance to cisplatin in ovarian cancer cell lines. Similarly to the work presented here a drug reducing STAT1 signaling increased sensitivity to the platinum drug AMD473.[9] Furthermore, prostate cancer cell lines selected for docetaxel resistance showed overexpression and activation of STAT1. The docetaxel resistance could be effectively reversed by STAT1 directed siRNA giving unequivocal proof of its importance in acquired docetaxel resistance.[33]

Not all ISRE containing genes are overexpressed in all examples of STAT1 associated resistance studied with microarrays.[9,31,32] This suggests that a subset of the genes involved in STAT1 signaling have more important functional roles in the resistant phenotypes. Antisense experiments from Kita *et al*. suggested that IFITM1 are required for radio-resistance in the human RSa cell line and Thahara *et al*. showed that G1P3 is antiapoptotic in the gastric cancer cell line TMK-1.[6,34] However, little is known about the molecular functions and biological processes of interferon stimulated genes downstream of STAT1.[35]

The ISRE containing genes are overexpressed in cells with acquired resistance to a wide variety of noxious challenges, such as drugs and radiation. Thus, it appears that they are a part of a response mechanism to adverse environmental conditions. As previously mentioned cisplatin resistance has been shown to increase STAT1 signaling and it can be modulated by reduced STAT1 signaling.[9] With this information in mind it is interesting to note that a synergistic effect of combining fludarabine and cisplatin is exerted through a DNA-repair-inhibitory effect of fludarabine.[36,37]

Some limitations of the study should be discussed. First, cell lines removed from their *in vivo* environment and selected for growth in culture differ from *in vivo* tumor cells. Therefore, the relevance of the identified pathway needs to be studied in additional settings, such as primary cultures of tumor cells from patients. Second, the effects of drugs and radiation were generated using single end point short-term assays. Drugs and radiation may induce dose dependent effects on different targets, leading to different modes of cell death, including apoptosis, necrosis and cell senescence.[38] However, short-term assays such as the trypan blue exclusion test are reliable, robust and correlates well with clonogenic assays.[39,40]

STAT1 mRNA expression has been reported to be increased in a number of human tumors including both hematopoetic malignancies and solid tumors such as breast cancer and squamous cell carcinoma of the head and neck.[41–43] Notably, STAT1 expression has been reported to be higher in tumors compared with corresponding normal tissue for a wide range of tumor types (www.oncomine.com). Therefore, treatment strategies targeting STAT1 signaling in combination with other treatments may be important for a wide variety of tumors.

The identification of STAT1 as a possible component and downstream effectors of resistance to radiation and cytotoxic drugs allows the development of compounds that can reduce crossresistance and provide higher efficacy and specificity. Fludarabine is a commonly used antitumor drug that can be combined with conventional drug treatment. Interestingly, in a recent study fludarabine was used as a second-line treatment for recurring chronic lymphoblastic leukemia (CLL) where primary treatment with doxorubicin had failed.[44] Based on the observations in our study, it is tempting to speculate that the treatment of recurring forms of CLL and possibly other types of tumors may benefit from inclusion of fludarabine, because of a reduced cellular resistance. In this context is also interesting to note that fludarabine has been shown to potentiate radiation treatment of solid tumors in mice.[45–47] Moreover, a phase I trial shows that fludarabine can safely be administered concurrently with radiation.[48] The present data support further development of therapies using fludarabine combined with radiation.

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