Molecular Pharmacology Fast Forward. Published on February 9, 2012 as doi:10.1124/mol.111.075994

Title page

Regulation of interferon-inducible proteins by doxorubicin via IFNγ-JAK-STAT signaling in tumor cells

J. Hussner, MS; S. Ameling, PhD; E. Hammer, PhD; S. Herzog, MS; L. Steil, PhD; M. Schwebe, MS; J. Niessen, PhD; H.W.S. Schroeder, MD; H.K. Kroemer, PhD; C.A. Ritter, PhD; U. Völker, PhD; S. Bien, PhD

Department of Pharmacology (S.B., J.H., S.H., J.N., M.S., H.K.K.), Interfaculty Institute for Genetics and Functional Genomics (S.A., E.H., L.S., U.V.), Institute of Pharmacy (C.A.R.) and Clinic of Neurosurgery (H.W.S.S.), Ernst-Moritz-Arndt University, Greifswald, Germany

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title Page

Running Title: IFNγ-JAK-STAT signaling upon doxorubicin

Address for correspondence: Sandra Bien, PhD
Institute of Pharmacology
Ernst-Moritz-Arndt University
Felix-Hausdorff-Str. 3
17487 Greifswald
Germany
Phone: +49 3834 865646
FAX: +49 3834 865631
Email: sbien@uni-greifswald.de

Text pages: 25

Tables: 2

Figures: 5

References: 37

Number of words:		
	Abstract:	245
	Introduction:	508
	Discussion:	1494

Abbreviations: DMSO, Dimethylsulfoxide (solvent); Dox, doxorubicin; STAT1, Signal Transducer and Activator of Transcription 1; IFI6-16, Interferon-Inducible Protein 6; IFI27, Interferon-Inducible Protein 27; IFI30, Interferon-Inducible Protein 30; IFI35, Interferon-Inducible Protein 35; IFIT4, Interferon-Induced Protein with tetratricopeptide repeats; IFITM1, Interferon-Induced Transmembrane Protein 1; ISGF3G, Interferon-Stimulated Transcription Factor 3 gamma; NMI, N-myc and STAT interactor; TFIID, Transcription Initiation Factor IID

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Activation of the immune system is a way for host tissue to defend itself against tumor growth. Hence, treatment strategies that are based on immunomodulation are on the rise. Conventional cytostatic drugs such as the anthracycline doxorubicin can also activate immune cell functions of macrophages and natural killer cells. In addition, cytotoxicity of doxorubicin can be enhanced by combining this drug with the cytokine IFNγ. Although doxorubicin is one of the most applied cytostatics, the molecular mechanisms of its immunomodulation ability are not investigated thoroughly. In microarray analyses of HeLa cells, a set of 19 genes related to interferon signaling was significantly overrepresented among genes regulated by doxorubicin exposure including STAT-1, -2, IRF9, NMI, and caspase 1. Regulation of these genes by doxorubicin was verified with Real-Time PCR and immunoblotting. An enhanced secretion of IFNγ was observed when HeLa cells were exposed to doxorubicin as compared to untreated cells. IFNγ neutralizing antibodies and inhibition of JAK-STAT signaling (ATA, AG490, STAT1 siRNA) significantly abolished doxorubicin-stimulated expression of interferon signaling-related genes. Furthermore, inhibition of JAK-STAT signaling significantly reduced doxorubicin induced caspase 3 activation and desensitized HeLa cells to doxorubicin cytotoxicity. In conclusion, we demonstrate that doxorubicin induces interferon-responsive genes via IFNγ-JAK-STAT1 signaling and that this pathway is relevant for doxorubicin's cytotoxicity in HeLa cells. As immunomodulation is a promising strategy in anticancer treatment, this novel mode of action of doxorubicin may help to further improve the use of this drug among different types of anticancer treatment strategies.

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Since its discovery in 1969 doxorubicin is one of the most effective anticancer drugs used in a wide variety of malignancies (Di Marco *et al.*, 1969). Despite its longstanding clinical application the mechanism of action of doxorubicin has not been completely revealed yet. At the cellular level, the cytotoxic effects of doxorubicin involve direct DNA intercalation with impaired DNA and RNA synthesis, topoisomerase II inhibition resulting in DNA strand breaks, generation of free oxygen radicals, lipid peroxidation, alterations in membrane structure and function (Minotti *et al.*, 2004) as well as induction of apoptosis (Gamen *et al.*, 1997). As early as 1973, Schwartz et al. proposed that immune defense mechanisms of the host against tumor antigens may contribute to the effectiveness of doxorubicin (Schwartz and Grindey, 1973). This suggestion was later proven by several groups who showed that doxorubicin induces specific immune functions and cytokine expression which leads to increased tumoricidal potential of cells of the macrophage and natural killer type in mice (Maccubbin *et al.*, 1992;Ehrke *et al.*, 1984). Ujhazy and co-workers could demonstrate that the greater anti-tumor potential of peritoneal cells from doxorubicin-treated mice is accompanied by enhanced production of cytokines such as TNF, IL1 and IFNγ (Ujhazy *et al.*, 2003b). Subsequently, it was shown that cytotoxic lymphocytes used in conjunction with doxorubicin are effective in treating murine renal cell cancer (Salup *et al.*, 1987). In addition, in patients receiving doxorubicin, increased cytotoxic T-cell function, IL-2 levels, numbers of immature monocytes and of $CD8^+$ T cells were found (Arinaga *et al.*, 1986). However, knowledge about molecular mechanisms involved in the doxorubicin-induced immunomodulation caused by alterations on the tumor cells themselves is still very limited.

In addition to the immunomodulatory effects of doxorubicin described above, an enhancement of the antitumoral efficacy of doxorubicin can be achieved by its combination with the cytokine interferon-γ (IFNγ) without increased toxicity (Eppstein *et al.*, 1988). IFNγ

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

itself is well known to play crucial roles in several aspects of the immune response or immunomodulation as well as in immunosurveillance of malignant cells (Kaplan *et al.*, 1998). Considering immune regulatory roles of IFNγ, doxorubicin-induced immunomodulation and the synergistic antitumoral effect of doxorubicin and IFNγ, we performed transcriptome analyses in the tumor cell line HeLa in order to investigate whether doxorubicin influences expression of genes with immune function. Using this microarray approach, we revealed up-regulation of a cluster of interferon-regulated genes with partially known immunomodulatory functions which was verified with Real-Time PCR and immunoblotting and compared with tumor cells of different origin. Pre-treatment of cells with AG490 and ATA, inhibitors of JAK-STAT signaling (Wang *et al.*, 1999b;Chen *et al.*, 2002), as well as neutralizing antibodies against IFNγ and IFNγ receptor 1 (IFNGR1) and silencing of STAT1 by the use of siRNA reduced the up-regulation of most of the investigated genes. Additionally, we demonstrate that doxorubicin treatment results in enhanced secretion of IFNγ as well as JAK1 and STAT1 activation. Therefore, we conclude that the IFNγ-JAK1-STAT1 cascade mediates doxorubicin-induced regulation of interferon-inducible genes and immunomodulatory effects that are associated with the administration of this cytostatic drug.

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Chemicals and Antibodies

Doxorubicin hydrochloride, aurintricarboxyl acid (ATA) and recombinant human IFNγ were obtained from Sigma Aldrich (Deisenhofen, Germany). AG490 was purchased from Calbiochem (Darmstadt, Germany). Trypsin and RPMI1640 were from PAN (Aidenbach, Germany) and fetal bovine serum was obtained from Invitrogen. Neutralizing anti-Human Interferon gamma Receptor Chain 1 (anti-IFNGR1) and neutralizing anti-Human Interferon gamma (anti-IFNγ) were from US Biological/Biomol (Hamburg, Germany). The following unconjugated primary antibodies were used for immunoblotting: anti-STAT1, anti-caspase 1 (both Santa Cruz Biotech), anti-IFI35, anti-NMI, anti-ISGF3G (all from Abnova, Heidelberg, Germany), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Biodesign Int., Saco, USA), and for detection of phosphorylated forms of STAT1, STAT2 and STAT3 the Phospho-STAT Antibody Sampler Kit (Cell Signaling Technology, Frankfurt am Main, Germany) was used.

Cell culture

The human cervix carcinoma cell line HeLa, the colon carcinoma cell line Caco2, the breast cancer cell line (MCF-7) and the hepatocarcinoma cell line HepG2 were obtained from the American Type Culture Collection and maintained by culture in RPMI 1640 or DMEM medium supplemented with 10% FCS, 1% glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. For expression analysis, cells were seeded 24 h before drug treatment in 6-well plates (300,000 cells per well) and were cultured for the respective time points with doxorubicin, IFNγ or DMSO and/or PBS as solvent. In some experiments, cells were pre-incubated for 1.5 h with ATA, AG490 or neutralizing antibodies.

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Transcriptional profiling experiments

Total RNA of one batch Doxorubicin treated and untreated (DMSO) HeLa cells for two timepoints (24 h, 48 h) was extracted using Trizol PeqGold RNA Pure (Peqlab, Erlangen, Germany) according to the manufacturer`s protocol. T7-RNA polymerase-mediated linear amplification was performed starting with 1 µg RNA (One cycle Target labelling protocol; Affymetrix, Santa Clara, CA, USA). Size-distribution of biotin-labeled amplified RNA was analyzed with an Agilent 2100 BioAnalyzer, and concentrations were photometrically determined. For transcriptome analysis, Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) were employed. After hybridization and washing, arrays were scanned using the GeneChip System Confocal Scanner 3000 (Affymetrix). Expression data have been submitted to GEO.

Gene Chip Operating software (GCOS) with MAS5.0 was used for data extraction. Expression raw data were transferred to GeneSpring GX version 7.3.1. (Agilent Technologies) and per-chip normalized. Genes which were flagged as present or marginal and exceeding a fold change of 2 in comparison to control were analysed in Ingenuity Pathway Analysis rel.8.5 (Ingenuity Systems) for functional classification.

Quantitative Real-Time PCR (qRT-PCR)

Following treatment with the respective substances, cells were harvested and total RNA was isolated using the PeqGold RNAPure reagent protocol. 500 ng of total RNA was used for cDNA synthesis in a 20 µl reaction volume. Real-Time PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). cDNA was amplified using Assays on Demand for STAT1 (Hs00234829_m1), STAT2 (Hs00237139_m1), IFI6 (Hs00242571_m1), IFI27 (Hs00271467_m1), IFI30 (Hs00173838_m1), IFI35 (Hs00413458_m1), IFIT4 (Hs00382744_m1), IFITM1 (Hs01652522_g1), ISGF3G/IRF9 (Hs00196051_m1), NMI (Hs00190768_m1), Caspase 1 (Hs00354832_m1), all conjugated

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

with fluorochrome FAM, and 18S rRNA (Pre-developed TaqMan® Assay Reagent) conjugated with fluorochrome VIC (Applied Biosystems). Quantitation was performed with the comparative $\Delta\Delta C_T$-method.

Immunoblot analysis

Following treatment with the respective substances, cells were solubilized in lysis buffer (pH 7.4) consisting of 1% Triton X-100, 100 mM NaCl, 50 mM Tris, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM aprotinin. The amount of protein was determined by the BCA method using bovine serum albumin. 50 µg of protein was separated on a 10% SDS-polyacrylamide gel electrophoresis followed by electrophoretic transfer to nitrocellulose membranes. The membranes were blocked using 5% non-fat milk in 150 mM NaCl, 10 mM Tris, pH 8.0 with 0.05% Tween 20 for 1 h followed by incubation with the primary antibodies for 1 h at room temperature (anti-GAPDH or overnight at 4°C (anti-STAT1, anti-caspase 1, anti-NMI, anti-IFI35, anti-ISGF3G or anti-phosphoSTATs, see *Chemicals And Antibodies*). Following washing, the membranes were incubated with peroxidase-conjugated secondary antibodies and developed using enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany).

Determination of JAK1 activation

For measurement of active JAK1, we used the JAK1 phospho (Tyr1022 / Tyr1023) Cell-based ELISA Kit, FACE™ from Active Motif.

Determination of transcriptional binding activity of nuclear STAT1

For assessment of binding ability of nuclear STAT1 to its respective consensus sequence, we used the *DuoSet IC STAT1 p91 activity assay* (R&D Systems) according to the manufacturer´s

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

protocol with preparation of nuclear extracts. Activity values were normalized to the nuclear protein content.

Measurement of IFNγ

Detection of IFNγ in supernatants of HeLa cells was performed by using Human IFN-gamma Quantikine ELISA Kit from R&D systems according to the manufacterer´s protocol. Values were normalized to the respective protein content of HeLa lysates.

siRNA-mediated silencing of STAT1 expression

For siRNA transfection cells were seeded at 75,000 cells/well in a 12-well multiplate. After reaching a confluence of 70-80%, cells were transfected with either SignalSilence® Control siRNA or the specific SignalSilence® Stat1 siRNA (both from Cell Signaling, each 100 nM) by use of Lipofectamine™ RNAiMAX Reagent according to the manufacturer´s protocol. 48 h later, cells were treated with either DMSO (solvent) or 1 µM doxorubicin for further 30 h followed by RNA isolation of the harvested cells, reverse transcription and quantitative RT-PCR.

Assays for Caspase 3 activity

Caspase 3 activity was measured in cell lysates with a commercially available Colorimetric assay kit (R&D Systems) according to the manufacturer´s instructions, normalized to the respective protein content and is shown as x-fold increase relative to DMSO control.

Cell viability assay

HeLa cells were seeded at 5,000 cells/well in a 96-well multiplate. The growth medium was changed after 1 day and replaced by DMSO or doxorubicin containing media with or without pre-incubation with ATA or AG490. After the respective incubation period, 1/10 of Alamar

Blue (Biosource) was added and analysis was performed according to the manufacturer´s instruction. Data were expressed as percentage of viability of DMSO treated control cells.

Statistical Analysis

Data represent at least three independent experiments and were expressed as the mean±SD. Data were analyzed using Student´s t test or One-way ANOVA. Statistical significance was defined as *p-value* < 0.05 (*<0.05, **<0.01 and ***<0.001).

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Functional categorization of differentially expressed genes upon doxorubicin treatment

For screening of changes at the transcriptome level we have performed comparative DNA microarray analysis of control and doxorubicin (1 µM) treated HeLa cells. After doxorubicin application for 48 h 2102 regulated genes (24 h, 1316 genes) were identified, displaying at least a 2 fold change. Functional classification of regulated genes at the timepoint 48 h by Ingenuity pathway analysis revealed the following most overrepresented categories: 1. Interferon signaling, 2. HGF (Hepatocyte Growth Factor) signaling, 3. Cdc42 (cell division control protein 42 homolog) signaling, 4. Activation of IRF (Interferon Regulatory Factors) signaling by Cytosolic Pattern Recognition Receptors and 5. Production of Nitric Oxide and Reactive Oxygen Species in Macrophages (Figure 1A+B and Supplemental Table 1). Obviously, many of the identified genes are related to immunomodulation and/or play a role in interferon signaling. In detail, among those genes related to interferon signaling three genes were down- and 16 were up-regulated. Likewise, among those genes related to Activation of IRF (Interferon Regulatory Factors) signaling six genes were down- and 24 up-regulated (Supplemental Table 1).

Effect of doxorubicin, IFNγ or doxorubicin plus IFNγ on IFN signaling regulatory genes

Since IFN-dependent signaling pathways were strongly affected upon doxorubicin (1 µM) we analyzed regulation of selected genes of this functional category in detail (STAT1, Signal Transducers and Activators of Transcription; IFI6, Interferon, alpha-inducible protein 6; IFI27, Interferon, alpha-inducible protein 27; IFI30, Interferon, gamma-inducible protein 30; IFI35, Interferon-induced protein 35; IFIT4, Interferon-induced protein with tetratricopeptide repeats 4; IFITM1, Interferon-induced transmembrane protein 1; ISGF3G/IRF9, Interferon-stimulated gene factor 3 gamma/Interferon Regulatory Factor 1; NMI, N-myc and Stat

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Interactor and Caspase 1). Expression data of these genes determined by microarray analysis of HeLa cells after treatment with doxorubicin for 24 or 48 h are displayed in Supplemental Table 2.

For independent validation of the genes with potential regulatory function in interferon signaling and immunomodulation, we performed quantitative RT-PCR analyses of the selected eleven genes which were differentially expressed in the presence of doxorubicin in our microarray analysis. When HeLa cells were incubated with 1 µM doxorubicin, mRNA expression increased in a time-dependent manner with a maximum at 48 h for most of the investigated genes except NMI, IFI35 (maxima at 24 h), and IFI27 (maximum at 72 h) (Figure 1C and Supplemental Table 2). Furthermore, quantitative RT-PCR assays demonstrated that the mRNA levels of all selected genes were dose-dependently up-regulated by IFNγ with the exception of IFI30 which was slightly reduced upon 50 U/ml IFNγ and unchanged upon 100 U/ml IFNγ (Supplemental figure 1).

Furthermore, we checked the consequence of co-administration of doxorubicin and IFNγ on mRNA expression of the genes of interest (Table 1). For all genes investigated, the combination of doxorubicin and IFNγ resulted in significantly higher mRNA expression than each of the compounds alone.

Release of IFNγ upon doxorubicin exposure and effects of neutralizing anti-IFNγ and anti-IFNγRI antibodies

Since IFNγ functions as a secreted cytokine by binding to its specific receptors, we tested whether exposure of HeLa cells with doxorubicin results in the release of IFNγ into the supernatant, too. As shown in Figure 2A, within 4 h of doxorubicin exposure the release of IFNγ into supernatant was significantly enhanced from 15.8 ± 0.62 (control) to 23.5 ± 2.89 pg/mg protein. Furthermore, using immunoblot analysis we were able to detect the IFNγ

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

receptor 1 (54 kDa) and 2 (37 kDa) in HeLa as well as in Caco2, HepG2 and MCF-7 cells (Figure 2D).

To determine whether doxorubicin triggers gene expression via the IFNγ/IFNγ-receptor pathway, we performed inhibition experiments for selected genes using neutralizing antibodies against IFNγ and IFNγR1 (raised against the IFNγ receptor subunit 1) followed by RNA extraction and quantitative RT-PCR. As shown in table 2, addition of neutralizing antibodies 1.5 hour before doxorubicin application resulted in significantly diminished doxorubicin-mediated mRNA induction of the investigated genes with the exception of STAT1, STAT2, and IFI30. While doxorubicin-induced STAT1 and IFI30 mRNA expression was reduced by the anti-IFNγ antibody only, STAT2 mRNA expression was not influenced by either of the neutralizing antibodies. As expected, IFNγ-mediated induction of selected immunomodulatory genes in HeLa cells was also clearly and significantly inhibited by the use of the neutralizing IFNγ antibody (data not shown).

Involvement of the JAK1-STAT1 pathway in the transcriptional response to doxorubicin

It is known that IFNγ signaling involves activation of JAKs and STATs such as JAK1 and STAT1. To test whether treatment with IFNγ (50 U/ml) or doxorubicin (1 µM) resulted in activation of JAK1 as primary target kinase of the interferon receptors in our HeLa cell model, we performed JAK1 specific ELISA. Using this assay, we were able to determine both the total as well as the phosphorylated JAK1 protein in HeLa cells. Whereas no changes in regulation of total JAK1 were observed (data not shown), JAK1 was clearly phosphorylated 1 h after IFNγ application and four hours after doxorubicin treatment (Figure 2B). This increase in phosphorylated JAK1 induced by doxorubicin and IFNγ was significantly diminished in the presence of ATA (30 µM), an inhibitor of JAK-STAT signaling (Figure 2B).

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

JAK1-regulated STAT1 phosphorylation is a prerequisite for STAT dimerization, nuclear translocation and finally gene activation. Therefore, we performed nuclear extraction and measured the phosphorylation of STAT1 on tyrosine 701, which is necessary for dimerization and nuclear translocation. Results shown in Figure 2C clearly demonstrate that nuclear extracts obtained from doxorubicin-treated cells after four and eight hours contained higher amounts of tyrosine phosphorylated STAT1 than those from DMSO-treated control cells. In contrast, phosphorylation of STAT2 and STAT3 was not affected by doxorubicin treatment. Additionally, the transcriptional binding activity of nuclear STAT1 to the respective consensus sequence was significantly enhanced about two-fold after treatment of HeLa cells with doxorubicin (Figure 2E). Application of IFNγ resulted also in a clearly amplified and significant STAT1 binding activity serving as a kind of positive control. An unlabeled competitor oligonucleotide demonstrated the specificity of the assay (data not shown).

To further analyze whether the doxorubicin-mediated regulation actually involves the activation of JAK-STAT cascade, we performed inhibition experiments with AG490 and ATA as inhibitors of JAK-STAT signaling (Wang *et al.*, 1999a) and determined the mRNA content of selected genes by Real Time RT-PCR. AG490 pre-treatment significantly suppressed doxorubicin-induced gene expression with the exception of ISGF3G. Pre-treatment with ATA also resulted in a significantly diminished doxorubicin-mediated induction of all investigated genes with the exception of STAT1 (Table 2).

Additionally, we investigated the effect of ATA and AG490 on doxorubicin-mediated changes of caspase 1, STAT1, NMI, IFI35 and ISGF3G levels by immunoblot analysis (Figure 3A). Densitometric analysis of protein levels is shown in Fig. 3B and demonstrates that treatment with doxorubicin for 48 h triggered significant increases in levels of STAT1 (DMSO: 0.20±0.14 vs Dox: 2.35±0.40), NMI (DMSO: 0.13±0.06 vs Dox: 0.84±0.11), ISGF3G (DMSO: 0.14±0.10 vs Dox: 0.58±0.13) and of Caspase 1 (DMSO: 0.43±0.40 vs Dox: 1.48±0.44). For IFI35 protein levels did not significantly change (DMSO: 0.48±0.03 vs

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Dox: 0.91±0.65) despite significantly increased mRNA levels. Pre-treatment with AG490 and ATA significantly reduced the doxorubicin-mediated increases of STAT1 and caspase 1 protein levels. For NMI, only pre-treatment with ATA prevented increase in protein levels upon doxorubicin and no significant influences of either AG490 or ATA were seen on doxorubicin-mediated effects on IFI35 and ISGF3G. The findings above suggest that doxorubicin is a potent activator of the JAK1-STAT1 pathway resulting in downstream regulation of interferon responsive genes which is in parts also reflected on protein level.

To further support the role of STAT1 in doxorubicin-mediated signaling and gene induction we decided to knockdown STAT1 expression by using a specific siRNA against STAT1. After 78 h, STAT1 expression was silenced to about 25% in DMSO treated and to 43% in doxorubicin exposed cells in STAT1 siRNA transfected cells in comparison to transfection with a non-targeting control siRNA. Additionally, with exception of IFI30, the doxorubicin-mediated induction of NMI, Caspase 1, IFI6 and IFI27 was clearly reduced in STAT1 silenced cells but did not reach the control level maybe because the remaining STAT1 expression is sufficient to mediate the transcriptional activation of the genes.

Effect of ATA and AG490 on doxorubicin mediated cell death

To test whether the doxorubicin-mediated changes have any consequences for cell viability we performed an Alamar Blue^{TM} assay and determined caspase 3 activity as a marker of apoptotic cell death.

As shown in Figure 4A, cytotoxicity of doxorubicin exposure for 72 h resulted in cell viability of 41.9 ± 3.3%. However, when HeLa cells were pre-treated with 30 and 50 µM ATA cell viability significantly increased to 73 ± 5.3% and 82 ± 6.6%, respectively. In contrast, pre-treatment with 30 and 50 µM AG-490 did not significantly reduce cytoxicity of doxorubicin (39 ± 4.8% and 50 ± 5.6%, respectively). Additionally, treatment with IFNγ sensitized HeLa cells to doxorubicin-induced loss of cell viability (41.9 ± 3.3% for doxorubicin and 24.2 ±

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

3.8% for doxorubicin plus IFNγ, Figure 4A). In contrast, cells treated with IFNγ alone (125.4 ± 1.4%) seemed to even increase proliferation compared with untreated control cells.

To further analyze whether doxorubicin-triggered cell death indeed involves apoptosis, we measured the activity of caspase 3. Treatment of HeLa cells with doxorubicin alone resulted in significant activation of caspase 3. Interestingly, both ATA and AG-490 pre-treatment of HeLa cells resulted in a significant reduction of doxorubicin-induced caspase 3 activity as shown in Figure 4B.

Effect of doxorubicin on IFNγ secretion and interferon-inducible genes in tumor cells of different origins

In order to analyze whether the regulation of interferon-inducible genes by doxorubicin is limited to HeLa cells, we further investigated three cell lines of different tumor entities namely Caco2 (colon carcinoma), MCF-7 (breast cancer) and HepG2 (hepatocarcinoma). First, we checked IFNγ secretion into supernatant upon treatment with doxorubicin (1 µM) in these cells. As shown in Figure 5A, doxorubicin treatment resulted in a significantly increased IFNγ secretion in all three tumor cell lines after 2 or 4 h.

Despite secretion of IFNγ upon doxorubicin and expression of the IFNγ receptor, the change in expression of the interferon-inducible genes IFI6, IFI30 and STAT1 48 h after doxorubicin application was somewhat different in the analyzed tumor cell lines (Caco2, HepG2, MCF-7) compared with that in HeLa cells (Figure 5B). In contrast to the doxorubicin-induced up-regulation of STAT1 expression in HeLa cells, up-regulation of STAT1 was not observed in HepG2, and even a reduced STAT1 mRNA expression was detected in Caco2 (0.5-fold) and MCF-7 cells (0.39-fold) (not statistically significant). This decrease in doxorubicin-mediated STAT1 expression was significantly amplified by pre-treatment with the JAK/STAT inhibitor AG490 in MCF-7 cells (0.08-fold of DMSO control). Concerning IFI6, in MCF-7 cells no

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

significant regulation upon doxorubicin application was seen whereas in Caco2 and HepG2 cells IFI6 expression was significantly increased 25.3-fold (Caco2) and 3.8-fold (HepG2) by doxorubicin. Interestingly, this increase in IFI6 expression was significantly reduced by pre-treatment with the neutralizing IFNγ antibody only in Caco2 cells (3.4-fold compared to 25.3-fold for only doxorubicin) but not in HepG2 cells (4.4-fold compared to 3.8-fold for only doxorubicin). Furthermore, pre-treatment with AG490 showed no effect on the doxorubicin-mediated up-regulation of IFI6 in both Caco2 (21.2-fold compared 25.3-fold for only doxorubicin) and HepG2 cells (4.8-fold compared to 3.8-fold for only doxorubicin) which is not in accordance with the results obtained in HeLa cells. IFI30 mRNA expression was slightly but not significantly elevated upon doxorubicin in MCF-7 cells (2.5-fold) whereas no obvious regulation was seen in Caco2 and HepG2 cells. Despite of this, a decrease of IFI30 expression was observed after pre-treatment with the neutralizing IFNγ antibody in Caco2 cells (to about 0.3-fold for both DMSO or doxorubicin) and after pre-treatment with AG490 in MCF-7 (0.54-fold for DMSO and 0.3-fold for doxorubicin) and HepG2 cells (0.22-fold for DMSO) (Figure 5B).

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Immune defense against tumors is complex and can be mediated by cells of the innate immune system such as macrophages or natural killer (NK) cells as well as by the adaptive immune system including B and T cells (Bhardwaj, 2007). Hence, immunobased therapeutic strategies that target tumors use these effector cells , particularly as infiltration of malignant tumors with NK or T cells was associated with a better clinical outcome (Ishigami *et al.*, 2000). Experimental studies have demonstrated that doxorubicin can augment immune responses that involve macrophage and NK cell activity (Ujhazy *et al.*, 2003a). Interestingly, antitumoral efficiency of some cytostatics including doxorubicin could be enhanced by co-administration of IFNγ and an improved antitumor efficacy by coadministration of doxorubicin and IL-12 is dependent on the accumulation of IFNγ in tumors (Wadler and Schwartz, 1990;Zhu *et al.*, 2007a). But to date, detailed studies on the effects of doxorubicin on the pattern of tumor cell gene expression linked to immunomodulation or interferon signaling are not reported. Therefore, the purpose of this study was to identify genes with potentially immunomodulatory functions regulated by doxorubicin, underlying regulatory mechanisms and potential consequences for doxorubicin's efficiency.

Screening of the effect of doxorubicin on the gene expression profile of the cervix carcinoma cell line HeLa revealed up-regulation of interferon-inducible and immunomodulatory genes including the interferon-inducible/stimulated proteins IRF1, IFI6, IFI27, IFI30, IFI35, IFITM1, IFIT4, ISGF3G, the transcription factors STAT1 and NMI as well as the protease caspase 1. For all of these genes an induction by interferons has previously been shown in different cell models. Results of the DNA microarray analysis were confirmed by RT-PCR and immunoblot analysis of selected genes. While such regulatory effects have already been reported for STAT1 and caspase 1 (Gupta *et al.*, 2001), regulation of the remaining genes upon doxorubicin is novel.

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

With exception of STAT1 and caspase 1, the functions of most of the regulated genes in infection, inflammation and innate immune system (Lamkanfi *et al.*, 2007;Matsukawa, 2007) are to date only partly described. IFI35 and NMI are similarly induced by IFNγ with IFI35 being stabilized by NMI (Zhou *et al.*, 2000). NMI itself can potentiate STAT-dependent transcription (Zhu *et al.*, 1999). While no antitumoral function has been shown for IFI35 by now, NMI inhibits Wnt/beta-catenin signaling and retards tumor growth (Fillmore *et al.*, 2009). Hence, up-regulation of these interferon-induced proteins by doxorubicin could be involved in its antitumoral effect. Concerning therapy of malignancies STAT1 is of potential interest since it mediates growth inhibitory signals and contributes to the host's rejection of tumors (Levy and Gilliland, 2000). Nuclear STAT1 was directly correlated with intratumoral T cell levels in colorectal cancer accompanied by a better patients' prognosis suggesting that doxorubicin mediated increase in STAT1 expression and nuclear localization could result in enhanced T cell migration into the tumor and may lead to recognition of tumor cells by the immune system. Beside, doxorubicin potentiates STAT1 activation in response to IFNγ resulting in enhanced apoptosis in breast cancer cells (Thomas *et al.*, 2004b) which is also supported by our findings. Otherwise, it was shown that constitutive overexpression of STAT1 correlates with the selection of doxorubicin- and IFNγ-resistant tumor cells raising the question of growth advantages for cells with increased STAT1 expression upon doxorubicin (Khodarev *et al.*, 2009).

Since most genes shown to be regulated by doxorubicin are also stimulated by IFNγ via the JAK-STAT signaling (Darnell, Jr., 1998), we investigated whether doxorubicin utilized this signaling axis for gene induction. Igarashi and colleagues demonstrated that binding of IFNγ to HeLa cells initiated a series of events that resulted in phosphorylation of not only JAKs but also the IFNγ receptor (Igarashi *et al.*, 1994) suggesting a fully intact IFNγ signaling in HeLa cells. Beside detection of the IFNγ receptor system, we were able to determine a substantial

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

induction of IFNγ secretion upon doxorubicin treatment in HeLa cells. Until now, the doxorubicin-dependent increase in IFNγ has only been described for peritoneal cells of doxorubicin-treated mice (Ujhazy *et al.*, 2003c) but not for tumor cells. Using neutralizing antibodies against the IFNγ cascade we could show that regulation of all investigated genes beside STAT2 was dependent on IFNγ.

To date, four mammalian JAKs and seven STAT family members have been identified, which may be activated individually or in combination (Leonard and O'Shea, 1998; Darnell, Jr., 1997). Upon doxorubicin treatment, activation of JAK1 was observed in HeLa cells which was inhibited by ATA underlining the role of ATA as JAK inhibitor. Further, enrichment of nuclear phosphorylated STAT1 and increased binding to its consensus sequence was seen in HeLa cells after doxorubicin application whereas no enhanced phosphorylation was seen for STAT2 and STAT3. These results indicate that in HeLa cells doxorubicin treatment results in activation of IFNγ signaling and has no influence on IFNα/β cascade which would lead to phosphorylation of both STAT1 and STAT2. This notion is supported by the doxorubicin-mediated up-regulation of IFN-regulatory factor 1 which is preferentially induced by IFNγ, whereas hypoxia-inducible factor 1 is primarily induced by IFNβ (Der *et al.*, 1998) and was not increased upon doxorubicin. However, one has to be aware of cell type dependent differences in STAT1 signaling upon doxorubicin treatment because STAT1 phosphorylation was not seen following doxorubicin exposure in the breast cancer cell line MDA-MB 435 (Thomas *et al.*, 2004).

In HeLa cells pre-treatment with the JAK inhibitor AG490 significantly reduced doxorubicin-mediated gene induction with the exception of ISGF3G, and pre-treatment with ATA, another inhibitor of JAK-STAT signaling, also resulted in a diminished induction of all investigated genes with the exception of STAT1. The discrepancy between the results obtained with ATA and AG490 could be based on different intervention in additional signaling pathways distinct

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

from JAK-STAT signaling which are in fact described in other cell models (Tsi *et al.*, 2002;Kwak *et al.*, 2008). However, the involvement of STAT1 in doxorubicin mediated gene regulation was further evidenced by silencing STAT1 expression using a specific siRNA resulting in a strong reduction of doxorubicin induced gene regulation.

To analyze whether regulation of interferon-inducible genes by doxorubicin is limited to HeLa cells, we further investigated three cell lines of different tumor entities namely Caco2 (colon carcinoma), MCF-7 (breast cancer) and HepG2 (hepatocarcinoma). Despite an increased secretion of IFNγ upon doxorubicin in all three cell lines, the influence of doxorubicin on expression of IFI6, IFI30 and STAT1 was somewhat different compared to HeLa cells. Therefore, it seems that induction of IFNγ secretion is not cell type specific whereas activation of the downstream JAK-STAT cascade and regulation of interferon-inducible genes is dependent on the intracellular repertoire of signaling mediators such as kinases or transcription factors. Therefore, immunomodulation upon doxorubicin treatment occurs in a cell-type specific manner. Complementary, it has to be noted that Zhu and colleagues (Zhu *et al.*, 2007) could demonstrate a doxorubicin-mediated increased immigration of IFNγ-secreting immune cells into tumor and a role of IFNγ/STAT signaling of the invaded immune cells in tumor growth inhibition. This finding argues for a dual modulation of the IFNγ cascade in both immune and tumor cells suggesting a potential interaction between these two cell types. However, in peripheral blood mononuclear cells (PBMC) from healthy volunteers we could not find such a regulation of immunomodulatory genes upon doxorubicin as seen in HeLa cells (data not shown).

The IFNγ-JAK-STAT signaling is described to induce cell inhibitory effects. Therefore, the effects of JAK-STAT inhibitors on doxorubicin mediated cell death were investigated yielding some interesting results. Whereas both inhibitors caused significantly reduced caspase 3 activation, only treatment with ATA improved cell viability upon doxorubicin

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

treatment. In fact, for AG490 both pro- (Wu *et al.*, 2009) and anti-apoptotic (Zhao *et al.*, 2009) mechanisms are described. In contrast, for ATA only anti-apoptotic actions are delineated (Cho *et al.*, 2004) which is in agreement with the observations made in this study. Failure of AG490 to rescue viability of HeLa cells could be caused by its pleiotropic action, e.g. not only blocking JAK1 and 2 but also JAK3 signaling which leads to inhibition of the growth promoting STAT3 (Bromberg and Darnell, Jr., 2000). Hence, JAK1/2-STAT1 mediated pro-apoptotic functions and JAK3-STAT3 anti-apoptotic components could leverage each other, and thus in sum no influence on overall cell survival was observed. In addition to these direct cytotoxic effects of the JAK1-STAT1 signaling, it seems possible that regulation of genes with potential immunomodulatory function could enhance the recognition by or activation of immune cells and thereby tumor defense mechanisms. This is in line with observations that doxorubicin treated cells are more prone to attack by peripheral mononuclear cells than the respective control cells (Supplemental figure 2). However, further work is needed to clarify this question.

Taken together, in this study we demonstrate that doxorubicin induces the secretion of IFNγ and modulates several interferon-responsive and immunmodulatory genes via IFNγ-JAK1-STAT1 signaling leading to apoptosis. These findings contribute to a better understanding of molecular mechanisms involved in the destruction of tumor cells by immune cells and chemotherapeutic drugs which could facilitate the selection of protocols with effective tumor killing.

Authorship Contributions

Participated in research design: S. Bien, C. Ritter, H.K. Kroemer, U. Voelker, E. Hammer, H.W.S. Schroeder
Conducted experiments: S. Bien, M. Schwebe, J. Hussner, E. Hammer, S. Herzog, L. Steil, J. Niessen, S. Ameling
Contributed new reagents or analytic tools:
Performed data analysis: S. Bien, E. Hammer, L. Steil, J., S. Herzog, S. Ameling, J. Hussner
Wrote or contributed to the writing of the manuscript: S. Bien, E. Hammer, U. Voelker, C. Ritter, H.K. Kroemer

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

References

Arinaga S, Akiyoshi T and Tsuji H (1986) Augmentation of the Generation of Cell-Mediated Cytotoxicity After a Single Dose of Adriamycin in Cancer Patients. *Cancer Res* **46**: pp 4213-4216.

Bhardwaj N (2007) Harnessing the Immune System to Treat Cancer. *J Clin Invest* **117**: pp 1130-1136.

Bromberg J and Darnell J E, Jr. (2000) The Role of STATs in Transcriptional Control and Their Impact on Cellular Function. *Oncogene* **19**: pp 2468-2473.

Chen CW, Chao Y, Chang Y H, Hsu M J and Lin W W (2002) Inhibition of Cytokine-Induced JAK-STAT Signalling Pathways by an Endonuclease Inhibitor Aurintricarboxylic Acid. *Br J Pharmacol* **137**: pp 1011-1020.

Cho H, Lee D Y, Shrestha S, Shim Y S, Kim K C, Kim M K, Lee K H, Won J and Kang J S (2004) Aurintricarboxylic Acid Translocates Across the Plasma Membrane, Inhibits Protein Tyrosine Phosphatase and Prevents Apoptosis in PC12 Cells. *Mol Cells* **18**: pp 46-52.

Darnell JE, Jr. (1997) STATs and Gene Regulation. *Science* **277**: pp 1630-1635.

Darnell JE, Jr. (1998) Studies of IFN-Induced Transcriptional Activation Uncover the Jak-Stat Pathway. *J Interferon Cytokine Res* **18**: pp 549-554.

Der SD, Zhou A, Williams B R and Silverman R H (1998) Identification of Genes Differentially Regulated by Interferon Alpha, Beta, or Gamma Using Oligonucleotide Arrays. *Proc Natl Acad Sci U S A* **95**: pp 15623-15628.

Di Marco A, Gaetani M and Scarpinato B (1969) Adriamycin (NSC-123,127): a New Antibiotic With Antitumor Activity. *Cancer Chemother Rep* **53**: pp 33-37.

Ehrke MJ, Ryoyama K and Cohen S A (1984) Cellular Basis for Adriamycin-Induced Augmentation of Cell-Mediated Cytotoxicity in Culture. *Cancer Res* **44**: pp 2497-2504.

Eppstein DA, van der Pas M A, Marsh Y V, Bruno N A and Kurahara C G (1988) Enhanced Antitumor Efficacy of Adriamycin in Combination With Interferon-Gamma: Effects on Macrophages. *J Interferon Res* **8**: pp 263-273.

Fillmore RA, Mitra A, Xi Y, Ju J, Scammell J, Shevde L A and Samant R S (2009) Nmi (N-Myc Interactor) Inhibits Wnt/Beta-Catenin Signaling and Retards Tumor Growth. *Int J Cancer* **125**: pp 556-564.

Gamen S, Anel A, Lasierra P, Alava M A, Martinez-Lorenzo M J, Pineiro A and Naval J (1997) Doxorubicin-Induced Apoptosis in Human T-Cell Leukemia Is Mediated by Caspase-3 Activation in a Fas-Independent Way. *FEBS Lett* **417**: pp 360-364.

Gupta S, Radha V, Furukawa Y and Swarup G (2001) Direct Transcriptional Activation of Human Caspase-1 by Tumor Suppressor P53. *J Biol Chem* **276**: pp 10585-10588.

Igarashi K, Garotta G, Ozmen L, Ziemiecki A, Wilks A F, Harpur A G, Larner A C and Finbloom D S (1994) Interferon-Gamma Induces Tyrosine Phosphorylation of Interferon-

Gamma Receptor and Regulated Association of Protein Tyrosine Kinases, Jak1 and Jak2, With Its Receptor. *J Biol Chem* **269**: pp 14333-14336.

Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Che X, Iwashige H, Aridome K, Hokita S and Aikou T (2000) Prognostic Value of Intratumoral Natural Killer Cells in Gastric Carcinoma. *Cancer* **88**: pp 577-583.

Kaplan DH, Shankaran V, Dighe A S, Stockert E, Aguet M, Old L J and Schreiber R D (1998) Demonstration of an Interferon Gamma-Dependent Tumor Surveillance System in Immunocompetent Mice. *Proc Natl Acad Sci U S A* **95**: pp 7556-7561.

Khodarev NN, Roach P, Pitroda S P, Golden D W, Bhayani M, Shao M Y, Darga T E, Beveridge M G, Sood R F, Sutton H G, Beckett M A, Mauceri H J, Posner M C and Weichselbaum R R (2009) STAT1 Pathway Mediates Amplification of Metastatic Potential and Resistance to Therapy. *PLoS One* **4**: pp e5821.

Kwak HB, Sun H M, Ha H, Lee J H, Kim H N and Lee Z H (2008) AG490, a Jak2-Specific Inhibitor, Induces Osteoclast Survival By Activating the Akt and ERK Signaling Pathway. *Mol Cells* **26**.

Lamkanfi M, Kanneganti T D, Franchi L and Nunez G (2007) Caspase-1 Inflammasomes in Infection and Inflammation. *J Leukoc Biol* **82**: pp 220-225.

Leonard WJ and O'Shea J J (1998) Jaks and STATs: Biological Implications. *Annu Rev Immunol* **16**: pp 293-322.

Levy DE and Gilliland D G (2000) Divergent Roles of STAT1 and STAT5 in Malignancy As Revealed by Gene Disruptions in Mice. *Oncogene* **19**: pp 2505-2510.

Maccubbin DL, Wing K R, Mace K F, Ho R L, Ehrke M J and Mihich E (1992) Adriamycin-Induced Modulation of Host Defenses in Tumor-Bearing Mice. *Cancer Res* **52**: pp 3572-3576.

Matsukawa A (2007) STAT Proteins in Innate Immunity During Sepsis: Lessons From Gene Knockout Mice. *Acta Med Okayama* **61**: pp 239-245.

Minotti G, Menna P, Salvatorelli E, Cairo G and Gianni L (2004) Anthracyclines: Molecular Advances and Pharmacologic Developments in Antitumor Activity and Cardiotoxicity. *Pharmacol Rev* **56**: pp 185-229.

Salup RR, Back T C and Wiltrout R H (1987) Successful Treatment of Advanced Murine Renal Cell Cancer by Bicompartmental Adoptive Chemoimmunotherapy. *J Immunol* **138**: pp 641-647.

Schwartz HS and Grindey G B (1973) Adriamycin and Daunorubicin: a Comparison of Antitumor Activities and Tissue Uptake in Mice Following Immunosuppression. *Cancer Res* **33**: pp 1837-1844.

Thomas M, Finnegan C E, Rogers K M, Purcell J W, Trimble A, Johnston P G and Boland M P (2004a) STAT1: a Modulator of Chemotherapy-Induced Apoptosis. *Cancer Res* **64**: pp 8357-8364.

Tsi CJ, Chao Y, Chen C W and Lin W W (2002) Aurintricarboxylic Acid Protects Against Cell Death Caused by Lipopolysaccharide in Macrophages by Decreasing Inducible Nitric-Oxide Synthase Induction Via IkappaB Kinase, Extracellular Signal-Regulated Kinase, and P38 Mitogen-Activated Protein Kinase Inhibition. *Mol Pharmacol* **62**: pp 90-101.

Ujhazy P, Zaleskis G, Mihich E, Ehrke M J and Berleth E S (2003b) Doxorubicin Induces Specific Immune Functions and Cytokine Expression in Peritoneal Cells. *Cancer Immunol Immunother* **52**: pp 463-472.

Wadler S and Schwartz E L (1990) Antineoplastic Activity of the Combination of Interferon and Cytotoxic Agents Against Experimental and Human Malignancies: a Review. *Cancer Res* **50**: pp 3473-3486.

Wang LH, Kirken R A, Erwin R A, Yu C R and Farrar W L (1999a) JAK3, STAT, and MAPK Signaling Pathways As Novel Molecular Targets for the Tyrphostin AG-490 Regulation of IL-2-Mediated T Cell Response. *J Immunol* **162**: pp 3897-3904.

Wu MH, Chen X Y and Cai K R (2009) Effects of a JAK Inhibitor, AG490, on Proliferation and Apoptosis of Human Nasopharyngeal Carcinoma Cell Line CNE-2Z. *Ai Zheng* **28**: pp 24-28.

Zhao LY, Huang C, Li Z F, Liu L, Ni L and Song T S (2009) STAT1/2 Is Involved in the Inhibition of Cell Growth Induced by U0126 in HeLa Cells. *Cell Mol Biol (Noisy -le-grand)* **55 Suppl**: pp OL1168-OL1174.

Zhou X, Liao J, Meyerdierks A, Feng L, Naumovski L, Bottger E C and Omary M B (2000) Interferon-Alpha Induces Nmi-IFP35 Heterodimeric Complex Formation That Is Affected by the Phosphorylation of IFP35. *J Biol Chem* **275**: pp 21364-21371.

Zhu M, John S, Berg M and Leonard W J (1999) Functional Association of Nmi With Stat5 and Stat1 in IL-2- and IFNgamma-Mediated Signaling. *Cell* **96**: pp 121-130.

Zhu S, Waguespack M, Barker S A and Li S (2007) Doxorubicin Directs the Accumulation of Interleukin-12 Induced IFN Gamma into Tumors for Enhancing STAT1 Dependent Antitumor Effect. *Clin Cancer Res* **13**: pp 4252-4260.

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Figure 1. Functional categorization of differentially expressed genes identified in HeLa cells upon doxorubicin treatment for 48h. Functional classification was performed for genes displaying ≥ 2 fold change, in doxorubicin treated compared to control cells (DMSO) by Ingenuity pathway analysis. (A) Display of categories overrepresented among the genes exhibiting differential expression in doxorubicin vs. DMSO treated cells (*p*-value <0.001). (B) Number of repressed and induced genes. (C) Time-dependency of transcriptional regulation of IFN-inducible genes by doxorubicin. HeLa cells were treated with DMSO (solvent) or 1 µM doxorubicin for the indicated time points, RNA was extracted, reverse transcribed and analyzed by qRT-PCR Mean+SD, n=3, * $p<0.05$ and *** $p<0.001$.

Figure 2. Involvement of the IFNγ-JAK1-STAT1 pathway in the transcriptional response to doxorubicin. (A) Measurement of IFNγ secretion. HeLa cells were treated for 2 and 4 h with 1 µM doxorubicin and the supernatant was used for determination of IFNγ levels with a specific ELISA. Mean±SD, n=3, * $p<0.05$. (B) Determination of phospho-JAK1 and total JAK1 was performed in HeLa lysate using a specific ELISA. Cells were pretreated for 1.5 h with or without ATA followed by incubation with PBS, DMSO, 50 U/ml IFNγ or 1 µM doxorubicin (Dox) for the indicated time points. Mean±SD, n=3, * $p<0.05$ and ** $p<0.01$. (C) Detection of phospho-STAT1, 2 and 3 was done by immunoblotting using nuclear extracts of DMSO or doxorubicin (Dox) treated HeLa cells. (D) Detection of the interferon-γ receptor system in Caco2, HeLa, HepG2 and MCF-7 cells by immunoblot analysis. (E) Assessment of transcriptional binding activity of nuclear STAT1 to its specific consensus sequence. Mean±SD, n=3, * $p<0.05$ ** and $p<0.01$, AU=Arbitrary Units.

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. Influence of ATA and AG490 on doxorubicin-induced changes on protein level. HeLa cells were pre-treated with NH_4OH (solvent) or 30 µM ATA or 50 µM AG490 for 1.5 h followed by treatment with 1 µM doxorubicin for 72 h and detection of Caspase 1, NMI, IFI35, ISGF3G, STAT1 and GAPDH in whole cell lysate by immunoblot analysis using specific antibodies. (A) Western Blot signals (B) Densitometric evaluation of protein levels with normalization to GAPDH as a housekeeping protein. Mean±SD, n=3, * $p<0.05$ and ** $p<0.01$.

Figure 4. siRNA mediated silencing of STAT1 and effects of JAK/STAT blockade on cell viability and caspase 3 activity. (A) Doxorubicin-mediated gene regulation in STAT1 siRNA silenced cells determined by RT-PCR. 48h after siRNA transfection, cells were incubated with 1 µM doxorubicin for further 30 h. Mean±SD, n=3 (B+C) HeLa cells were pre-treated with ATA or AG490 for 1.5 h and then incubated with 1 µM doxorubicin for 72 h. (B) Cell viability was determined using AlamarBlue assay. Mean±SD, n=3. (C) Caspase 3 activity was assayed using a commercially available colorimetric test and values were normalized to the protein content of each sample. Mean±SD, n=3. * $p<0.05$ and *** $p<0.001$.

Figure 5. Influence of doxorubicin on mRNA expression of interferon-inducible genes and IFNγ secretion in different tumor cell lines. (A) Caco2 (colon carcinoma), MCF-7 (breast carcinoma) and HepG2 (hepatocarcinoma) cells were pre-treated with anti-IFNγ-Ab or 50 µM AG490 for 1.5 h and then incubated with 1 µM doxorubicin for 48 h. mRNA expression was analyzed with RT-PCR and normalization to 18S rRNA. (B) Measurement of IFNγ secretion. Cells were treated for 2 and 4 h with 1 µM doxorubicin and the supernatant was used for determination of IFNγ levels with a specific ELISA. Mean±SD, n=3, * $p<0.05$.

Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Tables

Table 1. Effects of doxorubicin and IFNγ either alone or in combination on the expression levels of IFNγ regulated genes. HeLa cells were incubated for 48 h with DMSO, PBS, 1 µM doxorubicin, 50 U/ml IFNγ or 1 µM doxorubicin plus 50 U/ml IFNγ. Total RNA was extracted, reverse transcribed and analyzed by qRT-PCR. Presented values are normalized to the respective control (DMSO or PBS). Mean±SD, n=3, * $p<0.05$ vs. respective control, ** $p<0.01$ vs. respective control, *** $p<0.001$ vs. respective control, # $p<0.05$ vs. doxorubicin, ## $p<0.01$ vs. doxorubicin, ### $p<0.001$ vs. doxorubicin.

	Dox	IFNγ	Dox + IFNγ
STAT1	3.99 ± 0.95 *	7.57 ± 1.10 **	16.52 ± 5.87 #
STAT2	7.99 ± 1.97 *	3.14 ± 0.55 **	58.89 ± 9.97 #.
IFI6	85.86 ± 20.74 *	45.28 ± 3.28 ***	551.16 ± 58.39 ###
IFI27	1648 ± 359 *	98 ± 142 **	9424 ± 2036 #
IFI30/GILT	3.52 ± 0.12***	0.41 ± 0.50	5.56 ± 0.48 ###
IFI35	9.12 ± 2.81 *	7.23 ± 1.86 *	35.82 ± 6.08 ##
IFIT4	384.81 ± 141 *	122.41 ± 11.35 ***	1652.16 ± 162 ###
IFITM1	4.73 ± 0.81 *	2.55 ± 0.52 *	37.51 ± 10.28 #
ISGF3G/IRF9	8.11 ± 0.70 ***	7.76 ± 2.58 *	20.57 ± 7.33
NMI	5.45 ± 1.71 *	14.77 ± 2.88 *	23.74 ± 4.23 ##
Caspase 1	4.2 ± 0.46 ***	1.42 ± 0.11*	8.77 ± 2.99

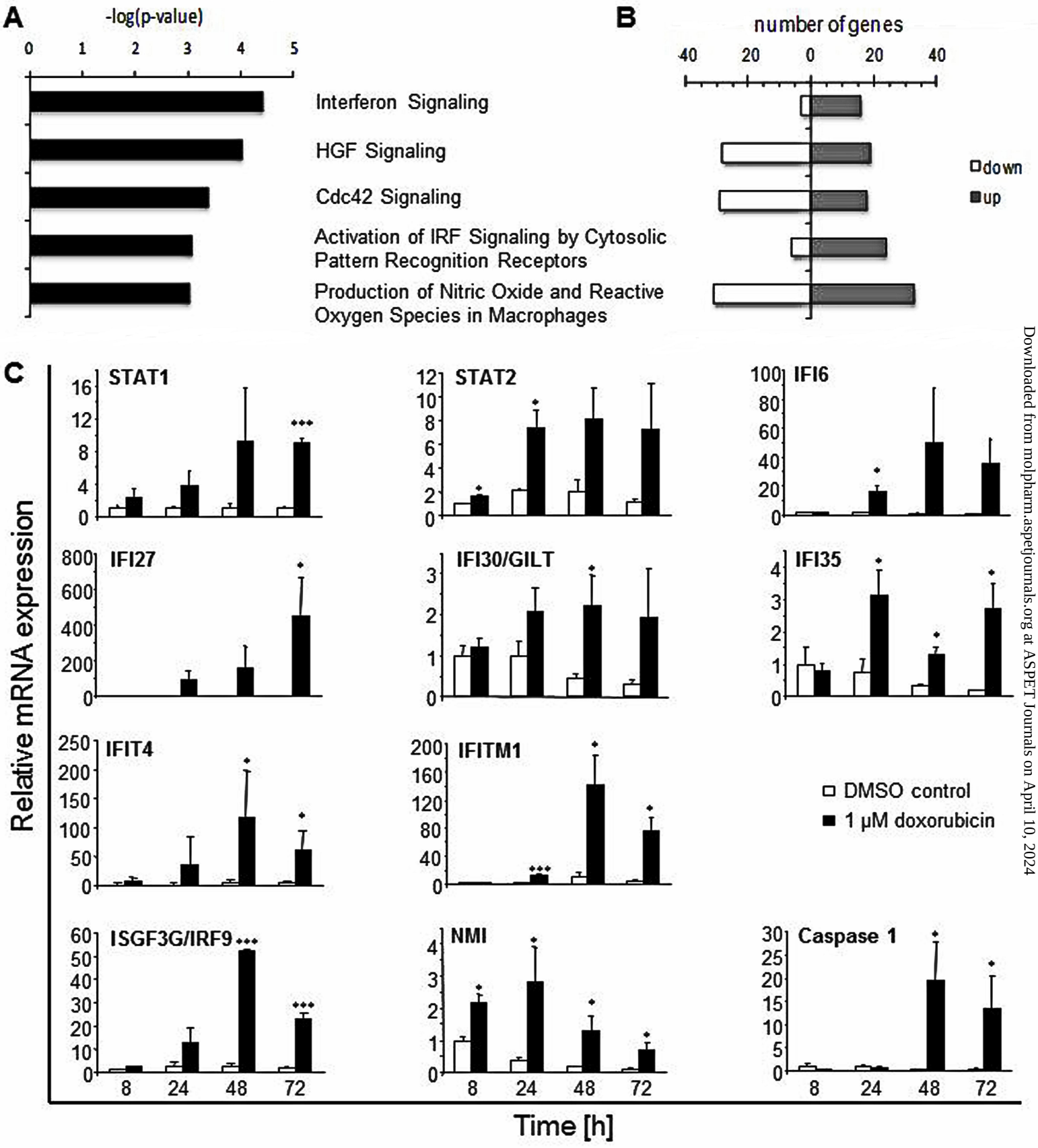
Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. Involvement of the IFNγ-JAK1-STAT1 pathway in the transcriptional response to doxorubicin. HeLa cells were pre-treated for 1.5 h with or without neutralizing antibodies against IFNγ or INFγ receptor 1, ATA (30 µM) or AG490 (50 µM) followed by treatment with 1 µM doxorubicin for 48 h. RNA was extracted, reverse transcribed and analyzed by qRT-PCR. Gene expression is calculated as the ratio of 18S normalized fluoerescence intensities measured in the respective DMSO controls. Gene expression levels upon doxorubicin exposure was set to 100% and assays containing inhibitors were normalized to this value. Mean±SD, n=3, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs doxorubicin.

Percentage of Dox	Dox + anti-IFNγ-Ab	Dox + anti-IFNγR1-Ab	Dox + 30 µM ATA	Dox + 50 µM AG490
STAT1	48.3 ± 3.0***	83.4 ± 23.5	178.5 ± 32.8	41.1 ± 4.8*
STAT2	86.2 ± 39.1	88.5 ± 43.9	61.9 ± 4.6**	53.0 ± 16.0*
IFI6-16	43.3 ± 16.8*	62.7 ± 14.4*	31.7 ± 2.7*	29.5 ± 7.8*
IFI30	43.9 ± 20.8*	55.5 ± 28.1	42.1 ± 17.9*	27.8 ± 7.3*
IFI35	34.6 ± 2.8*	56.2 ± 13.6*	22.0 ± 2.1**	48.0 ± 13.2*
IFITM1	23.0 ± 7.1*	32.7 ± 6.3*	37.0 ± 8.8*	48.7 ± 9.6**
ISGF3G/IRF9	36.3 ± 10.2**	39.4 ± 17.2*	63.4 ± 30.5*	195 ± 45.7
NMI	35.5 ± 12.6**	46.9 ± 9.2*	41.5 ± 5.6*	39.2 ± 5.9*
Caspase 1	24.2 ± 12.4*	45.6 ± 5.2*	32.6 ± 8.3**	74.2 ± 5.0*

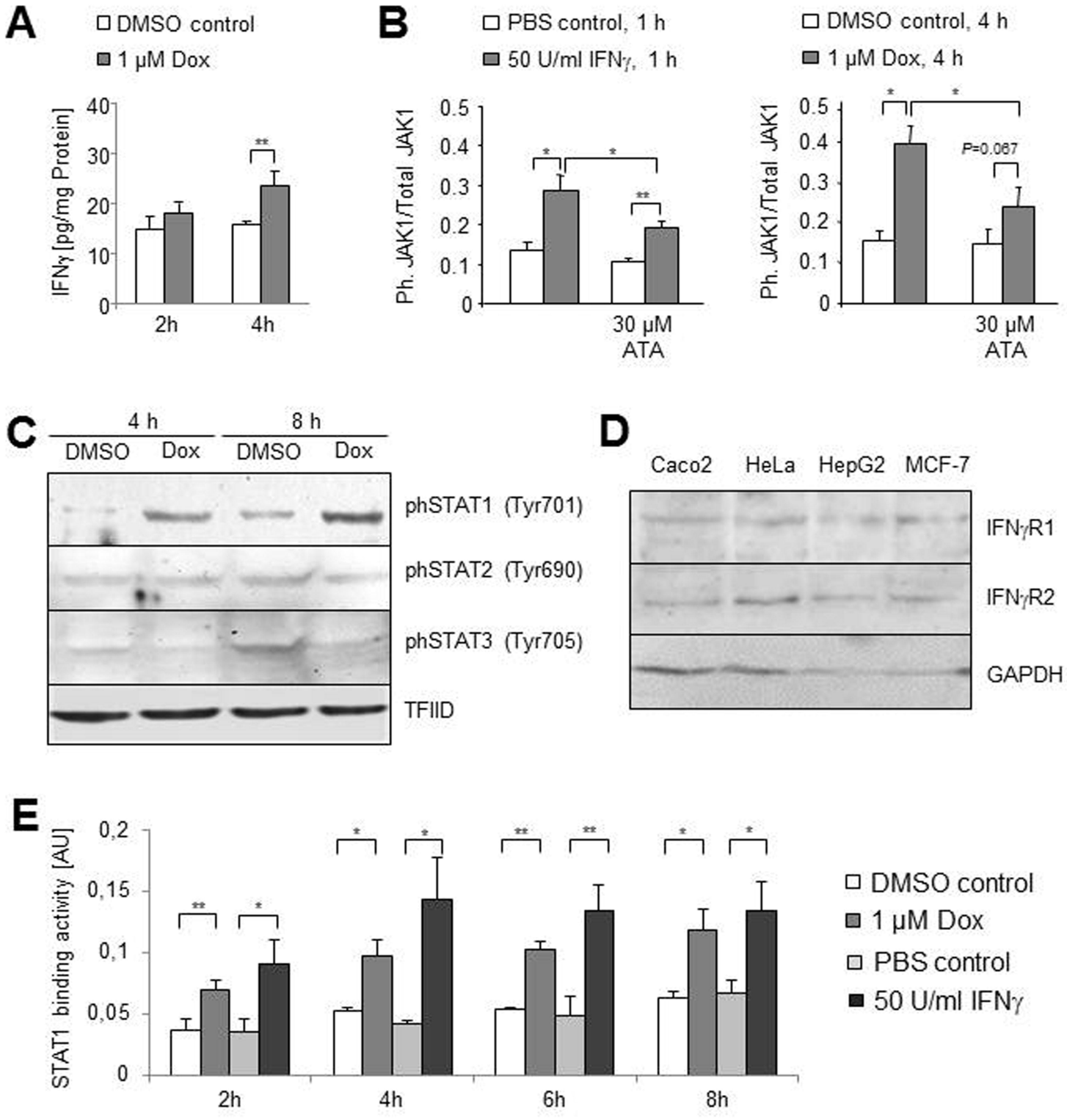
Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1



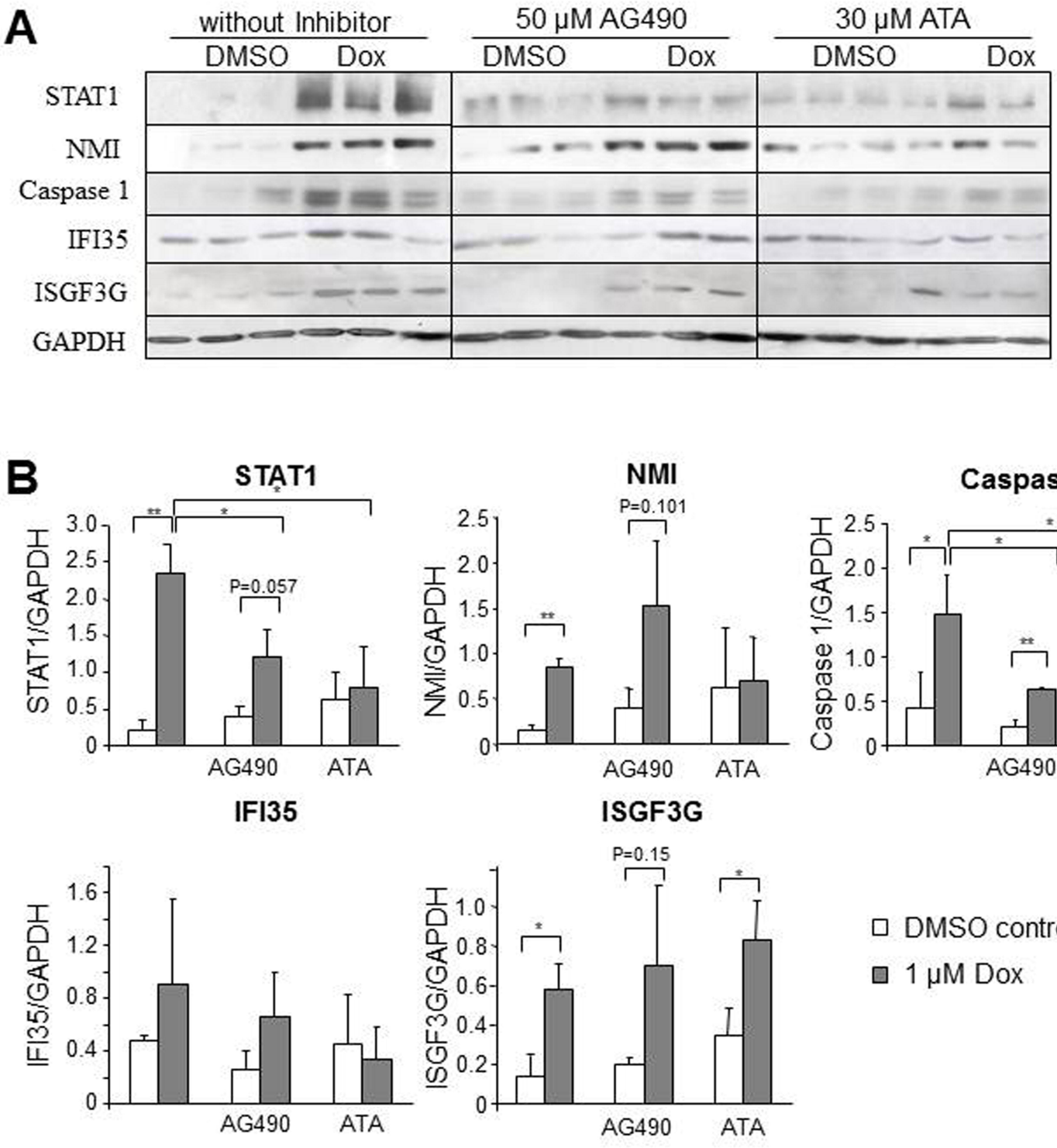
Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



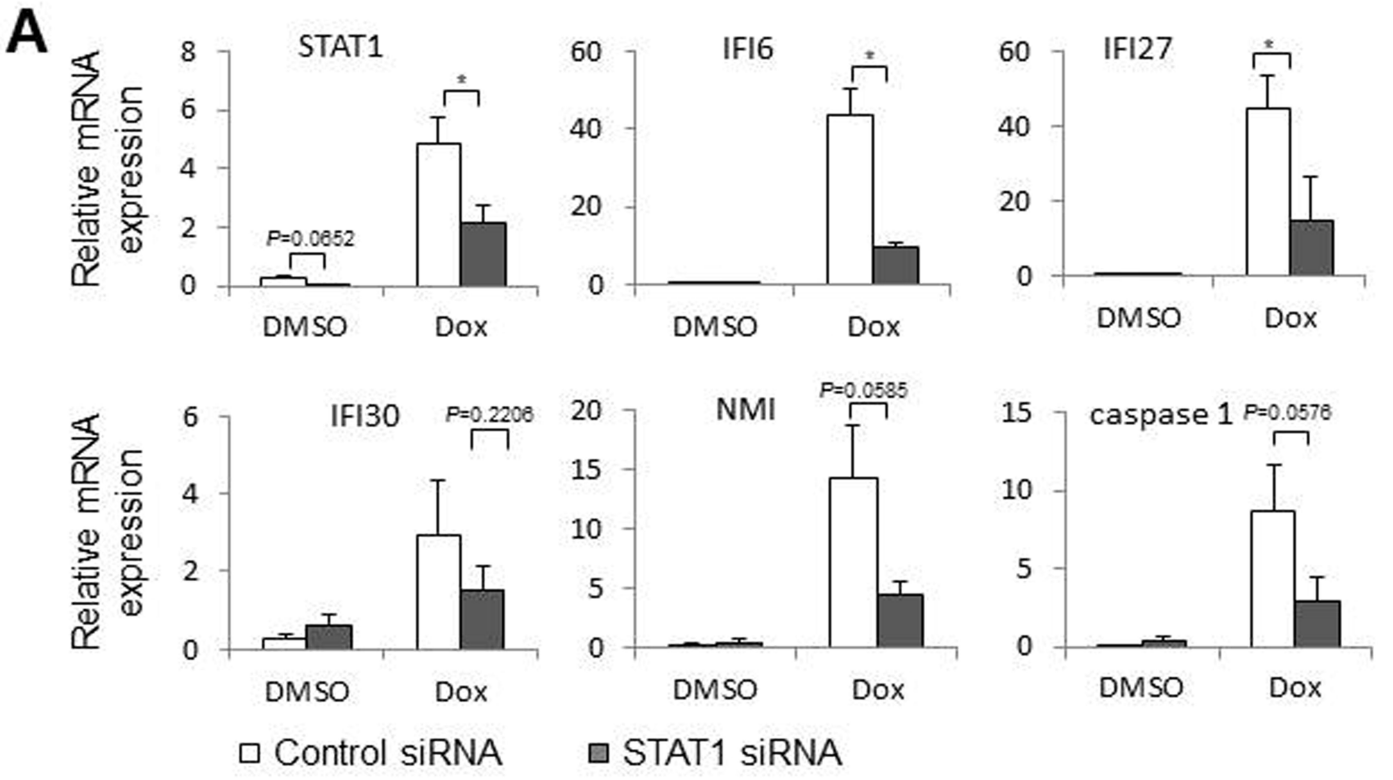
Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

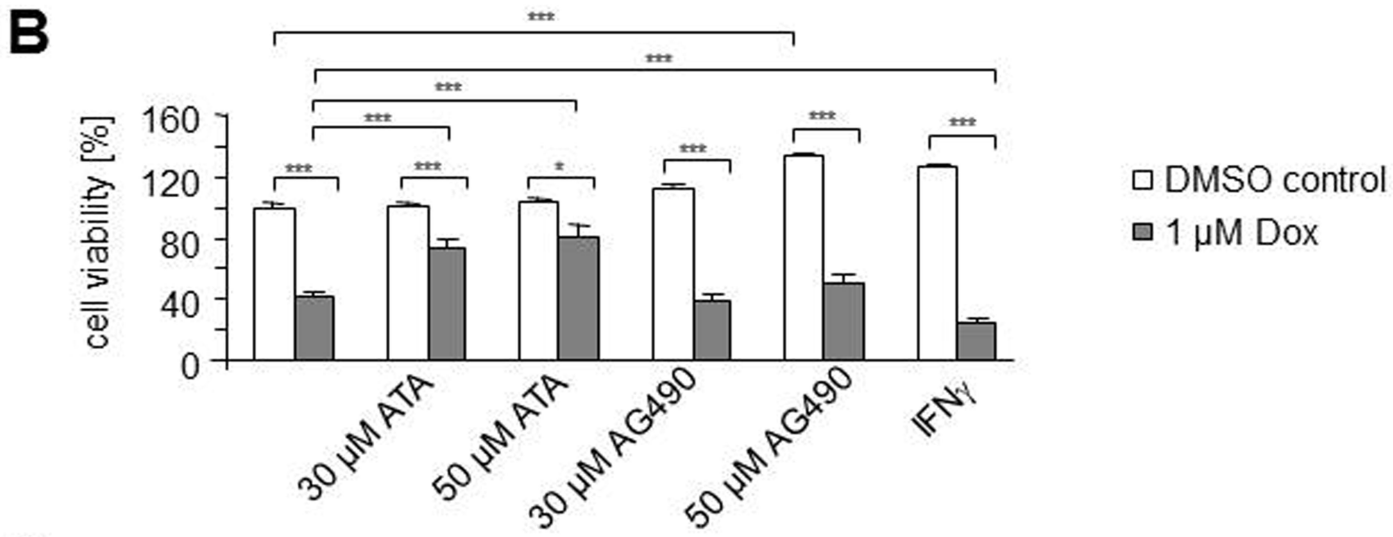
Figure 3

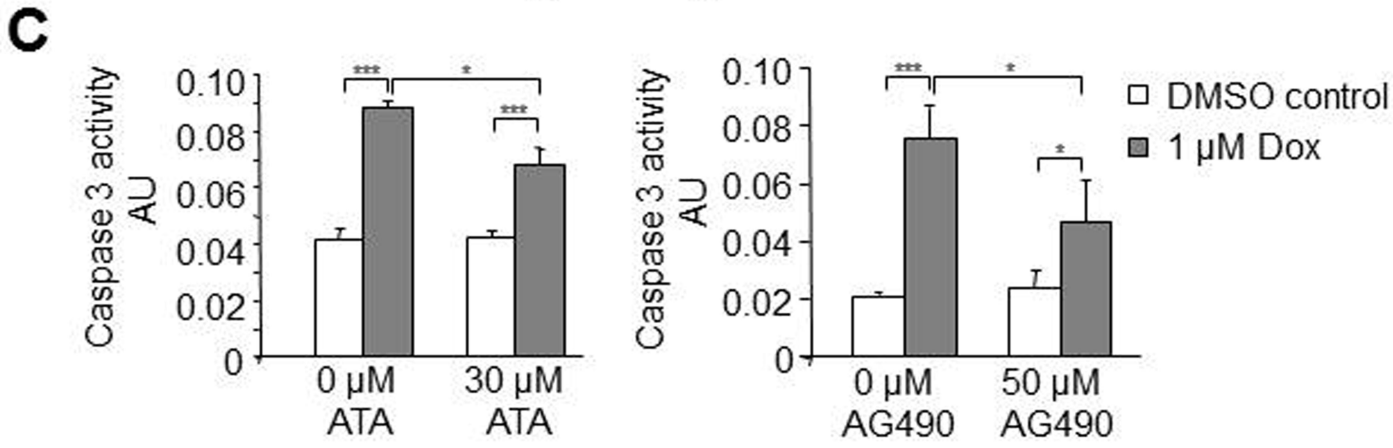


Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4

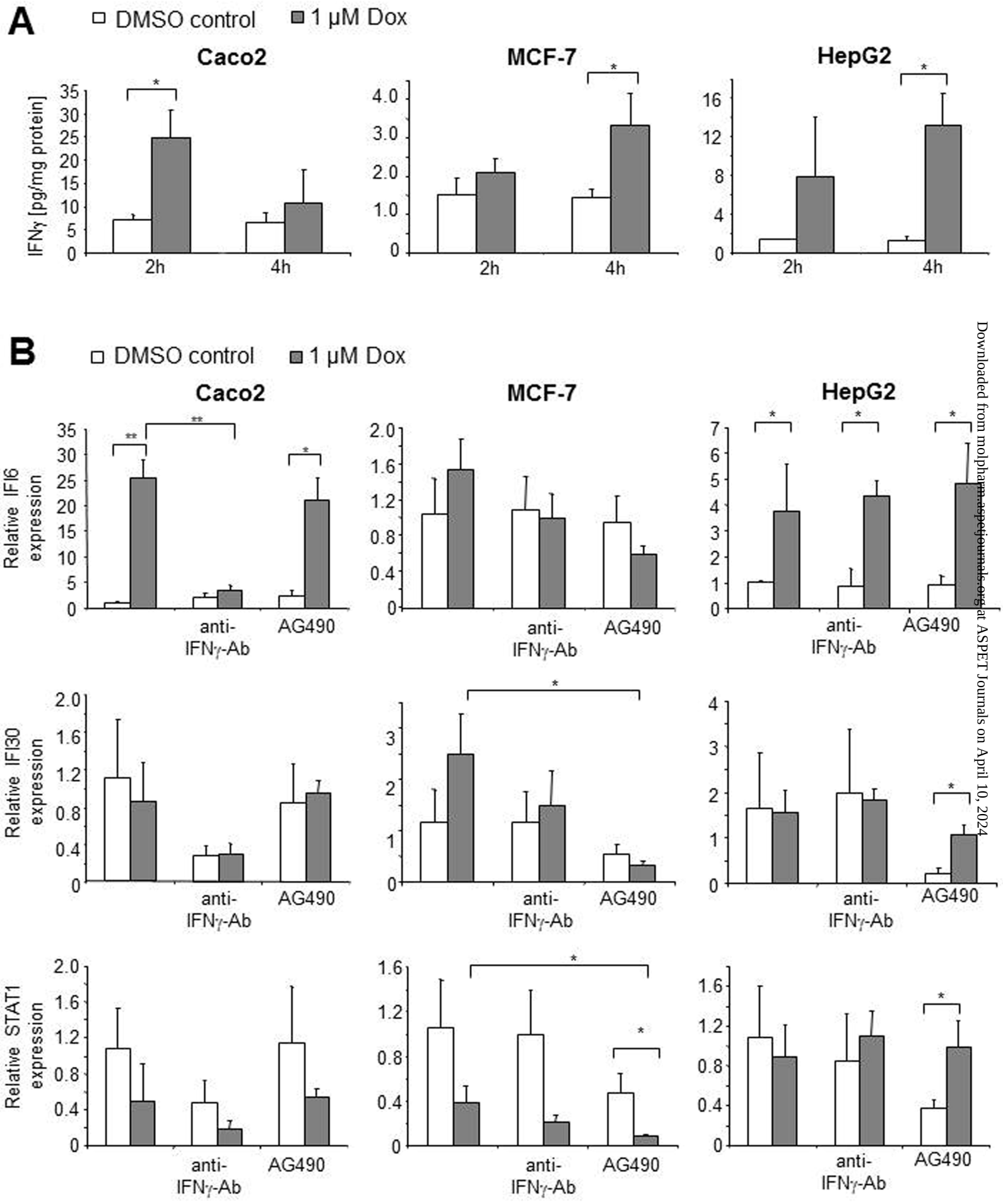






Molecular Pharmacology Fast Forward. Published on February 9, 2012 as DOI: 10.1124/mol.111.075994
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5



Supplements to the manuscript

Regulation of interferon-inducible proteins by doxorubicin via IFNγ-JAK-STAT signaling in tumor cells

J. Hussner, MS; S. Ameling, PhD; E. Hammer, PhD; S. Herzog, MS; L. Steil, PhD; M. Schwebe, MS; J. Niessen, PhD; H.W.S. Schroeder, MD; H.K. Kroemer, PhD; C.A. Ritter, PhD; U. Völker, PhD; S. Bien, PhD

Journal: Molecular Pharmacology

Supplemental Table 1. Functional categorization of differentially expressed genes identified in HeLa cells upon doxorubicin treatment after 24 and 48 h determined by microarray analysis (>2 fold change).

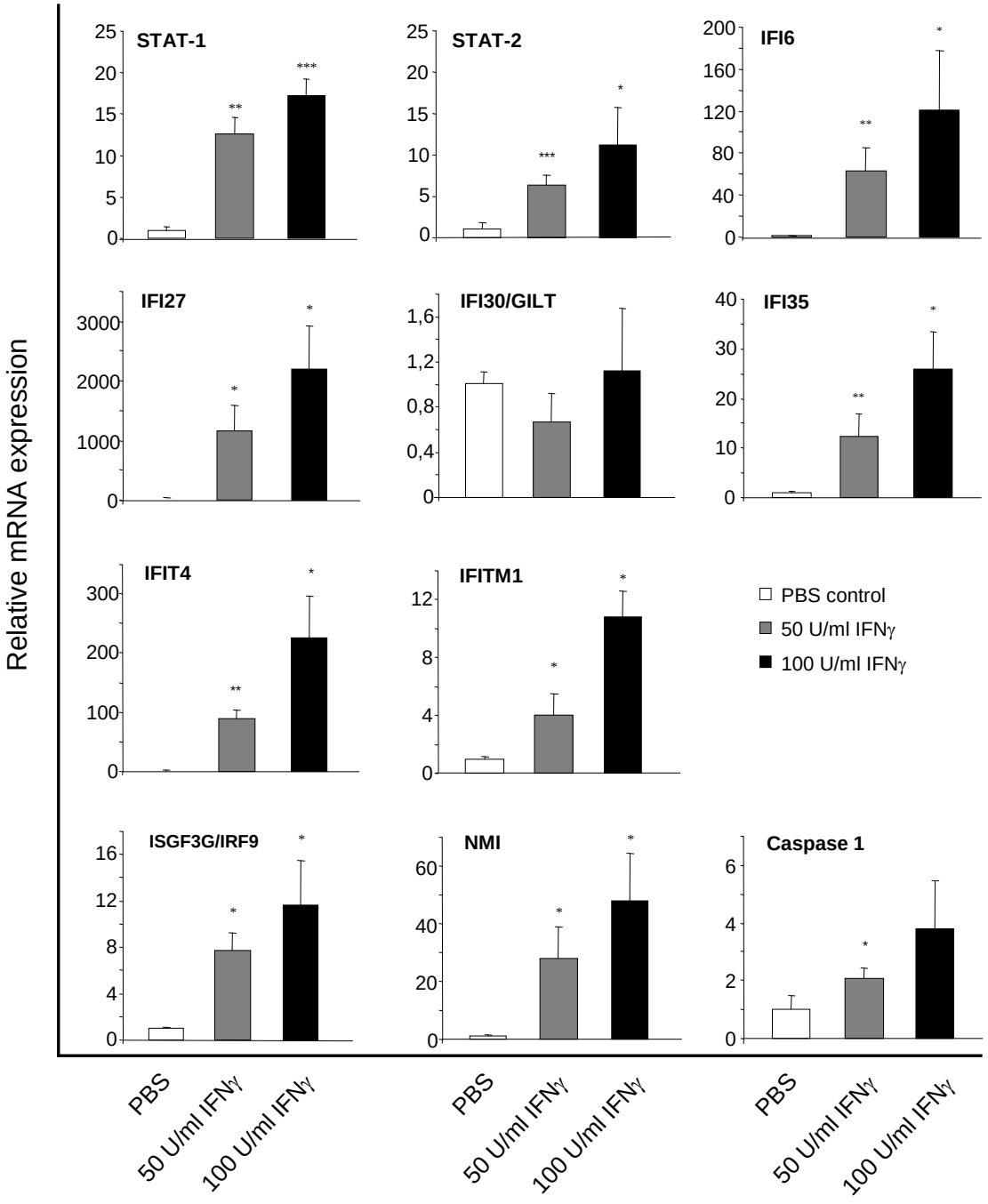
Ingenuity Canonical Pathways (*p*<0.001)	total molecules known to be associated to pathway	-log (p-value)	number of repressed genes upon Dox treatment	% down*	number of induced genes upon Dox treatment	% up**
Interferon Signaling	30	4.41	3	10	16	37
HGF Signaling	100	4.01	28	28	19	53
Cdc42 Signaling	120	3.37	29	24	18	61
Activation of IRF by Cytosolic Pattern Recognition Receptors	69	3.05	6	9	24	57
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	160	3.01	31	19	33	60
LPS-stimulated MAPK Signaling	75	2.87	17	23	16	56
IL-17 Signaling	74	2.82	14	19	20	54
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	334	2.73	42	13	69	67
PPAR Signaling	97	2.69	21	22	16	62
Prolactin Signaling	72	2.65	20	28	12	56
IL-6 Signaling	91	2.62	13	14	26	57
Glucocorticoid Receptor Signaling	268	2.6	42	16	52	65
Antigen Presentation Pathway	39	2.6	0	0	16	59
Molecular Mechanisms of Cancer	359	2.45	70	19	52	66
Type I Diabetes Mellitus Signaling	111	2.43	8	7	34	62
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	75	2.41	6	8	26	57
4-1BB Signaling in T Lymphocytes	33	2.39	5	15	11	52
mTOR Signaling	145	2.39	38	26	17	62
HMGB1 Signaling	96	2.38	20	21	20	58
PXR/RXR Activation	72	2.37	12	17	18	58
p53 Signaling	92	2.31	19	21	20	58
HER-2 Signaling in Breast Cancer	79	2.26	20	25	13	58
PPARα/RXRα Activation	174	2.26	33	19	27	66
AMPK Signaling	141	2.18	31	22	21	63
TR/RXR Activation	90	2.16	27	30	10	59
IL-8 Signaling	173	2.16	31	18	33	63
Dendritic Cell Maturation	167	2.14	18	11	38	66
CD40 Signaling	66	2.12	10	15	17	59

Glioma Signaling	107	2.1	28	26	12	63
B Cell Receptor Signaling	148	2.07	29	20	27	62
SAPK/JNK Signaling	97	2.06	24	25	13	62

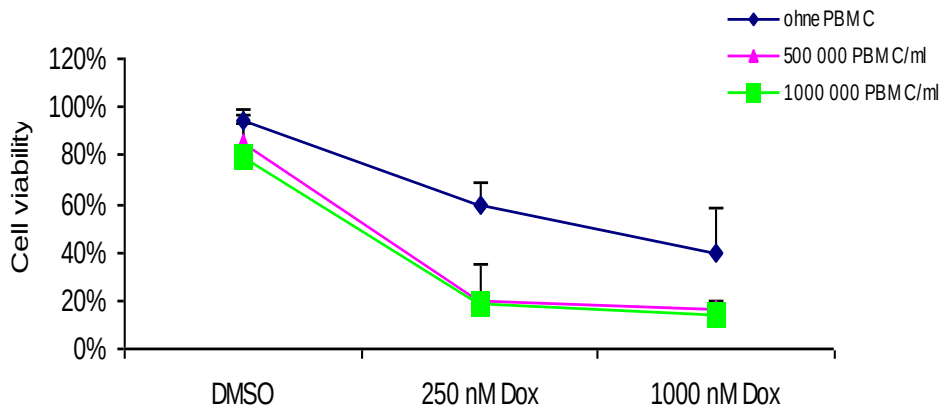
* percentage of all known molecules belonging to the pathway that is down regulated
** percentage of all known molecules belonging to the pathway that is up regulated

Supplemental Table 2. Expression levels of IFN-stimulatable genes in the presence or absence of doxorubicin. HeLa cells were incubated for 24 or 48 h with DMSO as solvent or 1 µM doxorubicin. Gene expression was determined either using DNA microarray or qRT-PCR relative to the DMSO control at the respective time point. Abbreviations: Dox, Doxorubicin; f.c., fold change

Gene symbol	Annotation	Probe set	f.c. 24 h Dox microarray	f.c. 24 h Dox qRT-PCR	f.c. 48 h Dox microarray	f.c. 48 h Dox qRT-PCR
STAT1	signal transducer and activator of transcription 1	AFFX-HUMISGF3A/M97935_5_at	5.4	3.9	10.6	9.6
STAT2	signal transducer and activator of transcription 2	225636_at	3.9	3.4	6.9	6.3
IFI6	interferon, alpha-inducible protein 6 (G1P3)	204415_at	8.0	14.3	23.3	58.9
IFI27	interferon, alpha-inducible protein 27	202411_at	15.0	89.9	1020	210
IFI30	interferon, alpha-inducible protein 30	201422_at	2.5	2.1	4.0	5.1
IFI35	interferon, alpha-inducible protein 35	209417_s_at	3.8	4.2	27.9	4.3
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	229450_at	33.7	48.3	84.0	30.4
IFITM1	interferon induced transmembrane protein 1 (9-27)	214022_s_at	2.3	6.3	7.5	13.8
IRF9	interferon regulatory factor 9	203882_at	4.3	5.2	9.5	21.0
NMI	N-myc (and STAT) interactor	203964_at	3.4	7.0	9.2	7.4
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	211367_s_at	3.4	0.5	23.9	51.0



Supplemental figure 1. Transcriptional regulation of doxorubicin stimulated genes upon IFNγ treatment. HeLa cells were treated with PBS (solvent), 50 U/ml or 100 U/ml IFNγ for 48 h, RNA was extracted, reverse transcribed and analyzed by qRT-PCR. Mean+SD, n=3, * $p<0.01$, ** $p<0.05$ and *** $p<0.001$.



Supplemental figure 2. Influence of PBMCs (peripheral blood mononuclear cells) isolated from healthy volunteers on doxorubicin induced HeLa cell killing in dependence of treatment with doxorubicin (Dox) at 250 nM and 1 µM. HeLa cells were seeded at a density of 5000 cells per well in 96-well plate and treated 24 h later with DMSO or Dox for further 24 h. Thereafter, IL-2 activated (48 h) PBMCs were added to HeLa cells at the indicated dilutions and 48 h later the supernatant containing PBMCs were removed, HeLa cells were washed with PBS for elimating all of the PBMCs and HeLa cell viability was determined using AlamarBlue assay according to the manufacturer´s instructions (Invitrogen). Mean and SD, n=3.