

STAT1: A Modulator of Chemotherapy-induced Apoptosis

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ABSTRACT

The anthracyclines, such as doxorubicin, are widely used in the treatment of breast cancer. Previously, we showed that these drugs could activate the transcription factor, nuclear factor κ B, in a DNA damage-dependent manner. We now show that these drugs can potentiate the activation of signal transducer and activator of transcription 1 (STAT1) in MDA-MB 435 breast cancer cells treated with IFN- γ . We observed that key markers of STAT1 activation, including tyrosine 701 and serine 727 phosphorylation, were enhanced in the presence of doxorubicin. This potentiation resulted in enhanced nuclear localization of activated STAT1 and led to an increase in the nuclear binding of activated STAT complexes. The observed potentiation was specific for STAT1 and IFN- γ , as no effects were observed with either STAT3 or STAT5. Furthermore, the type I IFNs (α and β) had little or no effect. The observed effects on STAT1 phosphorylation have previously been linked with maximal transcriptional activation and apoptosis. Cell viability was assessed by crystal violet staining followed by analysis with CalcuSyn to determine combination index values, a measure of synergy. We confirmed that significant synergy existed between IFN- γ and doxorubicin (combination index = 0.34) at doses lower than IC₅₀ values for this drug (0.67 μ mol/L). In support of this, we observed that apoptotic cell death was also enhanced by measuring poly(ADP-ribose) polymerase and caspase-3 cleavage. Finally, suppression of STAT1 expression by small-interfering RNA resulted in a loss of synergistic apoptotic cell death compared with cells, where no suppression of STAT1 expression was attained with scrambled small-interfering RNA control. We conclude that doxorubicin potentiates STAT1 activation in response to IFN- γ , and that this combination results in enhanced apoptosis in breast cancer cells.

INTRODUCTION

Members of the signal transducer and activator of transcription (STAT) family of transcription factors regulate the expression of a variety of genes involved in proliferation, differentiation, survival, and apoptosis (1, 2). There are seven family members known to date, which are nuclear and cytoplasmic in location and provide a direct link between signals generated at cell surface receptors and regulation of gene expression in the nucleus. Many cytokines, growth factors, and hormones can lead to simultaneous activation of two or more STAT factors; however, targeted deletion of specific members has revealed cell type-specific roles with, for example, STAT1 being identified as the major effector of IFN- γ signaling (3, 4). Activation of STAT1 occurs after phosphorylation on a conserved COOH-terminal tyrosine residue (701) in response to activated receptor-associated Janus-activated kinases (JAK). Active STAT1 dimers are then formed through reciprocal interactions between this tyrosine phosphorylated residue and the SH2 domain of the partner STAT1 monomer, triggering nuclear localization. A COOH-terminal serine

residue (727) is also phosphorylated, resulting in maximal transcriptional activity (5). Nuclear STAT1 dimers recognize specific sequences (GAS) in the promoters of genes and activate their transcription (2, 6). Down-regulation of STAT activation occurs in response to phosphatases (SHP family members), inhibitors (including members of the SOCS family), and ubiquitin-mediated proteolysis (1). STAT1 can also form heterodimeric interactions with STAT2 after activation by the type I IFNs, α and β . The formation of a complex termed ISGF3 is then triggered by the association of the STAT1/2 heterodimer with a third component, p48 (IRF9). This transcriptional complex subsequently enters the nucleus and binds IFN-stimulated response elements. An additional layer of complexity arises where IFN- γ , through STAT1-mediated expression of IRF1, can up-regulate the expression of type I IFNs, resulting in a chain of signaling events where genes containing both GAS and IFN-stimulated response elements are (directly and indirectly) regulated in response to IFN- γ (1).

The molecular events and signaling pathways that lie downstream of activated STATs have been largely determined from studies relevant to development and immune responses; however, recent years have seen the emergence of a role for select STAT family members in cancer (7). Normally, STAT activation is a transient and tightly regulated process as discussed previously. However, in cancer, transient regulation is often replaced by constitutive activation. In the case of STAT5, persistent activation is mediated by the Bcr-Abl fusion protein, an oncogenic receptor tyrosine kinase, and this has been implicated in the pathogenesis of chronic myelogenous leukemia (8). Constitutive activation of STAT3 and 5 has been observed in a variety of tumor types including solid tumors of the breast, prostate, head and neck, as well as many leukemias and lymphomas (9–12). Their role in cell growth and survival is underpinned by their diverse gene targets, which include genes encoding inhibitors of apoptosis, such as Bcl-2 family members, proto-oncogenes such as c-Myc, and proliferative markers such as Pim-1 (7). Furthermore, many reports describe how blocking constitutively activated STAT3 or STAT5 leads to apoptotic cell death in tumor cells (7, 9, 10). Not surprisingly, therapeutic approaches in targeting these STATs have focused primarily on disrupting their constitutive activation. This has been largely achieved through inhibition of JAK family members (13) and the use of phospho-peptide mimetics (7, 14).

In contrast, loss of STAT1 protein expression has been observed in cancer (7, 15), consistent with its role in IFN-mediated cell cycle arrest and apoptosis. In fact, the ability of IFN- γ to inhibit the growth of cells in culture is dependent on transcriptionally active STAT1 (15). This phenotype is reflected in the spectrum of gene targets it regulates, including proteins involved in death receptor signaling (Fas) and those involved in cell cycle arrest (p21^{WAF1}; refs. 3, 6). Furthermore, a recent study showed that mice deficient in both STAT1 and RAG2, a protein critical for V(D)J recombination, were predisposed to spontaneous mammary gland carcinomas (16). This increase in tumor incidence observed in the absence of STAT1 is also consistent with the observation that its down-regulation has been observed in some tumor types (7). Interestingly, in a recent study, the presence of activated STAT1 in a panel of breast cancers was shown to be a significant indicator of good prognosis, even after adjusting for

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known prognostic variables (lymph node status, stage of disease, estrogen receptor status, and cathepsin D; ref. 17). STAT1 has properties of a tumor suppressor protein and not surprisingly has been suggested to antagonize the activities of STAT3 and 5 (7, 18). Consistent with this and its antiproliferative role, STAT1 has been shown to down-regulate the expression of c-Myc protein, a target of STAT3 (19).

Given that STAT1 activation can promote cell death, it is a relevant target for therapeutic intervention. In this study, our overall aim was to investigate the link between STAT1 potentiation and enhanced apoptotic cell death, observed concurrently in breast cancer cells treated with topoisomerase II-targeted drugs and the type II cytokine, IFN- γ . Our preliminary observations are novel, describing for the first time how a subset of frequently used chemotherapeutic agents can potentiate the activation of STAT1 in cells treated with IFN- γ , and this results in enhanced cell death.

MATERIALS AND METHODS

Materials and Cell Culture. MDA-MB 435 breast cancer cells (ETACC) were grown as a monolayer culture in DMEM supplemented with 10% FCS, 1 mmol/L sodium pyruvate, and L-glutamine (final concentration, 1 mmol/L), all obtained from Invitrogen (Paisley, Scotland). MDA-MB 468 and MCF-7 breast cancer cells (ETACC) were cultured in RPMI 1640 (Invitrogen) with supplements as described above. Doxorubicin and mitoxantrone were purchased from Sigma (St. Louis, MO), and camptothecin purchased from Calbiochem (Nottingham, England). Cisplatin was supplied by Faulding Pharmaceuticals Ltd. (United Kingdom). IFN- γ was purchased from Calbiochem and tumor necrosis factor (TNF)- α from PeprTech EC Ltd. (London, England). Lyophilized oligonucleotides (STAT1, 5'-AACTAGTGGAGTGAAGCGGA-3' and Scrambled, 5'-AAGGGTCGAGAGAGAGCTGTA-3') for small-interfering RNA were from Qiagen (Crawley, West Sussex; synthesized by Xeragon). Small-interfering RNA duplexes were prepared as recommended by the manufacturers.

Cells in late log phase of growth were reseeded in fresh medium at a concentration of 4×10^5 /mL. After treatment with chemotherapeutic agents and/or IFN- γ , incubations were discontinued by the addition of ice-cold PBS (1 \times PBS) containing sodium orthovanadate (1 mmol/L), and whole cell extracts were prepared as described previously (20, 21). Protein determinations were made with the BSA reagent (Pierce, IL) with bovine albumin (Sigma) as standard.

Western Blot Analysis. Equal protein concentrations of whole cell lysates (as indicated) were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After transfer, blots were incubated with primary antibody overnight at 4°C at a dilution (see below) in 5% dry milk 1 \times PBS containing 0.1% Tween 20. Western blot analysis was done with anti-poly(ADP-ribose) polymerase (PARP; Oncogene, Nottingham, United Kingdom, and BD Bioscience, Cowley, Oxford, United Kingdom), anticaspase-3 (Oncogene), antiprocaspase-8 (QBioGene-Alexis Ltd., Nottingham, United Kingdom), anticaspase-9, and caspase-3 (Cell Signaling, Beverly, MA) or anti- β -actin (Sigma) antibodies, where required. Secondary horseradish peroxidase antibody (antimouse or antirabbit; Dako, Copenhagen, Denmark) was applied at a dilution of 1:2,000 for 1 hour at room temperature. Blots were developed by enhanced chemiluminescent detection and exposed to X-ray film (Fuji).

Crystal Violet Cytotoxicity Assays. Cells (2.0×10^6 /plate) were treated with a range of doses of doxorubicin (0.25 to 1.0 μ mol/L) and incubated for 72 hours in the presence or absence of IFN- γ (500 units). After a specified time, the media was discarded, and the remaining viable adherent cells were washed with 1 \times PBS before staining with crystal violet (0.2% crystal violet in 2% EtOH) for 10 minutes at room temperature (BDH, Poole, United Kingdom). The stain was solubilized by adding 1% SDS, and the absorbance of each plate was determined at 570 nm. Viable cells were calculated as a percentage of absorbance with respect to untreated control cells, in the case of treatments without IFN- γ , or normalized with respect to IFN- γ , where it was part of the treatment. Treatment interaction effects between IFN- γ and cytotoxic drug were assessed with the CalcuSyn program, based on the principle of

Chou and Talalay (22). This program returns combination index values that are indicative of synergy, additivity, or antagonism between two agents. Combination index values in the order of 0.1 to 0.3 units are deemed "strongly synergistic," where synergistic ranges lie between 0.3 and 0.7 units. Statistical significance was determined with ANOVA analysis.

Small-interfering RNA Transfection. MDA-MB 435 cells were seeded at 4.8×10^6 cells/90-mm dish. After overnight incubation, 18 μ L of Oligofectamine (Qiagen) was added to 1.050 mL of OptiMem1 (Invitrogen) media and incubated at room temperature for 5 minutes. An aliquot (30 μ L) of a 40 μ mol/L small-interfering RNA solution was then added, and incubation took place for an additional 30 minutes at room temperature, mixing occasionally. An additional 4.9 mL of OptiMem1 was added to the small-interfering RNA suspension before being layered onto the prewashed cell monolayer. After 4 hours, DMEM containing 30% FCS was added to the cells. This entire procedure was repeated the following day. After 24 hours, cells were trypsinized, and each pool of cells corresponding to either STAT1, scrambled, or mock transfected cells (control) was plated out at 1.5×10^6 cells/90-mm dish. This corresponded to "set-up" treatment. The cells were left for two days and then treated with IFN- γ and doxorubicin as described previously. Cells were harvested at 24, 48, and 72 hours, where a portion was reserved for viability staining by crystal violet and the remainder used to prepare protein extracts, both as described previously.

STAT1 Electrophoretic Mobility Shift Assays (EMSA). The 32 P-radio-labelled oligonucleotide probe corresponding to a high-affinity binding site (GAS) for STAT1 from the IRF-1 promoter (23) was used in EMSAs. After drug/IFN- γ treatments, nuclear extracts were prepared as described previously (20, 21), incubated with labeled oligonucleotide probe and resolved by PAGE as described previously. Retarded complexes on dried gels were visualized after autoradiography. Complexes corresponding to STAT1 homodimers were confirmed by supershift analysis with antibodies specific to this protein (Active Motif), where complete supershifting would be expected with homodimeric but not heterotrimeric complexes such as that corresponding to STAT1/STAT2/IRF-9 (ISGF3). Nuclear extracts were incubated with 5 μ L of antibody (as recommended) for 45 minutes at room temperature, before the addition of labeled probe. Specificity of binding was also determined by competition analysis with either unlabelled competitor (κ B site) or nonspecific probe (oct-1). These were added (0.0175 pmol/L) to nuclear extract/labeled oligonucleotide incubations at room temperature 30 minutes before electrophoresis.

Immunofluorescent Microscopy. After drug/IFN- γ treatments, cells were fixed in methanol (100%) at -20°C for 10 minutes followed by washes with 1 \times TBS (3 \times 5 minutes). Cells were then quenched for 5 minutes with 0.1% sodium borohydride in 1 \times TBS, rinsed as previously described, followed by incubation with block solution containing 5.5% normal calf serum and 0.1% Triton X-100 at room temperature to reduce nonspecific binding. Cells were then incubated with primary antibody [anti-phospho-STAT1 (Y701); Cell Signaling] for 1 hour at room temperature followed by rinses and additional incubation with Cy3-labeled goat antirabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were mounted in Vectashield with 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining (Vector Laboratories, Inc., Burlingame, CA). Fluorescence was monitored with a Zeiss Axiovert 200M inverted microscope, with DAPI and tetramethylrhodamine isothiocyanate (TRITC) filter sets, and an objective setting of 63 \times .

RESULTS

Doxorubicin Potentiates the Activation of STAT1 in MDA-MB 435 Breast Cancer Cells Treated with IFN- γ . Initially, we observed that IFN- γ (500 units) treatment of MDA-MB 435 breast cancer cells resulted in the expected increase in the tyrosine 701 phosphorylated form of STAT1 (Fig. 1A), required for STAT dimerization and nuclear translocation (1, 24). As expected, activation was transient, although still detectable at 6 and 8 hours posttreatment. Serine 727 may also become phosphorylated in response to both related and discrete signaling events (in the case of IFN- γ) and is required for maximal transcriptional activity (1, 25). The phosphorylation of both residues (Y701 and S727) after IFN- γ treatment was

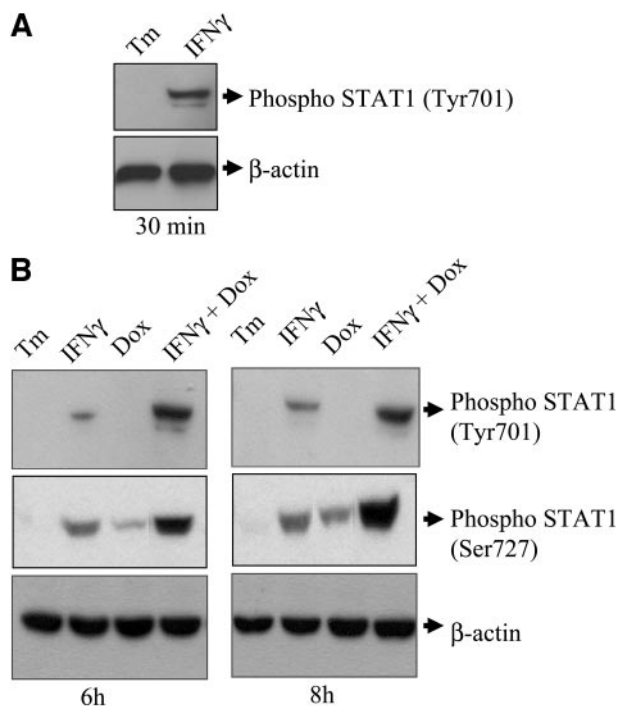


Fig. 1. Doxorubicin potentiates the activation of STAT1 in MDA-MB 435 breast cancer cells treated with IFN- γ . A. IFN- γ (500 units) treatment of MDA-MB 435 cells is associated with an increase in tyrosine 701 phosphorylation of STAT1 as determined by Western blot analysis. B. Doxorubicin (1 μ mol/L) treatment of cells has no effect on STAT1 phosphorylation but enhances both tyrosine 701 and serine 727 phosphorylation in response to IFN- γ . β -actin was used as loading control, and proteins (50 μ g/lane) were resolved on 8% SDS-PAGE gels.

enhanced in the presence of doxorubicin (Fig. 1B). Treatments with doxorubicin alone had no effect on tyrosine 701 phosphorylation but had a small effect on the serine 727 phosphorylation. We next investigated whether this potentiation resulted in enhanced nuclear localization of activated STAT1. Cells were treated with either IFN- γ (500 units) or drug alone (doxorubicin; 1 μ mol/L), and STAT1 localization was analyzed by immunofluorescent microscopy and compared with cells treated with IFN- γ and doxorubicin combined. We observed enhanced nuclear localization of the (tyrosine 701) phosphorylated form of STAT1 in cells treated with both agents, compared with IFN- γ and drug only treatments (Fig. 2A). In agreement with Western blot data, no effect was seen with drug only treatments, and nuclear staining was dramatically enhanced in cells treated with both agents. Finally, we examined whether enhanced activation and nuclear localization resulted in an increase in the nuclear binding of activated STAT complexes. Using EMSAs (Fig. 2B) and a labeled probe containing a consensus sequence for STAT1 binding (23), we observed a potentiation in the binding of activated nuclear complexes from cells treated with both agents compared with IFN- γ only treatments and drug alone (no binding). Specificity of binding was confirmed through competition analysis, where (cold) competitor but not nonspecific oligonucleotide resulted in a reduction in the presence of activated nuclear complexes. Furthermore, supershift analysis with anti-STAT1 antibody resulted in a complete retardation of activated complexes from treated cells, suggesting the presence of STAT1 monomers.

The Observed Potentiation of STAT1 Activation by Doxorubicin Is Specific for IFN- γ and STAT1 but Occurs in Other Breast Cancer Cell Lines. Reports have described how IFN- γ may activate STAT3 and 5, in addition to STAT1, in a cell type-specific manner (1). Therefore, we addressed whether this was the case in MDA-MB 435 breast cancer cells, to determine whether the observed potentia-

tion was specific for pathways leading to STAT1 activation. Neither STAT3 nor STAT5 were activated in response to IFN- γ in these cells (Fig. 3A), and no effect was observed with drug alone. Furthermore, potentiation appeared to be specific for STAT1 and IFN- γ , as the type I IFN, IFN- α had no effect on STAT1 activation in these cells.

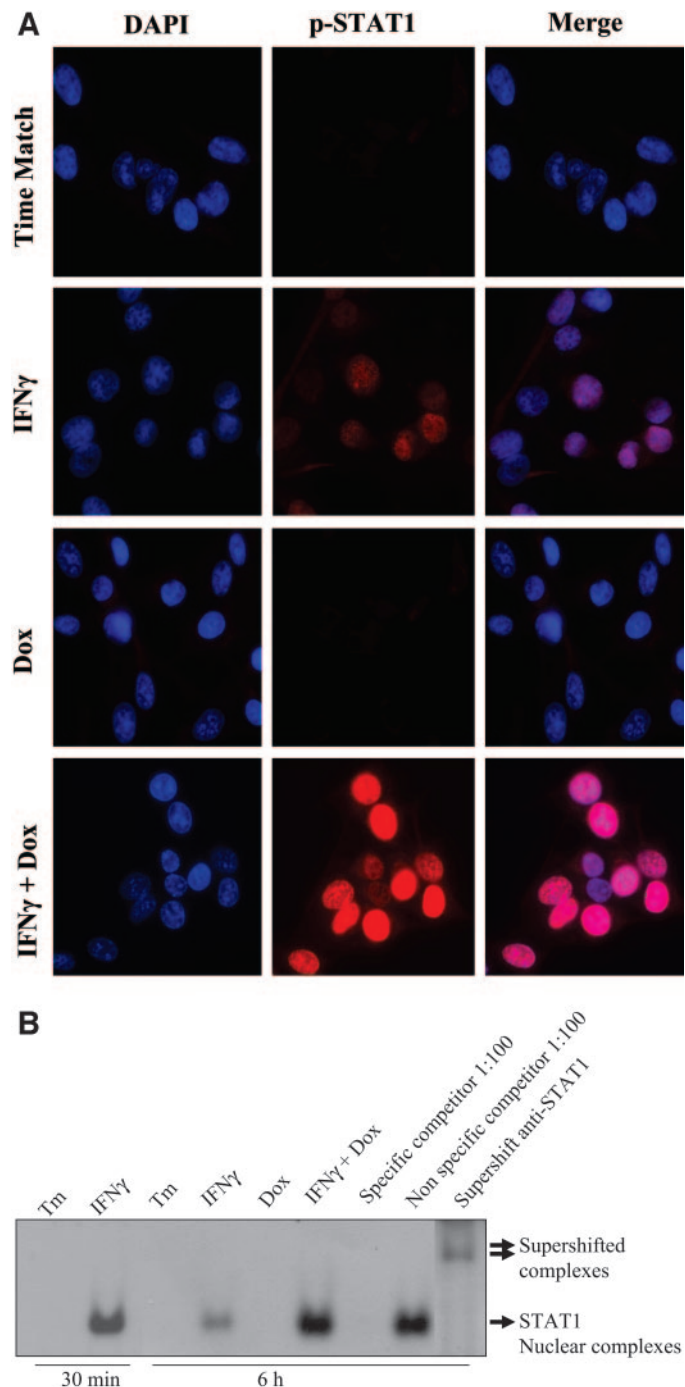


Fig. 2. Doxorubicin enhances the nuclear localization of active STAT1 and potentiates its binding to DNA in response to IFN- γ treatment of MDA-MB 435 cells. A. IFN- γ (500 units) treatment of MDA-MB 435 cells is associated with an increase in the nuclear content of activated STAT1 (tyr701 phosphorylated), as determined by immunofluorescent microscopy with a secondary antibody conjugated with Cy3. Doxorubicin treatment (1 μ mol/L) enhances nuclear content of activated STAT1 (tyr701 phosphorylated) in response to IFN- γ (500 units). Detection was made with DAPI and TRITC filter sets and a magnification of 63 \times . B. STAT1 DNA binding was determined by EMSA. Nuclear extracts were incubated with a labeled probe containing a consensus sequence for STAT1 binding. Competition studies were carried with 1:100 dilutions of cold specific and nonspecific probes. Supershift analysis was done by preincubating for 45 minutes at room temperature with an antibody to STAT1.

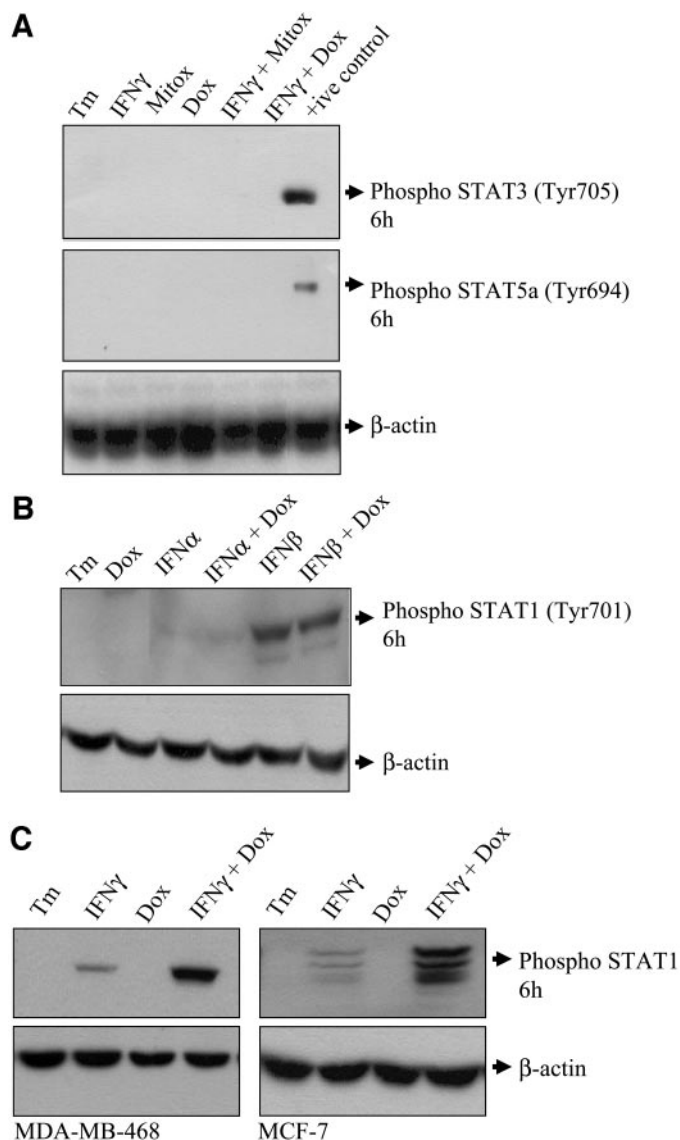


Fig. 3. Potentiation of STAT1 activation by doxorubicin is specific for IFN- γ and STAT1. **A**, MDA-MB 435 breast cancer cells were treated with doxorubicin ($1 \mu\text{mol/L}$) alone or in combination with IFN- γ (500 units). Proteins ($50 \mu\text{g/lane}$) were resolved on 8% SDS-PAGE gels and assayed for phospho-STAT3 (Tyr705) and phospho-STAT5 (Tyr694) by Western blot analysis. Positive controls are as follows: prolactin-treated mammary epithelial cells (STAT5) and IFN- α -treated HeLa cells (STAT3). Equal loading of protein was confirmed by probing blots for β -actin. **B**, MDA-MB 435 breast cancer cells were treated with IFN- α (500 units) or IFN- β (500 units) alone or in combination with doxorubicin ($1 \mu\text{mol/L}$). Proteins ($50 \mu\text{g/lane}$) were resolved on 8% SDS-PAGE gels and assayed for phospho-STAT1 by Western blot analysis. **C**, MDA-MB 468 and MCF-7 breast cancer cells were treated with IFN- γ (500 units) alone or in combination with doxorubicin ($1 \mu\text{mol/L}$). Proteins ($50 \mu\text{g/lane}$) were resolved on 8% SDS-PAGE gels and assayed for phospho-STAT1 by Western blot analysis. β -actin was used as loading control.

Although IFN- β induced STAT1 tyrosine 701 phosphorylation, it failed to synergize with doxorubicin (Fig. 3B). We next examined two other breast cancer cell lines, MDA-MB 468 and MCF-7, and showed that doxorubicin treatment could also lead to enhanced STAT1 activation in response to IFN- γ (Fig. 3C), suggesting that this phenomenon was not restricted to one cell type. We additionally addressed the specificity of this response by examining whether other distinct cytokine family members and a wider range of chemotherapeutic drugs could effect the potentiation of STAT1 activation in response to IFN- γ . Previous studies have shown synergy between the IFNs and the proinflammatory cytokine, TNF- α , in mediating a number of signaling pathways (26, 27). However, no effects on STAT1 activa-

tion were observed in MDA-MB 435 cells treated with both agents (Fig. 4A). Potentiation of STAT1 activation was not observed with the topo I-targeted drug, camptothecin, nor was it observed with either taxol or cisplatin, agents for which mechanism of action involves targeting of microtubules and DNA cross-linking, respectively (Fig. 4B). Additional analysis of topo II-targeted drugs revealed that mitoxantrone treatment of cells could also effect the potentiation STAT1 activation in response to IFN- γ ; however, etoposide was without effect (Fig. 4B). Collectively these data support the specificity of our observations.

Treatment of MDA-MB 435 Breast Cancer Cells with DNA Damaging Drugs and IFN- γ Combined Results in Synergistic Activation of Apoptotic Cell Death. The observed effects on STAT1 phosphorylation have previously been linked with maximal transcriptional activation and apoptosis (3, 28). We next examined whether enhanced STAT1 activation was accompanied by a potentiation in chemotherapy-induced cell death. Initially, we examined cell viability by assessing cytotoxicity with crystal violet staining of cells treated with either agent alone or in combination (Fig. 5, A and B). A >6-fold potentiation of cell death could be observed at doses that correlated with IC_{50} doses for doxorubicin ($0.67 \mu\text{mol/L}$) when combined with minimally toxic doses of STAT1 activator, IFN- γ (500 units), [IC_{50} doses previously determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; data not shown] at both 48 and 72 hours posttreatment. The treatment interaction effects were determined quantitatively by calculating the combination index values, according to the method of Chou and Talalay (22). We confirmed that synergism existed between IFN- γ and doxorubicin at doses reflecting IC_{50} values for this drug. Specifically,

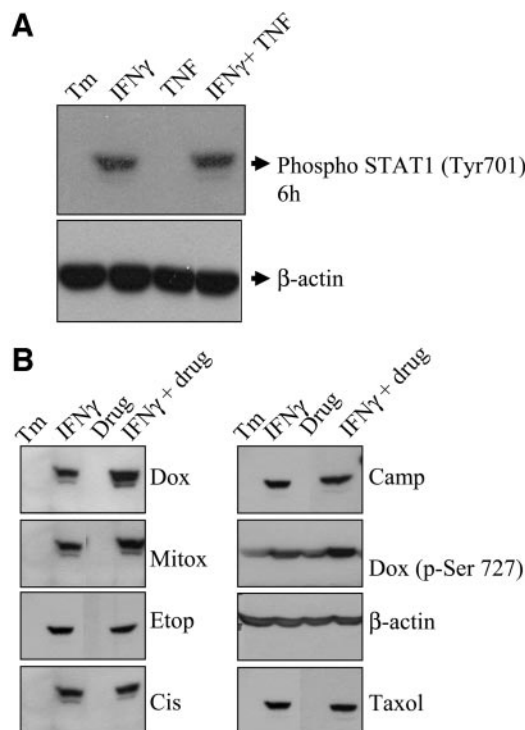


Fig. 4. STAT1 activation by IFN- γ is not potentiated by TNF- α but can be effected by other topoisomerase II-targeted drugs. **A**, MDA-MB 435 breast cancer cells were treated with TNF- α (20 units) alone or in combination with doxorubicin ($1 \mu\text{mol/L}$). Proteins ($50 \mu\text{g/lane}$) were resolved on 8% SDS-PAGE gels and assayed for phospho-STAT1 by Western blot analysis. **B**, MDA-MB 435 breast cancer cells were treated with a range of drugs [doxorubicin ($1 \mu\text{mol/L}$), mitoxantrone ($2 \mu\text{mol/L}$), etoposide ($1 \mu\text{mol/L}$), camptothecin ($1 \mu\text{mol/L}$), taxol (1nmol/L), and cisplatin ($1 \mu\text{mol/L}$)] alone and in combination with IFN- γ (500 units). Proteins ($50 \mu\text{g/lane}$) were resolved on 8% SDS-PAGE gels and assayed for tyrosine 701 (and serine 727 phosphorylated STAT1 for doxorubicin treatments only) by Western blot analysis. β -actin was used as loading control.

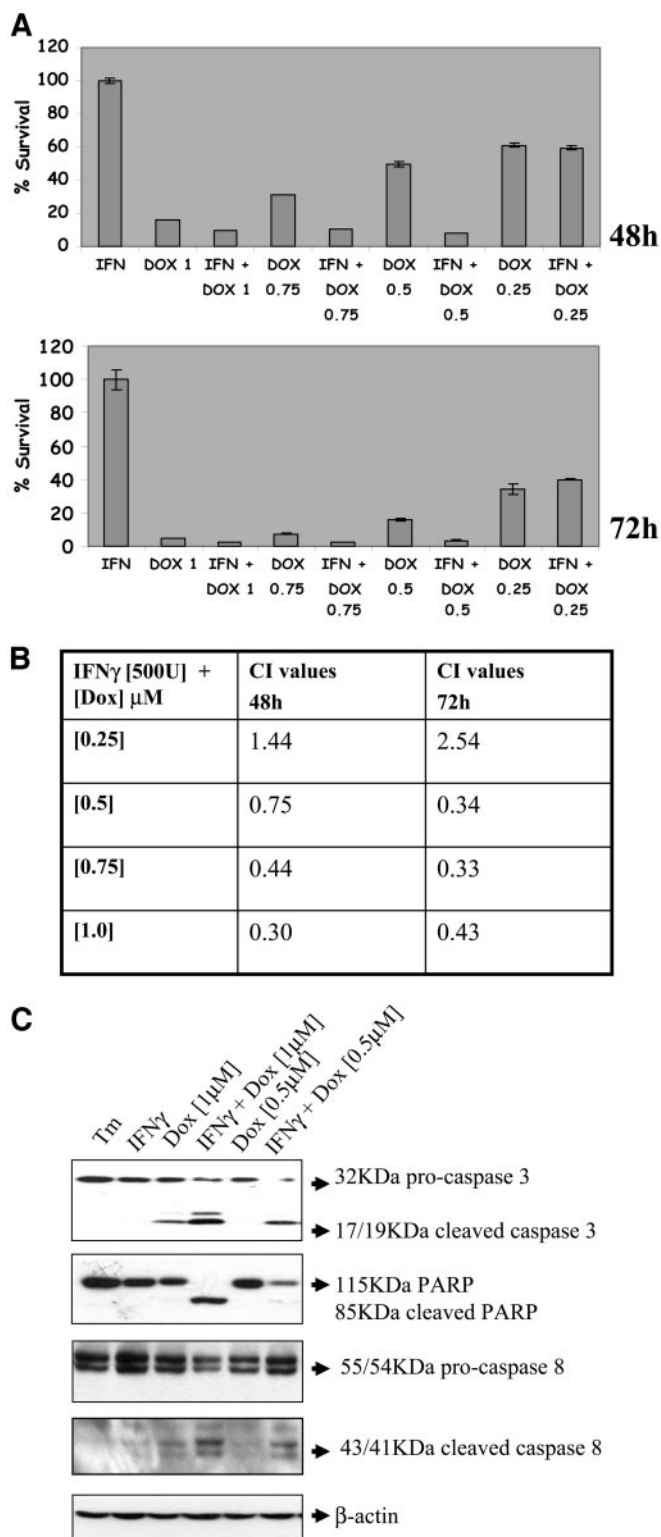


Fig. 5. Doxorubicin and IFN- γ treatment of MDA-MB 435 breast cancer cells results in a synergistic activation of apoptotic cell death. **A**. MDA-MB 435 breast cancer cells were treated with a range of doses of doxorubicin (0.25 to 1 μ mol/L) in the presence and absence of IFN- γ (500 units). After crystal violet staining, percentage of cell survival was determined. Data are representative of three separate experiments; bars, \pm SD. **B**. Synergy was determined for different drug combinations by calculating combination index values with Calcsyn (as outlined in Materials and Methods). Values of between 0.3 and 0.7 are considered synergistic, where 0.3 is bordering on highly synergistic. This program accounts for statistical significance. **C**. Treatment of MDA-435 breast cancer cells with doxorubicin alone [0.5, 1.0] μ mol/L and in combination with IFN- γ (500 units) results in enhanced apoptotic cell death. Proteins (20 μ g/lane) were resolved by SDS-PAGE and probed for caspase-3 and -8 (pro and cleaved forms), and PARP expression by Western blot analysis. β -actin was used as loading control.

combination index values indicative of synergy, 0.34 and 0.33, corresponding to [0.5] and [0.75] μ mol/L doxorubicin were calculated for this drug when used in combination with minimally toxic doses of IFN- γ (500 units; Fig. 3C).

We next investigated whether the observed potentiation in cell death was because of induction of apoptosis. Analysis of key markers of apoptosis revealed an increase in the presence of the cleaved form of PARP and caspase-3, compared with drug or IFN- γ only treatments (Fig. 5C), confirming qualitatively, synergy between these two agents. Additional analysis revealed an up-regulation in the expression of caspase-8, suggesting a possible mechanism underlying this potentiation (Fig. 5C). The activation and cleavage of caspase-8 indicated that apoptosis was mediated via the extrinsic death pathway; however, it does not exclude the involvement of the intrinsic mitochondrial death pathway. To investigate this we also probed extracts with an antibody to cleaved caspase-9, a specific marker of mitochondrial involvement in the death pathway. Very little cleaved caspase-9 was detected by Western blotting, suggesting little or no involvement of the intrinsic mitochondrial-mediated death pathway (data not shown).

Loss of STAT1 Expression in MDA-MB 435 Breast Cancer Cells Results in Loss of Synergistic Cell Death Seen with Combined Treatments of IFN- γ and Doxorubicin. On the basis of the reported role for active STAT1 in signaling cell death, our observations provided strong evidence to suggest that STAT1 could be synergistically activated by IFN- γ in the presence of certain topo II-targeted drugs, and that this could lead to enhanced apoptotic death. However, conclusive proof that these two events are linked required careful manipulation of the STAT1 response and examination of the outcome in cells after combined IFN- γ /drug treatments. Initially, we established that STAT1 expression could be suppressed by >90% with small-interfering RNA, compared with that seen with scrambled (or nonspecific) oligonucleotide (Fig. 6A), with maximal suppression observed 48 hours posttransfection. Cells were treated with doxorubicin and IFN- γ , alone and in combination, at this time, and additional analysis indicated that knockdown of STAT1 expression was sustained for at least 72 hours, correlating with viability staining and cleaved caspase-3 analysis.

To evaluate the effect of STAT1 inhibition on IFN- γ and doxorubicin-induced apoptosis, we carried out cell viability analysis by assessing cytotoxicity with crystal violet staining at 24, 48, and 72 hours posttreatment. Small-interfering RNA-based inhibition of STAT1 in MDA-MB 435 cells resulted in a substantial reduction in cell death mediated by IFN- γ and doxorubicin (Fig. 6B), compared with that seen in cells transfected with scrambled control oligonucleotide, at all of the time points tested. Pair-wise comparison of data recorded for STAT1 small-interfering RNA-treated breast cancer cells revealed that differences in cell survival for doxorubicin only treatments compared with those where IFN- γ was present were not statistically significant compared with data corresponding to cells treated with scrambled control (ANOVA analysis; Fig. 6C). This was particularly evident at 48 hours, where a 4-fold reduction in cell viability in cells where STAT1 was present (IFN- γ /doxorubicin compared with doxorubicin only treatments), compared with no significant change in cell survival ($P > 0.05$) in cells where STAT1 was suppressed. Loss of synergy was also observed in these cells when treated with 0.5 μ mol/L doxorubicin and IFN- γ compared with doxorubicin only, *i.e.*, 49.4% survival after treatment with doxorubicin and IFN- γ and 24.7% survival for the same treatment (48 hours) when STAT1 was present ($P < 0.001$). A dose range of 0.5 to 1.0 μ mol/L is within clinically relevant limits for doxorubicin (29) and correlates with IC₅₀ values determined for these cells.¹

These data were supported by analysis of key markers of apoptotic

¹ Unpublished data.

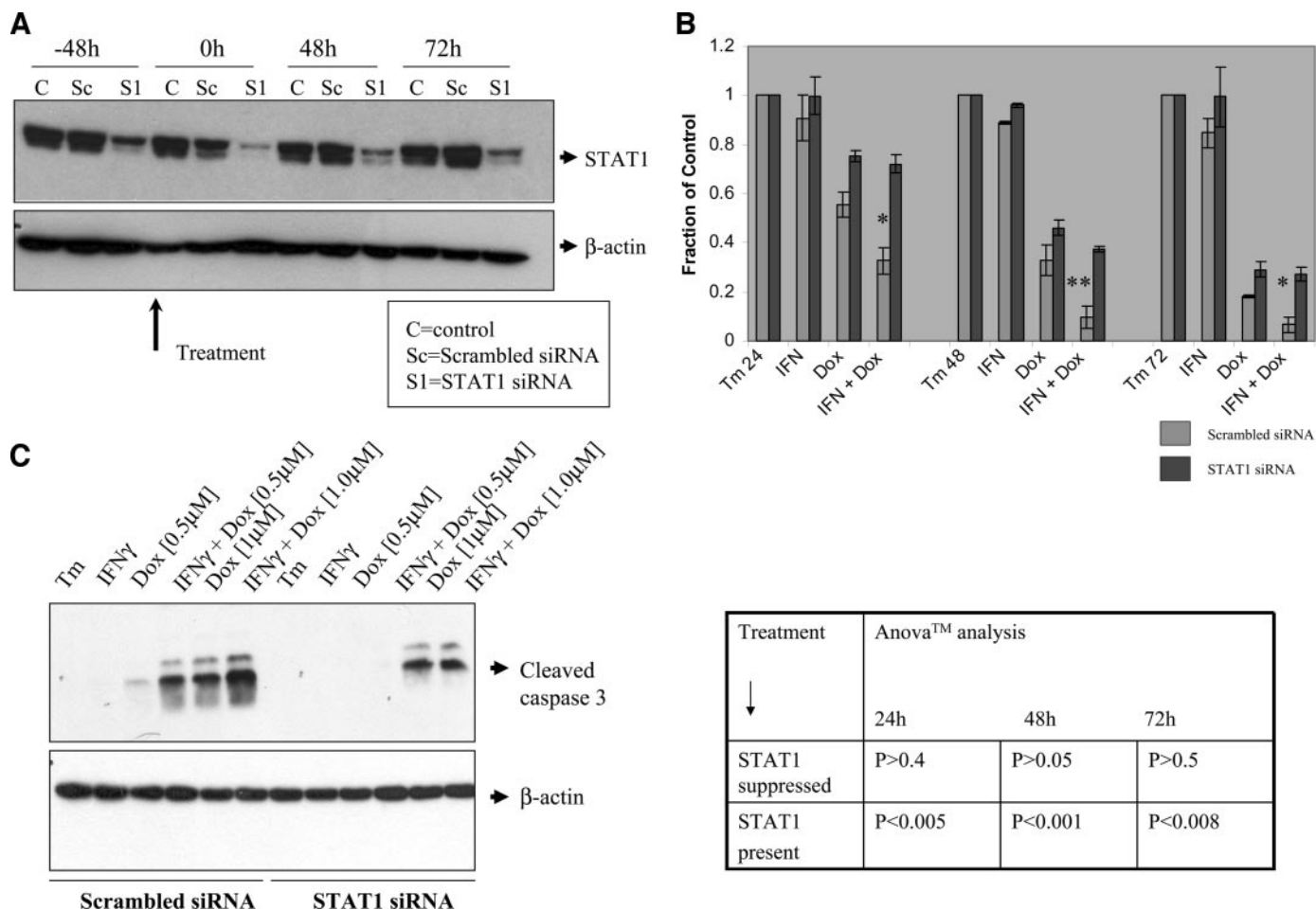


Fig. 6. Loss of STAT1 expression in MDA-MB 435 breast cancer cells results in loss of synergistic cell death seen with combined treatments of IFN- γ and doxorubicin. **A**, MDA-MB 435 cells were transfected with STAT1 (S1) or scrambled small-interfering RNA (Sc) duplexes as described. STAT1 protein expression was measured by Western blot analysis. Equal loading of protein was confirmed by probing blots for β -actin expression. **B**, Cells were treated (as indicated) with either doxorubicin (1 μ mol/L) or IFN- γ (500 units/mL) alone or in combination. Percentage of cell survival was determined after crystal violet staining at 24, 48, and 72 hours posttreatment. Columns of percentage cell survival (represented as fraction of control) are plotted, where scrambled small-interfering RNA treatments are represented by \square (Sc) and STAT1 small-interfering RNA treatments by \blacksquare (S1). The accompanying table is a summary of a pair-wise comparison between STAT1 S1/Sc cells made by comparing the statistical significance of differences in cell survival recorded for doxorubicin only *versus* doxorubicin/IFN- γ combined treatments by ANOVA (*, $P < 0.01$; significant, **, $P < 0.001$; highly significant, $P > 0.05$; not significant). The data shown is representative of three separate experiments; bars, \pm SD. **C**, Cell extracts were prepared from MDA-MB 435 cells (STAT1 S1/Sc) after drug/IFN- γ treatments (48 hours). Proteins (20 μ g/lane) were resolved by SDS-PAGE and probed for cleaved caspase-3 by Western blot analysis. β -actin was used as loading control.

cell death derived from parallel cell treatments. The loss of cleaved caspase-3 was observed in cells where STAT1 expression has been suppressed, compared with scrambled control (Fig. 6C). The effect was most evident at 0.5 μ mol/L doxorubicin, a dose closely correlating with its IC_{50} value in these cells (0.67 μ mol/L). Cell death, as indicated by cleaved caspase-3, was also lower compared with that seen in cells transfected with scrambled control small-interfering RNA, at 1.0 μ mol/L doxorubicin, and loss of potentiation in the presence of IFN- γ was evident.

Taken together, these data suggest that doxorubicin can potentiate STAT1 activation in response to IFN- γ , and that this potentiation results in synergistic cell death in cells treated with both agents.

DISCUSSION

We have observed that treatment of the breast cancer cell line, MDA-MB 435, with the anticancer drug, doxorubicin, results in a dramatic potentiation in STAT1 activation in response to IFN- γ . This was determined by measuring an increase in the phosphorylation of two key residues (tyrosine 701 and serine 727) and was accompanied by enhanced nuclear localization and DNA binding of STAT1. STAT1 activation is generally associated with cell cycle arrest and

apoptosis (3). In this regard, treatment of MDA-435 cells with IFN- γ together with doxorubicin resulted in significantly enhanced cell death as measured by caspase-3 and PARP cleavage (apoptosis), and this was supported by combination index values calculated from cell survival assays, indicative of synergistic interaction. Furthermore, loss of STAT1 expression by small-interfering RNA transfection resulted in loss of synergistic cell death in cells treated with both agents. Our data suggest that doxorubicin potentiates STAT1 activation in response to IFN- γ , and that this is the molecular basis underlying the observed synergistic cell death (apoptosis) with these agents.

IFN- γ is a member of a family of related cytokines that mediate a diverse range of functions in cells, including growth inhibitory and antiviral activities (30). Much effort has been focused on exploiting its therapeutic potential, in particular, its antitumor properties. Although its use as a single agent has been limited, studies have described synergistic activation of cell death (apoptosis) between IFN- γ and a range of agents, including TNF- α family members, some anticancer drugs (5-fluorouracil), and other nonrelated stimuli such as ischemia/reperfusion (3, 26, 27, 31–33). The molecular basis of this synergy has been ascribed to a number of different pathways, depending on the stimulus involved. For example, synergy has been shown to be ac-

accompanied by the enhanced expression of select proapoptotic genes, including TNF-related apoptosis-inducing ligand, FasL and IRF-1 (31, 34, 35), and furthermore, the latter has been linked with up-regulation of procaspase-8 expression (36). In fact, in a number of studies, IFN- γ was found to prime cells for death by up-regulating both initiator and effector caspases (including caspase-1, -3, and -8), albeit an indirect effect, thereby linking its effects with both death receptor and mitochondrial-mediated apoptosis (3). Our data also support a mechanism of enhanced cell death that is driven primarily by a pathway involving enhanced caspase-8 expression and cleavage. With respect to TNF- α , STAT1, activated in response to IFN- γ , has been reported to block its ability to activate nuclear factor κ B, a survival signal (37), thereby enhancing apoptotic death in cells treated with both cytokines. In a separate study, IRF-1, a downstream target of active STAT1, was shown to suppress nuclear factor- κ B-mediated transcriptional activity (27), resulting in a similar outcome. Previously, we have shown that topoisomerase II-targeted drugs could activate this transcription factor (20, 21) in a DNA damage-dependent manner; however, no effects of IFN- γ on inhibitor of nuclear factor κ B degradation, a marker of nuclear factor κ B activation, either alone or on that mediated by topo II-targeted drugs were observed (data not shown), ruling this out as a molecular basis for the enhanced cell death, and prompting us to explore other pathways. Despite a variety of molecular mechanisms, no reports to date have described a direct effect on signaling events leading to activated STAT1 as the molecular mechanism underlying synergistic cell death observed with IFN- γ when combined with cytotoxic drugs. Historically, molecular and biochemical analyses have revealed the importance of the JAK/STAT pathway in mediating the pleiotropic effects of IFNs, and therefore, it is not surprising that a synergistic effect might be targeted to a component of this pathway, as we have shown.

There are many examples supporting the role of STAT1 in inducing apoptosis, most notably evidence derived from cells lacking STAT1 expression, where IFN-mediated apoptotic induction is abolished compared with closely matched cells expressing functional STAT1 (3, 38). Studies indicate that these effects are most likely dependent on transcriptionally active STAT1 (13). However, a number of recent reports have shown that the COOH-terminal domain of STAT1, and not the entire protein, is necessary and sufficient for enhanced apoptosis seen when IFN- γ is combined with ischemia/reperfusion (39). It is suggested that its ability to interact with a variety of transcriptional regulators, thereby circumventing a requirement for its DNA binding domain, may underlie these observations. In support of this hypothesis, a recent study showed that phosphorylation of STAT1 on serine 727 after cisplatin treatment promoted its interaction with p53, resulting in the expression of selective p53 gene targets linked with apoptotic induction (32). A key difference with our data concerns the absence of a p53 response in our cell system because of a known mutation,² which may explain the lack of an observed effect with cisplatin in our studies. Furthermore, the authors do not reveal enhanced phosphorylation of STAT1 on tyrosine 701 (in addition to serine 727), suggesting that mechanistically, the modulation of STAT1 by cisplatin is distinct from our observations where both residues seem to be coordinately regulated. Finally, our data showing enhanced nuclear localization and DNA binding support a role for the dimerization and transcriptional activity of STAT1 alone. Additional support for this will require careful analysis of gene targets regulated in response to STAT1 potentiation.

In yet another series of reports highlighting potential cell type and stimuli specific effects, it was shown that STAT1 serine 727 phos-

phorylation is coupled to prior phosphorylation on tyrosine 701 in response to treatment with IFN- γ (5) but not after stress (39). Although we have yet to show that our two phosphorylation events are coupled, they are certainly concurrent. Although there is much evidence to support separate pathways mediating the phosphorylation of Ser727 and Tyr701, the potentiation we observe would seem to be coordinately regulated, suggesting that either DNA damage or oxidative stress mediated by the topo II-targeted drugs activates an apical mechanism that affects either a kinase or a phosphatase targeting each residue. One such candidate in this mechanism is the tyrosine kinase, c-Abl, which has previously been linked with both phosphorylations (40) and in separate studies, with DNA damage signaling (41), and which we are currently investigating. The lack of an observed effect with etoposide, an agent not known to cause oxidative stress in cells (42), might suggest that this is the important lesion that leads to STAT1 potentiation in cells treated with IFN- γ . We are pursuing this line of investigation in studies involving JAK1 and 2, where a mechanism involving STAT potentiation is likely to be mediated by one or both kinase.

Additional studies focusing on the COOH-terminal phosphorylation events have shown the importance of STAT1 serine 727 phosphorylation in mediating an apoptotic pathway in cells (39). In most instances, this phosphorylation results in enhanced transcriptional activity and has been additionally suggested to promote the selective transcription of a subgroup of STAT1 target genes (5). For example, serine 727 phosphorylation of STAT1 has been shown to mediate cardiomyocyte-induced cell death by a mechanism involving induction of Fas and its ligand (35). Furthermore, negative as well as positive regulation of gene expression has been reported in response to activated STAT1 (6, 19). Gene targets that are regulated that may reflect the pleiotropic effects of IFN- γ include proteins involved in death receptor signaling (Fas) and those involved in cell cycle arrest (p21^{WAF1}). Active STAT1 dimers bind to gamma-activated sequence (GAS) elements and subsequently activate the expression of genes through interaction with transcriptional coactivators such as CBP/p300, MCM-5, as well as with other transcription factors including p48 (IRF-9) and SP1 (43). In fact, we have already shown that IRF-1 is up-regulated in response to IFN- γ but not in response to drugs alone (data not shown). IRF-1 has been linked with STAT1-mediated apoptosis in a variety of studies, and therefore, its expression may be enhanced, correlating with STAT1 potentiation and enhanced cell death. Interestingly, active STAT1 can negatively regulate the c-Myc promoter in response to IFN- γ , consistent with its role as a mediator of growth suppression (19).

Our data are novel, describing for the first time how STAT1 potentiation is the basis of synergistic cell death observed in cells treated with both IFN- γ and doxorubicin. Because cell death triggered by chemotherapy is a key event in determining tumor growth and survival, our studies may uncover novel mechanisms, whereby clinically used anticancer drugs enhance cell death by modulation of STAT transcriptional activity. These findings may complement the use of small molecule inhibitors that specifically inhibit constitutively active STAT3 and 5, key players in promoting tumor growth and survival (14). The merit of these studies is underpinned by recent data supporting the prognostic value of activated STAT1 in breast cancer (17).

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² R. Kennedy, personal communication.

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