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Suppression of Survivin Phosphorylation on Thr34 by Flavopiridol Enhances Tumor Cell Apoptosis

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ABSTRACT

Survivin is a member of the inhibitor of apoptosis gene family that is expressed in most human cancers and may facilitate evasion from apoptosis and aberrant mitotic progression. Here, exposure of breast carcinoma MCF-7 or cervical carcinoma HeLa cells to anticancer agents, including Adriamycin, Taxol, or UVB resulted in a 4–5-fold increased survivin expression. Changes in survivin levels after anticancer treatment did not involve modulation of survivin mRNA expression and were independent of de novo gene transcription. Conversely, inhibition of survivin phosphorylation on Thr34 by the cyclin-dependent kinase inhibitor flavopiridol resulted in loss of survivin expression, and nonphosphorylatable survivin Thr34 → Ala exhibited accelerated clearance as compared with wild-type survivin. Sequential ablation of survivin phosphorylation on Thr34 enhanced tumor cell apoptosis induced by anticancer agents independently of p53 and suppressed tumor growth without toxicity in a breast cancer xenograft model in vivo. These data suggest that Thr34 phosphorylation critically regulates survivin levels in tumor cells and that sequential ablation of p34<sup>cdc2</sup>-dependent kinase activity may remove the survivin viability checkpoint and enhance apoptosis in tumor cells.

INTRODUCTION

The ability of cells to evade apoptosis, or programmed cell death (1), is a molecular trait perhaps common to all human cancers (2). This results in aberrantly extended cell viability, which translates in increased risk of transforming mutations, accelerated disease progression, and resistance to therapy (3). Among the regulators of apoptosis involved in cancer, interest has been recently focused on survivin (4), a member of the IAP gene family (5). Abundantly expressed during embryonic development but undetectable in most normal adult tissues, survivin is dramatically overexpressed in most human cancers and correlates with abbreviated survival, unfavorable prognosis, resistance to therapy, and accelerated rates of recurrences (4). Common molecular alterations of cancer, including loss of p53 (6, 7), changes in chromatin accessibility, *i.e.*, demethylation (8), or gain of the survivin locus on 17q25 (9), have been implicated in deregulation of survivin gene expression in transformed cells (7). Survivin has been associated with both cytoprotection and preservation of microtubule integrity (4), and its expression in malignant cells may facilitate evasion from apoptosis and promote aberrant mitotic progression, thus exacerbating aneuploidy.

The possibility of exploiting the survivin pathway for cancer therapy has been intensely investigated. In these studies, molecular antagonists of survivin including antisense or dominant negative mutants or generation of survivin-specific cytolytic T cells caused tumor cell apoptosis, enhanced chemotherapy-induced cell death, and resulted in anticancer activity in *vivo* (10–12). One of the critical requirements for survivin function was recently identified in the phosphorylation on Thr34 by the mitotic kinase p34<sup>cdc2</sup>-cyclin B1 (13), and a phosphorylation-mimetic survivin mutant strongly inhibited p53-induced apoptosis (6). This step has also been exploited for anticancer therapy, and inducible expression (14) or adenoviral delivery (15) of nonphosphorylatable survivin Thr34 → Ala prevented phosphorylation of endogenous survivin, which resulted in caspase-9-dependent apoptosis and anticancer activity in *vivo* (14, 15). However, the mechanism(s) by which Thr34 phosphorylation participates in survivin function has not been elucidated.

Using flavopiridol as a model of a Cdk inhibitor (16), we found that phosphorylation on Thr34 is required to maintain survivin expression/stability in cancer cells and that ablation of p34<sup>cdc2</sup>-kinase in mitotically arrested cells results in loss of survivin levels and dramatic enhancement of chemotherapy-induced anticancer activity in *vivo*.

MATERIALS AND METHODS

Cell Cultures, Proteins, and Antibodies. Breast carcinoma MCF-7 cells carrying wild-type p53 or cervical carcinoma HeLa cells with functionally inactivated p53 (American Type Culture Collection, Manassas, VA) were incubated with vehicle (DMSO), Taxol (2 µM; Sigma, St. Louis, MO), Adriamycin (100 nM, Sigma), or cisplatin (3 µM; Sigma) or exposed to UVB irradiation at 50 or 300 J/m<sup>2</sup>. To pharmacologically target survivin phosphorylation by p34<sup>cdc2</sup>- Thr34 enhanced tumor cell apoptosis induced by anticancer agents independently of p53 and suppressed tumor growth without toxicity in a breast cancer xenograft model in vivo. These data suggest that Thr34 phosphorylation critically regulates survivin levels in tumor cells and that sequential ablation of p34<sup>cdc2</sup>- dependent kinase activity may remove the survivin viability checkpoint and enhance apoptosis in tumor cells.

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GFP-survivin or GFP-survivin(T34A) by LipofectAMINE, as described. After a 16-h interval, cells were incubated with cycloheximide (20 μM) to prevent further expression of transfected plasmids plus the broad-spectrum caspase inhibitor Z-VAD-fmk (20 μM) to prevent loss of cell viability associated with survivin(T34A) expression. Aliquots of the various cultures were harvested 0–120 h after cycloheximide block and analyzed for expression of GFP-containing fusion proteins by Western blotting with an antibody to GFP.

**Survivin Promoter-Luciferase Reporter Expression.** MCF-7 cells (1–2 × 10^6 cells/well) were transfected with a minimal survivin promoter upstream of a luciferase reporter gene (pLuc-cyc1.2) by LipofectAMINE as described previously (18). Cells were treated with Taxol or Adriamycin or exposed to UVB irradiation as described, and luciferase activity was determined after 0–24 h of incubation at 37°C on a Lumat luminometer (LB9510), with normalization to β-galactosidase activity.

**MPM-2 Mitotic Phosphoepitope Expression.** For detection of mitotic phosphoproteins (19), MCF-7 cells (1–2 × 10^6 cells/60-mm dish) were treated with Taxol or Adriamycin or exposed to UVB irradiation as described and cultured for 0, 8, 16, 24, or 36 h at 37°C. Cells were fixed in 70% ethanol and labeled with MPM-2 antibody (6 μg/ml) followed by the addition of goat antimouse FITC (Boehringer Mannheim) for 1 h at 22°C in the presence of 5 μg/ml PI containing 50 μg/ml RNase A. Samples were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) using CellQuest software.

**Xenograft Breast Cancer Model.** All experiments involving animals were approved by the Institutional Animal Care and Use Committee. Breast carcinoma MCF-7 xenografts were developed in 5-week-old female CB.17 SCID/beige mice (Taconic Farms, Germantown, NY) as described previously (15). Each mouse received 2.5 × 10^6 exponentially growing MCF-7 cells (in 100 μl of sterile 1/10 PBS) s.c. in the right flank area. Tumors became palpable (25–75 mm³) within 5 days of tumor cell injection, after which groups of five animals were randomized and assigned to different treatment groups. Tumor size was measured in three dimensions with a caliper. Animals were sacrificed once their tumor burden reached 3000 mm³. Animals were given i.p. injections with Adriamycin alone (1.0, 2.0, or 4.0 mg/kg), flavopiridol alone (15 mg/kg), or the sequential combination of Adriamycin/flavopiridol for two consecutive days, each divided by a day with no treatment. For single-agent treatment, vehicle was given in place of Adriamycin or flavopiridol with the same schedule. Each complete cycle was separated by 2 days without treatment.

**Statistical Analysis.** All *in vitro* experiments were repeated at least three times unless otherwise indicated. For *in vivo* studies, each X value (time) shows the fraction still alive. We calculated survival fractions using the product limit or Kaplan-Meier method. The survival curves were compared using the log-rank test. This test generates a P value testing the null hypothesis that the survival curves are identical in the overall populations.

**RESULTS**

**Regulation of Survivin Levels by Thr^34^ Phosphorylation.** To target survivin phosphorylation on Thr^34^, we used the broad-spectrum Cdk inhibitor flavopiridol (16). Incubation of baculovirus-expressed p34^cdc2^-cyclin B1 with 100 nM flavopiridol abolished phosphorylation of ~32-kDa histone H1 and wild-type survivin in a kinase assay *in vitro* (Fig. 1A). In contrast, no phosphorylation of survivin(T34A) by p34^cdc2^-cyclin B1 was observed in the presence or absence of flavopiridol (Fig. 1A). Treatment of HeLa cells with flavopiridol resulted in concentration-dependent inhibition of survivin phosphorylation on Thr^34^ by Western blotting of survivin immunoprecipitates with a Thr^34^-phosphospecific antibody (α-survivin T34^*^; Fig. 1B). Inhibi-
tion of survivin phosphorylation on Thr\textsuperscript{34} was also associated with progressive loss of survivin expression at increasing flavopiridol concentrations by Western blotting with an antibody to survivin (Fig. 1B). Consistent with these observations, exposure of HeLa cells to flavopiridol resulted in progressive decrease in endogenous survivin levels by Western blotting (Fig. 1C). In control experiments, flavopiridol did not affect the expression of antiapoptotic Bcl-2, whereas the flavone genistein, which does not inhibit Cdk activity (20), or TNF-\alpha did not affect survivin expression by Western blotting (data not shown; Fig. 1D). To confirm that Thr\textsuperscript{34} phosphorylation increased survivin stability, we used cycloheximide block in MCF-7 cells transfected with survivin cDNAs fused to GFP. To overcome apoptosis induced by expression of survivin(T34A) (13), a broad-spectrum caspase inhibitor, Z-VAD-fmk (20 \muM), was also added. MCF-7 cells transfected with wild-type survivin exhibited time-dependent expression of a GFP-containing fusion protein that remained sustained for up to 96 h after transfection by Western blotting (Fig. 1E). In contrast, nonphosphorylatable survivin(T34A) was rapidly cleared from MCF-7 cells and nearly entirely depleted 72 h after transfection (Fig. 1E).

**Modulation of Survivin Expression by Anticancer Agents.** To determine the potential relevance of targeting survivin phosphorylation on Thr\textsuperscript{34} for tumor cell apoptosis, we first analyzed survivin levels in breast carcinoma MCF-7 cells treated with various anticancer drugs. Exposure of MCF-7 cells to Adriamycin (100 nM), Taxol (2 \muM), or 50 J/m\textsuperscript{2} UVB irradiation resulted in a 4–5-fold increase in survivin expression by Western blotting (Fig. 2A). In contrast, cisplatin (3 \muM) or 300 J/m\textsuperscript{2} UVB irradiation did not significantly affect survivin levels in MCF-7 cells (Fig. 2A). The increase in survivin expression by anticancer drugs was not accompanied by changes in survivin mRNA levels at various time intervals after Adriamycin treatment by Northern hybridization (Fig. 2B). Similar results were obtained by reverse transcription-PCR amplification of survivin transcript(s) in Adriamycin-treated MCF-7 cells (data not shown). When directly analyzed for potential changes in survivin gene expression, treatment of MCF-7 cells with the various anticancer drugs resulted in significant suppression of survivin promoter activity, as determined by analysis of MCF-7 cells transfected with a minimal survivin promoter upstream of a luciferase reporter gene (Fig. 2C).

**Role of Thr\textsuperscript{34} Phosphorylation on Survivin Levels during Anticancer Treatment.** Because increased survivin levels induced by anticancer treatment did not involve changes in survivin mRNA or promoter activity, we investigated a potential role of Thr\textsuperscript{34} phosphorylation in regulating survivin stability/expression (Fig. 1) under these conditions. In a kinase assay, p34\textsuperscript{cdc2} immunoprecipitated from Adriamycin-treated cells phosphorylated \(~32\)-kDa histone H1 (Fig. 3A). Under these experimental conditions, p34\textsuperscript{cdc2} immunoprecipitates from Adriamycin-treated cells also contained a phosphorylated \(~16.5\)-kDa band, which was identified as survivin by Western blotting with an antibody to survivin (Fig. 3A) and in agreement with the physical association of survivin with p34\textsuperscript{cdc2} (13). The presence of functional p34\textsuperscript{cdc2} activity in Adriamycin-treated cells correlated with the presence of a residual MCF-7 cell population (20–25\%) exhibiting elevated MPM-2 mitotic phosphoepitope expression (Fig. 3B), in agreement with previous observations (21).

**Suppression of Thr\textsuperscript{34} Phosphorylation of Survivin Enhances Anticancer Treatment.** For the role of Thr\textsuperscript{34} phosphorylation in stabilizing survivin levels during anticancer treatment, we next asked whether sequential ablation of p34\textsuperscript{cdc2} kinase activity could function to reduce survivin expression and enhance tumor cell apoptosis. Treatment of MCF-7 cells with 100 nM Adriamycin or 50 J/m\textsuperscript{2} UVB irradiation resulted in a 4.2- and 3.1-fold increase in survivin expression, respectively, by Western blotting (Fig. 4A) and in agreement with the data presented above (Fig. 2). At the concentration and time intervals used, flavopiridol alone did not significantly affect survivin levels in MCF-7 cells (Fig. 4A). In contrast, sequential treatment of MCF-7 cells with Adriamycin or UVB irradiation for 18 h followed by flavopiridol for 72 h significantly inhibited the increase in survivin expression, which approached background levels of untreated cultures (Fig. 4A). Sequential flavopiridol treatment was also associated with suppression of survivin phosphorylation on Thr\textsuperscript{34} by Western blotting of survivin immunoprecipitates with a Thr\textsuperscript{34}-phosphospecific antibody (Fig. 4B). Under these experimental conditions, loss of survivin expression in sequentially treated cultures was associated with significantly enhanced apoptosis induced by UVB (2\%, 5\%, and 12\%) and Adriamycin (2\%, 3\%, and 10\%) to 12\%, 26\%, and 46\% and 22\%, 45\%, 60\%, respectively, as compared with single-agent treatment alone (Fig. 4C).
Sequential Inhibition of Survivin Phosphorylation on Thr 34 as a Novel Anticancer Regimen, in Vivo. We next asked whether sequential suppression of Thr 34 phosphorylation could provide an effective anticancer regimen in vivo. Treatment of SCID/bg mice bearing MCF-7 xenograft tumors (70–100 mm³) with Adriamycin (2 mg/kg) or flavopiridol (15 mg/kg) alone did not affect tumor growth, as compared with animals given vehicle (Fig. 5A). In contrast, sequential combination therapy of Adriamycin (2 mg/kg/day over 2 days) followed by flavopiridol (15 mg/kg/day over 2 days) arrested tumor growth and resulted in indefinite survival of all treated animals (Fig. 5A). Significantly increased survival (P < 0.0001) was also observed upon suspension of sequential Adriamycin-flavopiridol treatment, when tumors exhibited de novo growth comparable to that of animals receiving single anticancer regimens (Fig. 5B). Mice treated with the sequential administration of Adriamycin-flavopiridol did not exhibit overt signs of systemic toxicity (weight loss, diarrhea, and so forth).

DISCUSSION

In this study, we have shown that survivin levels in tumor cells are critically regulated by phosphorylation on Thr 34. Conversely, timed suppression of survivin phosphorylation on Thr 34 by a broad-spectrum Cdk inhibitor, flavopiridol, resulted in loss of survivin expression, enhanced chemotherapy-induced apoptosis, and anticancer activity in vivo.

Protein phosphorylation has been implicated in the regulation of cell death pathways, influencing subcellular localization (22), cytoprotection (23, 24), and cell cycle transitions (25, 26). There is also ample precedent for a role of phosphorylation in controlling stability/expression of cell death regulators. In this context, phosphorylation of bcl-2 on Thr 20, Thr 21, and Ser 27 (27) or of p53 on Ser 15 and Ser 37 (28) has been implicated in preventing ubiquitin-dependent proteasome degradation. In the survivin crystal structure (29), Thr 34 is ideally positioned in an acidic knuckle to regulate the binding of potential client proteins controlling survivin stability and/or ubiquitin-dependent degradation. For IAP family proteins, including survivin (30), ubiquitin-dependent proteasome destruction has been recognized as a critical mechanism to regulate protein levels, influencing IAP-dependent cytoprotection (31). This suggests that the strong anticancer activity associated with overexpression of nonphosphorylatable survivin (T34A) may derive from inhibition of endogenous survivin phosphorylation (14), followed by fall of survivin levels and tumor cell apoptosis. A similar phenotype has been observed after treatment with survivin antisense oligonucleotides, which resulted in suppression of endogenous survivin levels, spontaneous tumor cell apoptosis, and enhancement of anticancer regimens in vitro and in vivo (10, 12, 17, 32).

Because adenoviral delivery of dominant negative survivin (T34A) may have only limited applications for cancer therapy (15), we tar-

![Fig. 3. Regulation of Thr 34 phosphorylation by Adriamycin treatment. A, kinase assay. MCF-7 cells were treated with Adriamycin (100 nM) for 0–48 h at 37°C, followed by immunoprecipitation of p34cdc2 and kinase assay in the presence of histone H1. p34cdc2 immunoprecipitates were analyzed by Western blotting (WB) with an antibody to survivin followed by chemiluminescence. B, MPM-2 expression. MCF-7 cells treated with the indicated anticancer agents were harvested after 0–36 h culture, stained with a MPM-2 antibody, and analyzed by flow cytometry.](https://example.com/f3.png)

![Fig. 4. Timed ablation of survivin phosphorylation on Thr 34 enhances genotoxic stress-induced apoptosis. A, modulation of survivin expression. MCF-7 cells (2 × 10⁶ cells/ml) were treated with Adriamycin (100 nM) or exposed to UVB irradiation (50 or 300 J/m²) in the presence or absence of flavopiridol (100 nM) for 18 h and analyzed for expression of survivin or β-actin by Western blotting. Band intensities were normalized by densitometry and expressed as fold increase over control. B, inhibition of survivin phosphorylation on Thr 34. Survivin was immunoprecipitated from MCF-7 cells treated as described in A, and immune complexes were analyzed by Western blotting (WB) with an antibody to survivin or Thr 34-phosphorylated survivin (α-survivin-T34*). C, enhancement of apoptosis. The experimental conditions are as described in A, except that MCF-7 cells treated with the indicated anticancer agents alone or in sequential combination with flavopiridol were analyzed for DNA content by PI staining and flow cytometry. Results represent the mean ± SD of three different experiments.](https://example.com/f4.png)
The findings described here may have practical implications for anticancer strategies. A simplistic model that survivin may be exclusively involved in mitotic regulation (45) has been discounted by overwhelming experimental evidence demonstrating that survivin targeting provides a viable anticancer approach for potently inducing apoptosis in vivo (4). Sequential therapy has recently emerged as a strategy to rationally improve the efficacy of anticancer combination therapy. Taken together, our findings may provide a molecular basis for the previously reported efficacy of flavopiridol to enhance chemotherapy-induced apoptosis in a strict sequence-dependent manner (46, 47) and the recent implementation of sequential combination therapy using flavopiridol in clinical protocols (48). Indeed, results from Phase II clinical trials using flavopiridol as a single-agent treatment were essentially negative in a variety of solid tumors with unacceptable systemic toxicity. Consistent with the approach presented here, this has prompted a strategy for combining flavopiridol with established chemotherapeutic regimens, i.e., taxanes, for clinical treatment protocols (48). For the emerging role of kinase inhibitors, including Cdk antagonists, in cancer treatment (33, 34) and the extreme sensitivity of tumor cells to manipulation of the survivin pathway (4), sequential ablation of p34^cdk2^ kinase activity after administration of genotoxic agents (this study) or spindle poisons, i.e., taxanes (44), may provide a rational approach to destabilize survivin levels in tumor cells and enhance the efficacy of common anticancer regimens in patients.

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9. Islam, A., Kageyama, H., Takada, N., Kawamoto, T., Takayasu, H., Isogai, E., Ohira, M., Hashizume, K., Kobayashi, H., Kaneko, Y., and Nakagawara, A. High expression transcription of the survivin gene was actually repressed by anticancer agents, which is consistent with similar findings observed with other G2^M^-regulated genes containing, like survivin, cell-cycle dependent element (CDE)/cell cycle homology region (CHR) promoter elements (42). Whether this pathway of survivin protein stabilization by anticancer drugs may facilitate the insurgence of chemoresistance, which has been consistently associated with the presence of survivin in tumors, in vivo (4) is currently not known. However, sequential ablation of survivin phosphorylation on Thr^34^ following Adriamycin treatment resulted in enhanced tumor cell apoptosis, in agreement with previous observations (43), and significantly increased anticancer activity in a xenograft breast cancer model in vivo. Similar results were obtained when p34^cdk2^ kinase activity was sequentially suppressed after Taxol treatment, consistent with dephosphorylation of survivin on Thr^34^ in mitotically arrested cells and apoptosis (44). On the other hand, UVB-induced G2^M^- arrest resulted in increased survivin expression despite the absence of MPM-2 phosphoepitope expression and lack of p34^cdk2^ kinase activity, suggesting that p34^cdk2^ may not be the only kinase inhibited by flavopiridol and required for survivin stability at G2^M^.

Fig. 5. Sequential ablation of survivin phosphorylation on Thr^34^ as a new anticancer regimen in vivo. A, kinetics of tumor growth. MCF-7 cells (2.5 × 10^6^) were grown as xenograft tumors in SCID mice. Animals (10 animals/group) were treated with the indicated concentrations of Adriamycin (A, Adria), flavopiridol (F, Flavo), or the sequential treatment of Adriamycin followed by flavopiridol. Tumor growth was monitored in three dimensions with a caliper. B, survivorship of SCID mice bearing MCF-7 tumors. Kaplan-Meier curves were based on tumor burdens of 3000 mm^3^, at which animals were sacrificed. gated the step of survivin phosphorylation on Thr^34^ using a broad-spectrum kinase inhibitor, flavopiridol. Kinase inhibitors, including Cdk antagonists, have recently emerged as promising anticancer agents (33, 34), and flavopiridol has entered clinical trials (35, 36) for its ability to trigger apoptosis (37, 38) and exert anticancer activity in tumor models (35, 39). Despite intense investigation, the molecular basis of flavopiridol-mediated anticancer activity has not been completely elucidated and may involve mechanisms unrelated to p34^cdk2^ inhibition, including global suppression of gene transcription by interfering with Cdk9/cyclin T function (40). In addition, flavopiridol functions as a relatively broad inhibitor of various Cdks as well as non-cell cycle-regulated kinases (40), thus potentially producing multiple effects on cell cycle progression, gene expression, or general signaling pathways. Despite these potential limitations, it is tempting to speculate that one of the mechanisms by which flavopiridol may exert its proapoptotic function may involve loss of survivin expression due to suppression of phosphorylation on Thr^34^, This model may be consistent with recent observations of the ability of flavopiridol to reduce the expression of other IAP family proteins (41), suggesting that global repression of cytoprotective molecules, including survivin, may contribute to flavopiridol-mediated apoptosis in vivo.

Whereas flavopiridol alone was sufficient to induce loss of survivin expression and apoptosis, the most significant enhancement of tumor cell apoptosis was obtained in sequential combination with selected chemotherapeutic agents. In this context, anticancer drugs inducing G2^M^- arrest with elevated (taxanes) or residual (Adriamycin) chemotherapeutic agents. In this context, anticancer drugs inducing cell apoptosis was obtained in sequential combination with selected expression and apoptosis, the most significant enhancement of tumor may contribute to flavopiridol-mediated apoptosis that global repression of cytoprotective molecules, including survivin, reduce the expression of other IAP family proteins (41), suggesting that flavopiridol-mediated anticancer activity has not been comprehensively elucidated and may involve mechanisms unrelated to p34^cdk2^.


