Histone deacetylase inhibitor enhances 5-fluorouracil cytotoxicity by down-regulating thymidylate synthase in human cancer cells

Ju-Hee Lee,[1,2] Jung-Hyun Park,[1,2] Yeonjoo Jung,[1,2] Jee-Hyun Kim,[4] Hyun-Soon Jong,[1,2] Tae-You Kim,[1,2,3] and Yung-Jue Bang[1,2,3]

[1]National Research Laboratory for Cancer Epigenetics, Cancer Research Institute; Departments of [2]Tumor Biology and [3]Internal Medicine, Seoul National University College of Medicine, Seoul, Korea; and [4]Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam, Korea

Abstract

Thymidylate synthase (TS) overexpression is a key determinant of 5-fluorouracil (5-FU) resistance in human cancer cells. TS is also acutely up-regulated with 5-FU treatment, and, thus, novel strategies targeting TS down-regulation seem to be promising in terms of modulating 5-FU resistance. Here, we report that histone deacetylase inhibitors can reverse 5-FU resistance by down-regulating TS. By using cDNA microarrays and validation experiments, we found that trichostatin A reduced the expression of both *TS* mRNA and TS protein. Cotreatment with trichostatin A and cycloheximide restored *TS* mRNA expression, suggesting that *TS* mRNA is repressed through new protein synthesis. On the other hand, TS protein expression was significantly reduced by lower doses of trichostatin A (50 nmol/L). Mechanistically, TS protein was found to interact with heat shock protein (Hsp) complex, and trichostatin A treatment induced chaperonic Hsp90 acetylation and subsequently enhanced Hsp70 binding to TS, which led to the proteasomal degradation of TS protein. Of note, combined treatment with low-dose trichostatin A and 5-FU enhanced 5-FU–mediated cytotoxicity in 5-FU–resistant cancer cells in accordance with TS protein down-regulation. We conclude that a combinatorial approach using histone deacetylase inhibitors may be useful at overcoming 5-FU resistance. [Mol Cancer Ther 2006;5(12):3085–95]

Introduction

Thymidylate synthase (TS; EC 2.1.1.45) is a folate-dependent enzyme that catalyzes the enzymatic reaction to produce intracellular *de novo* source of thymidylate, an essential precursor of DNA biosynthesis (1). TS catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate by transferring a methylene group from the cofactor CH_2H_4 folate to generate deoxythymidine-5′-monophosphate, which is subsequently phosphorylated to 2′-deoxythymidine-5′-triphosphate (2). 5-Fluorouracil (5-FU) is widely used to treat colorectal, breast, and aerodigestive tract cancers (3), and is converted intracellularly to various active metabolites, which inhibit RNA synthesis and TS activity. In particular, the 5-FU metabolite 5-fluoro-deoxyuridine-monophosphate binds to TS and forms a stable tertiary complex composed of TS, 5-fluoro-2′-deoxyuridine-monophosphate, and tetrahydrofolate, and thereby inhibits TS (2). This inhibition of TS induces an accumulation of 2′-deoxyuridine-5′-monophosphate, which may be subsequently incorporated into DNA, and causes DNA strand breakage and cell death.

Several studies indicate that TS expression or activity functions as a key determinant of 5-FU sensitivity, and preclinical *in vitro* and *in vivo* studies have shown an inverse relationship between TS expression in cancer cells and 5-FU sensitivity (4–10). Importantly, these findings have been extended to clinical settings where low tumoral TS expression is predictive of a response to 5-FU–based chemotherapy for colorectal and breast cancer (4, 8). Conversely, increased TS expression and activity are viewed as being mechanistic drivers of 5-FU resistance in cancer cells (11, 12). Given that TS determines response to 5-FU, various efforts have been made to enhance 5-FU response or overcome 5-FU resistance. In this context, the use of reduced folate leucovorin in combination with 5-FU increased ternary complex stability (13). Moreover, *in vitro* studies have confirmed that 5-FU cytotoxicity is significantly enhanced in the presence of added leucovorin (14). The majority of clinical trials have also shown that the 5-FU and leucovorin combination is associated with a better response than 5-FU alone (15). In addition, newer, more potent TS inhibitors than 5-FU, such as raltitrexed, nolatrexed, and ZD9331, have been developed (16–18). However, despite their potent and specific TS inhibitory

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Requests for reprints: Tae-You Kim, Division of Hematology/Medical Oncology, Department of Internal Medicine, Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-744, Korea. Phone: 82-2-2072-3943; Fax: 82-2-762-9662. E-mail: kimty@snu.ac.kr

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effects, these inhibitors acutely up-regulate TS expression when they are administered to cancer cells (19), and this reaction is regarded to be the main reason for the development of clinical resistance to 5-FU.

In addition to the well-established concept that TS is a target of fluoropyrimidine, a recent study suggested that TS may function as an oncogene (20). It was shown that the forced overexpression of human TS transforms immortalized murine cells to a malignant phenotype. In general, TS protein and mRNA expressions are higher in cervical, breast, kidney, bladder, lung, and gastric tumor tissues than in their normal counterparts (4–10), and patients showing high tumor TS expression have significantly poorer prognoses (21–24). These findings suggest that the overexpression of TS not only reflects drug resistance to fluoropyrimidine but also indicates the biological aggressiveness of cancer cells. Therefore, the development of a new therapeutic strategy that reduces TS expression would be of much interest.

Histone deacetylase inhibitors (HDACI) have been shown to acetylate the nucleosomal histones of condensed chromatin, and thus to cause the reactivation of genes silenced by hyperacetylating histones (25). HDACIs induce cell cycle arrest at G_1 and/or at G_2-M, differentiation, and apoptosis; they also have a potent antitumor activity against a variety of tumor cells both *in vitro* and *in vivo* (25–27). Several HDACIs are currently undergoing clinical trials and some of these show promising antitumor activity (28–33). In addition to histones, HDACIs acetylate nonhistone protein targets involved in transcription, cytoskeletal formation, DNA repair, signal transduction, and chaperone heat shock protein (Hsp; refs. 25, 34–37). The acetylation of nonhistone proteins seems to be an important requirement for the antitumor effects of HDACIs. In particular, recent studies have shown that HDACIs cause the depletion of several oncoproteins that are stabilized by chaperonic Hsp. In addition, Hsp90 is a key regulator of several client oncoproteins, such as, ErbB2, Akt, Bcr-Abl, and Raf. HDACI depsipeptide treatment promotes Hsp90 acetylation in parallel with the time-dependent stabilization of Hsp90-client protein complexes (37). Similarly, LAQ824 down-regulates ErbB2 by acetylating Hsp90 and, further, sensitizes breast cancer cells to chemotherapeutic agents (38).

In the present study, we show that HDACI can reverse 5-FU resistance by down-regulating TS. Trichostatin A treatment acetylated Hsp90 and subsequently enhanced the proteasomal degradation of TS protein, which sensitized cancer cells to 5-FU. Thus, combined treatment with trichostatin A and 5-FU is potentially promising in terms of overcoming 5-FU resistance.

Materials and Methods

Cell Culture

Human gastric cancer cells (SNU-1, SNU-5, SNU-16, SNU-484, SNU-601, SNU-620, SNU-638, SNU-668, and SNU-719) were purchased from the Korean Cell Line Bank (Seoul, Korea; ref. 39). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (WelGENE, Inc., Daegu, Korea) and gentamicin (10 μg/mL). Human breast cancer cells (SkBr3, MCF7) and colon cancer cells (LOVO, HCT116) were purchased from the American Type Culture Collection (Manassas, VA), and grown in RPMI 1640 (SkBr3, MCF7, LOVO) or DMEM (HCT116) supplemented with 10% fetal bovine serum and gentamicin (10 μg/mL). All cancer cells were incubated under standard culture condition (20% O_2 and 5% CO_2 at 37°C).

Reagents

Anti-Hsp70 and anti-Hsp90 monoclonal antibodies were purchased from Stress-Gen Biotechnologies Corp. (Victoria, British Columbia, Canada). Anti–TS monoclonal antibody was purchased from NeoMarkers (Fremont, CA). Antibodies to acetylated histone H3, acetylated histone H4, and dimethyl-histone H3 K9 were purchased from Upstate Biotechnology (Waltham, MA). Normal rabbit IgG antibody (a negative control) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antiacetylated lysine polyclonal antibody was from Cell Signaling (Beverly, MA). Trichostatin A, cycloheximide, and MG-132 were purchased from Sigma (St. Louis, MO), and 5-FU was from Choong Woe Co., Ltd (Seoul, Korea). Suberoylanilide hydroxamic acid (SAHA), oxamflatin, valproic acid, and sodium butyrate were kindly provided by Dr. Dae-Kee Kim (In2Gen, Seoul, Korea).

Western Blot Analysis

Cells were collected after trichostatin A and/or 5-FU treatment, washed with ice-cold PBS, and suspended in an extraction buffer [20 mmol/L Tris-Cl (pH 7.4), 100 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 5 mmol/L $MgCl_2$, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L pepstatin A, 0.1 mmol/L antipain, 0.1 mmol/L chymostatin, 0.2 mmol/L leupeptin, 10 mg/mL aprotinin, 0.5 mg/mL soybean trypsin inhibitor, and 1 mmol/L benzamidine] on ice for 15 min. Lysates were cleared by centrifugation at 13,000 rpm for 20 min. Equal amounts of cell extracts were resolved on 10% SDS-polyacrylamide denaturing gels, transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), and probed with an appropriate primary antibody and horseradish peroxidase–conjugated secondary antibody. Detection was done using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ; ref. 40).

Immunoprecipitation

Cells were collected after trichostatin A treatment and washed with PBS. They were then suspended in an immunoprecipitation extraction buffer [50 mmol/L Tris-Cl (pH 7.5), 250 mmol/L NaCl, 0.1% NP40, 5 mmol/L EDTA, 50 mmol/L NaF, 0.1 mmol/L $NaVO_4$, 100 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L leupeptin, 10 μg/mL aprotinin, 0.1 mmol/L pepstatin A, and 0.1 mmol/L antipain] and incubated on ice for 15 min. One microgram of each antibody (Hsp90 and TS) was added to 500 μg of each cell extract in 700 μL of extraction buffer and incubated overnight at 4°C with continuous

agitation. To collect immune complexes, 30 μL of protein A/G agarose were added to mixtures, which were then incubated for 4 h. Immune complexes were centrifuged at 1,200 rpm for 2 min, and precipitates were washed thrice with extraction buffer. Proteins were eluted with SDS sample loading buffer before immunoblot analysis (40).

Reverse Transcription-PCR and Quantitative Real-time Reverse Transcription-PCR Analysis

Total RNA was isolated with TRI reagent (Molecular Research Center, Inc. Cincinnati, OH), and cDNAs were synthesized from 1 μg of total RNA using ImProm-II Reverse Transcriptase (Promega Corporation, Madison, WI) using random hexamers. The primers used in the PCR reaction were as follows: TS, forward primer 5′-TCTG-GAAGGGTGTTTTGGAG-3′ and reverse primer 5′-CCTCC-ACTGGAAGCCATAAA-3′; and β-actin, forward primer 5′-CCACACTGTGCCCATCTACG-3′ and reverse primer 5′-AGGATCTTCATGAGGTAGTCAGTCAG-3′. Amplification reactions were done in 20 μL volumes for 94°C for 5 min, 25 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, and finally 10 min at 72°C. To estimate the efficiency of cDNA synthesis from each cell line, β-actin was used as an internal control. PCR products were resolved on 1.2% SeaKem agarose gels, and quantitative PCR was done using an iCycler IQ detection system (Bio-Rad Laboratories, Hercules, CA) using SYBR green I as a double-stranded, DNA-specific binding dye. Thermocycling was done in a final volume of 20 μL containing 4 μL cDNA sample, 10 pmol of each primer, 0.125 mmol/L deoxynucleotide triphosphate mixture, 0.25 mg/mL bovine serum albumin, 0.05% Tween 20, 1× rTaq reaction buffer containing 1.5 mmol/L $MgCl_2$ (Takara Bio., Otsu, Japan), 1 unit rTaq DNA polymerase (Takara), and 1× SYBR green I (Roche Diagnostics, Mannheim, Germany). After an initial denaturation at 95°C for 10 min, 40 cycles at 94°C for 30 s, annealing at 53°C for 30 s, and 72°C for 30 s were carried out. All cDNA samples were synthesized in parallel, and PCR reactions were run in triplicate. mRNA levels were derived from standard curves and are expressed as relative changes after normalization versus β-actin mRNA levels.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was done using antibodies specific for acetylated histone H3 and H4, as described previously (41). Briefly, SNU-484 cells, in a 10-cm dish, were fixed by adding formaldehyde to a final concentration of 1% and then incubated for 10 min at 37°C. Glycine (0.125 mol/L) was then added to quench the reaction (5 min at room temperature). The cells were suspended in SDS lysis buffer [1% SDS/10 mmol/L EDTA/50 mmol/L Tris-HCl (pH 8.1)], and the lysates were sonicated to shear the DNA into lengths of 200 to 500 bp. For preclearing, 50% protein A/G Sepharose slurry, containing 20 μg of sonicated salmon sperm DNA and 1 mg/mL bovine serum albumin, was added and the mixture was incubated by rocking for 30 min at 4°C. Precleared supernatants were immunoprecipitated using antibodies specific for acetylated histone H3, acetylated histone H4, or normal rabbit IgG (5 μg of each) and incubated overnight at 4°C. No antibody controls were included in the chromatin immunoprecipitation assays, and no precipitation was observed. Antibody/protein complexes were collected as salmon sperm DNA/protein A agarose slurries for 2 h at 4°C, washed five times for 5 min, and eluted twice with 250 μL of elution buffer (0.1 mol/L $NaHCO_3$/1% SDS). RNase A (0.03 mg/mL) and NaCl (0.3 mol/L) were added, and crosslinks were reversed by incubation for 4 h at 65°C. Samples were digested with Proteinase K (0.24 mg/mL) for 1 h at 45°C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The recovered DNA was analyzed by PCR using *TS* promoter–specific primer pairs, namely, forward primer 5′-CCTGGGCTCCGTTCTGTG-3′ and reverse primer 5′-CCGCGCCATGCCTGTGGCCGGCTCG-GAGCT-3′. The size of the amplified product was 270 bp. The conditions used were 95°C for 5 min, 35 cycles at 96°C for 1 min, annealing at 60°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min.

Cell Growth Inhibition Assay

Cells (5×10^3–7×10^3 in 50 μL/well) were seeded on 96-well plates and incubated for 24 h at 37°C and treated with drugs (i.e., trichostatin A or 5-FU) for 1 to 4 days at 37°C. After drug treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well, cells were incubated for 4 h at 37°C, and the medium was then removed. DMSO was then added and shaken for 30 min at room temperature. Absorbance was analyzed using a microplate reader (Molecular Devices, Sunnyvale, CA; ref. 42). In some experiments, proliferating cancer cells were incubated in the presence of 50 nmol/L trichostatin A for 24 h, and then medium was removed. Cells were then washed thoroughly with PBS, and fresh medium containing 1 μmol/L 5-FU was added. The cells were then incubated for an additional 3 days. Graphs were generated by nonlinear regression of the data points to a four-variable logistic curve using SigmaPlot software (Statistical Package for the Social Sciences, Inc., Chicago, IL).

Colony Formation Assay

For colony formation assays, cells were grown exponentially to ~40% confluency and exposed to trichostatin A for 24 h or mock-treated with DMSO. After 24 h, cells were trypsinized, washed in PBS, counted, and plated in growth medium in six-well plates at densities of 2×10^3 or 4×10^3 cells per well for SNU-484 or SNU-601, respectively. Cells were then incubated for 18 h until cells attach; 5-FU was added at concentrations of 0, 2, 4, 6, 8, or 10 μmol/L for 24 h; and drug-containing medium was decanted. Cells were then washed twice with PBS and incubated in growth medium for 10 to 12 days. Colonies produced were stained with 0.1% Coomassie brilliant blue R-250 (Bio-Rad Laboratories) in 30% methanol and 10% acetic acid. Colonies (>500 cells or 1 mm in diameter) were counted by two individuals. Graphs were constructed using SigmaPlot software (Statistical Package for the Social Sciences).

Combination Index by IC_{50} Isobologram

The effect of combined trichostatin A and 5-FU on cell growth inhibition was analyzed by isobologram method of Steel and Peckham (43). Based on dose-response curves of trichostatin A in combination with 5-FU, three isoeffect curves (modes I, IIa, and IIb) were drawn and the envelope of additivity was surrounded by modes I and II (44). When the data points of the combination fell within the envelope, the combination was considered additive. If the data points fell below the envelope of additivity, the combination was considered synergistic. Data points on the right side of the envelope were regarded as antagonistic. Sequential treatment was done in the same way used in cell growth inhibition assay. For concurrent treatment, cells were incubated for 3 days in the presence of both trichostatin A and 5-FU.

Statistical Analyses

Data were expressed as mean ± SE. Comparisons used Student's *t* test or ANOVA, as appropriate. *P* values of <0.05 were assigned significant.

Results

Transcriptional Down-Regulation of *TS* mRNA by Trichostatin A

To identify novel target genes regulated by HDACIs, we previously did cDNA microarrays to determine the effects of trichostatin A in SNU-601 gastric cancer and MDA-MB-231 breast cancer cells. It was found that trichostatin A significantly repressed *TS* mRNA by 9-fold in both cell lines (data not shown). To validate these findings, we first examined the expression of *TS* mRNA in gastric cancer cells. Semiquantitative reverse transcription-PCR (RT-PCR) showed that *TS* mRNA was expressed in all cancer cells, but its expression was at a higher level in SNU-1, SNU-484, and SNU-719 cells (Fig. 1A). To further validate the microarray results, *TS* mRNA expression was analyzed in SNU-484 cells after trichostatin A treatment. These cells were treated for the indicated concentrations (*left*) for 24 h or indicated times with 300 nmol/L trichostatin A (Fig. 1B, *right*). Treatment with trichostatin A was found to reduce *TS* gene expression in a dose- and time-dependent

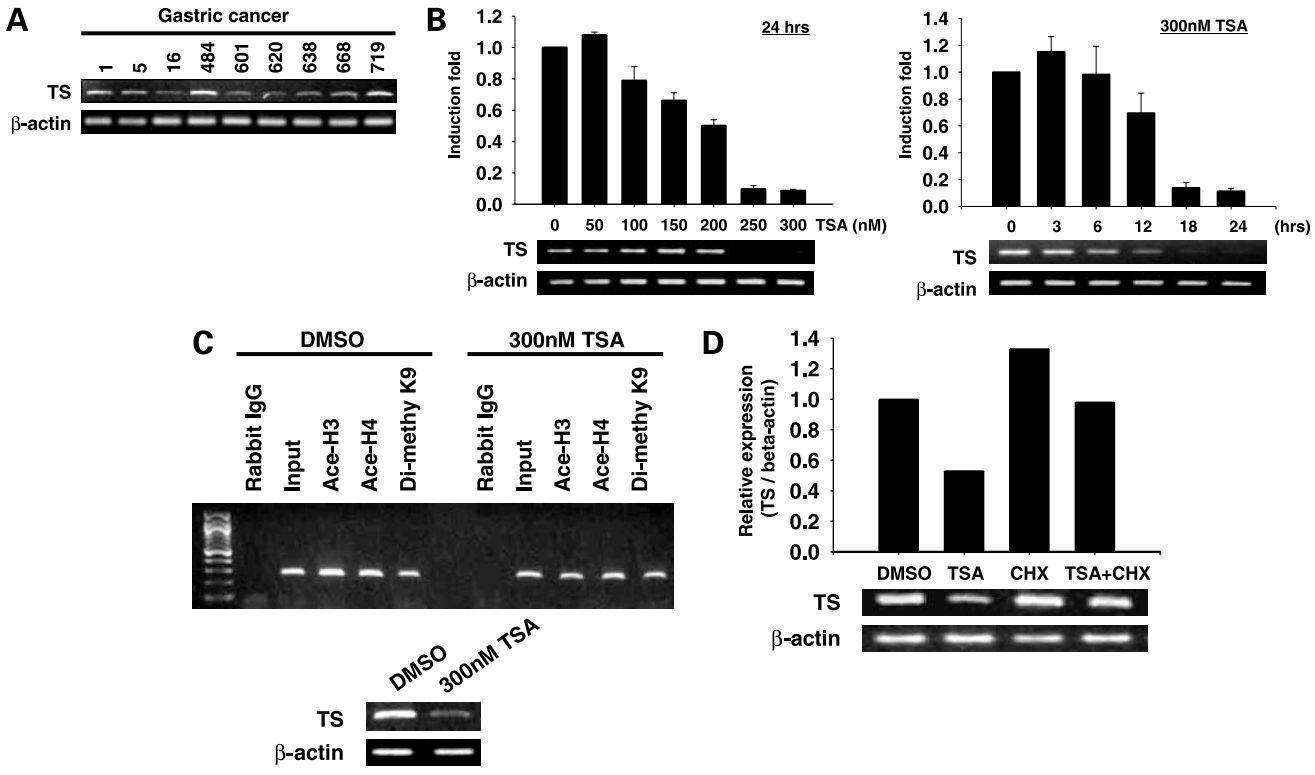

Figure 1. Transcriptional down-regulation of *TS* mRNA by trichostatin A. **A,** RT-PCR analysis screening for the expression of TS in human gastric cancer cells. β-Actin was used as a loading control. **B,** SNU-484 cells were treated for the indicated concentrations (*left*) for 24 h or indicated times with 300 nmol/L trichostatin A (*TSA, right*). *TS* mRNA expressions were determined by RT-PCR and quantitative real-time RT-PCR. β-Actin was used as a loading control. Results of quantitative real-time RT-PCR. *Columns,* mean of three independent experiments; *bars,* SE. **C,** trichostatin A did not induce the accumulation of modified histones in chromatin associated with *TS* gene. Soluble chromatin was immunoprecipitated with anti–acetylated histone H3 (*Ace-H3*), H4 (*Ace-H4*), and anti–dimethylated histone H3 (*Di-methy K9*) antibodies from untreated cells and 300 nmol/L trichostatin A–treated SNU-484 cells for 24 h. PCR primers for a region of the promoter of the *TS* gene were used to amplify the DNA isolated from immunoprecipitated chromatin. *Bottom,* expression of *TS* mRNA after treatment with 300 nmol/L trichostatin A. **D,** repression of TS by trichostatin A requires new protein synthesis, and the protein generated inhibits TS transcription and translation. Samples of 1 μg total cellular RNA were isolated from SNU-484 cells treated with trichostatin A (300 nmol/L) and/or cycloheximide (*CHX*; 50 μg/mL) for 24 h and analyzed by RT-PCR. Graphs were prepared by quantifying RT-PCR bands using TINA 2.0 software (Ray tests).

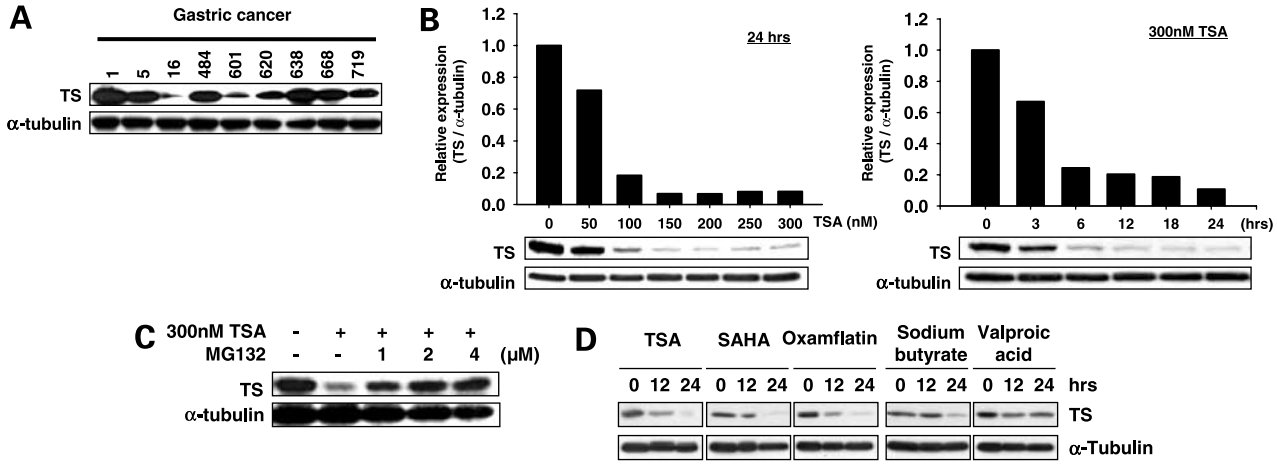

Figure 2. Trichostatin A depletes TS protein via proteasomal degradation. **A,** Western blotting was used to determine TS expressions in several human gastric cancer cells. α-Tubulin was used as a loading control. **B,** for Western blotting, SNU-484 cells were treated for the indicated concentrations for 24 h (*left*) or for indicated times with 300 nmol/L trichostatin A (*right*). Graphs were prepared by quantifying immunoblot bands using TINA 2.0 software (Ray tests). **C,** trichostatin A promotes the proteasomal degradation of TS. Samples for Western blotting were prepared from cells treated with 300 nmol/L trichostatin A and/or various doses of MG132 for 24 h. **D,** SNU-484 cells were exposed to trichostatin A (300 nmol/L), SAHA (1 μmol/L), oxamflatin (1 μmol/L), sodium butyrate (1 μmol/L), and valproic acid (1 μmol/L) for 12 or 24 h. α-Tubulin was used as a loading control.

manner, and *TS* mRNA expression was reduced below 20% of baseline by trichostatin A at 250 nmol/L. When cells were treated with 300 nmol/L trichostatin A, *TS* mRNA levels were observed to decrease after 12 h of treatment. With regard to transcriptional regulation, trichostatin A is known to activate gene transcription by increasing the acetylation of histones tethered to promoter regions. To determine whether *TS* promoter is directly associated with histone acetylation, we did chromatin immunoprecipitation assays using various antihistone antibodies after treatment with 300 nmol/L of trichostatin A for 24 h. It was found that *TS* promoter is associated with the acetylation of histones H3 and H4 even in the absence of trichostatin A, and that trichostatin A treatment did not alter the association between *TS* promoter and acetylated histones H3 and H4 in SNU-484 cells (Fig. 1C). Considering that trichostatin A tends to reduces *TS* gene transcription, these findings suggest that trichostatin A does not directly affect the histone acetylation of *TS* promoter, but rather that it indirectly regulates *TS* gene transcription. One possible explanation for this is that trichostatin A may induce a transcriptional factor that represses *TS* gene expression. To address this possibility, we tested the effect of cycloheximide on *TS* gene expression. As shown in Fig. 1D, cycloheximide, which inhibits new protein synthesis, effectively restored *TS* mRNA levels after trichostatin A treatment, which supports the indirect regulatory effect of trichostatin A on *TS* mRNA expression (i.e., that an unknown transcriptional repressor is induced by trichostatin A treatment and that it inhibits *TS* transcription).

Trichostatin A Depletes TS Protein via Proteasomal Degradation

In addition to gene expression analysis, the expression of TS protein was also analyzed in the same manner. As shown in Fig. 2A, TS protein was abundantly expressed by most gastric cancer cell lines. Moreover, although the expressions *TS* gene and TS protein were well-matched in general, TS protein was found to be overexpressed in SNU-638 and SNU-668 cells despite a low level of *TS* gene expression in these cells. This discrepancy suggested that TS protein expression may be determined by posttranscriptional regulation. Trichostatin A treatment also down-regulated TS protein in a time- and dose-dependent manner (Fig. 2B); however, in contrast to mRNA expression, down-regulation of TS protein occurred at a lower trichostatin A concentration. For example, trichostatin A at 100 nmol/L was found to down-regulate TS protein expression to below 20% of baseline, whereas the same dose had no observable affect on *TS* mRNA expression (Figs. 1B and 2B). Likewise, trichostatin A at 300 nmol/L significantly reduced TS protein expression in 6 h but this treatment had no effect of *TS* mRNA expression (Figs. 1B and 2B). Together with the observed differential expression patterns of *TS* mRNA and TS protein, these findings suggest that trichostatin A treatment down-regulates TS protein independently of the transcriptional regulation of *TS* mRNA. Moreover, this conclusion was supported by the observed inhibition of trichostatin A–mediated TS protein down-regulation by cotreatment with trichostatin A and MG132, a proteasomal inhibitor (Fig. 2C), indicating that TS protein is destabilized via a proteasomal pathway after trichostatin A treatment. These results suggest that trichostatin A treatment activates a proteasomal degradation pathway that subsequently degrades TS protein.

HDACIs are classified into hydroxamic acid, short-chain fatty acid, benzamide, cyclic tetrapeptide, and hybrid compounds according to their chemical structure (25, 45, 46). Recently, we reported that hydroxamic-based HDACIs

effectively inhibit HDACs (40). In present study, we found that hydroxamic acid–based HDACIs, such as trichostatin A, SAHA, and oxamflatin, significantly down-regulated TS protein compared with the effects of non-hydroxamic sodium butyrate and valproic acid (Fig. 2D). Given that hydroxamic acids are the most potent inhibitors of HDAC activity, these findings suggest that the degradation of TS is closely related to its HDAC-inhibiting activity (34, 40).

Trichostatin A Promotes the Proteasomal Degradation of TS Protein by Acetylating Chaperonic Hsp

It was recently reported that HDACIs acetylate Hsp90, and that this results in the degradation of client oncoproteins via the proteasomal pathway (37). Therefore, we attempted to determine whether TS is similarly affected by the acetylation of chaperonic Hsp. First, we found that TS protein interacts with Hsp90 and Hsp70 *in vivo* (Fig. 3A–C), and it was also found that trichostatin A affects the acetylation of Hsp90, but not that of Hsp70 (Fig. 3B and C). Immunoprecipitation also showed that trichostatin A treatment increases binding between Hsp70 and TS in accordance with the down-regulation of TS protein. Time-dependent observations more clearly showed increases in the amount of bound Hsp70, which is subject to proteasomal degradation of the client TS protein in cancer cells (Fig. 3D). Taken together, these findings show that TS protein is degraded by proteasome that is activated by Hsp90 acetylation after trichostatin A

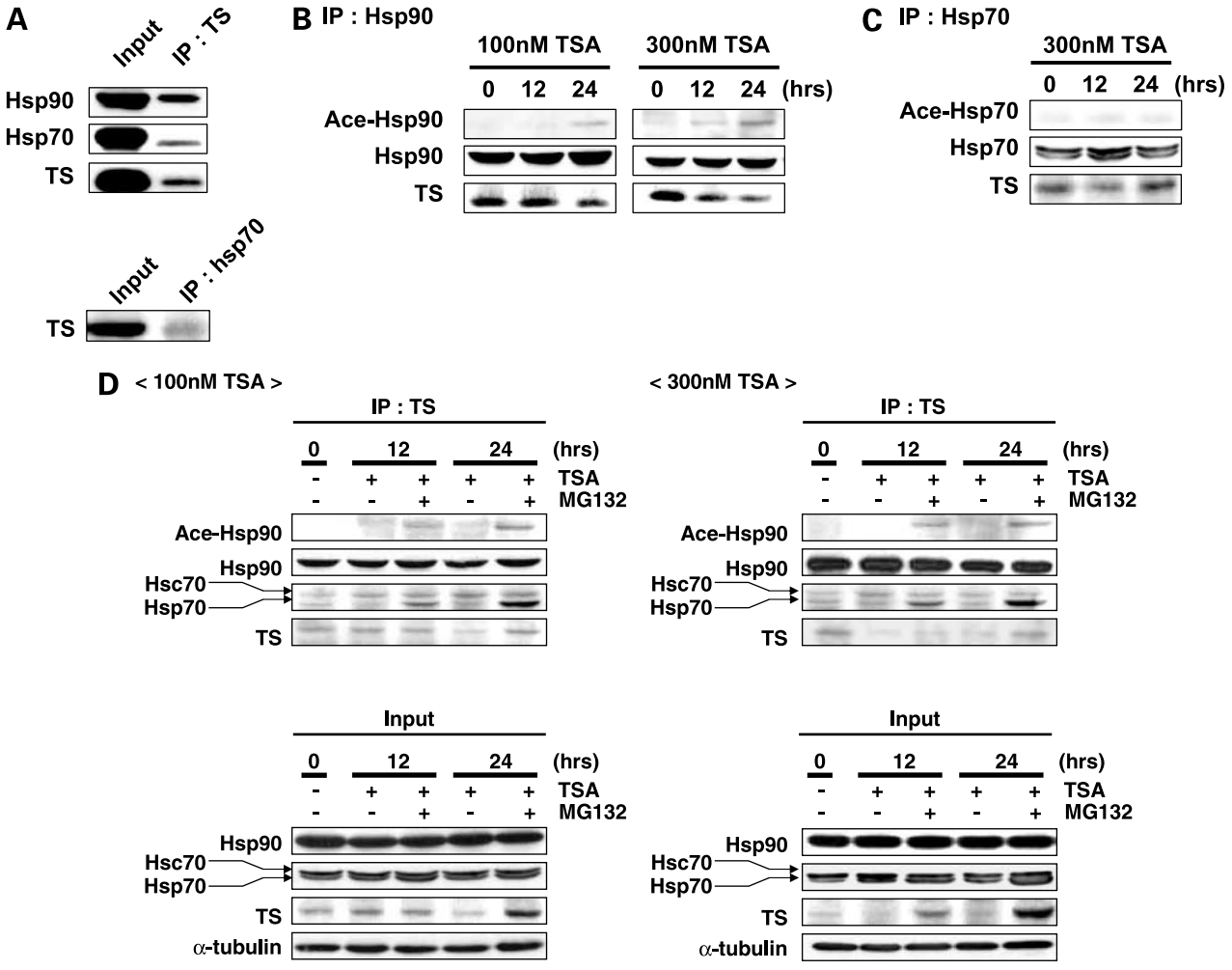

Figure 3. Trichostatin A degrades TS protein via Hsp acetylation. **A,** TS associates with Hsp90 and Hsp70. Whole-cell extracts were prepared from SNU-484 cells and equal amounts of extracts (500 μg) were immunoprecipitated using anti-TS antibodies or anti-Hsp70 antibodies. Blots were analyzed by immunoblotting with antibodies against TS, Hsp90, and Hsp70. **B** and **C,** Hsp90 acetylation was induced by trichostatin A. Cells were treated with trichostatin A for the indicated doses and times. Equal amounts of extracts (500 μg) were immunoprecipitated with anti-Hsp90 antibody or anti-Hsp70 antibody. Hsp90 acetylation was detected by immunoblotting using polyclonal antibodies against acetylated lysine 9. In contrast, trichostatin A treatment did not affect Hsp70 acetylation. **D,** trichostatin A enhanced the binding of Hsp70 to TS. SNU-484 cells were treated with trichostatin A at the indicated doses and/or MG132 (1 μmol/L) for the indicated times. TS immunoprecipitates from cell lysates were immunoblotted with acetylated Lys9, Hsp90, Hsp70, or TS antibody. *IP,* immunoprecipitation. Twenty micrograms of protein were used as an input control.

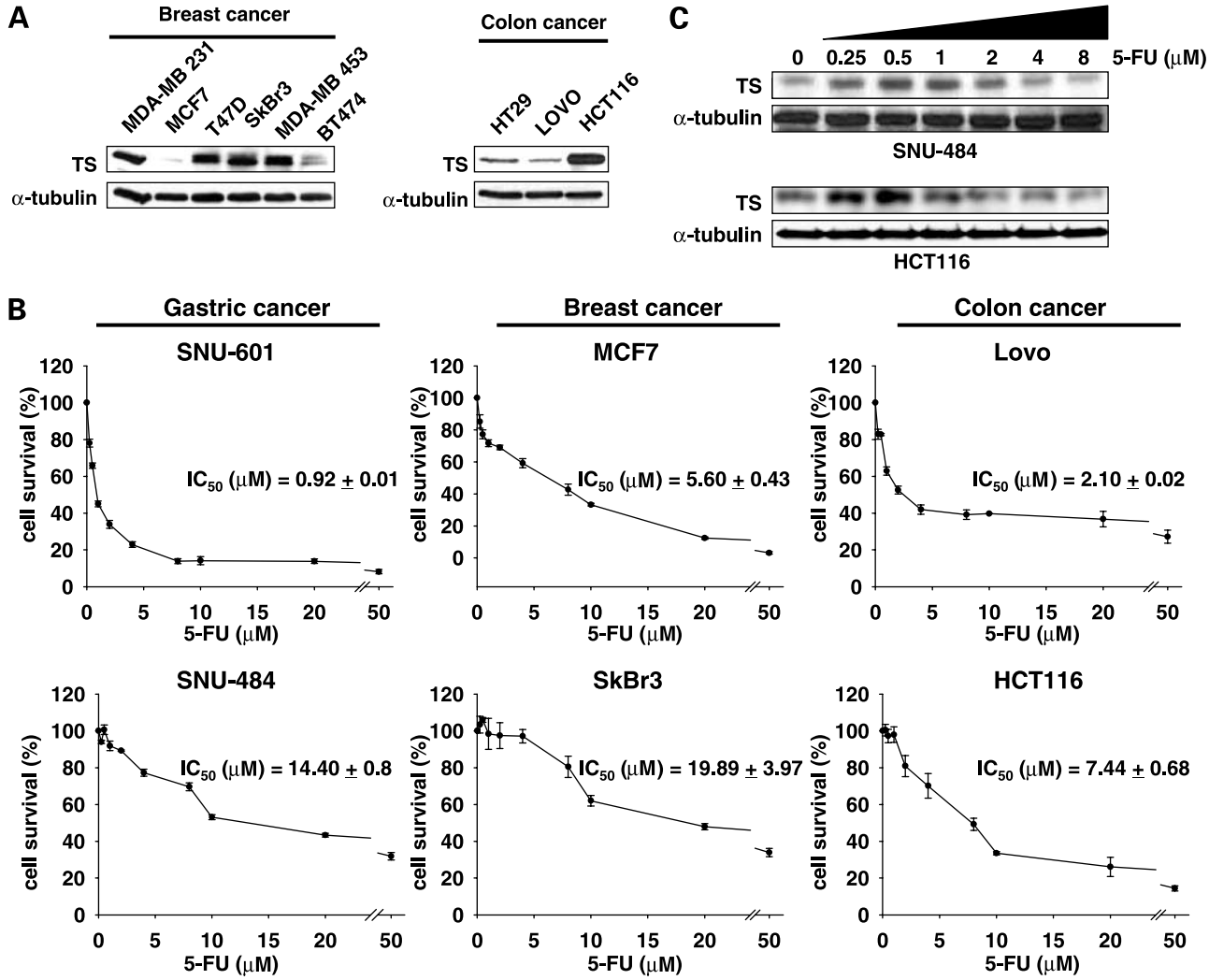

Figure 4. Relationship between TS expression and 5-FU cytotoxicity. **A,** Western blot analysis was used to examine TS expression in several human cancer cell lines by using α-tubulin as a loading control. **B,** MTT analysis was used to investigate 5-FU cytotoxicity. Cells were seeded into 96-well culture plates and treated with various doses of 5-FU for 3 d, and then treated with MTT for 4 h. Cell viabilities were determined by measuring the absorbance. *Points,* mean of three experiments; *bars,* SD. **C,** For Western blot analysis, cancer cells were treated with various doses of 5-FU for 24 h. α-Tubulin was used as a loading control.

treatment. Overall, we conclude that trichostatin A regulates TS expression via the indirect transcriptional repression of the *TS* gene or via the proteasomal degradation of TS protein mediated by Hsp90 acetylation.

Trichostatin A Overcomes 5-FU Resistance by Down-Regulating TS

In addition to gastric cancer, TS protein overexpressions were also observed in breast and colon cancer cells, whereas MCF-7 breast cancer cells, HT29, and LOVO colon cancer cells showed lower levels of TS expression (Fig. 4A). Moreover, the growth inhibitory effects of 5-FU were apparently dependent on basal TS expression level. The IC_{50} of 5-FU in cancer cells expressing low levels of TS protein was found to be lower than in cancer cells overexpressing TS (Fig. 4B). These results are consistent with the significant relation between TS protein overexpression and 5-FU resistance in these cancer cells. In addition to basal TS expression, cancer cells exposed to 5-FU showed rapid increases in TS protein expression, indicating that TS protein could be induced by 5-FU treatment (Fig. 4C). As shown in Fig. 4C, TS protein was up-regulated only with lower dosage of 5-FU (0.25–1 μmol/L), whereas, in dose range above 1 μmol/L, expression level of TS protein is not significantly changed (SNU-484 cells) or is somewhat decreased (HCT116 cells) compared with basal level of TS in these 5-FU–resistant cells. The molecular basis for differential TS response according to 5-FU concentration is not clear. Previous studies have shown that acute induction of TS is to maintain cell survival in response to exposure to 5-FU,

which is mediated by translational autoregulatory mechanism (2, 19). This is important because repeated 5-FU treatment may result in sustained TS expression and eventually lead to 5-FU resistance. Therefore, in view of the finding that TS overexpression is associated with 5-FU resistance in various cancer cells, trichostatin A–induced down-regulation of TS may have therapeutic implications in terms of overcoming 5-FU resistance. However, because trichostatin A is highly cytotoxic *in vitro* and *in vivo*, the use of relatively nontoxic lower doses of trichostatin A might be more acceptable in the treatment of 5-FU–resistant cancer cells. To determine the minimal trichostatin A dose able to down-regulate TS, we examined the cytotoxic effects and the TS expression induced by lower doses of trichostatin A. As shown in Fig. 5A, it was found that percentage of cell survival was ~80% with 50 nmol/L trichostatin A treatment, which reduced TS protein expression by 50%. Therefore, 50 nmol/L trichostatin A seems to be appropriate in terms of TS down-regulation and toxicity. We next determined the effects of trichostatin A on the cytotoxic effects of 5-FU on cancer cells. In HCT-116 colon cancer cells, which are resistant to 5-FU, sequential treatment with 50 nmol/L trichostatin A followed by 5-FU enhanced 5-FU cytotoxicity. Compared with untreated controls, 50% of HCT-116 cells survived trichostatin A (50 nmol/L) and 5-FU (1 μmol/L) treatment ($P = 0.02$), whereas 60% of 5-FU–sensitive LOVO cells survived 5-FU treatment ($P < 0.01$). As expected, TS protein expression was reduced in HCT-116 cells after trichostatin A treatment (Fig. 5B). Therefore, the sequential use of low-dose trichostatin A seemed to make 5-FU–resistant cells sensitive to 5-FU. Similarly, colony formation experiments showed that the sequential use of 50 nmol/L trichostatin A and various doses of 5-FU reduced colony formation by >30% versus 5-FU alone in 5-FU–resistant SNU-484 gastric cancer cells (Fig. 5C). Moreover, the growth-inhibitory effect of sequential trichostatin A and 5-FU in SNU-484 cells was found to be similar to that of 5-FU alone in 5-FU–sensitive SNU-601 cells. IC_{50} values were reduced to 4.63 μmol/L in SNU-484 cells, whereas the IC_{50} value of 5-FU was 13.22 μmol/L; the combination of trichostatin A potentiated the cytotoxicity of 5-FU to 3-fold. Isobologram analyses were used to determine the effects of sequential or concurrent treatment of trichostatin A and 5-FU in 5-FU–resistant SNU-484 cells. When SNU-484 cells were exposed to trichostatin A and varying doses of 5-FU (0.25–8 μmol/L) as a sequential or concurrent approach, the data points fell to the left side of envelope of additivity, indicating that each combination was synergistic (Fig. 5D). Taken together, these findings show that this combinatorial approach of HDACI and 5-FU seems to be effective in enhancing 5-FU cytotoxicity by down-regulating TS in 5-FU–resistant cancer cells.

Discussion

In the present study, we showed that low-dose trichostatin A down-regulates TS protein and as a result sensitizes 5-FU–resistant cancer cells to 5-FU. In terms of the molecular basis of TS down-regulation, we observed for the first time that following trichostatin A treatment, client TS protein of chaperonic Hsp90 is destabilized via the acetylation of Hsp90 and the subsequent recruitment of Hsp70. This finding is important in terms of overcoming 5-FU resistance. These observations suggest that HDACIs may be used in combination therapies with fluoropyrimidine to overcome the drug resistance associated with reduced TS protein expression in cancer cells.

5-FU was developed some 50 years ago but still remains one of the main anticancer agents used to treat colorectal, breast, gastric, esophageal, pancreatic, and head and neck cancers (4–10). In addition to 5-FU, various TS inhibitors have been developed that more specifically and potently inhibit TS. These inhibitors include raltitrexed (tomudex, ZD1674), pemetrexed (LY231514), nolatrexed (AG337), and ZD9331 (16–18), and all show potent inhibitory effect on TS and promising antitumor activity against different cancer cells. However, although some of these have shown improved efficacies in 5-FU–resistant cancer cells, TS overexpression also presents a main mechanism of resistance to these TS inhibitors (19). Moreover, cancer cells exposed to these TS inhibitors acutely up-regulate TS, which may eventually lead to drug resistance (2, 19). Therefore, a novel strategy aimed at inhibiting TS in a manner that does not induce TS may be a more reasonable approach to the overcoming drug resistance.

It is well known that TS is frequently overexpressed in cancer cells compared with normal tissues, and that tumors with high TS expression are generally resistant to fluoropyrimidine anticancer agents (11, 47). The present study also shows that high TS protein expressions in SNU-484 (gastric cancer), HCT-116 (colon cancer), and SkBr3 (breast cancer) are related with 5-FU resistance *in vitro* (Fig. 4). Moreover, preclinical and clinical studies have shown close relationships between *TS* mRNA/TS protein levels and response to therapy in colorectal cancer patients treated with fluoropyrimidine (5). Using a cDNA microarray approach, we previously observed that *TS* mRNA expression is down-regulated after trichostatin A treatment in breast cancer cells. In the present study, we confirmed that *TS* mRNA and TS protein expressions are decreased by trichostatin A treatment in a time- and dose-dependent manner in cancer cells (Figs. 1B and 2B). However, the mechanisms underlying the down-regulation of *TS* mRNA and TS protein seem to differ. More specifically, whereas trichostatin A did not alter the histone acetylation of *TS* promoter, it did repress *TS* gene transcription, possibly by inducing an unknown transcriptional factor that represses *TS* gene expression. Moreover, TS protein was down-regulated by <100 nmol/L of trichostatin A, but *TS* mRNA was not significantly affected at this dose. In theory, the down-regulation of TS protein, which can be achieved by using antisense oligodeoxynucleotides or small interference RNA, may sensitize cancer cells to TS-directed chemotherapeutic agents (48). In the present study, we showed that

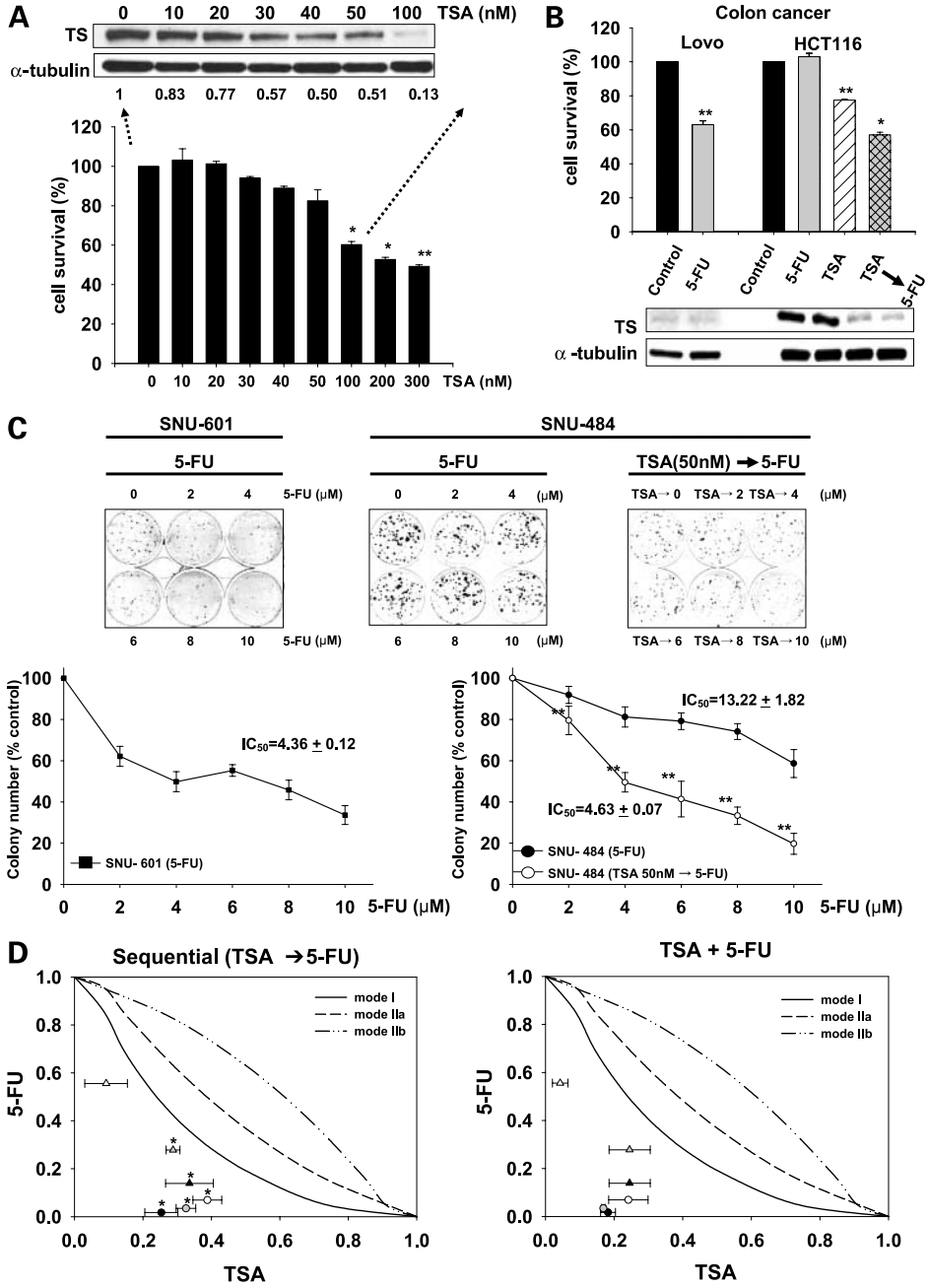

Figure 5. Trichostatin A overcomes 5-FU resistance by down-regulating TS. **A,** down-regulation of TS by low-dose trichostatin A. MTT assays were used to examine the effect of low-dose trichostatin A and 5-FU on cell viability. SNU-484 cells were treated with the indicated doses of trichostatin A for 1 d, and then incubated with normal growth medium for 3 d. Results were obtained after MTT treatment for 4 h. *P* values, 50 nmol/L trichostatin A ($P = 0.14$), 100 nmol/L trichostatin A ($P = 0.02$), 200 nmol/L trichostatin A ($P = 0.01$), and 300 nmol/L trichostatin A ($P < 0.01$). *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$. **, $P < 0.01$. For immunoblotting analysis, SNU-484 cells were treated with indicated doses of trichostatin A for 24 h. α-Tubulin was used as a loading control. **B,** combined treatment with low-dose trichostatin A and 5-FU enhanced the cytotoxic effect of 5-FU and down-regulated TS protein in 5-FU – resistant cancer cells using MTT assays and immunoblotting. *P* values, 1 μmol/L 5-FU ($P < 0.01$) in LOVO cells, 1 μmol/L 5-FU ($P = 0.27$), 50 nmol/L trichostatin A ($P < 0.01$), and trichostatin A→5-FU ($P = 0.02$) in HCT-116 cells. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$. **, $P < 0.01$. **C,** SNU-484 (5-FU – resistant) and SNU-601 (5-FU – sensitive) cells were exposed to increasing concentrations of 5-FU for 24 h, when medium was exchanged for growth medium. The cells were then allowed to form colonies over a period of 7 to 10 d. For combinatorial treatments, after pretreatment with 50 nmol/L trichostatin A for 24 h, trypsinized cells were attached by incubation (18 h) and then exposed to different doses of 5-FU for 24 h. Plates were then washed twice with growth medium and cells were allowed to form colonies over a period of 7 to 10 d. *Points,* mean of three independent experiments; *bars,* SD. **, $P < 0.01$. **D,** IC_{50} isobolograms in combination of trichostatin A and 5-FU in SNU-484 cells. *Left,* results of trichostatin A followed by 5-FU; *right,* effects of concurrent trichostatin A and 5-FU. These data points fell into the left of the envelope, indicating that each combination was synergistic. Doses of 5-FU: ●, 0.25 μmol/L; ◉, 0.5 μmol/L; ○, 1 μmol/L; ▲, 2 μmol/L; ▲, 4 μmol/L; △, 8 μmol/L. *, $P < 0.05$.

HDACIs deplete TS and sensitize cancer cells to 5-FU. Therefore, HDACIs may represent a novel class of TS inhibitors that deplete *TS* mRNA and TS protein and thereby inhibit TS function.

Then, how do HDACIs deplete TS protein levels? Studies have reported that HDACIs cause acetylation of several nonhistone proteins (25, 34–37). Of these, the main focus is on the acetylation of Hsp, because several prominent Hsp client proteins were intimately linked to human cancer. For example, cotreatment with SAHA and imatinib mesylate induced Bcr-Abl down-regulation and apoptosis more so than SAHA alone. LAQ824 lowered the expression, and promoted the proteasomal degradation, of Bcr-Abl. It also induced the apoptosis of imatinib refractory chronic myelogenous leukemia (49, 50). Similarly, the present study shows that TS binds to Hsp chaperonic complex (Fig. 3A–C) and that treatment with trichostatin A acetylated Hsp90 but not Hsp70. Moreover, along with Hsp90 acetylation, trichostatin A recruited Hsp70 to Hsp chaperonic complex containing client TS and resulted in the degradation of TS via a proteasomal pathway (Fig. 3B–D). Therefore, taken together, we believe that trichostatin A regulates TS expression in two ways—by indirect transcriptional repression of the *TS* gene and by the proteasomal degradation of TS protein mediated by Hsp90 acetylation. These results strongly suggest that HDACIs, like trichostatin A, can be used as a chemosensitizer of TS inhibitors. However, considering its significant toxicity, trichostatin A might not be suitable as a 5-FU modulator. About this, even low and relatively nontoxic dose of trichostatin A (<50 nmol/L) was found to down-regulate TS protein and sensitize drug-resistant cells to 5-FU (Fig. 5A–C). Besides low-dose trichostatin A, we found that relatively nontoxic SAHA and oxamflatin under clinical developments also down-regulate TS protein expression (Fig. 2D). Combination index from IC_{50} isobologram analysis showed that combinational treatments with trichostatin A and 5-FU, either sequential or concurrent, are synergistic in 5-FU–resistant SNU-484 cancer cells (Fig. 5D). Therefore, we expect that this combinatorial approach of HDACI and 5-FU can be potentially translated into clinical purpose.

In conclusion, the present study shows that HDACIs, in addition to regulating transcription, also exert nontranscriptional effects on nonhistone targets such as TS. Although TS inhibitors directly inhibit the enzymatic function of TS, HDACIs down-regulate TS expression and thus sensitize resistant cancer cells to 5-FU. In the aspect of enhancing 5-FU cytotoxicity, combined HDACI and 5-FU treatment seem to be a potentially promising strategy and clinical investigation using this approach is warranted.

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