Tunicamycin Enhances Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand–Induced Apoptosis in Human Prostate Cancer Cells

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Abstract
Death receptor 5 (DR5/TRAIL-R2) is an apoptosis-inducing membrane receptor for tumor necrosis factor–related apoptosis–inducing ligand (TRAIL/Apo2L). In this study, we showed that tunicamycin, a naturally occurring antibiotic, is a potent enhancer of TRAIL-induced apoptosis through up-regulation of DR5 expression. Tunicamycin significantly sensitized PC-3, androgen-independent human prostate cancer cells, to TRAIL-induced apoptosis. The tunicamycin-mediated enhancement of TRAIL-induced apoptosis was markedly blocked by a recombinant human DR5/Fc chimeric protein. Tunicamycin and TRAIL cooperatively activated caspase-8, -10, -9, and -3 and Bid cleavage and this activation was also blocked in the presence of the DR5/Fc chimera. Tunicamycin up-regulated DR5 expression at the mRNA and protein levels in a dose-dependent manner. Furthermore, the tunicamycin-mediated sensitization to TRAIL was efficiently reduced by DR5 small interfering RNA, suggesting that the sensitization was mediated through induction of DR5 expression. Tunicamycin increased DR5 promoter activity and this enhanced activity was diminished by mutation of a CHOP-binding site. In addition, suppression of CHOP expression by small interfering RNA reduced the tunicamycin-mediated induction of DR5. Of note, tunicamycin-mediated induction of CHOP and DR5 protein expression was not observed in normal human peripheral blood mononuclear cells. Moreover, tunicamycin did not sensitize the cells to TRAIL-induced apoptosis. Thus, combined treatment with tunicamycin and TRAIL may be a promising candidate for prostate cancer therapy. (Cancer Res 2005; 65(14): 6364-70)

Introduction
Prostate cancer is the most common malignancy and the second leading cause of male cancer-related death in the U.S. (1). Although androgen ablation is effective in treating prostate cancer, hormone-refractory tumor cells remain a major problem in current treatment protocols (2, 3). Hence, new strategies to overcome the resistance of hormone-refractory cancer cells are necessary to improve prostate cancer therapy.

Tumor necrosis factor–related apoptosis–inducing ligand (TRAIL/Apo2L) induces apoptosis selectively in cancer cells in vitro and in vivo and has little or no toxicity to normal cells (4–8). Therefore, TRAIL is a promising agent for cancer therapeutics. However, many tumors remain resistant to treatment with TRAIL.

Death receptor 5 (DR5; also called TRAIL-R2, Apo2, TRICK2, or KILLER) and death receptor 4 (DR4/TRAIL-R1) are members of the tumor necrosis factor receptor family that are activated by TRAIL (9–13). Each of these receptors contains an intracellular region designated as the death domain. Ligand-dependent and -independent activation of DR5 involves multimerization, with subsequent recruitment of intracellular adapter molecules, resulting in the activation of caspase-8, which can directly evoke cleavage of downstream effector caspases (14, 15). DR5 has been shown to be expressed more strongly in cancer cells than in normal cells (16, 17). Moreover, DR5 is a critical determinant of tumorigenicity and chemosensitivity (18). Therefore, DR5 is also considered an attractive candidate for use in cancer therapy.

Tunicamycin, a naturally occurring antibiotic, blocks the first step in the biosynthesis of N-linked oligosaccharides in cells (19). A previous report showed that tunicamycin increases the sensitivity of head and neck squamous cell carcinoma to cisplatin in vitro and in vivo (20). In the search for new strategies to overcome the resistance of hormone-refractory prostate cancer cells, we found that tunicamycin is a potent enhancer of TRAIL-induced apoptosis through induction of DR5 expression in human prostate cancer cells, but not in normal human peripheral blood mononuclear cells (PBMCs).

Materials and Methods
Reagents. Tunicamycin and soluble recombinant human TRAIL/Apo2L were purchased from Sigma (St. Louis, MO) and PeproTech (London, United Kingdom), respectively. Recombinant human DR5 (TRAIL-R2)/Fc chimera, and the caspase inhibitors zVAD-fmk, zDEVD-fmk, zETD-fmk, zLEHD-fmk, and zAEVD-fmk were purchased from R&D Systems (Minneapolis, MN).

Cell culture. The human prostate cancer cell line, PC-3, was maintained in RPMI 1640 with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO2. Normal human PBMCs were isolated as previously described (21).

Northern blot analysis and RNase protection assay. Northern blot analysis was done as previously described (21) using a full-length DR5 cDNA as a probe. The RNase protection assay was also done as previously described (21). The relative band intensity was assessed by densitometric analysis of digitalized autorgraphic images using Scion Image software (Scion Corporation, Frederick, MD).

Western blot analysis. Western blot analysis was done as previously described (21), using rabbit polyclonal anti-DR5 antibody (1:250; Cayman Chemical, Ann Arbor, MI), anti-CHOP antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-Bid (1:1,000), anti-caspase-8 (1:1,000), anti-caspase-9 (1:1,000), or anti-caspase-10 (1:1,000)
antibodies (MBL, Nagoya, Japan), or mouse monoclonal anti-procaspase-3 (1:10) antibody (Immunotech, Marseille, France). Enhanced chemiluminescence (Amersham Bioscience, Piscataway, NJ) was used for detection. The relative band intensity was assessed by densitometric analysis of digitalized autoradiographic images using Scion Image software (Scion Corporation).

**Plasmid preparation.** As previously described (22), the digested SacI-NcoI fragment from the DR5 promoter region of the human genomic DNA was subcloned into the SacI-NcoI site of the pGV82 luciferase assay vector (Toyok Inko, Tokyo, Japan) to produce pDR5PF. Deletion mutants of pDR5PF, termed pDR5/-1188, pDR5/-605, pDR5/-318, pDR5/-301, pDR5/-252, and pDR5/-198 were generated using the Mungbean-Exonuclease III system from the Kilo-sequence Deletion Kit (Takara, Tokyo, Japan). The construct pDR5/mitCHOP, which has a mutation in the CHOP binding site at -272/-269, was generated by site-directed mutagenesis using the Quick Change XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the following oligonucleotides: sense, 5'-CGCTTGGCGAG-GAGGTAGTTGACGAGAC-3'; and antisense, 5'-AGAGTCCTGTCAC- TACCTCCTCCGC-3'. Each sequence is identical to that of pDR5/-347, except for the sequence in boldface letters. All constructs were confirmed by sequencing.

**Transfection and luciferase assay.** A series of DR5 reporter plasmids and vacant vector plasmid (1.0 × 10⁵ cells) were transfected into PC-3 cells (1.5 × 10⁵ cells) using the DEAE-dextran method (CellPhect, Amersham Bioscience). After 24 hours, the cells were treated with or without tunicamycin (2 μg/mL) for 24 hours, and then harvested. Levels of luciferase activity were normalized with protein concentrations. Luciferase assays were carried out in triplicate, and the experiments were repeated several times. Data were analyzed using Student's t test, and differences between DMSO and tunicamycin treatment were considered significant when P < 0.05.

**Detection of apoptosis.** DNA fragmentation was quantified as the percentage of cells with hypodiploid DNA (sub-G1). Cells were fixed with 70% ethanol and treated with RNase A (Sigma). The nuclei were stained with propidium iodide (Sigma). The DNA content was measured using a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ). For all assays, 1.000 cells were counted.

**Small interfering RNAs.** The DR5, LacZ and CHOP small interfering RNA (siRNA) sequences were previously described (refs. 23, 24; synthesized by Proligo, Kyoto, Japan). The LacZ siRNA was used as a siRNA control. In brief, 1 day prior to the transfection, PC-3 cells were seeded without antibiotics at a density of 30% to 40%. DR5, LacZ, and CHOP siRNAs (25 nmol/L) were transfected into cells using a modified OligofectAMINE protocol (Invitrogen, Carlsbad, CA), in which the volume of OligofectAMINE was reduced to one-third of the recommended volume to limit toxic effects. Mock samples were treated with OligofectAMINE alone. Twenty hours after transfection, cells were treated with tunicamycin (2 μg/mL) and/or TRAIL (25 ng/mL) for 24 hours, and then harvested.

**Results**

**Tunicamycin potentiates TRAIL-induced apoptosis synergistically in PC-3, androgen-independent human prostate cancer cells.** To test the effect of tunicamycin on TRAIL-induced apoptosis, we first investigated the sensitivity of PC-3 cells to tunicamycin as a single agent. As shown in Fig. 1A, tunicamycin only moderately induced apoptosis in PC-3 cells. Surprisingly, we found that tunicamycin strongly enhanced TRAIL-induced apoptosis in a synergistic manner (Fig. 1B).

**Enhancement of TRAIL-induced apoptosis, caspase activation and Bid cleavage by tunicamycin is blocked in the presence of a DR5/Fc chimeric protein in PC-3 cells.** To elucidate whether the sensitization to TRAIL-induced apoptosis by tunicamycin occurred via a specific interaction between TRAIL and its receptors, we used a recombinant human DR5/Fc chimeric protein, which has a dominant-negative effect by competing with endogenous DR5. As shown in Fig. 2A, the tunicamycin-mediated enhancement of TRAIL-induced apoptosis was markedly blocked by the DR5/Fc chimera, indicating that the sensitization by tunicamycin was mediated through interactions of TRAIL with its receptors.

Next, we carried out a Western blot analysis to confirm the effect of tunicamycin on the TRAIL-induced activation of caspases. As shown in Fig. 2B, tunicamycin and TRAIL cooperated in the activation of caspase-8, -10, -9 and -3, and cleavage of Bid. In addition, the DR5/Fc chimera efficiently blocked the activation of these caspases and cleavage of Bid. These results suggest that the sensitization to TRAIL-induced apoptosis caused by tunicamycin was mediated through the death receptor pathway.

We also showed that the pan-caspase inhibitor zVAD-fmk efficiently blocked apoptosis induced by cotreatment with tunicamycin and TRAIL (Fig. 2C). These results indicate that the tunicamycin-mediated sensitization to TRAIL occurred in a caspase-dependent manner. Moreover, caspase-8, -10, -9 and -3 inhibitors also interrupted the apoptosis induced by the combined treatment with tunicamycin and TRAIL. These findings are consistent with previous reports on the role of caspases in TRAIL-mediated apoptosis (14, 25–27).

**Tunicamycin up-regulates DR5 expression in PC-3 cells.** To elucidate the mechanism of tunicamycin-mediated enhancement of TRAIL-induced apoptosis, we examined the expression of death receptors using Scion Image software (Scion Corporation). Results showed that both TRAIL and tunicamycin strongly induced expression of DR5, as shown in Fig. 3A. As expected, cotreatment with TRAIL and tunicamycin further increased DR5 expression, as shown in Fig. 3B. These phenomena were consistent with the enhancement of TRAIL-induced apoptosis caused by tunicamycin. Tunicamycin and TRAIL up-regulated DR5 expression in a dose-dependent manner (Fig. 3C). These results indicate that the sensitization to TRAIL-induced apoptosis by tunicamycin was mediated through the death receptor pathway.

To confirm that the sensitization to TRAIL-induced apoptosis by tunicamycin was mediated through interactions of TRAIL with its receptors, we tested the effect of the DR5/Fc chimera on the sensitization by tunicamycin. As shown in Fig. 4A, the DR5/Fc chimera efficiently blocked the sensitization to TRAIL-induced apoptosis caused by tunicamycin. These findings suggest that the sensitization to TRAIL-induced apoptosis caused by tunicamycin was mediated through the death receptor pathway.
receptor–related genes following tunicamycin treatment using a
RNase protection assay. As shown in Fig. 3A, tunicamycin
induced DR5 mRNA expression in PC-3 cells. This result is
consistent with a previous report of microarray data in tunicamycin-treated SH-SY5Y neuroblastoma cells (28). The
expression of other genes, including those for DR4, DcR2, and
TRAIL, were not markedly altered. Fas and caspase-8 expression
levels were slightly decreased.

To confirm the up-regulation of DR5 mRNA expression, we did a
Northern blot analysis. As shown in Fig. 3B, tunicamycin increased
DR5 mRNA expression in a dose-dependent manner. Furthermore,
we showed that tunicamycin also induced DR5 protein expression
in a dose- and time-dependent manner using Western blotting
(Fig. 3C and D). Previous reports have shown that these two bands
are consistent with DR5 protein (21, 29).

**Up-regulation of DR5 by tunicamycin contributes to the
enhancement of TRAIL-induced apoptosis in PC-3 cells.** Next,
we tested whether or not up-regulation of DR5 expression by
tunicamycin would have an effect on TRAIL-induced apoptosis in
PC-3 cells. The expression of DR5 protein was efficiently reduced
by transiently transfected DR5 siRNA (Fig. 4A). Importantly,
suppression of DR5 expression by siRNA prevented the sensitiza-
tion to TRAIL-induced apoptosis by tunicamycin (Fig. 4B). These
results suggest that the up-regulation of DR5 expression accounts,
at least in part, for the synergistic enhancement of TRAIL-induced
apoptosis by tunicamycin in PC-3 cells.

**Figure 2.** Tunicamycin-mediated sensitization to TRAIL-induced
apoptosis requires specific interaction between TRAIL and death
receptors in PC-3 cells. A, DR5/Fc chimeric protein blocks the
enhancement of TRAIL-induced apoptosis by tunicamycin. PC-3 cells
were treated with tunicamycin (2 μg/mL), TRAIL (25 ng/mL), and/or DR5/
Fc chimera (1 μg/mL) for 24 hours. Apoptosis was determined by FACS
analysis of the DNA fragmentation of propidium iodide-stained nuclei as
described in Materials and Methods. Columns, means; bars, SD. B,
enhancement of TRAIL-induced caspase activation by tunicamycin. PC-3
cells were treated with tunicamycin (2 μg/mL), TRAIL (25 ng/mL), and/or
DR5/Fc chimera (1 μg/mL) for 24 hours. Cleavage of caspase-8, -10, -9,
and Bid was assessed by Western blotting. β-Actin was used to
ensure equal gel loading. The band intensity was measured and
normalized by β-actin, and the protein levels relative to those of control
cells are noted at the bottom of the blot. C, caspase inhibitors reduce the
sensitization to TRAIL-induced apoptosis by tunicamycin in PC-3 cells.
PC-3 cells were treated with tunicamycin (2 μg/mL), TRAIL (25 ng/mL),
and/or various caspase inhibitors (20 μmol/L). VAD, zVAD-fmk
pancaspase inhibitor; C-8, zIETD-fmk caspase-8 inhibitor; C-10,
zAEVD-fmk caspase-10 inhibitor; C-9, zLEHD-fmk caspase-9 inhibitor;
C-3, zDEVD-fmk caspase-3 inhibitor. Apoptosis was determined by
FACS analysis of the DNA fragmentation of propidium iodide-stained
nuclei as described in Materials and Methods. Columns, means;
bars, SD.
**Tunicamycin Enhances TRAIL-Induced Apoptosis**

**Discussion**

New strategies are necessary to improve the survival of prostate cancer patients because many current treatment protocols are not effective against hormone-refractory cancer cells. In the search for new strategies, we found that tunicamycin is a potent enhancer of TRAIL-induced apoptosis through induction of DR5 expression specifically in prostate cancer cells. In this study, we obtained

**Figure 3.** Tunicamycin up-regulates DR5 expression in PC-3 cells. A, effect of tunicamycin on death receptor-related genes. RNase protection assay was done as described in Materials and Methods. Lane 1, 1 of 10 probes without RNase treatment; lane 2, RNase-protected probes following hybridization with yeast tRNA; lanes 3 and 4, RNase-protected probes following hybridization with total RNA from PC-3 cells treated with or without tunicamycin (2 μg/mL) for 24 hours. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L32 are shown as controls. The band intensity was measured and normalized by GAPDH, and the mRNA levels relative to those of control cells are noted at the bottom of the blot. B, tunicamycin up-regulates DR5 mRNA expression. PC-3 cells were treated with tunicamycin at the indicated concentrations for 24 hours. Northern blotting was done as described in Materials and Methods. Ethidium bromide-stained 28S and 18S rRNA are shown as controls. UT, untreated; CT, treated with DMSO (control). The band intensity was measured and normalized by 28S rRNA, and the mRNA levels relative to those of control cells (CT) are noted at the bottom of the blot. **C**, and **D**, tunicamycin up-regulates DR5 protein expression. PC-3 cells were treated with tunicamycin at the indicated concentrations for 24 hours (C), and with tunicamycin (2 μg/mL) for the indicated periods (D). Western blotting was done as described in Materials and Methods. β-Actin was used to ensure equal gel loading. UT, untreated; CT, treated with DMSO (control). The band intensity was measured and normalized by β-actin, and the protein levels relative to those of control cells (CT) are noted at the bottom of the blot.

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**CHOP** is involved in the induction of DR5 expression by tunicamycin in PC-3 cells. DR5 mRNA expression was increased by tunicamycin treatment in PC-3 cells (Fig. 3A and B). Therefore, we investigated whether tunicamycin could activate the DR5 promoter using a series of DR5 reporter plasmids. As shown in Fig. 5A and B, luciferase activity from pDR5/−301 as well as pDR5SF (−2.5 kbp) was increased by tunicamycin. On the other hand, pDR5/−252 showed a lack of response following tunicamycin treatment. These results suggested that the major tunicamycin-responsive elements are located within a 50-bp region between −301 and −252 relative to the first base of the translation initiation codon. This region contains a potential CHOP-binding site and a previous report has shown that the transcription factor CHOP is induced by tunicamycin (28).

To determine whether this CHOP-binding site is responsible for the transactivation of the DR5 promoter by tunicamycin, we generated a mutant of the deleted construct, pDR5/−347, by mutating the CHOP site, and called it pDR5/mtCHOP. As shown in Fig. 5B, the mutation abolished the activation of the DR5 promoter by tunicamycin. Therefore, we hypothesized that CHOP is involved in the tunicamycin-mediated induction of DR5. Indeed, tunicamycin induced CHOP expression in a dose-dependent manner in PC-3 cells (Fig. 5C). Furthermore, the induction of DR5 protein by tunicamycin was efficiently reduced by transiently transfected CHOP siRNA (Fig. 5D). These findings together suggest that CHOP is involved in the up-regulation of DR5 expression by tunicamycin.

**Tunicamycin does not induce CHOP and DR5 protein expression and fails to enhance TRAIL-induced apoptosis in normal human peripheral blood mononuclear cells.** We showed that tunicamycin up-regulated DR5 expression via an induction of CHOP and enhanced TRAIL-induced apoptosis in PC-3 cells. Moreover, tunicamycin also induced CHOP and DR5 protein expression and enhanced TRAIL-induced apoptosis in DU145, another androgen-independent human prostate cancer cell line (data not shown). These results prompted us to examine the effect of tunicamycin treatment on normal cells. As shown in Fig. 6A, the level of DR5 expression was significantly lower in normal human PBMCs than in PC-3 cells. Interestingly, tunicamycin induced neither CHOP nor DR5 protein expression in PBMCs. Furthermore, tunicamycin did not enhance TRAIL-induced apoptosis in PBMCs (Fig. 6B).
several pieces of evidence supporting this conclusion.

Induction of DR5 expression by tunicamycin treatment resulted in the sensitization of PC-3 cells to TRAIL-induced apoptosis. Indeed, other members of the death receptor family, including DR4, were not induced by tunicamycin (Fig. 3A). Furthermore, caspase-8, -10, -9, and -3, and Bid were activated only when tunicamycin and TRAIL were given together, and the activation of these caspases was blocked in the presence of the DR5/Fc chimera (Fig. 2B). It is

![Figure 4](image-url)

Figure 4. Down-regulation of DR5 reduces tunicamycin-mediated TRAIL-induced apoptosis in PC-3 cells. A, reduction of DR5 protein by DR5 siRNA. PC-3 cells were treated with DR5 siRNA, LacZ siRNA or transfection reagent (Oligofectamine) alone (Mock). Twenty-four hours after the transfection, cells were treated with tunicamycin (2 μg/mL) for 24 hours. Western blotting was done as described in Materials and Methods. β-Actin was used to ensure equal gel loading. Columns, means; bars, SD. B, inhibition of DR5 expression reduces the sensitization to TRAIL-induced apoptosis by tunicamycin. PC-3 cells were treated with DR5 siRNA, LacZ siRNA or transfection reagent (Oligofectamine) alone (Mock). Twenty-four hours after transfection, cells were treated with tunicamycin (2 μg/mL) and/or TRAIL (25 ng/mL) for 24 hours. Apoptosis was determined by FACS analysis of the DNA fragmentation of propidium iodide-stained nuclei as described in Materials and Methods. Columns, means; bars, SD.

![Figure 5](image-url)

Figure 5. CHOP is associated with tunicamycin-mediated induction of DR5. A and B, luciferase activity in transiently transfected PC-3 cells treated with or without tunicamycin (2 μg/mL) for 24 hours. Luciferase assays were done as described in Materials and Methods. Columns, means; bars, SD; *, P < 0.05. C, tunicamycin up-regulates CHOP protein expression in PC-3 cells. PC-3 cells were treated with tunicamycin at the indicated concentrations for 24 hours. Western blotting was done as described in Materials and Methods. β-Actin was used to ensure equal gel loading. The band intensity was measured and normalized by β-actin, and the protein levels relative to those of control cells are noted at the bottom of the blot. D, CHOP siRNA reduces the induction of DR5 protein by tunicamycin. PC-3 cells were treated with CHOP siRNA or LacZ siRNA. Twenty-four hours after transfection, cells were treated with tunicamycin (2 μg/mL) for 24 hours. Western blotting was done as described in Materials and Methods. β-Actin was used to ensure equal gel loading. The band intensity was measured and normalized by β-actin, and the protein levels relative to those of control cells are noted at the bottom of the blot.
recognized that caspase-8 and -10 are direct downstream molecules of DR5 and that Bid is a mediator connecting the death receptor pathway to the mitochondrial apoptotic pathway (30–32). Importantly, down-regulation of DR5 expression using DR5 siRNA molecules from the endoplasmic reticulum to the Golgi apparatus, brefeldin A, which inhibits the translocation of proteins from the endoplasmic reticulum stress (37). We confirmed that an inducer of endoplasmic reticulum stress, brefeldin A, which inhibits the translocation of proteins from the endoplasmic reticulum to the Golgi apparatus, also up-regulated CHOP and DR5 protein expression in PC-3 cells and sensitized them to TRAIL-induced apoptosis (data not shown).

Interestingly, tunicamycin induced both CHOP and DR5 proteins in tumor cells but not in PBMCs (Fig. 6), even though other normal organs were not examined. This result raises the possibility that the differential sensitivity of tumor versus normal cells toward the sensitization effect by tunicamycin may be due to the differential induction ability of CHOP. Therefore, we postulate that the CHOP molecule is one of the good targets of a new TRAIL-sensitizing drug which specifically affects tumor cells.

In conclusion, we have shown that tunicamycin synergistically enhances TRAIL-induced apoptosis through the induction of DR5 expression in prostate cancer cells but not in PBMCs. These observations raise the possibility that the combination of tunicamycin and TRAIL may be promising for the treatment of hormone-refractory prostate cancer.

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References


Figure 6. Up-regulation of DR5 and CHOP expression and sensitization to TRAIL-induced apoptosis by tunicamycin are not observed in normal human PBMCs. A, tunicamycin does not enhance CHOP and DR5 protein expression in normal human PBMCs. PC-3 cells and normal human PBMCs were treated with tunicamycin (2 μg/mL) for 24 hours. Western blotting was done as described in Materials and Methods. β-Actin was used to ensure equal gel loading. The band intensity was measured and normalized by β-actin, and the protein levels relative to those of control cells are noted at the bottom of the blot. B, tunicamycin does not enhance TRAIL-induced apoptosis in normal human PBMCs. Normal human PBMCs were treated with tunicamycin (2 μg/mL) and/or TRAIL (25 ng/mL) for 24 hours. Apoptosis was determined by FACS analysis of the DNA fragmentation of propidium iodide-stained nuclei as described in Materials and Methods. Columns, means; bars, SD.
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