In the United States, transitional cell carcinoma (TCC) of bladder is estimated to affect 67,160 new cases, with 13,750 deaths in 2007 (1). Conventional cytotoxic chemotherapy (such as methotrexate, vinblastine, doxorubicin, and cisplatin) is commonly used as a main regimen for advanced stages of bladder cancer (2, 3). However, drug resistance due to tumor heterogeneity (4) and serious side effects from current treatment regimens have made outcome unsatisfactory. Thus, developing novel effective chemotherapeutic regimens with fewer side effects are definitely needed to decrease the morbidity and mortality of TCC.

Epigenetic regulation refers as gene expression controlled by sequence per se (5). These changes, including DNA methylation and histone modifications (such as acetylation), are potentially reversible. Overwhelming evidence (6–8) indicate that altered epigenetic regulation is associated with cancer development. Thus, targeting epigenetic machinery with different inhibitors (9, 10) has become a new avenue of cancer therapy. Recently, we have shown a potent in vitro growth inhibitory effect of DNA hypomethylating agent (e.g., 5-azacytidine) and romidepsin on several human TCC cell lines (11). Using xenograft models, we concluded that romidepsin is a promising agent for human bladder cancer (11).

It is well known that drug resistance often emerges from single-agent regimen; therefore, developing rationalized combination strategy with higher therapeutic efficacy and acceptable drug toxicity becomes a critical issue for metastatic and refractory TCC. To enhance the effect of romidepsin, we have developed a high throughput system based on the induction of histone deacetylase inhibitor (HDACI)–mediated reporter gene activity and found tranilast as a potential agent from screening a chemical library (12). Using tranilast as a lead compound, we searched several analogues and found that 2,3-dimethoxycinnamoyl azide (DMCA; Supplementary Fig. S1) seems to be more potent than tranilast on enhancing the gene activity and found tranilast as a potential agent from screening a chemical library (12). Using tranilast as a lead compound, we searched several analogues and found that 2,3-dimethoxycinnamoyl azide (DMCA; Supplementary Fig. S1) seems to be more potent than tranilast on enhancing the antitumor effect of romidepsin without causing any more weight loss than romidepsin alone.

Conclusion: DMCA is able to enhance the antitumor effect of romidepsin on bladder cancer from in vitro and in vivo.
Materials and Methods

**Cells and reagents.** Human TCC cell lines T24, TCC-SUP, and UMUC3 were purchased from American Type Culture Collection. Cells were maintained in T medium (Invitrogen) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO₂. T24, TCC-SUP, and UMUC3 were derived from patients with grade 3 tumor (American Type Culture Collection).

Romidepsin (FR901228) was provided by Fujisawa Pharmaceutical Co., Ltd. transtast was purchased from LKT Laboratories; trans-2,3-dimethoxycinnamic acid and Na₃N₃ were purchased from Sigma-Aldrich.

Antibodies used in this study are as follows: poly(ADP-ribose) polymerase (1:2,000; Roche), actin (1:5,000; Sigma), p21 (1:400; BD Pharmingen), Rb(1:400; BD Pharmingen), p53 (1:400; BD Pharmingen), acetylated histone (1:3,500; Upstate).

**Synthesis of DMCA.** Because DMCA is currently not commercially available, a chemical synthesis process was designed. All chemicals were of reagent grade and used as received: trans-2,3-dimethoxycinnamic acid (97.0%), acetonitrile (anhydrous, 99.8%), thionyl chloride (99.5%), sodium azide (99.0%), acetonitrile (99.8%), thionyl chloride (99.5%), sodium aside (99.5%), and acetone (99.5%) were purchased from Sigma-Aldrich.

The 1H-NMR and 13C-NMR spectra were recorded on a Varian Inova 400 MHz spectrometer; the IR spectra of the product, which was prepared as solid thin films by dropping a mixture of the product with diethyl ether to a plate, were recorded on a Perkin-Elmer 1000 series FTIR; the low-resolution mass spectra were acquired on a Shimadzu QP5000 GC/MS using the electron impact ionization method.

The elemental analysis was conducted by Galbraith Laboratories, Inc. The chemical purity of DMCA was determined by a high-performance liquid chromatography system equipped with a Waters 600 Multi-solvent Delivery pump and a Waters 2996 Photodiode Array detector on an Xterra RP18 column (5 μm, 4.6 × 150mm). The samples were run by a gradient protocol: 100% H₂O containing 0.01% trifluoroacetyl or trifluoroacetic acid to 100% acetonitrile containing 0.1% trifluoroacetic acid in 50 min at a flow rate of 1.0 mL/min. Under this high-performance liquid chromatography condition, the retention times of trans-2,3-dimethoxycinnamic acid and DMCA were 22.7 and 36.5 min, respectively.

DMCA was synthesized by modifying the published procedures of preparing trans-3,4-dimethoxycinnamic azide (13, 14). Briefly, thionyl chloride (12 mL, 164.5 mmol) was added to a solution of trans-2,3-dimethoxycinnamic acid (4.0 g, 19.2 mmol) in anhydrous acetonitrile (150 mL). The resulting solution was refluxed for 3 h. Removal of the excess thionyl chloride and solvent under reduced pressure gave trans-2,3-dimethoxycinnamyl chloride (4.3 g, 19.0 mmol), then it was dissolved in acetone (125 mL) and cooled down to 0°C by an ice/water bath. Sodium azide (5.3 g, 80.8 mmol) dissolved in 15 mL of distilled water was added into this intermediate solution, and the mixture was stirred at 0°C for 1.5 h and then poured into an ice-cold sodium carbonate solution (0.5 mol/L, 200 mL). The product was extracted with dichloromethane (2 × 150 mL). The combined organic layers were washed with the sodium carbonate solution (2 × 200 mL) and then distilled water (200 mL). After drying over sodium sulfate, the extract was evaporated under reduced pressure to yield the desired product (brown powder): DMCA (3.8 g, 16.3 mmol; yield, 84.9%; purity by high-performance liquid chromatography, >99%), mp = 47 to 48°C (brown powder); DMCA (3.8 g, 16.3 mmol; yield, 84.9%; purity by high-performance liquid chromatography, >99%), mp = 47 to 48°C (brown powder); DMCA (3.8 g, 16.3 mmol; yield, 84.9%; purity by high-performance liquid chromatography, >99%), mp = 47 to 48°C (brown powder); DMCA (3.8 g, 16.3 mmol; yield, 84.9%; purity by high-performance liquid chromatography, >99%), mp = 47 to 48°C (brown powder).

**Cell growth assay and IC₅₀ calculation.** Cells were seeded in 48-well plates at a concentration of 2 × 10⁴ to 4 × 10⁴ cells in 0.5 mL of medium per well. After 24 h, the medium was aspirated and new medium containing different concentrations of drug was added; each treatment condition was carried out in quadruplicate. At the indicated time, total cell number was determined using crystal violet assay. Briefly, the medium was aspirated, and 150 μL of 1% glutaraldehyde (Sigma-Aldrich) in PBS was added for 15-min incubation. After removing glutaraldehyde, 0.5% crystal violet (Sigma-Aldrich) was added for 15 min, then plates were rinsed thrice with H₂O and air-dried at room temperature. Once crystal violet was eluted from cells with 300 μL of Sorensen’s solution (8.967 g trisodium citrate in 305 mL of distilled water, 195 mL 0.1 N HCl, 500 mL 90% ethanol) after a 30-min incubation, the A₅₄₀ nm of each sample was determined using ELX800 microplate reader (Bio-Tek Instruments).

IC₅₀ values for 5-Aza, FK228, and TSA on day 4 in each of TCC cell lines were calculated using Origin version 7.5 (OriginLab) software. IC₅₀ was considered as the drug concentration that decreases the cell count by 50%. Nonlinear regression curve fitting was done. The data were fitted to an exponential first-order decay function.

**Cell cycle analysis.** T24, TCC-SUP, and UMUC3 cells (1-2 × 10⁵) were plated in a 100-mm plate for 24 h, then different agents were added. After 2 days of treatment, the suspension cells were collected, and then the attached cells were also collected by trypsinization. Cells were washed twice with cold PBS and resuspended in 0.5 mL of cold PBS and fixed with 4.5 mL of 70% ethanol. The next day, ethanol was removed, and cells were incubated for 15 min at 37°C with 1 mL propidium iodide solution (100 mL of 0.1% v/v Triton X-100 in PBS, 20 mg DNase-free RNase A, and 2 mg of propidium iodide). Cell cycle distribution was measured with flow cytometry using FACScan (Becton Dickinson).

**Western blot analysis.** Cells were seeded (2-4 × 10⁴) in 100-mm plates, and treatments were applied 24 h after cell plating. Two days after plating, the attached cells were washed with PBS twice then lysed with protein lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L MgCl₂] containing protease inhibitor cocktail. Also, the detached cells were spun down, and the cell pellet was washed twice with ice-cold PBS then lysed with protein lysis buffer. Twenty micrograms of protein were subjected to a 10% SDS-polyacrylamide gel (NuPAGE 10% bis-Tris gel, Invitrogen). After transferring onto a nitrocellulose membrane (Osmonics), the membrane was blocked for 1 h with PBS containing 5% dry milk and 0.1% Tween 20, then incubated with the primary antibody overnight at 4°C, followed by secondary antibody. After extensive washing, the membrane was developed using ECL Plus (Amersham) or SuperSignal West Dura Extended Duration Substrate (Pierce).

**RNA isolation and real-time reverse transcription—PCR.** Total cellular RNA was extracted with RNAeasy mini kit (Qiagen) plus RNase-free DNase I (Qiagen). Total RNA (1 μg) was subjected to cDNA synthesis kit (Bio-Rad). The first strand of cDNA (4 μL) was subjected to real-time reverse transcription—PCR using primers p21 forward 5’TACCCGTGCTGGTCCCTCAG-3’, p21 reverse 5’CGCCGTTTG- GAATGGTCTAGA-3’ and E2F-1 forward 5’CAGATCTCCCTAAGACG-3’, E2F-1 reverse 5’TATCGAGAAGGCTCTG-3’. A 50-μL PCR reaction was carried out in iCycler Thermal Cycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) with a denaturation step at 95°C for 3 min followed by 40 cycles of amplification at 95°C for 30 s, 56°C for 30 s, and 72°C for 1 s. The 18S rRNA cDNA [18S F (5-GGAAA- TTTACCCGAAAGGCACCC-3’) and 18S R (5’-GGTGACGCGGGGACATCTAAGG-3’)] was used as an internal control. All experiments were repeated at least twice with sample duplication each time. Fold of induction of p21 mRNA was determined by normalizing the threshold of cycle (Cₜ) value of p21 cDNA with the xC value of 18S rRNA cDNA of each sample.

**Chromatin immunoprecipitation assay.** A chromatin immunoprecipitation assay was used to determine the effect of romidespin or DMCA.
on the level of acetylated histone H3 on p21 gene promoter region using EZ-ChIP kit from Upstate Biotechnology. T24 cells (2 × 10^6) were plated in a 100-mm plate 1 day before drug treatment. Cells were treated with different agents for 48 h, and then cell numbers were counted. An equal number of cells were subjected to chromatin immunoprecipitation assay according to the manufacturer's protocol. DNA fragments were subjected to PCR with the first primer set (forward 5'-TTTCCCTGGAGATCCAGTG-3', reverse 5'-ACATTTCCCGCAGGTG-3'). The purified DNA and ThermalAce DNA polymerase (Invitrogen) were added in a 50-μl volume PCR reaction with 59°C annealing temperature for 40 cycles. The PCR product was further subjected to a real-time PCR with the second primer set (F 5'-GGTGTCAGTAGGCCTCCG-3', R 5'-GGACATCTCCAGGAGACAC-3'). To compare the amount of acetylated histone associated with p21gene promoter among samples, the C_i value of precipitated DNA was normalized with the C_i value of input DNA from each sample. The fold of induction was calculated by normalizing with control (=1).

**Xenograft animal model and treatment schedule.** T24-tumorigenic cell line, a highly tumorigenic clone, was derived from T24 subcutaneous animal model. To generate xenograft tumors, athymic nude mice were injected with a 100-μl cell suspension containing 1 × 10^6 T24-tumorigenic cells on the flank (two sites per animal). When the tumors became palpable, different treatments were applied on randomized animals (six animals per treatment group) with similar tumor volume. Romidepsin was dissolved in 100% ethanol and further diluted with 5% glucose solution at 1:39 ratio before injection. DMCA (1.0 mg/kg) was administrated by i.p. injection weekly for 3 weeks. Tumor were measured weekly with a caliper, and tumor volume (mm³) was calculated using the ellipsoid formula (π/6 × length × width × depth). After 3 weeks of treatment, one animal from each treatment group was sacrificed and lysate prepared from tumor tissue was subjected to Western blot analysis probed with either p21 or histone H3 antibody. All the animal experiments were approved by Institution Animal Care and Usage Committee.

**Statistical analysis.** All numerical data were expressed as mean ± SD. Statistical significance was determined by conducting a paired Student’s t test. Results with P value of <0.05 were considered statistically significant.

**Result**

**Growth inhibitory effect on TCC cell lines by single agent (romidepsin, DMCA) or combination.** Based on the individual IC_{50} of romidepsin on three individual TCC cell lines (IC_{50}: T24 1.0 ng/mL, TCC-SUP 0.5 ng/mL, UMUC3 0.8 ng/mL; data not shown), we decided to examine the combination effect of romidepsin/DMCA using concentration of romidepsin below the IC_{50}. As shown in Fig. 1, 10 or 25 μmol/L DMCA alone did not elicit any cytotoxicity in all three cells, except 25 μmol/L DMCA elicited ~20% toxicity in UMUC3. Noticeably, either 10 or 25 μmol/L DMCA can greatly enhance the effect of romidepsin on all three cells 4 days after treatment, and this effect is significantly higher than single agent. In addition, no cell recovery was observed in combination treatment for all three cell lines 4 days after treatment, suggesting that this combination could provide a prolonged cytotoxic effect (data not shown). Structurally, tranilast, 2,3-dimethoxycinnamic acid, NaN_3, and DMCA share some similarity. Thus, we decided to determine whether tranilast, 2,3-dimethoxycinnamic acid, or NaN_3 could enhance the growth inhibitory of romidepsin and found that all three compounds (Supplementary Fig. S2) failed to achieve the same growth inhibitory effect as DMCA (Fig. 1).

**Romidepsin/DMCA induces apoptosis and G1 cell cycle arrested in TCC cells.** To understand the biological effect of romidepsin/DMCA combination on TCC cells, we investigated its effect on cell cycle distribution or apoptosis from three TCC cell lines. For example, romidepsin (0.75 ng/mL) caused 21.4% cell death in T24 cell line (Fig. 2A), and DMCA alone did not change cell cycle distribution compared with control. In contrast, the combination dramatically increased dead cell population to 89.5% (Fig. 2A), and these dead cells were due to apoptosis

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**Fig. 1.** Growth inhibitory effect of romidepsin and/or DMCA on TCC cell lines. Cells were seeded in 48-well plates for 24 h before adding drug. T24 and UMUC3 cell lines were treated with romidepsin at 0.75 ng/mL, and TCC was treated with 0.3 ng/mL. All three TCC cell lines were treated with DMCA at 10 and 25 μmol/L. Relative cell number from each treatment (n = 4) assessed using crystal violet assay was determined at day 4 after drug treatment, and the cell viability was calculated as percentage of control. Columns, mean; bars, SD. *, significant difference between combination and DMCA (P < 0.05); #, significant difference between combination and DMCA (P < 0.05). FK, romidepsin.
confirmed by the appearance of cleavage fragments from 89 kDa poly(ADP-ribose) polymerase (Fig. 2B).

Similar results can be seen in UMUC3 and TCC-SUP cells treated with DMCA/romidepsin combination (Fig. 2A). Apparently, this combination can induce apoptosis in these cell lines, and this combination can effectively target cells at either G0-G1 or S phase (Fig. 2A), suggesting that this combination could have better potency than the chemotherapeutic agent targeting only the mitotic cell population.

The effect of romidepsin/DMCA on cell cycle regulators. To understand the underlying mechanism of these agents on modulating cell cycle–related proteins, we have profiled the steady-state levels of several key regulators (Rb, E2F-1, p21, and p53) in these cells. As shown in Fig. 3, romidepsin, but not DMCA, alone could up-regulate p21 protein expressions in all three cell lines. The combination further enhanced the expression of p21 correlated with G1-S cell cycle arrest and then apoptosis detected by cell cycle analysis and poly(ADP-ribose) polymerase cleavages. However, this process seems to be p53-independent because wild-type p53 expression was diminished in these three cell lines with known mutated p53 (15). In addition, we noticed that the hypophosphorylated Rb (active form) levels became dominant in two Rb-positive TCC cell lines after the combination treatment, which dramatically reduced the hyperphosphorylated Rb protein. Consistently, the diminished expression of E2F-1 was also found in both Rb-positive cells after combination treatment, which may be caused by the binding of active Rb.

To further understand whether increased p21 and decreased E2F-1 by combination treatment is due to transcriptional or posttranscriptional regulation, we did quantitative reverse transcription–PCR to determine mRNA level of p21 and E2F-1 in these three TCC cells. Romidepsin alone induced p21 mRNA expression, whereas the combination can further enhance p21 mRNA expression level in all three cell lines tested (Fig. 4A). On the other hand, the combination can suppress E2F-1 mRNA (Fig. 4B) or protein (Fig. 3) levels in both T24 and UMUC3 cell lines but not in TCC-SUP cell.

The epigenetic effect of romidepsin enhanced by DMCA. As a typical HDACI, romidepsin is known to induce histone acetylation in vitro and in vivo in several human cancer cell lines (16–18); in general, the level of acetylation of histone H3 represents the inhibitory effect of histone deacetylase activity. To better understand the effect of this combination on the histone deacetylase activity, the steady-state levels of acetylated H3 in all three TCC cell lines were determined. As shown in Fig. 5A, romidepsin induced acetylated histone H3, whereas DMCA alone (either 10 or 25 μmol/L) did not.
change acetylated histone H3 levels in these cell lines after 2 days of treatment. Noticeably, the combination exhibited a higher induction of acetylated histone H3 than romidepsin alone.

Meanwhile, we also determined whether the increased p21 mRNA expression is due to the increased histone H3 acetylation on p21 gene promoter region. Using chromatin immunoprecipitation assay, an elevated acetylated H3 level (ca. 1.87-fold) associated with p21 gene promoter in T24 cells treated by romidepsin alone, whereas DMCA alone did not change acetylated H3 levels under the same condition (Fig. 5B). Nevertheless, the combination exhibited a significant elevation (ca. 3.6-fold) of acetylated H3 level associated with p21 promoter gene promoter in T24 cells (Fig. 5B).

The antitumor activity of romidepsin/DMCA combination. In our recent publication (11), we have shown that romidepsin is a potent antitumor agent for TCC in vivo. We then evaluated whether DMCA can further enhance tumor growth inhibition effect of romidepsin in vivo. By delivering romidepsin i.v. and DMCA i.p. into athymic nude mice bearing subcutaneous T24-tumorigenic tumors, romidepsin as a single agent could inhibit T24 tumor growth compared with the control group (Fig. 6A). As we expect, DMCA did not exhibit any tumor inhibition in vivo; however, DMCA significantly enhanced the antitumor effect of romidepsin (Fig. 6A). Accordingly, marked p21 and acetylated histone H3 expression were seen in tumor specimens treated with the combination (Fig. 6B). In addition, we noticed that the combination did not elicit any more total body weight loss from host compared with romidepsin alone, suggesting that adding DMCA into romidepsin treatment might not cause more toxicity (Supplementary Fig. S3).

Discussion

The long-term disease-free survival in patients with metastatic TCC is still considerably low. Novel chemotherapeutic agents with new mechanism of action are needed to reduce the morbidity and mortality of TCC. In addition to gene mutations associated with cancer cells, it becomes more apparent that altered epigenetic regulation is associated with malignant cells. For instance, up-regulated histone deacetylase activity has been documented in many human malignancies resulting in the repression of tumor suppressor genes and the promotion of tumorigenesis (19, 20). Romidepsin, a novel bicyclic depsipeptide as a potent HDACI, is undergoing clinical trials.5 Based on data from xenograft models (11), we believe that romidepsin is a promising agent for TCC. Nevertheless, emerging drug resistance from a single agent–romidepsin treatment has been reported (21, 22). In this study, we explored new compound(s) to enhance antitumor effect of romidepsin on TCC.

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5 http://www.gloucesterpharma.com/clinical/index.html
Although the exact mechanism of action of romidepsin has not been well defined, it has been suggested that the antitumor activity of HDACIs is exerted through multiple mechanisms, such as apoptosis, cell cycle arrest, and differentiation via the modulation of the gene expression (23, 24). In our study, romidepsin inhibits TCC growth by inducing both G1-S cell cycle arrest and apoptosis (Fig. 2); these results are consistent with other reports showing that HDACIs caused cell cycle arrest at the G1 phase and increased apoptosis (25, 26). Noticeably, DMCA can enhance the romidepsin effect and result in more G1-S cell cycle arrest and apoptosis.

Furthermore, we have analyzed the steady-state levels of various cell cycle regulators. A significant induction of p21 is associated with romidepsin treatment; this induction is independent of p53-mediated pathway (Fig. 3; refs. 27, 28). Using real-time reverse transcription–PCR and chromatin immunoprecipitation assays (Figs. 4 and 5), it seems that the induction of p21 by romidepsin alone or the combination is due to transcriptional activation of p21 gene evidenced by an increased acetylated H3 levels associated with p21 gene promoter. Taken together, we conclude that DMCA is able to enhance HDACI activity of romidepsin.

Interestingly, mutant p53 was depleted by romidepsin treatment in these three TCC cell lines harboring mutant p53 gene (15); similar results were noticed previously (17, 29). It is postulated that the degradation of mutant p53 by romidepsin is due to partially restoration of wild-type p53 protein function (17). Sudden restoration of p53-like functions could be highly cytotoxic to cancer cells expressing mutant p53, which may explain the selectivity of romidepsin on cancer cells (17). In all three TCC cells tested, DMCA significantly enhanced the effects of romidepsin on several cell cycle regulators. At 10 μmol/L, DMCA could enhance the effect of romidepsin on the induction of p21, the depletion of mutant p53, and increase of hypophosphorylation of Rb (Fig. 3), indicating that there are coordinated changes in cell cycle regulators leading to cell cycle arrest and apoptosis induced by romidepsin and further enhanced by DMCA.

For E2F-1 expression, other HDACIs, such as suberoylanilide hydroxamic acid, can also down-regulate E2F-1 in human multiple myeloma cells (30). However, the diminished expression of E2F-1 by romidepsin alone or the combination was only observed in T24 and UMUC3 cells but not in TCC-SUP cells (Figs. 3 and 4). Although, the detailed mechanism of this event is still unclear, one could speculate that the increased p21 expression may play a partial role in suppressing E2F-1 gene expression as observed in A549 human bronchogenic carcinoma cell line (31). Also, the activation status of Rb may be involved in regulating the steady-state levels of E2F-1 protein because Rb was not detectable in TCC-SUP cells.

Data (Fig. 6A) from the subcutaneous tumor model clearly indicate that this combination exhibits a significant tumor suppression, which is accompanied by marked elevation of p21.
and acetylated histone H3 from these tumor tissues (Fig. 6B). The induction of p21 seems to be a hallmark of histone deacetylation (32), and p21 expression is associated with the antiproliferative effect of HDACI (11, 33). Based on data from this study, we believe that p21 induction is critical for the growth inhibition and apoptosis of TCC. In summary, DMCA is able to enhance the growth inhibitory effect of romidepsin on TCC in vitro and in vivo. Multiple effects on cell cycle regulators signify the potency of this combination, and the induction of p21 gene expression is highly associated with the antitumor effect of HDACI and its combination with DMCA.

Acknowledgments

We thank Drs. Zolitan Kovacs and You-Fu Zhou for their assistance in the characterization of DMCA and Mary Barnes for editorial assistance.

References


Effect of *Trans*-2,3-Dimethoxycinnamoyl Azide on Enhancing Antitumor Activity of Romidepsin on Human Bladder Cancer

Jinhai Fan, Jennifer Stanfield, Yi Guo, et al.

*Clin Cancer Res* 2008;14:1200-1207.

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