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R-Flurbiprofen Reverses Multidrug Resistance, Proliferation and Metastasis in Gastric Cancer Cells by p75^{NTR} Induction†

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Received August 4, 2009; Revised Manuscript Received November 13, 2009; Accepted November 16, 2009

Abstract: Nonsteroidal anti-inflammatory drugs (NSAIDs) can inhibit cell growth and metastasis, and induce cell apoptosis in cancerous cells. They have been shown to reduce incidence and mortality of gastric cancer by an unknown mechanism. NSAIDs often exert their effects by Cox-2 inhibition, and Cox-2 is overexpressed in gastric cancer cells. Nevertheless, when gastric cancer cells were treated with different NSAIDs, the non-Cox-2-inhibiting *R*-flurbiprofen was most effective at reducing proliferation of gastric cancer cells in vitro. *R*-Flurbiprofen prevented the metastatic characteristics of gastric cancer cells in vitro, and reduced tumor size and metastasis in vitro, when gastric cancer cells were injected into nude mice. *R*-Flurbiprofen also affected multidrug resistance, increasing the sensitivity of resistant gastric cancer cells to chemotherapeutic agents. Mechanistically, *R*-flurbiprofen was found to have pleiotropic effects, changing levels of cell cycle factors like Cyclin D1 and CKD4, apoptotic protwins like caspase3 and Bcl-2, and protwins that affect metastasis, like metalloproteases. Consistent with reports on other cancer cell types, NSAID treatment with *R*-flurbiprofen increased levels of the tumor suppressor neurotrophin receptor (p75^{NTR}) in gastric cancer cells. The anticancer effects of *R*-flurbiprofen were found to require induction of p75^{NTR} via the p38 signaling pathway, suggesting a possible mechanism of action.

Keywords: *R*-Flurbiprofen; gastric cancer; p75

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) inhibit cyclooxygenase (Cox) enzymes, thereby reducing the production of inflammation-mediating prostaglandins. They have also been shown to have anticancer effects, reducing cell growth and metastasis, and inducing apoptosis.[1] The mechanism by which NSAIDs affect cancer progression may not be through Cox enzyme inhibition, however. Anticancer effects require levels of NSAIDs much higher than those required for Cox inhibition.[2] In addition, NSAIDs that do not have Cox-inhibiting activities, like *R*-flurbiprofen (*R*-flur, also known as Ansaid or generically as tarenflurbil), have anticancer effects in vivo and in vitro. *R*-Flur inhibits

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† Grant support: National Natural Science Foundation of China No. 30973423.

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10.1021/mp900189x
Published on Web 11/16/2009

progression of prostate cancer and colon cancer in mice[3−5] and inhibits survival of colon and prostate cancer cell lines in vitro.[6,7] Thus, the precise mechanism by which NSAIDs exert anticancer effects and the effects of NSAIDs on different types of cancerous cells are still under investigation.

NSAIDs may work at the level of the cell cycle. The activity of the cell cycle regulator cylin-dependent kinase 2, and the expression of cyclins D1, A and B are reduced by NSAID treatment, and flurbiprofens caused G1 cell cycle arrest in human colon carcinoma cells.[6] NSAIDs may also exert their anticancer effects by stimulating apoptosis. NSAIDs have been shown to target Bcl-2[8] and caspases.[9,10] NSAIDs also prevent metastasis through effects on the zinc-dependent matrix metalloproteases that are crucial for degrading extracellular matrix for migration and invasion.[11,12] NSAIDs may also act by altering expression of key genes associated with proliferation and angiogenesis, for example by reducing transcription of vascular endothelial growth factor (VEGF) by inducing degradation of Sp1 transcription factor.[13] In addition to antiproliferative and antimetastatic effects, NSAIDs may be able to alleviate multidrug resistance (MDR).[14]

Regardless of the overall mechanism by which NSAIDs act on cancerous cells, the specific intracellular targets of NSAIDs are being elucidated. In a lung cancer model, the NSAID NS398 was shown to inhibit Ras/c-Raf interaction and upregulate mitogen-activated protein kinase phosphatases, resulting in inhibition of the growth-factor stimulated ERK signaling pathway.[15] In several cancer cell lines, including lines derived from prostate, ovarian, bladder, glioblastoma, neuroblastoma and colon cancer, NSAIDs such as *R*-flur, ibuprofen and aspirin caused increased expression of the tumor suppressor neutrotrophin receptor ($p75^{NTR}$), at both the protein and mRNA levels, while also inducing apoptosis. Additionally, the p38 MAPK pathway could mediate *R*-flur or ibuprofen-induced mRNA stability of $p75^{NTR}$ [16] and mediate carprofen-induced $p75^{NTR}$-dependent apoptosis in prostate cancer cells.[17] Dominant-negative alleles or siRNA against $p75^{NTR}$ prevented the anticancer effect of NSAIDs, suggesting that NSAIDs act through this tumor suppressor.[18]

Human $p75^{NTR}$ is a cell-surface protein that is a member of the tumor necrosis factor (TNF) receptor family; $p75^{NTR}$ has an apoptosis-inducing "death domain" and, like others in the TNF receptor family, has apoptotic activity. $p75^{NTR}$ is responsible for caspase-mediated apoptosis in bladder cancer cells.[19] $p75^{NTR}$ levels are lower in gastric cancer cells, both in cultured lines and in primary cells, than in normal cells. In vitro increases to $p75^{NTR}$ levels correlated with a wide range of anticancer effects, including reduction in in vivo tumorigenicity, and cell cycle progression arrest that

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corresponded to reduction in the levels of cell cycle promoters.[20] Reduction of $p75^{NTR}$ levels in vitro caused an increase in the levels of proteases associated with metastasis, and correspondingly increased the in vitro migratory properties of gastric cancer cells.[21] The precise mechanism by which $p75^{NTR}$ effected these changes in gastric cancer cells is still unknown.

NSAIDs reduce the incidence and mortality of gastric cancer,[22] but by an unknown mechanism. Cox-2 is overexpressed in gastric cancer cells,[23,24] and the specific Cox-2 inhibitor celecoxib inhibits cell proliferation in both chemosensitive and chemoresistant gastric cancer cell lines.[25] Therefore, we investigated the mechanism of NSAIDs on gastic cancer cells in vitro using a panel of NSAIDs, some of which inhibited Cox-2 and some of which did not. The NSAID *R*-flur was most effective at inhibiting the proliferative and metastatic characteristics of gastric cancer cells, both in vitro and in vivo. In addition, *R*-flur increased the sensitivity of MDR cells to anticancer agents, suggesting an ability to reverse MDR. *R*-Flur does not inhibit Cox-2, but it effectively induced expression of $p75^{NTR}$ in a p38-dependent manner, suggesting a possible mechanism for its anticancer effects.

Materials and Methods

Cell Culture. The human gastric cancer cell lines SGC7901, MKN45, SGC7901/ADR and SGC7901/VCR described[21,26,27] were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin.

Drugs and Treatment. NSAIDs and chemotherapeutic agents were obtained from the Institute of Traditional Chinese Medicine at concentrations of 96%. They were diluted in dimethyl sulfoxide (DMSO) to a final concentration of 2 mM and 5 mg/L, respectively. SB202190 (Calbiochem) were dissolved in DMSO and used at final concentrations of 20 μmol/L. These solutions was filtered through a 0.22 μm filter and stored at 4 °C, and then diluted further in the cell culture medium. Cells were seeded in flasks or dishes. NSAID treatment at different doses (0, 2.5, 5, 10, 20 μM) was for 24, 48, 72, or 96 h. The control group received an equal volume of DMSO only. Cells were measured after treatments.

In Vitro Drug Sensitivity Test. Cells were seeded in 96-well plates at a density of 8×10^3 cells per well. Twenty-four hours later, freshly prepared anticancer drugs including ADR, 5-fluorouracil (5-FU), cisplatin (CDDP), mitomycin (MMC), and VCR were added, with the final concentrations being 0.01, 0.1, 1, and 10 times the human peak plasma concentration for each drug. The peak serum concentrations of various anticancer drugs are 0.4 μg/mL for ADR, 10 μg/mL for 5-FU, 2.0 μg/mL for CDDP, 1.0 μg/mL for MMC, and 0.5 μg/mL for VCR.[28,29] Cell viability was measured 72 h after addition of drugs with CCK-8 (Dojindo, Kumamoto, Japan). Briefly, CCK-8 reagent (10 μL) was added to each well, and the plates were incubated at 37 °C. Optical density was read 3 h later at a wavelength of 450 nm using a model 550 microplate reader (Biohit BP-800, Biohit Plc, Helsinki, Finland). The concentration at which each drug produced 50% inhibition of growth (IC_{50}) was estimated by the relative survival curves. The test for each drug concentration was performed three times, and cell viability was measured in quadruplicate in each test.

Adriamycin Accumulation. As described previously,[28] cells were cultured in 6-well culture plates at 37 °C for 48 h, and ADM was added to a final concentration of 5 mg/L. After further culturing for 1 h, PBS was used as negative control. Cells were harvested and suspended in cold PBS; intracellular adriamycin fluorescence intensity was determined by flow cytometric analysis with the stimulative and acceptant wavelength at 488 and 575 nm, respectively.

Transient Transfection. The dominant-negative antagonist p75NTR (ΔICD) was respectively sponsored by gifts of Moses V. Chao in New York University School of Medicine. siRNAs targeting p38a (5′-AAA CAA UGU UCU UCC AGU CAA CAG C-3′ and 5′-GCU GUU GAC UGG AAG AAC AUU GUU U-3′), p38b (5′-GCG AAG UGU ACU UGG UGA C-3′ and 5′-GUC ACC AAG UAC ACU UCG C-3′), and siRNAs targeting $p75^{NTR}$ (5′- GCU ACU ACC AGG AUG AGA CdTdT-3′ and 5′-GUC UCA UCC UGG UAG UAG CdTdT-3′) and negative control siRNA were obtained from GenePharma (Shang Hai, China). Cell transfection was performed with Lipofectamine2000 (Invitrogen, Carlsbad, CA) as described in the manufacturer's

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protocol. Cells were seeded in 60 mm dishes to reach 30% to 50% confluency after 24 h of incubation, and transfected with a total of 100 nmol/L siRNA using Lipofectamine2000 in antibiotic free medium, according to the manufacturer's instructions. Five hours after transfection, the medium was replaced with fresh DMEM. The cells were used for experiments after 30−48 h of further incubation.

Monolayer Growth Rate. Monolayer culture growth rate was determined as described previously by conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) to water-insoluble formazan by viable cells.[20] Wells of a 96-well plate were seeded with 3000 cells in 200 μL of medium and grown under normal conditions. Cultures were assayed at 0, 1, 2, 3, 4, and 5 days and absorbance values determined by enzyme-linked immunosorbent assay, read by a plate-reader at 490 nm (DASIT, Milan, Italy). Growth curves from cells were generated in parallel for comparison. Each experiment was performed in triplicate.

Plate Colony Formation Assay. Plate colony formation assay was determined as described previously.[30] For colony formation assays, 1×10^3 cells were seeded into 60 mm dishes with 5 mL of DMEM supplemented with 10% FBS. After 14 days, the resulting colonies were rinsed with PBS, fixed with methanol at −20 °C for 5 min, and stained with Giemsa (Sigma-Aldrich Co., St. Louis, MO) for 20 min. Only the colonies clearly visible (diameter > 50 μm) were counted.

Soft Agar Clonogenic Assay. Anchorage-independent growth as a characteristic of in vitro tumorigenicity was assessed by soft agar clonogenic assay as described previously.[20] Briefly, cells were detached and plated in 0.3% agarose with a 0.5% agarose underlay at 1×10^4 cells/well in 6-well plates. The number of foci were counted after 17 days. Each experiment was performed in triplicate.

Tumorigenicity in Nude Mice. Tumorigenicity assays were as described previously.[20] Four groups of five mice each were injected subcutaneously at a single site with SGC7901/ADR or SGC7901 cells. Tumor onset was scored visually and by palpitation at the sight of injection by two trained laboratory employees, at different times on the same day. Average tumor size was estimated by physical measurement of the excised tumor at the time of sacrifice. With the exception of mice with large tumor burdens, animals were sacrificed 4 weeks after injection. Tumors were verified by hematoxylin and eosin (HE) staining. Blocks were available for further analysis.

Hoechst Assay. Hoechst assay was as described previously.[8] Nuclear morphology was assessed using Hoechst staining. Mice were injected subcutaneously at a single site with SGC7901/ADR, and each group was treated with *R*-flur only, ADR only and *R*-flur + ADR. After the mice were sacrificed, the tumors were made into formalin-fixed paraffin-embedded specimens. 4 μm sections of formalin-fixed paraffin-embedded specimens were made. 0.5% Triton X-100 was added to the sample and left for 5 min at room temperature. Slides were washed twice with PBS and stained for 30 min at 37 °C in Hoechst 33258 1 μg/mL (Sigma Chemical); then, they were rinsed with PBS and examined with a fluorescence microscope (UV light). Fragmented nuclei were expressed as percentage of fragmented Hoechst-positive nuclei versus the total Hoechst-positive nuclei.

Flow Cytometry Analysis. Flow cytometry analysis was performed as described previously.[20] Cells were seeded into 60 mm diameter plates in complete medium overnight, placed in serum-free medium for 48 h to synchronize the cells, and then cultured again in complete medium. At 24 h, cells were harvested. After washing with ice-cold phosphate buffered saline (PBS), cells were suspended in about 0.5 mL of 70% alcohol and kept at 4 °C for 30 min. The suspension was filtered through 50 μm nylon mesh, and the DNA content of stained nuclei was analyzed by a flow cytometer (EPICS XL, Coulter, Miami FL). The cell cycle was analyzed using Multicycle-DNA Cell Cycle Analyzed Software. The proliferous indexes (PI) were calculated: PI = (S + G2)/(S + G2 + G1). Each experiment was performed in triplicate.

Immunohistochemical Assay. A two-step immunohistochemical staining technique was applied to detect the expression of $p75^{NTR}$ and microvessel density at tumor sites. Tissue sections of 4 μm thickness were used for immunohistochemical analysis and stained with H&E to determine the tumor structure and extent of liver and lung metastases according to the procedures briefly described below. 4 μm sections of formalin-fixed paraffin-embedded specimens were made. Slides were dewaxed, rehydrated, incubated in 10% normal goat serum and 0.3% Triton X-100 in phosphate buffer saline (PBS) for 1 h and then incubated with monoclonal anti-p75NTR antibody (1:100, Sigma, Swampscott, MA) and monoclonal antifactor VIII antibody (1:300, Sigma, Swampscott, MA). The slides were washed in PBS 3 times for 5 min each. The tissues were incubated in biotin-labeled rabbit anti-mouse serum (1:200) for 30 min, rinsed with PBS and incubated with avidin−biotin−peroxidase complex for 1 h. The signal was detected using 3,3-diaminobenzidine as the chromogen. Negative control slides using anti-6His as the primary antibody were included in all assays.

Invasion and Migration Assay. Cell invasion assays were performed as described[21] using Transwells (8 μm pore size, Corning Costar Corp). Matrigel was diluted to a concentration of 2 mg/mL, and 50 μL was placed on the lower surface of a polycarbonate filter and air-dried. After rinsing with PBS, the filters were placed into wells and 700 μL of DMEM containing 10% bovine serum was added into the lower compartment. Freshly trypsinized and washed cells were suspended at 2×10^5 cells/mL in DMEM containing 1% bovine serum and preincubated for 10 min with or without blocking antibodies. The cell suspension (150 μL) was placed in the upper compartments, and cells were allowed to invade for 36 h at 37 °C in a 5% CO_2 humidified incubator. After

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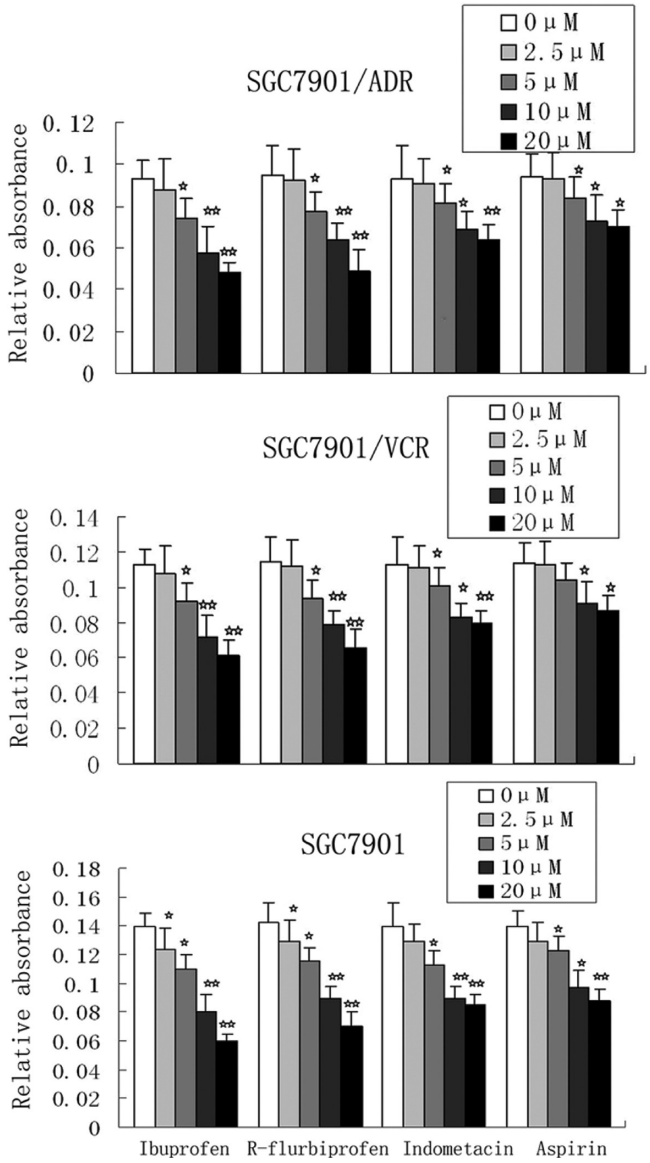

Figure 1. Effects of *R*-flur on the in vitro proliferation of gastric cancer cells SGC7901 and MDR cells SGC7901/ADR and SGC7901/VCR. Monolayer growth rates of cells after treatment with the indicated concentration of *R*-flur for 24 h were determined by MTT assays. Values represent the mean (SEM) from at least three separate experiments. $^*p < 0.05$.

incubation, cells were removed from the upper surface of the filter with a cotton swab. Cells that had invaded the bottom surface of the filter were fixed with methanol and stained with hematoxylin. The invasive ability was determined by counting penetrating cells under a microscope at 200× magnification in 10 random fields from each well. Each experiment was performed in triplicate. The in vitro migration assay was performed as previously described[21] using Transwells [8 μm pore size, Corning Costar Corp] without Matrigel, and otherwise similar to the invasion assay. Cells were suspended at 2×10^4/mL, and incubation time was 24 h.

Wound-Healing Assay. For monolayer wound-healing assays, a total of 2×10^5 cells was collected and plated in a 12-well plate. At 100% confluence, two parallel wounds of 1 mm were made using a pipet tip. Wound size after 24 h was measured using Zeiss LSM Image Browser software, version 3.1, for three independent experiments.

Tail Vein Metastatic Assay. The tail vein metastatic assay was performed as previously described.[21] Nude mice were handled using humane practices and were cared for in accordance with NIH Animal Care and Use Committee guidelines. Cells were harvested from tissue culture flasks using trypsin and washed three times with PBS. Mice were injected with 1×10^6 cells in 0.1 mL of PBS through the tail vein. The mice were then monitored for overall health and total body weight. Four weeks after injection, the mice were sacrificed. The liver and lung tissues were visually observed, and the number of visible tumors on the liver and lung surface were counted. Liver and lung tissues serial sections were made and dyed with HE and observed under a light microscope. Experimental and control groups contained six to ten mice.

Reverse Transcription PCR. RT-PCR was performed as described previously.[31] RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription PCR (RT-PCR) was done with the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) using equal amounts of RNA according to the following program: 47 °C for 30 min; 94 °C for 2 min; 30 cycles of 94 °C for 1 min, 60 °C for 1 min,and 72 °C for 1 min; and 72 °C for 5 min. Forward and reverse primers for p75NTR were 5′-AGCCTTCAAGAGGTGGAACAG-3′ and 5′-CTGTCGCTGTGGAGTTTTTCTC-3′, respectively, and gave an approximate 0.45 kb product. Forward and reverse primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-GCCTCAAGATCATCAGCAAT-3′ and 5′-AGGTCCACCACTGACACGTT-3′, respectively, and gave an approximate 0.58 kb product.

Western Blot. Cells were washed twice with Hanks's balanced salt solution and lysed directly in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na_3VO_4). The lysates were centrifuged at 14,000 rpm for 30 min at 4 °C and supernatants collected. To detect the expression of the secreted active form of MMPs in supernatants, 10 mL of conditioned medium was concentrated 100-fold in 10 kDa microcentrifuge concentrators (Millipore). Cell lysate (60 μg) or supernatant proteins (10 μg) were separated by SDS−PAGE, blotted onto nitrocellulose membranes, and incubated with primary antibody. Antibodies were mouse monoclonal anti-human p75NTR (diluted 1:300; Sigma Chemical Co.), mouse monoclonal anti-uPA (diluted 1:500; Sigma Chemical Co.), mouse monoclonal anti-MMP2 and anti-MMP9 (diluted 1:300;

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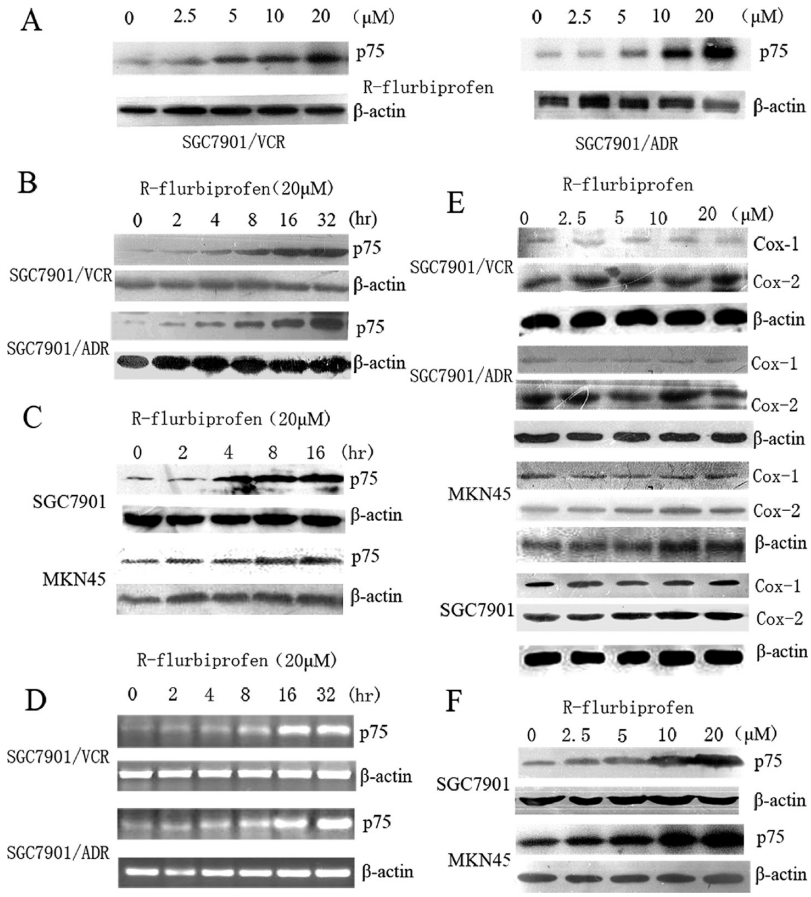

Figure 2. The effect of *R*-flur on p75^{NTR}, Cox-1 and Cox-2 by Western blot or RT-PCR. All examined gene expression levels were quantitatively analyzed and expressed as the ratios over β-actin. Shown is a representative experiment out of three with similar results. (A) Expression of p75^{NTR} in MDR SGC7901/ADR and SGC7901/VCR cell lines was evaluated by Western blot after treatment with *R*-flur at the indicated doses. β-Actin was used as an internal control. (B and C) Expression of p75^{NTR} in SGC7901/ADR, SGC7901/VCR, SGC7901 and MKN45 gastric cancer cells was evaluated after treatment with *R*-flur at different times by Western blot at the indicated doses. β-Actin was used as an internal control. (D) Expression of p75^{NTR} in SGC7901/ADR and SGC7901/VCR was evaluated after treatment with *R*-flur at different times by RT-PCR at the indicated doses. (E) Expression of Cox-1 and Cox-2 in SGC7901/ADR, SGC7901/VCR, SGC7901 and MKN45 cells was evaluated after treatment with *R*-flur at the indicated doses for 24 h by Western blot. β-Actin was used as an internal control. (F) Expression of p75^{NTR} in SGC7901 and MKN45 gastric cancer cells was evaluated after treatment with *R*-flur at different doses for 24 h by Western blot. β-Actin was used as an internal control.

Santa Cruz Biotechnology, Santa Cruz, CA) or mouse monoclonal anti-TIMP1 and anti-TIMP2 (diluted 1:300; Santa Cruz Biotechnology, Santa Cruz, CA.) Also used were monoclonal antibodies against P-gp, Bcl-2, Caspase3, Cyclin D1, CDK4, VEGF, phosphorylated p38 (p-p38), p38 (all diluted 1:500; BD Biosciences, San Jose, CA) and antibodies against Cox-1 and Cox-2 (diluted 1:500; Cell Signaling Technology, Beverly, MA). β-Actin was used in all the Western blots as a loading control (diluted 1:5000, Sigma, St. Louis, MO). After repeated washing, the membranes were incubated with horseradish-peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology), diluted 1:2000. Bands were visualized using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

Statistical Analysis. Each experiment was repeated at least three times. Bands from Western blot were quantified by Quantity One software (BioRad). Relative protein levels were calculated relative to β-actin. Numerical data are presented as the mean $\pm$ standard error of the mean (SEM). The difference between means was performed with ANOVA and a posthoc test. All statistical analyses were performed using SPSS11.0 software (Chicago, IL). $p < 0.05$ was considered statistically significant.

Results

NSAIDs Reduce Gastric Cancer Cell Proliferation. The human gastric carcinoma cell lines SGC7901 and MKN45 were used to test the effects of NSAIDs on proliferation and metastasis. The cell lines SGC7901/ADR and SGC7901/VCR,[28,31] which are resistant to adriamycin hydrochloride (ADR) and vincristine (VDR), respectively, were used as models for MDR. Cells were treated for 24 h with *R*-flur, ibuprofen, indomethacin and aspirin at a variety of concentrations, and MTT assays were performed to detect the effects of these

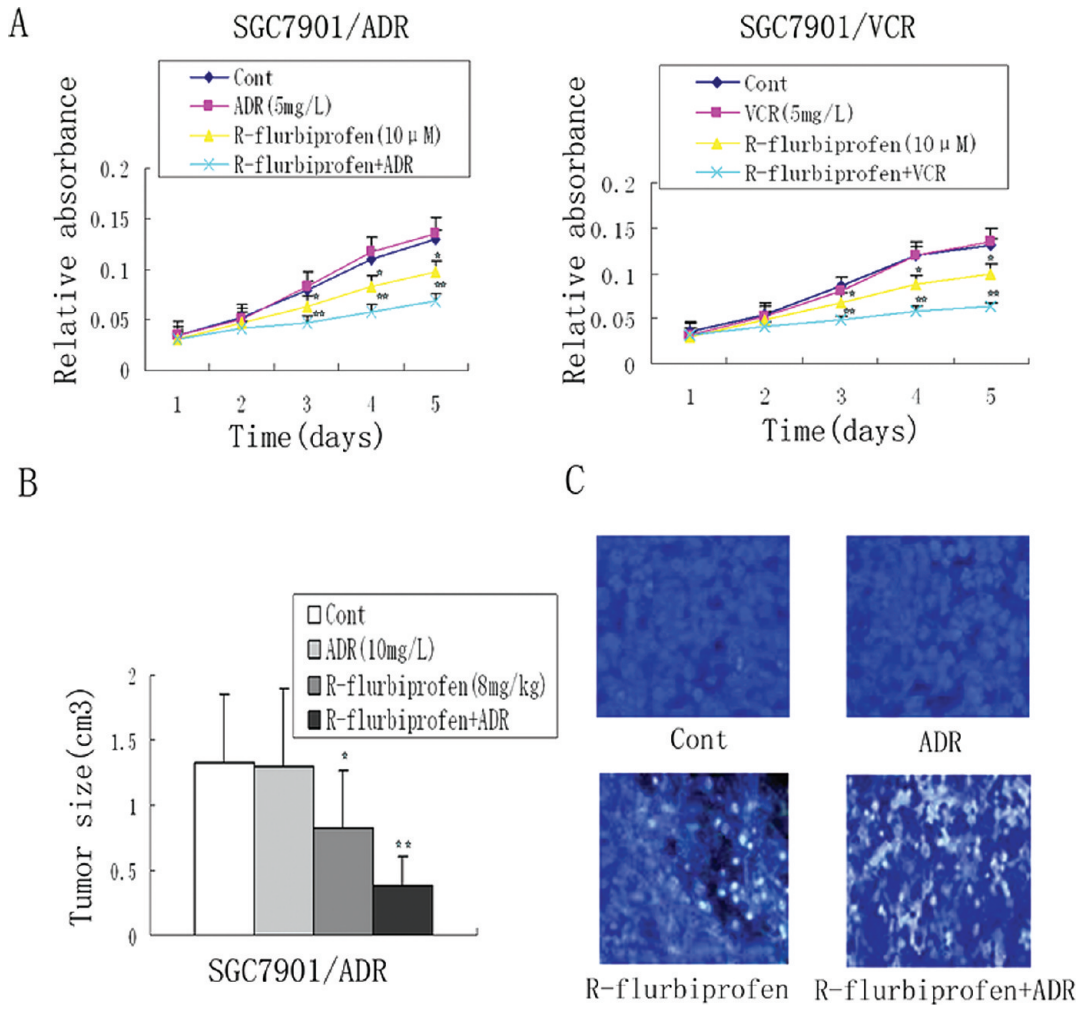

Figure 3. Reversal of MDR in SGC7901/ADR and SGC7901/VCR gastric cancer cells by *R*-flur. (A) Monolayer growth rates of cells were determined by MTT assays. Treatment was continuous over the 5 days. Values represent the mean (SEM) from at least three separate experiments. $^*p < 0.05$. (B) Reversal of MDR by *R*-flur for 8 mg/kg, at 30 days, in vivo, by reduction in tumor size (tumor size quantitation) after introduction of gastric cancer cells SGC7901/ADR in nude mice. (C) Hoechst assays of tumors from nude mice.

drugs on cell proliferation (Figure 1). All NSAIDs tested reduced proliferation by the MTT assay in both chemosensitive and MDR cell lines, but *R*-flur was the most effective at all concentrations.

Since *R*-flur cannot be acting through Cox inhibition, and previous studies showed that *R*-flur and other NSAIDs induce expression of the tumor suppressor $p75^{NTR}$ in several different cancer cell lines and cancer types,[17–19] we tested expression of $p75^{NTR}$ in gastric cancer cells treated with *R*-flur for 24 h. Western blots showed that *R*-flur showed a significant effect on $p75^{NTR}$ levels (Figure 2A), corresponding to an increase in $p75^{NTR}$ in both non-MDR and MDR strains (Figure 2B,C). The effect on $p75^{NTR}$ was at the transcript level, because PCR of a time-course of cDNA collected from the *R*-flur-treated MDR gastric cancer cells showed that $p75^{NTR}$ mRNA increased relative to the β-actin control (Figure 2D). Since gastric cancer cells express high levels of Cox-2,[21,22] we examined the effect of *R*-flur on Cox protein levels. Neither Cox-1 nor Cox-2 levels were affected by *R*-flur (Figure 2E). Induction of $p75^{NTR}$ by *R*-flur was also dose-dependent (Figure 2F). In summary, all NSAIDs tested increased $p75^{NTR}$ levels, and *R*-flur was the most effective, increasing $p75^{NTR}$ at both the mRNA and protein levels, in both non-MDR and MDR strains, in a dose- and time-dependent manner.

***R*-Flurbiprofen Reverses Multidrug Resistance in Gastric Cells in Vitro and in Vivo.** *R*-Flur treatment appeared to be effective at reducing cell proliferation in both drug-sensitive and drug-resistant gastric cancer cells. NSAIDs have been implicated in reversal of drug resistance in leukemia cells, by reducing expression of the efflux transporter *MRP1*,[14] so we investigated whether *R*-flur treatment increased the sensitivity of drug-resistant gastric cancer lines to ADR and VCR. SGC7901/ADR and SGC7901/VCR cell lines were completely resistant to the drugs used to select them. *R*-Flur reduced cell proliferation, and the combination of *R*-flur and ADR or VCR was more effective at inhibiting cell growth than either treatment alone (Figure 3A).This indicated that *R*-flur reduced the MDR of SGC7901 ADR- and VCR-resistant cells.

Table 1. Fluorescence Intensity of Intracellular ADR and ADR-Releasing Index of MDR Gastric Cancer Cells SGC7901/ADR and SGC7901/VCR[a]

R-flurbiprofen (μM)	accumulation	retention	releasing index
0	18.54 ± 6.81	4.15 ± 0.61	0.95 ± 0.096
2.5	20.78 ± 4.78	6.24 ± 1.21*	0.84 ± 0.061*
5	24.56 ± 5.88*	10.52 ± 2.78*	0.62 ± 0.078*
10	30.15 ± 7.25*	15.17 ± 4.35**	0.48 ± 0.035*
20	34.46 ± 8.15**	18.46 ± 6.25**	0.31 ± 0.032**
20 +ΔICD	21.55 ± 5.21	6.45 ± 0.57	0.88 ± 0.091
20 +p75si	19.23 ± 4.82	4.85 ± 0.63	0.91 ± 0.067

[a] For further information, see *Adriamycin Accumulation* in *Materials and Methods*. *$p < 0.05$ and **$p < 0.05$ vs control cells.

The reversal effects of *R*-flur on MDR of SGC7901/VCR were validated in vivo by treating tumors of SGC7901/ADR-resistant cells in nude mice. Quantitation of tumor sizes indicated that the cells were drug-resistant in vivo and *R*-flur reduced tumor size, but the most dramatic effects came from the combination of *R*-flur and ADR (Figure 3B). Hoechst staining of the tumors gave insights on the mechanism by which *R*-flur and ADR decreased tumor size. Very little difference was seen between the control and ADR1-treated tumors. The *R*-flur group showed that *R*-flur could significantly induce apoptosis of SGC7901/ADR tumors in nude mice, with even more significant effects in the *R*-flur + ADR group (Figure 3C). This suggested that *R*-flur could reverse the MDR of SGC7901/ADR. Fluorescence measurements of ADR uptake by SGC7901/ADR cells indicated that *R*-flur treatment indeed reversed the resistance of these cells to ADR (Table 1). *R*-Flur treatment reversed the MDR of these cells, as the sensitivity of these cells to other chemotherapeutic agents also increased, indicated by reduced IC_{50} values (Table 2).

***R*-Flurbiprofen Effects on Proliferation, Migration, MDR and Apoptosis Are ^{p75NTR}-Dependent.** To examine a mechanistic relationship between *R*-flur induction of $p75^{NTR}$ protein and its anticancer effects, the gastric cancer cell lines were transfected before *R*-flur treatment with a dominant-negative form of $p75^{NTR}$ (ΔICD) to antagonize $p75^{NTR}$ activity,[20,21] or $p75^{NTR}$ siRNA to prevent $p75^{NTR}$ protein expression. The ΔICD allele and the anti-p75 siRNA reduced or abolished the antiproliferative effect of *R*-flur on SGC7901 cells, when tested by three different assays: MTT assay of monolayer growth (Figure 4Aa), plate colony formation (Figure 4Ab) and soft agar colony formation (Figure 4Ac). Western blots confirmed the antagonistic effects of the ΔICD allele and specific siRNA, while a nonspecific control affected neither cell growth nor the increase in $p75^{NTR}$ levels. These results indicated that induction of $p75^{NTR}$ was necessary for *R*-flur effects on cell proliferation. The effects of *R*-flur on the MDR phenotype of SGC7901/ADR cells was also $p75^{NTR}$-dependent, as $p75^{NTR}$-reducing strategies inhibited the ability of *R*-flur to increase intracellular ADR accumulation (Table 1), and sensitivity to a panel of anticancer agents (Table 2).

In vivo, the size of SGC7901 cell tumors in nude mice was reduced in a dose-dependent manner by *R*-flur (Figure 4B). Immunohistochemistry on the tumors (Figure 4C) showed that *R*-flur treatment caused a dose-dependent induction of $p75^{NTR}$ expression in vivo. *R*-Flur also reduced the formation of microvessels in vivo, as shown by immunohistochemistry for factor VIII to visualize microvessel density in the tumors (Figure 4D). The immunohistochemistry suggested that *R*-flur not only induced apoptosis in vivo (Figure 3C) but also could inhibit tumor growth by inhibiting angiogenesis.

The antiproliferative effect of *R*-flur was at the cell cycle level, because it caused G1 cell cycle arrest in gastric cancer cells. This effect was also $p75^{NTR}$-dependent (Table 3). Apoptotic induction was measured as an increased percent of apoptotic cells, detected by Hoechst dye and flow cell cytometry. This effect was not seen with p75-diminishing treatments.

We also tested the effects of *R*-flur on the metastatic characteristics of gastric cancer cells. In vitro, *R*-flur had a dose-dependent inhibitory effect on MKN45 cell migration in a wound-healing assay (Figure 5A), as well as Transwell migration (Figure 5Ba) and invasion assays (Figure 5Bb). The antimetastatic effects of *R*-flur were reduced or eliminated in the absence of $p75^{NTR}$ induction (Figure 5B). In vivo, metastasis was evaluated by measuring metastasis to the liver and lungs (Figure 5C−F), of MKN45 cells injected into the tail veins of nude mice. H&E staining showed the

Table 2. IC_{50} (mg/L) Values of *R*-Flur-Treated Cells with Chemotherapeutic Drugs[a]

	IC_{50} (mg/L) (means ± SD)				
R-flurbiprofen (μM)	5-fluorouracil	cisplatin	mitomycin	adriamycin	vincristine
0	8.21 ± 1.02	11.13 ± 2.28	12.38 ± 1.69	7.78 ± 2.76	8.48 ± 2.45
2.5	7.15 ± 1.13	10.35 ± 2.09	10.26 ± 1.59*	6.22 ± 0.57	6.71 ± 0.77*
5	6.31 ± 2.02*	8.31 ± 1.65*	8.31 ± 0.68*	4.34 ± 0.48*	4.06 ± 0.56*
10	4.23 ± 0.78**	5.33 ± 0.98**	6.18 ± 0.68*	2.13 ± 0.36**	2.13 ± 0.24**
20	2.34 ± 0.29**	3.74 ± 0.69**	4.24 ± 0.58**	1.24 ± 0.28**	1.04 ± 0.16**
20 +ΔICD	7.29 ± 1.58	9.89 ± 3.11	10.02 ± 2.04	6.13 ± 1.56	6.88 ± 2.03
20 +p75si	7.42 ± 2.08	11.09 ± 2.81	12.01 ± 1.89	7.16 ± 2.16	7.92 ± 2.73

[a] The sensitivity of gastric cancer cells to chemotherapeutic drugs was evaluated using the colony-forming assay as described in *Materials and Methods*. The concentration of each drug that caused a 50% reduction in the number of colonies (IC_{50}) was calculated. Relative resistance = the IC_{50} value of the transfected gastric cells/the IC_{50} value of the nontransfected gastric cells. *$p < 0.05$ and **$p < 0.05$ vs control cells.

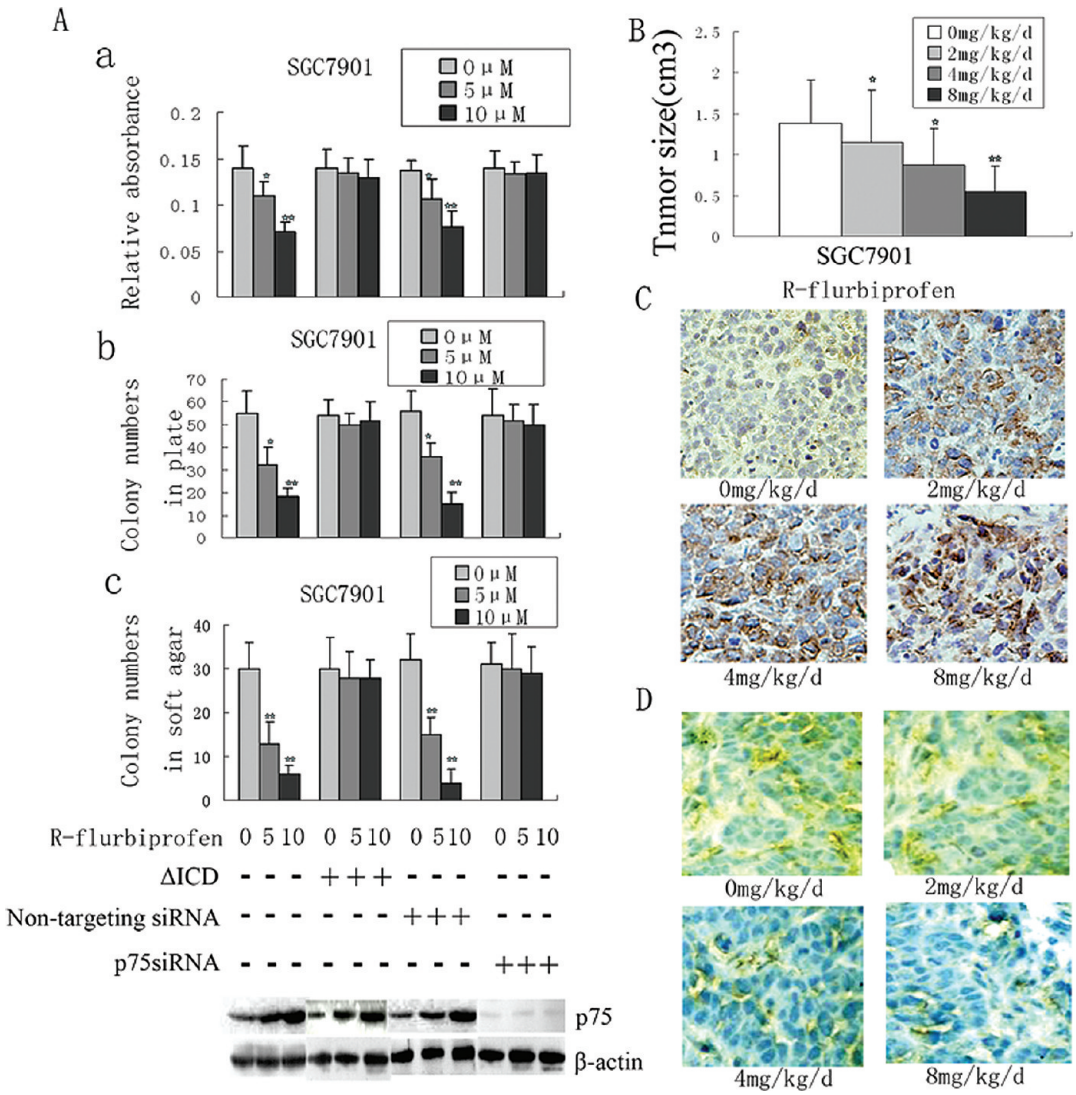

Figure 4. p75^{NTR}-dependence of *R*-flur effects on cell proliferation and tumorigenicity of gastric cancer cells. (A) (a) After treatmeant with *R*-flur at the indicated doses, monolayer cell growth rates were determined by MTT assays, for untreated cells, or cells transfected with a dominant-negative allele of p75NTR (ΔICD), control nontargeting siRNA, or anti-p75 siRNA. Values represent the mean (SEM) from at least three separate experiments. *$p < 0.05$. (b) Effect of *R*-flur on plate colony formation of SGC7901 cells, treated as in (a). Cells, treated as in (a) were placed in medium and incubated for 7 days. The number of foci >100 μm were counted. Values represent the mean (SEM) from at least three separate experiments, each conducted in triplicate. **$p = 0.000$. *$p < 0.01$. (c) Effect of *R*-flur on soft agar colony formation of SGC7901 cells. Cells treated as in (a) were placed in medium containing soft agar and incubated for 15 days. The number of foci >100 μm were counted. Values represent the mean (SEM) from at least three separate experiments, each conducted in triplicate. **$p = 0.000$. *$p < 0.01$. After treatment with *R*-flur at the indicated doses, the expression of p75^{NTR} was evaluated by Western blot. β-Actin was used as an internal control. (B) Effect of *R*-flur on tumorigenicity in nude mice of SGC7901 cells. Average tumor size was estimated by physical measurement of the excised tumor at the time of sacrifice. These tumors were verified as being gastric cancer by H&E staining. *$p < 0.01$. (C) Immunohistochemical staining of p75^{NTR} in gastric cancer tumors in nude mice, after 30 days of treatment with the indicated dose of *R*-flur. Monoclonal anti-p75^{NTR} antibodies were used to stain paraffin sections. (D) Immunohistochemical staining of factor VIII in tumor of gastric cancer in nude mice to detect microvessel density.

tumor structure and extent of liver and lung metastases according to the procedures briefly described below.

***R*-Flurbiprofen Induction of p^{75NTR} Affects Cell Cycle, MDR, Gene Regulation, Apoptotic and Metastatic Factors.** To investigate the cellular mechanisms of *R*-flur, the cell cycle proteins Cyclin D1 and CDK4, vascular epithelial growth factor (VEGF), the apoptosis factors Bcl-2 and Caspase3, and the MDR efflux transporter P-gp, the products of the *MDR1* gene were investigated after *R*-flur treatment. Consistent with the cell cycle arrest and apoptosis seen in Figure 4G,H, Western blots determined that Cyclin D1, CDK4 and Bcl-2 levels were reduced in both drug-sensitive SGC7901 and MNK45 cells, and drug-resistant SGC7901/VCR cells. Levels of the apoptosis

Table 3. The Effect of *R*-Flur on Cell Cycle Arrest and Apoptosis Induction in SGC7901[a]

%	0 μM	20 μM	ΔICD	p75siRNA
G1	51.81 ± 9.02	63.15 ± 8.76*	52.87 ± 7.79	50.29 ± 10.33
S	43.32 ± 6.57	31.9 ± 5.67*	40.72 ± 7.91	42.82 ± 6.73
apoptotic cells	6.28 ± 3.29	33.61 ± 5.29*	9.82 ± 4.19	9.98 ± 4.07

[a] *$p < 0.05$ vs control cells.

inducer Caspase3 were increased (Figure 6A,B). Consistent with the in vivo effects of *R*-flur on angiogenesis, VEGF levels were reduced. P-gp, the ABC transporter responsible for MDR, was also reduced, consistent with the MDR-reversing effects of *R*-flur seen in Figure 3. A molecular explanation of the antimigratory effects of *R*-flur is offered by the reduced levels of uPA and pro-MMP9, and increased levels of the matrix protease inhibitor TIMP1 after *R*-flur treatment for 24 h. (Figure 6B,C). All of these effects were eliminated by preventing $p75^{NTR}$ induction with the ΔICD dominant-negative allele or p75-specific siRNA (Figure 6A−C). Both the ΔICD allele and the specific siRNA rescued untreated expression of the cell cycle, apoptotic, metastatic, and MDR factors.

***R*-Flurbiprofen Induction of p^{75NTR} Requires the MAPK p38.** Inhibition of the p38 mitogen-activated protein kinase is known to increase sensitivity of MDR SGC7901cells to chemotherapeutic agents.[26] In addition, the p38 MAPK pathway could mediate *R*-flur or ibuprofen-induced mRNA stability of p75NTR[16] and mediate carprofen-induced p75NTR-dependent apoptosis in prostate cancer cells.[17] Therefore, we hypothesized that this signaling pathway might be involved in the *R*-flur mechanism. Activation of p38 by phosphorylation was measured using a phospho-p38-specific antibody. Indeed, *R*-flur increased phosphorylated p38 protein levels relative to total p38 and β-actin levels (Figure 6D). The increase in activated p38 increased over time with *R*-flur treatment (Figure 6E).

The dependence of the $p75^{NTR}$ response on activated p38 was tested using p38-specific siRNAs, and SB20219, a p38 specific inhibitor. Induction of p75*NTR* was dependent on p38 activation, as this effect of *R*-flur was lost with addition of 20 μM of SB20219. Specific anti-p38 siRNAs had less dramatic effects on the levels of phosphorylated p38 (Figure 6F), and correspondingly weaker effects on induction of $p75^{NTR}$ expression. In summary, p38 is activated, as measured by phosphorylation, by *R*-flur. The increase in p38 was required for induction of p75 expression, indicating a possible mechanism by which *R*-flur exerts anticancerous effects.

Discussion

In vitro results with prostate and other cancer cells suggested that long-term NSAID treatment could be effective at slowing or halting cancerous growth.[7,18] In this study, the NSAIDs *R*-flur, ibuprofen, indomethacin and aspirin inhibited gastric cancer cell proliferation in vitro, as measured by MTT assays, and activated p38, as measured by reactivity with antiphospho-p38 antibody. Consistent with results seen in the bladder cancer T24 cell line, *R*-flur was the most effective of the tested NSAIDs at increasing $p75^{NTR}$ expression.[18] *R*-flur was also the most effective at antiproliferation, showing dose- and time-dependent inhibition of gastric cancer cell growth by MTT assay, soft agar colony formation, plate colony formation and flow-cytometry cell cycle evaluation. *R*-Flur is not a Cox-2 inhibitor, so the NSAID effects on gastric cancer cells appear to be Cox-2-independent. Consistent with previous results that $p75^{NTR}$ levels are low in metastatic gastric primary and cultured cancer cells compared to normal cells,[20,21] the effects of *R*-flur corresponded to upregulation of $p75^{NTR}$ at the mRNA and protein levels. *R*-Flur treatment caused dose- and time-dependent increases in $p75^{NTR}$ levels, but did not affect levels of Cox-2.

Several results suggest pathways to be investigated in future mechanistic studies on the broad range of anticarcinogenic effects generated by *R*-flur. In vivo, *R*-flur reduced tumor size and induced apoptotic characteristics, as well as causing expression of $p75^{NTR}$ in a dose-dependent manner, in SGC7901 cell tumors in nude mice. Consistent with our previous analysis,[20] in vitro results with the same cell line showed a $p75^{NTR}$-dependent decrease in expression of Cyclin D1. In addition, $p75^{NTR}$-dependent changes in CDK4 were observed, as well as G1 cell cycle arrest. Previously, phosphorylation to inactivate the retinoblastoma tumor suppressor was associated with $p75^{NTR}$,[20] suggesting a mechanism for the in vivo results on tumors and the in vitro results on cell cycle progression. In vitro, Bcl-2 expression decreased, while Caspase3 levels increased. In vivo, *R*-flur inhibited angiogenesis and metastasis, and in vitro results suggested target pathways for these effects. For example, VEGF expression was decreased by *R*-flur, consistent with reduced microvessel density in tumors. Recently, X. Chi et al. reported that flurbiprofen was also demonstrated to downregulate MMP-9 expression in human colon cancer HT29 cells by induction of the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), an inhibitor of MMP-9.[32] In confirmation of our earlier study,[21] levels of matrix metalloprotease MMP9 and its activator uPA were reduced, and TIMP1 levels increased in human gastric cell line. ERK, p38 and JNK have been shown to get involved in (Ac)(5)GP-induced glioma cell apoptosis and elevation of p75. Elevation of p75 mRNA induced by (Ac)(5)GP was decreased by treatment of PD98059 (ERK-specific inhibitor), SB203580 (p38 MAPK inhibitor)

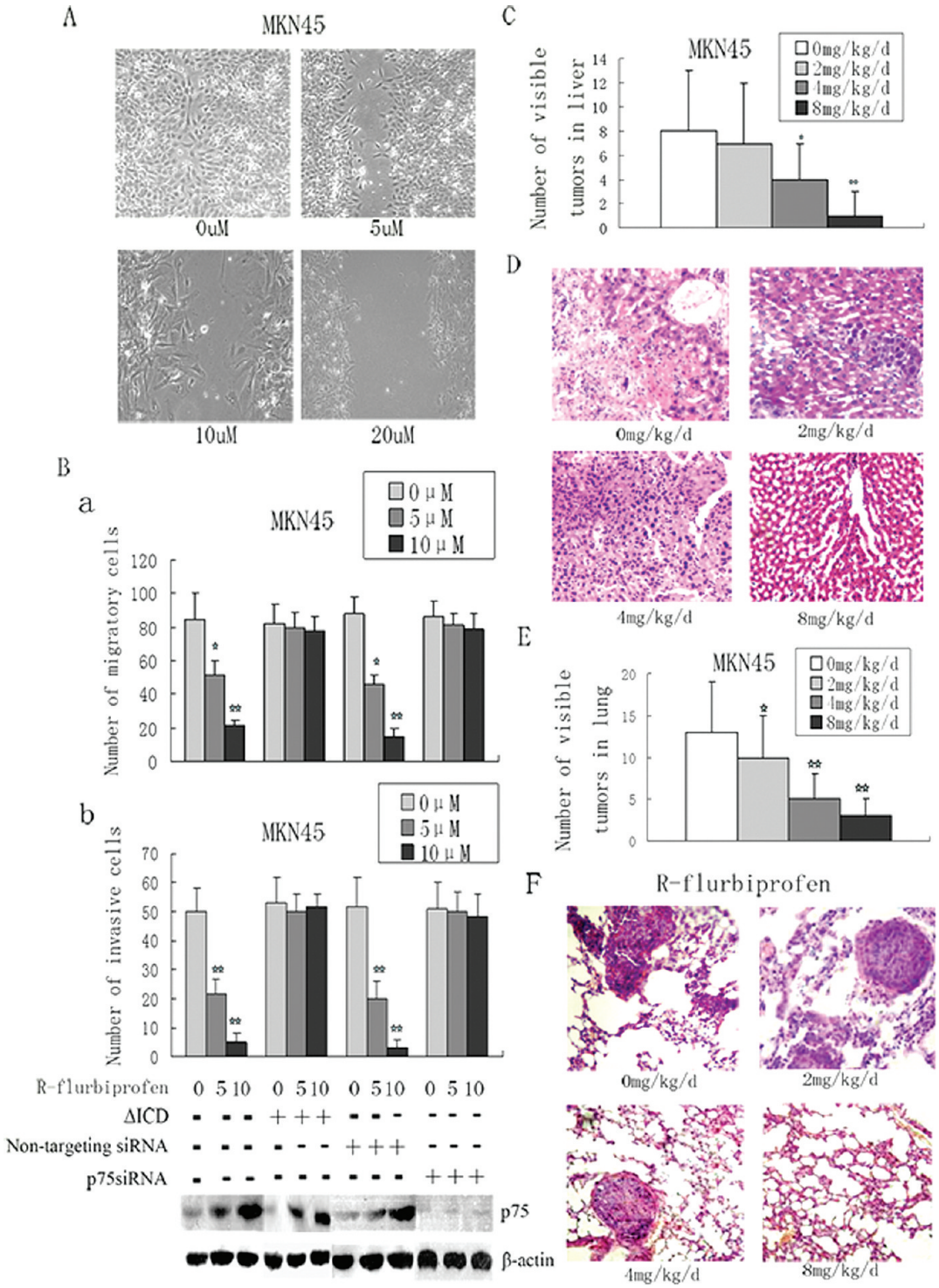

Figure 5. p75 dependence of *R*-flur effects on migration, invasion and metastasis of gastric cancer cells MKN45. (A) Migratory ability was evaluated by wound healing assay with cells treated with the indicated dose of *R*-flur for 12 h. (B) (a) Migratory ability was evaluated by counting untreated cells, or cells transfected with a dominant-negative allele of $p75^{NTR}$ (ΔICD), control nontargeting siRNA, or anti-p75 siRNA, in a 8 μm pore Transwell assay after treatment with the indicated dose of *R*-flur for 24 h. $*p < 0.05$ and $**p < 0.01$. (b) Invasive ability was evaluated by the Transwell assay in (a) with Matrigel, and cells treated as in (a). $*p < 0.05$ and $**p < 0.01$. After treatment with *R*-flur at different doses, $p75^{NTR}$ levels were evaluated by Western blot. β-Actin was used as an internal control. (C and D) Mice were injected with 1×10^6 cells through the tail vein. Both experimental and control groups contained 10 mice. Four weeks later, the mice were sacrificed and liver tissues observed visually. The number of visible tumors on the lung surface were counted (E). (F) Serial sections of lung tissues from mice treated with the indicated dose of *R*-flur for 45 days were dyed with H&E and observed under a light microscope.

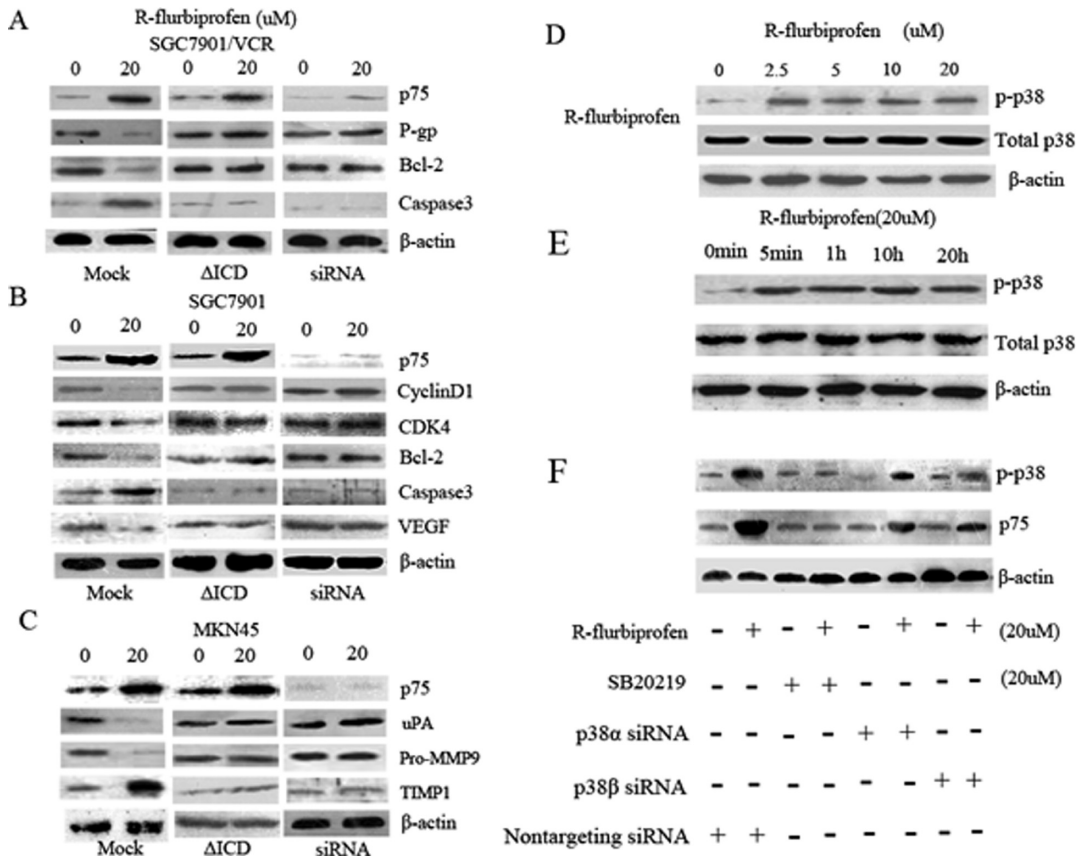

Figure 6. The effect of *R*-flur on proliferation and metastasis effectors. All gene expression levels were quantitatively analyzed and expressed as the ratios over β-actin. For all blots, ss-actin was used as an internal control. (A) The expression of P-gp, Bcl-2 and Caspase3 in SGC7901/VCR was evaluated by Western blot after treatment with *R*-flur at the indicated concentration, for 48 h before and after transient transfection with a dominant-negative allele of $p75^{NTR}$ (ΔICD) or anti-p75 siRNA. (B) The expression of Cyclin D1, CDK4, Bcl-2, Caspase3 and VEGF in SGC7901 was evaluated by Western blot as in (A). (C) The expression of uPA, pro-MMP9, TIMP1 was evaluated by Western blot after treatment as in (A). (D) The levels of p-p38 and total p38 were evaluated by Western blot after treatment with *R*-flur at the indicated dosages. (E) The expression of p-p38 and total p38 were evaluated by Western blot after treatment with *R*-flur for the indicated times. (F) The activation of p38 and expression of p75 were evaluated by Western blot with and without *R*-flur treatment, and with or without treatment with the specific p-p38 inhibitor SB20219, or siRNA against p38a siRNA or p38b siRNA.

and SP600125 (JNK inhibitor), indicating possible transcription regulation of p75 by MAPKs.[33] That was consistent with our data that induction of $p75^{NTR}$ is partially dependent on the p38 pathway. These results could mechanically implicate that the reduced migration in vitro and metastasis in vivo after *R*-flur treatment, and these phenomena were also $p75^{NTR}$-dependent.

R-Flur induction of the $p75^{NTR}$ protein correlated with its ability to reverse the MDR of gastric cancer drug-resistant sublines, and with the reduced expression of P-gp, a multidrug efflux transporter in the ATP-binding cassette family that is upregulated in MDR.[34] Previous studies noted that the NSAIDs were less effective at reducing cell proliferation and inducing $p75^{NTR}$ expression in drug-resistant cells.[18] However, in the gastric cancer model used here, cell growth of SCG7901 cells that were completely resistant to ADR or VCR was reduced in the presence of ADR or VCR by the addition of *R*-flur. This suggested that *R*-flur affected both cell division and MDR. Cotreatment of *R*-flur with a panel of anticancer agents decreased their IC_{50} in a p75-dependent manner, showing this was a general effect. Since

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overexpression of the *MDR1* gene, which encodes the ABC transporter P-gp, is responsible for MDR in SGC7901/VCR cells,[35] we examined if *R*-flur treatment affected the expression of P-gp. *R*-Flur reduced P-gp levels, suggesting a mechanism by which *R*-flur can help sensitize cells to anticancer therapeutic agents. This decrease required $p75^{NTR}$ induction. Other NSAIDs have also reduced MDR. Zatelli et al.,[36] working with a medullary thyroid carcinoma cell line that is resistant to the anticancer agent doxorubicin, showed that the NSAID rofecoxib increased the effectiveness of doxorubicin to the same extent as the P-gp inhibitor verapamil. The effects were independent of PGE_2, a Cox-2 product. Kang et al.[2] showed Cox-2 and PGE_2-independent downregulation of *MRP1* in human lung cancer cell lines. The specific Cox-2 inhibitor celecoxib was shown to reduce P-gp levels in a variety of MDR cell lines,[8] including the MDR gastric cancer cell lines used in this study.[23] It also downregulated other ABC-transporters in oral squamous cell carcinoma cells.[10] NSAIDs have been shown to reduce the activity of MRP2 and MPR4 efflux transporters.[37] Combined with the results of this study, in which a non-Cox-inhibiting NSAID decreased P-gp protein levels and increased chemosensitivity of MDR gastric cancer cells, we hypothesize that NSAIDs universally counteract MDR by downregulating ABC transporter levels, through a pathway that does not involve COX-2. NSAIDs may work by modulation of the transcription NF-kB, as suggested by Wijngaarden et al.[38] in a study of cultured breast cancer cells, showing that the Cox-2 inhibitor celecoxib affects accumulation of the chemotherapeutic drug doxorubicin while also regulating the intracellular localization of NF-kB. $p75^{NTR}$ suppresses NF-kB in gastric cancer cells,[21] and preliminary data in these cells suggests that *R*-flur can mediate this suppression in a Cox-2-independent manner (Jin et al., unpublished).

Taken together, these results indicate a complex cellular response to NSAID treatment, which involves the MAPK kinase p38 and the tumor suppressor $p75^{NTR}$, and affects gene expression and protein levels for MDR, cell cycle, metastatic and apoptotic factors. Thus, NSAIDs have potential for the treatment of MDR, proliferation and metastasis of gastric carcinoma. *R*-Flur is the non-Cox-inhibiting enantiomer of the 2-arylpropionic NSAID flurbiprofen. At clinically relevant concentrations, bioconversion to the Cox-inhibiting *S*-enantiomer is inefficient, so *R*-flur might not cause the gastric irritation associated with other NSAIDs. Finally, $p75^{NTR}$ appears to be an available therapeutic target for reversing the malignant phenotype of gastric cancer.

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