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A mechanistic study of the proapoptotic effect of tolfenamic acid; involvement of NF-κB activation

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Running head: Tolfenamic acid induces apoptosis via NF-kB

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Abstract

Recent studies demonstrate that tolfenamic acid (TA) induces apoptosis and suppresses the development and progression of several types of cancers. However, the underlying mechanisms are complex and remain to be fully elucidated. NF-κB plays a critical role in inflammation, cancer development, and progression. Although non-steroidal anti-inflammatory drugs (NSAIDs) modulate NF-κB signaling pathway in different ways, the link between NF-κB and TA-induced apoptosis of colorectal cancer cells has yet to be thoroughly investigated. In the present study, we examined the effects of TA on the NF-κB pathway and apoptosis. TA activated NF-κB transcriptional activity and binding affinity of NF-κB to DNA. TA-induced NF-κB activation was mediated by an increased phosphorylation and proteosomal degradation of IκB-α and subsequent p65 nuclear translocation. We also observed that TA stabilized p65 and increased nuclear accumulation via an increase of p65 phosphorylation at Ser276 residue, which was mediated by p38 MAPK and ERK. The knockout of p53 blocked TA-induced transcriptional activation of NF-κB, but not the p65 nuclear accumulation. TA increased transcriptional activity of p53 and the binding affinity of p53 with p65, which are mediated by p38 MAPK and ERK-stimulated Ser276 phosphorylation. TA-induced apoptosis was ameliorated by the knockout of p65 and p53 and the point mutation of p65 at Ser276 residue. We demonstrate a novel molecular mechanism by which TA induced the NF-κB and apoptosis in human colorectal cancer cells.

Keywords: apoptosis; colorectal cancer; NF-κB; tolfenamic acid

Introduction

Colorectal cancer is the third leading cause of cancer-related morbidity and mortality in both males and females in the United States (1). Chemoprevention is an inherently attractive approach to treat this disease, and epidemiological studies have revealed that there is an inverse correlation between the long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) and colorectal cancer incidence and mortality (2). It is estimated that there is a 40-50% reduction in the relative risk of colorectal cancer in subjects taking NSAIDs (3). Thus, the use of NSAIDs has been regarded as an effective chemopreventive strategy for colorectal cancer (4). The proposed mechanisms of NSAIDs' anti-cancer activity include increased cell cycle arrest and apoptosis (5-7), and inhibition of angiogenesis, invasion and metastasis (8,9), and these studies would provide criteria for rationally designing novel agents for chemoprevention and therapy.

Tolfenamic acid (TA), one of the fenamic acid-derived NSAIDs, has been used for the treatment of migraines (10). Recent evidences show that TA possesses anti-cancer activity in several cancers such as pancreatic cancer (11), head and neck cancer (12), and colorectal cancer (13,14). Like other NSAIDs, TA inhibits cyclooxygenase (COX) and prostaglandin biosynthesis, which contributes to the anti-cancer activity of NSAIDs (15). However, anti-cancer activity of NSAIDs is associated with COX-independent mechanisms and other molecular targets (16-19).

One of the potential molecular targets for the anti-cancer activity of NSAIDs is NF-kappaB (NF-κB) signal transduction (20-24). In the absence of stimuli, the NF-κB transcription factor formed by p50 and p65 is normally sequestered in the cytoplasm by binding to an inhibitory protein, IκB-α. In response to the cellular stimulation of NF-κB pathway, IκB-α is phosphorylated by the IκB-α kinase (IKK), ubiquitinated and then degraded by the 26S proteasome. Dissociation from IκB-α results in a release of NF-κB from the cytoplasmic NF-κB–IκBα complex and allows NF-κB to translocate to the nucleus. The translocated NF-κB contributes to the co-ordinated transcription of several genes involved in inflammation, cell proliferation, and apoptosis (25).

The roles of NF-κB in cancer progression and anti-cancer therapeutics are complex, as there are evidences to suggest that the NF-κB activation is associated with the increased survival of cancer cells and resistance to chemotherapy, showing that inhibition of NF-κB activity is regarded as the target for anti-cancer therapy (26,27). However, NF-κB activation promotes a proapoptotic response under different circumstances (28,29). For example, the retinoid-related compounds, 3-Cl-AHPC and CD437 induce apoptosis by activation of NF-κB transcriptional activity in prostate cancer cells (30,31). Another evidences supporting a proapoptotic role for NF-κB activation in cancer chemotherapy is that aspirin and betulinic acid require activation of NF-κB to induce apoptosis in cancer cells (32,33). Therefore, it is suggested that NF-κB activation may be a promising cancer preventive and therapeutic target.

In this study, we aimed to elucidate the role of NF-κB in TA-mediated apoptosis and we demonstrated that TA induces nuclear accumulation of NF-κB p65 and subsequent activation of NF-κB transcriptional activity in p53-dependent manner in colorectal cancer cells. Furthermore, NF-κB activation is required for TA-induced apoptosis. These studies identify an intrinsic activity of TA that is highly relevant to colon cancer chemoprevention.

Materials and methods

Chemicals and Materials

Cell culture media, Dulbecco's Modified Eagle medium (DMEM), RPMI1640, Ham's F-12 and McCoy's 5A were purchased from Lonza (Walkersville, MD), and tolfenamic acid (TA) was purchased from Cayman Chemical (Ann Arbor, Michigan). SB203580 and PD98059 were purchased from Calbiochem (San Diego, CA) and recombinant human TNF-α was purchased from R&D systems Inc. (Minneapolis, MN). Antibodies against p65 (D14E12, #8242), IκB-α (L35A5, #4814), phospho-IκB-α (Ser32) (14D4, #2859), PARP (#9542),TBP (#8515), FasL (#4273) and β-actin (#5125) were purchased from Cell Signaling (Danvers, MA). p53 (Bp53-12, sc-263) and p-p65 (Ser276) (ab30623) antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and

Abcam (Cambridge, MA), respectively. Control- (#6201) and p65-siRNA (#6261) were purchased from Cell Signaling. p53-siRNA (sc-29453) was purchased from Santa Cruz Biotechnology Inc. All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Cell culture and treatment

Human colorectal cancer cell lines, HCT116, SW480, LoVo, and Caco-2 cells were purchased from American Type Culture Collection (Manassas, VA) and grown in McCoy's 5A (for HCT116 cells), RPMI1640 (for SW480 cells), Ham's F-12 (for LoVo cells) and Dulbecco's modified Eagle medium (DMEM) (for Caco-2 cells) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were maintained at 37 ^{o}C under a humidified atmosphere of 5% CO_2. TA was dissolved in dimethyl sulfoxide (DMSO) and then treated to cells. DMSO was used as a vehicle and the final DMSO concentration was not exceeded 0.1% (v/v).

Expression vectors

Wild type EGFP-p65 expression vector (WT) was kindly provided from Dr. Warner Greene (J David Gladstone Institute). Point mutation of (Ser276Gly) of EGFP-p65 (MT) was constructed from wild type EGFP-p65 using the QuikChange II mutagenesis kit (Stratagene, La Jolla, CA) with primers as followed: forward 5'-ctgcggcggcctgccgaccggg-3' and reverse 5'-cccggtcggcaggccgccgcag-3'.

Isolation of cytosol and nucleus fraction

Cytosol and nuclear fractions were prepared according to the manufacturer's protocols of a nuclear extract kit (Active Motif, Carlsbad, CA). Briefly, after washing with ice-cold PBS containing phosphatase inhibitors, cells were harvested with 1×hypotonic buffer containing detergent and then incubated at 4 ^{o}C for 15 min. The supernatants (cytoplasmic fraction) were collected after centrifugation at 14,000 × g for 1 min at 4°C, and stored at -80 ^{o}C. Nuclear fractions were collected by suspending nuclear pellet with lysis buffer at 4 ^{o}C for 30 min and centrifugation at 14,000 × g for 10 min at 4 ^{o}C.

SDS-PAGE and Western blot

Cells were washed with 1 × phosphate-buffered saline (PBS), centrifuged at 12,000 × g for 10 min at 4 ^{o}C and cell pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Sigma Aldrich). The cell suspension was centrifuged at 12,000 × g for 10 min at 4°C. Protein content was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). The proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes and blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1h at room temperature. Membranes were probed with specific primary antibodies in 5% nonfat dry milk at 4 °C overnight and then with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature. Chemiluminescence was detected with Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL) and visualized by ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA).

Immunoprecipitation

HCT116 cells were lysed in immunoprecipitation lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) supplemented with protease inhibitor cocktail (Sigma Aldrich) and phenylmethanesulfonylfluoride (PMSF, Sigma Aldrich). The cell suspension was centrifuged at 12,000 × g for 20 min at 4°C. Protein content was measured by the bicinchoninic acid (BCA) protein assay (Pierce). A total of 500 µg of pre-cleared cell lysates were incubated with anti-p65 monoclonal antibody, normal rabbit IgG (Santa Cruze Biotechnology) and Protein A/G PLUS-Agarose (Santa Cruze Biotechnology), and rotated at 4°C overnight. The pellets were washed five times with lysis buffer and boiled in 2 × loading buffer and subjected to Western blot using an anti-p65 or anti-p53 antibody.

Immunofluorescence

HCT116 cells grown on sterile coverslips were transfected with expression vectors containing wild type and Ser276Gly mutated-p65 constructs. After 24 h, cells were treated with TA for the additional 24 h. Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with PBS containing 0.5% Triton X-100 for 5 min and washed three times with PBS. After several washes, Cells were mounted with ProLong Gold antifade reagent containing DAPI (Invitrogen, Grand Island, NY) and images were acquired on fluorescent microscopy (Nikon Eclipse Ti-U, Nikon Instruments Inc., Melville, NY) at 20 × total magnification.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed according to the manufacturer's protocol of LightShift Chemiluminescent EMSA kit (Thermo Scientific). Biotin-labeled NF-κB-specific oligonucleotides 5'-AGTTGAGGGGACTTTCCCAGGC-3' (sense) and 5'-GCCTGGGAAAGTCCCCTCAACT-3' (antisense) were used for EMSA. Binding reactions were performed for 30 min at the room temperature in 20 µl buffer (1×binding buffer, 2.5% glycerol, 5 mM $MgCl_2$, 0.05% NP-40) containing 5 µg nuclear extract protein, 50 ng poly(dI:dC) and 1 µM biotin-labeled NF-κB-specific oligonucleotides. Binding complexes were resolved by electrophoresis in vertical nondenaturing 6% polyacrylamide gels using 0.5×TBE as running buffer and then transferred to nylon membrane. After UV-crosslink, the membrane was blocked with blocking buffer for 15 min and then incubated with conjugate/blocking buffer solution containing stabilized streptavidin-horseradish peroxidase conjugate for 15 min. After washing, the membrane was incubated with substrate equilibration buffer for 5 min. Chemiluminescence was detected with working solution containing luminol/enhancer solution and stable peroxide solution and visualized by ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA).

Transient transfection and luciferase assay

Transient transfection for luciferase plasmids was performed using PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD) according to the manufacturer's instruction. Briefly, the cells were plated in 12-well plates at the

concentration of 2×10^5 cells/well and incubated overnight. Then, plasmid mixtures containing 1 μg of luciferase plasmid and 0.1 μg of *pRL-null* vector were transfected for 24 h at 37 ^{o}C under a humidified atmosphere of 5% CO_2. The transfected cells exposed to TA or TNF-α. The cells were harvested in 1 × luciferase lysis buffer, and luciferase activity was measured and normalized to the *pRL-null* luciferase activity using a dual-luciferase assay kit (Promega, Madison, WI).

Transfection of small interference RNA (siRNA)

The cells were plated in 6-well plates and incubated overnight. HCT116 cells were transfected with control siRNA and p65 siRNA for 48 h at a concentration of 100 nM using TransIT-TKO transfection reagent (Mirus Corp., Madison, WI). LoVo cells were transfected with control siRNA, p53 siRNA and p65 siRNA for 48 h at concentration of 100 nM using LoVo Transfection Kit (Altogen Biosystems, Las Vegas, NV) according to the manufacturer's instruction. Then the cells were treated with 50 μM of TA for 24 h.

Cell death assay

Cell death was performed using Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction. Briefly, LoVo cells were plated in 12-well plate. After 24 h, cells were transfected with control, p53 and p65 siRNA for 48 h, and then treated with TA for the additional 24 h. After TA treatment, the cytosol was prepared using Nuclear Extract Kit (Active Motif). Cytosolic extracts, immunoreagent containing anti-histone-biotin, and anti-DNA-POD were added to microplate well and incubated for 2 h under shaking. After washing, the ABTS solution was added to each well for 20 min and then the ABTS stop solution was added. The absorbance was recorded at 405 nm and 490 nm in an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc.).

Statistical analysis

A statistical analysis was performed with the Student's unpaired *t*-test, with statistical significance set at * $P < 0.05$.

Results

Effect of NSAIDs and dietary phytochemicals on cell growth and apoptosis

There are a number of evidences indicating that NSAIDs and dietary phytochemicals show anti-cancer activity in human colorectal cancer (4,34). To observe which NSAIDs and dietary phytochemicals effectively induce cell growth arrest and apoptosis, HCT116 cells were treated with 50 μM of various NSAIDs (tolfenamic acid, aspirin, diclofenac, SC-560 and celecoxib), and dietary phytochemicals (EGCG, resveratrol and genistein). As shown in Figure 1A, a significant cell growth arrest was observed in the cells treated with TA, diclofenac, celecoxib, EGCG, resveratrol and genistein. Apoptosis was also induced by TA, EGCG, resveratrol and genistein (Figure 1B). Among NSAIDs and dietary phytochemicals tested, TA showed the strongest growth arrest and pro-apoptotic activity among all other NSAIDs and phytochemicals tested. Thus, we chose TA for the further studies.

TA increases nuclear p65 level and transcriptional activity of NF-κB in human colorectal cancer cells

There is a growing body of evidence that NF-κB pathway could be a target of several chemotherapeutic agents. To test if TA affects NF-κB pathway, we measured transcriptional activity of NF-κB in HCT116 cells transfected with *NF-κB Luc* plasmid. As shown in Figure 2A, TA treatment resulted in a dose-dependent increase of NF-κB transcriptional activity by 1.8- and 3.4-fold at 30 and 50 μM, respectively. We also used TNF-α as a control, showing activated NF-κB transcriptional activity (Figure 2B). Since NF-κB transcriptional activity is associated with nuclear translocation of NF-κB p65 and subsequent DNA-binding of NF-κB, we performed Western blot for p65 in HCT116 cells treated with TA or TNF-α. Nuclear translocation of p65 was induced by TA and TNF-α in HCT116 (Figure 2C and 2D). Time course experiment showed that induction of p65 nuclear translocation by TA occurred after 6h stimulation (Figure 2E). In contrast to TA, TNF-α-mediated nuclear translocation of p65 occurred rapidly (Figure 2F). To test

whether TA- or TNF-α-induced p65 nuclear translocation correlates to NF-κB DNA-binding activity, we performed the electrophoretic mobility shift assay (EMSA) using nuclear extracts of HCT116 cells treated with TA or TNF-α. TA and TNF-α resulted in an increase of NF-κB DNA binding (Figure 2G). Overall, these data indicate that TA enhances nuclear translocation of p65, binding of p65 to DNA and transcriptional activity of NF-κB in human colorectal cancer cells.

TA-induced NF-κB activation requires IκB-α-dependent nuclear translocation of p65

IκB-α degradation is essential for nuclear translocation and subsequent transcriptional activation of NF-κB. Thus, we investigated whether NF-κB activation by TA is associated with IκB-α degradation. As shown in Figure 3A, TA treatment decreased the IκB-α level in HCT116 cells treated with 30 and 50 μM of TA and downregulation of IκB-α level was also observed in LoVo cells treated with TA (data not shown). A time course experiment further showed that IκB-α began to decrease markedly at 24 h after TA treatment in HCT116 cells. However, TNF-α mediated decrease of IκB-α level are very rapid in HCT116 cells (Figure 3B), suggesting that the mechanisms of TA- or TNF-α-mediated IκB-α degradation might not be same.

IκB-α is rapidly degraded by 26S proteasome after phosphorylation at serine 32 and serine 36 by IκB kinase complex (25). Thus, we tested whether the decrease of IκB-α protein by TA was mediated by proteosomal degradation. Pretreatment with MG-132 diminished TA-mediated degradation of IκB-α by inhibiting TA-induced IκB-α phosphorylation (Figure 3C) and ameliorated TA-induced p65 translocation (Figure 3D). In addition, inhibition of IκB-α degradation using a specific inhibitor, BAY 11-7085 attenuated p65 nuclear translocation mediated by TA (Figure 3E). In conclusion, these data indicate that TA-induced NF-κB activation might be associated with proteosomal degradation of IκB-α and subsequent nuclear translocation of p65.

TA stabilizes and increases nuclear p65 via p38 MAPK- and ERK-dependent phosphorylation at Ser276 residue

Interestingly, our findings indicated that TA-stimulated nuclear translocation of p65 is much more prominent than the reduction of cytoplasmic p65 by TA (Figure 2C and 2D). Moreover, the time point to increase nuclear p65 (6 hour after TA treatment) is earlier than that to start IκB-α degradation (24 hour after TA treatment) (Figure 2E and Figure 3B). Thus, we hypothesize that TA-induced increase of NF-κB in nucleus could be consequence of p65 nuclear accumulation. To address this question, we firstly investigated the effect of TA on degradation of exogenously expressed EGFP-p65 in HCT116 cells. As shown in Figure 4A, TA treatment increased both cytoplasmic and nucleus level of EGFP-p65 in a time-dependent manner. This result raised the possibility that TA may increase nuclear p65 protein by preventing p65 degradation.

According to a recent study (35), phosphorylation of p65 at Ser276 prevents degradation of p65 from the ubiquitin-proteasome machinery and subsequently induces transactivation of p65. To determine whether TA-mediated inhibition of p65 degradation is a result of Ser276 phosphorylation in p65, we investigated the effect of TA on Ser276 phosphorylation in HCT116 cells. As a result, p65 phosphorylation (Ser276) increased in both total cell lysate and nuclear fraction in the cells treated with TA (Figure 4B). We also observed that point mutation of Serine to glycine (Ser276Gly) diminished TA-mediated nucleus accumulation of p65 compared to HCT116 cells transfected wild type-p65 (Figure 4C). To verify the Western blot data, we used immunofluorescence imaging to directly visualize localization of EGFP after TA treatment in cells transfected with an expression vector encoding EGFP-wild type p65 (WT) or EGFP- Ser276Gly p65 (MT). As shown in Figure 4D, TA treatment increased the nuclear staining of GFP in wild type p65 transfected cells, however it was partially ameliorated in the Ser276Gly p65 transfected cells.

Since p65 phosphorylation is associated with p38 MAPK and ERK pathway (36,37), we tested if selective inhibitors of these pathways diminish TA-induced p65 phosphorylation and nuclear translocation. As shown in Figure 4E, pretreatment of SB203580 and PD98059 diminished TA-mediated phosphorylation of p65 (Ser276). In addition, to test the possibility that p38 MAPK and ERK-mediated p65 phosphorylation is associated with IκB-α-mediated NF-κB translocation, we tested if presence of

selective inhibitors for p38MAPK and ERK affect TA-induced IκB-α degradation (Figure 4F). The result showed that TA-induced p65 phosphorylation and protein stability is independent with IκB-α degradation by IκB-α phosphorylation and NF-κB translocation.

In conclusion, these results demonstrate that TA increased nuclear stability of p65 through p38 and ERK-dependent Ser276 phosphorylation, which contributes at least in part to increase of p65 accumulation in nucleus and transcriptional activation.

TA-induced activation of NF-κB is dependent on p53

A growing body of evidence suggests that p53 induces NF-κB activation (38,39). Therefore, to determine whether p53 is associated with TA-stimulated activation of NF-κB, we compared the effects of TA on NF-κB transcriptional activity and nuclear accumulation of p65 in HCT116 and HCT116 p53 null ($HCT116^{p53-/-}$) cells. As shown in Figure 5A (upper panel), TA treatment increased luciferase activity of NF-κB in dose-dependent manner in p53 wild type HCT116, but not in $HCT116^{p53-/-}$ cells, indicating that TA-mediated activation of NF-κB is dependent on p53. However, the knockout of p53 did not affect the amount of nuclear p65 (Figure 5A, lower panel), implying that TA-induced nuclear accumulation of p65 is not mediated by p53 status.

To further confirm the p53-dependency on TA-stimulated NF-κB transcriptional activity and nuclear accumulation of p65, we also observed the effect of TA on p65 nuclear accumulation and NF-κB transcriptional activity in different human colorectal cancer cells including LoVo (p53 wild type), SW480 (p53 mutant), and CaCo-2 (p53 null) cells. As a result, TA increased nuclear accumulation of p65 in all other colorectal cancer cell lines (Figure 5B, 5C and 5D, lower panel), while TA-induced NF-κB transcriptional activation was observed only in p53-wild type cell line (LoVo), but not in p53-mutant (SW480) or p53-null (CaCO-2) cell lines (Figure 5B, 5C and 5D, upper panel). To verify p53-dependent transcriptional activation of NF-κB, LoVo cells (p53 wild type) were knock-downed using p53 siRNA and transcriptional activity of NF-κB was measured. As shown in Figure 5B (upper panel), p53-knockdown decreased TA-stimulated NF-κB transcriptional activity. These results strongly indicate that p53 is necessary for TA-stimulated transcriptional activation of NF-κB, but not in TA-mediated

p65 accumulation. On the other hand, we observed that p53 did affect neither TNFα-induced transcriptional activity of NF-κB and p65 accumulation (data not shown), confirming that mechanisms of TA- or TNF-α-mediated NF-κB activation are different.

TA increases p53 transcriptional activity and binding affinity of p65 with p53

Another interesting finding is that TA stabilized p53 in the nucleus of HCT116 and LoVo cells (Figure 5A and 5B). Because nuclear p53 degradation is important mechanism of DNA damage and cell stress by anti-cancer compounds, we tested if TA affects transcriptional activity of p53 in HCT116 cells transfected with p53-MDM-luciferase plasmid. As shown in Figure 6A, TA increased the transcriptional activity of p53 by 1.9 and 2.5 fold at 30 μM and 50 μM, respectively.

According to a literature (40), ERK and p38MAPK phosphorylate p53 at serine 15 and subsequently induces transactivation of p53. In addition, these two kinases are required for NF-κB activation. To determine whether TA-mediated transactivation of p53 and NF-κB is dependent on p38MAPK and ERK, HCT116 cells transfected with p53-MDM-luciferase or NF-κB-luciferase plasmids were treated with TA in presence of SB203580 and PD98059. As a result (Figure 6B and 6C), the selective inhibitors for p38MAPK and ERK attenuated TA-stimulated transcriptional activities of p53 and NF-κB. On the other hand, p53 binds to p65 and subsequently regulates NF-κB transcriptional activity (41-43). Therefore, we examined the possibility that p53-dependent NF-κB activation by TA is associated with increased binding of p65 and p53 by performing immunoprecipitation. As a result, TA treatment increased the association between p65 and p53 in HCT116 cells (Figure 6D). However, pretreatment of SB203580 and PD98059 blocked the TA-induced binding of p53 to p65 (Figure 6D). We also observed that TA-induced binding of p53 to p65 was diminished in Ser276Gly mutant p65-transfected HCT116 cells compared to cells transfected with wild type-p65 (Figure 6E). These data imply that TA induce both transcriptional activities of p53 and NF-κB and that p53 plays a significant role in TA-induced NF-κB transcriptional activation, probably via enhancing binding affinity between p53 and p65 which might be associated with p38 MAPK and ERK-mediated phosphorylation of Ser276 in p65.

Both p65 and p53 mediate TA-induced apoptosis.

Having shown previously that TA induces apoptosis in human colorectal cancer cells (13), we investigated whether TA-mediated apoptosis is dependent on p53 and NF-κB activation. As shown in Figure 7A, TA treatment increased PARP cleavage in HCT116 cells expressing wild type p53, whereas TA-mediated PARP cleavage was ameliorated in HCT116$^{p53-/-}$ cells. In addition, knockdown of p65 using siRNA decreased TA-induced PARP cleavage compared to cells transfected with control siRNA in HCT116 (Figure 7B). The double knockout of p65 and p53 also decreased TA-induced apoptosis (Figure 7C). To confirm that TA-mediated apoptosis is dependent on p53 and p65, cleaved PARP and cell death were investigated in LoVo cells transfected with siRNAs of p65 and p53. As a result, knockdown of p65 and p53 decreased TA-induced PARP cleavage and cell death in LoVo cells (Figure 7D and 7E). Next, to determine if Ser276 phosphorylation of p65 affects TA-induced apoptosis, we transfected HCT116 cells with wild type-p65 (WT) or Ser276Gly (MT), and compared PARP cleavage by Western blot. As shown in Figure 7F, TA-induced PARP cleavage was attenuated in the cells transfected with Ser276Gly mutant compared with cells transfected with wild type-p65.

On the other hand, Fas (CD95/APO-1) ligand (FasL) is a p53-and NF-κB-target protein and induction of FasL is essential for p53- and NF-κB-dependent apoptosis (44-46). So, we also tested if TA affects FasL expression. As shown in Figure 7G and 7H, TA induced FasL expression in a dose- and time-dependent manner.

All together, these results demonstrate that p53 and p65 play a significant role in TA-induced apoptosis through Ser276 phosphorylation-mediated protein interaction and subsequent transcriptional activation of their target gene such as FasL.

Discussion

The transcription factor NF-κB is a key mediator of the cellular stress response upon anticancer therapy, and the activation of NF-κB can elicit a pro-death response (29). TA,

one of the traditional NSAIDs induces apoptosis in various cancer cells (11-13). In search for novel approaches to augment the therapeutic efficacy of TA, we investigated the role of NF-κB in TA-mediated apoptosis in colorectal cancer cells. In the present study, we for the first time provide evidence that TA induces apoptosis by activating NF-κB transcriptional activity in human colorectal cancer cells.

Here, we propose two mechanisms by which TA leads to an increase in nuclear p65 level and transcriptional activation. Firstly, nuclear accumulation of p65 by TA was mediated by the proteosomal degradation of IκB-α. This was accompanied by nuclear translocation of NF-κB subunit p65 and enhanced NF-κB/DNA binding, followed by an increase in NF-κB transcriptional activity, which was similar to NF-κB activation by the typical NF-κB inducer TNF-α. However, p65 translocation by TA and TNF-α markedly differed: While TA-stimulated IκB-α degradation occurred between 10 and 24 hours, TNF-α rapidly induced p65 translocation within minutes (Figure 3B). Secondly, in the present study, we observed that TA increased p65 nuclear accumulation through phosphorylation of Ser276 residue and subsequent stabilization of p65. TA increased protein level of ectopically expressing EGFP-p65 within 3 hours after TA treatment in wild type-p65 transfected cells, but not in cells transfected with Ser276Gly mutant (Figure 4A and 4C). These results suggest that phosphorylation of p65 at Ser276 residue may stabilize p65 and lead to nuclear accumulation and subsequent NF-κB transcriptional activation. We also observed that selective inhibitors of p38 MAPK and ERK attenuated TA-induced p65 phosphorylation and nuclear accumulation without affecting IκB-α degradation (Figure 4E and 4F), indicating that these two kinases may mediate TA-induced Ser276 phosphorylation and that nuclear accumulation of p65 provide another mechanism of NF-κB activation regardless of IκB-α-mediated NF-κB translocation. In our previous study, we observed that TA activated p38 MAPK and ERK pathways (14). Recently, several studies demonstrated that degradation of p65 via an ubiquitin-proteasome machinery in the nucleus leads to a negative regulation of NF-κB activation (47-50). Phosphorylation of p65 at Ser276 residue allows increased stability of p65 against ubiquitin-mediated degradation in the nucleus and whereby exerts activation of NF-κB signaling (35). In terms of the anti- or pro-apoptotic activity of p65,

Stark and Dunlop (24) reported that localization of p65 in nucleolus activates apoptosis while location in the nucleoplasm inactivates apoptosis, suggesting that nuclear localization of p65 determines apoptotic activity. Thus, further study is required to investigate if TA treatment affects nuclear localization of p65.

Another interesting finding of this study is that TA-induced transcriptional activity of NF-κB is dependent on p53. Interestingly, many proapoptotic stimuli such as DNA-damaging agents induce the transcriptional activation of both p53 and NF-κB. Ryan et al. suggested that p53 directly activates transcriptional activity of NF-κB which is required for p53-induced apoptosis (39). In our study, activation of NF-κB in response to TA treatment was observed in HCT116 and LoVo cells expressing wild type p53, however TA did not affect NF-κB activation in HCT116$^{p53-/-}$ and p53 mutant colorectal cancer cell lines, SW480 (mutant p53/Arg^{273}His) and CaCo-2 (p53 null) (Figure 5A-5D, upper panels). We also observed that TA-stimulated NF-κB activation was attenuated by p53 knockdown in LoVo cells (Figure 5B, upper panel). However, TA increases p65 nuclear accumulation in a p53-independent manner (Figure 5A-5D, lower panels). These results imply that p53 do not play a significant role in p65 translocation as well as nuclear accumulation. Taken together, it is possible that p53 may be essential for TA-induced NF-κB activation, but not in p65 stabilization.

We also found that TA increases p53 nuclear accumulation (Figure 5A and 5B, lower panels) and transcriptional activity (Figure 6A), and that TA-mediated p53 transcriptional activation is p38MAPK- and ERK-dependent (Figure 6B). Indeed, p38MAPK and ERK phosphorylated p53 at serine 15 (40) and Ser15 phosphorylation of p53 suppresses ubiquitination of p53 and subsequent 26S proteasome-mediated degradation by reducing the interaction of p53 with MDM2. As a result, Ser15 phosphorylation of p53 promotes the accumulation and transactivation (51,52). In the line of this notion, we propose that TA-mediated nuclear accumulation and transcriptional activation of p53 may be associated with p38MAPK- and ERK-mediated Ser15 phosphorylation of p53 by TA.

In terms of p53 involvement in NF-κB activation, we observed that TA treatment induced binding affinity of p65 with p53 and that TA-stimulated protein interactions and

NF-κB activation were blocked by selective inhibitors of p38 MAPK and ERK (Figure 6C and 6D), indicating that Ser276 phosphorylation of p65 may be important in TA-stimulated binding affinity of p65 with p53 and subsequent NF-κB activation. Furthermore, we tested whether Ser276Gly-mutation affects TA-mediated binding affinity of p65 with p53 and observed that TA-mediated binding affinity was diminished in cells transfected with Ser276Gly-mutant. These results indicate that Ser276 phosphorylation of p65 may be essential for TA-mediated binding affinity of p65 with p53 and subsequent NF-κB activation. However, we do not exclude the possibility that another p38MAPK and ERK-mediated p65 modifications are involved in p65-p53 bindings because point mutation of p65 (S276G) did not completely block the protein binding while chemical inhibitors of p38MAPK and ERK blocked protein interaction completely (Figure 6D and 6E).

Recent studies indicate that p53-dependent NF-κB activation could contribute to the apoptotic response (38,39), and the loss of p53 could abrogate NF-κB-mediated apoptotic response (53). In addition, p65 has been reported to promote apoptosis by actively repressing transcription of antiapoptotic genes through association of p65 with histone deacetylase-containing complexes acting as corepressor (54). In this study, we found that TA-induced apoptosis was significantly reduced in p53-null and p65-knockdown cells (Figure 7A-7E). We also observed that point mutation of p65 at Ser276 residue attenuated TA-induced apoptosis. These results indicate that TA-induced apoptosis may be mediated by Ser276 phosphorylation-mediated p65 stabilization and subsequent p53-dependent transcriptional activation of p65.

According to several studies, it is likely that p53 regulates NF-κB transcriptional activity via different pathways in normal and cancer cells. For example, the binding of p53 to p65 inhibits NF-κB activation in normal fibroblasts (42), while an increase of binding affinity stimulates NF-κB activation in cancer cells (43). In the line of this notion, TA-stimulated increase in binding affinity of p65 with p53 may contribute to apoptosis by TA in human colorectal cancer cells.

Both p53 and NF-κB regulate Fas (CD95/APO-1) ligand (FasL) activating an apoptosis signaling cascade by binding Fas on the target cell triggering cell death and

induction of FasL is essential for p53- and NF-κB-dependent apoptosis (44-46). So FasL could be a good target protein studying an apoptosis associated with p53 and p65. Thus, we test if TA affects FasL expression and observed TA-mediated FasL induction in a dose- and time-dependent manner (Figure 7G and 7H). In terms of time kinetics, we observed that the time point of p65 accumulation (6 h after TA treatment; Figure 2E) is earlier than that of FasL expression (10 h after TA treatment; Figure 7H). A number of studies indicate that FasL has NF-κB response elements on their promoters and p65 binds to these response elements (55,56). Thus, we claim that TA-stimulated p65 accumulation and transcriptional activation leads to an increase of FasL transcription.

Colorectal tumorigenesis is affected by many signaling pathways including cyclooxygenase (COX). TA activated NF-κB transcriptional activity in HCT116 (COX-negative) and LoVo cells (COX-negative) and but did not affect in Caco-2 (COX-negative) and CaCo-2 (COX-expressing) cells. It is likely that TA-induced apoptosis and NF-κB is COX-independent. However, we do not exclude the possibility that inflammatory status may affect TA's anti-cancer effects.

In summary, the data presented here show that TA induces p65 nuclear accumulation through IκBα-dependent nuclear translocation and increased stability of nuclear p65 through p38- and ERK-dependent Ser276 phosphorylation. Increased nuclear p65 directly interacts with p53 and activates NF-κB transcriptional activity, which is predominantly responsible for the ability of TA to induce apoptosis.

Acknowledgement

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Figure Legends

Figure 1. Inhibition of cell proliferation and induction of apoptosis by different NSAIDs and dietary phytochemicals. (A) HCT116 cells were treated with 50 μM of the indicated NSAIDs and phytochemicals in media containing 1% FBS for the indicated times. Cell proliferation was measured using CellTiter96 Aqueous One Solution Cell Proliferation Assay Kit and expressed as absorbance (A490). *p<0.05 compared to cells treated with DMSO. (B) HCT116 cells were treated with 50 μM of indicated NSAIDs and phytochemicals for 24 h. Total cell lysates were harvest and subsequently Western blot was performed for PARP and actin. (1, DMSO; 2, tolfenamic acid; 3, aspirin; 4, diclofenac; 5, SC-560; 6, celecoxib; 7, EGCG; 8, resveratrol; 9, genistein)

Figure 2. TA increase transcriptional activity of NF-κB, nuclear p65 and binding affinity of p65 to DNA. (A, B) The *pNF-κB-Luc* plasmid-transfected cells were treated with 0, 10, 30 and 50 μM of TA for 24 h or 0 and 10 ng/ml of TNF-α for 8 h. The cells were harvested, and luciferase activity was measured using a dual-luciferase assay kit. *p<0.05 compared to cells without TA or TNF-α treatment. (C, D) HCT116 cells were treated with 0 and 50 μM of TA for 24 h or 0 and 10 ng/ml TNF-α for 30 min. Cytosol (CE) and nucleus extracts (NE) were prepared and subsequently Western blot analysis was performed for p65, TBP (TATA-binding protein) and actin. TBP was used nuclear loading control. (E, F) HCT116 cells were treated with 50 μM of TA or 10 ng/ml TNF-α for the indicated time points. Cytosol (CE) and nucleus extracts (NE) were prepared and subsequently Western blot analysis was performed for p65, TBP and actin. (G) HCT116 cells were treated with 50 μM of TA for 24 h or 10 ng/ml TNF-α for 30 min. Nuclear extracts (5 μg) were prepared and incubated with biotin-labeled NF-κB oligonucleotide as described in Materials and methods. DNA-protein complexes were resolved by 6% nondenaturing polyacrylamide gel and developed using the protocol of LightShift Chemiluminescent EMSA kit.

Figure 3. TA increases translocation of p65 via proteosomal degradation of IκB-α. (A) HCT116 cells were treated with 0, 5, 10, 30 and 50 μM of TA for 24. Total cell lysates were harvested and subsequently Western blot analysis was performed for IκB-α and actin. (B) HCT116 cells were treated with 50 μM of TA or 10 ng/ml TNF-α for the indicated time points. Total cell lysates were harvested and subsequently Western blot analysis was performed for IκB-α and actin. (C, D) HCT116 cells were pretreated with 10 μM of MG-132 for 2 h and then co-treated with 50 μM of TA for 24 h. Whole cell lysates, cytosol (CE) and nuclear extracts (NE) were prepared and subsequently Western blot analysis was performed for IκB-α, p-IκB-α, p65, TBP and actin. (E) HCT116 cells were pretreated with 0, 1 and 5 μM of BAY 11-7085 for 2 h and co-treated with 50 μM of TA for 24 h. Cytosol (CE) and nuclear extracts (NE) were prepared and subsequently Western blot analysis was performed for p65, TBP and actin.

Figure 4. TA stabilizes p65 and increase nuclear accumulation via p38 MAPK and ERK-dependent phosphorylation of p65 at Ser276 residue. (A) HCT116 cells were transfected with EGFP-p65 for 24 h and then treated with 50 μM of TA for the indicated times. Cytosol (CE) and nuclear extracts (NE) were prepared and subsequently Western blot analysis was performed for EGFP, TBP and actin. (B) HCT116 cells were treated with 50 μM of TA for the indicated times (left panel) or 24 h (right panel). Whole cell lysates (WE), cytosol (CE) and nuclear extracts (NE) were prepared and subsequently Western blot analysis was performed for p65, p-p65 (Ser276), and actin. (C) HCT116 cells were transfected with wild type-p65 (WT) or Ser276Gly-mutated p65 (MT) for 24 h and then treated with 50 μM TA for 24 h. Cytosol (CE) and nuclear extracts (NE) were prepared and subsequently Western blot analysis was performed for EGFP, TBP and actin. (D) After transfection with wild type-p65 (WT) or Ser276Gly-mutated p65 (MT) for 24 h and treatment of TA, cells were fixed and mounted with ProLong Gold antifade reagent containing DAPI. DAPI staining was used to visualize the nucleus of the cells. (E, F) HCT116 cells were pretreated with 50 μM of selective inhibitors of p38MAPK (SB203580) and ERK (PD98059) for 2 h and then co-treated with 50 μM of TA for 24 h. Whole cell lysates (WE), cytosol (CE) and nuclear extracts

(NE) were prepared and subsequently Western blot analysis was performed for p65, p-p65 (Ser276), TBP, IκB-α, p-IκB-α (Ser32), and actin.

Figure 5. TA-stimulated NF-κB activation is dependent on p53. (Upper panel) Different cells (A, HCT116; B, LoVo; C, SW480; D, CaCo-2) were transfected with *pNF-κB-Luc* plasmid and then treated with TA for 24 h. The cells were harvested, and luciferase activity was measured using a dual-luciferase assay kit. *p<0.05 compared to cells without TA. (Lower panel) Different cells were treated with TA for 24 h. Cytosol (CE) and nuclear extracts (NE) were prepared and subsequently Western blot analysis was performed for p65, p53 and actin.

Figure 6. TA increases p53 transcriptional activity and binding affinity of p65 with p53 (A) HCT116 cells were transfected with *pNF-κB-Luc* plasmid and then treated with 0, 30, and 50 μM of TA for 24 h. (B,C) The *pNF-κB-Luc* plasmid or *p53-MDM-Luc* plasmid-transfected HCT116 cells were treated with TA for 24 h in absence or presence of selective inhibitors of p38MAPK (SB203580, 50 μM) and ERK (PD98059, 50 μM). The cells were harvested, and luciferase activity was measured using a dual-luciferase assay kit. *p<0.05 compared to cells without TA. (D) HCT116 cells were pretreated with 50 μM of SB203580 and PD98059 and then co-treated with TA for 24 h. Immunoprecipitation (IP) was performed by pull down of cellular protein with p65 antibody and immunoblotting with p53 antibody. (E) HCT116 cells were transfected with wild type-p65 (WT) or Ser276Gly-mutated p65 (MT) and then treated with 50 μM of TA for 24 h. IP was performed by pull down of cellular protein with EGFP antibody and immunoblotting with p53 antibody.

Figure 7. TA-induced apoptosis is dependent of p53 and p65. (A) HCT116 and HCT116$^{p53-/-}$ cells were treated with 50 μM of TA for 24 h. (B) HCT116 cells were transfected with control or p65 siRNA for 48 h and then treated with 50 μM of TA for 24 h. (C) HCT116 and HCT116$^{p53-/-}$ cells were transfected with control or p65 siRNA for 48 h and then treated with 50 μM of TA for 24 h. (D) Lovo cells were transfected with

control, p53 or p65 siRNA for 48 h and then treated with 50 μM of TA for 24 h. (E) Lovo cells were transfected with control, p53 or p65 siRNA for 48 h and then treated with 50 μM of TA for 24 h. The cell death was measured from cytosol fraction using the Cell Death Detection ELISA^{PLUS} Kit, and expressed as absorbance (A_{405}-A_{490}). *$p<0.05$ compared to cells without TA treatment. (F) HCT116 cells were transfected with wild type-p65 (WT) or Ser276Gly-mutated p65 (MT) and then treated with 50 μM of TA for 24 h. Western blot was performed against antibodies for EGFP, PARP and actin. (G,H) HCT116 cells were treated with indicated doses of TA for indicated times. Western blot was performed against antibodies for FasL and actin.

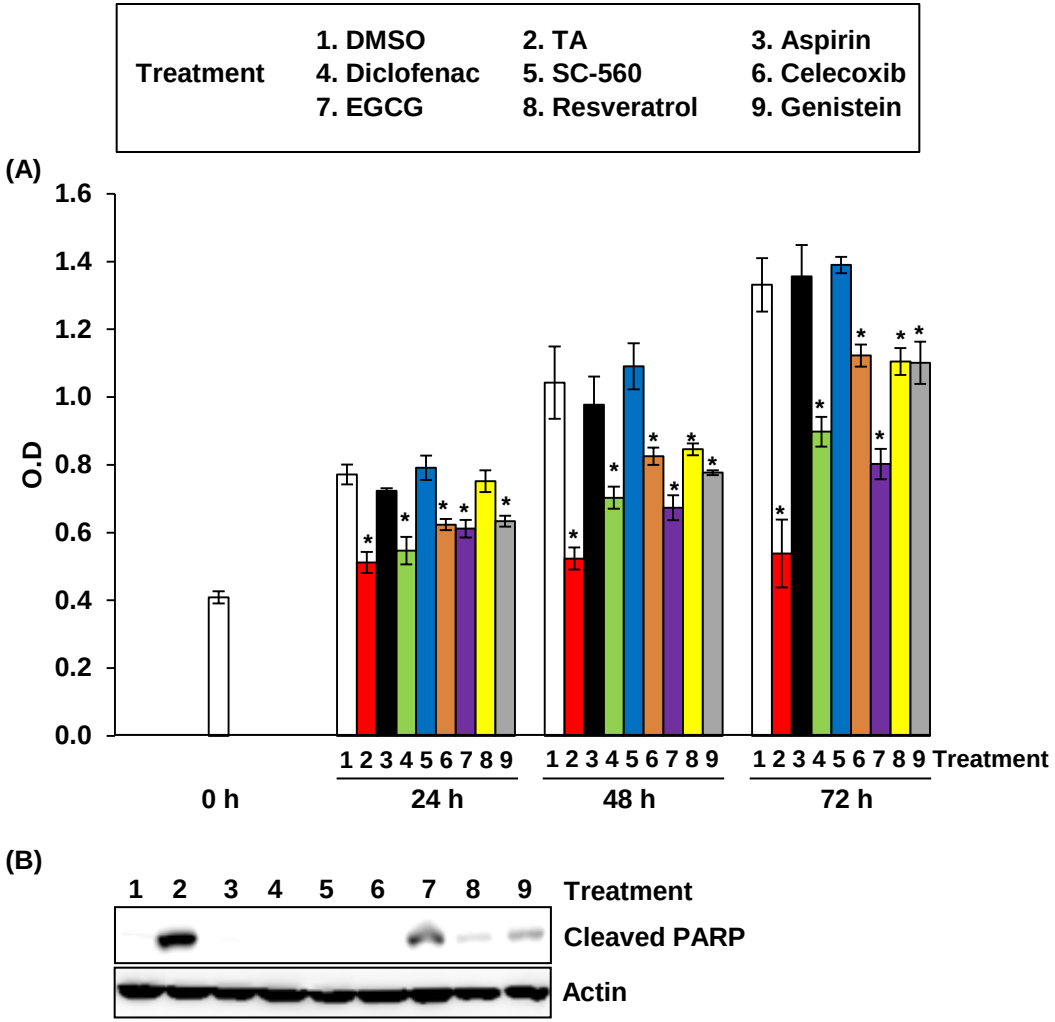

Figure 1

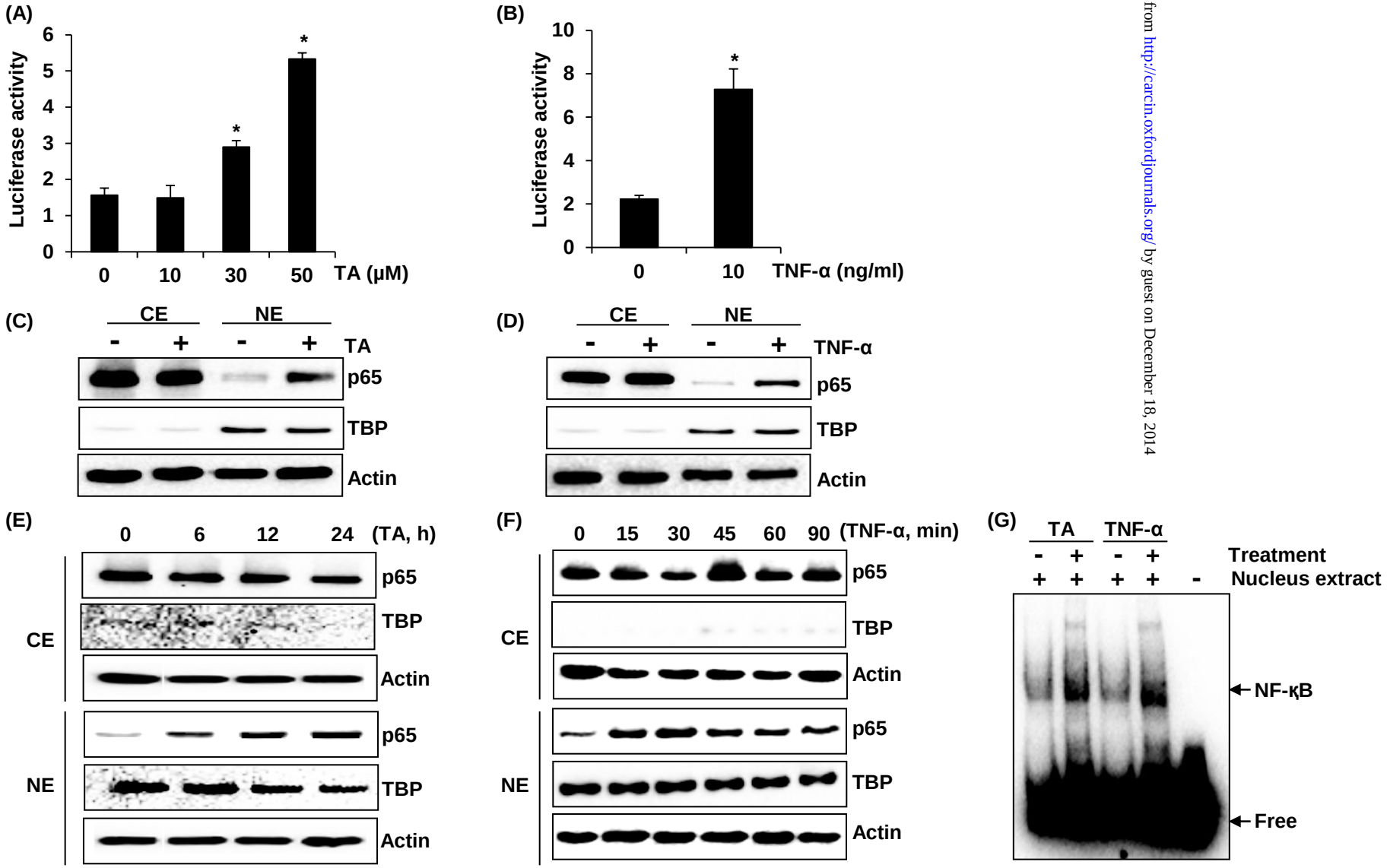

Figure 2

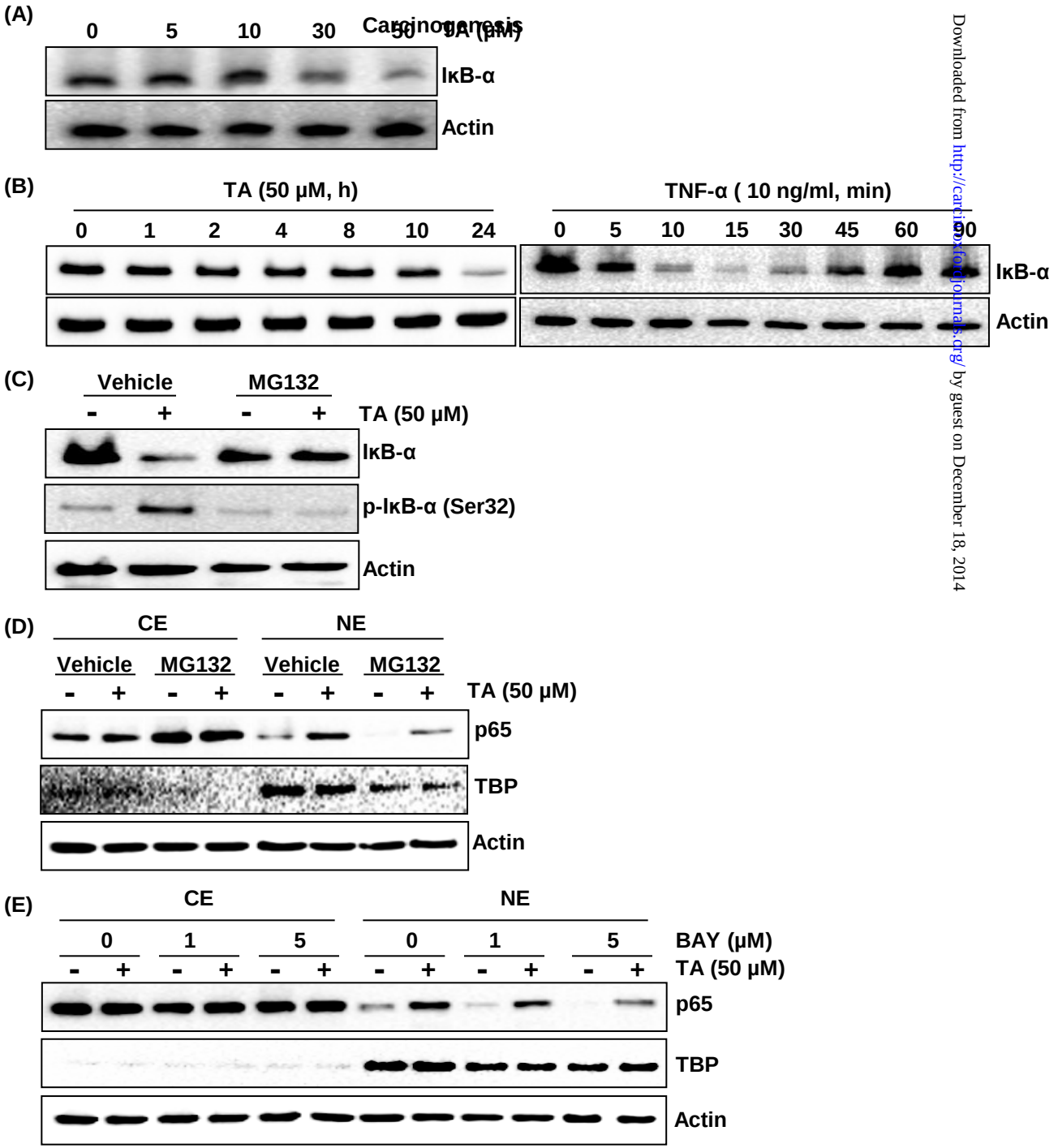

Figure 3

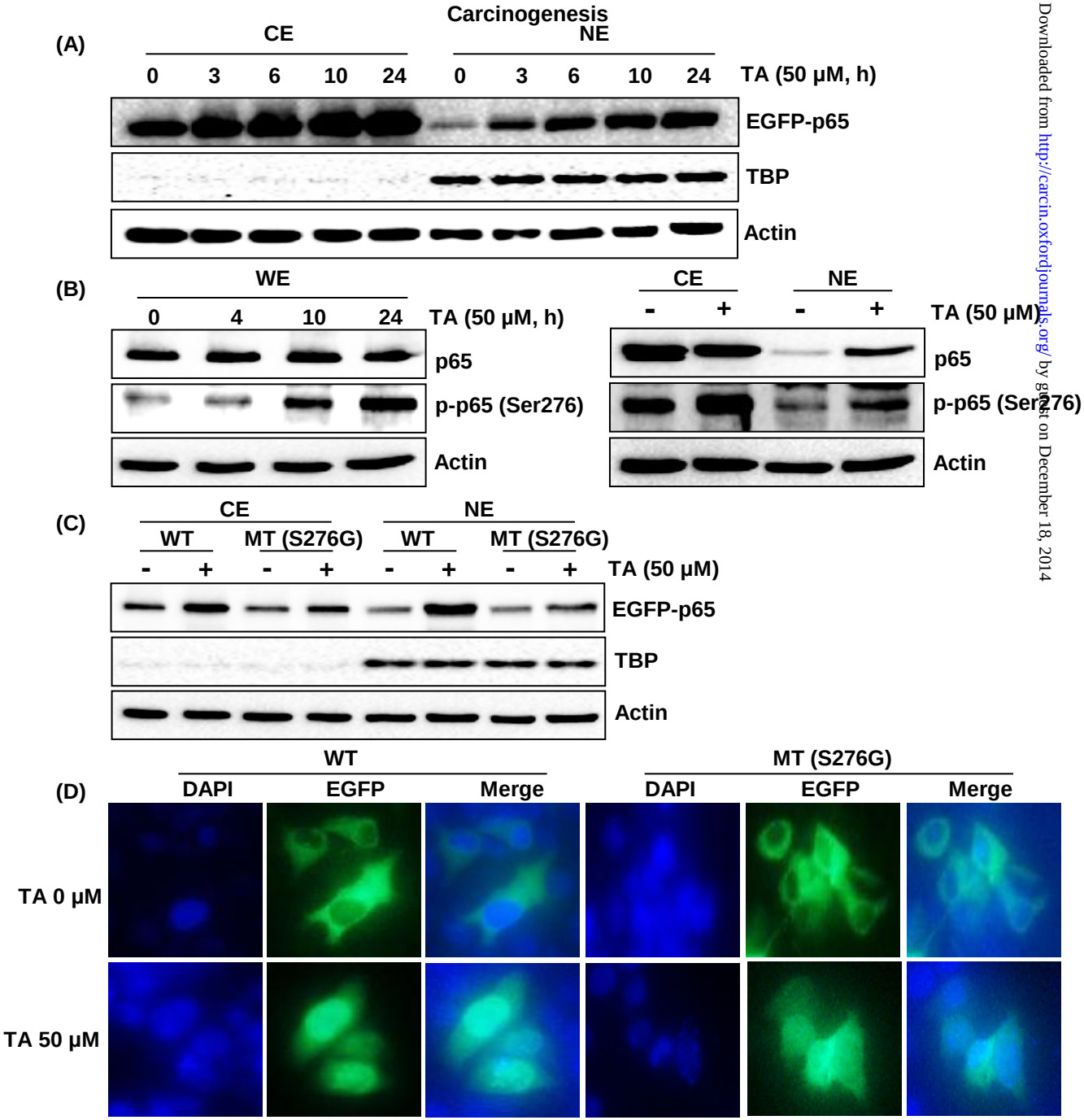

Figure 4

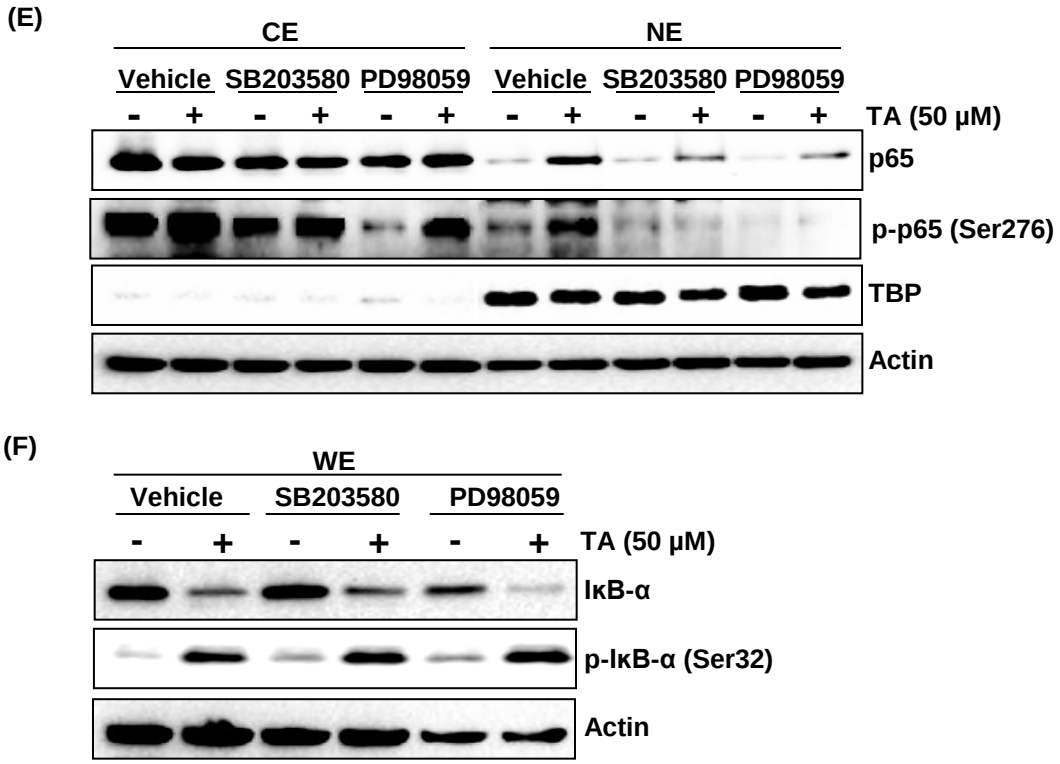

Figure 4

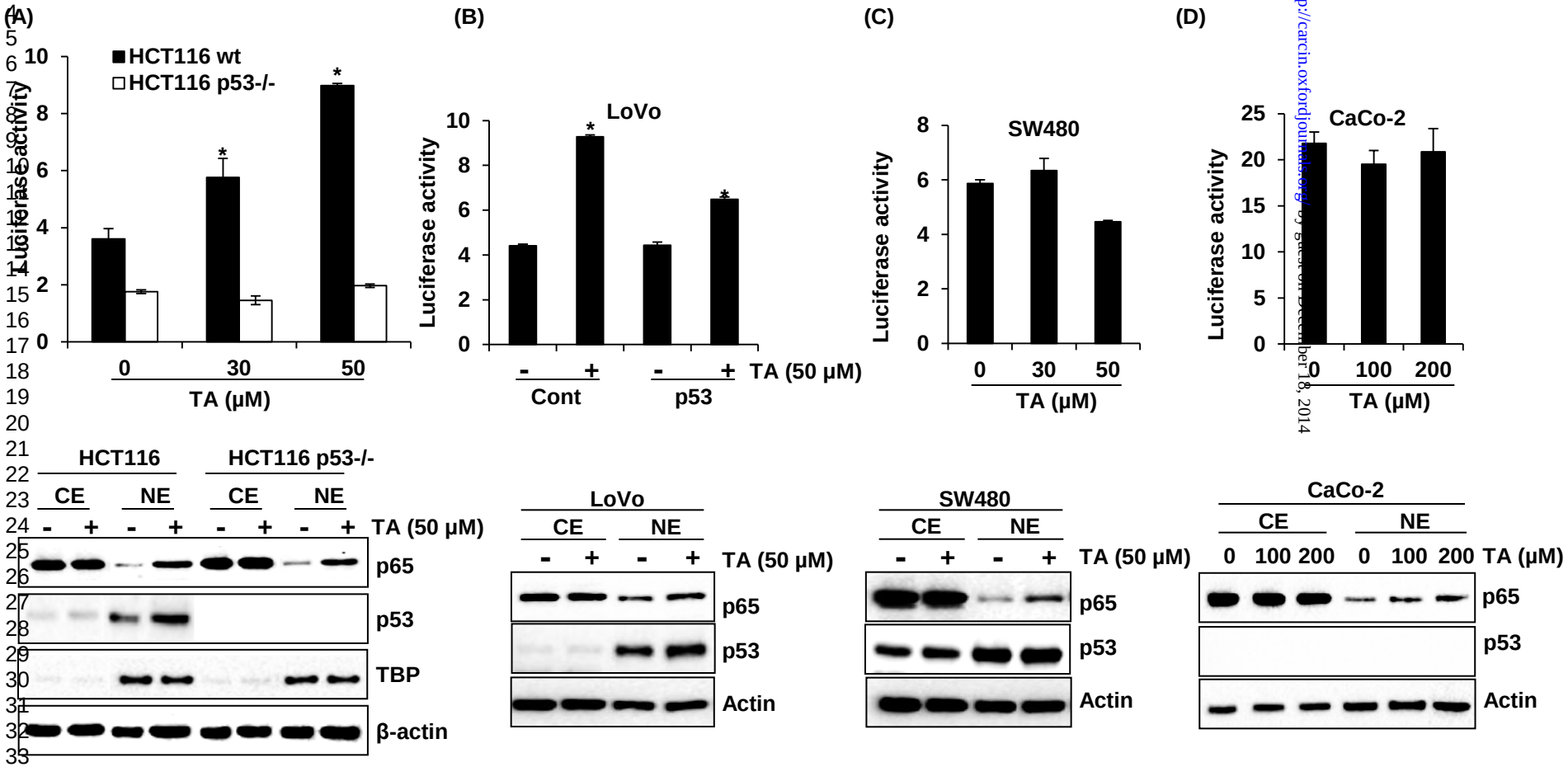

Figure 5

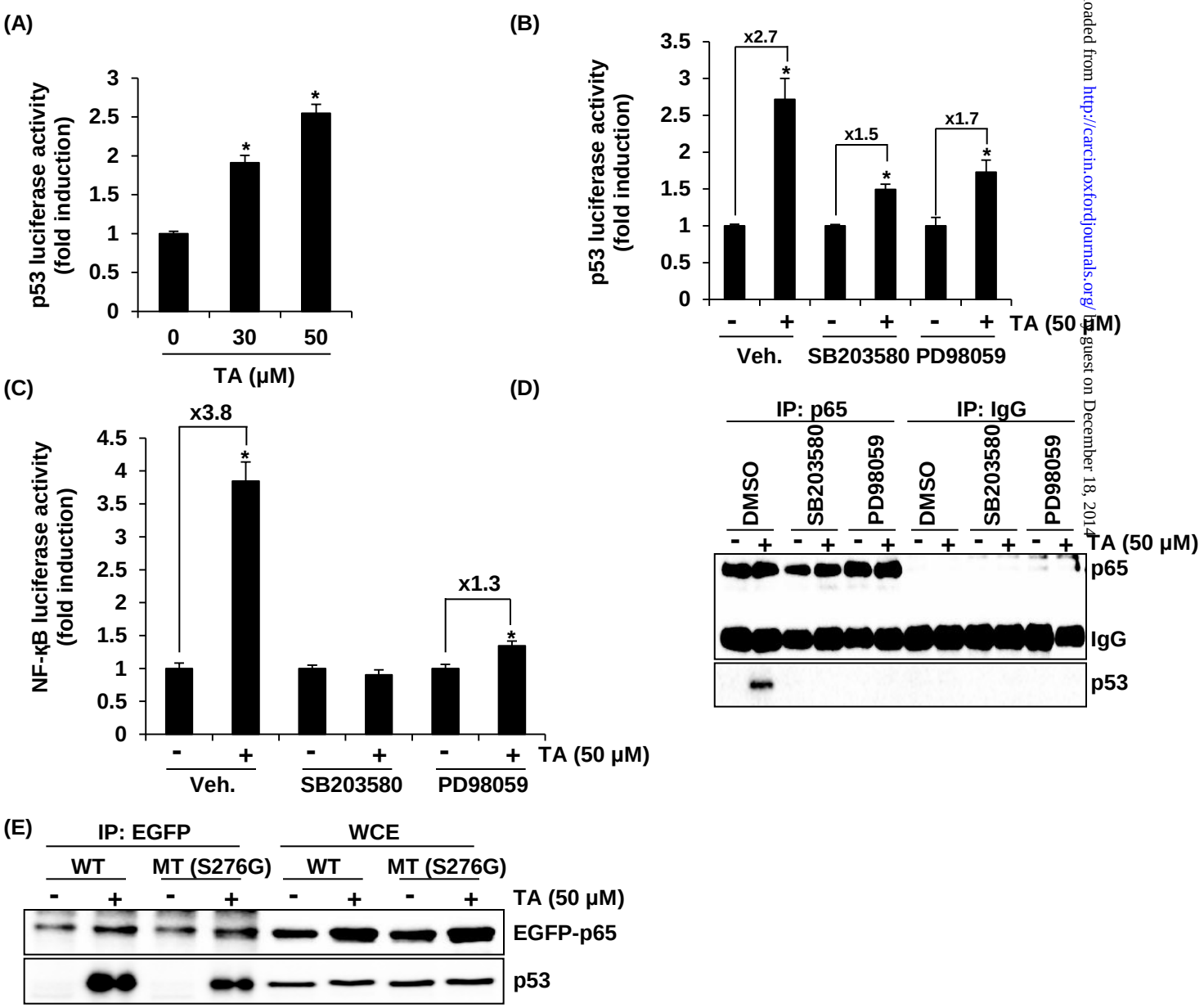

Figure 6

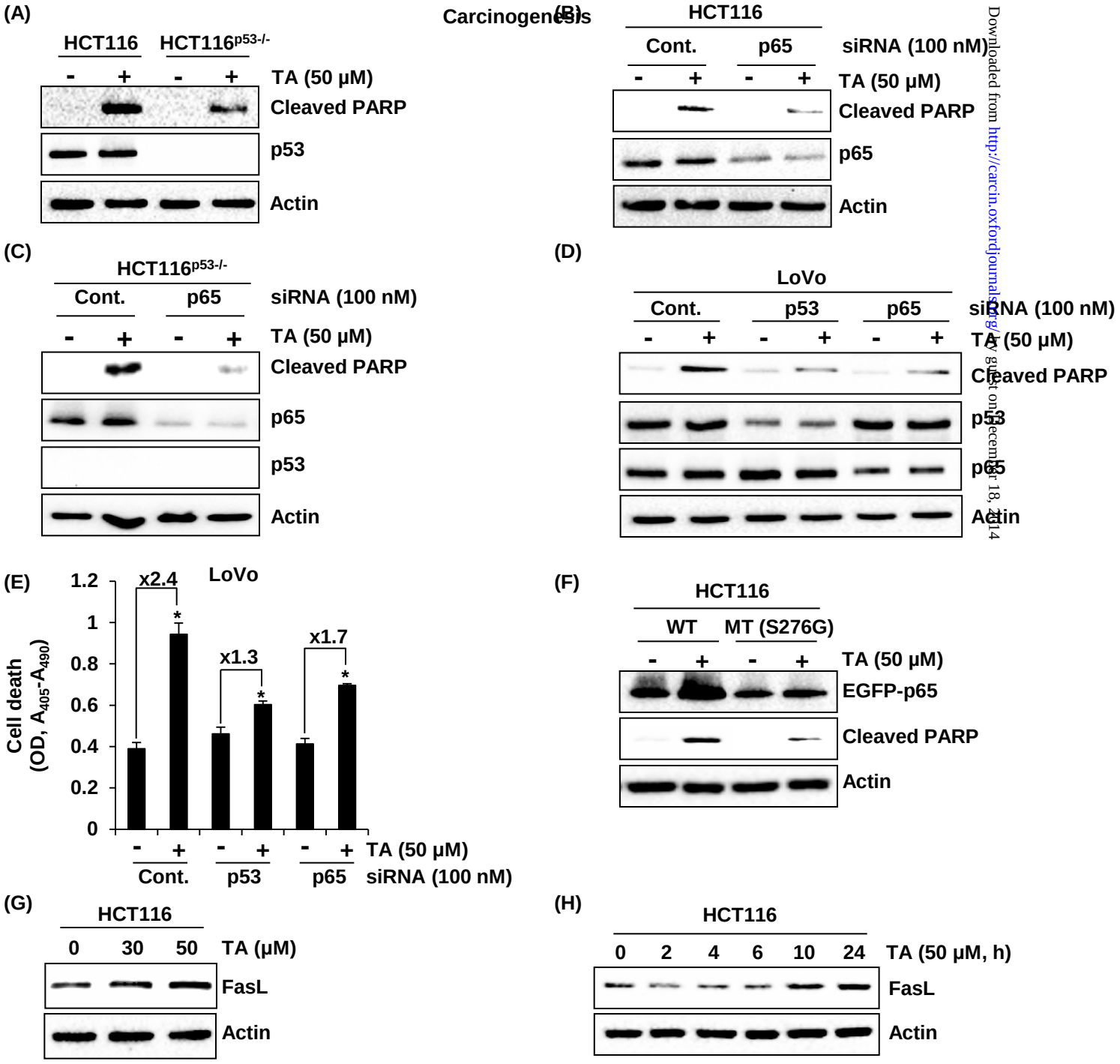

Figure 7