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Research Article

The molecular targets of diclofenac differs from ibuprofen to induce apoptosis and epithelial mesenchymal transition due to alternation on oxidative stress management p53 independently in PC3 prostate cancer cells

Elif D. Arisan*, Remzi O. Akar, Ozge Rencuzogullari, Pinar Obakan Yerlikaya, Ajda Coker Gurkan, Beyza Akın ☆, Elif Dener ☆, Ecem Kayhan ☆, Narcin Palavan Unsal

Istanbul Kültür University, Department of Molecular Biology and Genetics, Atakoy Campus, 34156, Istanbul

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ABSTRACT

Background: Prostate cancer is the most common type of cancer among men. Studies showed that the regular use of nonsteroidal antiinflammatory drugs might reduce disease progression risk for prostate cancer patients with prostate cancer. We evaluated the effects of ectopic expression of p53 on the biological functions of ibuprofen and diclofenac.

Materials and methods: For this purpose, We investigated cell death decision pathways related to survival and aggressive cellular phenotypes such as extrinsic/intrinsic apoptosis decision, Protein Kinase B/ Forkhead box O (AKT/FoxO) axis, mitogen-activated protein kinases (MAPKs), reactive oxygen species (ROS) generation, and EMT (epithelial mesenchymal transition) in wild type and p53 + PC3 prostate cancer cells.

Results and Conclusions: Ibuprofen (1 mM) and diclofenac (250 μM) effectively induced cell cycle arrest and led to apoptosis *via* modulating both extrinsic and intrinsic pathways. However, diclofenac was the only drug to generate ROS intermediates. Diclofenac triggered a typical EMT process with downregulated E-cadherin and upregulated N-cadherin, vimentin, and Snail in PC3 cells, regardless of p53 expression. In conclusion, although both drugs are effective on cell death mechanism, only diclofenac caused EMT because of increased ROS generation independent of p53. On the other hand, ibuprofen could inhibit metastasis *via* upregulating E-cadherin. The biological targets of both nonsteroidal antiinflammatory drugs are different to highlight their role in cell survival and death axis.

1. Introduction

Increasing evidence showed that poor progression of prostate cancer can be prevented through nonsteroidal antiinflammatory drugs (NSAIDs) that exert a chemopreventive and/or anticancer potential[1–5] through suppression of inflammation due to overexpression of cyclooxygenases (COXs).[6] Instead of COXs, NSAIDs have multiple targets in the cells such as phosphoinositol-3 kinase (PI3K) and Wnt/β-catenin signaling, and thereby NSAIDs gain importance in cancer therapy.[9] Recent reports approved that long-term use of low-dose NSAIDs improves prognosis of disease with chemopreventive efficiency.[5] Single or combinational administration of NSAIDs with chemotherapeutics induced apoptosis in leukemic, colon, prostate, and lung cancer cells.[7–12] Selective cyclooxygenase-2 (COX-2) inhibitor celecoxib-induced cell cycle arrest at G1 phase led to apoptosis in LNCaP and PC3 cells, which lack COX-2 expression, through inhibition of protein kinese B (AKT) and caspase-3 activation.[15,16]

In this study, we compared the molecular targets of nonselective COX inhibitors ibuprofen and diclofenac in PC3 prostate cancer cells through investigating their role in association with p53. Previous reports have shown that p53 induces COX-2 expression, and COX-2 inhibits p53-mediated or oxidative stress–mediated apoptosis.[17] Celecoxib increased the expression of p53 in LNCaP prostate cancer cells, and silencing of p53 in LNCaP cells prevented celecoxib-

* Corresponding author. Istanbul Kültür University, Department of Molecular Biology and Genetics, Atakoy Campus, 34156, Istanbul.
E-mail address: d.arisan@iku.edu.tr (E.D. Arisan).
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induced cell death.[18] In consequence, COX-2−expressing cancer cell lines showed diminished p53 response and activated AKT pathway. However, another study suggested that celecoxib decreased cell growth in LNCaP, DU145, and PC3 prostate cancer cell lines p53 independently.[19] It is not clear that how p53 regulated COX-2 expression in a positive or negative manner.[17,21]

2. Material and methods

2.1. Drugs, chemicals, and antibodies

Diclofenac and ibuprofen were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and dissolved in dimethyl sulfoxide as 10 mM stock solution, stored at −20 °C. Propidium iodide was purchased from AppliChem (Darmstadt, Germany) and 3,3′-dihexyloxacarbocyanine iodide (DiOC6) from Calbiochem (La Jolla, CA, USA). 4′,6-Diamidino-2-fenilindol (DAPI) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti−rabbit antibodies against p53, Mouse double minute 2 (Mdm-2), AKT, FoxO1, FoxO3a, Fas, caspase-8, caspase-7, caspase-2, RIP, TNF-R2, procaspase-9, procaspase-3, Bcl-2, Mcl-1, Bcl-x, Bax, BimEL, Puma, Bak, Bid, p38, SAPK/JNK, p-SAPK/JNK, Axin, Wnt5a/b, Dvl3, Dvl2, β-catenin, Wnt3a, LRP6, E-cadherin, N-cadherin, vimentin, Snail, Lamin A/C, GAPDH, and β-actin (each 1:500-1:1000) were bought from Cell Signalling Technology (CST, Danvers, MA, USA). Horseradish peroxidase−conjugated secondary anti−rabbit antibodies or anti−−mouse antibodies (1:5000) were obtained from CST.

2.2. Cell culture

Wild type (wt) PC3 cells (ATCC CRL-1435) were used in the study. To obtain p53+ PC3 cells, cells were stably transfected with pcDNA3 flag p53, which was a gift from Thomas Roberts (Addgene plasmid # 10838; http://n2t.net/addgene:10838; RRID:Addgene_10838) through neomycin selection. Both cell lines were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (PAN-Biotech, Aidenbach, Germany) supplemented with 10% Fetal Bovine Serum (FBS) (PAN-Biotech) and 100 U/100 mg ml penicillin/streptomycin of 100 U/100 mg ml^{-1} (Biological Industries, Kibbutz Beit-Haemek, Israel).

2.3. Cytotoxicity and cell cycle assay

Cytotoxic effects of NSAIDs on both cell lines were determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) cell viability assay in accordance with the manufacturer instructions. DiOC6 and DAPI costaining was used for mitochondria membrane potential detection. Cells were then analyzed by fluorescence microscopy at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

For cell cycle analysis, both floating and adherent cells were harvested, washed with 1× phosphate-buffered saline (PBS), and fixed with cold 70% ethyl alcohol at 4 °C. Cells were again washed with 1× PBS and incubated with RNase A (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. The staining procedure was completed with 2.5 μM propidium iodide for 30 min at room temperature at dark. Cell cycle analysis was read by flow cytometry (Accuri C6; BD Biosciences, Oxford, UK).

2.4. Immunoblotting and coimmunoprecipitation

For total protein isolation and nuclear isolation, samples were isolated with Mammalian Protein Extraction Reagent (M-PER) and Nuclear and Cytoplasmic Extraction Kit (NE-PER) , respectively (Pierce Biotechnology, Rockford, IL, USA). Protease inhibitor cocktail tablets (Roche Diagnostics, IN, USA) and the phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA) were supplementary to nuclear extraction reagent before use. To the sample, 30 μg protein was loaded and separated by 12−15% sodium dodecyl sulphate−polyacrylamide gel electrophoresis and then transferred onto polyvinyl difluoride membranes (Roche Diagnostics). After clogging procedure with 5% nonfat milk, dissolved in 0.1% tris-buffered saline with tween (TBS-T) (10 mM Tris-HCl and Tween 20), membranes were incubated with primary antibodies and horseradish peroxidase−conjugated secondary antibodies at 4C overnight. ChemiDoc™MP Imaging System (Bio-Rad Laboratories, Hercules, CA) was used to detect signals. All results were replicated at least three times and representative blots were given.

Cell lysates were isolated in 1% CHAPS buffer ((3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)) [5 mM $MgCl_2$, 137 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid), 1% CHAPS, and 20 mM Tris-HCl (pH 7.5) and protease inhibitors (Roche Diagnostics)]. Immunoprecipitation of Bim with Mcl-1 was performed by using Dynabeads (Invitrogen Co., Carlsbad, CA, US) in accordance with manufacturer recommendations. The samples were subsequently analyzed by immunoblotting with anti−Mcl-1 antibody (CST) to detect interaction potential of Mcl-1 and Bim.

2.5. Reactive oxygen species detection

After drug treatment, 2′,7′−dichlorofluorescin diacetate staining (5 μM) was applied to cells for 30 min, and trypsinized cells were prepared in 1× PBS. Cells were analyzed by flow cytometry (Accuri C6; BD Biosciences). At least 5000 events were analyzed to detect fluorescence intensity of dichloro-dihydro-fluorescein (DCF) on FL-1 channel (525 nm).

2.6. Soft Agar and scratch assay

The base agar was prepared creating a mixture with equal amounts of 2× Dulbecco's Modified Eagle's medium medium [20% FBS and 2% penicillin/streptomycin (GIBCO, Invitrogen Co., Carlsbad, CA, US)] and 0.5% agarose in PBS and the mixture was dispersed as 1 ml into each well in 6-well plates. After solidification of gel, 2.5×10^5 cells/ml in a 1:1 mix of 2× DMEM medium and 0.3% agarose were added on top of the base gel. After solidification, 500 μl media in the presence or absence of drugs was added on the gel and incubated at 37 oC for 15 days. Cells were stained with 0.05% crystal violet for 30 min. Results were also analyzed by fluorescence microscopy after DiOC6 staining (Olympus IX70, Tokyo, Japan).

After attachment of cells into 6-well plates, at least 80% confluent plates were scratched in a straight line using a 200-μl sterile plastic pipette tip. In the presence or absence of drugs, the motility and proliferation potential of cells was detected *via* an inverted microscope (Olympus IX70).

2.7. Statistical analysis

The results of the cell viability are shown in column graphics as mean ± standard deviation. The Student *t*-test was applied to evaluate the statistical meaningful differences. Alterations were observed as statistically significant when standard deviation values had a p value less than0.05.

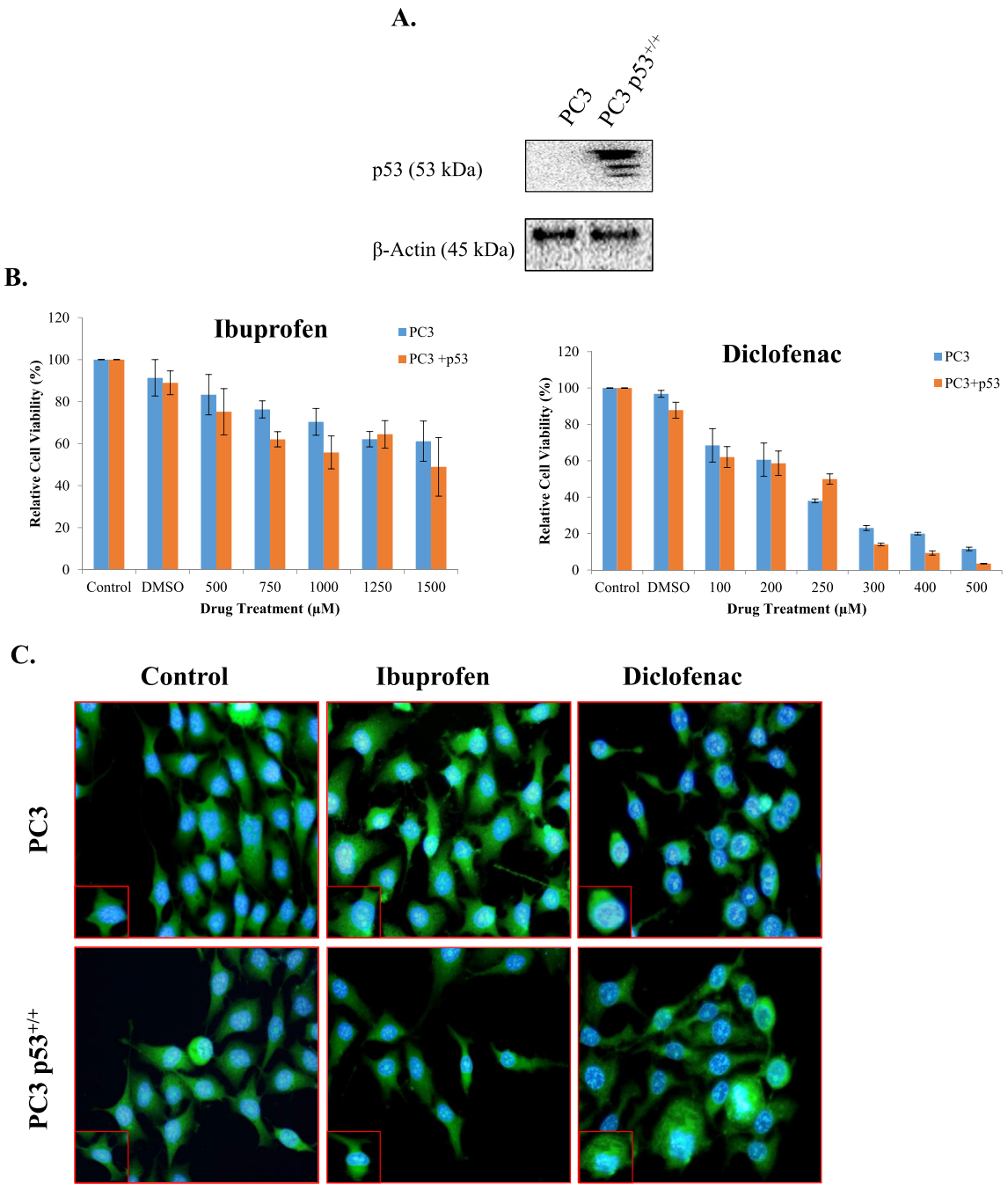
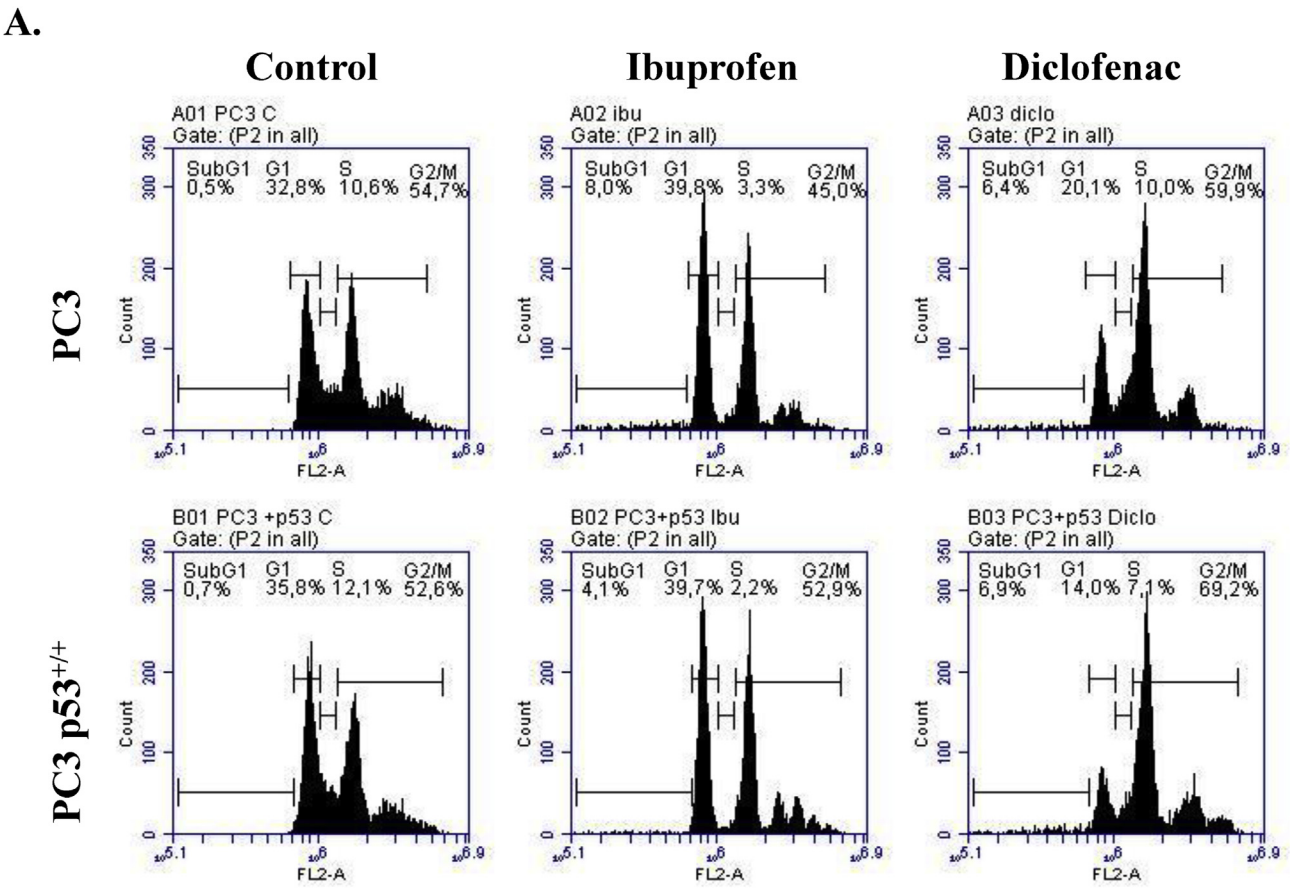


Fig. 1. Exposure of wt and p53 + PC3 cells to ibuprofen and diclofenac decreased cell viability in dose-dependent manner. (A) p53 plasmid transfection was investigated by immunoblotting with p53 antibody, and β-actin was used as loading control. (B) The effect of ibuprofen and diclofenac on cell viability was observed by using MTT assay. A total of 7×10^3 PC3 and PC3 $p53^{+/+}$ cells were cultured in 96-well plates. The cells were treated with ibuprofen (1000 μM) or diclofenac (250 μM) for 24 h. Subsequently, the absorbance data were determined at 570 nm with a microplate reader (iMark; Bio-Rad Laboratories, Hercules, CA, USA). (C) After 24 h treatment with ibuprofen and diclofenac, the cells were stained with DiOC6 and DAPI. Morphological alteration of the cells was detected by fluorescent microscopy (400×). MTT,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; wt, wild type; DiOC6, 3,3′-dihexyloxacarbocyanine iodide; DAPI, 4′,6-diamidino-2-fenilindol.

3. Results

3.1. Forced p53 expression increased the cytotoxic effect of NSAIDs in dose-dependent manner

After stable transfection of cells with wt p53 plasmid, expression levels of p53 were determined by immunoblotting (Fig. 1A). The increasing concentrations of ibuprofen (0-1500 μM) or diclofenac (0-500 μM) were exposed to cells for 24 h and then we used MTT cell viability assay (Fig. 1B). While ibuprofen at 1 mM concentration was reducing cell viability by 25% in wt cells, this ratio was 40% in p53 + PC3 prostate cancer cells (each condition vs untreated control samples $P < 0.05$ *vs* untreated control). Similarly, diclofenac (250 μM) decreased cell viability by 60% in wt and 50% in p53 + PC3 cells (each $P < 0.05$ *vs* untreated control).



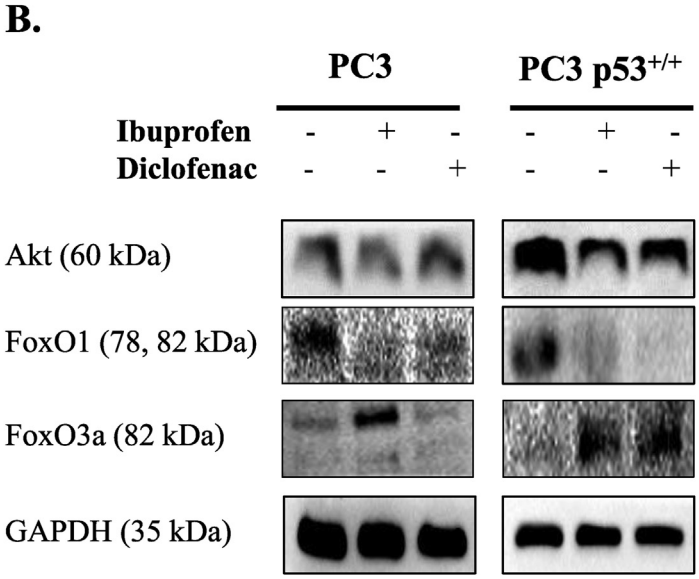


Fig. 2. Ibuprofen and diclofenac caused cell cycle arrest and modulated AKT–FoxO signaling axis in both cell lines. (A) Wt and p53 + PC3 prostate cancer cells were treated for 24 h with ibuprofen (1 mM) or diclofenac (250 μM) (A). Cells were labeled with propidium iodide and analyzed by using a FACS flow cytometer (BD Accuri) for 10x10. The image shown is representative of two experiments. (B) 60 μg of whole cell lysate were loaded in 12% SDS–PAGE gels and probed with AKT, FoxO1, and FoxO3. GAPDH was used as loading control. Wt, wild type; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FACS.

DiOC6 and DAPI costaining results confirmed the increased apoptotic cell death in both cell lines (Fig. 1C). DNA breaks were clearly determined after diclofenac treatment.

3.2. NSAIDs decreased AKT–-FoxO signaling axis

We found that ibuprofen and diclofenac treatment increased sub-G1 population by 7.5% and 6% compared with untreated cells in wt and p53 + PC3 prostate cancer cells, respectively. Exposure of cells to ibuprofen caused the cell cycle arrest at G1/S phase, but diclofenac was effective on G2/M phase to stop cell cycle in both cell lines (Fig. 2A).

We also checked the survival and cell death axis through investigating AKT and its downstream targets FoxO1 and FoxO3 in wt and p53 + PC3 cells. The basal expression levels of AKT were higher in PC3 p53 + cells compared with wt cells. NSAIDs down-regulated AKT expression levels, which led to diminished expression levels of FoxO1 in both cell lines. On the contrary, FoxO3 was upregulated after ibuprofen treatment in wt PC3 cells. Forced expression of p53 also potentiated the diclofenac-induced FoxO3 upregulation and ibuprofen treatment (Fig. 2B).

3.3. Ibuprofen and diclofenac caused apoptosis mechanism differs by presence of p53

Diclofenac induced apoptosis *via* activating caspase-8, which led to death domain kinase RIP cleavage in both cell lines; Fas expression level was further increased after ibuprofen treatment and both drugs could activate caspase-2. Therefore, we concluded that diclofenac treatment, but not ibuprofen, was effective to trigger intrinsic pathway of apoptosis through upregulation of Fas and cleavage of caspase-2. Both NSAIDs effectively trigger intrinsic apoptosis through inducing cleavage of caspase-9 and caspase-3 (Fig. 3A and B). We found that ibuprofen did not alter expression profile of Mcl-1 and Bcl-x, but diclofenac effectively downregulated expression levels of antiapoptotic Bcl-2 family members (Fig. 3C). Similar to these findings, we found that diclofenac upregulated Bax, Bak, and Puma. p53 + PC3 prostate cancer cells showed different expression levels for proapoptotic Bcl-2 family members. Although

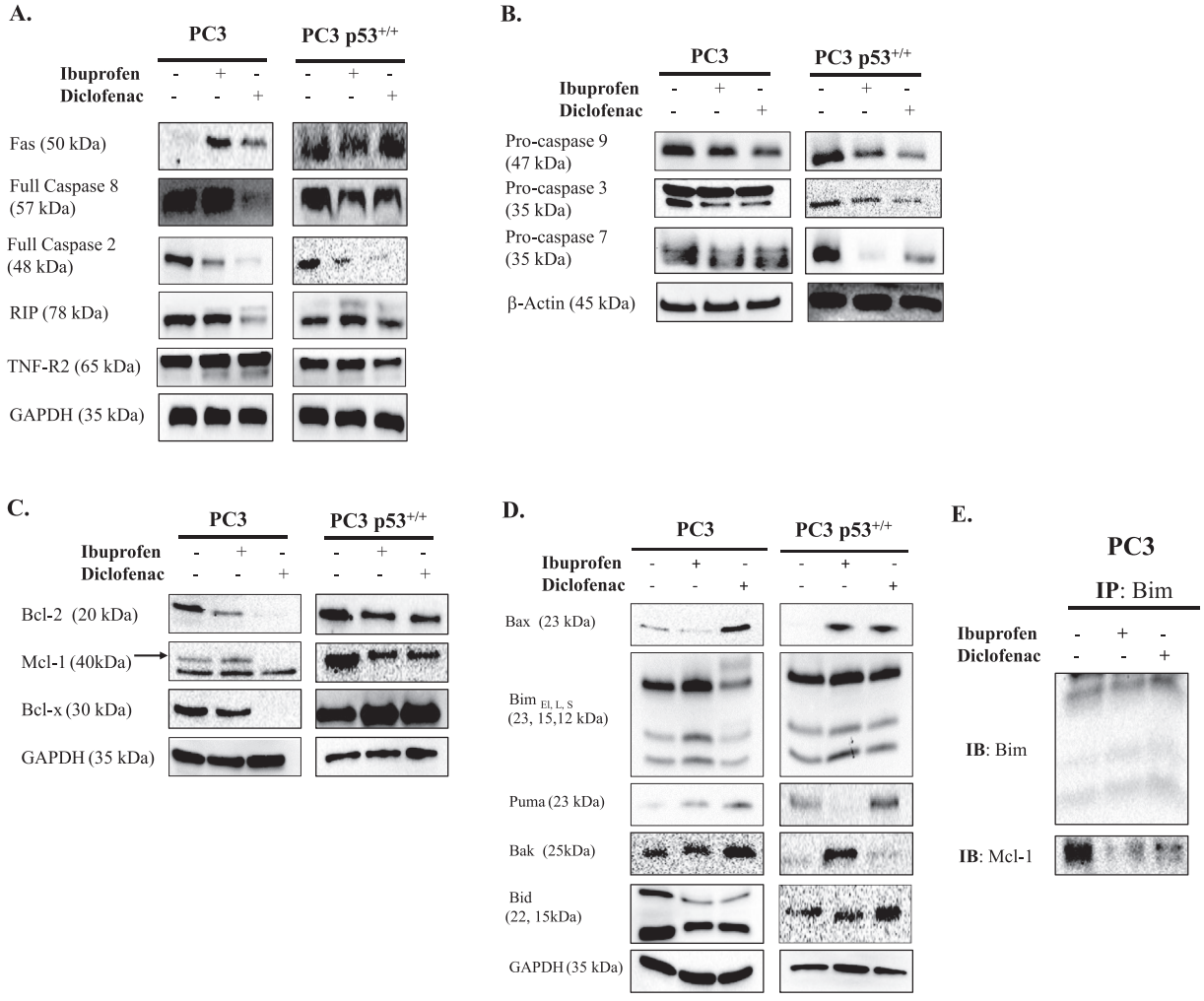


Fig. 3. Ibuprofen and diclofenac caused apoptosis mechanism differs by the presence of p53 expression. (A) To the sample, 60 μg of whole cell lysate were loaded in 12% SDS–PAGE gels and probed with Fas, full caspase-8, full caspase-2, TNF-R2, and RIP antibodies. GAPDH was used as loading control. (B) Same protocol (A) was used with procaspase-9 and procaspase-3. β-Actin was used as loading control. (C) Same protocol (A) was used with antiapoptotic Bcl-2 family members: Bcl-2, Mcl-1, and Bcl-x. GAPDH was used as loading control. (D) Same protocol (A) was used with proapoptotic protein antibodies: Bax, BimEL, Puma, Bak, Bid. (E) PC3 wt prostate cancer cells were subjected to lysis following drug treatment and immunoprecipitated with anti–Mcl-1 antibodies. Immunoprecipitated samples were observed at SDS–PAGE and Western blot analysis was performed for Bim. SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; wt, wild type.

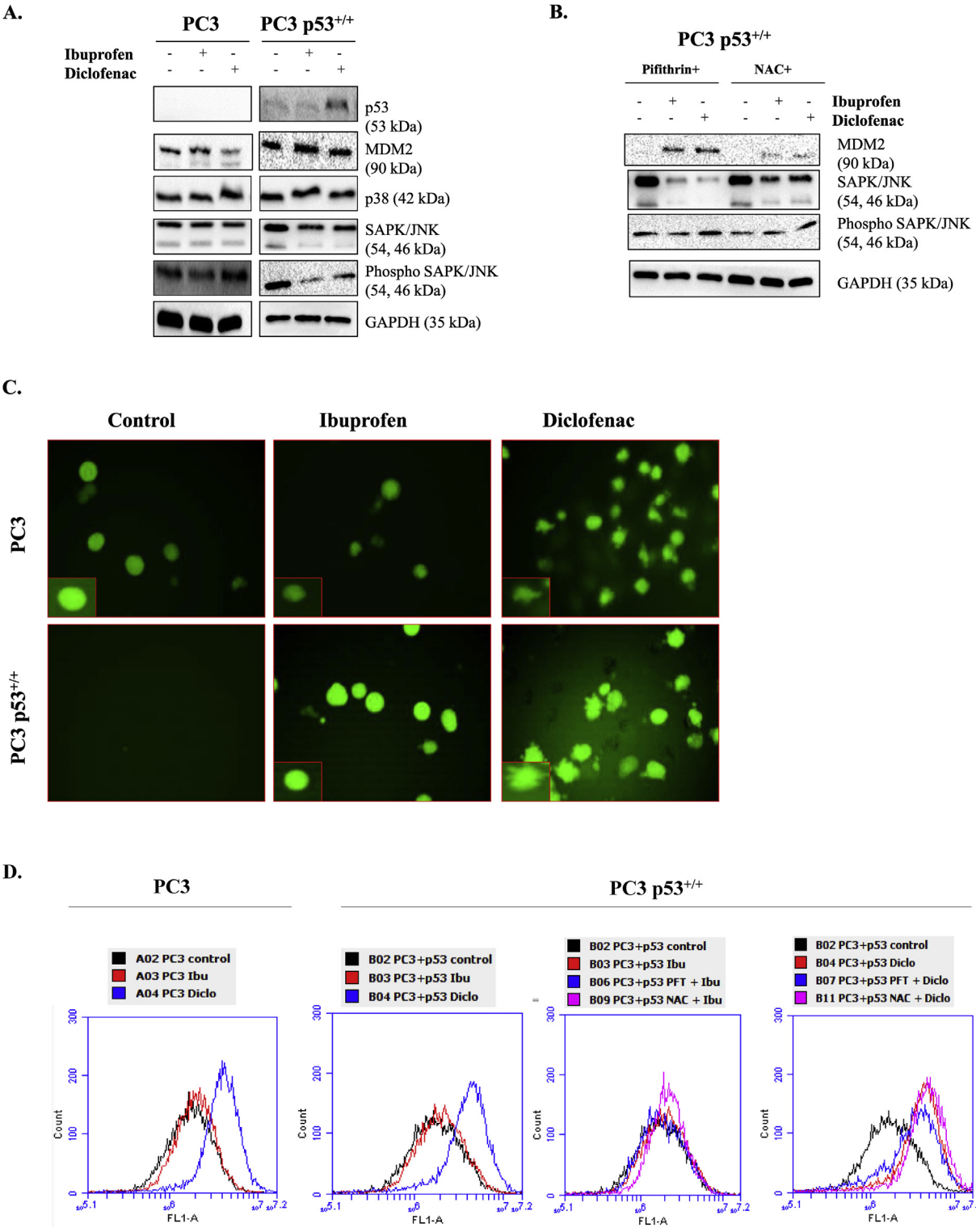


Fig. 4. Diclofenac but not ibuprofen induced ROS generation due to activation of SAPK/JNK. (A) To the sample, 60 μg of whole cell lysate were loaded in 12% SDS–PAGE gels and probed with p53, MDM-2, p38, SAPK/JNK, and phospho-SAPK/JNK (Thr183/Tyr185) in wt and p53 + PC3 cells. GAPDH was used as loading control. (B) Both cell lines were cotreated with pifithrin α (10 μM) or N-acetylcysteine (5 μM) with each NSAID for 24 h. The same protocol is applied as (A). (B) 2×10^5 wt and p53 + PC3 cells were cultured in 6-well plates and treated with ibuprofen and diclofenac for 24 h. After the treatment, cells were stained with 2′,7′–dichlorofluorescin diacetate (H2DCFDA) and 10000 events were analyzed by a flow cytometer. (C) After 24 h treatment with ibuprofen and diclofenac, cells were stained with H2DCFDA and screened by fluorescent microscopy (200×). NSAID, nonsteroidal antiinflammatory drug; ROS, rtecative oxygen species; wt, wild type.

p53 expression upregulated Bax in the presence of both NSAIDs, it was not effective in ibuprofen-induced Puma expression profile. In contrary, p53 prevented diclofenac-induced upregulation of Bak but forced p53 expression required ibuprofen-induced Bak upregulation. Bid cleavage was similar to caspase-2 activation. Ibuprofen upregulated BimL, but the presence of p53 prevented BimL upregulation in PC3 p53 + cells (Fig. 3D). We determined that both drugs induced cleavage of Mcl-1, which led to release of Bim to induce mitochondria-mediated apoptotic machinery (Fig. 3E).

3.4. Diclofenac but not ibuprofen-triggered reactive oxygen species generation led to activation of SAPK/JNK

Diclofenac is a potent Superoxide dismutase 2-2 inhibitor, led to higher generation of ROS, upregulated oxidative stress marker p53, and downregulated MDM-2 in remarkable in PC3 cells. Ibuprofen did not alter MDM-2 expression levels in both cell lines. The inhibition of SAPK/JNK activation was clear in p53 + PC3 cells (Fig. 4A). When we inhibited p53 activity and/or ROS generation *via* cotreated cells pifithrin-α (10 μM) or N-acetylcysteine (NAC, 5 μM), NAC cotreatment with each drug caused a sharp decrease in MDM-2 expression level. Pifithrin was less effective on MDM-2 expression, which confirmed the p53 independent effects of drugs in p53 + PC3 cells. On the contrary, NAC pifithrin cotreatment with NSAIDs downregulated SAPK/JNK levels (Fig. 4B). Diclofenac triggered ROS generation more than ibuprofen, regardless of p53 expression (Fig. 4C and D). Inhibition of p53 activity by pifithrin-α treatment or inhibition ROS generation by NAC treatment did not overcome diclofenac-induced ROS generation p53 + PC3 cells.

3.5. NSAIDs altered Wnt signalling and β-catenin nuclear translocation

While ectopic p53 expression increased Axin expression level compared with wt PC3 cells, ibuprofen overcame this effect in wt and p53 + PC3 cells. In contrary, diclofenac upregulated Axin expression levels in wt PC3 cells but not in p53 + PC3 cells. Concomitantly, p53 prevented Wnt5a/b overexpression profile in PC3 cells. Although NSAIDs did not alter Wnt5a/b expression profile, in the presence of p53, Wnt5a/b levels were downregulated after ibuprofen treatment. Dvl2 and Dvl3 are the homologous of dishvelled protein that were upregulated after drug treatment. Functional p53 decreased Dvl3 but not Dvl2, when the cells were exposed each NSAID (Fig. 5A). While diclofenac caused upregulation of β-catenin, ibuprofen did not exert similar effect on wt PC3 cells. The expression levels of Wnt11 were remarkably higher in wt PC3 cells than in p53 + cells, and diclofenac downregulated Wnt11. When the p53 + cells were exposed to ibuprofen, Wnt11 expression level was increased. Ibuprofen upregulated LRP6 expression levels in both cell lines. Thus, we figured out the potential effect of β-catenin nuclear translocation, which led to transcription of a number of genes related to Wnt signalling (Fig. 5B). After isolation of nuclear and cytoplasmic extracts in all experimental conditions, we found that NSAIDs effectively triggered β-catenin translocation into the nucleus in PC3 cells. Forced p53 expression in PC3 cells did not alter drug-induced β-catenin nuclear translocation. This effect was confirmed by epithelial mesenchymal transition (EMT) progression triggered by diclofenac by upregulation of N-cadherin, vimentin, and Snail and led to mesenchymal profile in residing cells in the absence of p53 (Fig. 6A and B). Diclofenac was more effective compared with ibuprofen treatment to prevent *in vitro* wound closure in wt PC3 cells but not in p53 + PC3 cells (Fig. 6B). Colony formation assay confirmed similar results (Fig. 7).

4. Discussion

Highly expressed COX-2 in cancer cells led to inhibition of p53 transcriptional activity, alteration of p53 subcellular localization, and apoptosis.[17,22] Ibuprofen triggered cell cycle arrest at G1/S phase, which led to apoptosis.[23] It was shown that both induction of a cell cycle blockage and apoptosis after ibuprofen treatment was

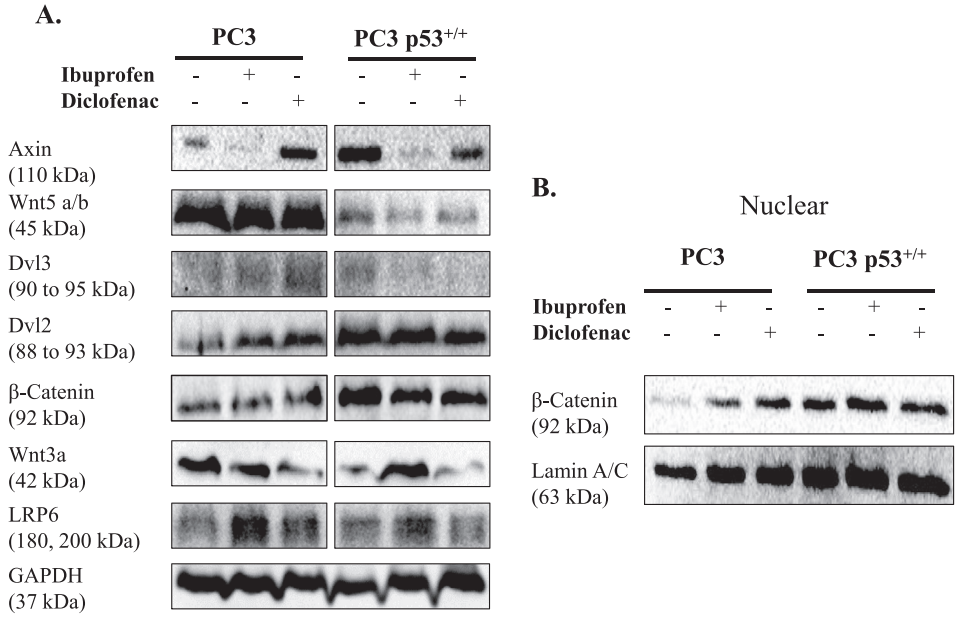


Fig. 5. p53 is effective to modulate Wnt signaling after ibuprofen and diclofenac treatment wt and p53 + PC3 prostate cells. (A) Immunoblotting results were shown for Wnt signaling pathway key targets: Axin, Wnt5a/b, Dvl3, Dvl2, b-catenin, Wnt11, and LRP6. GAPDH was used as loading control. (B) β-Catenin nuclear translocation was detected via immunoblotting method. Lamin A/C was used for nuclear loading control. Wt, wild type.

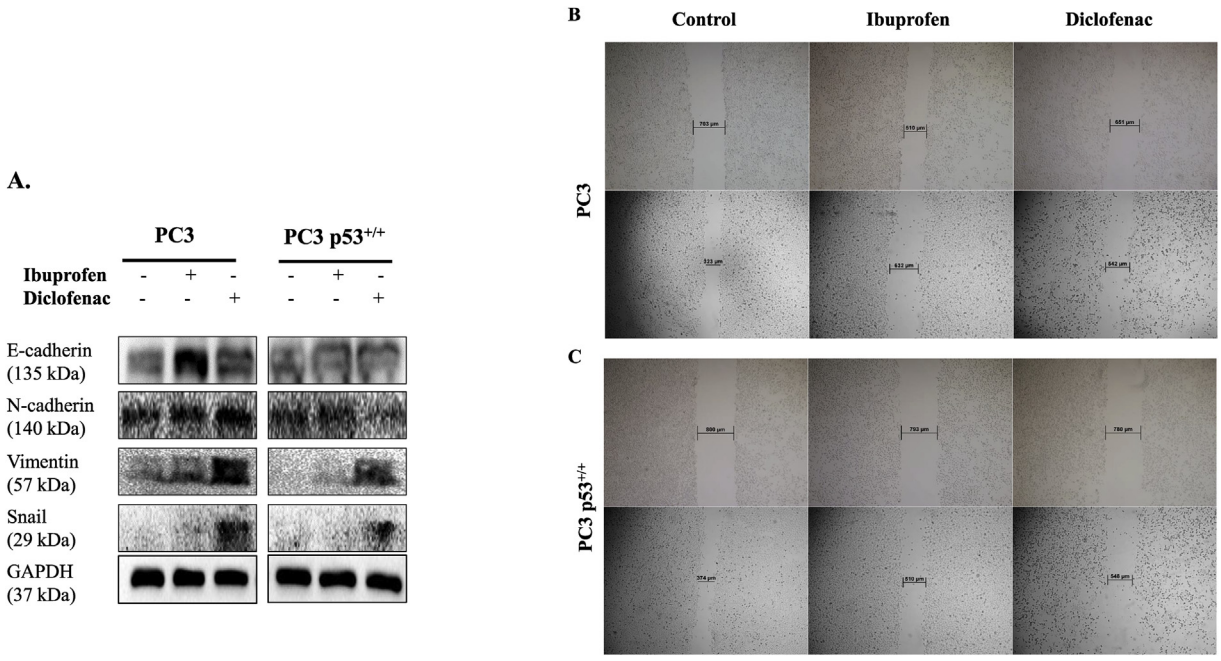


Fig. 6. The effect of p53 on ibuprofen and diclofenac-treated PC3 cells on EMT and wound closure. (A) To the sample, 60 μg of whole cell lysate were loaded in 12% SDS–PAGE gels and probed with E-cadherin, N-cadherin, vimentin, and Snail antibodies. GAPDH was used as equal loading control. (B–C) Images of wound closure assays (100× magnification). Cells were seeded into 6-well cell culture plates, cultured in RPMI1640 supplemented with 10% FBS, and allowed to grow to near confluence. Confluent monolayers were wounded via 200 μl micropipette tip, then the cellular debris was gently washed away with 1× PBS. The wounded cell monolayer for both wt (B) and p53+ (C) PC3 cells were treated with each drug for 24 h. All pictures were taken after 24 h drug treatment. Wound closure assay is repeated by two-independent assays and representative images are shown. SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EMT, epithelial mesenchymal transition; PBS, phosphate-buffered saline.

partly p53-dependent and accompanied by an accumulation of p53 and translocation of Bax to mitochondria.

Both drugs triggered similar responses in the cells to activate apoptosis through cell cycle arrest, increasing ROS generation and caspase activation and modulating Bcl-2 family members. Similar finding was shown in HeLa cervical cancer cells after celecoxib treatment.[27] The prevention of binding of Mcl-1 to Bim led to increased apoptotic ratio in PC3 cells. Because the activation of extrinsic pathway key players were clear following drug treatment, we concluded that NSAIDs were more effective on extrinsic pathways when the cells lacked p53 expression with high ROS generation profile.[11,28,29]

Downregulation of AKT was detected in our experiments, regardless of p53 expression. We found that downstream target of AKT, FoxO3a, was critical to distinguish the molecular targets of ibuprofen and diclofenac in PC3 cells in the presence or absence of p53. Although ibuprofen increased FoxO3a expression levels in wt and p53 + PC3 cells, diclofenac was only effective to increase FoxO3a levels in the presence of p53. Thus, we concluded that ectopic expression of p53 further increased FoxO3a expression levels after drug treatment. A previous study showed that a number of NSAIDs increased FoxO3a levels because of inhibition of AKT and led to upregulation of p27Kip to decrease human osteoblast cell proliferation.[24] Although FoxO family members could increase the SOD-2 expression level[25] to overcome oxidative stress p53 independently in C2C12 mouse myoblasts,[26] in our study, we found that existence of p53-activated diclofenac induced FoxO3a upregulation in PC3 prostate cancer cells.

COX inhibitors alone and in combination with chemotherapeutic agents downregulated p53 negative regulator MDM2 levels *via* reduction of p-MDM2 (Ser166) levels, which correlates with the early increase in p53 nuclear localization. Ibuprofen downregulated MDM2 but SOD-2 inhibitor diclofenac did not exert the same effect in PC3 cells, which might indicate its p53 independent therapeutic effect.

We found that diclofenac upregulated Axin, Dvl3, and Dvl2 and downregulated Wnt3a in PC3 prostate cancer cells. The presence of p53 in the cells caused higher β-catenin translocation into nucleus and decreased Axin and Dvl3 expression levels after diclofenac treatment. Ibuprofen exerted a different effect *via* downregulating Axin and upregulating LRP6 in PC3 prostate cancer cells. p53 did not alter ibuprofen-modulated gene expression profile. Axin is a negative regulator of Wnt signaling pathway and regulates the β-catenin levels in the cells. Diclofenac-mediated downregulation might increase the β-catenin nuclear translocation into nucleus.[31] Although FoxO transcription factors are reported to sequester β-catenin away from the T-cell factor (TCF/LEF) transcription factors.[32] It was also shown that the binding of β-catenin to FoxO family members caused cell cycle arrest.[33] Although β-catenin, in combination with its downstream TCF, has been implicated in poor prognosis, FoxO family members could counteract tumor progression by sequestering β-catenin away from TCF. However, ibuprofen and diclofenac treatment caused a similar β-catenin nuclear translocation profile in both cell lines, although they exerted opposite effect on Axin regulation. This finding was also contrast to previous findings shown in U87 and U251 glioblastoma multiforme cell lines with an attenuated[34] Wnt/β-catenin/TCF signaling pathway. Deregulated β-catenin led to decrease in tumor suppressor function of p53. Thereby, the elimination of p53 in the β-catenin high-expression profile in a number or cancer types is a

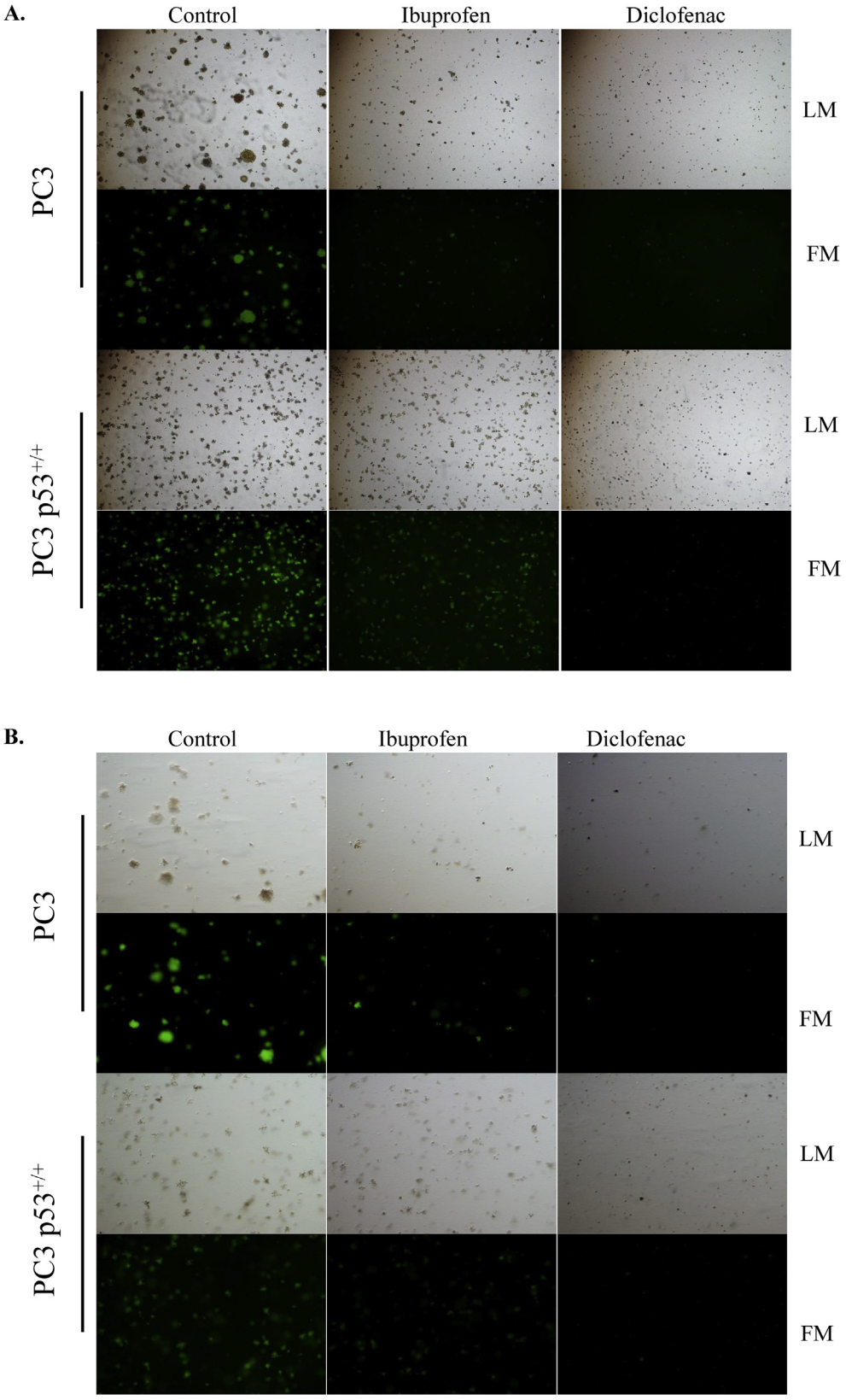


Fig. 7. p53 alters the effect of NSAIDs on colony formation. The effect of ibuprofen and diclofenac on colony formation in soft agar was observed by fluorescence microscopy. (A-B) 5×10^4 wt and p53 + PC3 cells were cultured in 6-well plates which contained soft agar and treated with ibuprofen and diclofenac for 24 h. After the treatment, cells were stained with DiOC6. After 24 h treatment with ibuprofen and diclofenac, media was changed with fresh media. Two (A) and seven days later (B), penetrated cells in soft agar and formed colonies were observed by fluorescence microscopy (FM). NSAIDs, nonsteroidal antiinflammatory drugs; DiOC6, 3,3′-dihexyloxacarbocyanine iodide.

difficulty. It might be a reason that low-expression levels of p53 did not negatively regulate β-catenin and the downregulation of Axin after diclofenac treatment in p53 + PC3 cell lines led to β-catenin translocation into nucleus.

p53 prevented diclofenac-induced EMT in wt PC3 prostate cancer cells. Similar finding with our observation showed diclofenac-delayed wound closure in connective or corneal tissue.[35] All these findings indicated that diclofenac altered metastatic phenotype, induced apoptosis, while cells were scattered to distant places. The less stained DiOC6 results confirmed the increased apoptosis in soft agar inner layer colonies. In addition, it is noteworthy that p53-expressed colonies were found invasive with higher diameter compared with wt PC3 cells. In conclusion, the distinct biological targets of ibuprofen and diclofenac should be elucidated to evaluate therapeutic potential in a clinical setting. The obtained results from this study indicate that both NSAIDs are promising therapeutic agents that target EMT pathway players to prevent invasion and metastasis capacity of cancer cells.

Conflict of interest

All authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.prnil.2019.09.003.

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