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

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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 through suppression of inflammation due to over-expression of cyclooxygenases (COXs). 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. Recent reports approved that long-term use of low-dose NSAIDs improves prognosis of disease with chemopreventive efficiency. Single or combinational administration of NSAIDs with chemotherapeutics induced apoptosis in leukemic, colon, prostate, and lung cancer cells. 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 kinase B (Akt) and caspase-3 activation. 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. Celecoxib increased the expression of p53 in LNCaP prostate cancer cells, and silencing of p53 in LNCaP cells prevented celecoxib-induced cell cycle arrest.
induced cell death. 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. It is not clear that how p53 regulated COX-2 expression in a positive or negative manner.

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-phenilindol (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, RIP, TNF-R2, pro-caspase-9, pro-caspase-3, Bcl-2, Mcl-1, Bcl-x, Bax, Bik, EMFL, Puma, Bak, Bid, p38, SAPK/JNK, p-SAPK/JNK, Axin, Wnt5a/b, Dvl3, Dvl2, β-catenin, Wnt3a, LRPS, 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-diphenyl-tetrazolium bromide (MTT, Sigma-Alrich, St. Louis, MO, USA) cell viability assay in accordance with the manufacturer instructions. DiOC6 and DAPI staining 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-Alrich, 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 horse-radish peroxidase–conjugated secondary antibodies at 4°C overnight. ChemiDoc™MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) 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 MgCl2, 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 × 105 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 °C 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 than 0.05.
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). 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 downregulated 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...
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 piptithrin (10 μM) or N-acetylcysteine (5 μM) with each NSAID for 24 h. The same protocol is applied as (A). (B) Both cell lines were cotreated with ibuprofen and diclofenac for 24 h. After the treatment, cells were stained with 2',7'-dichlorofluorescin diacetate (H2DCFDA) and 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, reactive 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 homologues of dishwelled 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 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.](image-url)
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+/C2 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 level25 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 b-catenin translocation into nucleus and decreased Axin and Dvl 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 b-catenin levels in the cells. Diclofenac-mediated downregulation might increase the b-catenin nuclear translocation into nucleus.31 Although FoxO transcription factors are reported to sequester b-catenin away from the T-cell factor (TCF/LEF) transcription factors.32 It was also shown that the binding of b-catenin to FoxO family members caused cell cycle arrest.33 Although b-catenin, in combination with its downstream TCF, has been implicated in poor prognosis, FoxO family members could counteract tumor progression by sequestering b-catenin away from TCF. However, ibuprofen and diclofenac treatment caused a similar b-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 attenuated34 Wnt/b-catenin/TCF signaling pathway. Deregulated b-catenin led to decrease in tumor suppressor function of p53. Thereby, the elimination of p53 in the b-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 × 10^5 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. 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 conclusion, it is noteworthy that p53-expressed colonies were found invasive with higher diameter compared with wt PC3 cells. In addition, it is noted that inflammation under supervision of EDA, is advisor.

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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