약학석사학위논문

NSAIDs inhibit autophagy flux and sensitize anti-tumor agent induced cell death to cancer cell lines

NSAIDs의 자가포식 억제에 의한 항암 화학요법제의 상승작용 규명

2018년 2월

서울대학교 대학원

약학과 예방약학전공

박 승 현

Abstract

NSAIDs inhibit autophagy flux and sensitize anti-tumor agent induced cell death to cancer cell lines

Seung Hyeon Park

Dept. of Pharmacy

The Graduate School

Seoul National University

Autophagy is a process in which double-membraned autophagosomes digest unnecessary organelles or proteins in cells and decompose them through lysosomes. If they do not function normally, the homeostasis of the cells is broken and causes various diseases. In addition, the activity of autophagy was significantly increased in the stress conditions such as hypoxia, chemotherapy, and irradiation in cancer cells, and the cancer cells had resistance to the anticancer drugs through the increased autophagy activity. Therefore, when a drug that inhibits autophagy is administered in

combination with an anticancer drug, an increased effect can be expected.

Previous studies have shown that diclofenac, one of the NSAIDs, inhibits autophagy. Therefore, the effect of the other 20 NSAIDs on the autophagy was confirmed by Western blotting with the half-value of IC50 in mouse normal hepatocyte AML12, and the autophagy markers LC3 and p62 were screened. Cytotoxicity of combination of 5 NSAIDs (diclofenac, aceclofenac, naproxen, dexibuprofen, flurbiprofen) with sorafenib, tamoxifen, 5-FU, and paclitaxel, in liver cancer cell lines HepG2 and Huh7, breast cancer cell line mcf7, colon cancer cell line HCT116 and lung cancer cell line A549 were observed through HCS instrument cyation. In addition, the combination of diclofenac and sorafenib in Huh7 was associated with changes in apoptosis through annexin V-PI assay, cleaved caspase 3, cleaved PARP and Bcl-2.

Most of the NSAIDs showed autophagy inhibitory activity as a result of screening in AML12. The combination of sorafenib or tamoxifen with diclofenac, aceclofenac, naproxen, dexibuprofen, and flurbiprofen in HepG2 and mcf7, combination of sorafenib and diclofenac in Huh7, diclofenac, aceclofenac, dexibuprofen and 5-FU in HCT116, a combination of dexibuprofen, naproxen and paclitaxel in A549, showed a large synergistic effect of cell death. In addition, the cotreatment of diclofenac and sorafenib in Huh7 significantly increased apoptosis by increased apoptosis positive cells and increased apoptosis marker cleaved caspase 3, cleaved PARP also

decrease of Bcl-2.

As a result, diclofenac inhibited autophagy in hepatocellular carcinoma cells with increased autophagy activity through anticancer drugs, and it was confirmed that this increased cancer cell death. In addition, other NSAIDs show autophagy inhibiting activity, and it is presumed that increasing cell death when combined with an anticancer agent is due to autophagy inhibition. Therefore, it is expected that the combined use of NSAIDs and anticancer drugs will increase the susceptibility of existing chemotherapeutic agents to cancer treatment.

keywords : autophagy, apoptosis, NSAIDs, diclofenac, sorafenib

Student Number : 2016-21816

Contents

List of Figures

List of Tables

Ⅰ. Introduction

Autophagy is an evolutionarily conserved cellular process that is used for the turnover of organelles and proteins or functions to generate sources of metabolic fuel under stress conditions (1, 2). Also several studies verified that autophagy functions as a mechanism of tumor suppression via the removal of defective premalignant factors in cells. Numerous evidence supports a major role for autophagic degradation in the maintenance of bioenergetic homeostasis under stress conditions, including hypoxia and nutrient deprivation (3). Additionally, autophagy has emerged as an important mechanism of resistance to radiation, chemotherapy, and anticancer agents by its ability to enhance the survival capacity of cancer cells (4–8). Therefore, there has been an attempt to increase the effectiveness of chemotherapy by inhibiting autophagy.

Chloroquine (CQ) and hydroxychloroquine (HCQ) have been used to treat malaria, rheumatoid arthritis, and lupus. And Chloroquine (CQ) and hydroxychloroquine (HCQ) also known for representatively disrupting lysosomal function and consequently inhibiting autophagy (6). These specific properties of CQ/HCQ spurred numerous preclinical investigations focused on establishing the safety and therapeutic benefit of inhibiting autophagy to increase the efficacy of a diverse range of anticancer agents (9, 10). Based on the positive impact of HCQ in this theory, a series of

phase I and phase I/II trials to investigate the safety and preliminary efficacy of the addition of HCQ to existing anticancer regimens were initiated. (11–16). Although preliminary efficacy has been observed in a small number of patients with the addition of HCQ generally safe and treated with HCQ–based therapy, HCQ fails to inhibit autophagy in acidic environments around 6.5, due to the decrease in the cellular uptake of the drug in these environments. The limitation of HCQ makes a demand for the production of more potent autophagy inhibitors.(17)

Therefore, the need for other autophagy inhibitors has been emphasized, and in a previous study conducted in the laboratory, diclofenac, a drug of NSAIDs, confirmed the autophagy inhibitory effect and screened the autophagy inhibitory activity against the entire NSAIDs.

Non–steroidal anti–inflammatory drugs (NSAIDs) are the most frequently used medicines worldwide (18). Several epidemiological studies have shown that long–term use of NSAIDs is associated with a low incidence of benign polyps or colon cancer. (19, 20)It is also associated with lowering the incidence of various cancers such as breast cancer, lung cancer, prostate cancer, and bladder cancer ovarian cancer as well as colorectal cancer. (21–26)It is also reported that clinical trials have been shown to reduce the incidence of advanced colorectal cancer or breast cancer and to inhibit metastasis of primary cancer.(27)

These anticancer effects of NSAIDs were thought to occur through inhibition of COX. Echosanoids such as PGE2 synthesized through COX are often observed to be increased in cancer tissues and are known to play roles such as cell division, angiogenesis and metastasis, and apoptosis. (28)

However, it has been suggested that COX inhibition is not a major mechanism of NSAIDs' antitumor activity through the fact that NSAIDs have much higher anticancer activity than COX activity inhibition. It has also been shown that NSAIDs have anticancer effects through the metabolism or isomerism of NSAIDs that have no COX inhibitory effect. Further studies revealed the COX-independent mechanisms which are Wnt / b-catenin signaling inhibition, cGMP phosphodiesterase inhibition, NF-kB, AMPK inhibition and increased activity of PPARg and PXRa.(29)

In this study, we have found additional autophagy inhibition as a possible antitumor drug for NSAIDs. And combination of anticancer drug with NSAIDs increased cytotoxicity synergistically. Therefore, we anticipate that the addition of NSAIDs to anticancer adjuvant therapy will provide more effective antitumor effects.

Ⅱ. Material and Methods

1. Reagents

cleaved caspase-3 kit, QuantiTect reverse transcription kit was acquired from Quiagen(Hilden, Germany) and EASY-BLUE was acquired from Intron biotechnology(Seongnam, Korea). All chemicals used were of the highest purity and grade available

2. Cell culture

AML12, HepG2, Huh7, MCF7, HCT116, A549 was obtained from American Type Culture Collection(ATCC, Rockville, MD). Every cell was grown at 37℃ in a 5% CO2 atmosphere. Growth medium for AML12 cells was DMEM(Gibco BRL, Grand Island, NY) with 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL, selenium(Insulin-Transferrin-Selenium, Gibco BRL), and 40 ng/mL dexamethasone(Sigma), and DMEM(Hyclone BRL) for HepG2, MCF7, HCT116 and RPMI1640(Gibco BRL) for Huh7 and A549. All medium was supplemented by 10% of heat inactivated fetal bovine serum(FBS; Gibco BRL), 50 units/ml of penicillin, and 50 units/ml of streptomycin(Antibiotic-antimycotic; Gibo BRL)

3. Cell viability measurement

AML12 cells were plated in 96-well plates at a density of 6000 cells per well and allowed to attach overnight. After treatment with chemical for 24hr, cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) cell proliferation assay. After washing the compound-containing medium, a working solution of MTT was added to each well and incubated for 1hr. Then the MTT solution was removed and DMSO(Dimethyl sulfoxide) was added and incubated for an additional 15 minutes and absorbance was measured at a wavelength of 540nm. The concentration required for 50% inhibition of growth (IC50) was determined by nonlinear regression analysis using the GraphPad PRISM statistics software package(Ver. 5.0; San Diego, CA)

4. Protein Extraction and Western Blot Analysis

Total cell lysate were prepared for Western immunoblotting using lysis buffer containing 50mM HEPES, 150mM NaCl, 1% Triton X-100. 5mM EGTA, 50mM glycerophosphate, 20mM NaF, 1mM Na3VO4 and 2mM PMSF. Equal amount of protein samples were seperated via SDS-polyacrylamid gel eletrophoresis and

transferred to a PVDF membrane (Millipore, Bedford, MA). Primary antibodies include anti-NBR1 from Santacruz (CA, USA); anti-LC3B, anti-p62, anti-B-actin, anti-PARP, anti-Caspase 3 and anti-Bcl-2 from Cell Signaling Technology (MA, USA) were used in immunoblot. The immunoreactive protein was detected using ECL Western blotting detection reagent (Amersham, Piscataway, NJ) with Universal hood ‖ Chemidoc (Bio-Rad, Hercules, CA, USA). Quantification of protein bands was done using Quantity One software (Ver. 4.6.2.) form Bio-Rad (Hercules, CA)

5. RNA Isolation and Quantitative Real Time - Polymerase Chain Reaction (qRT-PCR)

RNA was isolated using 0.5ml of EASY-BLUE per 10^5 cells and dissolved in nuclear free water. The quantity of total RNA from each condition was measured by ultraviolet spectrophotometry (ND-1000 spectrophotometer; Thermo, Wilmington, DE, USA), by absorption at 260 and 280 wavelengths. The quantified RNA was used to synthesize single-strand cDNA using QuantiTect Reverse Transcription kit First-Strand Synthesis System for RT-PCR (Quiagen, Hilden, Germany). Gene-specific primers were designed using Primer Premier (Premier Biosoft international, Palo Alto, CA, USA). Quantitative real time RT-PCR was performed using iTaq Universal SYBR® Green Supermix kit (Bio-Rad, Hercules, CA) in

a StepOne Real-Time PCR System (Applied Biosystems, Seoul, Korea) following manufacurer's protocol using specific primers for LC3B, p62, NBR1, B-actin. Relative changes in gene expression were expressed as the fold change using 2-⊿⊿CT method

6. GFP-LC3(mCherry GFP-LC3) transfection and imaging

6 well plates were seeded 5.0 x 10^5 cells per well. Cell were stablized for 24hr and incubated in OPTI-MEM medium (GIBCO BRL) for 1hr. 1.5ug of GFP-LC3 were transfected to cells using Lipofectamine 2000 reagent(invitrogen, CA) according to the supplier's protocol. After 4hr of incubation, medium was exchanged to complete medium containing 10% serum and antibiotics. The cells were additional 24hr an treated as indicated and observed under BioTek Cytation 3 Cell Imaging Multi-Mode Reader.

7. Imaging of cell proliferation and cytotoxicity using HCS

Each cell line was plated at 10000 cells per well in Nunc™ MicroWell™ 96-Well Optical-Bottom Plates with Polymer

Baseandculturedovernight. Only the inner 60 wells of 96-well microplates were used due to evaporation-related edge effects in the outside wells. The following day, each concentration of drug containing media with 100nM YOYO-1 was treated in 100uL per well. Each concentration was triplicated. Then incubate the plate for 30 min. After incubation, starting capture the images which was center of each well using bright filter and GFP filter for 48hours at every 2hrs by BioTek Cytation 3 Cell Imaging Multi-Mode Reader.

8. Calculation of cell death and cell proliferation

In Bright field images, all cell objects were counted and in GFP filter image YOYO-1 positive objects which mean dead cells were counted at each time point. Then "% of dead cell" is calculated by YOYO-1 positive objects per all cell objects. Using "% of dead cell", compare cytotoxicity of each drug or durg combination. Cell proliferation is calculated by dividing cell object number of each time point by the cell object number of the first time point (0hr) and then define the cell number of the first time point is 100%.

Ⅲ. Result

1. Most NSAIDs increases autophagy cargo receptor p62 protein.

To investigate activity of autophagy inhibition on NSAIDs, we determined treatment concentration using IC50 of NSAIDs. (Table 1) The half value of IC50 is maximum concentration which does not induce critical damage to cell and 0, 0.25, 0.5 and 0.75 of 0.5 IC50 were treated to AML12 cell for 24hr.

We used autophagosome marker LC3B and autophagy cargo receptor protein p62 as autophagy marker proteins. There are 4 classes of NSAIDs by structure. They are acetic acid derivatives, propionic acid derivatives, enolic acid(oxicam) derivatives and selecitve cox-2 inhibitors. Most tested NSAIDs increase p62 protein by concentration dependently. p62 is a substrate of autophagy so accumulation of p62 means inhibition of autophagy. (30) But patterns of LC3B changing were different by classes of NSAIDs. Acetic acid derivatives, enolic acid derivatives and selective cox-2 inhibitor generally increase LC3B protein. And propionic acid derivatives decrease LC3B.

Table 1. IC50 and 0.5 IC50 of NSAIDs IC50 of NSAIDs is determined by MTT assay in AML12 for 24hr. (n=6)

Class	Name	IC_{50}	0.5 IC_{50}
Acetic acid derivatives	Diclofenac	0.58mM	0.29mM
	Aceclofenac	1.6mM	0.8mM
	Etodolac	3.2mM	1.6mM
	Sulindac	3.2mM	1.6mM
	Indomethacin	2.3mM	1.15mM
	Ketolorac	≈10mM	5mM
	Nabumetone	2.6mM	1.3mM
Propionic acid derivatives	Dexibuprofen	7.2mM	3.6mM
	Naproxen	10.6mM	5.3mM
	Flurbiprofen	2.6mM	1.3mM
	Ketoprofen	8.7mM	4.4mM
	Loxoprofen	12.6mM	6.3mM
	Fenoprofen	1.4mM	0.7mM
	Oxaprozin	0.62mM	0.31mM
Enolic acid (Oxicam) derivatives	Piroxicam	4.8mM	2.4mM
	Lornoxicam	0.47mM	0.24mM
	Meloxicam	1.8mM	0.9mM
	Tenoxicam	2.4mM	1.2mM
Selective COX-2 inhibitor	Celecoxib	0.78mM	0.39mM

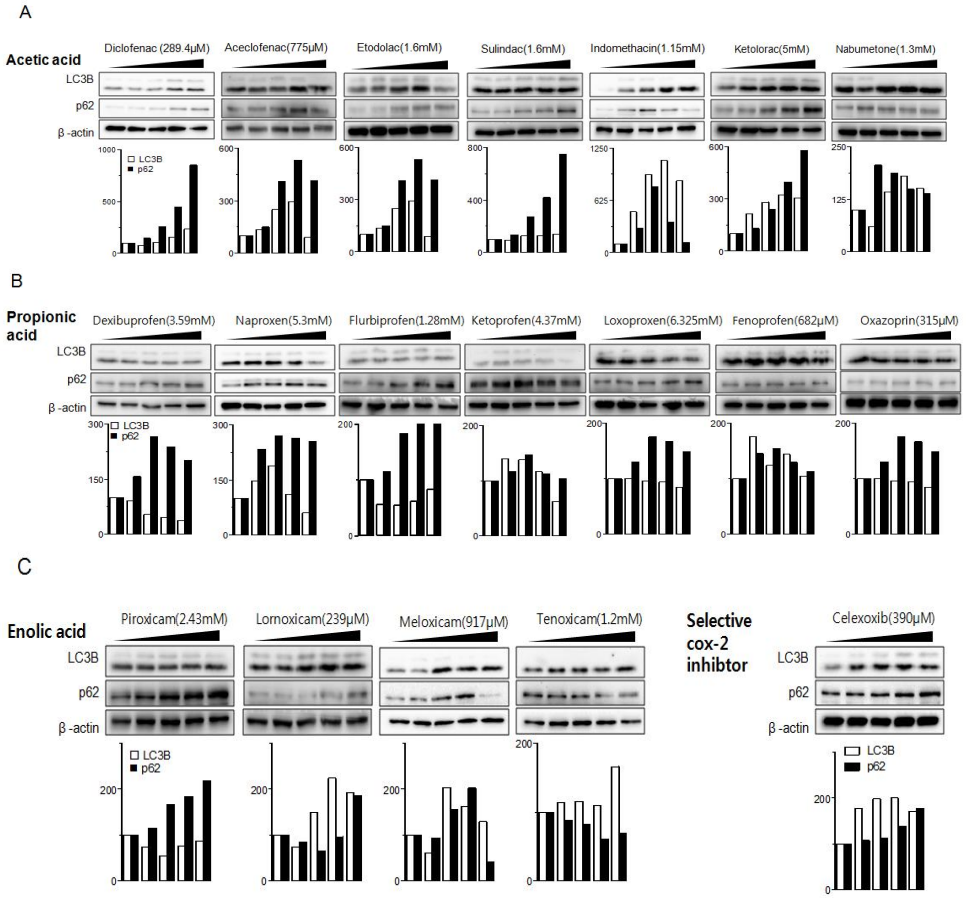


Figure 1. Most NSAIDs increases autophagy cargo receptor p62 protein.

(A) Autophagy marker LC3B, p62 is measured by western blot in AML12 for 24hrs. Concentration is 0, 0.25, 0.5, 0.75 and 1X of 0.5 IC50.(n=3) 7 acetic NSAIDs derivatives. (B) 7 propionic acid derivatives. (C) 4 enolic acid derivatives and 1 selecti cox-2 inhibitor

2. 6 acetic acid NSAIDs and 3 propionic acid NSAIDs inhibit autophagy flux.

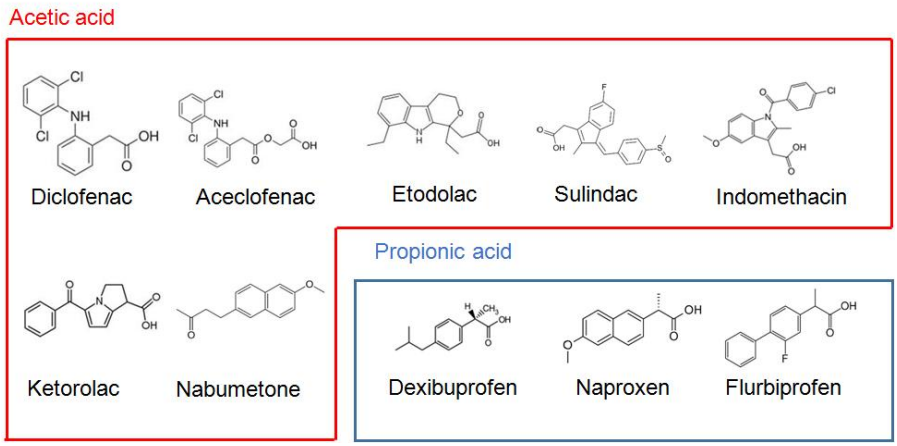
To further investigation of autophagy inhibition, we selected 6 acetic acid derivatives (aceclofenac, etodolac, sulindac, indomethacin, ketorolac and nabumetone) and 3 propionic acid(dexibuprofen, naproxen and flurbiprofen) derivatives NSAIDs which show strong autophgy inhibition acitiviy in western blot. (Figure 2A)

To confirm whether increased p62 protein is autophagy inhibition or upregulated gene expression, p62 mRNA expression was identified in 9 NSAIDs. There was no increased mRNA expression of p62 in propionic acid derivatives. But contrary to expectation, mRNA of p62 were upregulated in acetic acid derivatives. (Figure 2B) So we found another cargo receptor protein, NBR1. NBR1 acts as like p62 in autophagy process. We investigated protein level and mRNA expression of NBR1. Aceclofenac, indomethacin, ketorolac and dexibuprofen induced slight increase of NBR1 mRNA expression, but amount of increasing protein level was more than that of mRNA. (Figure 2C)

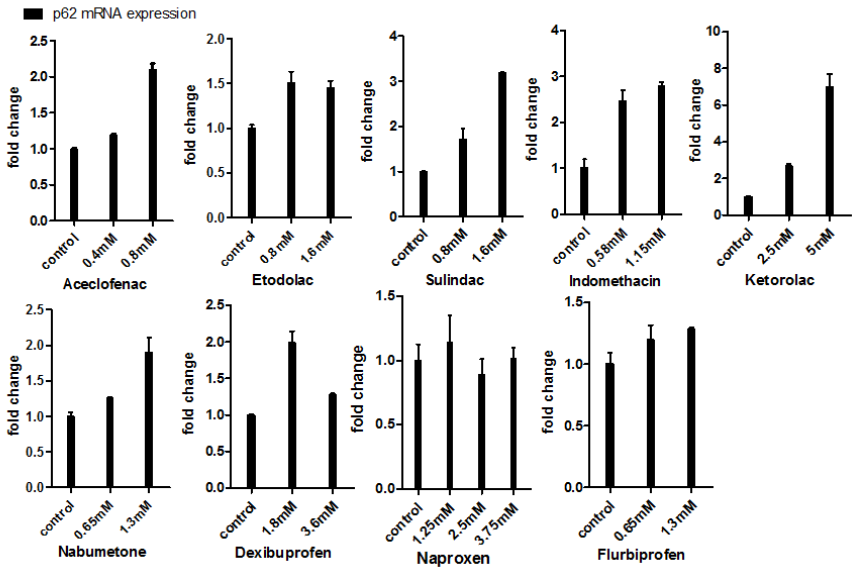
Then we verified autophagosome and autolysosome accumulation using GFP-LC3 vector and mCherry-GFP-LC3 vector. (Figure 2E, 2F) All acetic acid NSAIDs except nabumetone and flurbiprofen increased GFP dots(Figure 2E) and yellow dots. (Figure 2F) It

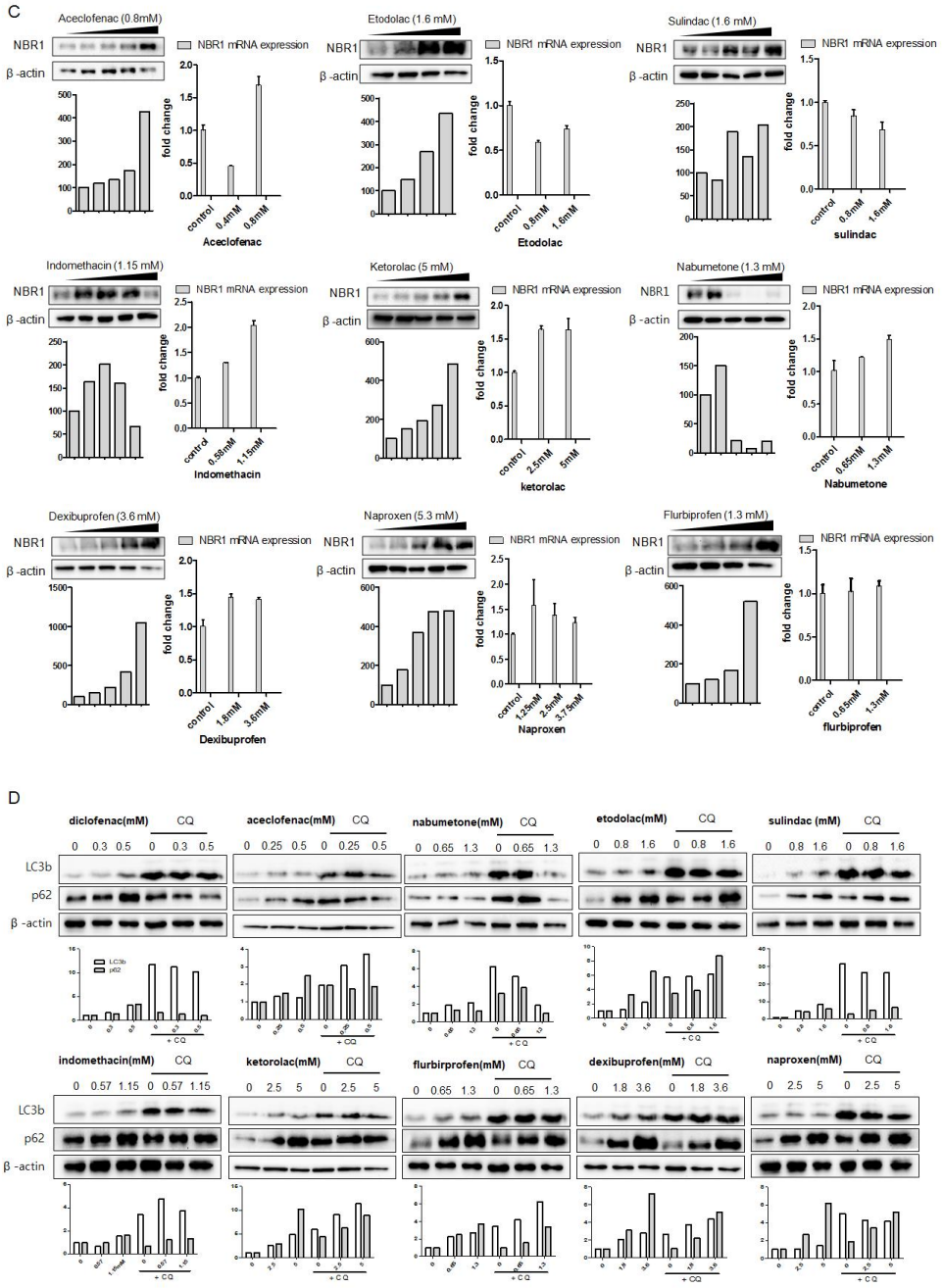
means that number of autophagosome increased but not autolysosome. This result reflected protein level of LC3B, except flurbiprofen.

A

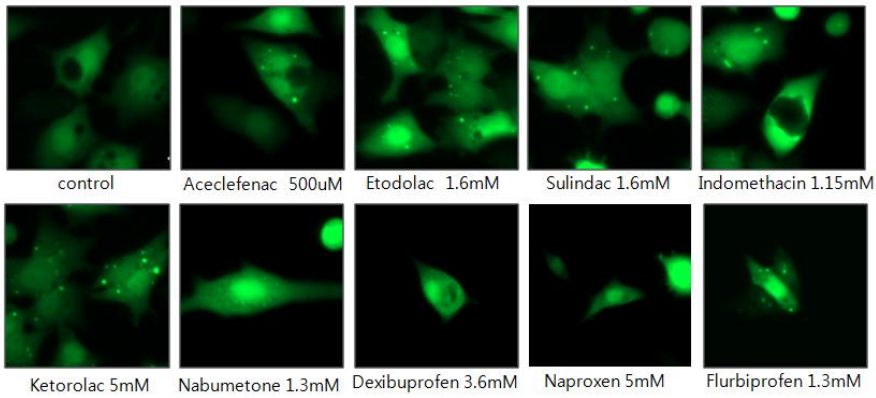

Acetic acid
Diclofenac
Aceclofenac
Etodolac
Sulindac
Indomethacin
Ketorolac
Nabumetone
Propionic acid
Dexibuprofen
Naproxen
Flurbiprofen

B


p62 mRNA expression
fold change
control
0.4mM
0.8mM
Aceclofenac
0.8mM
1.6mM
Etodolac
0.8mM
1.6mM
Sulindac
0.58mM
1.15mM
Indomethacin
2.5mM
5mM
Ketorolac
0.65mM
1.3mM
Nabumetone
1.8mM
3.6mM
Dexibuprofen
1.25mM
2.5mM
3.75mM
Naproxen
0.65mM
1.3mM
Flurbiprofen


C
Aceclofenac (0.8mM)
NBR1
β -actin
NBR1 mRNA expression
fold change
control
0.4mM
0.8mM
Aceclofenac
Etodolac (1.6 mM)
control
0.8mM
1.6mM
Etodolac
Sulindac (1.6 mM)
control
0.8mM
1.6mM
sulindac
Indomethacin (1.15 mM)
control
0.58mM
1.15mM
Indomethacin
Ketorolac (5 mM)
control
2.5mM
5mM
ketorolac
Nabumetone (1.3 mM)
control
0.65mM
1.3mM
Nabumetone
Dexibuprofen (3.6 mM)
control
1.8mM
3.6mM
Dexibuprofen
Naproxen (5.3 mM)
control
1.25mM
2.5mM
3.75mM
Naproxen
Flurbiprofen (1.3 mM)
control
0.65mM
1.3mM
flurbiprofen
D
diclofenac(mM)
CQ
0 0.3 0.5 0 0.3 0.5
aceclofenac(mM)
0 0.25 0.5 0 0.25 0.5
nabumetone(mM)
0 0.65 1.3 0 0.65 1.3
etodolac(mM)
0 0.8 1.6 0 0.8 1.6
sulindac (mM)
0 0.8 1.6 0 0.8 1.6
LC3b
p62
β -actin
+ CQ
indomethacin(mM)
0 0.57 1.15 0 0.57 1.15
ketorolac(mM)
0 2.5 5 0 2.5 5
flurbirprofen(mM)
0 0.65 1.3 0 0.65 1.3
dexibuprofen(mM)
0 1.8 3.6 0 1.8 3.6
naproxen(mM)
0 2.5 5 0 2.5 5

E


control
Aceclefenac 500uM
Etodolac 1.6mM
Sulindac 1.6mM
Indomethacin 1.15mM
Ketorolac 5mM
Nabumetone 1.3mM
Dexibuprofen 3.6mM
Naproxen 5mM
Flurbiprofen 1.3mM

F

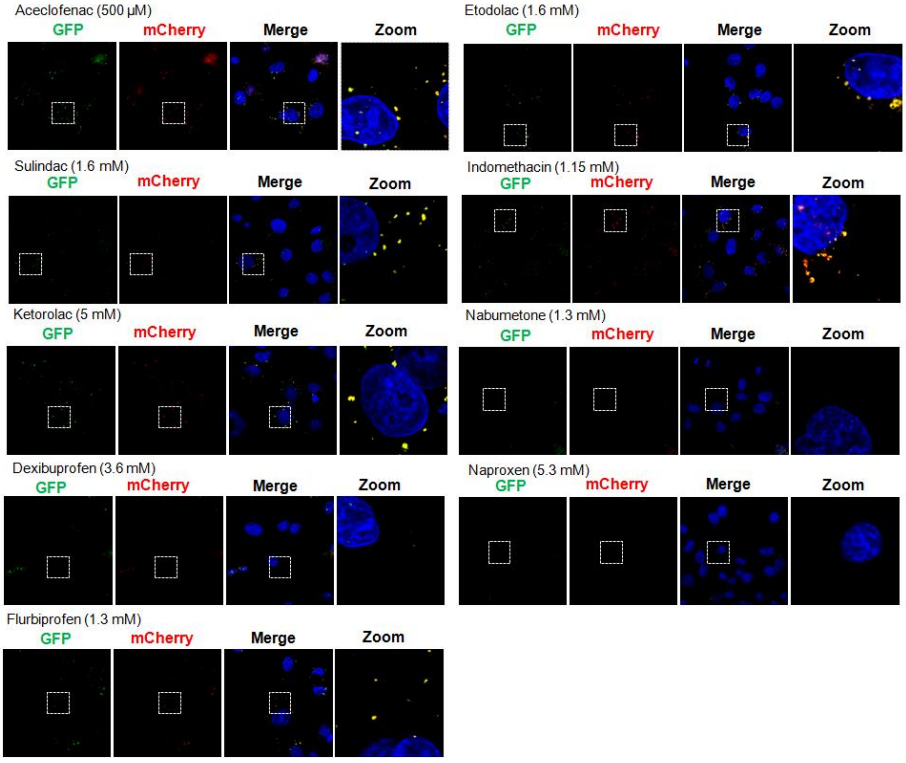

Aceclofenac (500 μM)
GFP
mCherry
Merge
Zoom
Etodolac (1.6 mM)
Sulindac (1.6 mM)
Indomethacin (1.15 mM)
Ketorolac (5 mM)
Nabumetone (1.3 mM)
Dexibuprofen (3.6 mM)
Naproxen (5.3 mM)
Flurbiprofen (1.3 mM)

Figure 2. 6 acetic acid NSAIDs and 3 propionic acid NSAIDs inhibit autophagy flux.

(A) The structure of selected NSAIDs. (B) Relative level of mRNA expression of p62 by NSAIDs in AML12 cell for 24hrs treatment. mRNA expression of b-actin is used for standardization. (C) Protein and mRNA levels of NBR1 by NSAIDs in AML12 cell for 24hrs treatment. (D) autophagy flux experiment, NSAIDs were treated for 24hsr and chloroquine was treated in 10uM concentration for 8hrs in AML12 cell. (E) HepG2 cells were transfected by GFP-LC3 vector then NSAIDs were treated for 24hrs. (F) HepG2 cells were transfected by mCherry-GFP-LC3 vector then NSAIDs were treated for 24hrs.

3. Combination of diclofenac and sorafenib increases apoptosis in liver cancer cell lines.

Sorafenib is a kinase inhibitor drug approved for the treatment of advanced primary liver cancer. Sorafenib is well known autophagy inducing drug and induced autophagy give tumor cells to resist against sorafenib. We examined autophagy marker changing in Huh7 cells by sorafenib. Sorafenib induce autophagy for increasing LC3B, reducing p62 and NBR1. (Figure 3A)

To demonstrate our hypothesis that autophagy inhibitor sensitizes anti cancer effect of chemothrapy, we treated sorafenib with diclofenac in liver cancer cell lines. Combination of diclofenac and sorafenib increase apoptosis synergically in HepG2 and Huh7. (Figure 3B) Also a mount of other apoptosis markers, cleaved caspase-3 and cleaved PARP increased by co treatment of the two drugs. (Figure 3C)

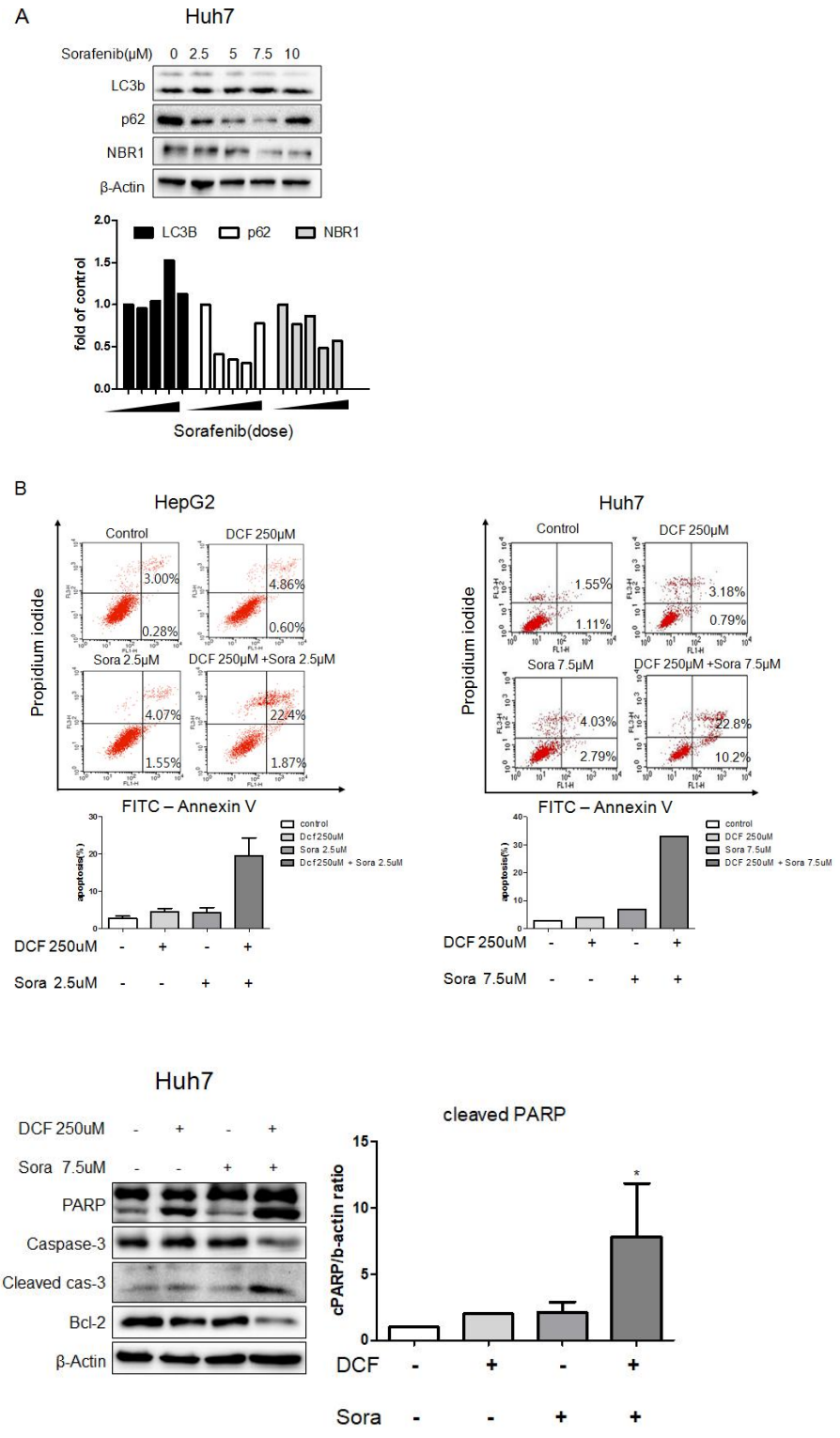

A
Huh7
Sorafenib(μM) 0 2.5 5 7.5 10
LC3b
p62
NBR1
β-Actin
LC3B
p62
NBR1
fold of control
Sorafenib(dose)
B
HepG2
Control
DCF 250μM
Sora 2.5μM
DCF 250μM +Sora 2.5μM
Propidium iodide
3.00%
0.28%
4.86%
0.60%
4.07%
1.55%
22.4%
1.87%
FITC – Annexin V
control
Dcf250uM
Sora 2.5uM
Dcf250uM + Sora 2.5uM
apoptosis(%)
DCF 250uM
Sora 2.5uM
Huh7
Control
DCF 250μM
Sora 7.5μM
DCF 250μM +Sora 7.5μM
1.55%
1.11%
3.18%
0.79%
4.03%
2.79%
22.8%
10.2%
FITC – Annexin V
control
DCF 250uM
Sora 7.5uM
DCF 250uM + Sora 7.5uM
DCF 250uM
Sora 7.5uM
C
Huh7
DCF 250uM
Sora 7.5uM
PARP
Caspase-3
Cleaved cas-3
Bcl-2
β-Actin
cleaved PARP
cPARP/b-actin ratio
DCF
Sora

Figure 3. Synergic effects of diclofenac and sorafenib in liver cancer cell lines

(A) Autophagy induction of soafenib in Huh7 cells. Sorafenib was treated for 24hrs. (B) Flow cytometric analysis of HepG2 cells and Huh7 cells undergoing apoptosis. percentage of Annexin V – positive cells were represended as bar. HepG2 cells were treated with 250uM diclofenac alone or cotreatment with 2.5uM sorafenib . And Huh7 cells were treated with 250uM diclofenac alone or cotreatment with 7.5uM sorafenib. After 24hr incubation, cells were collected by trypsinization and washed with cold DPBS. Then stained with annexin-V and PI and analyzed by flow cytometer. (C) Huh7 cells were treated with 250uM diclofenac alone or cotreatment with 7.5uM sorafenib for 24hrs. Western blot analysis showed that combination of diclofenac and sorafenib increases apoptosis markers, cleaved caspase 3 and cleaved PARP, whereas anti apoptotic protein Bcl-2 decreases.

4. Sorafenib and 5 NSAIDs have synergic effects in liver cancer cell lines.

We confrimed synergic cytotoxic effect of NSAIDs and anit cancer drug using cytation YOYO-1 system described in method. We choosed cancer cell lines and anit-tumor drug by each 4 popular cancers which are liver cancer, breast cancer, colon cancer and lung cancer. At liver cancer cells, we treated sorafenib with NSAIDs. In HepG2 cell, combination of sorafenib and diclofenac , aceclofenac, flurbiprofen, naproxen or dexibuprofen synergically killed cancer cells. (Figure 4A) In Huh7 cell, cotreatment of sorafenib and diclofenac induced enhanced cell death compared with each single drug only. (Figure 4B)

There are several concentration sets which show synergic effects. (Table 2, 3) Synergism is defined by Colby equation. (31)

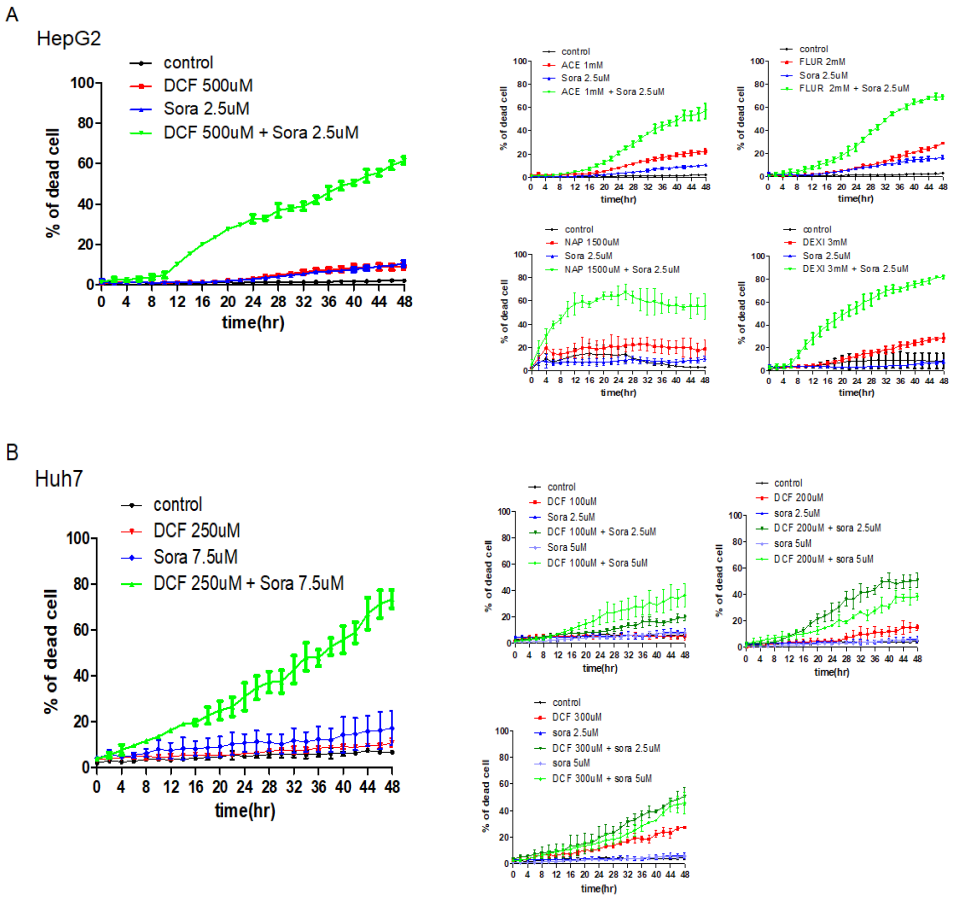


Figure 4. Sorafenib and 5 NSAIDs have synergic effects in liver cancer cell lines.

(A) In HepG2 cells, cytotoxicity of single drug or combination was assessed by "% of dead cell" for 48hrs. (B) In Huh7 cells, cytotoxicity of single drug or combination was assessed by "% of dead cell" for 48hrs.

Table 2. Synergism of drug combination in HepG2 cells

Synergism on HepG2

NSAIDs	Concentration	Cancer drug	A	B	E	R	Synergism
Aceclofenac	1mM	Sora 2.5uM	22.5	10.9	31	56.9	O
Diclofenac	100uM	Sora 2.5uM	2.9	13.1	15.7	42.2	O
	250uM	Sora 2.5uM	9.7	10.6	19.3	41.1	O
	500uM	Sora 2.5uM	9.2	10.6	18.8	61.6	O
Flurbiprofen	1mM	Sora 2.5uM	4	16.9	20.2	53.6	O
	2mM	Sora 2.5uM	29	16.9	41	69.1	O
Dexibuprofen	2mM	Sora 2.5uM	16.1	8.1	22.9	69.2	O
	3mM	Sora 2.5uM	28.7	8.1	34.5	81.8	O
Naproxen	500uM	Sora 2.5uM	5.2	10.4	15	31.2	O
	750uM	Sora 2.5uM	7	10.4	16.6	47.6	O
	1000uM	Sora 2.5uM	11.9	10.4	21	42.5	O
	1500uM	Sora 2.5uM	18.8	10.4	27.2	55.3	O

Table 3. Synergism of drug combination in Huh7 cells

Synergism on Huh7

NSAIDs	Concentration	Cancer drug	A	B	E	R	Synergism
Diclofenac	100uM	Sora 2.5uM	5.5	8.3	13.4	19.5	O
		Sora 5uM	5.5	7.8	12.9	36.3	O
	200uM	Sora 2.5uM	15.3	8.3	22.4	50.7	O
		Sora 5uM	15.3	7.8	21.9	38.5	O
	300uM	Sora 2.5uM	27.4	8.3	33.4	50.8	O
		Sora 5uM	27.4	7.8	33	45.6	O
	250uM	Sora 7.5uM	10.9	17.2	26.2	73.4	O

5. Tamoxifen and 5 NSAIDs have synergic effects in breast cancer cell, MCF7.

At breast cancer cells, MCF7 we treated tamoxifen with NSAIDs. In MCF7 cell, similarly at HepG2 combination of sorafenib and diclofenac, aceclofenac, flurbiprofen, naproxen or dexibuprofen synergically killed cancer cells. (Figure 5A) There are several concentration sets which show synergic effects. (Table 4) Synergism is defined by Colby equation.

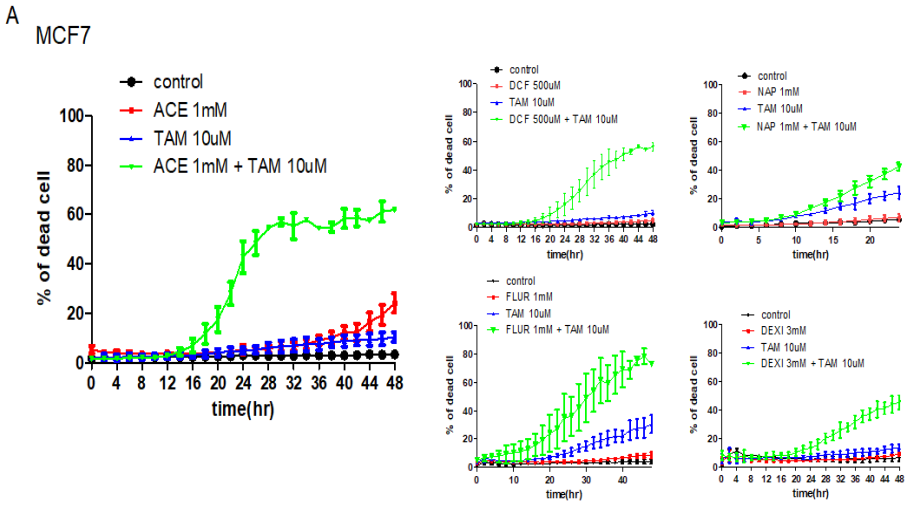


Figure 5. Tamoxifen and 5 NSAIDs have synergic effects in breast cancer cell line.

(A) In HepG2 cells, cytotoxicity of single drug or combination was assessed by "% of dead cell" for 48hrs.

Table 4. Synergism of drug combination in MCF7 cells

Synergism on MCF7

NSAIDs	Concentration	Cancer drug	A	B	E	R	Synergism
Diclofenac	500uM	Tam 10uM	5.2	10	14.7	56.1	O
Naproxen	1mM	Tam 10uM	7.2	24.5	30	42.9	O
	2mM	Tam 10uM	18.1	24.5	38.2	54.2	O
Aceclofenac	500uM	Tam 10uM	5.1	10.4	15	34.5	O
	800uM	Tam 10uM	4.9	10.4	14.8	49.3	O
	1mM	Tam 10uM	24.1	10.4	32	61.9	O
Flurbiprofen	750uM	Tam 10uM	4.7	30.6	33.8	50	O
	1mM	Tam 10uM	8.8	30.6	36.7	72.9	O
Dexibuprofen	3mM	Tam 10uM	9.1	13.4	21.3	45.3	O
	4mM	Tam 10uM	13.3	13.4	24.9	33.6	O

6. Dexibuprofen, diclofenac or naproxen have synergic effects with 5-FU in colon cancer cell, HCT116.

At colon cancer cell, HCT116 we treated 5-FU with NSAIDs. In HCT116, combination of 5-FU and diclofenac, dexibuprofen or naproxen synergically killed cancer cells. But the other NSAIDs aceclofenac and flurbiprofen can not show elevated cell death by cotreatment with 5-FU. (Figure 6A) There are several concentration sets which show synergic effects or not. (Table 5) Synergism is defined by Colby equation.

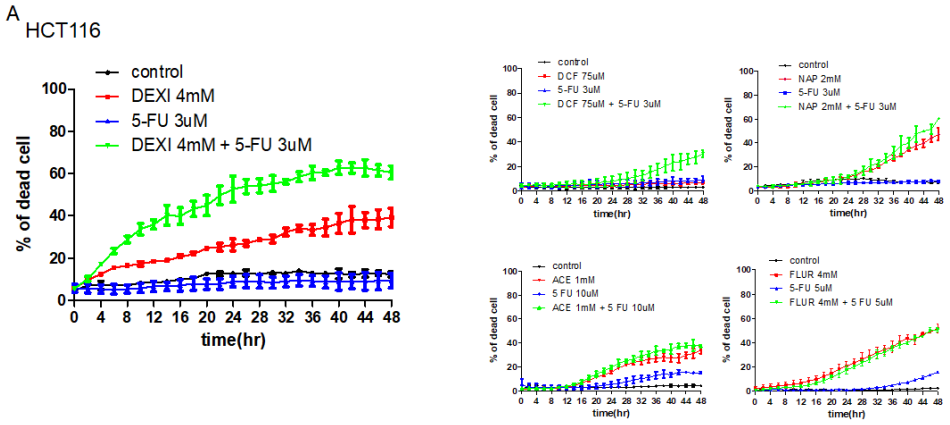


Figure 6. 5-FU and dexibuprofen or diclofenac or naproxen have synergic effects in colon cancer cell line

(A) In HCT116 cells, cytotoxicity of single drug or combination was assessed by "% of dead cell" for 48hrs.

Table 5. Synergism of drug combination in HCT116 cells

Synergism on HCT116

NSAIDs	Concentration	Cancer drug	A	B	E	R	Synergism
Diclofenac	75uM	5-FU 3uM	6.7	9.4	15.5	30.4	O
Dexibuprofen	3mM	5-FU 3uM	32	9.3	38.3	55	O
	4mM	5-FU 3uM	39.2	9.3	44.8	60.5	O
Naproxen	2mM	5-FU 3uM	47.2	7.8	51.3	60.5	O
Aceclofenac	1mM	5-FU 10uM	34.0	15.1	44.0	37.4	X
Flurbiprofen	4mM	5-FU 5uM	51.8	15.7	59.4	50.7	X

7. Dexibuprofen or naproxen have synergic effects with paclitaxel in lung cancer cell A549.

At lung cancer cell, A549 we treated paclitaxel with NSAIDs. In A549, combination of paclitaxel and dexibuprofen or naproxen synergically killed cancer cells. But the other NSAIDs declofenac, aceclofenac and flurbiprofen can not show elevated cell death by cotreatment with paclitaxel. (Figure 7A) There are several concentration sets which show synergic effects or not. (Table 6) Synergism is defined by Colby equation.

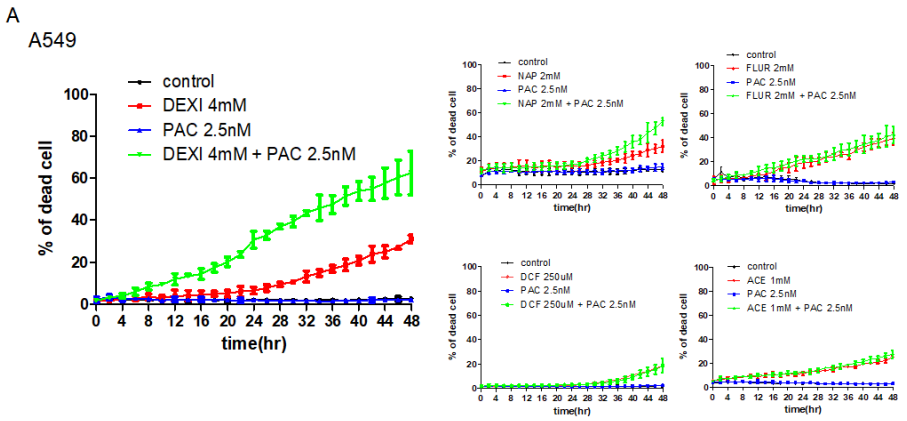


Figure 7. Paclitaxel and dexibuprofen or naproxen have synergic effects in lung cancer cell line

(A) In A549 cells, cytotoxicity of single drug or combination was assessed by "% of dead cell" for 48hrs.

Table 6. Synergism of drug combination in A549 cells

Synergism on A549.

NSAIDs	Concentration	Cancer drug	A	B	E	R	Synergism
Dexibuprofen	4mM	paclitaxel 2.5nM	30.9	2.2	32.4	62.5	O
Naproxen	2mM	paclitaxel 2.5nM	32.2	14.9	42.3	52.7	O
Flurbiprofen	2mM	paclitaxel 2.5nM	39.5	3.41	41.6	42.6	O
Diclofenac	250uM	paclitaxel 2.5nM	18.6	2.39	20.6	18.5	X
Aceclofenac	1mM	paclitaxel 2.5nM	25.2	2.89	27.3	27	X

Ⅳ. Discussion

Autophagy is one of the main mechanisms for maintaining cell homeostasis.(1) It plays a role in preventing the cancerization in the early stage of cancer development, but it plays a role in the survival of cancer cells after the cells are tumorized. There have been many reports that autophagy is increased in hypoxic condition, chemotherapy, and radiation therapy in various cancer cells and cancer cells have resistance against stress through autophagy.(3-8) Therefore, there has been a lot of attempts to use an autophagy inhibitor in combination with chemotherapy. Typical autophagy inhibitors such as HCQ and CQ are undergoing clinical trials.(11-16) Previous studies in the laboratory found that diclofenac, one of the NSAIDs, suppressed autophagy and further confirmed the effect of other NSAIDs on autophagy.

In the initial screening using autophagy markers, LC3B and p62, autophagy inhibiting activity were found in most of all four class of NSAIDs. At this time, accumulation of p62 was common, but the pattern of change of LC3B was different for each class. Propionic acid derivatives drugs showed no decrease or significant change in LC3B level, while other class of drugs showed LC3B increase. Therefore, it can be deduced that the mechanisms of autophagy inhibition are different in each class.

In order to confirm the autophagy inhibitory activity more precisely, all 6 acetic acid NSAIDs and 3 propionic acid drugs were

selected. And (1)mRNA expression of LC3b, p62 and NBR1. (2) autophagy flux test (3) Imaging of autophagosomes and lysosomes were observed. In contrast to the first anticipation, the mRNA expression of p62 itself was increased in all acetic acid derivatives so the cargo receptor NBR1 was found to accumulate without altering mRNA levels, confirming autophagy inhibition. When the NSAIDs were treated, the autophagosomes were not normally transferred to the autolysosome stage. The accumulation of autophagosomes was observed in all of the acetic acid NSAIDs and flurbiprofen except nabumetone. In the autophagy flux experiments, we confirmed that the increase of LC3B was the result of inhibition, not result of autophagy induction. In a series of results, it was concluded that all of the 8 selected drugs, except nabumetone, inhibited autophagy.

We next determined whether NSAIDs could increase the activity of anticancer drugs in cancer cells through autophagy inhibition. First, sorafenib, an anticancer drug for hepatocellular carcinoma, induced autophagy in Huh7 cells. We then examined the changes in apoptotic markers of annexin V-PI assay, caspase 3, cleaved PARP, and Bcl-2 when treated with sorafenib and diclofenac in HepG2 and Huh7. As a result, when the two drugs were treated together, the apoptotic factor was significantly increased and anti apoptotic protein was decreased.

Next, we examined whether the inhibition of autophagy by NSAIDs could increase the cytotoxicity of chemotherapy in various

cancer cells. HepG2, Huh7, MCF7, HCT116 and A549 cells were used in four types of cancer, liver, breast, colorectal and lung cancer. Sorafenib, tamoxifen, 5-FU and paclitaxel were used as anticancer agents.

Diclofenac, aceclofenac, dexibuprofen, naproxen, and fluriprofen, which showed a greater inhibitory effect on autophagy in the previous screening, were identified in combination with anticancer drugs in cancer cells. As a result, in HepG2 and MCF7 combinations showed synergistic effects with all NSAIDs. However, only dexibuprofen, diclofenac, naproxen in HCT116, and dexibuprofen and naproxen in A549 were effective. The reason for this result may be a lack of diversity of anticancer drug concentration settings. To confirm the effect dramatically, anticancer agents were fixed at a concentration that did not show toxicity alone. Therefore, in combination with higher concentrations of anticancer drugs, all of the NSAIDs may have a synergistic effect. The second reason may be that lack of autophagy inhibition to increase cytotoxicity may be lacking because the NSAIDs have different autophagy inhibitory activities and different sensitivities to each cell.

In conclusion, this study confirmed the autophagic inhibitory activity of NSAIDs and confirmed the synergistic effect of cytotoxicity with anti cancer agents in various cancer cell lines. These results will serve as a basis for future drug repositioning of NSAIDs as anticancer adjuvants in clinic.

Ⅴ. References

1. Rubinsztein DC, Codogno P, Levine B. Autophagy modulation as a potential therapeutic target for diverse diseases. Nat Rev Drug Discov 2012;11:709-30.
2. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 2010;22: 124-31.
3. White E. Deconvoluting the context-dependent role for autophagy in cancer. Nat Rev Cancer 2012;12:401-10.
4. Janku F, McConkey DJ, Hong DS, Kurzrock R. Autophagy as a target for anticancer therapy. Nat Rev Clin Oncol 2011;8:528-39.
5. Amaravadi RK, Lippincott-Schwartz J, Yin XM, Weiss WA, Takebe N, Timmer W, et al. Principles and current strategies for targeting autophagy for cancer treatment. Clin Cancer Res 2011;17:654-66.
6. Carew JS, Kelly KR, Nawrocki ST. Autophagy as a target for cancer therapy: new developments. Cancer Manag Res 2012;4:357-65.
7. Carew JS, Nawrocki ST, Cleveland JL. Modulating autophagy for therapeutic benefit. Autophagy 2007;3:464-7.
8. Chen S, Zhou L, Zhang Y, Leng Y, Pei XY, Lin H, et al. Targeting SQSTM1/ p62 induces cargo loading failure and converts autophagy to apoptosis via NBK/Bik. Mol Cell Biol 2014;34:3435-

49.

9. Rao R, Balusu R, FiskusW, Mudunuru U, Venkannagari S, Chauhan L, et al. Combination of pan-histone deacetylase inhibitor and autophagy inhibitor exerts superior efficacy against triple-negative human breast cancer cells. Mol Cancer Ther 2012;11:973-83.

10. Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Mycinduced model of lymphoma. J Clin Invest 2007;117:326-36.

11. Rangwala R, Chang YC, Hu J, Algazy KM, Evans TL, Fecher LA, et al. Combined MTOR and autophagy inhibition: phase I trial of hydroxychloroquine and temsirolimus in patients with advanced solid tumors and melanoma. Autophagy 2014;10:1391-402.

12. Vogl DT, Stadtmauer EA, Tan KS, Heitjan DF, Davis LE, Pontiggia L, et al. Combined autophagy and proteasome inhibition: a phase 1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma. Autophagy 2014;10:1380-90.

13. Rangwala R, Leone R, Chang YC, Fecher LA, Schuchter LM, Kramer A, et al. Phase I trial of hydroxychloroquine with dose-intense temozolomide in patients with advanced solid tumors and melanoma. Autophagy 2014;10:1369-79.

14. Mahalingam D, Mita M, Sarantopoulos J,WoodL,Amaravadi RK, Davis LE, et al. Combined autophagy and HDAC inhibition: a phase I safety, tolerability, pharmacokinetic, and pharmacodynamic

analysis of hydroxychloroquine in combination with the HDAC inhibitor vorinostat in patients with advanced solid tumors. Autophagy 2014;10:1403–14.

15. Rosenfeld MR, Ye X, Supko JG, Desideri S, Grossman SA, Brem S, et al. A phase I/II trial of hydroxychloroquine in conjunction with radiation therapy and concurrent and adjuvant temozolomide in patients with newly diagnosed glioblastoma multiforme. Autophagy 2014;10:1359–68.
16. Barnard RA, Wittenburg LA, Amaravadi RK, Gustafson DL, Thorburn A, Thamm DH. Phase I clinical trial and pharmacodynamic evaluation of combination hydroxychloroquine and doxorubicin treatment in pet dogs treated for spontaneously occurring lymphoma. Autophagy 2014;10: 1415–25.
17. Cynthia I. Chude and Ravi K. Amaravadi, Targeting Autophagy in Cancer: Update on Clinical Trials and Novel Inhibitors, Int. J. Mol. Sci. 2017, 18, 1279
18. Jadhav J, Juvekar A, Kurane R, Khanapure S, Salunkhe R, et al. (2013)Remarkable anti-breast cancer activity of ferrocene tagged multifunctionalized 1,4-dihydropyrimidines. Eur J Med Chem 65: 232-239.
19.Thun MJ, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical
issues. J Natl Cancer Inst 2002;94:252–66.

20. Ruder EH, Laiyemo AO, Graubard BI, Hollenbeck AR, Schatzkin A, Cross AJ. Non-steroidal anti-inflammatory drugs and colorectal cancer risk in a large, prospective cohort. Am J Gastroenterol 2011;106:1340-50.
21. Sharpe CR, Collet JP, McNutt M, Belzile E, Boivin JF, Hanley JA. Nested case-control study of the effects of non-steroidal anti-inflammatory drugs on breast cancer risk and stage. Br J Cancer 2000;83: 112-20.
22. Khuder SA, Mutgi AB. Breast cancer and NSAID use: a meta-analysis. Br J Cancer 2001;84:1188-92.
23. Muscat JE, Chen SQ, Richie JP Jr, Altorki NK, Citron M, Olson S, et al. Risk of lung carcinoma among users of nonsteroidal anti-inflammatory drugs. Cancer 2003;97:1732-6.
24. Norrish AE, Jackson RT, McRae CU. Non-steroidal anti-inflammatory drugs and prostate cancer progression. Int J Cancer 1998;77:511-5.
25. Thun MJ, Namboodiri MM, Calle EE, Flanders WD, Heath CW Jr Aspirin use and risk of fatal cancer. Cancer Res 1993;53:1322-7.
26. Cramer DW, Harlow BL, Titus-Ernstoff L, Bohlke K, Welch WR, Greenberg ER. Over-the-counter analgesics and risk of ovarian cancer. Lancet 1998;351:104-7.
27. Holmes MD, Chen WY, Li L, Hertzmark E, Spiegelman D, Hankinson SE. Aspirin intake and survival after breast cancer. J Clin Oncol 2010;28:1467-72.

28. Evrim Gurpinar ,WilliamE.Grizzle, GaryA.Piazza, COX-independent mechanisms of cancer chemoprevention by anti-inflammatory drugs Front Oncol: 2013; 3: 181.

29. Evrim Gurpinar1, William E. Grizzle2, and Gary A. Piazza3, NSAIDs Inhibit Tumorigenesis, but How? Clin Cancer Res; 2014; 20(5)

30. Noboru Mizushima, Tamotsu Yoshimorim, Beth Levine, Methods in Mammalian Autophagy Research *Cell*; 2010; 140(3): 313-326

31. Nancy Ferry, Dr. Bruce H. Stanley and Dr. Gregory Arme, DESIGN AND ANALYSIS OF BIOLOGICAL ASSAYS OF MIXTURES; Applied Statistics in Agriculture; 2005

국문초록

오토파지는 이중막을 가진 오토파고솜이 세포 내에 불필요한 소기관이나 단백질을 포식하여 리소좀을 통해 분해시키는 과정이며, 정상적으로 작동하지 않을 경우 세포의 항상성이 깨져 다양한 질병을 일으키는 원인이 된다. 또한 암세포에서 저산소, 항암화학요법, 방사선 조사와 같은 스트레스 상황에서 오토파지의 활성이 크게 증가되는 것이 관찰되었고 높아진 오토파지 활성을 통해 암세포는 항암약물에 대한 저항성을 가지게 된다. 따라서 오토파지를 억제하는 약물을 항암제와 병용투여하게 되면 상승된 효과를 기대할 수 있다.

이전 연구에서 NSAIDs 계열 약물 중 하나인 디클로페낙(diclofenac)이 오토파지를 억제한다는 것을 밝혔다. 그래서 다른 20여 가지의 NSAIDs들의 오토파지에 대한 작용을 마우스 정상 간세포주 AML12에서 IC50의 절반 값을 최고 농도로 하여 웨스턴 블롯을 통해 오토파지 마커인 LC3, p62를 확인하여 스크리닝하였다. NSAIDs중 5 가지 약물 diclofenac, aceclofenac, naproxen, dexibuprofen, flurbiprofen를 간암 세포주 HepG2와 Huh7, 유방암 세포주 mcf7, 대장암 세포주 HCT116, 폐암 세포주 A549에 각각 주로 항암제로 사용되는 sorafenib, tamoxifen, 5-FU, paclitaxel과의 병용투여를 통해 세포사멸과 세포증식의 변화를 HCS 장비인 cytation을 이용하여 관찰하였다. 또한 Huh7에서 diclofenac과 sorafenib의 병용투여했을 때 apoptosis의 변화를 annexin V-PI assay와 cleaved caspase 3, cleaved PARP, Bcl-2 등 apoptosis와 관련된 인자들을

통해 확인하였다.

AML12에서 스크리닝 결과 대부분의 NSAIDs들이 오토파지 억제 활성을 보였다. 그리고 암세포주에서 항암제와 병용투여 했을 때, HepG2와 MCF7에서는 sorafenib 또는 tamoxifen과 diclofenac, aceclofenac, naproxen, dexibuprofen, flurbiprofen의 조합, Huh7에서는 sorafenib와 diclofenac의 조합, HCT116에서는 diclofenac, aceclofenac, dexibuprofen과 5-FU의 조합, a549에서는 dexibuprofen, naproxen과 paclitaxel의 조합을 통해 세포사멸의 큰 상승효과 및 세포 증식의 억제를 확인할 수 있었다. 또한 Huh7에서 diclofenac과 sorafenib의 병용투여 했을 때 apoptosis가 크게 증가하는 것을 annexin V-PI assay에서 apoptosis positive cell의 증가와 apoptosis marker인 cleaved caspase 3, cleaved PARP의 증가와 Bcl-2의 감소를 통해 확인하였다.

결과적으로 diclofenac이 항암제를 통해 오토파지 활성이 증가된 간암세포에서 오토파지를 억제할 수 있고 이것은 암세포의 세포사멸을 증가시키는 것을 확인하였다. 그리고 다른 여러 NSAIDs들도 오토파지 억제 활성을 보이며 항암제와 병용처리 했을 때 세포사멸을 증가시키는 것이 오토파지 억제를 통한 것이라고 추측할 수 있다. 따라서 NSAIDs와 항암제의 병용투여를 통해 암 치료에서 기존 항암 화학요법제의 감수성을 증가시킬 수 있을 것이라 기대할 수 있다.