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Research Paper

COX-2 Inhibitors, a Potential Synergistic Effect with Antineoplastic Drugs in Lung Cancer

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Abstract

Background: Lung cancer represents the leading cause of cancer-related deaths worldwide and novel therapeutic approaches targeting crucial pathways are urgently needed to improve its treatment. Inflammation plays a critical role in multistage tumor development and increased evidence has supported the involvement of cyclooxygenase-2 expression in carcinogenesis. We investigated the potential use of COX-2 inhibitors in cancer proliferation and apoptosis.

Methods: Celecoxib, rofecoxib, etoricoxib, meloxicam, ibufrofen and indomethacin are the COX-2 inhibitors included in this study. Docetaxel and Cisplatin are the chemotherapeutic agents that we combined with COX-2 inhibitors. Lung cancer cell lines (NCI-H1048-Small cell lung cancer, A549-Non-small cell lung cancer) were purchased from ATCC LGC Standards. At indicated time-point, following 24h and 48h incubation, cell viability and apoptosis were measured with Annexin V staining by flow cytometry. Statistical analysis was performed by GraphPad Prism.

Results: In Small cell lung cancer cells, following 24h incubation, combinations of docetaxel and meloxicam, docetaxel and ibuprofen, docetaxel and indomethacin, showed increased apoptosis when compared to docetaxel alone ($p<0.0001$). In Non-small cell lung cancer cells, the 24h incubation was not enough to induce satisfactory apoptosis, but following 48h incubation, docetaxel plus indomethacin showed more cytotoxicity when compared to docetaxel alone ($p<0.0001$). In addition, the combination of cisplatin plus indomethacin was the only combination to be found with higher cytotoxicity when compared to cisplatin alone after 48h treatment ($p<0.0001$).

Conclusion: Depending on the drug, the synergistic effect of COX-2 inhibitors plus chemotherapeutic agents has been demonstrated in lung cancer. Our suggestion is that COX-2 inhibitors could be used as additive and maintenance treatment in combination to antineoplastic agents in lung cancer patients.

Key words: COX-2 inhibitors, lung cancer, *in vitro*.

Introduction

Lung cancer is still the most common type of cancer and the leading cause of cancer-related deaths worldwide [1]. Lung cancer is subdivided into two histological groups: Small Cell Lung Cancer (SCLC) and Non-Small Cell lung Cancers (NSCLC) which accounts for almost 85% of all lung cancers [2]. Small-cell lung carcinoma has an aggressive clinical course and metastases at diagnosis and widespread

dissemination. Although a platinum-etoposide combination combined with thoracic and prophylactic cranial irradiation has been shown to improve outcome in limited-stage SCLC (TxNxM0)) and in subgroups of extensive-stage SCLC (Tx,Nx,M1a/b) the clinical outcome for SCLC remains discouraging [3]. Similarly, NSCLC appears in the advanced stage at diagnosis in the majority of the patients with no surgical treatment option. Classical chemotherapy (platinum-doublet, taxanes, gemcitabine, pemetrexed) results in modest efficacy in NSCLC. At this point, molecular targeted drugs concerning the identification of molecular biomarkers including EGFR inhibitors, have led to personalized therapy in NSCLC, but mechanisms of resistance remain to be elucidated [4]. Furthermore, multimodal therapeutic strategy has become an important treating option for NSCLC patients, however, in several studies, two or more drug combinations were proven to have superior efficacy but at the expense of added toxicity [5] [6]. Thus, novel therapeutic approaches targeting crucial pathways are urgently needed to improve the treatment of lung cancer.

The last fifteen years, it has become clear that inflammation plays a critical role in multistage tumor development [7], and some of the molecular mechanisms involved have been elucidated [8]. Several studies have supported the involvement of cyclooxygenase-2 expression in carcinogenesis [9-13]. Indeed, increased expression of COX-2 and antiproliferative effects of COX-2 inhibitors were found in several types of cancer such as osteosarcoma [14], colorectal carcinomas [15, 16], urinary bladder cancer [17], breast cancer [18], prostate cancer [19] and lung cancer [11, 20]. Furthermore, preclinical studies in vivo have supported the benefit of COX-2 inhibition in cancer [21-25]. In addition, selective COX-2 inhibitors such as celecoxib have been included in clinical trials in non-small-cell lung cancer patients showing promising results [26-28]. Moreover, results from another clinical trial in adenoma cancer patients showed significant benefit effect in the celecoxib group of patients [29].

Taken together, these data have demonstrated a potential use of COX-2 inhibitors in cancer proliferation and apoptosis. In this study, we investigated the cytotoxicity of combined treatment of COX-2 inhibitors with anticancer agents on Small Cell Lung Cancer and Non-small cell lung cancer in vitro.

Materials and Methods

Cell cultures and reagents

The small cell lung cancer cell line [NCI-H1048 [H1048], ATCC® CRL-5853™) was purchased from ATCC LGC Standards. NCI-H1048 cells were cultured in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM):F12 Medium culture medium, supplemented with 5% Fetal Bovine Serum (FBS) and with the following components, 0.005 mg/ml Insulin, 0.01 mg/ml Transferrin, 30nM Sodium selenite (final conc.), 10nM Hydrocortisone (final conc.), 10nM beta-estradiol (final conc.), extra 2mM L-glutamine (for final conc. of 4.5 mM), 5% fetal bovine serum (final conc.), all purchased from SIGMA. The non-small cell lung cancer cell line (A549, ATCC® CCL185™) was also purchased from ATCC LGC Standards. A549 cell line was cultured in DMEM, supplemented with 5% FBS, 1mM Penicillin-Streptomycin and 2mM L-glutamin, all purchased from SIGMA.

Lung cancer cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2 [30] and cultured in Coming's tissue culture flasks (25 and 75 cm^2) according to the manufacturer's protocol. After cultures reached confluence, by microscope observation were then subcultured. Cells were detached with trypsin (1:250) 2.5 % and passaged. The indicated cell lines were seeded in 25 cm^2 flasks 0.7 × 10^6 cells at a seeding density of 10^6 cells for each cell. At confluence, at indicated time point, test compounds were added according to our protocol and after 24 h or 48h incubation apoptosis was measured (Table 1).

Table 1: Protocol of the experiment.

Incubation of the drugs in lung cancer cell lines
1. Addition if chemotherapeutic agents alone for 2h
2. Addition of COX-2 inhibitors alone for 2h
3. Addition of combinations: COX-2 inhibitors + chemotherapeutic agents for 2h
4. After 24h and 48h incubation, apoptosis was measured

Test compounds

Celecoxib (Celebrex® 100mg), rofecoxib (Vioxx® 25mg), etoricoxib (Arcox® 90mg), meloxicam (Movatec® 7.5mg), ibufrofen (Brufen® 200mg) and indomethacin (Fortathrin® 75mg) are the COX-2 inhibitors included in this study. These compounds were tested in several concentrations (5μM, 10μM, 20μM, 40μM, 80μM, 100μM, 200μM). Docetaxel (10mg/ml, 140mg/5.5L) and Cisplatin (1mg/ml, 90mg/5.5L) at concentration of 25μM are the chemotherapeutic agents that we combined with COX-2 inhibitors.

Analysis of the apoptotic cells with ANNEXIN V/ PI

Annexin V staining is used as a probe to detect cells that have expressed phosphatidylserine (PS) on

the cell surface, an event found in apoptosis as well as other forms of cell death. Propidium iodide (PI) is used as a DNA stain for both flow cytometry, to evaluate cell viability or DNA content in cell cycle analysis 17, and microscopy to visualize the nucleus and other DNA containing organelles. It can be used to differentiate necrotic, apoptotic and normal cells. The Annexin V kit used in this study was purchased from Immunostep (Salamanca, Spain). Data were acquired on a FACS Calibur (BD, Franklin Lakes, NJ, USA) instrument, and analyzed using the CellQuest Pro v6 software (BD) or FlowJo software vX.0.7 (Tree Star).

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Multiple comparisons between experimental groups for one or more variables were performed using one- or two-way ANOVA, respectively, with Tukey's post-hoc test. Values of $p \leq 0.05$ were considered as statistically significant. All the analyses were undertaken using PRISM version 6.01 (GraphPad software, version 6, San Diego, CA. USA).

Results

In SCLC, following 24h and 48h incubation, docetaxel was found more cytotoxic than cisplatin ($p<0.0001$), whereas in NSCLC only after 48h incubation. The combination of docetaxel and COX-2 inhibitors reduced viability significantly in both cell lines when compared to docetaxel alone. In contrast, the combination of cisplatin and COX-2 inhibitors was not as cytotoxic when compared to cisplatin alone except in the case of co-treatment of cisplatin plus indomethacin, cisplatin plus meloxicam and cisplatin plus ibuprofen in SCLC after 24h incubation ($p<0.0001$). In addition, the cytotoxicity was positively associated with drug concentrations, especially in the case of celecoxib (160μM). Specifically, in SCLC cell line, co-treatment of docetaxel and celecoxib (80μM $p<0.0001$) reduced viability more than docetaxel alone and more than cisplatin and celecoxib co-treatment after 24h (40μM $p<0.05$ and 80μM $p<0.0001$) and 48h (40μM 80μM and 160μM $p<0.0001$) incubation (Figure 1, Table 2). Similarly, combination of docetaxel and rofecoxib (40μM, $p<0.001$-24h incubation, $p<0.05$-48h incubation) was more cytotoxic than docetaxel alone. Furthermore, cytotoxicity was significantly increased in the docetaxel and etoricoxib combination (80μM, $p<0.001$) when compared to that of docetaxel alone (24h). Combinations (24h) of docetaxel and meloxicam (200μM, $p<0.0001$), docetaxel and ibuprofen (40μM, $p<0.0001$), docetaxel and indomethacin (100μM, 200μM, $p<0.0001$), showed increased apoptosis when compared to docetaxel alone. Following, 48h incubation docetaxel/ meloxicam (200μM, $p<0.01$) and docetaxel/ibuprofen combinations (40μM $p<0.001$), resulted in an increased number of late apoptotic cells ($p<0.001$) when compared to docetaxel alone. After 24h incubation, cisplatin/meloxicam, (200μM, $p<0.01$), cisplatin/ibuprofen (40μM, $p<0.0001$) and cisplatin/indomethacin co-incubation (200μM, $p<0.0001$), showed increased apoptotic levels when compared with cisplatin alone. No significant apoptosis was found on other combinations of cisplatin and COX-2 inhibitors after 48h incubation in SCLC cell line. Finally, in SCLC, the concentrations of COX-2 inhibitors alone (celecoxib 80μM, meloxicam 200μM, indomethacin 200μM) resulted in increased apoptosis when compared to untreated cells ($p<0.0001$, Figure 2, Table 3).

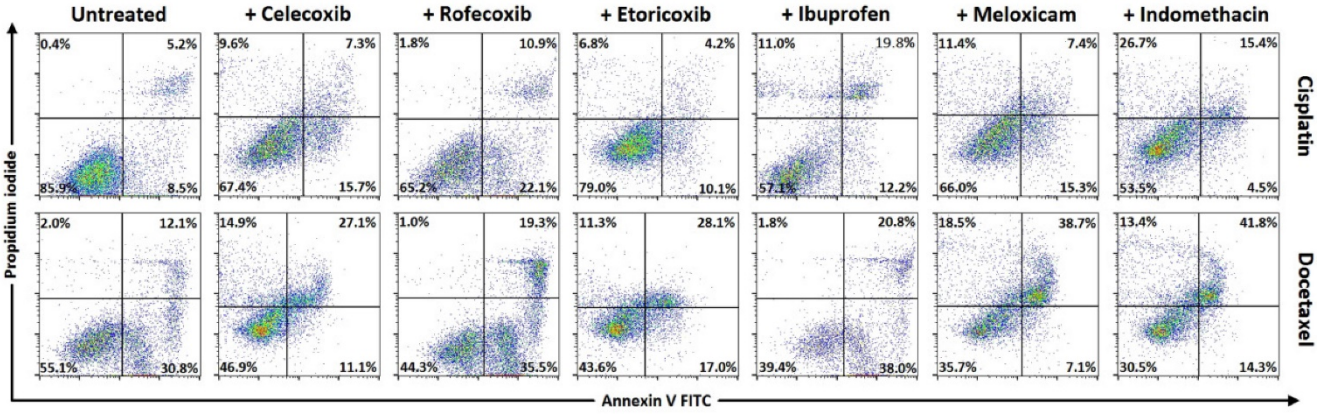

Figure 1: Cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry. Representative data for SCLC, after 24h incubation, combinations of cisplatin and docetaxel with celecoxib, rofecoxib, etoricoxib, ibuprofen, meloxicam, indomethacin.

Table 2: Means ± standard error of the mean (SEM) of percentages for cell viability and apoptosis with Annexin V/ Propidium iodide by flow cytometry in SCLC cell line after 24h incubation with drug combinations (representative data in accordance to Figure 1).

Drugs	Dead %	Late Apoptotic %	Early Apoptotic %	Live %
Control/ SCLC cells untreated	2±1.5	6 ±1.5	9±1.4	82±3.5
Cisplatin 25μM	3±1.3	5±1	13±2	78±3.3
Docetaxel 25μM	5±3.6	9±2.5	27±1.5	59±4.4
Celecoxib 80μM	9±2	4±1.5	14±1.5	73±2
Cisplatn + celecoxib 80μM	10±3	7±2	16±2	67±1.5
Docetaxel + celecoxib 80μM	15±2	27±3.4	11±2	47±2.5
Rofecoxib 40μM	1±1	3±1.5	6±2	90±2.5
Cisplatin + rofecoxib 40μM	1±0.5	7±3.5	14±8	65±0.5
Docetaxel + rofecoxib 40μM	1±0.5	20±1.4	36±2	44±3.2
Etoricoxib 80μM	9±1.5	4±1	18±2	70±3
Cisplatin + etoricoxib 80μM	7±1	4±1.2	10±1	79±2.5
Docetaxel + etoricoxib 80μM	11±2	28±3	17±3.5	44±2
Ibufrofen 40μM	1±0.5	6±1	14±2	79±3.4
Cisplatin + ibufrofen 40μM	9±1	27±2	11±1	53±3.5
Docetaxel + ibufrofen 40μM	2±0.5	21±2	38±2	39±4
Meloxicam 200μM	8±3	7±2.5	21±1.5	63±3
Cisplatin + meloxicam 200μM	12±1.4	8±1.3	16±2	65±3
Docetaxel + meloxicam 200μM	19±2	39±1.4	7±1	35±3
Indomethacin 200μM	8±1.3	13±2	19±3.1	60±2
Cisplatin + indomethacin 200μM	27±2	16±2	5±1	53±2
Docetaxel + indomethacin 200μM	14±3	42±2.3	15±2	30±2.5

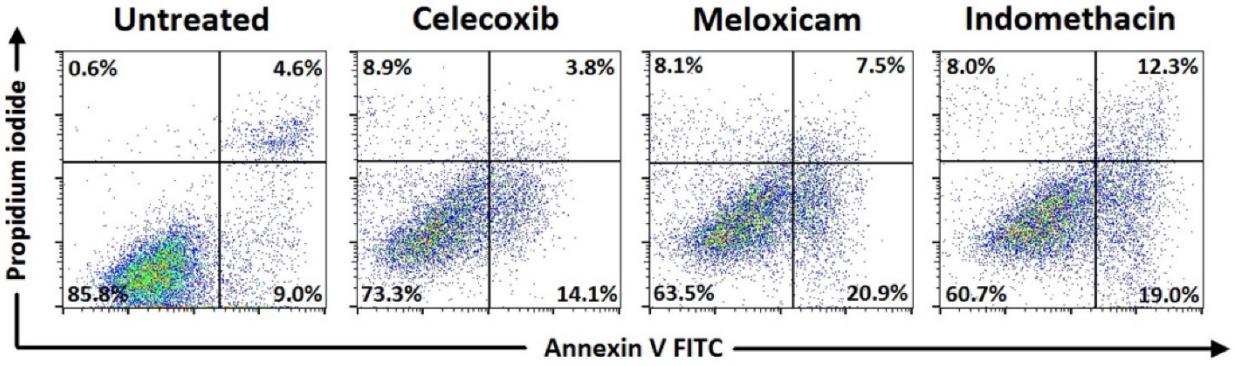

Figure 2: Cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry. Representative data for SCLC, after 24h incubation, celecoxib (80μM), meloxicam (200μM), indomethacin (200μM).

Table 3: Means ± standard error of the mean (SEM) of percentages for cell viability and apoptosis with Annexin V/ Propidium iodide by flow cytometry in SCLC cell line after 24h incubation with COX-2 inhibitors alone (representative data in accordance to Figure 2).

Drugs	Dead %	Late Apoptotic %	Early Apoptotic %	Live %
Untreated cells SCLC	2±1.5	6 ±1.5	9±1.4	82±3.5
Celecoxib 80μM	9±2	4±1.5	14±1.5	73±2
Meloxicam 200μM	8±3	7±2.5	21±1.5	63±3
Indomethacin 200μM	8±1.3	13±2	19±3.1	60±2

In NSCLC, the 24h incubation was not enough to induce satisfactory apoptosis. Results showed increased apoptosis particularly after 48h incubation of docetaxel plus indomethacin when compared to docetaxel alone ($p<0.0001$). In addition, the combination of cisplatin plus indomethacin ($p<0.0001$) was the only combination to be found with higher cytotoxicity when compared to cisplatin alone after 48h treatment (Figure 3, Table 4). Finally, in NSCLC, the concentrations of COX-2 inhibitors alone (celecoxib 80μM, meloxicam 200μM, indomethacin 200μM) resulted in increased apoptosis when compared to untreated cells ($p<0.0001$, Figure 4, Table 5).

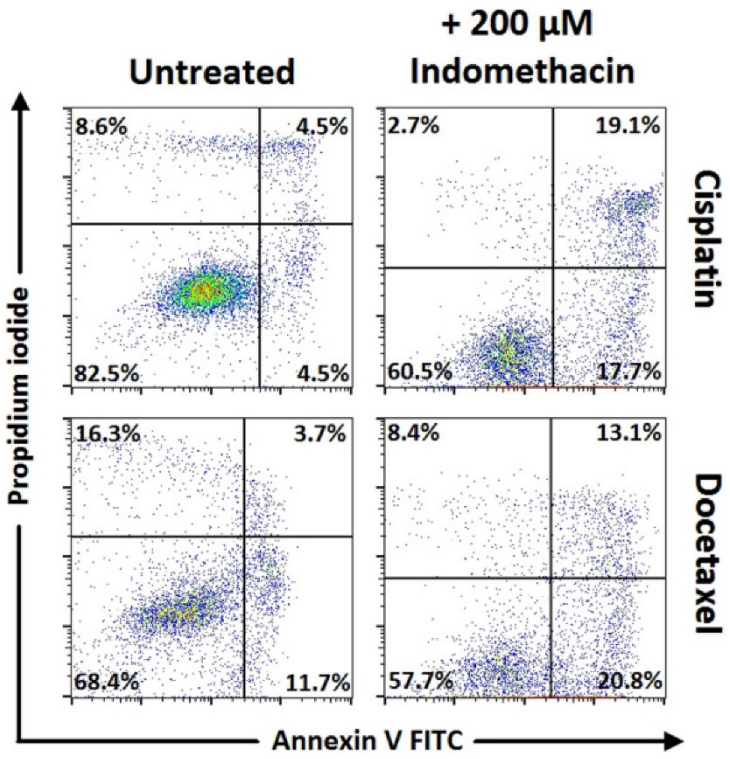

Figure 3: Cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry. Representative data for NSCLC, after 48h incubation. Combinations of cisplatin and docetaxel with indomethacin (200µM).

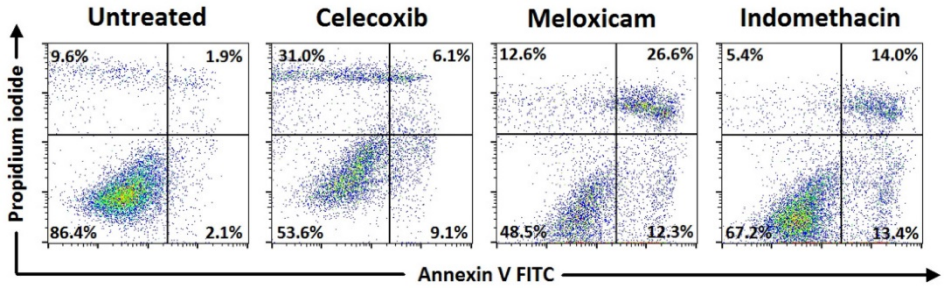

Figure 4: Cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry. Representative data for NSCLC, after 48h incubation, celecoxib (80µM), meloxicam (200µM), indomethacin (200µM).

Table 4: Means ± standard error of the mean (SEM) of percentages for cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry in NSCLC cell line after 48h incubation with drug combinations (representative data in accordance to Figure 3).

Drugs	Dead %	Late Apoptotic %	Early Apoptotic %	Live %
Control/NSCLC cells untreated	10±1.5	2±0.5	5±1.7	86±3
Cisplatin 25µM	9±1.8	5±0.8	5±0.8	82±3
Docetaxel 25µM	16±2.5	4±1.6	12±1	68±4
Indomethacin 200µM	6±0.5	14±2.3	13±2.1	67±3.5
Cisplatin + indomethacin 200µM	3±0.5	19±1.5	18±1.6	61±2.8
Docetaxel + indomethacin 200µM	8±1.9	13±1.4	21±2	58±2.3

Table 5: Means ± standard error of the mean (SEM) of percentages for cell viability and apoptosis with Annexin V/Propidium iodide by flow cytometry in NSCLC cell line after 48h incubation with COX-2 inhibitors alone (representative data in accordance to Figure 4).

Drugs	Dead %	Late Apoptotic %	Early Apoptotic %	Live %
Untreated cells NSCLC	10±1.5	2±0.5	5±1.7	86±3
Celecoxib 80µM	31±3	6±1.5	9±2.6	54±4
Meloxicam 200µM	13±2.9	27±2.7	12±1.8	49±3
Indomethacin 200µM	6±0.5	14±2.3	13±2.1	67±3.5

Discussion

According to the National Comprehensive Cancer Network practice guidelines, the etoposide–cisplatin (EC) regimen used concurrently with radiotherapy, has been the standard chemotherapy for decades for the first-line treatment of SCLC [31, 32]. Alternative treatment strategies include taxanes (paclitaxel and docetaxel) and platinum analogues (carboplatin, cisplatin). Similarly, radiotherapy and platinum-based chemotherapy has made no progress in the treatment of NSCLC. Clinical resistance is considered another obstacle in the treatment of NSCLC [33]. Thus, the need for novel treatment combinations has widespread interest.

For more than 30 years, nonsteroidal anti-inflammatory drugs (NSAIDs) have been the focus of attention as anticancer agents to reduce the clinical signs associated with inflammation [34]. The targets of traditional NSAIDs include the cyclooxygenases 1 and 2 (COX-1 and COX-2), enzymes involved in the production of prostaglandins from arachidonic acid. It has been reported that long-term use of traditional NSAIDs is associated with serious gastrointestinal side effects that have been attributed to COX-1 inhibition [35, 36]. As a result, COX-2 inhibitors were developed that had fewer gastrointestinal side effects according to clinical studies [37-41], but had anti-inflammatory activities that were similar to those of traditional NSAIDs. In the present study, we investigated the synergistic effect of COX-2 inhibitors, with chemotherapeutic agents, docetaxel and cisplatin on the apoptosis of lung cancer cell lines. Figure 5.

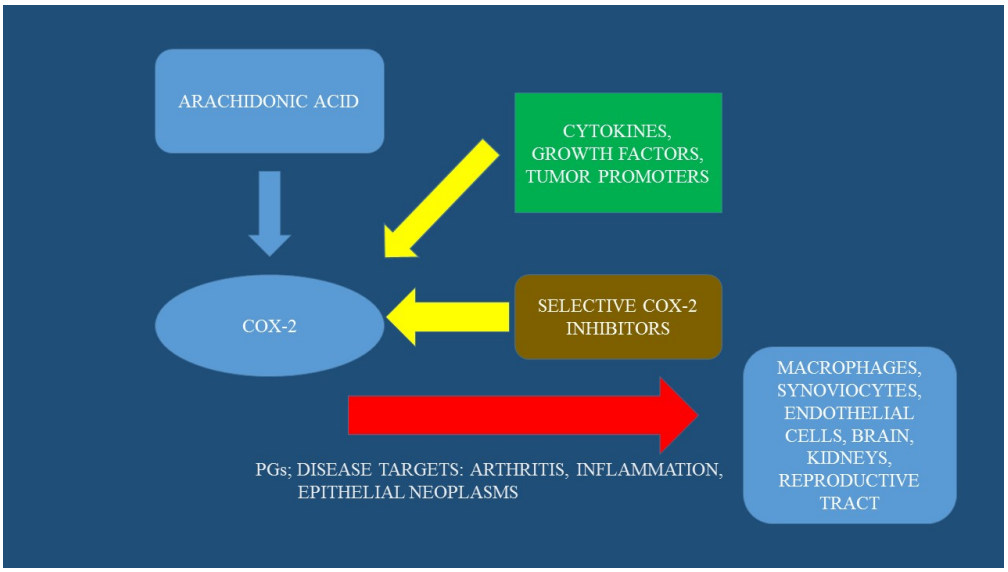

Figure 5. Mechanism of action of COX-2 inhibitors.

Traditional NSAIDs are nonselective inhibitors of both cyclooxygenase-1 (COX-1) and COX-2 which convert arachidonic acid to prostaglandin (H 2). COX-2 can generally be upregulated in response to cytokines, growth factors, tumor promoters, stress and other stimuli in various tissues including lung cancer [11]. In cancer, the regulation of COX-2 is abolished, so that both enzymes are overexpressed, leading to an increase in prostaglandin (PGE2) production in these cells [42]. PGE2 via stimulation of prostaglandin E2 receptor activates multiple pathways resulting in tumor cell proliferation, angiogenesis, survival and antiapoptosis [43-46]. In lung cancer cells, particularly in adenocarcinoma, according to clinical studies, overexpression of COX-2 is considered to be a negative predictive factor in the survival of the subpopulation [47]. Similarly, in colon cancer, overexpression of both cyclooxygenases in the early adenoma stage leads to one of the first steps for its development [48]. The patients showed a significant decrease in the multiplicity of polyps and induced regression of polyps after they received sulindac, a selective COX inhibitor, for 1 year [49-51].

The appearance of increased COX-2 expression might be involved in cancer development by inhibiting apoptosis, promoting cell division, altering cell adhesion and enhancing metastasis stimulating neovascularization, antitumor immune responses and inhibition of cellular protein synthesis [52]. Since traditional NSAIDs block these activities, their inhibition of COX-2 activity could be considered for their anticancer effects. Besides, apart from these mechanisms, according to several reports, COX-2 independent mechanisms may also participate in the

anticancer effects. Furthermore, each traditional NSAIDs such as celecoxib and rofecoxib may have their own more or less specific COX-independent target [53, 54]. In our study, indeed each COX-2 inhibitor showed different results in lung cancer cell lines. In particular, the combinations of meloxicam, ibuprofen and indomethacin with the chemotherapeutic agents showed more significant apoptosis when compared to the other combinations in both lung cancer cell lines. Furthermore, the combination of COX-2 inhibitors with chemotherapeutic drugs showed increased apoptosis in a time-dependent manner. SCLC showed higher sensitivity to the combination than NSCLC.

The use of taxanes in cancer is based on their interference with microtubule assembly, impairment of mitosis, and changes in the cytoskeleton [55]. They also stimulate mitogen-activated protein kinases (MAPKs) and the expression of numerous genes including COX-2 [56-58]. In particular, there is growing evidence that beyond the mechanisms that control COX-2 transcription [13], post-transcriptional mechanisms are also important [59, 60]. In our study, we observed that docetaxel had a synergistic effect with all the COX-2 inhibitors when compared with docetaxel alone in SCLC and NSCLC in vitro. It has been reported that taxanes stimulate COX-2 expression followed by increased PGE2 production, thus, resulting as expected in synergistic effect [13]. Indeed, in a phase II clinical trial on NSCLC patients, survival benefit with COX-2 inhibitor and chemotherapy was demonstrated in patients with moderate to high COX-2 expression [28]. Similarly, in our study, COX-2 inhibitors showed a synergistic effect with docetaxel on NSCLC cell line. However, in a recent phase II clinical trial the combination of docetaxel and apricoxib (COX-2 inhibitor) as second-line therapy on advanced NSCLC patients, showed negative results, implying that taxanes-driven augmentation of COX-2 expression might diminish the effect of COX-2 inhibitors [61].

Although, platinum-based doublet chemotherapeutic regimens include the primary therapeutic method for lung cancer, cisplatin resistance is a main clinical problem [33]. Thus, it is urgent the need for finding combination drugs to overcome this problem. In our study cisplatin combinations with COX-2 inhibitors showed inconsistent results in both lung cancer cell lines, however the synergistic effect was obvious. Similarly, in a phase II trial the combination of celecoxib plus platinum-based chemotherapy as first-line treatment COX-2 positive NSCLC patients, confirmed by immunohistochemical staining showed promising results [27]. Moreover, another phase II study suggested that celecoxib may safely be combined with etoposide for the treatment of extensive-stage in SCLC [62]. However, recently, Chen et al showed that celecoxib reduced an influx of cisplatin in gastric cancer in vitro, by antagonizing cisplatin-induced cytotoxicity and apoptosis in a COX-2 independent manner, suggesting the cautious selection of the combination drug with COX-2 inhibitor [63].

According to Yokouchi et al, results from several clinical trials indicate that patients that do not express COX-2 may have worse outcomes when treated with COX-2 inhibitor, perhaps due to the domination of COX-1 pathway in normal cells [64]. This study showed the importance to identify the subgroup of patients with activated COX-2 pathway. The selection of the appropriate method that will best determine the efficacy of the combination of COX-2 inhibition with chemotherapy is at need. Gitlitz et al attempted to use a patient-selection strategy in a randomized placebo-controlled study of a COX-2 inhibitor and erlotinib in NSCLC. They selected NSCLC patients based on a 50% decrease from baseline levels of a urinary metabolite of PGE2 (PGEM) in response to apricoxib, but the primary endpoint of the trial was not met, possibly due to the low cutpoint for the decline in PGEM [65]. Reckamp et al evaluated COX-2 expression in a phase II trial [66] by measuring levels of baseline PGEM based on results of their phase I trial in NSCLC [67, 68]. They reported that COX-2 pathway represents a novel mechanism of resistance to EGFR TKI therapy in NSCLC patients. Thus, in their phase II trial, they demonstrated that erlotinib and high-dose celecoxib led to an increase in progression free survival in selected patients with wild-type EGFR and COX-2 inhibition evaluated by elevated baseline PGEM [66]. In other studies, the evaluation of COX-2 and EGFR inhibition in small sample size and unselected patients in NSCLC showed limited benefit [69-72], high lightening the need of using biomarkers and genomic data.

Conclusion

The synergistic effect of the combination of COX-2 inhibitors and chemotherapeutic agents has been demonstrated. Our suggestion is that COX-2 inhibitors could be added as maintenance treatment in lung cancer patients. However, in clinical practice several issues should be considered for further investigation. Examination of genetic and epigenetic background and exploration of biomarkers defining a subset of lung cancer patients that would benefit by these combinations combined with better understanding of COX-2 biology would shed more light on how to achieve clinical improvement. Cox-2

inhibitors have been used with immunotherapeutic agents with effectiveness [73, 74], however; more trials are necessary for non-small cell lung cancer.

Conflict of Interest

None to declare.

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