



# Study of COX-independent chemopreventive activity of NSAIDs

NSAIDs chemopreventive action

Shokhan Tofiq

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Supervisors: Paolo Ciana and Staffan Tavelin

Thesis examiner: Olov Nilsson

## **Abstract**

Non steroidal anti-inflammatory (NSAID) drugs are a class of compounds displaying anti-inflammatory, mild analgesic and antipyretic effects. The anti-inflammatory activity of these drugs is due to their ability to bind and inhibit cyclooxygenase (COX) enzymes which transform arachidonic acid in prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), the first step in prostanoid biosynthesis. It has been known that cancer is a major public health problem and the global burden of this disease continues to rise. There are several factors which increase the risk of cancer such as tobacco smoking, excessive alcohol consumption, unhealthy diet habits and lifestyles. Prevention has been shown to produce a significant reduction of cancer death in the latest two decades; chemoprevention is certainly a promising strategy to reduce the general incidence of cancer, especially to the risk population similarly to what obtained in cardiovascular disease prevention.

The tumor suppressor p53 is a nodal switch turned on by different cellular stresses; this transcription factor controls the expression of several genetic programs that induce cell cycle arrest, apoptosis, senescence and DNA repair. p53 transcriptional activity is induced by post-transcriptional modifications such as phosphorylations and acetylations.

Previous study in the lab showed that the anti-proliferative effect of NSAIDs correlates with increased level of p53 acetylation on K382. In my thesis I provide evidence that the effect is COX-independent since sulindac sulfone, the sulindac metabolite, does not display anti-inflammatory activity while retains the ability to induce p53 activity in cancer cells.

If confirmed by future studies this initial evidence may lead to the development of a novel class of chemopreventive drugs without gastrointestinal side effects.

## **1. INTRODUCTION**

Non steroidal anti-inflammatory (NSAID) drugs are a class of compounds displaying anti-inflammatory, mild analgesic and antipyretic effects. The anti-inflammatory activity of these drugs is due to their ability to bind and inhibit cyclooxygenase (COX) enzymes (1) which transform arachidonic acid in prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), the first step in prostanoid biosynthesis. There are two different COX iso-enzymes: COX-1 and COX-2. COX-1 is responsible for the constitutive production of prostanoids and COX-2 for the elevated production of prostanoids that occurred in sites of disease and inflammation: many studies demonstrated the association between increasing level of COX-2 *in vivo* with inflammation, rheumatoid arthritis, seizures and ischemia (3). This is the reason why mainly COX-2 is the target for the anti-inflammatory drugs, however, many studies have shown that NSAIDs inhibit both COX-1 and COX-2 with a general tendency toward COX-1 selectivity. Inhibition of this isoenzyme is the main cause of gastrointestinal toxicity: the more COX-1-selective is a drug the more it seems to have the tendency to cause gastrointestinal damage. This is the rationale basis for development of selective inhibitors of COX-2 (3).

## **2. NSAIDs AND CANCER CHEMOPREVENTION**

The connection between the regular consumption of NSAIDs (especially aspirin) and a decrease in the incidence of colorectal cancer was demonstrated in the 1990s (1). It is not entirely clear how this protective effect of NSAIDs is exerted, but it was suggested to occur *via* inhibition of COX-2.

In studies of subjects with familial adenomatous polyposis, celecoxib and rofecoxib have been found to produce significant benefits not only in cancer development, but also in reduction of liver metastases as scored by endoscopy (1). The anti-neoplastic effects of NSAIDs is not limited to colon cancer cells but this activity was demonstrated in several type of neoplasia, such as gastric, esophageal and pancreatic cancer where the treatment of the anti-inflammatory drugs reduce tumor cell proliferation and invasiveness, increase of apoptosis (3). It has been known that cancer is a major public health problem and the global burden of this disease continues to rise. There are

several factors which increase the risk of cancer such as tobacco smoking, excessive alcohol consumption, unhealthy diet habits and lifestyles. Prevention has been shown to produce a significant reduction of cancer death in the latest two decades; chemoprevention is certainly a promising strategy to reduce the general incidence of cancer, especially to the risk population similarly to what obtained in cardiovascular disease prevention. Chemoprevention refers to the administration of chemical agents that occur naturally in foods (or have been synthesized) to help block tumor initiating and promoting events that are sequential stages of cancer development. Several epidemiological, clinical and experimental studies established NSAIDs especially the highly selective cyclooxygenase (COX)-2 inhibitors as promising cancer chemopreventive agents (11,8).

Randomized clinical trials have confirmed that two NSAIDs, the prodrug sulindac and the selective cyclooxygenase (COX)-2 inhibitor celecoxib, inhibit the growth of adenomatous polyps and cause regression of existing polyps in patients with the unusual hereditary condition familial adenomatous polyposis (FAP) (2). Several epidemiological studies have reported that long-term use of aspirin and other NSAIDs has been shown to reduce the risk of cancer of the colon and other gastrointestinal organs as well as of cancer of the breast, prostate, lung, and skin (10). Following this investigation, several other case-control studies likewise reported a protective effect of aspirin against colorectal cancer (10). Researchers also found that people who regularly used for years to prevent vascular events such as heart attack or stroke, reduces of 37% the mortality risk from multiple tumor types (10).

### **3. NSAID AND BREAST CANCER**

Breast cancer is the most common cause of cancer-related death in women. The basis of treatment is surgery, chemotherapy, radiotherapy, and drugs that counteract the estrogen activity at the receptor level like for example tamoxifen, or the newer generation of aromatase inhibitors, which inhibit the peripheral synthesis of oestrogens. Interest has grown in the past few years to know more about the link between NSAID use and breast cancer risk (4).

Several epidemiological evidence showed reduction of breast cancer risk in women taking NSAIDs. For example Coogan et al., in a hospital-based case-controlled study of 6558 patients, demonstrated a small reduction of breast cancer incidence in regular NSAID users (4). Harris et al. also found a significant reduction of breast cancer incidence in NSAIDs users in a prospective cohort study among 32505 women in central Ohio, USA (4). It is worth mentioning the genetic polymorphism of the COX-2 gene can have interaction with the effect of NSAIDs on breast cancer: in fact, a case-control study of 1067 patients and 1110 controls showed no reduction of breast cancer incidence for the three COX-2 variant alleles, but a reduced risk of breast cancer in those taking NSAIDs who had at least one of the variant C of the

COX-2 allele (4). Case-control and cohort studies show a modest reduction in the risk of breast cancer in women taking NSAIDs, especially aspirin, and for those who have been diagnosed with breast cancer can see reduced mortality of all those taking NSAIDs (4). A meta analysis of 38 studies (16 case – control studies, 18 cohort studies, 3 case – control studies nested in well defined cohorts, and 1 clinical trial) that included 2,788,715 subjects, suggest that overall, NSAID use was associated with reduced risk for breast cancer (6).

The picture emerging from the systematic review and meta-analysis carried out so far leads to the conclusion that that NSAID use is associated with a small reduction of breast cancer development with a marginally statistically significant difference: the reduction is slightly more obvious in the aspirin and ibuprofen use (5). According to these studies, NSAIDs can reduce breast cancer risk by 20% and there might be a role for NSAIDs in combination with endocrine therapies either an adjuvant or palliative treatment for women with established breast cancer (4).

#### **4. Breast cancer and chemoprevention**

In the last few years, we observed a reduction of mortality but an increased incidence of breast cancer. Therefore, the social and economic consequences of this disease continues to increase. Breast cancer is the most commonly diagnosed malignancy in women. The idea of prevention of breast cancer goes back to history. Researchers studied the correlation between environmental and individual factors that may increase the risk of breast cancer. Several advances have been made in understanding the underlying mechanisms of breast cancer development, and some drugs have recently been approved for the preventive approach of this disease.

Many clinical evidences showed that non-modifiable factors are able to increase breast cancer risk in women such as gender, age and family history; some other behavioral factor was shown to be associated with an increased risk of mammary tumors such as alcohol intake, fat, obesity in postmenopausal age, and hormonal stimuli. The exact mechanism that promotes breast cancer is not fully established, although estrogen hormones have been shown to play a significant role in almost 70% of cases. Estrogen hormones are well established as promoter of cell division in the mammary epithelium, this is the reason why breast cancer chemoprevention has focused heavily on endocrine intervention using selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs).

*SERMs*. This class of drugs includes in particular tamoxifen (TAM) and raloxifene, acting as both estrogen agonist and antagonists. Tamoxifen citrate is the first generation of SERMs that competes with circulating estrogen for binding the estrogen receptor (ER). Raloxifene, a second generation of SERMs, also has both estrogen agonist and antagonist properties, while it differs from tamoxifen principally by its lack of mitotic effects on

endometrium (7). TAM has been in use for breast cancer treatment for more than 30 years and was shown to reduce the risk of both recurrence and contralateral neoplasia; has indication as a chemopreventive agent, and several studies were conducted in last decades in this particular setting.

*Aromatase inhibitors* (AIs). Many studies have shown that high circulatory estrogen levels, and high aromatase levels in breast tissue, increase breast cancer risk. Therefore inhibition of aromatase would be expected to decrease estrogen production and ultimately estrogen-related breast carcinogenesis. Third generation of AIs (anastrozole, letrozole, and exemestane) in adjuvant has been found to superior to tamoxifen and to reduce the incidence of contralateral breast cancers. The use of these drugs have resulted in improved disease-free survival and were associated with fewer side effects than SERMs (7).

## **5. MECHANISM OF ACTION OF NSAID**

### **5.1 NSAIDs: Modulation of COX-Isoforms**

The mechanism initially proposed for the NSAIDs anti-cancer activity is based on the ability of these drugs to inhibit arachidonic acid (AA) metabolism via COX enzymes; COX inhibition reduces the synthesis of prostaglandins (PGs) which affect several cancer related functions including cell proliferation, apoptosis, neo-vascularization and immune responsiveness. NSAIDs prevents the formation of PGH<sub>2</sub>, the first committed step in the metabolism of AA into a complex cascade of signaling lipids, such as PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2</sub>α, PG I<sub>2</sub>, and thromboxane B<sub>2</sub>. In colon cancer, programmed cell death or apoptosis, is needed to maintain homeostasis in replicating tissues such as the intestine. NSAIDs seem to restore normal apoptosis and reduce cell proliferation in human adenomatous colorectal polyps, experimental colonic tumors, and in various cancer cell lines that have lost critical genes required for normal function (2).

### **5.2 NSAIDs:Modulation of COX-Independent Pathways**

Although inhibition of COX pathway may certainly explain at least in part the NSAID anti-cancer activity, several results seem to indicate that COX-independent mechanism may also play a role. Several studies pointed out that the products rather than the substrate of COX mediate its biologic effects: for example, in some experimental models, the concentration of free arachidonic

acid regulates apoptosis in colorectal epithelial cells (2). Other experimental models suggest that NSAIDs may affect apoptosis through a mixture of prostaglandin-dependent and prostaglandin-independent pathways (2). Sulindac sulfoxide is a prodrug that is either reversibly reduced to sulindac sulfide, which has potent antiprostaglandin synthetase activity due to its ability to inhibit cyclooxygenase I and II, or is irreversibly oxidized to the sulfone, which has been initially considered as an inactive metabolite that is eliminated from the body (9).

Sulindac sulfoxide and other NSAIDs have been reported to inhibit tumor formation in several models of experimentally induced cancer via inhibition of prostaglandin synthesis (16). Further studies on sulindac sulfone demonstrated that this metabolite is not inactive, but displayed a biological activity promoting cell growth arrest and inducing apoptosis independently from cyclooxygenase inhibition; furthermore, this drug was shown to inhibit development and to induce tumor regression in chemically induced mammary and colon cancer models *in vivo*. Another study shown that Exisulind (sulindac sulfone) is a proapoptotic drug that causes regression and prevents recurrence of polyps in patients with familial adenomatous polyposis (17). Exisulind and its analogs inhibit cell growth and induce apoptosis in colon tumor cells without cyclooxygenase (I or II) inhibition (17).

The discovery of a COX-independent anti-tumor activity of sulindac sulfone has a significant implication in relation to the use of NSAIDs for cancer chemoprevention, as one factor limiting their use is indeed gastro-intestinal toxicity.

## **6. PRELIMINARY DATA IN THE LAB**

The tumor suppressor p53 is a nodal switch turned on by different cellular stresses; this transcription factor controls the expression of several genetic programs that induce cell cycle arrest, apoptosis, senescence and DNA repair. p53 transcriptional activity is induced by post-transcriptional modifications such as phosphorylations and acetylations. In particular, p300 (12) and several deacetylases, including sirtuins (12) were shown to modulate p53 activity in response to carcinogens. Preliminary data in the laboratory demonstrated an anti-proliferative activity of nimesulide and other NSAIDs in MCF-7 and MDAMB-231 cell lines. The anti-proliferative effect of NSAIDs correlates with increased level of p53 acetylation on K382. Interestingly these effects appear to be COX-independent since sulindac sulfone displayed the same biological activities.

## **7. AIM OF THE STUDY**

To provide evidence of the COX-independent modulations of p53 activity by NSAIDs on mammary cancer cells. The study was aimed at testing p53 activity in Hela cells transiently transfected with a reporter of p53 activity before and after treatment with increasing concentrations of sulindac sulfone which is not able to inhibit COX activity.

## **8. MATERIAL AND METHODS**

### **8.1 Cell Culture**

Human Cancer Cell Lines. Hela cells (Human cervical cancer cell lines which express p53 wild type). Were maintained in MEM (minimum essential medium, Euroclone) containing 10% FBS and antibiotics (10000 units/ml penicillin G 1%, 5000  $\mu$ g/ml streptomycin); 1%, sodium pyruvate 100 mM. Cell cultures were carried out in Petri-dishes at 37°C in a 5% CO<sub>2</sub> fully humidified atmosphere and split two times per week.

### **8.2 Reagents**

Tested reagents were sulindac sulfone and etoposide powder purchased from Sigma Aldrich, dissolved in Dimethyl sulfoxide (DMSO) the final percentage of DMSO in each well is 0.1%

### **8.3 Protocol to prepare cells for transfection**

The performance is based on a transient transfection which is introducing nucleic acids into eukaryotic cells by nonviral method.

Two days before transfection cells were seeded using the following protocol:

1. Remove medium from the tissue culture dish. Add 5 ml of PBS (phosphate buffer saline) to cover the cell monolayer. Rock the plates to distribute the solution evenly. Remove the final wash. Add 1 ml of trypsin solution to cover the cell monolayer and remove it.
2. Place plates in a 37°C incubator until cells just begin to detach (usually 3–5 minutes).
3. Remove the petri dish from the incubator. View the cells under a microscope to check whether all cells have detached from the growth surface. If necessary, cells may be returned to the incubator for an additional 1–2 minute.

4. When all cells have detached, add MEM + 10% FBS medium to recollect. Gently pipet cells to break up cell clumps. Cells may be counted using trypan blue exclusion method for cell counting in Burker's chamber.
5. Seed  $4-6 \times 10^5$  cells per well of 24-well plate using 500  $\mu$ l of cell suspension plus growth medium and put the plate into the incubator.

#### **8.4 Transfection assay.**

Cells were grown to semi-confluence at the time of transfection.

A transfection mix solution was prepared with lipofectamine LTX, Invitrogen, to a solution containing DNA vectors (PGL13LUC, PCMV-LacZ), DNA filler (salmon sperm DNA), plus reagent and medium without antibiotics.

The transfection mix, vortexed and left it for 3 min at room temperature; then added lipofectamine, vortexed again and after 30 min added 95  $\mu$ l of transfection complex to each well of 24-well plate. The solution mixed gently by rocking the plate back and forth. Incubated the cells at 37° C in a incubator and after 5h the medium changed to MEM 10% DCC and the cells were ready next day for treatment or for assay for transgene expression or selection for stable transfection.

The day after transfection cells were ready for treatment.

#### **8.5 Cell lysates and luciferase assay**

This step was done to obtain protein extracts

A dilution of lysis buffer (promega) 1:5 made and stored it into ice, because lysis buffers sensitivity. The growth medium from the wells aspirated and the well washed with 500  $\mu$ l of PBS stored at room temperature. After washing

100 $\mu$ l of lysis buffer 1X added to each well and left at room temperature for 7 minutes. Last step was transferring 100  $\mu$ l from each of the 24 well to 96-well on ice and stores in freezer at -20°C.

#### **8.6 Luciferase assay**

To analyze the transfected extracts which express the reporter Luciferase it was carried out a Luciferase enzymatic assay. Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin (15).

#### **8.7 Beta-galactosidase assay**

The Beta-galactosidase was applied measures the LacZ enzymatic activity in the cells.

## **8.8 Bradford's assay for protein quantification**

The Bradford's assay is one of several simple methods commonly used to determine the total protein concentration of a sample(13). The method was based on the proportional binding of the dye Coomassie Brilliant Blue G-250 to proteins. Within the linear range of the assay (~5-25 mcg/mL), the more protein presented, the more Coomassie binds (13). Furthermore, the assay was colorimetric; as the protein concentration increases, the color of the test sample became darker. Coomassie absorbs at 595 nm(14).

The protein concentration of a test sample was determined by comparison to that of a standard curve made with Bovine Serum Albumin (BSA) at starting concentration of 2mg/ml (14).

Mind that the lysis buffer used to prepare extracts may interfere with the colorimetric reaction. Quantification was performed in 96-well plates. The first column was used for blank, the second and the third for the standard curve (prepared in double) (14).

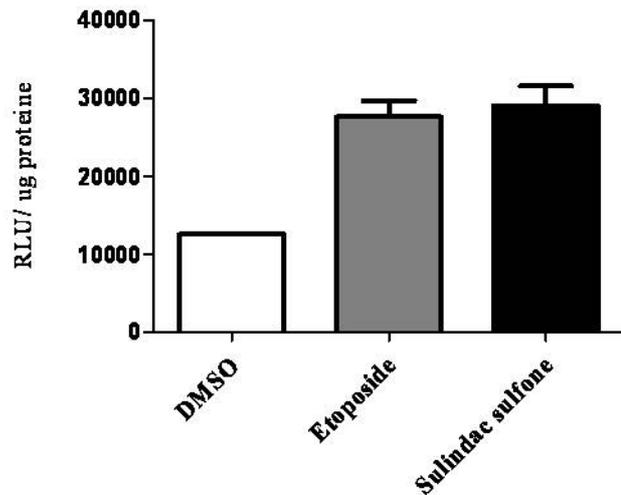
## **9. RESULTS**

### **9.1 Establishment of a reporter assay for p53 activity.**

Previous experiments from our and other laboratories demonstrated the ability of NSAIDs and of the sulindac metabolite, sulindac sulfone (unable to inhibit COX), to block cell proliferation (data not shown); preliminary data from our laboratory has demonstrated a correlation between the growth arrest induced by these compounds and p53 acetylation, raising the intriguing hypothesis of a common anti-proliferative mechanism of action shared by these compounds.

To gain further insight into the mechanism of p53 modulation by sulindac sulfone, the activity of this transcription factor was tested in transiently transfected Hela cells using a p53 reporter system (18). Initial experiments were carried out to set up the transfection system (data not shown), subsequently the functionality of the reporter assay was demonstrated by treating transfected cells with 20  $\mu$ M etoposide as positive control and with 90  $\mu$ M sulindac sulfone; previous data in the lab showed that at this concentration both compounds were able to induce p53 acetylation at K382 residue in Hela cells. The results of these experiments are summarized in Figure 1.

The result of data statistics checked with t-test and found that P value was significance different.

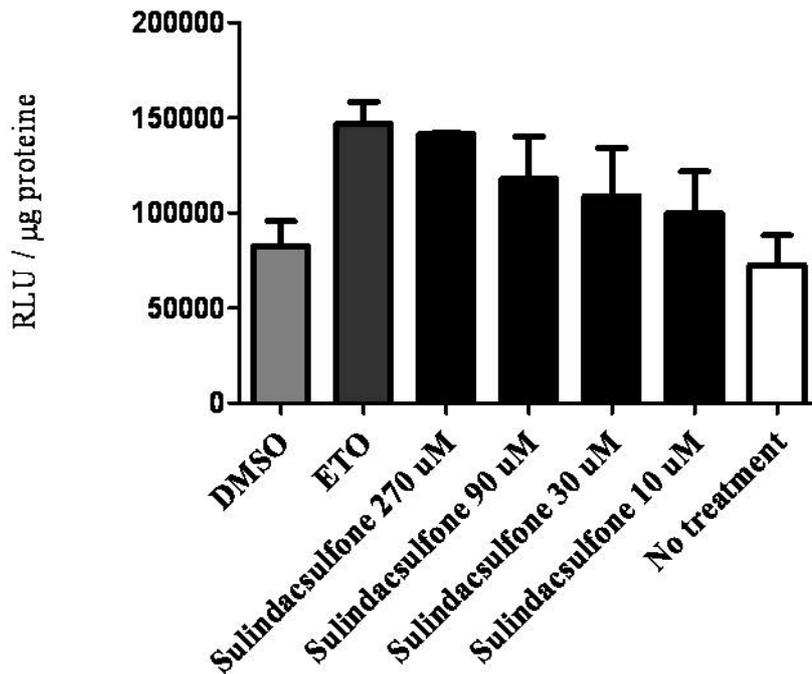


**Figure 1.** Sulindac sulfone increase P53 activity in HeLa cells.

HeLa cells were plated in 100mm dishes, transfected with the reporter system and then treated for 24 hours with 90  $\mu$ M sulindac sulfone, 90  $\mu$ M Etoposide or vehicle (DMSO).

## **9.2 Sulindac sulfone increases P53 activity in a concentration-dependent manner**

To verify that the increase P53 activity was a pharmacological effect of sulindac sulfone, HeLa cells transfected with the P53 reporter construct were treated with increasing doses of sulindac sulfone (Figure 2).



**Figure 2.** Hela cells transfected with the P53 reporter system were treated with increasing concentrations of sulindac sulfone and with DMSO and Etoposide as controls.

The results clearly demonstrated that sulindac sulfone was increasing P53 activity in a concentration-dependent manner suggesting a direct pharmacological effect of the compound on the modulation of the transcription factor action.

These data may suggest that the modulation of P53 activity by sulindac (not shown) and its metabolite is COX independent indicating that the anti-proliferative activity of these molecules might be due to a different pathway. The result of data statistics checked with t-test and found that P value was significance different, a P value 0,0474 gives that the result are significance different ( $P < 0,05$ ), se annex.

## 10. DISCUSSION

The tumor suppressor p53 is a nodal switch turned on by different cellular stresses; this transcription factor controls the expression of several genetic programs that induce cell cycle arrest, apoptosis, senescence and DNA repair(18). p53 transcriptional activity is induced by post-transcriptional modifications such as phosphorylations and acetylations. In particular, p300 and several deacetylases, including sirtuins were shown to modulate p53 activity in response to carcinogens (19). My result demonstrated that anti-proliferative effect of NSAIDs correlates with increased level of p53 acetylation on K382. Interestingly these effects appears to be COX-independent since sulindac sulfone displayed the same biological activities . Considerable excitement has been generated about the chemopreventative effects of NSAIDs on cancer. Unfortunately, sulindac , like most NSAIDs, can exhibit undesirable gastrointestinal side effects (16). My thesis provide evidence of the existence of a COX-independent mechanism for the anti-proliferative activity: along this line, future studies will further characterize, confirm and extend the mechanism responsible of this activity, a research which may lead to the development of a novel class of chemopreventive drugs without gastro-intestinal side effects.

The results reported in Figs 1 and 2. confirm and expand our preliminary report that sulindac sulfone, which has a minimal effect on either COX I or COX II activity in the prostaglandin biosynthetic pathway, significantly inhibits mammary carcinogenesis induced by either a low or a high dose. In fact, the levels of either sulindac sulfone required for chemopreventive activity against mammary carcinogenesis.

My data indicate that Sulindac sulfone is able to increase P53 activity and this could be a new COX-independent mechanism of chemoprevention.

Identification of COX-independent targets and mechanisms most important for the antineoplastic properties of these drugs can be used to develop more efficacious chemopreventive drugs without the gastrointestinal, renal, and cardiovascular side effects associated with NSAIDs and COX-2 inhibitors(9). Currently, sulindac derivatives have been developed that have antitumor activity without inhibiting COX-1 or COX-2 (11).

These experimental agents demonstrate the feasibility of developing safer and more efficacious drugs for chemoprevention by other targets without COX-inhibition represent other examples of separating COX-inhibitory activity and antitumor efficacy.

Some investigators have concluded that the mechanism responsible for the tumor cell growth inhibitory activity of NSAIDs may, in part or fully, be COX

independent, although alternative molecular targets have not yet been well defined and used to discover new drug candidates for chemoprevention. My results support the possibility that the mechanism responsible for the tumor cell growth inhibitory activity of sulindac sulfone is COX independent, which suggests the feasibility of developing safer and more efficacious drugs by chemically modifying sulindac to selectively design out its COX inhibitory activity.

Today chemoprevention in breast cancer is based on SERMS and aromatase inhibitors (8).

Research is needed to address the many unresolved issues related to the poor uptake of breast cancer chemoprevention agents in women who are at increased risk of breast cancer. For example the design of effective tools and approaches to educate providers on the option of chemoprevention, efficacious interventions that communicate to eligible women the risks and benefits of specific chemoprevention agents, the development of tools that more accurately identify women at increased risk, and a greater understanding of what disparities and barriers exist with regard to chemoprevention use among women at higher risk for breast cancer.

To interpret my data, I performed t-test to verify my hypothesis in section 9.1. I also performed a t-test of the data in section 9.2. Where i should have used a different statistical method, such as an ANOVA, as i compared several different conditions in the experiment. I could not do ANOVA because I do not really know how to do, I asked my supervisors from Milan, they tell me that the other tests can not fit to my data, since the P-value (and standard deviation) is too high, but I am aware of the risk of erroneous conclusion by simply using t-test in this situation.

## **11. Acknowledgement**

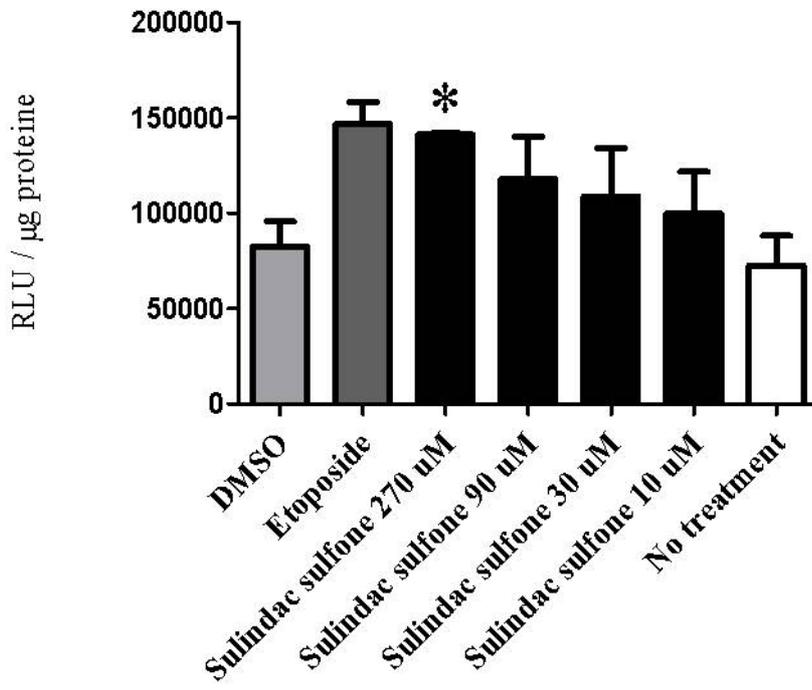
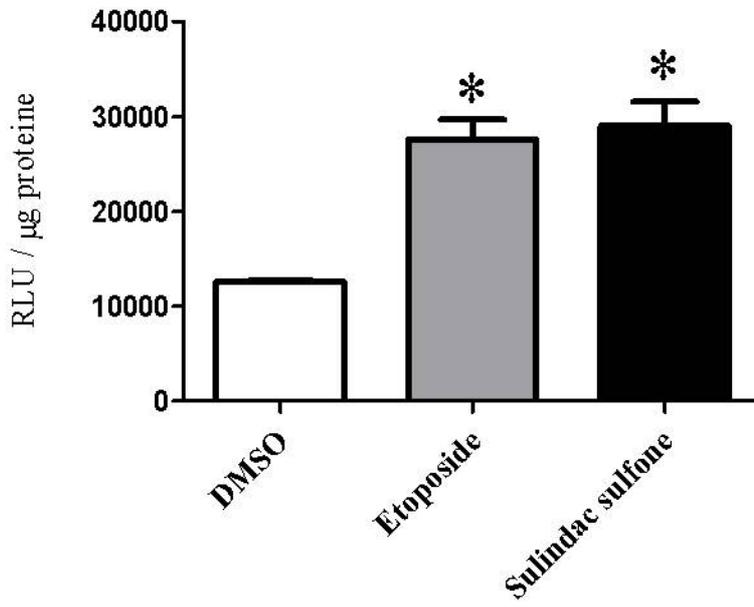
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## Annex 1

### Statistical analysis of figure 1

Parameter	
Table Analyzed	Data 1
Column A	DMSO
Vs	Vs
Column C	Sulindac sulfone
Unpaired t test	
P value	0,0029
P value summary	**
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.522 df=4
How big is the difference?	
Mean ± SEM of column A	12570 ± 122.3 N=3
Mean ± SEM of column C	29030 ± 2521 N=3
Difference between means	-16460 ± 2524
95% confidence interval	-23470 to -9455
R squared	0,9140
F test to compare variances	
F,DFn, Dfd	424.9, 2, 2
P value	0,0047
P value summary	**
Are variances significantly different?	Yes

### Statistical analysis of Figure 2

The only significance was found for the highest sulindac concentration

Table Analyzed	Data 1
Column A	DMSO
vs	Vs
Column C	Sulindac sulfone 270 uM
Unpaired t test	
P value	0,0474
P value summary	*
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.427 df=2
How big is the difference?	
Mean ± SEM of column A	82410 ± 13290 N=2
Mean ± SEM of column C	141300 ± 629.5 N=2
Difference between means	-58880 ± 13300
95% confidence interval	-116100 to -1644
R squared	0,9074



Department of Chemistry  
S-901 87 Umeå, Sweden  
Telephone +46 90 786 50 00  
Text telephone +46 90 786 59 00  
[www.umu.se](http://www.umu.se)