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IDENTIFICATION AND CHARACTERIZATION OF SIRT1 AS A NOVEL TARGET OF NSAIDS CHEMOPREVENTION

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A chi ha sempre creduto in me

Summary

ABSTRACT

English

Several epidemiological, clinical and experimental studies proposed non-steroidal antiinflammatory drugs (NSAIDs) as promising chemopreventive agents for many types of cancer. However, their use in chronic treatment is hampered by gastrointestinal, renal and cardiovascular side effects mainly due to the inhibition of cyclooxygenase (COX) isoenzymes. The development of NSAIDs as chemopreventive agents is prevented by the limited knowledge of the mechanism underlying the anti-cancer properties of these drugs. Inhibition of COX has been proposed as the mechanism underlying their anti-neoplastic activity; however, several lines of evidence challenged this simple view, among them the antitumor activity of non-COX inhibitory metabolites, enantiomers and derivatives are perhaps the most convincing.

The aim of the present project was to investigate a novel direct target of NSAIDs, which may provide a biochemical explanation of the multiplicity of the COX-independent effects. For that reason, molecular biology, cellular and in vivo tools, including innovative in vivo imaging and classical biochemical assays were applied.

Here we identify, for the first time sirtuin 1 (SIRT1) as a direct target of NSAIDs, showing that inhibition of this histone deacetylase may be responsible of their COX-independent antineoplastic activity. In particular, we demonstrated that many NSAIDs through SIRT1 inhibition: increased acetylation and activation of tumour suppressor p53 and increase the expression of antiproliferative gene p21 through SIRT1 inhibition. We showed that the mechanism occurs selectively in tumour tissue in cells, animal models and also in clinical setting. Activation of the p53 signaling produces an anti-proliferative effect, which prevents the early transformation steps of the mammary gland in an animal model of early breast cancer transformation. Increased local proliferation is not the only hallmark of cancer modulated by NSAIDs, interestingly our results indicate that the local immunosuppression promoted in the mammary gland by the exposure to a genotoxic agent can be efficiently counteracted by NSAIDs.

In conclusion, this thesis presents the discovery and characterization of the new target SIRT1 as effector of NSAIDs mediated chemopreventive activity. Our data disjoin the NSAIDs COX-dependent anti-inflammatory activity from the SIRT1-dependent anti-tumor activity, therefore suggesting a novel strategy to design molecules displaying anti-neoplastic chemoprevetinve activity without the COX-dependent side effects.

Italiano

Numerosi studi epidemiologici, sperimentali e clinici hanno proposto i farmaci antinfiammatori non steroidei (FANS) come promettenti agenti chemopreventivi per molti tipi di cancro. Tuttavia, il loro uso nel trattamento cronico è impedito dagli effetti collaterali tra cui eventi avversi gastrointestinali, renali e cardiovascolari, dovuti principalmente all'inibizione degli isoenzimi della ciclo-ossigenasi (COX). Lo sviluppo di FANS come agenti antitumorali, è limitato dalla mancata conoscenza del meccanismo d'azione alla base delle loro proprietà anticancro. L'inibizione delle COX è stata proposta come meccanismo responsabile, tuttavia evidenze di letteratura mettono in discussione questa visione, proponendo in modo più convincente, i metaboliti dei FANS che non hanno effetto su COX oppure i loro enantiomeri e derivati.

Lo scopo di questo progetto era quello di studiare un nuovo bersaglio molecolare per l'azione antitumorale dei FANS, fornendo così una spiegazione per tutti quegli effetti indipendenti dall'inibizione di COX. A questo proposito sono stati utilizzati metodi di biologia molecolare, cellulare e *in vivo*, tra cui l'imaging ottico *in vivo*.

Grazie allo studio svolto durante il mio dottorato è stato indentificato un nuovo bersaglio dei FANS, la sirtuina 1 (SIRT1). L'inibizione di questo enzima infatti, potrebbe spiegare l'attività antineoplastica dei FANS indipendente dall'inibizione di COX. In particolare, è stato dimostrato, in linee cellulari di tumore mammario, che l'inibizione di SIRT1 mediata dai FANS induce l'aumento dell'acetilazione e attivazione dell'oncosoppressore p53 e il conseguente aumento di espressione del gene p21 con azione antiproliferativa. Inoltre, l'effetto dell'inibizione di SIRT1 è stato dimostrato esclusivamente nei tessuti tumorali di modelli animali e pazienti affetti da cancro alla mammella. Nei modelli animali di carcinogenesi chimica utilizzati, si osserva un'iper-proliferazione a livello del tessuto tumorale. I nostri dati dimostrano che l'attivazione del segnale di p53, specificatamente nel tessuto tumorale, ha un effetto antiproliferativo, il quale impedisce al tumore di svilupparsi a partire dalle prime fasi di trasformazione neoplastica. L'aumento della proliferazione non è l'unica caratteristica tumorale modulata dai FANS. I nostri risultati indicano che l'immunosoppressione locale promossa dal genotossico dimetilbenzantrace (DMBA) nella ghiandola mammaria, può essere contrastata in modo efficace dai FANS.

In conclusione, questo lavoro di tesi ha portato alla scoperta e alla caratterizzazione di un nuovo target per l'attività chemopreventiva mediata dai FANS: SIRT1. I nostri dati separano gli effetti dei FANS tra l'attività antiinfiammatoria mediata dall'inibizione di COX, e l'attività

antitumorale mediata dall'inibizione di SIRT1. Questa scoperta suggerisce quindi una nuova strategia per lo sviluppo di nuove molecole che mantengano l'attività antitumorale dei FANS senza gli effetti collaterali COX dipendenti.

INTRODUCTION

CANCER IMPACT

Cancer is a generic term for diseases in which abnormal cells dived without control and can invade nearby tissues (PubMed Health Glossary). Despite significant advances in early diagnosis and the development of molecular targets, cancer remains the leading cause of mortality in the Western world[1,2].

In Italy prostate, lung, colorectal, bladder and kidney cancer are the most incident in men, while breast, colorectal, lung, corpus uteri and thyroid cancer are the most common among women[3] (Figure 1).

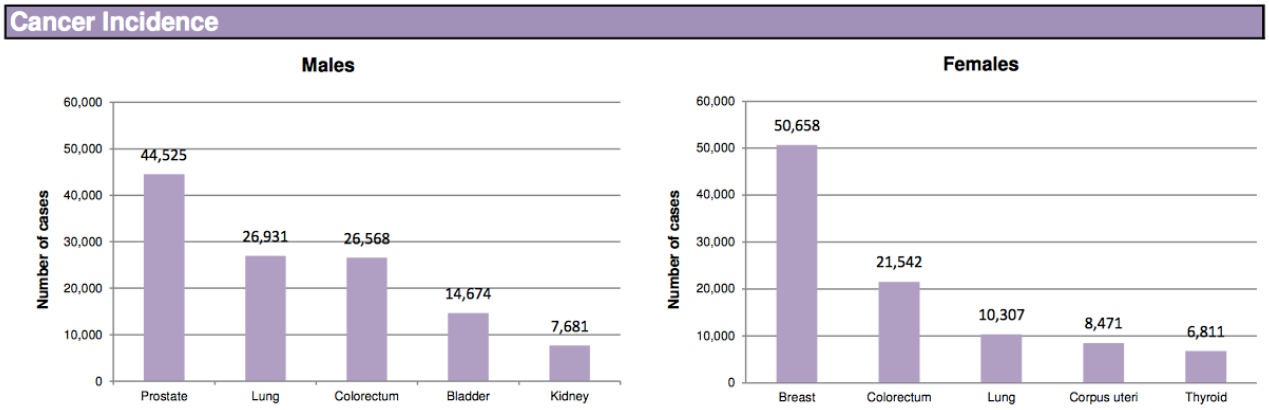


Figure1. Cancer incidence in Italy. World Health Organization – Cancer Country Profiles, 2014.

Mortality rates are influenced by incidence rates, and by the success in early diagnosis and treatments. New cancer cases are projected to increase in 42.47% between 2014 and 2035 (corresponding to an average annual increase of 1.63%). Moreover, this increase is disproportionately projected for males (48.42%) compared with females (36.41%). These massive changes in the absolute number of cancer cases are mainly a result of the growing and ageing population[4].

Hallmarks of cancer

The progressive evolution of healthy cell into a tumor cell is due to the acquisition of specific hallmarks that reflect the cancer phenotype. Instead, tumor formation is not the result of a single cell transformation, but tumor is represented by a complex tissue composed by different cell types, that interact with each other resulting in the expression of new functions. Usually, the cell journey of a normal cell towards full cancer transformation lasts several years and leads to the progressive acquisition of various neoplastic functions. Hanahan and

colleagues in 2001 have proposed six hallmarks of cancer that enable tumor growth and metastatic dissemination[5] (Figure 2), however during the following decade this notion has been solidified and extended, revealing that the biology of cancers must include the contributions of the tumor microenvironment to tumorigenesis[5].

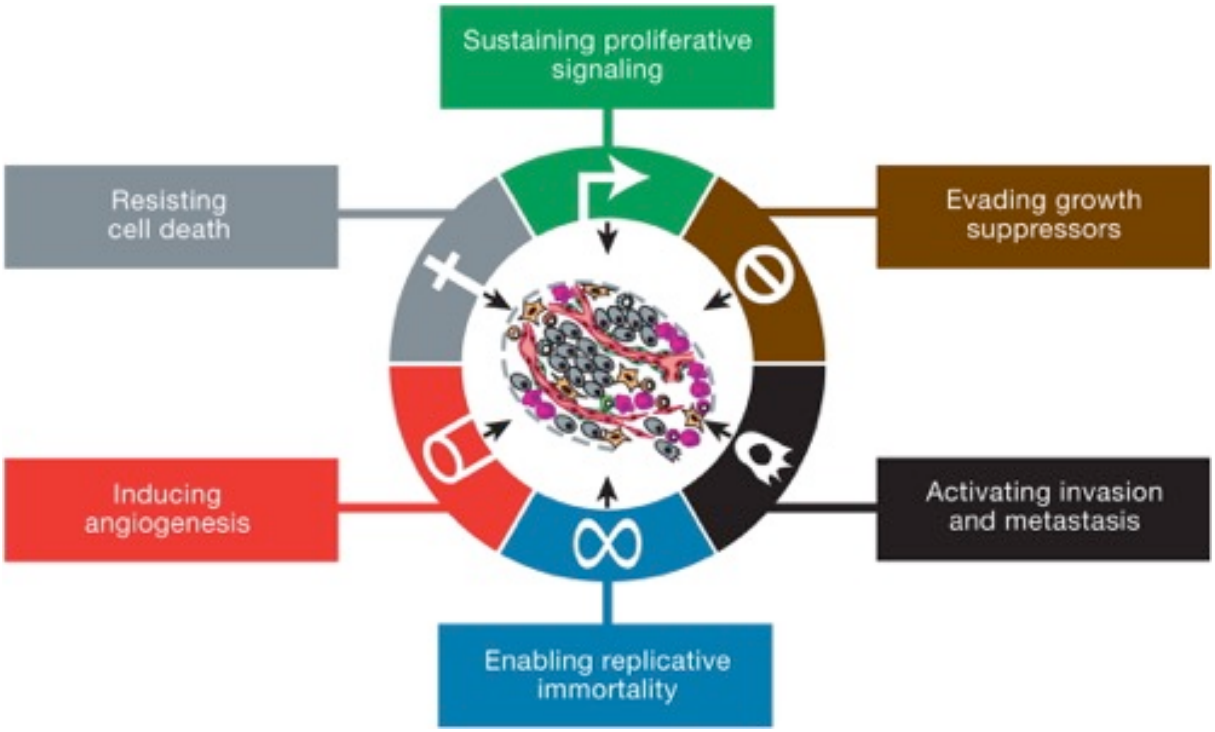


Figure 2. The Hallmarks of Cancer. This illustration encompasses the six hallmark capabilities, figure adapted from Hanahan 2011.

Sustaining proliferative signaling

Independent division and proliferation are the main characteristics of the cancer cells. A normal tissue regulates growth factors production and release that promote the initiation of mitosis and the cell cycle progression. The growth factors mostly interact with membrane tyrosine kinase receptor resulting in activation of pathways like cell cycle progression and cell growth. This condition is suppressed in the transformed cells, in fact, tumor cells are able to autoregulate themselves for replication. They explicate this function through several ways, mostly due to the genomic dysregulation[6]:

- By autocrine mechanism, tumor cells synthetize molecules that activates the growth factor receptors. Through this action, the cancer cell stimulates cell proliferation (Lemmon 2010);

- Tumour cells stimulate the surrounding cells to synthetize growth factors necessary for the tumour microenvironment[7];
- By increasing the number of the growth factor receptors on the surface of the tumour cells, this capability induces the cell to be more responsible for the increasing level of ligand;
- Somatic mutations of the transformed cells may result in constitutive receptor activation. This feature makes them able to activate the growth factors' pathways[8];
- Pathways that include cytoplasmic transduction and cell division are constitutively stimulated by eluding the ligand dependent activation[9].

Recent studies have underlined the importance of negative feedback loops that normally downregulate various types of signaling resulting in the homeostatic regulation of the flux of signals present through the intracellular circuity[10–13]. Enhanced proliferative signaling is permitted by defects in these feedback mechanisms. The model of this type of regulation involves the Ras oncoprotein: the oncogenic effects of Ras do not result from a hyperactivation of its signaling powers; conversely, the oncogenic mutations affecting Ras gene compromise Ras GTPase activity, which operates as intrinsic negative-feedback mechanism that usually certifies that active signal transmission is transitory[5].

Cell cycle division remains tight in check via regulatory proteins, even in the presence of growth signalling. For that reason, the deregulation of the cell cycle and checkpoint disruption are crucial for cancer cells to grow[14]. One key regulator is the retinoblastoma (RB) protein, that is usually inactivated in several tumours[15,16]. Another key regulator is the p53, whose gene is the most commonly mutated in care, with over 50% of sequenced tumours[17,18]. P53 is a stress detector, sensitive to a variety of stressor like genotoxic agents, nutrient deprivation, and hypoxia. Ones activated p53 induces cell cycle arrest and apoptosis, thus p53 mutation lead the tumour cells to the hyperproliferation state.

Evading growth suppressor

Besides sustaining proliferation, cancer cells must also contrast the regulated cell death; many of these programs depend on the actions of tumour suppressor genes. Thanks to cancer research several tumour suppressors have been discovered because of their function in limiting cell growth and proliferation. The two typical tumour suppressors encode the RB and p53 proteins; they operate as headquarter within key complementary cellular regulatory

pathways that manage the decisions of the cells to proliferate or activate senescence and apoptotic programs[5]. Cancer cell with defect in RB and p53 pathways are missing critical gatekeepers of cell-cycle progression whose absence permit persistent cell proliferation. Instead, during cell stress or irreparable damage to such cellular subsystem, p53 can induce apoptosis, however p53 is highly context dependent, changing by cell type as well as by the severity of cell stress conditions and genomic damage.

Mechanism of contact inhibition and its evasion

Confluent cell monolayers are allowed by cell to cell contacts formed by dense populations of normal cells propagated in two-dimensional culture operate to suppress further cell proliferation. Interestingly, "this contact inhibition" is abolished in various types of cancer cells in culture, suggesting that contact inhibition is an *in vitro* model of a mechanism that is present *in vivo* to ensure homeostasis in normal tissue, one that is abolished during carcinogenesis[5]. The mechanistic basis for this growth control is not clear yet however, mechanisms of contact inhibition are starting to emerge. One mechanism involves Merlin, the cytoplasmic product of NF2 gene that is the orchestrator of contact inhibition: this occurs via coupling molecules of cell surface adhesion like E-cadherin, to transmembrane receptor tyrosine kinases. Thus, Merlin intensify the adhesion of cadherin-mediated cell to cell interaction. Moreover, by sequestering growth factor receptors Merlin limits their ability to efficiently promote mitogenic pathways[19,20]. Another mechanism of contact inhibition involves LKB1 epithelial polarity protein, that helps the maintenance of tissue integrity by organizing epithelial structure. LKB1 has also been identified as an onco-suppressor since it can suppress hyperproliferation and it is lost in certain human cancer[21].

Finally, how often these two mechanisms of contact-mediated growth suppression are compromised in human cancer is yet to be discovered.

Resisting cell death

In the last two decades the concept that programmed cell death by apoptosis serves as a natural barrier to cancer development has been established by functional studies[2223,24]. The mechanism of apoptosis is induced by signalling imbalance resulting from elevated levels of oncogene signal and DNA damages associated with hyperproliferation. Moreover, other study has revealed how apoptosis is attenuated in high grade cancer that are commonly resistant to therapy[22,23]. The apoptotic machinery is composed of upstream regulators, that receiving and processing extracellular death-inducing signals and downstream effector components sensing and integrating a variety of intracellular signal[22]. Stimulation of apoptotic machinery culminates with the activation of caspases cascade responsible for the execution phase of apoptosis, in which the cell undergo to a progressive self-distribution process and the cell debris are finally internalized by the closet cells and by phagocytic cells[5]. Despite the cellular conditions that induce apoptosis remains to be fully elucidated, several abnormality sensors that play key roles in carcinogenesis have been identified[22,23]. Furthermore, tumour cells evolve several strategies to limit apoptosis, in fact they may reach similar scope by increasing expression of antiapoptotic or survival regulators, by downregulating pro-apoptotic factors, or by short-circuiting the extrinsic ligand-induced death pathway. The numbers of anti-apoptotic mechanism possibly reflect the diversity of signals that promote apoptosis in cancer cell population encounter during their evolution to the malignant state[5,25].

Inducing Angiogenesis

Angiogenesis consists in the new blood vessels formation. Endogenous local or systemic chemical signals manage endothelial cells and smooth muscle cells function to repair damage vessels, although the generation of new blood vessels is from pre-existing blood cells via endothelial cells sprouting[26,27] (Figure 3).

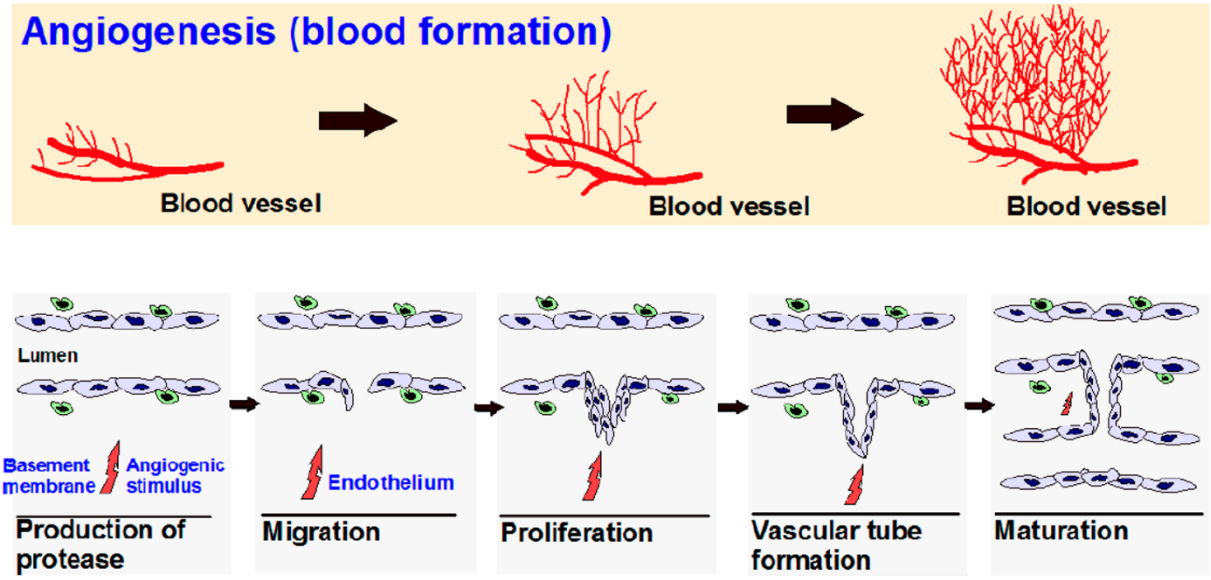


Figure 3. Angiogenesis steps. New blood vessels formation from pre-existing vessels. Figure adapted from Rajabi 2017.

Activated endothelial cells produce proteolytic enzyme which degrade the perivascular extracellular matrix (ECM) and the basement membrane. Then endothelial cells proliferate and migrate into perivascular area. Subsequent capillary loops are formed which is followed by synthesis of a new basement membrane and blood vessel maturation to compete tube-like structures through which blood can flow[28].

Among angiogenesis modulators, vascular endothelial growth factor (VEGF) is a powerful agent in neoplastic tissues, in fact secretion of VEGF leads the neighbour tissues to develop new blood vessels and the tumour cells will be able to feed[27].

Several drugs directed against VEGF signal have been developed in recent years. In addition, VEGF receptor tyrosine kinase inhibitors sorafenib and sunitinib have also been approved by the FDA for clinical use[29]. However, low-dose bevacizumab, a monoclonal antibody directed against VEGF, is associated with the increased risk of venous thromboembolism and some patients with a clinical response to VEGF blockade ultimately develop progressive disease. Therefore, numerous studies remain committed to a deep understating of the regulatory mechanism of VEGF, hoping to identify other synergistic agents able to inhibit excessive VEGF production observed in tumour cells[30].

Enabling replicative immortality

Cancer cells require unlimited replicative potential in order to generate macroscopic tumours[5]. In normal cells there are two distinct barriers to proliferation: senescence, a typically irreversible non-proliferative but viable state, and crisis, which involves cell death. Several studies indicate that telomeres protecting the ends of chromosomes are centrally involved in the capability for unlimited proliferation[31,32].

Activating invasion and metastasis

In the last two decades, it was clear that solid tumour evolved to higher pathological grades of malignancy through local invasion and distance metastasis. Cancer cells developed modifications in their morphology as well as in their attachment to other cells and to the ECM. The best characterized alteration regards the loss of E-cadherin by carcinoma cells, a key cell-to-cell adhesion molecule. Increased expression of E-cadherin leads the inhibition of invasion and metastasis, while reduced expression was known to potentiate these phenotypes. In human carcinomas, the observed downregulation and inhibitory mutation of E-cadherin provided strong support for its role as a key suppressor of this hallmark capability[5,33,34].

Invasion and metastasis multistep processes have been described as a consequence of different steps, called the invasion-metastasis cascade[35,36]. This process consists in a succession of cell biologic changes, starting with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by outflow of cancer cells from the lamina of this vessels into the parenchyma of distant tissues, the formation of small nodules of cancer cells and finally the growth of micro-metastatic into macroscopic tumours, this last phase is termed "colonization"[5].

Moreover, the developmental of a specific regulatory program termed epithelial-mesenchymal transition (EMT), has become prominently implicated during the process through which epithelial cells can acquire abilities to invade, to resist apoptosis and to disseminate[37–41]. Carcinoma cells can, in the same time, acquire multiple attributes that enable invasion and metastasis by co-opting a process involved in embryonic morphogenesis and wound healing steps.

Enabling characteristic and emerging hallmarks

Hanahan and Weimberg have defined the hallmarks of cancer as acquired functional capabilities that allow cancer cells to survive, proliferate and disseminate; these functions are acquired in different tumour types at various time during the course of carcinogenesis. Their acquisition is made possible by two enabling characteristics (Figure 4):

- Development of genomic instability that caused random mutations including chromosomal rearrangements. Among these are the rare genetic changes that can generate hallmarks capabilities.
- Inflammatory state of preliminary malignant lesions that is driven by immune system cells, in this case tumour cells promote inflammation.

Then other two emerging hallmarks of cancer cells have been proposed to be functionally important for the development of cancer[5,42–44] (Figure 4). The first involves major programming of cellular energy metabolism in order to support continuous cell growth, replacing the metabolic program that operates in most normal tissues and fuels the physiological operations of the associated cells. The second involves active evasion by cancer cells from attack and elimination by immune cells; this capability highlights the dichotomous roles of an immune system that both antagonized and enhances tumor development and progression[5].

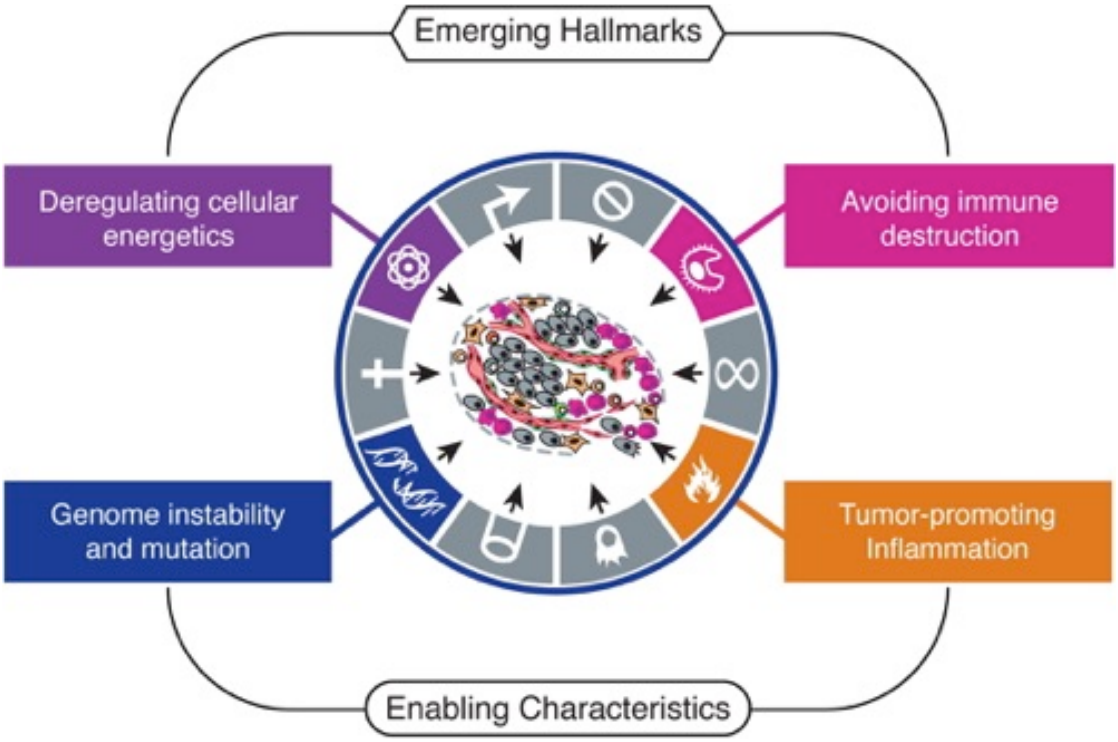


Figure 4. Emerging hallmarks and enabling characteristics. Figure adapted from Hanahan 2011.

Cancer immunoediting

Cancer immunoediting is a multistep mechanism described for the first time by Dunn et al. in 2004. Cancer immunoediting is a dynamic process by which the immune system attempts to destroy tumors, and it comprises three stages: elimination, equilibrium, and escape[45,46]. During the early stages of this process, immune-surveillance related lymphocytes (CD8+ T cells, CD4+ type-1 T-helper cells, and natural killer cells) are able to recognize and eliminate malignant cells, thus efficiently counteracting cancer proliferation. In the long-lasting equilibrium stage, tumor cells also begin to undergo a Darwinian selection which progressively favors the proliferation of the malignant clones that are more likely to evade immune recognition and elimination through the adoption of several biological strategies: tumor cells "become" invisible to host recognition and thus acquire the capacity to grow progressively[45,47]. Tumor cell escape may result from the establishment of an immunosuppressive state within the tumor microenvironment[48]. Tumor cells can promote the development of such a state by producing immunosuppressive cytokines such as VEGF, transforming growth factor-β(TGF-β), galectin, or indoleamine 2,3-dioxygenase (IDO) and/or by recruiting regulatory immune cells that function as the effectors of immunosuppression[49]. Regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) are two major types of immunosuppressive leukocyte populations that play key roles in inhibiting host-protective antitumor responses[45]. In addition, the overexpression of immune-checkpoint molecules, such as programmed cell death ligand-1/2 (PD-L1/PD-L2), vanishes the activity of immune cells which eventually still recognize transformed cells. The uncontrolled proliferation of Tregs and MDSCs as a consequence of the release of interleukin (IL)-10, IL-35, and TGF-β operated by cancer cells further contributes to the development of a strongly immunosuppressive microenvironment[47,50].

Solid tumors recruit also leukocytes and the major component of the infiltrate are macrophages, in all tumours[51]. Circulating precursors that are recruited into tumor tissues and subsequently differentiate into tumour-associated macrophages (TAMs) include conventional inflammatory monocytes and monocyte-related myeloid-derived suppressor cells (M-MDSCs). Chemoattractants involved in monocyte recruitment include chemokines (such as CCL2 and CCL5), and cytokines (for example, CSF-1 and members of the VEGF family), however TAMs themselves can be a source of CCL2 in cancer[52,53]. Indeed, such chemotactic factors act as more than attractants because they activate transcriptional programs that

contribute to the functional skewing of macrophages towards specific phenotypes[54]. CSF-1, in particular, is a monocyte attractant as well as a macrophage survival and polarization signal that drives TAM differentiation towards an immunosuppressive, tumour-promoting 'M2-like' phenotype[51,55]. Signals originating from tumour cells, T lymphocytes and B lymphocytes, and stromal cells influence TAM function and diversity. Classically activated 'M1' macrophages can kill tumour cells via extracellular mechanisms and thereby mediate tissue-destructive reactions involving the walls of blood[56].

TAMs can also promote the immunosuppressive activity of regulatory T (Treg) cells through a bidirectional interaction. With regard to the mechanisms underlying these effects, in the tumour context, macrophages produce immunosuppressive cytokines (IL-10 and TGFβ)[51]. In addition, the profile of amino acid metabolism by M2 or M2-like macrophages and TAMs results in metabolic starvation of T cells owing to the activity of arginase and/or the production of immunosuppressive metabolites via IDO pathway[57]. Moreover, prostaglandins produced by TAMs via arachidonic acid metabolism have immunosuppressive effects[58]. Finally, TAMs often express PD-L1 and PD-L2, which trigger the inhibitory PD-1-mediated immune checkpoint in T cells, as well as B7-H4 and VISTA, which might have similar functions[59].

Progress has been made in defining the molecular pathways involved in orchestration of tumorigenic mechanisms of both cancer cells lymphocytes and macrophages.

Breast cancer

Breast cancer is the most common cancer in women worldwide, in 2017, an estimated 252,710 new cases of invasive breast cancer will be diagnosed in US (American Cancer Society, Breast Cancer Facts and Figures 2017-2018). The latest census in Italy estimates 373 thousand cases that places breast cancer in the leading position among the most diffuse.

Advanced age is the biggest risk factor for breast cancer, moreover reproductive factors that increase exposure to endogenous estrogen, such as early menarche and late menopause, increase the risk, as does the use of combination estrogen-progesterone hormones after menopause (PDQ Cancer Information Summaries 2018). Women with a family history or personal history of invasive breast cancer, ductal carcinoma in situ or lobular carcinoma in situ, or a history of breast biopsies that show benign proliferative disease have an increased risk of breast cancer[60–63].

Moreover, increased breast density in mammography images has been associated with increased risk. This is often a heritable trait but is also seen more frequently in nulliparous women, women whose first pregnancy occurs late in life, and women who use postmenopausal hormones and alcohol (PDQ Cancer Information Summaries 2018). There are several types of breast cancer, divided in two broad categories: invasive and noninvasive. Moreover, genomic approach led to a more specific classification of breast cancers, based on their genetic phenotype (Figure 5).

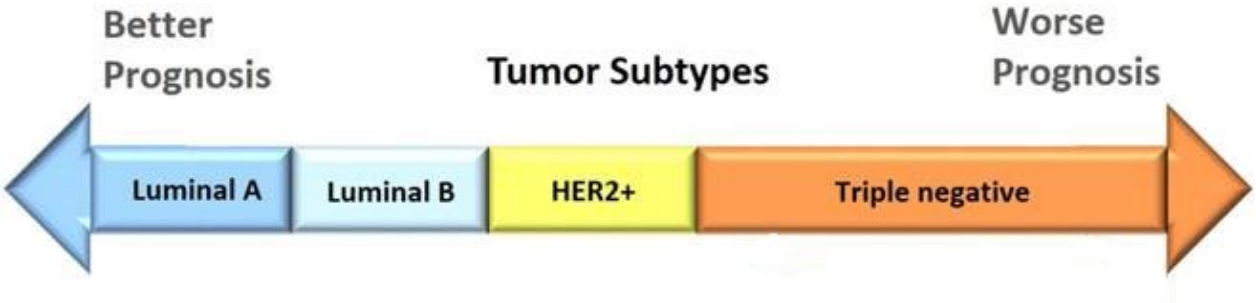


Figure 5. Breast cancer characterization. According to the status of ER, PR, HER2, breast cancer is classified as luminal A, luminal B, HER2 positive, and triple negative.

In situ carcinoma, are classified on the basis of the different site of tumor growth:

- Ductal carcinoma in situ (DCIS) is the most common, with the 83% of *in situ* cases diagnosed during 2010-2014. It refers to a condition in which abnormal cells replace the normal epithelial cells that line the breast ducts and may greatly expand the ducts

and lobules. DCIS may or may not progress to invasive cancer; in fact, sometimes DCIS grows so slowly that even without treatment it would not affect a woman's health. Long-term studies of women whose DCIS was untreated because it was originally misclassified as benign found that 20%-53% were diagnosed with an invasive breast cancer over the course of 10 or more years[64–69].

- Lobular carcinoma *in situ* (LCIS). This type of *in situ* breast cancer refers to abnormal cells growing within and expanding some of the lobules of the breast. LCIS is generally not thought to be a precursor of invasive cancer but is a strong risk factor for developing invasive cancers.

Invasive breast cancers are the most representative (80%), they originated and growth in ducts or glands, then they acquire the capability to go into surrounding breast tissue. There are more than twenty distinct histological subtypes and at least four different molecular subtypes that differ in terms of risk factors, presentation, response to treatment, and outcomes[69–71]. Classification of breast cancer in different molecular subtypes have been achieved using biological markers, that include the presence or absence of hormone (estrogen or progesterone) receptors (HR^+/HR^-) and high expression levels of human epidermal growth factor receptor 2 (HER2, a growth-promoting protein) and/or the presence of extra copies - measured by gene amplification- of the HER2 gene ($HER2^+/HER2^-$) [69,72]:

- Luminal A (HR^+/ $HER2^-$). Luminal A tumors are more responsive to hormone therapy, thus they are associated with the most favorable prognosis in the short term[73,74].
- Triple negative (HR^-/ $HER2^-$). They are estrogen receptorα (ERα) negative, progesterone receptor (PR) negative and HER2 negative and are also common in premenopausal women whom express BRCA1 gene mutation[75]. About 75% of triple negative breast cancers fall in to the basal-like subtype defined by gene expression profiling. They have a poorer short-term prognosis than other subtypes, unfortunately there are currently no targeted therapies for these tumors[76].
- Luminal B (HR^+/ $HER2^+$). This type of breast cancers is positive to the hormone receptor and are further classified as being highly positive for ki67 (marker for actively dividing cells) or HER2. Luminal B breast cancers are associated with poorer survival than luminal A cancer[74].
- HER2 positive (HR^-/ $HER2^+$). HER2-enriched cancers tend to grow and spread more aggressively than other subtypes and are associated with poorer short-term prognosis

compared to hormone positive breast cancers[74]. However, the introduction of Trastuzumab, a very powerful targeted therapies for HER2-enriched cancers has improved outcomes for these patients.

Breast cancer treatment is addressed to the specific subtype, although highly needed and still lacking is a targeted therapy for breast cancer chemoprevention.

Breast cancer prevention

There is not a sure way to prevent cancer, certainly keeping a healthy life style might lower this risk. For women with a higher risks of breast cancer, chemopreventive drugs are available for the prevention of relapses after surgery, but the balance between benefits and harms is not in favoring their use in healthy patients at high risk of developing a mammary neoplasia. The most used drugs for breast cancer prevention and treatment are selective estrogen receptor modulators (SERMs), they have features that can act as estrogen agonist or antagonist, depending on the target tissue[77]. Tamoxifen is the pioneering medicine[78] because is the precursors of the targeted treatments for cancer acting as ERα inhibitor in mammary cancer cells[79]. Initially, it was found to have a low affinity for estrogen receptor *in vitro*[80], however tamoxifen acts as a prodrug and is rapidly converted in the liver to a metabolite with high affinity and efficiently inhibiting ERα [79,81].

Several clinical trials were carried out to test the tamoxifen chemopreventive applications, the two most relevant are: The National Surgical Adjuvant Breast and Bowel Project (NSABP) initiated the Breast Cancer Prevention Trial (P-1) in 1993[82] and The International Breast Cancer Intervention Study (IBIS-I), was an international phase III chemoprevention trial comparing tamoxifen vs. placebo (IBIS investigator 2002). Taken together they reported that tamoxifen was able to lower the risk of developing ER-positive breast cancer in patients without a personal history of breast cancer, however the chemopreventive activity on breast cancer comes at the expense of well documented side effects, including an approximately 2-5 fold increase in uterine cancer[83,84], and 2–3 fold increase in thromboembolic disease but only in postmenopausal women. In addition, increased menopause symptoms, vaginal discharge and ocular abnormalities were reported in tamoxifen groups. All together the results suggested that chemoprevention with tamoxifen for high risk premenopausal women[79,85].

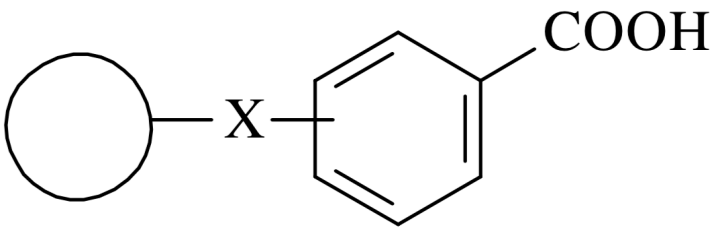
Non Steroidal Anti-inflammatory Drugs (NSAIDs)

As previously mentioned, despite emerging discovery in diagnosis and the development of specific molecular targeted drugs, cancer still remain the leading cause of mortality in the western world[2,86]. Individuals with precancerous lesions and high-risk populations are recommended to follow pharmaceuticals or dietary intervention in order to inhibit disease progression, however, the increasing chemopreventive strategies mandates safety and efficacy. NSAIDs are among the most promising chemopreventive agents for different cancer types and could give an important contribution to the control of neoplasia development especially in high risk groups[87]; a number of *in vivo* preclinical data demonstrated the cancer-protective activity of these drugs[88–92] and clinical studies assessed the protective effects of chronic or transient intraoperative treatments[93–96]. However, the use of NSAIDs in the treatment of a relatively healthy, at risk population is limited by the potentially serious adverse gastrointestinal and cardiovascular events.

NSAIDs classification

Non steroidal anti-inflammatory drugs are a chemically diverse family of drugs commonly used clinically to treat a variety of inflammatory conditions. A number of these drugs possess antipyretic activity in addition to having analgesic and anti-inflammatory action, and thus have utility in the treatment of pain and fever. Their anti-inflammatory activity is attributed to the cyclooxygenase (COX) inhibition[97], the enzyme that catalyze the arachidonic acid conversion into prostaglandin H2, the precursor for the synthesis of eicosanoids that include prostaglandins (PG), prostacyclin, and thromboxane A_2. Inflammation, pain and fever are promoted by the three major PG products of COX activity, PGE_2, PGD_2, and PGF_{2a}[86] . As first, Vane showed that aspirin inhibits inflammation by suppressing PG synthesis[98], however its COX inhibitory action responsible of this effect was later shown[99]. Eicosanoids, besides the role in inflammation, are critically involved in the homeostatic maintenance of gastrointestinal mucosa, blood clotting, regulation of blood flow, and kidney function. There are two isoforms of COX, COX-1 and COX-2 (Smith 1996, book), the first one is constitutively expressed in most tissues, whereas COX-2 is induced by inflammatory stimuli, mitogens, or growth factors, and is usually related with pathologic processes[100]. Usually NSAIDs inhibit both COX-1 and COX-2, however most of them are selective inhibitor of COX-1 such as aspirin, ketoprofen, indomethacin, sulindac, ibuprofen, diclofenac, while some others may be considered slightly

selective for COX-2 as etodolac, nabumetone and meloxicam[101]. The identification of COX-2 as the main mediator of inflammation, led to development of a new class of COX-2 selective inhibitors (coxibs) to elude gastrointestinal and renal toxicities associated with nonselective NSAIDs[86]. However, coxibs were later found to increase the risk of heart attack and stroke[102,103], resulting in the concept that all NSAIDs have risks of cardiovascular side effects. In general, NSAIDs structurally consist of an acidic moiety (carboxylic acid, enols) attached to a planar, aromatic functionality (Figure 5). Moreover, some analgesics also contain a polar linking group, which attaches the planar moiety to an additional lipophilic group.



NSAID General Structure

Figure 5. NSAIDs general structure. Figure adapted from DeRuiter 2002.

NSAIDs are characterized by chemical and pharmacologic properties and can be classified in:

- Salicylates
- Propionic Acids (Profens)
- Aryl and Heteroarylacetic Acids
- Oxicam (Enolic Acids)
- Phenylpyrazolones
- Anilides

Salicylates

This subclass are derivates of 2-hydroxybenzoic acid (salycilic acid) that was replaced therapeutically in the late 1800s by the acetylated derivate, acetylsalicylic acid (ASA) or aspirin. Therapeutic utility is enhanced by esterification of the phenolic hydroxyl group in aspirin, and by substitution of a hydrophobic/lipophilic group at C-5 as in diflunisal (Figure 6):

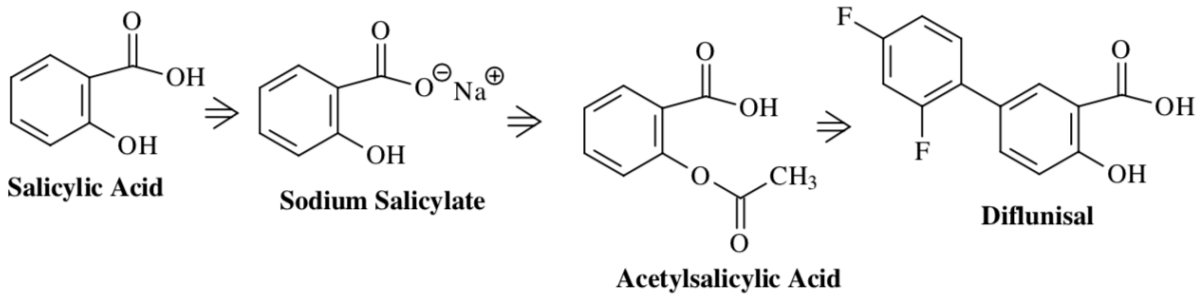


Figure 6. Salicylates Structure. Figure adapted from DeRuiter 2002.

The salicylates have potent anti-inflammatory activity with mild analgesic and antipyretic activities. These compounds are selective COX-1 inhibitor. Their toxicity includes gastrointestinal irritation, hypersensitivity reactions, inhibition of platelet aggregation. Therapeutic and toxic effect of aspirin could be ascribed to the COX irreversible inhibition in several tissues and to its ability to participate in trans-acetylation reaction *in vitro*[104].

Propionic Acid Derivates (Profens)

Propionic acid derivatives are often referred as “profens” based on the suffix of the prototype member, ibuprofen. Arylpropionic acids are characterized by the general structure Ar-CH(CH_3)-COOH, the a-CH3 substituent present in the profens increases COX inhibitory activity and reduce the toxicity.

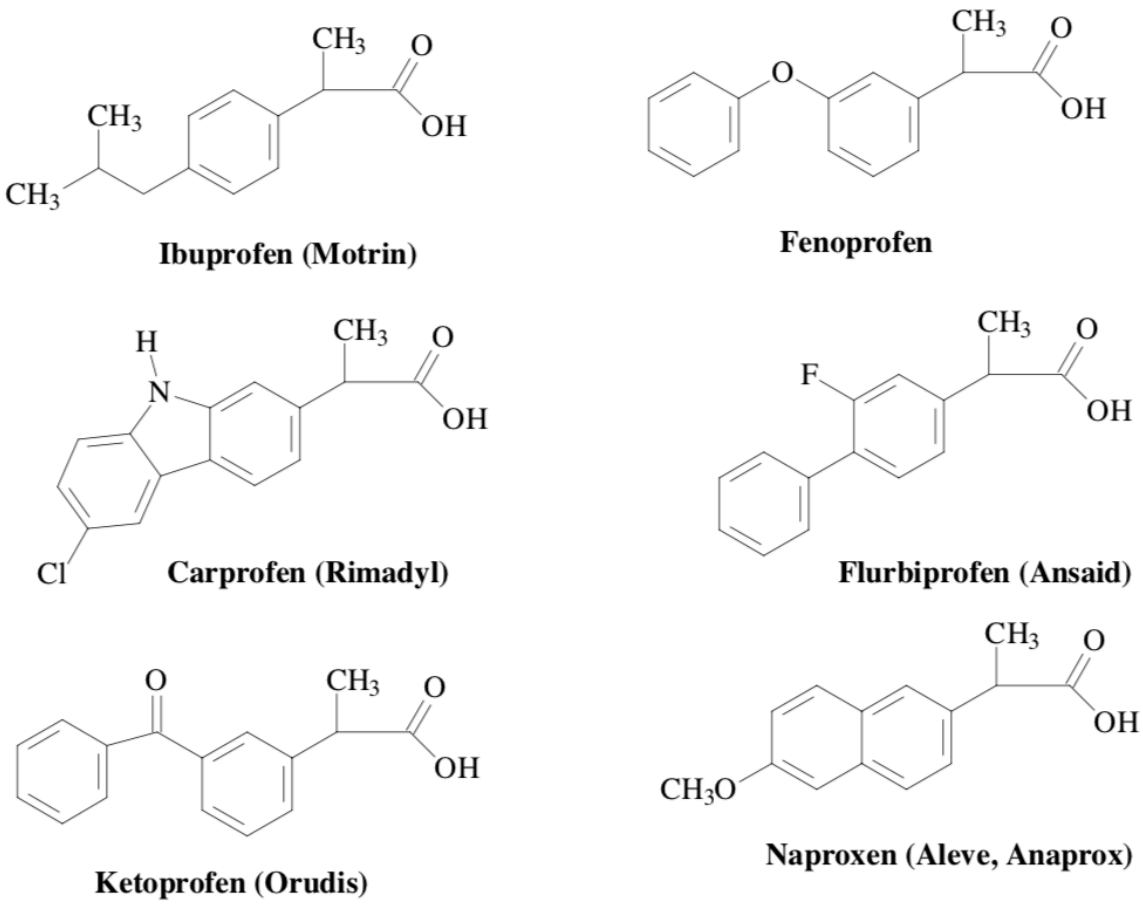


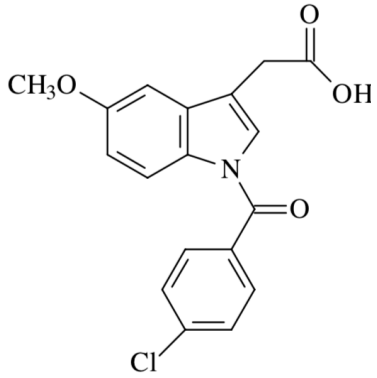
Figure 7. Propionic acid derivatives structure.

These compounds are anti-inflammatory agents with analgesic and antipyretic activity. Naxoprofen appears to be more selective for COX-2 than other members of the series. They are indicated for the treatment of rheumatoid arthritis, osteoarthritis and as analgesics and antipyretics. They should not be used during pregnancy or nursing because they can enter in fetal circulation and breast milk. They may cause thrombocytopenia, headache, dizziness, fluid retention edema.

Aryl and Heteroarylacetic Acids

Heterocyclic acetic acids are acetic acid derivatives with the substituent at the 2-position is a heterocycle or related carbon.

Indomethacin inhibit both COX-1 and COX-2 and it has produces anti-inflammatory, analgesic and antipyretic activity. It is used for rheumatic disease[105], to suppress uterine contraction (preterm labor)[106], and in premature infants with patent ductus arteriosus[107].



Indomethacin (Indocin)

Figure 8. Indometacin structure.

Another compound of this class is Sulindac, a prodrug (Figure 9), that is converted *in vivo* in the liver to an active sulfide compound by liver enzymes[108]. Sulindac is reduced to sulindac sulfide (S-sulfide) (Figure 9) which has analgesic and anti-inflammatory activities. Then sulindac sulfide is converted back to sulindac and further to sulindac sulfone, which is inactive on COXs but it has been recognized as a promising antiproliferative agent in colon cancer[109–112]. Sulindac is also indicated for acute or long-term use for osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, acute painful and acute gouty arthritis.

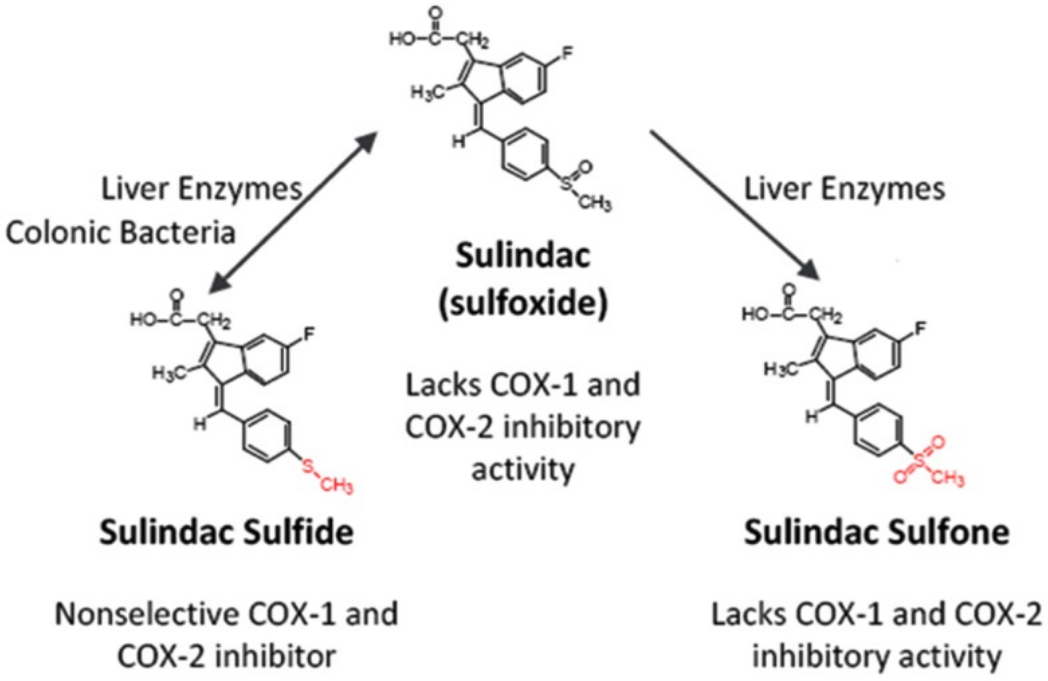
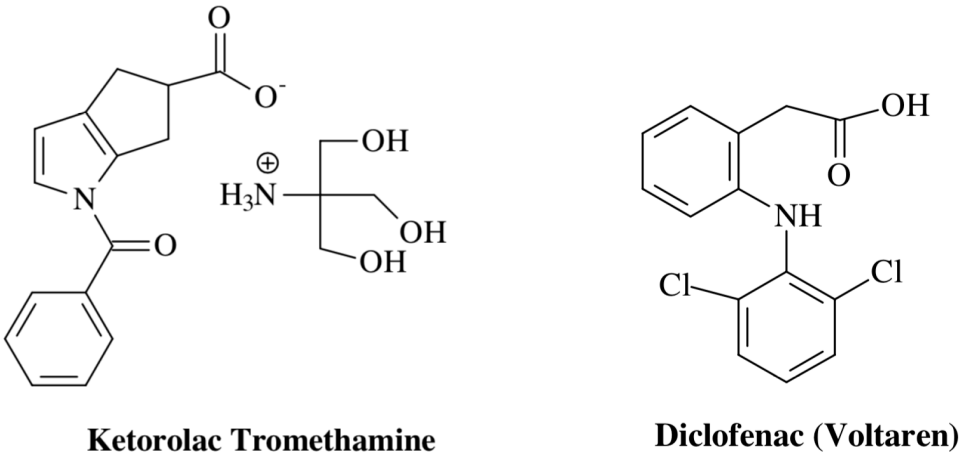


Figure 9. Chemical structure of Sulindac, Sulindac sulfide and Sulindac sulfone. Figure adapted from Gurpinar 2013.

Ketorolac is a synthetic pyrrolizine carboxylic acid derivative with anti-inflammatory, analgesic and antypiretic activities. It inhibits both COX-1 and COX-2, however the effect on COX-2, that is up-regulated at inflammation sites, prevents conversion of arachidonic acid to proinflammatory prostaglandins. The inhibition of COX-1 is correlated with gastrointestinal toxicity, nephrotoxicity, and the inhibition of platelet aggregation (DrugBank).

Diclofenac is a phenylacetic acid, its structure is composed by two chlorine atoms, produce maximal twisting of the phenyl ring resulting in a well fit in the binding pocket of COX enzyme[113,114]. Diclofenac is a COX-1 and COX-2 inhibitor and it has potent analgesic and anti-inflammatory properties[115–118].



Ketorolac Tromethamine **Diclofenac (Voltaren)**

Figure 10. Ketorolac and Diclofenac structures.

Oxicam

Oxicams are a class of NSAIDs related to the enolic acid class of 4-hydroxy-1,2-bezothiazine carboxamides, the first member of this class is piroxicam (Feldene), introduced by Pfizer in 1982[119]. After piroxicam, other oxicams were introduced including isoxicam, meloxicam, tenoxicam and lornoxicam. They are indicated to the treatment of acute and chronic inflammation by inhibiting both COX-1 and COX-2 enzymes.

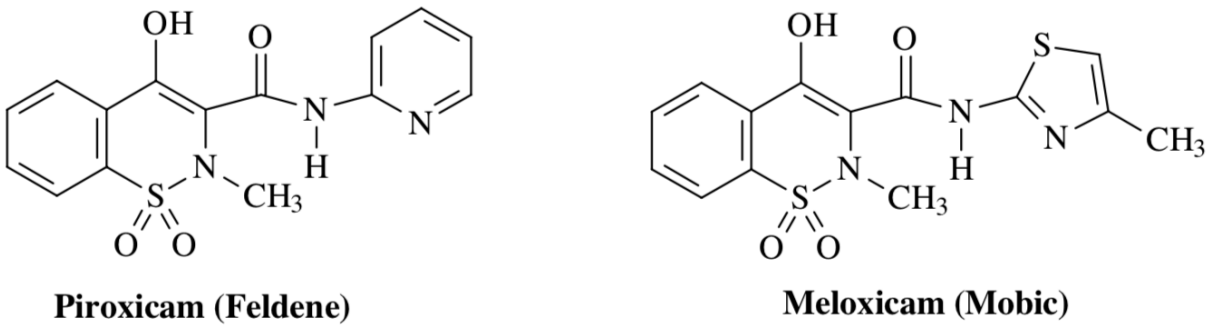


Figure 11. Piroxicam and Meloxicam structures.

Phenylpyrazolones

Phenylpyrazolones are a class of compounds characterized by the 1-aryl-3,5-pyrazolidinedione structure. Phenylbutazone and oxyphentobutazone are NSAIDs, antypiretic compounds useful in the management of inflammatory conditions (PubChem, DrugBank).

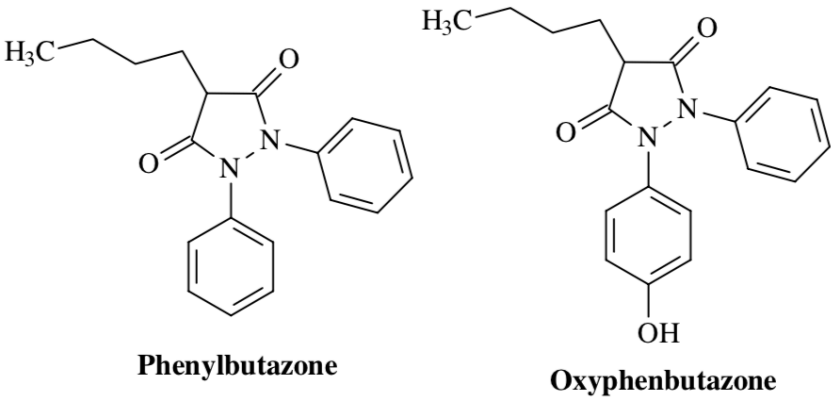
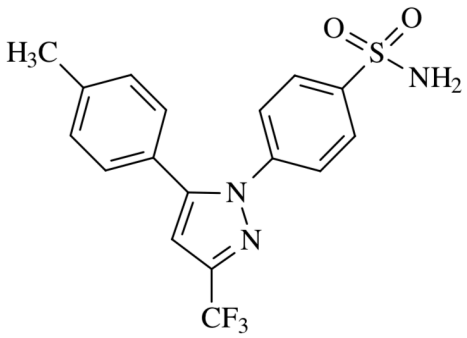


Figure 12. Phenylbutazone and Oxyphenbutazone structures.

COX-2 Selective inhibitors

To avoid the side effects due to the COX-1 inhibition, COX-2 selective inhibitors were developed[120].

Celecoxib is a COX-2 selective inhibitors with anti-inflammatory, analgesic and antipyretic properties. It is also indicated for the treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis and acute pain[121–123]. Interestingly, celecoxib has shown chemopreventive activity as an adjunct to surgery to reduce the number of adenomatous colorectal polyps in patient with familial adenomatous polyposis[124–126].

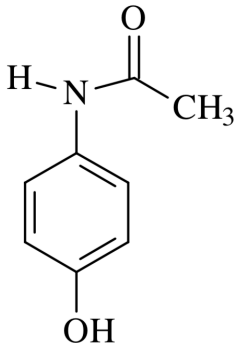


Celecoxib (Celebrex™)

Figure 13. Celecoxib chemical structure.

Anilides

The most representative compound of this class is N-acetyl-para-aminophenol also called acetaminophen or paracetamol (Figure 14). It has analgesic and antipyretic properties, however despite the typical NSAIDs it does not possess anti-inflammatory activity[127]. Years of studies reveal that paracetamol is a preferential inhibitor of COX-2 enzyme, however, its effect depends to a great extent on the state of environmental oxidation and reduction[128,129].



Acetaminophen

Figure 14. Acetaminophen (Paracetamol) chemical structure.

Nimesulide

Nimesulide is a 4-nitro-phenoxymethane-sulfonanilide (Figure 15) and for this reason, it belongs to the class of compounds sulfonanilide, unique for anti-inflammatory drugs. Nimesulide has good anti-inflammatory, analgesic and antipyretic properties. In addition, it is prescribed for arthritic disorders, musculoskeletal diseases, headache, gynecological and urological problems, post-surgical and cancer pain, vascular disease and airways inflammation[130].

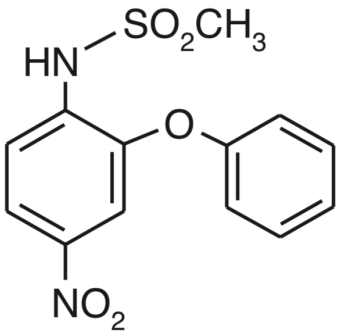


Figure 15. Nimesulide chemical structure

NSAIDs chemopreventive activity

In the last decades epidemiological and preclinical studies suggested that prolonged exposition to NSAIDs prevents the onset of cancer[90–92,94,96,131]. Clinical studies reported that in several types of cancer, NSAIDs protect high-risk group after long-term treatment or even after short-term exposition during operative analgesia[87,93–96,132]. Although these compelling evidence of NSAIDs anti-tumor activity, their use for cancer prevention in not recommended because of gastrointestinal, renal and cardiovascular side effects due to the COXs inhibition. Moreover, NSAIDs chemopreventive mechanism has not been fully elucidated, because of its complexity that involve multiple effects on cancer cells and their microenvironment[86]. Several mechanisms of action have been proposed to explain the antitumor properties of this structurally heterogeneous class of compounds: first of all, inhibition of COX-2 enzyme was proposed, however several studies have concluded that alternative targets may be involved[1,133–135]. The new targets that has been proposed include the modulation of cancer-related pathways (e.g. WNT/β-catenin or cGMP/PDE), the activity of transcription factors (e.g. p53, PPARγ, PPARδ, SP1, NFkB, RXR) and enzyme (AMPK, carbonic anhydrase, Ca^{++} ATPase, MMPs) involved in carcinogenesis. The hypothesis of COX-independent mechanism responsible of NSAIDs chemopreventive activity is supported by studies showing that NSAID metabolite or derivative that lack COX-inhibitory activity can retain antitumor activity. Sulindac sulfone (exisulind) is a protoptypical example of a non-COX-inhibitory NSAIDs derivative able to induce adenoma regression in familial and sporadic adenomatous polyposis, and to inhibit the formation of multiple tumor types in several preclinical studies[1,92,136–139].

Intraoperative Ketorolac delay breast cancer recurrence

One of the most intriguing clinical evidence of NSAIDs' chemopreventive action was reported by Forget and collaborators for Ketorolac[93]. They analysed the follow up of breast cancer patients treated with ketorolac or opiates as analgesic protocol during surgery. In this epidemiological study the showed that intraoperative administration of ketorolac was associated with significantly superior disease-free survival in the first 24 months after surgery. The expected prominent early relapse risk peak was completely absent in the ketorolac group (Figure 16). The few events in the ketorolac group showed a small bump in the first 10 months and then slowly rising until the 4th year when follow-up of this series end-up. After 24 months, the ketorolac group hazard rate pattern was indistinguishable from the corresponding pattern for the no-ketorolac group.

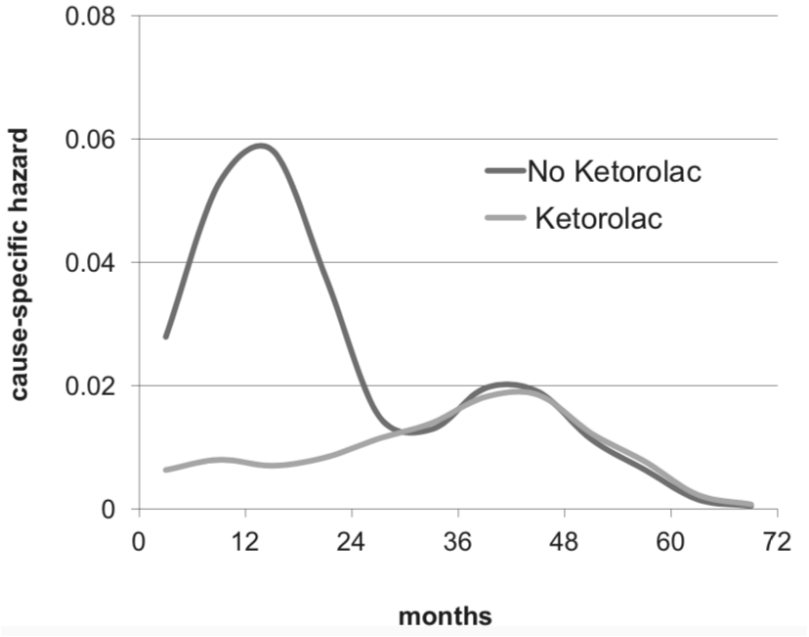


Figure 16. Data from Universite catholique de Lou-vain in Brussels, Belgium[93]. Relapse hazard is shown for mastectomy patients given ketorolac or not. Figure adapted from Retsky et al. 2013.

These data and other epidemiological studies demonstrated the association of intraoperative ketorolac with a dramatic reduction of the recurrences and overall survivals[140].

More recently, Desmedt and colleagues validate these findings. The results of their study showed that the reduction of distance recurrences of breast cancer significantly correlated with ketorolac intra-operative administration especially in those patients with increased body mass index (BMI)[132]. All these data are identifying ketorolac as a promising molecule for cancer prevention. However how ketorolac prevents the early relapses pick is not clear yet.

Understanding the mechanism underlying the keotorolac anti-tumour effects will reveal new pharmacological targets for the development of safer chemopreventive therapies.

Aspirin increase P53 acetylation in breast cancer cell line

Several mechanisms for NSAIDs chemopreventive activity alternative to COX inhibition were proposed: Alfonso and collaborators[104] showed that aspirin treatment in breast cancer cell line MDAMB-231, promotes p53 acetylation at residue K382 in a dose dependent manner (Figure 17).

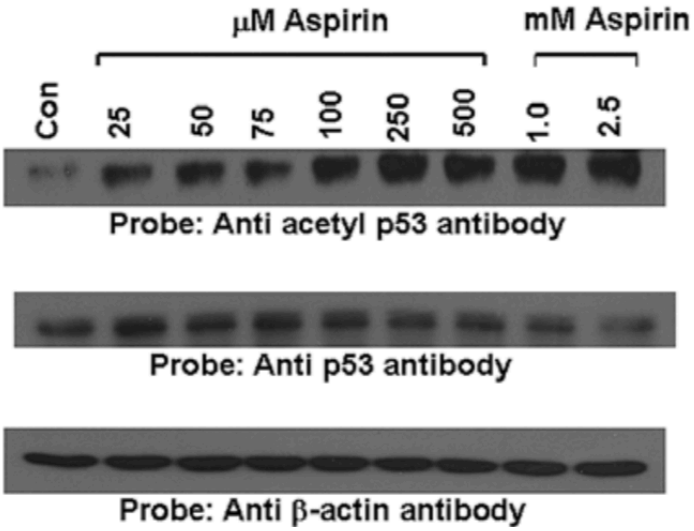


Figure 17. Aspirin acetylates p53 protein. Cells were left untreated or treated for 8 h with different concentrations of aspirin as indicated, lysates prepared and immunoblotted with anti-acetyl p53; anti-p53 antibody and anti-ß-actin antibodies. Figure adapted from Alfonso et al. 2009.

Moreover, they published that the increased acetylation induced by aspirin correlated with increasing transcriptional activity of p53, increase expression of its target gene p21 and increased p53-mediated apoptosis[104]. These observations were the starting point of our current work leading us to hypothesized that increased acetylation of p53 was actually a common mechanism shared by ore anti-inflammatory drugs.

Sirtuin 1 role in cancer

Sirtuins are class III histone deacetylase enzyme NAD^+ dependent[141,142]. These proteins have been shown to play a role against aging in a broad range of organism, from yeast to mammals[143]. Sirtuins activation is known to counteract the progression of ageing related disease, including neurodegeneration, diabetes, cardiovascular diseases and cancers[142,144]. The most studied member of sirtuins is the sirtuin 1 (SIRT1), which is activated during starvation and deacetylate various substrates, including histones, transcription factors, DNA-repair factors and signalling proteins[145,146]. SIRT1 has an important role in cell fate by deacetylating key regulators of inflammation, apoptosis and hypoxia such as NFkB, p53 and HIFs[147–149].

However, several studies reported a dual role of SIRT1 in cancer. It was shown to have both tumor-suppressor and oncogenic activity with somewhat controversial findings on different tumours and in different contexts. Indeed, SIRT1 expression is significantly different depending on tumour type[150]. In leukemia and lung cancer SIRT1 expression is significantly higher[150–152], while in prostate, bladder, ovarian cancers and glioblastoma present had a lower SIRT1 expression compared with normal tissues[153] . On the contrary, Huffman and colleagues found that the expression was higher in prostate cancer compared to a control group (Huffman 2007). The controversial role and expression of SIRT1 on the different type of cancer has been proposed to be dependent on the microenvironment[150].

Interestingly, by studying SIRT1 in breast cancer cells Jin and colleagues showed that the protein level was significantly increased in breast cancer tissues in comparison to adjacent normal breast tissues. They also reported that SIRT1 was up-regulated in breast cancer cell lines compared to normal breast epithelium cell line MCF10A, suggesting SIRT1 as oncogenic factor in breast cancer.

Tumour suppressor P53

The tumour suppressor p53, is a key transcription factor activated in response to several cellular stress resulting in inducing cell apoptosis and senescence. Functional inactivation or mutation of p53 is a common feature of human cancers[154,155]. Activation of p53 has been proposed as attractive cancer therapeutic strategy[156], mediated by DNA damage drugs or through regulation of protein expression and acetylation. Several p53 regulators have been identified, including the mouse double minute 2 homolog (MDM2), a negative regulator of p53 acting through degradation of the p53 protein via the ubiquitin-proteasome pathway or by directly blocking the p53 transactivation domain[155]. Many cancer therapies have been designed for p53 activation, through prevention of MDM2 mediated degradation or by inhibiting SIRT1 catalysed p53 deacetylation[155,156] (Figure 18). SIRT1 can strongly deacetylate and inhibit p53 *in vitro* and *in vivo*. SIRT1 mediated inhibition of p53 was shown to control the transcriptional activity of the oncosuppressor and the consequent activation of apoptosis in response to DNA-damage. In particular SIRT1, in presence of NAD, deacetylates the lysine 382 situated in the C-terminal regulatory domain of p53 protein[155].

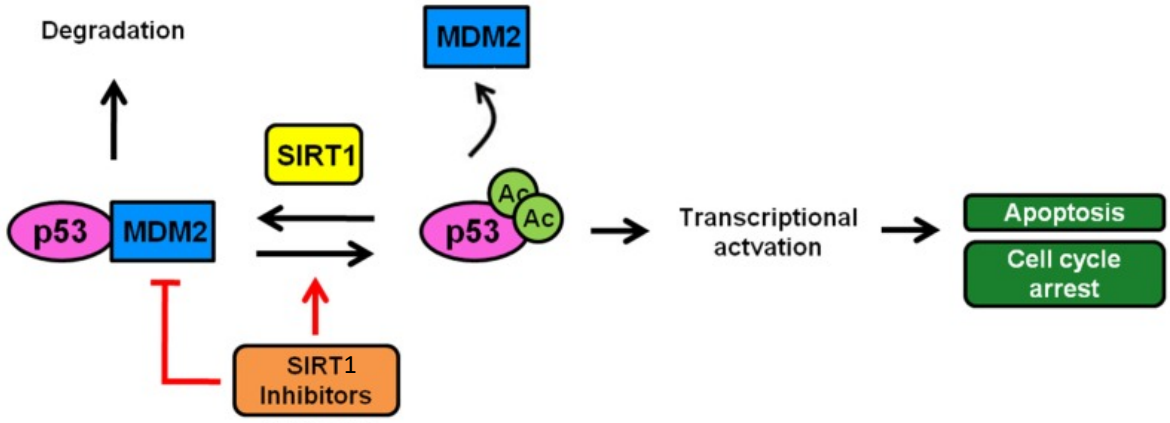


Figure 18. p53 activator scheme. A general scheme for p53 activator via reduction of MDM2 expression and SIRT1 inhibitors. New inhibitors could have double effect on SIRT1 and MDM2: inhibit SIRT1 and permit p53 increasing acetylation and activation, and they could also increase p53 by reducind MDM2 expression. Figure adapted from Park 2016.

AIM

Cancer incidence is projected to increase worldwide particularly in view of the ever-increasing population lifespan; novel strategies for prevention are therefore needed to decrease the personal, social and economic burden of this disease.

Non steroidal anti-inflammatory drugs are a chemically heterogeneous class of drugs commonly used clinically to treat a variety of inflammatory conditions. Several epidemiological, clinical and experimental studies established NSAIDs as the most promising chemopreventive agents[87]. A number of *in vivo* preclinical data demonstrated the cancer protective activity of ketorolac in chronic or transient intraoperative treatments[94,132]. Anticancer activity of NSAIDs is commonly attributed to COX-2 because of its higher expression in tumour tissues however, several lines of evidence challenged this simple view, including metabolites, enantiomers and derivatives[1] of NSAIDs that retain the anti-tumor activity without COX inhibition. In particular, the most characterized molecule of this type is sulindac sulfone (exisulind), a sulfone metabolite of sulindac. Exisulind lacks inhibitory activity on the two isoforms of cyclooxygenase, while displaying the ability of inducing a significant adenoma regression in familial or sporadic adenomatous polyposis, when evaluated in clinical trials[124]. Notwithstanding these positive clinical data, exisulind did not receive FDA approval because of its hepatotoxicity. Presently, the development of novel safer chemopreventive NSAIDs or derivatives is hampered by the lack of a clear picture concerning the mechanism of their anti-neoplastic effects.

Previous data showed that aspirin treatment promotes p53 acetylation at residue K382, increases the expression of p21 and induces apoptosis in the MDAMB-231 breast cancer cell line[104]. Aspirin treatment mediates activation of p53 through the increased acetylation of lysine 382 residue, which is deacetylated and negatively regulated by SIRT1. On the basis of these literature data, we hypothesized that NSAIDs could have a similar activity of aspirin on p53, thus we decided to study the interplay between these drugs, SIRT1 and p53 in the onset of breast cancer.

The aim of my PhD project was to investigate SIRT1 as novel potential target of NSAIDs, which could provide a biochemical explanation of the multiplicity of the COX-independent effects ascribed to NSAIDs and their metabolites, enantiomers and derivatives. This study, indeed identified SIRT1 as a direct target of NSAIDs, therefore disjoining the COX-dependent anti-inflammatory from the chemo-preventive activity of these drugs. This finding may contribute

to design novel molecules displaying anti-neoplastic activity without the COX-dependent side effects, which are currently hampering the use of NSAIDs as chemopreventive agents.

MATERIALS AND METHODS

Reagents

Unless otherwise specified reagents were purchased from Sigma-Aldrich.

Cell lines

MDAMB-231 and MCF-7 breast cancer cell lines were purchased from the American Type Culture Collection (ATCC) and grown in RPMI 1640 medium (Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and streptomycin-penicillin (50,000 IU plus 50 mg/l) (Gibco, Life Technologies).

hTERT-HME1 breast cancer cell lines were purchased from ATCC and grown in Dulbecco's Modified Eagle's Medium (Life Technologies) supplemented with 10% FBS (Sigma-Aldrich), 0.5 ng/ml hydrocortisone (Sigma-Aldrich), 0.1% insulin (Sigma-Aldrich), 100ng/µl Endothelial Growth Factor (EGF) (Sigma-Aldrich) and streptomycin-penicillin (50,000 IU plus 50 mg/l) (Gibco, Life Technologies).

P300, Sirt1 and COXs activity assay

The enzymatic activity of rhP300 was measured in the presence of increasing concentration (4.4-13.3-40-120 µM) nimesulide by using a commercial kit (Perkin-Elmer Life Sciences) based on the transfer of a radioactive ^{3}H-acetyl from ^{3}H-acetyl-CoA to a histone peptide; after the transfer the peptides are separated by a paper chromatography according to the manufacturer instructions. The amount of radioactivity incorporated by the peptides was determined with a β-counter.

The effect of increasing concentrations of NSAIDs, nicotinamide and exisulind on SIRT1 deacetylase activity was studied by using SIRT1 Activity Assay Kit (Fluorometric) (ab156065).

In addition, SIRT1 activity was also measured with a bioluminescent assay[157] (SIRT1-GLO assay, Promega) according to the manufacturer's instructions. Briefly, rhSIRT1 was incubated in the presence of NAD and of an acetylated SIRT1 substrate peptide covalently linked to luciferin (GLO). SIRT1 deacetylates the substrate peptide unmasking a site for a subsequent peptidase reaction, which can thus release free luciferin from the substrate: the amount of luciferin in the mixture is proportional to the deacetylase enzymatic activity and can be quantified by measuring the photon emission upon addition of the luciferase enzyme to the reaction mixture.

The effect of increasing concentrations of the new compounds RB2, RB4 and RB7 on COX1 and COX2 activity was studied by using COX Fluorescent Activity Assay Kit (Cayman 700200).

Quantitative measurement of SIRT1 activity in 5 µg of MDAMB-231 cell lysate cell lysates was carried out with a commercial kit (ab156065, Abcam) according to the manufacturer's protocol (Dell'Omo 2018 under revision).

Western blotting

Protein extracts were obtained by suspending pellet of cells in lysis buffer (10 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 15% glycerol, 1% Triton-X-100, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM NaF, protease inhibitor cocktail and 1 mM PMSF), disrupting cell membranes by freezing and thawing and collecting supernatant after 30 min minifuge centrifugation at the maximal speed. 15 µg of protein extracts were separated in a PAGE. After transfer, nitrocellulose membranes were incubated with specific antibodies overnight at 4°C and then with the secondary antibody conjugated with peroxidase for 1 h, at room temperature. Immunoblot assays were carried out using specific antibodies. The primary antibodies used were: acetylated p53 (Lys379 Cell Signaling 2570S), total p53 (Ab-7 Abcam), HIF1α (BD Bioscience 610958), β-actin (Sigma). Immunoreactivity was detected with an ECL Western Blotting Analysis System (Amersham) according to the manufacturer's instructions (Amersham).

P53 deacetylation assay

The SIRT1 deacetylase activity was tested on the K382 residue of native p53 present in the protein extract of MDAMB-231 cells pre-treated with 20 µM etoposide. rhSIRT1 (final dilution 1:60 from ab156065), 200 µM NAD and 270 µM inhibitors (NSAIDs or exilusind) were added to 15 µg protein extract of MDAMB-231, incubated for 30 min at room temperature and stopped with the addition of Laemmli's sample buffer. The entire reaction was loaded on a PAGE for immunoblot analysis; immunodetection was carried out using specific antibodies recognizing acetylated p53 (K382 residue) or total p53.

Stable transfection

MDAMB-231 cell clones that stably express a siRNA targeting human SIRT119 was generated by co-transfecting 1 µg pBABE SIRT1 siRNA (kindly provided by Prof. D.A. Sinclair) or 1 µg pBABE empty vector as a control, together with 1 µg renilla luciferase pRL-TK (E2241 Promega) and 0.1 µg pSV2Neo carrying the neomycin resistance for clone selection. Cells were transfected with Lipofectamine 2000 (Thermo Fisher) according to the manufacturer's

protocol. 48 hours after transfection different dilution (from $1*10^6$ to $1*10^5$/petri) of cells were seeded in petri dishes. After 3 weeks in selection medium containing 600 µg/ml G418, single clones were picked and tested for Renilla luciferase expression with an enzymatic assay on protein extract carried out according to manufacturer's protocol (Renilla-Glo Luciferase Assay System, Promega). Clones with higher levels of luciferase expression were further expanded and tested for SIRT1 expression by western blot analysis. Two clones displaying the lowest SIRT1 expression and two control clones (transfected with the empty vectors) were chowen for testing the effects of NSAIDs (Dell'Omo 2018 under revision).

Hypoxia

MCF7 and MDAMB-231 breast cancer cell lines were used were maintained in DMEM 25 g/l glucose (Life Technology) plus 10% FBS (Sigma-Aldrich). The day before the experiment were seeded in DMEM 1 g/l (low glucose) plus 10% FBS. Then the cells were treated with increasing concentration of ketorolac and nimesulide in normoxia or hypoxia condition (0.1% O_2) for 9 or 24 hours.

qPCR

Cells or breasts were homogenized in TRIzol® (Life Technologies, Carlsbad, CA) 6% (w/v). Total hepatic RNA was purified using RNeasy Mini Kit(QIAGEN, Milan, Italy), following the manufacturer's instructions and then was reverse transcribed to cDNA according to this procedure: 500 ng RNA was denatured at 75°C for 5 min in the presence of 0.75 µg of random primers (Promega) in 7.5 µl final volume. Deoxynucleotide triphosphate (GE Healthcare) and Moloney Murine Leukaemia Virus Reverse Transcriptase (MMLV-RT, Promega) were added at 0.25 mM and 4 U/µl final concentrations respectively, in a final volume of 12.5 µl. The reverse transcriptase reaction was performed at 37°C for 1 h and the enzyme was inactivated at 75°C for 5 min. Real-Time PCR experiments were performed SYBR Select Master Mix (Thermo Fisher) and the levels of mRNA transcripts were normalized on the constitutively expressed gene 36b4 or for hypoxia experiment with RPL11. The reactions were carried out according the manufacturer's protocol using 7900HT standard real-time PCR system (Applied Biosystems) and the thermal profiles used were: 2 min 50°C, 2 min 95°C, 40 cycles (15 sec at 95°C, 30 sec 55-60°C, 1 min 72°C). Data were analyzed using the Sequence Detection System Software v2.3 (Applied Biosystems) and the $2^{-\Delta\Delta Ct}$ method167.

The following primers were used for each gene:

Mouse genes:

36B4 mouse forward 5'-GGCGACCTGGAAGTCCAACT-3', reverse 5'-CCATCAGCACCACAGCCTTC-3';

P21 mouse forward 5'-GCCTGAAGACTGTGATGG-3', reverse 5'-GCCCTCAGCAAGAGTAAG-3'.

Human genes:

PUMA forward 5'-GAGCAGGGCAGGAAGTAACA-3' reverse 5'-CCTGGGGCCACAAATCTGG-3'

RPL11 forward: 5'-GCAAACTCTGTTCAACATCTG-3' reverse: 5'-CATACTCCCGCACTTTAGAC-3'

CA9 forward: 5'-CCTTTGCCAGAGTTGACGAG-3' reverse: 5'-GCAACTGCTCATAGGCACTG-3'.

Ethical approval animal experimentation

All animal experimentation was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in accordance with the European Guidelines for Animal Care and Use of Experimental Animals, approved by the Italian Ministry of the Research and University (MIUR) and controlled by the panel of experts of the Department of Pharmacological and Biomolecular Sciences (University of Milan, 20133 Milan, Italy). For the experiments before 2014 the MIUR authorization was DM 295/2012-A dated 20.12.2012 n. 10/2012, afterward experiments were done under MIUR authorization n. 611/2015 PR. All animal experimentation was carried out in the full observation of the Directive 2010/63/UE.

In vivo studies

Nimesulide, exisulind and ketoprofen and nicotinamide were dissolved in DMSO (270 mM stock solution); after appropriate dilution in water, they were given per os 15 mg/Kg/day and for nicotinamide 15mg/kg/day. DMBA was dissolved in acetone (12 mM solution). Sixteen female repTOPmitoIRE reporter mice (2-4 month old)[158] were divided in four groups and treated with nimesulide, ketoprofen, exisulind or vehicle (DMSO) for 8 days. At day 5, mice were subjected to a single s.c. intra-fat pad injection of 12 mM DMBA solution (left mammary gland) or acetone (vehicle, in the right mammary gland); at day 8, mice were sacrificed, the mammary glands explanted for *ex vivo* imaging and fixed for immunohistochemistry analysis or frozen for total RNA extraction.

Bioluminescence *in vivo* and *ex vivo* imaging

The procedure has been previously described[159]. Briefly, anesthetized animals were i.p. injected with 65 mg/Kg D-Luciferin (beetle luciferin potassium salt, Promega) before each *in vivo* imaging session. After 15 min luciferin distribution, photon emission was measured over

5 min-exposure time using a CCD camera (Night Owl Imaging Unit, Berthold Technologies, Germany). After the last *in vivo* imaging acquisition, animals were sacrificed, the mammary glands were excised and placed in a light-tight chamber for the *ex vivo* measurement. Pseudocolor images representative of photon emissions were generated by a Night Owl LB981 image processor and transferred *via* video cable to a PCI frame grabber using WinLight32 software (Berthold Technologies); grayscale and pseudocolor images were finally merged using WinLight version 32 software (color code from low to high photon emission: blue, green, red, yellow, and white). Light emission was expressed as integration of photon counts per time and per area unit ($p/s/cm^2/sr$). Normalization was performed using an external source of photons enabling to measure the instrumental efficiency of photon counting (Glowell Luxbiotech, Edinburgh, UK).

Immunohistochemistry

Mammary fat pads were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin blocks. Four µm thick sections obtained from these blocks were then immunostained with a primary rabbit monoclonal antibody against Ki-67 antigen (#RM-9106-S, LabVision) using a standard immunoperoxidase protocol (BA-1000 Biotinylated Goat Anti-Rabbit IgG Antibody and PK-6100 Vectastain Elite ABC kit, Vector Laboratories) followed by diaminobenzidine chromogen reaction (SK-4100 Peroxidase substrate kit DAB, Vector Laboratories). Serial sections incubated with normal goat serum instead of the primary antibody served as negative control.

Statistical Analysis

Data analyses were performed using GraphPad 5 Instat software® (GraphPad Prism Inc. San Diego, CA, USA), we have applied Bonferroni's and Student's t test analysis for determining statistical significance.

IC50 was calculated according to GraphPad 5 Curve Fitting Guide (GraphPad Software), with log(inhibitor) versus response–variable slope equation.

Ethics Approvals Human Material

All human tumor specimens were obtained in accordance with the Ethic Committee of the "European Institute of Oncology", Milan, Italy and the main tumor features are listed in Table1.

Table 1. Patients: diagnosis, treatments and tumour features. n.d. not detected

ID	Treatment before surgery	Grade	Type	Disease state	pT	pN	ERα%	PgR%	HER2 score	HER2 %	Ki67 %	Age
1	Control	2	Lobular carcinoma, NOS	Primary	1c	0(sn)	95	95	1+	80	19	73
2	Control	3	Infiltrating duct and lobular carcinoma	Primary	2	1a(sn)	60	30	2+	90	26	50
3	Control	2	Infiltrating duct carcinoma	Primary	2(m)	1	95	95	Neg	n.d.	18	61
4	Control	2	Lobular carcinoma, NOS	Primary	2(m,is)	1a	95	95	2+	20	21	58
5	Ketorolac	2	Lobular carcinoma, NOS	Primary	1c(m)	0(sn)	95	80	Neg	n.d	14	56
6	Ketorolac	1	Lobular carcinoma, NOS	Primary	1c(m)	0(sn)	90	85	1+	15	8	45
7	Ketorolac	2	Infiltrating duct carcinoma	Primary	1b(m)	0(sn)	95	25	Neg	0	21	61
8	Control	3	Infiltrating duct carcinoma	Primary	1c	0(sn)	15	15	3+	95	60	47
9	Control	3	Infiltrating duct and lobular carcinoma	Primary	2	1a	95	95	Neg	n.d.	21	44
10	Control	2	Infiltrating duct and lobular carcinoma	Primary	3(is)	1a	95	95	Neg	n.d.	18	54
11	Ketorolac	3	Infiltrating duct and lobular carcinoma	Primary	2	3a	95	80	2+	20	26	60
12	Ketorolac	3	Infiltrating duct carcinoma	Contralateral	1c	0(sn)	90	50	2+	40	24	72
13	Ketorolac	2	Infiltrating duct carcinoma	Primary	2	3a	95	95	Neg	n.d.	22	45

RESULTS

SIRT1 AS A NEW TARGET OF NSAIDS

NSAIDs and exisulind increase P53 acetylation in breast cancer cell line

In order to investigate the existence of a common mechanism for the chemopreventive activity of NSAIDs, we focussed our attention on the p53 signalling, based on the study by Alfonso et al reporting the ability of aspirin to increase the level of p53 acetylation at the K382 site, induce p21 expression and inhibit proliferation of MDAMB-231 breast cancer cells[104]. The level of K382 acetylation was initially measured by western blot analysis carried out on protein extracts obtained from MDAMB-231 cells treated with 90 µM of nimesulide, diclofenac, ketoprofen and exisulind at several time points.

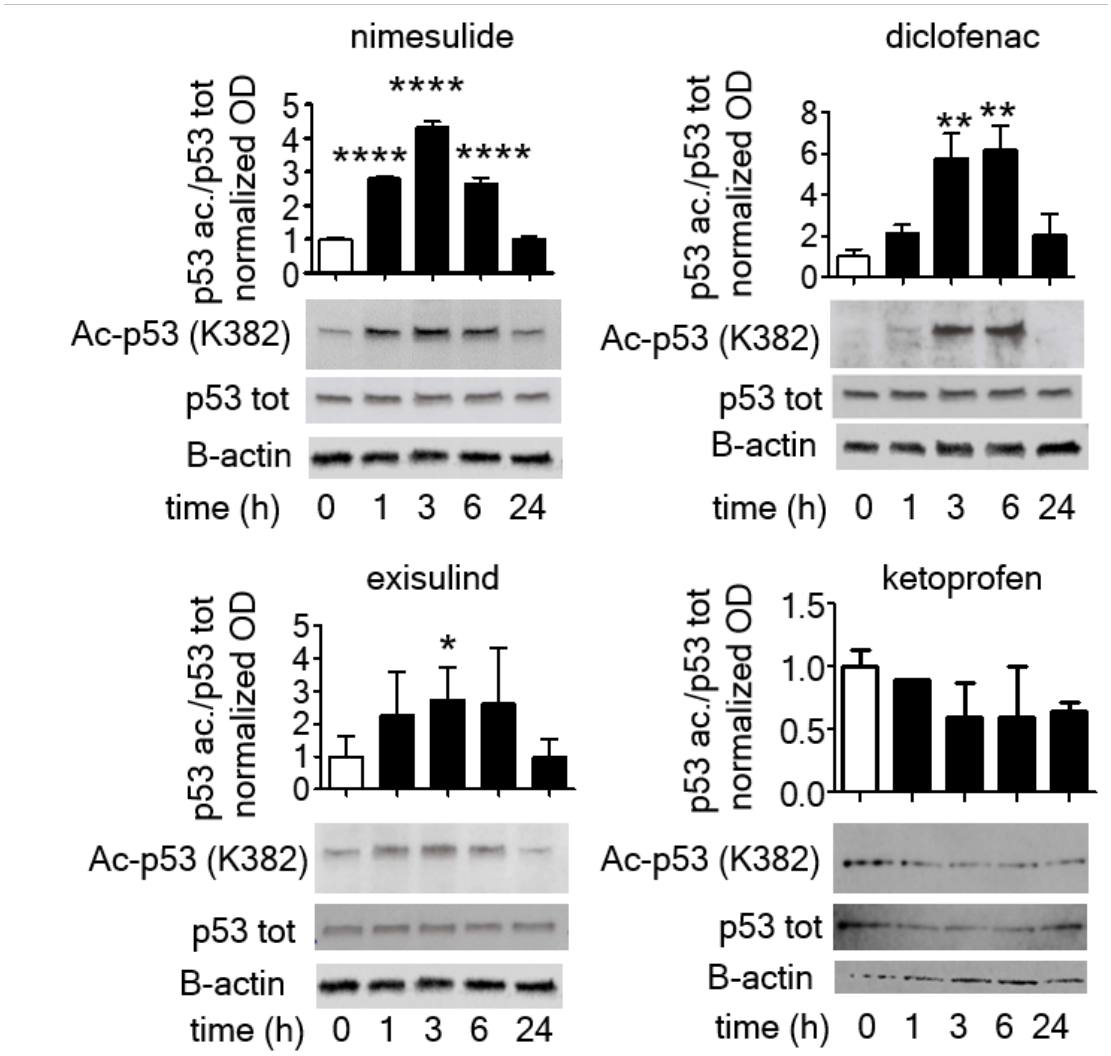
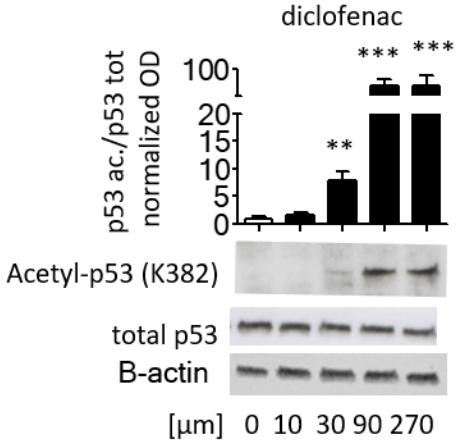
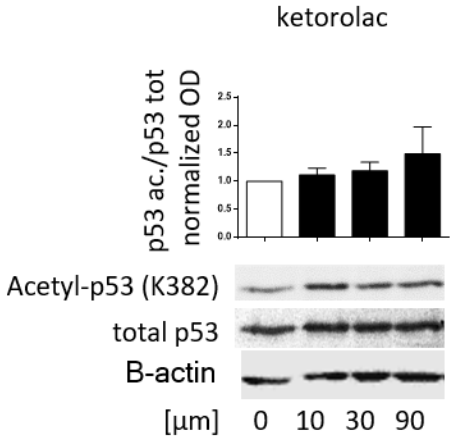


Figure 19. Treatment with nimesulide, diclofenac and exisulind, but not ketoprofen increase p53 acetylation at K382 residue. MDAMB-231 cells were treated with 90μM for each compound in the time course experiment. 30ug of protein extracts were analysed, confirmed by ponceau staining, immunoblot analysis was carried out using anti-acetyl (K382) p53 and anti-total p53 antibodies. Data are represented as mean ± SEM. Bars in the graphs represents densitometry quantifications of the autoradiographic signals (acetylated p53 vs total p53); *P< 0.05 **P< 0.01 ***P<0.001 ****P<0.0001 versus the baseline level; P values were calculated by one-way ANOVA. Figure adopted from Dell'Omo 2018 under revision.

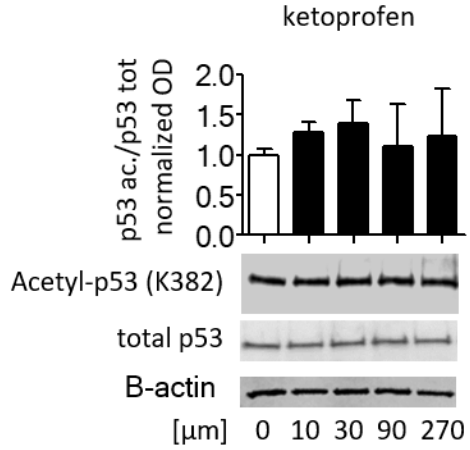
Ketoprofen was included in the study as negative control since a clear anti-proliferative activity of this drug has never been demonstrated[160]. The experiments showed that all compounds, but ketoprofen, significantly increased p53 acetylation 3-6 hours after exposure to the drugs (Figure 19). Interestingly, the sulindac metabolite exisulind, displaying anti-cancer properties, but unable to inhibit COXs and to produce an anti-inflammatory response, was increasing the p53 acetylation at the K382 site similarly to what observed for diclofenac and nimesulide. After these experiments, the analysis was extended by testing the effects of increasing concentration of NSAIDs belonging to different chemical classes including arylacetics (diclofenac, ketorolac), arylpropionics (ibuprofen, ketoprofen), arylsulfonamide (nimesulide), arylalkanoic acids (exisulind, sulindac sulfide) and NS-398 a COX-2 inhibitor used in the study of function of cyclooxigenases (PubChem); the effects on K382 acetylation was assayed by western blot analysis on MDAMB-231 protein extracts obtained after 3 hours of treatment.

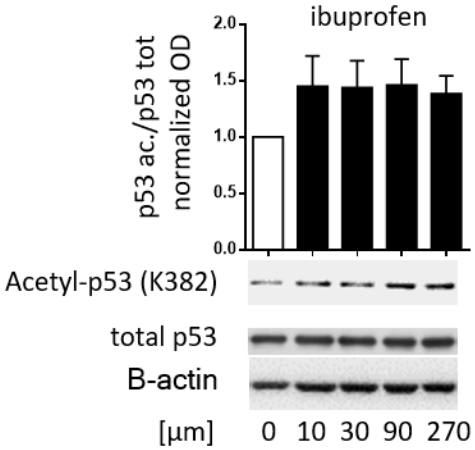
arylacetics



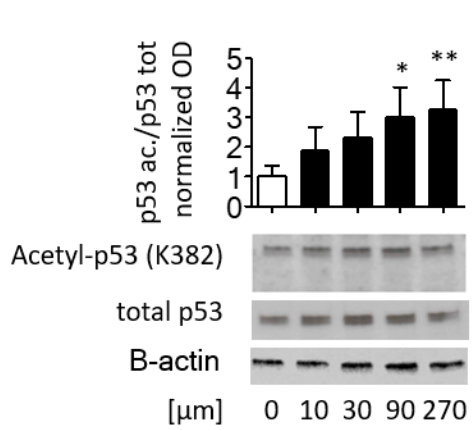


arylpropionics

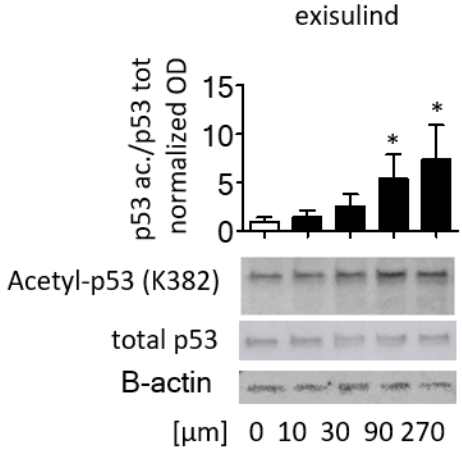




arylsulfonamide



arylalkanoic acid



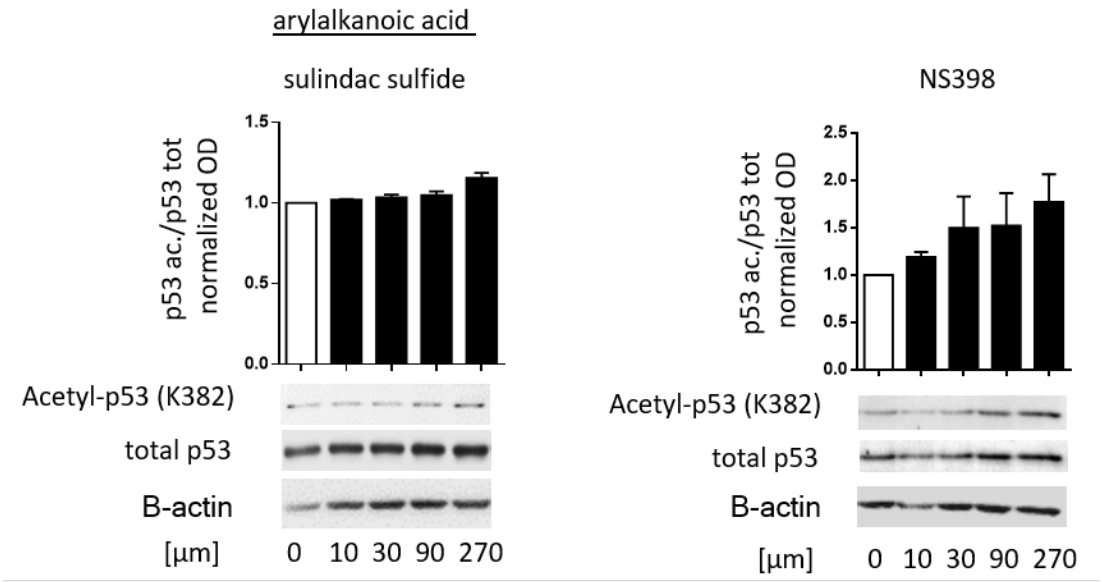


Figure 20. Treatment with NSAIDs and exisulind, but not ketoprofen increase p53 acetylation at K382 residue in dose dependent manner. MDAMB-231 cells were treated with increasing concentrations of each compound for 3 hours. 30ug of protein extracts were analysed, immunoblot analysis was carried out using anti-acetyl (K382) p53 anti-total p53 antibodies and β-actin as loading control. Data are represented as mean ± SEM. Bars in the graphs represents densitometry quantifications of the autoradiographic signals (acetylated p53 vs total p53); *P< 0.05 **P< 0.01 versus the baseline level; P values were calculated by one-way ANOVA. Figure adopted from Dell'Omo 2018 under revision.

The immunoblot analysis indicated that NSAIDs with different chemical structures and exisulind, shared the ability to increase the K382 acetylation of P53 in a concentration-dependent manner (figure 20). This activity was not observed when cells were treated with the negative control ketoprofen, suggesting that this property was independent form the cyclooxygenase inhibition activity.

NSAIDs directly inhibit SIRT1 deacetylase without affecting p300 activity

The experiments reported in Figures 19 and Figure 20 suggested that the increase of p53 acetylation was likely COX-independent for several reasons: i) ketoprofen, a COX-1/COX-2 inhibitor, was not able to increase p53 acetylation; ii) on the contrary, exisulind, a metabolite of sulindac without COX-inhibitory activity, was able to increase p53 acetylation; iii) the fast dynamic of p53 acetylation after NSAIDs treatment (the effects could be seen 1 h after treatment) was not compatible with an indirect mechanism involving several biochemical steps. These observations led us to hypothesize a COX-independent mechanism modulating the activity of enzymes able to deacetylate or acetylate the K382 residue; thus, we tested the effects of NSAIDs on the two enzymes known to add (P300) or remove (SIRT1) the acetyl

residue at the K382 site of p53. The study was done *in vitro* by using recombinant human P300 (rhP300) acetylase and the recombinant human SIRT1 deacetylase (rhSIRT1).

As first P300 was tested *in vitro* asking if nimesulide was able to increase the acetyl-transferase action. The assay was based on the ability of the enzyme rhP300 to transfer the radioactive ^{3}H-acetyl from ^{3}H-acetyl-CoA to a substrate histone peptide.

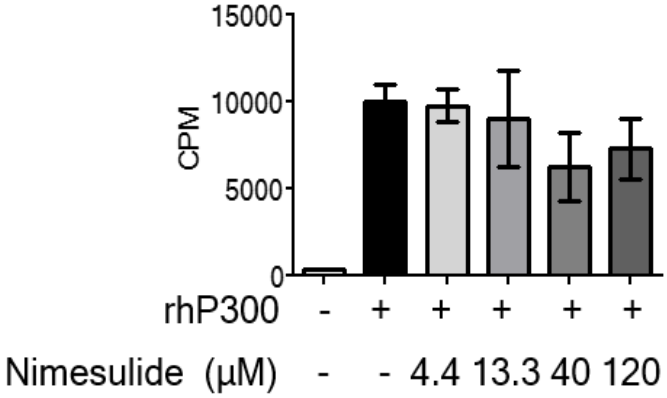


Figure 21. Nimesulide didn't increase rhP300 activity. The enzymatic activity of rhP300 was measured in the presence of nimesulide (4.4-13.3-40-120 μM) with a commercial kit (Perkin-Elmer Life Sciences) based on the transfer of a radioactive ^{3}H-acetyl from ^{3}H-acetyl-CoA to a histone peptide; after the transfer the peptides are separated by a paper chromatography according to the manufacturer instructions. The amount of radioactivity incorporated by the peptides was determined with a β-counter. The assay was carried out in duplicates and repeated twice. Figure adopted from Dell'Omo 2018 under revision.

The results indicated that rhP300 activity was not affected by nimesulide (Figure 21), suggesting that the NSAIDs-dependent increase of p53 acetylation was not caused by P300 regulation. Next, the attention was focused on the NSAIDs influence on rhSIRT1[156]. In order to assay the rhSIRT1 activity I measured the fluorescence produced by a two-step reaction: the first step is based on the presence of fluorophore and quencher coupled at the amino- and carboxyl-terminals, respectively, of the substrate peptide. In the presence of the SIRT1 deacetylase, the acetyl residue is removed from the substrate peptide that will become substrate to the action of a protease added simultaneously in the reaction; the proteolytic activity will separate quencher from fluorophore, generating a fluorescent emission whose intensity can be quantified by a fluorimeter. The assay has been carried out with a rhSIRT1 deacetylase in the presence of NAD^{+} (the physiological activator) and of increasing concentrations of different NSAIDs.

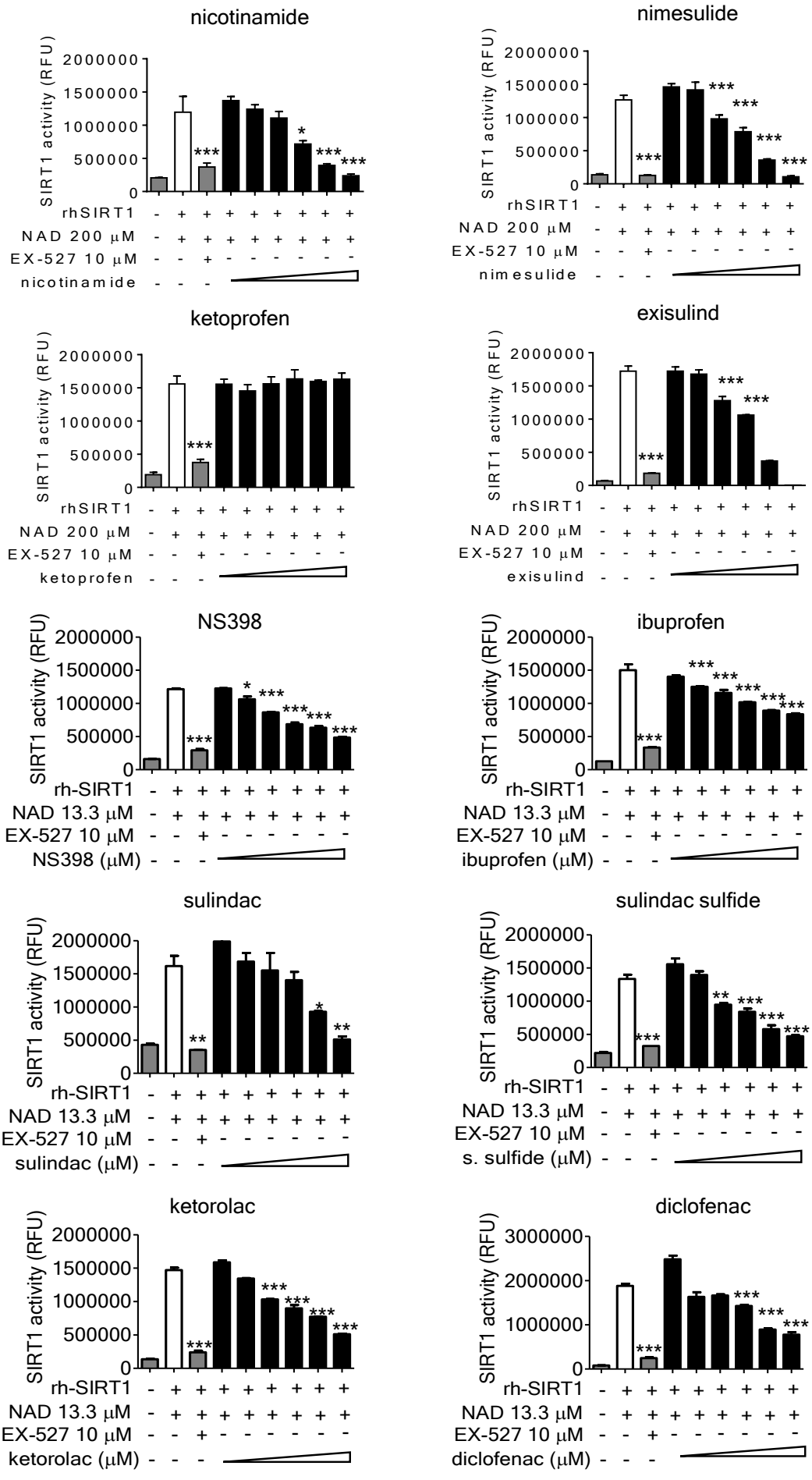

nicotinamide
SIRT1 activity (RFU)
2000000
1500000
1000000
500000
0
rhSIRT1 - + + + + + + + +
NAD 200 μM - + + + + + + + +
EX-527 10 μM - - + - - - - - -
nicotinamide - - -
nimesulide
rhSIRT1 - + + + + + + + +
NAD 200 μM - + + + + + + + +
EX-527 10 μM - - + - - - - - -
nimesulide - - -
ketoprofen
rhSIRT1 - + + + + + + + +
NAD 200 μM - + + + + + + + +
EX-527 10 μM - - + - - - - - -
ketoprofen - - -
exisulind
rhSIRT1 - + + + + + + + +
NAD 200 μM - + + + + + + + +
EX-527 10 μM - - + - - - - - -
exisulind - - -
NS398
rh-SIRT1 - + + + + + + + +
NAD 13.3 μM - + + + + + + + +
EX-527 10 μM - - + - - - - - -
NS398 (μM) - - -
ibuprofen
rh-SIRT1 - + + + + + + + +
NAD 13.3 μM - + + + + + + + +
EX-527 10 μM - - + - - - - - -
ibuprofen (μM) - - -
sulindac
rh-SIRT1 - + + + + + + + +
NAD 13.3 μM - + + + + + + + +
EX-527 10 μM - - + - - - - - -
sulindac (μM) - - -
sulindac sulfide
rh-SIRT1 - + + + + + + + +
NAD 13.3 μM - + + + + + + + +
EX-527 10 μM - - + - - - - - -
s. sulfide (μM) - - -
ketorolac
rh-SIRT1 - + + + + + + + +
NAD 13.3 μM - + + + + + + + +
EX-527 10 μM - - + - - - - - -
ketorolac (μM) - - -
diclofenac
3000000
2000000
1000000
0
rh-SIRT1 - + + + + + + + +
NAD 13.3 μM - + + + + + + + +
EX-527 10 μM - - + - - - - - -
diclofenac (μM) - - -

Figure 22. NSADs and exisulind inhibit SIRT1 in dose dependent manner. The enzymatic activity of rhSIRT1 was measured with a fluorescent assay according to the manufacturer instruction; the assay was carried out in the presence of increasing concentrations of the indicated NSAIDs. Bars represent the average values of rhSIRT1 activity measured in three independent experiments. Statistical analysis was done using two-way ANOVA followed by Bonferroni's analysis: *P<0.05 **P<0.01 ***P<0.001, rhSIRT1 activity in the presence of NAD versus rhSIRT1 activity in the presence of NAD and of increasing concentration of the indicated NSAIDs. Figure adopted from Dell'Omo 2018 under revision.

Compounds	**IC50**
Nicotinamide	374
Sulindac	969
Sulindac Sulfide	376
Exisulind	397
NS-398	862
Nimesulide	344
Ketoprofen	no activity
Ibuprofen	2152
Ketorolac	602
Diclofenac	554

Table 2. IC50 of NSAIDs and exisulind mediate SIRT1 inhibition. IC50 calculated according to GraphPad 5 Curve Fitting Guide (GraphPad Software), with log(inhibitor) versus response–variable slope equation. Table adopted from Dell'Omo 2018 under revision.

The results of these assays showed that all NSAIDs tested and exisulind, but not ketoprofen inhibited the rhSIRT1 activity in a concentration dependent manner (Figure 22) in line with the p53 acetylation activity observed in cells by the same compounds. Interestingly, for almost all the drugs tested, the calculated IC50 were in the same potency of the physiological inhibitor nicotinamide (Table 2).

Due to some possible limitations of the SIRT1 fluorescence-based assay[161,162] that could create potential artefact, we further tested the inhibitory action of nimesulide on rhSIRT1 using a bioluminescent assay[157]. Briefly, rhSIRT1 was incubated in the presence of NAD and of an acetylated SIRT1 substrate peptide covalently linked to luciferin (GLO). In the bioluminescent

reaction, SIRT1 deacetylated the substrate peptide unmasking a site for a subsequent peptidase reaction, which can thus release free luciferin: the amount of luciferin in the mixture is proportional to the deacetylase enzymatic activity and can be quantified by measuring the photon emission with a luminometer upon addition of the luciferase enzyme to the reaction mixture.

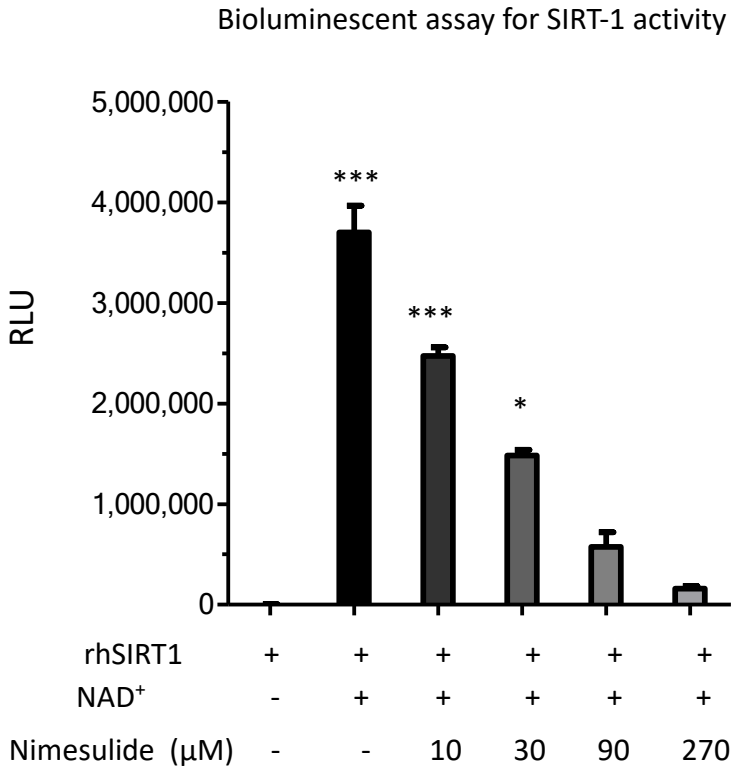


Figure 23. Nimesulide inhibit SIRT1 deacetylase activity. The deacetylase activity of rhSIRT1 was evaluated after treatment with 200 μM NAD and in the presence of the indicated nimesulide concentrations with a bioluminescent assay. The amount of photon emission was quantified with a luminometer and expressed as relative light units (RLU). Bars represent average value of two experiments carried out in triplicates. ***P<0.001, **P<0.01 * P<0.05 rhSIRT1 activity in the presence of NAD versus rhSIRT1 activity in the presence of NAD and of increasing concentrations of nimesulide. Statistical analysis was done using two-way ANOVA followed by Bonferroni's analysis. Figure adopted from Dell'Omo 2018 under revision.

The results obtained with the bioluminescent and fluorescent assays were superimposable (Figure 23), confirming the inhibitory effect of nimesulide on SIRT1 activity.

To confirm that the effects of NSAIDs on the recombinant SIRT1 could be observed also on the endogenous SIRT1, the fluorescent assay was used for testing the effects of the compounds on MDAMB-231 protein extracts.

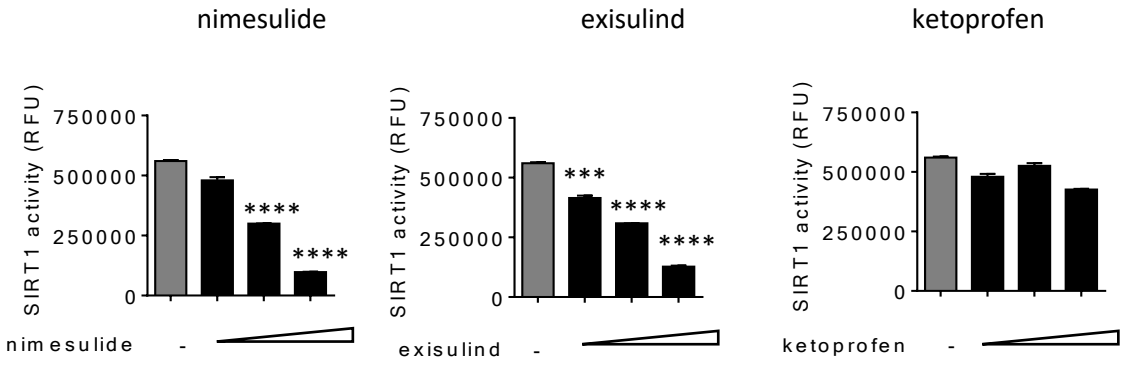
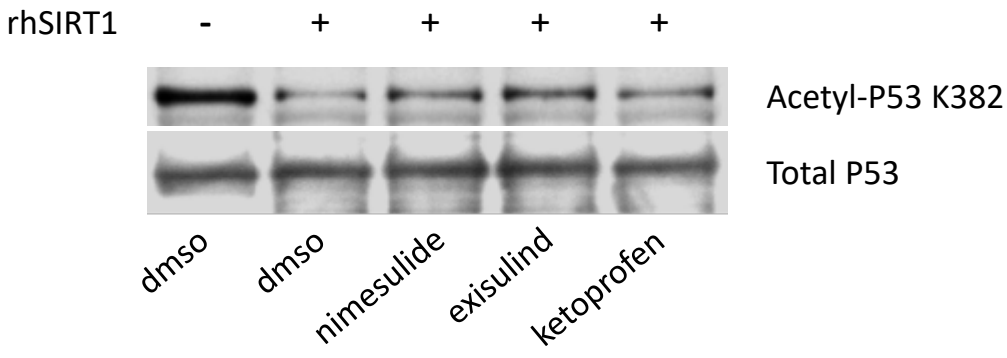


Figure 24. Nimesulide, diclofenac and exisulind, but not ketoprofen, inhibit rhSIRT1 activity *in vitro*. The SIRT1 activity was tested in MDA-MB-231 protein extracts treated with nimesulide, exisulind and ketoprofen at increasing concentrations (300, 900, 2700 µM). Data are represented as mean ± SEM. *P< 0.05 **P< 0.01 ***P<0.001 ****P<0.0001 versus the value of the NAD treated sample; P values were calculated by two-way ANOVA followed by Bonferroni's test. Figure adopted from Dell'Omo 2018 under revision.

The inhibitory activity of the NSAIDs and exisulind could be observed also using the MDAMB-231 protein extract as a source of SIRT1, suggesting that the observed activity on the deacetylase was not an artefact of the SIRT1 biochemical assay (Figure 24).

p53 acetylation at the K382 site is increased by the NSAIDs-mediated inhibition of SIRT1

As an evidence of the direct effect of the NSAIDs-mediated inhibition of SIRT1 on the K382 residue of p53, we investigated the deacetylation potential of rhSIRT1 on the acetylated p53 present in MDAMB-231 cells. Protein extract from this cell line was used as a source of native P53 and tested, by immunoblot analysis, whether NSAIDs were able to inhibit K382 deacetylation promoted by rhSIRT1 added to the reaction mix (procedure on materials and methods).



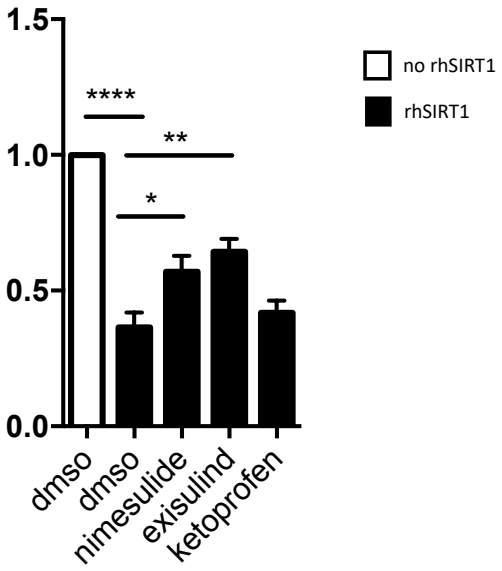


Figure 25. Deacetylation assay on native p53 protein. Picture shows a representative immunoblot analysis of native acetylated and total p53 present in the extracts of MDAMB231 6h before harvesting, cells were treated with etoposide 20μM to obtain sufficient amount of p53 acetylation; the same batch of protein extract was divided in 15μg aliquots and treated either with vehicle (dmso) or with 300 μM nimesulide, exisulind and ketoprofen, the extract was then treated with rhSIRT1 or with saline buffer. Quantification of the immunoblot signals are reported in the graph: acetylated p53 signal was normalized on the corresponding total p53 signal and referred to the control (no rhSIRT1/dmso). Bars represents the average ± SEM normalize values of three independent experiments. *$P < 0.05$ **$P< 0.01$ ***$P<0.001$ ****$P<0.0001$ P values were calculated by Student's t-test. Figure adopted from Dell'Omo 2018 under revision.

Data showed that the presence of rhSIRT1 alone significantly decreased by 64% the acetylation of P53. Interestingly, the activity of the enzyme was significantly inhibited by the addition of 300μM nimesulide or exisulind (Figure 25); once more, ketoprofen was inactive on SIRT1 activity. These results supported the notion that the effect of NSAIDs and exisulind on K382 acetylation is directly mediated by SIRT1.

Next, to further strengthen this conclusion, a loss of function approach using siRNA was adopted, generating stably transfected clones with an expression vector encoding for a siRNA directed against SIRT1 mRNA[163]. MDAMB231 transfection was carried out with lipofectamine technologies; after 21 days of G418 selection, siRNA expressing clones were initially identified with a coelenterazine-based procedure (details can be found in the Material and Methods section); the identified clones were amplified and the effective knock down of SIRT1 expression was demonstrated by western blot analysis; as expected from literature data[164], in the SIRT1 knocked down clones, a constitutive increment of the p53 basal acetylation at the K382 residue was observed in comparison with the cells transfect with the empty vector

(Figure 26).These knock down clones were used to firmly identify SIRT1 as the direct mediator for NSAIDs activity on P53 acetylation; to this aim, I treated the cells with 270 μM of each NSAIDs for 3 hours and measured the p53 acetylation by western blot analysis.

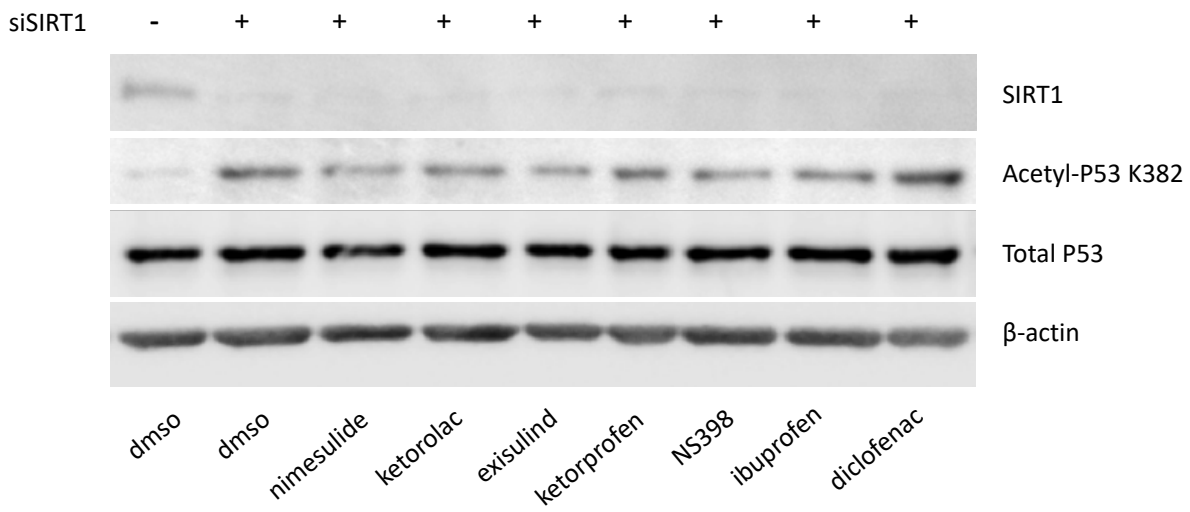


Figure 26. SIRT1 mediated NSAIDs effect on p53 acetylation. MDAMB231 stable transfected with pBABE or pBABEshSIRT1 were treated for 3 hours with dmso, MDAMB231 SIRT1 negative were also treated with 270μM of the reported compounds. Then 15 μg of protein lysate were analysed by western blot. SIRT1, acetylated p53 (Lys382), total p53 and β-actin were measured using specific antibody reported on materials and methods session. Figure adopted from Dell'Omo 2018 under revision.

After treatments with NSAIDs and exisulind (Figure 26), the basal acetylation remained unchanged providing a compelling demonstration of the direct role of SIRT1 in mediating the K382 acetylation induced by these compounds in breast cancer cells.

These *in vitro* data strongly indicate SIRT1 as a novel common target of NSAIDs with different chemical structures. Interestingly, this inhibition correlates with the anti-proliferative activity of these drugs previously reported other laboratories, thus indicating SIRT1 inhibition as a possible mechanism underlying the anticancer properties of NSAIDs. This prompted us to further characterize this mechanism in breast cancer cell line, in animal model and in clinical samples.

Functional role of the NSAIDs-mediated SIRT1 inhibition

p53 is activated upon the NSAIDs-mediated SIRT1 inhibition

During this part of my thesis, I studied and characterized the potential effect on the hallmarks of cancer of the NSAIDs-mediated SIRT1 inhibition. Since SIRT1 inhibition led to the increase P53 acetylation at the K382 site, it could be postulated that this might led to the activation of p53 transcriptional activity and to the increased expression of the anti-proliferative p21, a well-known p53 target gene[165], thus triggering an anti-proliferative pathway. This hypothesis was tested first in non-transformed cells, the hTERT-HME1, human mammary epithelium cell line

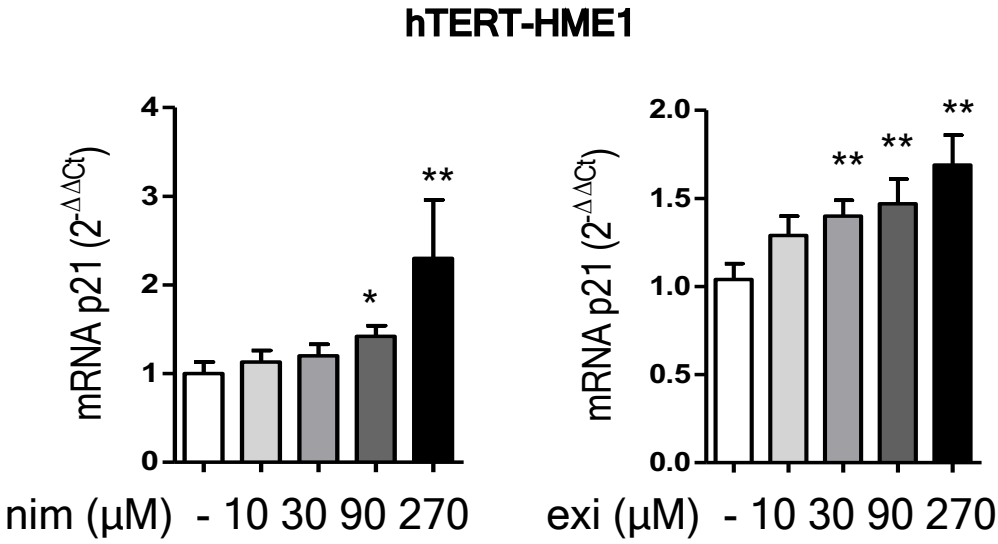
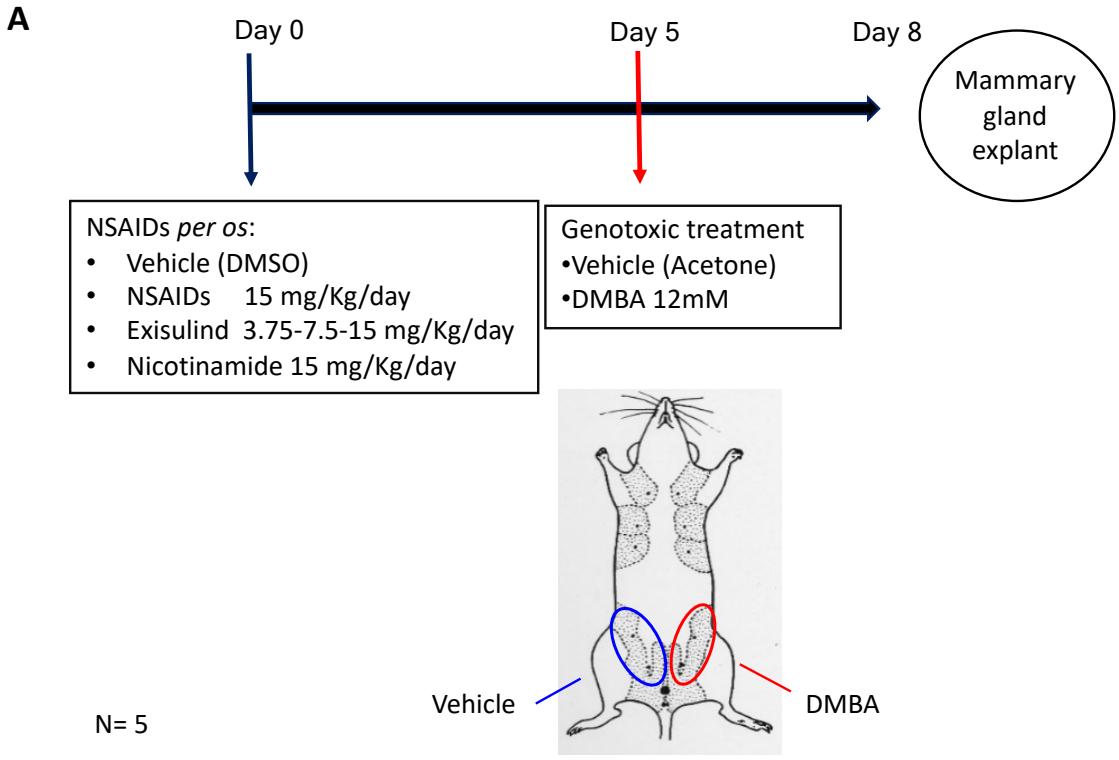


Figure 27. Nimesulide and exisulind increase p53 target gene expression. hTERT-HME1 are immortalized cells carrying wild type p53 and were treated with increasing concentrations of nimesulide (nim) and exisulind (exi) for 6 hours. The expression of p21 was measured by qPCR, bars represent average normalized values (quantified with the $2^{-\Delta\Delta Ct}$ method) of two independent experiments. Figure adopted from Dell'Omo 2018 under revision.

The hTERT-HME1 cells were treated with increasing concentration of nimesulide and exisulind for 6 h and the level of p21 mRNA was measured by qPCR. Results showed that upon treatment p21 expression increased in a concentration-dependent manner after treatment (Figure 27), suggesting that the oncosuppressor is indeed transcriptionally activated by these drugs.

NSAIDs-mediated SIRT1 inhibition results in the p53 activation in vivo, in a mouse model of the early steps of carcinogenesis

Based on the results obtained *in vitro*, the effect of the anti-inflammatory drugs was investigated in an animal model of the early transformation steps consisting in the topical treatment of the mammary gland with the carcinogen 7,12-Dimethylbenz(a)anthracene (DMBA). As showed in the Figure 28, mice were treated for 8 days *per os* with vehicle or 15 mg/Kg/day nimesulide, 15 mg/kg/day ketoprofen, 3.75-7.5-15 mg/Kg/day exisulind, 15 mg/Kg/day nicotinamide. Five days after the NSAIDs treatment, the left inguinal mammary gland was injected with DMBA, while the contralateral mammary gland was treated with acetone (vehicle). Three days after the genotoxic injection, mice were sacrificed and breast collected for gene expression analysis. In particular, p53 activation was quantified by the increased expression of its target gene p21, through a semiquantitative qPCR analysis carried out on the mRNA obtained from the breast tissue[166].



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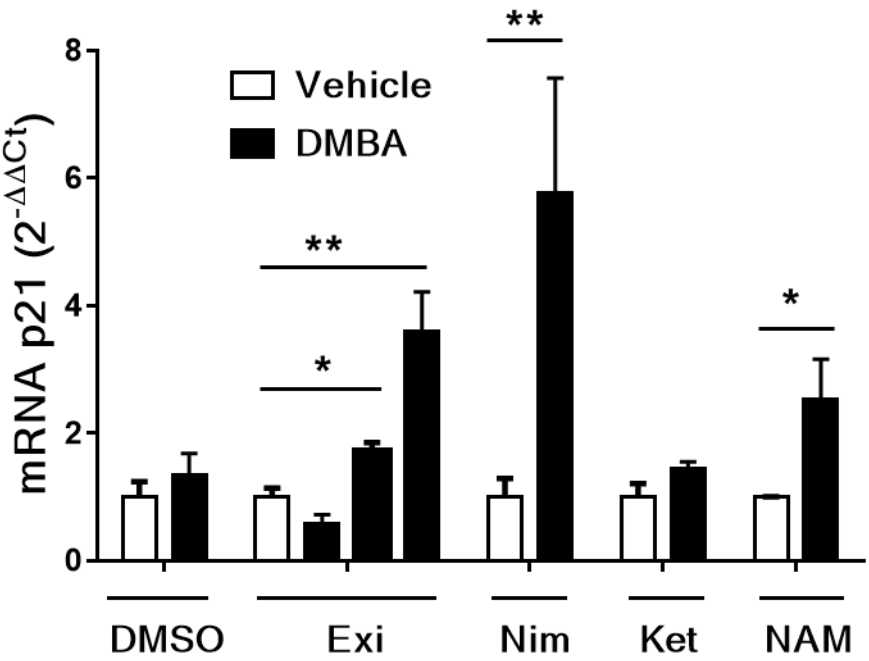


Figure 28. NSAIDs exisulind and nicotinamide treatment, but not ketoprofen increases p53 activity *in vivo.* (**A**) Five female mice per group were treated per os (gavage) with a daily dose of 3.75, 7.5, 15 mg/Kg exisulind (exi), 15 mg/Kg nimesulide (nim), 15 mg/Kg ketoprofen (ket), 15 mg/Kg nicotinamide or Dimethyl sulfoxide (DMSO, vehicle). Treatment was carried out for eight days; at day 5 a single dose of an acetone solution of 12mM DMBA (left mammary gland) or acetone (right mammary gland) was injected in the mammary fat pad of the animals. (**B**) p21 mRNA expression was determined by real time PCR; bars in the graph are the average ± SEM values quantified with the $2^{-\Delta\Delta Ct}$ method. * $P < 0.05$; ** $P < 0.01$ DMBA versus acetone treated breast. P values were calculated by Student's t-test. Figure adopted from Dell'Omo 2018 under revision.

The *in vivo* results showed that in the left mammary gland injected with DMBA, p21 mRNA expression was clearly induced in mice treated with nimesulide or exisulind (Figure 28B), however, this increment was not observed in mice treated with vehicle and ketoprofen. These experiments confirmed that NSAIDs (but not ketoprofen) and exisulind were able to increase p53 activity also *in vivo*; interestingly, the exisulind active dose (15 mg/Kg) was in the same order of magnitude of the dose reported to obtain polyp regression in humans[167]. To verify whether p53 activation *in vivo* could be indeed mediated by SIRT1 inhibition, the same experiment (figure 28A) was carried out with nicotinamide, the SIRT1 physiological inhibitor. The results showed that in the DMBA mammary gland, p53 activation increased in mice treated with nicotinamide (Figure 28B), suggesting that the NSAIDs-mediated p53 activation may occur *in vivo* through SIRT1 inhibition.

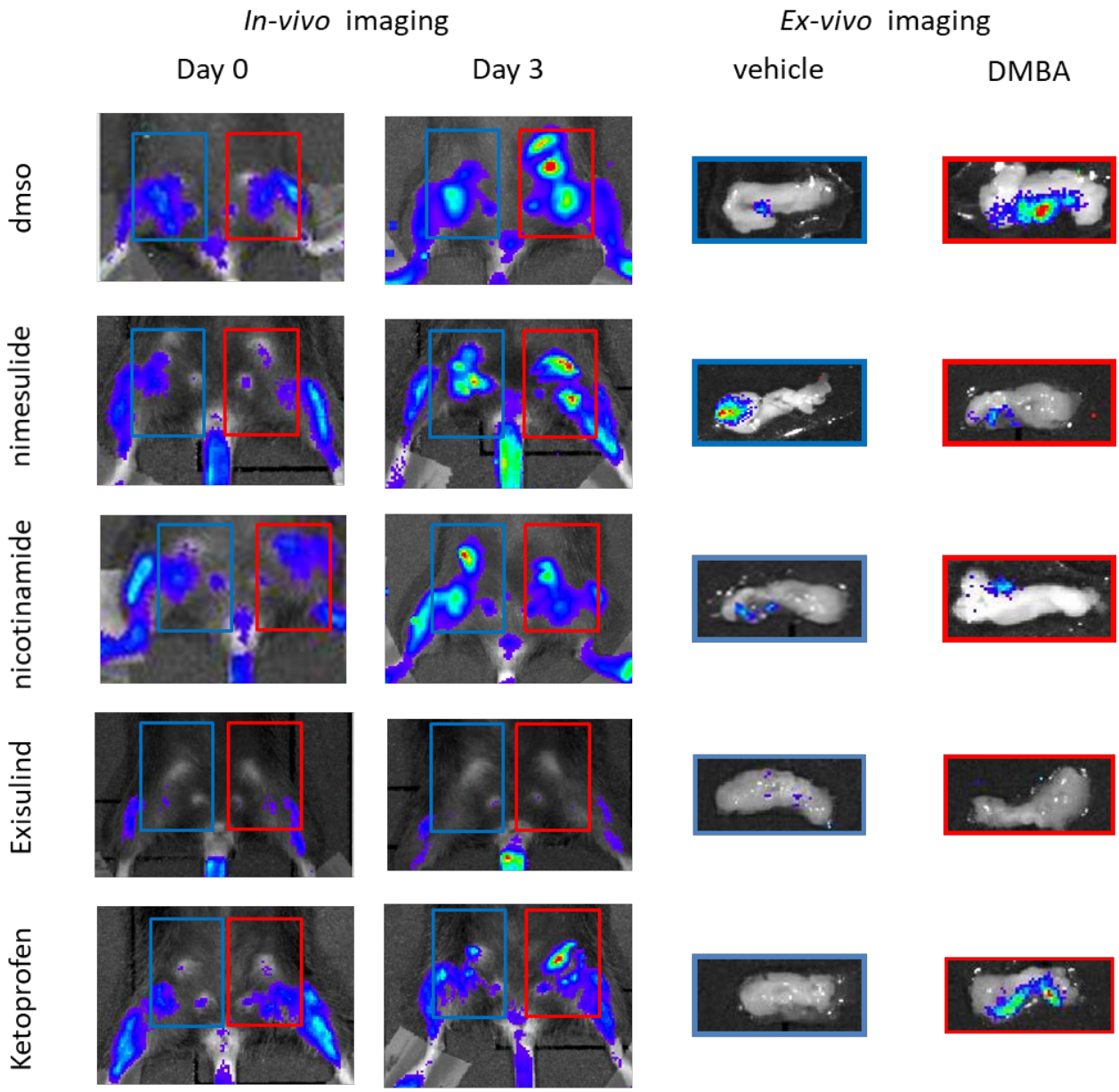
NSAIDs-mediated SIRT1 inhibition in early phases of Breast Cancer: functional effects on proliferation, immunosuppression and hypoxia

Once discovered and characterized SIRT1 as a new target for NSAIDs, we put our attention on the phenotypic consequences of SIRT1 inhibition in the tumour progression. For this reason, three of the major hallmarks of cancer were studied: increased proliferation, immunosuppression and hypoxia.

Proliferation

The effects on cell proliferation was investigated *in vivo* on the hyperproliferation produced by a genotoxic treatment; to this aim, we carried out the DMBA treatment as before (figure 28A). Thanks to the past expertise and tools developed by my lab, I was able to include in this thesis, the transgenic reporter mouse called MITO-Luc (for mitosis-luciferase), in which an NFY–dependent promoter controls luciferase expression. In these mice, bioluminescence imaging of NF-Y activity correlate with physiological cell proliferation and regeneration during response to injury[158]. With this tool, we had the opportunity to measure the effect of NSAIDs on the increased proliferation occurring upon treatment with a genotoxic agent, a condition that mimic the hyperproliferation during the early step of carcinogenesis. To this aim, we treated group of 5 MITO-Luc mice with an identical protocol reported in Figure 28 and measured the bioluminescent emission from the mammary gland *in vivo* and *ex vivo* by bioluminescence imaging (Figure 29)

A MITO-Luc reporter mouse - imaging



B MITO-Luc reporter mouse – *ex vivo* quantification of photon emission

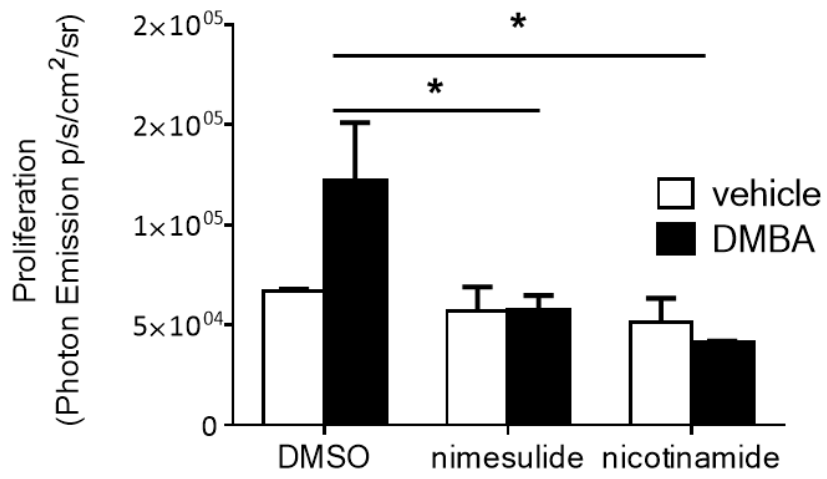


Figure 29. NSAIDs exisulind, nicotinamide treatment, but not ketoprofen decrease tissue proliferation *in vivo.* Five female repTOPmitoIRE mice/group were treated with nimesulide, nicotinamide, exisulid and ketoprofen following an identical protocol described in Figure 28A. (**A**) Pictures show the bioluminescence emission marking the proliferative activity in the repTOPmitoIRE reporter mouse or in the dissected mammary glands. (**B**) Quantification of the photon emission as a measure of tissue proliferation from the dissected mammary glands; bars are the average ± SEM values of the photon emission normalized over the area of the acquisition surface ($p/s/cm^2/sr$). *P<0.05 mammary glands treated with DMBA and NSAIDs versus DMSO treated animals; P values were calculated by Student's t-test. Figure adopted from Dell'Omo 2018 under revision.

Results showed that DMBA was able to increase the proliferation in breast tissue when compared to the contralateral breast, in DMSO treated mice (Figure 29), suggesting that cell hyperproliferation was induced by the treatment with the genotoxic agent. Interestingly, bioluminescent analysis of the photon emission demonstrated that cell proliferation was significantly influenced by NSAIDs treatment, in particular we were able to measure less signal in animals treated with nimesulide, nicotinamide and exisulind (Figure 29) compared to vehicle and ketoprofen (DMSO). Positively, results fitted with the previous p53 activations and increasing expression of p21 that triggers cell cycle arrest. Previous data of the lab demonstrated that the bioluminescent signal of the MITO-Luc reporter mice perfectly correlated with the Ki67 immunostaining[158]. This was confirmed by the immunohistochemistry staining of the breasts analyzed by *ex vivo* imaging with the Ki67 proliferation marker (Figure 30).

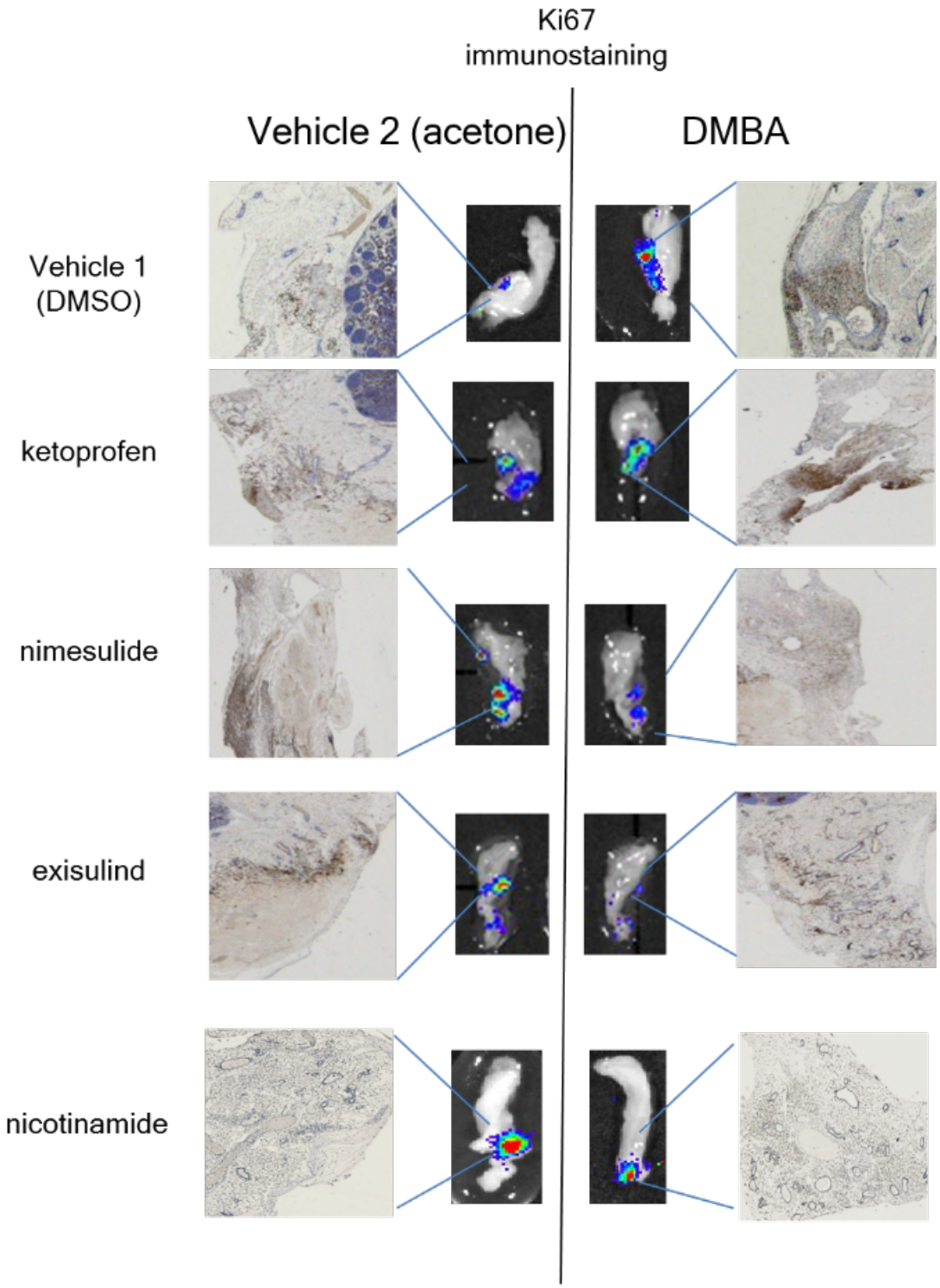


Figure 30. NSAIDs exisulind, nicotinamide treatment, but not ketoprofen decrease tissue proliferation *in vivo.* The bioluminescent signal reported in (figure 29) correlates with the Ki-67 immunostaining in the tissue of repTOPmitoIRE reporter mice. Pictures show the correlation between the bioluminescence emission and Ki67 staining in the same mammary glands (central and external set of pictures, respectively). After *ex vivo* imaging, mammary fat pads were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. 4µm thick sections obtained from these blocks were then immunostained with a primary antibody against Ki67 antigen. Figure adopted from Dell'Omo 2018 under revision.

The Ki67 staining was mirroring the bioluminescent analysis (Figure 29 and Figure 30).

These experiments indicated that the treatment with NSAIDs or exisulind attenuated the tissue hyperproliferation produced by the exposition to a genotoxic agent, a model recapitulating the initial hyperproliferation occurring during the initial stage of tumor transformation. Similar protective effects were observed for NSAIDs and nicotinamide, thus reinforcing the concept that they were mediated by SIRT1 inhibition (Figure 29 and Figure 30). Once more, ketoprofen did not have the same effect of NSAIDs.

Immunosuppression

Increased proliferation is not the only effect produced by the exposition to genotoxic agents: previous studies showed that local and systemic immunosuppression occurs after this exposure and is likely to participate in the early mechanism of neoplastic transformation[168]. Moreover, emerging data showed that sulindac mediated breast cancer inhibition as an immune modulator, by reducing the M2 macrophage influx *in vivo* [169]. To evaluate the effects of NSAIDs on the immune suppression induced by a genotoxic agent, the expression of cytokines and chemokines was measured in the mammary tissues of obtained from the same mice treated in the previous *in vivo* experiments (Figure 28A).

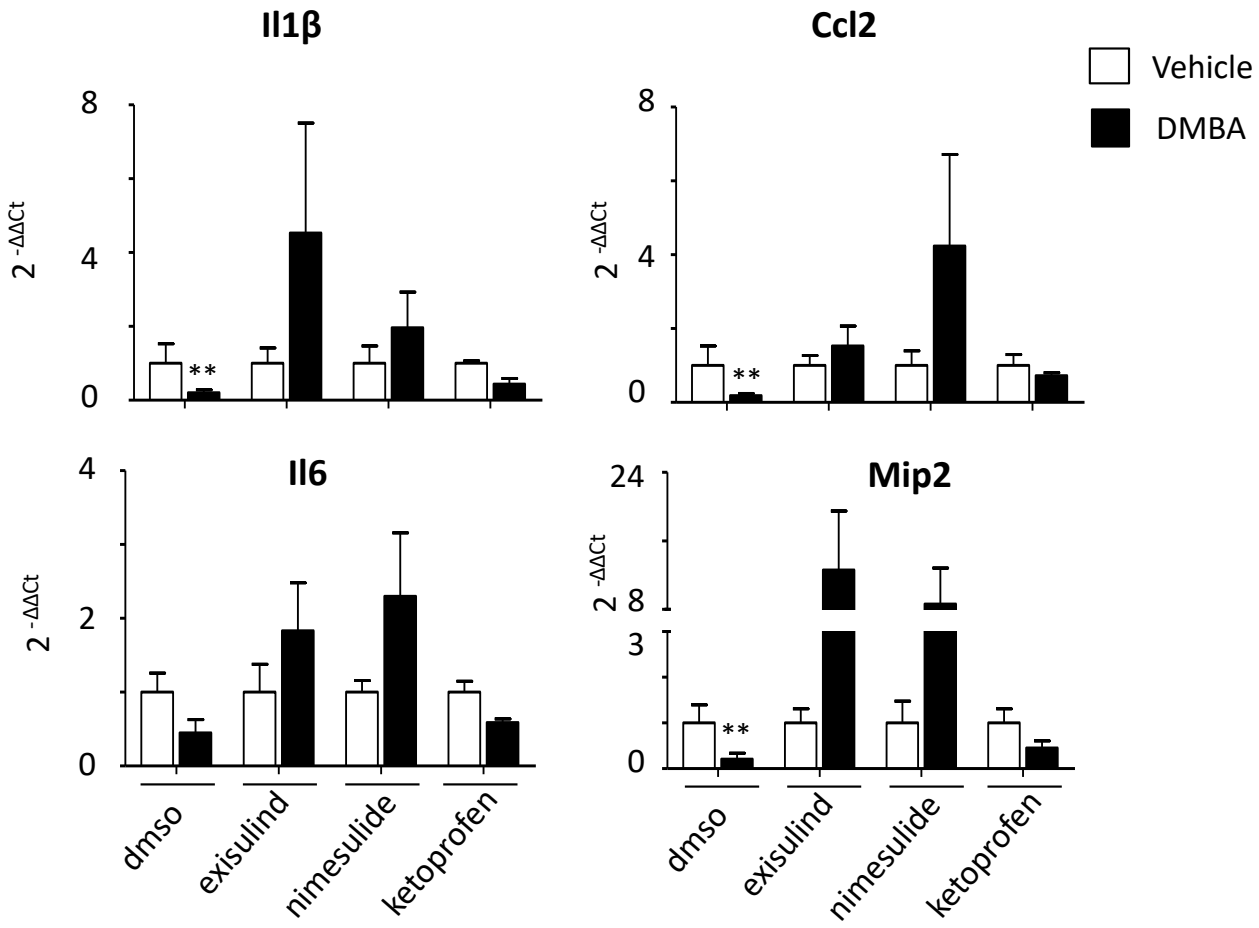


Figure 31. NSAIDs and exisulind treatment, but not ketoprofen counteract the genotoxic-induced immunosuppression. Quantification of the mRNA production of cytokines/chemokines in the mammary tissue of mice treated as reported in the text; mRNA level of expression of immune response markers (IL1β, CCL2, MIP2 and IL6) obtained by qPCR; bars in the graph are the average ± SEM values (quantified with the $2^{-\Delta\Delta Ct}$ method) of the relative amount of each specific mRNA. ** $P<0.01$ mammary gland treated with DMBA *versus* contralateral glands treated with vehicle; *P* values were calculated by Student's *t*-test.

In line with the previous literature data, qPCR demonstrated that DMBA was significantly decreasing the mRNA levels of several cytokines and chemokines involved in the immune response, including Il1β, Il6, CCL2, MIP2, mRNAs (Figure 30). Treatments with nimesulide and exisulind, but not with the vehicle (DMSO) and ketoprofen were efficiently counteracting this inhibitory effect of DMBA on the chemokine/cytokine expression (Figure 31), suggesting that SIRT1 inhibition was able to contrast the immunosuppression caused by the genotoxic injection. The effect of NSAIDs and exisulind on cytokine/chemokine expression could be explained, at least in part, by the reported SIRT1 ability to inhibit the NFkB-mediated transcription[151].

Hypoxia

Angiogenesis is one of the most relevant hallmarks of cancer and it is deregulated during early phases of breast cancer growth. Increasing evidence suggesting that NSAIDs treatment and consequently tumor cell death[92] was due to the inhibition of angiogenesis. However, molecular mechanism responsible for antiangiogenic actions of NSAIDs has not been defined. A major initiator of angiogenesis is hypoxia, which induces the activation of HIF1α. For these reasons we were asking if NSAIDs could have a role in hypoxia inducible response. Furthermore, SIRT1 inhibition impairs the transcriptional activity of HIF1α under hypoxia conditions[170] and it is induced by HIF1α in hypoxia, producing a positive feedback loop[171]. Overall these studies suggested the hypothesis that NSAIDs could influence hypoxia through SIRT1 modulation and HIF1α activity.

For this reason, I spent three months in the laboratory of Professor Adrian Harris at WIMM Department of Molecular Medicine, University of Oxford. His lab has a very long experience in hypoxia and breast cancer biology, thus I joined that lab to elucidate the interplay among NSAIDs, SIRT1 and HIF1α in breast cancer cell lines. As first experiment, I studied the NSAIDs effect on HIF1α target genes in breast cancer cell lines during hypoxia. Human breast carcinoma cells MCF7 ERα$^+$ and MDAMB-231 triple negative cell lines were cultivated for 24 hours in normoxia (21% oxygen) or hypoxia (1% oxygen) atmosphere[172] using an *in vivo* Hypoxia Work Station (Ruskinn Technology).

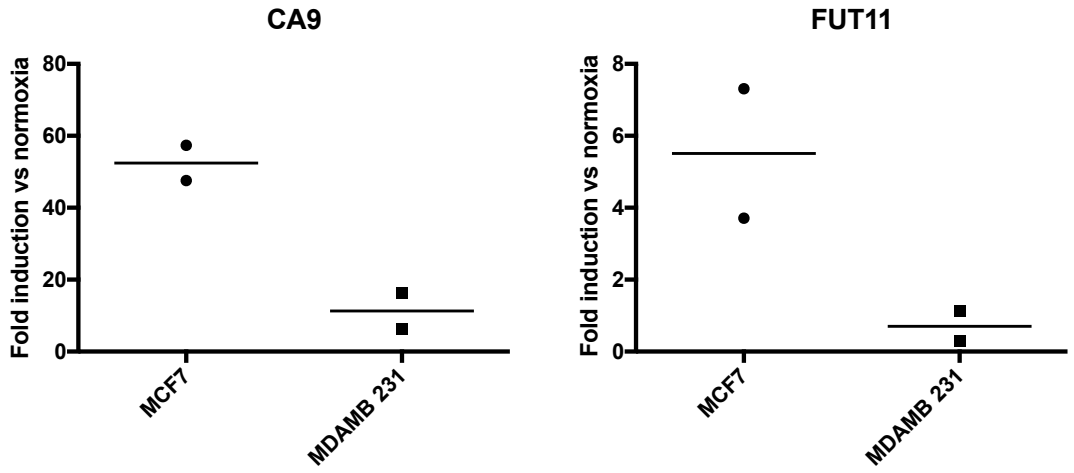


Figure 32. Hypoxia response in MCF7 and MDAB231 breast cancer cell lines. MCF7 and MDAMB-231 breast cancer cell lines were cultivated for 24 hours in either normoxia (21% oxygen) or hypoxia (0.1%

oxygen) atmosphere[172] using an *in vivo* Hypoxia Work Station (Ruskinn Technology). mRNA level of CA9 and FUT11 hypoxia responsible genes was obtained by qPCR; lines in the graph represent the average ± two biological replicates. Values were quantified with the $2^{-\Delta\Delta Ct}$ method and they are normalized on normoxia values.

Results from Figure 32 indicated that in both breast cancer cell lines, 24 hours at 0.1% oxygen induced hypoxia response, with different intensity between the two cell lines. MDAMB-231 seemed to be less activated than MFC7, a different response reflecting the more aggressive phenotype of the triple negative breast cancer cells that were shown to have the hypoxia genes already induced at the normal growth conditions[173]. Then I started to study the NSAIDs effect on hypoxia inducible response, I treated the two cell lines for 24 hours with increasing concentration of nimesulide and ketorolac in normoxia and hypoxia conditions.

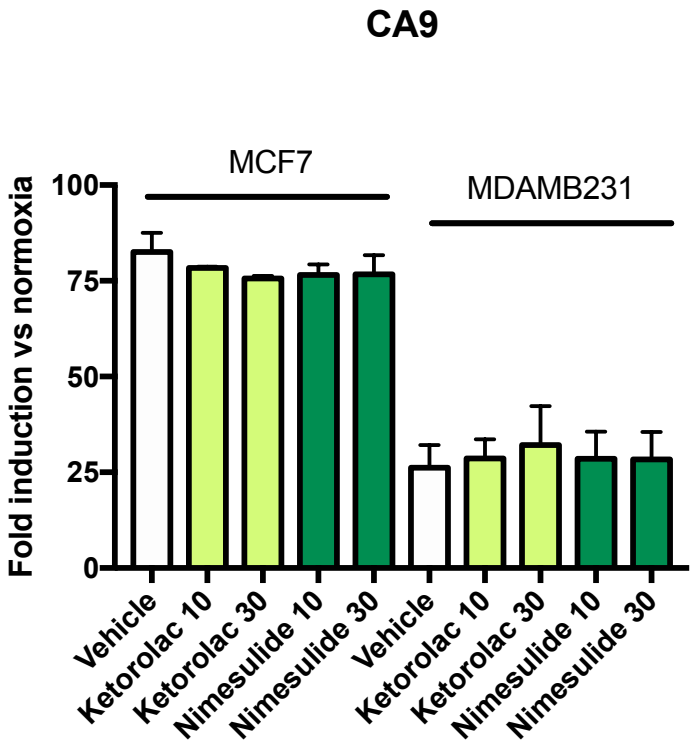


Figure 33. NSAIDs treatment and hypoxia response in MCF7 and MDAB231 breast cancer cell lines. MCF7 and MDAMB231 breast cancer cell lines were cultivated for 24 hours in either normoxia (21% oxygen) or hypoxia (0.1% oxygen) atmosphere and treated with 0, 10, 30 μM of nimesulide and ketorolac, doses in the same range of human plasma level[174]. mRNA level of CA9 and FUT11 hypoxia responsible genes was obtained by qPCR; bars in the graph represent the average ± SEM. Values were quantified with the $2^{-\Delta\Delta Ct}$ method and they are normalized on normoxia values. Data are from three independent experiments.

Results showed that the samples treated with vehicle were comparable with the data obtained from the pilot experiments, confirming the different response of the two cell lines

after hypoxia (Figure 33). However, no effect was measured after treatment with NSAIDs in both cell lines (Figure 33). To test the hypothesis whether NSAIDs could modulate the hypoxia response, at post-transcriptional level, I measured the protein level of HIF1α by western blot analysis and as control of NSAIDs activity I checked the level of P53 acetylation.

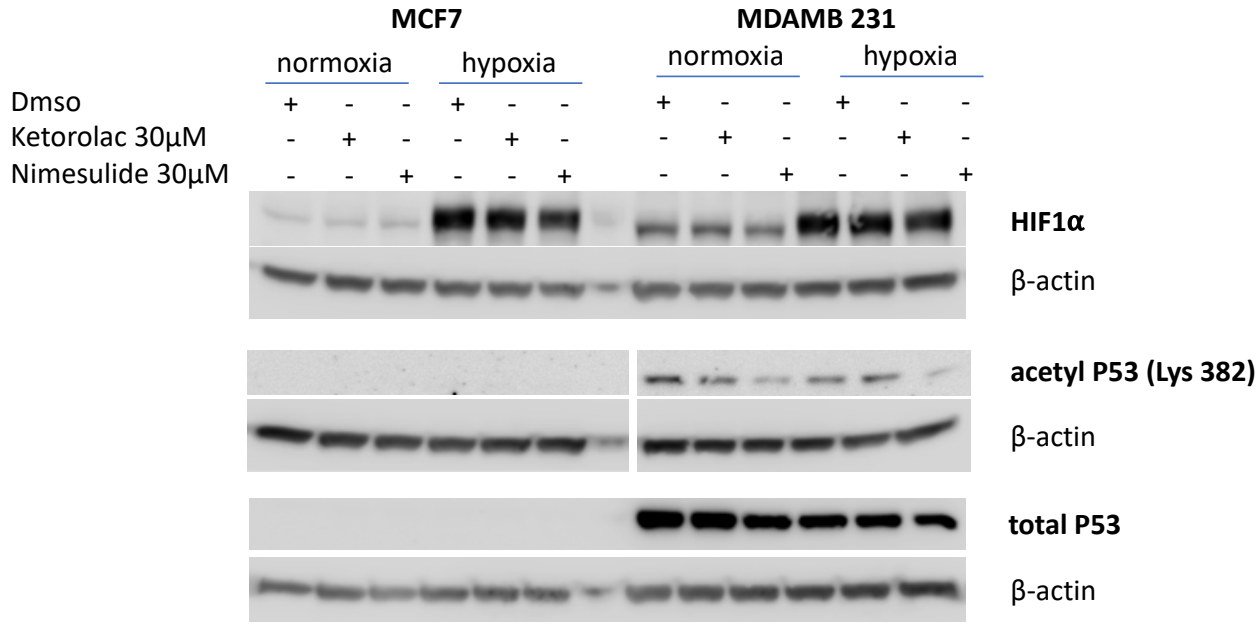


Figure 34. NSAIDs effects on hypoxia inducible response in breast cancer cell lines at post-transcriptional level. MCF7 and MDAMB-231 breast cancer cell lines were cultivated for 24 hours in either normoxia (21% oxygen) or hypoxia (0.1% oxygen) and treated with 0, 10, 30 μM of nimesulide and ketorolac, doses in the same range of human plasma level[174]. Protein level of HIF1α, acetyl P53 K382, total P53 were detected by western blot, β-actin represent loading control. Figure is representative of two independent experiments.

Data from the blot showed how HIF1α is upregulated after 24 hours of hypoxia in MCF7 and MDAMB-231 (Figure 34), however NSAIDs treatment did not modulate HIF1α expression. P53 was not detectable in MCF7, this observation was accord with the fact that in MCF7 breast cancer cell line the protein expression of P53 is very low[175]. In MDAMB-231 p53 expression was evaluable; however, p53 was not acetylated by the treatment. These negative results could have been due to several reasons: i) because of the low-concentration of the treatments, Palayoor and colleagues[176] report decreased expression of HIF1α protein after treatment with ibuprofen at 2mM, 70x than the dose used in my hypoxia experiments and I have detected p53 acetylation at higher concentration (>90uM); ii) because of the time-point, in fact the maximum effect of NSAIDs on P53 acetylation occurred after 3-6 hours (Figure 19), thus the NSAIDs effect could be lost at 24 hours in normoxia and hypoxia; iii) glucose could

influence the response during hypoxia, Harris lab experience reported that at high glucose concentration (25mM) the hypoxia response could be affected.

In order to answer to these hypotheses, I treated MDAMB-231 cell line at higher concentration of nimesulide, however I used 90 μM because is in the same range of human plasma level[174] (21 μM), in contrast with ibuprofen concentration of 2000 μM reported by Palayoor and colleagues. Furthermore, I decided to treat the cells for 9 hours according with the reported increased HIF1α expression in cancer cells after 9 hours of hypoxia[177]. Finally, the cells were treated in low glucose and high glucose condition to understand if glucose influenced the response to NSAIDs treatment in normoxia and hypoxia.

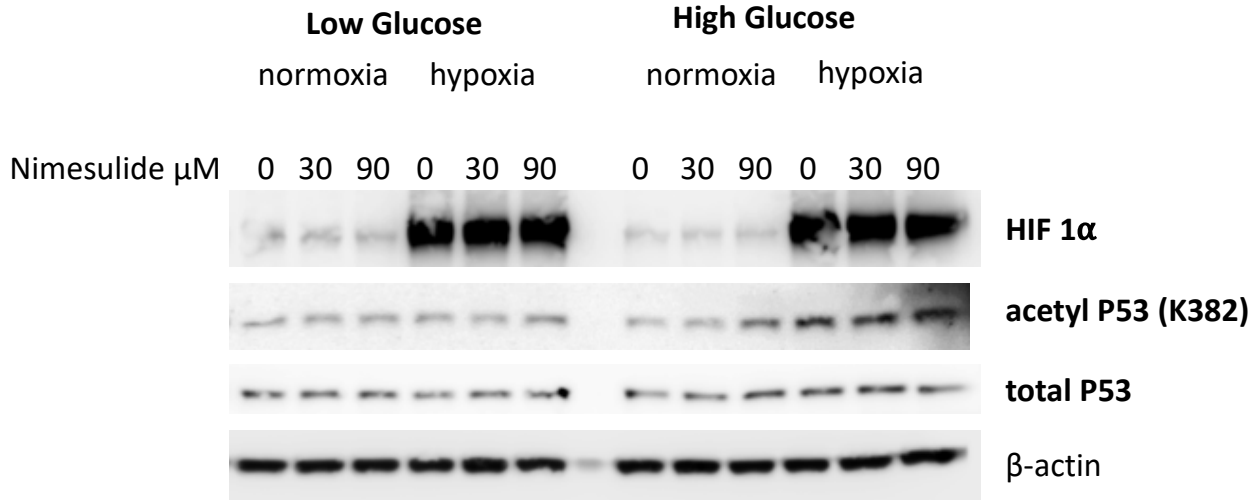


Figure 35. NSAIDs effects on hypoxia inducible response in MDAMB-231. MDAMB-231 breast cancer cell lines were cultivated in low (10mM) or high (25mM) glucose concentration for 9 hours in normoxia (21% oxygen) or hypoxia (0.1% oxygen) and treated with 0, 30, 90 μM of nimesulide. Protein level of HIF1α, acetyl P53 K382, total P53 were detected by western blot, β-actin represent loading control. Figure is representative of two independent experiments.

Results showed that in hypoxia condition nimesulide had no effect on HIF1α expression and P53 acetylation (Figure 35), however in normal condition, P53 acetylation increased in dose dependent manner after 9 hours of treatment. In particular p53 acetylation was more sensitive to nimesulide treatment under high glucose concentration (Figure 35), this effect could be explained by downregulation of SIRT1 expression in high glucose condition *in vitro*[178] and *in vivo* in diabetes rat model[179]. Thus, low expression of SIRT1 in high glucose condition could have enhanced the NSAIDs effect on SIRT1 resulting in higher acetylation of p53 after treatment with nimesulide.

All together these experiments showed that NSAIDs did not modulate the hypoxia response in breast cancer cells. However, the NSAIDs activity on angiogenesis is well described in literature, suggesting an effect of this drugs on different cell types like endothelial cells, stroma cells or inflammatory cells. In the next future this question could be unravel by studying the NSAIDs effect on tumour microenvironment, using *in vitro* and *in vivo* model.

NSAIDs mediated p53 acetylation in clinical samples

In a previous epidemiological study, Forget and collaborators suggested that an intraoperative treatment with the NSAID ketorolac was associated with a reduced relapse likelihood in the first 24 months, improved disease-free survival and overall survival in patients undergoing mastectomy[93]; interestingly, these beneficial effects were particularly relevant for high-body mass index group of patients[132]. Moreover, a recent report demonstrated the positive effects on survival of the intraoperative ketorolac also in the treatment in ovarian cancer[94]; interventional trials are underway to test the beneficial effects of this procedure. To investigate the potential clinical relevance of the mechanism described in our study, we verified whether this treatment could induce p53 acetylation at K382 site. Thanks to a collaboration with the European Institute of Oncology in Milan, the acetylation of p53 was measured in tumor samples of breast cancer patients treated with 20 mg ketorolac two hours before mastectomy.

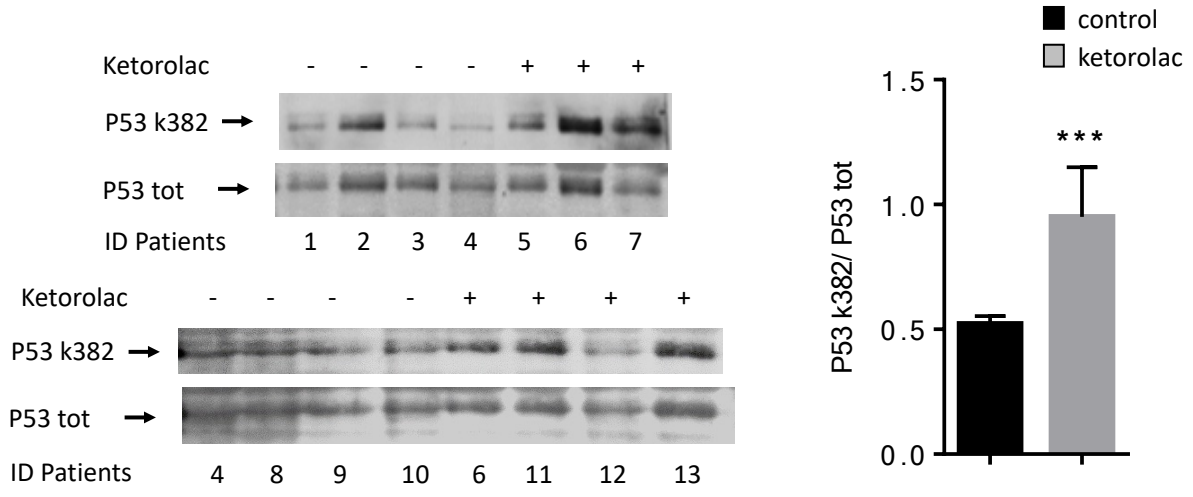


Figure 36. Intraoperative ketorolac induces p53 acetylation at the K382 site in clinical settings. Immunoblot analysis was carried out using anti-acetyl (K382) p53 and anti-total p53 antibodies and protein extracts obtained from 13 patients: six were treated with ketorolac (20 mg per *os*) 2 hours before surgery and seven with opiates for intraoperative analgesia. Bars in the graphs represents densitometry quantifications of the autoradiographic signals (acetylated p53 vs total p53); ***P< 0.001 versus the level of control; P values were calculated by Student's t-test.

The immunoblot analysis showed a significant increase of K382 acetylation in breast tumors obtained from patients (Table1 in material and methods session) treated with ketorolac as

compared to controls (opiates) indicating that SIRT1 inhibition could activate p53 by a single NSAID treatment during surgery (Figure 36). This result indicated that the NSAIDs-SIRT1-p53 pathway could be triggered in a clinical setting previously reported to have beneficial preventive effects against tumor relapse.

NEW NSAIDs DERIVATIVES FOR SIRT1 INHIBITION

During my PhD thesis we demonstrated that SIRT1 is a new molecular target for NSAIDs chemoprevention, then we would like to study the molecular interaction in order to develop new molecules that retain the ability to inhibit SIRT1 without effect on COXs. For these reasons, we collaborated with the laboratory of Computational Biochemistry and Biophysics (Ivano Eberini Group at the Department of Biomolecular and Pharmacological Sciences, University of Milan) and with the laboratory of Medicinal Chemistry (Professor Paola Conti Group at the Department of Pharmaceutical Sciences, University of Milan).

As the first step, in collaboration with Computational Biochemistry and Biophysics, we carried out a molecular docking on human SIRT1 (PDB ID: 4I5I) complexed with the NAD cofactor and EX-243 (EX-527 analog) inhibitor[180–182].

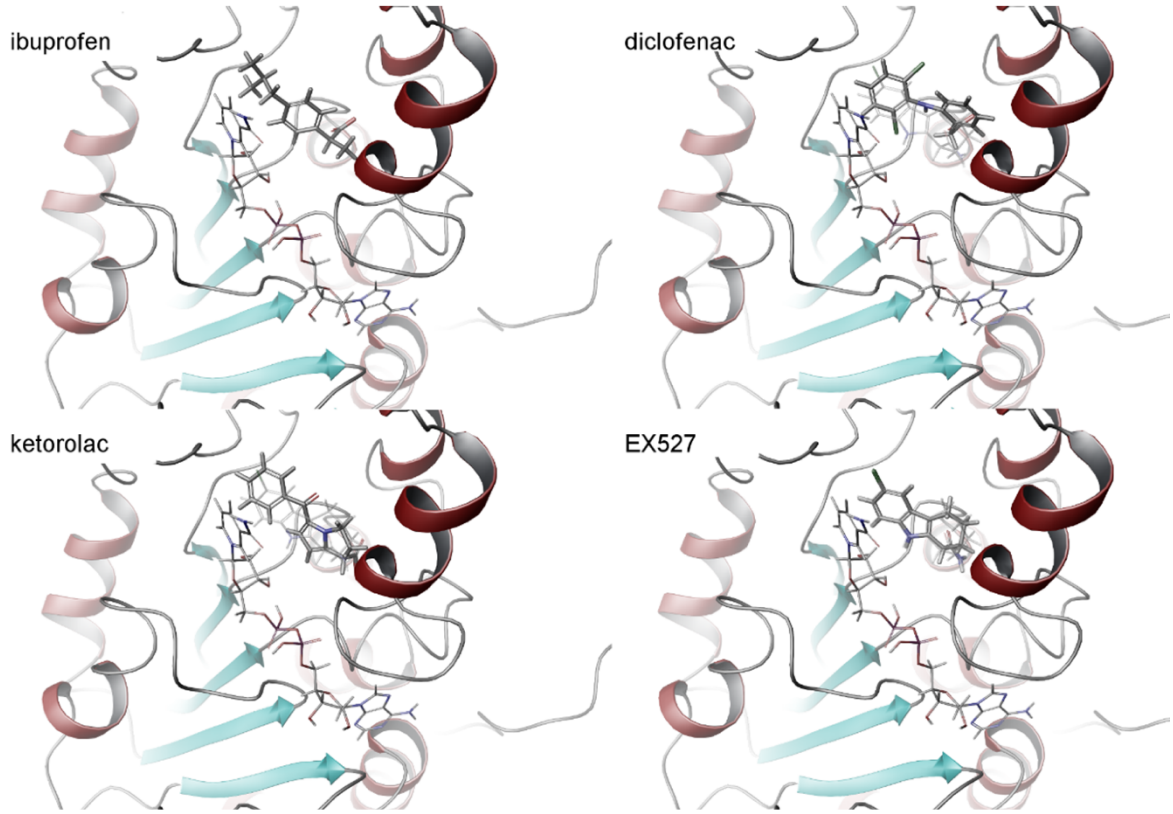


Figure 37. Best docking poses for selected SIRT-1 inhibitors. The enzyme is shown as ribbon, inhibitors are shown in stick representation. Ibuprofen, diclofenac, ketorolac and EX-527 overlap the EX-527-analog binding site.

The docking data showed that all tested compounds were able to bind the inhibitor pocket of SIRT1, most of them as EX-527 inhibitor. EX-527 inhibits SIRT1 by inducing an extended NAD

conformation and blocking the access to the channel of the acetylated lysine substrate[180]. Thus, we hypothesized a similar NAD-dependent inhibitory mechanism for the NSAIDs tested (Figure 37). In particular docking analysis for sulindac, sulindac sulfide and exisulind and energy evaluations -7.58 kcal/mol, -7.08 kcal/mol e -7.82 kcal/mol respectively, suggested that these compounds bind SIRT1 with high affinity and that exisulind is more active on SIRT1 than the others (Figure 38). Moreover, data from energy evaluation were comparable with EX-527 analog best docking conformation (-7.94 kcal/mol) (Figura 26) indicating sulindac and its metabolites as good SIRT1 inhibitors.

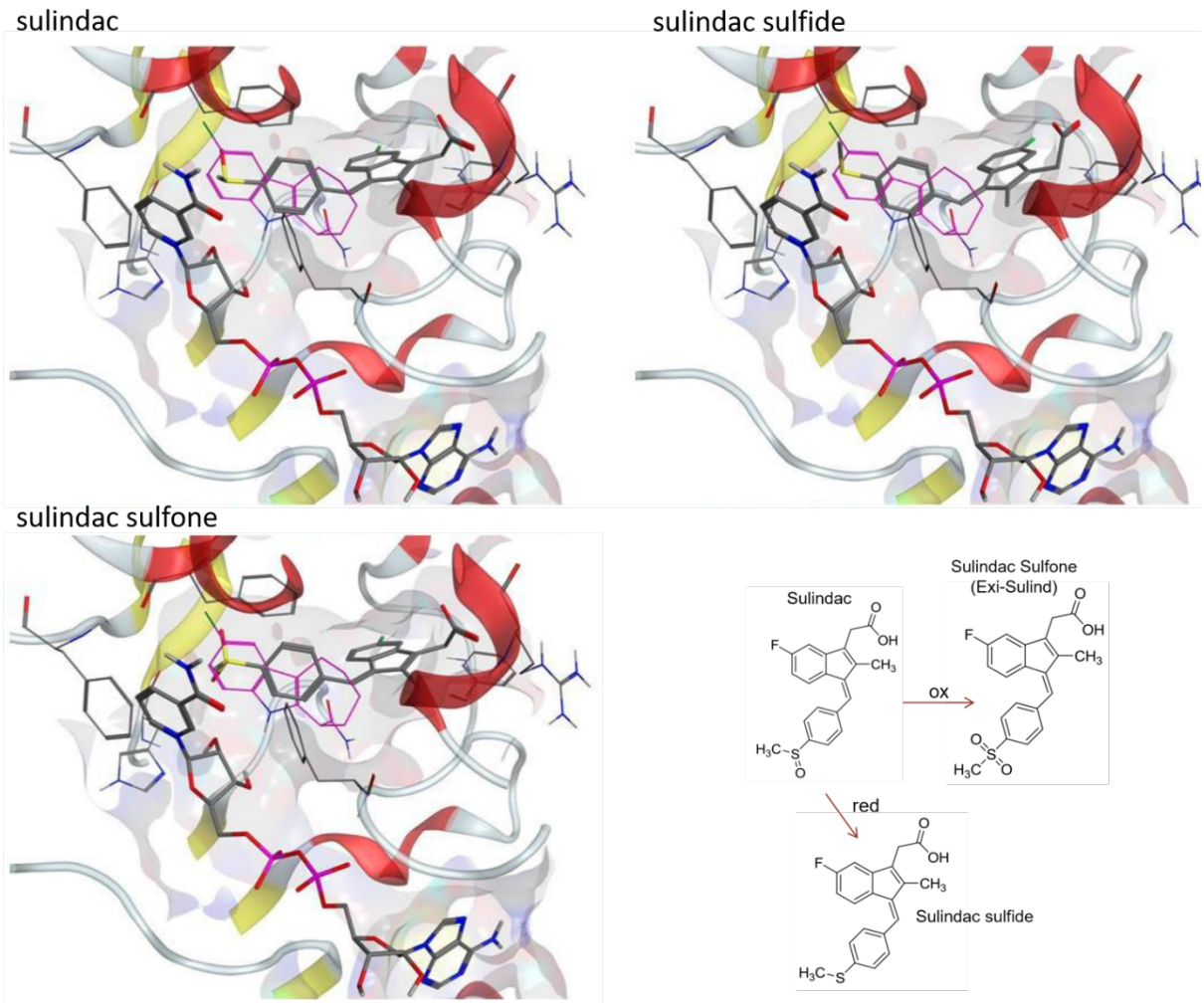


Figure 38. Best docking poses for selected SIRT-1 inhibitors. The enzyme is shown as ribbon, inhibitors are shown in stick representation, EX-527 analog in purple. Sulindac, sulindac sulfide and sulindac sulfone (exisulind) overlap the EX-527-analog binding site. Sulindac metabolites scheme on right bottom panel.

Altogether, docking data indicated that NSAIDs directly inhibit SIRT1 activity through the interaction with the NAD cleft, resulting in the stabilization of NAD non-productive conformation.

Our aim was to develop new NSAIDs like molecules that maintain SIRT1 inhibition activity without effect on cyclooxygenases, for this reason we set up a molecular docking assay between NSAIDs and COX for understanding which part of the molecular structure we can modify in order avoid the inhibitory activity of COX (Figure 39). COX1 crystal with indomethacin inhibitor has been used because COX1 is the constitutive isoform of cyclooxygenases and indomethacin is the sulindac precursor.

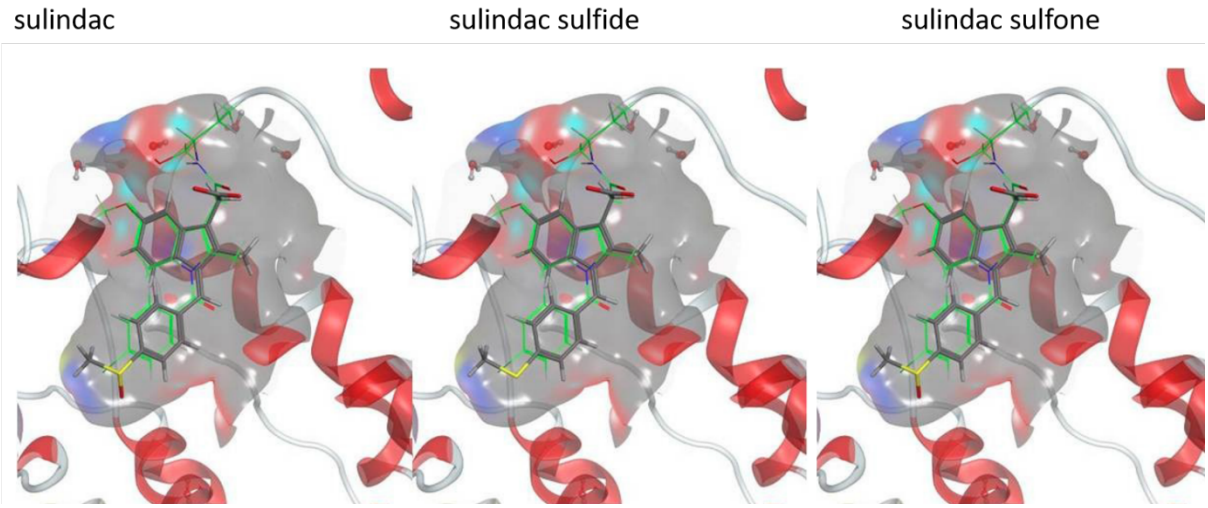


Figure 39. Best docking poses for selected COX-1 inhibitors. The enzyme is shown as ribbon, inhibitors are shown in stick representation, indomethacin in green. Sulindac, sulindac sulfide overlap the indomethacin binding site with the exception of sulindac sulfone (exisulind).

Data showed that the lowest energy poses for sulindac (-8.28 Kcal/mol) and its active metabolite sulindac sulfide (-8.03 Kcal/mol) were comparable with the best docking conformation for indomethacin indicating that, this conformation was necessary for COX1 inhibition. In contrast with these observations, sulindac sulfone molecular docking matched with a lowest energy (-5.27) than sulindac and sulindac sulfide (Figure 39). These results were in line with published evidences that indicate exisulind as inactive metabolite on COX[183].

Moreover, our collaborators suggested that a greater steric hindrance on the sulindac phenyl substituent corresponds to a reduction in the COX inhibitory activity. Thus we used these conclusions as starting point for design new NSAIDs derivatives.

Liedtke and colleagues[184] demonstrated that E-2'-des-methyl-sulindac analogs, with opposite conformation of double binding than sulindac, were able to inhibit proliferation in cancer cell line. However, the molecular mechanism of these new molecules wasn't clear yet. Interestingly *in silico* data demonstrated that the structural analogs of E-2'-des-methyl-sulindac maintained SIRT1 affinity but they interact less with COX1 than precursors (Data not shown). Thus, in collaboration with Medical Chemistry laboratory we decided to synthetize

three compounds from Liedtke library[184], which correlated with our mechanism of NSAIDs chemopreventive action.

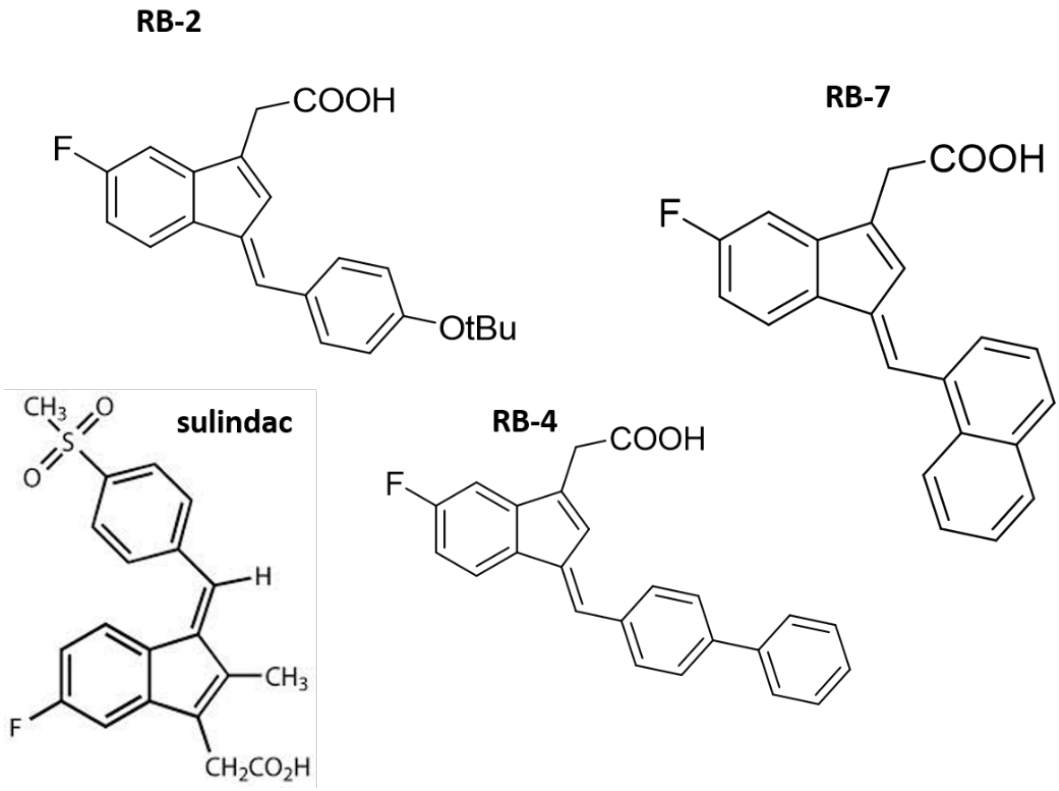


Figure 40. 2D structure of sulindac, rb2, rb4 and rb7.

From *in silico* studies these compounds should have a greater activity on SIRT1 with a decreased affinity for COX1. Thanks to the laboratory of Medicinal Chemistry we were able to test *in vitro* the effect of selected compounds on SIRT1 and COXs activity. We start with testing the activity of E-2'-des-methyl-sulindac analogs on SIRT1 through the enzymatic fluorescence assay previously used (Figure 22).

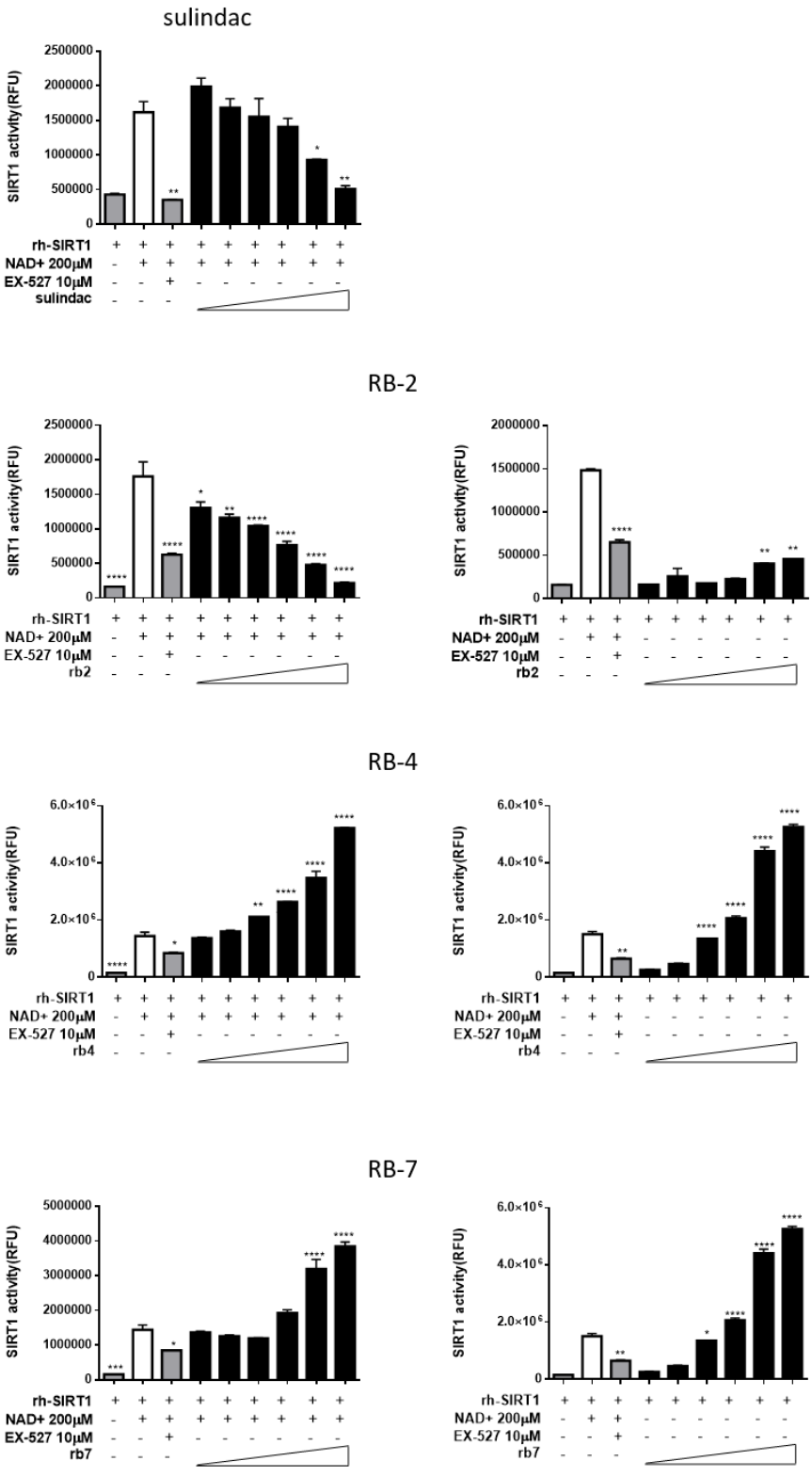


Figure 41. SIRT1 assay for sulindac, rb2, rb4, rb7. The enzymatic activity of rhSIRT1 was measured with a fluorescent assay according to the manufacturer instruction; the assay was carried out in the presence of increasing concentrations of the indicated compounds. Bars represent the average values of rhSIRT1 activity measured in three independent experiments. Statistical analysis was done using two-way ANOVA followed by Bonferroni's analysis: *P<0.05 **P<0.01 ***P<0.001, rhSIRT1 activity in the presence of NAD versus rhSIRT1 activity in the presence of NAD and of increasing concentration of the indicated compounds.

The results of these assays showed that RB2 was able to inhibit rhSIRT1 activity in a concentration dependent manner (Figure 41) and without NAD we didn't see any modulation of SIRT1 activity, in line with the *in silico* prediction. Instead we find a very interesting data from RB4 and RB7 because they were able to activate SIRT1 in the presence or absence of NAD, suggesting their possible role as acetyl acceptors. However these results were slight in contrast with *in silico* prediction.

Finally we tested the new compounds activity on COX1 and COX2. For that reason we set up *in vitro* assay allowed us to measure the activity of COXs by fluorescence (Figure 42, details on material and methods sessions). Interestingly, we find that RB2 lost the activity on COXs despite high concentration, confirming the *in silico* studies for which a greater steric hinder and E-sulindac isomer induce a reduction on COXs inhibitory activity (Figure 42). Also RB4 and RB7 showed a less inhibitory effect on COX than the precursor. Altogether COXs data validating the *in silico* predictions for the new synthetized compounds.

In conclusion, thanks to the collaboration, we design develop and synthetized new molecules that have a reduced activity on cyclooxygenases. Otherwise, differently from *in silico* evaluation we found a slight SIRT1 inhibitor (RB2) and two activators (RB4 and RB7). We are now working on the characterization of RB4 and RB7 mode of action that could mimic NAD cofactor resulting in the activation of SIRT1. If they are acetyl acceptors will be interesting to understand their implication on other sirtuins, which are involved in cancer prevention[185].

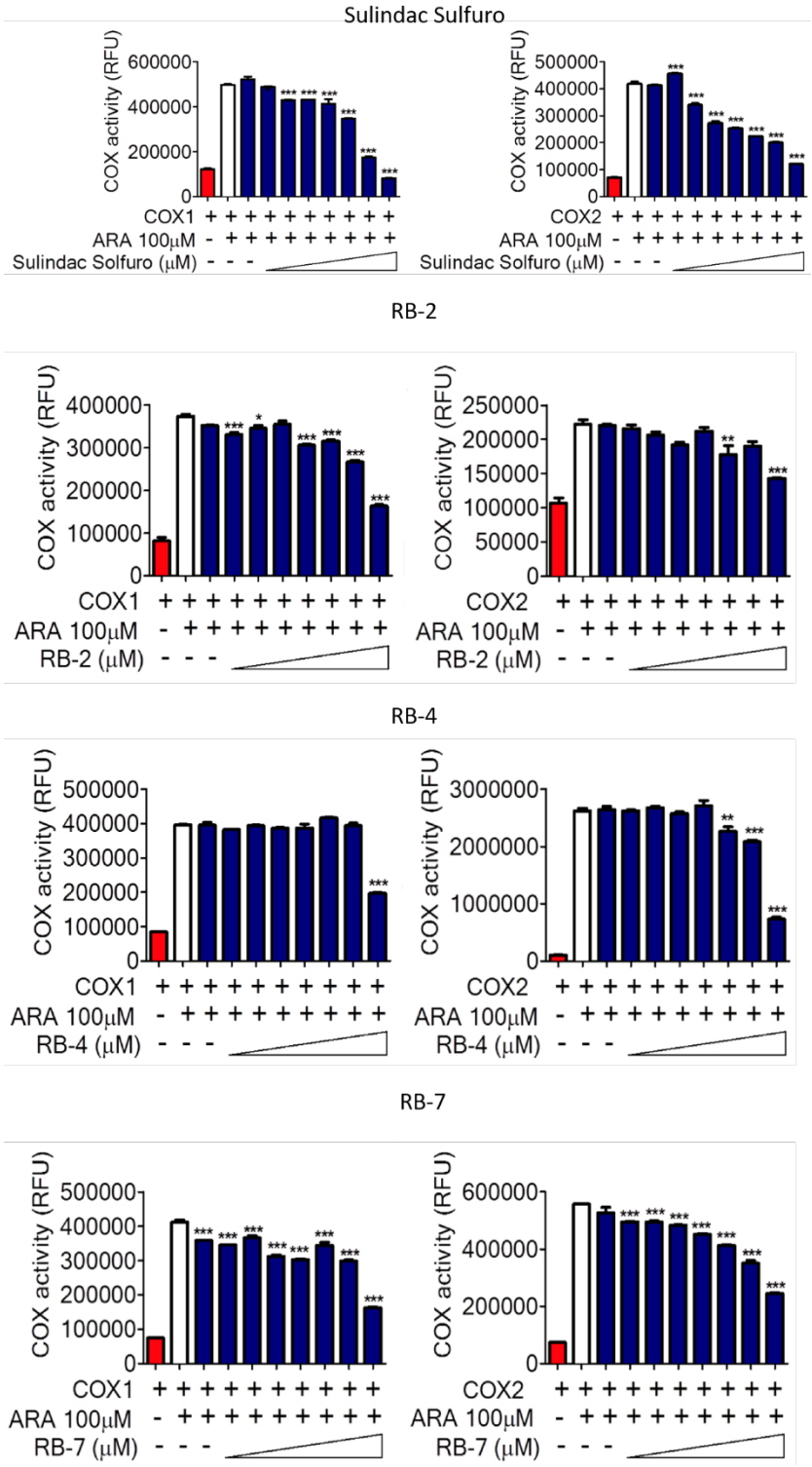


Figure 42. SIRT1 assay for sulindac solfuro, rb2, rb4, rb7. Fluorimetric assay that measure in vitro the activity of ovine recombinant COX1 and human recombinant COX2 in the presence of increasing concentration (0.0032 μM, 0.016 μM, 0.08 μM, 0.4 μM, 2 μM, 10 μM, 50 μM, 250 μM) of the indicated compounds. Bars represent the average values of recombinant enzymes activity measured in three independent experiments. Statistical analysis was done using two-way ANOVA followed by Bonferroni's analysis: *P<0.05 **P<0.01 ***P<0.001, rhSIRT1 activity in the presence of NAD versus rhSIRT1 activity in the presence of NAD and of increasing concentration of the indicated compounds.

DISCUSSION

Despite new discoveries and advances in diagnosis and therapies, cancer remains the leading cause of death in the modern age[2]. For this reason, it is essential to find novel strategies in order to decrease the health and economic burden of this disease. Epidemiologic, clinical and experimental studies point to non steroidal anti-inflammatory drugs as promising chemopreventive agents for many cancer types[86,87]. However, their use in chronic treatment is limited by side effects mainly due to COX inhibition (Dell'Omo 2018 under revision). The molecular and cellular mechanisms involved in the chemopreventive activity of NSAIDs are complex and likely include multiple effects on cancer cells and their microenvironment[86]. Thus, the identification of a master mediator of their antitumor properties will provide a strong starting point for the development of safer and more efficacious molecules, such as NSAIDs derivatives or new drugs.

My PhD project was focused on the identification of a new target for NSAIDs action, which could provide a biochemical explanation to their chemopreventive activity.

Starting from the observation published by Alfonso and colleagues[104] showing that aspirin increased the acetylation and activation of the onco-suppressor p53, we found that the ability to increase p53 acetylation in MDAMB-231 breast cancer cell line was shared by all the NSAIDs tested and by exisulind, with the only exception of ketoprofen (Figure 19 and Figure 20). However, this observation is in line with reports indicating that ketoprofen doesn't have chemopreventive activity[160]. Moreover, we demonstrate *in vitro* that NSAIDs treatment induced the expression of p21 (Figure 27), whose transcriptional upregulation by p53 results in a transient cell cycle arrest or cellular senescence[186]. Interestingly, the same pattern of activation of p53 was observed in a mouse model of chemical carcinogenesis, pointing to NSAIDs preventive effect during the initial stage of breast cancer (Figure 28B) (Dell'Omo 2018 under revision).

During my PhD we discovered a new target for NSAIDs chemoprevention: SIRT1. We showed, for the first time, that NSAIDs were able to inhibit SIRT1 activity *in vitro* (Figure 22). Moreover, through *in vitro* assays including stable transfection experiments, we demonstrate that SIRT1 is the mediator of NSAIDs effect on p53 acetylation (Figure 25 and Figure 26). Data presented in this thesis link the NSAIDs chemopreventive activity with the well-known SIRT1/p53/p21 anti-oncogenic pathway, suggesting a novel strategy for the design of tumor protective drugs. This finding supports the hypothesis that safer chemopreventive agents could be developed from NSAIDs derivatives specifically acting on SIRT1 without the COX-inhibitory activity. For

these reasons my laboratory started a collaboration with the laboratory of Computational Biochemistry and Biophysics and with the laboratory of Medicinal Chemistry at University of Milan. We are working for design and synthetize NSAIDs like molecules with improving SIRT1 inhibitory activity and reduced COX inhibition. Avoiding COXs inhibition, we are trying to nullify the side effects while preserving anti-cancer properties.

Studying the functional role of NSAIDs-mediated SIRT1 inhibition we demonstrate that in chemical murine models of cancer transformation, SIRT1 inhibition could prevent hyperproliferation and immunosuppression, two hallmarks of cancer.

In MITO-Luc model NSAIDs inhibit the hyperproliferation induced by the genotoxic agent DMBA (Figure 29 and Figure 30); this observation correlates with the increase in transcriptional activity of p53 occurring in mice treated with NSAIDs and nicotinamide, a physiological SIRT1 inhibitor now on phase III for skin cancer[187] (Figure 28B). Taken together, these data suggest that NSAID-mediated SIRT1 inhibition could be responsible for the activation of p53, with a consequent inhibition of hyperproliferation during the early phase on carcinogenesis.

We also found that, in *in vivo* model of early stage of breast cancer, pretreatment with NSAIDs increased the expression of immune-response genes (Figure 31). This suggests that a pro-inflammatory activation occurs after treatment with NSAIDs. Interestingly, this provocative observation is supported by literature, in which is reported that sulindac reduces the anti-inflammatory macrophage influx *in vivo*[169]. The NSAIDs pro-inflammatory action suggests an additional feature of NSAIDs chemopreventive activity, that could be further investigate in the next future.

Several studies demonstrate the ability of NSAIDs to inhibit[188] through a direct downregulation of pro-angiogenic factors and effectors[189,190]. However, our *in vitro* experiments revealed that NSAIDs were not able to modulate hypoxia response in MDAMB-231 breast cancer cell line (Figure 33, Figure 35), a model of an advanced stage of the disease. In addition, NSAIDs effect could be ascribed not directly to the tumour cells but to the tumour microenvironment. Thus, in the next future NSAIDs anti-angiogenic function should be studied *in vitro* on breast cancer cell line that are not fully transformed, such as DCIS like cells, and in *in vivo* models of early stage of breast cancer.

Results from clinical sample, demonstrate the ability of ketorolac to increase p53 k382 acetylation in mastectomy patients (Figure 36) suggesting a link between SIRT1 inhibition and the decreased relapses and increased survival associated with the treatment[93,132]. Thus, our results provide the rational basis for more prospective clinical trials aimed at demonstrating the beneficial effect of intraoperative ketorolac, as well as to assess the effect of the chronic nicotinamide administration to mastectomy patients. Both treatments eliciting SIRT1 inhibition with the potential of significantly improving management of breast cancer patients (Dell'Omo 2018 under revision).

In conclusion, the demonstration of a new target for NSAIDs chemoprevention provides a possible novel solution for an important medical need. In the last decades, prognostic markers characterizing populations at higher risk for several types of cancer were discovered, thus developing new preventive drugs for treating subjects at high risk of cancer is the future challenge.

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