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Ph.D. THESIS

"Valutazione delle proprietà antitumorali della capsaicina sul mesotelioma"

" Evaluation of capsaicin anticancer properties on mesothelioma"

Coordinatore: Prof. Paolo De Girolamo

Relatore: Prof. Roberto Ciarcia

Candidata: Dott.ssa Emanuela Andretta

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LIST OF ABBREVIATIONS

The list of abbreviations present in the text is indicated below in alphabetical order:

Atg: autophagy-related genes

BAP1: BRCA1-associated protein–1

CAPS: Capsaicin

CTLA-4: Cytotoxic T lymphocyte antigen-4

EPA: United States Environmental Protection Agency

EPD: extended pleurectomy decortication

EPP: extrapleural pneumonectomy

ER: endoplasmic reticulum

FBS: fetal bovine serum

FDA: U.S. Food and Drug Administration

FISH: fluorescence in situ hybridization

HMGB1: release high mobility group box 1

ICIs: immune-checkpoint inhibitors

IHC: immunohistochemistry

LC3: microtubule-associated light chain 3

MIS: mesothelioma *in situ*

MM*:* malignant mesothelioma

MMP: Matrix metalloproteinase

PARP: poly(ADP-ribose) polymerase

PD-1: Programmed cell death protein-1

PD-L1: programmed death-ligand 1

PD-L2: programmed death-ligand 2

PE: phosphatidylethanolamine

Pen/Strep: penicillin-streptomycin

PI: phosphatidylinositol

PI3K: phosphatidylinositol 3-kinases

PI3P: phosphatidyl inositol triphosphate

ROS: reactive oxygen species

SV40: simian virus 40

TNF-α: tumour necrosis factor-alpha

Vps34: vesicular protein sorting 34

WHO: World Health Organisation

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ABSTRACT

Background: Mesothelioma is an aggressive cancer with limited treatment options. Capsaicin (CAPS) is a compound known for its antitumor properties on several cancer lines. Anywaway, the effect of CAPS on proliferation and migration of mesothelioma cells needs to be investigated.

Methods: The effect of CAPS on the proliferation of several mesothelioma cells, representative of the main subtypes of mesothelioma, was evaluated by MTS assay. The *ratio* of phosphorylated and total form of both AKT and ERK1/2 and the levels of markers involved in apoptosis and autophagy were evaluated by western blotting. Furthermore, wound healing assays and transwell migration assays were performed to assess the anti-migration action of CAPS on different mesothelioma cell lines.

Results: In the present study we demonstrate that CAPS inhibits cell growth of both parental and cisplatin-resistant mesothelioma cells. CAPS exerts an antiproliferative action by disrupting the cell cycle and inducing S-phase arrest and reduces lateral motility and migration in various mesothelioma cell lines. Furthermore, CAPS treatment suppressed AKT and ERK1/2 activation in both MSTO-211H and NCI-H2052 cells. Preliminary results show that CAPS can impair the expression of markers involved in the autophagy process, such as LC3A/B II/I *ratio* and Beclin-1 level.

Conclusions: Our results demonstrate the antiproliferative and anti-migratory effect of CAPS on various mesothelioma cells. Since CAPS also reduces the proliferation of cisplatin-resistant mesothelioma cells, CAPS may represent a promising strategy to overcome drug-resistance and increase drug response in mesothelioma cells. Given the encouraging results obtained from this work, further studies are needed to evaluate the possible use of CAPS in the treatment of mesothelioma.

Keywords: Mesothelioma, Capsaicin, AKT, ERK1/2, migration, proliferation, cell cycle, cisplatin-resistance, autophagy.

1. INTRODUCTION

1.1 MESOTHELIOMA

Mesothelioma is a very aggressive cancer arising from the mesothelium which lines several body cavities, such as the peritoneum, pericardium and pleura (1).

Pleural mesothelioma is the most frequently encountered mesotelioma subtype in patients (73-85%). It affects males more than females with a 5:1 ratio, respectively. Its prevalence increases with age and the diagnosis mostly occurs in individuals over 65 years of age (2). However, mesothelioma has been found in an increasing number of young patients bearing germline mutations in BRCA1-associated protein 1 (*BAP1*) or other tumour suppressors genes (3).

In veterinary field, mesothelioma is a rare tumour that was reported in dogs (4), cats (5), cattle (6,7) horses (8), boars (9), deers (10) and lions (11). In dogs, some works have shown that the *Bouvier des Flandres* and *Golden Retriever* are the breeds most susceptible to the development of mesothelioma (12,13), while other studies have proven no correlation between sex or breed and increased risk of developping mesothelioma (14). In urban dogs with mesothelioma, the mean age of disease occurance was 10 years (14). According to records obtained at the Teaching Hospital of the University of California-Davis, mesothelioma in dogs affected the pericardium and pleura alone in 35% and 26% of cases, respectively, while 18% of dogs had both pleural and pericardial mesothelioma. In addition, in 9% of cases, mesothelioma was diagnosed in the peritoneum, whereas only 3% of dogs showed its presence in all three serous cavities. Finally, mesothelioma involved the scrotum in 9% of the analyzed dogs (15).

1.2 CAUSES OF MESOTHELIOMA

1.2.1 ASBESTOS AND MESOTHELIOMA

Several works demonstrated the strong correlation between mesothelioma occurrance and exposure to asbestos in both humans and animals (11,16–18).

Asbestos represents a group of natural fibrous silicates belonging to the amphibole (tremolite, actinolite, crocidolite, anthophyllite, and amosite) and serpentine (chrysotile) categories, which are characterized by a marked resistance to heat and traction. Because of these properties, asbestos has been used for years in construction and in the production of fireproof textiles (19). Based on their size, asbestos fibers can be divided into two types: long and short. Long asbestos fibers have a diameter up to 3 μm and a length ≥ 5 μm (20,21). Although long asbestos fibers are more dangerous to human health (21) than short ones (22), the detection of short asbestos fibers in the air can represent an optimal marker of the degradation of asbestos-containing products (20).

The main dangerous route of exposure to asbestos is through inhalation of dust containing asbestos fibers (23). In humans, exposure can be directly related to work occupation or occurs in domestic environments by handling contaminated clothing (24–26). Workers mostly exposed to asbestos are: miners, sailors, insulators, plumbers and workers employed in construction industries or refineries (27–31). According to the analysis of mesothelioma registries and the studies conducted in several countries, about 60%-85% of human patients diagnosed with mesothelioma had an occupational exposure to asbestos (32–38). The development of pleural mesothelioma can occur with a latency time from asbestos exposure ranging from 15 to 72 years (39).

In addition, geological soil characteristics such as the presence of naturally asbestos-containing minerals, urbanisation and the persistent presence of asbestos in buildings are also factors that promote environmental asbestos exposure (40).

In animals, mesothelioma has been often found in association with the presence of asbestos fibers detected in tissues or within the materials of the animals' shelter or soil where the animals live (9,11,18). Several works highlighted the importance of using animals as sentinel organisms to carry out epidemiologic studies and evalute the relation between asbestos exposure and risk of mesothelioma occurrance. In this context, wild animals, such as boar, can be helpful to evaluate environmental exposure to asbestos and its presence in the soil (9), while pet dogs represent a good system to monitor domestic and environmental exposure to asbestos (12).

Indeed, pet dogs having owners with an occupational exposure to asbestos exhibited an increased risk of developing mesothelioma (12).

In addition to the inhalation route, food consuption could represent another crucial source of asbestos exposure. Indeed, since asbestos fibers have been found in some fish, water and aquatic fauna control procedures should be essential to avoid a potential source of asbestos exposure through ingestion (41).

Around the world, 67 countries have banned the use of asbestos, but unfortunately in some countries the production, processing or import of asbestos-containing products is still permetted (42). In this regard, in April 2022, the *United States Environmental Protection Agency (EPA)* proposed to ban the use of asbestos, in particular chrysolite imported into the United States, to protect the health of workers exposed to asbestos fibers from an increased risk of cancer (43). The molecular mechanisms underlying asbestos-promoted mesothelioma involve the increase of inflammation and reactive oxygen species (ROS) levels in mesothelium cells (16,17).

- ***Inflammation and asbestos fibers:***

Asbestos is known to induce necrosis in mesothelial cells, which in turn release high mobility group box 1 (HMGB1) into the extracellular compartment. HMGB1 recruits macrophages that initiate the inflammatory process, increasing the levels of tumour necrosis factor-alpha (TNF-α) and pro-inflammatory cytokines. However, persistent high levels of HMGB1 allow mesothelial cells to overcome necrosis, through the induction of autophagy, thereby becoming transformated cells. (46).

- ***ROS levels and absestos fibers:***

One of the main mechanisms underlying asbestos fiber-induced carcinogenicity is increased oxidative stress. In fact, asbestos fibers, in the presence or absence of iron, can increase ROS levels in mesothelial cells, resulting in DNA damage (44,47). Indeed, exposure to crocidolite induces ROS-induced DNA fragmentation and activation of cell apoptosis in rat pleural mesothelium cells (48). After exposure to asbestos, ROS levels can be increased

either by the presence of “asbestos bodies” in tissues or through an indirect mechanism involving phagocytosis of asbestos fibers (44,49).
In this regard, coating crocidolite fibres with vitronectin, to promote phagocytosis of asbestos fibers in mesothelial cells, results in a further increase of intracellular ROS, DNA breakage, and leads to apoptosis and cell cycle arrest in G2-M. As a proof of concept, the inhibition of crocidolite uptake reduces the formation of crocidolite-induced DNA breaks and the rate of apoptosis (50).

1.2.2 NON-ASBESTOS CAUSES OF MESOTHELIOMA

Some studies have highlighted additional factors, which might contribute to the development of mesothelioma. In particular, the potential role of simian virus 40 (SV40) infection and exposure to various mineral fibers, such as erionite, fluoro-edenite, carbon nanotubes, in the development of mesothelioma has been discussed in the literature. In addition, the presence of germline mutations of BRCA1-associated protein–1 (*BAP1*) gene are also related to a syndrome that predisposes to the onset of several cancers, including mesothelioma (51).

- ***Mineral fibers other than asbestos***

Eryonite belongs to the zeolite family and shows physical properties similar to some minerals belonging to the amphibole category of asbestos. The carcinogenic action of erionite in the development of mesothelioma has been proven in studies in animal models (52). In addition, in regions where the presence of erionite was high, significant amounts of erionite fibers was found in the lungs of subjects with mesothelioma (51).

Fluoro-edenite is a non-asbestos mineral fiber with morphological characteristics similar to those of asbestos actinolite and tremolite fibers. It is considered as a carcinogen belonging to Group 1 by the International Agency for Research on Cancer. In fact, fluro-edenite induces DNA damage and the production of elevated ROS levels. In addition, exposure to fluoro-edenite promotes the development of mesothelioma in laboratory animals (51).

Carbon tubes, consisting of graphene cylinders, are widely used in industry. In laboratory animals their exposure was associated with the development of mesotelioma, but there is no evidence of the carcinogenic effect of carbon tubes on humans, and further studies are needed to assess the existence of a possible correlation between carbon tube exposure and mesothelioma (51).

- ***SV40 virus***

SV40 is a DNA polyomavirus, which infects the Asian macaqua monkey. Between 1954 and 1963, there was an important case of human infection with SV40 virus following the administration of vaccines with live and attenuated viruses produced from infected monkey cells.

SV40 is a virus able to induce transformation in cell cultures and promote tumor formation in animal models. T-antigen is a protein encoded by the viral genome known for its onogenic action to reduce the activation of some oncosuppressors such as retinoblastoma and p53.

Several studies have shown the presence of SV40 virus T antigen in human mesothelioma samples. However, the relationship between SV40 virus exposure and mesothelioma was not established due to some inconsistent results. Thus, the role of SV40 virus infection in the development of mesothelioma remains unresolved (51).

- ***BAP1 mutations***

BAP1 gene encodes deubiquitinate hydrolase enzyme which plays a key role in various cellular processes, such as DNA damage repair and cell cycle control. BAP1 protein acts as a tumor suppressor. Germline mutations of *BAP1* gene with an autosomal dominant inheritance have been associated with increased risk of developing mesothelioma in individuals carrying the *BAP1* mutations and their families.

Given the tumor suppressor role of *BAP1*, germline mutations in this gene can determine a hereditary syndrome that predisposes to the onset of cutaneous and uveal melanomas, clear cell renal cancer and mesotelioma (51).

1.3 CLASSIFICATION OF MESOTHELIOMA

Pleural mesothelioma is classified into three major histological subtypes: epithelioid, sarcomatoid and biphasic (53), collectively indicated as diffuse mesothelioma. Sarcomatoid and biphasic subtypes are commonly associated with worse prognosis than the epithelioid subtype (54).

In 2021 WHO introduced "mesothelioma *in situ*" (MIS) into the classification of pleural tumors. In addition, the prefix "malignant" was removed both from localized and diffuse mesotheliomas (55). The 2021 WHO classification of pleural tumors indicated the genes that are commonly impaired in mesothelioma, such as *BAP1*, *CDKN2A*, *NF2*, *TP53*, *SETD2*, and *SETDB1*, and defined the importance of immunohistochemistry (IHC) techniques to discriminate mesothelioma from a benign mesothelial proliferation (55).

- ***Mesothelioma in situ*" (MIS)**

MIS appears as a single layer of predominantly cuboid-shaped cells with inconspicuous nucleoli, located in the pleura. Its constituent cells exhibit an homozygous deletion of the *CDKN2A* gene identified by fluorescence in situ hybridization (FISH), or a loss of BAP1 and MTAP protein espression by IHC analysis. The diagnosis of MIS is based on the complete absence of invasive mesothelioma assessed by direct observation of the pleura or the use of imaging associated with pleura biopsy (55).

- ***Diffuse mesotelioma***

Epithelioid mesothelioma represents 80% of pleural mesotheliomas. It is characterized by the presence of round or polygonal epithelioid cells organized in a cuboidal cell layer. These cells infiltrate the pleura with a growth pattern that can be tubulo-papillary, trabecular, solid, micropapillary, adenomatoid, or microcystic (54). The above-mentioned WHO classification introduced the staging of epithelioid mesothelioma, based on the evaluation of mitotic rate, nuclear features, and the absence or presence of necrosis (55). In addition, further evaluation criteria introduced by this classification regarded pleomorphic

mesothelioma. Pleomorphic mesotelioma, composed of multinucleated giant cells and/or anaplastic tumor cells with prominent nuclei, has been recently classified as epithelioid, sarcomatoid, or biphasic depending on the morphological characteristic of coexisting cells (55).

Sarcomatoid mesothelioma is the second most common subtype of mesothelioma. It is characterized by spindle cells, organized in fascicles or haphazard patterns, able to invade lung parenchyma and/or adipose tissue. The presence of atypical mitoses is often observed in this subtype of mesothelioma (53). The diagnosis of sarcomatoid mesothelioma may result challenging especially when it is characterized by spindle cells with minimal atypia found, without a precise pattern, within a hyalinized stroma. This further variant is called desmoplastic mesothelioma (53). In this case, histologic identification of areas clearly typical of a sarcomatoid subtype as well as the detection of homozygous mutations in *CDKN2A* gene or the reduced expression of MTAP are particularly helpful to distinguish this variant from benign mesothelial tumors (54).

Biphasic mesothelioma is characterized by both epithelioid and sarcomatoid components. According to WHO classification, the presence of each component can be < 10% in small biopsies (55). However, it is recommended to indicate in histological reports the percentage of the sarcomatoid subtype and the eventual presence of transitional components in the epithelial subtype, since they are related to poor prognosis (54). The evaluation of cytokeratin expression is an important tool for determining the amount of sarcomatoid cells present in the specimen, as cytokeratin staining emphasizes spindle cell morphology. In cases of clinical doubt, IHC for BAP1 and the assessment of the presence of homozygous deletions in *CDKN2A* gene allow an appropriate distinction between benign or malignant spindle cell proliferation (53).

1.4 CURRENT THERAPEUTIC STRATEGIES FOR MESOTHELIOMA AND MAIN CHALLENGES

1.4.1 SURGERY

For human pleural mesotelioma patients, surgical strategies aim at either maximal macroscopic removal, such as extrapleural pneumonectomy (EPP), extended pleurectomy decortication (EPD), or palliative procedures such as partial pleurectomy or pleurectomy/decortication. According to the guidelines established by the European Respiratory Society/European Society of Thoracic Surgeons/European Association for Cardio-Thoracic Surgery/European Society for Radiotherapy and Oncology, the use of the EPD should be preferred to EPP, since the latter has been associated with a greater invasiveness and a higher post-operative mortality rate compared to EPD. Surgical procedures are only used in early stage patients and are not recommended for sarcomatoid mesothelioma (56). Instead, for patients with peritoneal mesothelioma, radical surgery is not supported by accepted guidelines. In contrast, cytoreduction surgery is commonly used as a therapeutic approach in combination with perioperative hyperthermic chemotherapy, which is based on intraperitoneal administration of a prewarmed mixture of chemotherapeutic agents, such as Cisplatin Plus Doxorubicin or Cisplatin Plus Mitomycin C (57,58).

In veterinary medicine, the use of surgical approaches for mesothelioma is limited. Pericardiectomy can be conducted in dogs with pericardiac effusions associated with mesothelioma. However, the formation of pleural effusions is among the main adverse effects resulting from this surgical procedure (59,60). In this case, thoracentesis or other options can be applied to remove pleural effusions. A case report demonstrated that an innovative thoroscopic implantation of a catheter after pericardiectomy was effective in draining pleural fluid and improving quality of life in a dog with mesothelioma (61).

1.4.2 NON-SURGICAL TREATMENT

Chemotherapy represents a therapeutic approach, which can increase the survival of both human and animal mesothelioma patients.

In 2004, the FDA approved the combined use of Cisplatin and Pemetrexed for human patients with pleural mesothelioma (62). Cisplatin binds DNA, blocking therefore the mechanisms of gene replication and transcription (63). On the other hand, Pemetrexed is an antifolate drug, and its use is commonly combined with the administration of vitamins and folic acid to reduce adverse effects resulting from the reduction of folic acid and vitamin B12 (64,65). A phase 3 study proved that treatment with Cisplatin plus Pemetrexed was more effective than Cisplatin monotherapy in increasing survival time (mean survival time: 12.1 months *versus* 9.3 months, respectively) and response rate (41.3% *versus* 16.7%, respectively) to therapy in patients with pleural mesothelioma (66). However, the main adverse effects are nausea, fatigue, diarrhea, stomatitis, and myelosuppression (62). In addition, Pemetrexed causes skin rashes that can be prevented by treatment with corticosteroids (67).

Recent studies are emphasizing the importance of immunotherapy as a promising approach for mesothelioma (68). In 2020, FDA approved the combined use of two immune-checkpoint inhibitors (ICIs), such as Ipilimumab, a cytotoxic T lymphocyte antigen-4 (CTLA-4) antibody, plus Nivolumab, a programmed cell death protein-1 (PD-1) monoclonal antibody, as first-line therapy for patients with unresectable pleural mesothelioma (69).

Cancer cells may have the ability to evade the body's immune system. The cytotoxic response of T-cells is inhibited when the PD-1 receptor is bound to its ligands, such as programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2). PD-1/PD-L1 pathway plays a key role in sarcomatoid pleural mesothelioma, since the increased expression level of PD-L1 is associated with reduced survival (68,70). CTLA-4 also acts as a negative regulator of the T-cell-regulated immune response. In fact, CTLA-4 antagonizes the action of cd28 by competing in the binding between cd28 and its ligands. In this way, CTLA-4 inhibits cd28-activated processes, such as T-cells proliferation and activation (71).

A phase 3 clinical trial (NCT02899299) indicated that the treatment with Nivolumab plus Ipilimumab resulted in higher overall survival than Pemetrexed plus platinum, as well as increased percentage of pleural mesothelioma patients reaching two-year survival (41% *vs* 27%, respectively). However, cardiac failure,

encephalitis, pneumonitis were fatal adverse effects in 1% of patients treated with Nivolumab plus Ipilimumab (72).

Unlike pleural mesothelioma, there are no FDA-approved therapies for peritoneal mesothelioma. Although cytoreduction surgery used in combination with perioperative hyperthermic chemotherapy is the main choice in case of patients with peritoneal mesothelioma, systemic chemotherapy can be exploited when surgery is not indicated or in case of recurrence (73). In patients with peritoneal mesothelioma, overall survival is less than 1 year in the absence of treatment, while it reaches 1 year following treatment with cisplatin combined with pemetrexed (73).

In the veterinary field, the choice of chemotherapeutic agent is strongly influenced by the type of animal to be treated and the known adverse effects after its administration. Cisplatin is not recommended in cats, as it can cause fatal pulmonary toxicity (74). In contrast, in dogs with pleural mesothelioma, intracavitary administration of Cisplatin is associated with low toxicity and complete resolution of effusions without tumor growth up over 306 days (75).
The treatment with chemotherapeutic agents, such as Cisplatin, Carboplatin and Doxorubicin, significantly increases survival in dogs with mesothelioma (76).

1.4.3 RESISTANCE TO CHEMOTHERAPY

Although chemotherapy is highly used in the treatment of mesothelioma, the onset of resistance to chemotherapeutic agents negatively affects patients' response to therapy (77).
Various studies conducted on different tissues proved a low ability of Cisplatin to penetrate deep tissues, and suggested the need for developing innovative strategies aimed at improving the delivery of cisplatin or its penetration (78–81). The limited ability of chemotherapy to penetrate through tissues could lead to the onset of drug resistance in various solid tumours (82).
Dysregulation of miRNAs expression levels could affect the response to platinum treatment (83). Furthermore, the presence of gene alterations in *BAP1* gene is

related to the development of cisplatin resistance in patients with pleural mesothelioma (84).

2. CAPSAICIN

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) (CAPS) (Figure 1) is the main phytochemical compound responsible for the spicy flavour of chilli peppers.

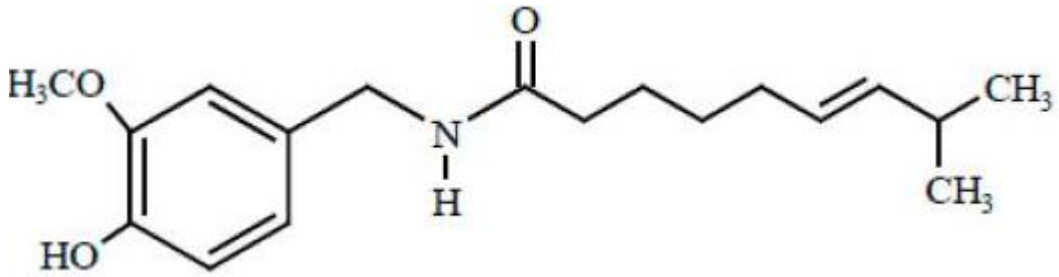

Figure 1: **The chemical structure of Capsaicin (CAPS)** (85).

Several studies proved the beneficial properties of CAPS on both human and animal health. In fact, CAPS exhibits cardio- and gastro-protective, anti-inflammatory, antibacterial, anti-viral and anticancer properties (86).

CAPS (Qutenza 8% path) was first approved by FDA in 2009 as topical treatment in patients with neuropathic pain associated with post-herpetic neuralgia (87) and in 2020 its use was indicated for neuropathic pain due to diabetic peripheral neuropathy of the feet (88). In 2017, a clinical trial (NCT02228928) demonstrated the efficacy of CAPS patch in significantly reducing peripheral neuropathic pain even at concentrations lower than those approved by the FDA. However, due to the small number of patients enrolled in this trial, further studies are essential to confirm the effects of lower CAPS concentrations and to evaluate their use as a potential replacement for currently approved concentrations (89). In cancer patients, peripheral neuropathic pain is a common adverse effect occurring with a prevalence rate commonly ranging from 19% to over 85% after treatment with chemotherapeutic agents, such as cisplatin, oxaliplatin and paclitaxel (90–93). Encouraging results have been associated with the use of CAPS patches in patients with oxaliplatin-induced peripheral neuropathy (94) and, currently, a phase II clinical trial (NCT03317613) is on going to evaluate the efficacy and the

safety of 8% CAPS patch on the painful zones in cancer patients with neuropathic pain.

2.1 ANTICANCER ACTION OF CAPSAICIN

CAPS exhibits antiproliferative effect against several cancers, such as melanoma; leukemia; breast, bladder, colon and prostate cancer cells (95–100). A phase 2 clinical trial is ongoing to evaluate the effect of CAPS, administered as a food additive, as chemopreventive agent for human patients with prostate cancer (NCT02037464).

In the veterinary field, studies in animal models proved the antitumor action of CAPS on prostate adenocarcinoma and lung cancer. In addition, the dietary administration of a CAPS-containing habanero pepper extract in dogs with different types of tumors reduced the size of the tumor mass (86).

The cellular mechanisms underlying the antitumor effect of CAPS involve the alteration of cell cycle, and the induction of autophagy and apoptosis in various cancer cell lines (101,102).

2.1.1 CAPSAICIN IMPAIRS CELL CYCLE IN CANCER CELLS

The cell cycle can be characterized by several phases: G1, S, G2, M. During the cell cycle, there are several checkpoints ensuring the integrity of DNA replication and promoting cell cycle blockage in the presence of DNA damage. CAPS acts in tumors by arresting cancer cell cycle at different phases, according to specific cell lines. It exhibits antiproliferative action, causing G0/G1 phase arrest in colon and bladder cancer cells and in osteosarcoma and leukemia cells (98,103–105). The arrest at this phase directly correlates with the reduction in levels of some regulators of G1-S transition, such as cyclin E, cyclin D1, CDK2, CDK4 and CDK6 that occurs following CAPS-treatment (105–108). CAPS and CAPS-like analogues can also promote cell blockade in the G2/M phase (109,110). Regarding the anticancer effect of CAPS on breast cancer cells, some works demonstrated cell cycle arrest in G0/G1 phase, while other studies proved S-phase or G2/M-phase blockade after CAPS treatment (108,111,112).

2.1.2 CAPSAICIN AND APOPTOSIS

CAPS induces apoptosis in many tumor types, such as gastric cancer (113), breast cancer (95), nasopharyngeal carcinoma (114), bladder cancer (96), urothelial cancer (115), acute lymphoblastic leukemia (99), small cell lung cancer (116), renal carcinoma (117) and hepatoma cells (118). Molecular mechanisms underlying CAPS-induced apoptosis in several cancer cells include: activation of caspases, such as caspase 3, 8 and 9 and PARP-1 cleavage; increased expression of pro-apoptotic proteins such as Bax, decreased levels of anti-apoptotic regulators such as Bcl2 and alteration of mitochondrial membrane potential (110,117,119,120).

In addition, CAPS treatment was associated with the impairment of oxidative stress balance in cancer cells. The regulation of oxidative balance plays a critical role in anticancer therapy. Tumour cells show elevated intracellular levels of ROS compared to non-tumour cells. The use of pro-oxidant agents that can further increase oxidative stress can promote cancer cell death (121). On the other hand, the lowering of ROS levels, aiming at preventing accumulation of ROS-induced DNA damage and accumulation of further mutations, represents another well known therapeutic anticancer strategy (122). While several works proved that CAPS promoted cell death by increasing oxidative stress and ROS production in various cancer cell lines, including hepatocellular carcinoma, pancreatic cancer and bladder cancer (118,123,124), other studies pointed out a reduction in ROS levels in other cancer cell lines, such as glioblastoma (125) and neuroblastoma cells (126,127) after treatment with CAPS. The dual effect of CAPS on ROS levels in cancer may be due to the different expression levels of genes coding for antioxidant or pro-oxidant factors, involved in cellular redox balance, as demonstrated by comparative proteomic studies performed on different cancer cell lines (126).

2.1.3 CAPSAICIN AND AUTHOPHAGY

Autophagy is a cellular process involved in the removal of damaged organelles, such as mitochondria, perixosomes, or endoplasmic reticulum (ER),

and misfolded proteins (128). In all autophagic processes, lysosomes, which contain lytic enzymes, play a key role in the degradation process of damaged cellular components. In chaperone-mediated autophagy, the translocation to lysosomes occurs selectively through the interaction of chaperone proteins with damaged cellular components, whereas both macro- and micro-autophagy rely on both selective and non-selective mechanisms to engulf the cellular components to be degraded (128). During microautophagy, cytoplasmic material is directly sequestered within the lysome. In contrast, macroautophagy is characterized by the formation of an intermediate structure, a double- membrane vesicle, named phagophore, which engulfs misfolded proteins and cellular organelles that need to be degraded. Then, the loaded phagophore, known as autophagosome, fuses with the lysosome, allowing degradation of the cytoplasmic material within it (129). The origin of the phagophore is not yet well known: it could develop either from the membranes of cytoplasmic structures such as the ER, the Golgi apparatus, endosomes or through a mechanism of *de* novo formation from cellular lipids (128). However, some regulators and factors underlie the process of phagophore and autophagosome formation. When vesicular protein sorting 34 (Vps34), a class III phosphatidylinositol 3-kinases (PI3K), is bound to Atg6/Beclin-1, it forms a complex that can promote phagophore formation. This complex promotes Vps34-mediated conversion of phosphatidylinositol (PI) to phosphatidyl inositol triphosphate (PIP3), which is critical for inducing phagophore enlargement (128). However, the activity of Vps34/Beclin-1 complex is strongly impaired when Bcl-2 binds to BH3 domains present on Beclin-1. In contrast, in nutrient deficiency, the activation of autophagy is promoted by Bcl-2 phosphorilation, which in turn induces the detachment of Bcl-2 from Beclin-1(130).

During macro-autophagy, microtubule-associated light chain 3 (LC3) protein, synthesized as a pre-cursor, is cleaved by autophagy-related genes (Atg) 4 protease forming LC3-I. After LC3-I activation by the E1 enzyme Atg7, LC3-I is bound to the carrier protein Atg3 and finally conjugated to phosphatidylethanolamine (PE), resulting in the formation of LC3-II (128,131). LC3-II recruitment to the phagophore promotes autophagosome formation. LC3-II in fact binds to p62/SQSTM1, an adaptor protein, that is itself linked to

damaged cellular components, resulting in selective engulfment of cytoplasmic material in the phagophore (128).

CAPS promotes autophagy in several cell lines, such as melanoma (97), glioma (132), hepatocellular carcinoma (133), nasopharyngeal carcinoma (134), breast cancer(135), renal and bladder cancer cells (136,137). Indeed, it induces autophagosome formation and increases the levels of autophagy markers, such as Beclin-1 and p62, and the cleavage of both LC3 and PARP-1 (97,132–137).

CAPS can induce both apoptosis and autophagy in different cancer cell lines (97,134,138). Anyway, the use of authophagy inhibitors promoted or increased apoptosis, suggesting autophagy as a survival mechanism used by cancer cells to counteract CAPS-induced cytotoxicity (97,132,135,137,138).

The role of autophagy is highly debated, and it exhibits a dual role since it can mediate both cell death and represent a mechanism of cell cytoprotection (139). Many anticancer drugs in fact act by inducing cell death through either apoptosis or autophagy, but the autophagic process may also underlie the onset of drug resi stance (140).

2.1.4 EFFECT OF CAPSAICIN ON CANCER CELL MIGRATION AND INVASION

CAPS reduces migration and invasion properties in several cancer cell lines, including cholangiocarcinoma, fibrosarcoma, renal, bladder and breast cancer cells (141–145).

The molecular processes underlying these antitumor properties involve the regulation of matrix metalloproteinases (MMPs), proteins with crucial roles in the process of tumor invasion, metastasis, and progression (146).

The expression of MMP9 is strongly upregulated in several tumors (146), while its level is significantly reduced following the treatment with CAPS (141,143–145).

2.1.5 EFFECT OF CAPSAICIN ON CANCER CELLS RESISTANT TO CONVENTIONAL CHEMOTHERAPEUTICS

The occurrence of drug resistance is a major problem affecting response to therapy in cancer patients.

Some researchers analyzed the effects of different natural compounds, including CAPS, on several cisplatin-resistant tumor lines, demonstrating their antitumor effect through the impairment of cell cycle. Interestingly, the natural compounds exhibited stronger action on resistant cells than the parental ones (147). In addition, the combination of CAPS with cisplatin promotes apoptosis in cisplatin-resistant gastric cancer cells (148). Furthermore, the combination of CAPS with other natural compounds can be particularly helpful in overcoming resistance of tumor cells even to chemotherapeutics other than cisplatin. In fact, the combined use of CAPS with piperine made multiresistant cells more sensitive to doxorubicin treatment (149). Therefore, CAPS represents a promising strategy to overcome the issue of drug resistance in cancer therapy.

2.1.6 CAPS AND COMBINATION WITH CONVENTIONAL DRUGS IN CANCER THERAPY

The use of CAPS in combination with chemotherapeutics represents an encouraging strategy to enhance the antitumor action exerted by conventional drugs (see Table 1)

Lipid-complexed cisplatin is the first-line therapy for patients with osteosarcoma (150,151). Low concentrations of CAPS in combination with cisplatin significantly reduce cancer cells proliferation and tumor mass size in xenograft models of osteosarcoma, thereby inducing greater antitumor response than treatment with cisplatin alone (152). Another promising example of such efficient combination is the synergistic effect of CAPS with Sorafenib, which is an approved drug for hepatocarcinoma. *In vitro* and *in vivo* studies carried out on mice xenotraplanted with hepatocellular carcinoma cells proved an even greater

antitumour effect of the combined treatment (CAPS with Sorafenib) than Sorafenib alone (153).

Table 1: The use of Capsaicin (CAPS) in combination with chemotherapeutics in different types of cancer

CAPS in combination with:	**Cancer**	**Type of study**	**Main Effect**	**Ref**
Cisplatin	osteosarcoma	*in vitro* in mice xenograft	↓proliferation ↓tumor size	(152)
Camptothecin	human small cell lung	*in vitro* xenograft in chicken chorioallantoic membrane model	↑apoptosis ↓Tumor weight	(154)
Sorafenib	Hepatocellular carcinoma	*in vitro* Xenograft mice	↓cellviability;↑apoptosis ↓Tumor weight	(153)

3. AIM

Mesothelioma is a rare cancer, mainly associated with asbestos exposure, affecting both humans and animals. Although surgery and chemotherapy increase the survival of mesothelioma patients, the prognosis is still poor. Therefore, the development of new safe pharmacological approaches able to improve the prognosis and increase survival of animal and human patients over time is a major challenge in the fight against mesothelioma.

Capsaicin (CAPS) is a compound exhibiting strong anticancer action against several types of cancer. Indeed, it negatively affects the viability and migration of several tumor lines and reduces tumor size in *in vivo* models. However, the effect of CAPS on mesothelioma cells has not been well explored. Thus, the aim of the present work was to evaluate the effect of CAPS on the cell cycle, proliferation and migration of mesothelioma cells. Furthermore, we determined the effect of CAPS on the regulation of some cellular pathways critical for proliferation and migration, such as AKT and ERK 1/2 activation.

Since the development of drug resistance negatively affects the response to oncological therapies and several works proved the antiproliferative action of CAPS on cancer cells resistant to conventional chemotherapy, a further objective of the work was to evaluate whether CAPS could reduce cell viability in cells resistant to cisplatin, chemotherapy drug of choice used in the treatment of mesothelioma.

Although *in vivo* studies will be essential to confirm the promising antitumor effect of CAPS, this study aimed to paving the way towards new pharmacological perspectives for the treatment of mesothelioma and overcoming drug resistance.

4. MATERIALS AND METHODS

4.1 CELL CULTURE

MSTO-211H (ATCC®CRL-2081), NCI-H2052 (ATCC®CRL-5915), NCI-H2452 (ATCC®CRL 5946) and NCI-H28 (ATCC®CRL-5820) mesothelioma cell lines were purchased by ATCC. All cell lines were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen/Strep).

4.2 CISPLATIN-RESISTANT CELLS LINES

Cisplatin-resistant MSTO-211H and NCI-H2052 cells were generated by treating the cells for prolonged times with increasing concentrations of cisplatin, such as 5 µM, 10 µM, 15 µM, 20 µM, 25 µM.

Following 72 h treatment with each concentration, cells were grown in drug-free medium for 10 days and then treated with next concentration of cisplatin up to 25 µM.

Selected resistant cells were maintained in RPMI-1640 medium supplemented with 20 µM cisplatin (IC50), 10% FBS, 1%Pen/Strep and 1% glutamine. Cells were kept at 37°C in a humidified atmosphere of 5% CO_2 and 95%air.

4.3 CAPSAICIN AND TREATMENT MEDIUM PREPARATION

Capsaicin (≥95%, from Capsicum sp) (M2028) was provided by Sigma-Aldrich. The powder was dissolved in Dimethyl sulfoxide (DMSO) to prepare a 200 mM stock solution. Then the stock solution was diluited in the medium to obtain the desidered CAPS concentration.

4.4 MTS

MSTO-211H, NCI-H2052, NCI-H2452 and NCI-H28 cells were seeded in 96 well plates at a density of 1000 cells/well (MSTO-211H) or 2000 cells/well (NCI-H2052, NCI-H2452 and NCI-H28). The day after the seeding, a 96-well plates were used to conduct proliferation assays on untreated cells, obtaining the absorbance at time zero. The same day, cells were treated with 5% FBS-RPMI

medium containing CAPS at concentrations ranging from 50 µM to 300 µM for 24, 48 and 72h in other 96-well plates.

To confirm that the effect of CAPS on cell proliferation was not due to a possible cytotoxicity of DMSO, vehicle control cells were treated for 24, 48 and 72 h with 5% serum-RPMI medium containing DMSO, used at the same *%v/v* as for cells treated with CAPS at maximum concentration.

Cell viability was evaluated by MTS assay (cat. no. G3582; CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Milan, Italy), following the manufacturer's instructions. The absorbance at 490nm was read with a plate reader.

To evaluate the effect of vehicle and CAPS on cell proliferation, the percentage of cell growth was calculated respect to time zero (% increase over time 0), according to the following formula: *((ABS Time X- ABS Time 0)/ABS Time 0) *100*, where ABS Time X was the absorbance read on the plate reader after 24, 48 or 72h of treatment and ABS Time 0 was the absorbance at time zero.

4.5 CYTOFLUORIMETRIC ANALYSIS OF CELL CYCLE

MSTO-211H (2.5×10^5 cells), NCI-H2052 (3×10^5 cells), NCI-H2452 (3×10^5 cells) and NCI-H28 (3×10^5 cells)cells were seeded onto 100 mm plates the day before starting the treatment. After washes in DPBS 1X, cells were treated with 5% serum-RMPI medium containing DMSO or CAPS for 24h, 48 and 72h. At the end of the treatment, cells were fixed in ice-cold 70% ethanol and stained with propidium iodide, according to the instructions of propidium iodide flow cytometry kit (Abcam, Cambridge, UK). Cell cycle analysis were carried out by FACS at the Wistar Institute Cytofluorimetry Core Facility (Philadelphia, United States).

4.6 WOUND HEALING ASSAY

A confluent monolayer of NCI-H2052, NCI-H2452 and NCI-H28 cells, seeded onto 6-well plates, was scratched with a 200µl-micropipette tip. After washes with DPBS 1X, cells were treated with DMSO or CAPS at concencentrations that did not affect cell viability. The images of woud area were

captured using a Leica microscope (4X objective) from time 0 (immediately after performing the scratch) for 24, 48 and 72 h of treatment, until the closure of the scratch. Pictures were analyzed by Wound_healing_size_tool update, an ImageJ/Fiji® plugin (155).

Wound closure rate was calculated through the following formula:

((Wound Area time 0- Wound Area time x)/Wound Area Time 0)*100). In this formula, Wound Area Time 0 represents the area of the woud after initial scratching, while Wound Area Time x representes the area of the woud at different time of treatment.

4.7 MIGRATION IN TRANSWELL ASSAY

MSTO-211H, NCI-H2052 and NCI-H28 were seeded in 300 µL SFM in the upper chamber of a 6.5 mm Transwell with 8.0 µm Pore Polyester Membrane Insert (Corning, REF 3464). Then, 700 µL of SFM (for NCI-H2052) or 5%serum-RMPI medium (for MSTO-211H and NCI-H28) were added in the lower chamber. DMSO or CAPS were introduced in both chambers of transwell. The time to allow cell migration depended on cell line: ON for NCI-H2052; 24h for NCI-H28 and 27.5h for MSTO-211H. The cells on the upper surface of the filter were removed with a cotton swab, while those on the lower surface of the filter were fixed with cold methanol, stained with Coomassie Brilliant Blue and counted under an inverted microscope (Leica, Wetzlar, Germany).

4.8 WESTERN BLOTTING

Western Blotting assay was performed to evaluate the effect of CAPS on the activation of AKT and ERK 1/2 and on the expression levels of authophagic markers.

To study the effect of CAPS on authophagy markers, treatments were performed in 5% FBS-medium. Instead, to induce the activation of AKT and ERK1/2 cells were starved in SFM for 5h and then treated for 15', 30' and 1h with RPMI medium containing 1% serum to promote cell proliferation.

After treatments cell were lysated using RIPA buffer (Termo Fisher Scientifc) supplemented with protease and phosphatase inhibitors (Termo Fisher Scientifc). Protein concentration was determined by BCA assay (Termo Fisher Scientifc).

30µg protein samples were run in polyacrylamide gel and transferred to nitrocellulose membranes. Nitrocellulose membranes were then incubated with the following primary antibodies: pAKT S473 (#4060), pan-AKT (#4691), pERK1/2 (#4370), ERK1/2 (#9102), Beclin-1 (D40C5) (#3495), LC3A/B (#4108) from Cell Signaling Technology (Danvers, MA, USA) and GAPDH (sc-365062) (Santa Cruz Biotechnology, Dallas, TX, USA). All primary antibody were used at the diluition of 1:1000. The following secondary antibodies were used: anti-rabbit HRP-linked (#7074) (Cell Signaling Technology) and m-IgGk BP-HRP antibody (Santa Cruz Biotechnology) (sc-516102) at the diluition of 1:5000.

5. RESULTS

5.1 EVALUATION OF CAPSAICIN ACTION (CAPS) ON CELL PROLIFERATION

To assess whether capsaicin inhibit proliferation of MM cells, we performed MTS assay and evaluated proliferation of several mesothelioma cell lines, representative of different subtypes of mesothelioma (Figure 2). Growth was assessed after 24, 48 and 72h of treatment. To confirm that the effect of CAPS was not affected by a possible cytotoxicity of the vehicle, cells were also treated with RPMI containing DMSO alone (DMSO) as control. We evaluated the increase in cell growth after 24, 48 and 72 hours of treatment compared to time zero and the data are expressed as % growth increase over time 0.

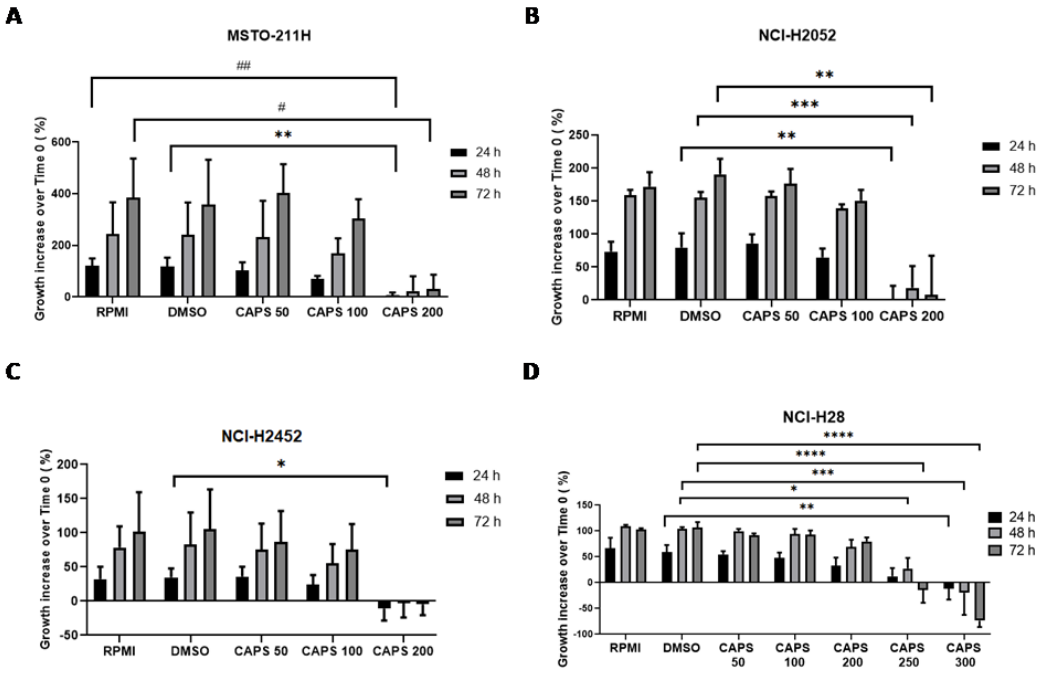

Figure 2: **The effect of Capsaicin (CAPS) on cell proliferation of parental mesothelioma cell lines.** MSTO-211H cells **(A)**, NCI-H2052 **(B)**, NCI-H2452 **(C);** NCI-H28 **(D)** cells were treated with: RPMI medium alone (RPMI); medium containing DMSO (DMSO) at the same %v/v as the maximum CAPS concentration; and various CAPS concentrations for 24, 48 and 72h. Data are expressed as percentage of growth increase over time 0 and the results are expressed as mean ± standard deviation of three indipendent experiments. Statistical differences were evaluated by one-way analysis of variance (ANOVA) ($^{*}p$-value ≤ 0.05, $^{**}p$-value < 0.01; $^{***}p$-value < 0.001; $^{****}p$-value < 0.0001 *versus* DMSO; $^{\#}p$-value ≤ 0.05 and $^{\#\#}p$-value < 0.01 *versus* RPMI).

Treatment with CAPS at the concentration of 50 µM and 100 µM did not reduce growth as compared with DMSO-treated cells in MSTO-211H, NCI-H2052 and NCI-H2452 cell lines. However, treatment with 200 µM CAPS significantly reduced growth of the cell lines mentioned (Figure 2 letter A, B, C, respectively).
Proliferation of NCI-H28 cell line was not instead affected by CAPS at the concentration of 100 µM and 200 µM as compared to DMSO-treated cells but CAPS 250 µM and 300 µM significantly reduced the growth as compared to controls, indicating that the antiproliferative action of CAPS requires higher concentration on cells of epithelioid origin.
Treatment with DMSO did not affect the growth of all mesothelioma cell lines.

Since the onset of resistance to conventional chemotherapeutic agents, including cisplatin, leads to therapy failure (77), it is necessary to develop new therapeutic drugs for mesothelioma. For this reason, we assessed if CAPS could affect the cell viability of cisplatin-resistant MSTO-211H and NCI-H2052 cells, generated in our laboratory after prolonged cisplatin treatment.

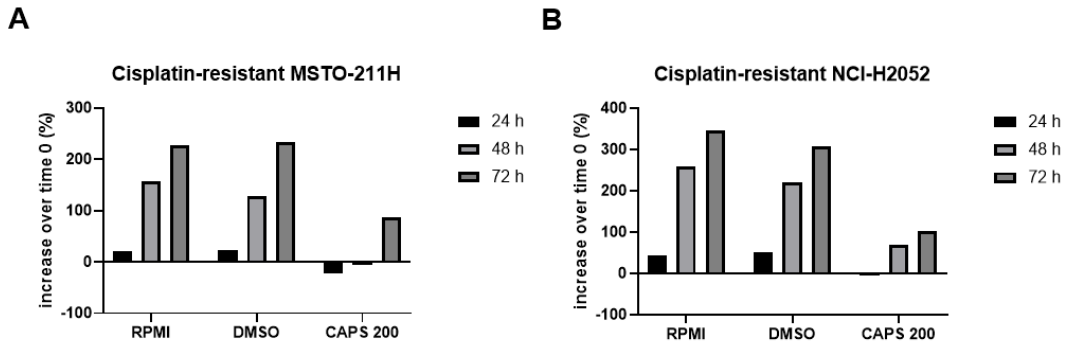

Figure 3: Effect of Capsaicin (CAPS) on cell proliferation of cisplatin-resistant mesothelioma cell lines. Cisplatin-resistant MSTO-211H **(A)** and NCI-H2052 **(B)** cells were treated with: RPMI medium alone (RPMI); medium containing DMSO (DMSO) at the same %v/v as the maximum CAPS concentration and CAPS at the concentration of 200 µM (CAPS 200) for 24, 48 and 72h. Data are expressed as percentage of growth increase over time 0 and represent the results obtained by one experiment performed in triplicates.

Cisplatin-resistant MSTO-211H and NCI-H2052 cells (Figure 3) cells treated with 200 µM CAPS showed a significantly reduced cell growth compared to treatment

with DMSO. These results suggest that CAPS could be effective in inhibiting cisplatin-resistant mesothelioma cells indicating a possible novel therapeutic approach for targeting resistant cells.

5.2 EFFECT OF CAPSAICIN ON THE CELL CYCLE IN MESOTHELIOMA CELLS

To evaluate the effects of CAPS on the cell cycle, we performed cytofluorimetric analysis on MSTO-211H, NCI-H2052, NCI-H2452, and NCI-H28 cells after 48 hours of treatment. As shown in Figure 4A. CAPS caused a reduction in the fraction of MSTO-211H cells in G0/G1 phase (33.85 ± 8.27 in CAPS 200 *vs* 72.5% ± 3.54 in DMSO, **p*-value ≤ 0.05), while determining a significant increase of the cell population in S phase (21.9 ± 0.566 in CAPS 200 *vs* 9.8 ± 3.39 in DMSO, **p*-value ≤ 0.05). In addition, MSTO-211H cells treated with CAPS 200 µM for 48 hours showed a rise in the number of apoptotic cells compared to cells treated with the medium containing DMSO alone.

A similar effect on the cell cycle was also observed in NCI-H2052 cells, in which CAPS induced S-phase cell cycle arrest (22.1 ± 3.68 in CAPS 200 *vs* 8.15 ± 1.91 in DMSO, **p*-value ≤ 0.05) (Figure 4 B). In NCI-H2452 cells, treatment with CAPS for 48 hours induced an increase, although not significant, in the number of cells in S phase compared to vehicle alone (Figure 4 C). In addition, in NCI-H28 cells the cell cycle was not significantly altered following the treatment for 48 hours with CAPS at both 200 µM and 250 µM concentrations. On the other hand, CAPS 300 µM induced a significant increase in the number of apoptotic cells after 48 hours (5.3 ± 2.69 in CAPS 300 *vs* 0.37 ± 0.55 in DMSO, **p*-value ≤ 0.05) (Figure 4 D).

Collectively these results suggest that CAPS effects on cell cycle might differ in cells derived from different mesothelioma subtypes.

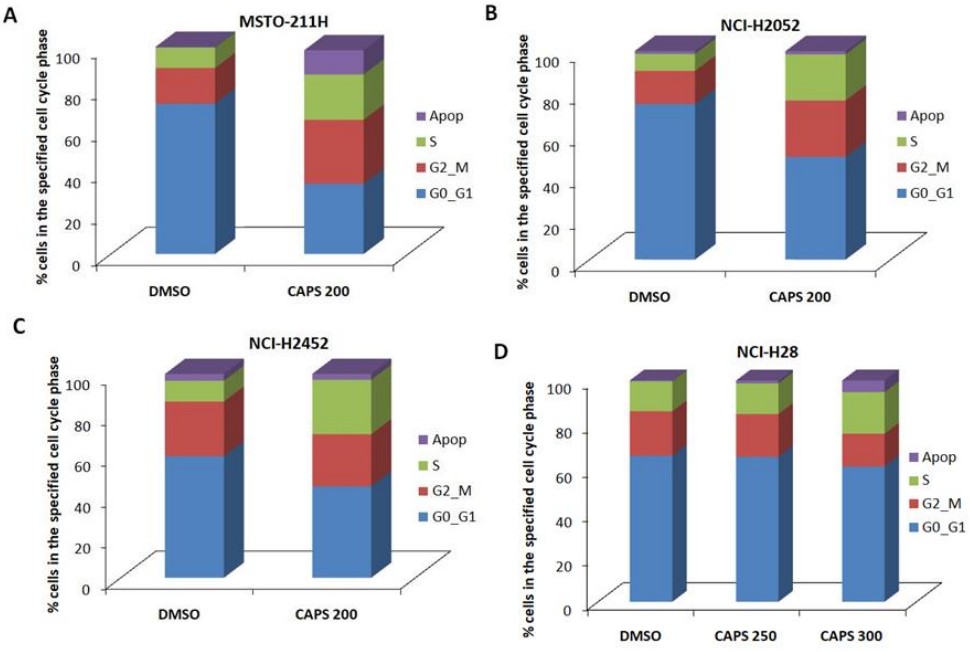

Figure 4: **Effect of capsaicin (CAPS) on the cell cycle of mesothelioma cells.** Cell cycle analysis was performed in duplicate on MSTO-211H **(A)**, NCI-H2052 **(B)**, NCI-H2452 **(C)** cells treated for 48 hours with medium containing DMSO alone or CAPS at a concentration of 200 µM (CAPS 200) and NCI-H28 **(D)** cells treated for 48 hours with medium containing DMSO alone or CAPS at a concentration of 250 µM and 300 µM (CAPS 250 and CAPS 300, respectively).

5.3 EVALUATION OF CAPSAICIN ON MIGRATION OF MESOTHELIOMA CELLS

Because enhanced migratory ability is one important feature of cancer cells (156,157), we tested the effect of CAPS on mesothelioma cell migration using two approaches: a woud healing assay, for lateral cell motility, and transwell assays. To rule out possible effects of CAPS on proliferation, for lateral motility assays we used 100 µM for MSTO-211H, NCI-H2052 and NCI-H2452 cells, and 200 µM for NCI-H28 cells, concentrations that had no effect on cell growth.

5.3.1 EVALUATION OF ANTI-MIGRATORY ACTIVITY OF CAPSAICIN (CAPS) ON MESOTHELIOMA CELLS BY WOUND HEALING ASSAY

The wound healing assay was conducted on NCI-H2452 (Figure 5), NCI-H28 (Figure 6) and NCI-H2052 (Figure 7) cells but not on MSTO-211H cells, as these cells have a tendency to detach when they reach confluence.

In NCI-H2452 cells, CAPS significantly reduced lateral motility at 72h of treatment compared to cells treated with DMSO alone as control. In fact, the mean value of % closure of the wound area was 69.62% *versus* 45.90%, following 72h of treatment with the medium containing DMSO alone or CAPS 100 µM, respectively (**p*-value < 0.05) (Figure 5).

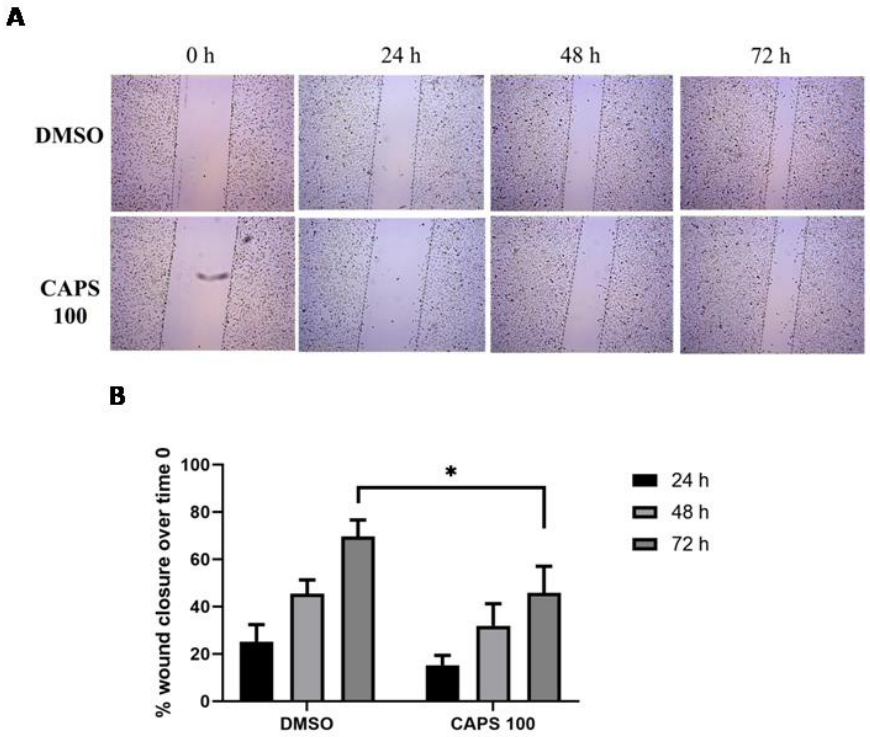

Figure 5: Effect of Capsaicin (CAPS) on lateral motility in NCI-H2452 cells. NCI-H2452 cells were treated with medium containing vehicle (DMSO) or CAPS at the concentration of 100 µM (CAPS 100) for 24, 48 and 72h. **(A)** Representative images of woud healing assay on NCI-H2452 cells. **(B)** Quantitative analysis of %wound closure. The data represent the percentage of closure of wound area compared to time 0. The results are expressed as mean ± standard deviation of three independent experiments. Statistical differences between DMSO and CAPS 100 µM were evaluated for each time of treatment (*p-value ≤0.05 *versus* DMSO).

In NCI-H28 cells, the treatment with 200 µM CAPS significantly reduced cell lateral motility compared to the group treated with medium containing DMSO alone (Figure 6).

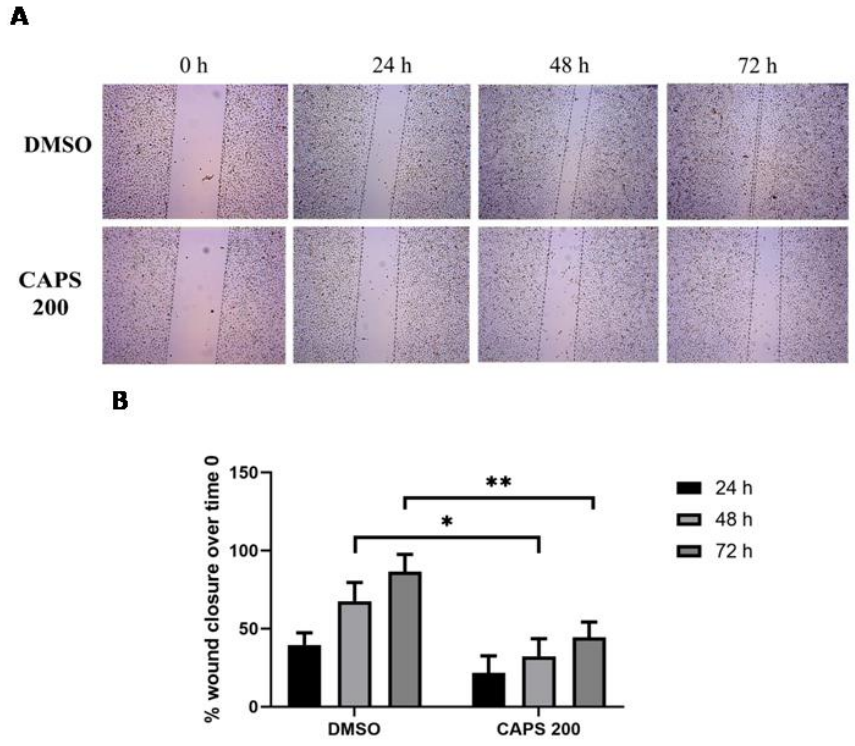

Figure 6: **Effect of Capsaicin (CAPS) on lateral motility in NCI-H28 cells.** NCI-H28 cells were treated with medium containing vehicle (DMSO) or CAPS at the concentration of 200 µM (CAPS 200) for 24, 48 and 72h. **(A)** Representative images of woud healing assay. **(B)** Quantitative analysis of %wound closure. Data represent the percentage of wound area closure compared to time 0. Results are expressed as mean ± standard deviation of three independent experiments. Statistical differences between DMSO and CAPS 200 µM were evaluated for each timepoint (**p*-value ≤0.05; ***p*-value < 0.01 *versus* DMSO).

After 48 h of treatment, the percentage of wound area closure was 67.56% in NCI-H28 cells with medium containing DMSO alone, while 32.34% in cells treated with CAPS 200 µM (**p*-value < 0.05) (Figure 6). Furthermore, after 72 h of treatment, the mean value of the wound area closure percentage was 44.44% in

200 µM CAPS group *versus* 86.62% in vehicle group (** p-value ≤ 0.001) (Figure 6).

Unlike the other mesothelioma cell lines, lateral motility of NCI-H2052 was not affected by CAPS at the concentration of 100 µM, as shown in Figure 7, suggesting that motility of cells of sarcomatoid origin is not affected by CAPS exposure.

Therefore, CAPS significantly reduced the lateral motility of several mesothelioma lines, such as epithelioid-type NCI-H28 and NCI-H2452 cell lines, but not sarcomatoid-type NCI-H2052 cells.

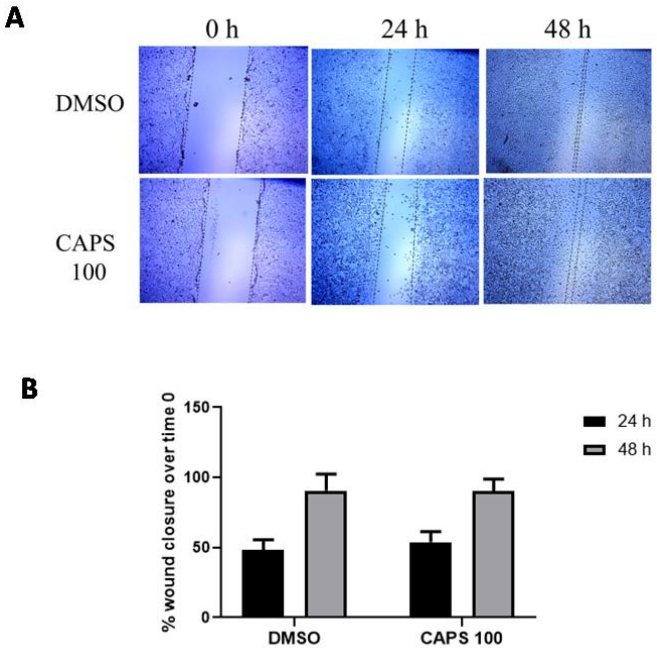

Figure 7: Effect of Capsaicin (CAPS) on lateral motility in NCI-H2052 cells. NCI-H2052 cells were treated with medium containing vehicle (DMSO) or CAPS at the concentration of 100 µM (CAPS 100) for 24 and 48. **(A)** Representative images of wound healing. **(B)** Quantitative analysis of %wound closure. Data represent the percentage of wound area closure compared to time 0. Results are expressed as mean ± standard deviation of three independent experiments. No statistical differences were found between DMSO and CAPS 100 µM at each time of treatment.

5.3.2 EVALUATION OF ANTI-MIGRATORY ACTIVITY OF CAPSAICIN (CAPS) ON MESOTHELIOMA CELLS BY TRANSWELL ASSAY

The effect of CAPS on migration activity was also evaluated by transwell assay on NCI-H2052 and MSTO-211H cells. These assays determine the ability of cancer cells to migrate without attachment

CAPS inhibited the migration of NCI-H2052 and MSTO-211H cells at concentration of 100 µM, which instead did not affect cell proliferation. In fact, CAPS reduced the percentage of NCI-H2052 and MSTO-211H cells able to migrate through the pores of the transwell membrane compared to treatment with medium containing DMSO alone (Figure 8 and Figure 9, respectively).

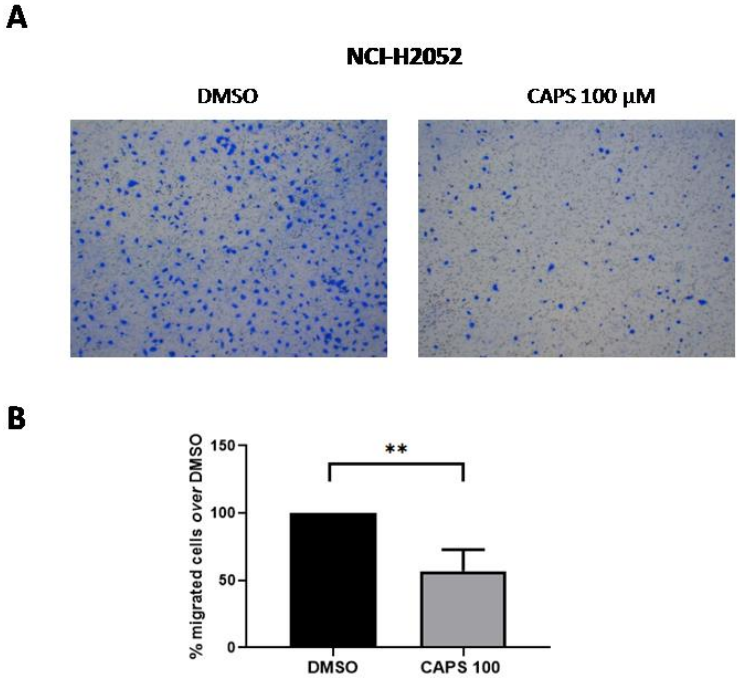

Figure 8: Effect of Capsaicin (CAPS) on transwell migration assay in NCI-H2052 cells. Migration of NCI-H2052 cells, treated with medium containing either DMSO alone (DMSO) or CAPS 100 µM (CAPS 100), was assessed by transwell assay, as described in Materials and methods. **(A)** Representative images of transwell migration assays on NCI-H2052 cells. **(B)** Quantitative analysis of the percentage of number of migrated cells compared to DMSO in transwell assay**.** Data are expressed as mean ± standard deviation of three indipendent experiments. Statistical differences between DMSO and CAPS 100 µM were evaluated by unpaired t-test (***p*-value < 0.01 *versus* DMSO)

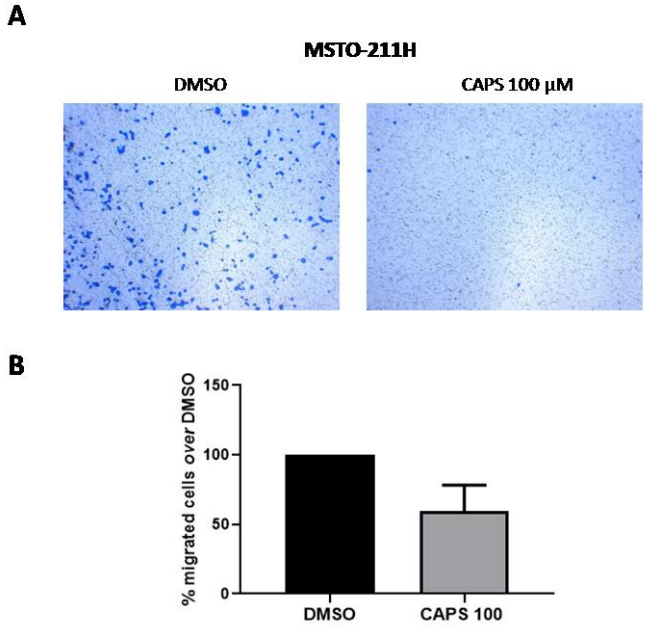

Figure 9: Effect of Capsaicin (CAPS) on transwell migration assay in MSTO-211H cells. Migration of MSTO-211H cells, treated with medium containing either DMSO alone (DMSO) or CAPS 100 µM (CAPS 100), was assessed by transwell assay, as described in Materials and methods. **(A)** Representative images of transwell migration assay on MSTO-211H cells. **(B)** Quantitative analysis of the percentage of the number of migrated cells compared to DMSO in transwell assay. Data are expressed as mean ± standard deviation of two indipendent experiments.

Collectively, these results indicate that CAPS exhibits anti-migratory effect in migration transwell assays in several mesothelioma lines, such as NCI-H2052 and MSTO-211H cells.

5.4 EFFECT OF CAPSAICIN (CAPS) ON AKT AND ERK1/2 ACTIVATION

AKT and ERK1/2 are serine-threonine kinases involved in the regulation of numerous cellular processes, such as proliferation, cell survival, metabolism and migration (158,159).

To assess whether the reduction in cell proliferation and migration promoted by CAPS in mesothelioma is dependent on a reduction in AKT and ERK1/2

activation, the levels of total and phosphorylated forms of AKT and ERK1/2 were assessed by western blotting assay in NCI-H2052 (Figure 10), MSTO-211H (Figure 11) and and NCI-H28 (Figure 12) cells.

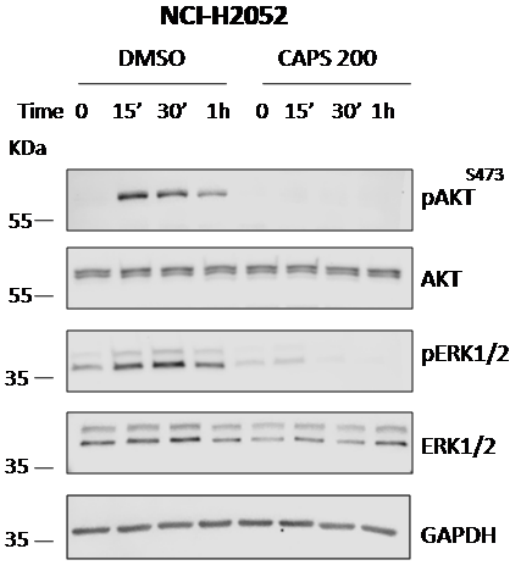

Figure 10: Capsaicin (CAPS) inhibited the activation of AKT and ERK1/2 in NCI-H2052 cells. Levels of total and phosphorylated AKT and ERK1/2 were assessed by western blotting in NCI-H2052 cells serum-starved for 5 h and then treated with medium containing 1% fetal bovin serum with DMSO alone (DMSO) or CAPS 200 µM (CAPS 200) for 15', 30' and 1h. Representative images of western blotting for phospho AKT (pAKT), total AKT (AKT), phospho ERK1/2 (pERK1/2), total ERK1/2 (ERK1/2) and GAPDH.

NCI-H2052 cells in serum-free medium supplemented with DMSO alone showed low AKT activation (phospho AKT) at baseline. Instead, the level of phospho AKT increased after 15' and 30'(min) of treatment with 1% fetal bovine serum-medium containing DMSO alone (Figure 10). Notably, we detected the same activation of ERK1/2 (phospho ERK1/2) after 15' of treatment with DMSO compared to time zero (Figure 10). These results demonstrate that in NCI-H2052 cells treated with DMSO alone there is an activation of both AKT and ERK1/2 pathways (Figure 10). In contrast, treatment with CAPS 200 µM inhibited the activation of both AKT and ERK1/2 in NCI-H2052 cells. Indeed, the levels of

phospho AKT and phospho ERK1/2 did not change following the treatment for 15', 30' or 1h with 1% serum-medium containing CAPS, compared to time zero in the absence of serum (Figure 10).

In addition, CAPS also suppressed the activation of AKT and ERK1/2 in MSTO-211H cells (Figure 11). Following the treatment with 1% serum medium, the levels of phospho AKT and phospho ERK1/2 as well as the *ratio* of phosphorylated over total form of both AKT and ERK1/2 increased in cells treated with DMSO. Conversely, AKT and ERK1/2 activation did not occur in cells treated with CAPS 200 µM, compared with the relative time zero in absence of serum (Figure 11 A and Figure 11 B).

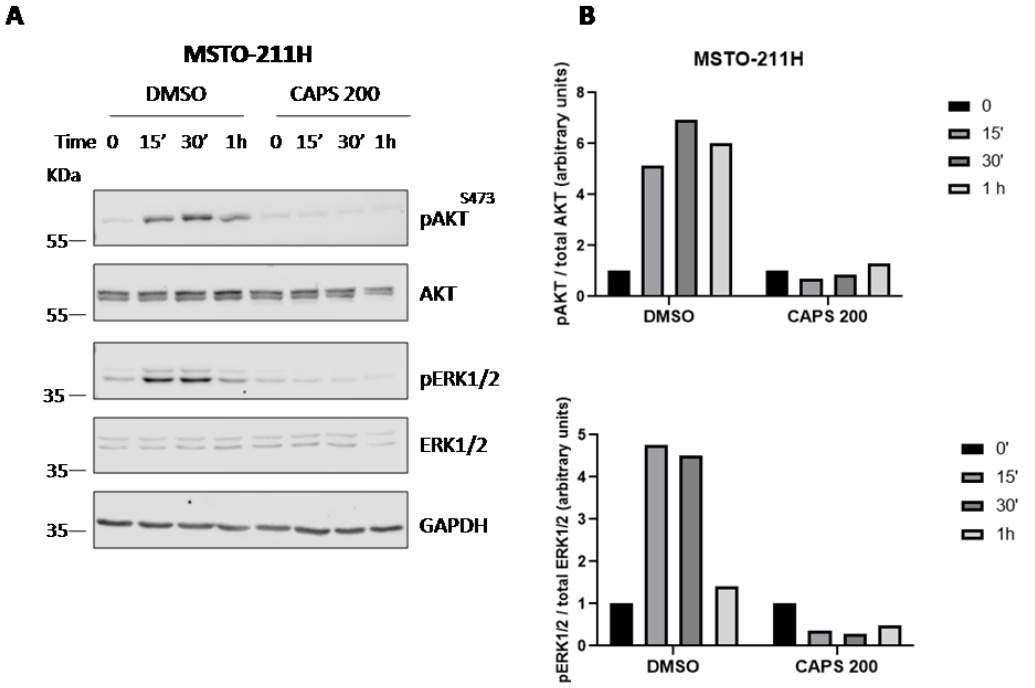

Figure 11: Capsaicin (CAPS) inhibited the activation of AKT and ERK1/2 in MSTO-211H cells. Levels of total and phosphorylated AKT and ERK1/2 were assessed by western blotting in MSTO-211H cells serum-starved for 5 h and then treated with medium containing 1% fetal bovin serum with DMSO alone (DMSO) or CAPS 200 µM (CAPS 200) for 15', 30' and 1h. **(A)** Representative images of western blotting for phospho AKT (pAKT), total AKT (AKT), phospho ERK1/2 (pERK1/2), total ERK1/2 (ERK1/2) and GAPDH. **(B)** Densitometric analysis of phosphoAKT/total AKT *ratio* and phospho ERK1/2 /total ERK1/2 *ratio*. The values are expressed as arbitray units.

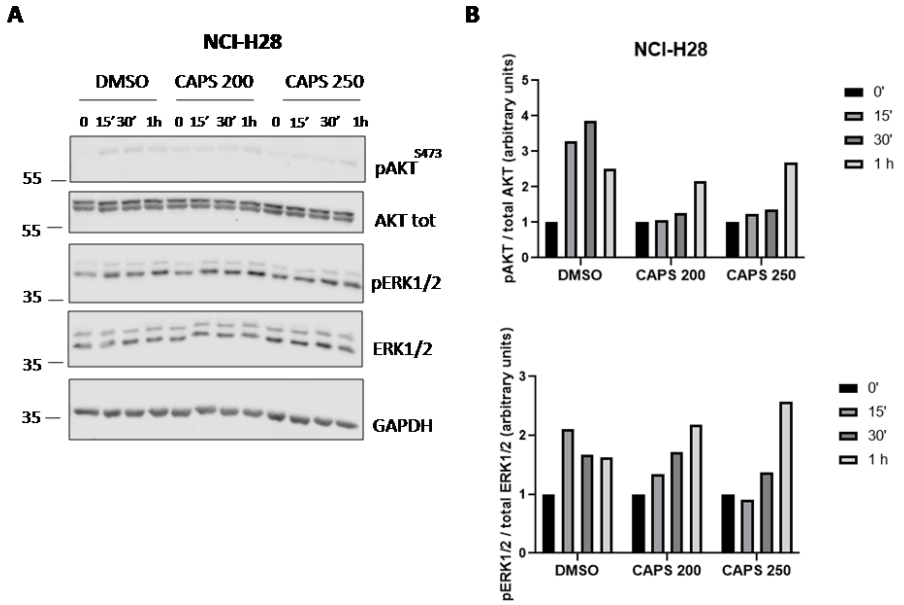

Figure 12: Effect of Capsaicin (CAPS) on the activation of AKT and ERK1/2 in NCI-H28 cells. Levels of total and phosphorylated AKT and ERK1/2 were assessed by western blotting in NCI-H28 serum-starved for 5 h and then treated with medium containing 1% fetal bovin serum with DMSO alone (DMSO) or CAPS at the concentration of 200 µM (CAPS 200) and 250 µM for 15', 30' and 1h. **(A)** Representative images of western blotting for phospho AKT (pAKT), total AKT (AKT), phospho ERK1/2 (pERK1/2), total ERK1/2 (ERK1/2) and GAPDH. **(B)** Densitometric analysis of phosphoAKT/total AKT *ratio* and phospho ERK1/2 /total ERK1/2 *ratio.*The values were expressed as arbitrary units.

Figure 12 shows the effect of CAPS on the activation of AKT and ERK1/2 in NCI-H28 cells. Cells treated with medium containing DMSO alone showed a fast activation of AKT after 15 min of treatment with 1% serum medium compared with baseline in the absence of serum. Instead, the treatment with CAPS 200 µM or CAPS 250 µM induced a delayed activation of AKT (Figure 12 A). In fact, the *ratio*-levels of phosphorylated to total AKT increased in NCI-H28 cells treated with CAPS at both concentrations only after 1 hour of treatment with 1% serum medium compared with the corresponding time zero (Figure 12 B).

Regarding ERK1/2, DMSO-treated cells exhibited increased levels of phospho ERK1/2 and the *ratio* of phosphorylated to total ERK1/2, even after 15' in presence of 1% serum medium compared to time zero, proving that DMSO did not affect ERK1/2 activation. Instead, the treatment with CAPS at the concentration of 200 µM did not reduce ERK1/2 activation. In addition, CAPS used at the concentration of 250 µM, capable of reducing the proliferation of NCI-H28 cells, only led to a delay in the activation of ERK1/2. Therefore, the effect of CAPS on the suppression of ERK1/2 activation was milder in NCI-H28 cells than in MSTO-211H and NCI-H2052 cells.

5.5 EFFECT OF CAPSAICIN ON APOPTOSIS AND AUTHOPHAGY MARKERS IN MSTO-211H CELLS

To further investigate the molecular mechanisms underlying CAPS-induced cytotoxicity on mesothelioma cells, we determined protein expression levels of markers involved in apoptosis, such as BAX, a pro-apoptotic factor, and BCL2, an anti-apoptotic factor, by western blotting in MSTO-211H cells.

MSTO-211H cells were chosen for the evaluation of apoptotic markers as these cells were highly responsive to CAPS treatment. In fact, as shown in section 5.2, cytofluorometry analysis showed the presence of apoptotic MSTO-211H cells after treatment for 48h. Furthermore, as shown in section 5.4, CAPS significantly reduced AKT and ERK1/2 activation.

Preliminary experiments show that treatment with CAPS at the concentration of 200 µM for 24h induced a slight increase in BAX/BCL2 *ratio* (Figure 13). However, the levels of this *ratio* did not significantly change after 48 hours of treatment with CAPS 200 µM compared to cells treated with medium containing vehicle alone, suggesting that CAPS might not induce cell death by regulating of BAX and Bcl2 expression levels.

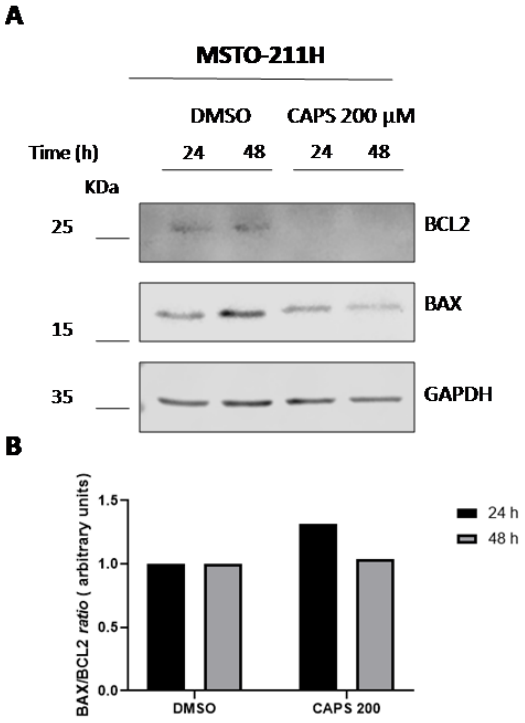

Figure 13: Effect of Capsaicin (CAPS) on BAX and BCL2 protein levels in MSTO-211H cells. MSTO-211H cells were treated with medium containing DMSO or CAPS 200 µM for 24 and 48 hours. **(A)** Representative images of Western Blotting for BCL2, BAX and GAPDH. **(B)** Densitometric analysis of BAX/BCL2 *ratio*. The results were obtained from a preliminary experiment. The values were normalised towards GAPDH and expressed as arbitrary units.

In order to assess the effect of CAPS on authophagy in MSTO-211H cells, we evaluated some authophagic markers such as LC3II/LC3I *ratio* and the level of Beclin-1 by Western Blotting analysis. As shown in Figure 14 Beclin-1 levels were reduced in a time-dependent manner following treatment with CAPS compared to medium containing DMSO alone (Figure 14 A). Moreover, treatment with CAPS reduced the LC3II/LC3I *ratio* (Figure 14 B), proving that CAPS affected the processing of LC3I into LC3II, which is required for autophagosome formation (128).

These preliminary results suggest that CAPS might reduce autophagy activation in MSTO-211H cells by reducing the levels of Beclin-1 and LC3II/I *ratio*.

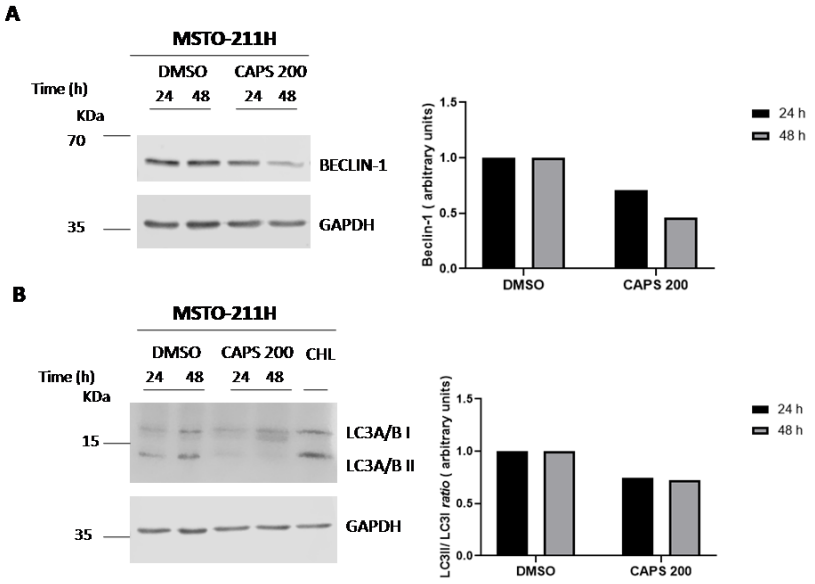

Figure 14: Effect of Capsaicin (CAPS) on authophagic markers levels in MSTO-211H cells. MSTO-211H cells were treated with medium containing DMSO or CAPS 200 µM for 24 and 48 hours. **(A)** Representative images of Western Blotting and densitometric analysis of Beclin-1 protein levels normalized with GAPDH. The results of densitometric analysis are expressed as arbitrary units. **(B)** Representative images of Western Blotting and densitometric analysis of LC3A/B I and LC3 A/B II. The levels of LC3A/B I and LC3A/B II were normalized towards GAPDH and the results of the LC3A/B II/I *ratio* were expressed as arbitrary units. Results were obtained in a preliminary experiment.

6 DISCUSSION

Mesothelioma is an aggressive cancer, mainly associated with asbestos exposure. Despite the use of surgical approaches or treatment with chemotherapeutic agents, the prognosis of both animals and humans affected by mesothelioma remains poor (4,160). For this reason, developing drugs that are safe for human and animal health is a major challenge in the fight against mesothelioma. Capsaicin (CAPS) has shown a strong antitumor action both *in vitro* and *in vivo* models on different types of tumors (101), but its effect on mesothelioma cells was not fully investigated. Therefore, the aim of this study was the evaluation of the effect of CAPS on proliferation, migration and cell cycle on different mesothelioma cell lines representative of all mesothelioma subtypes.

In this work we demonstrated that CAPS decreased cell proliferation in several mesothelioma cell lines. In detail, CAPS at the concentration of 200 µM exerted an inhibitory action on cell growth of MSTO-211H, NCI-H2052 and NCI-H2452 cells. Compared with other mesothelioma cell lines, NCI-H28 cells were more resistant to CAPS, as demonstrated by the absence of change in proliferation following CAPS 200 µM treatment. However, CAPS at the concentration of 250 µM or 300 µM significantly reduced the growth of NCI-H28 cells. The concentrations of CAPS that resulted effective for the experiments of this study (200 µM, 250 µM and 300 µM) fell within the range of concentrations known to exhibit an anticancer activity against several cancer cell lines (161). Unfortunately, resistance to conventional chemotherapeutic agents, such as cisplatin, influences the response to mesothelioma therapy (77).

We demostrated that CAPS significantly reduced cell growth of cisplatin-resistant MSTO-211H and NCI-H2052 cells. These results are in line with other studies proving the beneficial action of CAPS on cisplatin-resistant tumour cells. Indeed, Catanzaro *et al.* demonstrated that CAPS induced cell cycle arrest in cisplatin-resistant ovarian carcinoma and cervix squamous carcinoma cells and it even exhibited a stronger action on cisplatin-resistant cancer cells than on parental ones (147). In addition, CAPS used in combination with other natural agents made

multi-resistant cancer cells sensitive to the action of conventional chemotherapeutic agents, such as doxorubin, in various cancer cells (149).

CAPS exerts its anti-proliferation action on tumor cells by arresting the cell cycle at various phases depending on cell context (98,110). Our results evidenced that CAPS induced S-phase cell cycle arrest in various mesothelioma lines. These results are consistent with other studies proving an increase in the cell population in S-phase after CAPS-treatment in various cancer cell lines (112).

In addition to antiproliferative effect, our results demonstrated that CAPS had an anti-migratory action on several mesothelioma cells, even at concentrations that did not affect cell viability. Indeed, we obtained a significant reduction in the migration of NCI-H28, NCI-H2452 and MSTO-211H cells following CAPS-treatment. These results are in agreement with other studies proving that CAPS decreased the migration of different tumor cell lines (141–145).

Although CAPS did not impair lateral motility of NCI-H2052 cells, it reduced the migration of NCI-H2052 cells in a transwell assay. The different outcome obtained by wound healing assay and migration transwell assay could be due to the different nature and purpose of these migration assays (162).

Furthermore, we demonstrated that CAPS inhibited the activation of both AKT and ERK1/2 in MSTO-211H and NCI-H2052 cells. These results can explain the reduction in proliferation and migration associated with CAPS-treatment, since AKT and ERK1/2 are serine-threonine kinases involved in numerous cellular processes, including cell survival, proliferation and migration (158,159).

These results are in line with other studies showing the reduction of the levels of phospho ERK1/2 and phospho AKT following CAPS-treatment in other tumor cell lines (104,163,164). Furthermore, several studies proved the efficacy of various drugs in lowering the proliferation and migration of mesothelioma cells through the inhibition of AKT and ERK1/2 activation (165–167).

Compared to other mesothelioma cell lines, CAPS delayed AKT activation in NCI-H28 cells, but it did not inhibit ERK1/2 activation. In this regard, we can speculate that the different effect of CAPS on the regulation of ERK1/2 activation might be influenced by the mesothelioma subtype, since NCI-H28 are epithelioid-

type mesothelioma cells, whereas NCI-H2052 and MSTO-211H are sarcomatoid and biphasic type cells, respectively (168).

Since CAPS is known to promote apoptosis in several tumor cell lines by promoting caspase 3 activation, increasing levels of the pro-apoptotic factor BAX and decreasing the anti-apoptotic factor Bcl2 (110,117,119,120), we investigated the effects of CAPS on these apoptotic markers in mesothelioma cells. Preliminary data showed that CAPS did not promote caspase-3 cleavage in several mesothelioma lines (data not shown). These results were in agreement with Cömertpay *et al.* who noted the presence of the cleaved form of caspase-3 in CRL-5946 mesothelial cancer cells (coincident with NCI-H2452), but found no significant differences between cells treated with CAPS and those treated with medium containing vehicle alone, demonstrating that CAPS did not lead to a caspase-3-dependent cell death (169).

Preliminary results in MSTO-211H cells, showed only a slight increase in the BAX/Bcl2 *ratio* following 24 hours of treatment with CAPS, but this effect was not present after 48 hours. However, these results need to be carefully considered, since further experiments are essential to confirm these findings.

To date, there are no studies in the literature regarding the action of CAPS on markers of autophagy in mesothelioma cells. We demonstrated that the treatment with CAPS reduced the conversion of LC3A/B I to LC3A/B II and Beclin-1 levels in MSTO-211H cells. These preliminary data highlight the potential inhibitory action of CAPS on autophagy in MSTO-211H cells. Although many works proved that CAPS promoted autophagy in different tumor lines (114,136), our results are in line with Bort *et al* who reported the inhibitory effect of CAPS on autophagy in hepatocellular carcinoma cells (170). Autophagy can represent a mechanism used by cancer cells to evade apoptosis and reduce response to therapeutic agents (140). Indeed, the use of autophagy inhibitors sensitizes mesothelioma cells to various chemotherapeutic agents, thereby increasing mesothelioma cell apoptosis (171,172). Although further studies are needed to confirm the inhibition of autophagy promoted by CAPS in MSTO-211H cells, our preliminary results could pave the way toward new studies aimed at investigating the combined use of CAPS with therapeutic agents in mesothelioma therapy.

Regarding future perspectives, we will repeat experiments to confirm the data in autophagy and apoptosis markers after CAPS treatment. As we proved that CAPS arrested the cell cycle in S-phase, our future investigations will aim at evaluating the molecular mechanisms underlying S-phase cell cycle arrest in mesothelioma cells. For this purpose, we will use the EdU assay, which is based on the binding of EdU, a thymidine analogue, to newly synthesised DNA (173). In particular, after induction of G1-S-phase cell synchronisation with hydroxyurea, we will promote cell release in the presence or absence of CAPS. This will allow us to assess whether the S-phase arrest promoted by CAPS is due to defects occurring during DNA replication (174). CAPS in fact can lead to DNA fragmentation (175) and p53 activation in cancer cells (98,176).

Various cell cycle regulators will be studied, such as markers involved in the S-G2 transition checkpoint,such as ATR; the levels of p53, involved in DNA-damage response and the expression of cyclin A which plays a key role in phase S (177–180).

In this work, we demonstrated the anticancer effect of CAPS in reducing the proliferation and migration of several mesothelioma cells, representative of the main mesothelioma subtypes. In addition, CAPS inhibits cell growth of cisplatin-resistant cells. These results are encouraging and suggest the potential use of CAPS in mesothelioma therapy.

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