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THE HDAC INHIBITOR ITF2357 (GIVINOSTAT) AS A KEY PLAYER IN EPIGENETIC TARGETING OF MELANOMA AND COLON CANCER CELLS

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Dear Reader,

part of the data shown in this thesis are the object of the scientific papers “The Histone Deacetylase Inhibitor ITF2357 (Givinostat) Targets Oncogenic BRAF in Melanoma Cells and Promotes a Switch from Pro-Survival Autophagy to Apoptosis” (Celesia et al., *Biomedicines.* 2022 Aug 17; 10(8):1994. doi: 10.3390/biomedicines10081994) and “Oncogenic BRAF and p53 interplay in melanoma cells and the effects of the HDAC inhibitor ITF2357 (Givinostat)” (Celesia et al., *International Journal of Molecular Sciences,* 2023, *24*(11), 9148; https://doi.org/10.3390/ijms24119148).

Thanks for your kind attention, I hope you will find this work interesting and helpful.

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I. INTRODUCTION

1. Melanoma: etiology and epidemiology

Melanoma is a malignancy derived by the transformation of melanocytes, cells in the basal layer of the epidermis which produce the pigment melanin. Melanocytes are cells of neural crest origin and express many signalling molecules and factors that promote migration and metastasis after malignant transformation (figure 1). Melanoma accounts for over 80% of skin cancer deaths and can be divided into many clinical subtypes (Naik, 2021):

- the superficial spreading melanoma is the most common type among cutaneous melanomas, which has a good prognosis due to a low Breslow thickness, that depends on the earlier time of diagnosis;
- the acral lentiginous melanoma arises from the glabrous skin of the palms, soles and nailbeds, and is more likely to arise in darker-skinned ethnicities;
- melanoma from mucosal or uveal tissue is more rare and independent of sun exposure.

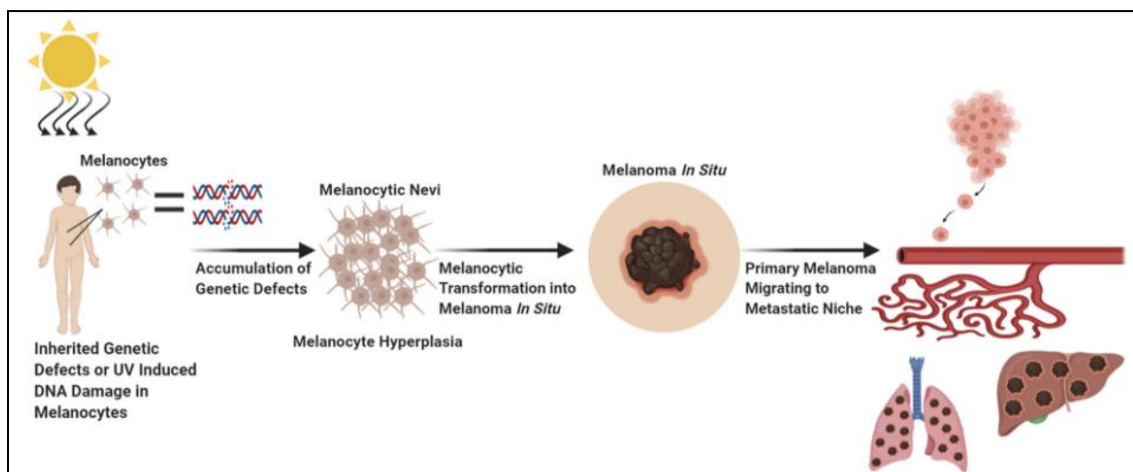


Figure 1: *Factors contributing to melanocytic transformation* (Eddy et al., 2021).

Melanoma is characterized by the accumulation of genetic mutations that lead to uncontrolled cell proliferation and invasiveness (figure 2). The genesis of melanoma initiates with precursor lesions in the form of benign melanocytic nevi and/or intermediate lesions (also known as dysplastic nevi) and then it proceeds through a series of increasingly malignant lesions. It generally starts as melanoma in radial growth phase, including melanoma in situ, evolving into melanoma in vertical growth phase (also known as invasive melanoma) and, finally, into metastatic melanoma with different degrees of aggressiveness. Not all melanomas pass through these steps, with a possible tumour development direct from transformed melanocytes (Guo et al., 2021). Moreover, dysplastic nevi have a broader spectrum of driving mutations than benign nevi. Interestingly, while most of the intermediate lesions display the $BRAF^{V600E}$ mutation, there is a subset of

dysplastic nevi harbouring different MAPK pathway activating mutations (NRASQ61K/R, BRAFV600K and BRAFK601E) (Melamed et al., 2017).

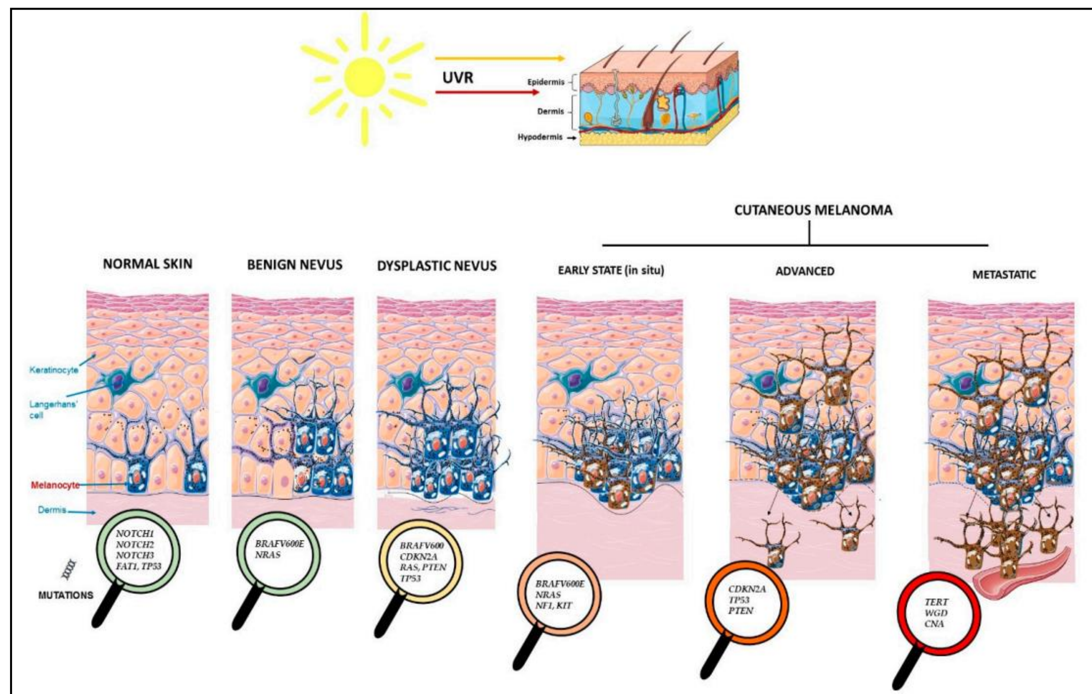


Figure 2: *Proposed steps of melanomagenesis: most frequently, melanoma develops from melanocytes at the basal layer of the epidermis. However, melanoma can also develop from pre-existing nevi (Loras et al., 2022).*

Approximately 30% of melanomas are associated with a nevus, although the malignant transformation rate of this lesion is a rare event (Pellegrini et al., 2021).

The incidence of melanoma varies among different countries and it causes about 55.000 deaths around the world annually. In Italy, recent data from AIRTUM (Associazione Italiana Registri Tumori) display how melanoma recorded the highest average annual increase in both men (+ 8.8%) and women (+ 7.1%). According to the "Istituto Superiore di Sanità", cutaneous melanoma is rare in children and generally affects adults between 30 and 60 years age. Women are often affected in young age, while men develop this cancer in adult age (Leonardi et al., 2018).

The incidence of malignant melanoma is rapidly increasing worldwide, especially in women. Melanoma is more common in Whites than in Blacks and Asians. Overall, melanoma is the fifth most common malignancy in men and the seventh most common malignancy in women. The average age at diagnosis is 57 years, and up to 75% of patients are younger than 70 years of age; therefore, melanoma affects young and middle-aged people. The highest incidence rate is found in sunny areas inhabited by North European

populations and cutaneous melanoma frequency is ten times higher in Caucasian people (Conforti and Zalaudek, 2021).

The high mutation rate in melanoma is primarily attributed to the mutagenic effect of UV radiation, with two distinct pathways (Davis et al., 2018):

- a nevus-prone pathway, promoted by intermittent sun exposure and sunburns;
- a chronic sun exposure pathway, restricted to sun-sensitive people who progressively accumulate UV-related DNA damage to the sites of future melanomas.

Besides gene mutations, some epigenetic events have a key role in the genesis of melanoma and its progression. These epigenetic alterations include: methylation or demethylation of specific genes, non-coding RNAs (including long non-coding RNAs and miRNAs), histone post-translational modifications (including variant histones) and chromatin remodelling by specific complexes as the polycomb-repressive complex PRC2 (Karami Fath et al., 2022).

1.1 Risk factors related to Melanoma

Throughout the years many risk factors related to melanoma have been identified and described in the literature. The following is a list of the most common acknowledged risk factors:

1) *UV exposure:* this is the primary risk factor for melanoma of the skin, which is modulated by genetics, melanin and UV wavelengths. UV light is known to induce DNA photoproducts (commonly thymidine-dimers) which, if unrepaired by nucleotide excision repair (NER), cause errors in DNA replication, subsequent mutations in cell signalling molecules and, finally, carcinogenesis. The location of UV-induced mutations varies with melanoma subtype, prognosis and response to treatment. Commonly mutated proteins include members of the mitogen-activated protein kinase (MAPK). Moreover, UV-induced mutations in tumour-suppressor p53 are more commonly seen in those with stage IV disease and are associated with a worse prognosis (Sample and He, 2018).

2) *Indoor Tanning*: tanning bed radiation is known as a carcinogen due to higher levels of UVA and UVB exposure than that of the daily sun. A dose–response relationship has been noted among years of tanning bed usage, total hours spent in a tanning bed, the number of sessions and melanoma risk (Ghiasvand et al., 2017).

3) *Immunosuppression*: low doses of UVA and UVB have been shown to decrease immunosurveillance by Langerhans and dendritic cells, impairing antigen-presentation and T-cell and NK-cell activation against aberrant melanoma cells. It has also been found that immunosuppressed patients have an increased risk of melanoma (Bogach et al., 2021).

4) *Moles, or nevi*: they are benign growths of melanocytes which are considered both precursors and markers of increased risk for melanoma. Notably, around 10% of patients with melanoma have a family history of the disease, although only few congenital syndromes have been characterized. Constant monitoring and mole resection is recommended in these cases (Roh et al., 2015).

5) *Obesity*: some studies showed an increased risk among people with a Body Mass Index (BMI) over 30. It seems that excessive body mass induces $BRAF^{V600E}$ oncogene activity through metabolic signalling and also disrupts immunosurveillance. This may explain why obese patients show above-average progression free survival and overall survival on BRAF inhibitor therapies and immunotherapies that specifically target these pathways (Smith et al., 2020).

According to tumour-node-metastases (TNM) classification, the stages of cutaneous melanoma (0 - IV) were defined as: early, locoregional and metastatic. According to this classification, the staging is the following:

- Stages 0 - IIC (early stages) = Cancer has persisted in the primary site and within the skin;
- Stage III (locoregional) = Cancer has spread to the skin or lymph nodes (LNs) or lymph vessel areas;
- Stage IV (metastatic) = Cancer has spread to other body parts and other organs.

If metastasis occurs, patients are given a diagnosis of stage III or IV. The lymph nodes are the most probable non-contiguous regions where cutaneous melanoma spreads and sentinel lymph nodes (SLNs) are first identified in the area where the primary cutaneous melanoma is located (Keung and Gershenwald, 2018).

1.2 Melanoma treatment

Nowadays, different therapeutic approaches are available based on the stage of the tumour. Treating stage I melanoma involves surgery to remove the melanoma and a small area of skin around it (surgical excision). In most cases, once melanoma has been removed no

further treatment is needed. Most people (80 to 90%) are monitored for 1 to 5 years and are then discharged with no further problems.

A sentinel lymph node biopsy is a procedure to test for the spread of cancer. If the procedure shows no spread to nearby lymph nodes, probably the patient will not have further problems. If the results confirm melanoma has spread nearby, the specialist will evaluate whether surgery is required. In stage III melanoma (that may be diagnosed by a sentinel node biopsy), melanoma has spread to nearby lymph nodes and surgery may be needed to remove them (Wong et al., 2018).

In stage IV, melanoma comes back or spreads to other organs. In the past, cure for stage IV melanoma was very rare but there are new encouraging treatments, such as immunotherapy and targeted treatments. Treatment for stage IV melanoma is not curative, but it is given to slow the cancer's growth, reduce symptoms and extend life expectancy.

Surgery may be also offered to remove melanomas that have grown away from the original site. Moreover, the patient may receive other treatments to help with the symptoms, such as radiotherapy and chemotherapy (Garbe et al., 2022).

Multiple chemotherapeutic agents have been evaluated for the treatment of advanced melanoma, but only Dacarbazine has been approved by the FDA.
Another chemotherapeutic agent evaluated for melanoma treatment is Temozolomide, the dacarbazine analog and derivative of triazene; its major advantage is the ease of dosing, given the oral formulation of the drug. Despite this advantage, Temozolomide alone has not achieved FDA approval for the treatment of metastatic melanoma.

A class of alkylating agents with documented activity in melanoma is represented by nitrosoureas, a family of compounds including fotemustine, carmustine and lomustine. While none of these agents was approved by the FDA for treatment of advanced melanoma, fotemustine was approved by some European regulators (Luke and Schwartz, 2013).

Immunotherapy is also used to treat advanced melanoma (stage IV) and it is sometimes offered to people with stage III melanoma as part of a clinical trial. Immunotherapy is used to help the immune system of the patient to find and kill melanoma cells (Davis et al., 2019). Moreover, the use of cancer vaccines to enhance cell-mediated immunity has been recently considered one of the most modern immunotherapy options for cancer treatment. In particular, mRNA vaccines are the most recent cancer vaccine options. Advantages of

mRNA cancer vaccines include their rapid production and low manufacturing costs. mRNA-based vaccines are also able to induce both humoral and cellular immune responses and they seem to be particularly promising for melanoma treatment (Bidram et al., 2021). However, these approaches are still under evaluation.

Melanoma is also particularly responsive to checkpoint-inhibitor immunotherapy, which consists of monoclonal antibodies that stimulate T-cells to recognize and destroy cancer cells (Ziogas et al., 2021). Among them, Ipilimumab (tradename Yervoy), a CTLA-4 inhibitor, received its first FDA approval in 2011, specifically for melanoma. Also PD-1 and PD-L1 inhibitors, such as pembrolizumab (Keytruda) and nivolumab (Opdivo), have been approved for stage III and IV disease (Menzies et al., 2017). A combination of PD-L1 inhibitor atezolizumab (Tecentriq) with BRAF inhibitor vemurafenib (Zelboraf) and MEK inhibitor cobimetinib (Cotellic) was approved in 2020 for unresectable or metastatic BRAF V600 positive melanoma (Ascierto et al., 2021).

Many patients with melanoma display oncogenic mutations in B-Raf proto-oncogene serine/threonine kinase (BRAF) that causes cells to grow and divide too quickly, thus promoting cancer development. Targeted medicine can therefore be used to target these mutations to slow or stop cancer cells growth.
These compounds may be recommended as a treatment for people who display specific gene mutations and have an aggressive type of localized melanoma or melanoma that has spread (Mishra et al., 2018). Treatments that target BRAF V600 (Val600) mutations using selective BRAF inhibitors combined with mitogen-activated protein kinase (MAPK) inhibitors have significantly improved response and overall survival.

Melanoma is considered intrinsically resistant to both radiotherapy and chemotherapy. The molecular basis of resistance to chemotherapy observed in melanoma depends on: defective drug transport system, deregulation of apoptosis and/or changes in enzymatic systems that mediate cellular metabolic machinery. Understanding of alterations in molecular processes involved in drug resistance may help in developing new therapeutic approaches to treat melanoma (Kalal et al., 2017).

Radiotherapy has been used as adjuvant therapy after the complete excision of primary melanoma and lymph nodes to reduce the rate of recurrences. After treatment, the patient will have regular follow-up (Gonzalez et al., 2010). Nevertheless, resistance to radiotherapy may be in relation with the constitutive activation of the MAPK pathway

and/or with the inactivation of tumour-suppressor p53 (Krayem et al., 2019; Rogers et al., 2019). Moreover, melanin pigment can protect normal melanocytes from ultraviolet radiation (UVR) and oxidative stress, but it can also make melanoma cells resistant to chemo or radiotherapy (Brożyna et al., 2016).

1.3 MAPK pathway involvement in Melanoma development

The MAPK signalling pathways are signal transduction pathways, which regulate fundamental cellular processes including cell proliferation, differentiation, senescence, survival, transformation and migration. These signalling pathways are characterized by the cascades of phosphorylation events, which spread from the cell membrane in the cytosol up to the nucleus. The MAPK/ERK signalling is essential for melanoma development and progression, because the most frequent driving mutations in the genesis of melanoma are the activating mutations of this pathway. Moreover, MAPK signalling reactivation by genetic and epigenetic events is the principal mechanism for acquired resistance to target therapy in this tumour (Sullivan and Flaherty, 2013).

The MAPK pathway is activated by extracellular signals, including mitogens, growth factors and cytokines, with specific plasma membrane receptors. This interaction triggers a cascade of phosphorylation that activates specific MAP kinases (MAPKs) and their catalytic activity determines the specific signalling response of a given pathway. In particular, the MAPK/ERK signalling pathway, mainly activated by growth factors, plays a crucial role in cancer development by promoting cell proliferation and migration (Czarnecka et al., 2020).

In detail, the MAPK/ERK signalling (figure 3) involves the activation of a growth factor/cytokine receptor tyrosine kinase (RTKs), which binds the ligand and then dimerizes and autophosphorylates, generating multiple docking sites for adaptors (mainly Grb2) that bind guanine nucleotide exchange factors (GEFs, mainly Sos) to RTKs. GEFs catalyze the dissociation of GDP from Ras-GTPases, thus favouring the conversion of Ras-GDP (inactive) to Ras-GTP (active) at the plasma membrane. On the other hand, GTPase activating proteins (GAPs) switch off Ras through their GTPase activity. GDIs (Guanosine nucleotide Dissociation Inhibitor) are regulators of RAS GTPases. The Ras family of small GTPases is composed of 39 members; among them, only three are frequently mutated in solid tumours: KRAS (about 85% of all RAS mutations), NRAS (about 15%), and HRAS (<1%). These Ras-GTPases favour the aggregation of ARaf, BRaf and CRaf members of the MAP3K family to form kinase-active homodimers or heterodimers. In particular, BRaf

is the key MAP3K in the genesis of melanoma, followed by CRaf. The Raf dimers phosphorylate at specific serine residues two MEKs (MAP/ERK Kinase 1 (MEK1/MAP2K1) and MEK2/MAP2K2). MEK1/2 proteins, in turn, catalyze a dual phosphorylation on threonine and tyrosine residues of a specific tripeptide sequence (Thr-Glu-Tyr) of the Extracellular signal-Regulated protein Kinase 1 (ERK1/MAPK1) and ERK2/MAPK2. Dephosphorylation by dual specificity phosphatases (DUSPs) modulates ERK activity, with DUSP5 inhibiting ERKs in the nucleus and DUSP6 in the cytoplasm, establishing a negative feedback loop to prevent over-activation of signalling outputs (Ullah et al., 2022).

Notably, ERK1/2 phosphorylate serine/threonine residues to many primary targets, both in the cytoplasm and in the nucleus. In the cytoplasm, ERKs phosphorylate cytoskeletal and adherens junction components, thus promoting cell detachment from the extracellular matrix and motility. In the nucleus, ERKs modulate the activity of proteins implicated in RNA transport/metabolism and act on transcription factors, including c-Fos, c-Myc and c-Jun. Their targets include proteins implicated in cell cycle regulation, in apoptosis and in many signalling pathways (Guo et al., 2021).

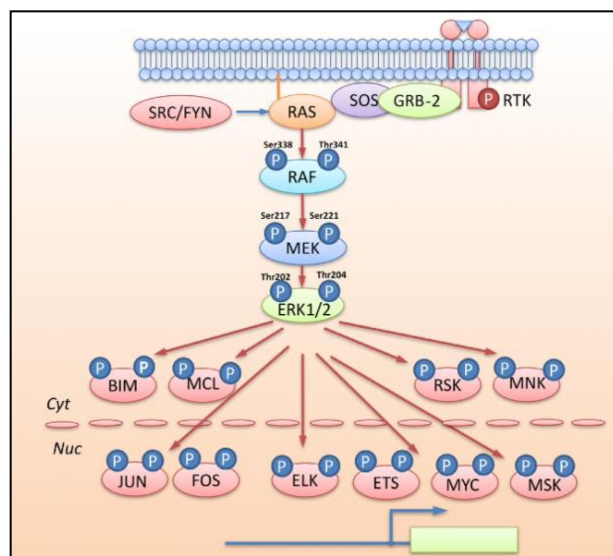


Figure 3: *The major downstream targets of ERK1/2 in the MAPK pathway. ERK regulates both cytosolic targets and nuclear transcription factors* (Liu et al., 2018).

Alongside RAF kinases (ARaf, BRaf and CRaf), additional major RAS effectors are the PI3K/AKT/PTEN pathway and the Ral/RalGDS pathway. Among them, PI3K and Ral pathways are frequently upregulated in melanoma, where they participate in the development of acquired drug resistance, frequently undermining BRAF/MEK target therapies. Furthermore, they crosstalk each other and with the MAPK pathway, thus

participating in melanoma malignant transformation (Barbosa et al., 2021; Cicenas et al., 2017). The constitutive activation of PI3K/AKT pathway in melanoma typically results from increased expression of RTK ligands or mutations in the genes encoding for their cognate receptors (including MET, EGFR, PDGFRβ, IGF1R and KIT, and/or, more frequently, in PI3K/AKT/PTEN/mTOR pathway genes). Loss-of-function mutations in PTEN are the most frequent somatic mutations of the signalling pathway, occurring in about 10% of melanomas and being associated with increased AKT signalling (Mercurio et al., 2021).

The MAPK pathway is particularly important for Melanoma development because of BRAF mutations broad occurrence, representing a critical therapeutic target in cancer therapy. In melanoma, BRAF mutation frequency varies on the basis of the histological subtype, the anatomical location of the tumour and the pattern of sun exposure (Aleksakhina and Imyanitov, 2021).

More than 20 BRAF mutations have been so far described; among them, $BRAF^{V600E}$ mutation is the most prevalent, accounting for 80–90% of all BRAF mutations in melanomas. In particular, the p.V600E mutation results in an amino acid substitution at the position 600 in the BRAF protein, from valine (V) to glutamic acid (E), as a result of the transversion c.1799T > A in exon 15; this mutation increases BRAF kinase activity over Wild Type BRAF. Other activating mutations observed in melanomas are the following: p.V600K (with a prevalence of 7.7%), p.V600R (1%), p.V600M (0.3%) and p.V600D (0.1%). Most of BRAF activating mutations are clustered in two regions of the molecule, specifically the activation domain near the DFG motif (where BRAFV600 mutations occur) and the P-loop (Teixido et al., 2021).

The mentioned activating BRAF mutations are usually mutually exclusive with other melanoma driver mutations. In metastatic lesions the presence of BRAF/NRAS mutations is associated with a shortened survival, while the presence of BRAF/NRAS mutations in primary tumours do not negatively impact progression free or overall survival (Zablocka et al., 2022). Notably, MEK1 mutations are often associated with either BRAF or NRAS mutations. Moreover, it has been suggested that MEK1 mutations in $BRAF^{V600E}$ melanomas are linked to both intrinsic and acquired resistance to BRAF inhibitors, although these studies were disputed by other evidences (Mehnert and Kluger, 2012).

1.4 BRAF inhibitors in melanoma treatment

Because of BRAF involvement in melanoma disease, oncogenic BRAF is targeted in melanoma therapy and BRAF inhibitors are widely used in melanoma treatment. The classification of these compounds is based on the functional conformation assumed by BRAF when the protein binds to the inhibitor (Noeparast et al., 2018):

- In type-I inhibitors (e.g. Vemurafenib, Dabrafenib and Encorafenib), the kinase occupies the ATP binding pocket and it is stabilized in its active conformation (DFG-in);
- In type-II inhibitors (e.g. AZ628, Belvarafenib, TAK-580 (MLN2480)) the kinase binds to a hydrophobic site, which is adjacent to the ATP binding pocket, and it is stabilized in its inactive conformation (DFG-out).

BRAF inhibitors could also activate RAF kinase isoforms (paradoxical effect). This mechanism consists in the association of BRAF with the inhibitor, thus promoting the formation of RAF homo/heterodimers (Peng et al., 2015). RAF dimerization triggers autophosphorylation events, thus potentiating the kinase activity of the complex by transactivation between protomers (Proietti et al., 2020). Notably, BRAF/CRAF heterodimers seem more active in phosphorylating MEK substrates than BRAF or CRAF homodimers. While BRAF inhibitors of both classes stimulate the formation of BRAF-CRAF dimers, type-II inhibitors are considerably less efficient than type-I inhibitors in stimulating the phosphorylation of downstream targets (i.e. MEK kinases) and in activating the MAPK pathway. Interestingly, type-II kinase inhibitors have the potential advantage of exerting a selective action because they are directed towards the most divergent regions of the kinases. Therefore, type-II inhibitors could be exploited to enhance BRAF inhibition as single agents or in combined therapies for melanoma treatment. Unfortunately, they did not hold up to the promises for melanoma treatment so far (Sforza et al., 2022).

The high-affinity chemical structure for BRAF kinase inactivation was identified in the 7-azaindole group, which stabilized the bound protomer in the DFG-in conformation, with the activation loop locked away from the ATP-binding site. Further characterizations identified the first candidate in the PLX4720, which had a high specificity in inhibiting the kinase activity of $BRAF^{V600E}$ mutant protein both in vitro and in vivo, displaying excellent oral bioavailability. Notably, PLX4720 induced a potent MAPK pathways paradoxical activation in treated cancer cell lines (Kim and Cohen, 2016). The following discovery of PLX4032 as a candidate inhibitor of the same class with superior pharmacokinetics in

higher mammals made this compound the most used drug for BRAF^{V600E} melanoma treatment and steered the clinical interest away from PLX4720 (Joseph et al., 2014).

Vemurafenib (PLX4032) is a type-I competitive, serine/threonine kinase inhibitor. It was approved for the treatment of metastatic melanoma by FDA in July 2011 and by EMA in February 2012. PLX4032 resulted efficacious at nanomolar concentrations. It was able to inhibit tumour growth in melanoma xenograft models and, at high dose, it induced tumour regression without toxicity (Lee et al., 2010). Vemurafenib is mainly eliminated by the liver, and it is metabolized by cytochrome P450 (CYP) 3A4 (Zhang et al., 2017). It has been evaluated in many clinical studies and its safety has been widely assessed (Larkin et al., 2014; Schadendorf et al., 2019).

Dabrafenib (GSK2118436) is a type-I kinase inhibitor that belongs to the class of sulfanilides. Dabrafenib was approved for advanced-stage melanoma treatment by both the FDA and EMA in 2013 (Menzies and Long, 2014). It has been recommended its use as a single agent or in combination with Trametinib (selective inhibitor of MEK 1 and MEK 2). Dabrafenib is mainly metabolized by CYP2C8 and CYP3A4 in the liver (Dhillon, 2016).

Encorafenib (LGX818) is a type-I inhibitor that belongs to the class of phenylpyrazoles. It was approved in a combination therapy with the MEK inhibitor Binimetinib by the FDA on June 2018 and by the EMA on September 2018 (Davis, PharmD, BCOP, CPP and Wayman, PharmD, 2022). It is metabolized in the liver mainly through CYP3A4 (Koelblinger et al., 2018). In both phase-I and phase-II trials, at least an adverse effect related to the treatment was always scored (Sun et al., 2018).

BRAF inhibitors achieved improved overall survival over chemotherapy and have been approved for the treatment of BRAF-mutated melanoma. However, most patients develop mechanisms of acquired resistance and about 15% of them do not achieve tumour regression at all, due to intrinsic resistance to therapy. Moreover, early adaptive responses limit the initial efficacy of BRAF inhibition, leading to incomplete responses that may favour the selection of a sub-population of resistant clones and the acquisition of alterations that cause tumour regrowth and progressive disease (Yadav et al., 2019). For these reasons, new compounds are investigated for melanoma treatment.

BRAF mutations are not a prerogative of melanoma, in fact they are also found in about 10% of patients with Colon Rectal Cancer (CRC). These mutations are associated with the following features: female gender, often right-sided, advanced stage, mucinous histology,

defective mismatch repair, and a serrated adenoma pathway (Caputo et al., 2019). Interestingly, BRAF-mutated CRCs are characterized by a dismal prognosis and resistance to standard therapies. Several clinical studies aimed to clarify the role of BRAF mutations as a potential prognostic biomarker in these patients. Current available data derive mainly from patients presenting $BRAF^{V600E}$ mutation, which represents the most common variant. Regardless of the stage of the disease, the presence of this mutation seems related with greater chemoresistance and worse prognosis (Nakayama et al., 2020). Therefore, Kopetz et al. performed a phase II study to investigate the effect of vemurafenib in patients with previously-treated BRAF-mutated Colon Rectal Cancer. Among the 21 patients enrolled, only one reached a partial response, while seven showed stable disease. These results are disappointing, and other studies confirmed the lack of efficacy of BRAF inhibitors in this model (Kopetz et al., 2015).

2. Colon Cancer: etiology and risk factors

Colon cancer is a malignancy derived by the transformation of the cells in the large intestine and it is particularly referred to the final part of the digestive tract. It is sometimes called colorectal cancer (CRC), a term combining colon and rectal cancer that begins in the rectum. In general, colon cancer develops when cells grow and divide uncontrollably. Colorectal cancer is considered the second most lethal cancer and the third most prevalent malignant tumour worldwide (Chen et al., 2020). This disease typically affects old adults, although it could arise at any age (Weinberg and Marshall, 2019). It starts with the polyps, which are small, noncancerous (benign) clumps of cells formed on the inside of the colon. Over time some of these polyps can degenerate and become colon cancers (Huck and Bohl, 2016). Polyps may be small and produce few symptoms. For this reason, it is recommended to make regular screening tests to prevent colon cancer by identifying and removing polyps before they turn into cancer (Stracci et al., 2014). If colon cancer develops, many treatments are available nowadays, including surgery, radiation therapy and drug treatments, such as chemotherapy, targeted therapy and immunotherapy.

Colon cancer can occur in any part of the colon. An examination of the entire colon, known as colonoscopy, is one way to detect colon cancer and polyps (Shine et al., 2020). Signs and symptoms of colon cancer include:

- A persistent change in the bowel habits, including diarrhoea or constipation;
- Rectal bleeding or blood in the stool;
- Persistent abdominal discomfort, such as cramps, gas or pain;

- A feeling that the bowel is not completely empty;
- Weakness or fatigue;
- Unexplained weight loss.

Many patients show no symptoms in the early stages of the disease. When symptoms appear, they depend on the size of the cancer and its location (Holtedahl et al., 2021).

Colon cancer starts in the mucosa, the innermost lining of the colon. It consists of cells that make and release mucus and other fluids. If these cells mutate or change, they may create a colon polyp. Over time, colon polyps may become cancerous. If the polyp is undetected and/or untreated, the cancer spreads through a layer of tissue, muscle and the outer layer of the colon. Finally, colon cancer may also spread and metastasize via lymph nodes or blood vessels (Hossain et al., 2022).

There are four stages of colon cancer carcinogenesis: initiation, promotion, progression and metastasis. The liver is the most common metastatic site, followed by the lung and bone (Riihimäki et al., 2016). It is difficult to determine the duration required for each stage, although decades are necessary to develop the colorectal cancer. These features are described in figure 4:

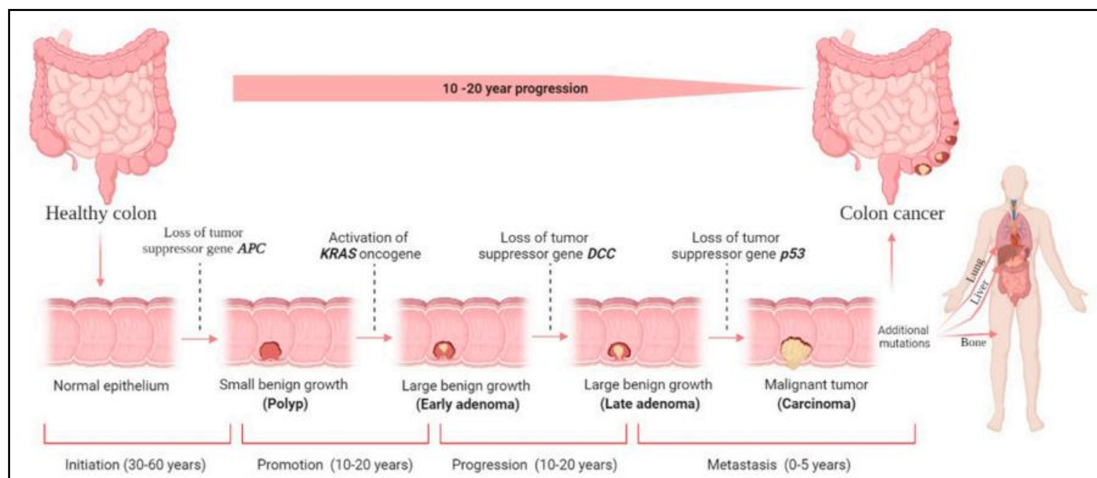


Figure 4: *Colorectal carcinogenesis and development* (Hossain et al., 2022).

It is widely known that certain risk factors increase the probability of developing precancerous polyps and colon cancer. Those risk factors take into account certain medical conditions, including inherited conditions and lifestyle choices. The presence of one or more risk factors for colon cancer implies that the patient has an increased risk of developing the disease. Among the risk factors responsible for colon cancer development, the following are acknowledged: smoking, excessive use of alcohol, obesity, excessive use of red meat and processed meat and, finally, a sedentary lifestyle that excludes physical activity. Alongside the mentioned risk factors, there are some medical conditions that can predispose to colon cancer, such as inflammatory bowel disease, inherited conditions, a family history of colon cancer and/or other kind of cancer, a family history of polyps

development and a high number of polyps (Lewandowska et al., 2022). Up to 70% of CRCs are sporadic and mainly associated with environmental and dietary factors. About 30% of patients with CRC have an inherited predisposition due to several genetic alterations, some of which can be already identified by specific tests (Xi and Xu, 2021).

As previously described for melanoma, colon cancer is also classified basing on the TNM (Tumour-Node-Metastasis) system, which includes 5 stages of colon cancer as described in figure 5. On the base of the gravity and the spread of the disease, the treatment is chosen (Li, 2014).

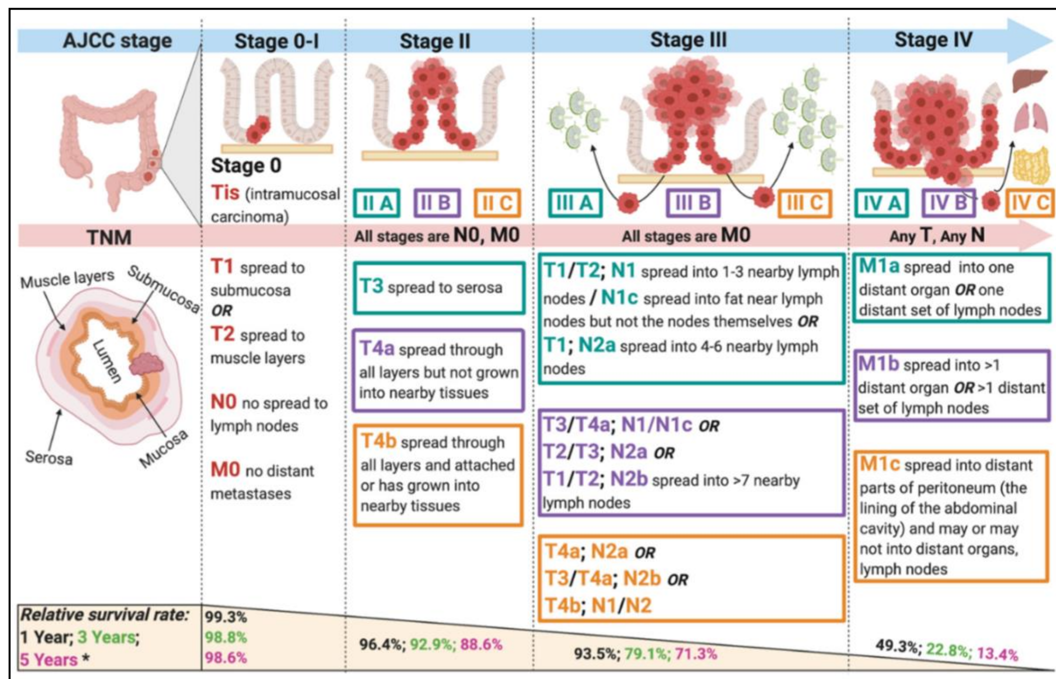


***Figure 5**: Colorectal cancer stages according to the American Joint Committee on Cancer (AJCC). TNM staging is based on the size of the tumour, the growth into lymph nodes and the distant metastases to organs and/or tissues* (Shek et al., 2021).

2.1 Colon Cancer: treatment

Surgery is the most common treatment for colon cancer, which includes polypectomy, partial colectomy, surgical resection with colostomy and radiofrequency ablation. Surgery is generally combined with adjuvant therapy, which is done before or after surgery in order to avoid cancer spread or recurrence. Treatments generally include chemotherapy or targeted therapy against cancer cells (Matsuda et al., 2018). The treatment depends on different factors, including the risk of the cancer coming back and whether there are any other medical conditions.

Common chemotherapy drugs for bowel cancer used before or after surgery are: capecitabine, fluorouracil (5FU), folinic acid (leucovorin or calcium folinate), fluorouracil

and oxaliplatin (FOLFOX), irinotecan (Campto), oxaliplatin and capecitabine (McQuade et al., 2017).

As widely known, chemotherapy uses cytotoxic drugs to destroy cancer cells and the drugs circulate throughout the body in the bloodstream, which makes this approach aspecific with many side effects. On the other hand, targeted therapies act on cancerous cells by directly inhibiting cell proliferation, differentiation and migration (Baudino, 2015). The tumour microenvironment, including local blood vessels and immune cells, might also be altered by targeted drugs to impede tumour growth and enact stronger immune surveillance and attack. Small molecules, such as monoclonal antibodies, are mainly used in targeted therapies (Roma-Rodrigues et al., 2019).

The first targeted agent for CRC approved by the Food and Drug Administration (FDA) was cetuximab in 2004, followed by bevacizumab in the same year, and emerging FDA-approved targeted drugs for CRC have been brought to market successively since then (Xie et al., 2020).

Targeted therapies are sometimes used to treat bowel cancers that have spread to other parts of the body. They may be given on their own or with chemotherapy. Targeted therapies used for colon cancer include: cetuximab (Erbitux®), panitumumab (Vectibix®), bevacizumab (Avastin®), aflibercept (Zaltrap®), ramucirumab (Cyramza®), regorafenib (Stivarga®).

Cetuximab and panitumumab are monoclonal antibodies. They are also called "cancer growth inhibitors" because some cancers have receptors on their surface called epidermal growth factor receptors (EGFRs). Proteins called Epidermal Growth Factors (EGFs) activate the receptors, causing cell growth. In detail, these drugs prevent that the factors bind to the receptors, therefore blocking the signals that allows the growth and division of cancer cells (Moriarity et al., 2016).

Before these drugs are used, cancer cells are analyzed for mutations in the RAS and BRAF genes, in order to decide whether cetuximab or panitumumab is appropriate. In fact, these drugs only work on bowel cancers that have a normal RAS gene and no changes in the BRAF gene (Di Nicolantonio et al., 2008).

Other drugs, such as bevacizumab, aflibercept, ramucirumab, regorafenib are targeted therapy called "angiogenesis inhibitors". They block the chemical signals that cells use to make blood vessels grow, thus making difficult for a tumour to develop the network of blood vessels that it needs to get a blood supply and the tumour does not get the oxygen and nutrients it needs. This finally slows the growth of the tumour (Hansen et al., 2021).

Finally, immunotherapy uses the immune system to better recognize and destroy cancer cells and this approach is used to treat patients with advanced colorectal cancer. Colorectal

cancer cells sometimes use the immune checkpoints to avoid being attacked by the immune system. Drugs that target these checkpoints help to restore the immune response against cancer cells and they are called "checkpoint inhibitors"; among them, Pembrolizumab, Nivolumab and Ipilimumab are known (Makaremi et al., 2021).

3. Epigenetic modifications

Epigenetic modifications influence gene expression without permanent changes in the genomic sequence. These modifications may contribute in tumour transformation, being reversible and quickly regulated compared to gene mutations. These features render epigenetic alterations early targetable. It is widely known that epigenetic modifications are not only related to cancer cell development, but also to the interactions between tumour cells and their Micro-Environment (Li et al., 2021). Epigenetic treatment may therefore benefit cancer patients as monotherapy or combinatorial therapy.

Epigenetic modifications consist in covalent binding of specific groups to DNA, RNA or histone tails. They can be generally categorized into three groups (figure 6):

1) DNA and RNA methylations;
2) histone modifications;
3) non-coding RNAs modifications.

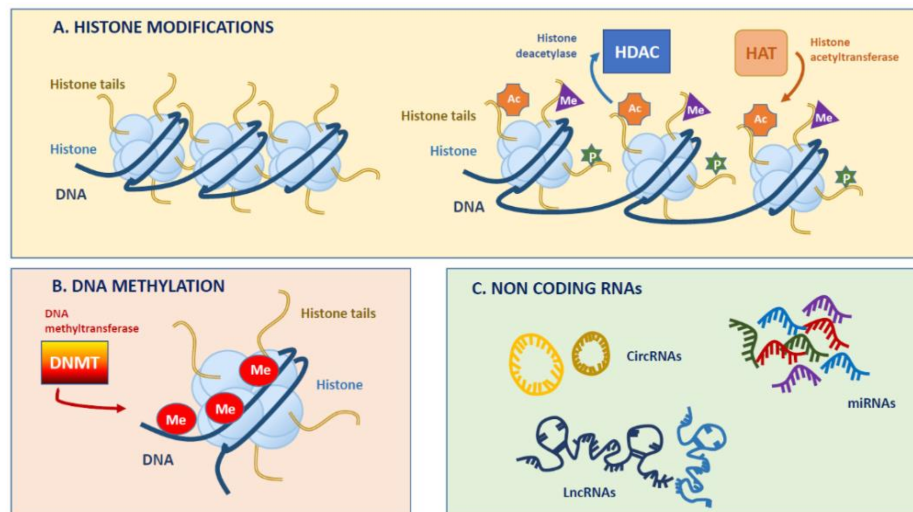


Figure 6: *Overview of the main epigenetic modifications: (A) histone modifications mediated by HDACs and HATs; (B) DNA methylation mediated by DNMTs; (C) Non coding RNAs are also involved in epigenetic modulations (Coco et al., 2019).*

DNA methylation occurs in CpG islands (CGIs), which are preferentially located at the 5′ promoter region of more than 50% of human genes. This mechanism is fundamental in developmental processes, including X chromosome inactivation, embryonic development, genomic imprinting, epigenetic reprogramming, cell identity establishment and lineage

specification. The methylation-dependent gene silencing is mediated via covalent addition of methyl groups from S-adenosylmethionine (SAM) to the 5 position of the cytosine pyrimidine ring. In particular, the structure of 5-methylcytosine (m5C) can either prevent access of Transcriptional Factors (TFs) to the binding sites of DNA, or recruit methyl-binding domain proteins (MBDs) in association with histone modifications to reconfigure chromatin, thus leading to repressive gene expression (Moore et al., 2013).

In detail, three DNA methyltransferases (DNMTs) are responsible for catalysing DNA methylation. DNMT1 is known as the "maintenance" DNA methyltransferase, which has a higher catalytic activity to preferentially methylate hemimethylated DNA during replication and is mostly responsible for maintaining DNA methylation status. DNA methylation status in the genome is instead generated by DNMT3a and DNMT3b, known as "de novo" methyltransferases, which display equal preference in binding to the previously unmethylated DNA independently of replication (Jin and Robertson, 2013). On the other hand, DNA demethylation recovers silenced genes affected by DNMTs. This process is catalysed by a family of Ten-eleven translocation methylcytosine dioxygenases (e.g., TET1, TET2 and TET3), which can turn 5mC to 5-hydroxymethylcytosine (5-hmC), then further oxidizing 5-hmC into 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). Homeostasis between genome demethylation and methylation incurs as a dynamic mechanism of gene expression in various types of cells (Kohli and Zhang, 2013).

Histone modifications also occur in eukaryotic cells. Eukaryotic chromatin organization implies DNA packaging into a compact structure wrapped with histone octamer, which form structures called "nucleosomes" that control the accessibility of DNA sequences. Each histone octamer is composed of a tetramer of two copies of histone 2A (H2A) and two copies of histone 2B (H2B), flanked by dimers of histone 3 (H3) and histone 4 (H4). These histone proteins contain a globular C-terminal domain and an extended N-terminal tail, which are subjected to Post Translational covalent Modifications, including methylation, acetylation, ubiquitylation, phosphorylation, SUMOylation, ADP ribosylation, citrullination and biotinylation at specific amino acidic residues. Among them, acetylation and methylation of lysine residues on H3 and H4 histones have been mostly studied. The mechanism of histone acetylation is based on the "charge neutralization model", according to which the positive charge of lysine residues on H3/H4 facilitates a tight packaging of negatively charged DNA with histones, whereas the addition of an acetyl group can lose up the tight configuration of chromatin, thus enabling the access of transcriptional factors (Krajewski, 2022). As regards histone methylation, this

process is dependent on targeted residues. The equilibrium between methylation, catalysed by different histone methyltransferases (HMTs), and demethylation, catalyzed by histone demethylases (HDMTs), alters the status of transcriptional activity (Bannister and Kouzarides, 2011).

Multiple enzymes are responsible for catalysing the addition and removal of acetyl groups, including histone acetyltransferases (HATs) and histone deacetylases (HDACs) respectively. Lysine acetylation is a post-translastional modification that requires the transfer of an acetyl group from acetyl CoA to a lysine residue, either by a reaction catalyzed by HATs or through an enzyme-free process. Acetylation can alter the biological properties of a protein as well as being the chemical signal for recognition by bromodomains, a protein-protein interaction subunit found in transcriptional activators (Javaid and Choi, 2017).

The reversal of lysine acetylation is accomplished by enzymatic cleavage catalyzed by the HDACs. In humans, there are 18 HDACs that fall into two families (figure 7) based on their catalytic mechanism:

- eleven HDACs (HDAC1-11) are zinc-dependent metalloenzymes that hydrolyze the amide bond using water as a nucleophile;
- seven sirtuins (SIRT 1-7) employ NAD^+ as a cofactor and transfer the acyl group to the C2 position of the ribose sugar.

Although both enzyme families perform the same chemical reaction, the term HDAC usually refers to the zinc-dependent enzymes (Milazzo et al., 2020).

The acetyllysine substrate sits in a narrow channel lined with hydrophobic residues, with a tyrosine residue flipping in conformation to enable hydrogen bonding with the carbonyl oxygen. Noteworthy, the substrate binding to the HDAC enzymes is predominantly through the acetyllysine side chain entering the hydrophobic channel rather than by recognition of the protein backbone.

The compact nature of substrate binding is an advantage for medicinal chemistry, as small molecule inhibitors need only to simulate the acetyllysine residue rather than a longer peptide sequence. Coordination between acetyllysine and the active site zinc cation is critical for substrate binding and catalysis. Indeed, high affinity HDAC inhibitors achieve their potency by zinc coordination, acting as either monodentate or bidentate ligands to mimic the tetrahedral oxyanion intermediate (Lombardi et al., 2011). Therefore, there are

significant variations in the geometry of the active site, the nature of the catalytic residues, the substrate channel, the presence or absence of the internal cavity and the positioning of adjacent protein loops. Selective inhibitors that discriminate between HDAC isoforms take advantage of such differences (Porter and Christianson, 2019).

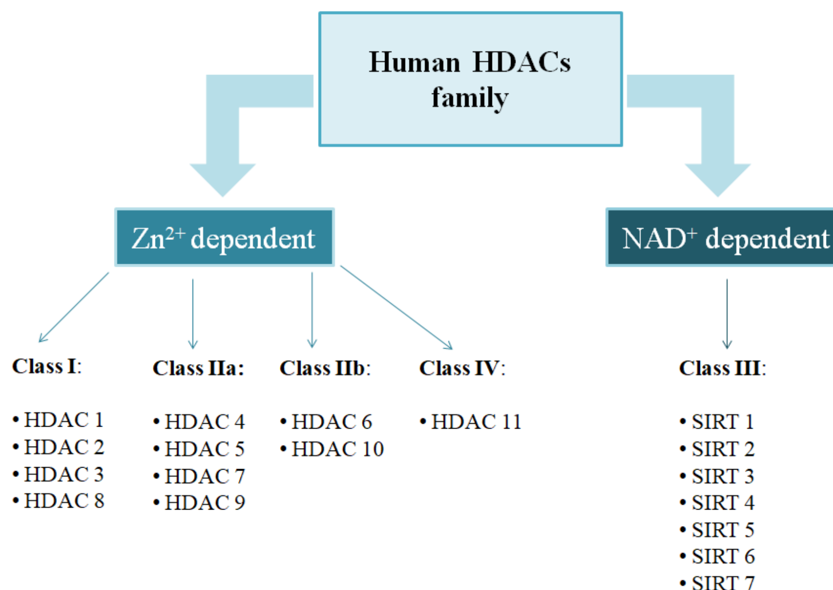


Figure 7: *HDACs classification.*

As mentioned, acetylation and methylation occur in the lysine-rich N-terminal tails of histone proteins, where they play a central role in the epigenetic regulation of chromatin structure and the recruitment of its binding partners to modulate gene transcription. Consequently, the enzymes involved in lysine acetylation and methylation and their removal became important targets for small molecule drug discovery.

3.1 Implications of epigenetics in cancer

Aberrant epigenetic alterations can lead to inappropriate onset of gene expression and promote tumorigenesis. Compounds that act as epigenetic modifiers are promising in multiple cancer therapies. The use of epi-drugs (alone or in combination with chemotherapy or immunotherapy) has shown promising outcomes, including augmentation of anti-tumour effects to overcome drug resistance and activation of host immune response (Nirmaladevi, 2020).

Epigenetic alterations activating oncogenes and/or suppressing tumour suppressor genes (TSGs) are involved in the onset of cancer (Han et al., 2019). DNA methylation acts as a switch controlling the “on” and “off” status of gene expression. The most acknowledged mechanism of epigenetic alterations in cancer cells is the hypermethylation of promoters in

CpG islands, which has been implicated in various cancer types. Many tumour-suppressor genes are under hypermethylation (such as PTEN in melanoma), along with additional genes involved in multiple pivotal cellular functions. By contrast, hypomethylation of oncogenes is commonly reported in multiple cancers, including RBBP6 in colorectal cancer (Lu et al., 2020).

Considering that chromatin is generally hypoacetylated in cancer cells, thus affecting the expression of oncogenes and tumour-suppressor genes, it is not surprising that changes in the equilibrium between the acetylated and deacetylated state of the chromatin may bring to neoplastic transformation. In fact, cancer cells are often characterized by a disequilibrium between the HATs and the HDACs activities and the over-expression of specific HDACs has been frequently found in many tumours. For example, HDAC5 and HDAC6 are specifically overexpressed in melanoma cells and their knockdown prevented proliferation and induced apoptosis in these cells (Liu et al., 2016). In another paper it was shown that exposure of melanoma cells to multiple stresses leads to an increase in HDAC8 activity and the adoption of a drug-resistant phenotype; in particular, HDAC8-mediated BRAF inhibitor resistance was driven via receptor tyrosine activation, leading to MAPK signalling (Emmons et al., 2019). Moreover, it was demonstrated that HDAC3 is overexpressed in colon-cancer and the reduction of its expression in colon cancer cells slows their proliferation and alters the expression of cell cycle regulatory proteins, including p21 (Godman et al., 2008). It was also found that HDAC6 is highly expressed in colon cancer and it is associated with poor prognosis; moreover, HDAC6 knockdown inhibits colon cancer cell growth and migration, partly through the MAPK/ERK pathway (Zhang et al., 2019).

Abnormal epigenetic changes may arise stochastically or be driven by transcriptional program, indicating that the mutations in epigenetic regulators (e.g. DNMTs, TETs, EZH2, etc.) or in specific signalling pathways (e.g. EGFR and KRAS signalling) can modify the epigenome. Moreover, the interactions among different epigenetic mechanisms, acting synergistically or antagonistically, can alter genetic expression (Baylin and Jones, 2016).

Recently, the studies on epigenetics have paid more attention to the Tumour Micro Environment (TME), which is composed of stromal cells, immune cells, extracellular matrix and cytokines, creating a favourable and immune-suppressive niche for tumour cells which is dependent on epigenetic reprogramming. Accumulating evidences indicated that extracellular vesicles (including exosomes, microvesicles, ectosomes, large oncosomes, exosome-like vesicles and apoptotic vesicles) are secreted by many cell types in TME.

Because of their content (DNA fragments, mRNAs and noncoding RNAs), they serve as communicating messengers between cells in early stages of premetastatic niche formation and are also associated with epithelial-mesenchymal transition and metastatic progression. Therefore, it is widely known that tumorigenesis requires an immune-suppressive environment, which enables tumour cells to escape from immune surveillance and T cells-killing (Yang and Wang, 2021).

Cancer cells generally use the inhibitory immune checkpoint pathway to prevent immune system response. The proteins involved in immune-checkpoints are engaged as receptor or ligands in the surface of both T cells and cancer cells (such as CTLA-4/CD80 or CD86, PD-1/PD-L1 or PD-L2, etc.). Induction of those checkpoint proteins can turn off T-cells, which are under epigenetic control in cancers. Repressive histone marks and DNA methylation marks are usually found at the promoters of checkpoint proteins and restoring the epigenetic balance is a strategy under evaluation (Perrier et al., 2020).

3.2 Epigenetic compounds in cancer therapy

The drugs that target the epigenome have been developed more than 40 years ago. They have been widely used in therapy and many epigenetic compounds have been tested in clinical trials for cancer treatments and displayed favourable outcomes (Ganesan et al., 2019).

3.2.1 DNMT inhibitors

DNA Methyltransferase Inhibitors (DNMTIs) are anticancer therapeutic agents that reverse the DNA hypermethylation status of Tumour Suppressor Genes (TSGs). DNMTIs are divided into cytosine analogue inhibitors and non-nucleotide analogue inhibitors. Cytosine analogues can be incorporated into the DNA or RNA backbone, thus disturbing the methylation and inducing DNMTs degradation. These compounds include 5-aza-cytidine (azacytidine), 5-aza-2′-deoxycytidine (decitabine), zebularine, SGI-110, fazarabine, pseudois cytidine, and others (Dan et al., 2019).

Azacytidine and decitabine are cytosine analogues and have been approved by the FDA for the treatment of hematologic malignancies (specifically myelodysplastic syndrome and acute myeloid leukaemia), but they are currently used in different solid tumours. Azacytidine is incorporated into RNA and DNA, while Decitabine is only incorporated into DNA. Decitabine is thus integrated and the decitabine-guanine dinucleotides trap DNMTs with irreversible covalent bindings, thus inactivating DNMTs and removing the

DNA methylation marks on the promoters of TSGs. Furthermore, DNA damage response is triggered and leads to cell cycle arrest, growth suppression and apoptosis. The anti-tumour activities of these two drugs have been determined at relatively low doses in clinical trials (Derissen et al., 2013).

Zebularine (ZEB), 6-thioguanine, and 4′-thio-2′-deoxycytidine, other cytosine analogues, act differently. The class of non-nucleotide analogue inhibitors include small molecules (hydralazine, EGCG, RG108, MG98, disulfiram, etc.) that bind to the catalytic site of DNMTs or to the CpG-rich sequences to prevent that DNMTs bind to the target sequences. Those epi-drugs have lower inhibitory effects to multiple cancer cells compared to the cytosine analogue inhibitors (Sachan and Kaur, 2015). In recent years, many selective DNMT inhibitors have emerged and resulted very promising in cancer therapy. For example, the DNMT1-selective inhibitor GSK-3685032 was shown, via crystallographic studies, to compete with the active-site loop of DNMT1 for penetration into hemi-methylated DNA between two CpG base pairs. GSK-3685032 induces loss of DNA methylation, transcriptional activation and cancer cell growth inhibition *in vitro*. Due to its improved *in vivo* tolerability compared with decitabine, it was found that this selective DNMT1 inhibitor yields superior tumour regression and survival in mouse models of acute myeloid leukemia (Pappalardi et al., 2021).

3.2.2 HDAC inhibitors

Another interesting class of epigenetic compounds is represented by HDAC inhibitors (HDACIs), which can modify the aberrant acetylation status of histones and non-histone proteins. Interestingly, cancer cells exhibit a high sensitivity in response to HDACIs-induced apoptosis and this makes them promising in cancer therapy. Based on their structure, HDACIs can be divided into four groups, as indicated in the following table:

Classification	**Examples**	**Specificity**
Hydroxamic acids	SAHA (Vorinostat) PXD101 (Belinostat) LBH589 (Panobinostat) ITF2357 (Givinostat) 4SC-201 (Resminostat) PCI 24781 (Abexinostat) Tubacin	Pan Inhibitor Pan Inhibitor Classes I and II Pan Inhibitor Pan Inhibitor Classes I and II Class IIb
Aliphatic fatty acids	Butyrate Valproic Acid	Classes I and IIa Classes I and IIa
Cyclic peptides	Depsipeptide/FK228 (Romidepsin) Apicidin	Class I Class I
Benzamide derivatives	MS-275 (Entinostat) MGCD0103 (Mocetinostat)	Class I Class I and IV

	CI-994 (Tacedinaline)	Class I

The widely studied hydroxamic acids contain an hydroxamic acid moiety that binds to a zinc atom, which is a component of the catalytic sites of HDACs, thus inactivating them (Shanmugam et al., 2022). Multiple studies demonstrated their efficacy in treating both hematological malignancies and other solid tumours. Currently, three general hydroxamic acid HDACIs have been approved by FDA (Bondarev et al., 2021):

1. Vorinostat is used for cutaneous T-cell lymphoma (CTCL) (Duvic and Vu, 2007);
2. Belinostat is used for peripheral T-cell lymphomas (PTCL) (Lee et al., 2015);
3. Panobinostat is used for multiple myeloma (Laubach et al., 2015).

In addition, there are other hydroxamic acid displaying inhibitory effects on HDACs either selectively or generally, including resminostat, givinostat, pracinostat, abexinostat and quisinostat. They have been implicated in phase I or II clinical trials for multiple cancers (Mottamal et al., 2015).

To inhibit a HDAC, the logical starting point is to mimic the tetrahedral oxyanion enzyme intermediate and design a molecule that coordinates the active site zinc cation with a slender hydrophobic linker to fit the substrate binding channel. In addition, it is helpful to anchor the molecule at the other end with a cap that can potentially engage in additional binding interactions with the rim of the enzyme (figure 8). In fact, this simple model for a HDAC pharmacophore composed of three elements (zinc binding group, linker and cap) is sufficiently powerful to account for the vast majority of HDAC inhibitors and it is widely used (Dańko et al., 2022).

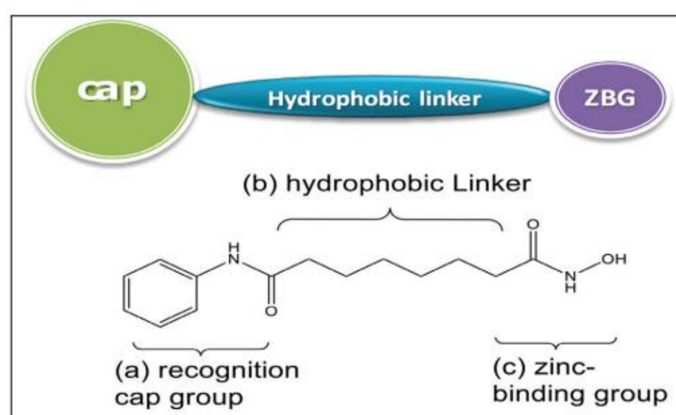


Figure 8: *Pharmacophore requirements for histone deacetylase inhibitors* (Yadav et al., 2019).

Thirty years ago was identified the Streptomyces metabolite **Trichostatin A**, the first potent HDAC inhibitor. Trichostatin A brings the HDAC pharmacophore, where the hydroxamic acid functions as a bidentate zinc chelator and the cap's tertiary amine

substituent, protruding out of the enzyme surface, presumably aids in drug solvation by the aqueous environment (Bouyahya et al., 2022). A masked thiol prodrug features as a reducible disulfide in the bacterial natural products **Romidepsin** (FK228). While trichostatin A, with its simple aromatic cap, is a nonselective HDAC inhibitor, the bicyclic peptide Romidepsin contains larger macrocyclic caps that contribute to their enzyme affinity as well as enable isoform discrimination (Panicker et al., 2010).

Hydroxamic Acids (HA) are intensively investigated in cancer therapy. The ability of the hydroxamic acid moiety to coordinate metals has been exploited for the development of inhibitors targeting metal-bearing enzymes. Their activity resides in the ability to coordinate the catalytic zinc ion in the enzyme binding site, as well as on the presence of specific structural decorations at the "linker" and "cap" regions driving isoform selectivity (Citarella et al., 2021). **Suberoylanilide hydroxamic acid** (SAHA; C14H20N2O3) is the first generation HDAC pan-inhibitor belonging to the hydroxamic acids group approved by FDA. The HDAC catalytic activity inhibition by SAHA is based on its binding to the zinc ion located in the enzyme catalytic domain. It has been demonstrated that SAHA shows anti-proliferative activity on human cancer cell lines (Wawruszak et al., 2021).

3.3 Focus on ITF2357 (Givinostat)

More recently, the general HDAC inhibitor Givinostat (ITF2357) has been synthesized by Italfarmaco. ITF2357 hydroxamic acid-derived compound (figure 9) is able to inhibit class I and II HDACs with anti-inflammatory properties. Previous studies have shown that ITF2357 is selective against HDACs 1, 2, 3, 4, 6, and 7 and it has demonstrated no specificity for class III or IV HDACs. Due to its effectiveness, Givinostat has been used in many clinical trials.

Givinostat was used in phase III clinical trials for the treatment of Duchenne muscular dystrophy. In particular, it was shown that Givinostat significantly reduces fibrosis in muscle tissue and promotes the increase of the cross-sectional area of muscles in mdx mice. Moreover, Licandro et al. demonstrated that *in vivo* treatment with Givinostat was effective in improving muscle morphology in both mdx and D2.B10 mice by reducing fibrosis (Licandro et al., 2021).

Givinostat was also used in a phase II clinical trial for children with active systemic onset juvenile idiopathic arthritis. In fact, it was evaluated as treatment option for this disease, demonstrating the safety of this orally active histone deacetylase inhibitor and its ability to

affect the disease. Givinostat therefore resulted safe and well tolerated, with adverse events being mild or moderate, of short duration and self-limited (Vojinovic et al., 2011).

Other studies indicated that ITF2357 is able to reduce the production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, and IFN-γ) at a low dose (1.0 mg/kg) without adverse cytotoxic effects and it has been demonstrated to be particularly efficacious in cancer treatment (Regna et al., 2014).

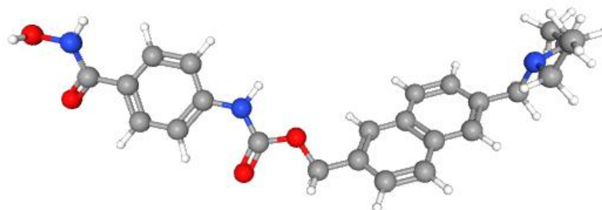


Figure 9: *The molecular structure of ITF2357 (Givinostat).*

Givinostat is a safe and tolerable panHDACi and its anti-tumoral activity has been reported in several hematologic and solid tumor models. At first, the activity of ITF2357 was investigated on multiple myeloma (MM) and acute myelogenous leukemia (AML) cells *in vitro* and *in vivo* by Golay et al.. They showed that ITF2357 has a potent anti-neoplastic activity through direct induction of apoptosis in leukemic cells. Furthermore, this drug inhibited the production of growth and angiogenic factors (in particular IL-6 and VEGF) by bone marrow stromal cells (Golay et al., 2007).

More recently, Di Martile et al. demonstrated that Givinostat induces a mitochondrial apoptosis in human sarcoma cells. Moreover, it enhances *in vitro* Doxorubicin's cytotoxicity in both established and patient-derived sarcoma cells and the combination treatment strongly impaired xenografts tumour growth *in vivo*, when compared to single treatments (Di Martile et al., 2018a).

In another study, Angeletti et al. reported the effect of Givinostat on human Glioblastoma cancer stem cells, taking into consideration the involvement of apoptosis and autophagy. In particular, they demonstrated the inhibitory activity of Givinostat against cancer stem cells proliferation and self-renewal, which allows to consider this compound as a novel possible adjuvant approach for Glioblastoma treatment. Their results also suggest that Givinostat therapeutic efficacy could be intensified by the association with autophagy inhibitors, since they could synergize and revert the resistance of cancer stem cells towards therapeutic interventions, thus reducing the drug concentration required to achieve a significant tumour mass reduction (Angeletti et al., 2016a).

4. Programmed cell death events in cancer

In order to develop effective treatments for cancer and other diseases characterised by abnormalities in cell death regulation, the biochemical mechanisms of specific cell death processes need to be elucidated. Programmed cell death includes a wide variety of processes ranging from classical apoptosis to caspase-independent mechanisms of cell death, including necroptosis and ferroptosis. Autophagy represents a dual function process that can be either pro-survival or death promoting. For this reason, it has been originally classified as type II cell death (Koren and Fuchs, 2021; Peng et al., 2022). Both autophagy and apoptosis will be discussed in the following paragraphs.

4.1 Apoptosis

The most well characterised form of programmed cell death is termed "apoptosis". The word apoptosis was first used in 1972 (Kerr et al., 1972) and the process was first distinguished from necrosis, a passive form of cell death that is induced by external injury and results in the rupture of the cell membrane and the subsequent release of the cell content into surrounding areas, thus activating inflammation (Szabó, 2005). In contrast, apoptosis represents a physiological and controlled death process (genetically determined) that culminates in the formation of apoptotic bodies, which are rapidly recognized by macrophages without a specific inflammatory reaction.

The initiation of apoptosis is dependent on the activation of specific proteins called caspases (cysteine-aspartic proteases). Based on their role, caspases are divided in initiator caspases and executioner caspases (Elmore, 2007). When apoptotic stimuli occur, initiator caspases (caspases 8 and 10) are activated from inactive procaspases and activate by proteolysis the executioner caspases (caspases 3, 6 and 7). The activation of executioner caspases initiates a cascade of events that results in DNA fragmentation, formation of apoptotic bodies and expression of ligands for phagocytic cells (Martinvalet et al., 2005; Poon et al., 2014). Although apoptotic bodies formation has primarily been observed in cell culture, *in vivo* cells, such as macrophages, often remove apoptotic cells before they fragment. It results in a containment of the injured tissue and reduces the risk of collateral damage to surrounding cells. Apoptosis can be initiated when the cell detects damage via intracellular sensors. Alternatively, apoptosis can result from the interaction between a cell of the immune system and a damaged cell (figure 10). Both pathways work synergistically to ensure that multi-cellular organisms remain healthy and defective cells are removed

from the body. Failure to regulate apoptosis can result in many diseases, including neurodegeneration and cancer (Oppenheim et al., 2001; Xu and Shi, 2007).

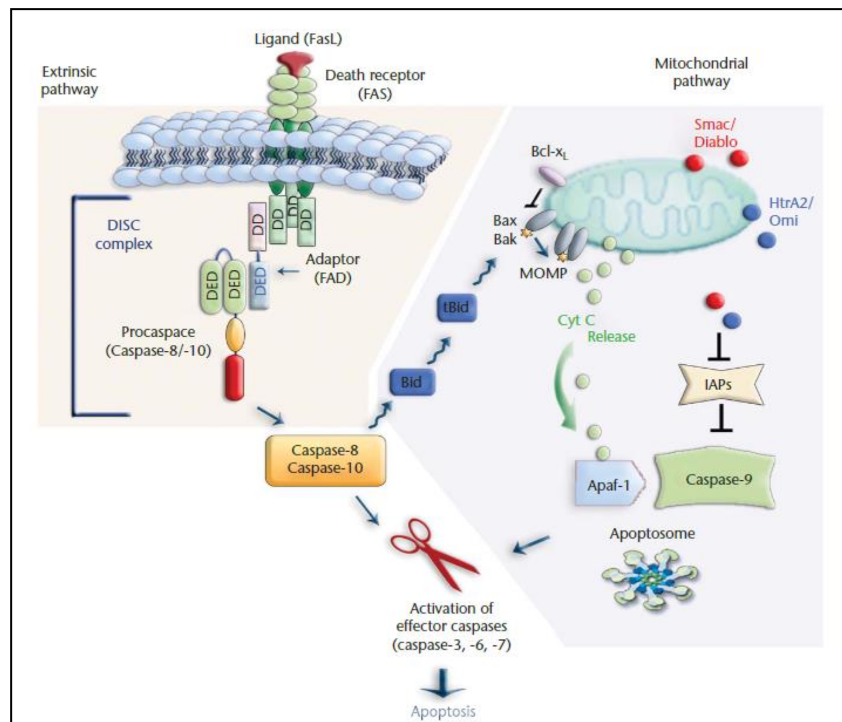


Figure 10: *Extrinsic and mitochondrial (intrinsic) pathways of Apoptosis* (Cairrão and Domingos, 2010).

The canonical *intrinsic pathway* (or mitochondrial pathway) is dependent on factors released from the mitochondria, including cytochrome c, Smac/Diablo and HtrA2/Omi (Igney and Krammer, 2002). Caspase 9 is the initiator caspase that triggers the intrinsic pathway. Specifically, pro-caspase 9 binds to adapter protein Apoptotic Protease Activating Factor 1 (APAF1), following exposure of its caspase recruitment domain (CARD domain). APAF1 in healthy cells is usually folded and its CARD domain is blocked, so that pro-caspase 9 is unable to bind to it. When apoptosis is induced, changes are triggered in the mitochondrial membrane, which result in the opening of the mitochondrial permeability transition pore. As a consequence, pro-apoptotic proteins are able to spread from the mitochondria to the cytoplasm and activate apoptosis (Cain et al., 2002). Cytochrome c induces apoptosis by binding to the WD domain of APAF1 monomers, which results in a conformational change of APAF1. APAF1 exposes a nucleotide binding and oligomerization domain that is able to bind deoxy ATP (dATP). This binding induces an additional conformational change in APAF1, exposing both its CARD and oligomerization domains, thus allowing several APAF1s to assemble into a complex known as “apoptosome” (Acehan et al., 2002). The apoptosome contains in its open centre several exposed CARD domains, which then recruit and activate pro-caspase 9 into active caspase 9. Active caspase 9 is able to activate the executioner pro-caspase 3,

and active caspase 3 can fully induce apoptosis (Wright et al., 2007). Smac/Diablo and HtrA2/Omi help to initiate apoptosis by inhibiting inhibitors of apoptosis proteins (IAPs), although without the release of cytochrome c, inhibiting IAPs alone is not sufficient to initiate apoptosis (Ekert and Vaux, 2005).

Differently from the intrinsic pathway, the *extrinsic pathway* (or death receptor (DR) pathway) is initiated by natural killer cells or macrophages when they produce death ligands, which, upon binding with DRs in the target cell membrane, induce the extrinsic pathway via activation of pro-caspase 8 to caspase 8 (Kim et al., 2005). DRs are members of the tumour necrosis factor (TNF) superfamily and include several members (Bossen et al., 2006), with each DR having a corresponding death ligand. To activate caspase 8, a death ligand binds to a DR, resulting in the recruitment of monomeric pro-caspase 8 via its death-inducing (DED) domain to a death-inducing signal complex (DISC) located on the cytoplasmic domain of the ligand-bound DR. The DISC also includes an adaptor protein known as FAS-associated death domain (FADD) or TNF receptor (TNFR)-associated death domain (TRADD), which facilitates the interaction of pro-caspase 8 to the DISC. The recruitment of several pro-caspase 8 monomers to the DISC results in their dimerization and activation, with the resultant activation of caspase 8, which can then trigger apoptosis execution.

Apoptosis tight regulation is particularly important in cancer. In fact, apoptosis failure in cancer cells might cause a detrimental and uncontrollable cell growth and proliferation (Philchenkov, 2004). It is relevant to consider alternative types of cell death such as autophagic cell death, necroptosis or ferroptosis, for those tumours that developed resistance to classic apoptosis.

4.1.1 Apoptosis induced by HDAC inhibitors

It has been widely demonstrated that HDACIs are able to induce cell death through apoptosis. Induction of apoptosis following HDACi-mediated histone acetylation is well documented and is considered the prevailing form of cell death induced by these compounds. In various tumour cell lines, including breast cancer, colon cancer and others, treatment with HDAC inhibitors induces apoptosis by sequential activation of caspases resulting from both intrinsic and extrinsic pathways (Bolden et al., 2013; Hsieh et al., 2015; Wilson et al., 2010). Moreover, HDAC inhibitors seem to influence the balance between pro- and anti- apoptotic proteins by modulating their expression and promoting the activation of caspases leading to cell death. HDACIs have been shown to selectively

induce apoptosis in transformed cells. For example, Emanuele et al. demonstrated that the pan-HDACI SAHA induces apoptosis in HepG2 and Huh6 hepatoma cells, but not in primary human hepatocytes (Emanuele et al., 2007). More recent studies also demonstrated that the novel HDAC6 inhibitor MPT0G612 preferentially induced apoptosis in colon cancer cells (Chen et al., 2019), and the new HDAC8-selective inhibitor HMC induced caspase-dependent apoptosis in MCF-7 breast cancer cells (Chiu et al., 2019).

4.2 Autophagy

Autophagy is a physiological process, which facilitates the removal and renewal of cellular components of eukaryotic cells and thereby balancing the cell's energy consumption and homeostasis. Deregulation of autophagy is considered a key feature contributing to the development of tumours. In recent years, the suppression of autophagy in combination with chemotherapeutic treatment has been approached as a novel therapy in cancer treatment. However, depending on the type of cancer and the context, interference with the autophagic machinery can either promote or disrupt tumorigenesis. Therefore, disclosure of the major signalling pathways that regulate autophagy and control tumorigenesis is crucial. To date, several tumour suppressor proteins and oncogenes have emerged as eminent regulators of autophagy, whose depletion or mutation favours tumour formation (Maiuri et al., 2009).

During autophagy many cellular components, such as macroproteins or even whole organelles, are sequestered into lysosomes for degradation (Shintani and Klionsky, 2004). The lysosomes are then able to digest these substrates, whose components can be recycled to create new cellular structures and/or organelles or alternatively can be further processed and used as a source of energy. Autophagy can be initiated by a variety of stressors, such as nutrient deprivation (caloric restriction), or it can result from signals present during cellular differentiation and embryogenesis and from signals on the surface of damaged organelles (Mizushima et al., 2008). Autophagy has also been shown to be involved in both adaptive and innate immune system, where it may degrade intracellular pathogens and deliver antigens to MHC class II holding compartments, thus initiating the transportation of viral nucleic acids to Toll-like receptors (Levine and Deretic, 2007). Although autophagy is often used to recycle cellular components, it can result in cell destruction and therefore it has been linked to the removal of senescent cells from aged tissues and destruction of neoplastic lesions. Failure of autophagy potentially allows cancer development, but it has also been associated with the accumulation of protein aggregates in

the neurons and the development of neurodegenerative conditions, including Alzheimer's disease (Nixon and Yang, 2011). Three different types of autophagy have been described (figure 11):

- in "macroautophagy", the most described form of autophagy, whole regions of the cell are enclosed in double-membrane vesicles referred to as autophagosomes. These autophagosomes then fuse with lysosomes to become autophagolysosomes and the contents are degraded by proteases present therein (Mizushima et al., 2010);
- in "microautophagy", the cargo (organelles or regions of the cytosol) directly interacts and fuses with the lysosomes. Therefore, it is more specific than macroautophagy and can be triggered by signalling molecules present on the surface of damaged organelles, such as mitochondria or peroxisomes, resulting in specific fusion of lysosomes with these organelles. Depending on which organelle is targeted, this process is specifically named, for example mitophagy for the mitochondria or peroxophagy for a peroxisome (Li et al., 2012);
- in the chaperone-mediated autophagy (CMA), proteins within the cytoplasm are targeted for the fusion with lysosomes by a cytosolic chaperone through interaction between the chaperone and a pentapeptide present within the amino acid sequence of the substrate. Substrate proteins then bind to a lysosomal receptor LAMP-2A and are carried into the lysosome for degradation (Dice, 2007).

Among them, the most investigated pathway is macroautophagy. Initially, macroautophagy is mediated by ULK1 complex and, in order to form a phagophore, a class III phosphoinositide 3- kinase (PI3K) complex is also required, which consists of 5 sub-units (ATG14L, Beclin 1, VSP34 and VSP15). A complex composed of ATG5, ATG12 and ATG16L, together with lipidated microtubule-associated protein light chain 3 (LC3II) stimulates the elongation of the phagophore and it is essential for the formation of the autophagosome. The protein p62 is another key factor that binds to ubiquitinated proteins and they are thus targeted for degradation (Emanuele et al., 2020). P62 binds to LC3II as the autophagosome is formed, and the target proteins and organelles become engulfed in the newly formed autophagosome. The autophagosome then fuses with the lysosome and the contents are digested (Ndoye and Weeraratna, 2016).

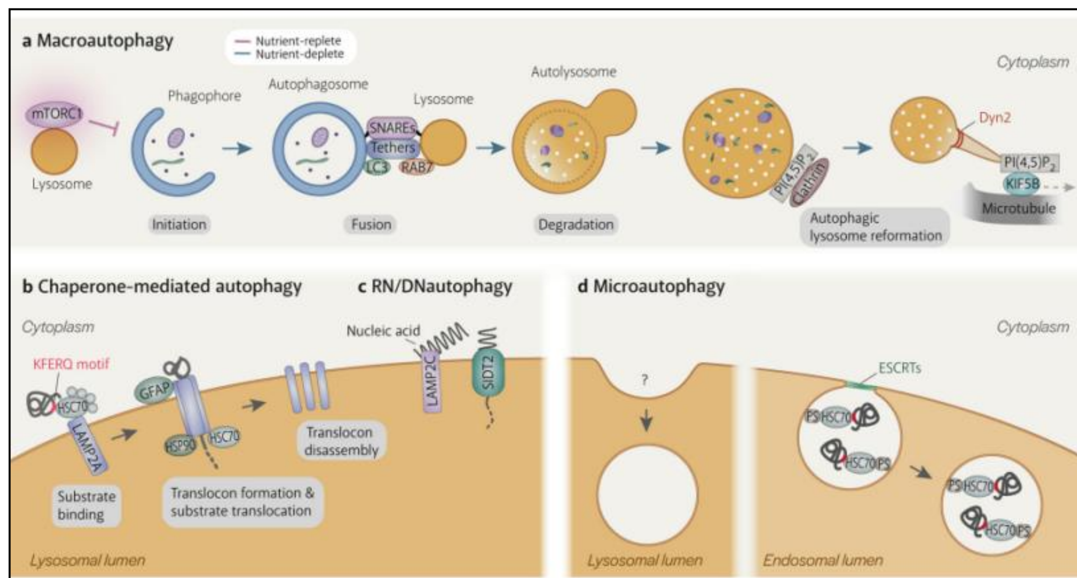


Figure 11: *Representation of the different forms of autophagy* (Yim and Mizushima, 2020).

4.2.1 "Oncojanus" role of Autophagy

Autophagy in cancer displays a double role, since it can be interpreted as a prosurvival process, associated to tumorigenesis and tumour progression, or a cell death process (type II programmed cell death). Autophagy constitutes a possible way to specifically target tumour cells that lost the ability to undergo classic apoptosis, thus displaying drug resistance (Yun et al., 2020).

The role of autophagy in cancer offers high potential for future therapy and is, therefore, currently intensively investigated. Interestingly, in apoptosis-resistant tumour cells autophagy takes on a tumour suppressive function, limiting tumour necrosis and inflammation. In this context, autophagy displays a protective pro-survival role that inhibits the onset of apoptotic and necrotic cell death. Moreover, it can help tumour cells dealing with metabolic stress and overcoming the cytotoxicity of chemotherapy (Chavez-Dominguez et al., 2020). When autophagy has a supportive function in cell death, however, its inhibition in tumour cells will promote tumour survival. Tumour cells could use autophagy for their survival, due to the higher turnover requirements of their metabolism. In this case, disruption of autophagy in combination with chemotherapeutic treatment has been approached intensively in cancer therapy (Sui et al., 2013). On the other hand, autophagic cell death may be exploited when other cell death mechanisms are not effective in cancer cells (Pellerito et al., 2020).

Autophagy generally occurs at a basal level and represents a tumour-suppressor mechanism, whose disruption causes oxidative stress, DNA damage and genomic

instability. Under stressful physiological conditions (including starvation, hypoxia, growth factor withdrawal and senescence, as well as pathological conditions such as tumour) autophagy can be stimulated above basal levels (Aman et al., 2021). Therefore, autophagy in cancer therapy could be either cytoprotective or cytotoxic, as emerged in recent studies. In fact, on one hand autophagy was considered indispensable in the elimination of SAHA-treated apoptosis-resistant uterine sarcoma cells or SAHA and OSU-HDAC42-treated hepatocellular carcinoma cells, and, on the other hand, it was demonstrated that inhibition of autophagy by RNA interference promoted SAHA-induced apoptosis in glioblastoma cells. Many signalling pathways activating or suppressing autophagy have been unveiled for HDACi-mediated autophagy (Mrakovcic et al., 2018). Moreover, in a previous study we demonstrated that autophagic cell death could be strictly related to other cellular processes, such as oxidative stress and ER stress, in colon cancer cells (Celesia et al., 2020).

5. The tumour suppressor protein 53 (TP53) and its key role in cancer

TP53 is a transcription factor that activates genes regulating many processes, including cell cycle regulators such as p21 and pro-apoptotic ones. p53 has occupied a central position in cancer research because of the frequent “loss of function” mutation of TP53 gene in humans and because of its involvement in a wide range of diverse cellular processes.

Originally, p53 was found to be activated in response to various types of DNA damages, but it can also respond to multiple upstream stress signals like oncogene activation, telomere erosion, ribosomal stress and hypoxia. Once activated, p53 can regulate lots of cellular processes like cell cycle arrest, DNA repair, apoptosis, ferroptosis, senescence or autophagy to promote cell survival or limit cell malignant transformation. p53 has a primary role in cancer suppression, and it is also involved in cell metabolism, pluripotency, epigenetic states and aging (Zilfou and Lowe, 2009).

p53 is a homotetrameric transcriptional factor. In its multidomain structure, there are six major protein domains: two intrinsically disordered N-terminal transactivation domains (TADs), a proline-rich domain (PRD), a central deoxyribonucleic acid (DNA)-binding domain (DBD), a tetramerization domain (TD) and an intrinsically disordered C-terminal regulatory domain (CTD) (figure 12). p53 can activate the expression of multiple genes by binding the responsive elements located at target genes’ promoters or enhancers (Okorokov et al., 2006).

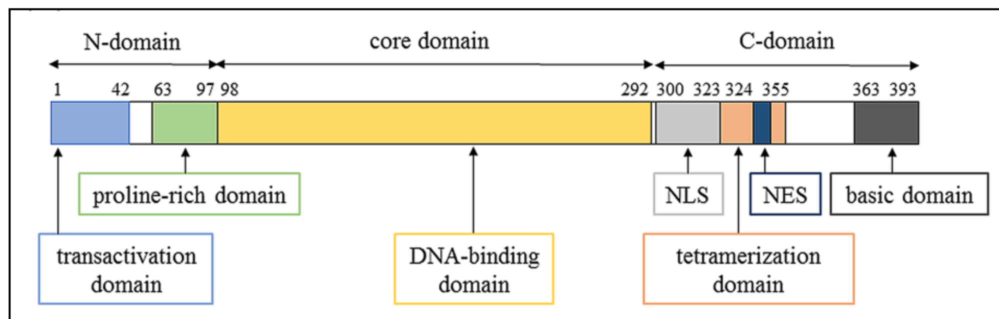


Figure 12: *p53 protein structure* (Tanaka et al., 2018).

p53 post-translational modifications (PTMs) represent the most effective type of regulation. p53 structure undergoes covalent modifications (phosphorylation, ubiquitination, acetylation, methylation, sumoylation, neddylation, O GlcNAcylation, ADP-ribosylation, hydroxylation, and β hydroxybutyrylation), which have some common features:

1. each modification type can occur on multiple sites and, for some amino acids, they can be modified by different chemical groups;
2. the modifications are site-, type- and context-dependent. The same modification at different sites may have different effects, or different modifications can exert similar functions;
3. these modifications are reversible, since for each modification there are one or more corresponding de-modification enzymes;
4. modifications can influence the effects of modifications at other sites (crosstalk).

The working mechanism of these modifications affects p53 stability and localization. This causes protein conformational changes, thus providing interacting partner docking motifs and altering the local electrostatic forces (Liu et al., 2019a).

In time, multiple p53 isoforms have been discovered. Unlike most other tumour suppressor genes, which usually carry inactivating mutations in tumours, most TP53 mutations (~75%) are missense mutations, that result in single amino acid substitutions and in the expression of a full-length protein, but functionally deficient. p53-missense mutations are mainly localized in the DNA binding domain, thus preventing DNA binding and the transactivation of downstream target genes (Kato et al., 2003).

Mutant p53 may also acquire "gain-of-function" activities (GOFs), which allow p53 to bind to other cellular proteins and transactivate new targets. Thus, TP53 mutation may result in tumour-promoting GOF effects and not only in loss-of-function of p53 tumour suppressor activity (Alvarado-Ortiz et al., 2021).

Genetic alterations of p53 tumour suppressor gene are frequent in human tumours. In particular, the prevailing type of mutation is represented by p53 missense mutations resulting in the loss of functional wild-type p53 and its tumour suppressor function. In addition, several mutant p53 variants may have a dominant-negative effect over the remaining wild-type allele or even lead to gain-of-function alleles that carry new oncogenic potential. Interestingly, tumours with a mutant gain-of-function variant of p53 are characterized by higher genomic instability and reduced chemotherapeutic response (Zhu et al., 2020).

Therapeutic targeting of mutant-p53 may have a great impact on cancer therapy. In tumours carrying missense mutant-p53, the main strategy is to restore the normal p53 conformation and function. In this regard, compounds reactivating mutant p53 were studied, such as APR-246, which is now being tested in the clinic (Synnott et al., 2018). It is important to consider that the target is heterogeneous, and the main types of missense p53 mutants are:

- DNA contact mutants (such as His273), that retain wild-type conformation but change amino acid residues that make direct contact with DNA;
- structural mutants (such as His175), that display amino acid substitutions in p53's core domain, thus causing unfolding of the protein and loss of specific DNA binding.

To create new DNA contacts and restore DNA binding is a plausible approach for DNA contact mutants, whereas thermodynamic stabilization should restore structural p53 mutants (Eriksson et al., 2019).

5.1 Targeting p53 in cancer

p53 is deregulated in various diseases and rescuing p53-protective function is a focus in drug discovery. In those patients retaining wild-type p53, its expression, stability, localization and activity can also be disrupted due to different mechanisms.

To recover p53 functions, many efforts have been made on targeting the negative modifications of p53 function. MDM2 (mouse double minute 2) is the major negative regulator of p53 that induces p53 ubiquitination and its subsequent proteasome-mediated degradation (Henningsen et al., 2021).

Many molecules targeting MDM2–p53 interaction have been developed. Among them, Nutlins are imidazoline derivatives that activate p53 pathway by binding MDM2 in the p53-binding pocket and preventing MDM2-mediated p53 degradation. Nutlin treatment

can therefore improve the effect of p53 in mediating cell cycle arrest, apoptosis and growth inhibition. RG7112, the analog of Nutlin, has become the first small-molecule clinically used as MDM2 inhibitor. Additionally, other MDM2 inhibitors (like RG7388, MK8242 and AMG232) have entered the clinic (Burgess et al., 2016). The MDM2-stuctural homologue MDMX has no ubiquitin ligase activity, but it can dimerize with MDM2 and strengthen its function. Therefore, targeting MDMX can promote p53 activation and the first MDMX inhibitor, SJ172550, was developed and shows a therapeutic effect. Moreover, inhibitors targeting both MDM2 and MDMX (such as ATSP-7041) were also developed. Besides inhibiting MDM2 and MDMX function on p53, de-ubiquitinating enzymes that regulate the stability of p53 E3 ligases can also be targeted (Jiang and Zawacka-Pankau, 2020; Klein et al., 2021).

The deacetylating enzymes may be targeted in order to activate p53, since acetylation is critical for p53 activity. A novel compound called Tenovin, which inhibits the deacetylating activities of Sirt1 and Sirt2 to activate p53, has been discovered. Tenovins can delay highly aggressive melanoma cell growth without significant general toxicity, showing a promising clinical potential (Singh et al., 2014).

One major obstacle in targeting p53 modifications is the specificity of the drug, and there are two key points to take into consideration:

- most of these enzymes belong to different groups of proteins with members similar in structure (with similar or different functions), like the DUB family, SIRT family and HDAC family. Moreover, a drug designed for an enzyme may also target another family member;
- most of these enzymes have a large list of substrates. For example, the inhibition of an HDAC may cause a global epigenetic and gene expression change. Therefore, drugs targeting p53 modifications must consider the potential off-target effects.

These compounds were mainly developed to induce wild-type p53 activity. In the future, gene therapy (like adenovirus-based or CRISPR-Cas9-based gene therapy) may serve as an ideal choice in patients bearing mutant modification sites, like K120 mutation in various cancers or R249S mutation in Hepatocellular Carcinoma (Liu et al., 2019a). On the molecular level, p53 activation is obtained by covalent modifications. Nevertheless, how p53 post-translational modifications specify its selectivity for each transcriptional target in order to induce apoptosis or autophagy is still unclear.

As regards autophagy, it was found that nuclear p53 activates autophagy, even though regulatory activities of cytoplasmic p53 protein related to autophagy have been discovered. Notably, p53 elicits either pro- or anti- autophagic responses, based on its localization (figure 13). Thus, p53 is activated and translocates to the nucleus under stress-conditions, where it stimulates pro-autophagic functions, while physiological p53 in the cytosol (that is generally involved in mitochondrial apoptosis) has an inhibitory effect on autophagy under normal conditions (Tasdemir et al., 2008).

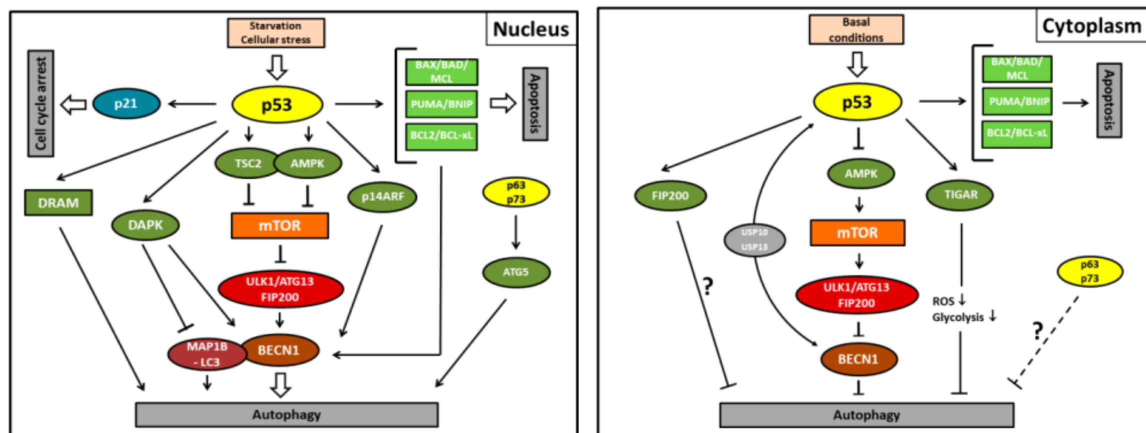


Figure 13: *Autophagy, apoptosis and cell cycle arrest mediated by the activity of nuclear p53 protein (p53) in its function as a transcription factor in the nucleus (under stress conditions) and the cytoplams (under basal conditions)* (Mrakovcic and Fröhlich, 2018).

The effect of HDAC inhibitors on histone deacetylation is associated with chromatin relaxation and re-expression of silenced genes including non-histone proteins, such as p53. Moreover, several p53 acetylation sites have been determined that either augment DNA binding or cause a loss of transcriptional activity. In example, loss of p53-dependent p21 transcription can be caused by deletion of C-terminal acetylation residues (Mrakovcic and Fröhlich, 2018).

In melanoma, p53 gene is rarely mutated (5-17%) and is frequently inactivated. p53 inactivation could be related to the overexpression of MDM2, but MDM2 inhibitor Nutlin-3 causes only modest p53-mediated cell death in melanoma. Moreover, phosphorylated nuclear iASPP (Inhibitor of apoptosis-stimulating protein of p53) has been shown to correlate with MDM2 overexpression in wild-type p53 melanoma cells; this strongly suggests to co-target both MDM2 and iASPP to reactivate p53. Finally, one of the key mechanism of p53 inactivation in melanoma is the upregulation of MDM4 (Gembarska et al., 2012), which renders most primary melanoma cultures immune to specific MDM2 inhibition. However, in mutant BRAF vemurafenib-resistant melanoma cell lines, MDM4

is found weakly expressed and these cells may use other mechanisms causing p53 inactivation (Box et al., 2014).
Aberrant expression of additional p53 co-factors (directly binding p53) and regulators (modulating p53 activity) has been also described in melanoma, including PIASy (protein inhibitor of activated STAT), the histone acetyl transferase Tip60 (HTATIP), Y box binding protein 1, p63 and p73. Interestingly, most p53 activators are selective, targeting one of the regulators and leaving the other free to interact with p53 and to operate. Mutated or inactivated p53 may therefore represent a key therapeutic target for melanoma (Krayem et al., 2019).

In conclusion, many p53 mutations have been described in melanoma cells and, interestingly, some of these mutations change p53 function and turn this protein into an oncogene. For example, in the literature is reported that mutational analysis of p53 gene in separate studies identified different p53 mutations in SK-MEL-28 melanoma cells (Abd Elmageed et al., 2009). In particular, it was shown that SK-MEL-28 cells display the "likely oncogenic mutation" L145R (Ikediobi et al., 2006). On the other hand, in another paper it is indicated that SK-MEL-28 cells display the "gain of oncogenic function" mutation R273H, a hot spot mutation resulting in the substitution of arginine for histidine at codon 273. This specific mutation leads to gain of function, loss of tumour suppressor functions and acquisition of new oncogenic activities (Vasconcelos et al., 2018). Therefore, p53 status may account for the drug-resistance towards anti-tumour drugs observed in some melanoma cell lines and needs to be further investigated.

II. OBJECTIVES AND EXPERIMENTAL MODELS

The general objective of this PhD project was to evaluate the effects of HDAC inhibitors (HDACIs) on oncogenic BRAF and autophagy in melanoma and colon cancer cells bearing the oncogenic $BRAF^{V600E}$ mutation. Specifically, ITF2357 was chosen as the HDAC inhibitor used in most experiments for its high efficacy. The specific objectives can be summarized as follows:

FIRST PART

1) To assess whether ITF2357 targets oncogenic BRAF in melanoma cells. For this purpose, SK-MEL-28 and A375 BRAF-mutated melanoma cells were used;
2) To evaluate autophagic/apoptotic effects in melanoma cells after treatment with ITF2357;
3) To examine the localization and the functional role of oncogenic BRAF;
4) To characterize a possible interplay between oncogenic BRAF and p53 and to assess whether p53 status in melanoma cells influences the response to ITF2357.

This part was mainly focused on melanoma models and represented the main part of the project. In particular, SK-MEL-28 and A375 cells were chosen, since they both display $BRAF^{V600E}$ mutation but differ in p53 status. These features are summarized in figure 1.

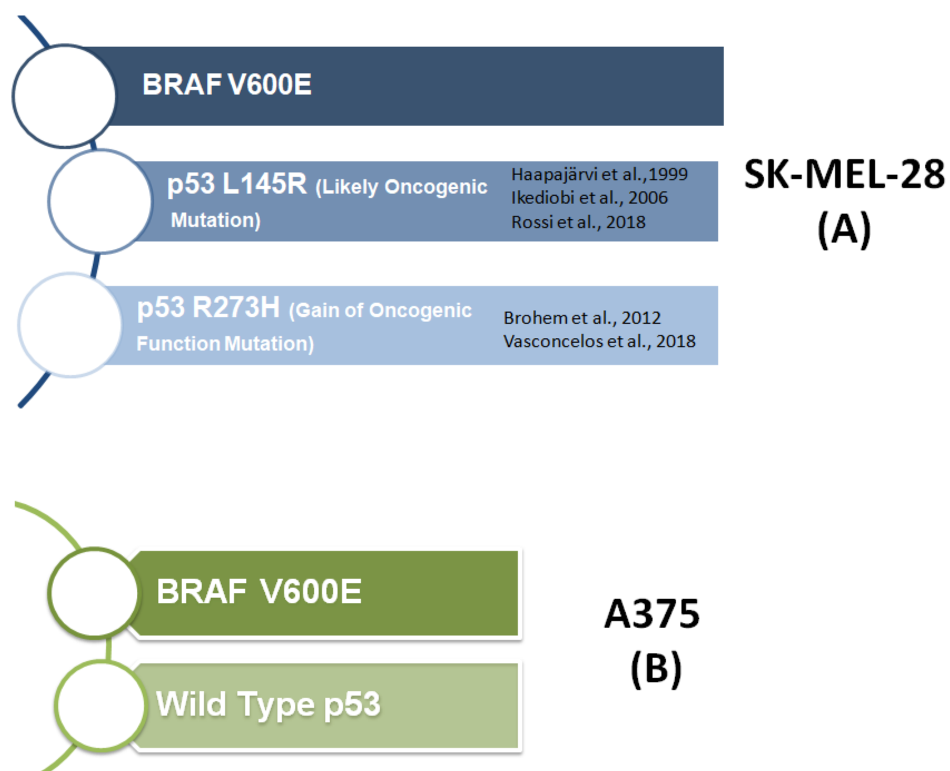


***Figure 1:** Genetic profile of SK-MEL-28 (A) and A375 (B) melanoma cell lines.*

SECOND PART

This part of the project was mainly focused on colon cancer and was developed during a six months period abroad during the third year of PhD. Three months were spent in Prof. Christopher Gregory's laboratory at the Centre for Inflammation Research (CIR, University of Edinburgh, U.K.) to focus on apoptosis. In particular, the objective of this part was to achieve a new technique to produce and isolate apoptotic extracellular vesicles (Apo-EVs) from colon cancer cells and to analyze them using the Nanoparticle Tracking Analysis (NTA).

For other three months, the project was carried out at the laboratory of epigenetics directed by Dr. Parinaz Mehdipour at the Ludwig Institute for Cancer Research (University of Oxford, U.K.). The general aim of this part of the project was to evaluate the efficacy of the combination of ITF2357 with other epi-drugs, including DNA methyltransferase inhibitors (DNMTIs) in colon cancer cells.

The specific objectives of this part of the project include:

1) To evaluate the effects of ITF2357 alone in colon cancer cells;
2) To assess whether generic and/or selective DNMT inhibitors increase the anti-tumour efficacy of ITF2357;
3) To characterize the immunogenic response induced by DNMTIs in combination with ITF2357, focusing on the modulation of Interferon-mediated genes and on the production of double stranded RNAs (dsRNAs) in colon cancer cells.

For these purposes, BRAF- and p53- Wild Type HCT116 and $BRAF^{V600E}$ and $p53^{R273H}$ HT29 colon cancer cells were used. The genetic features are reported in figure 2.

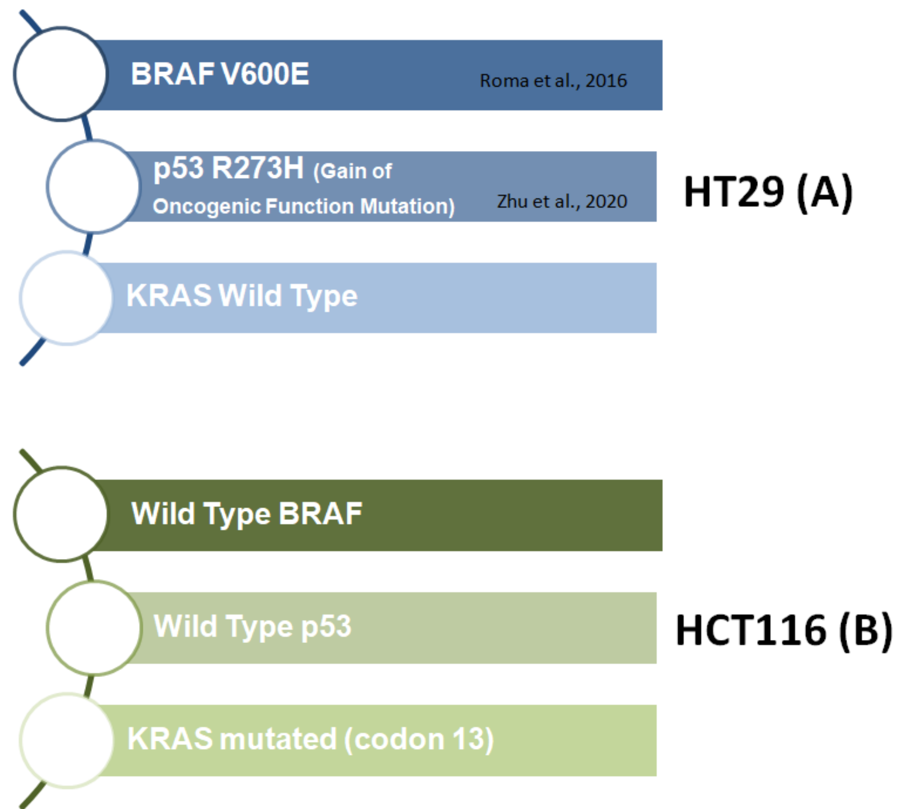


Figure 2: *Genetic profile of HT29 (A) and HCT116 (B) colon cancer cell lines.*

III. MATERIALS AND METHODS

1. *Chemicals and reagents*

ITF2357 (Givinostat) and SAHA (Vorinostat) were synthesized and kindly provided by Italfarmaco, Cinisello Balsamo, MI, Italy. For in vitro experiments, ITF2357 and SAHA were dissolved in DMSO (20 mM stock solution) and stored at -20°C. Prior to use, stock solution was diluted in DMEM culture medium, not exceeding 0.01% (v/v) DMSO to realize the proper final concentrations. Equal volumes of DMSO were added to untreated cells (control–CTR) as vehicle control. The autophagy inhibitors (Bafilomycin A1 and 3-methyladenine), the proteasome inhibitor (Bortezomib) and the protein synthesis inhibitor (Cycloheximide) were purchased from Sigma-Aldrich (Milan, Italy). The general caspase-inhibitor z-VAD-fmk was purchased from Promega (Milan, Italy). Prior to use, stock solutions were opportunely diluted in DMEM culture medium, not exceeding 0.01% (v/v) DMSO, to realize the proper final concentrations. To study the MAPK pathway, the MEK inhibitor U0126 was used (Merck S.r.l., Milan, Italy), which was diluted in DMEM culture medium prior to use. The DNMT inhibitor Decitabine (DAC – Sigma-Aldrich A3656) and the selective DNMT1 inhibitor (DNMT1i) GSK-3685032 (MedChemExpress MCE) were kindly provided by Dr. Parinaz Mehdipour (Ludwig Institute for Cancer Research, University of Oxford, U.K.). For in vitro experiments, DAC was dissolved in PBS and DNMT1i in DMSO (10 mM stock solution) and stored at -80°C. Prior to use, stock solution was diluted in PBS (DAC) or McCoy culture medium (DNMT1i).

2. *Cell Cultures*

Human melanoma SK-MEL-28 (American Type Culture Collection, ATCC, Milan Italy) and A375 (gently provided by Prof. Mario Allegra, University of Palermo, Palermo, Italy) cell lines were grown in monolayer in 75 cm^2 flasks in DMEM medium, supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 50 µg/mL streptomycin in a humidified atmosphere of 5% CO^2 in air at 37°C. For the experiments, cells were seeded at a density of 5×10^3 (SK-MEL-28) or 7×10^3 (A375) in 96-wells plates and at a density of 1.5×10^5/well (SK-MEL-28) or 1.8×10^5/well (A375) in 6-wells plates, respectively, and allowed to adhere overnight. Subsequently, cells were treated with the chemicals or vehicle only and the incubation was protracted for the established times. All materials for cell cultures were purchased from Euroclone (Pero, Italy) and Life Technologies Ltd. (Monza, Italy).

HCT116 and HT29 cells used in our lab (American Type Culture Collection, ATCC, Milan Italy) were also used in Dr. Parinaz Mehdipour's laboratory (Ludwig Institute for Cancer Research, University of Oxford, U.K.) during the period abroad. These cells were grown in

McCoy's 5A (Modified) Medium (Thermo Fisher Scientific) and were kept as previously described. For the experiments, 1.4×10^5 cells/well (HCT116) or 1.6×10^5 cells/well (HT29) were seeded in 6-wells plates.

3. *Evaluation of cell viability (MTT assay)*

The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a mono-tetrazolium yellow salt. Reduction of MTT results in the disruption of the core tetrazole ring and the formation of a violet-blue water-insoluble molecule called formazan. The MTT reagent can pass through the cell membrane as well as the mitochondrial inner membrane of viable cells (presumably due to its positive charge as well as its lipophilic structure) and it is reduced to formazan by metabolically active cells. The chromogenic nature of this redox chemical reaction provides a colorimetric-based measurement of intracellular formazan production, which is detected through a spectrophotometric read (Ghasemi et al., 2021). SK-MEL-28 and A375 cell viability was determined by MTT assay. For these evaluations, SK-MEL-28 and A375 melanoma cells were seeded in 96-wells plates and treated with various concentrations of ITF2357 and SAHA for different times. After treatment, MTT (Sigma-Aldrich, Milan, Italy) was solubilised in PBS (5.5 mg/mL) and 20 µL/well were added prior incubation at 37°C for 2 h. The medium was then removed and cells were lysed in 100 µL/well of lysis buffer (20% sodium dodecyl sulfate in 50% N,Ndimethylformamide). Finally, the absorbance of the formazan was measured at 490 nm with 630 nm as a reference wavelength using an automatic ELISA plate reader (OPSYS MR, Dynex Technologies, Chantilly, VA, USA) as previously described (Carlisi et al., 2015). Values reported in figures are expressed as percentage of the viability of treated cells compared with vehicle-treated (untreated control CTR, 100% viability). The experiments were performed in triplicate and data are shown as mean ± SD of three independent experiments. IC50 were determined using the IC50 Calculator AAT Bioquest.

4. *Evaluation of autophagy by Monodansylcadaverine (MDC staining)*

Monodansylcadaverine (MDC) staining was performed to evaluate the formation of autophagic vacuoles. SK-MEL-28 cells (7×10^3/200 µL culture medium) were seeded in 96-wells plates and treated with ITF2357 (5 µM). After 16 h treatment, cells were incubated with 50 µM MDC (Sigma Aldrich, Milan, Italy) for 10 min at 37°C in the dark. Cells were then washed with PBS and analyzed by fluorescence microscopy using a Leica DMR (Leica Microsystems, Milan, Italy) microscope equipped with a DAPI filter system (excitation wavelength of 372 nm and emission wavelength of 456 nm). Images were

acquired by computer imaging system (Leica DC300F camera, Milan, Italy). Three different visual fields were examined for each condition.

5. *Detection of chromatin condensation by Hoechst 33342 staining*

Cell death was assessed by staining the cells with the vital dye Hoechst 33342 (Sigma Aldrich, Milan, Italy), which shows nuclei and allows the detection of chromatin condensation and fragmentation. For these experiments, 7×10^3 cells/well were seeded in 96-wells plates, incubated with the compounds for the established times and then stained with Hoechst (2.5 µg/mL medium) in the dark for 30 min. After washing with PBS, cells were visualized using an inverted Leica fluorescent microscope (Leica Microsystems, Wetzlar, Germany) with a 4*0*,6-diamidino-2-phenylindole dihydrochloride (DAPI) filter (excitation wavelength of 372 nm and emission wavelength of 456 nm). Images were acquired through a computer imaging system (Leica DC300F camera, Milan, Italy). Three different visual fields were examined for each condition.

6. *Fluorescent staining with Annexin V-FITC/PI*

In most normal and viable eukaryotic cells, the negatively charged phospholipid phosphatidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer. PS redistribution from the inner to the outer leaflet is an early and widespread event during apoptosis. Annexin V is a 35 kDa phospholipid-binding protein and a major cell membrane component of macrophages and other phagocytic cell types that has a high affinity to PS in the presence of physiological concentrations of calcium (Ca^{2+}) (Crowley et al., 2016). This staining is used to detect apoptosis and necrosis. For these evaluations, we purchased the kit from Miltenyi Biotec (n. 130-092-052). In detail, SK-MEL-28 (1.8×10^5 cells/well) and A375 (2×10^5 cells/well) were seeded in 6-wells plates and, after 24 hours, were treated with ITF2357 5 µM (SK-MEL-28) or 2 µM (A375) for further 24 or 48 hours. Then, cells were counted and 1×10^6 cells per condition were stained with Annexin V and PI, as indicated in the datasheet. Then, approximately 10.000 gated events were acquired for each sample on a FACSCanto cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry data were analyzed using FlowJo software (v10; TreeStar, Ashland, OR, USA).

7. *Western Blot analysis*

Western Blot analysis is used to evaluate a target protein contained in a protein sample. In general, this analysis requires the following steps:

- the protein samples are run in a polyacrylamide gel, allowing protein discrimination based on the molecular weight;
- the proteins are transferred from the gel to a nitrocellulose membrane (blotting);
- the membrane is incubated in a blocking solution (5% milk in TBS-T) for 1 hour to avoid any aspecific signal;
- the membrane is incubated with the specific primary antibody (O.N. - 4°C), which binds the target protein, and then with the secondary antibody (1 hour - room temperature);
- the chemiluminescent signal relative to the protein of interest is detected as a band.

For Western blot analysis, whole-cell extracts were prepared in ice-cold lysis RIPA buffer (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS, pH 7.4), supplemented with a protease inhibitor cocktail (10X), and subjected to SDS PAGE and consequent immunoblot after inactivation at 95°C for 5 minutes. In these experiments, the correct protein loading was verified by both Ponceau red staining and housekeeping protein γ- or α-tubulin immunodetection as previously described (Celesia et al., 2020). The following is a table of the specific primary antibodies used for these evaluations:

Antibody	**Source**	**Supplier**	**Dilution**
BRAF (sc-6284)	Mouse	Santa Cruz Biotechnology (St.Cruz, CA, USA)	1:500
ERK1/2 (sc-514302)	Mouse	Santa Cruz Biotechnology (St.Cruz, CA, USA)	1:500
P-ERK1/2 (sc-81492)	Mouse	Santa Cruz Biotechnology (St.Cruz, CA, USA)	1:500
Caspase 3 (sc-7272)	Mouse	Santa Cruz Biotechnology (St.Cruz, CA, USA)	1:500
p53 DO1 (sc-126)	Mouse	Santa Cruz Biotechnology (St.Cruz, CA, USA)	1:500
H3 Histone (07690)	Rabbit	Upstate (Merck KGaA, Darmstadt, Germany)	1:1000
H4 Histone (sc-25260)	Rabbit	Santa Cruz Biotechnology (St.Cruz, CA, USA)	1:500
H3Ac-histone (sc-8655)	Mouse	Santa Cruz Biotechnology (St.Cruz, CA, USA)	1:500
H4 Ac-histone (sc-8661)	Rabbit	Santa Cruz Biotechnology (St.Cruz, CA, USA)	1:500
γ-tubulin (T6557)	Mouse	Sigma-Aldrich (Milan, Italy)	1:1000
LC3 (L8918)	Rabbit	Sigma-Aldrich (Milan, Italy)	1:1000

p62 (P0068)	Rabbit	Sigma-Aldrich (Milan, Italy)	1:1000
ATG7 (2631)	Rabbit	Cell Signaling Technology (Beverly, MA, USA)	1:1000
Beclin-1 (3738)	Rabbit	Cell Signaling Technology (Beverly, MA, USA)	1:1000
Caspase 9 (9502)	Rabbit	Cell Signaling Technology (Beverly, MA, USA)	1:1000
p-MDM2 (3521)	Rabbit	Cell Signaling Technology (Beverly, MA, USA)	1:1000
PARP (C2-10)	Rabbit	R&D Systems	1:1000
H3 Ac-histone (9677S)	Rabbit	Cell Signaling Technology (Beverly, MA, USA)	1:3000
H3 Histone (4499S)	Rabbit	Cell Signaling Technology (Beverly, MA, USA)	1:3000
DNMT1 (D63A6)	Rabbit	Cell Signaling Technology (Beverly, MA, USA)	1:3000
α-tubulin (T6199)	Mouse	Sigma-Aldrich (Milan, Italy)	1:3000

For the immunodetection, the secondary antibody anti-mouse (Promega – W4021) or anti-rabbit (Promega – W4011) were used, based on the source of the primary antibody. Immunodetection was carried out by electrochemical luminescence labelling system (ECL) using ChemiDoc, XR Image system (Bio-Rad Laboratories, Hercules, CA, USA). The intensity of the protein bands was quantified by using Quantity One Imaging Software (BioRad Laboratories) and reported as the ratio of the intensity of protein bands normalized to γ-tubulin or α-tubulin, versus the intensity of untreated control samples (CTR) with only vehicle, if not differently indicated. The blots shown in figures are representative of three or two independent experiments.

8. Semiquantitative RT-PCR

The reverse transcription-polymerase chain reaction (RT-PCR) is a technique that uses a very small amount of material (usually total RNA). The RNA is copied by reverse transcription (RT) to produce single-stranded, complementary DNA (also known as first-strand cDNA). The cDNA obtained is less prone to degradation than RNA and it can then be amplified by PCR and quantified to determine the relative abundance of expressed genes within and between sample groups (Walker et al., 2002).

RNA samples used in these analyses were extracted by using Direct Zol RNA Mini-Prep (Zymo research, Freiburg, Germany). A DNase I treatment step was included. One microgram of total RNA was reverse-transcribed in a final volume of 20 μL by using

QuantiTect® Reverse Transcription Kit (Qiagen, Germany). The resulting cDNAs were used for semi-quantitative PCR (RT-PCR) by using Euro Taq thermostable DNA polymerase kit (Euroclone, Mi, Italy), according to the manufacturer's instructions. Primers against BRAF were the following:

- BRAF-51F (forward 5*0*-CTACTGTTTTCCTTTACTTACTACACCTCAGA-3*0)*
- BRAF-176R (reverse 5*0*-ATCCAGACAACTGTTCAAACTGATG-3*0*)

The cycling protocol used was as follows: initial denaturation at 95℃ for 5 min, followed by denaturation at 95℃ for 30 sec, annealing at 62℃ for 50 sec, extension at 72℃ for 30 sec for 35 cycles and a final extension at 72℃ for 5 min. Primers against 18S rRNA were used as control to demonstrate the equal loading of RNA (initial denaturation at 95℃ for 3 min, denaturation at 95℃ for 1 min, annealing at 55℃ for 1 min, extension at 72℃ for 1 min for 25 cycles, followed by a final extension at 72℃ for 10 min). The amplified products were resolved by agarose gel electrophoresis (1% agarose, 0.5 µg/mL ethidium bromide; Sigma-Aldrich, Milan, Italy) and the bands were visualized and photographed with ChemiDoc XRS (Bio-Rad Laboratories Srl, Milan, Italy). Data processing and densitometric analysis were performed by using Quantity One Analysis Software from Bio-Rad Laboratories.

9. Extraction of cytosolic and nuclear fractions

SK-MEL-28 and A375 cells were seeded in 75 cm^2 flasks (1×10^6 cells/10 mL culture medium). Two days later, cells were treated with ITF2357 10 µM (SK-MEL-28) or 2 µM (A375) and, after 24 h, were lysed. To collect the extracts, cells were washed in PBS and scraped with 500 µl of subcellular fractionation buffer (250 mM Sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and protease inhibitors, pH 7.4) as previously described (Cernigliaro et al., 2019). Next, cells were passed 10 times through a needle of 25G and then kept on ice for 20 minutes. The homogenates were centrifuged at 720×g for 5 min at 4°C. The pellets were resuspended in lysis buffer and passed 10 times through a needle of 25G and centrifuged at 720×g for 10 min at 4°C. The pellets of the second centrifugation (nuclear fraction) were lysed with nuclear buffer (standard lysis buffer with 10% glycerol and 0.1% SDS - 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, inhibitors of proteases: 25 µg/mL aprotinin, 1 mM PMSF, 25 µg/mL leupeptin and 0.2 mM sodium pyrophosphate) and sonicated. The supernatants obtained from the first centrifugation were considered as cytosolic fractions. Nuclear and cytosolic fractions were used to evaluate B-Raf and p-ERK through Western

Blot analysis, while β-tubulin and H3 Histone (Sigma-Aldrich) were used as cytosolic and nuclear markers, respectively.

10. Immunoprecipitation analysis

Co-immunoprecipitation analysis is used to evaluate the possible interaction between a given protein and other proteins of interest. In general, the procedure requires an initial interaction between the protein of interest and its specific antibody to form the immunocomplex. The immunocomplexes are incubated with agarose beads and, after they are bound, they are centrifuged to remove the supernatant. The beads are then diluted in sample buffer 2X and the immunocomplexes are denaturated at 95°C for 5 minutes. Finally, the proteins contained in the immunocomplexes are analyzed by Western Blot analysis. The general protocol used for these evaluation was previously described (Blasio et al., 2019).

For these experiments, SK-MEL-28 and A375 cells were seeded in 75 cm^2 flask (1×10^6 cells/10 mL culture medium). Two days later, cells were treated with ITF2357 10 μM (SK-MEL-28) and 2 μM (A375) for 24 hours. The following day, cells were washed in PBS and lysed with modified RIPA buffer (50 mM HEPES, 150 mM NaCl, 1% TRITON, 10% glycerol, 1,5 mM MgCl2 and 1 mM EGTA and protease inhibitors). Cells were then scraped and the obtained lysates were collected and incubated on ice for 20 minutes. The lysates were passed 10 times through a needle of 25G and the homogenates were centrifuged at 13.000 x g for 10 min. Afterwards, the supernatants were used for a protein assay and, to immunoprecipitate the target protein, equal amounts of proteins (600 μg) were incubated with mouse monoclonal BRAF (Santa Cruz: sc-5284) or p53-DO1 (Santa Cruz: sc-126) antibody for 2 h on ice with a concentration of 1 μg antibody/1 mg protein in a final volume of 600 μl. Then, 30 μl of A/G agarose beads (Santa Cruz 5284) were added and the incubation was protracted overnight at 4°C to allow the interaction between the beads and the antibody-protein complexes. The following day, the immunocomplexes were centrifuged at 10.000 x *g* for 5 minutes and the beads were washed three times in PBS. Once all the PBS was removed, the immunocomplexes were dissolved in 2X sample buffer at 90°C for 5 min and the samples were centrifuged to separate the supernatants from the beads. The supernatants were collected for the gel loading and the proteins were analyzed by Western Blot analysis with different antibodies (BRAF or p53). The data shown are representative of two different experiments.

11. p53 siRNA transfection

The *short interfering RNAs* (siRNAs) are double stranded RNAs (21-22 nucleotides) that are complementary to their target mRNAs; it means that, once they are denaturated, they bind their targets and induce their degradation. Therefore, they regulate the gene expression of specific targets through a mechanism known as *RNA interference* (Dana et al., 2017). For these evaluations, 1.5×10^5 (SK-MEL-28) or 1.8×10^5 (A375) cells were seeded in 6-wells plates. Once reached 70% of confluency, 50 pmoles of siRNA (p53 siRNA (h2), Santa Cruz Biotechnology, Dallas, USA) were transfected in 1 ml of media (with 1% glutamine) without serum and antibiotics, as previously described (Di Fiore et al., 2014). Prior the transfection, siRNAs were incubated with the Transfection Reagent Lipofectamine™ 2000, Invitrogen™ (5 µl/well) for 30 minutes at room temperature. Then, cells were transfected for 6 hours with the p53-siRNA or with the siRNA *scramble* (SI03650318, Qiagen, Hilden, Germany), considered as a negative control of transfection. Afterwards, complete media was added to each well and cells were treated for further 24 hours. To confirm the silencing of the target protein, Western Blot analysis of p53 was performed. The transfected cells were used to perform MTT cell viability assays. The data shown are representative of two different experiments.

12. Clonogenic assay

For this evaluation, HCT116 cells were seeded in 6-wells plates (400 cells/well). Four days later, media was replaced and cells were treated with ITF2357. After treatment (6 days), clones were washed twice with PBS and then stained with methylene blue 1% in PBS/ethanol 50%. Once the clones were visible, the staining was removed and, after a wash in PBS, they were photographed and counted.

13. Trypan blue staining

Trypan blue is a dye that selectively marks dead cells. In fact, viable cells with intact membrane do not allow the access of the dye into the cytoplasm. On the other hand, in dead cells this dye enters into the cytoplasm and makes the cells blue.

For these evaluations, HCT116 and HT29 cells were treated with the compounds of interest and brightfield images were acquired (Nikon Eclipse Ts2 microscope). Then, cells were collected and centrifuged at 300 rpm for 3 minutes to obtain the pellets, which were adequately resuspended in PBS and an aliquot of cell suspension (10 µl) was mixed with an equal volume of trypan blue solution (Sigma- Aldrich). Cells were therefore counted in 10 µl of mixed cell suspension by using an automatic cell counter (TC20-Bio-Rad), which provided the percentage of viable and dead cells.

14. Real-Time PCR

The Real-Time polymerase chain reaction (RT-PCR) monitors the amplification of a targeted cDNA molecule (obtained from the mRNA of interest) during the PCR in real time. Real-time PCR can be used quantitatively (qPCR) to evaluate and compare the amount of mRNA contained in different samples. This technique requires a previous RNA reverse-transcription in cDNA, followed by the amplification (RT-PCR) of the cDNA derived from the target mRNA using specific primers.

For these experiments, cells were seeded in 6-wells plates and treated with the compounds of interest. After treatment, cells were collected and the RNA samples were obtained by using the RNeasy Mini Kit (Qiagen). RNA was then quantified at the Nanodrop™ (Thermo-Scientific) and 1000 ng of RNA was used for reverse-transcription. The reverse transcription was performed by using the SuperScript IV VILO (Thermo-Fisher) master mix. Once the cDNA was obtained, it was diluted in UP water to reach the final concentration of 2 ng/µl. 10 ng of cDNA were added to a solution containing SYBR green Master Mix, primers (forward and reverse) and water (up to 20 µl) to perform the Real Time PCR on a Step One™ Real-Time PCR (Applied Biosystem) machine. In these experiments, each sample was evaluated in triplicate. The following is a list of the primers used:

RPLP0 (Housekeeping)	Fw CAGACAGACACTGGCAACA Rv ACATCTCCCCCTTCTCCTT
ISG15	Fw GCCTCAGCTCTGACACC Rv CGAACTCATCTTTGCCAGTACA
DDX58	Fw CCAGCATTACTAGTCAGAAGGAA Rv CACAGTGCAATCTTGTCATCC

In these evaluations, the housekeeping gene RPLP0 was used as endogenous control. The PCR was run at 95°C for 20 sec, followed by 40 cycles at 95°C for 3 sec and 60°C for 30 sec. Relative changes in gene expression between control and treated samples were determined using the ΔΔCt method.

15. Double Stranded RNAs (dsRNAs) evaluation by Dot Blot analysis

RNA Dot Blot is a semi-quantitative analysis that allows to compare the quantity of a specific RNA contained in the same volume (1.5-3 µl) of different samples. At first, equal volumes and concentrations of RNA (at least 250 ng) are used to make dots of the same dimension on a Hybond N+ membrane (Amersham). The membrane is dried for few

minutes and then it is crosslinked in a UV stratalinker, in order to fix the RNA. At this point, the membrane is incubated with the antibody of interest and it is developed to observe the dots by chemiluminescence. Finally, to confirm that the same amount of RNA has been loaded, the colorimetric evaluation of the dots (used as control) is performed and the membrane is also stained in a methylene blue solution (0.3% W/V methylene blue + 30% V/V ethanol + 70% V/V H_2O).

For these evaluations, RNA samples from HCT116 and HT29 cells were obtained as previously described. The membranes were incubated with a blocking solution (5% milk in PBS-T) for 30 minutes at room temperature and then with the antibody J2 (Jena Bioscience, dilution 1:500) overnight at 4°C. After the incubation with the secondary antibody and the evaluation of the chemiluminescent signal at the Chemidoc (Bio-Rad), the membrane was stained with methylene blue to assess that the same amount of RNA in all the samples has been used. Densitometric analysis has been made as previously described.

16. dsRNAs evaluation by immunofluorescence (IF)

Double-stranded RNAs (dsRNAs) were also analyzed by immunofluorescence.

For this experiment, glass coverslips were placed in 24-wells plates and washed with 100% ethanol for 5 minutes at room temperature (RT). After treatment with the compounds of interest, cells were detached and seeded over the coverslips (100.000 cells/well). The following day, cells were fixed in ice-cold methanol for 15 minutes at -20°C. Then, after three washes with PBS, cells were incubated with the blocking solution (1% BSA in PBS at 37°C) for 1 hour. Cells were therefore stained with the primary antibody anti-dsRNA J2 (Jena Bioscience, dilution 1:500) at 4°C overnight. The next day, cells were washed at room temperature with PBS and incubated for 1 hour at RT with the secondary antibody (anti-mouse Alexa Fluor 647 Conjugate, Cell Signalling, dil. 1:1000). DNA was also marked after incubation with Hoechst staining (H1399 - Thermo Fisher Scientific, dil. 1:2000) in PBS for 5 minutes at RT. After further washes in PBS, each coverslip was mounted on a glass-slide by adding 10 μl of prolong gold antifade reagent with DAPI (Invitrogen, P36935) and the coverslips were sealed with nail polish and kept in the dark. Once the slides were dried, the images were captured using the confocal microscopy ZEISS LMS980. The images were acquired using the ZEISS ZEN 3.6 (blue edition) program.

17. Statistical analysis

Data were represented as mean ± S.D., and analysis was performed using the Student's *t*-test and one-way analysis of variance. Comparisons between untreated control vs. all treated samples were made. If a significant difference was detected by ANOVA analysis, this was re-evaluated by post hoc Bonferroni's test. GraphPad PrismTM 4.0 Software (GraphPad PrismTM Software Inc., San Diego, CA, USA) was used for statistical calculations. The statistical significance threshold was fixed at $p < 0.05$.

IV. RESULTS

PART I: MELANOMA

1. ITF2357 potently reduces melanoma cell viability and induces histone acetylation

For this study the HDAC inhibitor ITF2357 was chosen and its efficacy was evaluated in melanoma cells for the first time. Initially, the effects of different doses of ITF2357 were evaluated on BRAF-mutated melanoma cell viability by MTT assay in comparison with the well-known HDAC inhibitor SuberoylAnilide Hydroxamic Acid (SAHA). As shown in figure 1, after 48 hours treatment, both ITF2357 and SAHA reduced the viability of SK-MEL-28 and A375 cells in a dose-dependent manner. It is interesting to note that ITF2357 resulted much more effective than SAHA in both cell lines, as revealed by the histograms and by IC50 values reported in Table 1. Moreover, these evaluations indicated that A375 cells were more susceptible to both HDAC inhibitors than SK-MEL-28. Overall, these results indicate that ITF2357 can be considered an HDAC inhibitor with a particular anti-tumour efficacy in melanoma cells.

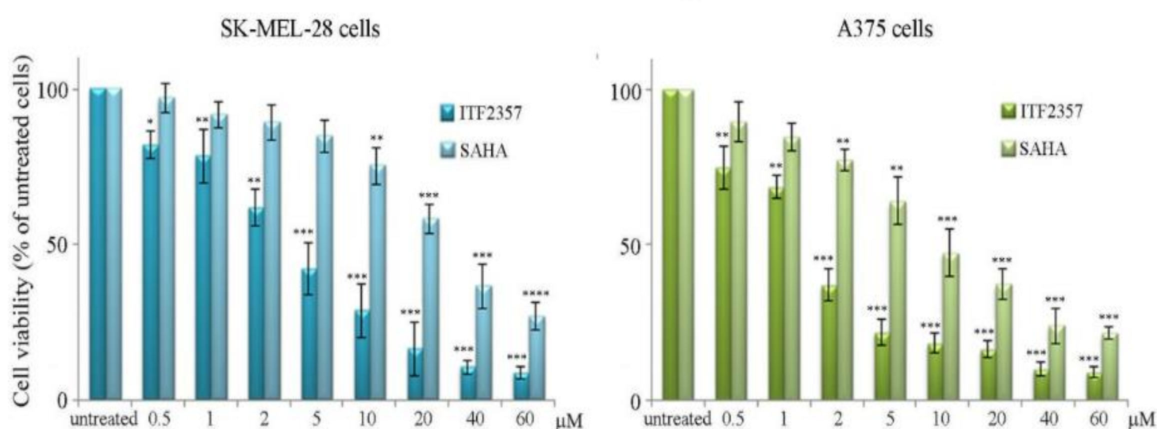


Figure 1: The effects of ITF2357 and SAHA on melanoma cell viability. For cell viability evaluations, SK-MEL-28 and A375 cells were treated with the indicated concentrations of ITF2357 or SAHA for 48 h. MTT analysis was then carried out as reported in Materials and Methods. The results reported in the histograms are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ with respect to untreated cells.

Table 1. IC50 values of ITF2357 and SAHA in SK-MEL-28 and A375 cells.

	IC50 Value	
	SK-MEL-28 Cells	**A375 Cells**
ITF2357	4.2 µM	1.7 µM
SAHA	26.9 µM	9.2 µM

The epigenetic effect of ITF2357 on HDAC activity was also evaluated. To verify whether it was capable of inducing histone acetylation in melanoma cells, western blot analysis of acetylated H3 and H4 histones was performed. As shown in figure 2, the compound markedly increased the levels of acetylated H3 and H4 histones in both SK-MEL-28 and A375 cell lines. This effect was already visible at 16 h treatment and maintained at 48 h, thus confirming that ITF2357 is capable of promoting HDAC inhibition in both melanoma cell lines.

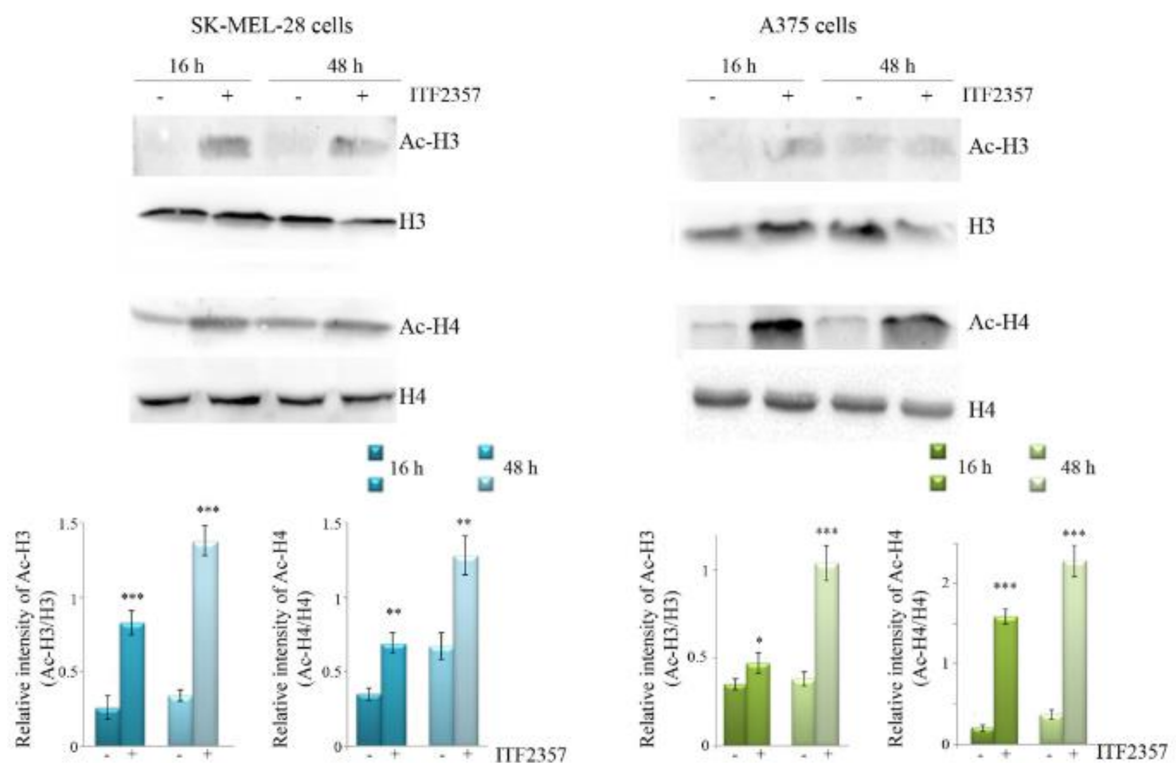


Figure 2: ITF2357 promotes histone acetylation in melanoma cells. Western blot analysis of acetylated H3 and H4 histones after treatment for 16 and 48 h with 5 μM (SK-MEL-28) or 2 μM (A375) ITF2357. The ratio between acetylated and total histone levels was quantified. Representative blots of three independent experiments and densitometric analysis are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to untreated cells.

2. *The effects of ITF2357 on oncogenic BRAF*

It is widely known that mutated BRAF^{V600E} exerts a key role in promoting melanoma growth and aggressiveness. Therefore, the first part of this project aimed to investigate whether ITF2357 is able to target this protein or epigenetically modify its expression levels. According to cell viability evaluations, the effect of ITF2357 on BRAF protein levels was initially evaluated in comparison with the effect of SAHA by western blot analysis (figure 3). Interestingly, both HDACIs reduced the level of oncogenic BRAF protein in a dose-dependent manner in both melanoma cell lines. In SK-MEL-28 cells, the

reducing effect was remarkable with 2 µM ITF2357 after 48 h treatment, while SAHA exerted a similar reduction in BRAF level with a dose of 40 µM, thus confirming the much higher efficacy of ITF2357 compared to SAHA. Notably, the BRAF band almost disappeared with 5 µM ITF2357 and was not visible at all with 10 µM. In A375 cells, which were more sensitive to both HDAC inhibitors, the reducing effects were remarkable even with 1 µM ITF2357. As regards the effects of SAHA, BRAF protein decrease was visible at much higher concentrations (from 10 µM to 40 µM). These data indicate for the first time that both HDAC inhibitors target oncogenic BRAF, although at different concentration range, being ITF2357 the most efficacious compound.

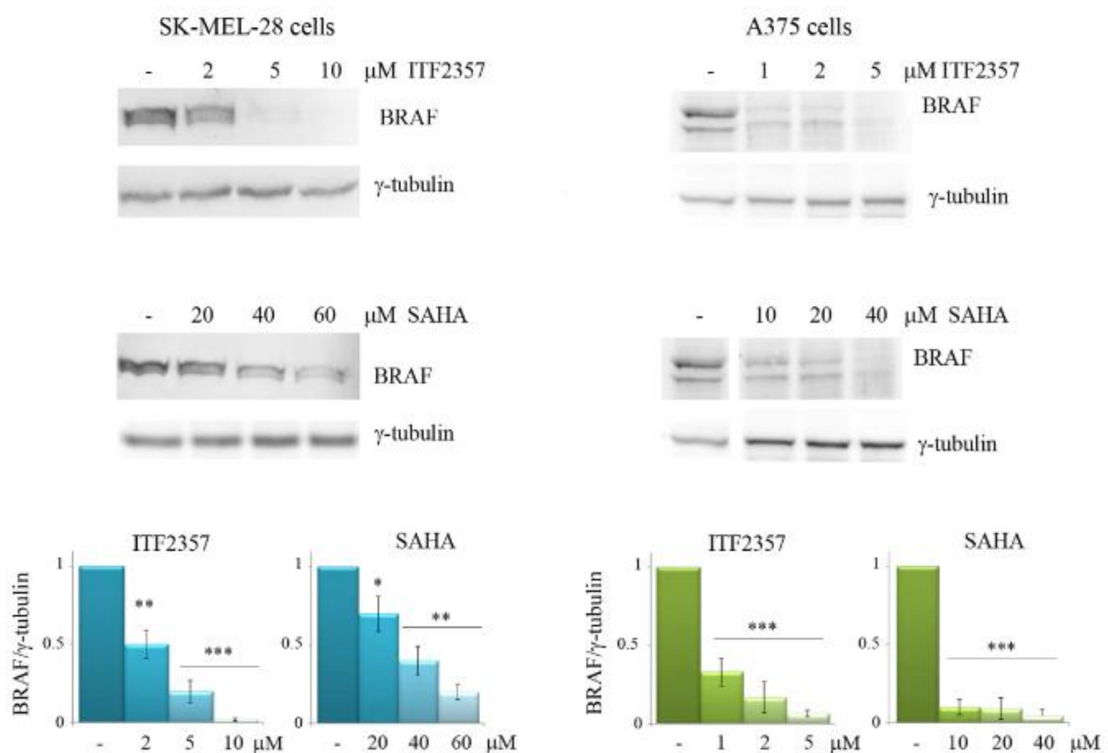


Figure 3: ITF2357 and SAHA dose-dependently decrease oncogenic BRAF levels. SK-MEL-28 and A375 cells were treated with the indicated concentrations of the two HDACIs for 48 h. Western blot analysis of BRAF was performed as reported in Materials and Methods. Representative blots of three independent experiments and densitometric analysis are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to untreated cells.

Based on these results, the attention was specifically focused on ITF2357 for subsequent experiments aimed to clarify the reason for the BRAF-decrease.

Considering that ITF2357 is an epigenetic compound, a possible effect on BRAF gene expression was considered. As shown in figure 4, semiquantitative RT-PCR analysis indicated that ITF2357 also determined a reduction in BRAF mRNA levels in both

melanoma cell lines. This was evidenced at 24 h treatment, a time that was considered proper since the cells were still viable and degradation processes did not occur yet.

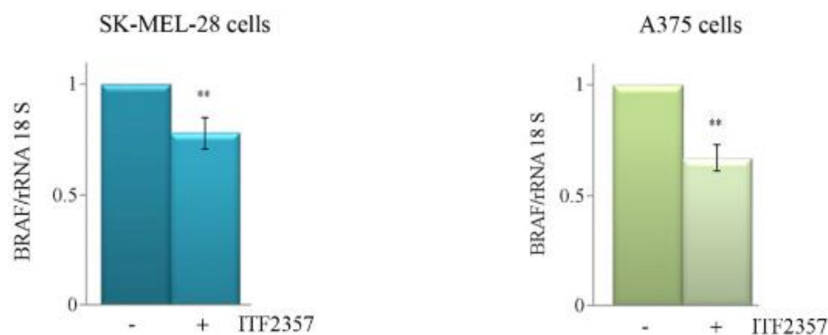


Figure 4: The effects of ITF2357 on BRAF expression. SK-MEL-28 and A375 cells were treated for 24 h with 5 μM and 2 μM ITF2357, respectively. RNA extraction and semiquantitative RT-PCR were then performed as reported in Materials and Methods. Histograms are representative of two independent experiments. ** $p < 0.01$ with respect to untreated cells.

However, since the dramatic BRAF protein decrease was observed at 48 h, in addition to reduced expression, protein degradation events could also be involved. Considering that BRAF can be degraded by the 26S proteasome (Chiappetta et al., 2012), we investigated whether the proteasome inhibitor Bortezomib could attenuate the BRAF reducing effects of ITF2357. For this purpose, cells were pre-treated with ITF2357 and Bortezomib was added after 24 h and maintained for additional 24 h. This experiment scheme was chosen to avoid synergistic interactions between the HDAC inhibitor and the proteasome inhibitor (Laporte et al., 2017) and to guarantee proteasome inhibition in the second phase of ITF2357 treatment, when BRAF tends to decrease. As shown in figure 5a, the addition of Bortezomib to ITF2357-treated cells consistently reduced the decrease of BRAF in both cell lines. These data suggest that proteasome-mediated degradation can also account for the remarkable BRAF protein level decrease observed with the HDAC inhibitor. To confirm the induction of a protein degradation process, the half-life of BRAF protein in the presence of the protein synthesis inhibitor cycloheximide was evaluated with or without ITF2357. The results shown in figure 5b indicate that ITF2357 reduced the half-life of BRAF, anticipating the decreasing effect under protein synthesis inhibition. These results confirm that degradative processes also account for ITF2357-induced BRAF decrease.

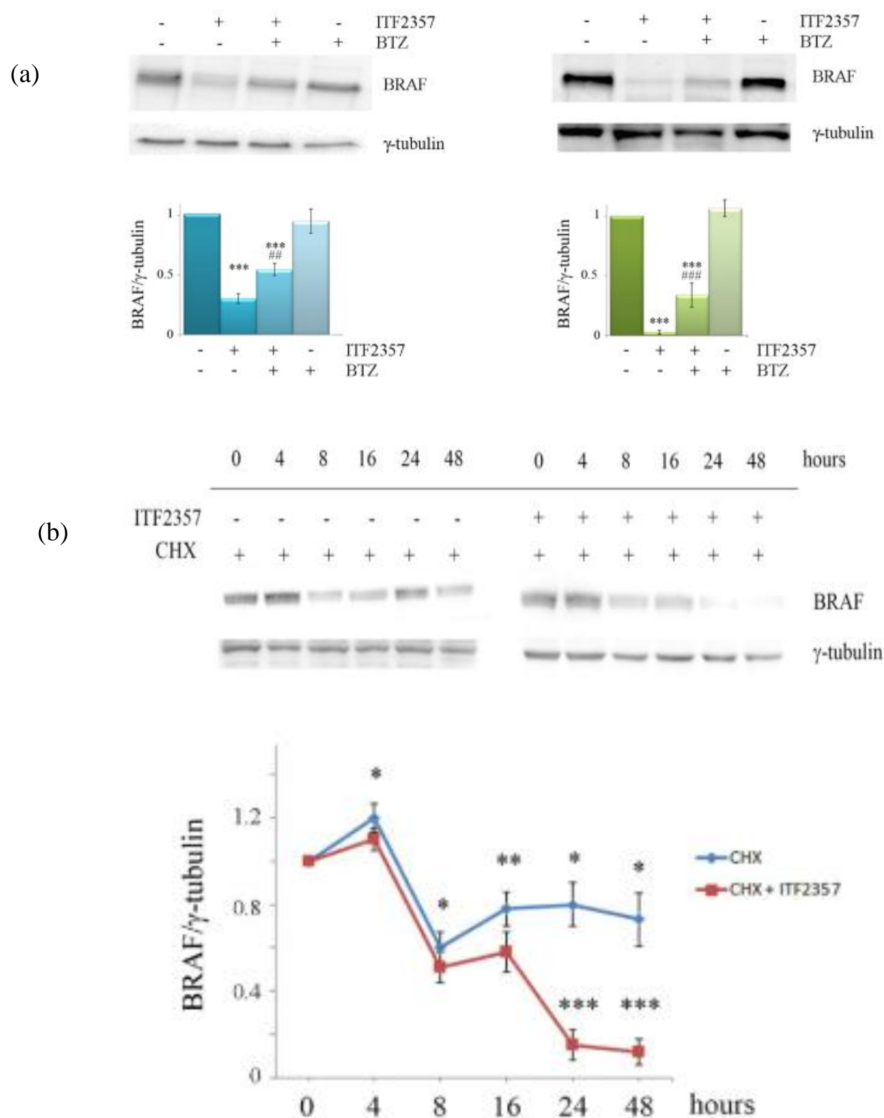


Figure 5: The effect of ITF2357 on BRAF protein degradation. (a) The effect of proteasome inhibition by bortezomib (BTZ) on the decreasing effect of ITF2357. SK-MEL-28 and A375 cells were treated for 24 h with 5 μM and 2 μM ITF2357, respectively; then BTZ (10 nM) was added, and the incubation was protracted for other 24 h. (b) BRAF half-life was determined using 100 μM cycloeximide for the indicated times in the presence or absence of ITF2357 in A375 cells. Western blot analysis of BRAF was performed as reported in Materials and Methods. Representative blots of three independent experiments and densitometric analysis are shown. * $p < 0.05$,** $p < 0.01$, *** $p < 0.001$ with respect to controls; ## $p < 0.01$, ### $p < 0.001$ with respect to ITF2357 treated cells.

3. *The effect of ITF2357 on BRAF mitogenic signalling cascade*

To investigate whether BRAF-targeting by ITF2357 was correlated with a reduction of BRAF-mediated mitogen activated kinase (MAPK) signalling, the levels of phospho-ERK1/2, the downstream kinase in this pathway, were evaluated. Western blot analysis performed at different treatment times showed that the level of phospho-ERK1/2 was not significantly modified after 16 h treatment with ITF2357, while it decreased at 48 h similarly to the level of BRAF (figure 6a). It is thus possible to hypothesize that the BRAF

decrease, which is a late event, accounts for a reduced activation of the MAPK mitogenic signalling. To confirm this hypothesis, we also evaluated the effect of U0126, a widely known inhibitor of MEK, the kinase that is directly activated by BRAF and promotes downstream ERK1/2 phosphorylation. As shown in the same figure, U0126 produced a cytostatic effect when used alone, but markedly potentiated the effect of ITF2357 on both cell viability (b) and cell morphology (panel c).

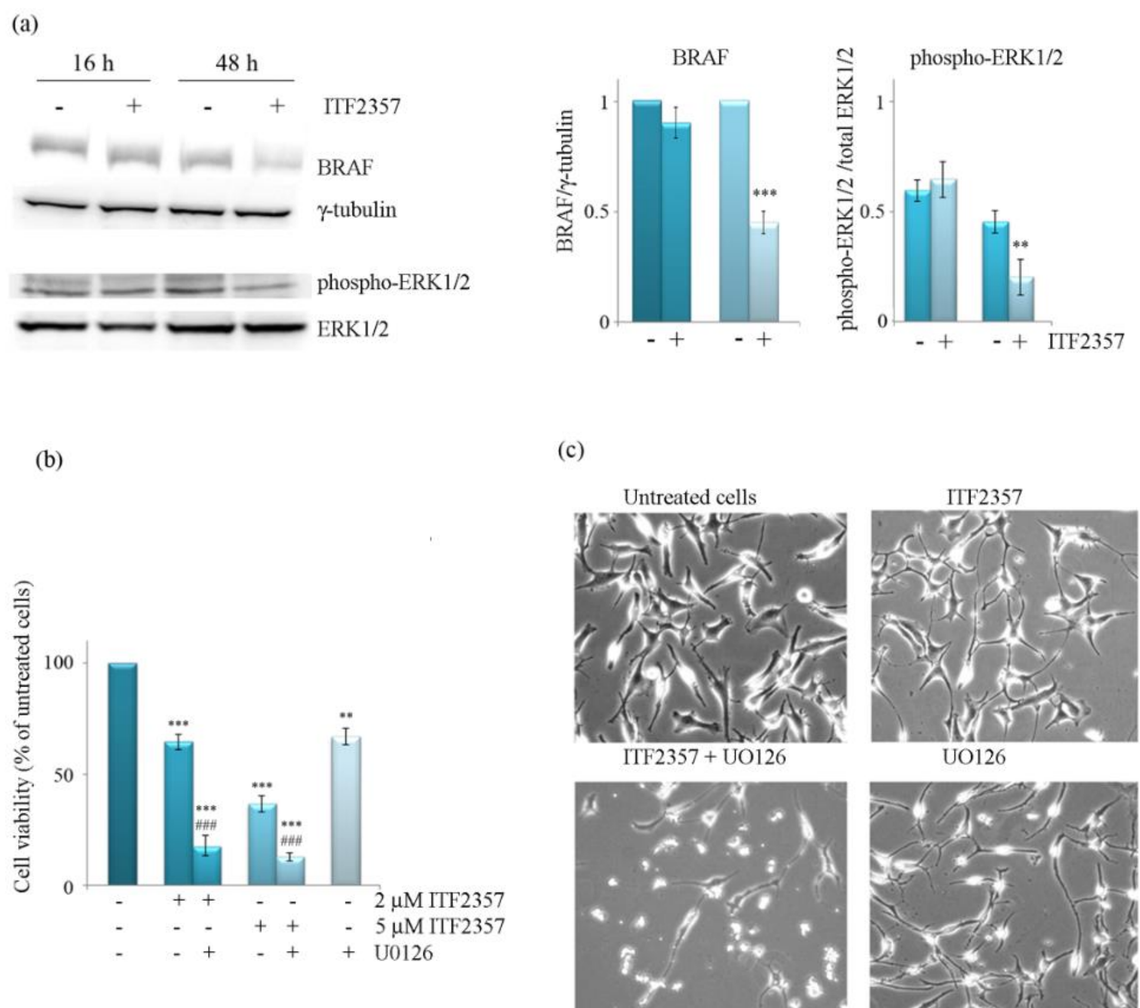


Figure 6: The effects of ITF2357 on phospho-ERK1/2 and its potentiation by the MEK inhibitor U0126. (a) Western blot analysis of BRAF and phospho-ERK1/2 after 16 and 48 h treatment of SK-MEL-28 cells with 5 μM ITF2357. The effects of ITF2357 in the presence of U0126 were also evaluated on cell viability (b) and cell morphology (c). Cells were treated with ITF2357 at the indicated concentrations in the absence or presence of U0126 (10 μM) for 48 h. Cell viability was then assessed by MTT assay as reported in Materials and Methods. For morphological analysis, cells were treated for 24 h with 5 μM ITF2357 in the absence or presence of 10 μM U0126. Cells were then visualized under light microscope at 200 X magnification and the pictures acquired by IM50 Leica Software (Leika Microsystems, Wetzlar, Germany). ** $p < 0.01$, *** $p < 0.001$ with respect to untreated cells, # $p < 0.05$, ### $p < 0.001$ with respect to ITF2357 treated cells.

It is also interesting to note that U0126 completely suppressed ERK phosphorylation and exacerbated the decreasing effect of ITF2357 on BRAF levels, as revealed by Western blot analysis (figure 7).

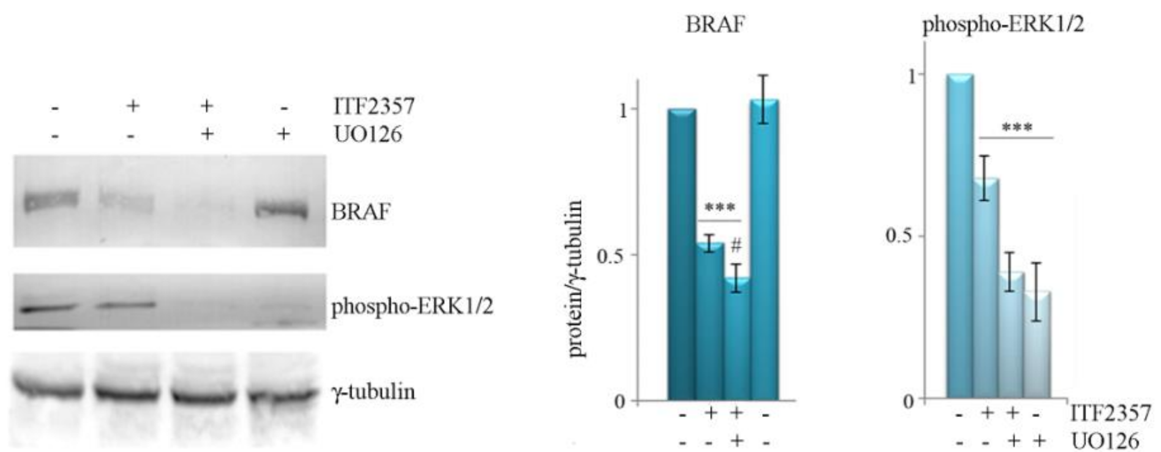


Figure 7: ITF2357 and U0126 effect on MAPK pathway. Western blot analysis of BRAF and phospho-ERK1/2 following ITF2357 treatment for 48 h in the absence or presence of U0126. Representative blots of three independent experiments and densitometric analysis are shown. *** $p < 0.001$ with respect to untreated cells, # $p < 0.05$ with respect to ITF2357 treated cells.

4. ITF2357 promotes a switch from autophagy to caspase-dependent apoptosis

From the literature it is known that oncogenic BRAF is involved in the promotion of pro-survival autophagy, thus favouring melanoma cell survival and tumour propagation (Li et al., 2019). In order to understand whether ITF2357 induces autophagy, first monodansylcadaverine (MDC) staining was performed to detect autophagosome formation. As shown in figure 8 (upper panel), a basal autophagy level was detected in SK-MEL-28 cells as revealed by green fluorescence. This effect markedly increased following ITF2357 treatment for 16 h, when brilliant dot-like structures were clearly visible, indicating the presence of autophagic vacuoles. Prolonging treatment-time up to 48 h revealed a dramatic reduction in green fluorescence intensity, whereas signs of chromatin condensation and fragmentation appeared as evidenced by nuclei staining with the vital Hoechst 33342 dye (lower panel).

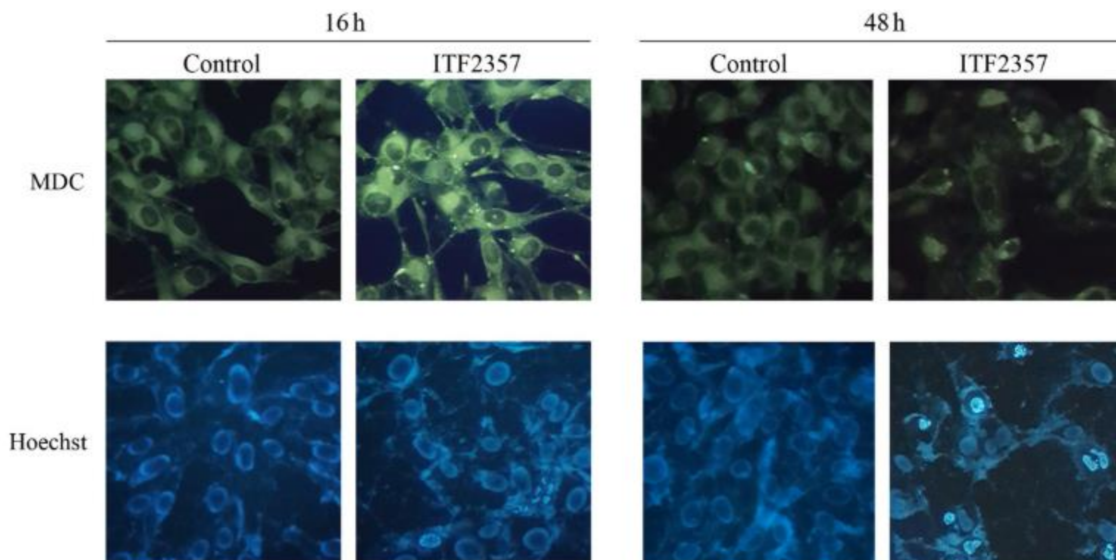


Figure 8: ITF2357 induces autophagic vacuolization and chromatin condensation. SK-MEL-28 cells were incubated for 16 or 48 h in the presence of 5 µM ITF2357. At the end of incubation, cells were stained with monodansylcadaverine (MDC), which highlights autophagic vacuoles, or Hoechst 33342, which permits the visualization of nuclei. Cells were then visualized under fluorescence microscope Leika equipped with a DAPI filter (Hoechst 33342) or FITC filter (MDC) at magnification of 400 X. Micrographs are representative of two different fields from two independent experiments.

To confirm the morphological evidence of autophagy and apoptosis, the effects of ITF2357 on autophagic and apoptotic markers were evaluated at two time points (16 and 48 h) in both melanoma cell lines. As shown in figure 9, ITF2357 induced the production of LC3-II from LC3-I, an effect that was particularly evident in A375 cells already at 16 h and increased at 48 h, indicating autophagosome formation. Another important marker of autophagy is p62, a well-known multifunctional protein involved in selective autophagy and usually studied to monitor the autophagic flux. In particular, p62 is considered as a marker of the autophagic flux because its levels generally increase early upon the stimulation of the process and then decrease when autophagy is completed, since the protein is degraded by autophagolysosomes (Emanuele et al., 2020; Liu et al., 2019b). ITF2357 induced a significant increase in p62 level, which was further enhanced at 48 h in both cell lines, most likely due to protein accumulation correlated with incomplete autophagy. Other two autophagic markers, beclin and Atg7, were also evaluated, and tended to decrease at 48 h. Such effects were more pronounced in A375 cells, which were more sensitive to ITF2357 than SK-MEL-28 cells. These data can be interpreted considering that the triggering of the autophagic flux most likely represents an adaptive cell response to the effects of this compound.

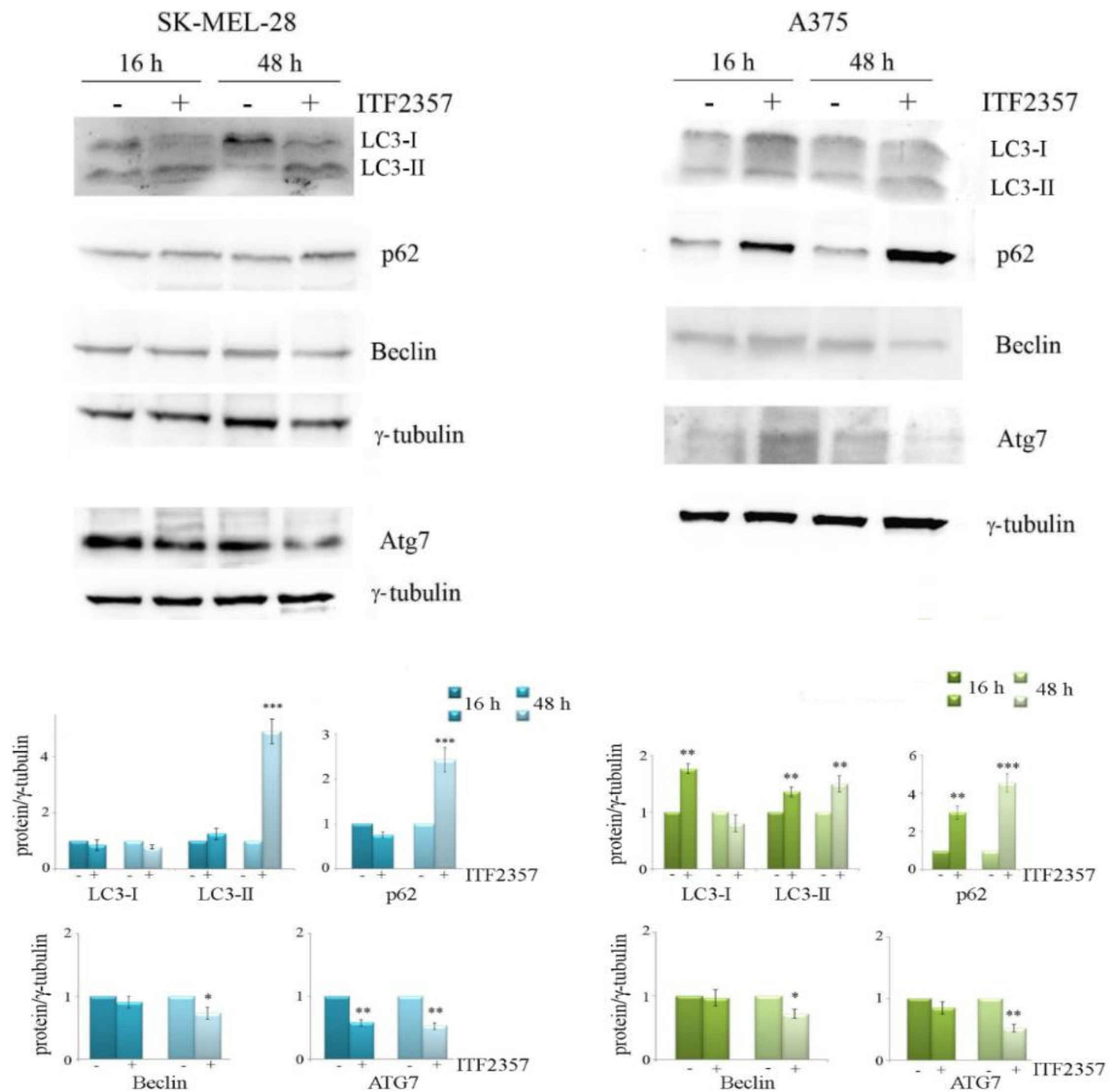


Figure 9: The effects of ITF2357 on autophagic markers. SK-MEL-28 and A375 cells were treated for 16 and 48 h with 5 μM and 2 μM ITF2357, respectively. Western blot analysis of autophagic proteins was performed as reported in Materials and Methods. Representative blots of three independent experiments and densitometric analysis are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to untreated cells.

At this point, to elucidate the nature of the autophagic process observed in response to ITF2357, cell viability was evaluated in the presence of two autophagy inhibitors: 3-methyladenine (3MA) and Bafilomycin A1. In detail, 3MA acts upstream, counteracting the formation of the autophagosome by inhibiting the complex PI3K of class III. On the other hand, Bafilomycin A1 acts downstream, inhibiting the fusion between the autophagosome and the lysosome blocking the lysosomal acidification and consequent degradation events. Cell viability data reported in the histograms (figure 10) show that the effect of ITF2357 was significantly potentiated by both autophagy inhibitors, thus suggesting that autophagy has a predominant pro-survival role in these cells. Interestingly, the general caspase inhibitor z-VADfmk almost completely prevented the effect of

ITF2357, supporting the hypothesis that caspase-dependent apoptosis accounts for ITF2357-induced cell death.

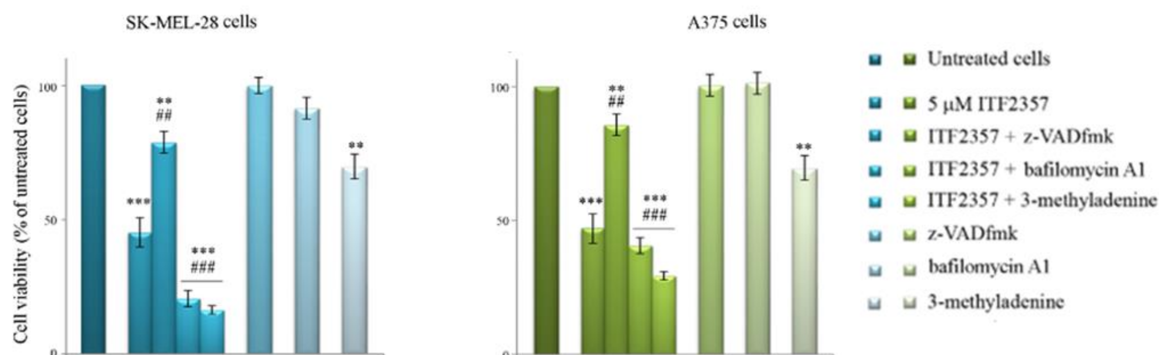


Figure 10: The influence of autophagy inhibitors and pan-caspase inhibitors on the effects of ITF2357. SK-MEL-28 and A375 cells were pre-treated for two hours with the autophagy inhibitors Bafilomycin A1 (20 nM) or 3-methyladenine (2.5 mM), then 5 µM ITF2357 was added, and the incubation was protracted for 48 h. Co-treatment of ITF2357 with the caspase inhibitor z-VADfmk (80 µM) was maintained for 48 h. MTT assay was performed as indicated in Materials and Methods to evaluate cell viability. The results in the histograms are representative of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ with respect to untreated cells; ## $p < 0.01$, ### $p < 0.001$ with respect to ITF2357 treated cells.

To confirm that ITF2357 induces apoptosis in melanoma cells, Annexin V/PI double staining was performed (figure 11). The results showed that the population of cells undergoing apoptosis significantly increased at 48 h treatment with the HDAC inhibitor in both cell lines. No significant apoptotic effects were observed at 24 h, thus confirming that apoptosis execution induced by ITF2357 is a late event.

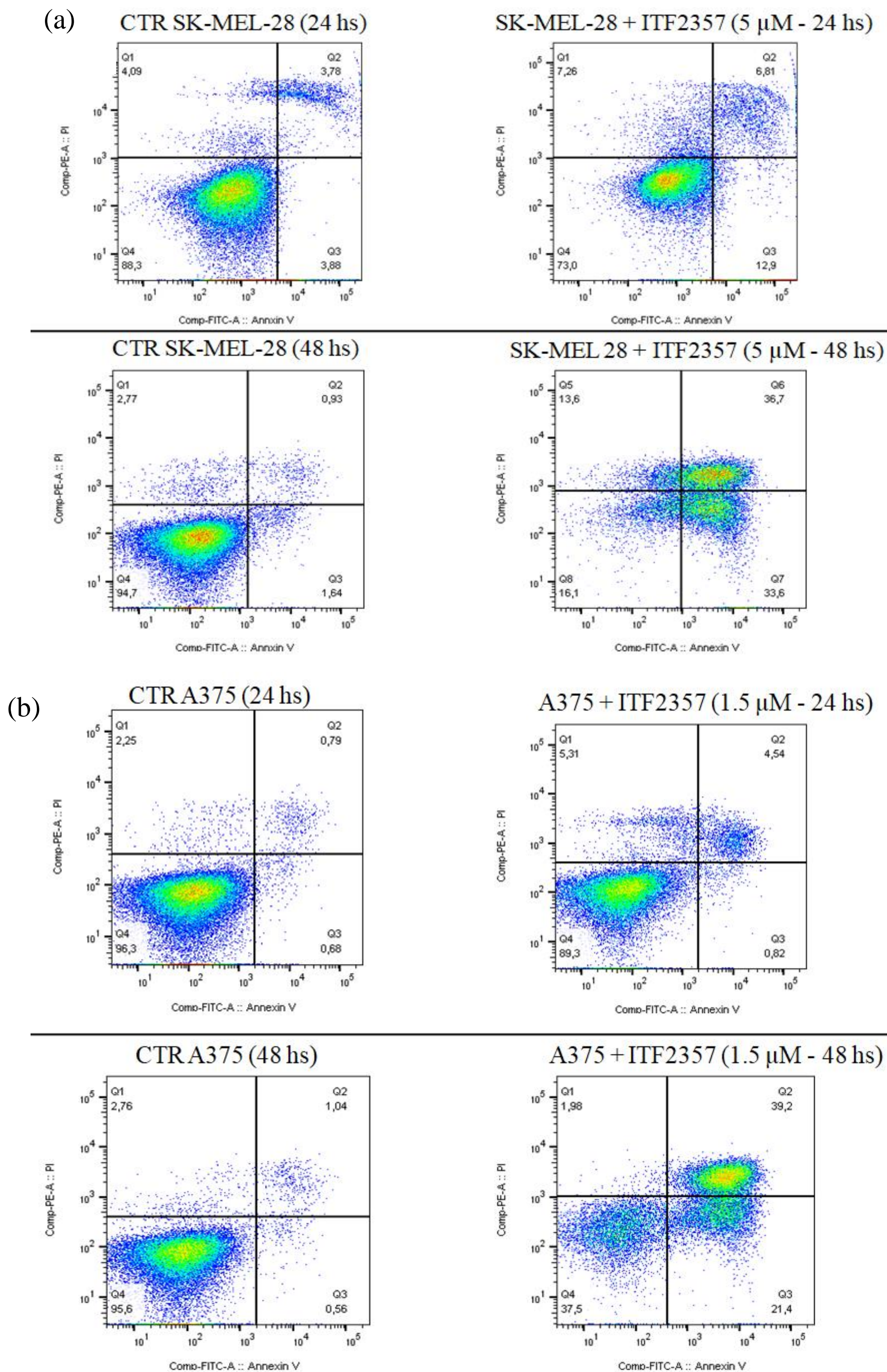


Figure 11: Annexin V evaluation in SK-MEL-28 and A375. Cells were stained with Annexin V and PI after 24 or 48 hours treatment with ITF2357 5 µM (SK-MEL-28 (a)) or 1.5 µM (A375 (b)). In these experiments, cells were stained with Annexin V to evaluate cells undergoing apoptosis and with Propidium Iodide to evaluate the dead cells. The samples were evaluated at the FACSCanto cytometer. These results are representative of two different experiments.

The apoptotic response induced by ITF2357 in melanoma cells was further confirmed by evaluating the protein levels of different apoptotic markers. In particular, caspase-9,

caspase-3 and PARP were considered in both cell lines. It is interesting to note that caspase activation occurred at 48 h, as evidenced by the remarkable decrease in pro-caspase 3 levels and the appearance of the active fragment of caspase-9 (figure 12). These effects were accompanied by PARP cleavage, detected by the 85 kDa fragment display. These data confirm that ITF2357 activates caspase-dependent apoptosis in both cell lines in the second phase of treatment.

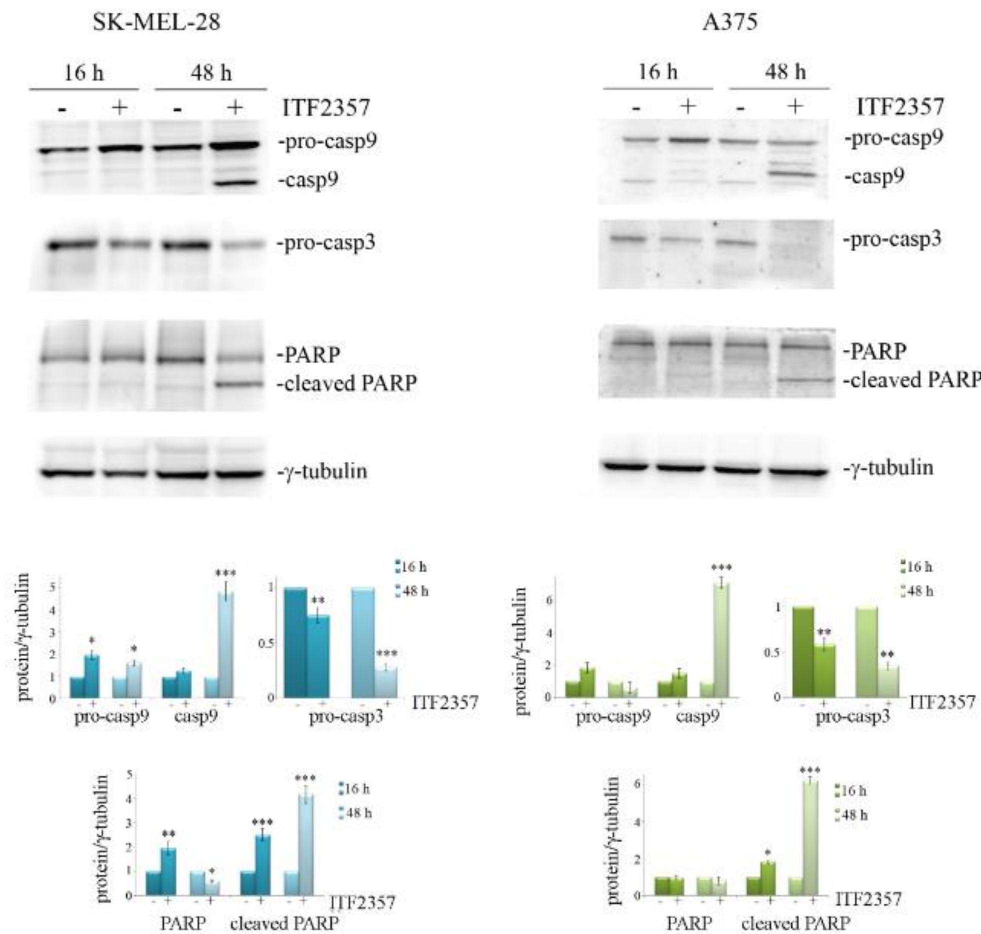


Figure 12: The effects of ITF2357 on apoptotic markers. SK-MEL-28 and A375 cells were treated for 16 and 48 h with 5 μM and 2 μM ITF2357, respectively. Western blot analysis of apoptotic proteins was performed as reported in Materials and Methods. Representative blots of three independent experiments and densitometric analysis are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to untreated cells.

5. *Oncogenic BRAF has a nuclear localization in melanoma cells and ITF2357 reduces BRAF protein level in both the cytosol and the nucleus*

It is widely known that BRAF is a component of the mitogenic pathway and it is usually localized in the cytosol. To investigate the BRAF oncogenic potential, its subcellular localization was evaluated focusing on the nuclear compartment. BRAF protein level was thus analyzed in both cytosolic and nuclear fractions obtained from SK-MEL-28 and A375 melanoma cells. The results showed that oncogenic BRAF is localized in both the cytosol

and the nucleus of these cells. Β-tubulin and H3 histone were also reported in the western blot analysis as cytosolic and nuclear markers respectively, to confirm the purity of the two fractions and to normalise the levels of BRAF by densitometric analysis in the two fractions. Interestingly, after 24 hours treatment, ITF2357 decreased BRAF protein level in both the cytosol and the nucleus of SK-MEL-28 and A375 melanoma cells (figure 13). This treatment time was chosen to avoid nuclear fragmentation due to full blown apoptosis, since these concentrations induced BRAF decreasing effect without provoking remarkable effects on cell viability. These data indicate that ITF2357 is able to target oncogenic BRAF in both the compartments.

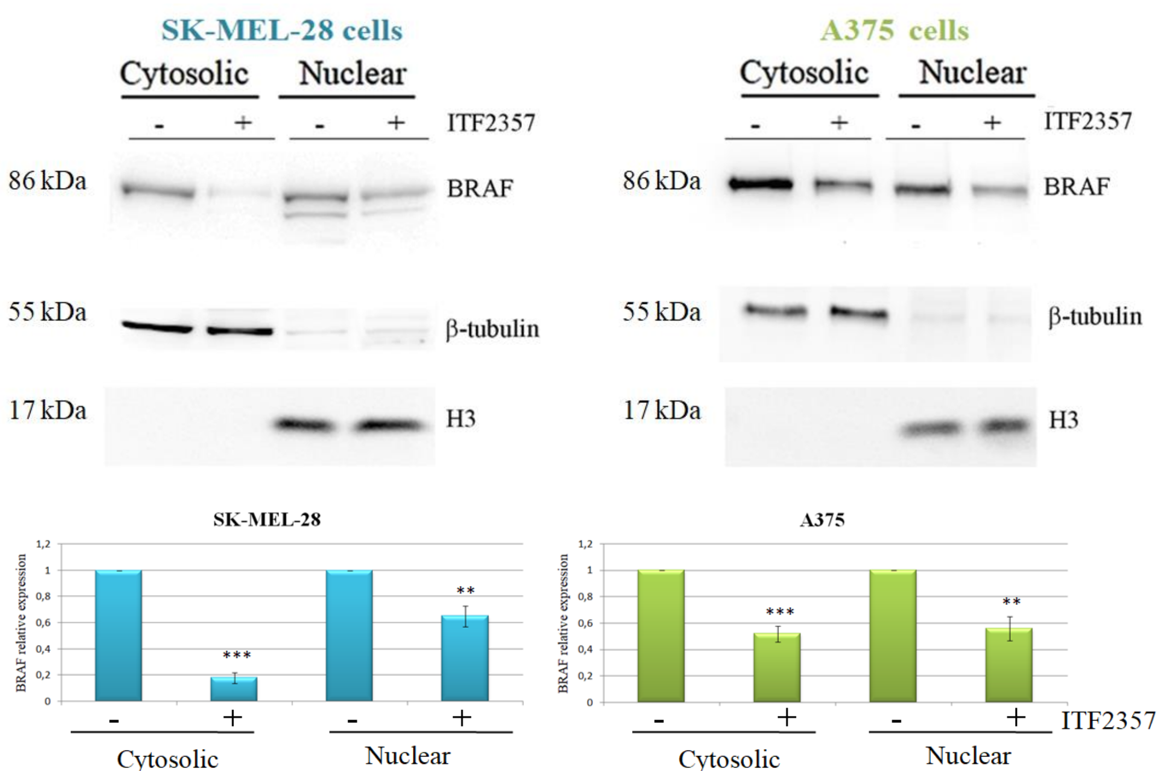


Figure 13: Oncogenic BRAF is localized in both the cytosol and the nucleus of melanoma cells. Evaluation of oncogenic BRAF level after 24 h treatment with ITF2357 in the cytosolic and nuclear fractions of SK-MEL-28 (ITF2357 10 μM) and A375 (ITF2357 2 μM) cells. In these Western Blot analyses, β-Tubulin was used as cytosolic marker, while H3 histone was used as nuclear marker. The blots are representative of three different experiments. Densitometric analysis is shown referred to beta tubulin for cytosolic fraction and H3 Histone for nuclear fraction. ** $p < 0.01$, *** $p < 0.001$ with respect to untreated cells.

Considering these data, it is possible to hypothesize that BRAF expresses its oncogenic potential at the nuclear level and that ITF2357 is also able to target the protein in the nucleus. To explain the nuclear localization of oncogenic BRAF, two main hypotheses were formulated as reported in figure 14.

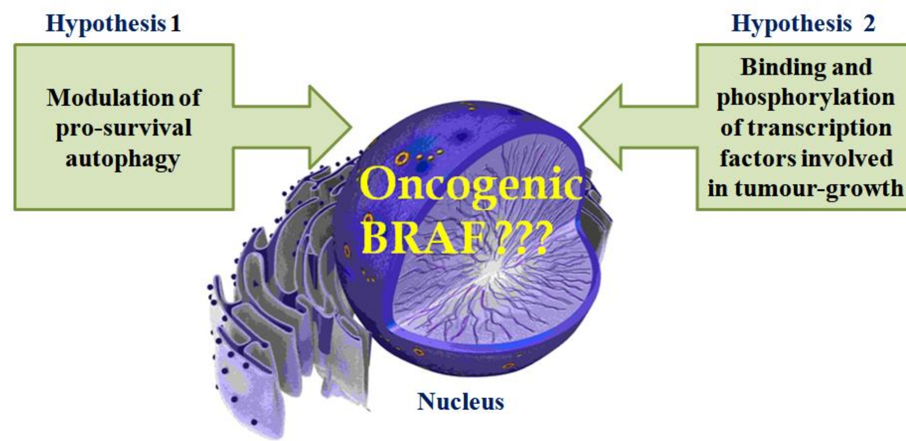


Figure 14: Hypotheses on the role of oncogenic BRAF in the nucleus.

The first hypothesis is that BRAF modulates pro-survival autophagy in melanoma cells, which is in line with the findings of other authors (Corazzari et al., 2015) and with results previously showed in this thesis (paragraph 4). The second hypothesis is that oncogenic BRAF binds and activates by phosphorylation transcription factors involved in tumour promotion and progression. Among the putative nuclear interactors of BRAF, p53 protein was considered. In particular, p53 was chosen because its status differs in SK-MEL-28 cells (that display oncogenic mutated p53) and A375 cells (displaying wild type p53). The attention was then focused on p53 in the two cell lines and a possible interplay with oncogenic BRAF was considered.

6. *ITF2357 targets oncogenic p53 in melanoma cells and induces p53 proteasome-mediated degradation*

As mentioned, p53 is found in a different status in the two melanoma cell lines that were considered. First, the effect of ITF2357 on oncogenic or wild type p53 protein level was evaluated. Interestingly, Western blot analysis performed at two time points (16 and 48 h) revealed that ITF2357 dramatically decreased oncogenic p53 protein level in SK-MEL-28 cells already at 16 h treatment. Differently, ITF2357 increased wild type p53 protein level in A375 cells at the same treatment conditions. At 48 hours, a decrease in p53 protein level was observed in both cell lines, most likely due to degradative processes caused by cell death (figure 15).

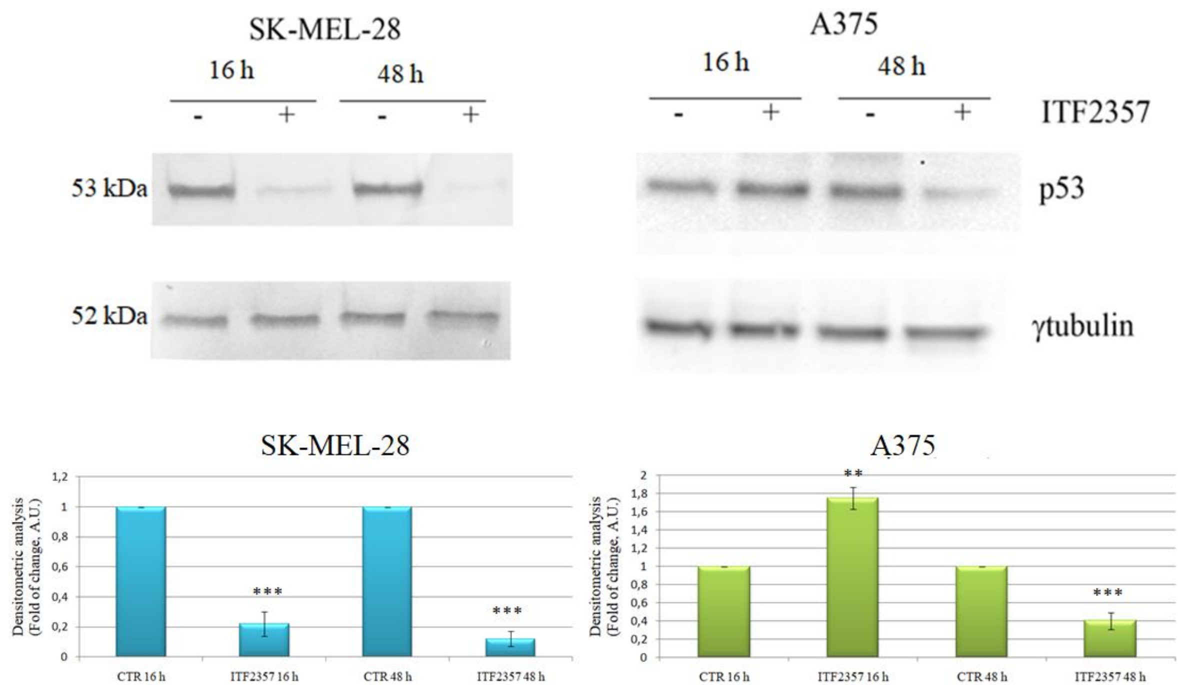


Figure 15: ITF2357 promotes p53 degradation in melanoma cells. Western Blot analysis was performed after treating SK-MEL-28 and A375 melanoma cells with ITF2357 (10 μM for SK-MEL-28 and 2 μM for A375 cells) for 16 and 48 h. Densitometric analysis is shown after normalization referred to γ-tubulin. The results are representative of three different experiments. ** $p < 0.01$, *** $p < 0.001$ with respect to untreated cells.

To understand the mechanism regulating the dramatic and precocious decrease of oncogenic p53 in SK-MEL-28, two processes involved in protein degradation were considered: autophagy and proteasome-mediated degradation. Western blot analysis demonstrated that the proteasome inhibitor Bortezomib completely prevented oncogenic p53 protein reduction induced by ITF2357 in SK-MEL-28 cells, while the autophagy inhibitor Bafilomycin A1 had no effect (figure 16a). To avoid any synergistic interaction with bortezomib, the concentration of ITF2357 was reduced to 5 μM in these experiments and a short treatment time (16 h) for the combinatory treatment was considered. This result suggests that ITF2357 promotes proteasome-mediated degradation of p53. Moreover, this event was accompanied by the increase in the levels of p-MDM2 (figure 16b), the ubiquitine ligase that is responsible for p53 targeting and subsequent degradation by the 26S proteasome (Haronikova et al., 2021). These results demostrate that ITF2357 promotes oncogenic p53 proteasome-mediated degradation.

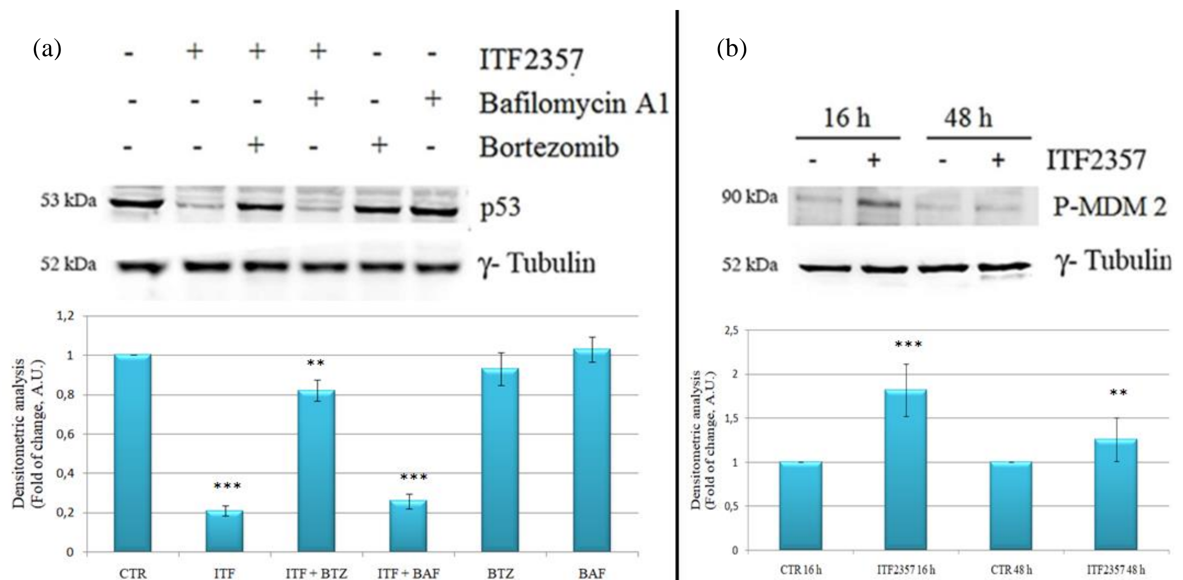


Figure 16: Degradation of oncogenic p53 in SK-MEL-28 is proteasome-mediated. (a) Western Blot analysis was performed after treating SK-MEL-28 with ITF2357 (5 µM) for 16 h. Cells were pre-treated with the inhibitors Bortezomib (15 nM) and Bafilomycin A1 (20 nM) for 1 h before adding ITF2357. (b) The effect of ITF2357 (10 µM) on p-MDM2 in SK-MEL-28. Densitometric analysis is shown after normalization referred to γ-tubulin. The results are representative of three different experiments. ** $p < 0.01$, *** $p < 0.001$ with respect to untreated cells.

7. Oncogenic BRAF preferentially interacts with mutated p53 in melanoma cells

Considering BRAF nuclear localization in melanoma cells and the targeting effect of ITF2357 on both oncogenic BRAF and oncogenic p53, a possible interplay between the two proteins was investigated. To this purpose, immunoprecipitation (IP) experiments were performed. The results demonstrated for the first time that BRAF interacts with oncogenic p53 in SK-MEL-28 cells, as demonstrated by the presence of p53 protein in BRAF immunoprecipitates (figure 17a – left panel). This result was confirmed by performing the opposite: immunoprecipitating p53 and evaluating BRAF (right panel).

It is also interesting to note that ITF2357 reduced BRAF levels and its interaction with oncogenic p53 in SK-MEL-28 cells. The same IP experiments performed in A375 cells (wild type p53) produced a weaker signal (figure 17b), thus suggesting that BRAF could preferentially interact with the oncogenic p53 form. Ongoing studies in collaboration with a computational analysis group aim to clarify whether the mutated (oncogenic) p53 form can early bind to oncogenic BRAF compared to the wild type p53.

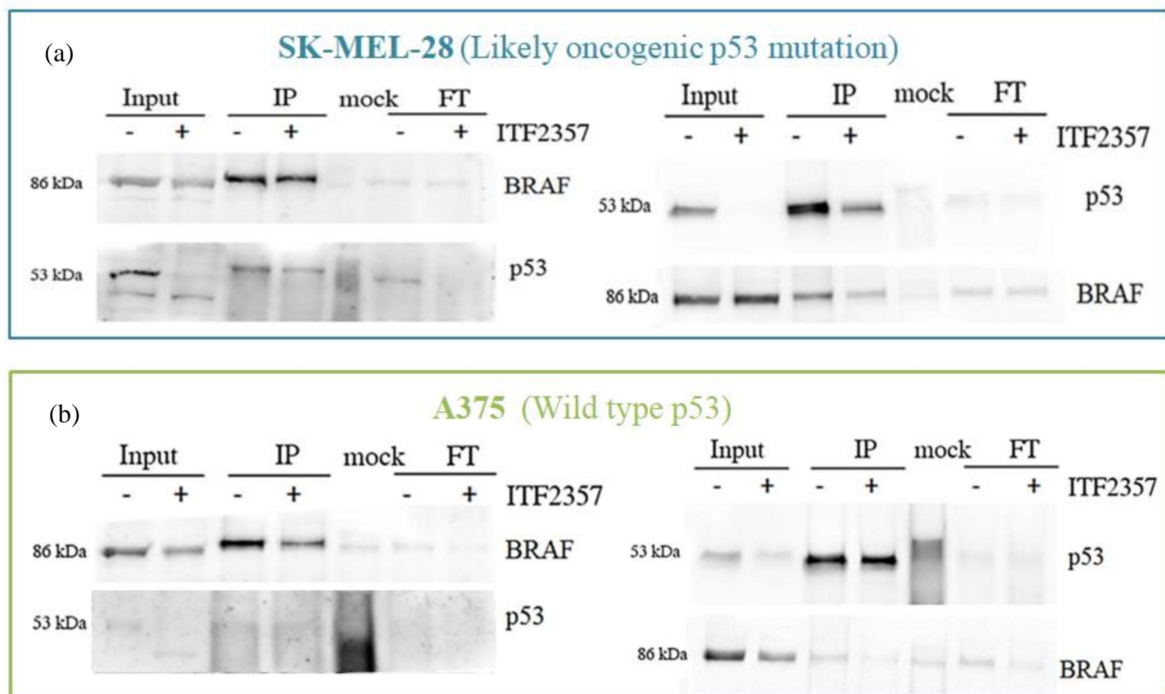


Figure 17: p53 immunoprecipitates with BRAF protein in BRAF^{V600E} mutated melanoma cells. BRAF (left) and p53 (right) immunoprecipitation was performed after treating SK-MEL-28 with ITF2357 10 µM (a) and A375 with 2 µM (b) for 24 hours. Western Blot analysis shows the input (sample cointaining all the proteins), the immunoprecipitate, the mock (sample containing the beads used for the immunoprecipitation) and the flow through (sample containing all the proteins except for the one that has been immunoprecipitated). The blots are representative of two different experiments.

8. Oncogenic p53 counteracts the effect of ITF2357 in melanoma cells

To validate the oncogenic role of p53 in SK-MEL-28 and to assess whether the effect of ITF2357 depends on p53 status, siRNA-mediated silencing of p53 was performed. For these evaluations, both SK-MEL-28 and A375 cells were transfected with a specific siRNA for p53 or with an aspecific siRNA *scramble* (SCR, used as negative control). After transfection, both cell lines were treated with ITF2357 for 24 h, and the concentrations effective in reducing p53 protein level were used in the two cell lines. In detail, p53-silenced SK-MEL-28 cells were treated with 5 µM ITF2357, while p53-silenced A375 cells were treated with 2 µM ITF2357 for 24 h. The silencing of p53 was confirmed by Western blot analysis, which showed that basal p53 level was reduced after siRNA transfection (figure 18a).

Cell viability evaluations in p53 silenced cells revealed that oncogenic p53 knockdown increased the effect of ITF2357 in SK-MEL-28; in fact, cell viability decreased from 86.8% with SCR to 58.7% with p53-siRNA (figure 18b). On the other hand, the knockdown of wild type p53 in A375 cells prevented the effect of ITF2357; in fact, in this case cell viability increased from 65.8% with SCR to 85% with p53-siRNA (figure 18b).

These data are interesting, because they corroborate the oncogenic role of p53 in SK-MEL-28 cells, while in A375 cells p53 displays its usual tumour-suppressor role. In addition, these data suggest that ITF2357 response in BRAF-mutated melanoma cells is dependent on p53 status, thus providing a rationale for melanoma targeted therapy.

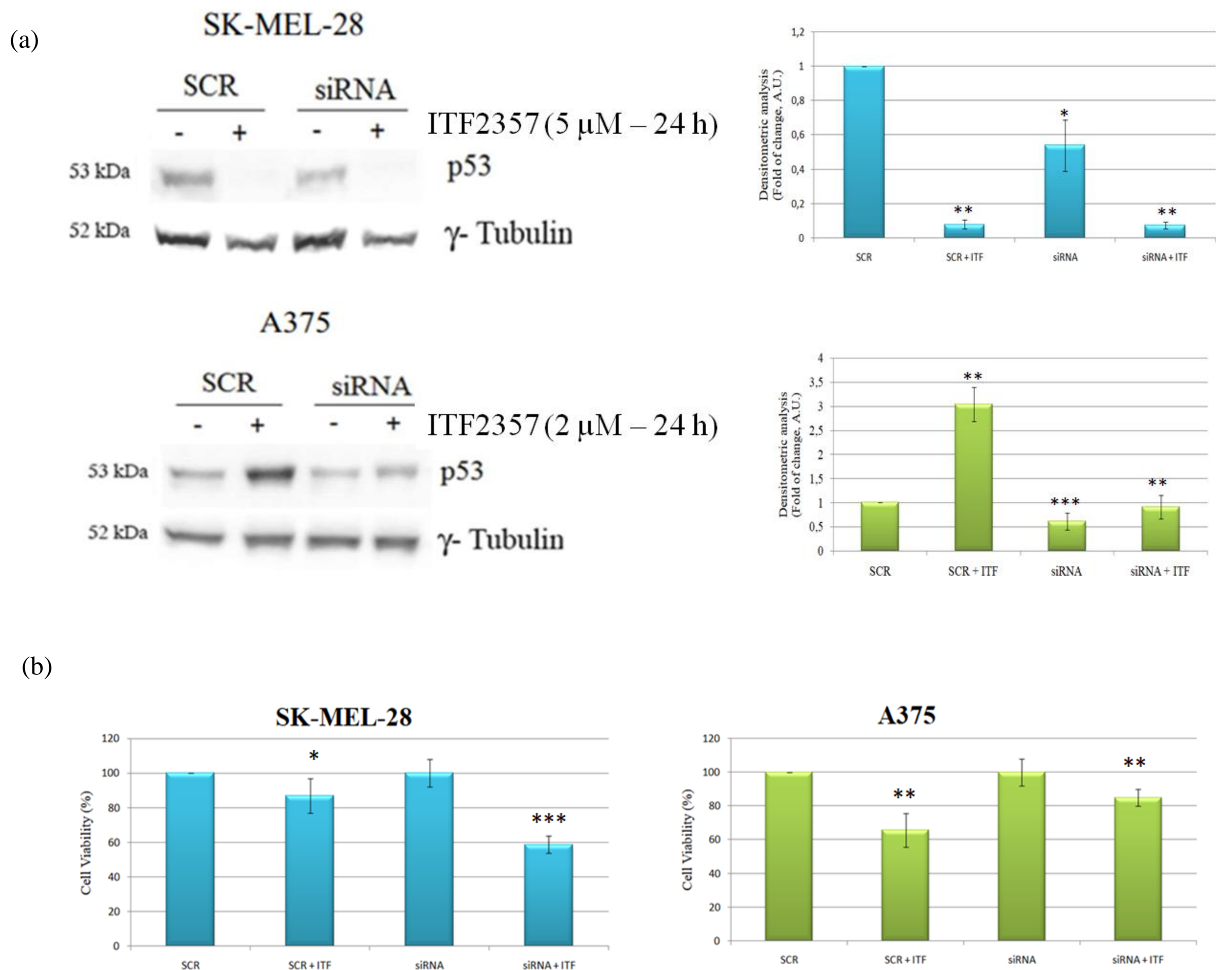


Figure 18: Oncogenic p53 counteracts the effect of ITF2357 in melanoma cells. (a) Western blot analysis of p53 in scramble (SCR) and p53-siRNA transfected SK-MEL-28 and A375 cells. Transfection was performed for 6 hours and then cells were treated with ITF2357 (5 µM in SK-MEL-28; 2 µM in A375). Densitometric analysis is shown after normalization referred to γ-tubulin. The results are representative of two different experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to SCR cells. (b) The effect of ITF2357 on cell viability in scramble and p53-siRNA transfected SK-MEL-28 and A375 cells. The results are representative of two different experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to SCR cells.

PART II: COLON CANCER

9. *ITF2357 is effective in colon cancer cells and potentiates the effect of DNMT inhibitors*

The second part of the project, that was mainly developed at the University of Oxford, was focused on the effects of ITF2357 in colon cancer cells. In particular, ITF2357 was used alone or in combination with other epi-drugs, DNA Methyltransferase (DNMT) inhibitors, in HCT116 and HT29 colon cancer cells.

First, cell viability was evaluated using different doses of the compounds in the two cell lines. As shown in figure 19, ITF2357 dose-dependently reduced cell viability in both colon cancer cell lines after 48 h, being more effective in HCT116 and resulting active at low doses (about 50% cell viability reduction with 1 µM) (figure 19a). HT29 cells, that display BRAF and p53 mutations, appeared to be more resistant to the effect of the compound.

Further analysis also indicated that ITF2357 affected the clonogenic ability of HCT116 cells (b). In detail, clonogenic assay was developed after 6 days of treatment with increasing concentrations of ITF2357. Data reported in figure 19 show that 0.25 µM ITF2357 reduced the number of clones of about 50%, and the maximum effect was obtained with 0.5 µM.

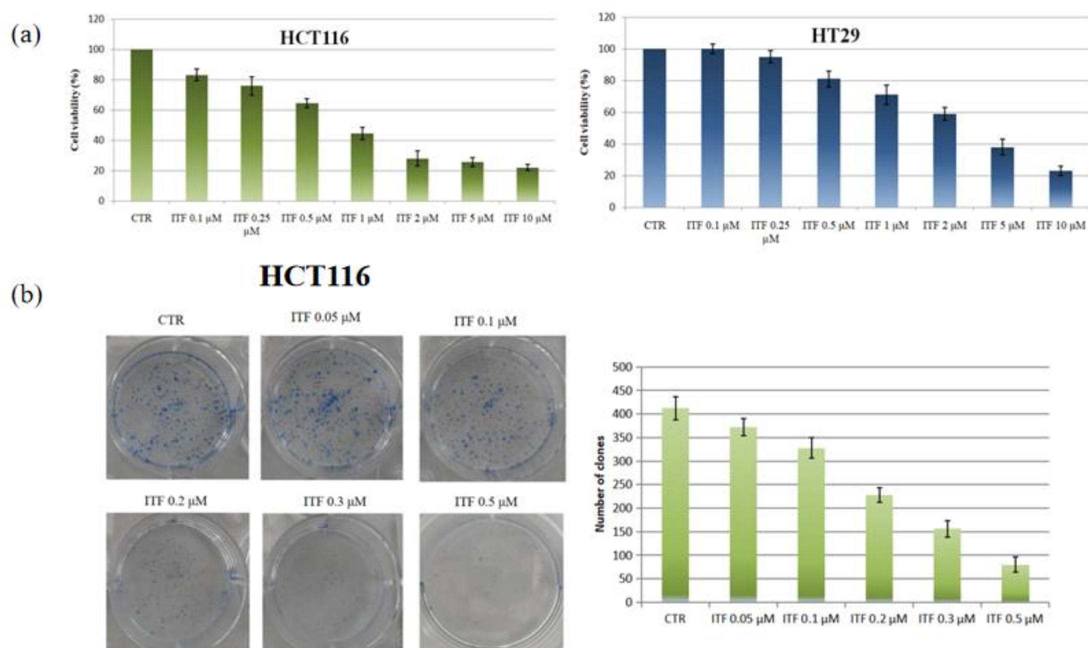


Figure 19: **The effects of ITF2357 in colon cancer cells**. (a) For cell viability evaluations, HCT116 and HT29 cells were treated with the indicated concentrations of ITF2357 for 48 h. At the end of the treatments, MTT analysis was performed. The results reported in the histograms are representative of two different experiments. (b) For clonogenic assays, HCT116 cells were seeded (400 cells/well) and, after 4 days, they were treated with ITF2357 as indicated. After further 6 days, the clones were stained as indicated in materials and methods. The images are representative of two different experiments.

The laboratory where I developed this second part of the project has a great experience in epigenetics and it has recently used DNA methyltransferase inhibitors (DNMTIs) as anti-tumour epi-drugs in colon cancer. In order to assess whether HDAC inhibitors potentiate the effects of DNMTIs, low doses of ITF2357 were combined with different DNMTIs in both HCT116 and HT29 cells. Specifically, the general DNMT inhibitor Decitabine (DAC - a nucleoside analogue) and the selective DNMT1 inhibitor (DNMT1i) GSK-3685032 were considered for these experiments.

As shown in figure 20 and in accordance with previous MTT analysis, 0.5 µM ITF2357 alone reduced cell viability of about 40% in HCT116 cells. Notably, ITF2357 potentiated the effect of both Decitabine (DAC) and DNMT1i on HCT116 cell viability reduction (a). The maximum effect was observed with the combination ITF 0.5 µM - DAC 600 nM (75% of cell viability reduction). Morphological evaluations also revealed that the effect of ITF2357 on cell viability was mainly due to cell number reduction, but most of the cells appeared viable (cytostatic effect), whereas clear signs of cell death appeared under the effect of the combination of ITF2357 with both DNMTIs (figure 20b).

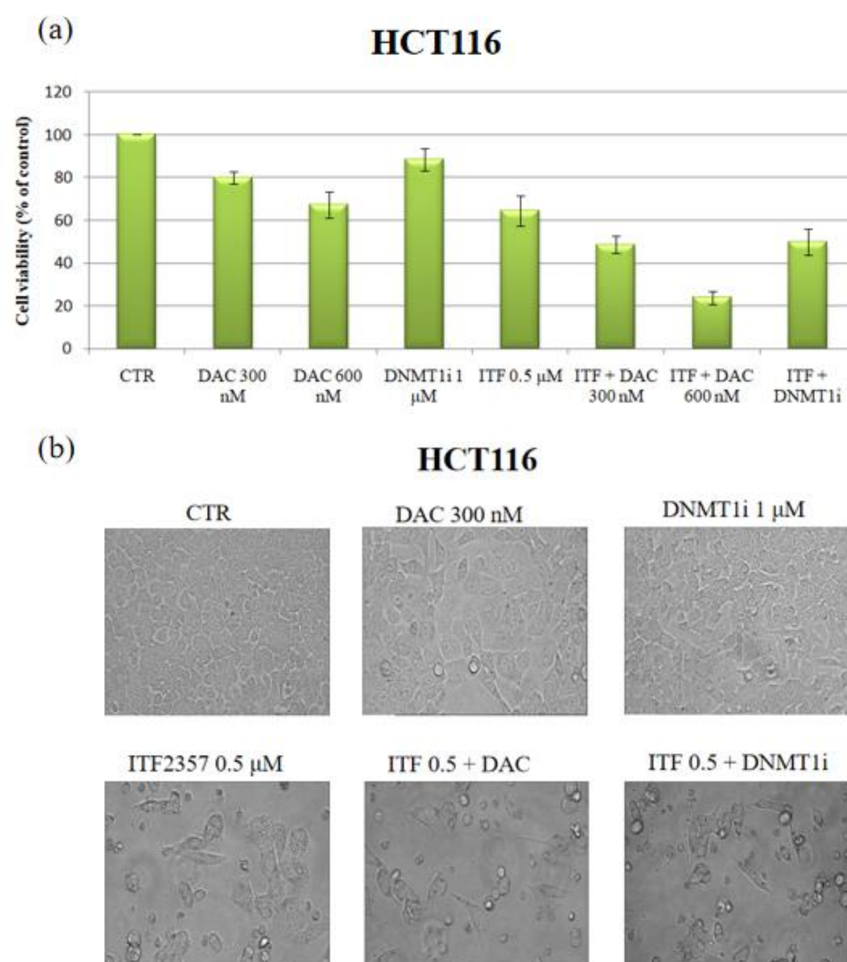


Figure 20: **The effects of ITF2357 and DNMT inhibitors in HCT116 colon cancer cells**. (a) For cell viability evaluations, HCT116 cells were treated with the indicated concentrations of Decitabine (DAC) or DNMT1 selective inhibitor (DNMT1i); after 24 h, ITF2357 was added and the treatment was prolonged for further 48 h. MTT analyses were then carried out using the Cell Proliferation Kit I (Roche®) as indicated in the datasheet. The results reported in the histograms are representative of two different experiments. (b) For morphological analysis, HCT116 were treated as previously indicated. After treatment, bright field images (400 X magnification) were acquired using the Nikon eclipse Ts2 microscope. The images are representative of two different experiments.

The same combinations of epi-drugs were also tested in BRAF and p53-mutated HT29 cells, which appeared much more resistant to the effects of ITF2357 either alone or in association with both DNMTIs, as shown in figure 21 by both MTT and morphological analysis.

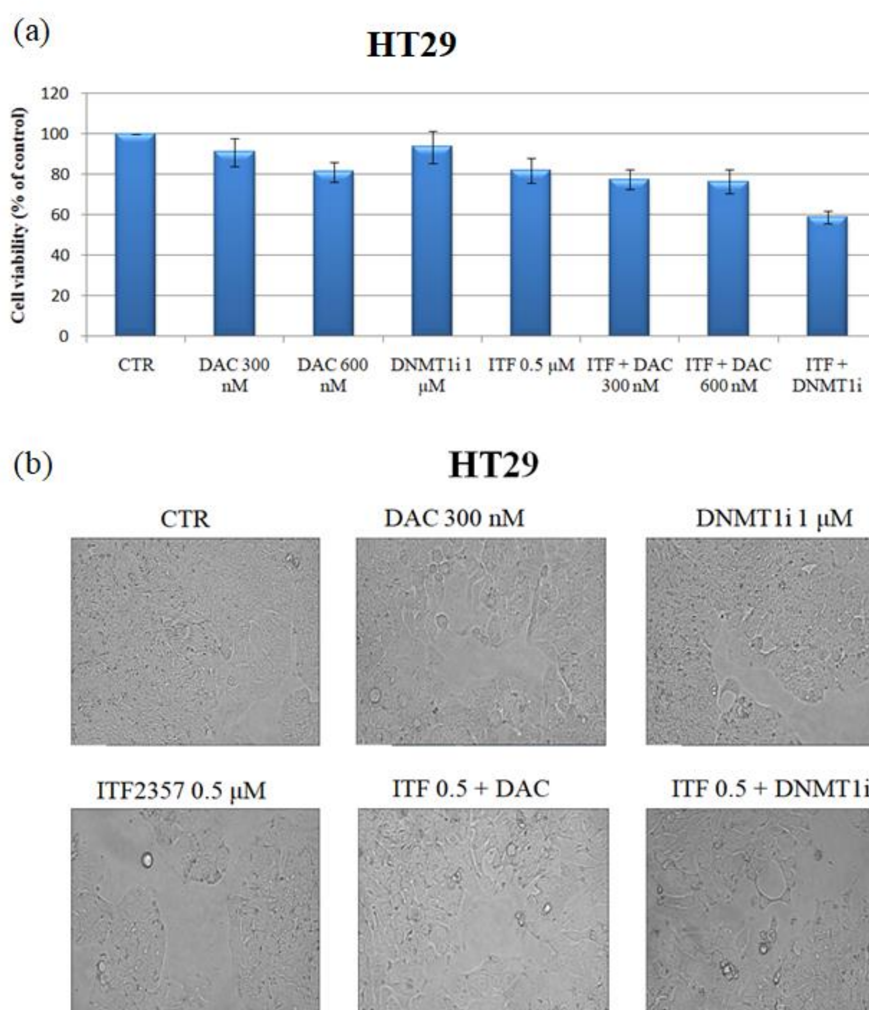


Figure 21: **The effects of ITF2357 and DNMT inhibitors in HT29 colon cancer cells**. (a) For cell viability evaluations, HT29 cells were treated with the indicated concentrations of Decitabine (DAC) or DNMT1 selective inhibitor (DNMT1i); after 24 h, ITF2357 was added and the treatment was prolonged for further 48 h. MTT analyses were then carried out using the Cell Proliferation Kit I (Roche®) as indicated in the datasheet. The results reported in the histograms are representative of two different experiments. (b) For morphological analysis, HT29 were treated as previously indicated. After treatment, bright field images (400 X magnification) were acquired using the Nikon eclipse Ts2 microscope. The images are representative of two different experiments.

These results were confirmed by trypan blue staining that is used to exclude dead cells from viable cells. As shown in figure 22, ITF2357 markedly increased the effect of DAC (from 3 to 59% reduction of viable cells) and DNMT1i (from 0 to 54%) in HCT116 cells (a). These effects were less evident in BRAF mutated HT29 cells (b), which showed a slight reduction of the percentage of viable cells with both DAC (from 15% of viable cell reduction with only DAC to 30% in combination with ITF2357) and DNMT1i (from 14% of viable cell reduction with only DNMT1i to 32% in combination with ITF2357).

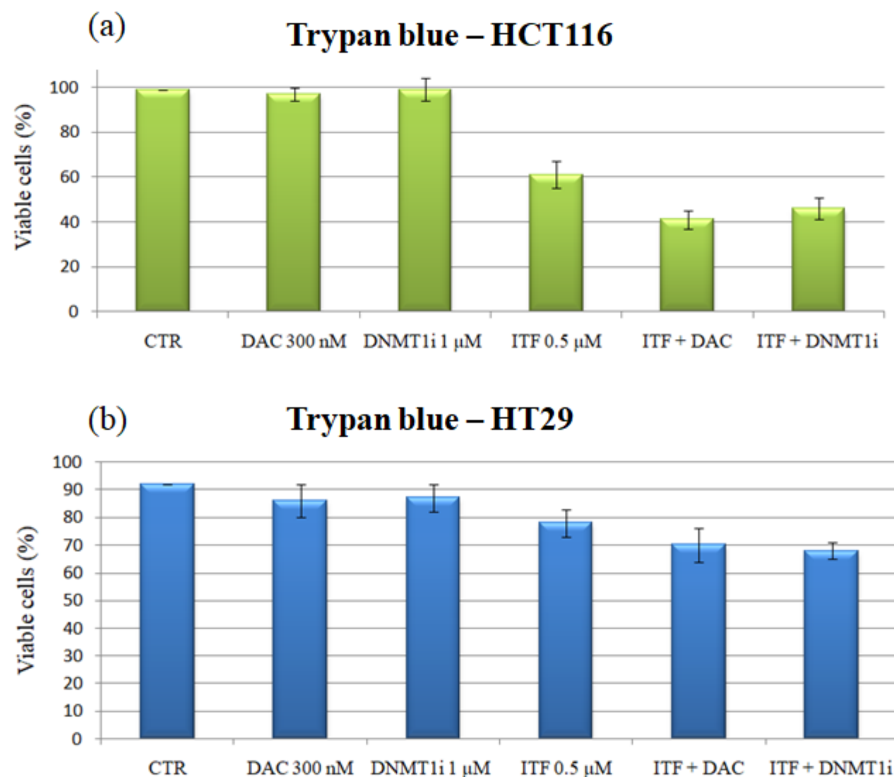


Figure 22: **The evaluation of colon cancer viable cells after treatment with ITF2357 and DNMT inhibitors**. For these evaluations, HCT116 and HT29 cells were treated with the indicated concentrations of Decitabine (DAC) or DNMT1 selective inhibitor (DNMT1i); after 24 h, ITF2357 was added and the treatment was prolonged for further 48 h. Then, cells were counted using an automatic cell counter (Bio-Rad). The results are relative to two different experiments.

10. The epigenetic effect of HDACIs and DNMTIs in colon cancer cells

To assess the epigenetic effects of ITF2357 and DNMTIs in our model, histone acetylation and DNMT1 protein levels were evaluated.

Western blot analysis (figure 23) showed that ITF2357 markedly increased the protein level of acetylated H3 histone in both HCT116 and HT29 cell lines, thus confirming its epigenetic effect as HDAC inhibitor in colon cancer cells. A similar effect was observed using another HDAC inhibitor, the cyclic peptide FK228 (2.5 nM), that was used as a positive control. Acetylation of H3 histone was also maintained in combination with

DNMT inhibitor Decitabine that, as expected, did not exert any effect on histone acetylation when used alone.

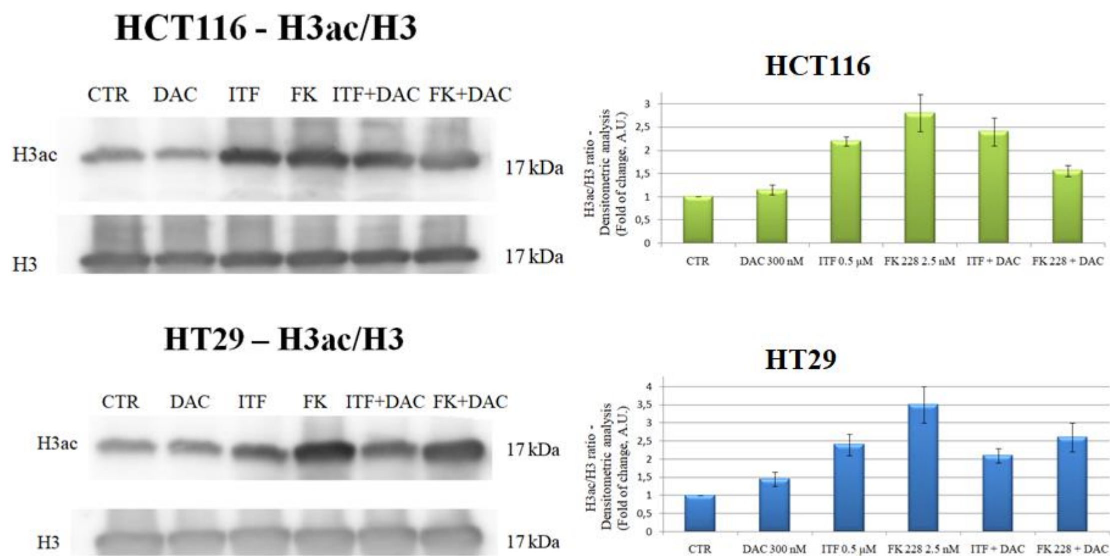
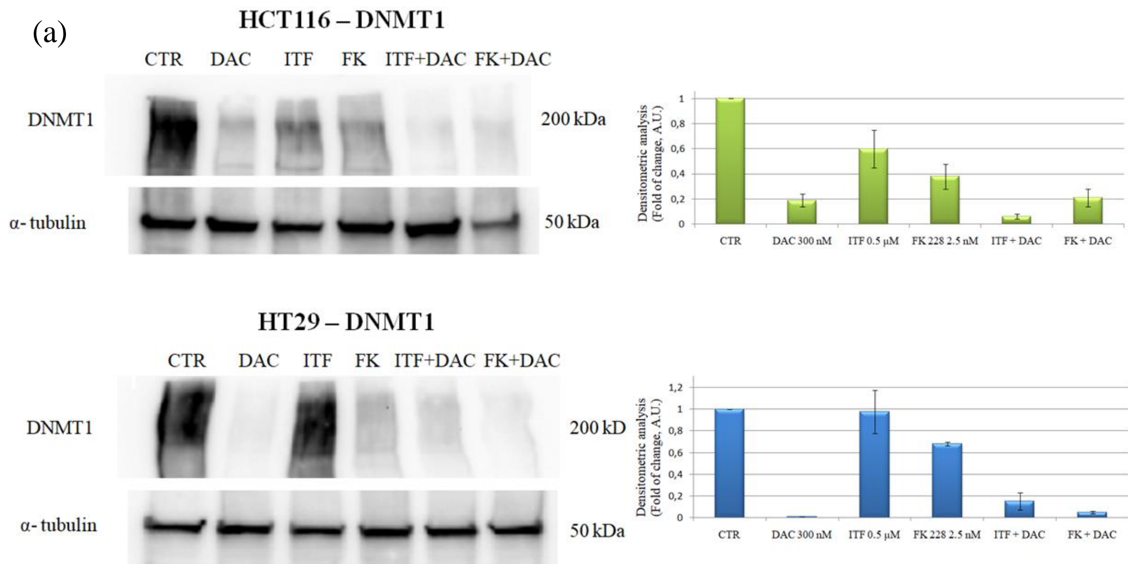


Figure 23: **ITF2357 promotes histone acetylation in HCT116 and HT29 cells**. Western blot analysis of acetylated H3 and H3 histone after 72 h treatment with DAC (300 nM) and 48 h with ITF2357 (0.5 µM) or FK228 (2.5 nM). The ratio between acetylated and total histone levels was quantified. Representative blots of two independent experiments are shown.

To validate the epigenetic effect of the DNMT inhibitors, the protein level of DNMT1 was also investigated. DNMT1 can be also considered a tumour target since it is often upregulated in tumour cells. Western blot analysis showed that Decitabine (DAC) was able to drastically reduce DNMT1 protein levels in both HCT116 and HT29 cells. Surprisingly, ITF2357 alone as well as the other HDAC inhibitor FK228 markedly decreased DNMT1 protein level in HCT116 cells. This reducing effect was dramatic when HDACIs were combined with DAC, the band almost disappearing. On the other hand, ITF2357 alone was not capable of producing a reduction of DNMT1 in HT29 cells whereas a dramatic effect was observed in the presence of DAC, either alone or in combination with the HDAC inhibitors (figure 24 – panel a).



The effect of Decitabine on DNMT1 protein levels was also compared to the effect of the selective DNMT1i (GSK-3685032). Western blot analysis demonstrated that DNMT1i was also efficacious in reducing DNMT1 protein level, although Decitabine had a stronger effect in both colon cancer cell lines. Moreover, ITF2357 exacerbated the reducing effect of DNMT inhibitors on DNMT1 protein level in HCT116 cells (figure 24 – panel b). Taken together, these data suggest that both HDACIs and DNMTIs target DNMT1, an activity that has been specifically correlated with tumour transformation and progression of colorectal cancer (Bowler et al., 2020).

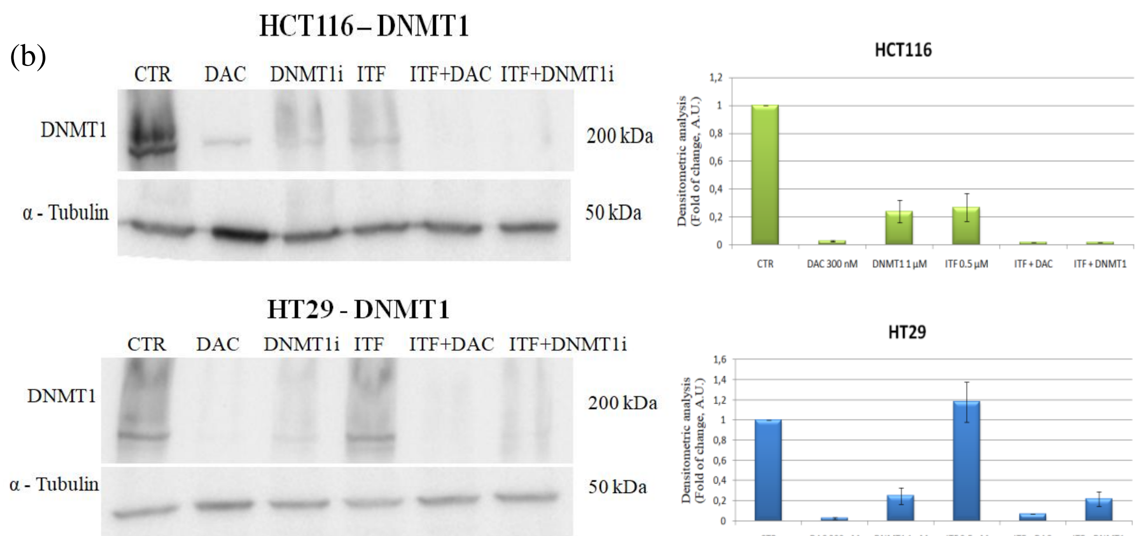


Figure 24. **The effect of ITF2357 on DNMT1 protein level in HCT116 and HT29 cells.** (a) Western blot analysis of DNMT1 protein after treatment for 72 h with DAC (300 nM) and 48 h with ITF2357 (0.5 µM) or FK228 (2.5 nM). (b) Western blot analysis of DNMT1 after treatment with DAC (300 nM) or DNMT1i (1 µM) for 24 h before adding ITF2357 as described in a. Representative blots of two independent experiments and densitometric analysis are shown.

11. The effect of DNMT inhibitors and ITF2357 on the induction of interferon-mediated genes

It is well known that tumour cells can evade immune response by different mechanisms. Interestingly, HDAC inhibitors have been shown to sensitize tumour cells to the attack of the immune system (Shen et al., 2023). The interest in the epigenetic laboratory coordinated by Dr. Mehdipour is also focused on the investigation of the immune system response to epigenetic drugs. For this reason, this part of the project aimed to evaluate the immunogenic response induced by ITF2357, either alone or in combination with DNMT inhibitors, in colon cancer cells.

From the literature it is known that DNMT inhibitors induce many interferon-mediated genes involved in immune response, which are exploited to regulate tumour immunity (Dan et al., 2019). In particular, in the first part of the study, the attention was focused on on two interferon-mediated genes with antitumour and immunoregulatory functions:

- ISG15 is a gene induced by type I interferon (IFN) that serves many functions, acting both as extracellular cytokine and intracellular protein modifier. Its functions include the promotion of Interferon gamma (IFN-II) production in lymphocytes, the ubiquitin-like conjugation to newly-synthesized proteins and the negative regulation of the IFN-I response (Perng and Lenschow, 2018). It was also found that it may trigger an anti-tumour response in tumour models; in fact, extracellular free ISG15 suppresses breast tumour growth and increases NK cell infiltration in nude mice, while intracellular free ISG15 enhances major histocompatibility complex (MHC) class I surface expression in breast cancer cells, thus concluding that free ISG15 may have antitumour and immunoregulatory function (Burks et al., 2015);
- DDX58 (RIG-I) is an innate immune receptor that senses viral nucleic acids in the cytoplasm, thus having a key role in sensing viral infection and activating a cascade of antiviral responses, such as type I interferons and proinflammatory cytokines. It can bind 5'-triphosphorylated ssRNA and dsRNA and short dsRNA (<1 kb in length) (Huang et al., 2022). Interestingly, it was shown that the activation of DDX58 inhibited the proliferation, migration and invasion of colon cancer cells, as well as tumour growth in a nude mouse xenograft model. In particular, DDX58 regulated the STAT3/CSE signalling pathway by interacting with STAT3 and consequently affecting the proliferation of tumour cells in colon cancer (Deng et al., 2022). Another study demonstrated that DDX58 upregulation triggered the extrinsic apoptosis pathway and pyroptosis, a highly immunogenic form of cell death in breast cancer cells. Moreover,

RIG-I agonist induced the expression of lymphocyte-recruiting chemokines and type I IFN, thus decreasing tumour growth and metastasis (Elion et al., 2018).

ISG15 and DDX58 gene expression was evaluated by Real-Time PCR following treatment with the epi-drugs. Specifically, the results indicated that Decitabine (DAC) induces the expression of both these genes in HCT116 and HT29 cells after 72 h treatment. At the same treatment-time, DNMT1i was able to slightly increase the mRNA level of ISG15, while it had no effect on DDX58 mRNA (figure 25).

Interestingly, ITF2357 significantly potentiated the effect of DNMT inhibitors, increasing both ISG15 and DDX58 levels in colon cancer cells.

In detail, ITF2357 combination with DNMT1i was particularly effective in exacerbating interferon-mediated gene expression in HCT116 cells (a), significantly increasing the mRNA levels of both ISG15 (from 3 fold with DNMT1i alone to 18 fold in combination) and DDX58 (from 1.7 fold with DNMT1i alone to 7.4 fold in combination with ITF2357).

On the other hand, in HT29 cells the combinatory effect was stronger with DAC (b); in fact, ITF2357 used in combination with DAC highly increased the mRNA levels of both ISG15 (from 2.6 fold for DAC alone to 7.6 fold in combination with ITF2357) and DDX58 (from 2.1 fold for DAC alone to 4.5 fold in combination with ITF2357). These results suggest that ITF2357 is able to increase the interferon-mediated response induced by DNMTIs in colon cancer cells.

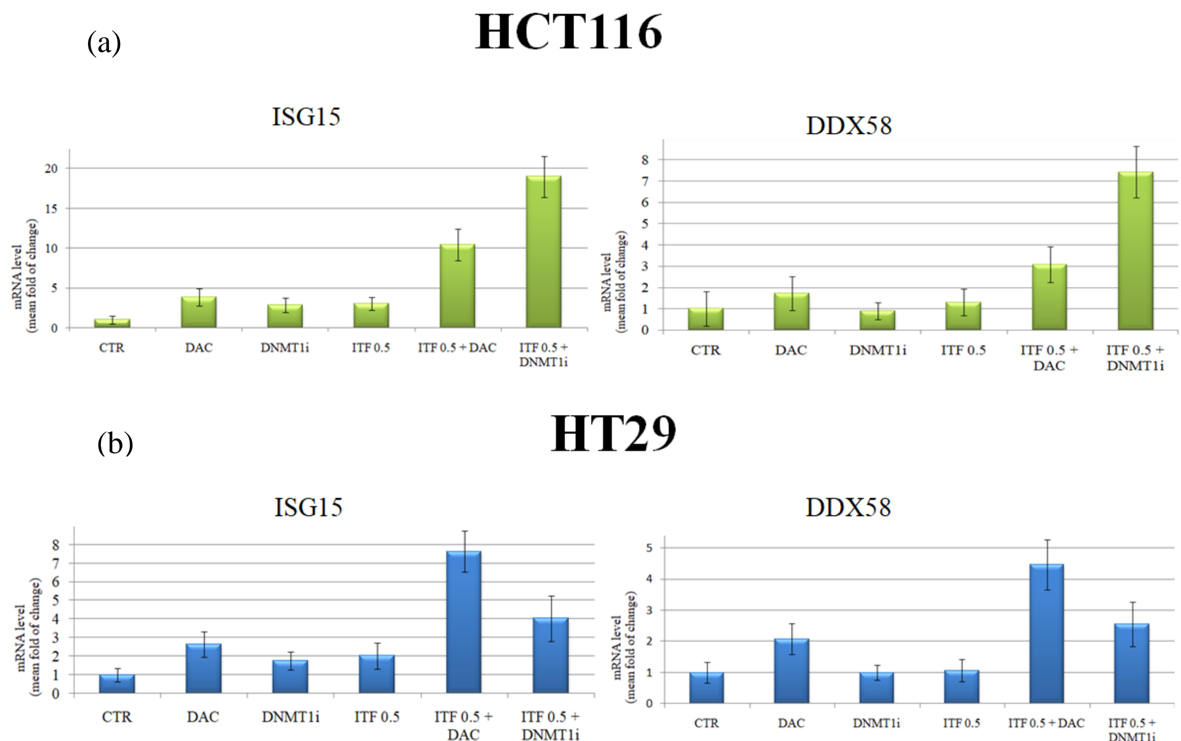


Figure 25: **The effect of ITF2357 on ISG15 and DDX58 in HCT116 and HT29 cells**. Real-Time PCR was performed after treatment for 72 h with DAC (300 nM) or DNMT1i (1 μM) and 48 h with ITF2357 (0.5 μM) in HCT116 cells (a) or HT29 cells (b). Data shown in the histograms are representative of two independent experiments.

12. The effect of DNMT inhibitors and ITF2357 on the induction of immunogenic dsRNAs

Mammalian cells can sense viral infection through detection of double - stranded RNAs (dsRNAs) by using specific receptors of the innate immune system that trigger antiviral and inflammatory immune responses. It has been recently demonstrated that dsRNAs are not limited to virally infected cells, but can be produced from endogenous sources, such as retroelements and mitochondrial DNA, in various pathophysiological states or after treatment with some epigenetic drugs. These endogenous dsRNAs can therefore signal misregulated cellular processes. Accordingly, innate immune and inflammatory responses to dsRNAs underlie diverse pathophysiologies (Chen and Hur, 2022). Moreover, hypomethylating agents (such as Azacytidine or Decitabine) also reactivate the transcription of endogenous dsRNAs that trigger the innate immune response and subsequent apoptosis via viral mimicry. Moreover, it has been demonstrated that the increase in dsRNAs is related to the response to hypomethylating agents. In fact, by analyzing the bone-marrow aspirates of myelodysplastic syndrome or acute myeloid leukemia patients who received the hypomethylating agents, a dramatic increase was found in total dsRNAs levels upon treatment only in patients who later benefited from the therapy. It has also been shown that in solid tumour cell lines the degree of dsRNA induction correlates with the effectiveness of Decitabine in most cases (Kang et al., 2022).

To evaluate a possible immunogenic response induced by DNMT inhibitors in combination with ITF2357, immunofluorescence (IF) analysis was first performed to evaluate the effect of epi-drugs on dsRNAs production. Using J2 antibody against dsRNAs greater than 40 bp, IF images showed that cytoplasmic dsRNAs are not detectable in untreated cells, while Decitabine (DAC) and DNMT1i notably increased dsRNAs levels (red) in both colon cancer cell lines after 72 h treatment (figure 26). As regards HCT116 cells, dsRNAs red signal was also detected after ITF2357 treatment. Moreover, the HDAC inhibitor was also capable of producing dsRNAs red signal when it was used in combination with DAC or DNMT1i. However, it has to be considered that, in this case, cell number was dramatically reduced due to cell death determined by the combinatory effect of the compounds (panel a).

Interestingly, in HT29 cells the intensity of dsRNAs signal increased after treatment with DAC or DNMT1i, an effect not observed after ITF2357 treatment alone. Nonethless, a clear induction of dsRNAs was observed when ITF2357 was used in combination with DAC or DNMT1i (panel b), thus suggesting that DNMT inhibitors are able to stimulate the dsRNAs production that is mildly induced by ITF2357 alone.

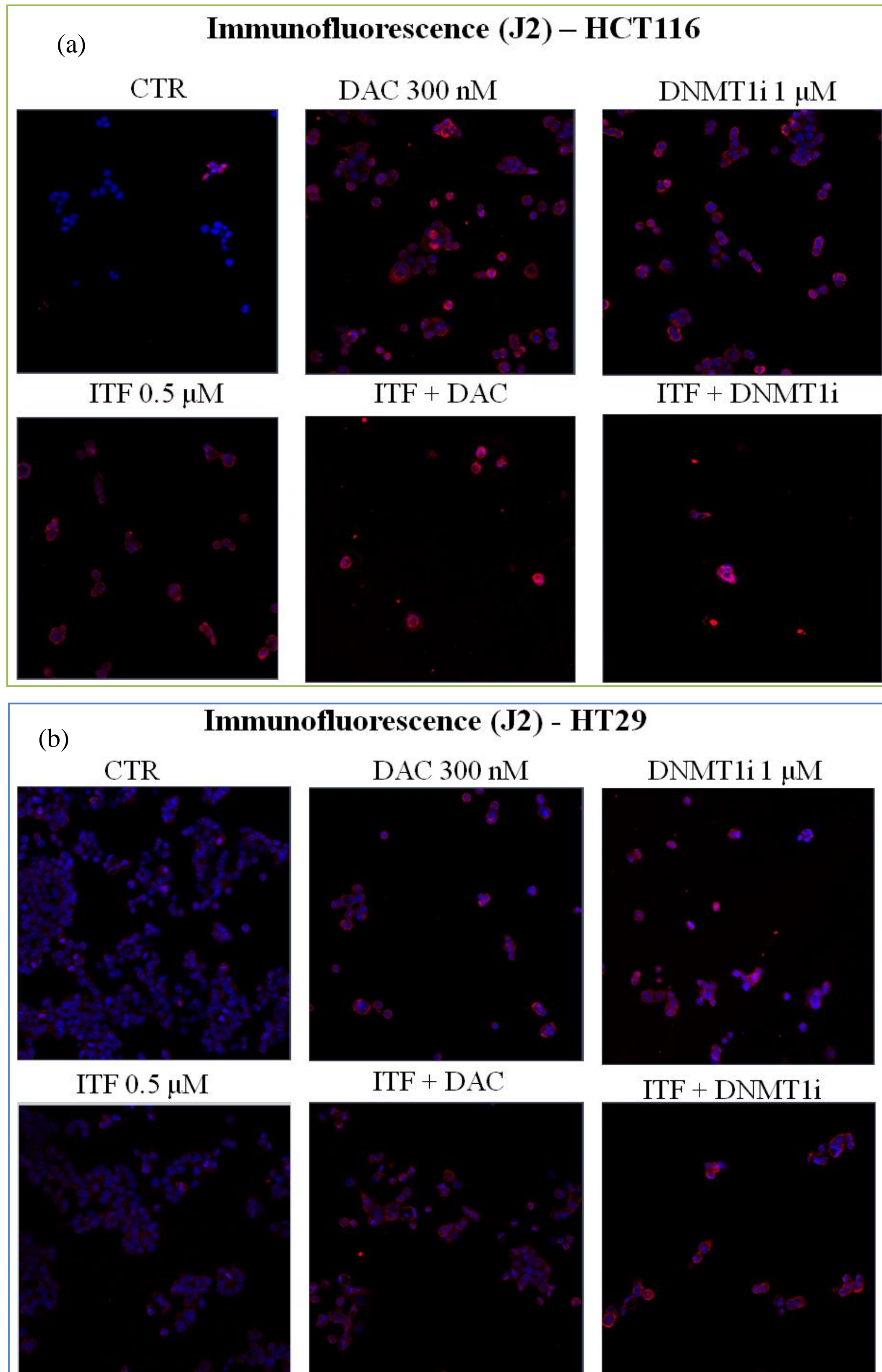


Figure 26: **Immunofluorescence (IF) analysis of dsRNAs in HCT116 and HT29 cells.** Cells were seeded after treatment for 72 h with DAC (300 nM) or DNMT1i (1 µM) and 48 h with ITF2357 (0.5 µM) in HCT116 or HT29 cells. dsRNAs signal was detected using J2 antibody (red) and nuclei were stained with Hoechst 33342 (blue). The IF images were acquired using the Zeiss LSM980 confocal microscopy and are representative of three different fields of a single experiment.

Immunogenic dsRNAs were also evaluated by dot blot analysis in RNA samples from HCT116 and HT29 cells using the J2 monoclonal antibody (the same used for immunofluorescence). As shown in figure 27, an increase in the amount of immunogenic dsRNAs compared to the control was observed after treatment with Decitabine and DNMT1i in both colon cancer cell lines, thus confirming what was previously observed by immunofluorescence.

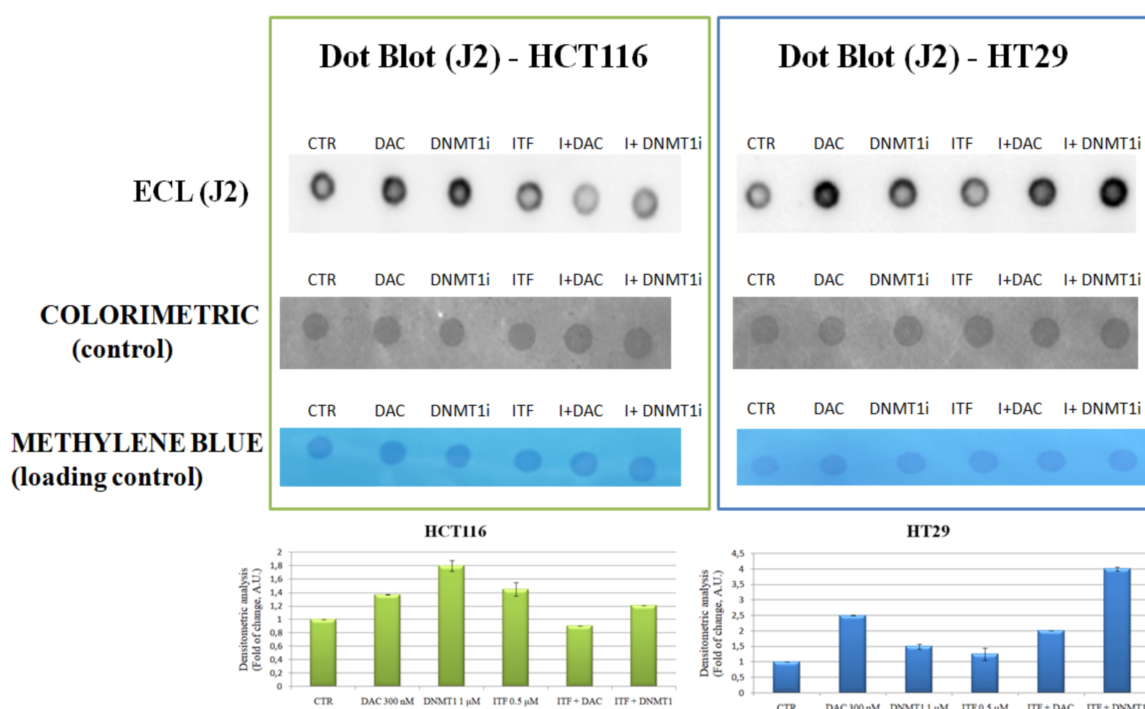


Figure 27: **Dot Blot analysis of dsRNAs in HCT116 and HT29 cells**. RNA samples were obtained after treatment for 72 h with DAC (300 nM) or DNMT1i (1 µM) and 48 h with ITF2357 (0.5 µM). RNA dots were detected using J2 antibody. Representative blots of two independent experiments are shown. The histograms show the densitometric analysis, where J2 signal was normalized for colorimetric, considered as control.

Overall, ITF2357 increased cytoplasmic J2 signal induced by DNMT inhibitors in HCT116 cells, as previously observed by immunofluorescence. However, the combinatory treatment remarkably reduced HCT116 cell number due to cell death, strongly suggesting that this effect accounts for the reduction in J2 signal observed by dot blot analysis.

An opposite effect was observed in HT29 cells, where the combination with DNMTIs increases the dsRNAs level compared to ITF2357 alone. This result can be explained

considering the lower efficacy of the combination on cell death, that guaranteed a more reliable result on dsRNA level.

All the evaluations shown in the second part of the project, that was developed during the visiting period abroad, require further replication experiments in order to provide a statistic significance. Considering the time restraint of the period spent abroad, data reported are in most cases the mean of two independent experiments. These preliminary data need to be corroborated by further investigations aimed to elucidate the immunogenic effect of ITF2357/DNMTIs combination in colon cancer cells. Moreover, future perspectives aim to clarify the role of oncogenic BRAF and p53 in immunogenic response induced by the epigenetic combination, considering that the two colon cancer cell lines used in this study differ in BRAF and p53 status.

V. DISCUSSION

PART I : MELANOMA

The first part of this study demonstrated, for the first time, that HDAC inhibitors ITF2357 and SAHA are effective in melanoma cells and target oncogenic BRAF. These findings are in line with the observation that HDAC inhibitors sensitize tumour cells bearing $BRAF^{V600E}$ mutation to the effect of BRAF inhibitors (Gallagher et al., 2018).

The decreasing effect on the oncogenic BRAF protein level was particularly evident with ITF2357, which resulted the most efficacious HDAC inhibitor in SK-MEL-28 and A375 melanoma cells. Importantly, this compound was also capable of inducing histone acetylation in melanoma cells, thus validating its epigenetic effect.

It is not clear yet whether histone acetylation can contribute to the BRAF decrease. However, it is possible to hypothesize that epigenetic changes caused by HDAC inhibitors promote modification in gene expression pattern reducing BRAF gene transcription. This hypothesis is sustained by our data, indicating that BRAF-mRNA levels decrease following ITF2357 treatment in both cell lines. Although histone acetylation usually favours chromatin relaxation and consequent gene expression, overall epigenetic changes may also result in gene repression. Accordingly, acetylation of specific histone tail residues, together with other histone modifications, has been shown to promote transcriptional repression (Mehrotra et al., 2014). It is thus not surprising that epigenetic changes may result in reduced BRAF expression, a result that was clear after 24 h treatment with ITF2357, a condition properly chosen to avoid degradative processes due to cell death. However, since the BRAF protein level dramatically decreased at 48 h, we also hypothesized that the effect of ITF2357 is related to degradative processes. In line with this hypothesis, our data demonstrated that proteasome-mediated degradation can also account for the remarkable BRAF protein level decrease, since the proteasome inhibitor Bortezomib partially prevented the decreasing effect of ITF2357 on BRAF levels in melanoma cells. This result is in accordance with the finding that BRAF can be degraded by the 26S proteasome (Ohoka et al., 2022). The evaluation of BRAF half-life using Cycloheximide confirmed that BRAF undergoes degradation under the effect of ITF2357, since its decrease was anticipated in the presence of the protein synthesis inhibitor.

Interestingly, BRAF decrease induced by ITF2357 was a quite late event observed at 48 hours, similarly to the decrease in phospho-ERK (p-ERK), its downstream signalling component. The reduction of p-ERK confirmed that BRAF targeting was accompanied by the inhibition of the mitogenic pathway. Moreover, our results showed that the MEK inhibitor U0126 markedly potentiated the effects of the HDAC inhibitor on both cell

viability and BRAF levels. These data also indicate that combining the HDAC inhibitor with the MEK inhibitor increases oncogenic BRAF-targeting and may represent a nice tool to potentiate melanoma cell death. These findings are sustained by recent evidences demonstrating that HDACIs can enhance the antitumor activity of MEK inhibitors in other tumour models, such as lung cancer (Yamada et al., 2018) and pancreatic cancer cells (Chao et al., 2019).

Several data present in the literature sustain that pro-survival autophagy can be promoted by oncogenic BRAF, thus favouring melanoma cell survival and propagation (Corazzari et al., 2015). More specifically, oncogenic BRAF mutations have been associated with autophagy-promoted tumorigenesis and tumour progression in other tumour models, including colon cancer (Goulielmaki et al., 2016; Koustas et al., 2018). Interestingly, it has been recently demonstrated that autophagy inhibition strongly potentiates the effectiveness of ITF2357 in human glioblastoma cancer stem cells (Angeletti et al., 2016b). In line with these data, our results showed that ITF2357 induces a pro-survival autophagic response in melanoma cells, since the autophagy inhibitors Bafilomycin A1 and 3-methyladenine markedly increased the cytotoxic effect of the HDAC inhibitor. Moreover, further evaluation of autophagic markers confirmed that they increased early (16 h) and tended to decrease later on (48 h), with an exception for p62, which accumulated at 48 h, thus suggesting that autophagy was not completed. Indeed, p62 is usually considered to monitor the autophagic flux since its degradation by the autophagosome is associated with completed autophagy while p62 degradation often indicates a blockage of the autophagic process (Emanuele et al., 2020). At 48 h, when autophagy was not observed, caspase-dependent apoptosis was detected and confirmed by evaluating apoptotic markers and the protective effect of the pan-caspase inhibitor z-VADfmk. Taken together, these results strongly indicate that ITF2357 promotes a switch from autophagy to classic apoptosis. The finding that ITF2357 induces apoptosis is in line with the observations of other authors, who described the pro-apoptotic effect of the compound in other tumour cell lines, such as sarcoma (Di Martile et al., 2018b) and meningioma (Zhang et al., 2022).

According to our data indicating that ITF2357 targets oncogenic BRAF, we specifically investigated BRAF localization in melanoma cells. It is widely recognized that BRAF usually localizes in the cytoplasm, where it takes part in the mitogenic MAPK-mediated pathway. Recent evidences indicate that oncogenic $BRAF^{V600E}$ is also found in the nucleus of melanoma cells, thus promoting cell proliferation and tumour growth (Zerfaoui et al., 2022). The results reported in this thesis demonstrate that oncogenic BRAF is localized in

both the cytosol and the nucleus of SK-MEL-28 and A375 melanoma cells and, most importantly, that ITF2357 is able to target the oncogenic protein $BRAF^{V600E}$ in both these compartments, thus reducing its protein level.

The nuclear localization of BRAF is most likely correlated with its oncogenic potential. Therefore, we hypothesized that nuclear oncogenic BRAF binds and phosphorylate transcription factors, thus favouring the expression of genes involved in tumour development and progression. Among the putative BRAF nuclear interactors, p53 was considered. This choice was motivated by the different p53 status in the two melanoma cell lines. Specifically, SK-MEL-28 cells display an oncogenic form of p53, while A375 cells display the wild type form of p53. Considering these premises, a possible interplay between oncogenic BRAF and p53 was thus considered.

First, we evaluated whether the different susceptibility of the two melanoma cell lines to ITF2357 depends on p53 status. Our results show that ITF2357 dramatically reduced the level of oncogenic p53 in SK-MEL-28 already at 16 h, while increasing the wild type p53 level in A375 at the same treatment condition. Prolonging treatment up to 48 h decreased p53 level in both cell lines, probably due to degradative events correlated with apoptosis. According to these data, it is plausible that the oncogenic p53 form present in SK-MEL-28 cells is somehow targeted by ITF2357. On the other hand, the wild type p53 present in A375 cells precociously increases most likely to promote the apoptotic response and is then degraded when apoptosis is completed. Further analysis demonstrated that oncogenic p53 reduction was completely prevented by the proteasome inhibitor Bortezomib, suggesting that oncogenic p53 in SK-MEL-28 cells is targeted via proteasome-mediated degradation, which is in line with the findings of other authors (Zhang and Zhang, 2008).
Interestingly, recent data suggest that selective degradation of mutated (oncogenic) p53 may occur and can represent a strategy to target tumour cells (Garg et al., 2023; Kong et al., 2023; Nishikawa and Iwakuma, 2023). On the other hand, wild type p53 stabilization is considered an approach to favour cell cycle arrest and apoptosis and it is exploited to counteract tumour progression and invasiveness (Yang et al., 2009; Zhou et al., 2023).
To unveil whether BRAF interacts with p53, immunoprecipitation experiments clearly showed that oncogenic p53 is present in BRAF immunoprecipitates obtained by SK-MEL-28 cells. This was confirmed by the presence of BRAF in p53 immunoprecipitates. The same evaluations in A375 cells did not produce an equally clear result, because wild type p53 was almost undetectable in BRAF immunoprecipitates. It is thus possible to deduce that BRAF might preferentially bind to oncogenic p53 and probably contribute to its

oncogenic potential. However, this hypothesis need further confirmation. Studies are ongoing in our laboratory in this regard.

To our knowledge, our data represent the first evidence about a possible $BRAF^{V600E}$/oncogenic p53 interplay in melanoma cells.

p53 status may also be determinant in melanoma onset and pharmacological response. Canonical p53 tumour suppressor function results to be compromised in melanoma by different mechanisms (Loureiro et al., 2021) and it is widely known that mutations changing p53 from a tumour suppressor to an oncogene strongly contribute in tumour transformation (Pitolli et al., 2019; Sigal and Rotter, 2000). Those p53 isoforms with enhanced expression and promoting resistance to BRAF inhibitors in melanoma cells are interesting, because targeting their functions may help to overcome resistance to BRAF inhibitors (Vlašić et al., 2022).

Although p53 mutations are common in diverse tumour types (Zhu et al., 2020), mutational alteration of p53 in melanoma is a non-frequent event (Loureiro et al., 2021). Abnormal p53 content and function often appear in melanoma and its knockdown has been shown to decrease cell proliferation in melanoma (Avery-Kiejda et al., 2011). In melanomas, p53 loss-of-function has been related with reduced cytokine expression, reduced migration and increased sensitivity to BRAF inhibition (Pandya et al., 2022), thus supporting the role of aberrantly accumulated p53 in melanoma. p53 impairment has been attributed in melanoma to either deregulation of mouse double minute 2 (MDM2), the major ubiquitin ligase involved in p53 degradation, or inactivation of the cyclin-dependent kinase inhibitor 2A (CDKN2A) locus, encoding p16INK4A and p14ARF tumour suppressors (Loureiro et al., 2021). It has also been shown that altered expression of p53 family isoforms impacts melanoma aggressiveness (Tadijan et al., 2021). Therefore, targeting p53 in melanomas is considered a valid strategy (Box et al., 2014; Loureiro et al., 2021).

This study provided the first evidence of an interplay between oncogenic p53 with oncogenic BRAF that may account for melanoma malignancy. Interestingly, data reported in this thesis also reveal the efficacy of ITF2357 in targeting both oncogenic factors and disrupting their interaction.

Moreover, we investigated whether p53 status may influence the response to ITF2357 in the two melanoma cell lines. To this purpose, p53 silencing and subsequent cell viability evaluations were performed and revealed that oncogenic p53 knockdown increased the effect of ITF2357 in SK-MEL-28. Similarly, in another paper was shown that mutant *gain of function*-p53 silencing cooperate with anti-tumour compounds (in this case, Cisplatin) in targeting tumour cells, such as 5637 bladder carcinoma cells (Zhu et al., 2013). On the

other hand, the knockdown of wild type p53 in A375 cells reduced the effect of ITF2357. These data demonstrate that the response of ITF2357 in BRAF-mutated melanoma cells is dependent on p53 status and provides a rationale for melanoma targeted therapy.

Overall, these data corroborate the oncogenic role of p53 in SK-MEL-28 cells, where it probably serves a pro-survival function, while in A375 cells wild type p53 maintains its usual tumour-suppressor role promoting apoptosis. Moreover, it has been shown by other authors that HDAC inhibitors promote p53-dependent apoptosis in different tumour models, such as neuroblastoma and osteosarcoma (Condorelli et al., 2008; Deng et al., 2016). The presence of wild type p53 in melanomas may therefore sustain HDAC inhibitors role as anti-tumour agents. Considering that targeting mutated p53 constitutes a key approach for melanomas, our finding that ITF2357 targets both oncogenic p53 and oncogenic BRAF in SK-MEL-28 cells lay the foundations to consider this compound as a potential epigenetic candidate for melanoma targeted therapy. The figure below shows the effects of ITF2357 in melanoma cells.

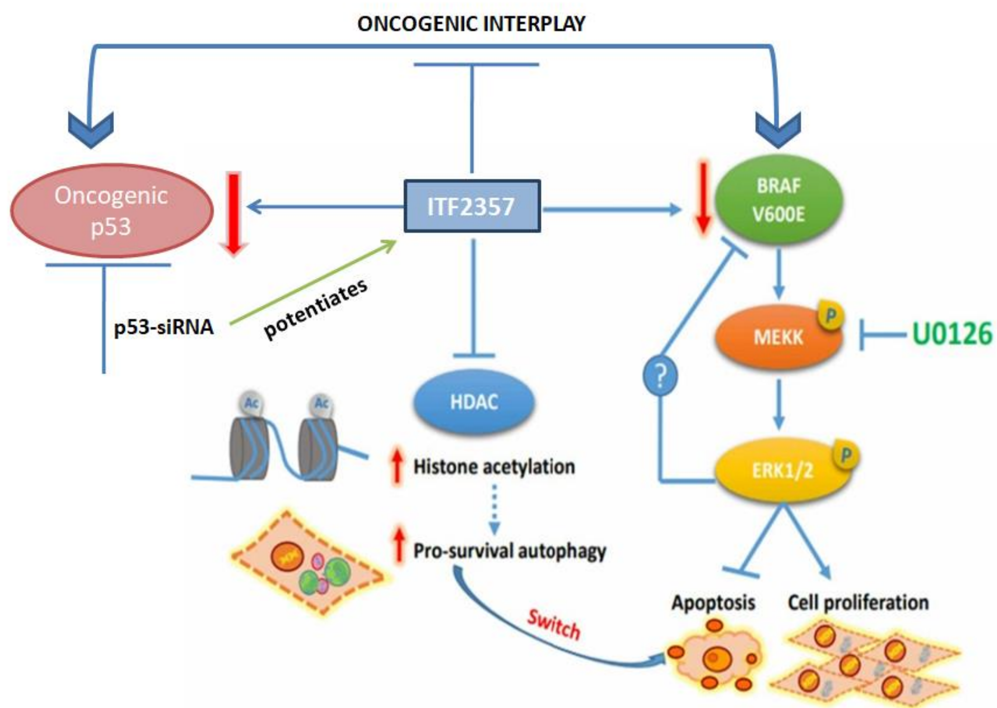


***Figure 1**: Schematic representation of the effects of ITF2357 in melanoma cells.*

PART II: COLON CANCER

The anti-tumour effects of ITF2357 have been widely described in hematological malignancies, but few data have been obtained in solid tumours so far. The second part of this study was then aimed to investigate the effects of ITF2357 in colon cancer cells and to evaluate the effects of its combination with other epigenetic drugs, including hypomethylating agents (HMA).

Many evidences in the literature indicate that HDAC inhibitors can synergize with DNMT inhibitors in cancer. For example, it was shown that, in acute myeloid leukemia, the DNMTi Decitabine (DAC) in combination with the HDACi panobinostat or valproic acid induces a massive downregulation of genes, including oncogenes (e.g., MYC) and epigenetic modifiers (e.g., KDM2B, SUV39H1) often overexpressed in cancer, that was associated predominantly with gene DNA demethylation and changes in acH3K9/27 (Blagitko-Dorfs et al., 2019).

First, cell viability evaluations shown in this thesis demonstrated that ITF2357 reduced HCT116 and HT29 cell viability in a dose-dependent manner after 48 h treatment. Interestingly, the compound was effective at very low concentration without showing toxic effects and resulted more efficacious in HCT116 than HT29 cells. Then, the attention was focused on the evaluation of the effect of ITF2357 in combination with two DNMT inhibitors, the general DNMT inhibitor DAC and the selective DNMT1 inhibitor GSK-3685032 (DNMT1i).

Cell viability results and morphological analysis indicated that DNMT inhibitors had no visible effect alone, while ITF2357 was effective and potentiated the effects of DNMT inhibitors in colon cancer cells. Interestingly, BRAF- mutated HT29 cells resulted more resistant than HCT116 cells to the effect of these epigenetic compounds. The low susceptibility of HT29 cells is in accordance with a study demonstrating that these cells are more resistant than HCT116 cells to the combination of the chemotherapeutic drug Irinotecan (CPT-11) with the epigenetic drug Decitabine (DAC) (Hakata et al., 2018).

Moreover, another study demonstrated that BRAF mutation in colon cancer is associated with the CpG island methylator phenotype, resulting in epigenetic silencing of key genes that may be associated with resistance to the BRAF inhibitor PLX4720. In detail, RKO, Colo205, LS411N, and HT29 were treated with the DNA methyltransferase inhibitor 5-azacytidine in combination with PLX4720 and it was found that 5-azacytidine had minimal effect on colon cancer cell growth *in vitro*. However, combination therapy resulted in

greater inhibition than PLX4720 alone and, in a HT29 xenograft, enhanced tumour growth inhibition was seen with 5-azacytidine combined with PLX4720 administration *in vivo*, compared to either agent alone (Mao et al., 2013).

Further analyses reported in this thesis demonstrated that ITF2357 was epigenetically effective, increasing H3 histone acetylation in both cell lines; the same effect was detected using FK228, another HDAC inhibitor that was chosen as positive control. To elucidate the epigenetic effect of DNMT inhibitors, their effect on DNMT1 levels was evaluated, which was markedly reduced by both DAC and DNMT1i. Interestingly, ITF2357 alone was also able to dramatically reduce DNMT1 level in HCT116 cells (but not in HT29 cells) and DNMT1 protein completely disappeared when ITF2357 was in combination with DAC or DNMT1i in both cell lines. These data strongly indicate that DNMT1 can be considered an interesting target in colon cancer cells and its inhibition results to be beneficial, as evidenced in HCT116 cells by other authors (J. Chen et al., 2019; Seo et al., 2017). Notably, some colon cancer cell lines, including HCT116, display an high basal level of DNMT1, while other cell lines, such as HT29, show very low basal level of this protein (Sharma et al., 2017). For this reason, the anti-tumour effects related to DNMT1 targeting may be more beneficial in cell lines presenting high basal level of DNMT1, thus supporting a rationale for colon cancer targeted therapy.

An interesting link exists between epigenetics and immune response in cancer. It is widely known that DNA hypomethylation reactivates the expression of genes aberrantly silenced by hypermethylation in cancer cells, including tumour suppressor genes. This is also valid for anti-tumour immunity modulators. For instance, by reverting DNA methylation-dependent repression of MHC-I genes and by inducing the expression of cancer/testis antigens, the availability of T-cell antigens on cancer cells can increase upon DNMT inhibitors stimulation. In addition, DNMT inhibitors induce a state of viral mimicry by activating the expression of human endogenous retroviruses (HERVs) that are normally silenced by DNA methylation. The transcription of HERV-derived double-stranded RNAs (dsRNAs) leads to increased expression of type I and/or type III interferons (IFNs), which display antineoplastic effects and support anti-tumour immunity. The process ends with the stimulation of innate and adaptive cytotoxic lymphocyte populations and negative regulation of cell types known to dampen anti-tumour immune responses, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Traynor et al., 2023). From the literature, it is therefore known that DNMT inhibitors display a remarkable immunogenic effect in cancer cells, thus regulating the interferon-mediated response (Chiappinelli et al.,

2015). Moreover, it has been demonstrated that combining DNMTIs and HDACIs can increase the anti-tumour immune signalling and decreases tumour burden (Moufarrij et al., 2020).

The results reported in this thesis show that DAC and DNMT1i are able to increase the gene expression of two interferon-mediated genes (ISG15 and DDX58) in colon cancer cells, and, most importantly, that this effect is significantly increased in combination with ITF2357. These data suggest that HDAC inhibitors can sustain the immunogenic effect of DNMT inhibitors. Moreover, these results are in line with a paper demonstrating that ITF2357 resulted more efficacious in combination with Azacitidine than other HDAC inhibitors, such as SAHA, in inducing interferon-stimulated genes in lung cancer (Topper et al., 2017), thus suggesting that a combinatory treatment with ITF2357 could be promising. Interestingly, another paper reported that clinically relevant doses of DNA methyltransferase and histone deacetylase inhibitors reduce the immune suppressive microenvironment through type I IFN signalling and improve response to immune checkpoint therapy (Stone et al., 2017).

Throughout the years it has been demonstrated that interferon production could be triggered by the accumulation of endogenous double-stranded RNAs (dsRNAs). For example, the depletion of DEAD-box RNA helicase 3X (DDX3X) triggers a tumor-intrinsic type I IFN response in breast cancer cells. Depletion or inhibition of DDX3X activity led to aberrant cytoplasmic accumulation of cellular endogenous dsRNAs, which triggered type I IFN production through the melanoma differentiation-associated gene 5 (MDA5)-mediated dsRNA-sensing pathway. Therefore, loss of DDX3X in mouse mammary tumours enhanced antitumor activity by increasing the tumour-intrinsic type I IFN response, antigen presentation, and tumour infiltration of cytotoxic T and dendritic cells, suggesting a novel therapeutic approach for breast cancer by targeting DDX3X in combination with immune-checkpoint blockade (Choi et al., 2021).

In the literature there are evidences that DNMT inhibitors target colorectal cancer cells through viral mimicry (Roulois et al., 2015), and, in accordance to these data, we found that DNMT inhibitors (DAC and DNMT1i) are able to increase the level of dsRNAs in colon cancer cells, as demonstrated by dot blot analysis and immunofluorescence (IF). Surprisingly, ITF2357 alone had no remarkable effect on dsRNAs induction. Nonetheless, IF images and dot blot analysis results indicated that the combination ITF2357/DNMTIs increase the level of dsRNAs compared to DNMTIs mono-treatment in HT29 cells. On the

other hand, the combinatory treatment seems to decrease dsRNAs level in HCT116 cells. In fact, IF images of cells treated with ITF2357 and DNMTIs (DAC or DNMT1i) showed an intense red signal related to cytoplasmic dsRNAs, although the cell number was dramatically reduced because of cell death. Importantly, this could also account for the decrease of dsRNAs signal detected in ITF2357/DNMTIs treated cells by dot blot.

Taken together, these results suggest that combining ITF2357 with DNMT inhibitors could be a good strategy to target colon cancer cells, although the immunogenic response induced by these compounds need to be further elucidated. We can also hypothesize that the different effect of these compounds observed in HCT116 and HT29 cells could be related to the mutational status of BRAF and p53 in HT29 cells, that make these cells more resistant in accordance with the findings of other authors (Mao et al., 2013). A general effect of ITF2357 in potentiating DNMTI-mediated immune response is represented in the figure below.

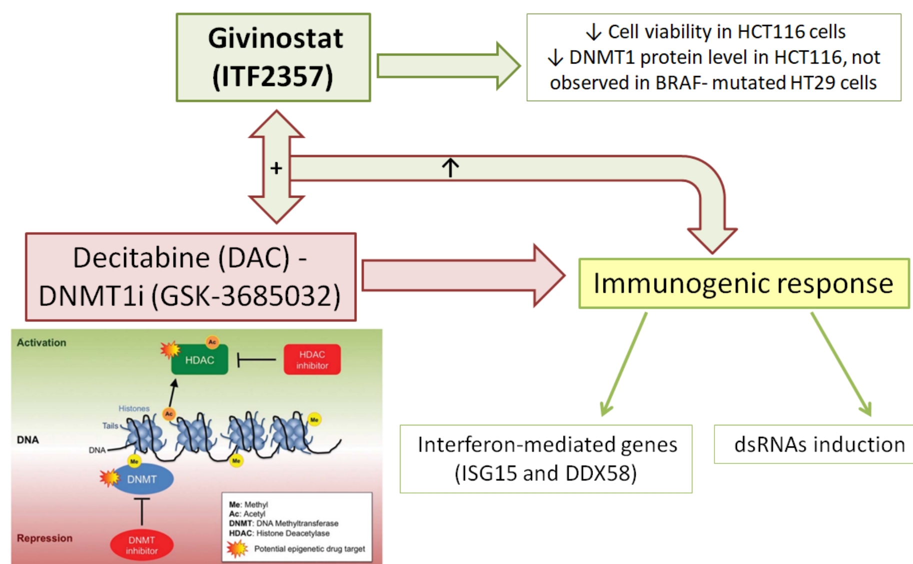


Figure 2*: Schematic representation of the effect of ITF2357 and DNMT inhibitors in colon cancer cells.*

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