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Epigenetic reprogramming of CatB in Colorectal cancer: a versatile prognostic marker

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DOTTORATO DI RICERCA IN MEDICINA TRASLAZIONALE

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1.0 ABSTRACT

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Colorectal cancer (CRC) is the third most common tumor worldwide and the fourth most common cause of death (1;2). Despite the screening has substantial net benefit and there have been improvements in therapies for the patient outcome, it can often spread leading to metastatic disease (mCRC) in non-responder or late diagnosed patients (3). For CRC patients, surgery and systemic chemotherapy have long been considered the standard treatment. However, patients especially with metastatic lesions have not a good prognosis, and targeted therapy is a recent optional approach successfully improving overall survival for CRC patients (1).

In the last decades the research has focused on extracellular matrix (ECM) potential role in CRC tumorigenesis. The ECM has distinctive biochemical and biomechanical properties, and it is one of the proximal structures that tumor cells of epithelial origin destroy to favor invasion and cell migration (4). Indeed, during the CRC progression, migration and invasion capability are mostly due to the presence of proteins capable of degrading ECM among them, the presence of cysteine- proteases known as Cathepsins. Cathepsins (CTS) are intra/extracellular enzymes recently identified as key elements in the pathological processes of tissue metastasis (5). In CRC among CTS, CTSB enzyme is overexpressed and associated with the late stages of the disease and with a short average survival (OS) and a reduced disease-free survival time (DSF) (6).

In addition, CTSB, as proteases, is a suitable tool in activating anticancer prodrugs in a site-specific manner to avoid side effects (7). CTSB expression in CRC can be significantly increased by treating the cells with epi-modulators, including histone deacetylase inhibitors (HDACi, such as *SAHA*) (8). CTSB cleavable cytotoxic prodrugs were designed and tested (alone or in combination with epi-modulator) to obtain a site-specific release of active chemotherapeutic agents to develop an alternative therapeutic strategy for CRC patients. TMA analysis was carried out to estimate CTSB expression in CRC tissue Protein and RNA evaluation by Western Blot and Realtime PCR, coupled with ELISA assay and probe based-fluorescence assay were used to monitor CTSB presence inside and outside the cells. Cell viability was evaluated in 2D and 3Dculture (organoids) testing epi-modulators in combination or pretreatment with prodrugs. This study supports the hypothesis of CTSB expression as a tumor biomarker in CRC. For this purpose, a tissue microarray analysis (TMA) was performed on CRC tissues clustered on criteria of stage disease after diagnosis, gender, left and right-side tumor, confirming CTSB hyper-expression in tumor (upregulation

of RNA/protein expression) in comparison to healthy counterpart, particularly in colon-rectal right-side tumors (considered the tumor with the worst prognosis) at late stages. Furthermore, the overexpression inside and outside the tumoral cells offer the possibility of using its enzymatic activity to improve activation of prodrugs. For this purpose, two new prodrugs have been designed and synthetized in collaboration with Prof. Christopher Phenix from University of Saskachewan and Prof. Salvatore di Maro from University of Campania "Luigi Vanvitelli", these two different prodrugs were *Doxorubicin* based targeted selectively by CTSB. In addition, it was demonstrated the epigenetic modulation of CTSB expression upon the treatment of CRC cell lines with epigenetic modulators such as *SAHA* (HDACi) and *JQ1* (BET inhibitor). The results revealed the potentiality to use epigenetic therapy in combination with synthetic compounds (prodrugs CTSB targeted) in 2D and 3D CRC systems in collaboration with Prof. Luca Primo from IRCSS in Candiolo, Italy. Indeed, by using CRC cell lines and patients derived organoids (PDO), there was observed decrease in cell vitality suggesting that *SAHA* not only induces apoptotic mechanism but also by increasing CTSB expression, it is possible to promote a more efficient release of the prodrug active part. These data corroborate the idea that is possible to improve the CRC therapy with the synergistic effect of an epigenetic modulator and a chemotherapeutic agent that can be delivered to the tumor cells avoiding side effects on healthy cells thus improving CRC patients' survival especially in non-responder cases.

1.0 SOMMARIO

Il cancro colorettale (CRC) è il terzo tumore più comune al mondo e la quarta causa di morte più frequente (1;2). Nonostante lo *screening* nei pazienti adulti di età compresa tra 50 e 75 anni abbia un beneficio netto sostanziale, e vi siano stati miglioramenti nelle terapie per la prognosi del paziente, spesso può diffondersi portando alla malattia metastatica (mCRC) in pazienti non responsivi o con diagnosi tardiva (3). Per i pazienti con CRC, la chirurgia e la chemioterapia sistemica sono state a lungo considerate il trattamento standard. Tuttavia, soprattutto per i pazienti con lesioni metastatiche, la prognosi del CRC non è mai stata soddisfacente e la terapia mirata rappresenta un nuovo approccio opzionale che migliora con successo la sopravvivenza globale per i pazienti con CRC (1).

Negli ultimi decenni la matrice extracellulare (ECM) ha suscitato un crescente interesse per il suo potenziale ruolo nella tumorigenesi del CRC. L'ECM ha proprietà biochimiche e biomeccaniche distintive ed è una delle strutture prossimali che le cellule tumorali di origine epiteliale distruggono per favorire l'invasione e la migrazione cellulare (4). Infatti, durante la progressione del CRC, la capacità di migrazione e invasione è principalmente dovuta alla presenza di proteine in grado di degradare la ECM tra di loro, la presenza di cistein-proteasi note come Catepsine. Le Catepsine (CTS) sono enzimi intra/extracellulari recentemente identificati come elementi chiave nei processi patologici delle metastasi tissutali (5). Nel CRC tra le Catepsine, l'enzima CTSB è over-espresso e associato agli stadi avanzati della malattia. La sua presenza è associata a una breve sopravvivenza media (OS) e a un ridotto tempo di sopravvivenza libera da malattia (DSF) (6).

Inoltre, la CTSB, come proteasi, è uno strumento adatto per attivare profarmaci antitumorali in modo sito-specifico per evitare effetti collaterali (7). L'espressione di CTSB nel CRC può essere significativamente aumentata trattando le cellule con epi-modulatori, inclusi gli inibitori dell'istone de-acetilasi (HDACi, *SAHA*) (8). I profarmaci riconosciuti dalla CTSB sono stati progettati e testati (come singoli agenti o in combinazione con epi-modulatori) per ottenere un rilascio sito-specifico di agenti chemioterapici attivi in modo da sviluppare una strategia terapeutica alternativa per il trattamento dei pazienti con CRC. Analisi di TMA da pazienti con tessuto CRC, valutazione di proteine e RNA mediante *Western Blot* e *Realtime PCR*, test *ELISA* e test di fluorescenza basata su sonda, sono stati utilizzati per monitorare la presenza di CTSB all'interno e all'esterno delle cellule. La vitalità cellulare è stata valutata in coltura 2D e

3D (organoidi), in collaborazione con prof. Luca Primo, IRCSS, Candiolo, testando epi-modulatori in combinazione o pretrattamento con profarmaci.

In questo studio è stato confermato che nel CRC tra tutte le Catepsine, vi è una over-espressione dell'enzima CTSB. Sono stati analizzati diversi campioni *ex-vivo* di CRC classificati secondo i criteri di *clustering* dei tessuti in base allo: stadio della malattia dopo la diagnosi, al sesso, al tumore del lato sinistro e destro, confermando l'iper-espressione di CTSB nel tumore (*up-regulation* dell'espressione di RNA/proteina) rispetto alla controparte sana, in particolare nel colon-retto destro -tumori laterali (considerati il tumore con la prognosi peggiore) in fase avanzata. Inoltre, l'over-espressione all'interno e all'esterno delle cellule tumorali offre la possibilità di utilizzare la sua attività enzimatica per migliorare l'attivazione dei profarmaci. Sono stati progettati e sintetizzato in collaborazione con il prof. Christopher Phenix dell'Università di Saskatchewan e il prof. Salvatore di Maro, dell'Università degli Studi della Campania "Luigi Vanvitelli" due diversi profarmaci (a base di Doxorubicina) presi di mira selettivamente dal CTSB. Inoltre, è stata dimostrata la modulazione epigenetica dell'espressione di CTSB attraverso l'uso di alcuni modulatori epigenetici come *SAHA* (HDACi) e JQ1 (BET inibitore). I risultati hanno rivelato la possibilità di utilizzare la terapia epigenetica in combinazione con composti sintetici (profarmaci mirati al CTSB) nei sistemi CRC 2D e 3D. Nelle linee cellulari CRC e nei pazienti derivati da organoidi, è stata osservata una diminuzione della vitalità cellulare suggerendo che *SAHA* non solo induce il meccanismo apoptotico ma che aumentando l'espressione di CTSB può promuovere un rilascio più efficiente della parte attiva del profarmaco. Questi dati corroborano l'idea di migliorare la terapia CRC con l'effetto sinergico di un modulatore epigenetico e un agente chemioterapico che può essere indirizzato alle cellule tumorali evitando effetti collaterali sulle cellule sane migliorando così la sopravvivenza dei pazienti con CRC soprattutto nei casi *non responder*.

2.0 INTRODUCTION

2.0 INTRODUCTION

Colorectal cancer (CRC) is estimated to be the second most common diagnosed cancer and the second leading cause of cancer death in Europe in 2020 (International Agency for Research on Cancer, WHO. Global cancer observatory. https://gco.iarc.fr), with 1.8 million of new cases and 1.2 million of death in the incoming ten years (9). This type of tumor occurs rarely in young people 40 years old age, and it increases afterwards. Improved treatments and early diagnosis in developed countries have contributed to decrease its incidence and mortality (10). However, it is predicted that the new cases will increase by 60% and reach 1.1 million of deaths estimated by 2030. In Italy CRC is reported to be the second most common cancer and the second cause of death for cancer in both genders. The 5-years-survival rate is homogenous between men and woman, and it is around 66% for colon cancer and 62% for rectum cancer (2). CRC constitutes not only a dramatic humanitarian burden, but also is critical problem causing health issue-based economic and financial trouble worldwide (World Health Organization, 2020).

Therapeutic options for stage I, II and III of the disease are surgery and the adjuvant chemotherapy used for selected patients with stage II and most patients with stage III. The radiotherapy strategy is used for patients with stage II and III for rectal cancers (11, 12). Patients with metastasis or stage IV generally not resettable receive palliative treatments to control symptoms and to increase their survival (13). CRC has a global poor prognosis, so there is the huge need to discover new diagnostic and prognostic markers, especially in early stage of the disease (14). In line with this, the most important prognostic factor is the time of the diagnosis at early disease stage. The 5-years survival rate increases if the tumor is detected at an early stage, and it decreases for advanced stages (15,16,17). The risk factors are independent of age, sex and family history but is it slightly lower among women than men. However, there are some predisposing factors: genetic and environmental agents play an important role in the etiology of CRC. More than 70% of cases are sporadic and related to the lifestyle whereas more than half of the cases are caused by the interaction between genetic and environmental agents (14). For this pathology other factors also play a fundamental role, such as low diet in vegetables and fruits, intake of red meat, alcohol, and tobacco (15). 5-6% of CRC cases are inherited forms like the Lynch syndrome which is the most common one accounting for the 3% of these cases. This syndrome is an autosomal dominant inherited, known as hereditary nonpolyposis CRC it is due to mutations in the ‘mismatch repair’ (MMR) genes

predominantly predisposing to CRC, endometrial cancer, and other cancers at a younger age than the general population (18,19). CRC as a solid tumor is a heterogeneous disease and different subtypes can be distinguished by specific clinical and molecular features. Sporadic CRCs, almost 85% show changes in chromosome number and structure with chromosomal instability (CIN) (20,21). The other cases (15%) report high-frequency microsatellite instability (MSI) phenotypes (22). From adenoma to carcinomas in sporadic and inherited forms of CRC there is a defined sequential acquisition of genetic and epigenetic alterations important for initiation and progression of this tumor (23). The evolution of CRC starts from a normal epithelium to a first benign adenoma then it progresses with the accumulation of multiple genetic and epigenetic aberrations (24). CRC is caused by the gradual loss of the regulatory mechanisms (25). In cancer progression which occur in multistep there is a sustained proliferation process constituted by the hyperactivation of mitogenic signalling pathways such as growth factor receptors, downstream effectors, and the inhibition of negative feedback control mechanisms (26,27). The tumorigenesis process and invasiveness are related to the essential hallmarks of this cancer (28): evading growth suppressors; replicative immortality; cell death resistance; sustaining proliferative signalling; inducing angiogenesis; activating invasion and metastasis; and reprogramming of energy metabolism; evading immune destruction (29). Malignant cells, indeed, are resistant to cell death blocking the cascades of intrinsic and extrinsic apoptosis process (30). Furthermore, cancer cells have the possibility to obtain a greater supply of nutrients and oxygen by a vascularization process, the neo angiogenesis, increasing the number of existing vessels (31). In CRC, there are not only several genetic issues but also epigenetic aberrations. Epigenetic modifications are defined as heritable changes in the gene expression occurring though the chromosomes rather than in the DNA sequences. These modifications are altered in several cancers including CRC and so contribute to the alterations of signalling pathways regulating behaviour of cancer during the progression from normal mucosa towards carcinoma (32, 33). In general, genetic mutations contribute to the modification of epigenetic control favouring genomic instability and mutagenesis (33). So far Next Generation Sequencing (NGS) techniques have contributed to determine unexpected genetic mutations associated with epigenetic alterations. The epigenetic aberrations include cytosine methylation, histone modifications, and nucleosome organization. Epigenetic silencing of DNA mismatch repair (MMR) genes frequently contributes to genomic instability leading to mutations of oncogene or tumor suppressor genes (34). The study on the epigenetic

changes have highlighted the link between CRC-specific gene expression patterns and the absence of genetic alterations. Microsatellite instability is for instance the result of a deficiency in the DNA mismatch repair (MMR) system, that is the consequence of a genetic mutation one of the MMR genes but also due to epigenetic silencing of the MLH1 gene by hypermethylation of its promoter (35). Chromosomal instability (CIN) is considered typical of CRC and is a consequence of global hypomethylation (36). In addition, microRNAs (miRNAs) that prevent protein expression and influence many cancer-related pathways at the post-transcriptional level play a role in CRC stages, from initiation to progression and metastasis. However, epigenetic markers such as enzymes involved in the histone modifications or in DNA methylation and lncRNAs are considered promising attractive therapeutic targets, for future therapies (32).

2.1 Genetic and Epigenetic aberrations in CRC

In CRC the genomic mutations occur on different genes such as p53, Ras, beta-catenin, transcription factors involved in embryogenesis, and DNA mismatch repair genes contributing to the progression of the disease from benign adenoma to malignant adenocarcinoma (37). In addition, three major classical pathways that contribute to the CRC carcinogenesis: genomic instability-microsatellite instability (MSI), chromosomal instability (CIN), and CpG island methylator phenotype (CIMP) (23). The aberrant methylation of tumor suppressor genes results in their inactivation and subsequent promotion of neoplasia (38). Over the past two decades, it is becoming evident that epigenetic alterations of the chromatin, particularly the chromatin components in the promoter regions of tumor suppressors and oncogenes, play key roles in CRC pathogenesis (39). Epigenetic processes fine-tune the accessibility of DNA orchestrating various physiological procedures (transcription, replication, repair) from developmental to differentiated stages (37). These processes regard modifications on chromatin that do not change the DNA sequence but are inheritable. The chromatin environment (eu- or heterochromatin) can be regulated by promoting or disrupting the DNA association to histone proteins (40). During tumor progression, epigenetic aberrations affect each step from initiation to metastasis (41). The deregulation in genetic and epigenetic events play important role during the progression from adenoma to carcinoma. The Vogelgram sequence implied different oncogenes (e.g., *KRAS*, *NRAS*, *BRAF*, and *PIK3CA*) and tumor suppressor genes (e.g., *APC*, *TP53*, *SMAD4*, and *PTEN*) leading to the deregulation key

signalling pathways driving disease progression, notably Wnt/β-catenin, the epidermal growth factor receptor (EGFR), downstream mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and transforming growth factor-beta (TGF-β) (42).

Different epigenetic mechanisms also have been correlated to the development of CRC: aberrant hypermethylation has been identified in the promoter regions of important tumor-suppressor genes such as *CDKN2A*, *MLH1* and *APC19*; hypermethylation have been reported outside of the CpG islands in gene bodies and it seems to be positively correlated with gene expression. The hypomethylation also has been correlated to the activation of oncogene: promoter regions leading to loss of gene imprinting (for example, IGF2) or direct activation of proto-oncogenes like *MYC* and *HRAS* (32).

The deregulation of histone modification patterns involved in the activation of oncogenes and silence tumor suppressor genes are involved in the etiology of CRC. Moreover, the alteration of histone modification patterns led to the deregulation of gene expression which plays pivotal roles in the formation of CRC (43). Therefore, it is important to investigate the mechanism and biological function of histone modifications in CRCto improve the clinical diagnosis and therapy of colorectal cancer.

2.2 ECM degradation in CRC

The identification of more valuable and versatile biomarkers for early diagnosis, prevention, and personalized therapy are considerably important and urgent (44), considering also that 50% of CRC patients will develop liver metastases during their lifespan and that there is no effective therapy for them (45). One of the main mechanisms involved in the metastatization process is the epithelial-mesenchymal transition (EMT). This process is characterized by the acquisition of epithelial cells of a mesenchymal phenotype in which tumor cells lost the tight junction and disrupted apical–basal polarity and reorganize the cytoskeletal architecture development. The spread of colon cancer cells through the human body is allowed by alterations in cell shape and cell-cell adhesion, as well as by attachment to the extracellular matrix (ECM), which allows cells to invade other tissues and spread to various districts of the body. For epithelial cells, this mechanism is called epithelial-mesenchymal transition (EMT) (46). The multiphase process of the metastasis includes the invasion of local tissues, intravasation into the blood and lymphatic vessels, the transit through the lymphatic or hematogenous system, as well as the extravasation and colonization of even distant tissues (46,47). In the last

decades a consistent interest is growing around ECM microenvironment role in CRC tumorigenesis and progression. ECM characteristics such as biochemical and biophysical properties are very peculiar and unique. Normal or pre-invasive cells segregated from vascular structures within the stroma by the basement membrane (BM) that consists of laminins, type IV collagen and surrounding epithelial cells. During tumorigenesis cancer cells interact with BM and the ECM and disrupt them favouring invasion and metastasis thanks to the presence of proteases: cysteine-, serine-, aspartic-, and metalloproteinases classified according to the essential catalytic component (usually an amino acid) in their active site (48,49). These proteolytic enzymes play a major role in a variety of physiological and pathological processes, such as protein turnover, tissue remodelling, wound repair, angiogenesis, destructive diseases, inflammatory disorders (48).

2.3 Cathepsins role in tumorigenesis

It has been reported that among all the proteinases, Cathepsins are known to be the most abundant proteases in the lysosome organelles (50). These proteins are categorized by the catalytic amino acids, Cathepsins (CTS) in this case are categorized as cysteine proteases (CTSB, C, F, H, L, K, O, S, W, and Z), serine proteases (CTSA and G) and aspartic proteases (CTSD and E). Among the Cathepsins CTSB, C, D, E, G, K, L, S and X/Z are the most widely studied in tumorigenesis, a process related to tissue/cell-specific expression of selective CTS. CTS are synthesized as inactive form as pre-proenzymes then processed in the rough endoplasmic reticulum by the removal of the pre-peptide, followed by glycosylation in the Golgi apparatus, finally transported to the lysosome and activated through controlled proteolysis of the pro-peptide (51). A compelling deliberation now arises whether all CTS are activated in the lysosome simultaneously. These enzymes indeed are active in the lysosomes preferring an acidic environment (52). Originally cathepsins means “to digest” thus relating this concept to the fact that these enzymes are involved in hydrolytic processes. These proteins participate in development and differentiation, apoptosis, immune responses under healthy physiological conditions, while their presence is also related to some diseases, such as osteoporosis, rheumatoid arthritis, Down syndrome, Alzheimer's disease, and digestive cancers, such as CRC (53). CTS are activated and translocated during tumor progression, contributing to at least three stages of cancers. Firstly, CTS participate in the activation of various cytokines promoting proliferative capacity of cancer cells. Secondly, they contribute to the degradation of the

vascular basement membrane and activate growth factors favouring angiogenesis. Furthermore, when these proteins are secreted into the pericellular environments they cleave laminin, collagen, elastin, E-cadherin, and other matrix proteins, degrading the junctions between cells and the ECM and allowing cancer cells to invade or metastasize (54). Among the CTS, Cathepsin B, it has been reported to play important role in cancer progression, so it has raised more attention in comparison to other cysteine-proteases. Its elevated activity has been detected in multiple human cancer cells lines as well as in all stages of tumorigenesis, from initiation to angiogenesis, invasion, and metastasis. Different studies reported among all the CTS that Cathepsin B (CTSB) in particular is expressed and correlated with a poor prognosis for CRC patients (55).

Cavallo-Medved et al., 2017 demonstrated that secretion of CTSB is increased in the extracellular environment of CRC playing an essential role in disrupting ECM barriers between tumors and surrounding tissue, thereby facilitating invasion and metastasis.

2.4 Cathepsin B contribution in CRC development

CTSB normally is in the epithelium of colorectal mucosa, and it is only active in the older cells at the colorectal surface. In contrast, in tumor tissues, CTSB is present at the base of cells close to the basement membrane. Furthermore, in most well-differentiated and half of moderately differentiated colon carcinomas its localization remains unchanged, whereas this protein is spread throughout the cytoplasm in poorly differentiated colon carcinomas (53). In primary and metastatic tumor specimens there have been observed increased level of CTSB (56): its overexpression was reported in prostate cancer (57) gliomas (58) melanomas (59), breast cancer (60) and lung squamous cell carcinoma (61). In CRC this protein is found in the extracellular compartment and regulates the cell invasion while intracellularly it contributes to the malignant properties. It has already demonstrated CTSB overexpression in CRC at different stages of the disease and it is reported its association to overall mortality. For this reason, CTSB can be considered as a potential CRC biomarker (6). Since CTSB activity favours tumor formation in the colon, it is not surprising that this protein maybe a biomarker of CRC progression (56). Cathepsin B is synthesized inactive as a pre-pro-enzyme subsequently processed to its active form. The first step is characterized by auto-processing consisting of the removal of the pro-peptide within acidic-mediated environment and followed by activation by lysosomal proteases leading to mature CTSB (6).

2.5 Epigenetic modulation of Cathepsin B in CRC *in vitro*

The active and silent epigenetic genes are modulated by the addition or removal of chemical modifications in the chromatin including a variety of post-translational histone modifications (acetylation, phosphorylation, etc.). Most of the epigenetic genes have been reported to be acetylated, playing critical roles in cell-cycle progression, differentiation, apoptosis, and autophagy. Recent papers reported the epigenetic modulation of apoptosis-related genes including CTSB (62,63). For instance, Han et al., 2017 demonstrated that *SAHA*, a pan HDAC inhibitor, induced the expression of several apoptosis and autophagy-related genes such as CTSB. Indeed, the authors reported CTSB activity increase in breast cancer cells, MDA-MB-231 and MCF-7 upon *Vorinostat* treatment (63).

Furthermore, Sakamaki et al., 2017 (62) described that knockdown of BRD4, belonging to BRD class of epigenetic readers (65), significantly upregulated the CTSB expression. In Cheriyath et al., 2011 is reported the potentiation of apoptosis and related mechanisms of HDACi and *Doxorubicin* combination in a panel of myeloma cell lines. It was highlighted that sodium butyrate (an HDACi) or *Doxorubicin* alone caused little apoptosis but in combination, potentiated apoptosis and synergistically reduced the viability of myeloma cells. In addition, the butyrate and *Doxorubicin* combination significantly increased the activity of cytoplasmic CTSB (69).

2.6 Treatment and management for CRC patients

Typically, the ideal CRC treatment consist in the removal of the tumor and metastases that mostly requires surgical intervention (1).

Generally, stage I CRC patients require only the surgical intervention of the tumor and eventually of the lymph nodes, stage II surgery and in some cases coupled with the adjuvant chemotherapy is recommended to impede cancer spreading to nearby organs. For patients at stage III usually treatment is surgical removal of the tumor followed by adjuvant chemotherapy, in case of rectal cancer, radiation therapy may be used with chemotherapy before or after surgery, along with adjuvant chemotherapy. The treatment for patients with Stage IV CRC have include combination of surgery, chemotherapy, immunotherapy, targeted therapy, and radiation (67).

Despite the emergence of numerous screening programs to reduce CRC incidence, 25% CRCs are diagnosed at an advanced stage with metastases (1). The patients with

unresectable lesions or intolerant to surgery, the goal is to reduce the tumor and suppression of further tumor spread and growth, so radiotherapy and chemotherapy are the leading strategies for controlling disease in such patients (68).

Surgery coupled with chemotherapeutic intervention surgery regards metastatic CRC treatment with the only purpose of enhance survival (69).

Current chemotherapeutic treatment consists of single agent such 5-fuorouracil (5-FU), and multiple agents with one ore multiple drugs such as capecitabine, tigafluorin, irinotecan, and oxaliplatin (1).

Nonetheless, data from research performed in recent decades show that using chemotherapy in patients with CRC, especially those with metastases, has pushed their OS time to almost 20 months, resulting in chemotherapy becoming the backbone of CRC treatment (70). However, chemotherapy option is associated with certain limitations, such as existing systemic toxicity, unsatisfying response rate, unpredictable innate and acquired resistance, and low tumor-specific selectivity (4). Indeed, most of the time the tumor cells acquire resistance to these chemotherapeutic drugs. Therefore, massive investments have been pledged to develop new approaches to refine or even replace existing CRC chemotherapy (70). In addition, the side effects relative to chemotherapeutic agents also limit the maximum allowable amount, resulting in a limited amount of drug accumulated in tumor tissue that is insufficient to reach the effective therapeutic concentration (71).

Furthermore, in the last 20 years the concept of targeted therapies has expanded a lot: these (macro) molecules trigger cancerous cells proliferation, differentiation, and migration; also the tumor microenvironment, including local blood vessels and immune cells can be altered by targeted drugs to impede growth (1,70) Different pathways mediating the initiation, progression, and migration of CRC, such as Wnt/β-catenin, Notch, Hedgehog, and TGF-β (transforming growth factor-β)/SMAD, as well as those capable of activating signalling cascades, such as phosphatidylinositol 3-kinase (PI3K)/AKT or RAS/rapidly accelerated fibrosarcoma (RAF), are ideal for targeted therapy (9). Despite its versatility, targeted therapy has difficulties in completely inhibiting specific biological interactions and not all CRC-related pathways can be successfully impaired: current data cover only a few pathways and a large group of targeted drugs remain in preclinical status or in phase I trials (1,3).

2.7 Prodrugs as a valid alternative approach for CRC treatment

The use of chemotherapeutic agents converted into prodrug is considered a valid strategy to overcome their different drawbacks. Prodrugs, which are chemically modified derivatives of an active drug require biological or chemical transformations for pharmacological responses (72,73). The prodrugs idea in cancer therapy is to reduce unintended side effects by designing compounds that interact with specific targets. (74). Furthermore, prodrug design can improve pharmacokinetic parameters, prolonged action, increased selectivity, increased membrane permeability and less adverse effects (72). Most prodrugs require one or two enzymatic or chemical transformation for yielding the active drug and is preferentially released at the site of action. The possibility of use prodrugs as novel agents in targeted therapy offers a wide range of advantages: reduced toxicity, improved specificity, and the avoidance of multidrug resistance. In targeted therapy the prodrug use is generally associated to the presence of tumor-associated cell surface markers, such as antigens or receptors, whose expression differs between normal and cancer cells (73,74). Cathepsin B as a hydrolytic enzyme can cleave prodrugs inducing the release of the active drug (75). In addition, since it is already reported that it is overexpressed in malignant tumors, this protease then can selectively activate these prodrugs directly at the tumor site. In line with these assumptions, we formed a collaboration with prof. Christopher Phenix from University of Saskachewan and prof. Salvatore di Maro from University of Campania “Luigi Vanvitelli”, who designed and synthetized two different prodrugs (*doxorubicin* based) that were selective substrates of CTSB. The prodrugs were relatively nontoxic until they were activated by CTSB. In addition, the results obtained from this project demonstrated the epigenetic modulation of CTSB expression could be accomplished using some epigenetic modulators such as *SAHA* (HDACi) and *JQ1* (BET inhibitor).

3.0 AIM OF THE STUDY

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The CRC is characterized by an unfortunate prognosis and the high incidence with therapeutic options still an unmet clinical need due to rates of high metastasis and, for this reason, the University of Campania "Luigi Vanvitelli" has started a research project focused on the evaluation of epigenetic changes that contribute to the development and maintenance of the CRC. This project is based on studying new molecular markers involved in the carcinogenesis of CRC and is carried out in accordance with the authorization of the Ethics Committee (No. 790 of 12/12/2018.), following the Italian directives that regulate the treatment of biological samples and their resulting information. This study evaluated CRC biomarkers using *in vitro* and *ex-vivo* models obtained from CRC patients following colectomy surgeries performed at the Hospital of University of Campania "Luigi Vanvitelli". The differences in biomarkers expression were analysed from different databases, integrating the results with data reported in the literature. This research, therefore, led to the identification of molecular gene and protein components involved in the development of CRC. In particular, a cysteine-protease, Cathepsin B (CTSB) known to be involved in ECM degradation and plays an important role in tumor invasion and metastasis. The aim of this study was then to confirm CTSB as biomarker in CRC and to investigate its epigenetic modulation upon *SAHA* treatment. In addition, since this protein is a suitable target capable of activating prodrugs in the tumor environment., CTSB cleavable cytotoxic prodrugs were designed and tested (alone or in combination with an epi-modulator) to obtain a site-specific release of active chemotherapeutic agents. The results obtained may lead to the development of potential alternative therapeutic strategy for treating CRC patients.

4.0 RESULTS

4.0 RESULTS

4.1 GEPIA expression profile for Cathepsin B in CRC patients

CTSB gene expression was first investigated by using GEPIA (Gene Expression Profiling Interactive Analysis) a web-based tool to deliver fast and customizable functionalities based on TCGA and GTEx data (76). GEPIA provides key interactive and customizable functions including differential expression analysis, profiling plotting, correlation analysis, patient survival analysis, similar gene detection and dimensionality reduction analysis. The GEPIA expression profile analysis for CTSB in box plots reported its overexpression in tumor tissues with a greater median expression level of this protein in CRC tissue in comparison to healthy part, normalized by the maximum median expression value across all box plots s. (**Figure 1**). GEPIA uses log-rank test, the Mantel–Cox test, for the evaluation of survival plot patients. The GEPIA analysis was then conducted on the expression profile of CTSB gene on survival patients' plot. The Cox proportional hazard ratio and the 95% confidence interval information included in the survival plot as shown in **Figure 2**, reported a negative correlation for patients having high CTSB expression in comparison to those with low expression. In patients CTSB overexpression is associated with overall or disease-free survival thus its presence is associated with risk of overall mortality.

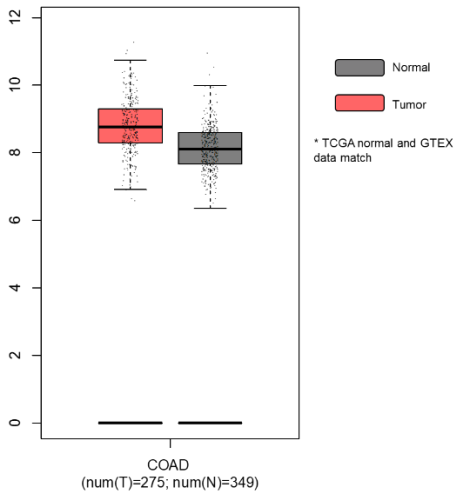


Figure 1: GEPIA box plots reported the CTSB overexpression in CRC samples in comparison to the healthy tissues.

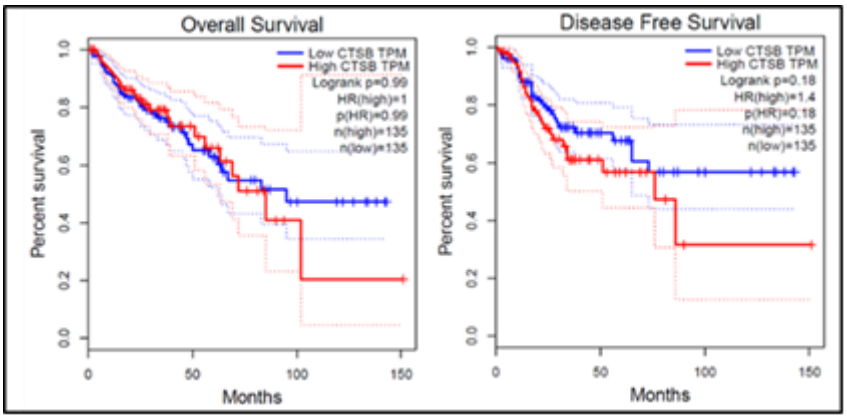


Figure 2: 82% of CRC patients shows CTSB over-expression (source: GEIPA, gene expression profiling interactive analysis). Overall Survival (OS) and Disease-Free Survival (DFS) decreases in CRC that overexpress CTSB.

4.2 TMA analysis for CRC patients

A Tissue Microarray Analysis (TMA) was performed to better characterize Cathepsin B as a biomarker for CRC. The immunohistochemistry analysis for this protein was carried out on forty-four CRC *ex-vivo* samples obtained in collaboration with Istituto Nazionale Tumori, IRCCS Fondazione "G. Pascale" of Naples, Italy. These samples consisted of forty-four CRC tissues paired with their colon healthy (non-tumoral) tissues. Particularly each sample was investigated for the CTSB protein expression (CTSB positivity) in both tumor samples and in the healthy counterpart. The TMA analysis confirmed CTSB overexpression in tumor: histograms reported that 66% of cases in tumor tissue express CTSB (CTSB-positive) (29/44) whereas 34% (15/44 specimens) that did not express CTSB (CTSB-negative) (Fischer exact test - p value < 0.001). (**Figure 3**).

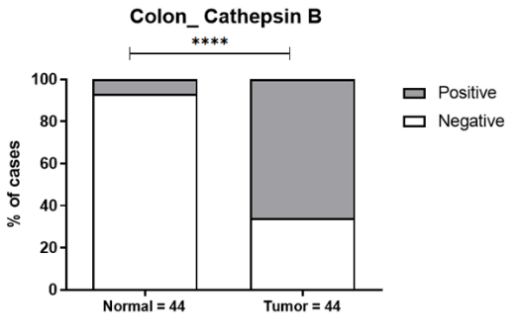


Figure 3: TMA analysis indicating % of cases with CTSB positivity and negativity in tumor and healthy counterpart of 44 samples. 29 patients (66%) reported CTSB expression (CTSB positive) while 15 patients were CTSB negative (34%) did not report protein expression.

As reported in **Figure 4** the CTSB protein was found strongly overexpressed in right-colon side tumor, considered generally to be the worst for patients' outcomes (56).

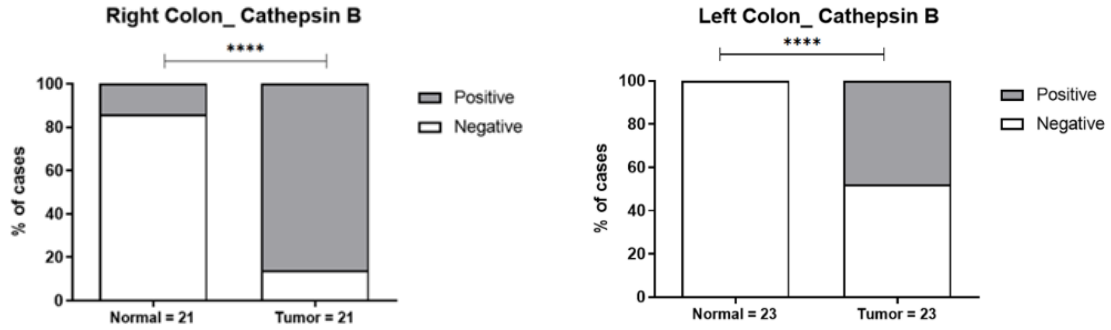


Figure 4: TMA analysis indicating % of cases in tumor and healthy tissues of *ex-vivo* samples (A) In right colon (number= 21 specimens) CTSB positive were 85% (18/ 21) while 15% (3/21) resulted CTSB negative. (B) in left colon (number= 23 specimens) CTSB positive were 47% (11/23) instead 53% (12/23) resulted CTSB negative.

These preliminary results suggests that CTSB is a biomarker of poor prognosis for CRC patients. Increases in CTSB expression was also correlated to reference tissues by comparing % positivity for early-stage samples (from 0 to IIa) to advanced (from IIb tIVa) tumor stages as reported in **Figure 5.**

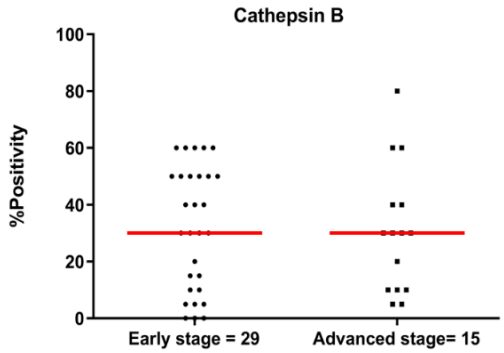


Figure 5: Comparative dot plot analysis of % CTSB positivity grouped in early (29 tissues) and advanced stages (15 tissues).

Cathepsin B positive and negative samples were evaluated against percent of survival: the Kaplan–Meier curve (**Figure 6**) reported for those CTSB positive patients had worst overall survival compared to those with negative results. These data support the hypothesis that CTSB overexpression is significantly associated with an increased risk of CRC and overall mortality

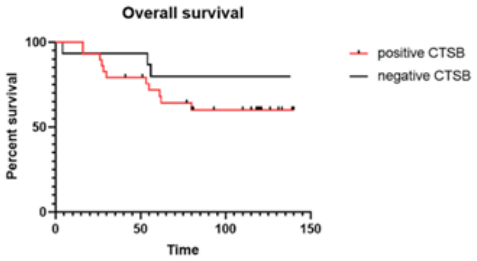


Figure 6: Kaplan–Meier curve for Overall Survival comparing CRC patients positive and negative for CTSB expression demonstrating that CTSB expression reported less OS.

4.3 CTSB localization in CRC patients' tissue

CTSB is a lysosomal cysteine protease, and it is synthetized in an inactive form then processed into a cleaved form and delivered to lysosome/extracellular compartments (77,78). CTSB differential expression was evaluated in cells (intracellularly), in the extracellular compartment (extracellularly) and in the *transitional mucosae* (TM) (this term describes alterations in the morphology and mucin histochemistry of large intestinal mucosa immediately adjacent to colorectal adenocarcinomas) (79). The results reported in **Figure 7**, demonstrated that accumulation of CTSB protein in the lysosomes and in the extracellular matrix (ECM) was measured in the cohort of forty-four samples (TMA collection annotated for the site, grade, and Astler and Coller stadiation).

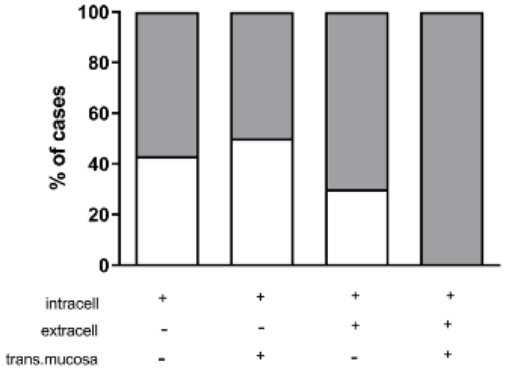


Figure 7: Histograms representing the % of cases that are CTSB positive (grey) and its localization (intracellular, ECM and transitional mucosa).

In addition, since the Cathepsin B protein is involved in tumor progression and metastasis and it facilitates rapid local invasion promoting angiogenesis (53), the CRC samples stained for this protein were evaluated by correlating CTSB levels with or without metastasis. The histograms in **Figure 8** report that CTSB positivity for these patients was associated both in primary and in tissue metastasis. All these data demonstrate that the presence of this protein as an important biomarker for CRC prognosis and patient outcomes.

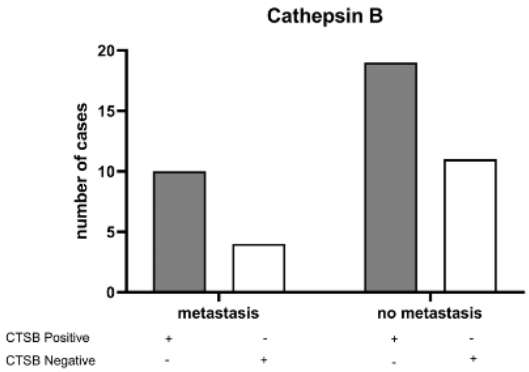


Figure 8: Cathepsin B prevalence (CTSB Positive and Negative samples) associated with primary or tissue metastasis.

4.4 Cathepsin B protein and mRNA expression evaluation in CRC tissue samples

To better investigate on CTSB overexpression in tissues of CRC patients, Western blot analysis was carried out starting from total protein extract of fourteen additional samples obtained in collaboration with Campobasso Hospital. Western blot results of all snap-frozen CRC samples reported an overall increase of Subexpression in tumor tissue in comparison with healthy and lymph node tissues. There bands were observed as the expected molecular weight for Cathepsin B. **Figure 9** summarizes the results from all protein extract obtained from patient tissue samples: the CTSB expression levels in tumor, healthy, and lymph node tissues from two different CRC patients. Case n#1 reported upregulation of both CTSB forms, inactive (Pro-CTSB) and active (CTSB) in tumor tissue, whereas case n#2 reported overexpression of cleaved (active) form in tumor and inactive form in healthy tissue.

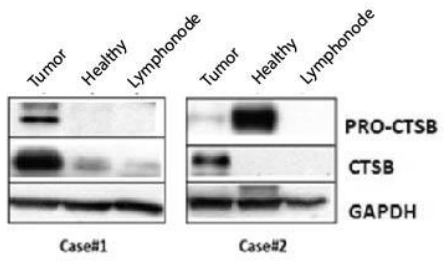


Figure 9: Western blot analysis of CTSB expression levels - in its inactive (PRO-CTSB) and active form (CSTB) in tumor, healthy and lymph node tissues from two different CRC patients.

The CTSB hyperexpression was then further analysed by Realtime PCR (**Figure 10**), confirming that mRNA expression of the CTSB gene in tissue samples is upregulated in CRC tumors compared with healthy and lymph node tissues (14 samples analysed - $p<0.01$).

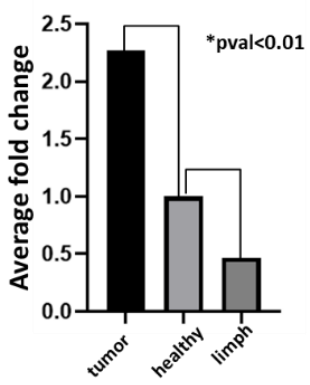


Figure 10: CTSB mRNA levels (average fold change) in CRC patients analysed by qPCR.

4.5 SAHA as an epigenetic agent in CRC

Histone deacetylases (HDACs) and histone acetyltransferase are responsible for the covalent modification of histone proteins and consequential changes in chromatin architecture and gene expression in different gastric cancer such as including CRC. Recent insights have suggested the importance of HDAC inhibitors (HDACi) as new therapeutic approach for the treatment of cancers. One HDACi, suberoylanilide hydroxamic acid (*SAHA*), also known as *Vorinostat* is a pan HDAC inhibitor approved by the Food and Drug Administration (FDA) for the treatment of cutaneous T cell lymphoma (CTCL) (79). This molecule has already demonstrated promising antiproliferative activity particularly it can induce apoptosis and sub-G1 arrest in colon and other cancers (80). For all these reasons, *Vorinostat* can be used as a sensitizing epigenetic agent in CRC in combination with other agents to reduce the CRC progression. In addition, *Doxorubicin* is one of the most common chemotherapeutic agents used in therapy, but suffers from side effects especially cardiotoxicity, was tested in CRC cells, HCT116 wt. These two drugs were tested both to verify cell viability performing the MTT assay. *Doxorubicin* plus *SAHA* combined, showed a reduction in viability and induction of cell death (**Figure 11-12**). This effect is in part be due to concomitant activation of apoptosis, autophagy and DNA damage pathways promoted and triggered by *SAHA* and *Doxorubicin*.

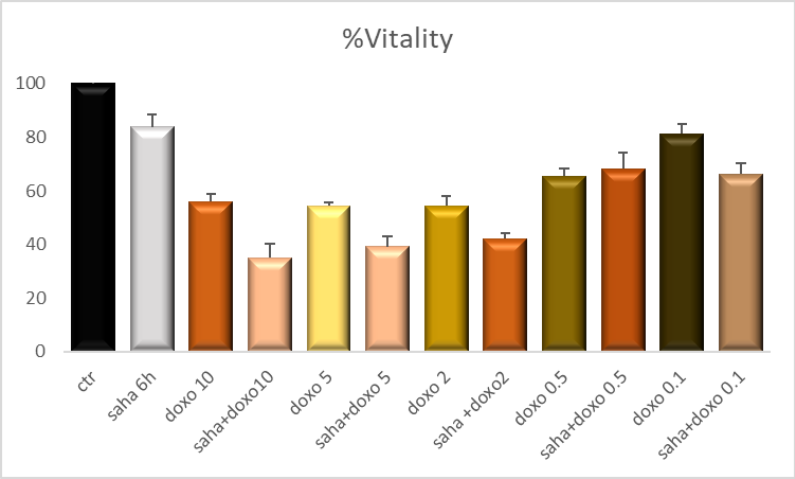


Figure 11: Cytotoxic effect in HCT116 by MTT assays indicating decrease in % cell vitality by treatment of cells with *Doxorubicin* alone (from 10 to 0.1 µM) for 24h and in pre-treatment with SAHA µM for 6h and then subsequently exposed to different concentration of *Doxorubicin* (from 10 to 0.1 µM).

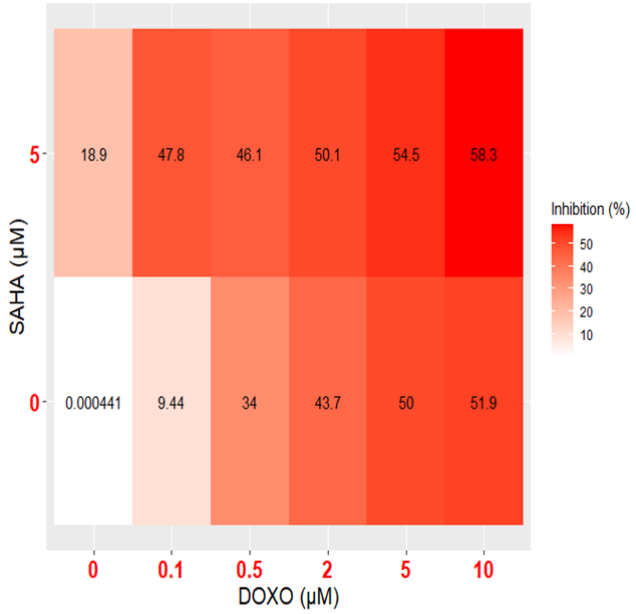


Figure 12: Dose-response matrix for Doxorubicin plus SAHA in colon cancer cell line. CI (cooperation index) = 0,2.

4.6 Epigenetic modulation of Cathepsin B in CRC cell lines

Epigenetic mechanisms are at the basis of CRC mantainance asepigenetic programs play pivotal roles in the CRC incidence as well as in its progression (81,82). Futhermore, given the substantial differences in enzymes and/or proteins involved in the regulation and modulation of epigenetic mechanisms, we evaluated the expression of Cathepsin B after the treatment of different CRC cell lines with epigenetic modulators. For this type of analysis HCT 116 wild type (wt) and HCT 116 p53-null and SW480 were used. These enzymes are responsible for maintaining the methylation pattern at the DNA level, fundamental for the regulation of gene expression during the development of cancer. Therefore, different epigenetic modulators have been chosen: *Vorinostat* (*SAHA*), pan-inhibitor of histone deacetyltransferases (HDACs); GSKJ4, KDM6B demethyltransferase inhibitor (UTX); JQ1, an inhibitor of bromodomain proteins (BET proteins) and finally UVI5008 (83) inhibitor of DNA methyltransferase (DMNTs), HDACs and Sirtuins (Sirt) in particular inhibitor of Sirt1 and Sirt 2. Following the treatment of several CRC cell lines with the aforementioned epi-drugs protein and mRNA expression for CTSB was evaluated. Western Blot experiment demonstrated that CTSBexpression increases especially after treatment with SAHA (**Figure 13**) and UVI5008.

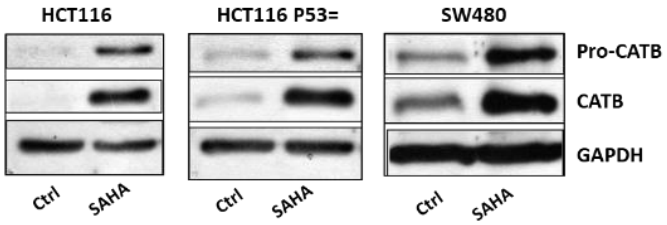


Figure 13: Western blot analysis demonstrating the CTSB protein modulation (both inactive and active form) upon 24h of SAHA treatment at 5 μM in different CRC cell lines.

These data were further confirmed by the evaluation of the transcriptional expression of mRNA using the Realtime PCR. In addition, the increased amount of CTSB in the intracellular compartment was also evaluated by isolating lysosomes: these organelles contain enzymes such as cysteine proteases, for the host defence. CTSB levels were measured after treatment with *SAHA* at 5 μM for 24h in HCT116 wt. For this purpose, lysosomes were isolated by using a Lysosome isolation kit from Abcam and Western Blot analysis was

conducted using protein extract (5γ). The results reported in **Figure 14** demonstrated that upon *Vorinostat* treatment increased the amount of lysosomal CTSB protein in a consistent manner when compared to the untreated cells.

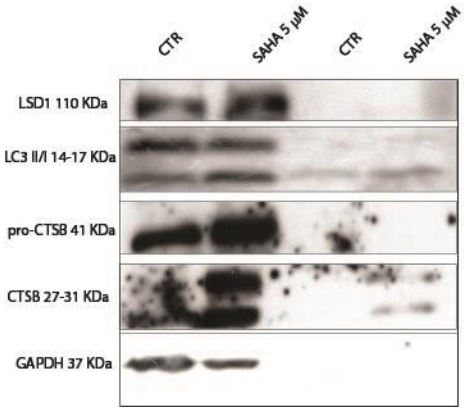


Figure 14: Western Blot results demonstrate the up regulation of both pro-CTSB and CTSB active form in total and lysosome extract upon SAHA 5μM treatment in comparison with untreated cells. LSD1 protein was used as a control for total extract while for lysosome extract, LC3 II/I antibody.

4.7 Extracellular increase of Cathepsin B upon SAHA treatment

Cathepsin B as a protease enzyme, plays important roles in the degradation of the extracellular compartment favouring invasion and metastasis. The increased expression of the protein and mRNA of this gene was observed in response to epigenetic modulation *via SAHA* treatment within intracellular compartments. For this reason, after the evaluation of its intracellular increase, it was then investigated whether, not only the epigenetic agent could affect intracellularly but also the extracellular content of the protease, known to be important in the degradation of the ECM. For this purpose, the HCT116 wt cells were again treated with SAHA for 24h at 5 μM and then the medium in which the cells were treated, the conditioned medium, was collected to be tested for an ELISA Cathepsin B assay (Abcam). This assay as reported in **Figure 15** demonstrated that CRC cells exhibited an increase in the extracellular amount of the cysteine protease after the treatment. Furthermore, to verify if the amount of the extracellular CTSB was able to maintain its enzymatic activity, two prodrugs inspired -probes were used to assess the activity of the protein after the epi-modulator treatment. The assay used was based on the of probes ability to selectively report on CTSB activity in cell lysates, living cells, and medical models. In line with this, the two probes were used to assess the enzymatic activity of CTSB in the intracellular and extracellular compartments. These

imaging probes consist of a peptide portion providing high affinity and specificity to CTSB, a self-immolative linker that spontaneously releases a reporter upon its enzymatic removal of the peptide, and a latent fluorophore becoming highly fluorescent once enzymatically freed from the intact probe. For this experiment, the cells were treated again for 24 h with SAHA at 5μM and then the medium was used as a sample and incubated with each of two prodrug-inspired fluorogenic peptides (84). The fluorescence increase in the cell lysates and in the conditioned medium demonstrated the efficacy of the epi-modulator (*SAHA*) in fuelling not only the intracellular increase of CTSB but also promoted its secretion extracellularly. Moreover, the enhancement of the two probes fluorescence was able to demonstrate that the epigenetic modulation upon *SAHA* treatment induced the release of the protein in its catalytically active form.

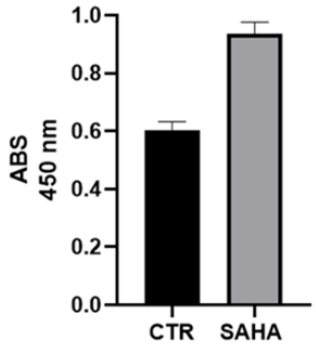


Figure 15: The extracellular amount of CTSB after SAHA treatment in HCT116 was detected by ELISA assay. Absorbance values were normalized on total protein extracts.

Additionally, Ca074 a selective inhibitor of CTSB, was used to confirm that probe hydrolysis was due to CTSB. Indeed, Ca074 treated lysates for 24h exhibited less fluorescence, confirming the specificity of each prodrug- inspired probes (**Figure 16**).

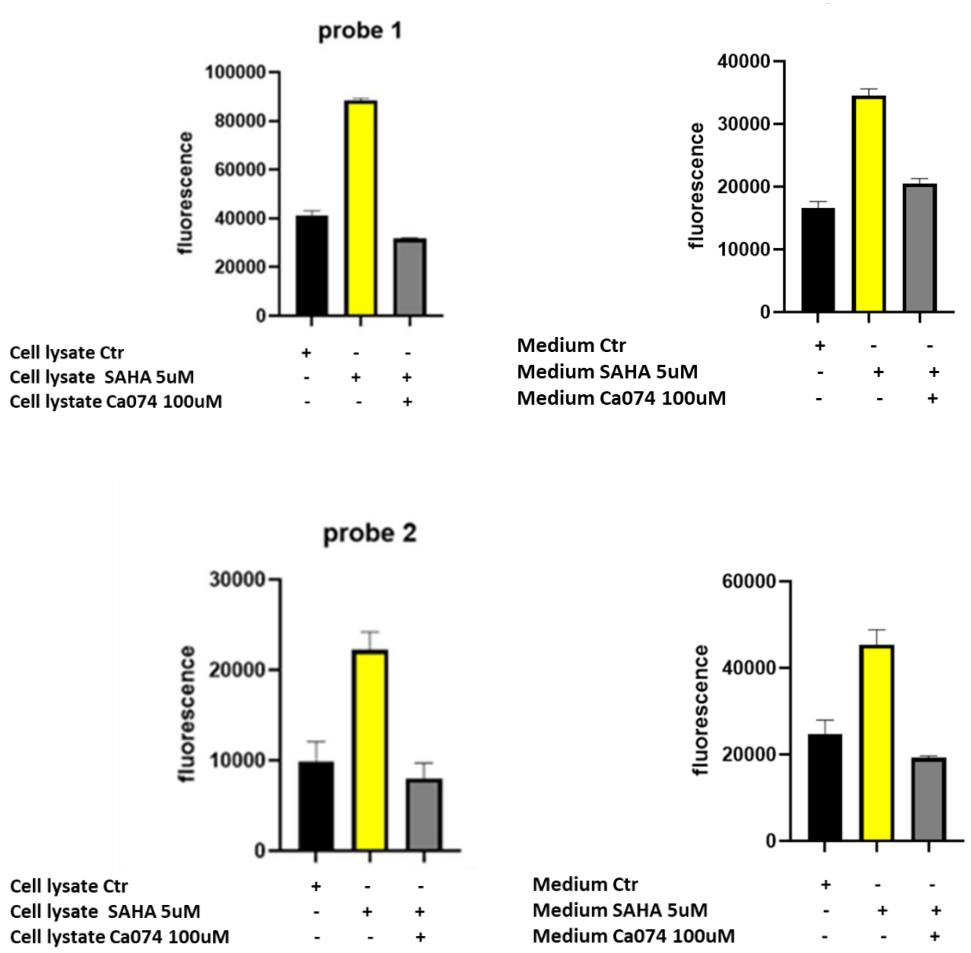
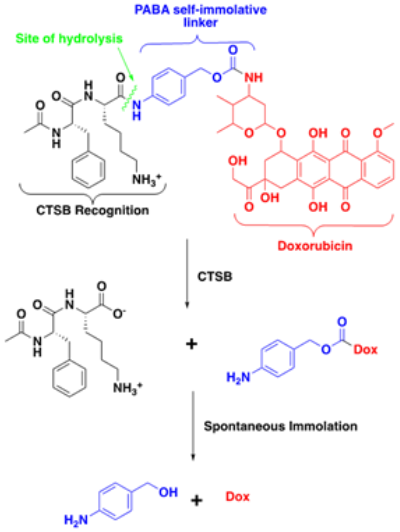


Figure 16: Activity assay obtained from cancer cell lysates and conditioned medium after treating cells for 24 h with SAHA and then incubated with probe 1 or probe 2 showed increases both intracellular and extracellular activity, demonstrating the epigenetic modulation increased CTSB in its active form. In contrast Ca074 cell lysates reported less fluorescence in comparison to the Ctr.

4.8 Cathepsin B as a suitable tool to activate prodrugs

Over the years the prodrug concept has raised considerable interest in cancer research due to its advantages in comparison to classical chemotherapeutic agents. Such prodrugs have indeed the potentiality to overcome common drawbacks associated with chemotherapy such as chemoresistance, side effects and low specificity (85). In addition, their site-specific release upon enzymatic activation renders these molecules advantageous to use in therapy. CTSB, as protease, is known to be a suitable target to activate many different anticancer drugs present in an inactive form as prodrugs, thus resulting in the release of the active molecule only in the surrounding of tumor cells avoiding healthy cells. The assumptions based on the previously results demonstrating CTSB overexpression in the tumor-specific layer allowed us to the

design of prodrugs carrying a Cathepsin B-cleavable entity. For this reason, a *Doxorubicin*-based prodrug that was reported in the literature, was synthesized, and tested to verify toxicity in CRC cells. Using this information, Ac-Phe-Lys-PABC-*Doxorubicin* (Dox) was obtained (7), combining tripeptide Ac-Phe-Lys, well-known tripeptide recognized by CTSB, and PABC (para-aminobenzylalcohol) as self-immolative linker and *Doxorubicin*, as potent non-specific cytotoxic anticancer drug to test the CTSB-prodrug efficacy in CRC (**Scheme 1**).



Scheme 1: Chemical structure of Ac-Phe-Lys-PABC-*Doxorubicin* whose dipeptide Ac-Phe-Lys (CTSB substrate) is conjugated to PABC (self-immolative linker) and then to *Doxorubicin* drug. CTSB hydrolysis activated PABC which then decomposes to the release cytotoxic *Doxorubicin*.

As shown in **Figure 17** the prodrug, Dox-CatB, had demonstrated at 1 μM its toxic effect in CRC cell line reducing of 30% of the cancer cell vitality. For this MTT assay *Doxorubicin* drug was used as control experiment to verify toxic effect of the free molecule. In addition, by pre-treating the cells with CTSB selective inhibitor, Ca074 at 100 μM for 3h and then adding the prodrug incubated for 24h, the effect on cell viability disappeared due to the inhibition of CTSB enzymatic activity (% of cell vitality was normalized on Ca074 treatment). The use of the selective inhibitor, Ca074 confirmed the selective cleavage of the prodrug by this protease.

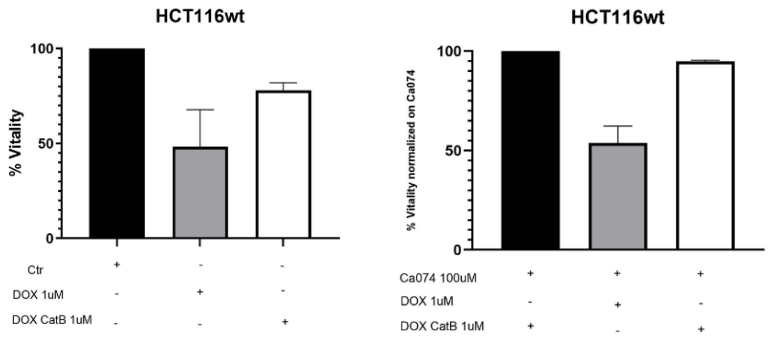


Figure 17: Cell viability assessed by MTT assays: cytotoxic effects of free *Doxorubicin* and *Doxorubicin* CTSB prodrug evaluated after 24h of treatment in HCT116. Cells pre-treated with Cathepsin B selective inhibitor Ca074 at 100µM for 3h were subsequently exposed to free *Doxorubicin* or to the *Doxorubicin* CTSB prodrug for 24h. (% viability normalized on Ca074 treatment).

4.9 SAHA pre-treatments increase CTSB favouring prodrug release

Considering that the expression of CTSB can be modulated epigenetically in CRC following treatment with *Vorinostat* (SAHA), the next step was to verify the synergistic effect of SAHA and the prodrug-CTSB targeted. SAHA as an epigenetic agent, promotes apoptotic mechanisms while also increasing the expression of the active form of CTSB in the tumor and in the tumor environment. This combination favoured a more efficient release of the active portion of prodrug (*Doxorubicin)* in the CRC. For this purpose, MTT experiments on different cell lines including one of CRC, HCT116 wt and two non-tumoral cell lines, H9C2, human cardiomyocytes and 3T3L1, murine fibroblasts, exhibited that the pre-treatment with SAHA at 5uM for 6h and the subsequent addition of prodrug affected cancer cell vitality to a greater extent compared to healthy (normal) cell lines (**Figure 18**).

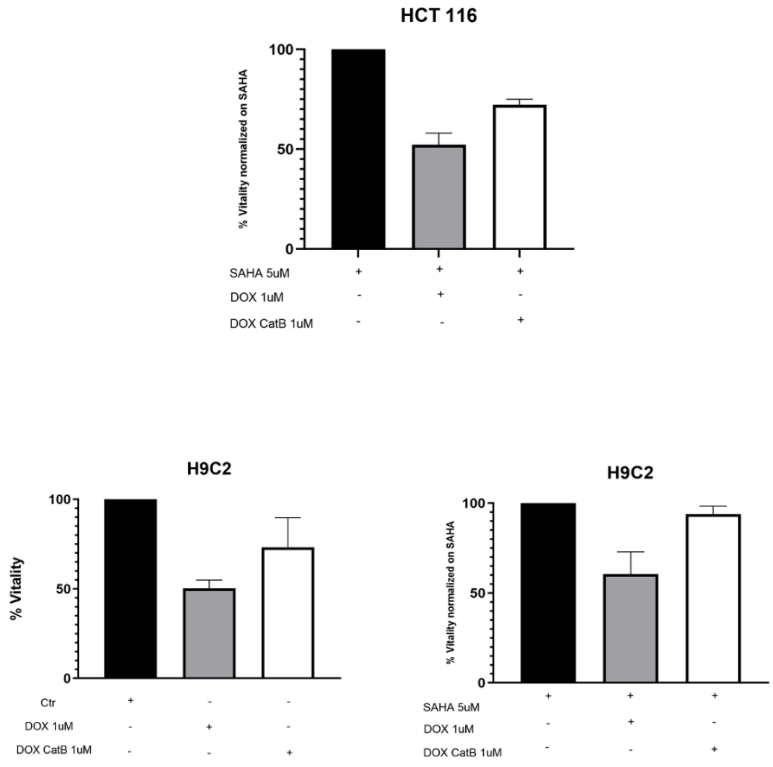


Figure 18: (A) % Cell viability normalized on SAHA treatment was assessed by MTT assays: cytotoxic effects of cells pre-treated with SAHA for 6h and then exposed for 24h with free *doxorubicin* and the *doxorubicin* CTSB prodrug in HCT116. (B) (Left panel) Cytotoxic effects on normal cells treated with free *doxorubicin* and *doxorubicin* CTSB prodrug after 24h of treatment was evaluated in H9C2 cardiac myocytes. (Right panel) Healthy cells viability was only affected by pre-treating with SAHA for 6h and with free *doxorubicin* for 24 and not with addition of *doxorubicin* CTSB prodrug. (% cell viability normalized on SAHA treatment).

4.10 Antiproliferative effect of CTSB targeted prodrug in 3D CRC systems

The anticancer effect of the CTSB prodrug (*Doxorubicin* based-DOX CatB) was also tested in different 3D patient's derived organoids (PDO), by Prof.Luca Primo in IRCCS in Candiolo, Italy. K-Ras mutated and wt 1257 and 1589 PDO systems both K-Ras mutated were tested with *doxorubicin*, *doxorubicin* CTSB prodrug and *doxorubicin* CTSB negative prodrug, a prodrug not cleavable from CTSB, the Ac-Phe-Lys (Boc)-PABC-Dox with an uncleavable CTSB peptide. The samples were both treated with each molecule at 5 µM for 7 days. As reported in **Figure 19**, the anticancer effect was exhibited when PDO specimens were treated only with free *doxorubicin* and with DOX CatB. Moreover, the area increase (% vs Ctr) of primary tumor cells showed a decrease in PDO volume after *doxorubicin* CTSB prodrug, whereas no affection from *doxorubicin* CTSB negative prodrug treatment was observed.

Noteworthy, when Ac-Phe-Lys-PABC-Dox is used in combination with SAHA, the anticancer effect is increased in CRC 3D patient's derived organoid. These results corroborated that SAHA pre-addition by upregulating CTSB in its active form, also sensitized a 3D system of CRC organoid to doxorubicin CTSB prodrug treatment.

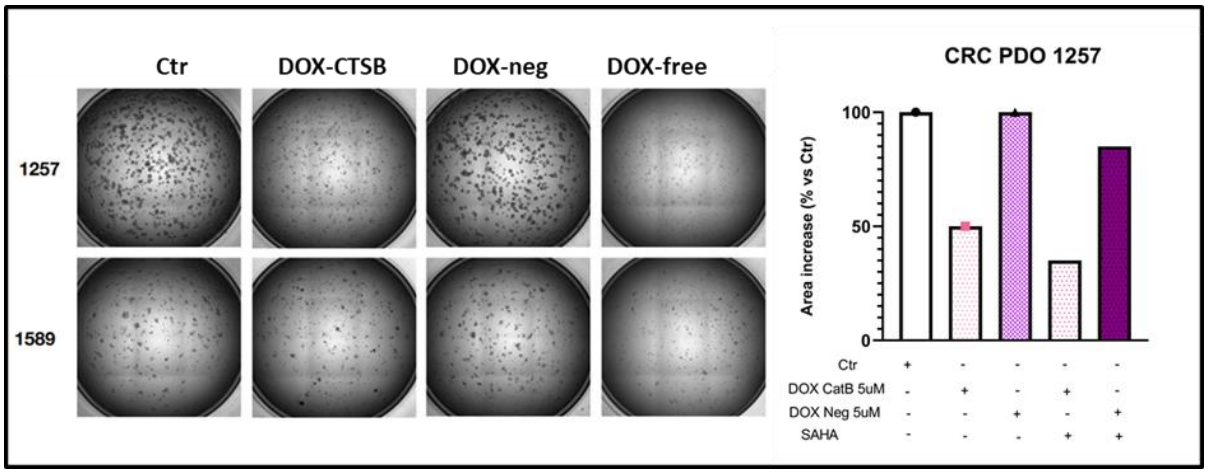


Figure 19: (A) CRC PDO morphology of 1257 and 1589 (both k-ras mutated) tested after *doxorubicin*, *doxorubicin* CTSB prodrug (DOX-CTSB) and *doxorubicin* CTSB negative prodrug (DOX-neg) treatment at 5 µM for 7 days. (B) Area increase (% vs Ctr) of PDO volume after DOX CTSB prodrug and DOX-neg. SAHA pre-addition sensitizes CRC organoid to CTSB prodrug treatment.

4.11 Synthesis of new CTSB prodrugs- Doxorubicin based

The published Ac-Phe-Lys-PABA-DOX is an efficient and selective substrate of CTSB over other CTS in cancer cell lysates and live cells. To increase the selectivity for CTSB, Cbz-Lys-Lys-PABA-Dox and Cbz-Lys-Lys-PABA-PABA-DOX, two new CTSB prodrugs doxorubicin-based agents were designed and synthetised by Prof. Phenix Christopher and PhD student Brady Vigliarolo from University of Saskachewan, Canada. In these modified prodrugs, Lys-Lys is a dipeptide specific for cathepsin B, and PABA (para-aminobenzoyl alcohol), the self-immolative spacer. For these two prodrugs, the Phenix lab determined kcat/KM values using recombinant human CTSB and an HPLC kinetic assay (**Table 1**). The substitution of Phe-Lys with Lys-Lys and the addition of a PABA group in one prodrug increased the selectivity for CTSB *in vitro* (**Figure20**).

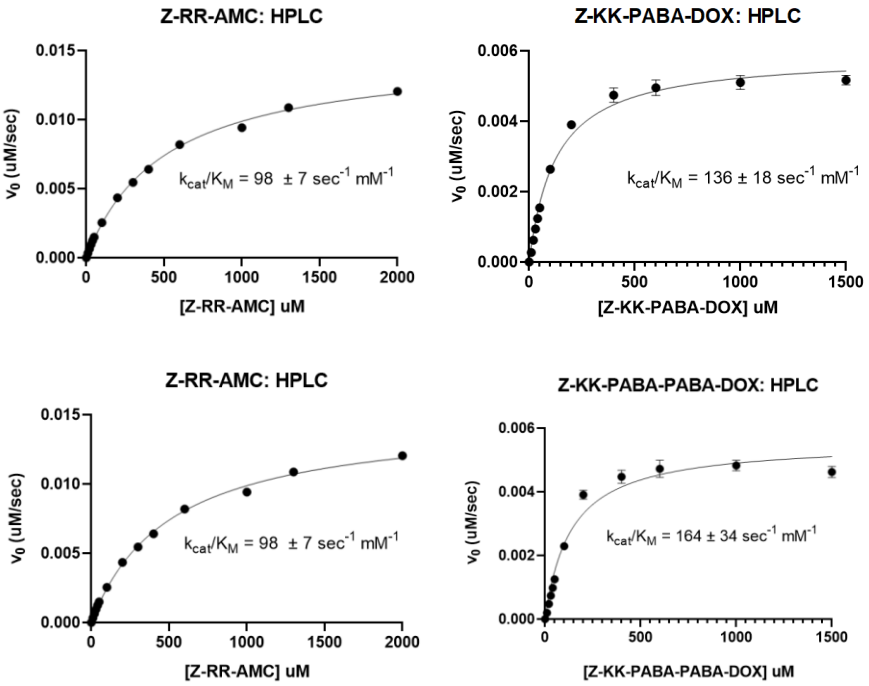


Figure20: Kinetic data obtained from HPLC enzymatic assay using CTSB protein and as substrate the two new synthetic prodrugs Z-KK-PABA and Z-KK-PABA-PABA reporting higher catalytic efficiency in comparison to the prodrug reference.

Probe	K_{cat} (sec^{-1})	K_M (uM)	K_{cat}/K_M (sec^{-1} mM^{-1})
Z-RR-AMC (Plate reader)	31.4 ± 1	277 ± 28	113 ± 12
Z-RR-AMC (HPLC)	49.5 ± 1.3	506 ± 32	98 ± 7
Z-KK-PABA-DOX	17.3 ± 0.6	127 ± 16	136 ± 18
Z-KK-PABA-PABA-DOX	22.3 ± 0.2	136 ± 28	164 ± 34

Table 1: Data obtained from Kinetic experiment of CTSB processing two new synthetic prodrugs.

4.12 SAHA treatment sensitizes CRC cells to exposure of two new CTSB-prodrugs

The epigenetic reprogramming of CTSB upon SAHA treatment demonstrated the possibility of modulating its expression and activity extracellularly, rendering cancer cells susceptible to prodrug treatment. In line with these assumptions the two new CTSB cleavable prodrugs were tested alone and in combination with *Vorinostat* in CRC. The prodrug designed means that the antitumor agent is inactive when there is lowthe protease activity thus avoiding the side

effects in normal tissues. The two new prodrugs were tested by MTT assay to verify their eventual effect on CRC viability. The MTT assay results demonstrated that these two prodrugs have no effect on cell vitality when there are used alone at different concentration starting from 10 to 0.5 μM (**Figure 21**) In addition upon SAHA pre-treatment for 6h there was observed a decrease in cell vitality at 5 μM of treatment with both prodrugs (**Figure 22**) The decrease of cell vitality upon SAHA pre-treatment is due to the upregulation of CTSB in its active form extracellularly, thus contributing to more efficient CTSB cleavage and release of the active drug (*Doxorubicin*). These results suggest that possibility to use an epigenetic modulation by SAHA to promote the CTSB expression may enhance protease activation of the prodrugs thus selectively killing cancer cells.

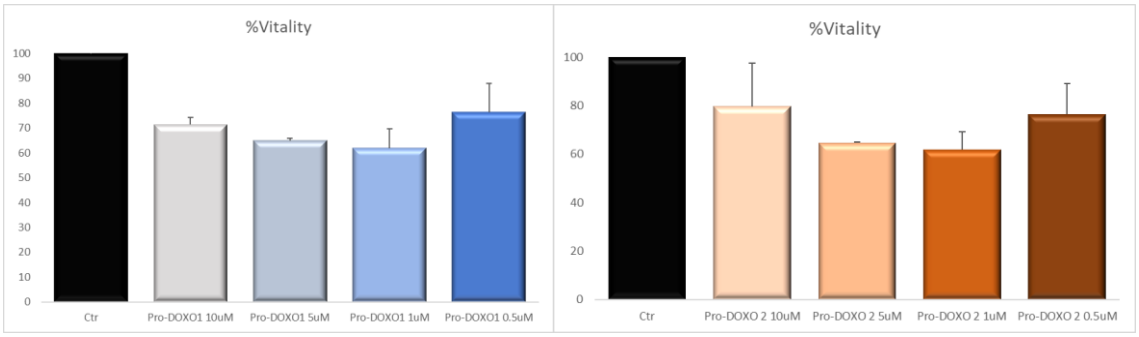


Figure 21: MTT assay in HCT116 showing cytotoxic effect of the two prodrugs at different concentrations starting from 10 to 0.5 μM effect of the two prodrugs (prodrug 1 and 2) by pre-treating the cells with SAHA at 5μM.

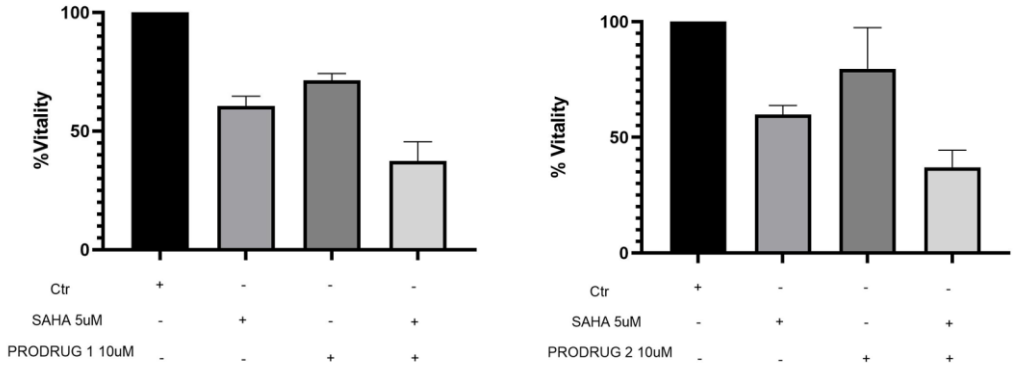


Figure 22: MTT assays in HCT116 showing the antiproliferative effect of the two prodrugs (prodrug 1 and 2) alone and by pre-treating the cells with SAHA at 5μM.

4.13 CTSB prodrugs cardiotoxicity effect

Doxorubicin is one of the most widely used anticancer agents for treating solid tumor in adult and pediatric patients such as in breast, lung, and bladder cancers and lymphoma (85). Despite its use and versatility, a major drawback is cardiotoxicity, a common side effect associated with this chemotherapy (85). However, *Doxorubicin* in the prodrug form (CTSB targeted) renders this molecule inactive until cleavage by CTSB. To test whether the new prodrugs were toxic to cardiac cells, AC16 were treated with 5 μM alone or by pre-treatment with SAHA. The epigenetic activity of *Vorinostat*, sensitizing the healthy cells to the prodrug's treatment was visible in this cell system (**Figure 23**). However, by comparing the antiproliferation activity of the two prodrugs in combination with *Vorinostat* with CRC cell line, there was observed quite no effect in cardiac cells (**Figure 24**). These results corroborate the idea that the epigenetic modulation of CTSB guarantees the up regulation of the protease in its active form only in the tumor avoiding the prodrug release in the normal cells.

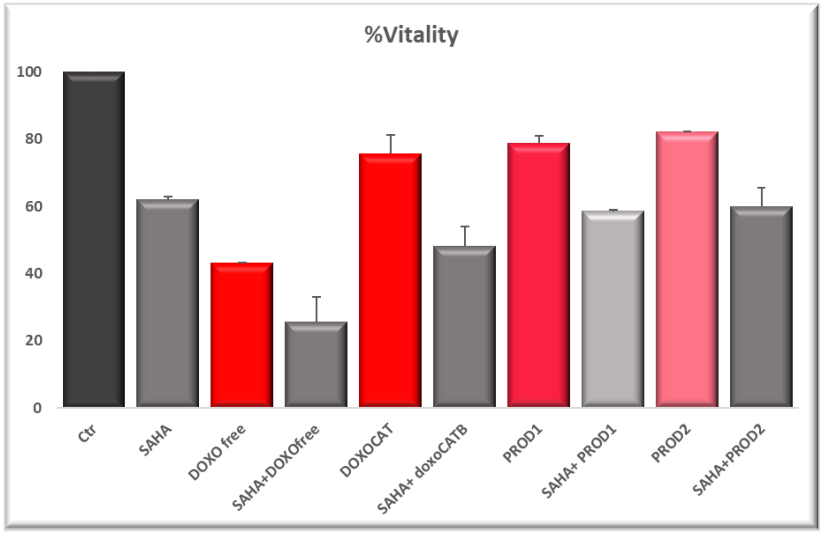


Figure 23: MTT assays in AC16 reporting the antiproliferative effect of the two prodrugs (prodrug 1 and 2) by pre-treating the cells with SAHA at 5μM.

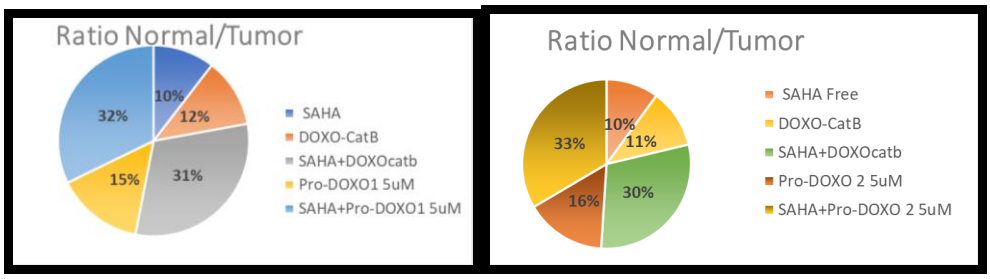


Figure 24: Pie charts indicating the antiproliferative activity of two prodrugs alone or in pre-treatment with SAHA by comparing the effects in healthy (AC16) vs tumor system (HCT116).

5.0 DISCUSSION

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Colorectal cancer (CRC) accounts for 10% of all yearly cancers diagnosed and cancer-related deaths worldwide. Indeed, it is considered the third most diagnosed malignancy and the fourth leading cause of cancer death in the world. In the literature, most studies comprising different states of the disease for colon and rectum cancers are combined (86). CRC is considered a major health issue but also an economic burden due to costs of patient care that is increasing worldwide. Particularly, the treatments for rectal cancer diagnosis are more complex (including for example, radiotherapy, temporary stoma), that requires longer hospitalization periods, thus affecting and influencing the initial phase of care (87). In addition, it is predicted that by 2035 worldwide, deaths from colon and rectal cancer are going to increase by 60.0% and 71.5%, respectively due to growth and aging in the population (88). Despite the substantial improvements in patient management and treatment outcomes, CRC can often spread leading to metastatic disease (mCRC) in non-responder or late diagnosed patients (89). mCRC is generally not a curable disease with median overall survival (OS) of around ~35 months (90). About 20% to 25% of CRC patients have advanced stage disease at diagnosis and systemic chemotherapy, alone or in combination with targeted/immune therapy, is the current standard treatment. For these patients, the identification of new biomarkers to reveal aggressive disease and new therapeutic regimes is an urgent and unmet need. The formation of metastases requires the degradation of extracellular matrix and the activation of the processes of invasiveness, survival, and cell death escape (91). Intra/extracellular protease enzymes, from different families have recently been identified as key elements in the pathological processes of tissue metastasis, facilitating cell cycle, local invasion, energy supply, and angiogenesis (92). In CRC, different cystein-proteases known as Cathepsins are involved in the degradation of the ECM barrier favouring invasion and metastasis. CTSB, a lysosomal cysteine protease, has been reported not only for its involvement in tumor progression and metastasis, but also in facilitating rapid local invasion and promoting angiogenesis. Consistent with its role in the promotion of metastasis, CTSB overexpression is significantly associated with an increased risk of CRC and overall mortality (93). Increases in CTSB expression is associated with the late stages of the disease (the most severe) and with a short average survival (OS) and reduced disease-free survival time (DSF) in patients with CRC (57). Because both primary and mCRCs show the upregulation of CTSB, this enzyme is a driver for tumor initiation and metastasis and, moreover, its catalytic activity may be used as a diagnostic, predictive and prognostic CRC biomarker (94,95). GEPIA analysis reported

CTSB overexpression associated with low OS while the TMA analysis confirmed its hyper-expression as a negative prognostic factor. Importantly, this protein is almost absent in the healthy tissue compartments; its expression is extremely low in healthy cells. Further, it is highly expressed in right side colon cancer, usually characterized by an inauspicious prognosis. In addition, reports in the literature indicate that CTSB can be epigenetically regulated using HDACi (8). *SAHA*, a well-known pan-HDAC inhibitor, induced the upregulation of CTSB in breast cancer. Our data demonstrated that some epi-modulators induce upregulation of CTSB in CRC cells; Most notably, the epi-modulator *SAHA* showed an increase in the active form of CTSB not only intracellularly but also in the extracellular compartment determined by ELISA, Western Blot from total extracts and lysosomes. In addition, fluorescence-based assays using prodrug inspired-probes corroborated these data indicating that the epigenetic modulation can augment CTSB secretion extracellularly in its active form. Furthermore, among the players involved in CRC, CTSB, has come to the limelight not only as diagnostic and prognostic biomarker, but also as biochemical target for the activation of prodrugs. Prodrugs enzymatically activable at the tumor site have the potentiality to overcome common drawbacks of chemotherapeutics such as toxicity and lack of selectivity. However, numerous enzymes as putative prodrug activators failed due to insufficient expression and low activity. To address this limitation, this project has proposed a combined strategy based on exploiting the hydrolytic activity of CTSB to activate cytotoxic and epigenetic prodrugs selectively and efficiently at the tumor site for targeted (m)CRC therapy. The synergistic effect of employing *SAHA* to induce local CTSB activity in the tumor and the tumor environment may be a novel strategy to treat aggressive CRC tumors through prodrug therapy. The synergistic effect of *SAHA* and CTSB targeted prodrugs induced a reduction of cell vitality in 2D and 3D CRC systems (CRC cell lines and PDO). Because the induction of apoptotic mechanisms generally promoted by *SAHA* is therefore accompanied by the over expression of CTSB, it is reasonable to think that this increase within cells induces cell death with the consequent release of the active form of CTSB in the extracellular space. Hypothetically, the administration of an epigenetic modulator, selectively and efficiently released at the tumor site, may induce the expression of CTSB thus making the tumour more susceptible to cytotoxic prodrugs more efficiently activated by CTSB. In this way, *SAHA* would act as a neoadjuvant treatment selectively priming the tumor for cytotoxic prodrug chemotherapy. However, these data support the possibility to use an epigenetic modulator such as *SAHA*, generally recognized as an anticancer drug that can be used in combination with conventional chemotherapy (including 5-fuorouracil, leucovorin, and oxaliplatin) has

already been tested in patients with advanced CRC. Once activated, drugs can enter in tumor cells, in which the apoptotic mechanism has already been partially triggered by the sensitizing pre-treatment with Vorinostat.

In conclusion proof of concepts of this project are: 1) Evaluation of the differential expression of CTSB between CRC and healthy tissues thus identifying this protein as a new predictive and diagnostic marker. 2) The possibility of using a CTSB-targeted prodrug that can only be activated in tumor cells, overcoming the common drawbacks of chemotherapy, such as low therapeutic index, high toxicity, side effects and lack of tumor selectivity. 3)The expression of CTSB can be modulated "epigenetically" to improve the activation of prodrugs and increase the concentration of active drugs at the tumor site. 4) Identification of novel prodrug strategies, that released drugs not considered suitable in clinical studies due to high systemic toxicity or low selectivity index can be reconsidered.

6.0 MATHERIAL AND METHODS

6.0 MATERIAL AND METHODS

6.1 Bioinformatics Analysis by GEPIA

Differential expression of Cathepsin B in CRC samples and normal samples was analysed using the online Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) database. The GEPIA survival analysis tool was used to analyse the relationship between CTSB mRNA expression and CRC prognosis. In addition, the expression level of CTSB was evaluated in tumor and normal COAD data set patients.

6.2 CRC patients for IHC analysis

Forty-four colorectal cancer patients were admitted to the National Cancer Institute "Giovanni Pascale" of Naples, between 2012 and 2017, were recruited for this study. All patients had provided written informed consent for the use of samples according to the institutional regulations and the study was approved by the ethics committee of the National Cancer Institute "G. Pascale". All CRCs cases have been reviewed by pathologist (FT) and graded and staged according to WHO 2010/AJCC 2017 classification criteria, on standard tissue sections. Medical records have been reviewed for clinical information, including histologic parameters, assessed on standard H&E-stained slides, and tumor location (right or left).

6.3 Immunohistochemistry Analysis

All selected samples derived from formalin-fixed, paraffin embedded tissues (FFPE) including tumor, non-neoplastic colonic mucosa, and adenomatous dysplastic modifications areas. It was built a Tissue Micro Array (TMA) using cores representative of all three components. Paraffin slides were then deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed with slides heated in 0.0.1 M citrate buffer (pH 6.0.) for 10 min at 110 °C. After antigen retrieval, the slides were allowed to cool. The slides were rinsed with TBS and the endogenous peroxidase has inactivated with 3% hydrogen peroxide. After protein block (BSA 5% in PBS 1×), the slides were incubated with primary antibody to human Cathepsin B, dilution 1:200 ab58802 overnight. Sections were incubated with goat anti-mouse secondary IgG biotinylated secondary antibody for 30 min. Immunoreactivity was visualized by means of avidin–biotin–peroxydase complex kit reagents (Novocastra, Newcastle, UK) as the chromogenic substrate. Finally, sections were weakly counterstained with haematoxylin and mounted.

6.4 Evaluation of Immunostaining

Antigen expression was evaluated by two experienced pathologists (FT) using light microscopy. All values of immunostaining were expressed only in percentage terms of positive cells. The percentage of positive cancer cells was evaluated in each sample by counting the number of positive cells over the total cancer cells in 10 non-overlapping fields using ×400 magnification.

6.5 Cell lines

Cell lines were tested and authenticated following the manufacturer's instructions: ATCC for HCT116, HCT-116 p53-/-, HCT116- DKO and SW480 colon cancer cell lines; normal cell lines H9C2 (human cardiomyocytes) and 3T3L1 (murine fibroblasts). All the cell lines were propagated in Dulbecco's Modified Eagle's Medium (Euroclone, Milan, Italy) with 10% fetal bovine serum (FBS) (Euroclone), 2 mM L-glutamine (Euroclone) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin: Euroclone) and amphotericin 25 μg/mL (Euroclone).

6.6 Western blotting for histones

Western blotting analysis was performed following the recommendations of antibody suppliers and loading 5 μg of histone extracts on 15% polyacrylamide gels. Antibodies used were: H3K9/14acetyl (Cell signalling) and histone H4 was used for normalization (Abcam). Semi-quantitative analysis was performed using ImageJ software.

6.7 Western blot analysis

After removal of the culture medium, the cells were washed with cold 1X PBS and were lysed using a lysis buffer supplemented with protease and phosphatase inhibitors: 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM beta glycerol phosphate. The lysis reaction was carried out for 15 min at 4 °C. The samples were then centrifuged at 13,000 rpm for 30 min at 4 °C and protein concentration quantified by Bradford assay (Bio-Rad). After centrifugation, each sample (total extract 50 μg whereas for histone and lysosome extract 5 μg) were loaded on 10% or 15% and electroblotting on nitrocellulose membrane. Immunoreactive signals were detected with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). All the antibodies were used according to the manufacturer’s protocol. Antibodies used were: Cathepsin B (Abcam), GAPDH (Santa Cruz) and beta-actin (Santa Cruz), LSD1 (abcam), LC3 II/I (abcam).

6.8 Western blot analysis from *ex vivo* samples

CRC ex vivo samples were obtained from the "University of Campania "Luigi Vanvitelli'' Hospital Department of Surgery in collaboration with Dr. Selvaggi. The use of human derived specimens was allowed by ethics committee (number of protocol n 790 del 12/12/2018 entitled: "I-CURE"). The collected CRC samples, including healthy (non-cancerous), tumor tissue and lymph nodes were immediately preserved after surgery at -80° C (snap frozen). Total protein extraction was performed from the healthy, tumor and lymph node tissues of 14 different CRC patients. Ex vivo samples were firstly fragmented in chill a mortar with liquid nitrogen, then grind small tissue pieces in the presence of liquid nitrogen to a fine powder. The powder was then treated with lysis buffer and total protein extraction was carried out as described above. Briefly, 50 ug of total protein were loaded on 10% of acrylamide gel and CTSB expression levels were monitored.

6.9 CTSB activity detection using prodrug-inspired probes

Cells were grown to ~80% confluency and washed with PBS, treated with lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA), and centrifuged 12 000 rpm in a microcentrifuge in a 1.5 mL tube. CTSB activity was evaluated by adding 100 μL of the lysis solution to a black 96-well plate and diluted with 100 μL of activation buffer (100 mM acetate−NaOH, pH 5.5, 5 mM DTT, 5 mM EDTA) followed by 15 min incubation at 37 °C. A 100 μL aliquot of the activated lysate was then withdrawn and added to a 50 μL solution containing 300 μM probe, in 30% DMSO and 70% buffer (100 mM acetate buffer, 5 mM DTT, 5 mM EDTA, pH 5.5). The enzymatic reaction proceeded at 37 °C for 1 h after which 50 μL was withdrawn and added to 100 μL of sodium chloroacetate (200 mM) to stop the reaction. The fluorescence at 460 nm was measured in a microplate reader, Infinite TECAN M200 and normalized to total protein concentration (Bradford Assay) and expressed as a percentage of AMC released from experiments using Cbz-Arg-Arg-AMC.

6.10 RNA isolation and quantification

Cells were centrifuged and resuspended in 1 mL of TRIzol reagent (Invitrogen, Monza and Brianza, Italy), vortexed, and stored overnight at −80 °C. Then, 100 μL of 2-bromo-3-chloro propane (Sigma Aldrich) were added to the samples, gently shaken, and incubated for 15 min at room temperature RT. After centrifugation at 12,000 rpm for 15 min at 4 °C, the supernatants were collected in fresh tubes supplemented with 500 μL of cold isopropyl

alcohol. The total RNA was precipitated for 1h at −80 °C followed by a centrifugation of 30 min at 12,000 rpm at 4 °C. The samples were resuspended in 1 mL of cold 70% ethanol and then centrifuged for 10 min at 7500 rpm at 4 °C. The RNA pellets were dried at 42 °C for a few minutes and resuspended in DEPC-treated H2O. The RNA samples were quantified using Nanodrop 1000 and their quality was checked using an Agilent RNA 6000 Nano Assay.

6.11 CTSB gene expression analysis

Real-time RT-PCR was performed to examine mRNA expression level of CTSB gene using the VILO cDNA Synthesis Kit (Invitrogen, Monza and Brianza, Italy) to convert RNA into cDNA. A 1X SYBR Green PCR Master Mix (Bio-Rad, Segrate, Milan, Italy) was used according to the manufacturer's instructions, using 50 ng of cDNA.

6.12 CTSB ELISA Assay

The ELISA assay for CTSB was performed as reported in the manufacturer 'protocol (ab119584). Briefly, conditioned medium (from untreated and SAHA treated samples) from 24h of treatment was dilution 1:2 with sample diluent buffer and then 100 µl were added in each well, the plate was covered with seal and incubated 90 min at 37 °C. Then the samples were discarded, and microtiter plate wells were coated with 100 ml of Biotinylated anti-Human Cathepsin B antibody into each well and then incubated at 37°C for 1 h. After each well was then extensively washed for three times with 300 µl of 0.01M PBS, each time let the washing buffer stay in the wells for one minute. Subsequently, the washing buffer was discarded and blotted the plate onto paper towels and then 100 µL of 1X Avidin-Biotin-Peroxidase Complex were added into each well and the plate incubated 30 minutes at 37°C. The plate was washed five times with 0.01M PBS, and each time washing buffer let stay in the wells for 1 - 2 minutes. The washing buffer was discarded and blot the plate onto paper towels. Finally, 90 µL of prepared TMB color developing agent were added into each well and the plate incubated 15 min at 37°C. 100 µL of prepared TMB Stop Solution were then added into each well. The absorbance was detected at 450 nm using a microplate reader, Infinite TECAN M200.

6.13 Lysosome Isolation from CRC cells

Lysosomes Isolation was permed by using Kit according to the manufacturer 'protocol (ab234047). Briefly, cell pellet from 2 x10^7 of HCT116 treated for 24 h with SAHA at 5 µM and untreated were collected by centrifugation at 600 x g for 10 minutes and then the supernatant was carefully removed and discarded. 500 µL of Lysosome Isolation Buffer were add to the pellet and vortex for 5 seconds, followed by incubation on ice for 2 minutes. The cells were homogenized using a precooled glass Dounce homogenizer. The sample was incubated 20-30 times on ice and then 500 µL of Lysosome Enrichment Buffer were added and the tubes were inverted several times to mix. After the samples were centrifuged at 500 x g for 10 min at 4°C. The supernatant was collected in a separate tube and keep on ice. Five gradient solutions were prepared using Lysosome Gradient and Lysosome Enrichment Buffer in five centrifuge tubes. The prepared cells were diluted 1:4 with Lysosome Gradient, by mixing 1 part of Lysosome Gradient with 3 parts of cell lysate. Carefully the diluted cell lysate was added to the top of the prepared density gradient. The sample tubes were centrifuged using an ultracentrifuge for 2 h at 145,000 x g at 4°C. Then the lysosome fraction band carefully taken by using an extra-long pipette tip starting from top of the gradient. This fraction was then washed with 2 volumes of PBS and centrifuged for 30 min at 18,000 x g at 4°C. Discard the supernatant and keep the pellet containing the purified lysosomes.

7.0 REFERENCES

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