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Discovering New Molecules for Diagnosis and Non-Toxic Differentiation Therapy in Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is the third most prevalent type of cancer worldwide. Therefore, better diagnostic tools and anticancer therapies have been some of the most sought out topics in CRC research. MicroRNAs (miRNAs), a type of short non-coding single strand RNA molecules capable of repressing mRNA translation, are rising as potential and powerful diagnostic and prognostic biomarkers in cancer. In CRC, differences in the expression pattern of certain miRNAs in tissue have been already described, suggesting that miRNAs could represent an effective diagnostic option for this particular disease. On the other hand, cancer stem cells (CSCs), a subpopulation of cells within tumors with phenotypic resemblance to normal stem cells, have been implicated in tumorigenesis, metastization and recurrence processes. Indeed, these undifferentiated cells are not entirely eliminated with the classical anti-proliferative approaches, being responsible for tumorigenic potential and rapid relapse. Thus, efficient therapies targeting this specific type of cancer cells are being developed, with special emphasis on combinatorial drug therapies, to sensitize cells to conventional anticancer treatments and therefore reduce the toxicity associated with high doses of chemotherapeutic treatments.

Two major objectives were pursued in this project: 1) to optimize methodologies to assess the diagnostic and prognostic potential of a panel of miRNAs in patients with CRC, prior to and after being submitted to chemoradiotherapy; and 2) to evaluate the effectiveness of two chemical compounds, from an in-house library, as potential non-toxic differentiation drivers in CRC cells and characterize their specific signaling pathways.

The qPCR analysis of human colonic samples revealed differential expression profiles for several miRNAs derived from normal and tumor tissues, as well as before and after chemoradiotherapy. Nevertheless, these data are still preliminary and further studies are necessary to validate the prognostic and diagnostic value of these miRNAs. In addition, we identified the biological effects of two potential candidate molecules, a spiropyrazoline oxindole (DS6) and a 3-piperidinyl-indole (SAS9), capable of promoting differentiation in human colorectal carcinoma cells. Although both compounds had impact on CRC cells, DS6 was particularly successful, being capable of reducing the levels of stemness and pluripotency markers, increasing differentiation, inhibiting self-renewal and exhibiting a slight synergistic effect in sensitizing cells to a classical chemotherapeutic, 5-fluorouracil.

Interestingly, by using isogenic $p53^{+/+}$ and $p53^{-/-}$ HCT116 cell lines, we were able to observe a tendency for DS6 to develop its differentiation effect only in $p53^{+/+}$ cells, suggesting a p53-dependent mechanism of action for this molecule. Further studies are still required to validate the DS6 mechanism of action, as well as consolidate the data on its therapeutic potential for differentiation in CRC.

Altogether, our results shed light on two aspects of CRC research, related with diagnosis and treatment- We open the way to optimize the discovery of novel miRNAs as biomarkers for diagnosis and prognosis and contribute to uncover new regulators of differentiation in CRC.

Keywords: Biomarkers; Cancer stem cells; Colorectal cancer; Differentiation therapy; MicroRNAs

Resumo

O cancro colorretal (CRC) é o terceiro tipo de cancro mais prevalente a nível mundial. Assim, o desenvolvimento de ferramentas de diagnóstico e terapias anticancerígenas mais eficazes têm sido alguns dos tópicos mais relevantes na área de investigação do CRC. Os microRNAs (miRNAs) são pequenas moléculas de RNA não-codificante de cadeia simples que inibem a tradução de RNA mensageiro e que, recentemente, têm sido vistos como potenciais biomarcadores de diagnóstico e prognóstico de várias doenças, como o cancro. De facto, alguns estudos em CRC já identificaram diferenças nos padrões de expressão de vários miRNAs, sugerindo que estes possam ser uma opção de diagnóstico menos invasiva e com melhor custo-benefício nesta doença. Por outro lado, células estaminais cancerígenas (CSCs), uma subpopulação de células tumorais fenotipicamente semelhantes às células estaminais, têm sido implicadas na tumorigénese, metastização e recidivas. De facto, estas células indiferenciadas não são completamente eliminadas pelas abordagens anti-proliferativas clássicas, sendo responsáveis pelo potencial tumorigénico e rápida reincidência. Deste modo, mais recentemente, têm-se desenvolvido terapias tendo como alvo específico esta população de CSCs, com especial ênfase para a terapia combinada de fármacos que sensibilizam as células para os tratamentos anticancerígenos convencionais e reduzem, assim, a toxicidade associada a altas doses de quimioterapêuticos.

Este projeto teve como objetivos: 1) otimizar metodologias que permitam investigar o potencial de um painel de miRNAs como marcadores de diagnóstico e de prognóstico em doentes com CRC, antes e após serem submetidos a quimioradioterapia; e 2) avaliar a eficácia de dois compostos químicos, provenientes de uma biblioteca interna de compostos, como potenciais indutores de diferenciação celular em células de CRC e caraterizar vias de sinalização.

A análise por qPCR de amostras de cólon de doentes com CRC revelou perfis de expressão distintos em vários miRNAs derivados de tecido normal e tumoral, bem como, antes e depois da quimioradioterapia. Contudo, os nossos resultados são ainda preliminares, sendo necessário mais estudos com um número mais elevado de amostras para validar o valor de marcadores de prognóstico e diagnóstico destes miRNAs. Adicionalmente, identificámos os efeitos biológicos de duas moléculas promissoras, um spiropirazolina oxindole (DS6) e um piperidinil-indole (SAS9), capazes de promover diferenciação numa linha celular de

CRC. Apesar de ambos os compostos químicos revelarem algum efeito nas células de CRC, o DS6 demonstrou ser particularmente mais eficaz, tendo reduzido os níveis de marcadores de estaminalidade e pluripotência, aumentando os níveis de diferenciação, inibindo a autorrenovação e demonstrando um ligeiro efeito sinergístico na indução de morte celular, quando combinado com o quimioterapêutico clássico, 5-fluorouracilo. Curiosamente, com a utilização de duas linhas celulares de cancro HCT116 isogénicas $p53^{+/+}$ e $p53^{-/-}$, foi-nos possível observar uma tendência no efeito pro-diferenciador do DS6 em células $p53^{+/+}$, sugerindo que o seu mecanismo de ação poderá ser dependente de p53. Estudos posteriores serão necessários para validade as caraterísticas do DS6, o seu mecanismo de ação, bem como o seu potencial terapêutico de diferenciação.

Em suma, estes resultados clarificam dois aspetos da investigação em CRC, relacionados com diagnóstico e tratamento. Por um lado, abrem caminho para a otimização de procedimentos de descoberta de potenciais miRNA biomarcadores de diagnóstico e o prognóstico desta doença e, por outro, contribuem para o progresso das terapias de diferenciação no CRC.

Palavras-chave: Biomarcadores; Células estaminais cancerígenas; Cancro colorretal; miRNAs; Terapia de diferenciação

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Abbreviations

5-FU	5-Fluorouracil
ABC	ATP-binding cassette transporter
ALDH1	Aldehyde dehydrogenase
APC	Adenomatous polyposis coli
APS	Ammonia persulfate
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CDI	Coefficient of drug interaction
cDNA	Complementary DNA
CK20	Cytokeratin 20
CR	Complete response
CRC	Colorectal cancer
CSC	Cancer stem cell
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
EMT	Epithelial-to-mesenchymal transition
FFPE	Formaldehyde-fixed paraffin-embedded
IAP	Inhibitor of apoptosis protein
MDM2	Mouse double-minute 2
miRNA	Micro RNA
MMR	Mismatch repair
MSS	Microsatellite stability
MSI	Microsatellite instability
nCR	No complete response
PBS	Phosphate buffer solution
SDS	Sodium dodecyl sulfate
TBS-T	Tris buffered saline-Tween
TEMED	Tetramethylethylenediamine

I. Introduction and Aims

1. Colorectal Cancer

Colorectal cancer (CRC) ranks as the third most commonly diagnosed cancer worldwide and recently became the second leading cause of cancer death. By the end of 2018, 1.8 million new CRC cases and almost 900,000 deaths attributed to this cause are already estimated to occur. The distribution of this type of cancer shows a clear pattern, in which the majority of cases are observed in more developed countries and the incidence is similar between both genders[1]. Curiously, CRC is seen as a clear marker of "cancer transition" in countries undergoing a rapid social and economic development, where these types of cancer are taking the place of infection-related cancers. In fact, an increase in CRC incidence and mortality has been observed in Eastern Europe, Asia and South America, areas with medium-to-high Human Development Index (HDI). In contrast, CRC incidence and mortality appears to be stabilizing or even decreasing in many of the highest HDI countries, such as Western European countries, USA, Australia, New Zealand and Japan[1–3]. The reasons underlying this last declining trend can be associated with a reduction of risk factors and an improvement in screening and treatment options[1,3–5]. Nevertheless, by 2030, the global burden of CRC is expected to increase, accounting for 2.2 million new cases and 1.1 million cancer deaths[2].

In terms of CRC prognosis, it has slowly but consistently improved in the past decades in many countries. In 2014, the 5-year relative survival rate in high-income regions, such as North America, Europe and Oceania, has reached around 65%, while in low-income countries it has remained lower than 50%. Of note, the age and stage at diagnosis are also important prognostic elements[6,7].

As for risk factors, there is not a single factor that could account for the majority of CRC cases. The following risk factors have already been established in CRC: family history of CRC[8], inflammatory bowel and metabolic diseases, including obesity and diabetes[9–11]; and certain behaviors, such as smoking, excessive alcohol consumption and high consumption of red and processed meat[12–14]. Growing evidence has also indicated a possible association between increased risk of CRC and infection with potential infectious agents, such as *Helicobacter pylori* or *Fusobacterium spp*, among others[15–17]. There are also established

preventive factors, which include the use of hormone replacement therapy[18], aspirin[19,20], physical exercise[21], endoscopy with removal of precancerous lesions[22,23] and, to a lower extent, diets rich in fruit, vegetables, cereal fiber and whole grains, dairy products[24] and even statin therapy[25].

According to the Union Internationale Contre le Cancer (UICC), to assert the most appropriate therapeutic decision in a more standardized way, CRC can be classified through a stage definition, that goes from stage 0 to IV. This classification is normally based on certain parameters, such as local invasion depth (T stage), lymph node involvement (N stage), and the existence of distant metastases (M stage)[26].

1.1. Molecular pathogenesis of CRC

The molecular mechanisms behind CRC are heterogenous and of great clinical relevance, as they can be crucial for prognosis and to predict treatment response[27,28]. It usually takes more than a decade for CRC to fully develop. Following the classic adenoma-carcinoma sequence, dysplastic adenomas are the most common type of premalignant lesion, being serrated adenomas another relevant form[29,30]. The adenoma-carcinoma sequence is usually associated with chromosomal instability (CIN) phenotype, where cells suffered extensive alterations to the structure and number of chromosomes[30,31]. There are several widely recognized steps for the formation and development of CRC through this pathway. Adenomatous polyposis coli (*APC*) gene inactivation occurs early and, in most cases, is pivotal in tumor initiation, accounting for more than 70% of sporadic adenomas. Other common mutations in adenomas with important roles in tumor progression are, for example, the activation of *KRAS* oncogene or the inactivation of the TP53 tumor suppressor gene[32,33].

Nevertheless, there is still a small but significant portion of sporadic cases that develop through a different stream of molecular events, such as those arising from serrated precursor lesions[34], with CpG island methylator phenotype (CIMP) and activation of the *BRAF* oncogene as "signature" mutations[30,35]. This portion is considered as the microsatellite instability (MSI) phenotype, which originates from a deficiency in DNA mismatch repair (MMR), that causes microsatellites (small nucleotide repeats of 1-6 base pairs susceptible to mutation) to be inserted or deleted in coding regions[36]. The cases which do not display this phenotype are considered microsatellite stable (MSS), associated with CIN, while others

exhibit high-level (MSI-H) and low-level MSI (MSI-L)[36,37]. This MMR deficiency is due to the silencing of MLH1 through hypermethylation of its promoter[38].

Hereditary or familial forms of CRC are also of great value as a good model to study

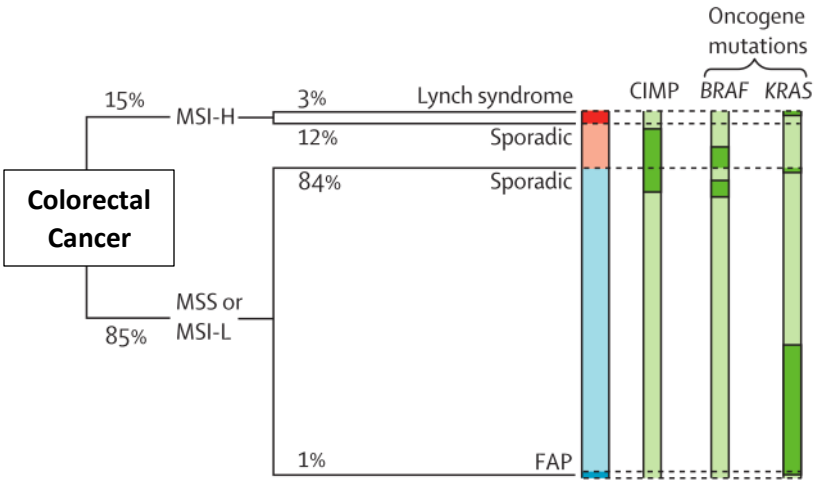

Figure 1 – Molecular subtyping of CRC. MSS or MSI-L are the most common phenotypes in CRC (85%, light blue and dark blue), characterized by chromosomal instability. Most of these cases develop through the classic adenoma–carcinoma sequence, but about 1% develop through FAP, an inherited form of CRC. MSI-H is present in about 15% of patients (red and pink) due to an impairment in DNA mismatch repair. About 3% of CRCs have MSI-H in a context of Lynch syndrome, another form of familial CRC, whereas 12% develop sporadically, being sessile serrated adenomas the typical precursor lesion. In green is represented the distribution of the most common molecular changes, such as CIMP and mutations of the BRAF or KRAS oncogenes. Dark green indicates the proportion of positive or mutant changes, while light green depicts the proportion of negative or wildtype changes. Adapted from *Brenner et al, 2014.*

the molecular pathogenesis associated to this type of cancer[39,40]. Two of these forms stand out as the most common: Lynch Syndrome, also known as hereditary non-polyposis colon cancer (HNPCC); and familial adenomatous polyposis coli (FAP)[40,41]. Lynch syndrome does not exhibit extensive polyposis[35] and is characterized by an inherited defect in DNA MMR, caused by an autosomal dominant germline mutation, with consequential loss of function of the allele, in one of the four main MMR genes – *MLHL1*, *MSH2*, *MSH6* or *PMS2*. This type of cancer arises following a somatic mutation in the remaining functional wild-type allele of the respective MMR gene. As a result of this MMR deficiency, this syndrome follows the molecular pathogenesis associated to MSI-H, a characteristic also manifested in this inherited type of cancer[41,42]. FAP, in turn, includes features such as the development of multiple colonic adenomas (>100 adenomas) and an early onset, being caused by an autosomal dominant germline mutation in the tumor suppressor gene *APC*. Its occurrence, as in the

previous case, results from a somatic event that leads to loss of function of the wild-type protein. This form of cancer, unlike Lynch syndrome, follows the other typical CRC molecular pathogenesis, the classic adenoma-carcinoma sequence, showing, as well, MSS and CIN[40,43].

All these molecular characteristics show good prognostic relevance, are valuable predictors of treatment efficacy. For example, CRC patients with MSI show significantly better prognosis when compared to patients with a functional MMR[44]. There is also evidence, although controversial, of microsatellite instability being linked to better response to irinotecan-based chemotherapy[45,46]. Among other relevant prognostic and predictive markers are the presence of tumor-infiltrating lymphocytes, which correlate with a better prognostic in MSI-H due to an improved anti-tumor response[47], and the mutational profile of each patient, as is the case of *KRAS* mutation turning the afflicted cells irresponsive to anti-EGFR immunotherapy[48].

1.2. Diagnosis and treatment of CRC

Diagnosis of CRC is made through histological analysis of biopsy samples taken during endoscopic procedures. Complete colonoscopy, sigmoidoscopy or CT colonography are also advisable in certain cases[49,50]. In addition, stool-based tests may also be used as noninvasive screening methods for CRC that, while being less specific and sensitive and still being optimized to be applied to a general public, are good cost-effective options to complement the standard means of diagnosis[50].

As for the treatment course, surgery is still the main curative treatment for patients with non-metastasized CRC. Staging prior to the procedure, as well as treatment selection and surgery quality reflect highly on the outcome for the patient[51]. In more advanced cases, such as T4 colon cancer or locally advanced cancer, neoadjuvant treatment can help reducing the tumor mass or even tumor stage, improving the chances of success of the procedure[52,53]. Typical neoadjuvant treatments encompass short-course radiotherapy prior to surgery or chemoradiotherapy with 5-fluorouracil (5-FU) or capecitabine (an oral fluoropyrimidine)[54,55]. On the other hand, adjuvant treatment is the accepted standard for stage III tumors, being used a combination of 5-FU with oxaliplatin[53]. Irinotecan, in turn, was seen as a promising option for metastatic CRC patients, but unfortunately it failed to

show improved results in the adjuvant setting[56,57]. Several targeted therapies are also being developed and employed in CRC. Angiogenesis inhibitors, such as bevacizumab[58], epidermal growth factor receptor inhibitors, such as cetuximab[59] and panitumumab[60], are examples of targeted therapies already being used in a clinical setting in CRC.

Being identified in the late 1990's, microRNAs (miRNAs) are short non-coding single strand RNA molecules with about 22 nucleotides in length, which act mainly as post-transcriptional regulators of gene expression and, thus, play important roles on several essential cellular processes, such as viability, differentiation or proliferation [61–63]. In combination with a ribonucleoprotein complex designated RNA-induced silencing complex (RISC), and through complementarity with given mRNA target, they can either repress mRNA translation or drive it for degradation, depending on the degree of complementarity[64,65].

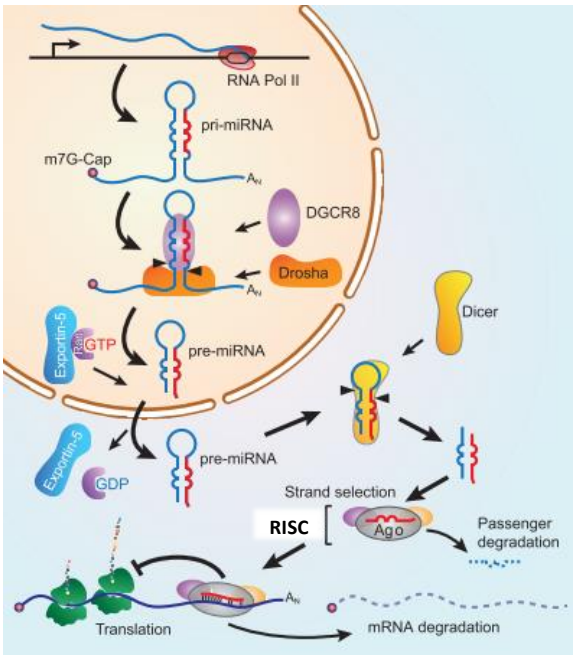

Figure 2 – miRNA biogenesis. This process begins with the transcription of the RNAs, subsequently capped and polyadenylated, designated primary miRNAs (pri-miRNA). A protein complex composed of nuclease Drosha and RNA-binding protein DGCR8 conducts the cleavage of the pri-miRNA, generating short-hairpin shaped structures, called pre-miRNAs. Following export from the nucleus, they are further processed by nuclease Dicer, creating a 21-23 nucleotide double-stranded miRNA, which is then loaded into the RNA-induced silencing complex (RISC). Through this loading process, the "guide" strand is selected, and the "passenger" strand is left for degradation. The guide strand pairs with a complementary sequence in the 3' UTR region of target mRNAs, resulting in inhibition of translation and subsequent degradation of the target mRNA. Adapted from *Strubberg et al, 2017.*

Different pathologies have been shown to exhibit aberrant miRNA expression profiles, including cancer. In these cases, they were shown to contribute to cancer by regulating either tumor suppressor genes (oncomiRs) or oncogenes (tumor suppressor miRNA)[62,63]. Curiously, a distinction between the miRNA expression levels in cancer samples and their normal tissue counterparts has been found for many miRNAs, making them prospective diagnostic biomarkers. In addition, these different expression profiles are characteristic of a given cancer type and stage, among other clinical variables, also showing potential as prognostic markers[62,66].

Beside biomarkers, and as they can have regulatory effects on several pathways related to tumor progression and even metastization, they could also prove to be novel therapeutic targets[67]. Indeed, several RNA-targeting therapeutics involving miRNAs are already being developed by different biopharmaceutical companies. While most are still in a preclinical stage[68,69], there is one currently in phase II clinical trials, for the treatment of hepatitis C virus infection, by targeting miR-122[70,71].

The MAP kinase pathway, for instance, crucial for cell proliferation and survival processes, has been associated with CRC, as *KRAS* and *BRAF* mutations are common events in this type of cancer[61]. miR-31, while being reported as a downstream target of the MAP kinase cascade, also functions as an activator of this pathway, as it has been shown to stimulate KRAS through negative regulation of one of its inhibitors, RASA1[72]. High expression levels of miRNA-31 have also been associated with *BRAF* mutations, showing another connection to this pathway[73].

The hyperactivation of the Wnt pathway, in turn, is seen as an hallmark of CRC[61], being its modulation a matter of extensive study. Linked to Wnt dysregulation are miRNAs, as they can repress several components of this pathway. As an example, miR-34 directly targets and represses various effector of this signaling pathway and is directly induced by p53, adding another layer to the tumor suppressive action of p53[74].

Regarding CRC, while routine colonoscopy still constitutes the standard and most successful preventive and diagnostic method, miRNA expression in blood could offer a good alternative for preliminary testing by being a cost-effective and less-invasive diagnostic approach[61]. There are already several studied miRNA expression “signatures” that could provide valuable diagnostic and prognostic information in CRC, such as miR-21[75], miR-

18a[76] or miR-92a[77], among many others. Thus, the analysis of a panel with these miRNAs would constitute a novel powerful noninvasive diagnostic method in plasma or blood samples. Nevertheless, since miRNAs are dysregulated in several cancer types and other pathologies[78,79], there is a pressing need to perform other non-plasma-based analysis, as these molecules can be non-specific to CRC[61,75]. It is thus necessary to find new and tissue-specific miRNAs to complement the analysis. Stool-based testing, which is already being widely adopted for CRC screening, has been optimized for miRNA analysis and can give a tissue-specific miRNA expression profile[80–82], overcoming this lack of specificity limitation.

2. Emerging CRC Therapies

Novel cancer therapeutics are trying to target fundamental cellular and physiological processes. An example of this is apoptosis, a type of regulated cell death, which is critical for normal tissue development and homeostasis. In fact, suppressed or defective apoptosis can lead to cancer and even resistance to conventional cancer treatment[83–85]. Activation of the immune system, on the other hand, is a novel trend emerging in cancer therapeutics[86].

Despite the development of new therapies falling largely in these two groups, there is a relatively recent line of investigation arising. Designated differentiation therapy, it stands out from the other paths by targeting the induction of differentiation of cancer cells, instead of inducing their death, contributing to a less malignant phenotype. While therapies targeting apoptosis can exhibit a certain degree of toxicity[87] and immunotherapy entails the risk of severe immune adverse effects[86], this approach is interesting, not only because it can sensitize the cancer cells to common chemotherapeutics, but also because differentiation treatments have inherently lower toxicity than conventional anticancer treatments[88].

With chemoresistance as one of the main problems nowadays, reducing therapeutic options and worsening a possible outcome, alternative treatments are necessary and of great focus to researchers. Cancer stem cells (CSCs), an undifferentiated subpopulation of cancer cells, are intrinsically connected with resistance to chemotherapy, due to their quiescence state and expression of drug membrane transporters[89,90]. As such, CSCs can be seen as desirable targets for the development of new anticancer therapies. In fact, being able to disrupt this population within a tumor mass could directly impact on recurrence and

metastization processes, while also improving the prognosis of patients which otherwise would not have any other options.

Most differentiation therapy strategies focus on targeting stemness. It has been reported that induction of terminal differentiation in small cell lung cancer cells, which exhibit high stemness and tumorigenicity, enabled cells to undergo autophagy and apoptosis[91]. Further, knockdown of CD44 in breast cancer cells successfully reduced the expression of genes related to stemness, metastization and tumorigenesis and sensitized the cells to conventional anticancer treatment[92].

3. Cancer Stem Cells

Stem cells are present in our body in various types of somatic tissues, playing important developmental roles. This population of cells is characterized by three fundamental properties: the ability to self-renew through asymmetric or even symmetric division (i.e., upon dividing, each cell can give rise to at least one daughter cell with the same biological properties of the parent one); the ability to differentiate into multiple lineages; and an indefinite proliferative potential[93,94].

It is hypothesized that tumors, like in many somatic tissues, possess a minor population of cells responsible for the replenishment of the tumor mass, giving rise to multiple clonal lineages and driving tumor progression. These are referred to as cancer stem cells (CSCs)[95]. The cancer stem cell hypothesis was first proven by studying acute myeloid leukemia (AML)[96], but it has been also proven in solid tumors, such as in breast[97], brain[98] and colon[99]. Despite of the designation “stem”, these cells can be originated either from a normal stem or progenitor cell that suffered a malignant transformation and obtained an aberrant phenotype, or from a differentiated mature cell that underwent sufficient mutations to acquire self-renewal capability and unlimited proliferative potential[100].

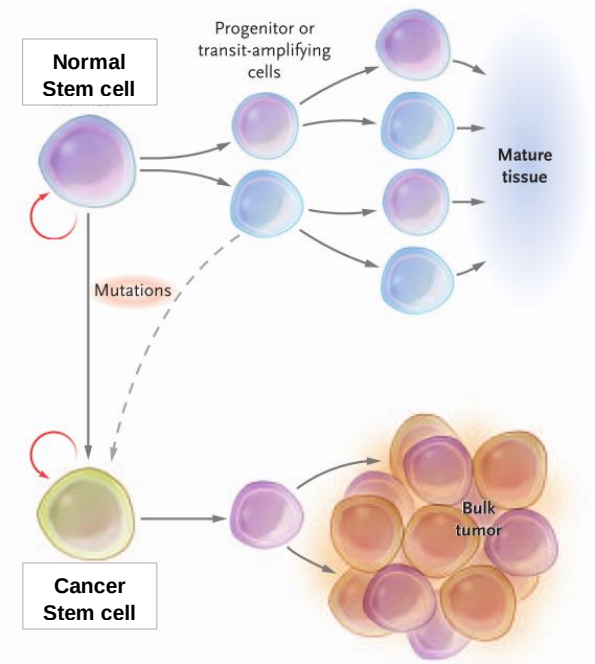

Figure 3 – Normal and cancer stem cells. Stem cells are the foundation from which normal tissues originate. CSCs arise from normal stem cells or progenitor cells that, upon mutation, grow uncontrollably and differentiate to generate the primary tumor. Much like its normal counterpart, CSCs are able to self-renew, generate different lineages and proliferate extensively. Adapted from *Jordan et al, 2006.*

The association between CSCs with therapeutic resistance, metastization and even recurrence could be linked to several factors. Much like their "healthy" counterpart, CSCs are not extensively proliferative in nature, acquiring a more quiescent behavior. Thus, they escape "unharmed" against conventional anti-proliferative chemotherapeutics and upon the end of the treatment they can divide and give rise to new tumor cells [89]. Other possible explanation for the therapeutic resistance of CSCs relies on the fact that normal stem cells and CSCs express different types of ATP-binding cassette (ABC) transporters involved in the protection against toxins and multi-drug resistance (MDR)[90].

3.1. CSC markers and pathways

Several molecular markers have already been specifically established for CSCs. They have been used to identify and isolate CSC populations in several cancer types, such as in CRC. Some of these stemness markers include surface protein markers, CD133[101], CD44[102], CD24[103], Lgr5[104], and also enzymes, such as the aldehyde dehydrogenase 1 (ALDH1)[105]. As some of them are not specific for CSCs, as CD133[106], combination with markers that show

tissue specific patterns of expression, as CD44 for CRC, enable a more accurate approach for CSC identification and isolation [102].

Many signaling pathways of normal stem cell self-renewal have already been identified to be mutated and dysregulated in cancer, being particularly associated with CSCs. In fact, pathways such as Wnt[107], Hedgehog[108], Notch[109] and phosphoinositide 3-kinase (PI3K)[110] are some of them. Interestingly, evidence of crosstalks between some of these signaling pathways has also been reported. This could be beneficial, as it has been reported that Notch inhibition also suppresses Wnt signaling[111], and Hedgehog inhibition may indirectly repress Notch signaling[112]. Nevertheless, this feature can also be a limitation, as compensatory mechanisms might overcome the specific inhibition of a pathway, providing a therapeutic escape route[109,113,114].

Epithelial-to-mesenchymal transition (EMT) is a cellular program intricately connected to CSCs. Through EMT, epithelial cells are converted in migratory cells with invasive capabilities. This process is normally necessary for tissue and organ generation during embryogenesis in both vertebrate and invertebrate animals[115]. Despite this, EMT has already been associated with pathological conditions, such as organ fibrosis[116] and cancer[117].

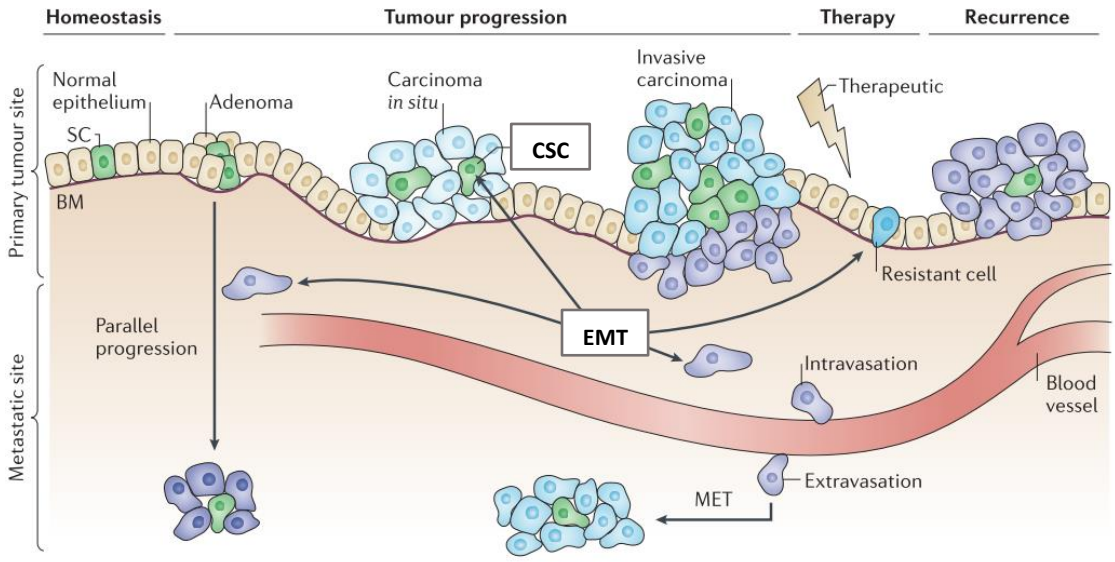

Figure 4 – EMT involvement in cancer progression. EMT can grant stem cell traits to dedifferentiated tumor cells or even drive resident stem cells into becoming CSCs. The most common association of EMT in cancer is the dissemination and subsequent migration of tumor cells from the solid tumor, leading to metastization. EMT characteristics can play a role in chemoresistance, leading to recurrence and worst prognosis. The degree of EMT activation along cancer progression may depend on the dysfunction of several regulatory networks associated with activated oncogenic pathways. Adapted from *Craene et al, 2013*.

Transcription factors associated to EMT have been reported to be involved not only in cancer migration and invasion, but also in suppression of senescence and apoptosis, cell cycle arrest and chemoresistance[115,118,119], all characteristics already associated with CSCs. In cancer, specifically in CRC, it has been reported that EMT occurs in the invasive front of the tumor, triggering events for metastization and tumor progression[116,120].

Another important piece in cellular homeostasis that is linked to CSCs is the proapoptotic and tumor suppressor protein p53[121]. Also designated as "guardian of the genome", p53 is necessary in maintaining genomic stability and regulates processes such as cell differentiation, self-renewal and plasticity, essential in stem cell sustainment[122–124] It is also involved in cell apoptosis and senescence, promotes DNA repair upon insults and can inhibit EMT[125,126]. In fact, mutations in p53 that lead to its inactivation are a fundamental step for cancer to overcome the dedifferentiation and CSCs generation barrier, as there is evidence that aggressive and poorly differentiated tumors exhibit an transcription signature similar to embryonic stem cells (ESCs), which included mutations of p53, while well differentiated tumors displayed an opposite signature[127–129].

The activity of p53 is regulated by modulation of its protein levels by the ubiquitin ligase mouse double-minute 2 (MDM2), which targets p53 for proteasome degradation through ubiquitination. Both proteins are maintained at basal levels through a negative regulatory loop, as MDM2 is itself a p53 target gene, being its expression induced upon p53 activation[130].

p53 is also known as a potent inhibitor of stemness. In fact, several reports showed that p53 is a negative regulator of Nanog, a homeodomain protein that induces pluripotency. p53 directly binds the *Nanog* promoter and negatively regulates its transcription upon DNA damage, in mouse ESCs[131]. p53 can also indirectly suppress *Nanog* expression through the inhibition of the Hedgehog pathway[132]. In induced pluripotent stem cell (iPSC) reprogramming, p53 was also shown to block this process by inhibiting the mesenchymal-to-epithelial transition (MET)[133], functioning as a barrier to the gain of stemness traits

Interestingly, p53 also seems to be activated by several oncogenes, such as β-catenin[134], which can be seen as a protective mechanism to a possible tumorigenic threat and initiate an inhibitory response either by inducing apoptosis or arresting cell proliferation, demonstrating its strong potential as a tumor suppressor[126].

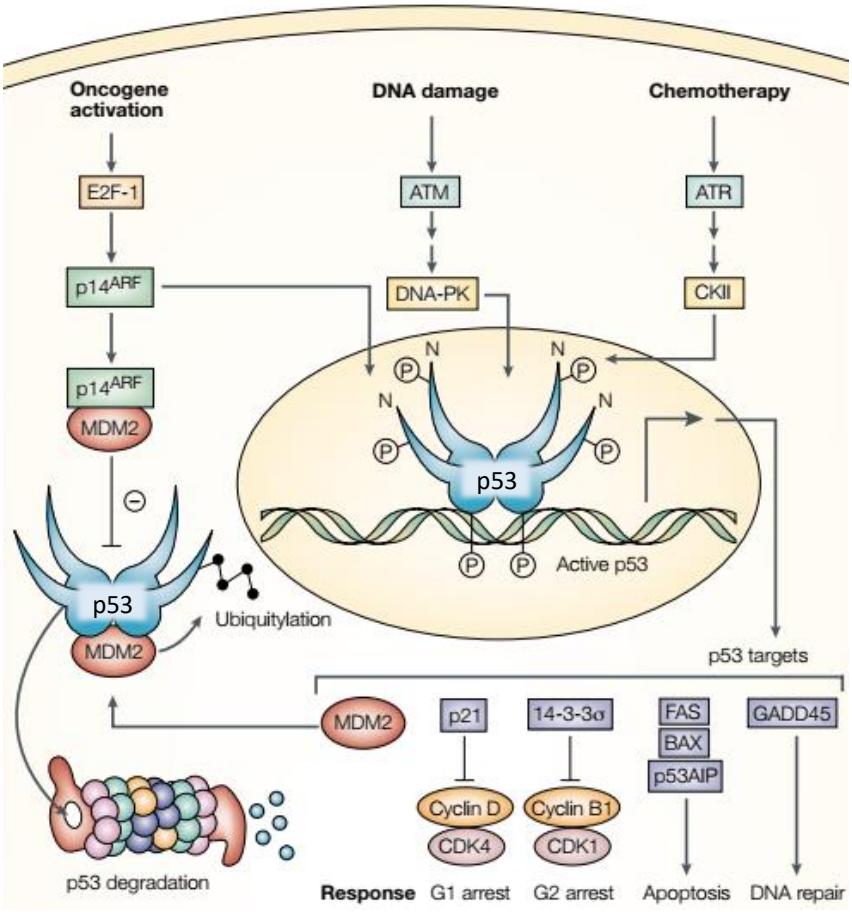

Figure 5 – The tumor suppressive function of p53. MDM2 controls p53 cellular levels through a negative feedback loop. Stimuli like DNA damage, oncogene activation or even the action of chemotherapeutics can prompt p53 phosphorylation and consequent activation or on the other hand promote MDM2 sequestration, impairing p53 suppression and degradation. These events lead to the activation of p53 target genes, initiating a series of cellular processes, such as cell cycle arrest, DNA repair, and even apoptosis, when repair is not possible or unsuccessful. Adapted from *Bullock et al, 2001*.

3.2. Non-toxic differentiation therapies

Regarding differentiation therapy, it has been shown that bone morphogenetic proteins (BMPs) are expressed in differentiated CRC tissue, but not in the CSC subpopulation, and that treatment of CSCs with exogenous BMP4 promotes the differentiation of tumor cells[135]. BMPs play important roles in the differentiation program of the normal gut, counterbalancing stem cell expansion and enabling epithelial cell

differentiation in the intestine[136,137]. BMPs were already shown to boost chemotherapeutic potency when used in combination therapy with oxaliplatin and 5-FU, showing a synergistic effect.

Other possible target in promoting differentiation of CSCs is the induction of endoplasmic reticulum (ER) stress by the unfolded protein response (UPR). *In vitro* activation of UPR resulted in differentiation of colon CSCs and sensitization of malignant cells to chemotherapeutic treatments, when combined with oxaliplatin alone[138].

Concerning CSC targeting therapies, there is a compound worth highlighting, compound which is salinomycin, a ionophore drug with antibiotic and anticancer properties[139,140]. It was initially shown to selectively target CSCs in breast cancer[141], but then salinomycin was also shown to sensitize several other types of cancer and even MDR cancer cells[142]. Although its mechanism of action is not fully understood, several targets and pathways have already been connected to salinomycin, depending on the type of cancer. It has been shown that salinomycin reduces ABC transporter activity in leukemia stem cells, inhibits Wnt pathway in CRC cells[143], induces reactive oxygen species (ROS) accumulation in prostate cancer cells, as well as growth and migration inhibition[144,145].

Importantly, salinomycin also diminishes a cisplatin-resistant mesenchymal-like subpopulation of cells in squamous cell carcinoma *in vitro*[146], dysregulates apoptotic and anti-apoptotic gene expression in SW620 CRC cell line[147], promotes autophagy response in prostate and breast cancer[148], and also induces apoptosis in hepatocellular carcinoma cells[149]. Regarding differentiation therapy, the use of salinomycin has shown promising results, with increased epithelial differentiation of cancer cells[141].

However, while having a wider range of action and increased efficacy over other common treatments, it has a particularly important drawback to clinical use, as it exhibits toxicity *in vivo*[150]. In fact, different toxic effects have been described, such as toxicity to the male reproductive system[151], neural damage[152], and even death, in some cases[153].

4. Aims

The work was undertaken with two different goals. The first goal was to optimize methodologies to identify miRNA biomarkers in CRC with improved diagnostic and prognostic value. The second goal was to evaluate the effectiveness of a set of chemical

compounds, from an in-house library, as potential differentiation inducers and chemotherapy sensitizers of CRC cells, while dissecting their mechanism of action.

Specifically, our aims were translated into work packages (WPs) with specific tasks, rationales, methodology and outcomes.

WP1: Optimize methodologies to evaluate the expression profiles of a set of miRNAs in CRC using tumor and normal colon tissue from patients, prior and after chemoradiotherapy.

WP2: Investigate the non-toxic differentiation potential of two small molecules in CRC, making use of an *in vitro* CSC-like model of a human colorectal carcinoma cell line, and by testing their effects on modulating the stemness and differentiation status of these cells.

WP3: Test the efficiency of these molecules in sensitizing tumor cell death in combination therapy treatments, by assessing a possible synergetic effect, when compared with a conventional chemotherapeutic alone.

This study may contribute to the improvement of our understanding of which methods can be used to evaluate miRNA expression as tools in CRC prognosis and diagnosis, while also suggesting a promising differentiation agent for the emerging non-toxic differentiation therapy.

II. Materials and Methods

1.1. Tissue RNA extraction

The total RNA was extracted from formaldehyde-fixed paraffin-embedded (FFPE) slices of human colon cancer tissue samples, which are part of a larger research project led by Dr. Susana Ourô (Hospital Beatriz Ângelo, Loures). Normal tissue and tumor tissue prior to chemoradiotherapy were obtained through biopsy; after treatment, tissue was obtained through surgical resection. Sample collection followed institutional ethical approval procedures.

For deparaffinization, each tissue slice was washed twice with 100% xylene for 3 min at 50ºC, suspended in 100% ethanol for hydration steps and homogenized using a motorized tissue homogenizer. The samples were then centrifuged at the maximum speed for 5 min and the pellet recovered. A final wash was performed with 95% ethanol for 10 min, followed by a centrifugation at maximum speed for 5 min. The pellet was air dried at 37ºC for 15 min. For protein digestion, 500 µg/mL proteinase K (Thermo Fischer Scientific, Waltham, Massachusetts, USA) in protease digestion buffer (20 mM Tris-HCl (pH 8.0); 1 mM CaCl2; 0.5% SDS) was added to each sample for 1-3 h at 55º C.

For the RNA extraction procedure, RiboZol™ (VWR, Radnor, Pennsylvania, USA) was added to each sample for at least 5 min at room temperature (RT). Chloroform was then added to each tube, which were vigorously mixed for 15 sec, incubated for 2-3 min at RT and centrifuged at 12 000 g for 15 min at 4ºC. Afterwards, the supernatant was transferred to a new tube, and isopropanol was added. The tubes were mixed and incubated for 10 min at RT. After centrifugation at 12 000 g for 10 min at 4ºC, the supernatants were discarded and the pellets were washed with 75% ethanol and centrifuged at 7 500 g for 5 min at 4ºC. Finally, the supernatants were discarded while the dried pellets were resuspended in RNase-free water.

1.2. TaqMan® Advanced miRNA Assays

The TaqMan® Advanced miRNA assays (Thermo Fischer Scientific) allow the measurement and amplification of multiple miRNAs from a single sample. This is particularly useful for the analysis of precious samples, such as human serum, plasma and

other biological fluids. The TaqMan® Advanced miRNA assay was performed according to the manufacturer's instructions, involving several reactions, starting with the poly(A) tailing reaction. Firstly, to a sample volume, a master mix composed of 10x Poli(A) buffer, ATP, poly(A) enzyme and RNase-free water is added, proceeding to vortex and spin down. The reaction tubes were placed in the thermal cycler and incubated using the following settings: polyadenylation at 27ºC for 45 min; stopping the reaction at 65ºC for 10 min; holding the samples at 4ºC.

The second reaction was the adaptor ligation reaction. Another master mix constituted by 5x DNA ligase buffer, 50% PEG 8000, 25x ligation adapter, RNA ligase and RNase-free water was added to each tube containing the product of the previous reactions. After the vortex and spin down, the tubes were placed in the thermal cycler and incubated with the following settings: ligation at 16ºC for 60 min; holding the samples at 4ºC.

The next reaction was the reverse transcription (RT). The next reaction mix contained 5x RT buffer, dNTP mix, 20x universal RT primer, 10x RT enzyme mix and RNase-free water and, upon adding to the product of the previous reaction it was further vortexed and spin down. The incubation in the thermal cycler was done using the following settings: reverse transcription at 42ºC for 15 min; stopping the reaction at 85ºC for 5 min; holding the samples at 4ºC.

The last step before the real-time PCR was the miR-Amp reaction. The mix for this reaction was composed by 2x miR-Amp mix, 20x miR-Amp primer mix and RNase-free water and added to new tubes with the product of the previous reaction, being vortexed and spin down. The incubation in the thermal cycler for this reaction was as follows: 1 cycle of enzyme activation at 95ºC for 5 min; 14 cycles consisting of 3 sec of denaturation at 95ºC and 30 sec of annealing/extending at 60ºC; stopping the reaction at 99ºC for 10 min; holding the samples at 4ºC. Each of the previous reaction step was performed on a UnoCycler VWR 732-1200 thermal cycler (VWR).

To prepare for the real-time PCR, a 1:10 dilution of cDNA template was done, by diluting a volume of the product of the miR-Amp reaction in TE buffer. The reaction mix for each miRNA was prepared with TaqMan® Fast Advanced master mix (2x), TaqMan® Advanced miRNA assay (20x) and RNase-free water, this time being added to a volume of the cDNA dilution previously prepared, sealing the reaction plate, which was then vortexed

and spin down. The plate was then loaded in the QuantStudio 7 Flex Real-Time PCR System (Thermo Fischer Scientific) and the real-time PCR run on the following settings: 1 cycle of enzyme activation at 95ºC for 20 sec; 40 cycles consisting of 1 sec of denaturation at 95ºC and 20 sec of annealing/extending at 60ºC.

2.1. Cell culture

HCT116 and SW620 human colorectal carcinoma cell lines were obtained from ECACC, (Porton Down, Wiltshire, UK). HCT116 p53 wild-type ($p53^{+/+}$) and p53 null ($p53^{-/-}$) isogenic cell lines were provided by the Genetic Resources Core Facility (GRCF) Biorepository and Cell Center (Johns Hopkins University, School of Medicine, Baltimore, MD, USA). HCT 116 (p53-wt and -null) were cultured in McCoy's 5A modified medium, as described previously[154], and SW620 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), both supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (all from Gibco, Life Technologies, Thermo Fisher Scientific). The cell lines were maintained at 37ºC in a humidified atmosphere of 5% CO_2. For RT-qPCR and Western blot analysis, as well as for flow cytometry, cells were plated at 1.5 x 105 cells/mL, in 6-well plates, while for chemotherapy resistance assays, cells were plated at 0.5 x 105 cells/mL, in 96-well plate.

2.2. Cell treatment

Cells were treated with either 1.25 µM Oxaliplatin, 5 µM Salinomycin (Sigma-Aldrich, Merck), 2.5 µM DS6 or 0.5 µM SAS9, after 24h of plating, for different periods of times. Oxaliplatin, a conventional chemotherapeutic agent, was kindly provided by Hospital São Francisco Xavier (Lisboa, Portugal), while DS6 and SAS9 were kindly provided by Dr. Maria Santos and Professor Alexandra Paulo, both members of the Medicinal Chemistry group at iMed.ULisboa. All stock solutions were prepared in dimethyl sulfoxide (DMSO). The concentrations chosen for DS6 and SAS9 were based on preliminary data from our group in CRC cell lines and further optimization in order to avoid extensive cell death. Cells were exposed to the compounds for 24 h (flow cytometry and RT-qPCR) or 48 h (Western blot). For chemotherapy resistance related assays, we have also used 10 µM Fluorouracil (5-FU), a standard chemotherapeutic used in the treatment of CRC, provided by Hospital de Santa

Maria (Lisboa, Portugal). In this case, cells were treated with either 5-FU, Salinomycin, DS6, SAS9, 5-FU plus Salinomycin, 5-FU plus DS6 or 5-FU plus SAS9 for 48 h. 0.1% DMSO was used as control.

2.3. Sphere formation assays

Tumor sphere-forming assays were performed, as described[155]. To assess the effect of the compounds on sphere-forming ability, or number, the cells were plated at 1.5 x 105 cells/mL, in 6-well plates, for 48 h and exposed to each compound for additional 24 h. Then, cells were collected and replated without further treatments, at 500 cells/mL, in ultra-low attachment 24-well plates (Corning, Corning, New York, USA) using DMEM/F12 stem cell medium (Gibco, Life Technologies, Thermo Fisher Scientific), supplemented with nonessential amino acids, sodium pyruvate, penicillin-streptomycin, N2 supplement, B27 supplement, 4 μg/mL heparin, 40 ng/mL human epidermal growth factor (EGF) and 20 ng/mL basic fibroblast growth factor (bFGF)), and incubated in a humidified atmosphere with 5% CO2 at 37ºC, for 7 days. After the 7 days, the number of spheres in each well was counted using a PrimoVert microscope with an Axiocam 105 color (Carl Zeiss Microscopy, Oberkochen, Germany) and dissociated to assess the total number of cells per well.

To assess the effect of the compounds on sphere size (number of cells per sphere), single cells were seeded at the same conditions described above for 7 days. Upon that period, the spheres were treated with each compound for 72 h. The number of spheres in each well was counted and then dissociated to count the total number of cells per well.

2.4. Flow cytometry

The activity of ALDH1 and the expression of cell-surface CD133, both stemness markers for colon CSCs, was evaluated by flow cytometry, as previously described[156–158]. For ALDH1 activity, we performed the ALDEFLUOR™ assay (Stemcell Technologies, Vancouver, British Columbia, Canada), according to the manufacturer instructions. It consisted in dissociating the plated cells, after 24h of treatment, with Accutase® (Gibco, Life Technologies, Thermo Fisher Scientific), washing with Phosphate-buffered saline (PBS) and resuspending in Assay buffer. A negative control tube for the fluorescence reaction was also prepared. For that, an inhibitor of ALDH activity (DEAB) was added to a new tube. Aldefluor

substrate, which through the activity of the ALDH enzyme resulted in the emission of fluorescence, was then added to the test cell tube, and, after a quick mix, a small amount of this cell suspension was transferred to the negative control tube. Both tubes were left to incubate for 40 min at 37ºC, centrifuged at 500 g for 5 min and finally resuspended in ice-cold Assay buffer. For CD133 staining, the plated cells were also dissociated with Accutase® for 5 min, 24 h after treatment, and washed with PBS. Cells were then resuspended in of PBS with bovine serum albumin (BSA) and EDTA and CD133/1 (AC133)-VioBright FITC antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) and left to incubate for 10 min at 4ºC, in the dark. After a final washed with PBS, cells were finally resuspended in PBS with BSA and EDTA. Sample acquisition and analysis for both procedures were performed in a Guava easyCyte 5HT flow cytometer (Merck Millipore, Merck).

2.5. Total protein isolation and quantification

Total protein isolation was performed as previously described[159,160]. The cells were collected with TrypLE and lysed using an ice-cold lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM MgCl2, 1.5 mM potassium acetate, 1% Nonidet P-40, 2 mM DTT, and protease inhibitors) for 30 min in ice. Samples were then sonicated for 30 sec in ultrasounds, the lysate was centrifuged at 10 000 g at 4ºC for 10 min, and the supernatant recovered. Protein content was also measured by the Bio-Rad protein assay kit, according to the manufacturer's specifications, using BSA as standard[161]. Absorbance was measured in a GloMax-Multi+ Detection System (Promega), at 595 nm, being the protein concentrations determined by using a linear regression standard curve. The samples were frozen at -80ºC until further use.

2.6. Western Blotting

The steady-state levels of pluripotency-related proteins Sox2, Oct4, Nanog and C-Myc, as well as the levels of differentiation-related proteins BMP-4 and CK20 were evaluated by Western Blot, as described[162].

Briefly, 40 µg of total protein extracts were separated in a discontinuous gel system of a 4% stacking gel and a 10% sodium dodecyl sulphate-polyacrylamide electrophoresis gel (SDS-PAGE), comprised by 30% (w/v) protogel, mili-Q water, upper/lower gel buffers, ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). The run was

performed using a Bio-Rad Mini Protean II Electrophoresis System (Bio-Rad) and covered in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS), running at 80-140 V until the bromophenol blue reached the end of the gel. The PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific) was used as a molecular weight marker.

Gel transfer to blot was performed by using a transfer buffer (250 ml 4X transfer buffer, 200 ml of methanol and H2O to 1 L) upon hydration of sponges, filter paper, and 8.5 x 6.5 cm hybond-C nitrocellulose membrane. The transference occurred in cold room packed ice at a constant amperage of 0.3 A for 90 min. To proceed to immunolabeling of the blots the membrane was previously incubated with 5% nonfat dry milk in TBS (blocking solution) at least 30 min at RT to avoid any non-specific binding of the antibodies. Then, it followed an overnight incubation at 4ºC with the primary monoclonal antibodies anti-BMP4 (sc-393329, 1:500); anti-C-myc (sc-40, 1:500); anti-Nanog (sc-29321, 1:750) (Santa Cruz Biotechnology, Dallas, Texas, USA) or rabbit polyclonal antibodies anti-CK20 (sc-25725, 1:200); anti-Oct4 (#2750, 1:1000) (Cell Signaling Technology); anti-Sox2 (AB5603, 1:500) (Merck Millipore, Merck). Incubations were in blocking solution and 0.5% sodium azide or 5% BSA in TBS. After washing, blots were subsequently incubated with secondary antibodies conjugated with anti-mouse or anti-rabbit IgG, and with horseradish peroxidase (HRP) for 2 h at RT. Finally, membranes were processed for protein detection using Immobilon™ Western Chemiluminescent HRP Substrate (Merck Millipore, Merck) or Super Signal™ West Femto substrate (Thermo Fisher Scientific, Inc.) in a ChemiDoc™ MP System (Bio-Rad Laboratories) with approximately 1 min of exposure. Ponceau S (P7170; Sigma-Aldrich Corp.), a sodium salt of a diazo dye of a light red color, was used to reversible stain total protein bands as well as loading control.

2.7. Cell RNA extraction

The RNA isolation was performed using the RiboZol™ (VWR) manufacturer's protocol, as described previously[163]. The RNA was resuspended in RNase-free water and quantified through the Qubit fluorometer using the Qubit RNA assay kits (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. The samples were frozen at -80ºC, until further use.

2.8. RT-qPCR

Following the RNA isolation and quantification, quantitative RT-PCR (RT-qPCR) reaction were performed to assess the expression levels of pluripotency and differentiation genes, as described[163], using the primer sequences as shown in Table 1.

First, the samples were treated with DNase I recombinant (Roche Applied Science, Penzberg, Germany) to eliminate DNA in all samples and therefore over-estimations in gene expression analysis. Then, a reverse transcription reaction was performed to convert the extracted RNA into cDNA using random hexamers and dNTP mix (NZYTech, Lisboa, Portugal). A thermal cycler step at 65ºC for 5 min, was performed to avoid the potential primer dimers of random hexamers. After this, a 1:1 dilution of reverse transcriptase with 1x reaction buffer and 10x reaction buffer (NZYTech). The samples were again submitted to further thermal cycler steps, for a cycle of 10 min at 25ºC, 50 min at 50ºC and 5 min at 85ºC.

The qPCR was then performed, adding a mix of SensiFAST™ SYBR Hi-ROX (Bioline Reagents, Meridian Life Science, Tennessee, USA), the forward and reverse primers and nuclease-free water to the cDNA. The thermal cycler program is as follows: 1 cycle of 2 min at 50ºC and 10 min at 95ºC; 40 cycles of 15 s at 95ºC and 1 min at 60ºC (; 1 cycle of 15 s at 95ºC, 1 min at 60ºC and 15 s at 95ºC. The DNase and reverse transcription reactions were performed on a UnoCycler VWR 732-1200 thermal cycler (VWR), while the qPCR was done on a QuantStudio 7 Flex Real-Time PCR System (ThermoFischer Scientific).

Table 1 - Primer sequences used for RT-qPCR analysis in HCT116 cell line

Nanog	Forward: 5'- TCTGGACACTGGCTGAATCCT -3' Reverse: 5'- CGCTGATTAGGCTCCAACCAT -3'
Oct4	Forward: 5'- TCGAGAACCGAGTGAGAGG -3' Reverse: 5'- GAACCACACTCGGACCACA -3'
Sox2	Forward: 5'- GCTAGTCTCCAAGCGACGAA -3' Reverse: 5'- GCAAGAAGCCTCTCCTTGAA -3'
β-actin	Forward: 5'- CTGGAACGGTGAAGGTGACA -3' Reverse: 5'- AAGGGACTTCCTGTAACAACGCA -3'
GAPDH	Forward: 5'- CGCTCTCTGCTCCTCCTGTT -3' Reverse: 5'- CCATGGTGTCTGAGCGATGT -3'
Krt20	Forward: 5'- GAACCTAAATGACCGTCTAGCG -3' Reverse: 5'- GGTTTCGTACCACTGCTTGATT -3'
Ada	Forward: 5'- GCCTTCGACAAGCCCAAAGTA -3' Reverse: 5'- CTCTGCTGTGTTAGCTGGGAG -3'
Aqp3	Forward: 5'- CCGTGACCTTTGCCATGTG -3' Reverse: 5'- CGAAGTGCCAGATTGCATCATAA -3'

2.9. Cell pair assay

To evaluate the effect of the compounds in inducing differentiation in CRC cells, we analyzed, through immunocytochemistry, the pattern of symmetric and/or asymmetric cell division in low-density conditions to allow the identification of mitotic pairs.

Briefly, cells were plated at 0.075 x 10^5 cells/mL, in 35 mm culture dishes (BD Falcon, Franklin Lakes, New Jersey, USA) and treated at the same time with the compounds previously described. After 24 h, the medium was removed, the cells washed with PBS and fixed with 4% paraformaldehyde (PFA) for 30 min. The cells were then washed 3x with PBS and incubated with the blocking solution (10% Donkey serum, 1% FBS, 0.1% Triton X-100, in PBS) for at least 30 min.

After removal of the blocking solution, cells were incubated with a primary antibody solution (rabbit polyclonal anti-ALDH1/2 sc-50385 (Santa Cruz Biotechnology), diluted 1:100 in Blocking solution), overnight, at 4ºC. Cells were washed with PBS and incubated for 2 h, at RT in the dark, with the secondary antibody (goat anti-Rabbit IgG (H+L) secondary antibody Alexa Fluor 594 #R37117 (ThermoFischer Scientific), diluted 1:100 in blocking solution).

After three washes with PBS, cells were labelled with Hoechst (Sigma-Aldrich, Merck) for 5 min and again washed with PBS. Samples were mounted using Mowiol® 4-88 anti-fading mounting medium (Sigma-Aldrich, Merck) and 20 cell pairs were counted and evaluated in each condition. The cellular staining for ALDH1 was observed in an Axio Scope.A1 microscope coupled with an AxioCam 105 camera (Carl Zeiss Microscopy).

2.10. Viability assays

Cell viability was measured by two different approaches, through the use of ToxiLight Non-destructive Cytotoxicity BioAssay (Lonza, Basel, Switzerland) and the PrestoBlue Cell Viability assay (ThermoFischer Scientific), according with manufacturer's instructions. The signals were measured using the GloMax-Multi+ Detection System (Promega). In addition, we have evaluated the coefficient of drug interaction (CDI), as described[164]. Through this coefficient, it is possible to assess the synergistic effect of a certain drug combination. CDI is calculated as follows: $\mathrm{CDI} = \frac{\text{viability rate (AB)}}{\text{viability rate (A)} \times \text{viability rate (B)}}$, being

AB the combination treatment, and A or B the single agent treatments. CDI values < 1, = 1 or > 1 indicate that the drug effects were, respectively, synergistic, additive or antagonistic.

2.11. Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the Image Lab™ version 5.0 densitometric analysis program (Bio-Rad Laboratories). The results from different groups were compared using an unpaired Student's t test. Values of $p < 0.05$ were considered statistically significant. All statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software, La Jola, California, USA).

III. Results and Discussion

1. Identification of miRNAs as biomarkers in CRC

1.1. Differential miRNA expression profiles in human CRC samples in response to chemoradiotherapy

With the development of screening and diagnostic methods as effective options to prevent cancer mortality, miRNAs emerged as promising candidates for diagnostic and prognostic biomarkers[62]. In CRC, several miRNAs have already been found differentially expressed, not only showing potential diagnostic value, but also emerging as promising therapeutic targets[165].

Initially, we selected a set of miRNAs, miR-16-5p, -145-5p, -335-5p, -135b-5p, -34-5p and -561-3p, to investigate their expression levels, through the TaqMan® Advanced miRNA assay, in human colon cancer FFPE tissue samples. This selection was based on previous studies indicating their potential as CRC biomarkers and indicators of chemoradiotherapy sensitivity[166–169]. However, miR-34-5p and -561-3p were excluded, for not amplifying in any sample tested (data not showed). Since the use of TaqMan® Advanced miRNA assay is not compatible with the use of the endogenous control RNU6B, a small-nucleolar RNA commonly used for miRNA normalization, we also had to select a set of 3 miRNAs as possible endogenous controls. Upon preliminary optimization, only miR-484

showed expression levels most consistent with RNU6B (data not shown). Therefore, miR-484 was the only endogenous control used in the following experiments.

Normal and tumor colonic tissue were collected from 6 CRC patients, by biopsy and surgical resection, prior to and after chemoradiotherapy treatment, respectively. Normal tissue prior to treatment was used as the control condition (Figure 6). Importantly, to better understand whether some of these miRNAs could be predictive biomarkers for response to therapy, the collected samples were also divided in two main groups, samples derived from 3 patients that did not achieve a complete therapeutic response to chemoradiotherapy (nCR) (Figure 6 – A.1; B.1; C.1; D.1) and 3 patients that achieved complete response (CR) (Figure 6 – A.2; B.2; C.2; D.2), assessed by the absence or presence of tumor tissue after chemoradiotherapy, respectively.

MiR-16-5p was already shown to be downregulated in CRC[170]. In fact, this miRNA has been associated with a tumor suppressor role, by repressing the oncogene KRAS[171] and regulating the p53 signaling pathway[172]. Our results also pointed to this idea, but only a trend in miR-16-5p reduction was observed (Figure 6 – A.1; A.2) in pre-treatment tumor tissue from both CR and nCR. In fact, chemoradiotherapy increased miR-16-5p in normal tissues but further diminished this miRNA in tumor tissue in nCR patients (Figure 6 – A.1). In patients that had a complete response to treatment, miR-16-5p levels decreased in normal tissue (Figure 6 – A.2). This miRNA has already been evaluated as a prognostic indicator in CRC and, despite being downregulated in this type of cancer, a stratification of patients into low and high expressing miR-16-5p levels has resulted in a correlation with higher expression levels and a better outcome[170]. It was not possible to establish the same conclusion with our data, being necessary to further increase the sample size for such stratification analysis. Nonetheless, we might think that the increase upon treatment in nCR patients (Figure 6 – A.1) may rely, in part, on a compensatory mechanism of the normal cells to inhibit tumor growth and induce apoptosis, as it has been described[172]. In addition, we can also speculate that the basal levels of miR-16-5p expression in normal tissue prior to treatment, in the CR compared with nCR group (Figure 6 – A.2), could be indicative of colon tumor outcome after treatment.

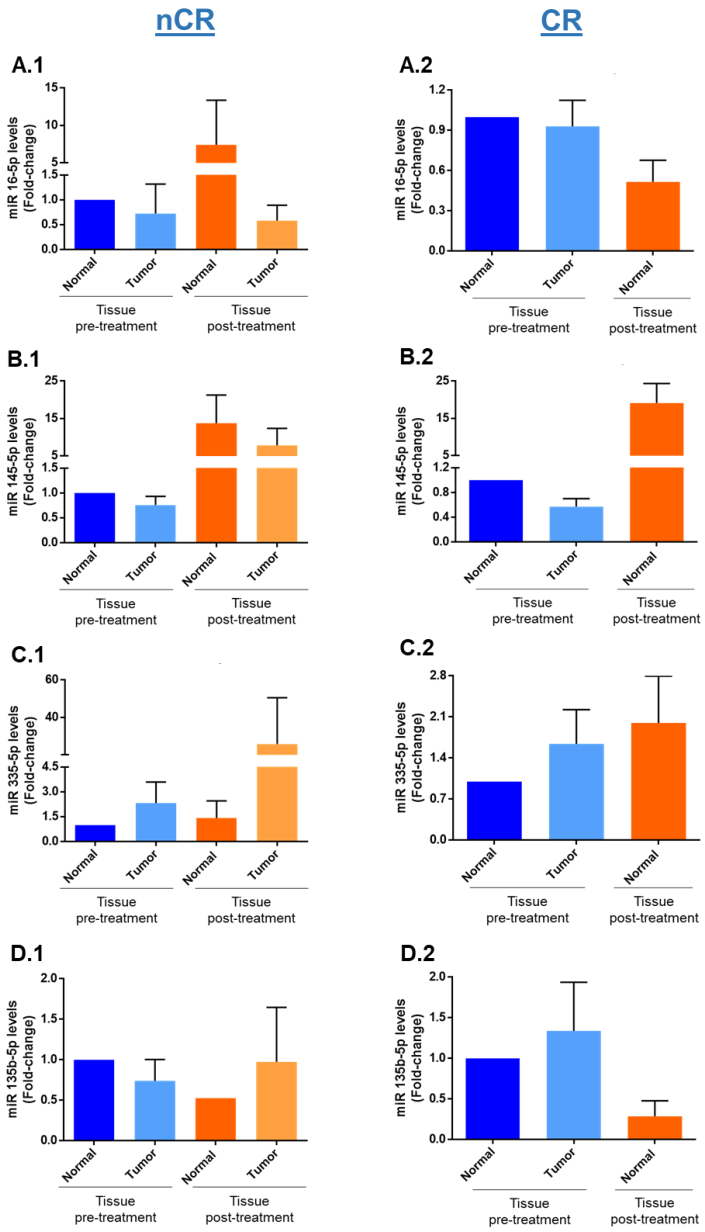

Figure 6 – Modulation of miRNA expression levels with chemoradiotherapy in CRC. Human FFPE tissue samples from CRC tumor tissue (when available) and normal colonic tissue, before and after chemoradiotherapy treatment, was used to extract total RNA. miRNA expression levels for miR-16-5p **(A.1; A.2)**, miR-145-5p **(B.1; B.2)**, miR-335-5p **(C.1; C.2)** and miR-135b-5p **(D.1; D.2)**, were determined using TaqMan® Advanced miRNA assay. Patient samples were divided in two groups: a group where patients did not achieve a complete response to treatment - nCR **(A.1; B.1; C.1; D.1)** and a group where patients had a complete response - CR **(A.2; B.2; C.2; D.2)**, which is evidenced by the absence of tumor tissue post-treatment. Results are expressed as mean ± SEM fold-change to normal pre-treatment tissue control for at least 3 different human samples.

Regarding miR-145-5p, it is also considered a tumor suppressor miRNA, since it has been shown to be downregulated in various types of cancer, namely in CRC[173,174]. In fact, miR-145-5p was shown to target oncogenes such as c-Myc[175] or IRS-1[176], being also

associated with colon CSC regulation[177]. Our results were in accordance with this idea, since a reduction in miR-145-5p expression levels was always observed prior treatment in tumor tissues from both groups of patients (Figure 6 – B.1; B.2). No differences were detected for miR-145-5p expression levels in normal tissues, for both groups (Figure 6 – B.1; B.2), being both upregulated with chemoradiotherapy treatment. Indeed, this trend in miR-145-5p increased levels after chemoradiotherapy has already been reported in CRC[168], also showing a slight association between better outcomes and higher post-treatment expression levels. Thus, this miRNA might have a potential as a prognosis and response predictor marker of CRC.

Concerning miR-335-5p expression levels, there is contradictory data in the literature. It has been demonstrated that miR-335-5p is involved in cell invasion and cell viability in astrocytoma, specifically by targeting Damm1, a cytoskeletal regulator, and acting downstream of the Wnt pathway[178]. In fact, in lung cancer, downregulation of miR-335-5p has been associated with reduced cell growth and migratory properties[179]. On the other hand, downregulation of this miRNA has also been reported in breast cancer[180] and osteosarcoma[181]. In rectal cancer, it has been associated with better survival[182]. This data may suggest a cell specific and/or context-dependent function for this specific miRNA.

Our results showed a tendency for increased miR-335-5p levels in tumor tissues from both groups of patients prior to treatment (Figure 6 – C.1; C.2). Interestingly, after chemoradiotherapy, patients without complete response did not show an increase in miR-335-5p levels in normal tissue, while patients with a complete response did (Figure 6 – C.1) Thus, experiments with an increased number of human samples will help clarify if miR-335-5p could indeed be an effective prognostic marker or can be associated with a favorable CRC prognosis, as previously suggested[182]. Our results appear to indicate that miR-335-5p acts as a tumor suppressor molecule.

Finally, miR-135b-5p has been closely associated with several cancer types, including CRC[183,184]. However, it has also been shown to be downregulated in lung cancer[185], as well as in breast and prostate cancer in a hormonal receptor-dependent manner[186], indicating a cell specific role. In CRC, miR-135b-5p has been described as a regulator of tumor suppressor genes such as TGFβR2[187], LATS2[188] and APC[189], and higher expression of this miRNA has been associated with worse outcome[184].

In the nCR patient group, the expression levels of miR-135b-5p were slightly decreased in tumor tissue before treatment, while in CR patients the tendency of its expression was to increase (Figure 6 – D.1; D.2). As a matter of fact, this miR-135b-5p pattern of expression was in disagreement with those previously described for CRC[184]. This could be, in part, explained by some technical issue or by having a small number of samples, not entirely representative of CRC patients. After chemoradiotherapy treatment, a tendency for miR-135b-5p reduction in normal tissue of both patient groups was observed, being particularly lower in the CR group (Figure 6 – D.1; D.2), which can be indicative of complete response to chemoradiotherapy.

In the tumor tissue of the nCR patient group, chemoradiotherapy seemed to increase miR-135b-5p expression to levels similar or slightly increased when compared to normal control tissue. This might rely on the chemoresistant phenotype triggered by treatment, as described for miR-135b-5p upregulation[188].

Taken together, several limitations should be considered during this experimental procedure. First, the TaqMan® Advanced miRNA assay was a possible technical limitation; this advanced assay does not allow the amplification of other small non-coding RNAs, such as U6 or RNU6B, which usually function as good endogenous controls. This required that miRNAs with unaltered expression needed to be found to normalize RNA quantity among samples.

In addition, the inclusion of more miRNAs with potential diagnostic and prognostic value would also enrich this study. However, the low number of samples included in this optimization study appeared to be a major limitation. In this respect, a bigger sample size would certainly allow us to increase the statistical power of the data, stratify patients and uncover any distinct expression patterns, such as gender dependency, as described for other types of cancer[190].

Nevertheless, this study is an important first step in optimization procedure to develop a panel of new biomarkers in CRC that could allow, in the future, improved diagnosis, as well as a better evaluation of treatment response and outcome.

2. Non-toxic differentiation potential of two small molecules in CRC therapy

2.1. Reduction of functional stemness markers in CRC by chemical compounds

Chemoresistance is a preponderant factor on the outcome and survival of cancer patients[191]. CSCs, as their normal counterpart, are able to resist conventional treatments dependent or not on their differentiation status[90,192]. Thus, as CSCs present a more undifferentiated phenotype, differentiation therapy could represent a potential strategy to sensitize this tumor cell subpopulation[193].

To discover a solution for this reality in CRC, we decided to study the potential of two small molecules, as possible therapeutics for non-toxic differentiation in CRC cells. Indeed, both molecules were already proved to be pro-differentiation and /or anti-tumoral agents in previous experiments in our laboratory. The compound DS6 is a spiropyrazoline oxindole molecule[194]. Some different compounds of this family have already been reported to exhibit high antitumoral activities in CRC and beside having low cytotoxicity towards non-malignant cells they also induce apoptosis by disrupting p53-MDM2 interaction[195]. The other molecule, SAS9 is a 3-piperidinyl-indole molecule, a compound studied for antimalarial therapy[196] and, in studies performed by our group, a possible necroptosis inducer for anticancer treatment[197]. Both molecules were kindly provided by the Medicinal Chemistry group at iMed.ULisboa.

We started this part of the study by testing these compounds in two distinct CRC cell lines: in HCT116, a colorectal carcinoma cell line from a primary tumor, known to be highly aggressive. This cell line has reduced or absent differentiation capability and is presumed to be highly enriched in CSCs[198]; and in SW620, also a colorectal carcinoma cell line derived from a lymph node metastasis. Based on the assumption that metastization is attributed to CSCs[199], we assumed that as this cell line derives from a metastasis, it could be enriched in CSCs. Since other spiropyrazoline oxindoles develop their antitumoral activity by impairing the p53-MDM2 interaction[195] and the mechanism of SAS9 was still unclear, decided to test their effect in these particular cell lines, knowing that HCT116 cells contain a wild-type p53, while SW620 cells a mutated p53[200].

For that, we first evaluated by flow cytometry the effect of these compounds in reducing the expression of functional stemness markers, the cell-surface marker CD113 and the enzyme ALDH1 activity in both cell lines (Figure 7).

Interestingly, DS6 was shown to significantly reduce both stemness markers ($p < 0.01$ for CD133 staining; $p < 0.005$ for ALDH1 activity) in HCT116 cell line, while having no effect in SW620 cells (Figure 7). Also, although in a lower extent, SAS9 showed also a tendency to reduce the expression of both stemness markers in HCT116 cells, but not in SW620 cells (Figure 7). Thus, these results demonstrate that, at least, DS6 might act by a p53-dependent manner.

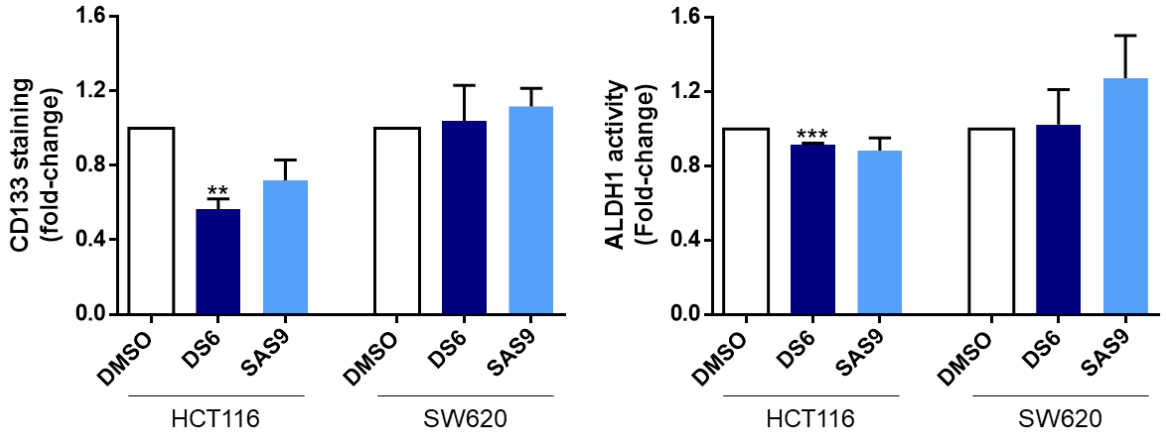

Figure 7 – DS6 was able to reduce functional stemness markers in a CRC cell line. HCT116 and SW620 cells were exposed to DS6 or SAS9, as described in Materials and Methods. DMSO was used as vehicle control. After 24 h of treatment, cells were collected for flow cytometry analysis of CD133 staining and ALDH1 activity. Results are expressed as mean ± SEM fold-change to vehicle control of at least 3 experiments. $^{**}p < 0.01$ and $^{***}p < 0.005$ from DMSO.

Since p53 was already shown to induce differentiation in different cell types[131,201,202], also acting as a negative transcriptional regulatory mechanism of CD133 [203], it would be interesting to evaluate the differentiation effects of DS6 in this cellular context and clarify whether p53 is, or not, the driver of DS6 differentiation capability.

2.2. Modulation of pluripotency and differentiation genes in CRC by chemical compounds

Since pluripotency is a key factor for the maintenance of stemness properties, and thus for the undifferentiated status of cells[105], we decided to investigate whether these two compounds could interfere with the equilibrium pluripotency *versus* differentiation either

through the reduction of the first or through the increase of the latter markers. The gene expression of pluripotency markers commonly connected to CSCs (Figure 8), also referred as Yamanaka factors, *Nanog*, *Sox2* and *Oct4*[204–206], were analyzed through RT-qPCR 24 hours after compound incubation. We did not use the SW620 cell line, as no differences were still observed with both treatments (data not shown).

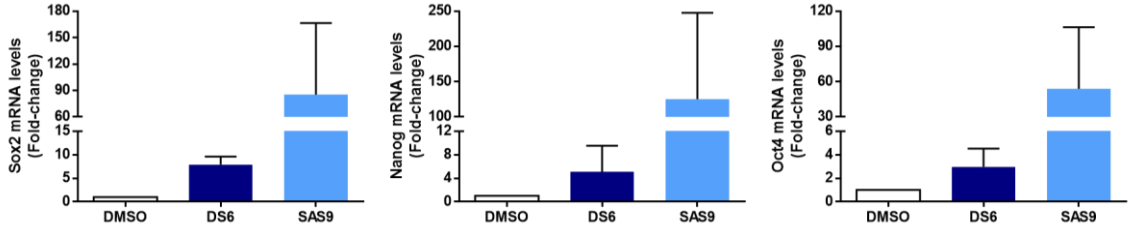

Figure 8 – DS6 and SAS9 increased the gene expression of pluripotency markers in HCT116 cell line. HCT116 cells were exposed to DS6 or SAS9, as described in Materials and Methods. DMSO was used as vehicle control. After 24 h of treatment, cells were collected for total RNA extraction. mRNA levels were evaluated through RT-qPCR. Results are expressed as mean ± SEM fold-change to vehicle control of at least 3 experiments.

Surprisingly, despite not being significant, both chemical compounds appeared to strongly increase the expression of the three pluripotency genes (Figure 8), suggesting an increase in the stemness levels. This was also in disagreement with the idea that downregulation of CD133 reduces the expression of these pluripotent markers[203].

Regarding differentiation-related genes, we decided to investigate the expression profiles of three differentiation-related markers, such as *Ada*, *Aqp3* and *Krt20* (Figure 9). *Ada* codes for an adenosine deaminase, an enzyme that induces lymphocyte differentiation[207]. Aquaporin 3, coded by *Aqp3* gene, is a water channel-forming protein shown to be associated with the differentiation degree of lung cancer[208]. *Krt20* gene codes for cytokeratin 20 (CK20), an epithelial cell marker associated with lower-grade CRC[209].

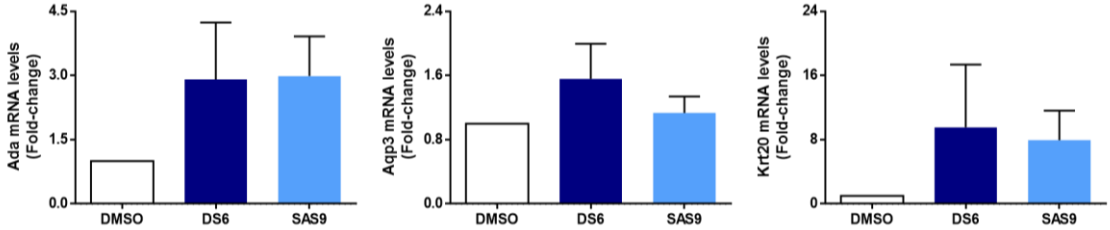

Figure 9 – DS6 and SAS9 increased mRNA levels of differentiation markers in HCT116 cell line. HCT116 cells were exposed to DS6 or SAS9, as described in Materials and Methods. DMSO was used as vehicle control. After 24 h of treatment, cells were collected for total RNA extraction. mRNA levels were evaluated through qPCR. Results are expressed as mean ± SEM fold-change to vehicle control of at least 3 experiments.

In fact, our results showed a strong increase in the expression of *ADA*, *Aqp3* and *Krt20* by both compounds (Figure 9), which were not significant due to different augment magnitudes observed for all differentiation-related genes. These results were in line with the first experiment (Figure 8), indicating that both compounds might develop their effect by favoring differentiation and reducing stemness.

2.3. Modulation of pluripotency and differentiation proteins in CRC by chemical compounds

Based on the fact that mRNA levels not always reflect phenotypic variations inside the cells, and since pluripotency genes were surprisingly upregulated in CSCs after DS6 and SAS9 exposure, we next evaluated by Western blot the protein levels of pluripotency and differentiation-related proteins after 48 hours of compound exposure. This time, and to get more accurate conclusions regarding the real impact of p53 in the mechanism triggered by both chemical compounds, we used two isogenic HCT116 cell lines, one expressing a wild-type p53 ($p53^{+/+}$) and the other having a p53 deletion ($p53^{-/-}$) (Figure 10). To confirm that p53 phenotype in these two cell lines were as described, we analyzed the protein levels of p53 by Western blot (Figure 10 – B) and confirmed that $p53^{-/-}$ cell line does not express p53.

We also used oxaliplatin and salinomycin as negative and positive controls, respectively. Oxaliplatin is a standard chemotherapeutic for colon cancer and shown to increase CSCs in CRC cell lines that are resistant to conventional chemotherapeutics[210]. Salinomycin, in turn, is a compound with high antitumor activity that targets specifically CSCs[141].

Interestingly, our results showed that, at least pluripotency is downregulated by both compounds at the post-transcriptional level (Figure 10 - A). Indeed, the majority of pluripotency-related proteins were reduced by DS6 in the $p53^{+/+}$ cell line.

DS6 significantly reduced the protein levels of Sox2 and Nanog ($p < 0.05$ for both), also showing a tendency to reduce C-Myc protein levels, in the $p53^{+/+}$ cell line. Notably, this DS6 effect was completely absent in p53 null cells (Figure 10 - A), corroborating the hypothesis that DS6 operate in a p53-dependent manner.

SAS9, in turn, did not show the same efficacy of DS6, only significantly reducing c-Myc protein levels ($p < 0.05$). However, the total levels of Sox2, and Oct4 tend to also be

diminished in the p53 wild-type cells after SAS9 (Figure 10 - A). This also indicates a certain level of selectivity of SAS9 to act through a p53-dependent manner, with the exception of Nanog which increased in both colon cell lines (Figure 10 - A).

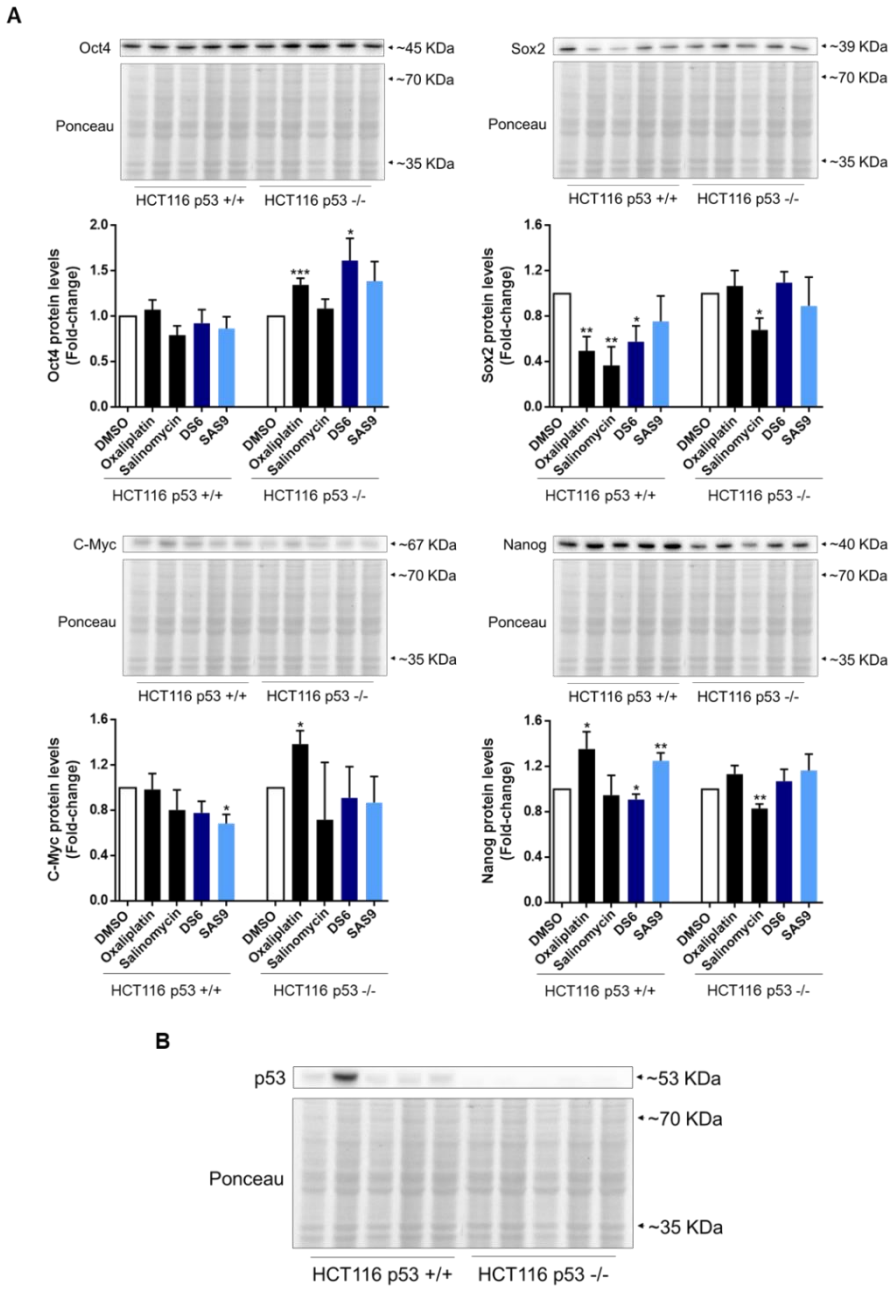

Figure 10 – Both DS6 and SAS9 reduced the majority of pluripotency related proteins in HCT116 p53$^{+/+}$ cells. HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells were exposed to oxaliplatin, salinomycin, DS6 or SAS9, as described in Materials and Methods. DMSO was used as vehicle control. 48 h upon treatment, the cells were harvested for total protein extraction. **(A)** Protein levels were evaluated through Western blot. Representative blots are shown. **(B)** p53 representative blot shown to validate p53 expression in those cell lines. Results were normalized to Ponceau and are expressed as mean ± SEM fold-change to vehicle control of at least 3 experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$ from DMSO.

Thus, despite not inducing an effect in pluripotency gene expression at 24 h after treatment, DS6, and in a lower extend SAS9 developed a significant effect on the pluripotency proteins in these CRC cells at later time-points, further confirming their potential as possible differentiation inducers.

Regarding the differentiation-related proteins, we noticed that surprisingly both compounds significantly reduced the CK20 protein levels ($p < 0.05$) in $p53^{+/+}$ CRC cells (Figure 11). Although we would not expect this decrease by looking to previous data on mRNA levels, and beside the RT-qPCR analysis performed 24 h before the CK20 protein analysis, it is possible that CRC cells might have to increase mRNA expression to compensate this protein decrease and assure differentiation and stemness suppression. On the other hand, DS6 appeared to increase CK20 expression in $p53^{-/-}$ cells. Although this increase was not significant, it may suggest that DS6-induced CK20 protein expression is not a p53-dependent mechanism.

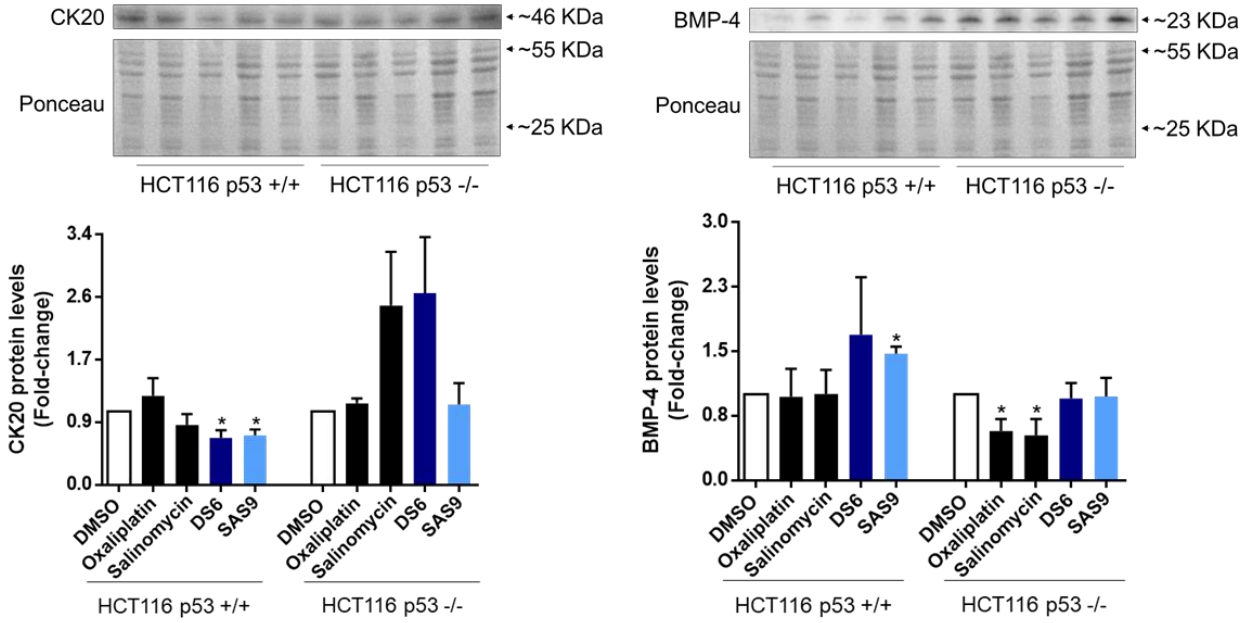

Figure 11 – SAS9 and DS6 induced an increase in BMP-4 protein levels in HCT116 p53 wild-type cells. HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were treated with oxaliplatin, salinomycin, DS6 or SAS9, as described in Materials and Methods. DMSO was used as vehicle control. 48 h upon treatment, the cells were harvested for total protein extraction. Protein levels were evaluated through Western blot. Representative blots are shown. Results were normalized to Ponceau and are expressed as mean ± SEM fold-change to vehicle control of at least 3 experiments. *$p < 0.05$ from DMSO.

Nevertheless, our results also revealed that the differentiation marker BMP-4 was significantly increased by SAS9 in $p53^{+/+}$ CRC cells ($p < 0.05$) and that DS6 incubation also

resulted in a slight tendency for a positive regulation of this marker in these cells. Again, the chemical induction of BMP-4 levels by both compounds still was completely abolished in p53 null cells, corroborating the possible involvement of p53 in this specific regulatory mechanism by DS6 and SAS9 (Figure 11).

Therefore, further analysis of a larger set of stemness and differentiation related proteins specific to colon and CRC would give better clues on the real impact of DS6 and SAS9 in differentiation in CRC cells.

LGR5 could be a specific and valuable stemness marker for colon CSCs. Although presenting some disadvantages, as it is also expressed by normal stem cells of the intestine and colon[104], LGR5 plays important roles in the CSC phenotype[211]. Unfortunately, the promoter of its gene is methylated and silenced in HCT116 cell line[104], meaning that further investigations of the potential modulation of LGR5 by DS6 and SAS9 would require the use of different CRC cell lines.

2.4. Inhibition of sphere forming ability of CRC cells by chemical inhibitors

Tumor sphere-forming assay allows the evaluation of the aggressiveness of a cancer cell line, namely its enrichment in CSCs for further studies[212]. The first goal was to evaluate the precise effect of compounds on sphere formation (Figure 12). For that, we treated CRC cells in adherent conditions and then replated them for sphere-forming conditions. This allow us to also test indirectly the role of DS6 and SAS9 in inducing differentiation, since it has been shown that cells more differentiated have lower ability to survive anoikis and reestablish a new sphere population[213]. Notably, our results showed that both small molecules were able to significantly reduce the number of spheres formed in p53 wild-type cell lines ($p < 0.05$ for DS6; $p < 0.01$ for SAS9) (Figure 12 – A). These data indicate that both compounds are able to target CSCs, differentiate them and induce a less malignant phenotype by effectively impairing their ability to self-renew and form other tumor populations. The absence of DS6 and SAS9 inhibitory effect in spheres derived from $p53^{-/-}$ cell line, once again, is a further evidence of the p53 participation in the DS6 and SAS9 mechanisms of action.

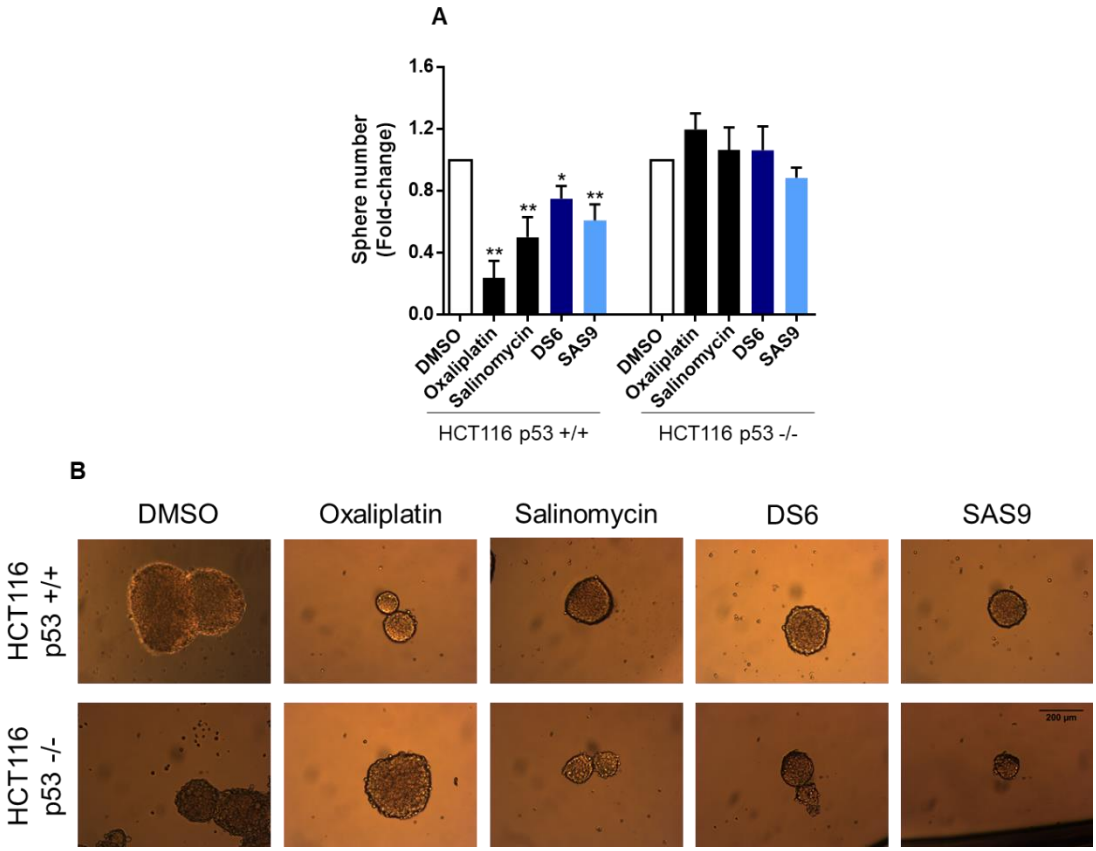

Figure 12 – Both compounds successfully impaired sphere formation capability in HCT116 p53$^{+/+}$ cells. HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells were incubated with oxaliplatin, salinomycin, DS6 or SAS9, as described in Materials and Methods. DMSO was used as vehicle control. **(A)** 7 days after plating, the spheres were counted to assess sphere number. **(B)** Representative photos of each condition are shown, at 10x magnification. Scale bar, 200 µm. Results are expressed as mean ± SEM fold-change to vehicle control of at least 3 experiments. *$p < 0.05$ and **$p < 0.01$ from DMSO.

The loss of sphere-forming ability is also an indicator of self-renewal decrease, probably resulting from the cell disability to give rise to more undifferentiated and stem-like cells through symmetrical divisions. In this respect, a tumor sphere-forming efficiency over three generations was also performed using spheres treated with the compounds when first plated. Unfortunately, we were not able to take any conclusion from these experiments (data not shown).

Finally, we also assessed the effect of the compounds on cell death and sphere size after their formation (Figure 13). Our results showed that neither cell death or sphere size reduction were observed in both cell lines after exposure with DS6 and SAS9 (Figure 13 - A). This, in turn, indicates that both chemical compounds target CSCs and induce cellular alterations without triggering toxicity or cell loss.

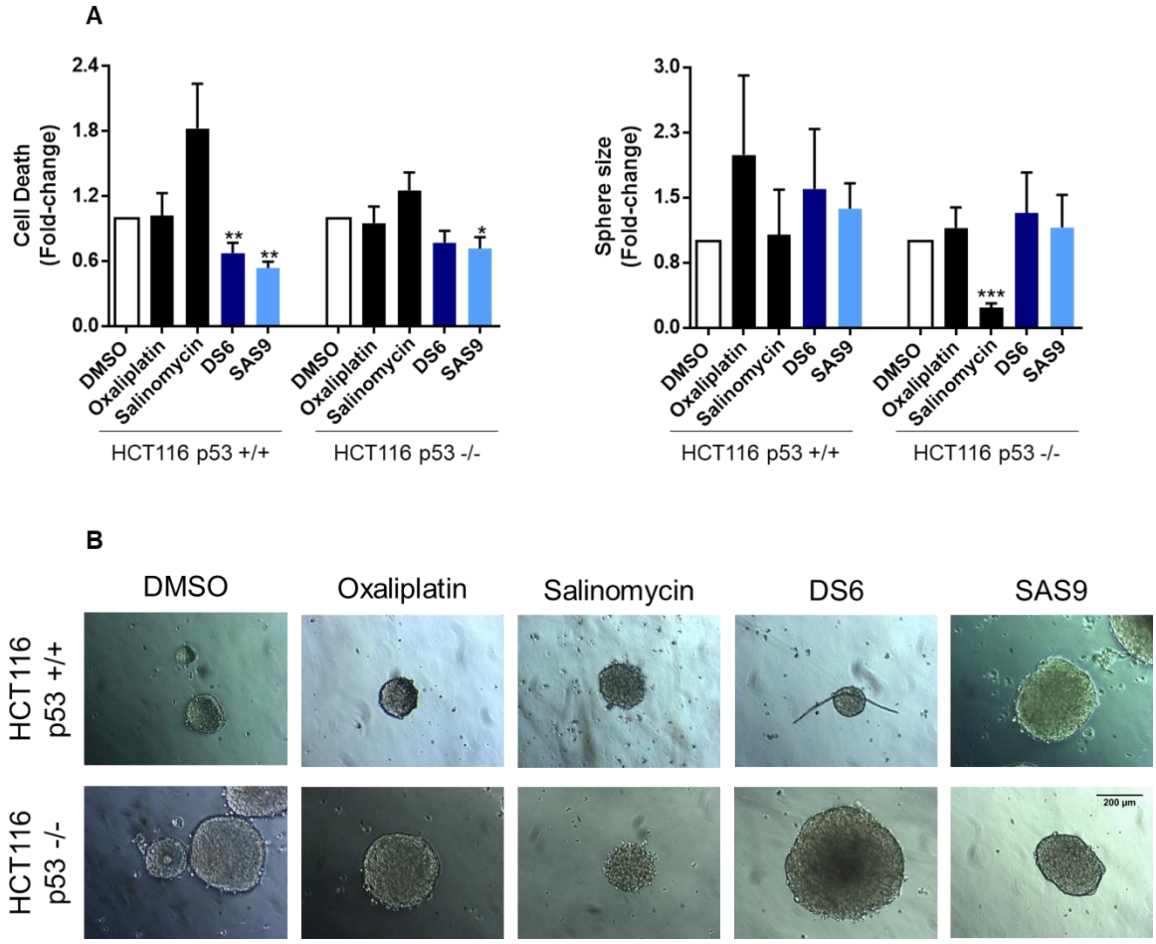

Figure 13 – DS6 and SAS9 had no discernible effect on tumor sphere size. HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were incubated with oxaliplatin, salinomycin, DS6 or SAS9, as described in Materials and Methods. DMSO was used as vehicle control. **(A)** 72 h upon treatment, a volume of the medium is removed to perform the ToxiLight cell death assay. The spheres were counted and afterwards dissociated into single cells, to count the cells to calculate the size of the spheres. **(B)** Representative photos of each condition are shown, at 10x magnification. Scale bar, 200 µm. Results are expressed as mean ± SEM fold-change to vehicle control of at least 3 experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$ from DMSO.

2.5. Modulation of self-renewal ability of CRC cells by chemical compounds

To go deeper into the mechanisms by which DS6 and SAS9 reduce sphere-forming ability, we decided to reevaluate self-renewal in our cell lines through a different methodology. Briefly, symmetrical and asymmetrical divisions were analyzed through the cell pair assay by culturing CRC cells in low density, in the presence or absence of the DS6 and SAS9, and evaluating the expression of the stemness marker ALDH1 in mitotic cell pairs through immunocytochemistry (Figure 14).

In accordance with decreased sphere number, lower levels of symmetrical stem cell divisions ALDH$^{+/+}$ were detected after 24 h of DS6 incubation, when compared with control cells ($p < 0.01$) (Figure 14 – B). In agreement with our previous data, the effect of DS6 in reducing self-renewal was abolished in p53 mutant cell lines, supporting a role for p53 in this process. SAS9 only induced a mild effect in reducing symmetrical stem cell divisions and increasing symmetrical differentiation divisions, in both cell lines (Figure 14 – B). These results support the role of DS6 as a differentiation-inducer molecule in CRC cells.

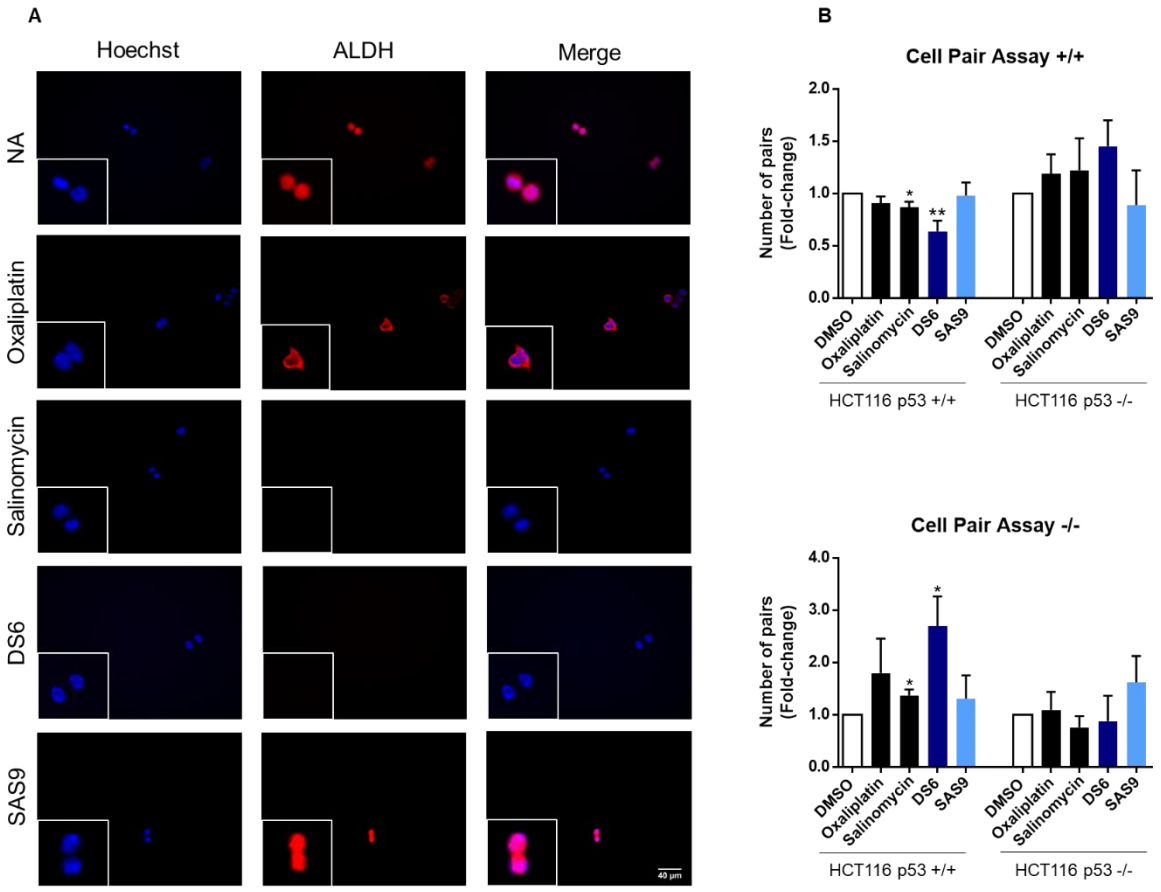

Figure 14 – DS6 disrupts self-renewal and induces differentiation in HCT116 p53$^{+/+}$ cells. HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells were incubated with oxaliplatin, salinomycin, DS6 or SAS9, as described in Materials and Methods. DMSO was used as vehicle control. **(A)** Representative photos of each condition are shown, at 40x magnification. Scale bar, 40 µm. **(B)** 24 h after treatment, the cells were fixed and underwent an immunocytochemistry protocol, being visualized in a fluorescence microscope. Results are expressed as mean ± SEM fold-change to vehicle control of at least 3 experiments. *$p < 0.05$ and **$p < 0.01$ from DMSO.

2.6. Chemotherapy sensitization of CRC cells by DS6 and SAS9.

Our final goal was to test the potential of both compounds in sensitizing CRC cells to the conventional fluorouracil (5-FU). 5-FU is a standard drug for the treatment of metastatic CRC that has been afflicted with the problem of chemoresistance[214]. Most chemotherapeutics act by damaging DNA and inducing apoptosis[215]. Since DNA repair capability[216] and resistance to apoptosis[217] are two key characteristics of CSCs, combining with a therapy capable of targeting CSCs, theoretically, would be sufficient to overcome that barrier and effectively achieve a treatment of cancer. In fact, this is the same experimental setting as that already described for compounds of DS6 family[195].

In our analysis, we evaluated cell viability and cell death after treatments alone or in combination (Figure 15). As shown in Figure 15, and as we expected, DS6 and SAS9 alone did not reduce cell viability nor significantly increased cell death in either cell line.

We also observed that exposure of 5-FU alone induced a significant reduction in cell viability in both cell lines ($p < 0.005$ for both), as well as a significant increase in cell death in HCT116 $p53^{+/+}$ cells ($p < 0.05$), not inducing cell death in p53 null cells (Figure 15). Indeed, it is possible that 5-FU requires p53 to induce cell death.

More importantly, when CRC cells were incubated with a combination of DS6 and 5-FU, or SAS9 and 5-FU, for 24 h, a tendency for cell viability decline was observed when compared to 5-FU treatment alone (Figure 15). Further, combination therapy with DS6 and with SAS9 exerted no significant effect in cell viability and cell death in $p53^{-/-}$ cells, when compared to 5-FU (Figure 15). The high concentration of 5-FU used in our experiments might partially explain the absence of chemoresistance, while also masking potential synergistic effects by combination therapies. Further optimizations of the 5-FU concentration, namely by assessing IC_{50} of 5-FU, could be helpful in the future.

Nevertheless, to effectively assess synergy, we resorted to the coefficient of drug interaction (CDI), previously described[164,195], and calculated for DS6 and SAS9 combination therapy. In fact, our analysis revealed that DS6 induces a slight synergistic effect when combined with 5-FU (CDI=0.91) in the p53 wild-type cell line, but not in the p53 null cells (CDI=1.02). These results still support the p53-dependent mechanism rationale for DS6. As

for SAS9 combination, no synergistic effect was observed in both $p53^{+/+}$ and $p53^{-/-}$ cell lines (CDI=0.96 for both cell lines).

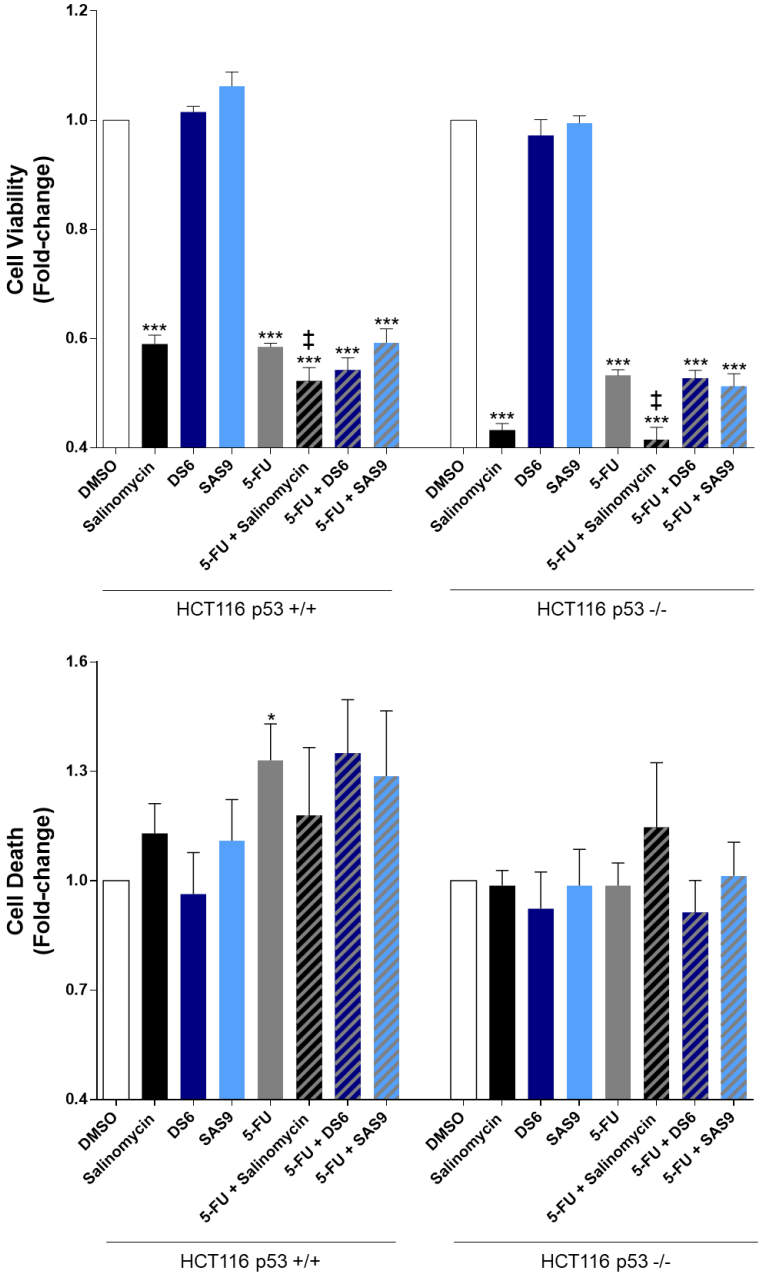

Figure 15 – DS6 shows a tendency to work synergistically in reducing cell viability in HCT116 $p53^{+/+}$ cells. HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were incubated with 5-FU, Salinomycin, DS6, SAS9, 5-FU + Salinomycin, 5-FU + DS6 or 5-FU + SAS9, as described in Materials and Methods. DMSO was used as vehicle control. 48 h after treatment, the cells are evaluated for cell death through ToxiLight assay, and cell viability through PrestoBlue assay. Results are expressed as mean ± SEM fold-change to vehicle control of at least 3 experiments. *$p < 0.05$ and ***$p < 0.005$ from DMSO; ‡$p < 0.05$ from 5-FU monotherapy.

Further experiments, including other concentrations and time-points, are needed to validate, or not, this small degree of synergistic effect promoted by DS6 and understand whether these two compounds are promising in CRC differentiation therapy.

IV. Conclusion and Future perspectives

Regarding the first aim of this project, to optimize the experimental procedure to determine tissue miRNA and uncover specific miRNAs that could be potential diagnostic and prognostic biomarkers in CRC, we only opened a road for future investigations and the small number of samples did not allow us to take strong conclusions. However, through the molecular analysis of a previously selected set of miRNAs in human CRC samples obtained at different stages of chemoradiotherapy treatment, we were able to observe variations consistent with what was previously described in the literature for several miRNAs. We also identified other differential patterns of expression that allowed us to take some preliminary conclusions about the prognostic value of these miRNAs. Although requiring further experiments and a larger cohort of samples to validate these assumptions, our preliminary results show a step in the right direction.

Regarding our second aim in identifying new molecules with non-toxic differentiation potential for differentiation therapy, we evaluated the therapeutic potential of DS6, a spiropyrazoline oxindole molecule, and SAS9, a 3-piperidinyl-indole molecule. While affecting cellular function through different mechanisms of action, we aimed to see their efficacy in inducing differentiation in CSCs, using CRC cell lines, and sensitizing cancer cells to common chemotherapeutics.

Regarding SAS9, the results were not so promising, as it only showed effects on the pluripotency and differentiation modulation in few markers and minor effects on tumor sphere-forming ability, which might be due to its pro-necroptotic role, thus overcoming a possible differentiation induction.

In contrast, DS6 showed more promising results, possibly acting by a p53-dependent mechanism. In fact, DS6 exposure resulted in significant reduction of several stemness and pluripotency markers, increased expression of differentiation markers, inhibition of tumor sphere-forming ability, significant suppression of self-renewal and, finally, a slight synergistic sensitization of the chemotherapeutic response in combination with 5-FU.

Nevertheless, further experiments are necessary to understand its potential in preclinical studies. For this reason, some remaining points should be addressed in the future.

The validation of the CD133 and p53 regulatory link, using the HCT116 $p53^{+/+}$ and $p53^{-/-}$ cell lines, will allow us to investigate whether CD133 is suppressed by a p53-dependent mechanism, another possible pathway through which DS6 may promote a reduction in stemness. In addition, investigation of the DS6 differentiation potential in Caco-2 cells, a cell line originated from colon adenocarcinoma that undergoes spontaneous differentiation in culture[218] will also be interesting to confirm the therapeutic efficacy of DS6.

It would also be important to further dissect the mechanism of DS6 and uncover its influence on pathways commonly dysregulated in CRC. Finding the association with stem-like traits, not only will allow further therapeutic enhancement of this compound, but it could also shed light on novel drugable targets for differentiation therapy in cancer.

Finally, the *in vivo* assessment of DS6 therapeutic effect by using appropriate animal models could also clarify if *in vitro* results could be translated to *in vivo* models. Determination of pharmacokinetics and pharmacodynamics of DS6 will also be important aspects of its use in preclinical trials.

Altogether, our results provided helpful preliminary data to tackle CRC, both at diagnostic and therapeutic levels. Continuing with this line of work could prove invaluable for a future better CRC diagnosis and improved therapeutic options.

V. Bibliography

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VI. Annex

Supplemental data S1: Chemical structures a spiropyrazoline oxindole (chemical family of DS6) (Figure 16 – A) and of a 3-piperidinyl-indole (chemical family of SAS9) (Figure 16 – B).

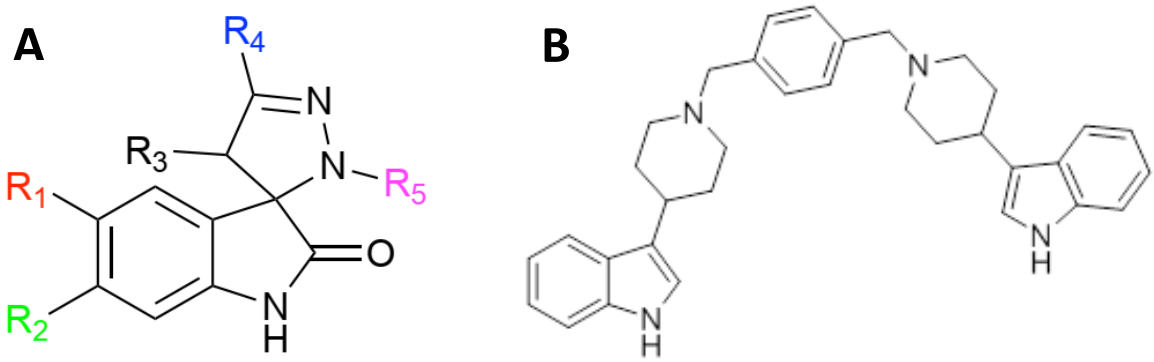

Figure 16 - Schematics of the chemical structures of a spiropyrazoline oxindole (A) and of a 3-piperidinyl-indole (B)