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Escola de Ciências

Helena Paula Fernandes Pereira

Study of the role of monocarboxylate transporters in colorectal carcinoma

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DECLARAÇÃO

Nome

Helena Paula Fernandes Pereira

Endereço electrónico: helenapaula@portugalmail.pt Telefone: 00351966867670

Número do Bilhete de Identidade: 9877872

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SUMMARY

Study of the role of monocarboxylate transporters in colorectal carcinoma

Colorectal carcinoma (CRC) is the second leading cause of cancer-related death in industrialized countries. Despite 5-fluorouracil (5-FU) being one of the most efficient therapeutics in the treatment of CRC, new strategies of combination of other agents have been developed, aiming to improve its anti-tumour efficacy and decrease side-effects. Similar to most malignant tumours, CRC is highly glycolytic, producing large amounts of lactic acid, which is effluxed to the tumour microenvironment via lactate transporters, namely monocarboxylate transporters (MCTs). MCTs are potential therapeutic targets in solid tumours including CRCs.

The aim of this work was to explore the role of MCTs in CRC, by evaluating the effects of MCT inhibitors on human colon carcinoma cell viability, migration, lactate efflux, and MCTs expression. Furthermore, we intended to evaluate the effect of the combination of the MCT inhibitor alfa-cyano-4-hidroxycinnamic (CHC), with 5-FU in human colon carcinoma cell viability and migration.

Our results showed that CHC has an inhibitory effect on HCT-15 and Co-115 colon carcinoma cell viability being this effect greater in HCT-15 cells than in Co-115 cells. CHC also inhibited HCT-15 cell migration, reinforcing the sensitivity of these cells to CHC. Furthermore, our results suggested that CHC was also able to decrease lactate efflux and probably MCT1 expression in HCT-15 cells. On the other hand, AR-C155858 had no effect on both HCT-15 and Co-115 cell viability, as indicated by MTT assay and morphological analysis. However, it will be necessary to evaluate its effect on cell viability, by trypan blue assay, and cell proliferation.

In this work, it was also demonstrated, for the first time, that CHC potentiated the cytotoxic effect of 5-FU in HCT-15 and Co-115 colon carcinoma cells.

Our findings provide important evidence for the role of MCTs in CRC as well as for the exploitation of MCTs as therapeutic targets in CRC. Additionally, our results show evidences for the benefit of combining MCT inhibitors with conventional anticancer chemotherapy.

RESUMO

Estudo do papel dos transportadores de monocarboxilatos no carcinoma colorectal

O carcinoma colorectal (CCR) é a segunda causa de morte por cancro nos países industrializados. Apesar do 5-fluorouracilo (5-FU) ser uma das terapêuticas mais eficazes no tratamento do CCR, novas estratégias de combinação de outros agentes têm sido desenvolvidas, no sentido de diminuir as suas reacções adversas. Tal como a maioria dos tumores malignos, o CCR é altamente glicolítico e produz grandes quantidades de ácido láctico, que é transportado para o microambiente tumoral através de transportadores de lactato, nomeadamente os transportadores de monocarboxilatos (MCTs). Os MCTs são potenciais alvos terapêuticos em tumores sólidos, inclusive nos CCRs.

O objectivo deste trabalho foi explorar o papel dos MCTs no CCR, através da avaliação dos efeitos dos inibidores dos MCTs na viabilidade, migração, efluxo de lactato e expressão dos MCTs em células humanas do carcinoma do cólon. Além disso, avaliou-se o efeito da combinação do inibidor dos MCTs, ácido alfa-ciano-4-hidroxicinâmico (CHC), com o 5-FU, na viabilidade e migração em células humanas do carcinoma do cólon.

Os nossos resultados mostraram que o CHC tem um efeito inibitório na viabilidade das células HCT-15 e Co-115, sendo este efeito maior nas células HCT-15. O CHC inibiu também a migração das células HCT-15, reforçando a sua sensibilidade ao CHC. Além disso, os nossos resultados também sugeriram que o CHC foi capaz de diminuir o efluxo de lactato e provavelmente a expressão do MCT1. Por outro lado, o AR-C155858 não teve efeito na viabilidade de ambas as linhas celulares, HCT-15 e Co-115, como indicado pelo ensaio MTT. Contudo, será necessário avaliar o seu efeito na viabilidade celular, pelo ensaio do *trypan blue*, bem como na proliferação celular.

Neste trabalho, foi também demonstrado, pela primeira vez, que o CHC potenciou o efeito citotóxico do 5-FU nas células HCT-15 e Co-155.

Os nossos resultados fornecem evidências importantes para o papel dos MCTs no CCR, bem como para a exploração dos MCTs como alvos terapêuticos. Além disso, este estudo mostra evidências para o benefício da combinação de inibidores dos MCTs com a quimioterapia convencional.

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Abbreviations

CHC	Alpha-cyano-4-hydroxycinnamate
CRC	Colorectal carcinoma
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulphonate
DMSO	Dimethylsulfoxide
FBS	Fetal bovine serum
FDG-PET	Fluorodeoxyglucose positron emission tomography
5-FU	5-fluorouracil
GLUTs	Glucose transporters
HIF	Hypoxia-inducible factor
IC50	50% inhibition concentration
MCTs	Monocarboxylate transporters
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NHE1	Na^+/H^+ exchangers
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
pCMBS	p-chloromercuribenzene sulphonate
SDS	Sodium dodecyl sulphate
TBS	Tris Buffered Saline
VEGF	Vascular endothelial growth factor

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1 Introduction

1.1 Colorectal Cancer

Colorectal carcinoma (CRC) is the third most common type of cancer in both men and women and the second leading cause of cancer related-death in developed countries [1]. The incidence of CRC in Europe is 58/100 000 per year and the mortality is 30/100 000 per year [2]. Worldwide, the mortality rate is 8.9 per 1000 people for men and 8.1 for women. These rates vary from 4.6 and 3.9, in less developed regions, respectively to 27.5 and 25.1, in more developed regions, respectively [3]. The probability of developing colorectal cancer rises sharply with age. In the younger population, the risk of CRC is very low; between the ages of 45 and 49 years, the incidence rate is approximately 20 per 100,000 for both males and females [4]. Among those over 75 years of age, the incidence rate for CRC is over 300/100 000 per year for males and over 200 for females [4]. The median age of patients at diagnosis is over 70 years [4].

1.1.1 Risk factors

Hereditary, experimental and epidemiological studies [5, 6] suggest that colorectal cancer results from complex interactions between genetic and environmental factors.

Approximately 75 percent of colorectal tumours are sporadic and occur in people with no specific risk factors. The remaining 25 percent of cases develop in people with significant risk factors. Most (15-20%) colorectal cancers occur in people with either a personal history or a positive family history of polyps or colorectal cancer [7]. The remaining cases develop in people with genetic predispositions, such as hereditary non-polyposis colorectal cancer (HNPCC, 4-7%) or familial adenomatous polyposis (FAP, 1%) or in people with inflammatory bowel disease (IBD, 1%) [8]. A diet that is high in red meat and fat and low in vegetables, folate and fibre may increase the risk of CRC [11]. A high intake of animal fat in the diet is linked with an increase in

faecal bile acids, such as cholic and deoxycholic acid. These bile acids act as potential carcinogens on the colonic mucosa. In contrast to fat, fibre decreases bowel transit time and therefore exposure of the bowel to these carcinogens [9]. Other risk factor associated with colon cancer is lack of physical activity.

1.1.2 Colorectal Carcinogenesis

Colorectal cancer includes cancerous lesions in the colon, rectum and appendix. Cancer cells can invade nearby tissues and organs and subsequently spread to distant parts of the body (metastases). The liver and the lungs are common metastatic sites of CRC [10].

The large majority of colorectal cancers develop from premalignant polyps, commonly referred to as adenomas. These lesions consist in well-demarcated masses of epithelial dysplasia, with uncontrolled cell division; most of them remain benign, but a small fraction may evolve to malignancy. The malignant potential of adenomas of the colon and rectum varies with size, histological type and grade of epithelial atypia. The adenomatous polyp is usually small and has a low malignant potential, whereas tumours with a villous structure are usually larger and have a much higher cancer rate. Severe atypia is more common in villous adenomas than in adenomatous polyps [11].

Colorectal cancer typically progresses from normal epithelium through dysplasia and adenoma stages to carcinoma *in situ* and finally to invasive cancer. Generally, CRC results from the cumulative effect of multiple sequential genetic alterations (multistep carcinogenesis) (**Figure 1**). These alterations can either be acquired, as happens in the sporadic forms, or be inherited, as in genetic cancer predisposition syndromes [12, 13]. It is now widely accepted that activation of certain oncogenes and simultaneous inactivation of a variety of tumour suppressor and DNA-repair genes are required for tumour development and progression. In addition, epigenetic alterations by promoter methylation have been found to play a major role in carcinogenesis of a large proportion of sporadic colon cancers. Consequently, it is now apparent that multiple molecular pathways exist in colorectal carcinogenesis in addition to the classic mechanisms [14].

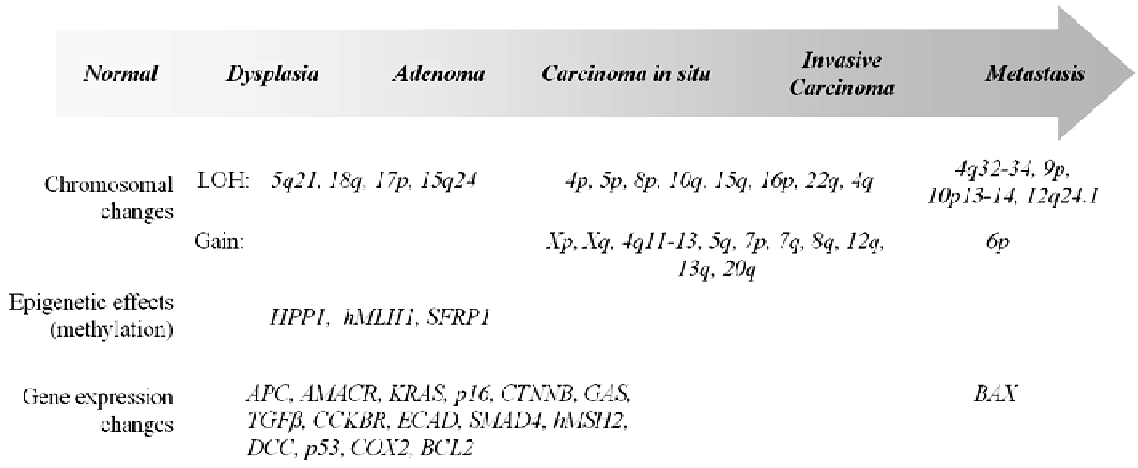

Figure 1. Progression model of colorectal cancer. The changes that occur in the progression of colorectal cancer are outlined in this figure [Garnis *et al*, 2004].

Inactivating mutations of both copies (alleles) of the *adenomatous polyposis coli* (*APC*) gene, a tumour-suppressor gene on chromosome 5q, mark one of the earliest events in colon cancer formation [15]. The APC protein interacts with the intracellular protein ß-catenin, which, when activated, translocates to the nucleus and stimulates cell proliferation by transcriptional activation of *c-myc, cyclin D1* and the *peroxisome-proliferator-activated receptor delta (PPAR*δ). Once ß-catenin levels increase, a cell proliferation program is activated. In concert with other factors, the APC protein stops cellular proliferation and promotes apoptosis by phosphorylating ß-catenin, leading to its ubiquitination and degradation through the proteosome pathway (**Figure 2**) [16]. In the case of an inactivating mutation, APC-mediated ß-catenin degradation is lost and nuclear concentrations of ß-catenin remain high, which results in the formation of the adenoma. Germline mutation of the *APC* gene and subsequent somatic mutation of the second *APC* allele cause the inherited familial adenomatous polyposis syndrome (FAP) [17, 18]. This syndrome is characterized by the existence of hundreds to thousands of colonic adenomatous polyps. If these polyps are left untreated, colorectal cancer develops. Somatic (or acquired) *APC* alterations are seen in most (85–90%) colorectal neoplasms, whether these cancers are of familial or sporadic origin [19].

Mutation leading to dysregulation of the *K-RAS* oncogene is also thought to be an early event in colorectal carcinogenesis [20]. The oncogene *K-RAS* on chromosome 12 encodes for a protein which transmits extracellular growth signals to the nucleus.

Mutations in *K-RAS* are always activating missense alterations that lead to continuous growth signals. It has been demonstrated that mutations in *K-RAS* correlate with the size of lesions and progression to dysplasia [21, 22]. Mutations of *K-RAS* are found in 50% of large polyps and colorectal cancers [23].

Conversely, loss of heterozygosity (LOH) on the long arm of chromosome 18 (18q) occurs later in the sequence of development from adenoma to carcinoma. Loss of the 18q region is thought to contribute to inactivation of the *deleted in colorectal carcinoma gene* (*DCC)* tumour-suppressor gene [20]. More recent evidence suggests that allelic loss on chromosome 18q may also contribute to the inactivation of *SMAD-4* and *SMAD-2* tumour-suppressor genes [21]. LOH on 18q occurs in approximately 70% of all colon cancers, and this mutation may predict poor prognosis [20].

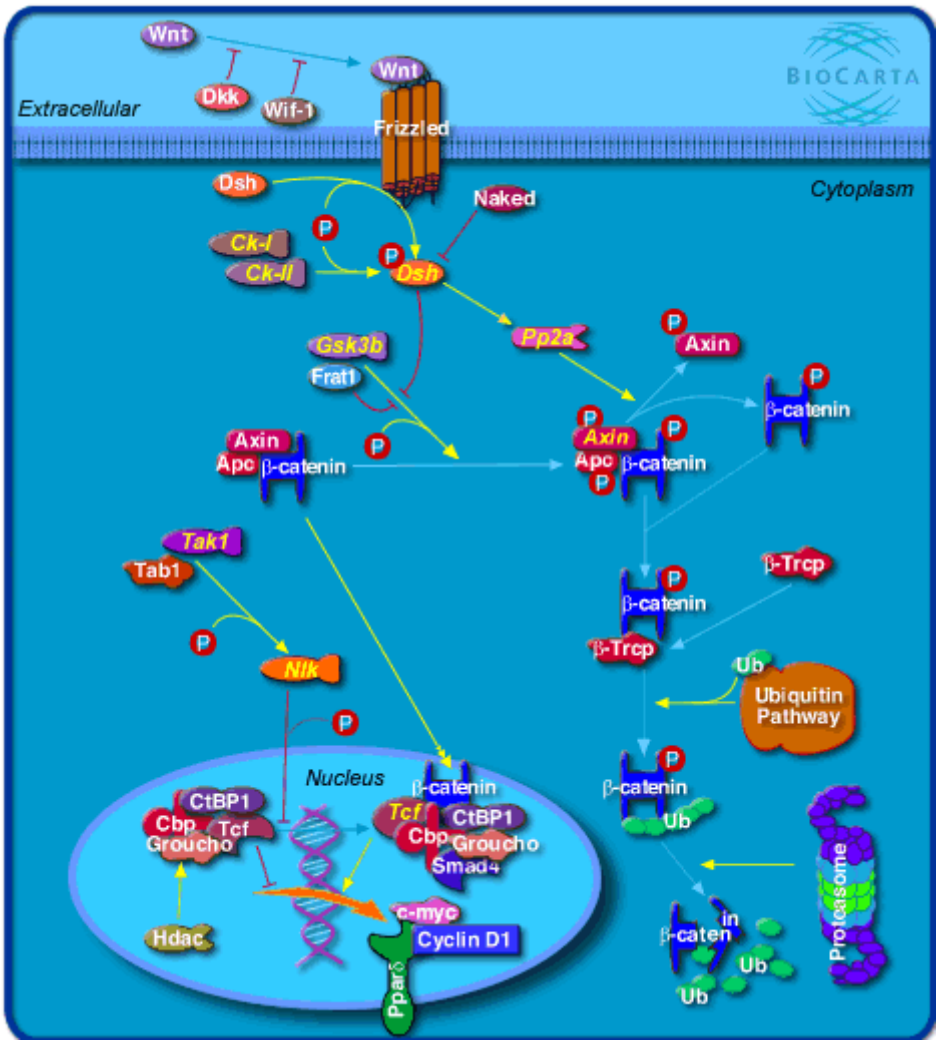

Figure 2. Overview of the Wnt signaling pathway [BIOCARTA, www.biocarta.com].

In addition, mutation of the tumour suppressor gene *TP53* on chromosome 17p appears to be a late phenomenon in tumorigenesis of colorectal cancer and can be found in 50-60% of sporadic colorectal carcinomas [20, 24]. This mutation may allow the growing tumour with multiple genetic alterations to evade cell cycle arrest and apoptosis.

Subsequently, a third class of genes, DNA repair genes, has been implicated in colorectal carcinogenesis [11]. DNA mismatch repair deficiency, due to germline mutation of the *hMSH2*, *hMLH1*, *hMSH6*, *hPMS1*, *hPMS2,* or *hMLH3* genes, contributes to development of hereditary nonpolyposis colorectal cancer (HNPCC) [25]. Loss of DNA mismatch repair genes leads to a hypermutable state in which simple repetitive DNA sequences, called *microsatellites*, are unstable during DNA replication, giving rise to widespread alterations in these repeats [26]. Most of these alterations occur in noncoding region of the genome, but a few in coding or promoter region of genes involved in regulation of cell growth, such as type II TGF-ß receptor [27] and *BAX* [28]. The majority of tumours in patients with HNPCC and 10% to 15% of sporadic colon cancers exhibit *microsatellite instability* (MSI) [25].

Moreover, hypomethylation or hypermethylation of DNA sequences may alter gene expression without nucleic acid mutation. The idea that epigenetic changes can be a mechanism for altering gene expression and driving tumorigenesis has been supported by previous work [29, 30]. In 1998, evidence begun to accumulate that *hMLH1* was inactivated by epigenetic modification (hypermethylation) of its promoter in most of the sporadic tumours with MSI [31, 32].

1.1.3 Histopathology

The most common colorectal cancer cell type is adenocarcinoma which accounts for 95% of cases. Other, rarer types include lymphoma, leiomyosarcoma and squamous cell carcinoma [33, 34].

Adenocarcinoma is a malignant epithelial tumour, originating from glandular epithelium of the colorectal mucosa. This tumour invades the wall, infiltrating the *muscularis mucosae*, the submucosa and the *muscularis propria* (**Figure 3**) [35]. Adenocarcinoma cells describe irregular tubular structures, presenting multiple lumens,

and reduced stroma ("back to back" aspect). Occasionally, tumour cells secrete mucus, which invades the interstitium producing large pools of mucus (optically "empty" spaces) - Mucinous adenocarcinoma, poorly differentiated. If the mucus remains inside the tumour cell, it pushes the nucleus at the periphery ("signet-ring cell") - Signet Ring Cell adenocarcinoma. Depending on glandular architecture, cellular pleomorphism, and mucosecretion of the predominant pattern, adenocarcinoma may present three degrees of differentiation: well, moderately, and poorly differentiated [35, 36].

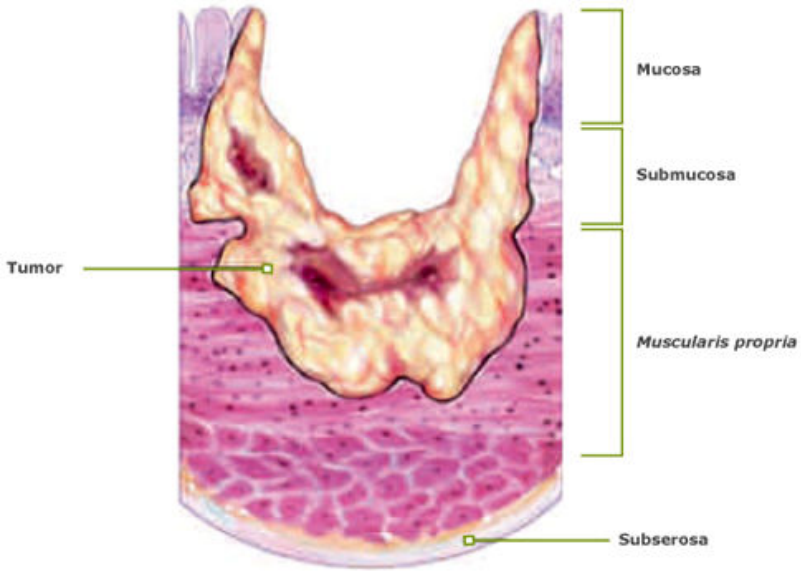

Figure 3. Adenocarcinoma progression [portal de oncologia português, www.pop.eu.com].

1.1.4 Treatment

The treatment for CRC depends, to a large extent, on the cancer stage. For early cancer, treatment may consist of surgery alone while for more advanced cancers, other treatments such as chemotherapy or radiation therapy may also be required [37].

Curative surgical treatment can be offered if the tumour is localized. In case of multiple metastases, palliative (non curative) resection of the primary tumour is still

offered in order to reduce further morbidity caused by tumour bleeding, invasion, and its catabolic effect [38].

Radiation therapy can be delivered preoperatively, intraoperatively, or postoperatively and sometimes chemotherapy agents are used to increase the effectiveness of radiation by sensitizing tumour cells [39].

Chemotherapy is used to reduce the likelihood of metastasis development, decrease tumour size, or slow tumour growth. Chemotherapy is often applied after surgery (adjuvant), before surgery (neo-adjuvant), or as the primary therapy (palliative) [40]. The chemotherapeutic treatment of CRC has undergone revolutionary changes in the last 5-10 years, with a variety of new drugs and regimens being either approved or under investigation [41].

1.1.4.1 Chemotherapeutic agents

5-fluorouracil (5-FU), an antimetabolite fluoropyrimidine analogue, is one of the most effective chemotherapeutic agents for colorectal carcinoma and has been used to treat this type of tumour for about 50 years [42]. 5-FU and its metabolites possess a number of different mechanisms of action (**Figure 4**). In vivo, fluorouracil is converted to the active metabolite 5-fluoroxyuridine monophosphate (F-UMP); replacing uracil, F-UMP incorporates into RNA and inhibits RNA processing, thereby inhibiting cell growth. Another active metabolite, 5-5-fluoro-2'-deoxyuridine-5'-O-monophosphate (F-dUMP), inhibits the activity of thymidylate synthase, resulting in the depletion of thymidine triphosphate (TTP) and leading to inhibition of DNA synthesis and G1/S cell cycle arrest [43]. Some data [44, 45] strongly supports the hypothesis that cell death in intestinal epithelia or in human epithelial colon cancer cell lines requires 5-FU metabolites to be incorporated into RNA. Cell death therefore occurs by p53-dependent apoptosis.

As a single agent, 5-FU is only modestly active, producing a response rate of 15% in advanced colorectal cancer. Strategies of combination of other agents have been developed, aiming to enhance the therapeutic efficiency of 5-FU and reduce the side-effects [46]. In the 1980s, studies showed that the addition of leucovorin (LV; also known as folinic acid) improved the efficacy of 5-FU without a large increase in

toxicity, thus 5-FU/LV became the standard of treatment for CRC [47, 48]. Today, however, the chemotherapy for CRC is more likely to consist of 5-FU/LV in combination with oxaliplatin, a third-generation platinum cytotoxic compound [49], or irinotecan, an inhibitor of topoisomerase I [50]. Another drug, capecitabine, may also be included. This is an orally administered fluropyrimidine, and a prodrug of 5-FU [48].

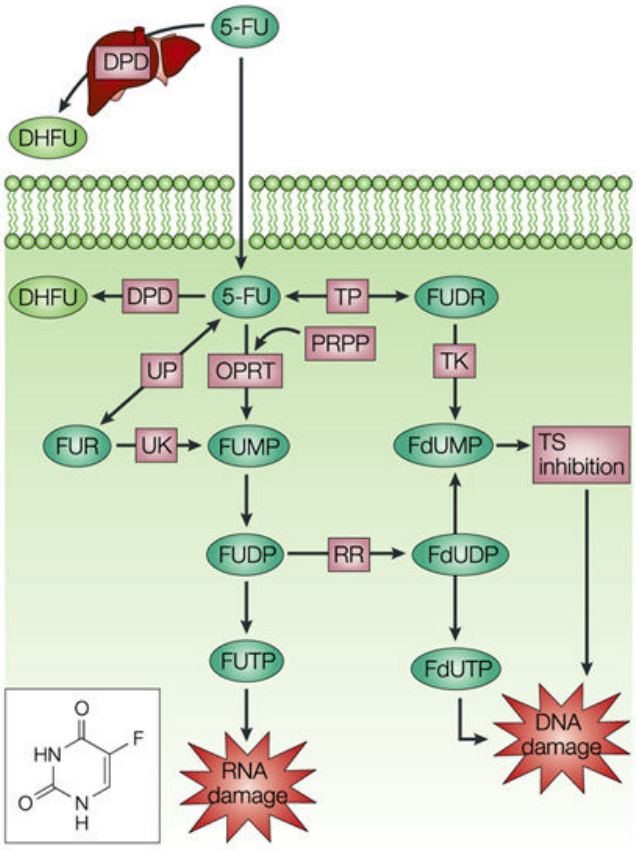

Figure 4. Mechanisms of action of 5-FU and its metabolites [Longley *et al*, 2003].

Recently, novel chemotherapeutic agents who target specific proteins in the pathway of carcinogenesis such as cetuximab and bevacizumab have demonstrated potential benefit. Bevacizumab is a recombinant humanised monoclonal antibody that targets vascular endothelial growth factor (VEGF). It is thought that bevacizumab inhibits angiogenesis (the formation of new blood vessels) by binding to VEGF. Bevacizumab is currently licensed in combination with intravenous 5-FU/LV or irinotecan plus intravenous 5-FU/LV in the first-line treatment of patients with

metastatic cancer of the colon or rectum [51, 52]. Cetuximab, a monoclonal antibody that targets a protein called the epidermal growth factor receptor (EGFR), used in combination with irinotecan, is indicated for the second- and subsequent-line treatment of EGFR-expressing metastatic colorectal cancer in patients who are refractory to irinotecan-based chemotherapy [53, 54]. EGFR is an ideal target as it is expressed in 25-77% of colorectal tumours, and it regulates cell division, repair, survival, and metastasis. [53].

Moreover, panitumumab was approved in 2006 for the treatment of EGFR-expressing metastatic colorectal cancer in patients who have failed prior therapy [55]. Panitumumab is the first fully human monoclonal antibody that binds to EGFR. Of the FDA-approved monoclonal antibodies targeting EGFR, panitumumab has a higher binding affinity, is more potent in inhibiting EGFR, has a longer half-life, and is less immunogenic than cetuximab [56].

1.1.4.2 The need of new therapeutic strategies

Despite advances in medical practices and the progress obtained with the introduction of new cytotoxic agents, survival rates in cases of colorectal cancer have changed little over the last 20 years [57]. Furthermore, for patients diagnosed with metastatic CRC, the median survival time remains below 2 years and healing is often an elusive goal. These facts highlight the need for more effective systemic therapies.

Proteomic analysis [58] and other published protein expression pattern changes in CRC suggest that alterations of the following major metabolic pathways are involved in the CRC tumorigenesis: elevated glycolysis, down-regulated gluconeogenesis, decreased glucuronate metabolism, and impaired tricarboxylic acid cycle (Krebs cycle). Furthermore, upregulation of glycolysis is an almost universal property of primary and metastatic cancers and represents a major biochemical alteration associated with malignant transformation. This alteration in cancer cell metabolism gives excellent opportunities for the development of therapeutic strategies to preferentially induce cancer cell death by targeting the glycolytic pathway [59]. In addition, cell culture studies demonstrated that glycolytic tumour cells are uniquely sensitive to inhibition of

glycolysis, unlike their normal counterparts, suggesting a potential therapeutic window [60].

1.2 Glucose Metabolism and Cancer

Well over a century ago, Pasteur first noted the inverse relationship between two modes of glucose metabolism, finding that the absence of oxygen resulted in the inhibition of oxidative phosphorylation (OXPHOS) and a switch to glycolysis for ATP generation (the 'Pasteur effect' or anaerobic glycolysis) [61]. Under normoxic conditions glucose is metabolized in mitochondria to H_2O and CO_2 producing about 36 moles of ATP per mole of glucose. Under anaerobic conditions glucose is metabolized to lactic acid producing only 2 moles of ATP per mole of glucose (**Figure 5**). Note that acid is produced by both processes but is increased in glycolytic pathways.

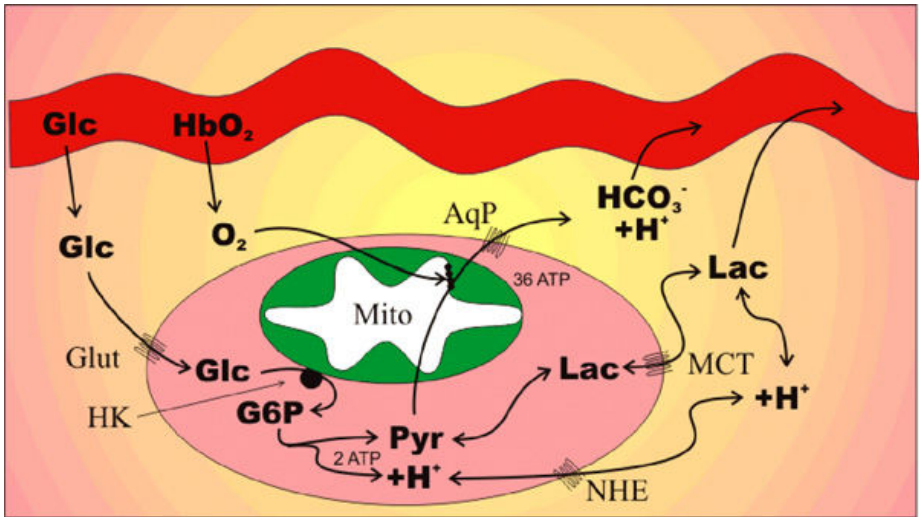

Figure 5. Transport of glucose and its metabolites in mammalian cells [Gatenby and Gillies, 2007]

Cancer cells are known to undergo a metabolic shift from oxidative to anaerobic glycolysis as a response to intratumoral hypoxia. The inefficiency of anaerobic metabolism is compensated by a several-fold increase in glucose flux. This phenomenon is now routinely exploited for tumour imaging through FDG-PET (^{18}fluorodeoxyglucose positron emission tomography) [62]. FDG injected into the

bloodstream is taken up by glucose transporters on the cell surface and then phosphorylated by hexokinases to form FDG-phosphate, thereby enabling visualization of the tissues with the greatest glucose uptake and hexokinase activity (**Figure 6**) [63]. PET has confirmed that the great majority (>90%) of human primary and metastatic tumours demonstrate increased glucose uptake indicating abnormal metabolism [64].

Figure 6. Positron-emission tomography imaging with ^{18}fluorodeoxyglucose of a patient with lymphoma. The mediastinal nodes (purple arrow) and supra clavicular nodes (green arrows) show high uptake of ^{18}fluorodeoxyglucose (FdG), showing that tumours in these nodes have high levels of FdG uptake. The bladder (yellow arrow) also has high activity, because of excretion of the radionuclide [Gatenby and Gillies, 2004].

1.2.1 Prevalence of aerobic glycolysis in cancer

In the 1920s, Otto Warburg made the surprising finding that tumour cells, unlike their normal counterparts, utilize glycolysis instead of mitochondrial oxidative phosphorylation for glucose metabolism even when in normoxia, a phenomenon historically known as the Warburg effect or aerobic glycolysis [65].

However, the proliferative advantages gained from altered glucose metabolism are far from clear. Firstly, anaerobic respiration is more than an order of magnitude less efficient than its aerobic counterpart, producing only 2 ATP per glucose in comparison to approximately 36 ATP (**Figure 5**). Secondly, glycolysis increases acid production resulting in a highly acidic extra-cellular environment [66]. This results in local toxicity including cell death and extra-cellular matrix degradation due to release of proteolytic enzymes [67]. Thus, the dynamics that leads to adoption of aerobic glycolysis as a typical component of the malignant phenotype remains unknown.

Recently, Gatenby and Gillies [68] proposed that the glycolytic phenotype initially arises as an adaptation to local hypoxia due to disordered angiogenesis and blood flow (**Figure 7**). Persistent or cyclical hypoxia subsequently induces constitutive upregulation of glycolysis even in the presence of oxygen, through mutations or epigenetic changes. Increased acid production from upregulation of glycolysis results in microenvironmental acidosis and requires further adaptation to phenotypes resistant to acid-induced toxicity. Cell populations that emerge from this evolutionary sequence have a powerful growth advantage, as they alter their environment through increased glycolysis in a way that is toxic to normal populations, but harmless to themselves. The environmental acidosis also facilitates invasion through destruction of adjacent normal cells, degradation of the extracellular matrix and promotion of angiogenesis (**Figure 7**) [68].

Increased glycolysis may also confer adaptive advantages if it allows excess pyruvate to be available for lipid synthesis or providing essential anabolic substrates, such as ribose for nucleic acid synthesis [69]. Glucose consumption through the pentose pathway may also provide essential reducing equivalents (NADPH) to reduce the toxicity of reactive oxygen species conferring resistance to senescence [70, 71].

These evolutionary advantages can explain the significant prevalence of aerobic glycolysis in human cancers. This conceptual model is supported by empirical studies that demonstrate constitutive upregulation of glycolysis is consistently observed during the transition from premalignant lesions and invasive cancer [63, 72, 73, 74]. Additionally, Smallbone *et al*. [75] developed a hybrid cellular automation approach to investigate the cell-microenvironmental interactions that mediate somatic evolution of cancer cells. That study supported the hypothesis that regional variations in oxygen, glucose and H^+ levels drive the final stages of somatic evolution during carcinogenesis.

They proposed that the phenotypic adaptations to the sequence of hypoxia-glycolysis-acidosis are necessary to form an invasive cancer [75].

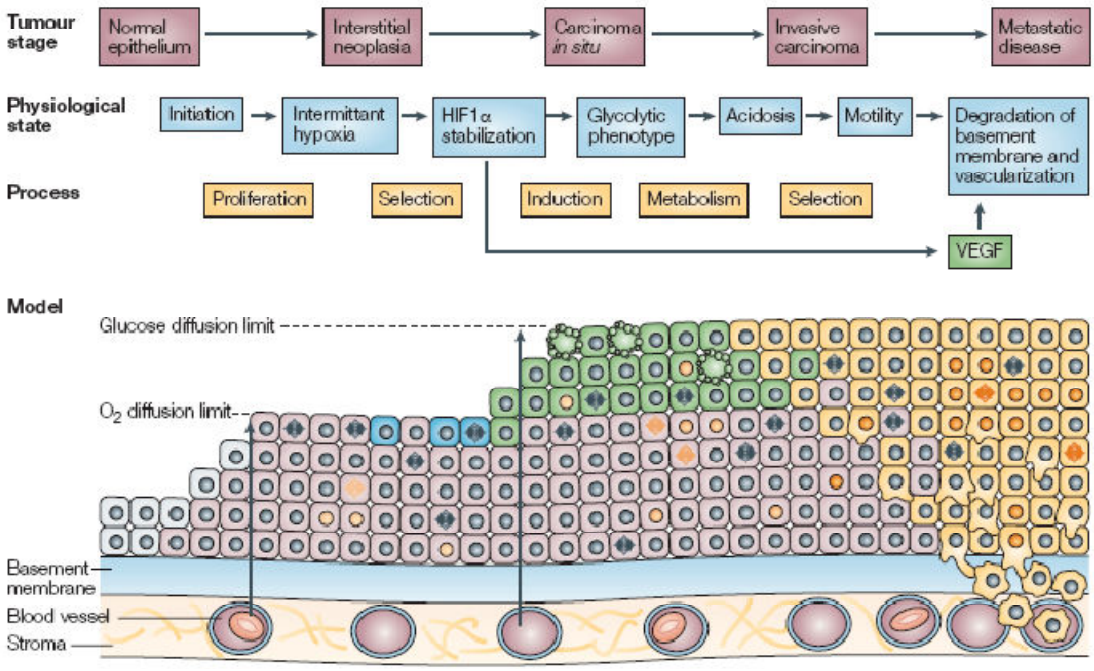

Figure 7. Model for cell–environment interactions during carcinogenesis, giving the stages of tumour growth and their associated physiological states. Normal epithelial (grey), hyperplastic (pink), hypoxic (blue), glycolytic (green) and motile (yellow) cells [Gatenby and Gillies, 2004].

These results suggest that tumour prevention strategies aimed at interrupting glycolysis and the resulting cellular adaptations could be explored to delay or prevent transition of in situ to invasive cancer. Furthermore, in advanced metastatic cancers, understanding of the molecular and physiological causes and consequences of upregulated glycolysis may lead to targeted therapies [76].

1.2.2 Molecular mechanisms

The increase in tumour glucose uptake is achieved by up-regulating the expression of glycolytic enzymes and glucose transporters [77]. The molecular basis for evolution of the glycolytic phenotype has been clarified by recent advances in

understanding the hypoxia-inducible factor, HIF-1, system [78]. Hypoxia and HIF-1 increase virtually all the enzymes in the glycolytic pathway, as well as the glucose transporters 1 and 3 (GLUT1, GLUT3) [79], and other hypoxia-related genes such as those involved in angiogenesis, iron metabolism and cell proliferation/survival (**Figure 8**) [80].

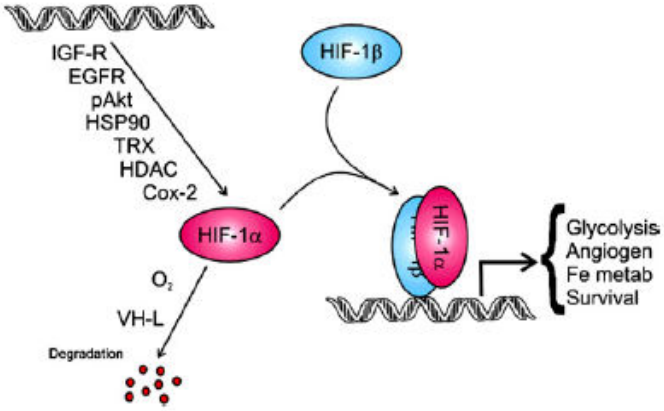

Figure 8. A summary of the HIF system demonstrating several factors which govern the level of HIF-1α and some of its protean effects on cell metabolism, survival, proliferation and their microenvironment [Gatenby and Gillies, 2007].

HIF-1 is a heterodimeric complex consisting of a hypoxically inducible subunit HIF-1α and a constitutively expressed subunit HIF-1β [81]. Although HIF-1β is constitutively expressed and its mRNA and protein are maintained at constant levels regardless of oxygen availability [82], HIF-1α protein has a short half-life ($t_{1/2}$ ~ 5 min) and is highly regulated by oxygen [83]. The transcription and synthesis of HIF-1α are constitutive and seem not to be affected by oxygen [81, 82, 84]. However, in normoxia, the HIF-1α proteins are rapidly degraded, resulting in essentially no detectable HIF-1α protein [81]. During hypoxia, HIF-1α becomes stabilized and translocates from the cytoplasm to the nucleus, where it dimerizes with HIF-1β, and the HIF complex formed becomes transcriptional active [82, 85]. The activated HIF complex then associates with hypoxia response elements (HREs) in the regulatory regions of target genes and binds the transcriptional coactivators to induce gene expression [86].

Furthermore, the glycolysis metabolic products, such as lactate and pyruvate, have been reported to cause HIF-1α accumulation under normoxia and regulate hypoxia-inducible gene expression, establishing a potential positive feedback loop which may be critical to aerobic glycolysis [87]. However, HIF activity can also be stabilized in the presence of oxygen by growth factors (**Figure 8**) that also participate in carcinogenesis, including RAS, HSP 90, Cox 2, HER, and the AKT/mTOR pathway [88]. Because of this, HIF can be characterized as being reversibly increased by physiologic stress (e.g., hypoxia) or by hormonal growth factor stimulation or as being constitutively active under normoxic conditions through heritable alterations, such as activated oncogenes.

1.2.3 Consequences of increased glucose metabolism

One consequence of this dependence on glycolysis is the need for metabolically active tumours to efflux the accumulating lactic acid to the tumour microenvironment to prevent intracellular acidosis [89, 90]. Cellular acidosis has been shown to be a trigger in the early phase of apoptosis and leads to activation of endonucleases inducing DNA fragmentation. In addition, cellular pH is crucial for biological functions such as cell proliferation [90], invasion [91], metastasis [92] and drug resistance [93]. The observation that the intracellular pH of tumours is more alkaline than that of normal tissues [94, 95], even in the presence of an acidic tumour microenvironment, suggests the existence of an active mechanism for lactate/H^+ efflux. Although several transporters including Na^+/H^+ exchangers (NHE1), vacuolar adenosine triphosphatases, anion exchangers, and lactate/H^+ symporters (monocarboxylate transporters [MCTs]) are involved in pH homeostasis in mammalian cells [89], MCTs are thought to be most important for pH regulation within rapidly metabolizing, highly glycolytic tumour cells [96, 97].

1.3 Monocarboxylate Transporters (MCTs)

MCTs are members of the SLC16 gene family [98]. This family includes 14 proteins related to each other by sequence homology. The first four members, MCT1-

MCT4 have been experimentally demonstrated to transport aliphatic monocarboxylates, a diverse group of compounds which includes many important metabolites such as lactate, pyruvate and ketone bodies. This transport is controlled by hydrogen ion and substrate gradients, thus facilitating transport of MCTs either in (influx) or out (efflux) of the cell. Lactate efflux is especially important for cells and tissues which generate large quantities of lactic acid as a result of glycolysis (e.g. red and white blood cells, skeletal muscle, and most tumour cells). Lactate influx is of value to tissues where it is being utilized (via pyruvate) as a respiratory fuel (brain, heart, red skeletal muscle), as a gluconeogenic substrate in liver, or under fasting conditions, as a glycerol-neogenic substrate in white adipose tissue [99]. Since lactate is a substrate for MCT1-MCT4 transporters, considerable attention has been given to the potential role of these transporters in the handling of lactate in tumour cells.

MCTs have 12 transmembrane domains with the N- and C-termini located in the cytoplasm [98, 100]. The transmembrane domains (TMDs) are highly conserved between isoforms with the greatest sequence variations observed in the C-terminus and the large intracellular loop between TMDs 6 and 7, which has a range of 29–105 amino acid residues (**Figure 9**) [100]. This observed variability is common to transporters with 12 TMDs and it is thought that these sequence variations are related to substrate specificity or regulation of transport activity [100, 101].

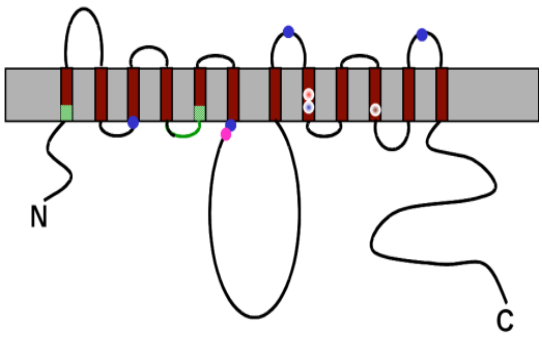

Figure 9. Proposed membrane topology of the MCT family. Adapted from Halestrap and Meredith, 2004.

Regulation of MCTs has been demonstrated to occur via transcriptional, translational and post-transcriptional mechanisms [102-104]. These regulatory pathways

appear to be age- and tissue dependent, which further complicates the understanding of these pathways [103, 104]. Some MCTs require an ancillary protein which can be involved in cellular localization [105] or protein–protein interactions [106]; however, the role of these accessory proteins in overall transporter function is not yet completely understood [105].

1.3.1 MCT1

MCT1 is the most well-studied and functionally characterized member of the MCT family, largely due to the fact that it is the only monocarboxylate transporter expressed in human erythrocytes, and it also has the widest tissue distribution. MCT1 is ubiquitously expressed but is especially prominent in heart and red muscle where it is upregulated in response to increased work, suggesting an important role in lactic acid oxidation [101, 107, 108, 109].

Transport kinetics have been thoroughly explored using lactate for this isoform and have demonstrated that it functions as a proton-dependent cotransporter/exchanger [110, 111]. Transport occurs by ordered sequential binding with association of a proton followed by lactate binding. The complex is translocated across the membrane and the lactate and proton are released sequentially. Since the transporter functions as an exchanger, transport can occur bidirectionally; however, it is primarily responsible for the uptake of substrates [101].

Relatively few studies have been conducted to assess the regulation of MCTs. Studies have indicated that altered physiological conditions and the presence of xenobiotics may alter the regulation of MCTs, in addition to altered expression at different developmental stages (112–114). MCT1 expression undergoes transcriptional, post-transcriptional and post-translational regulation and appears to be regulated in a tissue-specific manner [102-104]. In colonic epithelium, exposure to butyrate resulted in a concentration- and time dependent increase in MCT1 mRNA, protein expression and a corresponding increase in butyrate transport [115]. These data suggest the possibility of altered transcriptional regulation; however, the authors further demonstrated increased transcript stability indicating additional post-transcriptional regulation mechanisms [115]. High concentrations of lactate have also been demonstrated to increase MCT1

mRNA and protein levels in L6 cells [116]. In contrast, treatment with testosterone resulted in increased skeletal muscle MCT1 protein expression and lactate transport in the absence of mRNA changes suggesting the importance of post-transcriptional regulation [103].

MCT1 is further regulated by its association with the cell surface glycoprotein CD147 (Basigin), which has a single transmembrane domain with the C-terminus located in the cytosol (**Figure 10**) [117, 118]. Topology studies suggest that one MCT1 molecule interacts with a single CD147 molecule with subsequent dimerization with another MCT1/CD147 pair (**Figure 11**) [111]. The initial association of CD147 and MCT1 is required for the translocation of MCT1 to the plasma membrane [117]. Furthermore, more recent studies indicate that CD147 is also required for catalytic activity of MCT1 [119].

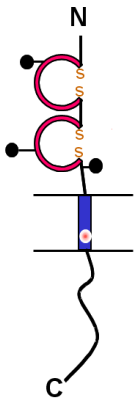

Figure 10. The proposed topology of CD147, the ancillary protein that associates with MCT1 and MCT4. Adapted from Halestrap and Meredith, 2004.

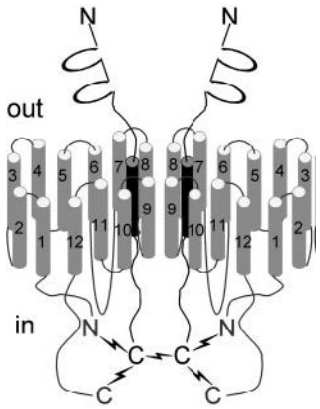

Figure 11. Hypothetical model of the topology of CD147 and MCT1 in the plasma membrane [Wilson et al, 2002].

1.3.2 MCT2

MCT2 is a high-affinity transporter for monocarboxylates and its expression under normal conditions is restricted to tissues such as the liver, kidney, and neurons that take up lactate rather than release it. In humans, expression of MCT2 is more restricted than MCT1, with the greatest expression observed in the testis [120]. In both rodents and humans, MCT2 splice variants have been detected in a species and tissue-

dependent manner suggesting that transcriptional and post-transcriptional regulation pathways play an important role in the tissue specificity of this isoform [120-122]. Similar to MCT1, MCT2 requires an accessory protein for translocation to the plasma membrane. However, MCT2 requires gp70 (EMBIGIN), not CD147 [105]. MCT2 possesses high affinity for lactate and most likely functions in the import of lactate into cells.

1.3.3 MCT3

MCT3 is believed to have the most restricted distribution of any MCT with expression in the basolateral membrane of the retinal pigment epithelium (RPE) and the choroid plexus epithelium (CPE) in humans, rodents and chickens [123-125]. However, recent studies demonstrated MCT3 expression in vascular smooth muscle cell lines [126], human aorta [126] and human kidney [127], suggesting that MCT3 mRNA may be more widely distributed than originally thought.

1.3.4 MCT4

In contrast to MCT1, MCT4 is predominantly expressed in highly glycolytic cells such as white muscle and white blood cells suggesting that its physiological function is lactate efflux [101, 128, 129]. MCT4 localization at the plasma membrane is dependent on CD147 expression, which is consistent with results obtained for MCT1 [114]. The role of MCT4 in lactate efflux is further supported by its high expression in the placenta where it is involved in the transfer of lactate into the maternal circulation [98]. While there is a great degree of overlap in the substrate specificity of MCT1 and MCT4, these two isoforms differ in their substrate affinities with MCT4 having lower affinities for a range of monocarboxylates.

1.3.5 Regulation of MCTs in cancer

MCT1 is the most widely expressed member of its family in normal tissues and is expressed, also, in a variety of human cancer cell lines and in human cancers,

including breast, head and neck, and lung cancers [130] as well as neuroblastoma [113], brain [131], alveolar sarcoma of soft tissues [132], colon [130, 133, 134], melanoma [135], pancreatic [136], cervical [137], and gastric [138] cancers.

MCT4 is expressed under normal conditions in tissues that are glycolytic in nature with resultant increased production of lactate [98]. It has low affinity for lactate, thus making it an ideal transporter for export of lactate when this metabolite is generated at high levels as occurs in glycolytic tissues. In spite of the fact that the kinetic features of MCT4 are suitable for lactate export, the expression of this transporter in cancer is controversial. Some studies have found no evidence of cancer-associated expression of MCT4 [133, 139]. However, more recent studies have demonstrated that MCT4 is upregulated in cancer and in tumour cells [114, 134, 137]. In accordance with these recent findings, the gene coding for MCT4 has been found to be upregulated by hypoxia through a HIF-1α-dependent mechanism [140].

The expression of MCT2 is reduced in tumour cells [133, 134]. Since tumour cells release lactate rather than import it, downregulation of MCT2 in tumour cells correlates well with the metabolic phenotype of tumour cells. Thus, MCT2, which is involved in the uptake of monocarboxylates into the cells in normal metabolism [141], does not appear to have an important role in highly glycolytic cancer cells. Furthermore, the gene coding for MCT2 lacks binding sites for HIF-1 [140].

Broad MCT distribution among human cancers opens promising therapeutic perspectives for the development and clinical evaluation of pharmacological MCT inhibitors. Furthermore, a number of reports have attributed to MCTs a role in lactate efflux in tumour cells with active aerobic glycolysis [113, 142, 143, 144, 145]. These studies showed further evidence of lethal intracellular acidification upon MCT inhibition *in vitro*. Thus, MCTs can constitute attractive targets for cancer therapy.

1.3.6 Inhibitors of MCTs

Since MCTs are transmembrane proteins exposed to the extracellular milieu, they are prone to targeting by systemic application of small-molecule inhibitors. Several chemicals are known to inhibit the function of MCTs (**Table 1**) [110]. Those inhibitors fall into four broad categories: (1) bulky or aromatic monocarboxylates which act as

competitive inhibitors (e.g. phenyl-pyruvate and α-cyano-4-hydroxycinnamate (CHC)); (2) amphiphilic compounds with divergent structures (e.g. quercetin and phloretin); (3) some 4,4'-substituted stilbene-2,2'-disulphonates (e.g. DIDS); and (4) miscellaneous inhibitors including thiol reagents such as p-chloromercuribenzene sulphonate (pCMBS) and amino reagents (e.g. pyridoxal phosphate and phenylglyoxal [98].

Table 1. Inhibitors of monocarboxylate transporters.

MCT Isoform	Inhibitor	References
MCT1	CHC	[147]
	pCMBS	[105]
	Phloretin	[110]
	Quercetin	[110]
	DIDS	[150]
	AR-C155858	[149]
MCT2	CHC	[147]
	Phloretin	[110]
MCT4	CHC	[147]
	pCMBS	[105]
	Phroretin	[110]
	Fluvastatin	[151]
	Atorvastatin	[151]
	Lovastatin	[151]
	Simvastatin	[151]

1.3.6.1 α-cyano-4-hydroxycinnamic acid (ACCA; CHC)

Some "classic" inhibitors of monocarboxylate transporters are derivatives of cinnamic acid, first identified by Halestrap and co-workers for their effect on isolated mitochondrial pyruvate transport [146] and by Lehninger and co-workers on intact Ehrich ascites tumour [147]. Others studies [142, 144] have indicated cinnamic acid derivates to be competitive inhibitors of lactate transport in tumours, with α-cyano-4-hydroxycinnamic acid (ACCA; CHC) (**Figure 12**) as one of the most potent inhibitors of lactate transport, with a K_i of 0.5 mM. A study [148] evaluating its therapeutic efficacy against malignant glioma indicates CHC to be an effective cytotoxic and cytostatic agent both *in vitro* and *in vivo*, but at mM concentrations (≥ 10 mM) due to its high k_i against lactate. This study on glioma cells also indicated that CHC remains

extracellular. Thus, the inhibitory effect of CHC was restricted to the glioma plasma membrane monocarboxylate transport. Intracellular transporters known to be strongly inhibited by CHC, i.e., the mitochondrial pyruvate carrier (MPC) were not affected. A recent *in vivo* study [130] demonstrated that the inhibitory effect of CHC is restricted to tumour cells expressing MCT1 at the plasma membrane. That study also showed that concentrations of CHC up to 125 mM were not toxic in mice (animal models).

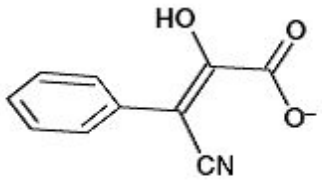

Figure 12. Structure of α-cyano-4-hydroxy cinnamic acid (CHC).

1.3.6.2 Other inhibitors

The stilbene derivatives such as 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS) act as reversible inhibitors of MCT1 in erythrocytes. Inhibition by DIDS eventually becomes irreversible on prolonged incubation, reflecting covalent modification of the transporter [150]. In addition, MCT1 and MCT4 are inhibited by thiol and amino reagents, especially the organomercurial thiol reagent p-chloromercuribenzene sulphonate (pCMBS). Interestingly it has been shown that this inhibition is not of MCT1 and MCT4 directly, but of the ancillary protein CD147 [105]. In contrast to other MCTs, lactate transport via MCT4 is inhibited by a range of statin drugs which may play a role in cytotoxicities observed with statin administration [151].

1.3.6.3 New MCT1 specific inhibitors

A group of small molecular weight compounds, synthesized by AstraZeneca, were recently identified as potent and selective inhibitors of MCT1 in activated human T cells. These compounds produced specific inhibition of human T lymphocyte

proliferation *in vitro* with nanomolar potency [149]. This study also demonstrated that the effects of MCT1 inhibitors in activated T cells, which rely on aerobic glycolysis for energy, seem to be mediated by the blockade in lactate efflux rather than by effects on pH, by transport of alternative substrates or via CD147 [149].

1.4 MCT1 and MCT4 as potential targets for CRC therapy

CRCs, in common with many other malignancies, have enhanced glucose utilization and glycolytic metabolism [152-154], producing high amounts of lactic acid, which is effluxed to the tumour microenvironment via lactate transporters, namely monocarboxylate transporters (MCTs).

There are evidences for the upregulation of MCTs in colorectal carcinomas [130, 133, 134]. Koukourakis et al. [133] reported an increase in MCT1 expression in tumour cells, which is supported by the metabolic alterations induced by anaerobic glycolysis. That study also assessed the expression of MCT2 and MCT4, finding a strong cytoplasmic expression of MCT2 in cancer cells, but a weak expression of MCT4 in the tumour environment. Pinheiro et al. [134] reported an increase in expression of MCT1, MCT2 and MCT4 in tumour cells when comparing with the adjacent normal epithelium. In that study, the authors also found a significant gain of membrane expression for MCT1 and MCT4 and loss of plasma membrane expression for MCT2 in tumour cells. Taking into consideration the lactate affinities to the different MCT isoforms, MCT1 and MCT4 expression in the membrane is in agreement with the metabolic alterations observed in colorectal cancer cells. That is, MCT1 and MCT4 need to be at the plasma membrane to export the accumulating acids in highly glycolytic tumour cells [134].

Those findings point to MCT1 and MCT4 as playing an important role in highly glycolytic CRC cells. Thus, targeting MCT1 and MCT4 activity may debilitate such tumours via disruption of aerobic glycolysis and promoting apoptosis, suggesting that MCT inhibitors may be particularly effective against CRC. Moreover, inhibiting MCTs would not only induce apoptosis due to cellular acidosis, but would also lead to reduction in tumour angiogenesis [155], invasion [91], and metastasis [92].

1.5 Aims

The first purpose of this work was to explore the role of MCTs in CRC, by evaluating the effects of MCT inhibitors in human colon carcinoma cell viability, migration, lactate efflux, and MCT expression. The second purpose was to investigate the effects of combination of CHC (MCT inhibitor) with 5-FU (conventional chemotherapy) in human colon carcinoma cell viability and migration.

Thus, the specific aims were:

- Characterization of MCT1, MCT4 and CD147 expression in colorectal carcinoma cell lines by Western blotting and immunocytochemistry.
- Evaluate the effect of MCT inhibitors on:
    a) Cell viability by MTT assay and/or trypan blue assay;
    b) Cell migration by wound-healing assay;
    c) MCT activity;
    d) MCT expression by Western blotting and immunocytochemistry.
- Investigate the effect of the combined treatment with CHC and 5-FU on:
    a) Cell viability by trypan blue assay;
    b) Cell migration by wound-healing assay.

2 Materials and methods

2.1 Cell lines

The human colon carcinoma-derived cell lines HCT-15 and Co-115 were kindly provided by Dr. Raquel Seruca, IPATIMUP, Oporto.

2.2 Chemicals

5-FU, a commonly used anti-cancer drug for the treatment of colorectal carcinoma, and CHC, a competitive inhibitor of lactate/pyruvate transporters, were purchased from Sigma-Aldrich (St. Louis, MO, USA). AR-C155858, a specific monocarboxylate transporter-1 (MCT-1) inhibitor, was synthesized and gently provided by AstraZeneca (R&D Charnwood, UK).

5-FU, CHC and AR-C155858 were dissolved in 100% dimethylsulfoxide (DMSO) at the concentrations of 2 M, 5 M and 10 mM, respectively, and further diluted in RPMI 1640 medium (Gibco, Invitrogen, USA) to the desired final concentrations. The pH of the CHC solutions were adjusted to 7.4 with NaOH and all solutions were sterilized by filtration (0.20 μm syringe filter units, Starstedt, Germany) before use. Control cells were incubated with vehicle (DMSO). The final concentration of DMSO was maintained at 0.5%.

2.3 Cell culture conditions

HCT-15 and Co-115 colon carcinoma cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Invitrogen, USA) and 1% (v/v) penicillin-streptomycin solution (Invitrogen, USA) and incubated at 37 ºC in a humidified atmosphere containing 5% CO_2. Cells were subcultured every three days and maintained in a log-phase growth.

2.4 Cell viability assays

Cell viability was measured with both MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) dye reduction [Cell Proliferation Kit I (MTT), Roche, Mannheim, Germany] and Trypan Blue dye exclusion assays.

2.4.1 MTT dye reduction assay

This assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH.

HCT-15 and Co-115 cells were seeded in 96-well plates (6×10^3/100 µl/well) and incubated at 37 ºC in a 5% CO_2 atmosphere for 24 hours. To examine the effects of the treatment with 5-FU, CHC or AR-C155858, the spent media were removed and 0.01-10 mM 5-FU, 5-25 mM CHC, or 1-10 µM AR-C155858 were added. Controls were performed with DMSO alone. After 24 hours of incubation, 10 µl MTT labelling reagent (final concentration 0.5 mg/ml) was added to each well and microplates were incubated for 4 hours at 37ºC. Subsequently, 100 µl of solubilisation solution (10% SDS in 0.01 M HCl) were added to each well for solubilisation of the purple formazan crystals and the mixture incubated at 37 ºC overnight. Spectrophotometrical absorbances were measured using a microplate reader (Model 450, Bio-Rad). The wavelength to measure absorbance of the formazan product and the reference wavelength were 570 nm and 750 nm, respectively. To determine cell viability, percent viability was calculated as (OD experiment/OD control) × 100 (%). Results are presented as mean ± SD of three independent experiments.

2.4.2 Trypan Blue dye exclusion assay

Trypan Blue is a dye which cannot enter healthy cells, but can permeate compromised membranes of dying cells. Dye exclusion was measured with 0.4% trypan blue in 0.85% saline (Invitrogen).

HCT-15 and Co-115 cells were seeded in 24-well plates (2.5×10^4/500 µl/well) and incubated at 37 ºC in a 5% CO_2 atmosphere for 24 hours. Spent media were removed and to examine the effects of the treatment with CHC, cells were treated with 5-25 mM CHC for 24 hours. To determine the effect of CHC exposure time, HCT-15 cells were treated with 10 mM CHC for 6-48 hours. To address the effects of 5-FU and CHC co-treatment, cells were treated with 1 mM 5-FU (IC50) and increasing concentrations of CHC (5-25 mM) for 24 hours. Controls were performed with DMSO alone. The supernatant from each well was recovered and 75 µl trypsin (0.05% trypsin, 0.53 mM ethylenediamine tetra-acetic acid·4Na, Invitrogen) was added to each well and incubated at 37ºC for 10-20 minutes. Subsequently, 150 µl of fresh medium was added to quench the trypsin activity, and cells were recovered and pooled with the culture supernatant. Aliquots of the cell suspensions were diluted 1:1 in trypan blue solution. Clear and blue cells were counted with a hemocytometer under a bright-field microscope, and the viable fraction was estimated by dividing the number of clear cells by the total number of cells. Results are presented as mean ± SD of three independent experiments.

2.5 Western blotting

The characterization of MCT1, MCT4 and CD147 protein expression in untreated HCT-15 and Co-115 cells and HCT-15 CHC treated cells were evaluated by western blotting.

HCT-15 and Co-115 cells were cultured in 6-well plates at a density of 3×10^5 cells per well and incubated at 37 ºC in a 5% CO_2 atmosphere for 48 hours. To evaluate the effect of CHC on MCT1, MCT4 and CD147 protein expressions, HCT-15 cells were treated with 10 mM CHC and incubated for further 24 hours. Controls were performed with DMSO alone. Cells were washed with PBS and quickly scraped from the well with 50 µl lysing buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1% Triton X-100, 1% Nonidet P-40), supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany), and homogenized 10 minutes on ice. Cells were then centrifuged at 3000 rpm, for 15 minutes at 4ºC, to remove cell debris. Supernatants were collected and protein concentrations were quantified using a Bio-Rad DC protein assay (Bio-Rad

Laboratories, Inc., Hercules, CA, USA). BSA was used as a protein standard. Twenty micrograms of total protein from each cell lysate were separated on a 10% (w/v) polyacrylamide gel and electrotransferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked with 0.1% Tween-20, 5% (w/v) non-fat dry milk in TBS for 1 hour at room temperature. Membranes were then incubated overnight at 4ºC with primary polyclonal antibodies for MCT1 (AB3538P, Chemicon International, Temecula, CA, USA), MCT4 (AB3316P, Chemicon International), and CD147 (18-7344, Zymed, Invitrogen, Carsbad, CA, USA) diluted 1:200 for MCT1 and MCT4, and 1:750 for CD147, in TBS, 0.1% Tween-20 and 1% (w/v) non-fat dry milk. After washing, membranes were incubated 1 hour at room temperature with secondary antibody conjugated with IgG horseradish peroxidise (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:10 000 in TBS, 0.1% Tween-20 and 1% (w/v) non-fat dry milk. Subsequently, immunoreactive bands were visualized by chemiluminescence (Supersignal West Femto kit, Pierce, Rockford, IL, USA). Band area intensity was quantified using ImageJ/Java, a free software downloaded from http://rsb.info.nih.gov/ij/. ß-actin was used as loading control.

2.6 Immunocytochemistry

Characterization of MCT1 and MCT4 protein expression in untreated HCT-15 and Co-115 cells and HCT-15 CHC treated cells were evaluated by immunocytochemistry.

HCT-15 and Co-115 cells were grown on coverslips at 30% confluence. To evaluate the effect of CHC in MCT1 and MCT4 protein expression, HCT-15 cells were treated with 10 mM CHC for 24 hours at 37 ºC. Controls were performed with DMSO alone. Untreated and treated cells grown on coverslips were fixed with PBS containing 4% (w/v) paraformaldehyde for 15 minutes at room temperature and then permeabilized in PBS containing 0.05% Triton X-100 for 4 minutes. Cells were incubated with 0.3% hydrogen peroxide in methanol for 30 minutes, to inactivate endogenous peroxidases, and washed in PBS. Cells were then incubated for 20 minutes with Normal Horse Serum (Vector, Burlingame, CA, USA), to block non-specific binding, and incubated

overnight at room temperature with primary polyclonal antibodies for MCT1 (AB3538P, Chemicon International) and MCT4 (AB3316P, Chemicon International), diluted 1:200. Cells were then sequentially washed in PBS and incubated with biotinylated universal secondary antibody (Vector) for 30 minutes, R.T.U. Vectastain ABC elite reagent (Vector), for 45 minutes at 37 ºC and developed with 3,3'- Diamino-benzidine (DAB+ Substrate System, DakoCytomation, Carpinteria, CA, USA) for 10 minutes. Cells were counterstained with haematoxylin for 5 seconds and coverslips were permanently mounted on slides using an aqueous solution (Biomeda corp., Foster City, CA, USA). Images were acquired in an Olympus BX 61 microscope (Tokyo, Japan).

2.7 Lactate determination

An enzymatic colorimetric assay (Lactate, LO-POD. Enzymatic colorimetric, Spinreact, S.A.U., St. Esteve de Bas, Spain) was used for lactate determination in CHC treated and untreated cell culture media.

HCT-15 cells were seeded in 24-well plates (2.5×10^4/500 µl/well) and incubated at 37 ºC in a 5% CO_2 atmosphere for 24 hours. To examine the effects of the treatment with CHC, spent media was removed and cells were incubated with 10 mM CHC. Controls were performed with DMSO alone. Supernatant was collected at 2, 6, and 24 hours and assayed using the enzymatic colorimetric assay. In 96-well plates, 2 µl of each sample were mixed with 200 µl of working reagent and incubated for 10 min. at room temperature (15-25ºC). Spectrophotometrical absorbances were measured at 490 nm using a microplate reader (Model 450, Bio-Rad). Results are presented as mean ± SD of three independent experiments.

2.8 Wound-healing assay

In vitro wound healing assays were used to assess the effect of 5-FU and CHC, alone and in combination, on HCT-15 cell migration. This method mimics cell migration during wound healing *in vivo*.

HCT-15 cells were seeded in 12-well plates (4.5×10^5/well) in RPMI containing 10% FBS and grown until 90% confluent. Cells were then starved for 24 hours, and two linear wounds were scratched with a p200 pipette tip. Cells were then washed with PBS and to examine the effect of the treatment with CHC alone, cells were treated with 5 - 15 mM CHC. To address the effects of 5-FU and CHC co-treatment, cells were treated with the combination 1mM 5-FU and 10 mM CHC. Controls were performed with DMSO alone. Wounds were photographed using a microscope coupled with a camera at 0 and 24 hours post-treatment. Wound regions were marked and photographed at the same region each time. The images acquired for each sample were analyzed quantitatively using the ImageJ/Java software (http://rsb.info.nih.gov/ij/). Wound sizes were measured at five distinct sites from the microphotographs taken at 24 h and expressed as a percentage of the value at 0 h. Results are presented as mean ± SD of three independent experiments.

2.9 Statistical analysis

Data were expressed as mean ± standard deviation (SD). One-way ANOVA, followed by Bonferroni post test was used to perform statistical analysis, using GraphPad Prism 4.0 software, and *P*-values <0.05 were considered statistically significant in all experiments.

3 Results

3.1 Assessment of MCT1, MCT4 and CD147 expression in colon carcinoma cells

Western blotting and immunocytochemistry were performed to assess MCT1/MCT4 expression in HCT-15 and Co-115 colon carcinoma cell lines. Total protein extracts from HCT-15 and Co-115 colon carcinoma-derived cell lines were profiled. Western blot analysis demonstrated that HCT-15 and Co-115 cells expressed both MCT1 and MCT4 (**Figure 13**). Protein bands were detected around 50 kDa, which is consistent with the molecular weight described for these two MCT isoforms [131].

Functional MCT transporter complexes require association with ancillary proteins. It was reported that CD147 forms complexes with MCT1 and MCT4, which is essential for MCT membrane expression and catalytic activity [117]. Thus, expression of CD147 was also evaluated. Western analysis demonstrated that both HCT-15 and Co-115 cells expressed CD147 (**Figure 14**). Both high glycosylated (HG) CD147 and low glycosylated (LG) CD147 were detected in the blots.

HCT-15 and Co-115 cells were also analyzed for MCT1 and MCT4 immunocytochemical expression. MCT1 and MCT4 were present in both HCT-15 and Co-115 cell lines (**Figure 14**). MCT1 and MCT4 were expressed in the cytoplasm of both cell lines, however, a plasma membrane expression of MCT1 was only observed in HCT-15 cells (**Figure 14 A**).

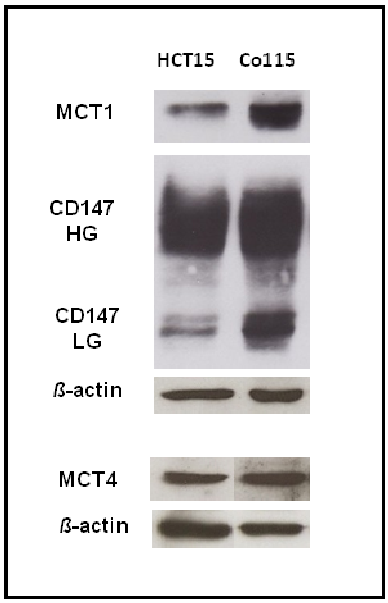

Figure 13. Western blot analysis of MCT1, MCT4 and CD147 in HCT-15 and Co-115 colon carcinoma cell lines. *β*-actin was used as internal loading control.

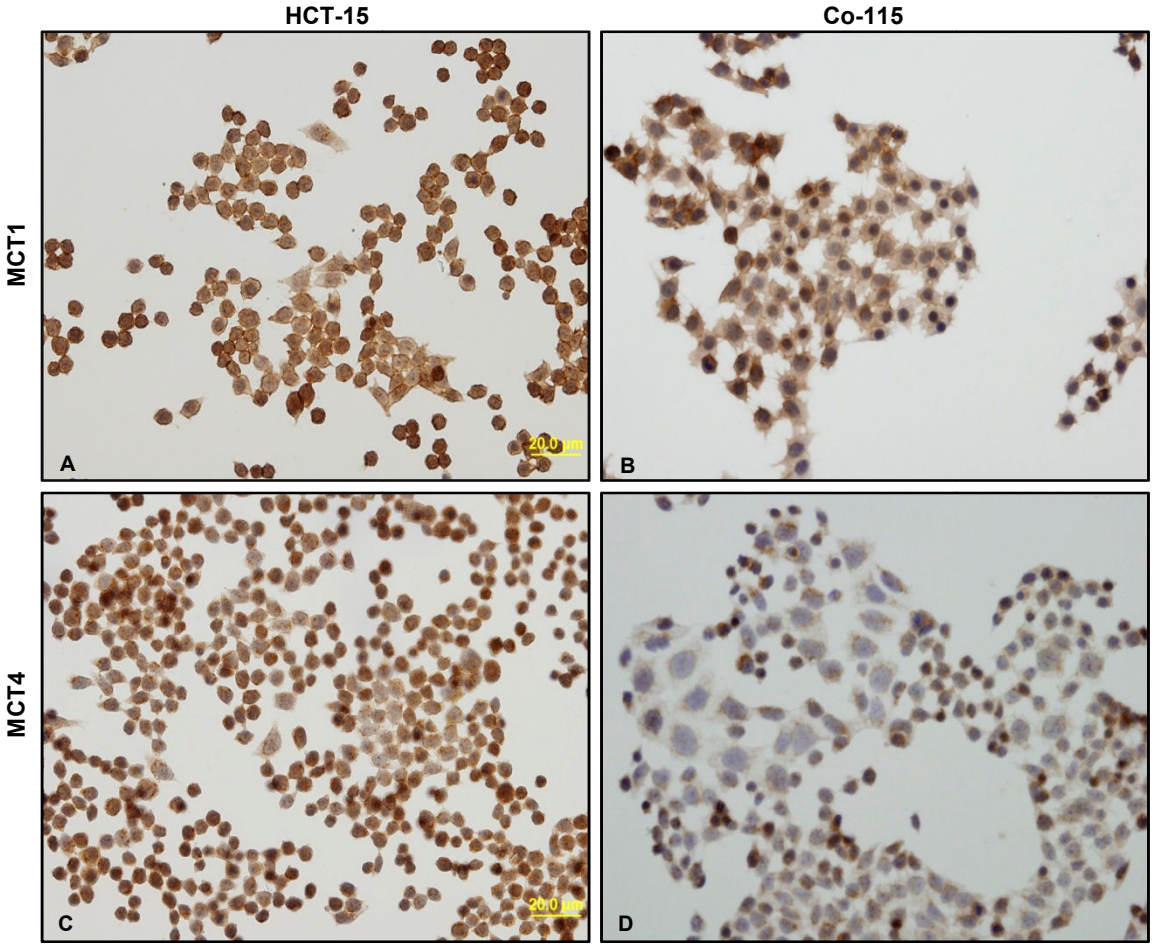

Figure 14. Immunocytochemical expression of MCT1 and MCT4 in HCT-15 and Co-115 colon carcinoma cells. MCT1 plasma membrane staining (A) was observed in HCT-15 cells.

3.2 Effect of CHC on the viability of colon carcinoma cells

CHC, a competitive inhibitor of MCTs, has an inhibitory effect in the viability of some cancer cells [130, 144]. To evaluate the effect of CHC on the viability of HCT-15 and Co-115 colon carcinoma cells, both cell lines were treated with different concentrations of CHC (5-25 mM) and cell viability was assessed by MTT assay.

CHC was able to decrease both HCT-15 and Co-115 cell viability in a dose-dependent manner (**Figure 15**). When treated with 5-25 mM CHC, the viability of HCT-15 cells decreased from 95.3% to 4.0% (**Figure 15 A**). In Co-115 cells, the viability of cells for the same concentrations decreased from 93.1% to 34.0% (**Figure 15 B**). A significant decrease in the percentage of viable cells was observed for HCT-15 and Co-115 cells treated with 15 mM, 20 mM, and 25 mM CHC ($p<0.05$). The CHC concentrations responsible for 50% growth inhibition (IC50) were 16.77 mM and 19.24 mM for HCT-15 and Co-115 cells, respectively.

MTT dye reduction assay was reported to give a fourfold higher level of viability than trypan blue dye exclusion assay in MCT-target cells [141]. Thus, cell viability of CHC-treated cells was further evaluated by trypan blue assay to compare with MTT assay data. In general, trypan blue assays indicated lower cell viabilities when compared with MTT assays (**Figure 15 C** and **D**). When treated with 5-25 mM CHC, the viability of HCT-15 cells decreased from 78.0% to 4.6% (**Figure 15 C**). In Co-115 cells, the viability of cells for 10-25 mM CHC decreased from 73.5% to 32.0% (**Figure 15 D**). A significant decrease in HCT-15 and Co-115 cell viability could be observed when treated with 10 mM to 25 mm CHC ($p<0.05$). The inhibitory effect of CHC was higher in HCT-15 cells when compared with Co-115 cells, being the CHC IC50 values 9.99 mM and 16.18 mM for HCT-15 and Co-115 cells, respectively.

The influence of CHC exposure time was also evaluated in HCT-15 cells. The trypan blue assay was used since it indicated lower viabilities when compared with MTT assay in CHC-treated cells. A significant decrease in cell viability was achieved from 12 to 48 hours ($p<0.05$) of treatment with 10 mM CHC (**Figure 16**). The lowest percentage in viable cells was obtained after 24 hours of exposure.

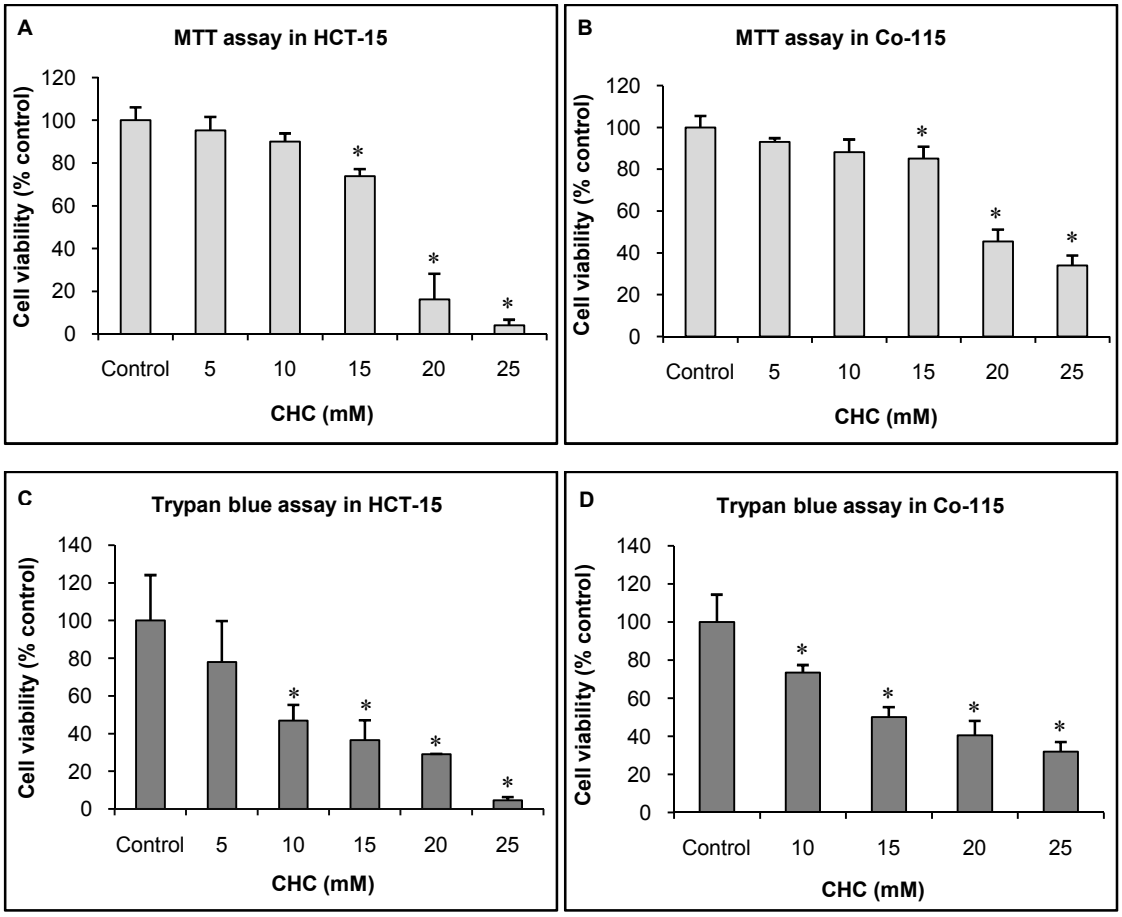

Figure 15. Effect of CHC on colon carcinoma cell viability. HCT-15 (A and C) and Co-115 (B and D) colon carcinoma cells were incubated with different CHC concentrations for 24 hours. Cell viability was determined by MTT assay (A and B) and trypan blue assay (C and D). Values are means ± SD of three independent experiments performed in triplicates. *p<0.05, compared to control.

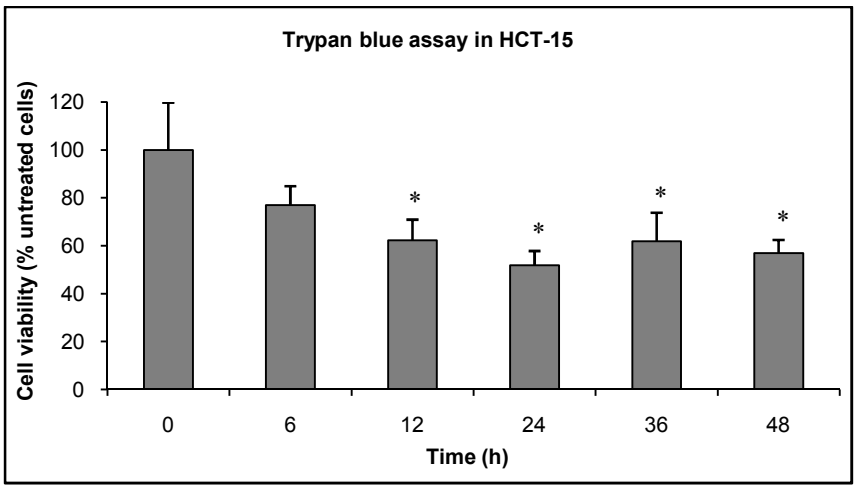

Figure 16. Effect of CHC on colon carcinoma cell viability. HCT-15 colon carcinoma cells were incubated with 10 mM CHC up to 48 hours. Cell viability was determined by trypan blue assay. Values are means ± SD of one experiment performed in triplicate. *p<0.05, compared to 0 hours.

3.3 Effect of AR-C155858 on the viability of colon carcinoma cells

The effect of AR-C155858, a specific inhibitor of MCT1, on the viability of HCT-15 and Co-115 was also tested. Twenty-four hours after exposure to AR-C155858, cell viability was assessed by MTT assay. Incubations with 1 μM, 5 μM, and 10 μM AR-C155858 did not have any effect on the viability of HCT-15 and Co-115 cells (**Figure 17**). Analysis by light microscopy also revealed no changes in morphology and cell number in AR-C155858 treated cells when compared with untreated cells (**data not shown**), confirming the results obtained in MTT assay.

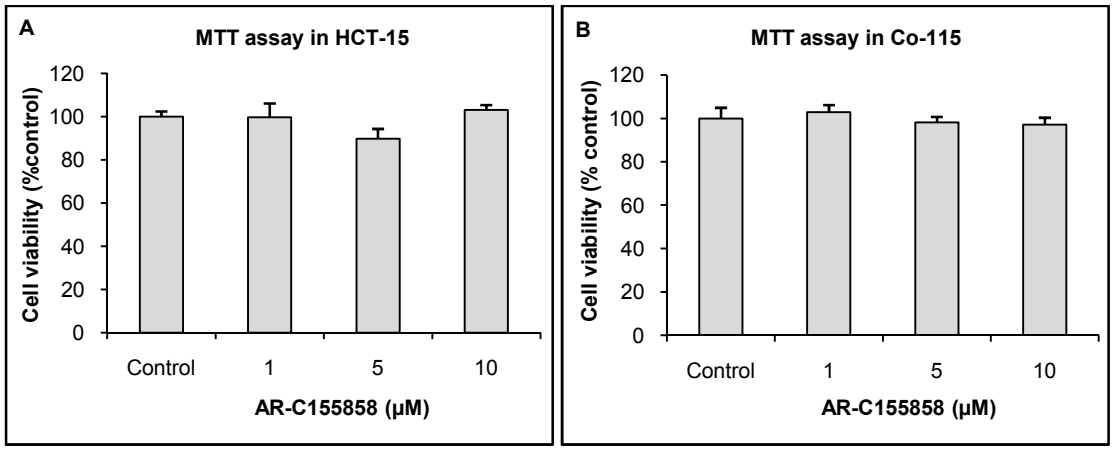

Figure 17. Effect of AR-C155858 on colon carcinoma cells viability. HCT-15 (A) and Co-115 (B) colon carcinoma cells were incubated with different AR-C155858 concentrations for 24 hours. Cell viability was determined by MTT assay. Values are means ± SD of three independent experiments performed in triplicates.

3.4 Effect of CHC on HCT-15 cell migration

To evaluate the influence of CHC on the migration capacity of HCT-15 cells (more sensitive to CHC) a wound-healing assay was performed. Cells were treated with different concentrations of CHC (5-15 mM) for 24 hours and the wound size was compared between untreated (control) and CHC treated cells. As shown in **Figure 18** and **19**, after treatment with 10 mM or 15 mM CHC (24 hours) the wound size was 47.6% ± 8.4% and 52.9% ± 5.2%, respectively, whereas in the untreated control, the wound was 24% ± 6.6% of its original size. Thus, a significant increase in wound size was observed for CHC treated cells (10, 15 mM) in comparison to control ($p<0.05$).

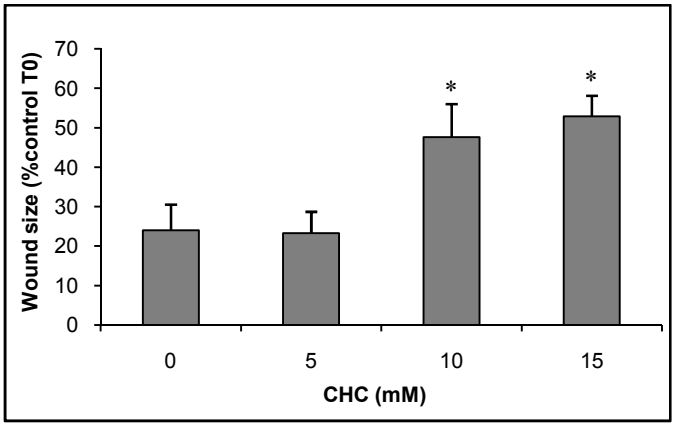

Figure 18. Migration of HCT-15 cells in response to 5-15 mM CHC was measured in a wound-healing assay. The width of the wound was measured at five distinct sites from the microphotographs taken at 24 h and expressed as percentage of the value at 0 h. Data are means ± SD of three independent experiments, each performed in triplicate. *$p<0.05$, compared to 0 mM CHC.

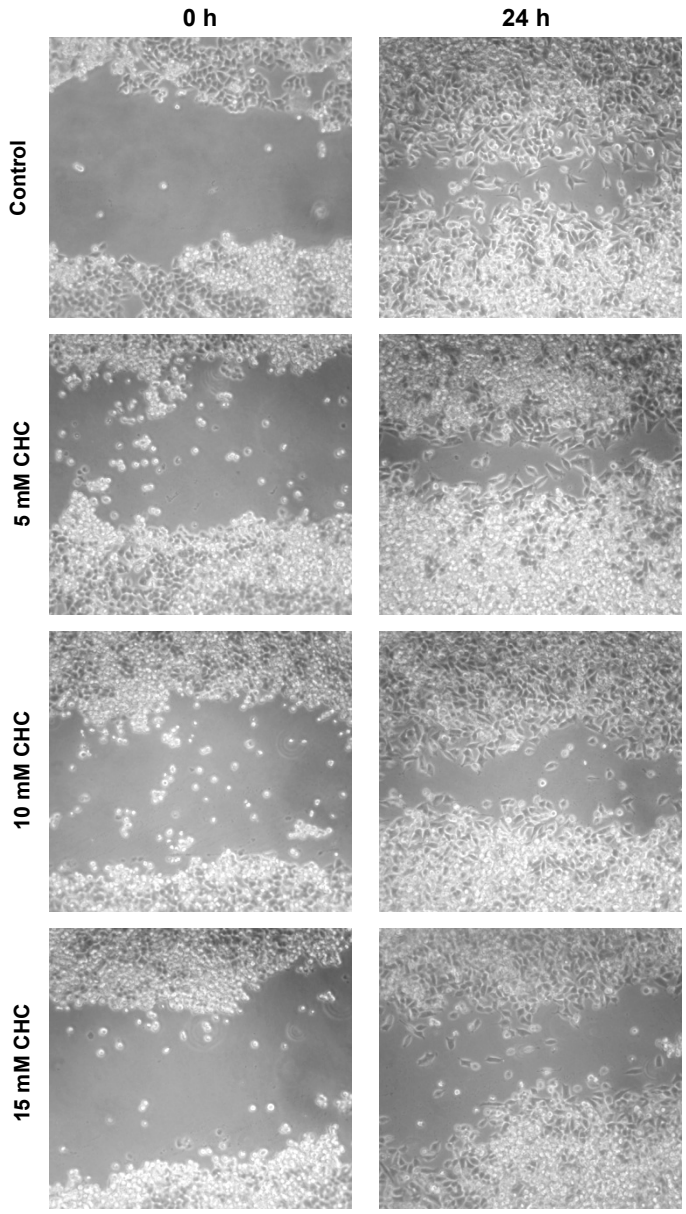

Figure 19. Cell migration *in vitro* in the presence of CHC. Representative images of HCT-15 control cells (untreated) and HCT-15 cells after exposure to 5 mM, 10 mM and 15 mM CHC for 24 hours. Magnification x200.

3.5 Effect of CHC on HCT-15 cells lactate export

Glycolysis is substantially enhanced in tumour cells compared with their non-tumorigenic counterparts and increased glycolysis leads to excessive glucose consumption and lactate production [76]. Lactate produced is excreted through the plasma membrane by lactate transporters, namely MCTs and CHC is known to inhibit its function. To determine the effect of CHC on lactate export, HCT-15 cells were treated with 10 mM CHC for 24 hours. Extracellular lactate concentration was assayed in the supernatant of untreated (control) and treated cells at 2, 6, and 24 hours. As shown in **Figure 20**, treatment with 10 mM CHC for 24 hours resulted in a significant decrease in extracellular lactate concentration as compared to control ($p<0.05$). A 40% reduction of extracellular lactate was observed, suggesting that CHC inhibited lactate export in HCT-15 cells.

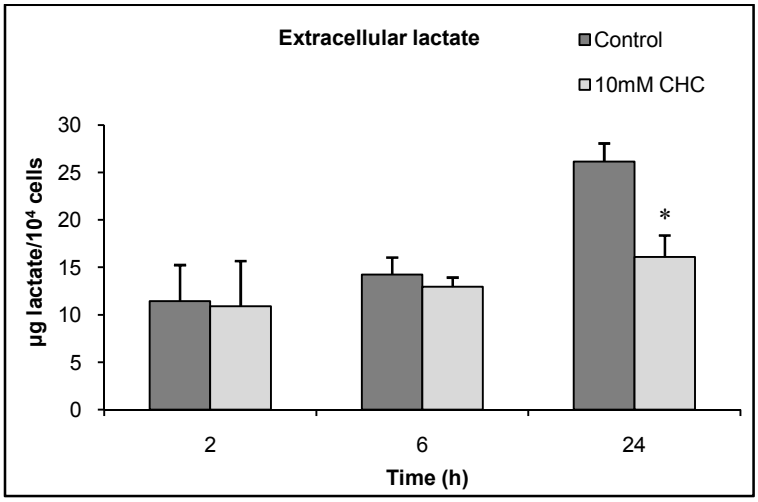

Figure 20. Effect of CHC on lactate export in colon carcinoma cells. Concentration of lactate was assessed in supernatant of HCT-15 cells, untreated (control) and treated with 10 mM CHC, over a period of 24 hours at the time points indicated. Values are means ± SD of three independent experiments performed in triplicates. *$p<0.05$, compared to control at each time point.

3.6 Effect of CHC on MCT1, MCT4 and CD147 protein expression levels in HCT-15 cells

To check if the effects of CHC reflect directly on MCT1, MCT4 and CD147 protein expression levels, expression of these proteins was assessed by western blot and immunocytochemistry in HCT-15 cells untreated and treated with 10 mM CHC for 24 hours. As shown in **Figure 21**, by western blot, CHC decreased the expression of MCT1 but not both MCT4 and CD147. The decrease of MCT1 expression in CHC treated cells was of 40% compared with untreated (control) cells.

The effect of CHC on MCT1 and MCT4 protein expressions was also analyzed by immunocytochemistry. MCT1 and MCT4 immunoexpressions were apparently similar in untreated and CHC treated cells (**Figure 22**). In contrast to the results from western blot the decrease of MCT1 expression in CHC treated cells was not evident. Furthermore, the decrease of MCT1 plasma membrane expression was not observed either.

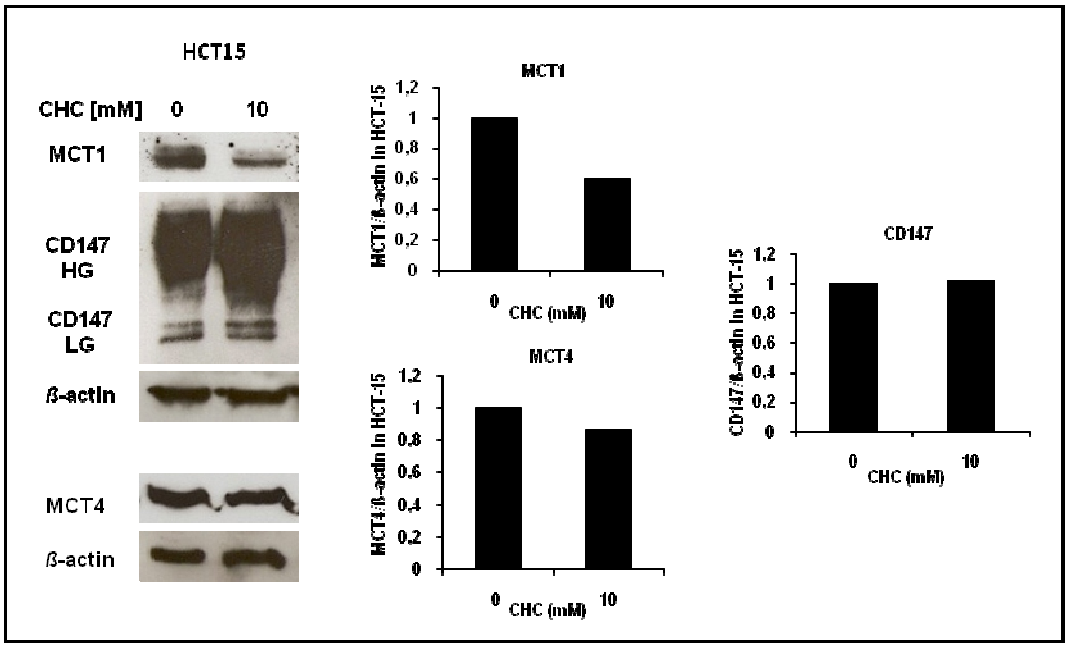

Figure 21. Effect of treatment with 10 mM CHC (24 hours) on MCT1, MCT4 and CD147 expressions in HCT-15 cells, using western blot. *β*-actin was used as loading control.

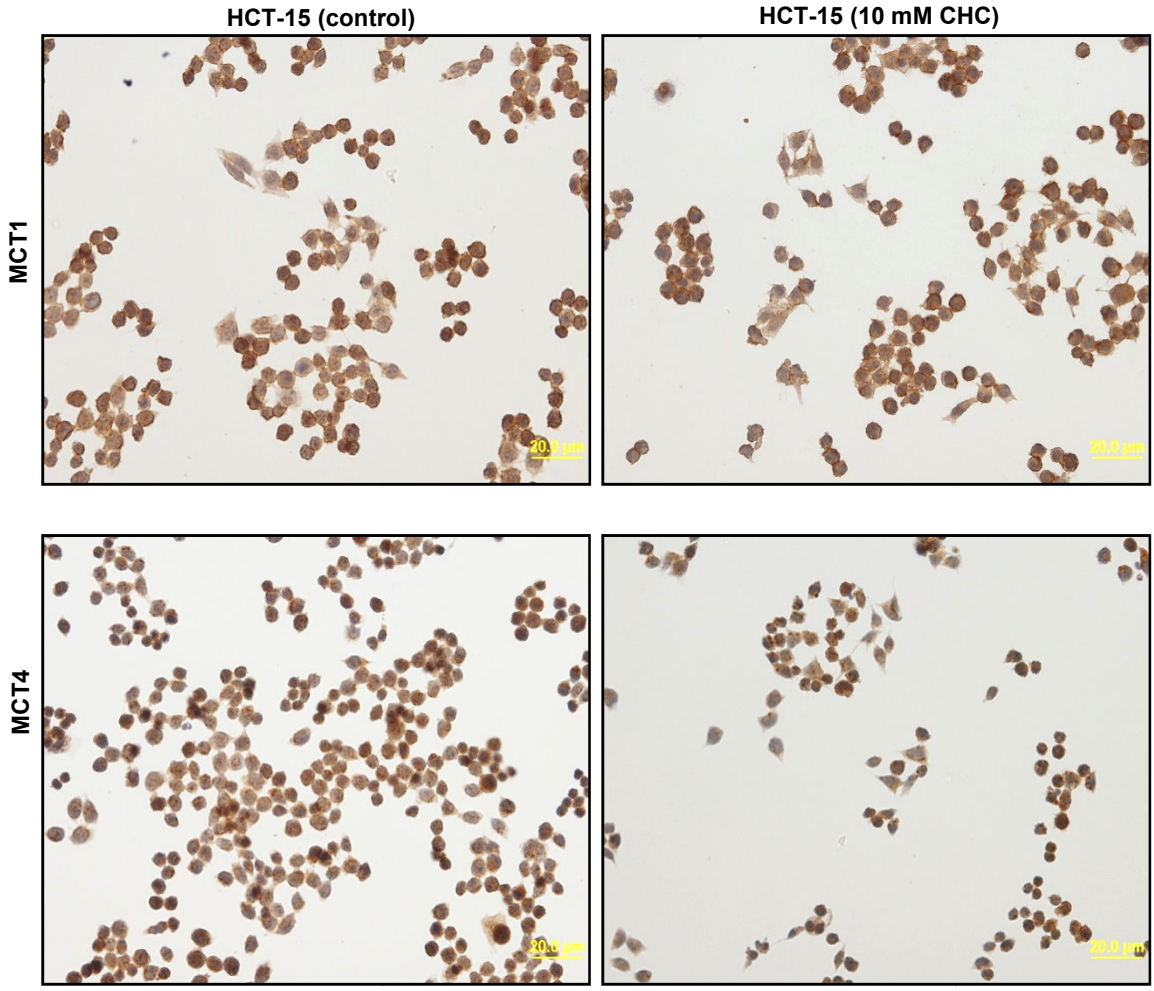

Figure 22. Immunicytochemical expression of MCT1 and MCT4 in the presence of CHC. Representative images of HCT-15 control cells (untreated) and HCT-15 cells after exposure to 10 mM CHC for 24 hours.

3.7 Effect of CHC and 5-FU combined treatment on the viability of colon carcinoma cells

To address the question whether CHC may potenciate the cytotoxic effect of 5-FU in colon carcinoma cells, the effect of combined treatment with CHC and 5-FU on the viability of HCT-15 and Co-115 cells was assessed. Thus, as a first step, IC50 values were determined for HCT-15 and Co-115 cells treated with 5-FU and then, the effect of 5-FU and CHC co-treatment on viability of both cell lines was evaluated.

3.7.1 Effect of 5-FU on the viability of HCT-15 and Co-115 cells

To assess the cytotoxic effect of 5-FU, various concentrations of 5-FU were added to the media of HCT-15 and Co-115 colon carcinoma cells. Twenty-four hours after exposure to 5-FU, cell viability was assessed by MTT assay. 5-FU decreased HCT-15 and Co-115 cell viability in a dose-dependent manner (**Figure 23**). When treated with 0.01-10 mM 5-FU, the viability of HCT-15 cells decreased from 79.0% to 35.0% (**Figure 23 A**). HCT-15 cells treated with 0.01 to 10 mM 5-FU presented a significant decrease in the percentage of viable cells relative to control ($p<0.05$). In Co-115 cells, the viability of cells for the same concentrations of 5-FU decreased from 82.1% to 34.4% (**Figure 23 B**). Treatment of Co-115 cells with 0.1 to 10 mM 5-FU induced a significant decrease in cell viability ($p<0.05$). The cytotoxic effect of 5-FU was similar in both cell lines, being the 5-FU IC50 values 1.6 mM and 1.5 mM for HCT-15 and Co-115 cells, respectively.

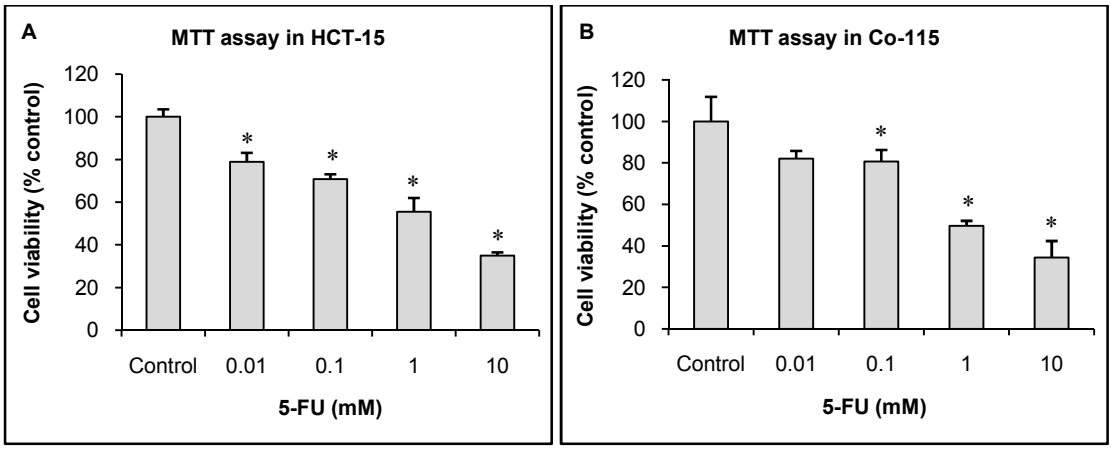

Figure 23. Effect of 5-FU on colon carcinoma cell viability. HCT-15 (A) and Co-115 (B) colon carcinoma cells were incubated with different 5-FU concentrations (0.01-10 mM) for 24 hours. Cell viability was determined by MTT assay. Values are means ± SD of three independent experiments performed in triplicate. *$p<0.05$, compared to control.

3.7.2 Effect of 5-FU combined with CHC on the viability of HCT-15 and Co-115 cells

To evaluate the effect of the combined treatment with 5-FU and CHC on cell viability, HCT-15 and Co-115 cells were treated with 1 mM 5-FU (IC50) and different concentrations of CHC. Twenty-four hours after treatment, cell viability was assessed by trypan blue assay.

CHC increased the cytotoxic effect of 5-FU in HCT-15 and Co-115 cell viability in a dose-dependent manner (**Figure 24**). Treatment of HCT-15 cells with 1 mM 5-FU combined with CHC decreased significantly the percentage of cell viability compared with 1 mM 5-FU alone for all concentrations tested ($p<0.05$). Co-115 cells treated with 1 mM 5-FU combined with CHC presented a significant decrease in the percentage of viable cells relative to 1 mM 5-FU alone only for 25 mM CHC ($p<0.05$). Thus, the cytotoxic effect of the combined treatment (5-FU plus CHC) was more evident for HCT-15 than for Co-115 cells.

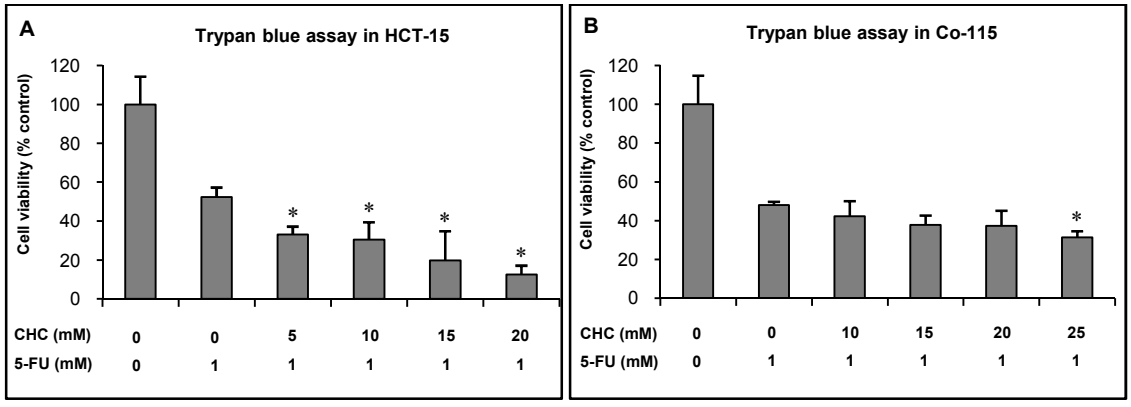

Figure 24. Effect of 5-FU + CHC on colon carcinoma cell viability. HCT-15 (A) and Co-115 (B) colon carcinoma cells were incubated with 1 mM 5-FU and different CHC concentrations for 24 hours. Cell viability was determined by Trypan blue assay. Values are means ± SD of three independent experiments performed in triplicates. *$p<0.05$, compared to 1 mM 5-FU (0 mM CHC).

3.8 Effect of CHC and 5-FU combined treatment on HCT-15 cell migration

In order to assess the effect of combined treatment of 5-FU and CHC on cell migration, a wound-healing assay was performed. HCT-15 cells were treated with 1 mM 5-FU and 10 mM CHC, alone and in combination, for 24 hours. As shown in **Figure 25** and **26**, after incubation with either 10 mM CHC or 1 mM 5-FU, a significant increase in wound size was observed in comparison to control ($p<0.05$). After treatment with 10 mM CHC or 1 mM 5-FU, the wound size was 47.6% ± 8.4% and 57.6% ± 2.4%, respectively, whereas in the untreated control, the wound was 24% ± 6.6% of its original size. However, with the combined treatment with 5-FU and CHC, the wound size did not increase significantly when compared with either 5-FU or CHC alone.

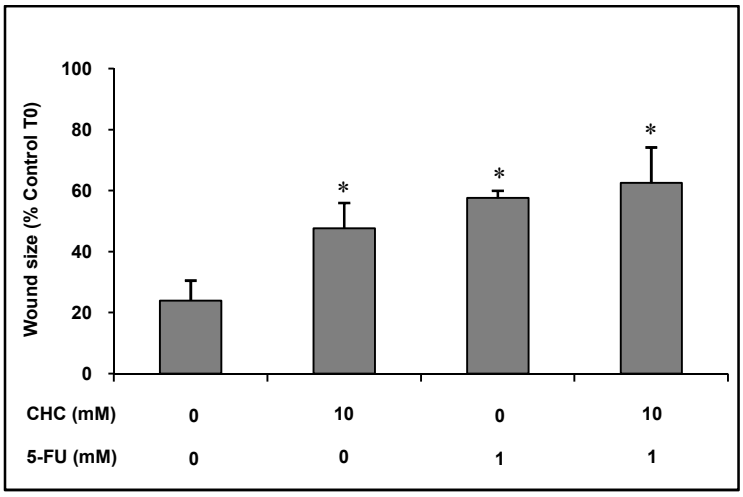

Figure 25. Migration of HCT-15 cells in response to 10 mM CHC and/or 1 mM 5-FU was measured in a wound-healing assay. The width of the wound was measured at five distinct sites from the microphotographs taken at 24 h and expressed as a percentage of the value at 0 h. Data are the means ± SD of three independent experiments each performed in triplicate. *$p<0.05$, compared to control (no treatment).

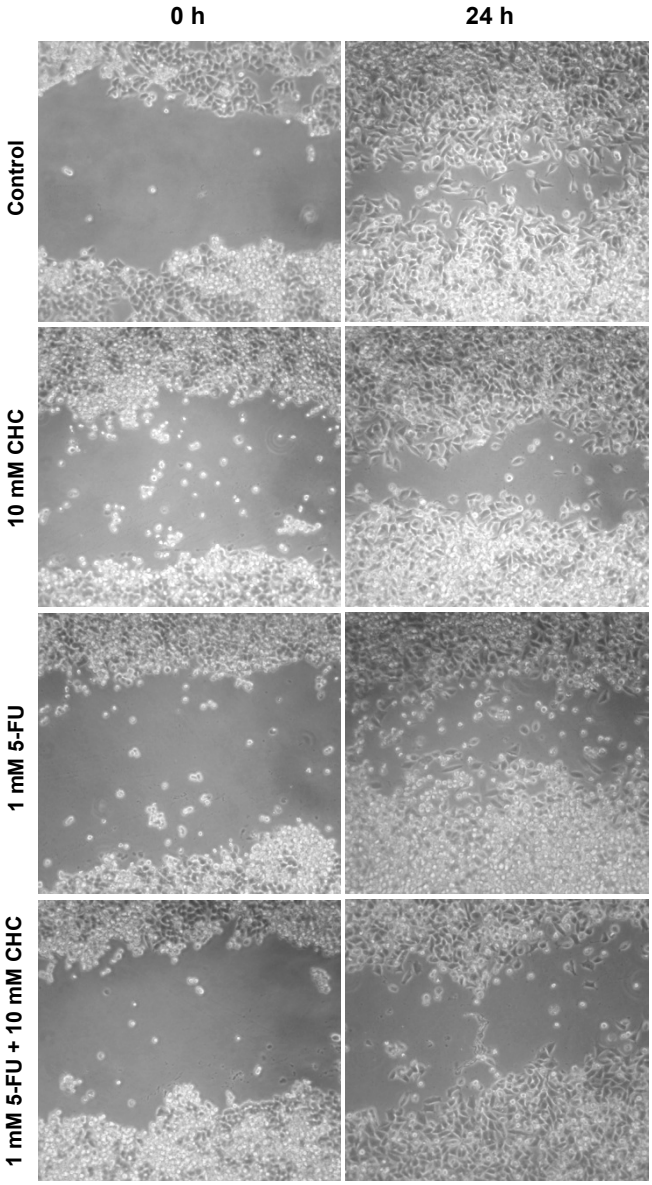

Figure 26. Cell migration *in vitro* in the presence of 5-FU and/or CHC. Representative images of HCT-15 control cells (untreated) and HCT-15 cells after exposure to 10 mM CHC and 1 mM 5-FU, alone and in combination, for 24 hours. Magnification x200.

4 Discussion

Similar to most malignant tumours, colorectal carcinoma (CRC) is highly glycolytic, producing large amounts of lactic acid, which is effluxed to the tumour microenvironment via lactate transporters, namely monocarboxylate transporters (MCTs). Thus, inhibition of lactate efflux in these tumours to cause internal acidification presents an attractive therapeutic approach. The consequent increase in extracellular pH will also lead to reduction in tumour angiogenesis [155], invasion [91], and metastasis [92]. Some findings point to MCT1 and MCT4 as playing important roles in highly glycolytic CRC cells [133, 134]. Thus, MCT inhibitors may be particularly effective against those tumours. CHC, a low affinity competitive inhibitor of MCTs, has been reported to have an inhibitory effect in cell viability of highly glycolytic tumours [130, 148]. AR-C155858 was recently identified as a potent and selective inhibitor of MCT1 in activated T cells [149]. In this study, AR-C155858 showed a potent inhibitory effect on T lymphocyte proliferation *in vitro* and *in vivo*, resulting in significant suppression of alloimmune responses in immune-mediated animal models of disease [149]. However, there are no reports on the effect of this compound in cancer. AR-C155858 was provided by AstraZeneca for the present study.

In this work, the effects of CHC and AR-C155858 were studied in two different human colon carcinoma cell lines. First, MCT1, MCT4 and CD147 expressions were assessed in both colon carcinoma cell lines and after demonstrating their expression in both HCT-15 and Co-115 cells, the effect of CHC and AR-C155858 on cell viability was assessed. Both MTT and trypan blue assays indicated that CHC decreased the viability of both HCT-15 and Co-115 cell lines in a dose-dependent manner, although a higher level of viability was indicated by MTT assay. Mathupala et al [145] also observed a disparity between the two cell viability assays in MCT-targeted cells. They then assumed that the higher level of viability obtained by MTT may indicate an increased level of NADH in MCT-targeted cells, resulting from changes in the pyruvate-lactate equilibrium that follow accumulating intracellular lactate levels. From these findings and considering the results of Mathupala *et al* [145], it was decided to use trypan blue assay in subsequent CHC experiments. The inhibitory effect of CHC was higher in HCT-15 cells than in Co-115 cells, as indicated by the trypan blue assay. It

was reported that the antitumour efficacy of CHC, known to inhibit MCT1 with approximately 10-fold selectivity compared to other MCTs, is restricted to tumour cells expressing MCT1 at the plasma membrane [129, 130]. In this study, we observed that MCT1 was expressed in the cytoplasm of both cell lines but only in the plasma membrane of HCT-15 cells. Thus, the presence of MCT1 in HCT-15 plasma membrane could probably explain the higher sensitivity of these cells to CHC. We also observed that the migration of HCT-15 cells was inhibited by CHC, reinforcing the sensitivity of these cells to CHC, this time on the migration capacity of cells. AR-C155858 had no impact on both HCT-15 and Co-115 cell viability, as indicated by MTT assay, and morphological analysis. However, trypan blue assay demonstrated be more suitable to assess MCT-target cell viability. It would then be important to further evaluate the effect of this drug on viability of HCT-15 and Co-115 cells with trypan blue assay, as well as on proliferation.

Another purpose of the present study was to assess if the inhibitory effect of CHC in HCT-15 cells viability was mediated by MCTs. Thus, the effects of CHC on both lactate export and MCT1, MCT4 and CD147 (MCT1/4 chaperone) protein expression levels were assessed. In the present study, CHC decreased extracellular lactate in HCT-15 cells after 24 hours of treatment, suggesting that CHC inhibited lactate efflux in these cells. Our results are similar to the study by Colen *et al* [148]. According to that study, CHC inhibited the glioma cells capacity to efflux lactate. In the present work, CHC also decreased the expression of MCT1, as indicated by western blot analysis. Our results suggest that CHC has a direct effect both in function and expression of MCT1. However, only one experiment was done in western blot analysis. Thus, the effect of CHC on MCT protein expression levels will have to be confirmed in a future work. A number of reports have attributed to MCTs a role in lactate efflux in tumour cells [113, 142, 143, 144, 145], and those studies showed further evidence of lethal intracellular acidification upon MCT inhibition. Thus, we can deduce that this inhibition of both lactate efflux and MCT1 expression by CHC may induce an increase in intracellular pH promoting a decrease of HCT-15 cell viability. In contrast, by immunocytochemistry, the decrease of MCT1 expression was not evident, neither in the plasma membrane.

Although 5-FU is currently the first-line agent for colorectal cancer, the response rate to this chemotherapeutic agent in adjuvant treatment is less than 15%. This lack of

acceptable response has stimulated intensive efforts to develop new cancer drugs and new combined regimens in colorectal cancer treatment [46]. Recently, a combined CHC/radiation therapy in glioma cells showed an enhanced level of radiation-induced cell kill [148]. Other recent study showed that sulindac sulfide, an active metabolite of sulindac, potentiated the inhibitory effect of 5-FU on colorectal cancer cell survival, parallel to the induction of apoptosis [156]. On the other hand, Lim *et al* [157] demonstrated that celecoxib, a cyclooxygenase-2 (COX-2) specific inhibitor, attenuated cytotoxic effect of 5-FU in colon cancer cells, parallel to prevention of apoptosis. The inhibitory properties of CHC, observed in the present work, suggest that CHC may potentiate the cytotoxic effect of 5-FU in colon cancer cells. The final purpose of this study was then to examine the effect of CHC on 5-FU-induced decrease in cell viability in HCT-15 and Co-115 cell lines. As expected, 5-FU given alone inhibited both HCT-15 and Co-115 cell viability. Addition of CHC to 1 mM 5-FU increased the cytotoxic effect of 5-FU in a dose-dependent manner, being the cytotoxic effect of the combination more evident in HCT-15 cells. Thus, we also studied the effect of the combination in HCT-15 cell migration. The studied compounds given alone were able to inhibit the migratory capacity of cells, however the combined treatment did not increase the inhibitory effect of 5-FU. The potentiation of 5-FU cytotoxic effect by CHC is not surprising as by itself it has inhibitory potential in colon cancer cell viability. Thus, when 5-FU and CHC are applied simultaneously they may mutually enhance their activity against CRC cells.

To further characterize the therapeutic value of CHC as well as the combination 5-FU plus CHC, it would be also important to evaluate their effect on other tumour features, such as proliferation (e.g. BrDU assay), apoptosis and invasion.

5 Conclusion

The current work was based on the fact that MCT1 and MCT4 expression is increased in CRC [133, 134], representing attractive targets in CRC drug therapy. Additionally, CHC, a competitive inhibitor of MCTs, has already been explored as potential anticancer drug [130, 148]. In the present study, it was demonstrated that CHC has an inhibitory effect on colon cancer cell viability, cell migration, lactate efflux, and probably on MCT1 expression.

In this study, we also investigated, for the first time, whether CHC has the potential to be used with 5-FU as a combined chemotherapeutic agent for the treatment of CRC. CHC was found to potentiate the cytotoxic effect of 5-FU in human colon cancer cells viability. Our study shows evidence for the benefit of combining MCT inhibitors with conventional anticancer chemotherapy.

Although additional studies are necessary concerning other aspects of tumour cells, such as proliferation, invasion, and apoptosis, our results showed important evidence for the role of MCTs, especially MCT1, in CRC as well as for the exploitation of MCTs as therapeutic targets in CRC.

6 Future perspectives

In the near future it will be necessary to carry out further experiments to complement the work here presented, such as:

- Evaluation of AR-C155858 effects on cell viability, by trypan blue assay, lactate efflux, cell migration, and MCTs expression, in colon carcinoma cells.
- Evaluation of the effects of both MCT inhibitors alone and in combination with 5-FU on other tumour features such as proliferation (e.g. BrDU assay), invasion (Transwell migration assay), and apoptosis (Anexin V/PI by flow cytometry).
- Assessment of the effects of both MCT inhibitors on cell viability and proliferation of normal colon cells, so as to evaluate the selectivity of the proposed treatment for cancer cells.

7 References

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