

Università degli Studi di Catania Scuola Superiore di Catania

International PhD

in

STEM CELLS

XXIII cycle

IDENTIFICATION OF NOVEL THERAPEUTIC TARGETS FOR COLON ADENOCARCINOMA

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Abstract

Colorectal cancer (CRC) is the third most common form of cancer in the Western world. Despite the emergence of new targeted agents and the use of various therapeutic combinations, none of the treatment options available is curative in patients with advanced cancer. A growing body of evidence is increasingly supporting the idea that malignancies originate from a small fraction of cancer cells, called Cancer Stem Cells (CSC), that show self-renewal and pluripotency and are capable of initiating and sustaining tumor growth. Several studies have shown that, with respect to the bulk of tumor cells, CSC possesses a higher degree of resistance to chemotherapy and radiotherapy that could explain the inefficacy of current therapies. The ability to isolate and study these tumor cells provided a powerful tool for the investigation of drug- and radio-resistance mechanisms thus paving the way for the development of novel targeted therapies aimed at the tumor complete eradication.

The aim of this PhD thesis was to use CSC lines, derived from CRC specimens, to individuate new potential molecular targets for the development of novel therapies. To this end four colon-CSC lines were subjected to phosphoproteomic analysis by RPPA (*Reverse Phase Protein Array*) technology. Through this analysis phosphorylation levels of various protein kinases and their substrates were evaluated in order to create an activation map of the main colon-CSC proliferation and cell survival pathways. In parallel colon-CSC lines have been screened *in vitro* to the action of 80 commercially available protein-kinase inhibitors. This screening has revealed a partial correlation between *in vitro* sensitivity and phosphoproteomic analysis, but in this study, was not possible to identify predictive factors to infer colon-CSC sensitivity to specific kinase inhibitors. Colon-CSC was sensitive to the inhibition of protein kinase C (PKC), known regulator of cell proliferation and survival. Among PKC inhibitors, the most interesting was the UCN-01, a staurosporine derivative that can also inhibit PDK1 and Chk1. This compound has been already used in clinical trials as antineoplastic agent in combination with conventional chemotherapy. The *in vitro* treatment of colon-CSC with UCN-01 has demonstrated its ability to enhance the irinotecan cytotoxicity by increasing the apoptotic response. The combined action of UCN-01 and irinotecan caused a marked reduction in the levels of antiapoptotic proteins such as Bcl-XL and Mcl-1 and the activation of caspase 3.

The *in vivo* administration of UCN-01/irinotecan combination, in a mouse model of subcutaneous xenograft, confirmed the observations obtained *in vitro*, leading to a significant reduction in tumor growth compared to the single treatments. UCN-01 has also

shown efficacy in the inhibition of Chk1, as demonstrated by the reduction of the phosphorylation of its target protein cdc25. Inhibition of Chk1, an important regulator of cell cycle, in combination with chemotherapy, could help in reducing the viability of colon-CSC, thus preventing cell cycle arrest and repair DNA damage induced by irinotecan. Although UCN-01 exerts its effect by inhibiting the activity of various protein kinases, this reduced selectivity could be the basis of its effectiveness. The present study demonstrated that it is possible to identify, among the commercially available compounds, those that interfere with processes that regulate colon-CSC survival or proliferation and therefore are potentially able to interfere with tumor growth. The use of newly developed inhibitors, combined with the analysis of genetic alterations or phosphoproteomic, will identify factors predictive of response to therapy and lead to the possibility of developing individualized therapeutic strategies, increasing the likelihood of success of targeted therapy.

Introduction

1. Colorectal Cancer

Colorectal cancer (CRC) is the third most common form of tumor, after lung and breast cancer. Each year more than a million people worldwide develop this type of neoplasm and in Europe about 2.5×10^5 people are diagnosed every year, covering approximately 9% of new cases [1]. In Italy, the annual incidence is about 50×10^5 for men, 38×10^5 for women. The incidence of this tumor follows an age-dependent distribution: under 45 years there is a low incidence (2×10^5), and then it progressively raises in the 45-54 years age group (20×10^5), reaching 55×10^5 cases between 54 and 64 years, 150×10^5 in the range 65-74 years and more than 250×10^5 in patients over 75 years. Similarly the 5-year survival rate changes in an age dependent manner, from 63% in the younger age groups (15-45 years) to 49% in older patients (over 75 years) [1].

1.1 Risk factors

The majority of CRC cases occurs sporadically and only 5% of cases can be attributed to inherited mutations. Epidemiological studies have identified an association between several environmental factors and CRC onset risk. In addition to the male sex and age these include, a high fat diet, red meat consumption, inadequate fiber intake, a sedentary lifestyle and smoking. Together with the environmental risk factors, a genetic

predisposition has been found and it was estimated that at least 20% of CRC cases have a family history. Inherited syndromes that may lead to CRC include familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome). These diseases occur in individuals who have inherited heterozygous mutations in specific genes in which the acquisition of a second mutation, in the normal allele, leads to the loss of the corresponding protein. In FAP cases, homozygous mutations lead to excessive proliferation of intestinal epithelial cells with a subsequent polyp formation. Lynch syndrome is, on the other hand, characterized by the lack of DNA mismatch repair system components that leads to the acquisition of a "mutator phenotype". This is in turn associated with an increased risk of neoplastic transformation [1].

1.2 Pathology

One of the most important CRC progression feature is the "adenoma to carcinoma sequence", a widely used term to describe the multistep pathogenesis processes that characterize this tumor. This involves the progressive acquisition of genetic alterations in intestinal epithelial cells which is reflected in the histological progression from normal epithelium to a more malignant phenotype passing through several degrees of dysplasia with increasing severity and carcinoma in-situ (CIS) [3]. In particular, the majority of colorectal cancers arise from malignant transformation of polyps. A polypoid lesion may develop from a normal mucosa in about five years and it will require at least two more years to turn into CIS stage. Three more years are finally needed to reach the stage of invasive cancer.

The main colorectal cancer histotype is the adenocarcinoma which accounts for 90-95% of all large intestine cancers, while 17% is represented by mucinous adenocarcinomas [1].

The ability to identify adenomatous polyps or pre-neoplastic lesions, together with the high survival rate associated with earlier stages of the disease, make colorectal cancer an ideal target for screening programs, whose main purpose is to prevent its sporadic forms (more than 90% of cases) [1, 2].

1.3 Pathogenesis

In 1990 Fearon and Vogelstein proposed a genetic model to describe colorectal tumorigenesis [6]. According to this model colon cancer development is driven by mutations in oncogenes and tumor suppressor genes, whose total accumulation, rather than the order of appearance, is considered important in tumor development, even though their

acquisition sequence often reflect tumor clinical progression (Figure 1). Genetic mutations responsible for sporadic colorectal cancer are the same that characterize the hereditary forms and it is possible to distinguish two major pathogenetic pathways: the APC/ β -catenin pathway and the microsatellites instability pathway.

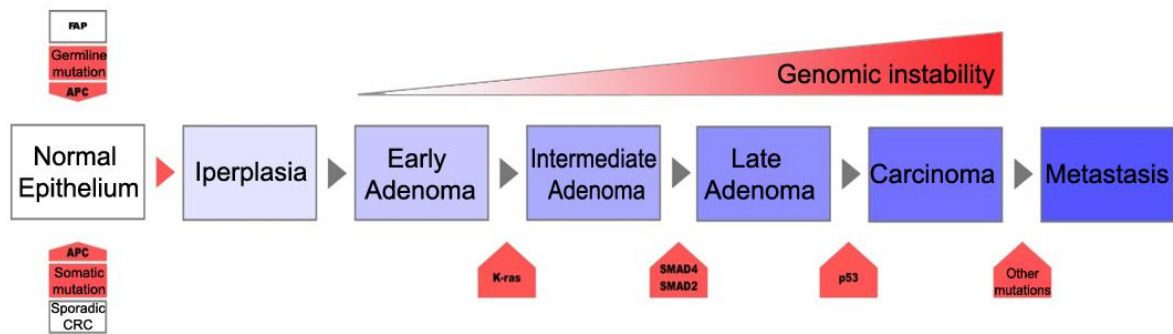


Figure 1. Fearon and Vogelstein model for colorectal carcinogenesis [6]. Patients with FAP inherit a mutation in APC and develop numerous dysplastic foci in colon crypts. Progression towards a more malignant phenotype is then driven by the acquisition of other mutations (e.g. K-Ras, SMAD4/2 and p53) and increased genomic instability.

1.3.1 APC/ β -catenin pathway and chromosomal instability

APC/ β -catenin pathway is altered in 85% of sporadic CRC cases. APC (adenomatous polyposis coli) gene mutation is one of the earliest events in the adenoma-carcinoma sequence. Heterozygous mutations in this gene are responsible for FAP and, if untreated, this disease inevitably evolves in CRC [3]. An APC key function is β -catenin intracellular level control. Indeed, APC takes part in Wnt signal transduction pathway involved in regulation of cell proliferation and survival (Figure 2). This pathway is activated by Wnt binding to its receptor Frizzled (FZD) that interacts, at a cytoplasmatic level, with a protein complex formed by Axin, APC and GSK-3 β (destruction complex). In the absence of Wnt signal, this complex is responsible for β -catenin degradation via the ubiquitin-proteasome pathway. APC mutations impair protein ability to interact with the destruction complex, leading to β -catenin stabilization and its accumulation in the nucleus. The nuclear functions of this protein lie in its ability to act as a co-activator of the TCF family of transcription factors activating the transcription of specific genes, including c-MYC and

Cyclin D1 (CCND1). Mutations in the APC gene were observed in 40-80% of colorectal cancers reinforcing the belief that this mutation is one of the earliest events in adenoma-carcinoma sequence. Finally about half of tumors bearing a wild type APC gene show mutations in the β -catenin gene, thus suggesting that these latter mutations can replace those in APC [3].

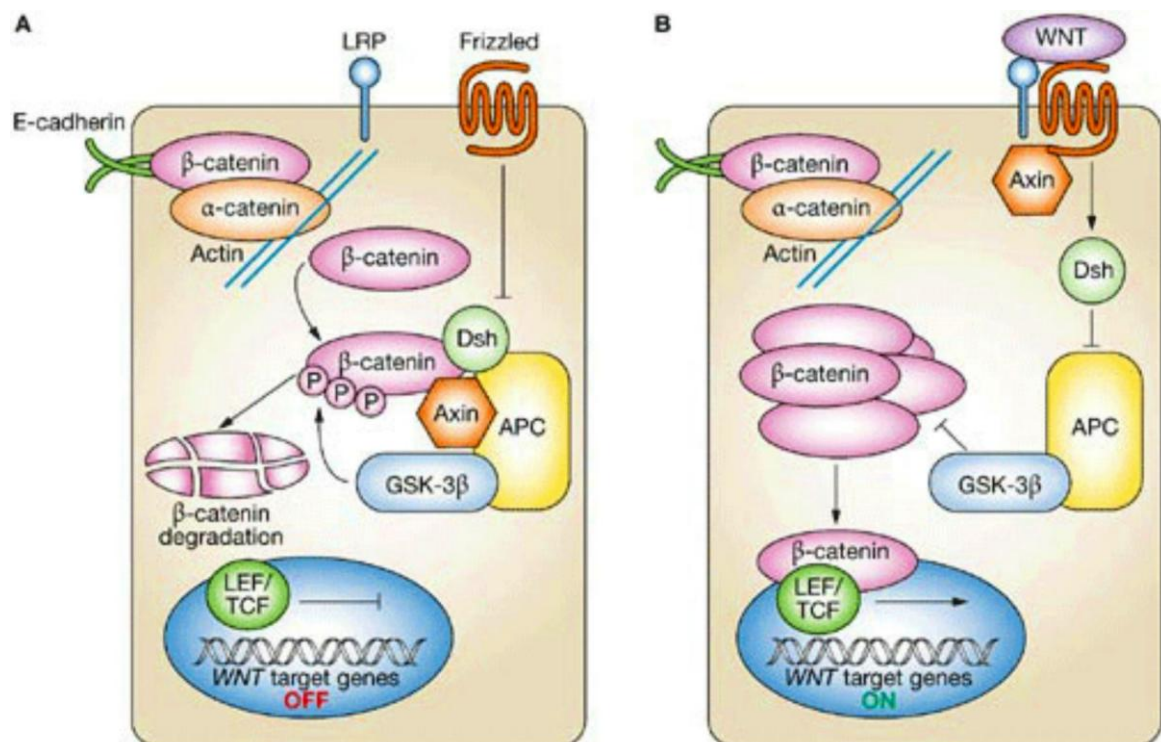


Figure 2. Wnt signal transduction pathway. (A) In the absence of Wnt the cytosolic β -catenin is degraded through the APC/Axin/GSK-3 β complex. (B) When Wnt binds to the receptor, DSH (Dishevelled) is activated by preventing the binding of β -catenin to the complex, the β -catenin is stabilized and, moving into the nucleus, can associate with transcription factors fostering the expression of target genes [8].

Although APC mutation is an early event this alteration seems not to be self-sufficient for adenomas progression [9]. Each new mutation leads additional control genes lost (chromosomal instability) and is correlated with a greater tendency for the acquisition of new malignant aberrations. Another frequent genetic alteration that occurs early in the

adenoma-carcinoma sequence is the KRAS activating mutation [3]. This gene, belonging to the RAS family (KRAS, HRAS and NRAS), encodes for a small GTPase localized on cell membrane cytoplasmic side and is involved in proliferation and differentiation signals coming from receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) [9]. These receptors are coupled to the RAS family proteins through a series of adaptor proteins known as GRB2/SOS and the downstream signal transduction pathway elements consist in the serine-threonine kinase Raf and the MAP (mitogen activate protein) kinase cascade (Figure 3).

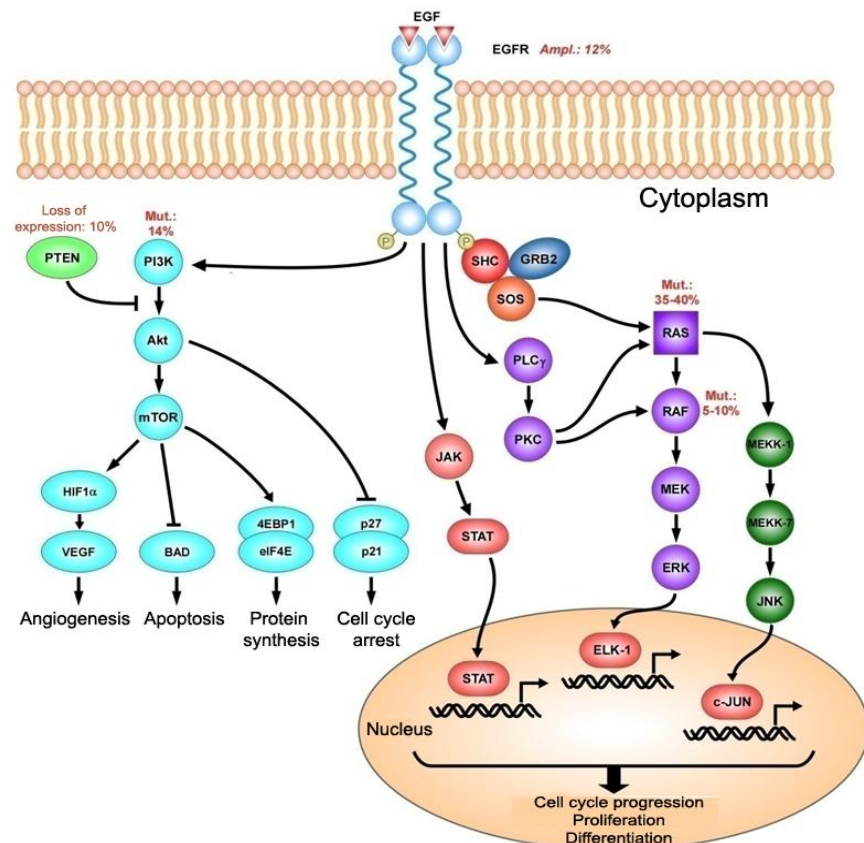


Figure 3. Signal transduction pathways activated by EGF binding to its receptor (EGFR). Frequencies of different mutations observed in colorectal carcinoma are shown.

RAS proteins are activated by GTP binding and inactivated by their intrinsic GTPase activity, through the action of GAP (GTPase Activating Protein) proteins, called p120, RasGAP and neurofibromin (NF1 gene product). All of the KRAS oncogene mutations

associated with cancer are located in the GTP binding domain and are responsible for a reduction in GTPase activity, causing constitutive protein activation [3]. Activating KRAS mutations characterize the 35-42% of CRCs and adenomas and the percentage tends to increase in advanced lesions. The high genetic alteration frequency found in adenomas suggests that this is a quite early event in tumor development. However, the low occurrence frequency of this mutation in small adenomas suggests that RAS altered function could not be a tumor initiating factor but rather has a role in conferring a proliferative advantage to cells that have already acquired a mutation in the APC gene [9]. The deletion of a chromosome 18 region is another important genetic alteration in the CRC pathogenesis, found in about 70% of cases. Its frequency shows an increase proportional to tumor staging: 10-30% of early adenomas and 60% of the most advanced ones. Initially, the DCC gene (Deleted in Colorectal Cancer), contained within this region, was considered a tumor suppressor associated with tumorigenesis. Experiments on mutant mice bearing a homozygous deletion, however, did not confirm this hypothesis [3]. Other tumor suppressor genes have been further identified in this region. Among these SMAD2 and SMAD4 (Small Mother Against Decapentaplegic 2-4), whose protein products act as mediators of intracellular TGF- β (transforming growth factor β) signal. TGF- β transduction pathway regulates various processes such as cell growth, differentiation, extracellular matrix production and apoptosis in various cell types. Mutations in SMAD2/4 and TGF- β were found in several human neoplasms, including colorectal cancer, in which the deletion of SMAD4 is known to abolish the antiproliferative TGF- β signals [3]. The short arm region of chromosome 17 is frequently subject to deletion in CRC, an event that involves TP53 gene lost. TP53 gene mutation or deletion is one of the most common events found in human cancers. This gene alterations or 17p region lost are observed in 4-26% of adenomas, in approximately 50% of adenomatous polyps and in 50-75% of adenocarcinomas [3]. p53 protein plays a major function as a transcription factor regulating physiological processes such as stress response, DNA damage repair, cell cycle regulation and apoptosis. In particular, genotoxic stresses, trigger cell cycle arrest mediated by p53, that for this function, has been described as the "the genome guardian" [10].

1.3.2 Microsatellites instability

About 15% of CRC cases show high level of microsatellites instability (MIN) that results in frame shift mutations and base substitutions. This phenomenon is indicative of a reduced efficiency of DNA mismatch repair (MMR). MIN has been found in almost all HNPCC patients, and only in 10-15% of sporadic cases.

To date, there are five known genes involved in MMR: MSH2, MLH1, PMS1, PMS2, and MSH6. The majority of HNPCC patients bear a heterozygous somatic mutation in at least one of these genes, whereas in sporadic cancers somatic mutations in MMR genes are much less frequent. Noteworthy is the high mutation frequency in the TGF- β RII (TGF- β receptor type II) gene associated with MIN-positive CRC. The gene encoding this receptor contains sequence similar to microsatellite repeats and therefore particularly sensitive to defects in MMR. Inactivating mutations of the TGF- β RII were observed in 90% of MIN-positive CRC [3].

1.4 Therapy

1.4.1 Surgery, chemotherapy and radiotherapy

The most important therapeutic treatment for CRC is surgery. This procedure aims to remove the affected bowel section and its lymphatic system. The currently used screening programs, allows an early diagnosis (adenomatous polyps), ensuring, after surgery, disease resolution in about 50% of cases [1]. Systemically administered chemotherapy is used as adjuvant therapy for tumors that have reached the third stage of progression (invasion of serosa and lymph node involvement), with the aim of reducing recurrence risk. Moreover, it represents the first-line treatment in metastatic patients and aims to prolong survival and improve quality of life. Unfortunately, drug treatments generally produce only a partial and short-termed clinical response [11].

The most widely used anticancer drugs in CRC treatment are 5-fluorouracil (5-FU), oxaliplatin, irinotecan and their possible combinations. 5-FU is a pro-drug that is converted by the cell in fluorodeoxyuridin-5-monophosphate (FdUMP), which can interfere with the activity of the enzyme thymidylate synthase, responsible for the conversion of dUMP to dTMP, a precursor of dTTP, thus resulting in a dTTP production impairment and consequently in DNA synthesis inhibition [12]. As a single agent, 5-FU shows little activity against the most advanced forms of cancer [11, 13]. Although the initial response rate is improved by combining 5-FU with folinic acid (leucovorin), thanks to its ability to

inhibit thymidylate synthase [14], there is not a significant increase in survival rate [15]. This led to the development of new drugs with an analogous mechanism of action such as capecitabine, which used alone or in combination with leucovorin, induces a better response rate with a lower toxicity profile [16].

Oxaliplatin was developed as an analogue of cisplatin in order to achieve greater therapeutic efficacy. Like all alkylating compounds, oxaliplatin is able to form guanine-guanine or adenine-guanine adducts between DNA complementary strands. These adducts hinder DNA polymerase progression during replication, thus interfering with normal cell division processes. Clinical studies have proven oxaliplatin effectiveness either as CRC first-line therapy or as a secondary treatment of 5-FU refractory cancers [17, 18].

Irinotecan is a camptothecin derivative that acts by introducing double-stranded breaks in DNA stabilizing topoisomerase I-DNA complex and preventing replication fork progress during DNA synthesis [21, 22]. Clinical studies have shown that in patients insensitive to 5-FU treatment, irinotecan produced a response rate of 13.5% and tumor stabilization in 44% of cases with a median survival of 45 weeks [23]. This has led to irinotecan acquisition as secondary treatment of 5-FU insensitive CRC patients.

Studies investigating oxaliplatin/5-FU/leucovorin (FOLFOX) combination benefits started after the observation of a synergistic effect of these drugs in vitro and in mice models [24]. In a recent clinical trial (MOSAIC) patients have shown a significant increase in 3 years disease-free survival, with a 23% reduction in recurrence risk compared to control and a moderate toxicity profile [25].

Some studies on metastatic patients [32, 33] evaluated the irinotecan/5-FU/leucovorin combination effectiveness (FOLFIRI). Compared to irinotecan alone, the results have shown a 21% to 39% increase in response rate, a 4.3 to 7 months increase in progression free survival and a 12.6 to 14.8 months increase of median survival. In cases of unresponsiveness to first-line therapy it is possible to proceed with palliative chemo/radiotherapy treatment to reduce bleeding and pain. For radiotherapy, there is no standard protocol and the system is determined by the patient's general condition and severity of symptoms.

Liver is the most common CRC metastatization site. Liver metastases tend to appear within two years after surgical removal of primary tumor in 70-80% of cases [26, 27]. The main therapeutic approach in the treatment of liver metastases involves the surgical removal of liver affected tissue, followed by chemotherapy. The 5-year survival does not exceed 40-50% [28-31].

1.4.2 New therapies for CRC

Over the past 30 years many signal transduction pathways regulating tumor progression processes such as cell division, growth, survival, programmed death and angiogenesis have been described. For this reason in the last decade we have seen an overwhelming development of new therapeutic strategies aimed at the selective inhibition of cancer cells deregulated pathways.

Human genome sequencing has revealed that 20% of the approximately 32,000 human genes encoding proteins are involved in signal transduction. Some of these proteins are frequently mutated in cancer and, among these, protein kinases and phosphatases play a central role [34]. In 1.3.1 paragraph a classical example of signal transduction pathway (EGFR) has already been described representing how its deregulation is involved in colorectal carcinogenesis. Signal transduction involved protein kinases are classified in tyrosine and serine/threonine kinases depending on their specific enzymatic activity. Generally the former are receptors with intrinsic tyrosine kinase activity and are essential for plasma membrane originating signal triggering. Serine/threonine kinases are instead generally responsible for signal amplification via intracellular phosphorylation cascade events. These signaling cascades usually converge in transcription factor activation with consequent specific gene expression. Activating mutations of one or more kinases result in deregulated proliferation, growth or cell survival signals.

Cancer research ultimate goal is the selective eradication of the tumor cell compartment preserving normal cell counterpart. Selective deregulated pathway inhibition is therefore, the basis for a large proportion of both clinical and basic research studies. There are two main mechanisms for kinases activity inhibition: monoclonal antibodies directed against receptor tyrosine kinases or highly specific small molecules able to block the catalytic activity. Once bound to its specific receptor, monoclonal antibodies can either prevent receptor-ligand interaction or cause receptor degradation. Monoclonal antibodies can also sequester the receptor ligand avoiding their interaction [35]. Small molecules of natural or synthetic origin act by competing with ATP (adenosine triphosphate) for binding to kinases catalytic site. Other small molecules block kinases catalytic activity by interacting with allosteric regulation sites [35].

Targeted therapy protocols have also been considered in CRC treatment. Since in about 80% of colon cancers there is an EGFR overexpression [35] blocking its increased signals has recently been an investigational therapeutic approach. The chimeric monoclonal

antibody cetuximab can specifically bind to EGFR extracellular domain, thereby preventing its ligand binding. In a phase III clinical trial Cetuximab has demonstrated efficacy leading to an increase in both overall and disease free survival with respect to active comparators [36]. In addition, cetuximab is able to increase oxaliplatin and irinotecan sensitivity to chemotherapy in otherwise refractory tumors, probably by abolishing EGFR antiapoptotic functions [37, 38]. In a second Phase III clinical study based on cetuximab/irinotecan combination it has been shown a significant increase in objective response rate from 4.2% to 16.4% and progression-free survival from 2.6 to 4 months compared with irinotecan alone [39]. In the CRYSTAL trial Cetuximab was administered as first-line treatment to metastatic patients in combination with FOLFIRI protocol. This resulted in a significant increase in both the objective response and progression free survival [40]. However, Cetuximab effectiveness has however been questioned in tumors bearing a constitutively active RAS. In these contexts the antibody antiproliferative effect is missing and this was clinically confirmed [41]. The explanation lies in RAS downstream position that guarantees a constitutive proliferation signal even in the absence of receptor function thus, eliminating Cetuximab effects. KRAS activating mutations have therefore been proposed as an anti-EGFR therapy predictive marker of resistance. PI3K/Akt represents a second signal transduction pathway which may be the target of treatment (Figure 3). PI3K (phosphatidylinositol 3-kinase) can be activated either directly by receptor tyrosine kinases such as EGFR or upon interaction with the RAS family proteins. The main role of PI3K is the phosphorylation of the lipid membrane by creating sites for recruitment and activation of various proteins including protein kinase B/Akt, an important regulator of survival, growth and cell proliferation. PI3K mutations occur in 15-30% of CRC cases in which the kinase acquires the ability to constitutively activate PKB/Akt in absence of growth factors. Similarly, PTEN (phosphatase and tensin homologue) tumor suppressor gene is frequently mutated or deleted in CRC (approximately 30% of cases) [42]. PTEN is a phosphatase that antagonizes PI3K activity and whose mutation or no function causes Akt hyperactivation. Several small molecules inhibitors that target PI3K or mTOR (mammalian target of rapamycin, Akt activation main target) are currently in clinical trials for CRC as a single treatment or in combination with chemotherapy [42].

Tumor growth and development can also be blocked by intervening on cancer cells ability to stimulate angiogenesis. Angiogenesis primary regulator is Vascular Endothelial Growth Factor (VEGF) produced by tumor cells, which can bind to its specific tyrosine kinase

receptor (VEGFR) expressed by endothelial cells. VEGF binding promotes the VEGFR phosphorylation triggering a signal transduction cascade that promotes endothelial cells proliferation, survival and migration [43]. Bevacizumab, a humanized monoclonal antibody directed against VEGF, is one of the clinically proven therapies in CRC. Several clinical studies have evaluated the effectiveness of bevacizumab combined with conventional chemotherapy protocols such as FOLFIRI and FOLFOX leading to a significant increase in overall and progression-free survival [44, 45]. VEGFR block by using anti-angiogenic small molecules is an alternative approach for CRC treatment. Sorafenib is a VEGFR, PDGFR (platelet derived receptor growth factor) and RAF inhibitor whose use has been approved for hepatocellular carcinoma treatment [46]. The combination of sorafenib/cetuximab/irinotecan in patients with metastatic CRC is currently under clinical evaluation [42]. Sunitinib is another VEGFR, PDGFR and c-kit (stem cell factor tyrosine kinase receptor) inhibitor approved for renal cell carcinoma treatment [47] and its combination in FOLFIRI and FOLFOX regimens is now under study for CRC treatment [42].

In recent years the development of new treatment protocols has improved patients prognosis in malignancies such as CRC, but we are still looking for a definitive strategy. Many tumors, indeed, after an initial positive response, tend to recur with a frequency that varies according their histological type and progression staging [48].

2. Cancer stem cells

Normal adult stem cells have been described in small quantities in most of the body tissues, where they carry out functions of tissue maintenance and regeneration [49]. There are two properties that help to define a stem cell:

- self-renewal. Stem cells have unlimited replicative potential.
- multipotency. Stem cells have the ability to differentiate into the different cell types that compose the organ of origin.

These properties are due to the ability of stem cells to perform both symmetrical and asymmetrical mitotic divisions. The former produces two identical daughter stem cells, while the latter give rise to a stem and to a more differentiated cell. This allows stem cells to provide cell replacement for the whole lifespan.

The idea that a subpopulation of stem cells may guide the development of the tumor dates back from the early twentieth century [50-52]. This hypothesis arises from studies that

assessed cancer cells heterogeneity in terms of both morphological and proliferative potential [53]. Indeed, in cancer cell population only a small fraction is able to form colonies in semisolid culture. It is also known that xenotransplantation experiments (into immunocompromised mice) requires a large number of cells, indicating that only a small fraction of cells is equipped with tumorigenic capacity [54]. Two models have been proposed to explain this heterogeneity (Figure 4). The "stochastic" model proposes that all the cells within a tumor have tumorigenic capacity. The explanation why only a small part of them is able to regenerate the tumor in vitro and in vivo is therefore due to extrinsic factors such as experimental or microenvironmental conditions.

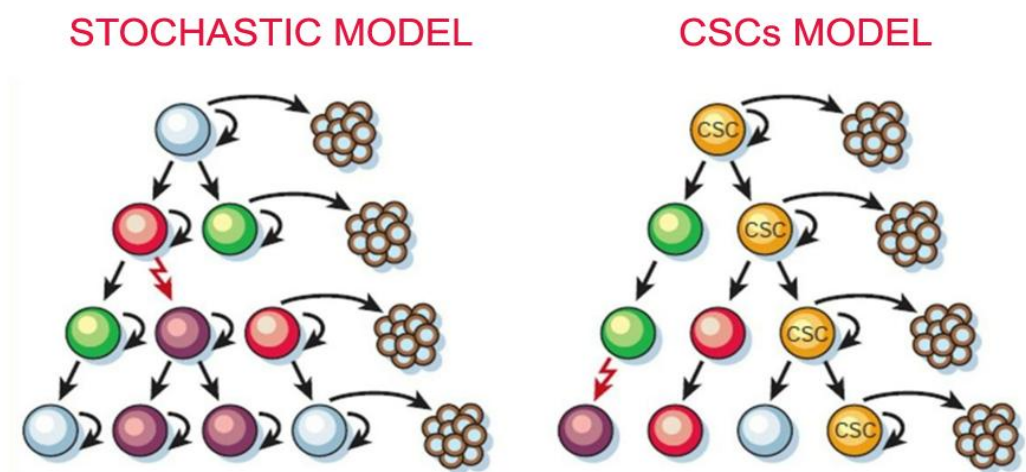


Figure 4. Two general models to explain cellular heterogeneity in solid tumors. According to the stochastic model there is a degree of heterogeneity among the tumor cells and most of them have the ability to form new tumors. According to the CSC model only a small population of cells (represented in yellow) can give rise to new tumors [55].

According to "cancer stem cells" (CSC) model, instead, there is a specific subpopulation of cells capable of expressing tumorigenic properties. CSC in fact shares with normal tissue stem cells the ability to self-renew and generate differentiated progeny responsible for tumor formation and maintenance.

CSC existence was confirmed in 1997 by John Dick and colleagues who first isolated human leukemic CSC [59]. The experimental procedure adopted is analogous to that used for hematopoietic stem cells (HSC) isolation [60, 61]. CD34⁺/CD38⁻ cells were isolated by FACS (Fluorescence Activated Cell Sorting) from patients with AML (acute myeloid leukemia) and inoculated in immunodeficient mice (NOD/SCID) to test their tumorigenicity. The authors found that CD34⁺/CD38⁻ were able to regenerate the leukemic compartment whereas, the CD34⁺/CD38⁺ more differentiated population, lack this ability. This study was followed by other leading to the possibility to identify CSC populations also in solid tumors including breast, central nervous system, prostate, lung, liver and colon cancer (Table 3).

Table 3. Cancer stem cells associated markers.

Tumor Type	Marker
Leukaemia	CD34 ⁺ , CD38 ⁺
Breast	CD44 ⁺ , CD24 ^{low} , ALDH ⁺
Brain	CD133 ⁺
Melanoma	CD20 ⁺
Lung	Sca1 ⁺ , CD45 ⁺ Pecam ⁺ , CD133 ⁺
Liver	CD133 ⁺
Head and Neck	CD44 ⁺
Colon	CD133 ⁺
Stomach	CD44 ⁺
Pancreas	CD44 ⁺ , CD24 ⁺ , ESA ⁺
Ovary	CD44 ⁺

Once having identified cancer cells with stem-like properties several assumptions about their possible role in cancer development have been made. One possibility is that mutations occur that alter self-renewing regulation in normal stem cells given the ability of stem cells to self-renew indefinitely while maintaining replicative potential. A second

hypothesis is that these cells are more likely to accumulate mutations over time increasing chances of neoplastic transformation [83]. CSC model identification has, however, led to new approaches in cancer experimental research. This is true especially in light of recent works that have been demonstrated CSC resistance to chemotherapy and radiotherapy. One of the most commonly used methods for CSC enrichment from primary cultures of tumor cells, is the Hoechst 33342 dye exclusion [88-90]. Goodell and colleagues demonstrated that this phenomenon involves the multidrug resistance transporter (MDR1), a member of the ABC (ATP Binding Cassette) family transporters [91]. Several studies [92, 93] showed that the CSC associated chemoresistance is due to increased expression or activity of MDR1. Other gene expression studies have identified a correlation between CSC chemotherapy resistance and high expression levels of BCRP1 (Breast Cancer Resistance Protein) multidrug resistance gene. Similarly, genes involved in mismatch repair system in DNA as MGMT (methyl-guanine methyl transferase) and genes encoding proteins such as antiapoptotic Bcl-2, Bcl-XL and FLIP have been associated with chemoresistance [94]. As with chemotherapy, radiotherapy resistance has been linked to the CSC. In a recent paper [95] has been shown that the stem cell subpopulation contributes to tumor radioresistance through more efficient checkpoint activation by DNA damage, compared to the remaining tumor population. For the aforementioned reasons, tumor CSC subpopulation can be considered responsible for tumor initiation, progression and spreading to other organs. Thus, CSC must be considered as a new target for therapy aimed at complete tumor healing (Figure 5).

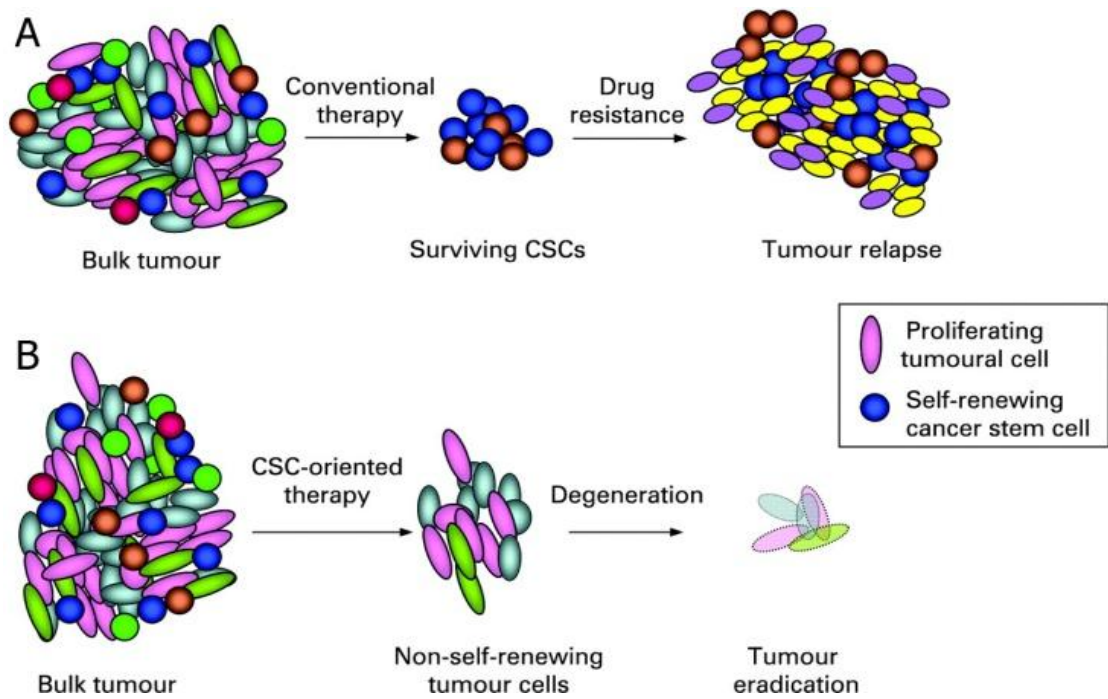


Figure 5. (A) Conventional therapy was mainly addressed towards the more mitotically active cells, thus resulting less effective on resting cells such as CSC. After an initial shrinkage the tumor tends to recur because of the small stem cell population remained alive. (B) A targeted therapy can selectively kill CSC thus eliminating tumor regenerative capacity [96].

2.1 Colon cancer stem cells

Colon epithelium is formed by a columnar cell monolayer organized into functional units called crypts of Lieberkühn. Crypt based resident multipotent stem cells are able to generate actively proliferating progenitors which, in turns, gives rise to three main terminally differentiated colonic cell types: enterocytes, goblet cells and enteroendocrine cells. Differentiated cells are continuously produced and migrate to the apical end of the crypts, which are shed into the intestinal lumen (Figure 6a) [97].

The stem cells compartment existence in the intestine has been inferred through DNA label retention experiments (e.g. tritiated thymidine or bromodeoxyuridine), by virtue of reduced mitotic activity [98, 99].

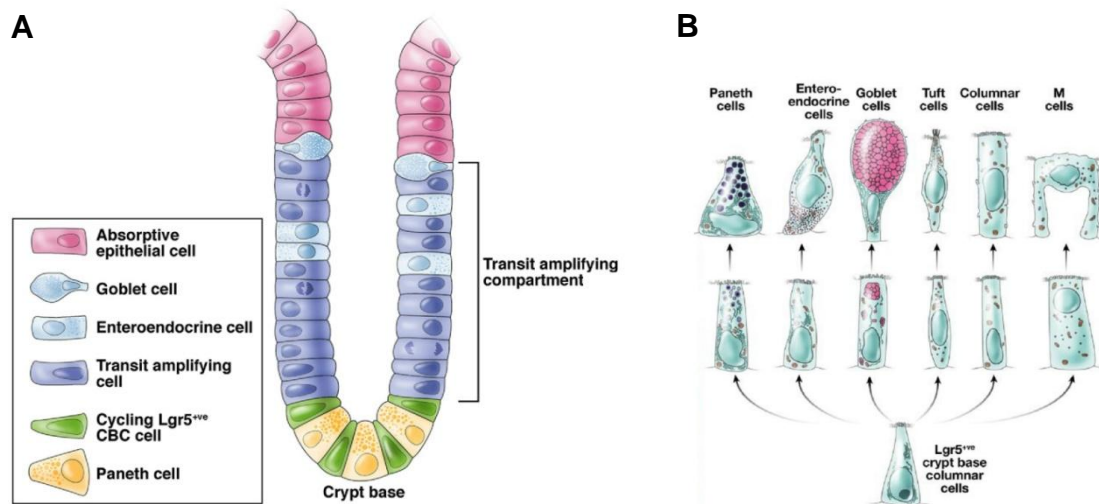


Figure 6. (A) Schematic representation of the crypt of Lieberkühn. (B) CBC Lgr5⁺ cells can differentiate and give rise to all differentiated colon epithelial cells [100].

Such studies conducted over the past 30 years to identify intestine normal stem cells have led to a first model, known as "+4 position" model [101, 102]. In this model the intestinal stem cells are localized in position +4 from the base of the crypt, just above the Paneth cells. These stem cells divide at a relatively slow rate for the whole lifespan, giving rise to cells able to divide at a higher rate ("transit amplifying-cells"). These in turn can differentiate into enterocytes, goblet cells and enteroendocrine cells, which migrate to the apex of the crypt, or Paneth cells that are located below the +4 position at the base of the crypt.

Recently, the characterization of a rare undifferentiated cell population, interspersed between the Paneth cells named Crypt Base Columnar Cells, CBC, [103, 104], lead to the definition of an alternative model, the "stem cell compartment" model (Figure 6b). According to this model the CBC are the normal intestinal stem cells [105, 106]. Stem cell compartment maintenance at the crypt base is dependent on a variety of signals from surrounding mesenchymal cells, which control self-renewal, migration, proliferation, differentiation and apoptosis. Among these signal transduction pathways Wnt plays a major role [107-109]. In a recent study [111] it has been demonstrated in mice that the expression of the LGR5 (Leucine Rich Repeats Receptor 5 G protein-coupled) gene, is Wnt-dependent and is limited to the stem cell population at the base of the crypts. In particular, the authors demonstrated that the expression of Lgr5⁺ is restricted to CBCs. Lgr5⁺ cells were then proposed as a stem cell population of normal colon in a murine system. Despite these evidences the true colon stem cells have not yet been uniquely identified due to lack of specific biomarkers.

In a recent study related to humans, Lgr5 overexpression has been placed in relation to colorectal carcinogenesis and correlated with Wnt/ β -catenin aberrant pathway activation [112]. Lgr5 expression was observed in rare cells at the crypt base in normal mucosa. Significantly higher expression was observed in adenomas and in CRC cases. Another study [113] showed, in a murine tumor model system, that stem cell regulation operated by Wnt signal, is a dynamic property also influenced by extrinsic factors such as the microenvironment. In particular, authors demonstrated that myofibroblasts present in the crypt surrounding stroma, are capable of regulating the Wnt activation through hepatocyte growth factor (HGF) secretion. HGF binding to its receptor (c-Met) present on crypt base cell membrane is responsible for the increased nuclear levels of β -catenin, through a molecular mechanism not yet clear. Wnt pathway activation cause increased expression of Lgr5 and maintenance of the tumorigenic potential of colon-CSC.

In recent years several studies have evaluated, by flow cytometry, the functionality of some biomarkers in the identification of colon-CSC (Table 3).

In a first study conducted in our laboratory [75] we proposed CD133 as a colon-CSC marker. This protein, also known as Prominin-1, is a glycoprotein expressed in neural, hematopoietic, epithelial and endothelial cells. Using surgically removed samples from primary CRC, we identified, in a large number of cases, a small population of cells (about 2.5%) expressing CD133 marker. CD133+ cells do not express cytokeratin 20 (CK20), a component of intermediate filaments expressed in differentiated intestinal epithelial cells. The next step was the isolation, from primary tumors, of a population enriched in CD133+ cells, to test the tumorigenicity by subcutaneous injection in NOD/SCID mice. Differently from CD133-, CD133+ cells gave rise to tumors that faithfully reproduced parental tumor. CD133+ cells can be maintained in culture, in an appropriate serum free medium enriched with growth factors. In these condition, cells proliferate indefinitely maintaining their tumorigenic ability. Colon-CSC can be induced to differentiate *in vitro* by removing growth factors and supplementing the culture medium with serum. Differentiation involves a morphological change and increased expression of CK20, with a concomitant decrease of CD133. To assess the long term tumorigenic ability of the colon-CSCs, we submitted the CD133+ cells to serial transplantation in mice, showing that they were able to maintain tumorigenicity even after several *in vivo* passages. Following these observations, the CD133+ population has been associated with the initiation and maintenance of CBC and was therefore proposed as a preferential target of antineoplastic therapy.

A second working group has parallelly come to the same conclusion through a similar experimental procedure [74]. Cells obtained from human primary CRC specimens were inoculated into the renal capsule of NOD/SCID mice resulting in the formation of tumors that replicate the parental tumor in terms of morphology and expression of CRC marker such as CEA and p53. Using the CD133 membrane marker, the tumor cell population was split into two subsets by FACS CD133+ and CD133-, which are respectively 2% and 98% of the total. These two subpopulations were inoculated into immunodeficient mice to test the possible enrichment of the tumor initiating cells. Only the CD133+ population was able to generate a tumor xenografts. Both papers identified in the CD133+ sub-population colon cancer initiating cells but do not provide any information about their contribution to metastases formation. More recently it has been shown that the expression of CD133 marker is unable to distinguish unambiguously the population of CSC in metastatic CRC, since both CD133+ and CD133- subpopulations, contained cells with tumorigenic ability [114].

Dalerba and coworkers [76] have proposed an alternative protocol for colon-CSC isolation, by means of CD44 and EpCAM (Epithelial cell adhesion molecule) surface markers. The injection in NOD/SCID mice of CD44+/EpCAMhigh cells, purified by FACS, produced tumor formation with a high frequency, whereas the CD44-/EpCAMlow population did not show any tumorigenic ability.

2.2 Cancer stem cells therapeutic implication

CSC ability to maintain multipotency and tumorigenicity and their ability to faithfully reproduce the parental tumor in mice models of xenotransplantation, makes them an excellent model to study tumorigenesis and to evaluate the effectiveness of new cancer therapies. The development of new targeted therapy strategies will require a better CSC characterization in terms of their genomic and proteomic characteristics. The era of large scale genetic analysis has led to valuable new information on the molecular aberrations that contribute to tumorigenesis and has increased the sample size that is possible to analyze [115]. However, even though this approach could allow a better patient stratification [116, 117] and provide potential prognostic or predictive factors, genome analysis was not able to overcome the limitations of traditional cancer therapy.

Emerging technologies are not only able to qualitatively infer protein translation but also to quantify protein expression levels and measure the amount of proteins in an active state. Proteins activity is indeed regulated through post-translational modifications such as

phosphorylation and cleavage. Phosphorylation, in particular, plays a major role in the regulation of signal transduction pathways that control proliferation, survival and cell death processes.

RPPA (Reverse Phase Protein Array) represents a novel approach designed to conduct large-scale phosphoproteomic quantitative analysis. [119, 120] (Figure 7). This technology is a larger scale extension of western or dot blotting. Nano-amounts of protein lysates or laser capture microdissected biological sections are automatically spotted on nitrocellulose coated slides and then proteins of interest are detected by using specific antibodies. The high detection sensitivity permits to print on each spot less than one microgram of material, much less than that required in a Western blotting experiment. In addition this technology allows users to analyze hundreds of samples simultaneously on a single slide in a relatively short time. RPPA great potential lies in the possibility of molecularly characterize a large number of tumor samples, allowing the identification of prognostic/diagnostic factors as well as potential targets for novel therapies. In recent years, research interest has focused on the possibility to asses personalized therapy calibrating treatments on the basis of anticancer drugs response predictive biomarkers. Tumors of different patients are in fact considered as distinct molecular entities, whose development could be guided by several deregulated signal transduction pathways leading to a different responsiveness to the same treatment. RPPA analysis offers the opportunity to analyze individual signal transduction activation profile providing new molecular targets and opening new therapeutic ways.

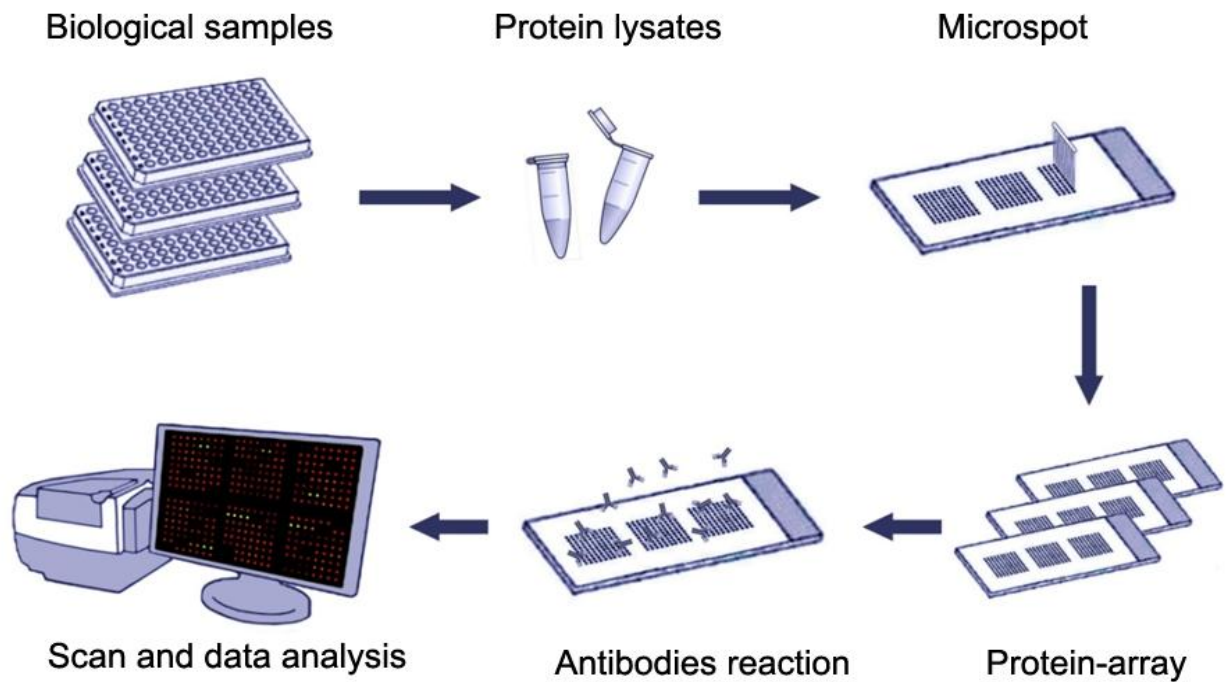


Figure 7. Schematic representation of Reverse Phase Protein Array protocol.

An RPPA technology important application is its association with *in vitro* and *in vivo* screening of potential new drugs for cancer therapy. In our laboratory we analyzed several samples of CSC, including those derived from CRC, in order to create an activation profile of different signal transduction pathways. The purpose of these studies is to correlate pathways signal transduction activation levels with the response to *in vitro* and *in vivo* treatments. A colon-CSC RPPA preliminary profile analysis of susceptibility to inhibitors of protein kinase, gave positive indications on the association between molecular activity and sensitivity to inhibitors. Even though this analysis must be confirmed on a larger patients number, the great potential of this technology for novel anticancer therapies finding is already emerged.

Methods

1. Colon cancer stem cells isolation and cultivation

In this study we used four different colon-CSC lines isolated from colorectal carcinoma tissue specimens. Tissues were mechanically dissociated with sterile scissors and forceps in DMEM (Dulbecco's Modified Eagle Medium, Gibco Invitrogen Inc., BRL, Rockville, MD) containing Streptomycin 500 g/mL, Penicillin 500 U/mL (PAA Laboratories Inc.) and Amphotericin-B 5 g/mL (Fungizone, Gibco). Then tissues were dissociated enzymatically at 37°C using type II collagenase 1.5 mg/mL (Gibco Invitrogen Inc.) DNase I 20 g/mL (Roche, Mannheim, Germany) for a time depending on piece size. Cell suspension was then passed through a sterile 100 µm filter. Following a further series of washes in PBS cells were then placed in culture in a DMEM-F12 (Hepes15mM L-glutamine, Invitrogen Life Technologies Inc., Grand Island, NY) containing medium and supplemented with b-FGF (Fibroblastic Growth Factor type II) 10 ng/mL and EGF (Epidermal Growth Factor) 20 ng/mL (PeproTech, Rocky Hill, NJ). Culture medium was replaced twice a week until the formation of spheroids in suspension was observed. Spheroids were passaged by mechanical dissociation followed by replating of individual cells and small aggregates in fresh medium.

CD133⁺ cells isolation were performed from freshly dissociated samples or from established cultures cell suspension. CD133 staining was performed by incubating cells for 45 minutes at 4 ° C with an anti-CD133/1-PE antibody (Miltenyi Biotec Inc., Bergisch Gladbach, Germany). After a series of washes in PBS CD133⁺ population was separated using fluorescence activated cell sorter FACSARIAIII™ (Becton Dickinson, San Jose, CA).

2. Kinase inhibitors library *in vitro* screening

For *in vitro* screening experiments colon-CSC spheroids were subjected to enzymatic dissociation with recombinant trypsin (TrypLE Express™, Gibco Invitrogen Inc.) for 3 minutes at 37 ° C. After being counted with Trypan Blue dye in a Burker chamber to exclude death cells, 5000 cells per well were plated onto 96-well microplates in 80 µL of culture medium. Each different treatment were performed 24 hours after plating by adding 20 µL of culture medium.

Inhibitors included in the Biomol Library (Enzo Life Sciences/Biomol http://www.enzolifesciences.com/BmL_-2832/kinase-inhibitor-library) were initially tested

at a 5 μ M concentration. All inhibitors were resuspended in DMSO (dimethyl sulfoxide, Sigma-Aldrich Inc., Saint Louis, MO). Some samples were therefore treated with DMSO 0.1% as a vehicle control. Staurosporine was used as positive control.

3. Positive hits titration and combination with chemotherapeutics or commercially available inhibitors

Compounds that resulted effective in the first screening were then titrated using three scalar concentrations (1 μ M, 500 nM, 250 nM), in addition to the vehicle control (0.1% DMSO).

Selected compounds were tested *in vitro* in combination with irinotecan (Sigma-Aldrich Inc.) at 250 nM concentration: ERK inhibitor and its commercial negative control (ERK inhibitor FR180204 II, ERK inhibitor II-Negative control, Calbiochem Inc., Merck, Germany) were both used at 1 μ M, 500 nM, 250 nM and 125 nM; Akt and PKC commercially available inhibitors and the multiple inhibitor (Akt inhibitor II and X, PKC inhibitor Set, PDK1/Akt/Flt Dual Pathway Inhibitor, Merck Calbiochem Inc.) were used at 10 μ M, 5 μ M, 2,5 μ M, 1 μ M and 500 nM; PDK1 commercially available inhibitor (OSU-03012, Alexis Biochemicals, San Diego, CA) was used at 5 μ M. In the same experiment, cells were treated individually and in combination with Akt inhibitor X at 5 μ M and PDK1/Akt/Flt Dual Pathway Inhibitor at 125 μ M. DMSO 0.1% was used as a vehicle control.

UCN-01 (Sigma-Aldrich Inc.) dose-response treatment was conducted for the four lines of colon-CSC at 1 μ M, 500 nM, 250 nM, 125 nM and 62.5 nM. In a second step of experiments we performed the following treatments (single and in combination): irinotecan 50 μ M, oxaliplatin (Sigma-Aldrich Inc.) 10 μ M, UCN-01 1 μ M. DMSO 0.1% was used as a vehicle control.

4. Viability assay

In vitro viability was assayed measuring cellular ATP content by luminometry. To this end we used the chemiluminescence assay CellTiter-Glo™ (Promega Inc., Madison, WI) following manufacturer instructions. This method takes advantage of cells ATP content to convert luciferin, the substrate of the enzyme luciferase, in an unstable compound. Before undergoing spontaneous oxidation the compound is able to emit photons in the 510-650 nm range of wavelength.

Viability was tested at different times of treatment. The intensity of the luminescence, proportional to cell viability, was measured by Victor 2™ (Wallac, Perkin Elmer Inc., Norwalk, CT) microplate reader. Vehicle control (DMSO 0.1%) luminescence values were averaged and arbitrarily set to 100%. The absolute values of luminescence for each treatment were then normalized with respect to vehicle control and then expressed as a percentage.

5. Mice models of xenotransplantation

In vivo experiments were conducted with immunodeficient NOD/SCID mice. Before injection in both flanks, colon-CSC spheroids were mixed in Matrigel (Becton Dickinson) in a 1:1 ratio. In two independent experiments, mice were injected with a suspension of 2×10^6 CSCs. Established tumors were regularly monitored over a four weeks period. When the tumors reached a 2-5 mm diameter, mice were divided into three groups and treated respectively with DMSO (vehicle), irinotecan 10 mg/kg (irinotecan group), administered only on the first day of treatment, UCN-01 5 mg/kg (UCN-01 group), administered from the first to the sixth day and the combination irinotecan/UCN-01 (combo group). Tumor growth was monitored regularly by measuring the major and minor axes during treatment and for seven days following the end of treatment. Tumor volume was then extrapolated using the modified ellipsoid volume formula [121]. Growth ratio with respect to the control was then calculated.

6. Western blotting

To determine caspase 3, phospho-Akt (S473), phospho-PKC α/β II (T638/641), Bcl-XL, Mcl-1, phospho-PDK1 (S241) and phospho-cdc25 expression levels, colon-CSC spheroids were subjected to enzymatic dissociation, after being counted 5×10^5 cells were plated 6-well in 2 mL of culture medium. Samples were subsequently treated with 0.1% DMSO (Sigma-Aldrich), UCN-01 (Sigma-Aldrich) 1 μ M, irinotecan (Sigma-Aldrich) 50 μ M, oxaliplatin (Sigma-Aldrich) 10 μ M and their combinations. Protein lysates were prepared by resuspending cells in a T-PER lysis buffer (Pierce) with 300 mM NaCl and Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails I and II (Sigma-Aldrich) according to manufacturer instruction. After a 30 minutes of-ice incubation, cells were centrifuged

for 10 minutes at 13000 RPM at +4°C. The recovered supernatant was then directly used for Western blotting experiments or stored at -80°C.

Protein concentration has been assessed using Bradford protein assay (Bio-Rad Laboratories, Richmond, CA), which is based on Coomassie Blue G-250 dye ability to change its maximum absorption wavelength from 465 nm to 595 nm in response to protein binding.

Proteins were subjected to electrophoresis on acrylamide gel containing SDS (Sodium Dodecyl Sulfate). NuPage Novex Bis-Tris gel 1.0 mm x 15 well 4-12% (Gibco Invitrogen Inc) were used. An equivalent of 20 µg of protein, supplemented with loading buffer NuPage LDS sample buffer (25mM Tris-HCl, pH 6.8, SDS 10%, 50% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue, (Gibco Invitrogen Inc.) was incubated at 95°C for 3 minutes and loaded. SeeBlue Plus 2 (Gibco Invitrogen Inc.) was used as molecular weight marker. Electrophoresis were performed in MOPS (3-[N-morpholino]-propansulfonico) buffer at 120 V for about 2 hours.

List of antibodies used in immunoblotting experiments

Antibody	Manufacturer	Species/isotype	Cat. #	Dilution
Primary antibodies				
Caspasi 3	Upstate	rabbit polyclonal IgG	06-735	1:1000
Bcl-X _L	Santa Cruz	mouse monoclonal IgG	8392	1:250
Mcl-1	Santa Cruz	rabbit polyclonal IgG	sc-819	1:500
Nucleolina	Santa Cruz	mouse monoclonal IgG	sc-8031	1:1000
p-Akt (S473)	Cell Signaling	rabbit monoclonal IgG	4058	1:1000
p-PKCα/βII (T638/641)	Cell Signaling	rabbit polyclonal IgG	9375	1:1000
p-PDK1 (S241)	Cell Signaling	rabbit monoclonal IgG	3061	1:1000
p-cdc25 (S216)	Cell Signaling	rabbit monoclonal IgG	4901	1:1000
Secondary antibodies				
Anti-rabbit IgG HRP-linked	Amersham		NA934	1:4000
Anti-mouse IgG HRP-linked	Amersham		NA931	1:4000

Protein transfer was made by using an elettroblot (Bio-Rad Laboratories) filled with transfer buffer (25mM Tris-HCl pH 8, 200 mM glycine and 20% methanol) at 80 V for 2 hours at 4°C. Membrane was saturated using TBST (25mM Tris-HCl pH 8, 150 mM NaCl, 0.2% Tween-20) containing 5% powder milk (Blottin-Grade Blocker non- fat dry milk, Bio-Rad). Primary antibodies incubation was performed overnight at 4°C. After the

appropriate secondary-HRP conjugated antibodies incubations, protein signals were visualized by chemiluminescence (Pierce Super Signal West Pico).

7. Phosphoproteomic analysis

A RPPA (Reverse Phase Protein Array) module consists of a solid support that can quantify proteins of interest previously immobilized onto nitrocellulose coated slides through the use of specific antibodies. Sample proteins immobilization was obtained through the use of an "Arrayer" tool that deposit (printing) microspot (about 30 nanoliters of sample) onto nitrocellulose slides. The immunostaining procedure, (staining) is automatically performed by an Autostainer. A portion of the printed slides are processed to detect the amount of total protein (used to normalize the antibody signal) by a fluorescence method. Once generated, signal, is detected by a high resolution scanner. The images obtained are analyzed by a software that can automatically identify the spots and quantify their relative intensity.

Printing

This procedure is performed by an Aushon 2470 Arrayer that takes advantage from a 20 "pin" head (5x4) that permits samples spotting onto nitrocellulose coated slides. Samples are diluted 1:2 with print loading buffer (Tris-glycine 2x SDS, Invitrogen) containing 2.5% β -mercaptoethanol (Sigma-Aldrich). After being heated at 100°C for 5 minutes and loaded in four different dilutions (1:1, 1:2, 1:4 and 1:8) on a 384-well plate (Genetix), samples are then spotted on the appropriate slide (FAST slides, Whatman, Fisher) by the instrument. Protein concentration is one of the most important parameter, in fact, too small amounts of protein per-spot can give rise to false negative results, while to high amounts can cause signal saturation.

Protein quantification

All protein signals were normalized on the basis of total protein values. This parameter has been estimated by SYPRO Ruby Protein Blot Stain (Invitrogen). After slides printing some of them were selected for total protein determination as described below:

- Fixing solution incubation (7% acetic acid and 10% methanol).
- Washes (water) and SYPRO Ruby Blot Stain incubation for 30 minutes.

- One minute wash.

Slides are then dried at room temperature and then scanned with a fluorescence scanner (Vidar Systems Corporation, Revolution 4550).

Staining

Staining was performed using the TSA and DAB (Tyramide Signal Amplification, Diaminobenzidine, DAKO, Denmark) system of signal amplification. In this procedure, a primary antibody is detected with a biotinylated secondary antibody. This process allows a significant signal amplification useful for the detection of femtomolar antigen amount. Before the immunostaining, slides are incubated for 15 minutes with a stripping solution (Reblot mild antibody stripping solution, Chemicon) to promote antigenic sites exposure. After two PBS washes slides are treated for two hours with PBS containing powder I-block 0.2% (Applied Biosystems/Tropix) and Tween-20 0.1%, in order to saturate nitrocellulose aspecific binding sites. Before primary antibody staining, slides are subjected to a streptavidin pretreatment in order to saturate endogenous biotin eventually present in the sample. The entire staining procedure is automatically carried out using a DAKO Autostainer.

Data analysis

After staining, slides are scanned with a flatbed scanner (UMAX PowerLook, UMAX, Dallas, TX) at a 1800 dpi resolution and saved as *.TIFF image file using Photoshop 6.0 (Adobe, San Jose, CA). Images are then analyzed with the MicroVigene software 2.9.9.9 (Vigen Tech, North Bedford, MA).

Expression levels of each protein in the four lines of colon-CSC were "standardized" as follows: $(x_n - \mu) / \sigma$, where x_n represents the intensity of the single protein in the sample n , μ is the mean value and σ the standard deviation of the individual proteins intensity calculated for all samples analyzed. In this way data are represented as relative expression values between the four lines in a range between -1.5 and 1.5 standard deviations. Data hierarchical clustering was performed through the T-MEV (<http://www.tm4.org>) open-source program.

Anticorpi utilizzati nell'analisi fosfoproteomica

Nome	Ditta	Specie	Cat. #
Histone H3 (S10) Mitosis Marker	Upstate	Rabbit	06-570
ASK1 (S83)	CellSig	Rabbit	3761
Akt (S473)	CellSig	Rabbit	9271
Akt (T308)	CellSig	Rabbit	9275
Bad (S136)	CellSig	Rabbit	9295
c-Abl (T735)	CellSig	Rabbit	2864
Smad2 (S465/467)	CellSig	Rabbit	3101
FAK (Y397) (18)	BD	Mouse	611806
PAK1 (S199/204)/PAK2 (S192/197)	CellSig	Rabbit	2605
EGFR (Y1068)	CellSig	Rabbit	2234
c-Raf (S338) (S6A6)	CellSig	RmAb	9427
EGFR (Y992)	CellSig	Rabbit	2235
ERK 1/2 (T202/Y204)	CellSig	Rabbit	9101
Met (Y1234/1235)	CellSig	Rabbit	3126
4E-BP1 (T70)	CellSig	Rabbit	9455
S6 Ribosomal Protein (S240/244)	CellSig	Rabbit	2215
PRAS40 (T246)	BioSource	Rabbit	44-1100
ErbB2/HER2 (Y1248)	CellSig	Rabbit	2247
Jak1 (Y1022/1023)	CellSig	Rabbit	3331
p70 S6 Kinase (T389)	CellSig	Rabbit	9205
PLCgamma1 (Y783)	CellSig	Rabbit	2821
4E-BP1 (S65)	CellSig	Rabbit	9451
EGFR (Y1045)	CellSig	Rabbit	2237
mTOR (S2481)	CellSig	Rabbit	2974
B-Raf (S445)	CellSig	Rabbit	2696
Adducin (S662)	Upstate	Rabbit	06-820
EGFR (Y1173)	BioSource	Rabbit	44-794
PKC alpha (S657)	Upstate	Rabbit	06-822
FAK (Y576/577)	CellSig	Rabbit	3281
Stat3 (Y705) (D3A7)	CellSig	RmAb	9145
PKA C (T197)	CellSig	Rabbit	4781
PKC zeta/lambda (T410/403)	CellSig	Rabbit	9378
GSK-3alpha (Y279)/beta (Y216)	BioSource	Rabbit	44-604
eIF4G (S1108)	CellSig	Rabbit	2441
PDK1 (S241)	CellSig	Rabbit	3061
Catenin (beta) (T41/S45)	CellSig	Rabbit	9565
NF-kappaB p65 (S536)	CellSig	Rabbit	3031
MARCKS (S152/156)	CellSig	Rabbit	2741
Ras-GRF1 (S916)	CellSig	Rabbit	3321
GSK-3beta (S9)	CellSig	Rabbit	9336
IkappaB-alpha (S32/36) (S45)	CellSig	Mouse	9246
c-Kit (Y719)	CellSig	Rabbit	3391
IRS-1 (S612)	CellSig	Rabbit	2386
VEGFR 2 (Y996)	CellSig	Rabbit	2474
Stat1 (Y701)	CellSig	Rabbit	9171
CREB (S133)	CellSig	Rabbit	9191
Bad (S112)	CellSig	Rabbit	9291
Pyk2 (Y402)	CellSig	Rabbit	3291
Survivin (71G4)	CellSig	RmAb	2808
p27 (T187)	Zymed	Rabbit	71-7700
Stat5 (Y694)	CellSig	Rabbit	9351
p90RSK (S380)	CellSig	Rabbit	9341
Bcl-2 (S70) (S42)	CellSig	RmAb	2827
MEK1/2 (S217/221)	CellSig	Rabbit	9121
SAPK/JNK (T183/Y185)	CellSig	Rabbit	9251
Estrogen Receptor alpha (S118)	CellSig	Rabbit	2515
PTEN	CellSig	Rabbit	9552
PTEN (S380)	CellSig	Rabbit	9551
p38 MAP Kinase (T180/Y182)	CellSig	Rabbit	9211
EGFR (Y1148)	CellSig	Rabbit	4404
Stat3 (S727)	CellSig	Rabbit	9134
Shc (Y317)	Upstate	Rabbit	07-206
FKHR (T24)/FKHRL1 (T32)	CellSig	Rabbit	9464
PDGF Receptor beta (Y716)	Upstate	Rabbit	07-021
VEGFR 2 (Y1175) (19A10)	CellSig	RmAb	2478
FKHR (S256)	CellSig	Rabbit	9461
c-Abl (Y245)	CellSig	Rabbit	2861
p70 S6 Kinase (S371)	CellSig	Rabbit	9208
MSK1 (S360)	CellSig	Rabbit	9594
PKC alpha/beta II (T638/641)	CellSig	Rabbit	9375
mTOR (S2448)	CellSig	Rabbit	2971

8. Statistical analysis

All statistical analyses were conducted using GraphPad Prism 4 program (GraphPad Software Inc., www.graphpad.com).

In vitro experiments statistical significance was calculated by ANOVA test while for *in vivo* experiments we used Student t test. In figures 17 and 19, the significance is indicated by asterisks: one asterisk represented a p-value ≤ 0.05 , two asterisks corresponded to $p \leq 0.01$, and three asterisks corresponded to $p \leq 0.001$.

Results

1. Phosphoproteomic analysis of Colon-CSC

We performed an RPPA analysis on a group of representative protein kinases, tested on four lines of colon-CSC (Figure 8). This analysis revealed a complex panel of kinase activities and was not possible to identify a single target protein common to all colon-CSC lines. These data reflect problems in identifying molecular targets for multifactorial diseases such as cancer, in which various individuals with different genetic abnormalities share similar histology and clinical course. To better understand the different characteristics of each of our colon-CSC line in terms of kinase activation and phosphorylation state we decided to directly test their *in-vitro* sensitivity to a panel of kinase inhibitors, to functionally test the importance of various molecular targets belonging to different signal transduction pathways for colon-CSC.

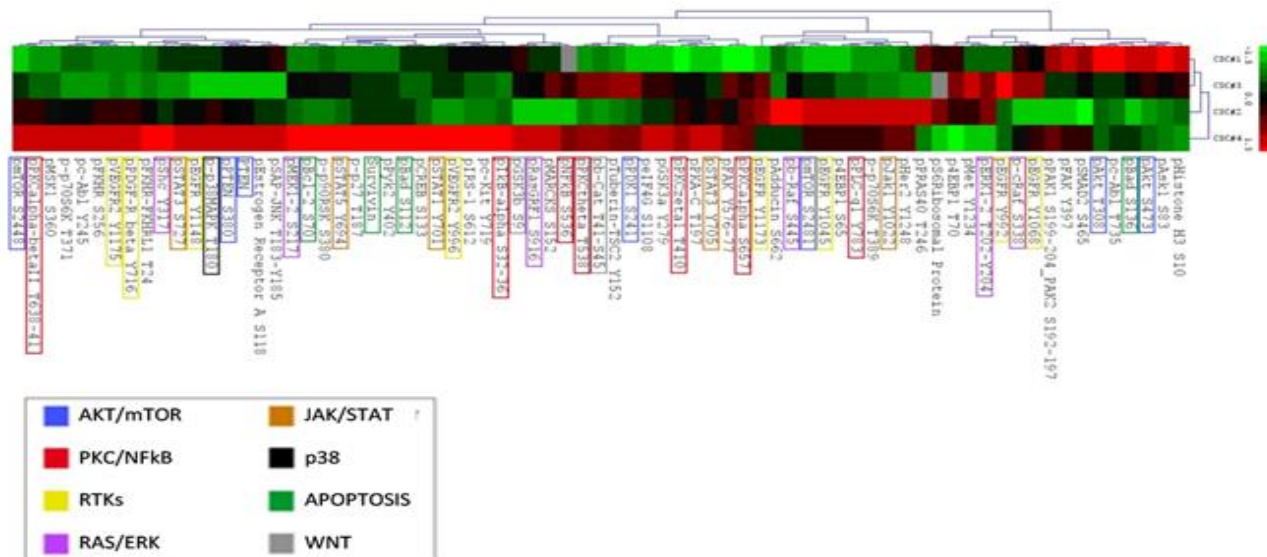


Figure 8: Hierarchical clustering of a panel of phosphoproteins expression in the four lines of colon-CSC. Color scale represents the normalized intensity of expression (values from $-1.5 \leq$ to ≥ 1.5 standard deviations). The signal transduction pathways which are known to be involved in the phosphoproteins investigated are listed below.

2. *In vitro* screening of a library of kinase inhibitors on colon-CSC

Libraries of synthetic compounds with known specificity have been screened *in-vitro* with the aim to study the sensitivity of tumor cells to the inhibition of a specific signal transduction pathway and the consequent development of targeted drug therapies. The ability to test *in-vitro* such a particular cell model, as the colon-CSC, with a library of kinase inhibitors, provides additional benefit to the study of aberrations in signal transduction pathways. This experimental approach allowed to assess and discover the importance of pathways involved in tumor progression and maintenance, in a cellular system that is the ideal target for cancer therapy.

In this study we tested the *in-vitro* sensitivity of colon-CSC to the action of a series of 80 kinase inhibitors that belong to a commercially available chemical library (Enzo Life Sciences/Biomol <http://www.enzolifesciences.com/BML-2832/kinase-inhibitor-library>). This library specifically contains inhibitors of BTK, CaM Kinase, CDK, CKI/II, EGFR, GSK, IKK, Insulin Receptor, JAK, JNK, MAPK, MEK, MLCK, PI 3-kinase, PDGFR, PKA, PKC, RAF, SAPK, Src-family, and VEGFR. Table 4 shows the names of the compounds in the library with their corresponding molecular target.

The wide coverage of most studied signal transduction pathways, mainly implicated in tumorigenesis, provides a good chance to identify possible relations between sensitivity to the compounds and molecular aberrations directly involved with neoplastic processes.

Table4: List of inhibitors included in the Biomol library and their relative target pathways

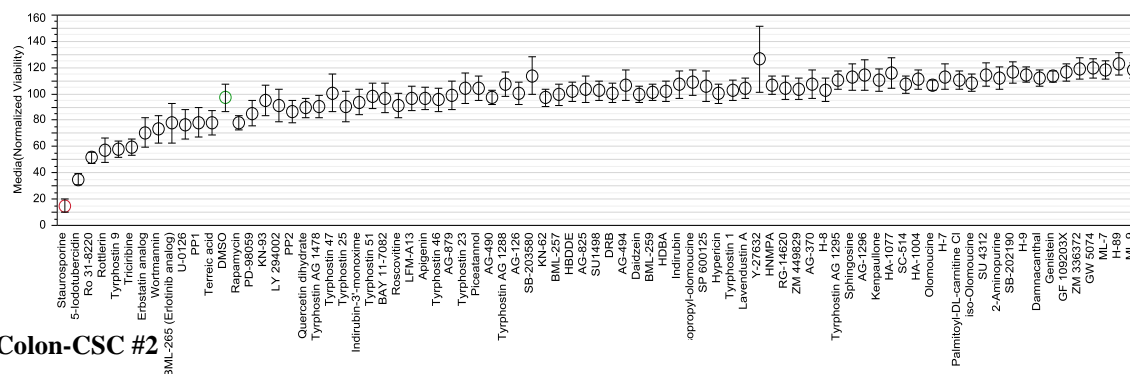
INHIBITOR NAME	MOLECULAR TARGET
2-Aminopurine	p58 PITSRE β 1
5-Iodotubercidin	ERK2, adenosine kinase, CK1, CK2,
AG-126	IRAK
AG-1296	PDGFRK
AG-370	PDGFRK
AG-490	JAK-2
AG-494	EGFRK, PDGFRK
AG-825	HER1-2
AG-879	NGFRK
Apigenin	CK-II
BAY 11-7082	IKK pathway
BML-257	Akt
BML-259	Cdk5/p25
BML-265 (Erlotinib analog)	EGFRK
Daidzein	Negative control for Genistein.
Damnacanthal	p56 lck
DRB (5,6-Dichloro-1-b-D-ribofuranosylbenzimidazole)	CK II
Erbstatin analog	EGFRK
Genistein	Tyrosine Kinases
GF 109203X	PKC
GW 5074	cRAF
H-7	PKA, PKG, MLCK, and PKC.
H-8	PKA, PKG
H-89	PKA
H-9	PKA, PKG, MLCK, and PKC.
HA-1004	PKA, PKG
HA-1077	PKA, PKG
HBDDE (2,2',3,3',4,4'-Hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether)	PKC α , PKC γ
HDBA (2-Hydroxy-5-(2,5-dihydroxybenzylamino)benzoic acid)	EGFRK, CaMK II
HNMPA (Hydroxy-2-naphthalenylmethylphosphonic acid)	IRK
Hypericin	PKC
Indirubin	GSK-3 β , CDK5
Indirubin-3'-monoxime	GSK-3 β
iso-Olomoucine	Negative control for olomoucine.
Kenpaullone	GSK-3 β
KN-62	CaMK II
KN-93	CaMK II
Lavendustin A	EGFRK
LFM-A13	BTK
LY 294002	PI 3-K
ML-7	MLCK
ML-9	MLCK

N9-Isopropyl-olomoucine	CDK
Olomoucine	CDK
Palmitoyl-DL-carnitine Cl	PKC
PD-98059	MEK
Piceatannol	Syk
PP1	Sre family
PP2	Sre family
Quercetin dihydrate	PI 3-K
Rapamycin	mTOR
RG-14620	EGFRK
Ro 31-8220	PKC
Roscovitine	CDK
Rottlerin	PKCδ
SB-202190	p38 MAPK
SB-203580	p38 MAPK
SC-514	IKK2
SP 600125	JNK
Sphingosine	PKC
Staurosporine	Pan-specific
SU 4312	Fik1
SU1498	Fik1
Terreic acid	BTK
Triciribine	Akt signaling pathway
Tyrphostin 1	Negative control for tyrosine kinase inhibitors.
Tyrphostin 23	EGFRK
Tyrphostin 25	EGFRK
Tyrphostin 46	EGFRK, PDGFRK
Tyrphostin 47	EGFRK
Tyrphostin 51	EGFRK
Tyrphostin 9	PDGFRK
Tyrphostin AG 1288	Tyrosine kinases
Tyrphostin AG 1295	Tyrosine kinases
Tyrphostin AG 1478	EGFRK
U-0126	MEK
Wortmannin	PI 3-K
Y-27632	ROCK
ZM 336372	cRAF
ZM 449829	JAK-3

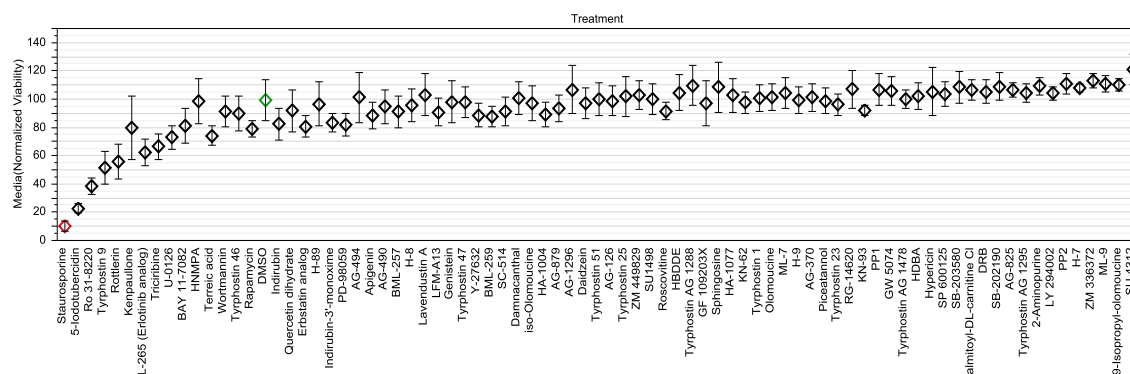
Colon-CSC spheroids were subjected to enzymatic dissociation in order to obtain a single-cell suspension ensuring reproducibility in the number of cells plated in each well (coefficients of variation < 10%). Colon-CSC were then plated in quadruplicate for each treatment and the 80 Biomol inhibitors were added the following day. Colon-CSC viability has been assessed after 48h of treatment through a method based on the quantification of cellular ATP. Figure 9 shows the viability for each of the four colon-CSC line tested. Following a supervised analysis, different inhibitors were found to significantly influence

the viability of colon-CSC 48h after treatment *in-vitro*. Inhibitors that showed a significant effect on colon-CSC, led to either a reduction or an increase in viability. Even though both interesting biological phenomena, we have chosen to continue with the study of inhibitors that led to a viability reduction. This choice was dictated by our experimental design, since the objective of this study was to identify possible new therapies aimed at eradicating colorectal cancer.

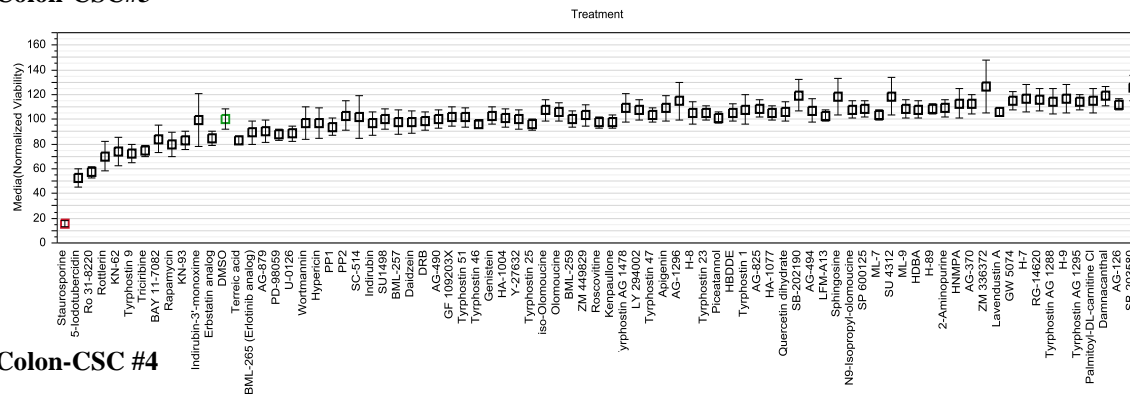
Colon-CSC #1



Colon-CSC #2



Colon-CSC #3



Colon-CSC #4

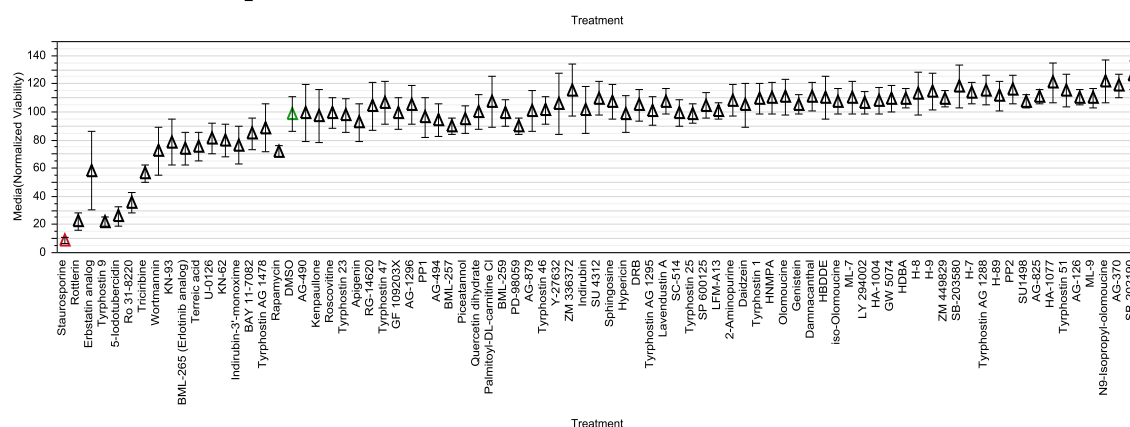


Figure 9. Viability chart after 48h of treatment with the compounds included in the library of kinase inhibitors (5 μ M concentration). Each symbol represents the average of at least three independent experiments and each error bars showed one standard deviation. Positive and negative controls are respectively in red and in green.

In order to summarize the data and obtaining a clear overview of inhibitors effects on colon-CSC, we created a scale of sensitivity and results of all experiments have been plotted in a two-dimensional map as showed in the Figure 10.

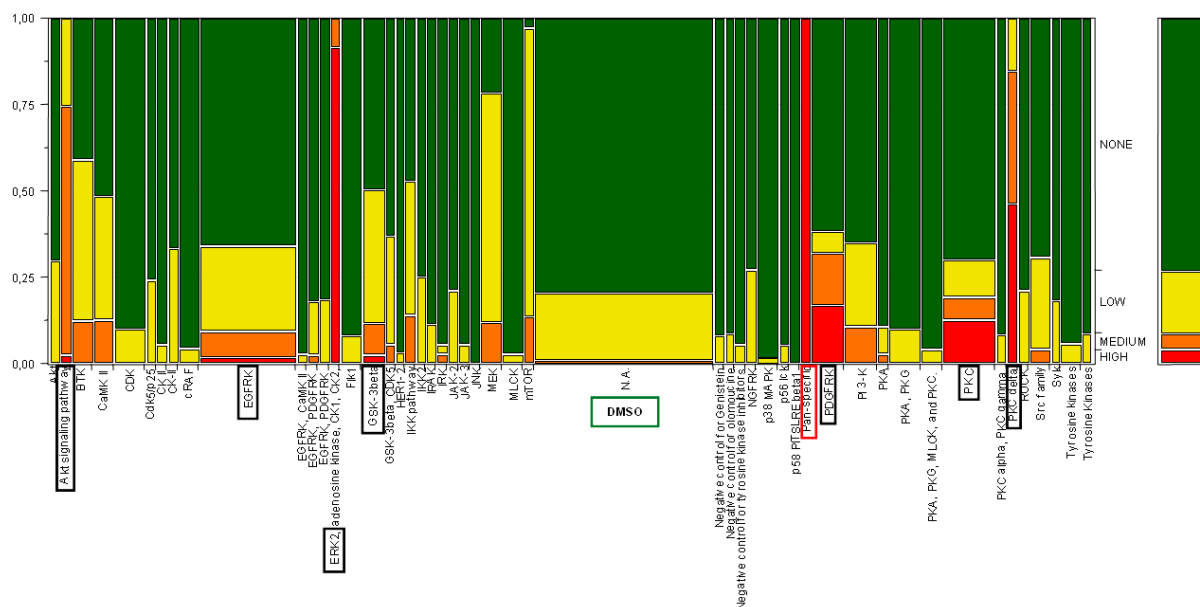


Figure 10. Synthetic map indicating the cumulative sensitivity of 4 colon-CSCs lines to the library of kinase inhibitors. The scale has been calculated comparing the viability data as follows: NO EFFECT (green) > 90%, 70% <LOW (yellow) <90%, 50% <Medium (orange) <70%, HI (red) <50%.

As shown in the boxes in Figure 10 the most important signal transduction pathways responsible for colon-CSC viability include EGFR, AKT, ERK, PKC, GSK3 β and PDGFR. It is clearly evident that most of compounds included in the library had no significant effect in reducing the viability of colon-CSC. Such a result was expected: in fact the number of inhibitors that cause an effect, on large scale studies, is related to the concentration used.

3. Titration of positive target on colon-CSC

The specificity of kinase inhibitors is not only due to their tridimensional structure, but also to the concentration at which they are used. The vast majority of synthetic inhibitors interacts directly with the kinase ATP-binding pocket (ATP-competitive), or avoids the conversion from the inactive to the active state. The ATP-binding pocket is an enzymatic site that possesses highly conserved amino acid modules among the hundreds of kinases expressed by eukaryotic cells. Therefore the concentration used in each treatment may be crucial in influencing the spectrum of specificity of each inhibitor. The aim of this study is to identify selective inhibitors of kinases responsible for survival and proliferation of colon-CSC, therefore we screened lower doses of the inhibitors previously seen to be effective at a 5 μ M concentration. Figure 11 shows a viability chart of the four lines of colon-CSC treated with three different concentrations of inhibitors.

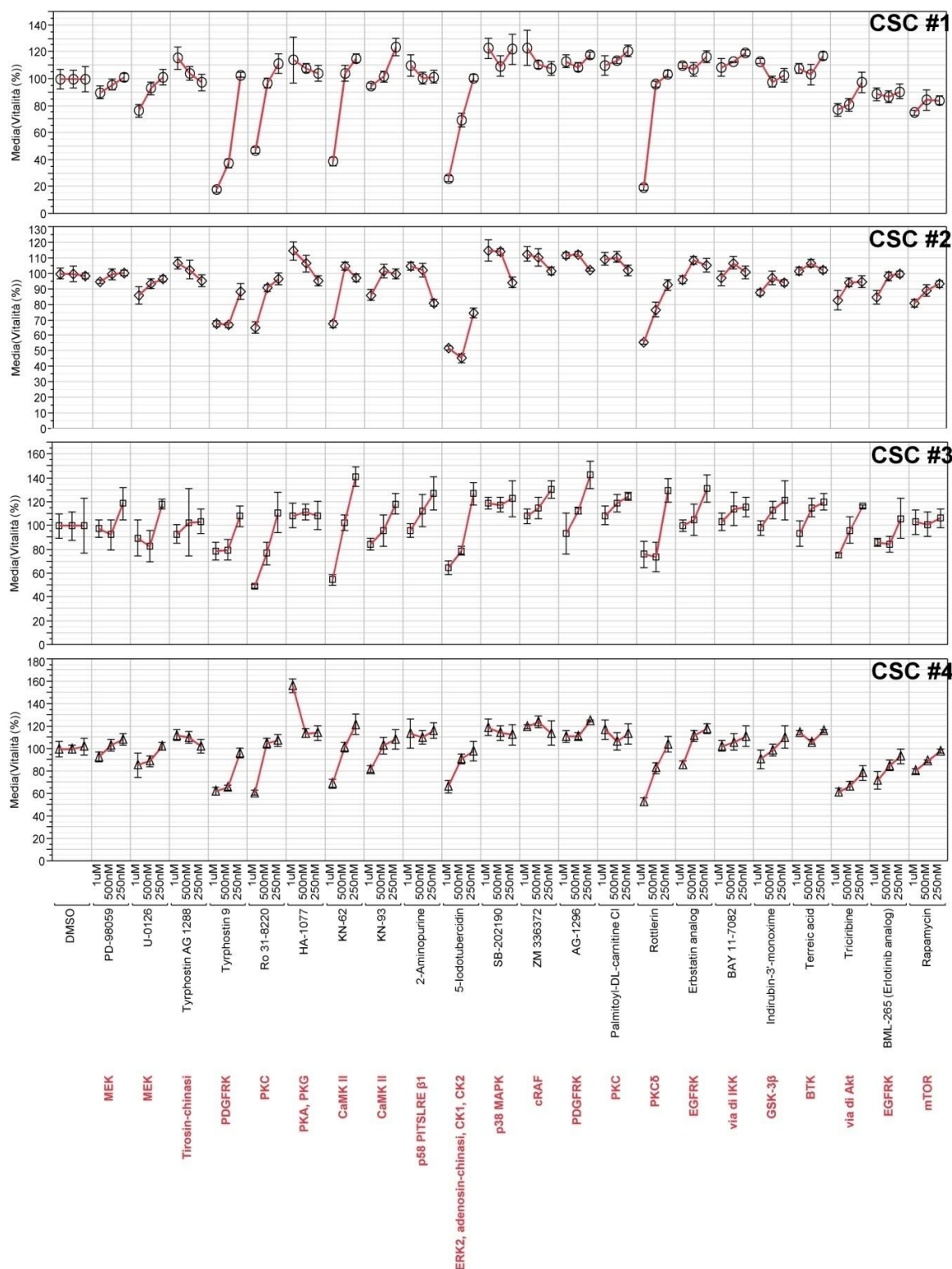


Figure 11. Viability chart after 48h of treatment with the library of kinase inhibitors (concentrations 1μM, 0.5μM and 0.25μM). Each symbol represents the average of at least three independent experiments and each error bar is calculated using one standard deviation. Vehicle control is represented by 0.1% DMSO.

The chart shows that the 4 lines of colon-CSC responded similarly, even with different degrees of sensitivity, to the lower doses of the inhibitors. The concentration decrease

brought to the disappearance of growth inhibition effects or death due to some inhibitors thus narrowing the pool of effective compounds and indicating that effect observed at higher concentrations could be due to involvement of pathways different from the one they were specific for. Signal transduction pathways that remained critical at lower concentrations were: PDGFR, ERK, GSK3 β , Akt and PKC.

4. Combination with chemotherapeutic agents

Since the actual potential of new compounds in the therapy of colon cancer is likely the association with established chemotherapeutic regimens, we assessed these compounds *in-vitro* ability in potentiating chemotherapy effect on colon-CSC. The rationale behind these regimens implicates that is possible to achieve better effects while maintaining doses tolerated by the patients. With this in mind we evaluated whether low concentrations of kinase inhibitors, although not effective alone, could enhance the Irinotecan cytotoxic or cytostatic effects.

Unfortunately after 72h of treatment none of the combinations used resulted in a significant impairment of cell viability compared with administration of single agents alone or with Irinotecan (Figure 12).

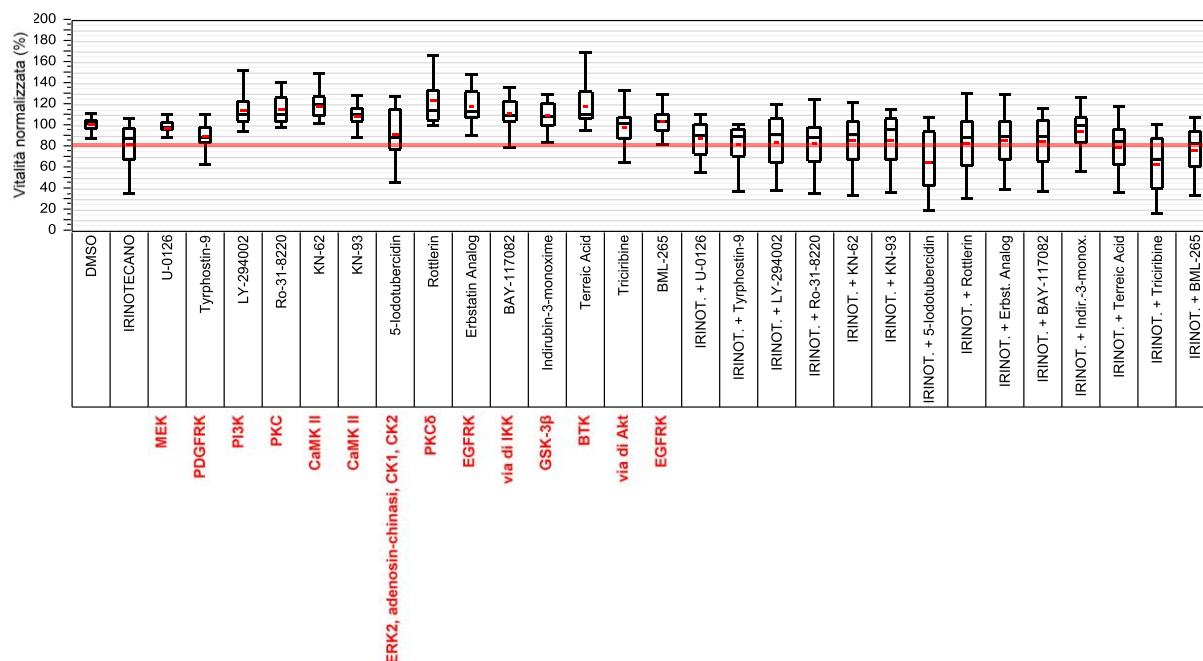


Figure 12. Viability chart after 72h of treatment with a set of inhibitors used alone or in combination with Irinotecan. The working concentration was 250nM for both inhibitors and Irinotecan. Vehicle control is represented by 0.1% DMSO. The thickened bar has been included as reference values of a single treatment with chemotherapy.

5. Definition of signal transduction pathways involved in survival/proliferation of colon-CSC

In order to assess the actual significance of a signaling pathway in CSC survival and proliferation we decided to use different inhibitors acting at different levels of the same pathway thus producing a more complete block. We were particularly interested in approaches with a relatively straight possibility of translation to clinical settings and therefore we performed a literature search, considering separately each of these molecular targets, to find molecules already in clinical use that could be used to confirm our data. For the PDGFR, some inhibitors were already approved by the FDA (Food and Drug Administration) such as Imatinib and Sunitinib. These are known to inhibit the tyrosine

kinase activity of this receptor but not in a specific and selective way: in fact they have at least another important target (c-kit for Imatinib and VEGFR for Sunitinib).

GSK-3 β inhibitors are currently used in phase I/II clinical trials (NCT00948259) for Alzheimer's disease, but their use for cancer treatment is hampered by the low bioavailability of currently used molecules.

Even if the pharmacological inhibition of ERK is possible through the use of MEK inhibitors, its upstream kinase, which mediates its activation, we excluded the importance of this target in colon-CSC since two major inhibitors of MEK (PD98059 and U-126) already used *in-vitro* has been shown to be not active in this study. The low relevance of ERK pathway in colon-CSC was confirmed by using by using a specific ERK inhibitor peptide *in-vitro* (Figure 13).

The inhibition of Akt and PKC may finally represent a therapeutic option for colon cancer. With the aim of confirming the positive data obtained previously we expanded our set of specific inhibitors for these two kinases (Figure 14).

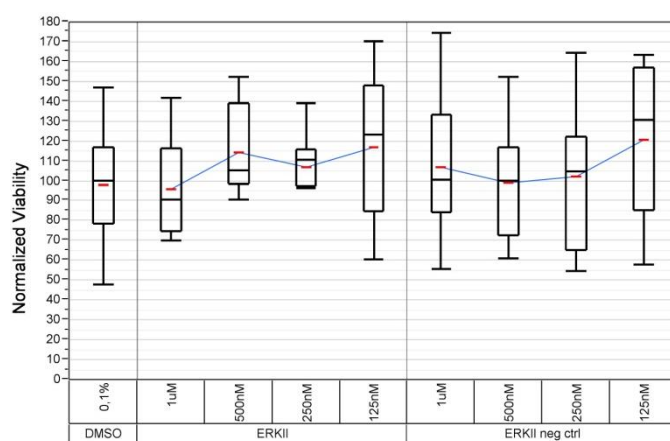


Figure 13. Viability chart after 48h of treatment with an ERK peptide inhibitor. Results are referred to 4 lines of colon-CSC. Vehicle control is represented by DMSO while negative control is represented by a random peptide sequence.

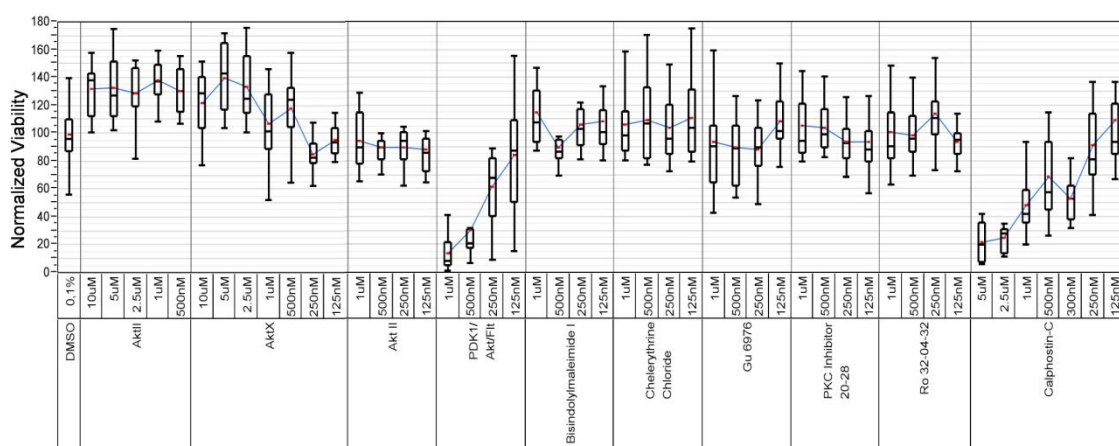


Figure 14. Viability chart after 48h of treatment with PKC and Akt commercially available inhibitors. Vehicle control is represented by DMSO.

The use of inhibitors other than those initially included in the library allowed for a better understanding of the mechanisms that regulates colon-CSC survival and proliferation. Treating these cells with the PKC inhibitor “Calphostin C”, an inhibitor of the regulatory site for diacylglycerol (DAG) binding and phorbol esters, was effective in all lines. On the other hand, inhibition of Akt was not effective if not through the use of a multiple inhibitor that can block both Akt, Phosphoinositol-3-kinase-dependent 1 (PDK1) and VEGFR (Flt1) at the same time. Therefore these results suggested the importance of PKC pathway, one of two main signal transduction cascade that rely on PDK1, for colon-CSC.

PKC key role was further demonstrated by the combined inhibition of Akt and a specific compound that block PDK1, namely OSU-03012 (Figure 15). The simultaneous treatment of colon-CSCs with Akt and PDK1 inhibitors has not been shown to be more effective than the single agents or the combination with the multiple inhibitor (PDK/Akt/Flt).

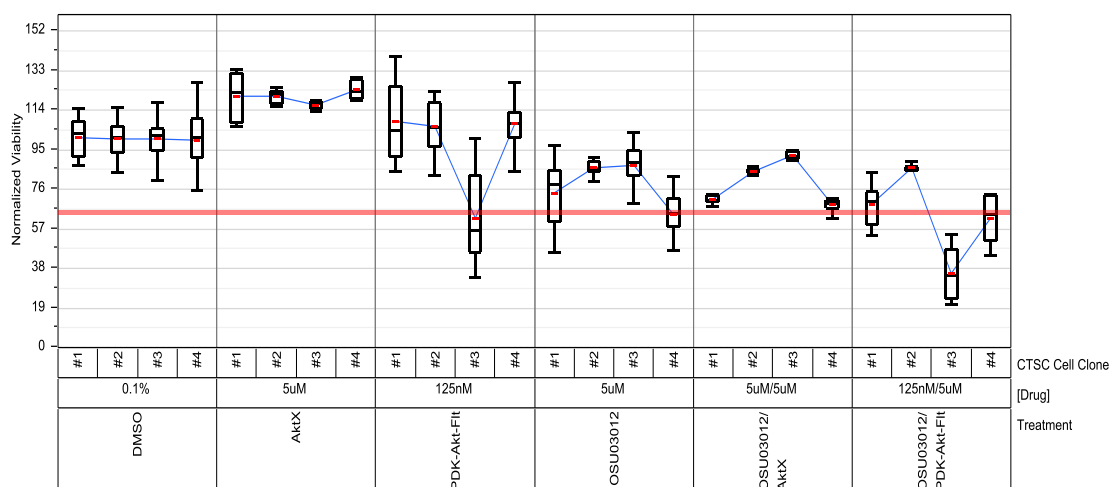


Figure 15. Boxplot of viability after 48h of treatment with inhibitors of Akt and PDK1. The results are shown for each line of colon-CSCs. Vehicle control is represented by DMSO. The thickened bar has been included as reference values of a single treatment with OSU-03012.

The low effectiveness of PDK1 inhibitor and the *in-vivo* toxicity of the multiple inhibitor, have determined a further shift towards other inhibitors of PDK1 and/or PKC. The only inhibitor that meets the needs of the experimental work conducted so far was the UCN-01. This molecule is a staurosporine derivative with a 100 times higher *in-vitro* IC50 and is well tolerated *in-vivo*. Among the known molecular targets for the UCN-01, there are PDK1, PKC and Chk1 (Checkpoint Kinase 1). UCN-01 *in-vitro* treatment of colon-CSC was effective albeit with a wide range of sensitivity (Figure 16).

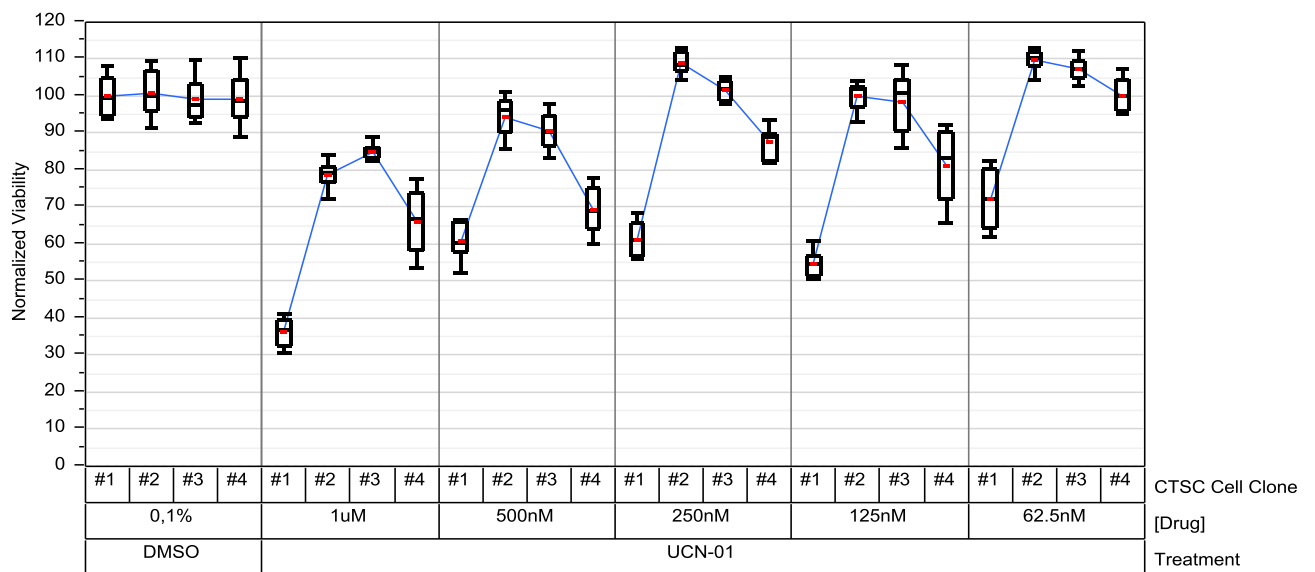


Figure 16. Boxplot of cells viability after 48h of treatment with UCN-01. Results are shown for each line of colon-CSC. Vehicle control is represented by DMSO.

To investigate the potentiating effect of this compound we decided to treat our colon-CSC with commonly used chemotherapeutics in association with UCN-01 (Figure 17).

We evaluated by western blot the effect of combined treatment from a molecular point of view. Figure 18 shows the results of immunoblot for caspase 3, Bcl-XL, Mcl-1 and PKC on lysates representing two colon-CSCs treated for 24 hours. The combination with UCN-01 enhances Irinotecan but not Oxaliplatin proapoptotic effect, causing an increased activation of caspase 3. Upon treatment Akt activation, which is the substrate of PDK1, is greatly diminished. Only in one line of colon-CSCs the combination led to an increase in this protein activation compared to individual treatments.

The potentiating effect is further confirmed by the decrease, in the combination sample, of phospho-PKC α/β II signal. This result could be a consequence of the PKC autoregulatory system. In turn, this reduction of PKC phosphorylation results in diminished expression of some important antiapoptotic proteins such as Bcl-XL, Bcl-XS and Mcl-1, in line with the activation of caspase 3.

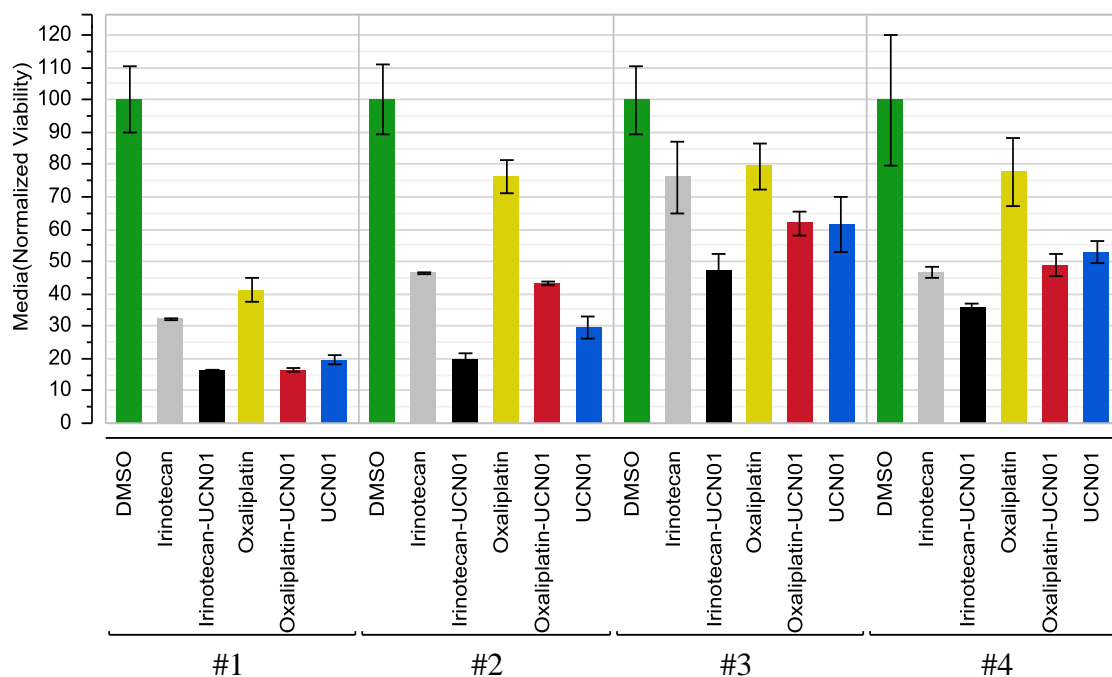


Figure 17. Bar graph depicting cells viability after 72h of treatment with UCN-01 (1 μ M), Irinotecan (50 μ M), Oxaliplatin (10 μ M) and their combination. Results are shown for each line of colon-CSC. Values are referred to the average of at least three independent experiments and each error bar is calculated using one standard deviation. Vehicle control is represented by DMSO.

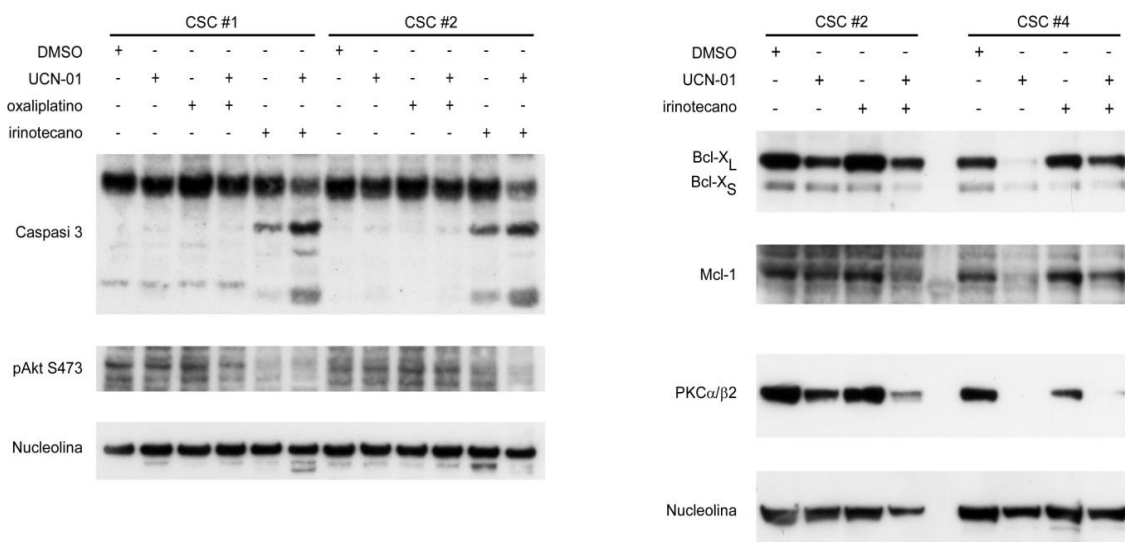


Figure 18. Immunoblot of colon-CSC lysates treated with chemotherapy, UCN-01 and their combination for 24 h. Nucleolin has been used as loading control.

The highest potentiating effect has been observed by combining UCN-01 with Irinotecan and this led to the use of this combination *in-vivo* in a xenograft model. In these models colon-CSC are subcutaneously injected into immunodeficient mice leading to the growth of a tumor mass in the mouse flank. This model represents a significant qualitative leap in the study of tumor biology because it allows to the analysis of preclinical parameters concerning new experimental drugs directly on target cells.

Therefore we proceeded *in-vivo* treating xenograft bearing mice with UCN-01 (5mg/kg), Irinotecan (10mg/kg) and their combination. By comparing (nonparametric Wilcoxon test) tumors growth rate we found a significant impairment in tumor growth only in the Irinotecan and in the combination groups respect to the vehicle control, with a p-value of 0,03 and 0,01 respectively (Figure 19).

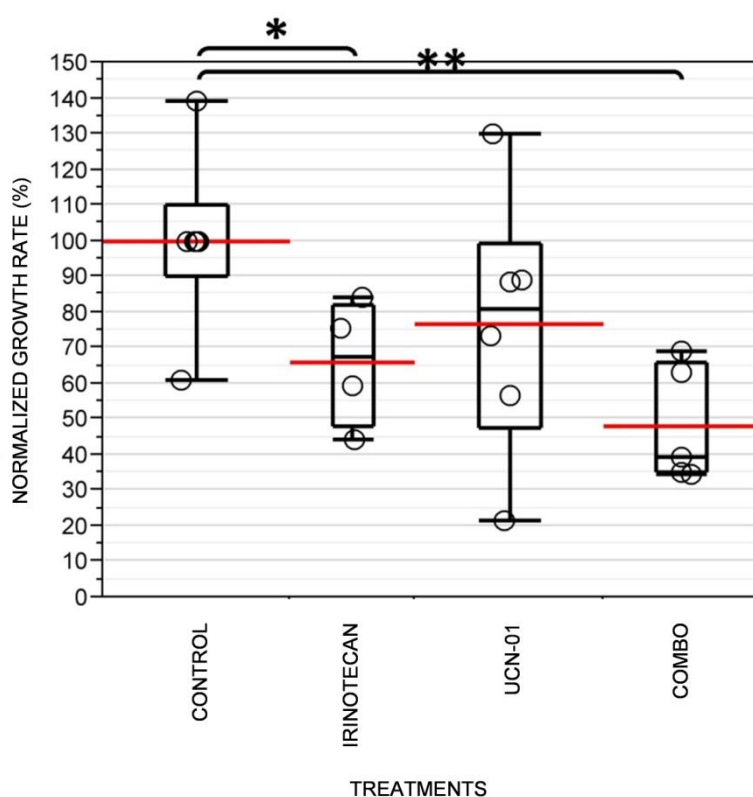


Figure 19. Boxplot graph showing the percentage of xenograft growth compared to control in NOD/SCID mice treated with Irinotecan, UCN-01 and the combination of these two agents. The values represented were obtained from measurements of subcutaneous tumors of two independent experiments on the seventh day after treatment start. Statistically significant different results are connected by bars with asterisks to indicate the relative level of significance (* $p < 0.05$; ** $p < 0.001$).

Discussion

In recent years there has been a rapid increase in therapeutically available approaches for CRC and this, in turn, has led to an overall patient's survival improvement. Even if

irinotecan and oxaliplatin have been introduced into the clinical system more than ten years ago, these two compounds remain the main therapeutic options for CRC patients. Unfortunately response to these treatments is not always definitive, with a mean 5-year survival rate of about 40% [1, 146]. For these reasons new therapies are increasingly oriented towards the use of targeted approaches able to specifically interfere with the major signal transduction pathways that control cell proliferation and survival that are crucial in tumorigenesis and tumor progression.

Recently characterized CRC aberrant/hyperactive signaling pathways allowed the understanding of the molecular mechanism underlying neoplasm initiation and progression, thus permitting the identification of potential molecular targets and predictors for new therapies. Automated platforms for phosphoproteomic analysis, as RPPA ("Reverse Phase Protein Array") technology, provided important support to tumors molecular characterization. By means of antibodies that can specifically recognize the phosphorylated form of different proteins, RPPA can quantify phosphorylation/activation levels for a large number of kinases from different samples in short time.

Protein kinases represent an important group of enzymes crucially involved in controlling survival and tumor progression signals. Their inhibition could be obtained by two main classes of compounds: monoclonal antibodies and small molecules inhibitors of natural or synthetic origin. The use of these compounds has also been applied to CRC treatment, in order to selectively inhibit tumor-associated activity of kinases such as EGFR, VEGFR, PDGFR, PI3K and mTOR. Although in CRC patients targeted therapy has produced a significant increase in progression free survival rate present clinical protocols are still ineffective in definitively eradicating the tumor [2]. The limited efficacy of currently available anti-cancer therapies has recently been linked to the possibility, supported by a growing body of evidence, that cancer is a stem cell disease. In particular, the recent identification of cell populations with stem cells characteristics in human tumors, including CRC, suggested that these might originate from a small fraction of tumor cells, known as "cancer stem cells" (CSC). CSCs are characterized by self-renewal ability, multipotency and tumorigenicity. For these reasons CSC should be the target of choice for new selective therapies aimed at a complete tumor eradication. Since targeted inhibition of colon-CSC activated protein kinases would induce their efficient elimination, leading to a complete tumor regression, the understanding of CSC behavior and, in particular, the molecular mechanisms that regulate self-renewal, survival, apoptosis, proliferation and differentiation is of major interest.

In this thesis, we performed an RPPA analysis on four colon-CSC lines. Results showed a great molecular heterogeneity among different patients, thus hindering the identification of a single common potential therapeutic target. Therefore, using a reverse approach, CSC lines were subsequently subjected to an *in vitro* screening of 80 protein kinase inhibitors with known targets and mechanisms of action. This analysis led to the identification of several pathways crucially involved in proliferation and survival of colon-CSC such as EGFR, AKT, ERK, PKC, PDGFR and GSK3 β . Although preliminary data suggested a certain degree of correlation between *in vitro* and RPPA positive hits, such correspondences will require further studies aimed at defining molecular biomarkers predictive of drug sensitivity.

In a second part of the study we scaled down compounds concentration in order to increase the specificity of our experimental setting. This resulted in colon-CSC sensitivity reduction to EGFR inhibitors letting us excluding them from the subsequent phases. In addition, due to the already described low degree of specificity of clinically used PDGFR inhibitors and to the low effectiveness of GSK-3 β inhibitors we excluded these two proteins from further screening. On the other hand, PKC inhibition resulted in a colon-CSC viability significant reduction in contrast to that observed following ERK and Akt inhibition. PKC serine/threonine kinases family includes more than ten isoforms which differ in structure, function and tissue distribution. [147-149]. Each PKC isoform is specifically involved in survival and death control regulation of different cell types. Some studies have shown that PKC may play both pro-and antiapoptotic functions in relation to cell type or the extent of the stimulus [147, 150-152]. Among CRC related alterations there are also those affecting PKC isoforms transduction pathways [155]. It is well known that CRC carcinogenesis in both rodents and humans is accompanied by specific alterations in PKC isoforms expression [155-159]. PKC β II increased expression, for example, is an early event in CRC carcinogenesis [160] and is associated with the acquisition of a hyperproliferative phenotype of colon epithelium [156]. PKC potential role in CRC tumorigenesis is supported by a series of scientific papers in which HGF (hepatocyte growth factor) signal transduction pathway have been investigated. In particular, PKC α activity has been shown to be important for colon-CSC stemness and tumorigenicity.

Colon-CSC marked sensitivity to PKC inhibition has prompted us towards a better investigation of UCN-01 effects. This is a Staurosporine derivative initially identified as a PKC inhibitor that is also able to inhibit PDK1 (3-phosphoinositide-dependent kinase 1) and Chk1 (Checkpoint Kinase 1) kinase. This compound is currently used as antineoplastic

agent, in combination with chemotherapy, in phase I clinical trials for solid tumors treatment [140, 141, 162]. All colon-CSC lines were *in vitro* sensitive to UCN-01 which has also been proven to significantly increase irinotecan cytotoxic effects. The molecular basis for this synergism may be due to the increased apoptotic response following irinotecan/UCN-01 combined treatment. In general, chemotherapy induced genotoxic stresses produced a response that varies depending on damage extent [163]. Genomic damages caused by irinotecan originated from topoisomerase I inhibition that is able, by itself, to trigger an apoptotic response as suggested from caspase 3 activation. Therefore, the interference with PKC and PDK1 pathways in a context in which genotoxic stresses are already present, might move the balance between cell survival and death programs towards the latter. Indeed, we observed an increased caspase 3 activity and a parallel reduction of the expression of the anti-apoptotic protein Bcl-XL and Mcl-1, following irinotecan/UCN-01 combined treatment, confirming an enhancement of apoptotic signals.

UCN-01 effects on PKC inhibition, and in particular on PKC α and PKC β II isoforms, were based on a reduction of these proteins autophosphorylation processes (threonine 638 and 641), which are necessary for catalytic activity maintenance [164, 165]. Our experimental data demonstrated that irinotecan/UCN-01 combination is able to enhance the inhibitory effects already observed with UCN-01 single treatment. PKC α isoform is known to exert its function as a promoter of cell survival by modulating the expression or the activity of antiapoptotic proteins Bcl-2 and Bcl-XL [147, 166]. Following irinotecan/UCN-01 combined treatment we observed a reduction of Bcl-XL and Mcl-1 proteins. Since in some cellular context PKC β II isoform is known to regulate NF κ B mediated survival, UCN-01 could also interfere with colon-CSC proliferation through this mechanism [167]. PDK1 protein kinase, an UCN-01 secondary target, is known to transduce signals involved in PI3K/Akt pathway thus promoting Bcl-2 protein family expression [168]. UCN-01-induced PDK1 reduced activity, was linked to the decrease of its autophosphorylation (Serine 241), further explaining the enhanced apoptotic response obtained with irinotecan/UCN-01 combined treatment in colon-CSCs. The analysis of UCN-01 sensitivity, in murine xenograft models, confirmed our previous *in vitro* observations, demonstrating that irinotecan/UCN-01 combination is able to significantly impairs tumor growth compared to chemotherapy alone.

The ability of UCN-01 to promote colon-CSC growth impairment could also, at least in part, be attributed to Chk1 kinase inhibition, as demonstrated by the reduced levels of phosphorylation of the Chk1 target, cdc25 phosphatase. In response to DNA damage, Chk1

phosphorylates cdc25 thus preventing cell cycle advancement. Under this light the synergism showed by irinotecan/UCN-01 combination could be explained as a result of the alteration in cell cycle blocking mechanisms after genotoxic stresses produced by chemotherapy. Therefore colon-CSC inability in repairing chemotherapy induced DNA damages, mainly due to Chk1 inhibition, could be another mechanism through which UCN-01/irinotecan combination exerts its effect.

UCN-01 possesses a much higher activity and a much lower toxicity than Staurosporine, however its ability to interfere with various protein kinases activity (PKC, PDK1 and Chk1) could be the real basis for its effects.

Therefore a better molecular characterization of UCN-01 molecular mechanism of action, in order to understand the relative weight of these processes, will be of major interest. To this aim, in the near future, we will perform RNA-interference experiments to selectively downmodulate PKC or PDK1 isoforms and we will analyze UCN-01 role on colon-CSC cell cycle block impairment. A further RPPA analysis on a wider CRC patient's population will be performed either to associate protein activation to patient's prognosis or to infer the efficacy of specific small molecule inhibitors.

CSC are thought to be the ultimate responsible for tumor maintenance and progression. The experimental framework described in this thesis allowed the identification, among the vast number of pharmaceutical active compounds, of those that could interfere with CSC survival and proliferation processes. For this reason only a clear patients stratification, based on mutational and/or phosphoproteomics status, will improve success rate in the search for better targeted therapies.

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