University of Milano-Bicocca

PhD in Molecular and Translational Medicine DIMET

Characterization of a novel isoform of Bruton's tyrosine kinase involved in resistance to drug-induced apoptosis of carcinoma cells

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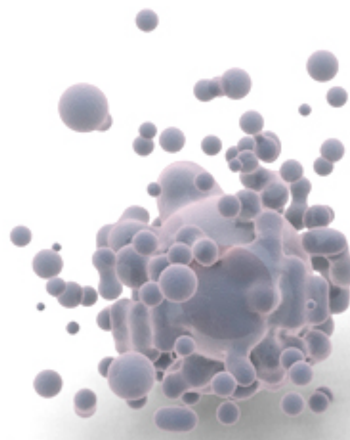
1. INTRODUCTION

1.1 APOPTOSIS

In all Eukaryotic organisms, both during development and in the adult life, the total number of cells is tightly regulated by a fine balance between proliferating and dying cells and a disruption of this balance can lead to the development of a number of diseases among which malignancies. The hypothesis that tumour development and progression could be influenced by apoptosis came first from the original work from Kerr, Wyllie and Currie [1972]. In their work they showed that the growth rate of tumours was less than the predicted rate as a result of a high level of endogenous tumour cell apoptosis. Extensive studies that followed this first work by Kerr et al. not only confirmed that apoptosis played an important part in tumour growth, but showed also that its malfunction and/or inhibition play pivotal roles in cancer progression and resistance to therapy [Kerr et al., 1994].

Apoptosis is a genetically controlled process that occurs not only in eukaryotic but also in unicellular organisms and whose basic mechanisms are conserved throughout the evolution. Characteristic morphological and biochemical

changes occur during the ongoing of the apoptotic process: the first step is cell shrinkage, followed by chromatin condensation, nuclear fragmentation, and membrane blebbing leading to the formation of vesicles containing cytoplasm, organelles and nuclear fragments. These vesicles, called apoptotic bodies [Hengartner MO et al., 2000] are initially released from the dying cell until it completely breaks up giving raise to several apoptotic bodies, eventually cleared by phagocytosis in the absence of an inflammatory response.



Apoptotic cell

Molecular mechanisms of the apoptotic process

Caspases

Responsible for the fragmentation process of the dying cell is the massive activation of caspases, a family of cystein aspartate specific proteases that proteolitically digests pivotal molecules such as the components of the cytoskeleton and other structural proteins, transcription factors, enzymes, etc..., eventually leading to the demise of the cell [Earnshaw et al., 1999; Nicholson DW., 1999]. Caspases are activated according to a hierarchical cascade and based on their function; they are classified into three subtypes:

-Initiator - caspases that possess long pro- domains, containing either a death effector domain (caspase-8 and -10) or a caspases activation and recruitment domain (caspase-2 and -9), which mediates the interaction with adaptor molecules.

-Effector - executor caspases (caspase-3, -6 and -7) that are typically processed and activated by upstream caspases and perform the execution steps of apoptosis by cleaving multiple cellular substrates [Degterev A et al., 2003].

-Inflammatory - caspases such as caspase-1, -4, -5, -11, -12, -13 and -14 involved in inflammation instead of apoptosis.

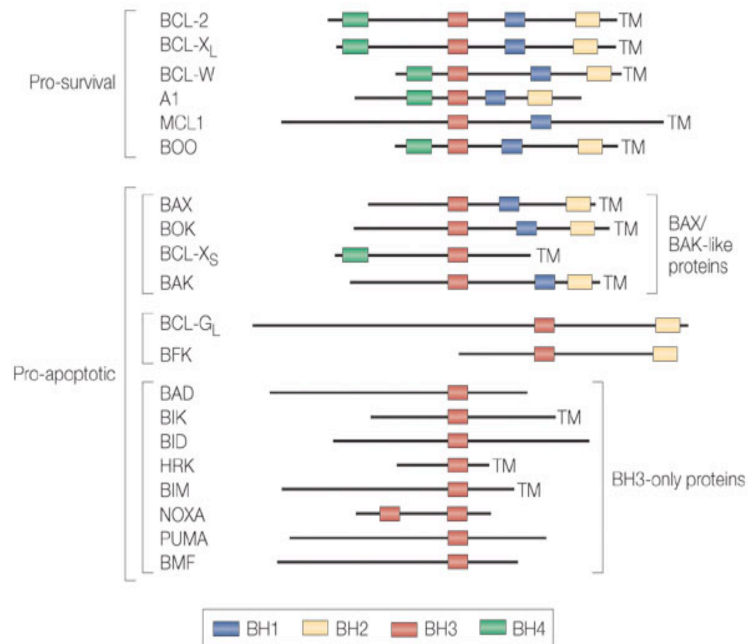
Caspases are always expressed at high levels inside the cell in an inactive form (pro-caspases) and undergo activation upon caspase-mediated proteolytic cleavage (auto- or trans-activation) when apoptosis is induced.

Bcl2 family

It comprises more than 20 members containing common motifs (Bcl-2 Homology – BH – domains) and can be grouped into 3 subfamilies, according to their structures and functions:

Anti-apoptotic (or Bcl2-like) subfamily: formed by Bcl-2, Bcl-xl, Mcl-1, Bcl-w e A1, very similar among them and characterized by the presence of 4 BH domains. They protect cells from several different apoptotic stimuli by inhibiting mitochondrial activation.

Pro-apoptotic (or Bax) subfamily: formed by only 2 members, Bax and Bak, very similar to each other and containing only 3 BH domains. They trigger mitochondrial activation thus leading to caspase activation.



Bcl-2 family members

"BH3-only" subfamily: it's the bigger group with more than 10 members including Bad, Bid, Bik, Bim, Bmf, Hrk, Noxa e Puma [Adams & Cory, 1998; Huang & Strasser, 2000]. The only conserved domain is the BH3 domain and outside this domain they share very low level of homology. They exert a pro-apoptotic action but only when Bax or Bak are present.

The balance between pro- and anti-apoptotic members, mediated by protein-protein interaction, is pivotal in

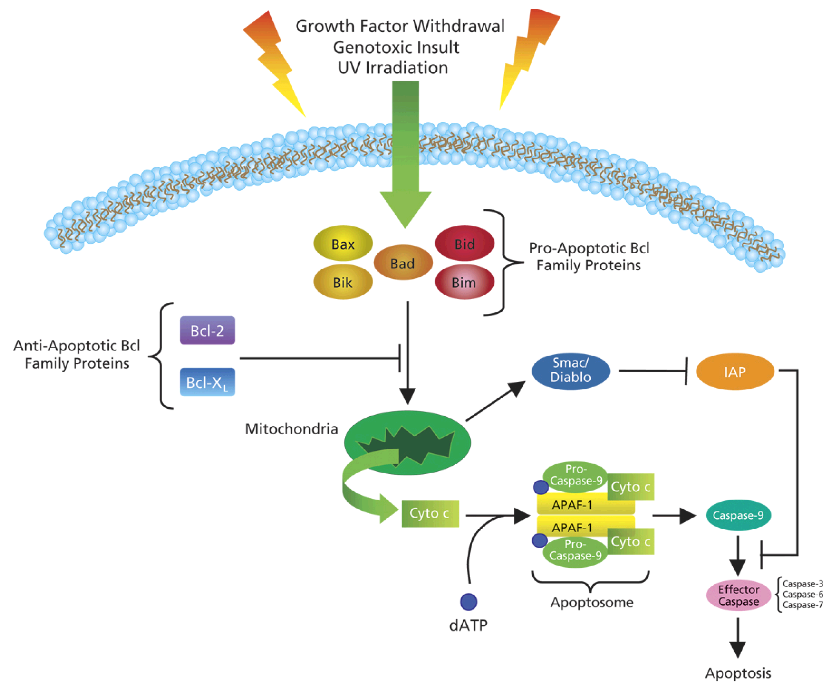
determining cell fate (life vs. death). Usually Bax and Bak are kept in check by anti-apoptotic members: when BH3-only proteins are activated they displace the Bcl2-like proteins, thus freeing Bax and Bak that can trigger mitochondrial activation.

Mitochondrial activation and the intrinsic pathway of apoptosis

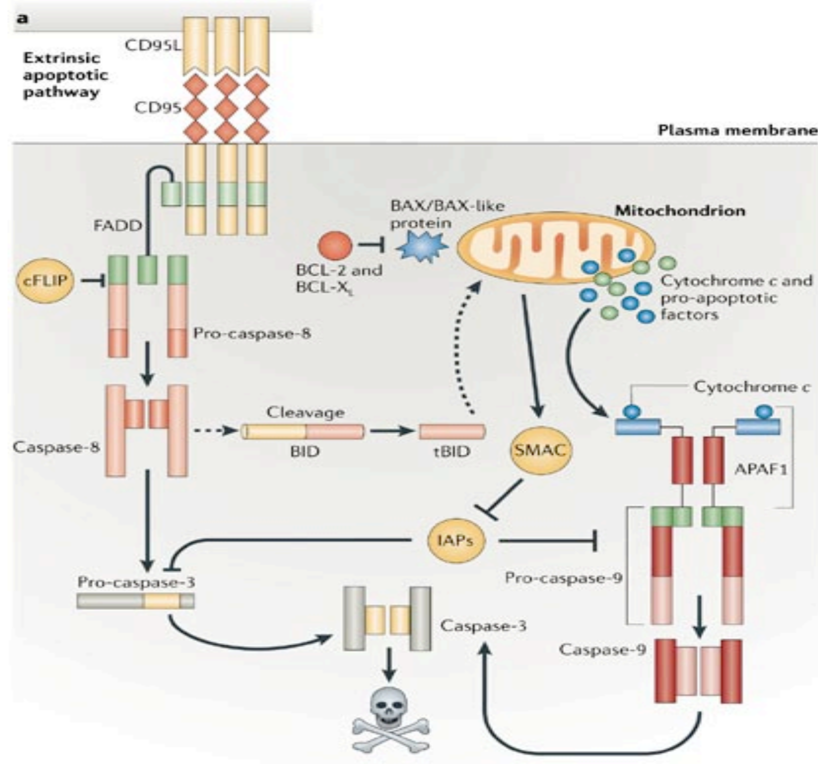
This term indicates a process where, as a result of mitochondrial membrane permeabilization (MMP), several mitochondrial proteins are released to the cytosol, including cytochrome-c, HtRA2/Omi and Smac/DIABLO (second mitochondria-derived activator of caspase/ direct inhibitor of apoptosis protein binding protein with low pI). and starts after a BH3-only protein has released Bax or Bak from Bcl2-like family members-mediated inhibition. Bax or Bak in the mitochondrial membrane starts their own oligomerization to form a pore-like structure and facilitating the release of mitochondrial proteins [Cheng EH et al., 2001]. Once released from the mitochondrion cytochrome-c (cyt-c) together with apoptosis protease activating factor-1 (Apaf-1) form the apoptosome, an activating platform responsible for recruiting and activating the inactive pro-caspase-9. Once activated, this

caspase can then activate the effector caspases.

The chain of events described above characterize what is referred to as the intrinsic pathway of apoptosis, usually triggered when cells are exposed to genotoxic insults, such as UV irradiation, gamma-rays, DNA-damaging agents (chemotherapeutic drugs included) and following growth factors withdrawal.



The extrinsic pathway of apoptosis



This apoptotic pathway is receptor-mediated and dependent from the activation of receptors belonging to the tumour necrosis factor (TNF) receptor superfamily: besides, TNFR itself, Fas (CD95) and TNF-related apoptosis induced ligand (TRAIL) receptors. Upon binding by their ligands a

trimerization of the receptors occurs leading to the recruitment at the internal face of the plasma membrane of the so-called “death-inducing signalling complex” (DISC) in which an adaptor protein, Fas-associated death domain protein (FADD) operates as a bridge to bring two molecules of pro-caspase-8 in close proximity. This event allows the transactivation of caspase-8 at the DISC: activated caspase-8 in turn cleaves and activates effector caspases, caspase-3, -6 and -7 [Ashkenazi A et al., 1998]. This pathway may be antagonised by cellular c-FLIP, by disabling DISC-mediated processing and abolishing the release of active caspase-8 [Krueger A et al., 2001]. Only in the case of TNFR, at the DISC one more adapter is recruited: TRADD, or TNFR1-associated death domain protein, interacts with the cytoplasmic tail of TNFR1 and recruits FADD which in turn recruits pro-caspase-8 as described above. Beside recruiting molecules involved in apoptosis signal transduction TRADD is able to interact also with proteins leading to NFkB activation and transducing proliferative signals: therefore the outcome of the signal initiated at the TNFR1 may be different from apoptosis, depending of which other molecules are recruited into the signalling complex associated at the cytoplasmic tail of the receptor.

In some type of cells the extrinsic pathway operates at very low efficiency, that is the levels of active caspase-8 produced are not sufficient for allowing enough effector caspase activation to bring upon the death process. However, the levels of active caspase-8 are enough to cleave, and therefore activate, a BH3-only protein: Bid. Truncated Bid (t-Bid) subsequently translocates from the cytosol to the outer mitochondrial membrane (OMM) and triggers mitochondrial activation and the intrinsic pathway.

Other regulatory mechanisms in apoptosis.

Caspase activation can be downregulated or completely abolished by inhibitors of apoptotic proteins (IAPs) that bind to them, even after their cleavage, and inhibit their proteolytic activity [Zhivotovsky B et al., 2003]. Moreover, due to their intrinsic Ub-ligase activity IAPs also promote caspase degradation. Members of the IAPs-family of proteins include: XIAP, c-IAP1, c-IAP2, NAIP, survivin, livin, Ts-IAP, and BRUCE [Salvesen GS et al., 2002].

IAPs action can be in turn antagonized by other molecules such as SMAC/DIABLO and HtrA2/Omi. These molecules,

normally localized inside the mitochondrion, after mitochondrial activation translocate to the cytoplasm where they bind to and inactivate IAPs by displacing them from their caspase binding [Holcik M et al., 2001].

The tumour suppressor gene p53 mediates G1 growth arrest by inducing the cyclin-dependent kinase inhibitor p21/waf1/cip1. p53 also regulates the intrinsic pathway in apoptosis by trans-activating pro-apoptotic Bcl-2 family members and repressing anti-apoptotic Bcl-2 proteins and IAPs including survivin [Wu Y et al., 2001]. Mutations in cancer cells commonly disrupt the intrinsic apoptotic pathway by affecting p53 or Bcl-2-family of anti-apoptotic proteins, promoting survival of transformed cells.

1.2 CHEMOTHERAPY AND RESISTANCE IN COLORECTAL CANCER

Colorectal Cancer

Cancer is a multistep process, which requires 7 to 10 mutations and/or genetic inactivations to occur in order to allow the cell to become fully oncogenic [Hanahan et al., 2000]. Very often, genes affected by such mutations encode for molecules belonging to the pathways that allow the repair of genetic damage or that control the apoptotic process, endowing the cell with metastatic potential [Lynch et al., 2002].

Colorectal cancer (CRC) is the third most frequently diagnosed cancer in men and women and represents a major public health problem accounting for over 1 million cases of new cancers and about half a million deaths worldwide [Parkin DM et al., 2005]. After breast cancer and lung cancer it's the most common case of mortality in developed countries. Moreover colon cancer seems to have higher prevalence in women while rectal cancer is more widespread in the population of men [Steward et al., 2003]. This type of cancer

occurs when abnormal cells grow in the lining of the large intestine (colon) or rectum.

Notwithstanding the overall improvements in CRC therapy, our understanding of why individual patients respond to therapy and others do not, and why some patients relapse, whereas others do not, remains poor. Despite curative surgery in those presenting early, the risk of recurrence is significantly high. Hence much work has been done in search of effective adjuvant therapy for the eradication of metastases.

In colon cancer, chemotherapy is the principal adjuvant therapy and the addition of radiotherapy to chemotherapy has not been shown to improve outcome [Martenson JA, Jr. et al., 2004]. Because colorectal cancer is stealthy, screenings are the key to early detection and successful diagnosis. Beginning at age 50, most people should have a colonoscopy every 10 years. These tests not only find tumours early, but also can actually prevent the progression of disease by removing polyps. Yet, colon carcinoma when found early, it is highly curable.

Classification of colon carcinomas

Classification of colon carcinoma prepared by American Joint Committee on Cancer [AJCC, 2002] is based on the TNM system, where T describes tumour dimensions, N - lymph nodes engagement and M - the metastasis to distant sites.

Dukes classification was proposed by Dr. Cuthbert Dukes [Dukes CE et al., 1956] and divides the progression of colon carcinoma into 4 stages:

A - Tumour confined to the intestinal wall (muscularis propria). High survival prognosis.

B - Tumour invading through the intestinal wall (perforation of peritoneum but lymph nodes are not involved)

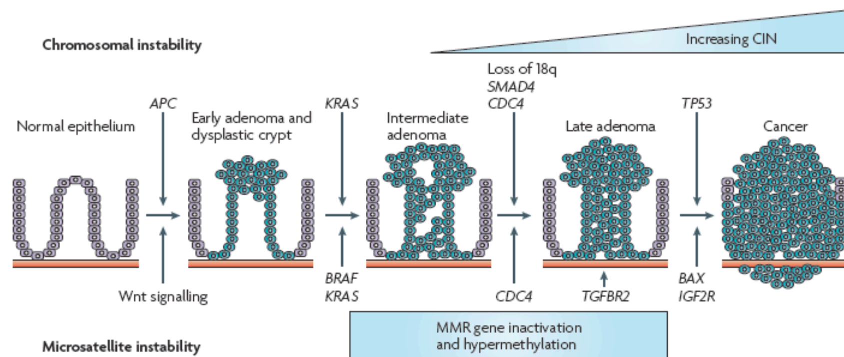
C - With lymph node(s) involvement (this is further subdivided into C1 lymph node involvement where the apical node is not involved and C2 where the apical lymph node is involved). No metastasis.

D - Distant metastasis, low survival prognosis.

AJCC stage		TNM stage	TNM stage criteria
Stage 0		Tis N0 M0	Tis: The most superficial of all the lesions and is limited to the mucosa without invasion of the lamina propria. Because of its superficial nature, the surgical procedure may be limited.
Stage I		T1 N0 M0	T1: Tumour invades submucosa
		T2 N0 M0	T2: Tumour invades muscularis propria
Stage II	**A**	T3 N0 M0	T3: Tumour invades subserosa or beyond (without other organs involved)
	B	T4 N0 M0	T4: Tumour invades adjacent organs or perforates the visceral peritoneum
Stage III	**A**	T1-2 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T1 or T2.
	B	T3-4 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T3 or T4.
	C	any T, N2 M0	N2: Metastasis to 4 or more regional lymph nodes. Any T.
Stage IV		anyT, any N, M1	M1: Distant metastases present (to the liver, bones, lungs, adrenal glands). Any T, any N.

Molecular events underlying neoplastic transformation and their contribution to chemoresistance.

During the carcinogenesis mutations in two molecular pathways can be detected: gatekeeper pathway and caretaker pathway [Kinzler and Vogelstein, 1997]. In gatekeeper pathway mutations in genes accounting for cellular growth are observe whereas in caretaker pathway mutations affect cellular systems of DNA repair.



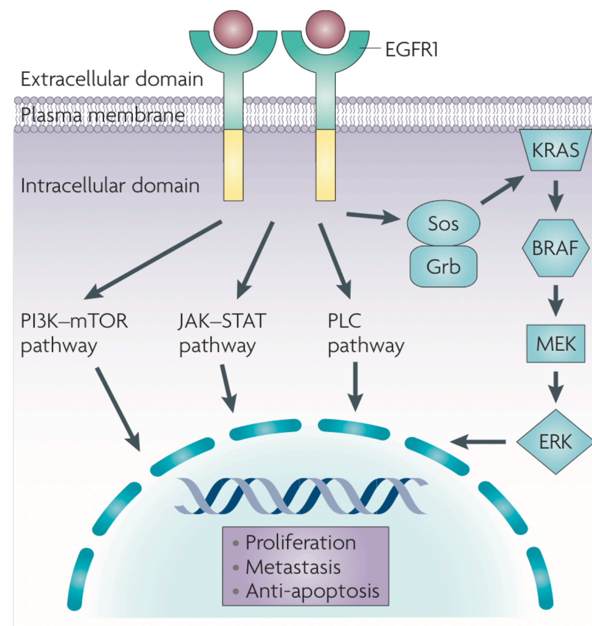
Molecular events occuring in colon carcinoma

Gatekeeper pathway

Mutations in KRAS and BRAF

K-Ras gene codifies for a protein involved in transduction of signals necessary for normal cellular differentiation and proliferation [Bourne et al., 1990]. RAS is anchored to the intracellular membrane and has the intrinsic GTPase activity regulated by other proteins [King et al., 2000]. As a consequence of GTP binding RAS becomes activated. Biological effects of RAS are mediated by three different signal transduction pathways and activate different kinases, like RAF, MEF and MAPK, leading to signals regulating many transcription factors [Malumbres M et al., 2003]. KRAS mutations are an early event in the adenoma–carcinoma sequence, although they are demonstrable in only one-third of CRCs [Andreyev et al., 1998]. Many studies evaluate KRAS mutations in exon 2 and in exon 3 for their association with CRC outcome. Mutations in each of codons compromise the ability of GTPase-activating proteins to effect the inactivating hydrolysis of Ras-bound GTP to GDP [King et al., 2000]. RASCAL, the largest international effort to combine data from different groups found that KRAS mutations generally confer a worse prognosis for the survival [Wang C et al., 2003].

More recently KRAS mutation status has been established as a predictive marker for treatment with epidermal growth factor receptor (EGFR) inhibitors.



Schematic EGFR signalling pathway

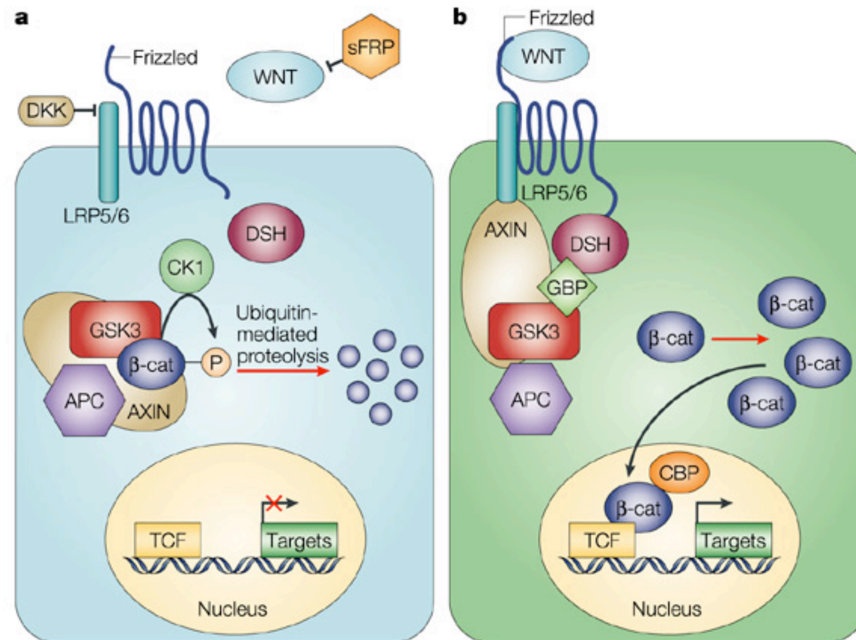
Nearly 99% of patients with mutated KRAS do not respond to EGFR inhibition [Karapetis, C. S. et al., 2008]. Moreover, in 80 KRAS wt patients treated with cetuximab, 11 patients with the BRAF v600e mutation did not respond to the treatment [Di Nicolantonio, F. et al., 2008]. The serine-threonine kinase BRAF is the principal effector of KRAS and mutations in the two genes seem to be mutually exclusive [Rajagopalan, H. et al., 2002].

Therefore, B-Raf could be a protein affecting the same pathway [Di Nicolantonio F et al., 2008], and together with K-Ras are important determinants of resistance to EGFR-specific therapies.

Alterations in Wnt pathway: Mutations in Adenomatous Polyposis Coli or beta-catenin.

In the absence of signalling beta-catenin is inactivated by a complex of APC/Axin/GSK3-beta. Ligands of WNT-type regulate various stages of development in mammals [Senda T et al., 2005]. They bind to frizzled receptors and activate Dishevelled (Dsh), which in turn inhibits GSK3-beta kinase. When the above-mentioned kinase is inactive beta-catenin cannot be degraded in cytosol and thus can translocate to nucleus, where it binds the TCF/LEF transcription cofactor. This binding regulates transcription of more than 500 genes, such as c-myc and cyclins, necessary for cell cycle progression [Sansom OJ et al., 2004]. Gene for APC is localized on chromosome 5 and is a part of Wnt signalling pathway, often deregulated in colon cancer [Fodde R et al., 2001]. Product of APC gene acts to promote the degradation of beta-catenin and limits the transcription of Wnt target genes engaged in the progression the cell cycle [Fodde R

et al., 2001]. Experiments with animal models show that knocking-out the APC gene in mice is responsible for abnormal organization of villi, increased number of mitoses and the arrest of the differentiation of cells that in result become poliploid [Sansom OJ et al., 2004]. In addition to mitosis also apoptosis increases dramatically during first days after APC ablation, causing death of normal epithelial cells of colon but not affecting clones that gained resistant phenotype. It is known that mutations in this gene in the germline accounts for Familial Adenomatous Polyposis (FAP) – an autosomal dominant disease characterized by the development of numerous colorectal adenomas from the early life stages (adolescence). Wnt pathway is important to colorectal tumorigenesis, and more than 90% of patients have alterations that affect it [Thorstensen, L. et al., 2005]. APC mutation could contain prognostic information - mutations that abolish β-catenin-binding sites of APC may be associated with poorer prognosis [Lovig, T. et al., 2002]. Determining the cellular localization of overexpressed β-catenin could also hold prognostic information [Hugh, T. J. et al., 1999]. Unfortunately changes in APC and β-catenin are insufficiently validated in patients to have a role in clinical practice.



Wnt pathway

Loss of functional tumour suppressor p53.

Loss of heterozygosity at chromosome 17p has been associated with 75% of adenocarcinomas [Leslie et al., 2002], and many studies have focused on the 17p region containing the p53 tumour suppressor gene. The role of p53 can be described as a 'guardian of the genome'. This role can be described as maintaining DNA integrity by activation of genes, such as

CDKN1A and GADD45α, which induce cell-cycle arrest as a response to a mild DNA damage [Lane DP et al., 1992]. This process depends on the cellular context and the nature of the DNA damage. When the DNA damage is to heavy to be repaired in fact p53 can induce elimination of the damaged cells by apoptosis through the induction of pro-apoptotic genes. As a transcription factor, p53 regulates the transcription of different apoptotic targets. Perhaps the most represented targets are those belonging to the bcl-2 family members: besides downregulating the anti-apoptotic BCL2 [Miyashita T et al., 1994], p53 positively regulates the expression of BAX in both in vitro and in vivo systems [Zhan et al 1994] and is the main inducer of puma, noxa, bid [Roos WP et al., 2006]. In addition to controlling members of the Bcl-2 family, p53 controls the expression of other key apoptosis-regulating proteins, such as genes codifying for the death receptors and their ligands (Fas/CD95, FasL, TRAIL, DR5) and genes involved in the mitochondrial pathway such as APAF-1 [Vogelstein et al., 2000].

A number of in vitro studies have shown that loss of p53 function reduces sensitivity to 5-FU treatment [Bunz F et al., 1999]. It has been demonstrated that deletion of both alleles of either TP53 or its downstream target BAX in colon cancer cell

lines made these cells resistant to apoptosis induced by 5-FU [Zhang L et al., 2000].

Usually mutations in the region encoding for p53 represent a late event occurring during the transition from adenoma to carcinoma. Many studies have demonstrated that inactivation of p53 and of the proteins downstream in this pathway contribute to the transformation and chemoresistant phenotype in mammals due to alterations in programmed cell death [Vogelstein et al., 2002]. On contrary wild-type expression of p53 in leukaemia cells is able to induce apoptosis [Yonish-Rouach et al., 1991]. In early nineties p53 was already recognized as a tumour suppressor gene and was known to be mutated in 85% of cases of colorectal cancer [Vousden KH et al., 2002].

It is known that some oncogenes such as myc, E1A, E2F or cytotoxic agents used to treat cancer can induce p53 expression. The oncogenic induction of p53 is p14ARF-mediated. Inactivation of p14ARF promotes Myc-mediated carcinogenesis as a result of inactivation in apoptotic response [Schmitt CA et al., 2002]. Induction of p53 by DNA damage is not p14ARF-dependent and tumours, where p53 is inactive due to p14ARF mutations retain the sensitive phenotype to chemotherapy-

induced DNA damage [Lowe SW et al., 2004].

Finally, p53 is capable of negatively controlling some survival pathways. For example p53 can negatively regulate activation of AKT by PI-3K via induction of PTEN phosphatase [Stambolic V, 2001]. Anti-apoptotic properties of AKT manifests with inhibitory phosphorylation of Capase-9, FAS/CD95, BAD and BAX and activation of NFkB which in consequence translocates to the nucleus to promote transcription of anti-apoptotic proteins, such as XIAP, BclXL and IAP [Pommier , 2004]. Mutations in p53 signalling allow the cell to proliferate continuously in the uncontrolled manner. Moreover this 'out-of-control' cell division and proliferation coincide with altered apoptotic mechanisms. This phenomenon of the abnormally proliferating cell population has been used as a basis for the chemotherapy – neoplastic cells that have high proliferative activity are more sensitive to chemotherapeutic agents inducing apoptosis leaving most of the normally proliferating cells intact [Lowe SW et al., 2004]. Activation of p53 can be triggered by the stimuli that control both cell proliferation and cell death [Lowe SW et al., 2004]. This is why p53 is considered the critical for the neoplastic transformation and the key molecule for the response to chemo- and radiotherapy.

Loss of 18q

Deletion of the long arm of chromosome 18 is the most common cytogenetic abnormality in CRC and has been associated with poor prognosis [Popat S et al., 2005]. Many studies have investigated the genes found in this region as prognostic markers, in particular the oncosuppressor indicated as ‘deleted in colorectal carcinoma’ (DCC). This gene encodes a protein with significant homology to the cell adhesion family of molecules.

SMAD4, a known CRC predisposition gene that is located on 18q, is a member of the transforming growth factor-β (TGFβ) signalling pathway, and decreased SMAD4 mRNA levels seem to be associated with a worse prognosis [Alhopuro, P. et al., 2005] and poorer response to 5-FU [Boulay, J. L. et al., 2002]. Furthermore, it seems that loss of 18q is a marker of chromosomal instability [Rowan, A. et al., 2005] whereas the other common mutation in the TGFβ pathway in patients with CRC is almost exclusively linked to microsatellite instability [Olschwang, S. et al., 1997].

Caretaker pathway

Microsatellite and chromosomal instability

Mutations in this pathway alter cellular mechanisms of DNA repair (Mismatch repair, MMR) especially in the microsatellite regions and are common in hereditary non-polyposis colorectal cancer (HNPCC) [Grady WM et al., 2004].

Therapeutic approaches in colorectal cancer treatment

Primary chemotherapy is used when colorectal cancer is advanced and has already spread to different sites. In this situation, surgery cannot eliminate the cancer, so chemotherapy may contribute to tumour shrinkage, alleviate symptoms, and prolong life.

Adjuvant chemotherapy is used after the cancer is surgically removed. The surgery may not eliminate all the cancer, so the adjuvant chemotherapy treatment is used to kill any cancer cells

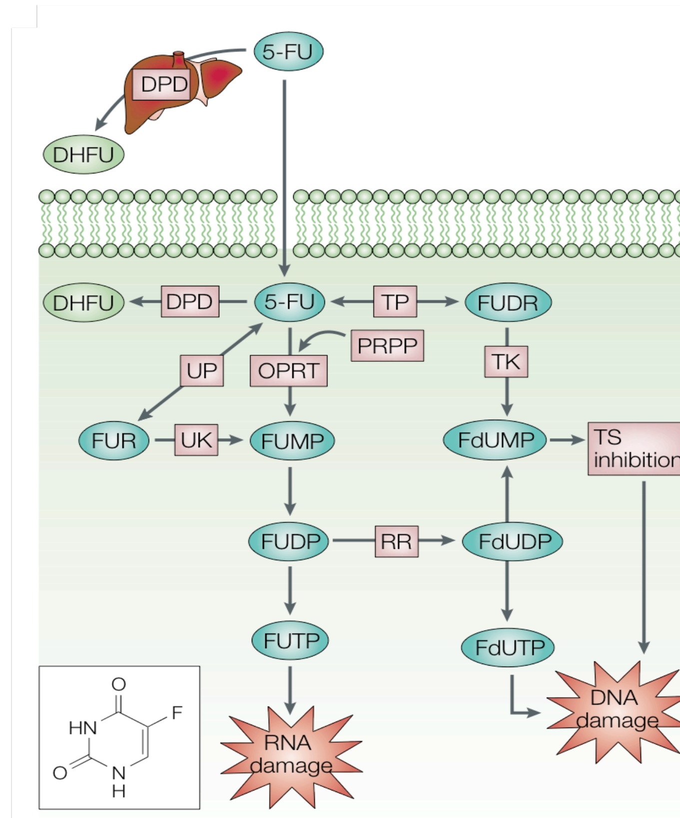
that may have been missed, such as cells that may have metastasized or spread to the liver.

Neoadjuvant chemotherapy is chemotherapy given before surgery. Chemotherapy drugs may be given prior to surgery in order to shrink the tumour so that the surgeon can completely remove it with fewer complications.

Most commonly used chemotherapeutic drugs in CRC

5-Fluorouracil and capecitabine

5-Fluorouracil (5-FU) has been the first-choice chemotherapy drug for colorectal cancer. Over the past 20 years, increased understanding of the mechanism of action of 5-FU has led to the development of strategies that increase its anticancer activity. Despite these advances, drug resistance remains a significant limitation to the clinical use of 5-FU [Longley DB et al., 2003].



Mechanism of action of 5-FU

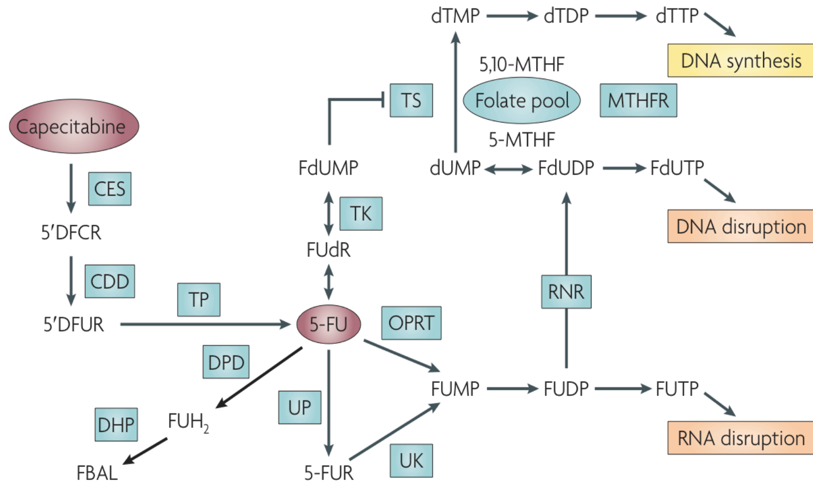
5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen. It rapidly enters the cell using the same facilitated transport mechanism as uracil [Wohlhueter, R. M., 1980]. 5-FU is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). 5-FU, which has been in use against

cancer for about 40 years, acts in several ways, but principally as a thymidylate synthase inhibitor (TS). Thymidylate synthetase is thought to be the dominant target for the active principle of 5-FU, fluorodeoxyuridine monophosphate (5-FdUMP). Interrupting the action of this enzyme blocks synthesis of the pyrimidine thymidine, which is a nucleotide required for DNA replication [Walther A et al., 2009]. A meta-analysis of TS expression suggested that higher expression of TS is associated with a poorer overall survival rate [Popat S et al., 2004]. The high expression variants have been associated with decreased survival in patients treated with 5-FU [Marsh S et al., 2001]. The rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD), which converts 5-FU to dihydrofluorouracil (DHFU). More than 80% of administered 5-FU is normally catabolized primarily in the liver, but its levels of activity can vary widely between individuals. There are more than 30 polymorphisms that cause DPD deficiency [Diasio, R. B., 2000], leading to severe, sometimes life-threatening, toxicity after 5-FU treatment [Wei X., 1996]. Despite extensive investigation, the pharmacogenetic basis of varied DPD activity remains still to be understood. The 5-FU metabolite FUTP is extensively incorporated into RNA, disrupting normal RNA

processing and function. Significant correlations between 5-FU misincorporation into RNA and loss of clonogenic potential have been shown in human colon cancer cell [Glazer RI et al., 1982] Reduced MTHFR (Methylenetetrahydrofolate reductase) activity creates variation in folate pools, indirectly increasing sensitivity to 5-FU. Increased response to 5-FU treatment has been associated with the 677T allele and the 1298C allele [Jakobsen, A., 2005]. Recent studies suggest that these polymorphisms affect capecitabine toxicity, as well as the efficacy of 5-FU. However, clinical data do not unequivocally support the influence of the MTHFR genotype on 5-FU responsiveness, toxicity and patient clinical outcome [Etienne-Grimaldi, M. C., 2007].

5-FU is given intravenously. Recently, a pill form of 5-FU has been developed, called Xeloda, which is used for colorectal cancer that has spread to other organs. Xeloda is also being used as neoadjuvant therapy with radiation in patients with rectal cancers to heighten the effect of radiation.

Capecitabine is an oral fluoropyrimidine that undergoes a three-step enzymatic conversion to 5-FU with the last step occurring in the tumour cell.

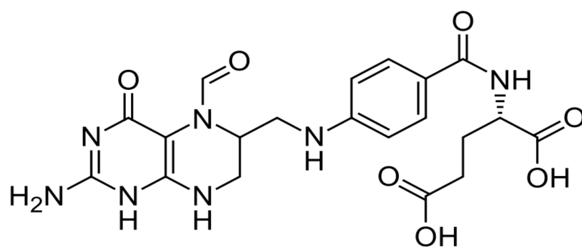


Mechanism of action of 5-FU

Inherited polymorphisms have the potential to greatly affect treatment: for conventional chemotherapy agents with a narrow therapeutic window, subtle genomic changes can modulate drug-specific pharmacokinetics and substantially affect responses and toxicity after chemotherapy. Almost all chemotherapy regimes for CRC incorporate 5-FU or its oral pro-drug, capecitabine, and the ability to tailor therapy to take into account variation in their metabolisms have the potential to translate into beneficial effects for a large patient population. Administration of 5-FU is often combined with leucovorin (folinic acid), which makes 5-FU more effective.

Folinic acid (Leucovorin).

Folinic acid is a 5-formyl derivative of tetrahydrofolic acid. It is readily converted to other reduced folic acid derivatives (e.g. tetrahydrofolate), and thus has vitamin activity, which is equivalent to folic acid. However, since it does not require the action of dihydrofolate reductase for its conversion, its function as a vitamin is unaffected by inhibition of this enzyme by drugs such as methotrexate. Folinic acid, therefore, allows for some purine/pyrimidine synthesis to occur in the presence of dihydrofolate reductase inhibition, so that some normal DNA replication and RNA transcription processes can proceed.

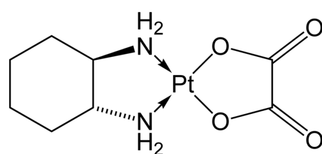


Chemical structure of Folinic acid.

Folinic acid is used in combination with the chemotherapy agent 5-FU in treating colon cancer. In this case, folinic acid is used to enhance the effect of 5-FU by inhibiting thymidylate synthase.

Oxaliplatin.

Oxaliplatin is a coordination complex classified as an alkylating agent, although is actually an alkylating group (they function by a similar mechanism) [Jung Y et al., 2007]. The cytotoxicity of platinum compounds is thought to result from inhibition of DNA synthesis in cancer cells. In vivo studies showed that Oxaliplatin has anti-tumour activity against colon carcinoma through its (non-targeted) cytotoxic effects. The primary anti-tumour mechanism of platinum derivatives is the formation of DNA adducts, which interfere with DNA replication and require the activity of DNA repair enzymes to avoid cell death. Oxaliplatin is typically administered with fluorouracil and leucovorin in a combination known as FOLFOX for the treatment of colorectal cancer.



Chemical structure of Oxaliplatin.

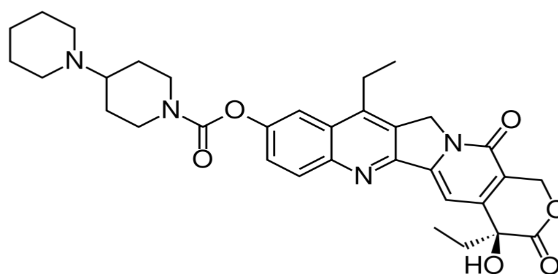
Oxaliplatin has been compared with other platinum compounds (Cisplatin, Carboplatin) in advanced cancers (gastric, ovarian). In clinical studies, Oxaliplatin by itself has modest activity

against advanced colorectal cancer [Becouarn Y et al., 1998]. It has been extensively studied in combination with Fluorouracil and Folinic Acid. There is some evidence to suggest that genetic polymorphisms in the genes that encode detoxifying enzymes and DNA repair proteins have an important role in determining the treatment response to the DNA binding agent oxaliplatin. Decreased sensitivity to platinum agents has been attributed to diminished cellular drug accumulation, increased intracellular drug detoxification and increased DNA repair [Mishima, M., 2002]. Glutathione-S-transferases (GSTs) are detoxification enzymes that target a wide variety of drugs for excretion by conjugation with glutathione. There are several isoenzymes and polymorphisms in this class of enzymes, with varying specificities, activities and tissue localizations [McIlwain, C. C., 2006]. The isoenzyme GSTP1 is the primary enzyme for the detoxification of platinum derivatives. There is no evidence for any isoenzymes or alleles (including null alleles) being associated with prognosis.

Irinotecan.

The hydrochloride salt of a derivative of camptothecin, a cytotoxic, alkaloid extracted from the *Camptotheca acuminata.* Irinotecan, a pro-drug, is converted to a biologically active

metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38) by a carboxylesterase-converting enzyme. One thousand-fold more potent than its parent compound irinotecan, SN-38 inhibits topoisomerase I activity by stabilizing the cleavable complex between topoisomerase I and DNA, resulting in DNA breaks, that inhibit DNA replication and trigger apoptotic cell death [Torigoe S et al., 2009]. Irinotecan is activated by hydrolysis to SN-38, an inhibitor of topoisomerase I. The inhibition of topoisomerase I by the active metabolite SN-38 eventually leads to inhibition of both DNA replication and transcription. Blocking this enzyme leads to breaks in the DNA, which leads to cell death. Because cancer cells divide faster than normal cells, they are more likely than normal cells to be affected by irinotecan.



Irinotecan.

Oblimersen Sodium

Oblimersen sodium is an antisense oligonucleotide inhibitor of Bcl-2. It targets the first six codons of the Bcl-2 mRNA, blocking the production of the target protein [Kang MH et al., 2009]. Recently published data from clinical trials using oblimersen sodium in combination with irinotecan in patients with metastatic CRC demonstrated the safety of the therapy, reduction in levels of Bcl-2 in peripheral blood mononuclear cells and stable disease in patients lasting 2.5 – 10months [Mita MM et al., 2006]. Immunohistochemistry studies done on FFPE tumour blocks obtained from all of the patients participating in trial confirmed anti-tumour efficacy of the combination of oblimersen and irinotecan and showed no correlation between Bcl-2, Bax and Bcl-XL expression [Mita MM et al., 2006].

Biological agents.

EGFR inhibitors: Cetuximab and Trastuzumab

EGFR is overexpressed in 77% of CRC and is involved in tumour growth and metastasis through interference in mechanisms of cell proliferation, apoptosis and neoangiogenesis [Mendelsohn J et al., 2002].

Cetuximab is a chimeric (mouse/human) monoclonal antibody, an epidermal growth factor receptor (EGFR) inhibitor, given by intravenous infusion for treatment of metastatic colorectal cancer and head and neck cancer. Cetuximab inhibits ligand- induced tyrosine kinase-dependent phosphorylation and downstream signalling of the EGFR, effecting an inhibition of cell proliferation in several different human tumour lines in vitro and in xenograft tumour models in vivo [Mendelsohn J et al., 1997]. This agent is based on Dr. John Mendelsohn's 1980s hypothesis that monoclonal antibodies against EGFR could block receptor activation. Cetuximab is indicated for the treatment of patients with epidermal growth factor receptor (EGFR)-expressing, KRAS wild-type metastatic colorectal cancer (mCRC), in combination with chemotherapy, and as a single agent in patients who have failed oxaliplatin- and irinotecan-

based therapy and who are intolerant to irinotecan.

Trastuzumab is a humanized monoclonal antibody that binds to the domain IV of the [extracellular segment of the HER2/neu receptor. Cells treated with trastuzumab undergo arrest during the G1 phase of the cell cycle so there is reduced proliferation. It has been suggested that trastuzumab induces some of its effect by downregulation of HER2/neu leading to disruption of receptor dimerization and signalling through the downstream PI3K cascade. P27Kip1 is then not phosphorylated and is able to enter the nucleus and inhibit cdk2 activity, causing cell cycle arrest [Kute T et al., 2004]. Also, trastuzumab suppresses angiogenesis by both induction of antiangiogenic factors and repression of proangiogenic factors. It is thought that a contribution to the unregulated growth observed in cancer could be due to proteolytic cleavage of HER2/neu that results in the release of the extracellular domain.

Bevacizumab (VEGF)

Bevacizumab is a recombinant humanized monoclonal antibody to VEGF. It has shown inhibition of growth in several tumor types in animal models. After several phase III studies

indicated that its use together with classical chemotherapy in metastatic patients prolonged their survival in 2005 bevacizumab has been approved for use in Europe and it's now being used as a first line therapy together 5-FU/folinic acid or 5-FU/folinic acid/irinotecan.

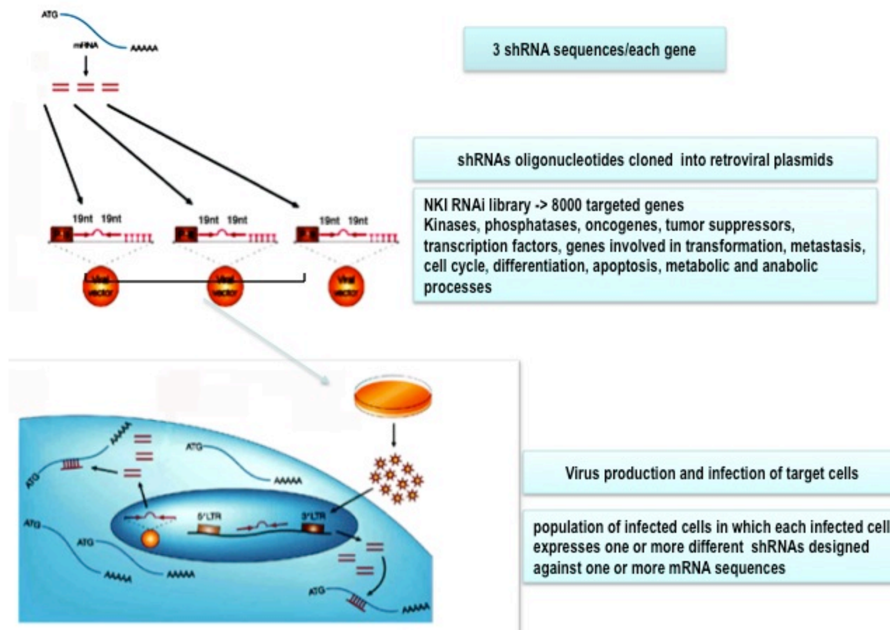
Chemotherapy and drug-resistance

Both in vitro and in vivo studies have demonstrated, that activation of the apoptotic pathways contributes to the cytotoxic action of most chemotherapeutic drugs [Hannun YA et al., 1997]. Studies suggest that chemotherapy-induced apoptosis involves activation of either the extrinsic or intrinsic pathway

[Fulda S et al., 2001]. The ability of chemotherapeutic agents to trigger activation of extrinsic pathway appears to be a very important determinant of sensitivity or resistance to cytotoxic and cytostatic therapies [Kaufmann SH et al., 2000]. Alteration in individual proteins involved in apoptosis such as p53, Bcl-2, TRAIL and survivin can alter sensitivity of CRC to chemotherapy.

1.3 IDENTIFICATION OF NEW MOLECULAR TARGETS TO OVERCOME RESISTANCE IN TUMORS

The discovery of the RNA interference (RNAi) process and its employment for developing RNAi-based in vitro techniques has made possible to efficiently generate stable loss-of-function phenotypes [Meister et al., 2004; Hannon et al., 2004]. The Bernards' laboratory has previously generated a retroviral expression vector whose transcription allows the synthesis of siRNAs, able to trigger the RNAi process of specific mRNAs, depending on the 19-mer sequences cloned into it [Brummelkamp et al., 2002]. As a development, the same group has generated a set of 25,000 RNAi retroviral vectors pRETROSUPER, puromycin selectable, that target 8300 human transcripts (3 vectors are designed against each transcript to ensure efficient knockdown of gene expression).



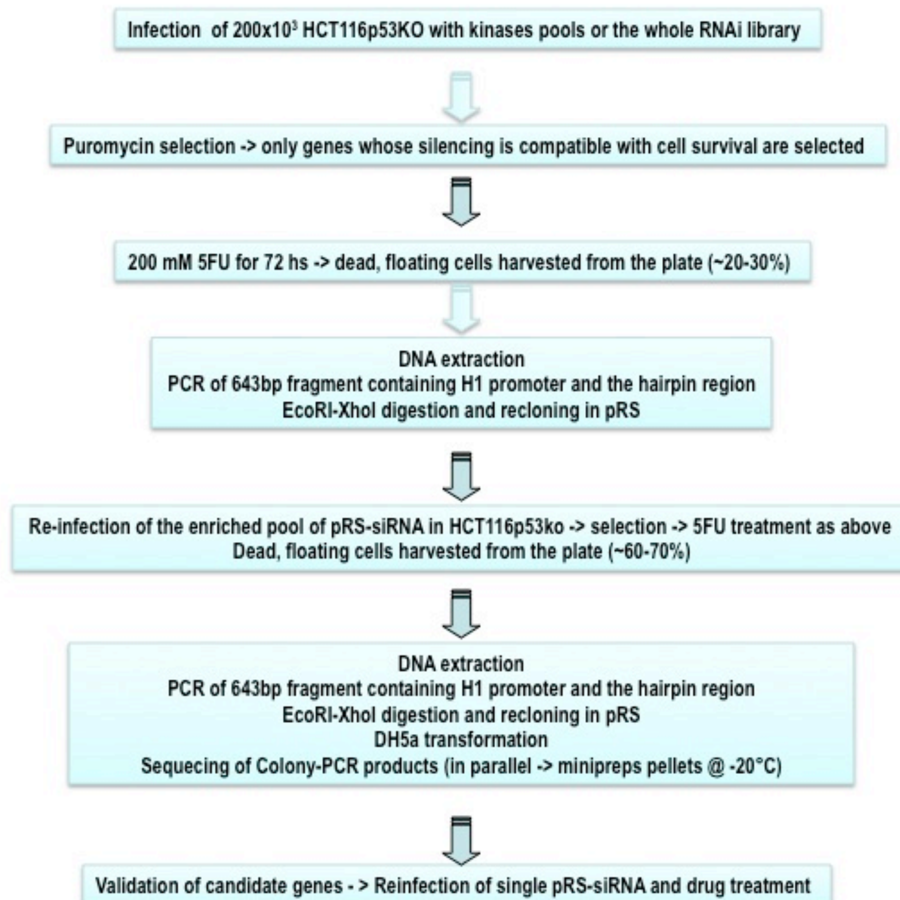
Experimental procedure of RNAi-library mediated screen

The 19-mer target gene-specific insert of the siRNA vector is unique in sequence. Introduction of such a vector into mammalian cells therefore results in the creation of a tagged knock-down cell carrying a permanent gene-specific identifier, easily isolated by PCR amplification using vector-derived PCR primers flanking the hairpin- encoding DNA sequence [Berns et al., 2004]. In order to identify genes whose silencing overcome resistance to drug-induced apoptosis in colon carcinoma cells a RNAi-based phenotype screen has been performed in our lab

using Bernards' library (Grassilli et al, manuscript in preparation).

The experimental model employed is a colon carcinoma cell line, HCT116p53KO, rendered p53-null by genetic inactivation of both alleles of the gene. As a consequence, the cell line is resistant to FU-induced apoptosis: experiments in our lab showed that 72hs treatment with 200μM FU induced less than 10% apoptosis in p53-null cells whereas parental, p53wt HCT116 treated in the same way underwent massive apoptosis (>95). Since the escape of only a few cells from drug-induced death is sufficient to allow tumour re-growth to test that the reversion of the resistant phenotype was not transient p53-null cells have been tested also in long-term experiment: 12 hs exposure to 200 μM 5-FU has been established as being sufficient to induce cell death in parental p53-proficient HCT116 cells: removal of the drug thereafter and subsequent incubation in drug-free medium does not allow recovery of viable cells neither in short (72 hs) nor in long (2 weeks) term. At variance, similar treatment of HCT116p53KO did not lead to any measurable effect on viability in the short term and allowed several colonies to grow in colony formation assay.

HCT116p53KO have been infected with the kinase pools of the NKI shRNA library and puromycin selected. Selected populations of HCT116p53KO (drug-resistant cells) have been treated with 200μM FU and floating dead cells harvested in order to rescue, by PCR, the shRNA inserts. As a result, only infected cells, bearing siRNA for genes whose silencing is not compatible with cell survival and proliferation have been used for the experiments. PCR products have been ligated in retroviral vectors and the plasmids from all the colonies obtained from the ligation reaction have been sequenced to identify the genes whose silencing bypass drug-resistance.



To validate the genes identified in the screen single plasmids have been re-infected in HCT116p53KO.and evaluation of drug-resistance reversion was assessed as colony formation capability after FU treatment (as above).

VALIDATION OF SINGLE GENE KNOCKOUT

Infection of HCT116p53KO with single pRS shRNA vectors

Puromycin selection

200 mM 5FU for 12 hs

Trypsinization -> reseeding at low density (colony assay)

Validation of a single gene knockout

As discussed in the previous section, colon cancer is the ultimate result of a long multistep progression process during which a number of genetic alterations accumulate, being p53 mutations usually a late event which contribute to drug resistance. In order to identify those targets able to modulate drug resistance independently of a particular combination of genetic defects, except p53 loss (or loss of function), colony assay experiments have been performed also in two other p53

mutant/drug-resistant cell lines, DLD-1 and SW480, characterized by a different combination of genetic defects. For both cells lines inhibition of the expression of a number of kinases reverted resistance to FU-induced cell death. In several cases the reduction in the number of colonies was not as striking as for HCT116p53KO; however, taken together all the experiments done in our lab suggest that downregulation of several kinases revert the resistant phenotype in p53-deficient cell lines.

	HCT-116p53KO	DLD-1	SW480
p53	null	mutated	mutated
Ras	mutated	mutated	mutated
APC	wild-type	mutated	mutated
beta catenin	mutated	wild-type	wild-type
MMR	MLH1-/+	MSH6-/-	wild-type
p14(arf)	mutated	meth. promoter	unmeth. promoter

Among the kinases identified in our lab Bruton's Agammaglobulinemia Tyrosine Kinase (BTK) gained particular attention by virtue of the fact that all the data from the literature indicate that BTK is expressed in a limited number of lineages, such as lymphocytes B, mastocytes, myeloid cells (mononcytes/macrophages, granulocytes and dendritic cells) erythroid precursors, trombocytes/platelets and some leukaemias (Chronic Myeloid Leukaemia, Chronic Lymphoid

Leukaemia) [Hantschel O et al., 2007] and its expression in the epithelial lineages has never been reported before. Therefore this novelty drove us to further investigate the role of BTK in colon carcinomas, with particular attention to its involvement in the drug-resistant phenotype.

1.4 BRUTON’S AGAMMAGLOBULINEMIA TYROSINE KINASE

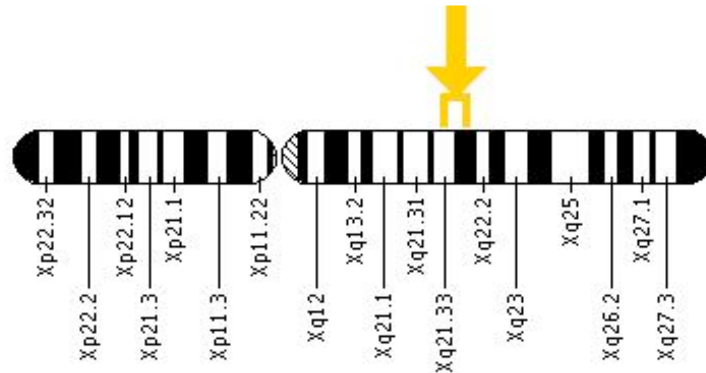
Structure of p77BTK.

The BTK protein is a 77 kDa protein of 659 amino acids. (NCBI Reference Sequence: NP_000052.1).

```
  1 maavilesif lkrsqqkkkt splnfkkrlf lltvhklsyy eydfergrrg skkgsidvek
 61 itcvetvvpe knppperqip rrgeesseme qisiierfpy pfqvvydegp lyvfspteel
121 rkrwihqlkn virynsdlvq kyhpcfwidg qylccsqtak namgcqilen rngslkpgss
181 hrktkkplpp tpeedqilkk plppepaaap vstselkkvv alydympmna ndlqlrkgde
241 yfileesnlp wwrardkngq egyipsnyvt eaedsiemye wyskhmtrsq aeqllkqegk
301 eggfivrdss kagkytvsvf akstgdpqgv irhyvvcstp qsqyylaekh lfstipelin
361 yhqhnsagli srlkypvsqq nknapstagl gygsweidpk dltflkelgt gqfgvvkygk
421 wrgqydvaik mikegsmsed efieeakvmm nlsheklvql ygvctkqrpi fiiteymang
481 cllnylremr hrfqtqqlle mckdvceame yleskqflhr dlaarnclvn dqgvvkvsdf
541 glsryvldde ytssvgskfp vrwsppevlm yskfssksdi wafgvlmwei yslgkmpyer
601 ftnsetaehi aqglrlyrph lasekvytim yscwhekade rptfkillsn ildvmdees
```

Amino acid sequence of BTK

The gene is composed of 19 exons. Translation of the p77BTK transcript starts at the ATG site that is located in exon 2 (…nt 194) and ends in exon 19 (nt 2173…). 5’UTR (untranslated region) starts from nt 1 and ends on nt 193. 3’UTR starts at nt 2174 and ands at nt 2611. mRNA coding for p77BTK is composed of 2611 bp and can be found under NCBI Reference Sequence: NM_000061.2.



Molecular localization on the chromosome X

Bruton's tyrosine kinase is encoded by the gene btk, localized on chromosome X (Xq21.33-q22), that when mutated causes the primary immunodeficiency disease X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice [Lindvall JM et al., 2005]. Btk-deficiency in the mouse is associated with a very mild early B cell developmental block, but with impaired maturation and poor survival of peripheral B cells [Khan WN, 1995].

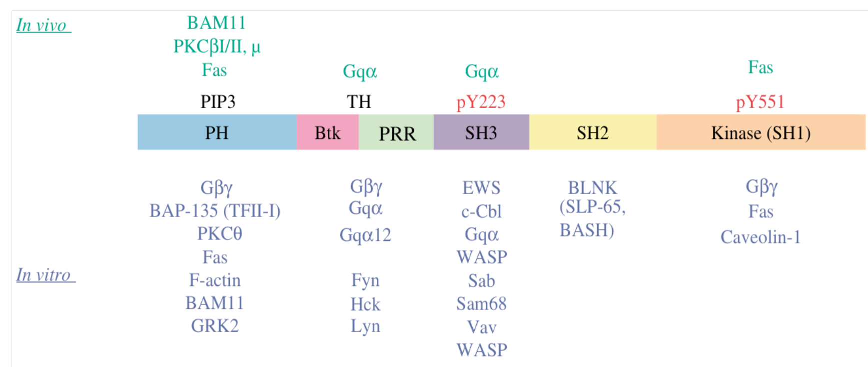
p77BTK is a member of TEC family, which consists of five members (Bmx/Etk, Btk, Itk, Rlk, and Tec) and constitutes the second-largest family of non-receptor protein tyrosine kinases in the immune system [Mohamed AJ et al., 2009]. Some Tec kinases have been shown to be major regulators of antigen receptor signalling in PBMCs, and deficiencies in Tec family

kinases cause several immunological defects in humans and mice [Koprulu AD. et al., 2009].

```
1    aactgagtgg ctgtgaaagg gtggggtttg ctcagactgt ccttcctctc tggactgtaa
61   gaatatgtct ccagggccag tgtctgctgc gatcgagtcc caccttccaa gtcctggcat
121  ctcaatgcat ctgggaagct acctgcatta agtcaggact gagcacacag gtgaactcca
181  gaaagaagaa gctatggccg cagtgattct ggagagcatc tttctgaagc gatcccaaca
241  gaaaaagaaa acatcacctc taaacttcaa gaagcgcctg tttctcttga ccgtgcacaa
301  actctcctac tatgagtatg actttgaacg tgggagaaga ggcagtaaga agggttcaat
361  agatgttgag aagatcactt gtgttgaaac agtggttcct gaaaaaaatc ctcctccaga
421  aagacagatt ccgagaagag gtgaagagtc cagtgaaatg gagcaaattt caatcattga
481  aaggttccct tatcccttcc aggttgtata tgatgaaggg cctctctacg tcttctcccc
541  aactgaagaa ctaaggaagc ggtggattca ccagctcaaa aacgtaatcc ggtacaacag
601  tgatctggtt cagaaatatc acccttgctt ctggatcgat gggcagtatc tctgctgctc
661  tcagacagcc aaaaatgcta tgggctgcca aattttggag aacaggaatg gaagcttaaa
721  acctgggagt tctcaccgga agacaaaaaa gcctcttccc ccaacgcctg aggaggacca
781  gatcttgaaa aagccactac cgcctgagcc agcagcagca ccagtctcca caagtgagct
841  gaaaaaggtt gtggcccttt atgattacat gccaatgaat gcaaatgatc tacagctgcg
901  gaagggtgat gaatatttta tcttggagga aagcaactta ccatggtgga gagcacgaga
961  taaaaatggg caggaaggct acattcctag taactatgtc actgaagcag aagactccat
1021 agaaatgtat gagtggtatt ccaaacacat gactcggagt caggctgagc aactgctaaa
1081 gcaagagggg aaagaaggag gtttcattgt cagagactcc agcaaagctg gcaaatatac
1141 agtgtctgtg tttgctaaat ccacagggga ccctcaaggg gtgatacgtc attatgttgt
1201 gtgttccaca cctcagagcc agtattacct ggctgagaag caccttttca gcaccatccc
1261 tgagctcatt aactaccatc agcacaactc tgcaggactc atatccaggc tcaaatatcc
1321 agtgtctcaa caaaacaaga atgcaccttc cactgcaggc ctgggatacg gatcatggga
1381 aattgatcca aaggacctga ccttcttgaa ggagctgggg actggacaat ttggggtagt
1441 gaagtatggg aaatggagag gccagtacga cgtggccatc aagatgatca aagaaggctc
1501 catgtctgaa gatgaattca ttgaagaagc caaagtcatg atgaatcttt cccatgagaa
1561 gctggtgcag ttgtatggcg tctgcaccaa gcagcgcccc atcttcatca tcactgagta
1621 catggccaat ggctgcctcc tgaactacct gagggagatg cgccaccgct tccagactca
1681 gcagctgcta gagatgtgca aggatgtctg tgaagccatg gaatacctgg agtcaaagca
1741 gttccttcac cgagacctgg cagctcgaaa ctgtttggta aacgatcaag gagttgttaa
1801 agtatctgat ttcggcctgt ccaggtatgt cctggatgat gaatacacaa gctcagtagg
1861 ctccaaattt ccagtccggt ggtccccacc ggaagtcctg atgtatagca agttcagcag
1921 caaatctgac atttgggctt ttggggtttt gatgtgggaa atttactccc tggggaagat
1981 gccatatgag agatttacta acagtgagac tgctgaacac attgcccaag gcctacgtct
2041 ctacaggcct catctggctt cagagaaggt atataccatc atgtacagtt gctggcatga
2101 gaaagcagat gagcgtccca ctttcaaaat tcttctgagc aatattctag atgtcatgga
2161 tgaagaatcc tgagctcgcc aataagcttc ttggttctac ttctcttctc cacaagcccc
2221 aatttcactt tctcagagga aatcccaagc ttaggagccc tggagccttt gtgctcccac
2281 tcaatacaaa aaggcccctc tctacatctg ggaatgcacc tcttctttga ttccctggga
2341 tagtggcttc tgagcaaagg ccaagaaatt attgtgcctg aaatttcccg agagaattaa
2401 gacagactga atttgcgatg aaaatatttt ttaggaggga ggatgtaaat agccgcacaa
2461 aggggtccaa cagctctttg agtaggcatt tggtagagct tgggggtgtg tgtgtggggg
2521 tggaccgaat ttggcaagaa tgaaatggtg tcataaagat gggaggggag ggtgttttga
2581 taaaataaaa ttactagaaa gcttgaaagt c
```

mRNA coding for p77BTK

p77BTK is expressed in all cell lineages of the hematopoietic system, except for T cells: lymphocytes B, mastocytes, myeloid cells (mononcytes/macrophages, granulocytes and dendritic cells) erythroid precursors, trombocytes/platelets and some leukemias [Vihinen M et al., 1994].



Schematic model of Btk organization, showing different domains and interacting molecules in vitro and in vivo.

5 domains, possessing specific characteristics and playing specific roles can be recognized in p77BTK:

I. Pleckstrin Homology Domain (PH) is located in the N-terminal region and counts 120 amino acids; PH has been found in a wide variety of signalling molecules ranging from protein kinases, phospholipases, GTPases to adaptor proteins as

well as cytoskeleton proteins [Rebecchi and Scarlata, 1998]. The last 27 amino acids are highly conserved (BTK motif) and contain one histidine and 3 cysteins, forming 'zinc fingers'. Proline rich region (PRR) can be found downstream from BTK motif [Smith et al., 2001]. PH domains are multifunctional signalling modules which are able to bind to phospholipids as well as many protein partners including heterotrimeric G-proteins, PKC isoforms, Stat 3, F-actin, Fas and FAK. The mutations in PH domain of Btk have been shown to associate with XLA suggesting the important role of PH domain in regulation of Btk kinase activity [Rawlings et al., 1993].

II. Tec Homology Domain is characteristic to all members of Tec family.

III. Src Homology 3 (SH3) domain is composed of 60 residues. SH3 domains are protein modules that recognize proline-rich motifs present in many proteins. Mutations in the SH3 domain of several proto-oncogenes such as c-Src and c-Abl increase their transforming potential, suggesting SH3 domains may down-regulate their kinase activity [Meyn MA et al., 2006]. Two tyrosine phosphorylation sites are located at the positions Y223 and Y551. Both phosphorylation sites play a pivotal role in the activation of BTK. Y551 is transphosphorylated by Syk (or

Lyn) kinases, which promotes the catalytic activity of BTK, with subsequent autophosphorylation at position Y223 [Wahl MI et al., 1997]. Mutation of Y233 or deletion of SH3 domain increases the transforming potential of Btk mutant (E41K) without affecting its kinase activity directly, suggesting that the SH3 domain regulates Btk function through binding to regulatory proteins or internal folding [Afar et al., 1996].

IV. Src Homology 2 (SH2) is composed of 100 residues. SH2 domains recognize phosphotyrosine containing peptides and proteins. Mutations in the SH2 domain of Btk are associated with impaired B cell function and may cause the XLA immunodeficiency in humans, possibly due to disruption of phosphotyrosine binding sites [Vihinen et al., 1999]. Several proteins have been shown to interact with the SH2 domains of Btk kinases and to modulate their functions. The SH2 domain of Btk is shown to be required for the activation of PLCg2 in DT40 cells [Takata and Kurosaki, 1996], although Btk has not been shown to bind to PLCg directly.

V. SH1 domain, called also catalytic or kinase domain is located in the C-terminal region and is highly conserved, sharing about 70% similarity with other kinase domains [Qiu Y et al., 1998]. Phosphorylation of a highly conserved tyrosine residue

(Y551 of Btk and Y566 of Bmx) by the Src family kinases has been shown to be required for activation of these kinases in vivo [Tsai et al., 2000].

BTK is usually localized in the cytoplasm and upon receptor signalling its activation results in its translocation to the membrane. Phosphoinositide-3 kinase (PI3K) is thought to activate the tyrosine kinase Btk [Suzuki H, 2003]. The membrane association of BTK is dependent on the interaction of PH domain with PIP3 that is synthesized from by PI3K. The translocation to the membrane brings the BTK protein in close proximity to the Lyn and Syk kinases that transphosphorylate BTK at tyrosine Y551.

Functional role of BTK

BTK is involved in numerous cellular processes of signal transduction in response to virtually all types of extracellular stimuli, which are transmitted by growth factor receptors, cytokine receptors, G-protein coupled receptors, antigen-receptors, integrins and death receptors.

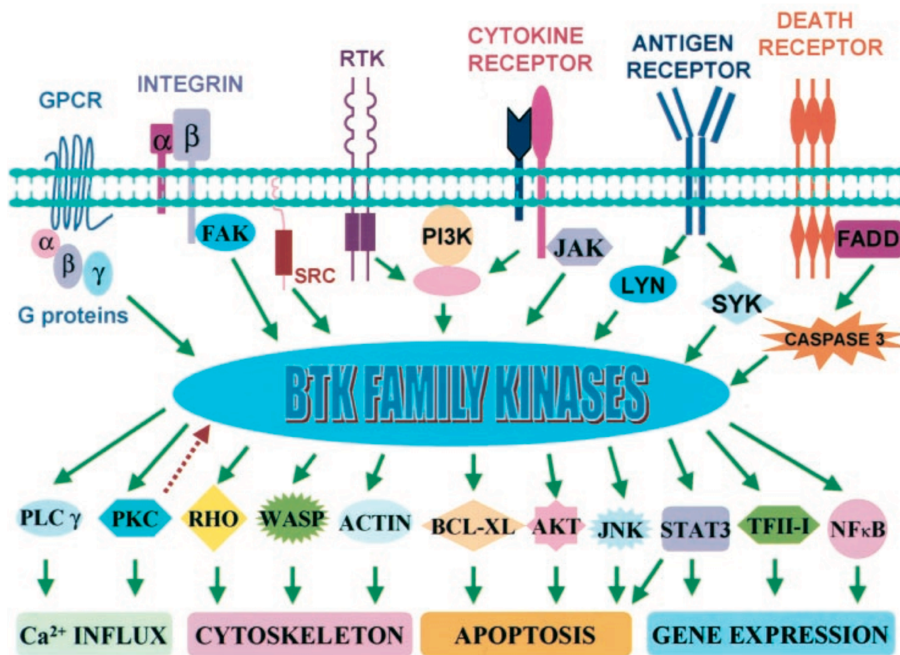
Signaling cascade	Cell type
(Pre-)B cell receptor	B cells
CD38	Activated B cells
TRAIL-R1	Erythroid cels
Epo Receptor	Erythroid cells
Glycoprotein (GP) Ib-IX-V complex, GPVI	Platelets
IL-5R	B cells, eosinophils, basophils
IL-6R gp130	Activated B cells, plasma cells
IL-10R	Activated T and NK cells
FcεR	Mast cells, basophils
TLR2 (MyD88 adapter-like (Mal))	Macrophage, DC
TLR4 - LPS (Mal)	Phagocytic cells, DC
CCL5	Microglia (brain)
CXCL12 (SDF1)	B cells
RANK	Osteoclasts
CD303 (BDCA2)	pDC

Its multi-domain structure allows BTK to interact with diverse cellular structures and proteins. The Btk family kinases were originally recognised for their requirement in the signalling by T-cell receptor, B-cell receptor and mast cell receptor (FceRI) [Tsukada et al., 1993]. These receptors themselves are not tyrosine kinases, but invariably activate Src family kinases as well as Syk family kinases. Btk was found to be directly phosphorylated and activated by Src [Rawlings et al., 1996]. Syk, which do not directly participate in the phosphorylation of Btk, apparently plays a crucial role in Btk's activation, since in Syk-/- cells, the Btk activity is drastically reduced, despite the presence of normal active Lyn kinase, an Src-family kinase [Kawakami et al., 2000]. As described above,

Btk activation also requires PI3K signal. The activation of PI3K is provided by Lyn kinase, which directly associates with the p85 subunit of PI3K, and at least in vitro it activates the activity of PI3K [Pleiman et al., 1994]. Another important role of BTK is activation of NFkB, a transcriptional factor essential for activation and proliferation of B-cells. It has been demonstrated that active form of BTK is capable of phosphorylating IkB - the main inhibitor of NFkB [Bajpai et al., 2000]. These data are supported by experiments in Xid mouse model: in absence of BTK there is no activation of NFkB upon BCR stimulation.

Among the cytokines that can activate Btk family kinases we can list IL-2, IL-3, IL-6, GCSF and erythropoietin. Although some of these receptors engage the Src family kinases, a common signal transducer of these receptors is the Jak family of kinases comprising Jak1, Jak2 or Tyk2 [Ihle and Kerr, 1995]. IL6 was shown to activate Btk and Tec in B-lymphocytes and hepatocytes respectively. The activated IL6 receptors form stable complexes with Btk and Tec via Jak family kinases. It was further shown that this association leads to direct phosphorylation of Btk by Jak1 [Takahashi-Tezuka et al., 1997]. The activation of Btk and Bmx/Etk by IL3 and GM-CSF in mast

cells has been demonstrated and both involve the activation by PI3K [Ekman et al., 2000].



The signals transmitted by Btk family kinases

X-linked agammaglobulinemia.

In 1993 BTK was identified as the gene defective in the human immunodeficiency disease X-linked agammaglobulinemia (XLA). The XLA disease, which was first

described by Dr. O.C. Bruton in 1952, is characterised by recurrent bacterial infections. Bruton's agammaglobulinemia is characterised by loss of function of BTK, due to missense mutations in the DNA region codifying for PH domain. Mutations in that region account for X-linked immunodeficiency, called XLA in humans and XID in mice [Rawlings et al., 1993]. The above-mentioned mutation affects Arg28, a residue conserved among all the members of the Tec family and in a number of proteins that possess PH domain. XLA Patients have less than 1% of the normal number of peripheral B cells. Serum levels of all Ig classes are very low due to the lack of plasma cells in the secondary lymphoid organs. In XLA patients, B cell development is almost completely arrested at the pre-B cell stage: the pre-B cell fraction mainly consists of small cells, suggesting that Btk is necessary for their proliferative expansion [Sideras et al., 1995]. Nevertheless the absence of functional BTK does not influence other lineages. Patients with XLA, being immunocompromised, are extremely sensible to any bacterial infection due to lack of humoral immunity response (HIR). During past decades various mutations in the BTK have been described, both in structural domains and non-codifying regions. Those mutations seem to be

equally distributed with the of the SH3 domain, where missense mutations has never been identified. Missense mutations account for 40% of total mutations, while nonsense mutations and deletions for around 20%. Insertions are about 7% of total number of mutations in BTK [Valiaho et al., 2006]. Mutations in BTK are grouped in many databases. An international registry for XLA (http://bioinf.uta.fi/BTKbase/) shows that mutations in all domains of the BTK gene cause the disease. BTKbase represent data collected from more than 1000 non-related patients [Piirila et al., 2006]. The effects of mutational events are different depending of the region of BTK. In the SH2 domain mutations often occur in the residues forming the linkage for phosphotyrosines. In the catalytic domain mutations hit sites recognizing ATP or substrates.

Double-faced role of BTK in apoptosis.

The overall consequences of the various signals transmitted by the Btk family kinases are varied including growth, differentiation and apoptosis. Overexpression of Btk was shown to inhibit Fas-mediated apoptosis [Uckun F, 1998]. The B-cells lacking Btk are prone to apoptosis, suggesting that

Btk exerts a protective role. The protective role of Btk family kinases could be due to their ability to activate Akt and NF-kB [Bajpai et al., 2000]. The activation of BTK leads to expression of BCL-XL via activation of another transcription factor - STAT5 [Dumon et al., 1999]. BTK can activate AKT in B-cells in both normal and oxidative stress conditions [Lindvall et al., 2002]. AKT when activated phosphorylates a number of proteins involved in apoptosis, such as Bad, FoxO1, FHKRL, caspase-9, showing a strong anti-apoptotic action [Thompson et al., 2005].

On the other hand BTK can be activated as a response to exposition to ionizing radiation thus leading to apoptosis [Uckun F. et al., 1998]. It has been recently demonstrated that BTK can antagonise the anti-apoptotic action of STAT3 via phosphorylation [Uckun F. et al., 2007]. In the oxidative stress conditions STAT3 protects the cell from apoptosis by increasing the expression of BCL-XL and survivin [Takeda et al., 1998].

Different regulatory mechanisms underlying pro- and anti-apoptotic action of are likely to be the consequence of the multitude of signals the Btk family kinases transmits, some pro-apoptotic (e.g., p38MAPK) and other anti-apoptotic (e.g., Akt and NF-kB). Depending on the balance of these signals, the overall consequence can be either pro- or anti-apoptotic.

Another plausible mechanism is that the kinase, through post-translational modifications, may exist in two forms, one pro-apoptotic and one anti-apoptotic. For instance, it was found that Etk/Bmx has a caspase-3 cleavage site at the SH3 domain, and can be cleaved into a protein, which contains only the SH2 and kinase domain. It is conceivable that the wild-type functions to protect the cells from apoptosis by activating Akt, whereas the truncated form may be engaged in apoptosis pathway. In either case, accumulating evidence suggests that the Btk family kinases are intimately involved in the interaction with the cellular apoptotic machinery and might serve as an apoptosis switch [Qiu Y et al., 2000].

BTK chemical inhibitors

LFMA13 (2-Cyano-N-(2,5-dibromophenyl)-3-hydroxy-2-butenamide) is a potent inhibitor of BTK and Polo-like kinases. It inhibits recombinant BTK with an IC_{50} value of 2.5 μM and has no activity on other protein kinases (JAK1, JAK3, HCK, EGFR kinase and insulin receptor kinase) at concentrations of up to 278 μM [Uckun F et al., 2007].

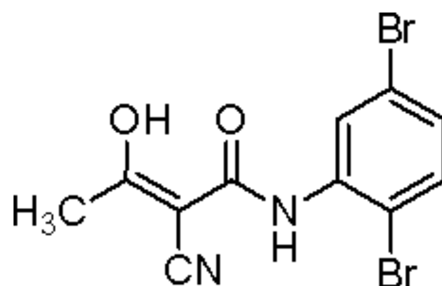
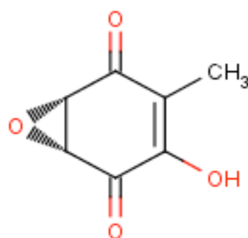


Fig Chemical structure of LFMA13.

Terreic acid (3-hydroxy-4-methyl-7-oxabicyclo (4.1.0) hept-3-ene- 2,5-dione) is quinone epoxide specifically inhibiting the enzymatic activity of Btk. Inhibits the interaction between PKCbII and BTK (IC50 ~ 30 mM) and the catalytic activity of BTK but does not affect the activity of PKC. It has little effect on the activities of Lyn, Syk, PKA, casein kinase I, ERK1, ERK2 and p38 kinases [Kawakami Y et al., 1999].



Chemical structure of terreic acid.

2. AIM OF THE STUDY

Inability of tumour cells to undergo chemotherapy-induced apoptosis is a main mechanism of drug resistance.

The aim of my project is to study the role of one of the targets identified in the RNAi-mediated phenotypic screen previously described, i.e., the Bruton's tyrosine kinase (BTK), so far thought to be expressed only in hematopoietic lineages. Moreover, in HCT116p53KO cells BTK has an apparent molecular weight around 65 kDa as opposed to the reported 77kDa in hematopoietic cells.

My principal aims are therefore:

1) Study the involvement of BTK in chemotherapy-induced resistance to apoptosis;

2) Clone and characterize this novel form of BTK;

3) Explain the molecular mechanisms underlying synthesis of novel BTK isoform in colon carcinomas.

3. MATERIALS AND METHODS

Cell lines and tissue samples. HCT116, DLD-1, SW480, HT29, MCF-7, SkBr-3, ZR-75-1, BT474, BT549, MDA-MB-231, CaOv, SkOv, Colo, A549, NCIH209, NCIH460, Calu-1, Calu-3, NCIH69, NCIH1299, 293T, NALM6 were purchased from American Tissue Type Collection (ATTC).

HCT116p53KO cell line was a kind gift of Prof. Bert Vogelstein from The Ludwig Center for Cancer Genetics and Therapeutics, Howard Hughes Medical Institute and Sidney Kimmel Cancer Center at the Johns Hopkins Medical Institutions, Baltimore, Maryland, USA.

CCSC lysates were from Prof. Ruggero De Maria from Istituto Superiore di Sanità in Rome, Italy.

FFPE solid tumor samples were provided by Prof. Biagio Eugenio Leone from Department of Surgical Sciences, University of Milano-Bicocca, Milano, Italy.

Drugs. 5-FU and OX were from the Oncology Department of San Gerardo Hospital, Monza. LFM-A13 was purchased from Calbiochem.

Cell cultures. Epithelial cells were maintained in McCoy5A or DMEM medium supplemented with 10% FBS and penicillin/streptomycin according to ATTC protocols. Leukemic lines and PBMCs were cultured in RPMI1940 supplemented with 10% FBS and penicillin/ streptomycin (all Gibco).

Antibodies. Antibodies anti-BTK M138, anti-hnRNPK, anti-cytochrome C, anti-AIF, anti-Smac were from Santa Cruz Biotechnology (Santa Cruz, USA).

Antibody anti-vinculin, and anti-β actins were from Sigma (Sigma-Aldrich, St. Louis, USA).

Antibody anti caspase-8 was from Calbiochem.

Antibody anti-NPM was home-made antiserum from European Institute of Oncology at the IFOM-IEO Campus, Milan, Italy.

Antibody anti-GFP was from Invitrogen.

Secondary antibodies were from Amersham (mouse, rabbit) and Abcam (goat).

siRNAs and PCR Primers. Short interfering RNAs were from Eurofinns MWG Operon (Ebersberg, Germany). Sequences directed against common for both isoforms 5th and 6th exons [Heinonen JE, FEBS Letters, 2002]:

N2 (5th exon) 5'-GGGAAAGAAGGAGGUUUCAUU-3',

N3 (6th exon) 5'-GAAGCUUAAAACCUGGGAGUU-3';

Sequences designed by me, directed against novel 1st alternative exon (p65BTK only):

Alt 1 5'-CACCUUUCGCAGCAAACUGTT-3',

Alt 2 5'-GUUGGUCCAUUCAACAAAUTT-3',

Alt 3 5'-ACUGCUAAUUCAAUGAAGATT-3'.

Sequences designed in our lab, directed against classic 1st exon (p77BTK only):

E1 5'-GUCCUGGCAUCUCAAUGCATT-3',

E2 5'-UGCAUCUGGGAAGCUACCUTT-3',

E3 5’-GUCCUGGCAUCUCAAUGCATT-3’,

Control sequence (luciferase):

LUC 5’-CGUACGCGGAAUACUUCGATT-3’.

Sequence directed against hnRNPK:

5'-AAUAUUAAGGCUCUCCGUACA-3'
[Moumen et al., Cell 2005].

All PCR primers were from Sigma (Sigma-Aldrich):

Primes specific p77BTK only:

FLFW 5’-CTCAGACTGTCCTTCCTCTC-3’

Primes specific for both isoforms of BTK:

2F 5’-TAGGGTTGTCTTTTTCTTTTGTA-3’

8F 5’-TAGAACTTTTTCGGTGATGGC-3’

12F 5’-CAACTATGCAGTAATACAACACACAA-3’

14F 5’-CGAGTTTATAGGTCACAGAGTTGT-3’

8R 5’-TTGCTTTCCTCCAAGATAAAATA-3’

14R 5’-ATCATGACTTTGGCTTCTTCAATG-3’

17R 5’-CTTTAACAACTCCTTGATCGTTTA-3’

19R 5’-GCTCAGGATTCTTCATCCATGACAT-3’

Primers used in 5'RACE RLM PCR:

BTK-GSP1B 5’-TGGCAGCCCATAGCATT-3’

1Rev 5’-GAGTTTGTGCACGGTCAAGAGA-3’

2Rev 5’-TATTGAACCCTTCTTACTGCC-3’

UAP 5’-CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC-3’

AAP 5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′

Primers specific for the novel 1st exon of p65BTK:

33A FW 5’-ACTCTGCTACGTAGTGGCGTTC-3’

56° FW 5’-CATTCCTGTTCCACCTCAAG-3’

68A FW 5’-TAGGGTCGAATGAAGGGGTC-3’

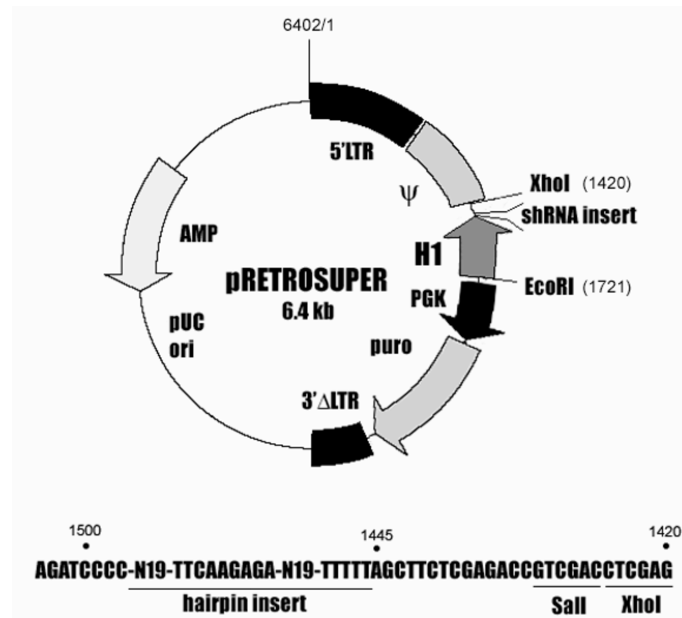
86° FW 5’-ATGCCCATTATGTGGCAGGC-3’

Control primers:

GAPDH FW 5’-TTAGCACCCCTGGCCAAG-3’

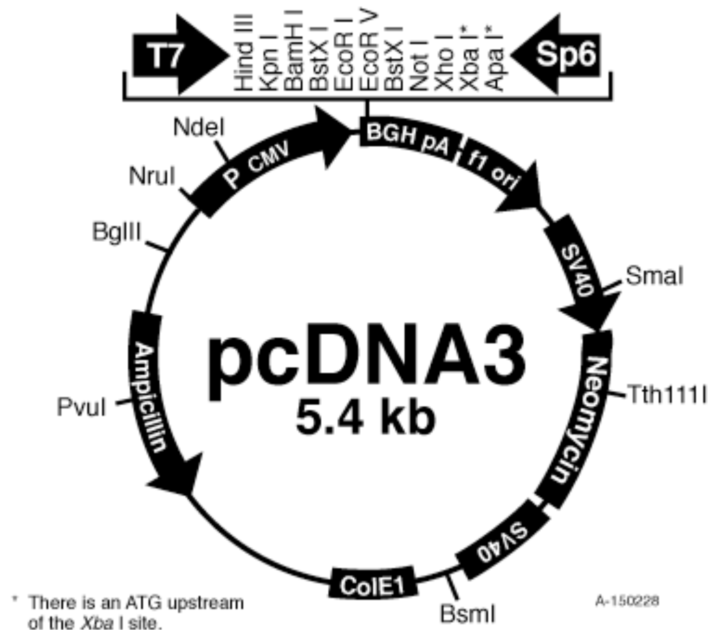
GAPDH REV 5’-CTTACTCCTTGGAGGCCATG-3’

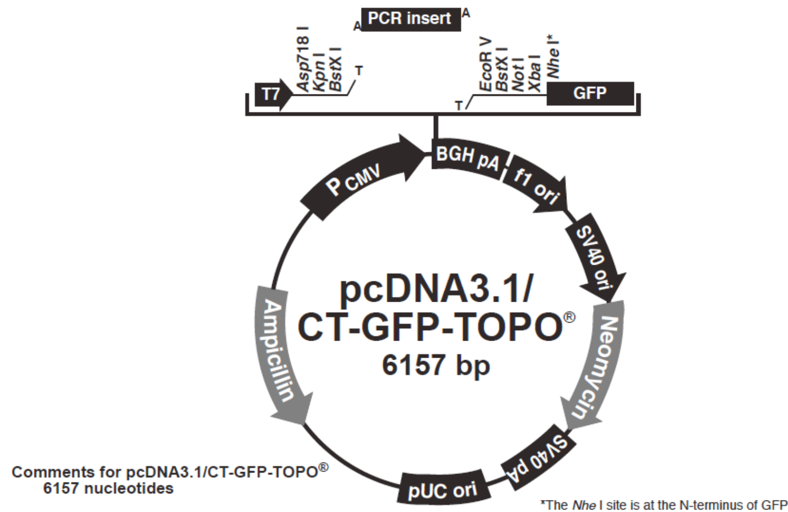
Design of short-hairpin RNA and construction of BTK sh vector. The pRETROSUPER (pRS) vector driving the expression of a specific shRNA for BTK has been recovered from the Bernards' library [Bernards R et al., 2004)



Generation of pcDNA3 p65/p77BTK constructs for the overexpression experiments. Briefly, full-length (FL, p77BTK), alternative (Alt, p65BTK), ATG1, ATG2, 5' Classic UTR-GFP and 5' Alternative UTR-GFP sequences were cloned

into pcDNA3.1 or pcDNA3.1/CT-GFP-TOPO between KpnI and XbaI cloning sites. DNA constructs were expressed in DH5α cells and were purified using Plasmid Maxi Kit (Qiagen). Constructs were subsequently transfected into HCT116p53KO cells using Lipofectamine 2000 (Invitrogen).





Plasmid DNA Transfection using Lipofectamine 2000 Reagent. For transfection, cells were seeded in a 6-well plate at a concentration of 1×10^6 cells per well and allowed overnight growth to reach 80–90% confluence. Cells were then transfected with the mixture of 3 μg plasmid DNA and 10 μL of Lipofectamine 2000 (Invitrogen) in 500 μL serum-free medium. For stable transfection at 48 h the medium was replaced by selective medium containing puromycin (Invitrogen), 10% FBS and Pen/strep), and the non transfected cells were eliminated within 10 days after transfection. For transient transfection after 48 h cells were ready for further experiments.

Immunofluorescence of cells transfected with GFP constructs. Briefly, transiently transfected cells were seeded onto sterile coverslips coated with polylysine (Sigma) in PBS (1:10) and after 24 h fixed with 4% PFA in PBS and mounted with mowiol.

Colony-forming Assay. Subconfluent growing cells transfected with pRS and pRS BTK were seeded at a density of 0.3×10^6 in a 6-well plate and the day after were treated with 200μM 5-FU for 12 h. Twenty-four hours after 5-FU addition cells were trypsinized, counted, and reseeded at a low density (5000 cells in a 6-well plate) in triplicate together with their untreated controls (2000 cells in a 6-well plate). Medium was replaced every 3 days, and after 2 weeks colonies were fixed and stained in 1% crystal violet in 35% ethanol.

RNA interference. Cells were seeded in a 6-well plate at a concentration of 5×10^5 cells per well and allowed overnight growth to reach 30-50% confluence. Cells were then transfected

with the mixture of 100 pmol of siRNA and 5 µL of Lipofectamine 2000 (Invitrogen) in 500 µL serum-free medium (final concentration of RNA when added to the cells is 33 nM). Cells were incubated at 37°C in a CO_2 (5%) incubator for 24 hours until they were ready to be assayed for gene knockdown.

Determining cell viability with Trypan Blue exclusion test. Cells were seeded in triplicates onto 96-well plate one day prior to the addition of the drug, so that they reached 60-70% confluence on the day of treatment. 24 hours after plating the treatment was started by addition of 5-FU (200µM) or OxPt (50µM) or LFMA13 (200µM) or a combination of all. After 72 hours floating cells were pooled with those harvested by trypsinization and stained with 0.4% Trypan Blue solution (Sigma) in 1:1 ratio. Stained cells were then loaded on Burker's chamber and both viable and dead cells were counted (in duplicate) under the light microscope. The percentage of dead cells has been calculated and presented on the charts.

Immunoblotting. Cells were lysed in high-salt E1A lysis buffer (Hepes 50mM, pH 7.5, NaCl 500 mM, DTT 1mM, EDTA 1mM,

0.1% NP40) supplemented with 1% protease inhibitor cocktail (PIC, Sigma). Protein concentration in cleared supernatants (obtained by centrifugation at 12 000 rpm at 4°C) has been determined by using the Bio-Rad Bradford reagent and read at 595nm wavelength with spectrophotometer (Eppendorf). Samples containing 30-100μg of protein lysates (depending on the experiment) were then mixed with 5X LB buffer (2% SDS, 50mM Tris pH 6.8, BBF 0.2 mg/ml, 0.1M DTT, glycerol 50%) and boiled for 5 minutes at 100°C. Samples where then loaded on 10 or 12% SDS-PAGE gel lysates (depending on the experiment) and run in 1XRunning Buffer (250mM glycine, 30mM Tris Base, 0.1% SDS) in Bio-Rad Mini Protean apparatus (0.08 A for 3 h).

Separated proteins were transferred onto nitrocellulose membranes (Hybond, GE Healthcare) in a chilled to 4°C 1XTransfer Buffer (20% methanol, 192 mM glycine, 25 mM Tris Base) in Bio-Rad Mini Protean (0.30 A for 1.5h). After the transfer procedure was complete the nitrocellulose membrane was soaked in Ponceau Red solution (2% Ponceau Red, 30% trichloroacetic acid, 30% sulphosalicylic acid) to visualise the efficiency of transfer. Ponceau Red was washed away with double-distilled water and the membrane was washed 3 times in TBS-T (20 mM Tris, pH 7.5, 0.1 M NaCl, 0.1 % Tween 20).

Next the membrane was incubated in 5% skimmed milk solution in TBS-T for 1h to block the aspecific binding. After blocking the primary antibody (raised in mouse, rabbit or goat) was added (1h or O/N incubation time) in a dilution determined experimentally or recommended by the supplier (1:200-1000). After the incubation with primary antibody the membrane was washed 3X for 5 minutes with 1XTBS-T. Next the incubation with secondary antibody conjugated with HRP was performed for 1h at RT. Secondary antibody was raised in mouse, rabbit or goat against host species of the primary antibody host. Incubation was followed by 3X washes with 1XTBS-T. Membranes were developed in a darkroom using ECL Chemiluminescent Assay and fotosensitive Hyperfilm (GE Healthcare) with different times of exposition depending on antibody performance. To develop and fix films I used KODAK developing and fixing solutions.

Caspase 3/7 Activation Assay. Cells were seeded onto white-walled 96-well plate one day prior to the addition of the drug, so that they reached 60-70% confluence on the day of treatment. 24 hours after plating the treatment was started by addition of 5-FU (200μM) or LFMA13 (200μM) or a combination of all in

100μl of culture medium. After 72 and 96 hours plates containing cells were removed from the incubator and allowed to equilibrate to room temperature. Next 100μl of Caspase-Glo 3/7 Reagent (Promega) were added to each well of a white-walled 96-well plate containing 100μl of blank, negative control cells or treated cells in culture medium. Plate was mixed gently using a plate shaker at 500rpm for 30 seconds and incubated at RT for 1h. The luminescence of each sample was measured in a plate-reading luminometer as directed by the luminometer's manufacturer.

Cytoplasmic and Nuclear Protein Extraction. The National Cancer Institute at Frederick's Flow Cytometry Facility Core (NCI ETI) protocol was used as follows: cells were removed from their dishes by trypsinization and washed twice in cold PBS before resuspension in cold nuclei extraction buffer (320mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES, 1% Triton X-100 at pH 7.4) supplemented with 1% protease inhibitors cocktail (PIC, Sigma) at approximately 1 ml per 1 million cells. The cells were then gently vortexed for 10 seconds and allowed to incubate on ice for 10 minutes. No dounce homogenization was necessary. Nuclei were then pelleted by centrifugation at 2000x

g and washed twice with nuclei wash buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES at pH 7.4) supplemented with 1% protease inhibitors cocktail.

RNA extraction from cells growing in monolayer using TRIzol Reagent. To proceed with RNA extraction the culture medium was removed from the plate, cells were washed once with PBS and trypsinized to detach them from the plate surface. Pellets were obtained by centrifugation at 1500 rpm for 5 minutes and cells were lysed in TRIzol Reagent by repetitive pipetting. 1 ml of the reagent was used per 5-10 × 10^6 of mammalian cells plus 0.2 ml of chloroform (Sigma) per every 1 ml of TRIzol Reagent. Tubes were shaken vigorously by hand for 15 seconds and incubated at RT for 2 to 3 minutes. Then samples were centrifuged at 12,000 × g for 15 minutes at 4 °C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase, which was harvested and transferred to a new tube. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 ml of isopropyl alcohol were used every 1 ml of TRIzol Reagent used for the initial homogenization and

samples incubated at RT for 10 minutes before centrifugation at 12,000 × g for 10 minutes at 4°C. After removal of the supernatant RNA pellet was washed once with 75% ethanol, adding 1 ml of 75% ethanol per every 1 ml of TRIzol Reagent used for the initial homogenization. After briefly vortexing and centrifugation at 7,500 × g for 5 minutes at 4°CRNA pellet was briefly dried and dissolved in RNase-free water by incubating for 10 minutes at 55 °C. RNA was stored at -80°C for further analyses.

Extraction of RNA from formaldehyde-fixed paraffin embedded tumor samples with paraffin removal. 5-10 serial sections paraffin embedded were mixed with 1 ml of Xylene vortexed, and left for 10' at RT. After a centrifugation for 5' at 14000 rpm xylene wash has been repeated once and followed by a wash with 100% EtOH plus centrifugation for 5' at 14000 rpm. Washes followed by centrifugation were then repeated with 90% EtOH and 70% EtOH. After a final centrifugation for 5' at 14000 rpm the sample-containing tubes have been left open under the hood to let the EtOH evaporate before performing RNA extraction RNA extraction was done by adding 850 ul of denaturation solution (4M guanidinum isothiocyanate, 0.25M

Sodium citrate, 0.5% Sarkosyl, 0.1M Beta-mercaptoethanol)and pipetting up and down sevral times before the addition of 250 ul of Proteinase K (20 mg/ml). Samples were incubated O/N in a shaking thermoblock (1400 rpm.) at 55° C, The following day samples were centrifuged for 5' at 14000 rpm, at 4°C, paraffin tap removed with a needle, and supernatant transferred in a fresh tube to be extracted using the TRIzol Reagent protocol as already described.

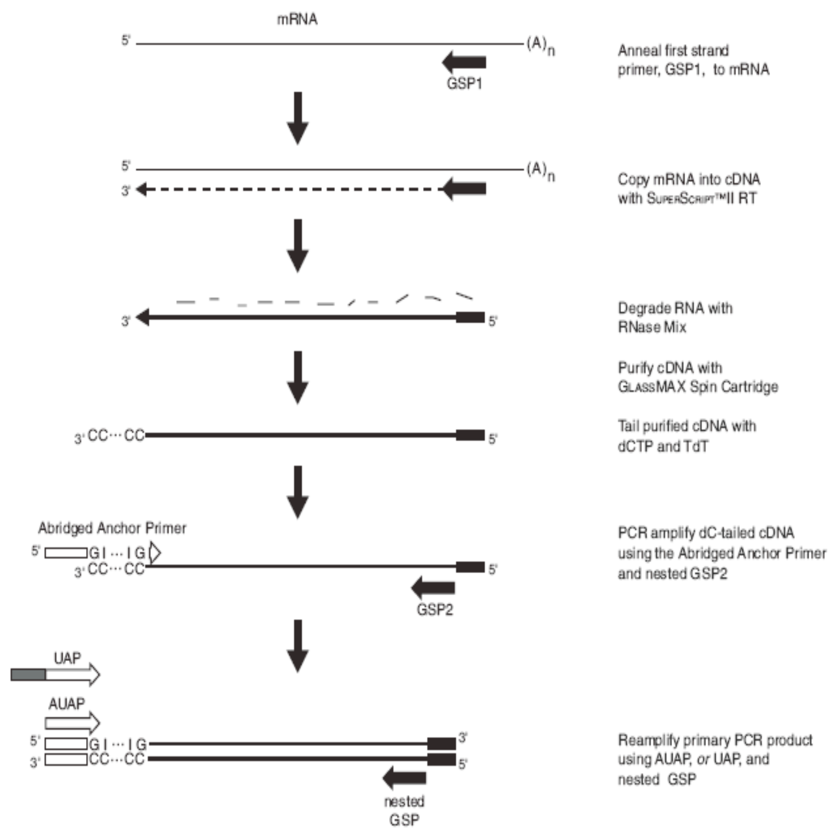
RT-PCR. Reverse transcription-PCR (RT-PCR) was performed on total RNA prepared by the guanidinium thiocyanate-acid phenol or TRIzol method. After DNase treatment, 5 µg of RNA were used for cDNA synthesis. In a 50-µl reaction volume were mixed RNA (denatured 1 min at 95°C), dithiothreitol (10 mM), deoxynucleoside triphosphates (0.25 mM each), RNasin (8 U), Superscript II with the provided buffer and random hexamers or oligo dT's (all reagents from Invitrogen). Reaction mixtures were incubated at room temperature for 15 min, at 42°C for 50 min, and finally at 95°C for 5 min to inactivate the reverse transcriptase.

PCR was performed on 1-2 µl (TRIzol extraction) or 3-5 µl (guanidinium thiocyanate-acid phenol method) of the 50-µl cDNA samples. In addition, a PCR sample contained deoxynucleoside triphosphates 50 µM each, buffer with $MgCl_2$ (1.5 mM final concentration), primers (0.2 µM each), *Taq* polymerase (1 U; Roche), and water (to a final volume of 30 µl). PCR was performed using Verity Thermocycler (Applied Biosystems) with the following scheme: initial step 95 C for 5 minutes, 40 cycles of: denaturation at 95 C - 30 seconds, annealing of primers at 56-65 C - 45 seconds, elongation 72 C - 1 minute, final elongation step was performed at 72 C for 7 minutes.

To enhance very low signal particularly from FFPE samples nested PCR was performed. Briefly, the procedure as above on the first PCR product was repeated using primers annealing to the internal sequences of the PCR product. PCR products were visualized by agarose gel electrophoresis

5'-Rapid Amplification of cDNA Ends (5'RACE). PolyA mRNA was isolated from HCT116p53KO, DLD-1 and SW480 cells by using the TRIzol reagent method (Invitrogen). Firstly, a classic

retrotranscription has been performed using 5μg of total RNA and 2,5 pMol (100nM) of primer GSP1β (5'-TGG CAG CCC ATA GCA TT-3'. Next samples were incubated at 70°C for 15 minutes and 1μL of RNase added and incubated for 30 minutes at 37 C. Then cDNA has been purified with S.N.A.P columns. Next deoxytocin has been added to permit 5' tailing. Afterwards incubation with Terminal-deoxynucleotidil-trasferase has been done followed by amplification of "tailed cDNA" using 1Rev (5'-GAG TTT GTG CAC GGT CAA GAG A-3'and 2Rev (5'-TAT TGA ACC CTT CTT ACT GCC-3') and AAP 5'-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3') to perform a nested PCR. Then another nested PCR has been performed using 1REV, 2REV and UAP (Universal Amplification Primer: 5'-CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC-3'), that anneals to primer AAP. The resulting DNA fragments were eluted from agarose gel and were analyzed by sequencing with an ABI PRISM DNA sequencing system (Applied Biosystems) using primers: UAP, 1REV e 2REV.



5'RACE RLM scheme

3.1 ABBREVIATIONS

Alt	Alternative
APS	Ammonium persulfate
BBF	Bromophenol Blue
bp	base pair
BSA	Bovine Serum Albumin
CCSC	Colorectal Cancer Stem Cells
Class	Classic
DMEM	Dulbecco-Modified Eagle's Medium
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
ex	exon
FFPE	Formaldehyde-Fixed Paraffin Embedded
FU	5-fluorouracil
GFP	Green Fluorescent Protein
Glu	L-glutamine
IF	Immunofluorescence
IP	Immunoprecipitation
kb	kilo base
kDa	kilo Dalton

KO	Knock-Out
LFM-A13	2-Cyano-N-(2,5-dibromophenyl)-3-hydroxy-2-butenamide
Luc	Luciferase
O.D.	Optical density
ORF	Open Reading Frame
OxPt/OX	Oxaliplatin
PAGE	Polyacrilamide gel electrophoresis
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PIC	Protease Inhibitor Cocktail
Pen/Strep	Penicillin/streptomycin
5'RACE	Rapid Amplification of 5' Complementary DNA ends
RLU	Relative Luminescence Units
RNAi	RNA interference
rpm	revolutions per minute
RT	Retrotranscription
UTR	Untranslated Region
WB	Western Blot

4. RESULTS

4.1 BTK INHIBITION ABOLISHES DRUG RESISTANCE IN COLON CARCINOMAS.

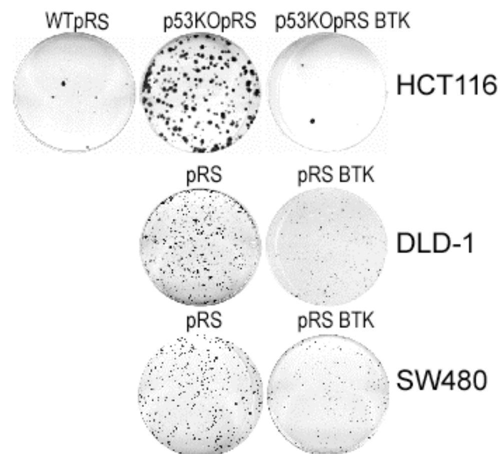


Fig 1 Colony-forming assay of p53 null colon carcinomas after treatment with 200μM of 5-FU.

To confirm that BTK absence reverted 5-FU-resistance of p53-null colon carcinoma cells I performed a colony-forming assay (CFA). To this end I used three different colon carcinoma cell lines, all p53-null: DLD-1 and SW480 bear mutated p53 whereas HCT116p53KO, have been knocked-out for both alleles of p53. These three carcinoma lines represent three different progression stages: Duke's A (HCT116), Duke's B (SW480) and Duke's C (DLD-1). As a control I used parental HCT116 wild-type cells bearing normal p53 alleles. p53-null carcinoma cell

lines were stably transfected with pRETROSUPER empty (pRS) or pRETROSUPER BTK (pRSBTK) and the interference of BTK was confirmed by Western Blot. Cells with strongly reduced levels of BTK have been reseeded at very low density after 12 hours exposure to 200 μM 5-FU and let grow for two weeks before colony staining and counting. As shown in Fig.1, and as expected, cells transfected with empty vector, being resistant to the cytotoxic action of 5-FU, grew normally forming several colonies. Strong inhibition of growth was instead evident in all 3 carcinomas, where BTK has been interfered (pRSBTK) as well as in parental HCT116. These results confirm that BTK silencing is enough to abolish 5-FU resistance of p53-null colon carcinoma cells. However, cells expressing very low levels of BTK due to stable silencing might in some way adapt their response to different stimuli due to the fact that they have been "forced" to live continuously in an unnatural condition (i.e., the very low level of BTK). So to exclude such a possibility I decided to further confirm that reduction of BTK levels or activity revert resistance to 5-FU treatment using two different approaches both of which rely on "acute" and transient rather than a "chronic" inhibition of BTK.

First, I decided to silence transiently BTK by using short-interfering RNA oligonucleotides (siRNA) directed against the 5th and 6th exon of BTK before treating them with 200 μM 5-FU for 72 hs (fig 2).

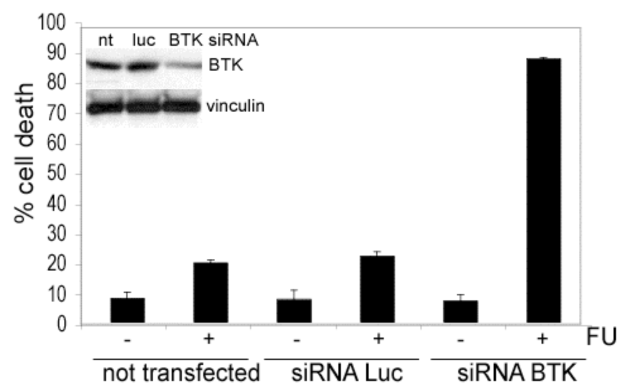


Fig 2. Percentage of cell death 72h after addition of 5-FU in HCT116p53KO cells after silencing BTK by siRNA.

A good reduction in BTK protein levels was obtained also by this method, as shown by the western blot in the inset of fig.2. Notably, only cells transiently transfected with BTK-specific siRNAs responded to 5-FU treatment with almost 90% of cell death, whereas cells transfected with siRNA against luciferase (luc) or untreated control (nt) showed only basal levels of spontaneous apoptosis.

Secondly, since BTK is a kinase I decided to further validate our hypothesis by using a potent and selective commercially available BTK inhibitor, LFMA13 (2-Cyano-N-(2,5-dibromophenyl)-3-hydroxy-2-butenamide). HCT116p53KO cells were treated with 5-FU (200μM); LFMA13 (200μM) or a combination of both for 72h and the percentage of dead cells was assessed by Trypan Blue exclusion test. (Fig 3)

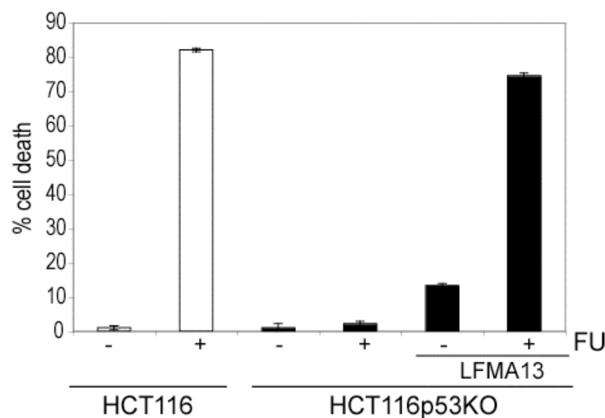


Fig 3 Percentage of cell death 72h after addition of 5-FU in HCT116p53KO cells in the presence or absence of LFMA13

According to what already observed when BTK was inhibited by other means, also upon chemical inhibition resistant cells lost their resistance to the cytotoxic action of 5-FU. Notably, LFMA13 is very mildly toxic to the cells when used alone as indicated by the very low level of cell death. Moreover, the fact

that the chemical inhibitor duplicate the results obtained by silencing indicates that the kinase activity of BTK is required for conferring resistance to chemotherapy. Finally, I further confirmed this last finding by using LFM-A13 in presence or absence of 5-FU on the other p53-null resistant cell lines DLD-1 and SW480 (fig 4).

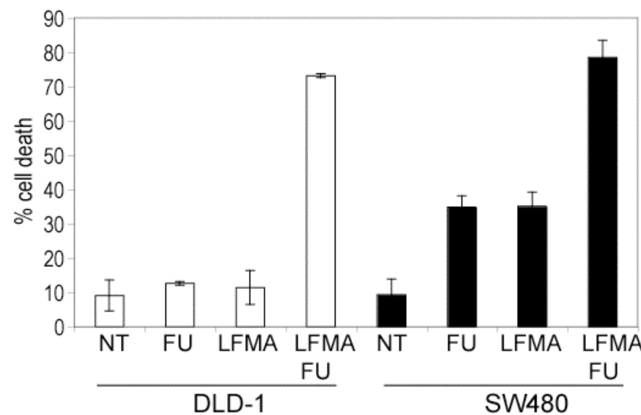


Fig 4 Percentage of cell death 72h after addition of 5-FU in different colon carcinoma cells after use of a BTK inhibitor (LFMA13).

I further tested the protective role of BTK by treating cells with 50 μM oxaliplatin (OxPt) - a drug often used in combination with 5-FU to treat CRC - in presence or absence of LFM-A13 (fig. 5, left). A significant increase in OxPt-induced cell death occurred when BTK was inhibited concomitantly to drug treatment in HCT116p53KO and DLD1.

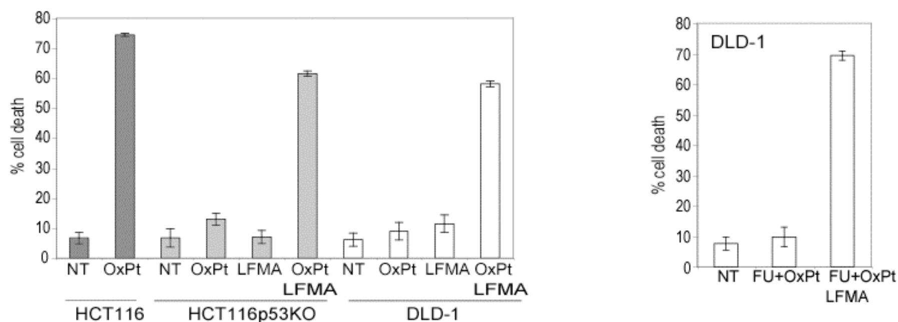


Fig 5 Percentage of cell death in different colon carcinoma cells 72h after addition of OxPt in the presence or absence of LFM-A13 (left) and 72h after addition of 5-FU+OxPt and in the presence or absence of LFM-A13 (right).

In colon carcinoma therapy, OxPt is usually given in combination with 5-FU. Despite the fact that patients who are given OxPt+ 5-FU are more likely to have a response and longer progression-free survival than patients not treated with OxPt, they don't survive longer [de Gramont et al., 2000]. This suggests that resistance eventually also occurs even in the case of combined therapy. The DLD-1 cell line is a good cellular model for testing drug resistance, because more than 60% of cells are still alive after 72 hours following treatment with 200 μM 5-FU + 50 μM OxPt. BTK inhibition is able to revert this resistance as demonstrated by the percentage of dead cells being > 80% after 72hs of combined treatment (fig 5, right).

4.2 BTK IS EXPRESSED AND REVERTS DRUG-RESISTANCE IN CARCINOMA CELLS DERIVED FROM DIFFERENT EPITHELIA.

The experiments from our lab demonstrating for the first time the expression of BTK outside of the bone marrow-derived lineages prompted me to further investigate BTK expression both in more colon carcinoma-derived cells (fig.6) and in other carcinomas derived from different epithelia (fig 7).

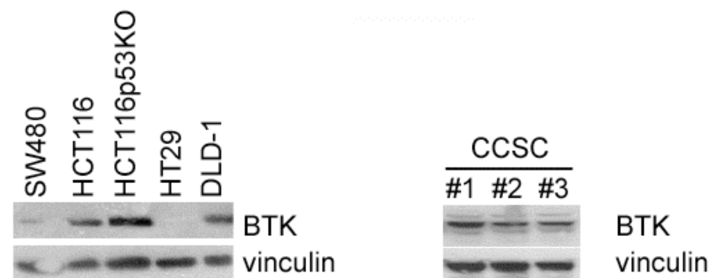


Fig 6 Expression of BTK in different colon carcinoma lines (left) and in colon carcinoma stem cells freshly isolated from patients

Beside confirming BTK expression in the established and routinely employed cell lines already used in the previous experiments (left), fig. 6 shows that BTK is expressed at comparable levels also in colon cancer stem cells (CCSC) freshly isolated from patients (right). Briefly, CCSC have been isolated

in the lab of Prof. R. De Maria (ISS, Rome) by FACS-sorting CD133+ cells dissociated from samples of tumours obtained from patients during surgery. Then, frozen pellets of sorted cells have been sent to our lab for western blot analysis. The expression of BTK by CCSC is particularly interesting since one of the most widely accepted and current hypotheses concerning drug resistance indicate in the cancerous stem cell compartment the reservoir of resistant cells [Jordan CT et al., 2006, Baguley BC et al., 2010].

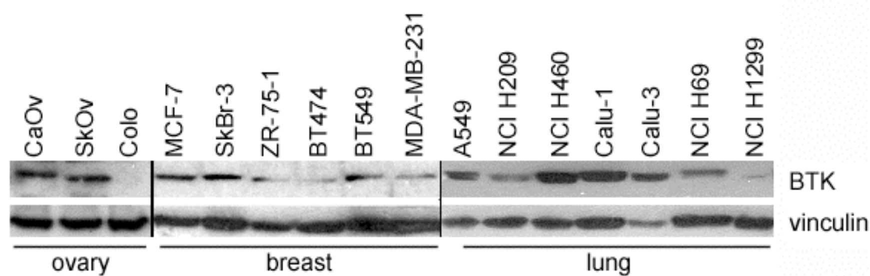


Fig 7 Expression of BTK in ovary, breast and lung cancer lines

Fig. 7 shows that BTK is widely expressed in cancer cell lines derived form different carcinomas such as ovarian, breast and lung carcinomas. Next, I decided to investigate whether expression/activity of BTK in the abovementioned carcinomas was also related to the drug-resistant phenotype, as already

shown for colon carcinoma cell lines. To this end, I performed the same biological assays done in colon carcinoma cell lines in two representative resistant cell lines from the panel of fig. 7, SKOV and A549 (fig. 8).

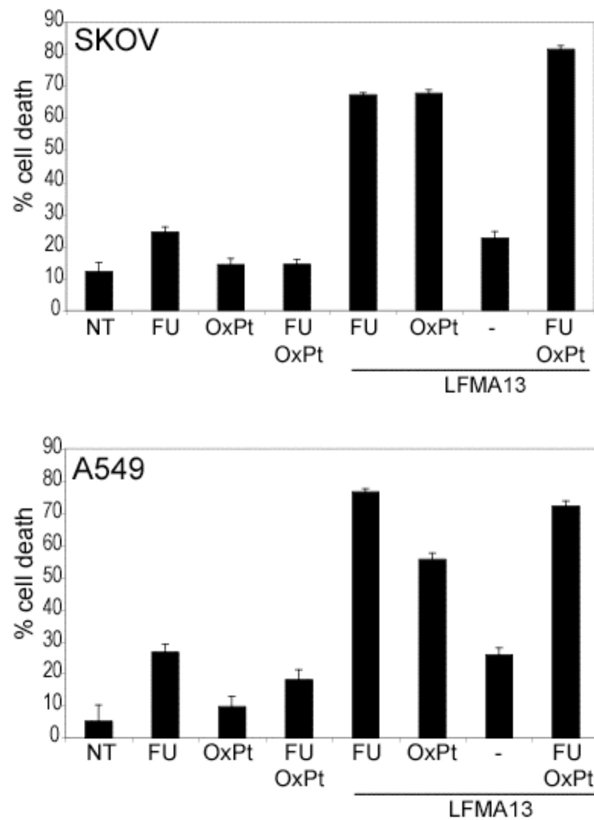


Fig 8. Percentage of cell death 72h after addition of 5-FU, OxPt, 5-FU/OxPt in the presence or absence of LFMA13 in SKOV (left) and A549 (right) cell cultures

Chemical inhibition of BTK, whereas mildly toxic alone, allows the reversion of the chemoresistant phenotype also in SKOV and A549 cell lines suggesting that BTK's role in preventing drug-induced cell death might be a general feature of drug-resistant carcinomas.

4.3 BTK INHIBITION ABOLISHES DRUG RESISTANCE BY TRIGGERING CASPASE-DEPENDENT APOPTOSIS

It is known that most anticancer drugs induce tumour cells to die by apoptosis [Woynarowska BA et al., 2002; Peters GJ et al., 2000]. To investigate which kind of cell death is induced by 5-FU when BTK is inhibited I checked a hallmark of apoptosis such as caspase activation by means of a chemiluminescent enzymatic assay (fig. 9).

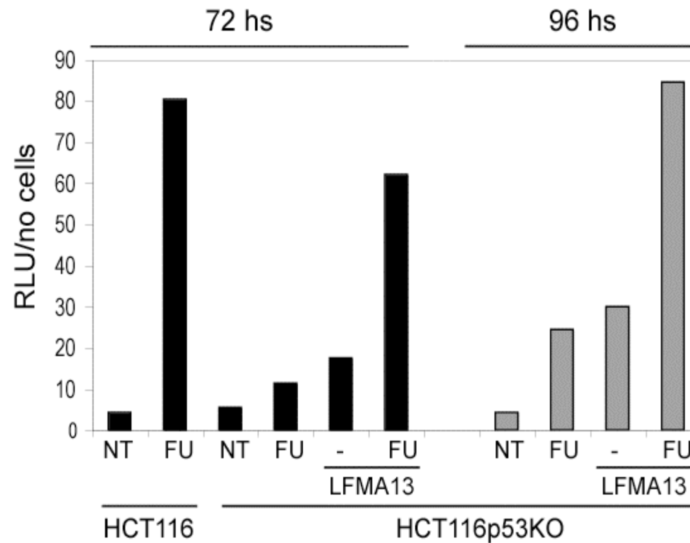


Fig 9 Caspase 3/7 activation assay of colon carcinoma cells 72h and 96h after treatment with 5-FU in presence or absence of LFM-A13.

Data clearly indicate effector caspase activation in HCT116p53KO drug-resistant cells after 72 hours of 5-FU treatment plus BTK inhibition. To note that the level of activation is still lower than that recorded in parental p53wt, drug-sensitive HCT116 cells, used as a positive control, and that to reach a similar level of caspase activation longer times (96 hs) are needed, suggesting that the apoptotic process triggered by 5-FU in absence of p53 and upon BTK inhibition is slower than that induced when p53 is active.

Next, to investigate which apoptotic pathway was triggered by 5-FU in absence of BTK activity I first checked the re-localization from mitochondria to the cytoplasm of some mitochondrial proteins involved in apoptotic signalling through the intrinsic pathway (fig.10).

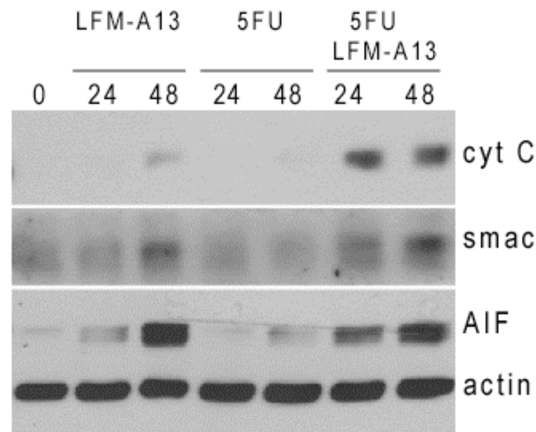


Fig 10. Western blot analysis of cytochrome C, smac, AIF in cytoplasmic fractions of HCT116p53KO cells, isolated at different times after LFMA, 5-FU or combined treatment. Actin expression was checked as a loading control and as a cytoplasmic marker

Upon 5-FU+LFMA13 treatment all the mitochondrial proteins studied are re-localized in the cytoplasm indicative that the mitochondrial pathway is activated. Notably, even though LFM-A13 alone does not affect viability (see fig.3), it

seems to induce some changes in mitochondrial membrane permeability as suggested by the fact that quite strong expression of AIF and smac is found in the cytoplasm of LFM-A13-treated cells. At variance, significant amounts of cytochrome C are instead released only when BTK inhibition is applied together with 5-FU treatment.

It has been demonstrated by several labs that 5-FU can induce apoptosis in colon cancer cells by triggering either the intrinsic or the extrinsic pathway or both. I therefore checked the extrinsic pathway by examining in western blot the appearance of active fragments of caspase-8 (fig. 11)

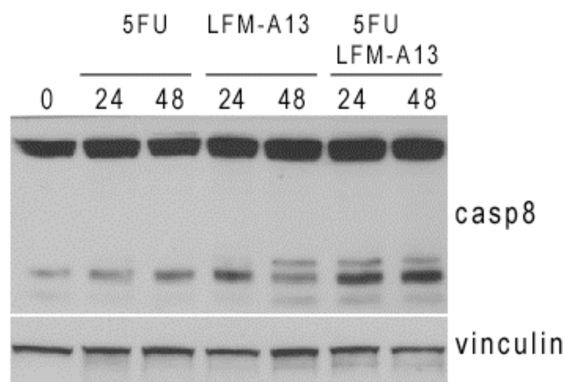


Fig 11 Western Blot analysis of caspase 8.

As evident from the blot a slight increase in active fragments of caspase-8 is induced either by 5-FU alone or, a little bit more intensely, by LFM-A13 alone; however, in both cases this increase is not enough to kill the cells as demonstrated by the very low levels of apoptosis corresponding to the single treatments illustrated in the experiments of fig 3. BTK inhibition and 5-FU treatment together further stimulate the processing of caspase-8 as indicated by the increasing intensity of the bands corresponding to the processed fragments, likely reaching a threshold that allows apoptosis to proceed.

Taken all together, these results clearly indicate that in absence of BTK activity 5-FU stimulates apoptotic cell death by triggering both the intrinsic and the extrinsic pathways.

4.4 IN CARCINOMA CELLS BTK HAS LOWER MOLECULAR WEIGHT (~65 kDa) THAN IN LYMPHOID CELLS AND IS ENCODED BY mRNA FEATURING A NOVEL 1st EXON IN THE 5'UTR.

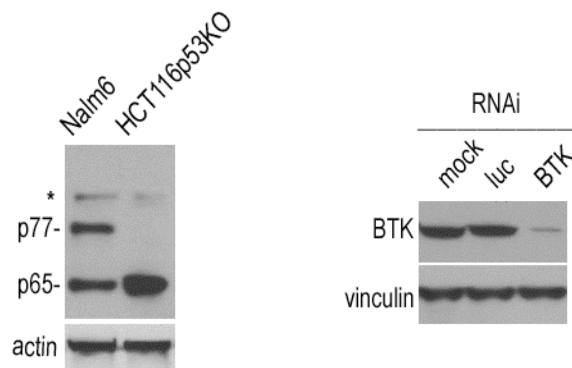


Fig 12 Western Blot analysis of p65BTK in HCT116p53KO and NALM6 (left). RNA interference of p65BTK in HCT116p53KO cells (right).. * = Aspecific band

Western blot analysis of cellular lysates from HCT116p53KO using a BTK specific antibody (M138, Santa Cruz Biotechnology) showed that in HCT116p53KO cells BTK has an apparent molecular weight of ~65 kDa (Fig 12, left). A band of the same molecular weight, together with the expected 'classic' band of 77 kDa, is also present in the leukemic B-cell line NALM-6. The 65 kDa band, called from here on 'alternative' or p65BTK is

specific, since it almost completely abolished upon interference (Fig 12, right) with siRNA targeted to the middle of BTK's mRNA sequence (5th and 6th exons).

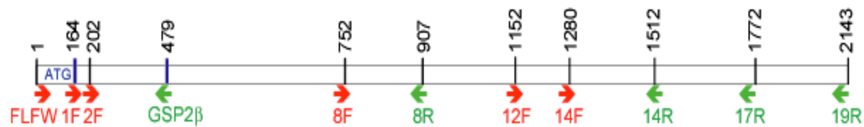


Fig 13 Scheme of the primers used to PCR out BTK in HCT116p53KO vs. NALM-6 cells and to perform RACE experiments

In order to understand, whether the shorter, p65BTK protein was encoded by an alternatively spliced mRNA, RT-PCR using different pairs of primers (Fig 13) spanning throughout the whole BTK messenger was performed, using RNA isolated from HCT116p53KO and NALM-6 (positive control)(fig 14).

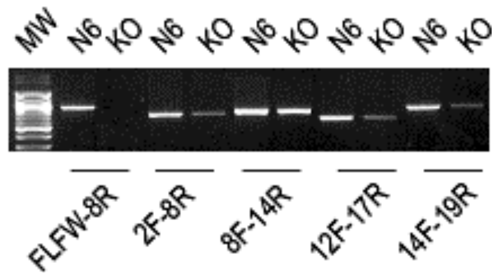


Fig 14 PCR amplifying different parts of BTK performed in HCT116p53KO vs. NALM-6 cells.

In case of NALM6 I succeeded in amplifying a fragment of the expected length using all pair of primers. In HCT116p53KO all primers used gave the same result as in Nalms6 but for the one annealing to nt 1- nt 21 in the first exon. Comparison of different PCR products in fact indicated that the sequence upstream of nucleotide 202 is different or missing in the mRNA encoding p65BTK (Fig 14).

By means of bioinformatic tools I identified a second ATG (ATG2) in position nt 428 (4th exon), in frame with the canonical ATG (ATG1) in position nt 164 (2nd exon) of the p77BTK transcript (NCBI Database RefSeq.: NM_000061.2). As predicted by the bioinformatic approach the protein translated starting from ATG2 should have MW around 65 kDa and that prediction was compatible with the apparent MW detected by western blot, where a band of ~65 kDa is observed both in HCT116p53KO and NALM6.

To verify that the sequence upstream nt 202 in the messenger encoding p65BTK is missing I used the 5' Rapid Amplification of cDNA Ends (RACE) technique.

Using as a template RNA isolated from HCT116p53KO and NALM-6 (5'RACE PCR fragments (around 400 bp long) were

generated that were subsequently cloned and sequenced. Since by western blot analysis it was evident that BTK had an apparent MW around 65 kDa in all carcinoma cell lines examined (see figs 6 and 7) and its inhibition gave the same phenotype in all cell lines studied I decided, as a further control, to perform 5' RACE also on RNA isolated from DLD-1 and SW480 cells. 8-10 clones obtained from the amplification product derived from each cell line were sequenced. All products revealed a ~ 400bp long fragment and the comparison of the sequences among them revealed that the sequence was the same for all the clones.

```
CLUSTAL 2.0.5 multiple sequence alignment

NM_000061       ------------------------------------------------------------
alternative     TTTTGGTGGACTCTGCTACGTAGTGGCGTTCAGTGAAGGGAGCAGTGTTTTTCCCAGATC 60

NM_000061       ------------------------------------------------------------
alternative     CTCTGGCCTCCCCGTCCCCGAGGGAAGCCAGGACTAGGGTCGAATGAAGGGGTCCTCCAC 120

NM_000061       ---------------------------------------------------CTCAGACTG 9
alternative     CTCCACGTTCCATTCCTGTTCCACCTCAAGGTCACTGGGAACACCTTTCGCAGCAAACTG 180
                                                                     ** ****

NM_000061       TCCTTCCTCTCTGGACTGTAAGAATATGTCTCCAGGGCCAGT--GTCTGCTGCG-ATCGA 66
alternative     CTAATTCAATGAAGACCTGGAGGGA--GCCAATTGTTCCAGTTCATCTATCACATGGCCA 238
                    * *  *   ***    **     * *    *  *****   ***    *    * *

NM_000061       GTCCCACCTTCCAAGTCCTGGCATCTCAATGCATCTGGGAAGCTACCTGCATTAAGTCAG 126
alternative     GTTGGTCCATTCAACAAATGGTTATTGGATGC--CCATTATGTGGCAGGCACTGTTCCGG 296
                **    ** * ***    ***    *  ****  *    * *   *  *** *    * *

NM_000061       GACTGAGCACACAGGTGAACTCCAGAAAGAAGAAGCTATGGCCGCAGTGATTCTGGAGAG 186
alternative     GGGAGAGCACACAGGTGAACTCCAGAAAGAAGAAGCTATGGCCGCAGTGATTCTGGAGAG 356
                *   ********************************************************
```

Fig 15 ClustalW alignment of the classic p77BTK (NCBI Nucleotide Database RefSeq NM_000061) with p65BTK sequence identified by 5'RACE PCR in HCT116p53KO (indicated as 'alternative')

Moreover, ClustalW comparison of the sequence identified (marked as "alternative" in fig. 15) with the RefSeq of BTK NM_000061 revealed that the alignment started at nucleotide 303 of p65BTK, i.e., at the beginning of the 2nd exon in p77BTK sequence, suggesting the presence of a novel, alternative first exon in the mRNA coding for p65BTK. To confirm that the identified sequence is indeed a novel, alternative exon I run the sequence through the BLAST database to pull out the genomic region coding for it.

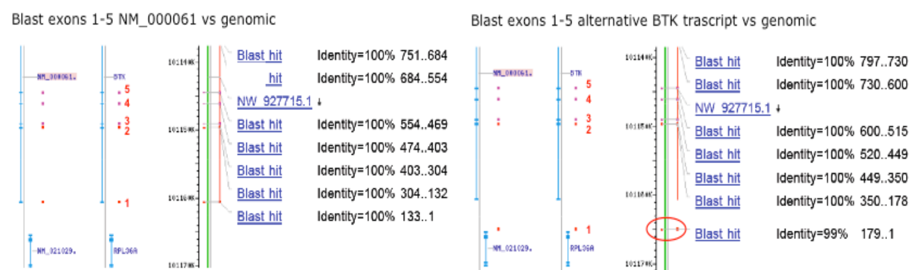


Fig 16 BLAST alignment of p77BTK and p65BTK isoform vs. genomic DNA.

As evident from the comparison in fig 16 the last 102 nt of the sequence obtained by 5'RACE PCR map in the p77BTK locus while the remaining 300 nt map in the region found 15194 nt upstream of the p77BTK locus. This data confirm that the novel p65BTK isoform is encoded by a messenger bearing an

alternative and different, hitherto unknown 1st exon. Notably, the first exon in the mRNA coding for p77BTK is known to be outside the coding sequence and being part of the 5'UTR. Also the novel alternative 1st exon is 5'UTR, since, as hypothesized at the beginning of the chapter, the putative ATG (ATG2) recognized in order to translate p65 is located in the 4th exon. This implies that the presence of the alternative 1st exon in the BTK mRNA causes the "extension" of the length of the 5'UTR.

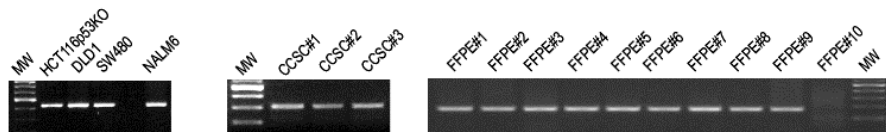


Fig 17 Detection of p65BTK's 'alternative' transcript, using primers specific for the novel 1st exon in: colon carcinoma cell lines and leukaemia cell lines NALM-6 (left), Colon Carcinoma Stem Cells (CCSC, middle) and formaldehyde-fixed paraffin embedded (FFPE) samples (right) from colon carcinoma tissue obtained from 10 patients at time of surgery.

Next, I decided to confirm the presence of the alternative transcript in several settings by means of RT-PCR using a forward primer annealing to the first 'alternative' exon and a reverse primer annealing to the second exon, common to the mRNAs coding for both BTK proteins (p65BTK and p77BTK). As expected, the expression of the novel BTK transcript is

confirmed in all cell lines whose RNA has been used for the 5' RACE experiments (fig 17, left) and in CCSC, already shown to express p65BTK (fig 17, middle). Moreover, the alternative BTK mRNA is expressed also in samples from cancerous tissue taken from patients at time of surgery (fig 17, right). Altogether, these data show that the messenger coding for p65BTK is expressed not only in colon carcinomas cell lines (HCT116, DLD-1, SW480), but also in tumour samples from cancer patients indicating that the presence of the novel transcript is not a consequence of cell culture conditions or adaptation but rather reflects the in vivo situation.

4.5 p65BTK ISOFORM IS TRANSLATED FROM THE mRNA CONTAINING THE ALTERNATIVE FIRST EXON.

To formally prove that shorter p65BTK derives from the newly identified, messenger and that the putative CDS in this mRNA starts from ATG2 I cloned the sequence coding for p65BTK starting from the newly identified 5'UTR and ending with the stop codon and the putative CDS starting from ATG2

into expression vector pcDNA3. Moreover, as control I also cloned the sequence coding for p77BTK starting from 5'UTR and ending with the stop codon (Class) and the corresponding CDS starting from ATG1. Finally, I transiently overexpressed all of them in HCT116p53KO (already expressing endogenous p65BTK) to compare the protein produced by each plasmid (fig. 18).

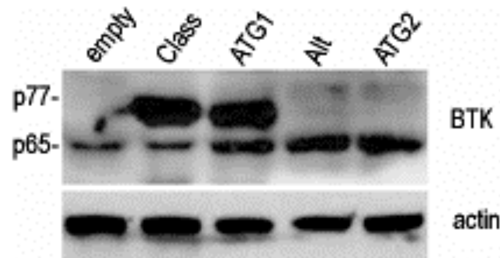


Fig 18 BTK expression in HCT116p53KO cells upon transfection with pcDNA3 vector alone (empty), encoding the sequence coding for p77BTK starting from 5'UTR and ending with the stop codon (Class) the coding sequence for classic BTK (ATG1), the sequence coding for p65BTK starting from 5'UTR and ending with the stop codon (Alt) and the putative CDS for p65 (ATG2).

The transfection of pcDNA3Class and pcDNAATG1 gave the expected band of 77 kDa, whereas, the transfection of pcDNA3Alt and ATG2 increased the levels of the 65 kDa band. To note that also in ATG1-transfected cells the levels of the p65 band is increased compared to the empty vector-transfected

cells where only the endogenous levels of p65 BTK are evident. These results demonstrate that p65 is translated from the sequence cloned in pcDNA and that the coding sequence of this messenger starts from ATG2.

To further ascertain that p77 is encoded by the sequence cloned in pcDNA3Class and that p65 is encoded by the sequence cloned in pcDNA3Alt I decided to transfect these same plasmids in cells not expressing endogenous BTK, such as 293T, and to use siRNA specific for the 1st alternative and for the 1st classic exons to silence the overexpressed protein (fig 19). SiRNA specific for exons 5 and 6, which are conserved in both forms of BTK, were used as a control.

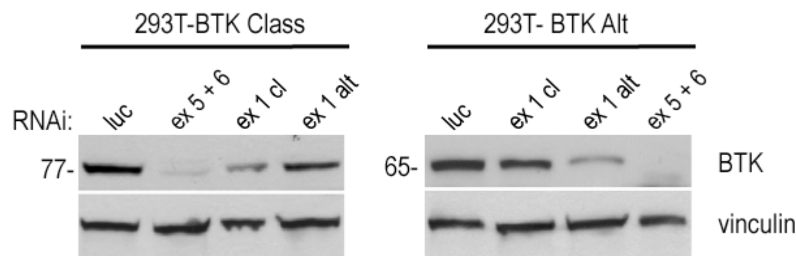


Fig 19. RNA interference of 293T cells overexpressing p77 and p65 cells with siRNA specific for 1st exon of both classic p77 (ex 1 cl) and alternative p65 (ex 1 alt). siRNAs for exons 5th and 6th (ex 5+6, common for both proteins) were used as an internal control whereas siRNAs for luciferase (luc) were used as non specific oligos. Vinculin was used a loading control.

As expected using siRNAs directed to exons 5 and 6 both p77 and p65 overexpressed proteins were silenced. Notably, overexpressed p77 was strongly, even though non completely, diminished by siRNA specific for the 1st classic exon but not by those specific for the 1st alternative exon. Vice versa, overexpressed p65 was almost completely silenced by siRNAs directed to the 1st alternative exon but not by those directed to the 1st classic exon. Altogether these data undoubtedly demonstrate that p65 BTK is translated from the sequence containing the novel exon.

Next, in order to be able to completely and successfully silence p65 expression I designed several siRNAs directed to different parts of the 1st alternative exon and tested them experimentally (fig 20). Endogenous p65BTK expressed by resistant HCT116p53KO can be successfully interfered using all designed siRNAs (oligonucleotides Alt1, Alt2, Alt3, fig. 20, left) and, as expected, this leads to the reversion of the resistant phenotype as demonstrated by the corresponding increase in cell death recorded upon 5-FU treatment (fig 20, right)

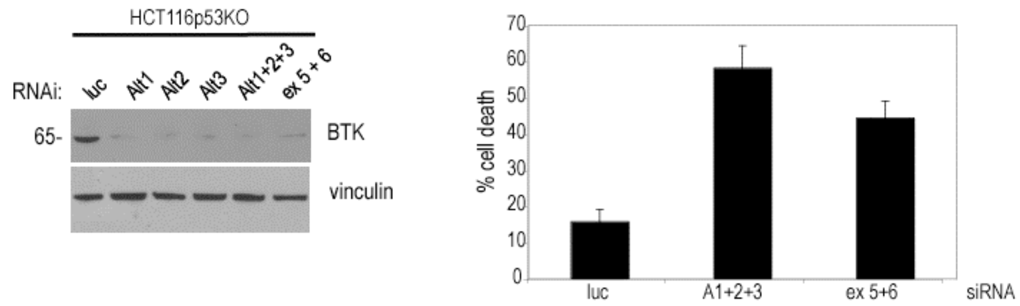


Fig 20. Left: RNA silencing of p65BTK in HCT116p53KO using specific siRNA directed to different part of the 1st alternative exons alone or in combination (Alt1, Alt2, Alt3 and Alt1+2+3). SiRNAs against exons 5 and 6 were used as a control. . Right: Percentage of cell death after 72 h of 200 µM 5-FU treatment in HCT116p53KO cells upon BTK silencing with siRNA tested on the left.

4.6 p65BTK EXPRESSION AND p65BTK-MEDIATED RESISTANCE TO DRUG-INDUCED APOPTOSIS ARE REGULATED BY hnRNP K.

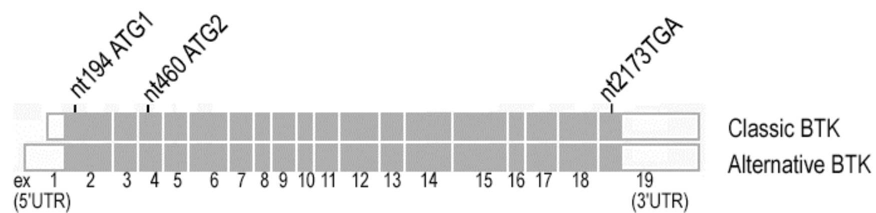


Fig 21. Scheme of mRNA coding for p77 (classic) and p65 (alternative) BTK

As previously mentioned, the 1st exon in the mRNA coding for p77BTK is 5'UTR, being the starting ATG in the second exon. The data so far obtained (either by bioinformatic or experimental approach) support the hypothesis that the presence of the 1st alternative exon in the messenger coding for p65BTK make it possible that a downstream ATG (ATG2) is used for the translation thus "extending" the 5'UTR until the 4th exon. If this is true, the 1st alternative exon should contain regulatory sequences known to regulate translation. To support this hypothesis I performed a bioinformatic analysis of p65BTK

5'UTR using UTRscan software (utrdb.ba.itb.cnr.it) and found the presence of a cytidine-rich 15-lipoxygenase differentiation control element (15-LOX-DICE) sequence able to bind the KH domain of Heterogeneous ribonucleoprotein K (hnRNP K) (Fig 22).

```
TCTTTTCCTCCACTCTCCTACCTACTCCCCTTCACTCAACCCACCACTCTTTTTCCCACATCCTCTCCCCTCCCCCTCCCCCACCCAAC
CCAGGACTAGGGTCGAATGAAGGGGTCCTCCACCTCCACGTTCCATTCCTGTTCCACCTCAAGGTCACTGGGAACACCTTTCGCAGCAA
ACTGCTAATTCAATGAAGACCTGGAGGGAGCCAATTGTTCCAGTTCATCTATCACATGGCCAGTTGGTCCATTCAACAAATGGTTATTG
GATGCCCATTATGTGGCAGGCACTGTTCCGGGGGAGAGCACACAGGTGAACTCCAGAAAGAAGAAGCTATGGCCGCAGTGATTCTGGAG
AGCATCTTTCTGAAGCGATCCCAACAGAAAAAGAAAACATCACCTCTAAACTTCAAGAAGCGCCTGTTTCTCTTGACCGTGCACAAACT
CTCCTACTATGAGTATGACTTTGAACGTGGGAGAAGAGGCAGTAAGAAGGGTTCAATAGATGTTGAGAAGATCACTTGTGTTGAAACAG
TGGTTCCTGAAAAAAATCCTCCTCCAGAAAGACAGATTCCGAGAAGAGGTGAAGAGTCCAGTGAAATG
```

Fig 22 Sequence of 5’ UTR region of new p65BTK isoform showing a putative hnRNPK biding site (red), ATG's (yellow-underlined) and stop codons (gray). Kozak consensus sequence is marked in yellow.

hnRNP K is a member of the hnRNP family which has several different cellular roles including transcription, mRNA shuttling, RNA editing and translation and can regulate these processes both positively and negatively. Moreover, several cellular functions strongly indicate that hnRNP K is involved in tumorigenesis [Michelotti EF et al., 1996, Mandal et al., 2001, Revil T et al., 2009].

First I wanted to ascertain whether 5'UTR sequence of p65BTK had a regulatory potential. To this end I cloned p65BTK 5'UTR and p77BTK 5'UTR (until ATG1) into pcDNA3.1/CT-GFP-TOPO plasmid to test whether the different UTRs could regulate the expression of an exogenous protein (GFP)(fig 23).

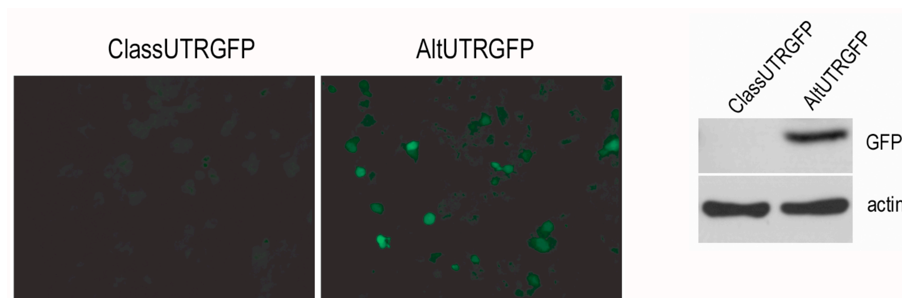


Fig 23 Overexpression of pcDNA3-GFP with classic 5'UTR and alternative 5'UTR in HCT116p53KO.

As evident either from immunofluorescence (fig 23, left) or by western blot analysis (fig 23, right) 5'UTR of p65BTK (5'AltUTR) strongly induced the expression of the GFP as opposed to 5'UTR of p77BTK (5'ClassUTR). These data confirm that the p65BTK 5'UTR possess indeed a regulatory potential.

Next, I wanted to check whether hnRNP K might influence p65BTK expression and to this purpose I overexpressed p65BTK in HCT116p53KO cells 24 hours after the interference of hnRNP K (Figure 24).

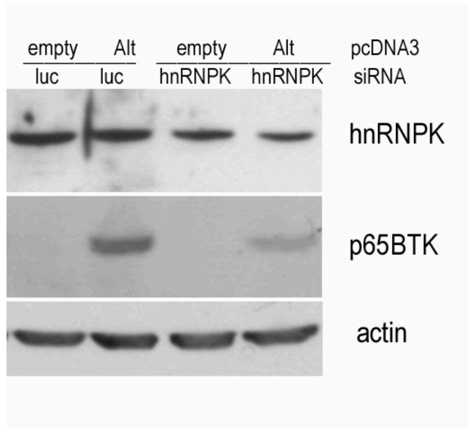


Fig 24. Western blot analysis of p65BTK expression after RNA interference of hnRNPK in HCT116p53KO cells. Transfection of pcDNA3Alt was done 24 hs after silencing hnRNP K and cells were lysed 36 hs after p65 transfection. To make it possible to appreciate the expression of only exogenous p65 BTK 10 mg of total lysate have been loaded and the exposure time of the film was very short (less than 1 min).

Silencing of hnRNP K was only partial, being the protein reduced only by the half: however, this decrease was enough to suppress the expression of exogenous p65BTK, driven by a strong promoter (pcDNA3 bear a CMV promoter) thus confirming that hnRNP K regulates p65 BTK expression.

Finally, to further confirm the involvement of hnRNP K in the regulation of p65BTK expression I decided to check the expression of endogenous p65BTK and the biological consequences on the response to 5-FU upon silencing of hnRNP K in HCT116p53KO cells (fig. 25)

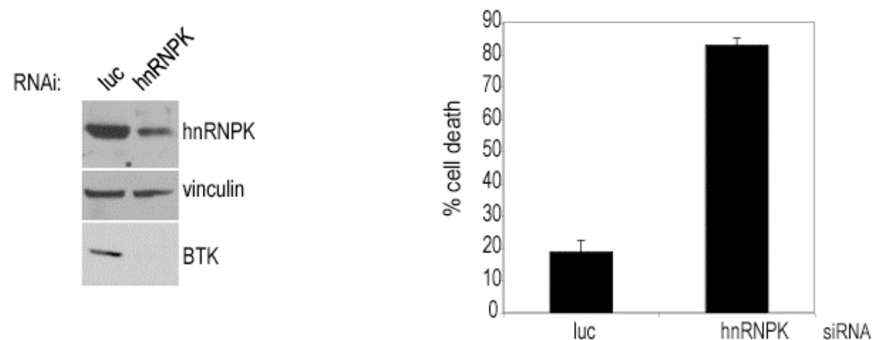


Fig 25 Western blot analysis of hnRNP K and p65BTK levels upon hnRNP K silencing; vinculin was used as a loading control (left). Percentage of cell death of HCT116p53KO cells upon hnRNPK silencing and after 72h of 200 μM 5-FU treatment (right).

As a confirmation of the previous experiment, the decrease in hnRNP K levels abolishes completely the expression of endogenous p65BTK (fig. 25, left), strongly suggesting that hnRNP K is necessary for the translation of p65BTK from the messenger RNA containing the 1st alternative exon. Accordingly to the lack of BTK protein due to hnRNP K silencing,

HCT116p53KO cells lost resistance to 5-FU treatment (fig. 25, right).

Altogether, these data clearly show that hnRNP K regulates p65BTK expression in HCT116p53KO colon carcinoma cells and suggest that hnRNP K-driven expression of p65BTK is ultimately responsible for protecting these cells from the apoptotic effect of 5-FU.

5. DISCUSSION

Data shown in this PhD thesis demonstrate for the first time BTK expression outside of the hematopoietic compartment and uncover its pivotal role as a modulator of drug resistance in colon carcinoma cells. Moreover, evidences are provided that the protective effect against drug-induced apoptosis is mediated by a novel isoform derived by alternative splicing and including a hitherto unknown first exon. Several data strongly support the hypothesis that the inclusion in the 5'UTR of this alternative exon is responsible for shifting the start of translation of the protein downstream of the canonical ATG in such a way, that a p65 BTK is produced instead of the "classical" p77BTK. Finally, the demonstration is also given that p65 expression is dependent on intact levels of hnRNP K protein, likely by interacting with a consensus sequence present in the first alternative exon.

p65BTK is expressed in carcinoma cells derived from different epithelia and is an important modulator of chemo-resistance. The first evidence that BTK plays a role in sustaining resistance to drug-induced apoptosis in p53-null colon carcinoma cells came as a result from a phenotypic shRNA library-mediated screening performed previously in our laboratory and aimed at identifying genes whose silencing

reverts drug-resistance. The main reason why this kinase gained our attention was because until that moment BTK has always been considered to be expressed only in bone marrow-derived lineages, where it's involved in B cell-specific cellular processes, such as BCR signalling and plasma cells maturation, in platelets activation and in degranulation of mast cells [Schmidt U et al., 2004]. In B cells, in particular, BTK has also been identified as a dual-function regulator of apoptosis being either pro- or anti-apoptotic depending on the setting and on the stimulus [Uckun F., 1998; Schmidt et al., 2004; Bajpai et al., 2000; Anderson JS et al., 1996]. Our findings that BTK is expressed in a wide variety of carcinoma cell lines derived from different epithelia (figs 6 and 7) and that abolishing its function, either by protein depletion (fig 2) or by kinase activity inhibition (figs 3, 4, 5, 8), in all drug-resistant cell lines tested, challenged with different drugs (figs 2, 3, 4, 5, 8), always reverted the drug-resistant phenotype, assign to BTK a novel and very important function outside of the hematopoietic lineages. Moreover, since the escape of only a few cells from drug-induced death is sufficient to allow tumour re-growth, particularly relevant is the demonstration that BTK inhibition not only allow resistant cells to respond to chemotherapy in the

short term (comparable levels of cell death at 72hs in figs 2, 3, 4, 5, 8) but also block the re-growth of colonies on the long term, even upon a short exposure to the drug (fig. 1). Notably, BTK inhibition by itself, in the absence of a chemotherapeutic drug is only mildly toxic (see figs. 2, 3, 4, 5, 8) suggesting that, at variance with B cells, it does not play a significant role in carcinoma cell physiology. On the whole these findings render BTK attractive as a novel drug target to overcome drug-resistance of cancer cells. Further fact strengthening this suggestion is also the observation of BTK expression in colon CSC isolated from tumours excised from patients undergoing surgery (fig. 6). In fact, recent evidences suggest that CSC are not only the reservoir of the heterogeneous populations of differentiated cancer cells constituting the tumour bulk, but also that they are responsible for the resistance to conventional therapy [Scopelliti et al, 2009]. A corollary descending from this is that selective targeting of CSC would be the ultimate goal to overcome drug-resistance. It is therefore tempting to speculate that BTK would be a good candidate for such strategy.

In carcinoma cell lines a novel p65BTK isoform is expressed which is encoded by an mRNA featuring a novel first exon in the 5'UTR. The second major finding of this thesis

is the identification of a novel isoform of BTK, which has been dubbed in our laboratory as p65BTK, due to its apparent molecular on SDS-PAGE. The possibility of an aspecific, cross-reactive band has been ruled out immediately by the finding that the band with apparent MW of 65kDa strongly diminished upon using siRNA specific for BTK (see fig. 2, but also figs 12, 19, 20), known from the literature to be working well [Heinonen JE et al., 2002]. This conclusion is further supported by the results obtained in experiments shown in figs 2 and 3, where the same phenotype (i.e., reversal of drug-resistance) was obtained either by silencing BTK or by inhibiting it by means of a specific inhibitor. 5'RACE experiments on mRNA from cells expressing p65BTK followed by bioinformatic study with ClustalW and BLAST programs allowed the identification of a novel mRNA transcript containing a different 1st exon in 5'UTR. Moreover, analysis of the features of the genomic sequences that precede those coding for the alternative exon indicate that they seem to have characteristics of a promoter region, with CpG island, an enhancer region and a TATAA box 200 nt upstream from the beginning of the exon. This suggests the presence also of a novel promoter governing the transcription of the novel messenger RNA containing the alternative 1st exon. Examples of transcripts

of the same gene being regulated by different promoters are reported in the literature, such as the Oct-1 transcription factor mRNA that can be initiated from 2 different promoters, in tissue-specific manner [Zhenilo SV et al., 2003]. Another example is the caspase-2 gene, where 2 promoters can be distinguished that drive initiation of transcription from 2 different initial exons giving rise to mRNA coding for short or long isoform. Surprisingly, the short isoform may also be initiated at a downstream AUG codon within the same exon. Thus, promoter strength, alternative transcriptional initiation and 5'-splicing events regulate the expression of the main caspase-2 isoforms that may be translated from alternative translation initiation codons [Logette E et al., 2003]. Similarly to what happens for caspase-2, also in the BTK cDNA sequence a second ATG (ATG2) located in the 4th exon, and in frame with the canonical ATG (ATG1) in the 2nd exon is found. Moreover, the putative protein translated starting from ATG2 should be of around 65 kDa, compatible with the apparent MW observed for our novel isoform. The hypothesis that p65BTK is translated from this second putative ATG is strongly supported by results obtained when the putative CDS has been cloned in pcDNA3 and overexpressed in HCT116p53KO cells in parallel with the a

pcDNA3 in which the longer sequence containing also all the 5'UTR is cloned (fig. 18). In both cases, in fact, the overexpressed product had the same MW around 65kDa and co-migrated with endogenous p65BTK.

p65BTK expression is regulated by hnRNP K. It's well known that 5'- and 3' untranslated regions (UTR) in a transcript may play regulatory roles influencing the translation of the messenger itself, usually via the interaction of protein recognizing and binding to specific consensus sequences or via miRNA-mediated silencing [Pickering BM et al., 2004; Le Quesne JP et al., 2010]. Since the presence of only a single different 1st exon in BTK mRNA determines the production of the novel p65 isoform instead of the classic p77, we first hypothesized a regulatory potential endowed in the 1st alternative exon, which was subsequently verified by fusing it in frame to GFP and performing a reporter assay (fig. 23). Bioinformatic analysis of the 5'UTR of p65BTK-encoding transcript identified a stretch of poly-(C) repeats, corresponding to the binding site for the KH domain of hnRNP K. hnRNP K belongs to the broad family of hnRNPs, which participate in the processing of pre-mRNAs and mRNA export from the nucleus [Dreyfuss et al., 2002]. HnRNP K contains three K homology (KH) domains that mediate

RNA/DNA binding [Ostrowski et al., 2002] and is involved in positive and negative regulation of multiple processes of gene expression including chromatin remodelling, transcription, RNA splicing, mRNA stability and translation [Bomsztyk K et al., 2004]. In particular, hnRNP K regulates the expression of many genes involved in tumorigenesis, such as oncogenes like c-Src, eIF4E, and c-myc [Barber JL et al., 1999] and has been recently reported as being itself overexpressed in colon carcinomas, where the increased levels correlated with poor prognosis, not necessarily related to p53 status [Carpenter B et al., 2006]. Overexpression of hnRNP K increases translation initiation, cell division, and neoplastic transformation in an eIF4E-dependent manner [Lynch M et al., 2005] and increases the activity of the c-myc gene promoter [Michelotti EF et al., 1996]. All these data from the literature together with the results obtained with the reporter assay suggested a possible role for hnRNP K in regulating p65BTK expression. In fact RNAi experiments revealed that hnRNP K levels are critical for p65BTK expression, since even a partial reduction abolishes not only endogenous p65BTK levels, but also the higher levels obtained upon overexpression using pcDNA3Alt (see figs. 24 and 25). Accordingly to its effects on BTK expression also siRNA-

mediated reduction of hnRNP K levels was able to revert resistance to 5-FU-induced apoptosis of HCT116p53KO cells (fig. 25). Still, the question of how hnRNPK regulates p65BTK expression remains an open issue. It is very likely that hnRNPK act by binding to the poly-C stretch present in the 1st alternative exon, as direct binding to its consensus sequence is the usual mode of action. To formally prove that, several experiments are ongoing in the lab: 1) pcDNA3Alt mutants for the poly-C stretch are being made to be transfected in HCT116p53KO cells silenced for hnRNPK; 2) RNA immunoprecipitation experiments and REMSA experiments are being set up to verify the direct interaction of hnRNPK and 5'UTR of p65BTK-encoding mRNA. Finally, experiments are in progress to understand, whether hnRNPK, besides regulating p65BTK levels, also dictates the choice of ATG2 as starting codon for the translation.

In conclusion, by identifying a novel isoform of BTK expressed in carcinomas and able to protect from the cytotoxic effect of anticancer drugs, this study indicates the specific targeting of p65BTK as a possible translational approach to overcome drug resistance.

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7. ACKNOWLEDGEMENTS

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