DECIPHERING 5-FLUOROURACIL MEDIATED MOLECULAR MECHANISMS REQUIRED FOR CELL DEATH

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To my family...

ABSTRACT

DECIPHERING 5-FLUOROURACIL MEDIATED MOLECULAR MECHANISMS REQUIRED FOR CELL DEATH

The chemotherapy agent 5-Fluorouracil (5-FU) is an antimetabolite that has been in use to treat several cancers for decades. In cells, it is converted into three distinct fluoro-based nucleotide analogues which interfere with DNA-synthesis and repair leading to impairment of the genome and, eventually apoptotic cell death. Current knowledge also state that 5-FU induced damage is signaling through a p53-dependent induction of death inducing complex (DISC) formation and further caspase-8 activation in certain cell types and members of the TNF-receptor family has been proposes to be required for the process. Here, we introduce calcium (Ca^{2+}) as a messenger for p53 activation in the cellular response triggered by 5-FU. Using a combination of pharmacological and genetic approaches, we show that treatment of cultured colon carcinoma cells stimulates entry of extracellular Ca^{2+} through L-type plasma membrane channels and that this event direct posttranslational phosphorylation of at least two specific p53 serine residues (ser15 and ser33) by means of Calmodulin (CaM) activity. Obstructing this pathway by the Ca^{2+}-chelator BAPTA or by two different inhibitors of CaM efficiently blocks 5-FU-induced cell death. The fact that a widely used therapeutic drug, such as 5-FU, is signaling by these means could provide new therapeutic intervention points, or specify new combinatorial treatment regimes.

ÖZET

5-FLOROURASİLİN TETİKLEDİĞİ HÜCRESEL ÖLÜMDE MOLEKÜLER MEKANİZMALARIN BELİRLENMESİ

Bir antimetabolit olan 5-Florourasil (5-FU), kanser tedavisinde uzun yıllardır yaygın olarak kullanılan bir kemoterapi ajanıdır. 5-FU, hücre içerisinde 3 farklı floro-temelli nükleotid analoglarına dönüşür ve DNA sentez ve tamir mekanizmasını etkileyerek genomun stabil yapısını kaybetmesine ve son olarak apoptotik hücre ölümüne neden olur. Belli hücre tiplerinde, 5-FU ile indüklenmiş hücre hasarında p53'e bağlı olarak Death Inducing Signaling Complex (DISC)'inin oluşumu, kaspaz-8 aktivasyonu ve TNF-reseptör ailesi moleküllerinin görev aldığı bilinmektedir. Bu çalışmada, 5-FU ile indüklenmiş hücre ölümünde kalsiyum (Ca2+)'un p53 aktivasyonu için gerekli olan bir mesajcı molekül olduğu gösterilmiştir. Farmokolojik ve genetik yaklaşımların kombinasyonları kullanılarak, 5-FU, kolon karsinoma hücrelerinde ekstraselüler Ca2+'un L-tipi plazma membrane kanalları yolu ile hücre içerisine girmesini uyarır ve kalmodulin akitivitesi ile de en az iki spesifik p53 serin artıklarının (ser15 ve ser33) postranslasyonel fosforilasyonuna neden olur. Ca2+ şelatlayıcısı BAPTA ya da iki farklı kalmodulin inhibitörleri kullanılarak 5-FU ile indüklenmiş hücre ölümü etkin bir şekilde önelnmiştir. Yaygın bir şekilde tedavi amaçlı olarak kullanılan 5-FU ajanı, bu sinyal ileti yollarının bilinmesi ile yeni tedavi yöntemleri ve kombinasyon tedavilerinin ortaya konmasında etkin bir rol oyanayabilir.

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CHAPTER 1

INTRODUCTION

1.1. Colorectal Carcinoma

Colorectal cancer (CRC) is the second most common cancer in Europe with more than 400,000 persons diagnosed each year (Jemal et al. 2008). In its early stages (stages 0-I), CRC is one of the most curable cancers but in more advanced stages (stages II-IV) the possibilities for a complete recover are drastically reduced (Markowitz et al. 2002). Generally, stage I and II are curable using surgical treatments. Stage III is characterized with the spreading to the lymph node and in stage IV the original tumor is metastasizing to distance regions of the body (Markowitz et al. 2002). The cause of colorectal cancer do not differ from other tumor types and include common factors such as age, diet, alcohol, smoking, environment, gender, the immune system and genetics (Harrison and Benziger 2011). Colon cancer cells are, also like many other tumor cells, characterized by genomic instability that may be a result of tumor-associated mutations, such as in tumor suppressor gene p53 and/or genes regulating DNA repair (Markowitz and Bertagnolli 2009). In addition, genes maintaining chromosome stability can be inactivated leading to a malfunctioned cell replication process and aberrant cell division (Peltomaki 2001). In this aspect, oncogenes such as RAS or BRAF and various mutation in the PI3K pathway has been proposed to play a role (Wong et al. 2010). Less common genetic alterations in the PI3K pathway is loss of the PI3K inhibitor, pTEN, an event that may cause an increase in Akt levels in these cell types, thus promoting resistance to chemotherapeutic agents (Vivanco and Sawyers 2002).

1.1.1. Treatment Strategies for Colorectal Carcinoma

Generally, surgery represents the only curative treatment and the aims of post-operative chemotherapy are to terminate microscopic metastases and to minimize the risk of recurrence. For stage III CRC patients, chemotherapy has been shown to improve overall survival rates and is recommended as standard therapy (Andre et al. 2004). The value for patients with stage II disease is, however, controversial (Lombardi et al. 2010). Thus, improved strategies for screening and more efficient chemotherapeutic options are central in order to increase CRC survival. Infusion of the antimetabolite 5-fluorouracil (5-FU) and leucovorin (LV) in combination with oxaliplatin (OHP) or irinotecan (CPT-11) are the current treatment regimens used for advanced CRC (Andre et al. 2004). While LV is an adjuvant with synergistic effects, the others are chemotherapeutic agents able to kill cancer cells, primarily through induction of DNA damage and initiation of apoptosis (Piedbois et al. 1992). In cells, 5-FU is metabolized into three main fluoronucleotide analogues causing an unbalanced nucleotide pool and, ultimately, irreversible DNA damage (Goyle and Maraveyas 2005; Longley et al. 2003). OHP, on the other hand, is a member of the platinum anticancer drug family including compounds that induce apoptosis by binding to DNA, forming structural adducts and triggering cellular responses, one of which is the inhibition of transcription (Machover et al. 1996; Todd and Lippard 2009). Finally, CPT-11 is a topoisomerase 1 inhibitor, which prevents DNA from unwinding (Cunningham et al. 1998). Engagement of apoptosis occurring in response to severe DNA damage usually requires activation of ATM/ATR–Chk1/Chk2–p53 signaling and, accordingly, mutations of genes contained in this complex network, which also controls various DNA repair systems and regulates cell cycle, can produce multiple drug-resistant phenotypes (Bakkenist and Kastan 2003; Maya et al. 2001; Bartek and Lukas 2003; Niida and Nakanishi 2006).

1.2. 5-Fluorouracil

The chemotherapy agent 5-FU (fluorouracil, Adrucil®) is an antimetabolite, which has been in use against cancer for about 40 years. Some of its principal uses are in colorectal and gastrointestinal cancers but also in treatment of aggressive forms of

breast cancer, head and neck cancer and ovarian cancer (Reed et al. 1992; Yoshimoto et al. 2003; Ijichi et al. 2008). 5-FU inhibits normal function of DNA and RNA by interfering with uracil metabolism and inhibiting nucleotide synthesis. 5-FU is a first line therapy; response rates are very low, especially in late stages of the disease (Johnston and Kaye 2001). Therefore, 5-FU is applied to patients in combination with oxaliplatin and irinocetan to improve treatment outcome (Cavanna et al. 2006). Although cancer cells may develop resistance to 5-FU, it is still widely used therapeutic option. For this reason, strategies to increase the activity of 5-FU by various combinatorial treatment regimens is of outermost importance.

1.2.1. 5-Fluorouracil Metabolism

In cells, 5-FU is converted into three main fluoronucleotide analogues: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP), each able to induce cellular stress by distinct mechanisms (Diasio and Harris 1989). FdUMP acts as a covalent thymidylate synthase (TS) inhibitor and its activity may result in a thymineless cell-state due to reduced deoxyuridine monophosphate (dUMP) methylation (Aherne et al. 1996). The lack of dTTP follows by an accumulation of FdUTP and dUTP pools which may overwhelm steady state repair systems that normally exclude uracil from DNA. As a consequence of a depleted thymine pool, forced incorporation of uracil and subsequent impairment of DNA replication and repair may then trigger activation of specific cell death pathways (Longley, Harkin, and Johnston 2003).

1.2.2. Chemotherapeutic Effects of 5-FU in Cancer Models

5-FU acts in several ways but principally as a thymidylate synthase (TS) inhibitor, leading to aberrant methylation of deoxyuridine monophosphate (dUMP) into thymidine monophosphate (dTMP). Thus, interrupting the action of TS blocks synthesis of the pyrimidine thymidine, required for DNA replication and repair, with sequential impairment of DNA, resulting in cell death (Longley, Harkin, and Johnston 2003). In addition, early works described that loss of carcinogenic properties in colon and breast cancer cell lines may be caused by misincorporation of the 5-FU metabolite

fluorouridine triphosphate (FUTP) into nuclear RNA (Kufe and Major 1981; Glazer and Lloyd 1982). It is generally accepted that cell death, mediated by the hierarchically-ordered ATM/ATR–Chk1/Chk2–p53 signaling pathway, can occur in response to severe DNA damage. In comparison, significantly less has been reported regarding death signaling pathways originating from RNA damage, although it has been suggested that transcriptional stress can lead to p53 activation (Derheimer et al. 2007).

1.3. The Role of Ca^{+2} in Cells

In cells calcium signaling is involved in a multitude of cellular mechanisms. For example, movement of the calcium ion Ca^{2+} into and out of the cytoplasm functions as a signal for many cellular processes, such as apoptosis, motility, transcription and excitability (Clapham 2007). The positive charge of Ca^{+2} can also affects the charge of proteins which might lead to an altered tertiary structure, and thereby obstruct protein function (Westheimer 1987). The main Ca^{+2} storage organelle within the cell is the endoplamic reticulum (ER), which also is capable to release its content in response to distinct signaling events. This event occurs by the action of G-coupled receptors which mediate 1,4,5-inositol trisphosphate (IP3) formation and binding of IP3 to IP3 receptors allowing for release of ER Ca^{+2} to the cytoplasm (Mikoshiba 2007). In more detail, when a ligand binds to a G protein-coupled receptor that is conjugated to a Gq heterotrimeric G protein, the α-subunit of Gq can further bind to and induce PLC isozyme (PLC-β) activity, which results in the cleavage of PIP_2 into IP_3 and DAG (Clapham 2007). In addition to the ER, the mitochondria can contain elevated levels of Ca^{+2}, especially under certain pathological conditions. Ca^{+2},which play a role mitochondria viability and function can pass the mitochondrial outer membrane freely by passive diffusion but is unable to pass through the inner membrane without active transporters, which in turn requires a proton gradient for their function. Upon enhancement in mitochondrial Ca^{+2} levels, dehydrogenases from Krebs cycle (isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase), which are sensitive to Ca^{+2}, stimulate ATP production (McCormack et al. 1990).

1.3.1. The Calcium Calmodulin Pathway

Calcium is a ubiquitous second messenger which appears to be involved in many cellular processes, including cell death. Calcium fluxes are determined by the activity of membrane channels that normally are under tight control. Thus, elevation in cytoplasmic Ca^{2+}-concentration can result, either from extracellular influx or from organelle release. Voltage-Operated-Calcium- Channels (VOCC), Receptor-Operated-Calcium-Channels (ROCC) or Store-Operated-Calcium-Channels (SOCC) are channels mediating Ca^{2+}-influx from the extracellular space (Lewis 2007). Many of the second messenger effects of Ca^{2+} are mediated through the ubiquitous Ca^{2+} sensing protein, Calmodulin (CaM). Of the many downstream targets of CaM, a family of enzymes known as the calmodulin-dependent kinases (CaM-kinases) is one of the best characterized (Hoeflich and Ikura 2002). Among them, the Death-associated protein kinase (DAPK) is a pro-apoptotic serine/threonine protein kinase that is deregulated in a wide variety of cancers (Michie et al. 2010).

1.3.1.1. ATF3

The activating transcription factor (ATF) family represents a large group of basic-region leucine zipper (bZIP) transcription factors. ATF/cyclic AMP response element-binding (CREB) family members include ATF1 (also known as TREB36), CREB, CREM, ATF2 (also known as CRE-BP1), ATF3, ATF4, ATF5 (also known as ATFX), ATF6, ATF7, and B-ATF. The common feature that these proteins share is the bZIP element. The basic region in this domain is responsible for specific DNA binding, while the leucine zipper region is responsible for forming homodimers or heterodimers with other bZIP-containing proteins such as the AP-1, C/EBP, or Maf families of proteins. ATF/CREB proteins were initially identified for their binding to the cyclic AMP response element (CRE) in various promoters, which has the consensus sequence TGACGTCA (Persengiev and Green 2003).

ATF family members normally found low levels in quiescent state cells. ATF3 can be activated via genotoxic agents, cytokines or physiological stresses. In contrast to other ATF family members, ATF3 is involved in ATF3 host defense against invading pathogens and in cancer. ATF3 is capable to regulate transcription of pro-inflammatory

cytokines negatively. On the other hand, it has been proposed that ATF3 has dual role in cancer cells; of which one is oncogenic since has been found to be overexpressed in such cancer types that leads to metastasis and also promote cell proliferation. On the contrary, ATF3 can also promote tumor suppression, inhibit metastasis and induce apoptosis *in vivo* and *in vitro* (Thompson et al. 2009). This paradox, however, is still unclear.

Another ATF family member (ATF4) is the main transcriptional regulator of the unfolded protein response and helps to restore ER function. ATF4 is associated with resistance to chemotherapeutic agents and result in a decrease in antitumor activity of chemotherapeutics.

1.4. Apoptosis

Evasion of apoptosis is interpreted as a hallmark of cancers. This cell death modality is driven by caspases and distinct upstream regulatory factors that have been defined as an oncogenes and tumor suppressors, which direct their proteolytic activity, (Olsson and Zhivotovsky 2011). Apoptotic pathways can be divided into two main routes; the intrinsic mitochondrial and the extrinsic receptor-mediated pathways. In the extrinsic pathway, extracellular ligands stimulate oligomerization of members from the tumor necrosis factor (TNF) receptor family (Fas/Apo1, TNF, DR4, DR5) leading to death inducing signaling complex (DISC) assembly, whereas in the intrinsic pathway, proteins from the Bcl-2 family control the release of factors involved in apoptosome formation through preservation or disruption of mitochondrial integrity. Both pathways lead to activation of effector caspases (caspases-3, -6, and 7) and an initiator caspase (caspases-2, -8, -9 and -10) is required for this process. Being active, effector caspases target a broad spectrum of cellular proteins, ultimately leading to cell death. The DISC, the apoptosome and the PIDDosome are protein assembly activation platforms for caspase-8/-10, caspase-9 and caspase-2, respectively.

1.4.1. p53 Signaling and Apoptosis

p53 is a tumor suppressor phosphoprotein. In the 1990s, it was revealed that p53 is a transcriptional factor with a DNA-binding domain. Subsequently, it was shown that

p53 levels were increased in response to DNA-damaging agents (Kastan et al. 1991). Lees-Miller et al. found that p53 is phosphorylated by a DNA-activated protein kinase at Ser15 and Ser37 residues (Lees-Miller et al. 1992). The phosphorylation of the Ser15 residue is crucial for p53 activity since it facilitates dissociation from MDM2, thus preventing p53 from degradation. MDM2 is thereby one of the critical molecules with respect to p53 activation. In normally dividing cells, MDM2 sequesters p53 and ubiquitylation leads to proteasomal p53 degradation. MDM2 itself is a target for p53 transactivation during stress conditions. Forced MDM2 silencing by siRNA automatically results in increased p53 levels and transactivation of target genes. Moreover, p53 is modified by approximately fifty post-translational events, including phosphorylations and acetylations which individually or cooperatively regulate transcriptional activity and protein-protein interactions. p53 co-activator proteins p300 and CBP have two different roles with respect to p53 regulation, to promote p53 degradation via ubiquitylation by MDM2 and to activate transcriptional properties, respectively. They can also inhibit p53 degradation by means of acetylation of certain residues in the C-terminus which normally is ubiquitylated.

It is obvious that Ser15 phosphorylation is a crucial point for the initiation of other p53 modifications, thus determining triggering of discrete signaling pathways in response to specific stress conditions. In contrast, it is not clear how acetylation of different residues effect the precise protein-protein interactions in signaling. On the other hand, amino terminal phosphorylation promotes transcription. The fully understand mechanism of p53 post-translational modifications will be beneficial to understand DNA damage response and tumor suppressor.

1.4.2. DISC Complex

Radiation therapy and many chemotherapeutic agents used in cancer treatment have been reported to trigger apoptosis in cells through the intrinsic pathway as a consequence of double-stranded breaks of nuclear DNA. Engagement of this pathway usually requires p53 function and mutations within the gene or its signaling pathway can produce multiple drug-resistant phenotypes *in vitro* and *in vivo* (O'Connor et al. 1997). Also members of the TNF superfamily including Fas ligand, TNF and TNF-related apoptosis-inducing ligand (TRAIL) have been identified as targets for cancer

biotherapy. In this respect, TRAIL is certainly of high interest since it preferentially induces apoptosis in cancer cells while exhibiting little or no toxicity in normal cells (Ashkenazi et al. 2008) To date, research has focused on the mechanism of apoptosis induced by TRAIL and the processes involved in the development of TRAIL resistance, and it has been shown that resistant tumors can be re-sensitized to TRAIL by chemotherapeutics or irradiation. Importantly, TRAIL triggers apoptosis in cancers, irrespective of the p53 status and appears to have a therapeutic index in preclinical studies (Abdulghani and El-Deiry 2010). To signal DISC mediated cell death, TRAIL trimerizes and binds to its receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2, Killer), thereby recruiting Fas-Associated protein with Death Domain (FADD). In turn FADD recruits caspase-8 or -10 through its death effector domain (DED). Since dimerization of apical caspases is sufficient for their activation, DISC aggregation of caspases -8 or -10 *per se* initiates their autocatalytic processing which in turn serve to stabilize the proteolytic activity (Boatright et al. 2003). Levels of FADD-like interleukin converting enzyme-like inhibitory protein (FLIP), receptor glycosylation and caspase-8 ubiquitination are examples of regulatory events in TRAIL induced apoptosis (Jin et al. 2009; Shirley and Micheau 2010; Wagner et al. 2007) . In type I cells, sufficient amount of active caspase-8 is generated at the DISC to directly process effector caspase-3, ultimately leading to apoptosis. In type II cells, however, the amount of caspase-8 processed in the DISC is not sufficient to directly activate downstream effector caspases and the signal is therefore amplified through the intrinsic mitochondrial pathway by means of Bid cleavage and Bax/Bak activity (Barnhart et al. 2003). Despite knowledge that treatment of tumor cells with some chemotherapeutic drugs, including 5-FU, can induce DR5 mediated cell death (Wang and El-Deiry 2004; Longley et al. 2006), the mechanisms underlying the significance of this particular pathway is not well established.

1.5. The Aims of the Study

The aims of the present study was initially to extend the knowledge concerning 5-FU induced apoptotic signaling by analyzing potential regulatory factors upstream the caspase cascade. Once the calcium-calmodulin dependent apoptotic signaling required

for specific phosphor-activation of p53 and subsequent DISC formation was identified, our preliminary aim has been to characterize this event in further detail.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

Treatment of cells with the antimetabolite 5-FU (Teva Pharmaceutical Industries, Petach Tikva, Israel), Thapsigargin, BAPTA (Invitrogen), Verapamil, Pepstatin A (Sigma-Aldrich, St. Louis, USA), Calmidazolium chloride, Fluphenazine-N-2-chloroethane (Santa Cruz Biotechnology, Santa Cruz, USA), PKC412 (Novartis International, Basel, Switzerland), ATM inhibitor KU-55933 (Selleck Chemicals LLC, Houston, USA), CA-074 Me, Trolox, Necrostatin-1 (Nec-1) and its inactive control Nec-1i (Merck, Darmstadt, Germany), were performed as indicated in figures.

2.2. Cell Lines and Culture Conditions

The HCT116 parental cell line and its variants, deficient in p53 or Chk2 were cultured in Dulbecco's modified Eagle's medium (DMEM) and the A549 cell line in RPMI1640 medium, both supplemented with 10% heat-inactivated fetal bovine serum and PenStrep (100 U/ml penicillin, 100 mg/ml streptomycin). Cell culture reagents were purchased from GIBCO (Invitrogen, San Diego, USA). Cells were grown in a humidified 5% CO_2 atmosphere at 37°C and maintained in a logarithmic growth phase during all experiments.

2.3. Gel Electrophoresis and Immunoblotting

Cells were treated as indicated, washed in Phosphate Buffer Saline (PBS) and lysed for 5min in Complete Lysis-M (Roche Diagnostics, Mannheim, Germany) containing 1x Complete Protease Inhibitor Cocktail (Roche Diagnostics) and 1x Phosphatase inhibitor Cocktail 2 (Sigma-Aldrich). The BCA Protein assay (Thermo Fisher Scientific, Lafayette, USA) was used to determine protein concentration and

subsequent to denaturation, equal quantities from each sample were subjected to SDS–PAGE at 40mA followed by electro blotting to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, USA) for 2h at 100V. Membranes were then blocked for 1h with 5% non-fat milk in PBS and probed with the primary antibody of interest at 4°C overnight. Finally, membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies for 1h at room temperature, revealed by ECL (GE Healthcare Biosciences, Uppsala, Sweden) and exposed to SuperRX X-ray films (Fujifilm Corporation, Tokyo, Japan). Three successive washes in PBS were performed after incubating membranes with primary and secondary antibodies.

2.4. Immunofluorescence

HCT116 and HCT116 p53-/- cells were seeded on coverslips, treated as indicated in figures and then fixed for 30min using 4% formaldehyde in PBS at 4°C. Permeabilization and blocking was done in PBS containing 0.3% Triton X-100 and 1% BSA for 1h. Incubations with primary antibodies (1:400), previously blocked (1:10) in PBS containing 5% BSA for 2h, and secondary antibodies (1:200) were performed at 4°C overnight in a humid chamber and at room temperature for 60min, respectively. Nuclei were counterstained for 10min with Hoechst 33342 (10µg/ml in PBS). Between all steps, cells were washed for 3×10min in PBS. Finally, the coverslips were mounted using Vectashield H-1000 (Vector Laboratories Inc., Peterborough, UK) and examined under a Zeiss LSM 510 META confocal laser scanner microscope (Carl Zeiss MicroImaging, Göttingen, Germany).

2.5. Antibodies

The following primary antibodies were used in western blotting: p53 mAb, clone DO1; GADPH pAb, Tom40 pAb, CD95 pAb (Santa Cruz), Phospho-p53 pAb's (Ser6, 9, 15, 33, 37, 46 and 382), Cleaved-Caspase-3 pAb, ATM mAb, clone D2E2; Phospho-H2A.X (Ser139) pAb (Cell Signaling, Danvers, USA), α-Tubulin mAb, clone B-5-1-2; DR5 pAb (Sigma-Aldrich), PARP mAb, clone 4C10-5; Bax mAb, clone 6A7; Caspase-7 mAb, clone B94-1 (BD Biosciences, Franklin Lakes, USA), FADD pAb (Upstate Biotechnologies, Lake Placid, USA) and Caspase-8 mAb, clone C15 (kindly provided

by Prof. PH Krammer, German Cancer Research Center, Heidelberg, Germany). All primary antibodies were diluted in PBS containing 1% BSA and 0.015% NaN3. Horseradish peroxidase-conjugated secondary antibodies (Thermo Fisher) were diluted in PBS containing 5% non-fat milk. Analysis of DR5 in immunofluorescence (IF) was performed using the mAb clone 11/B4 (kindly provided by Prof. L. Anděra, Academy of Sciences of the Czech Republic, Prague, Czech Republic). For IF-detection of p53, Phospho-p53 (Ser15 and 33), FADD, Phospho-H2A.X and CD95, the antibodies described above were used. Fluorescent secondary antibodies directed to mouse rabbit (Alexa488 and Alexa594) were purchased from Molecular Probes (Invitrogen).

2.6. RNAi Methodology

Silencing of protein expression in HCT116 cells was accomplished by transfection of 21-nucleotide RNA-duplexes purchased from Dharmacon (Thermo Fisher). Transfection of CD95 (L-003776-00), DR5 (L-004448-00) and control (D-001810-10) ON-TARGET-plus SMARTpool siRNAs was performed using the INTERFERin transfection reagent (Polyplus transfection, Illkirch, France) according to the instructions of the manufacturer. Briefly, 4×105 cells were transfected in normal cell medium using 10μM siRNA and 3,65μl/ml INTERFERin. Levels of target proteins were controlled by SDS–PAGE and their downregulation was normally detected as early as 24□h post-transfection. 5-FU-treatments of cells were initiated after 36h.

2.7. Calcium Measurements

Intracellular calcium levels were monitored by using the Fluo-4 AM fluorescent indicator (Invitrogen). In brief, 4μM of the calcium probe was added to cells 30min in advance of 5-FU treatment. Time-laps analysis of living cells was then performed using the Zeiss LSM 510 META confocal laser scanner microscope or the FACSCalibur system in combination with the CellQuest v.3.3 software (Becton-Dickinson, San Jose, USA).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Death Receptors

3.1.1. DR5 and Fas are Activated Death Receptors by 5FU

Immunostaining of both TNF-receptors DR5 and CD95 accumulates in the plasma membrane in response to 5-FU (Figure 3.1), indicating that either one of them or both could play a vital role for efficient apoptosis.

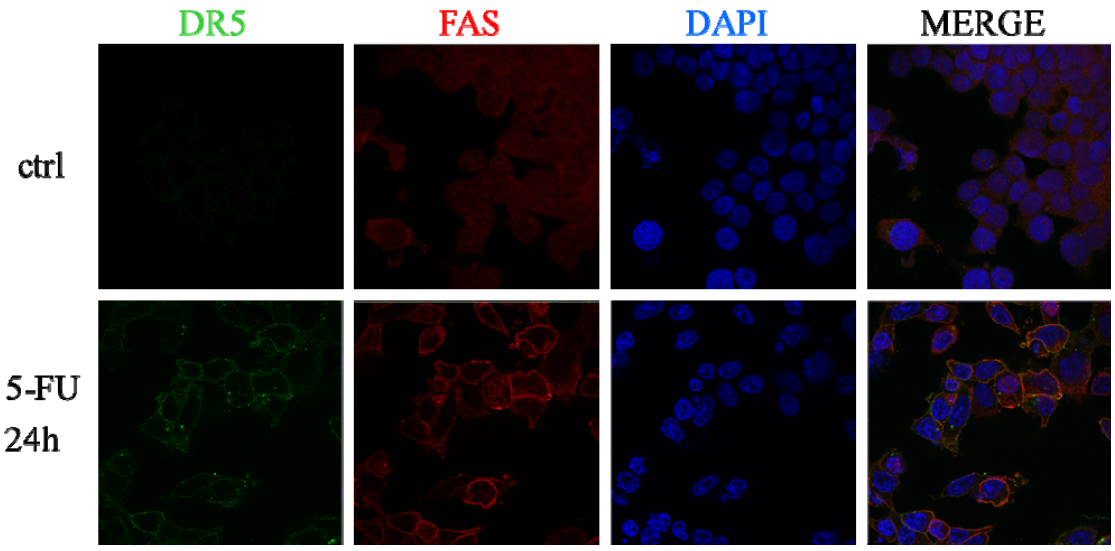

Figure 3.1. Localization of death receptors on plasma membrane after 5-fu treatment

3.1.2. DR5 but Not Fas Receptor is Implicated in 5FU Induced Apoptosis

Since conflicting evidences exists in this matter (Longley et al. 2006; Borralho et al. 2007; Longley et al. 2004), we decided to assess the individual contribution of each receptor to initiator caspase-8 and effector caspase-3 activation by means of siRNA technology. Inconsistent to previous reports (Borralho et al. 2007), siRNA experiments

clearly stated that DR5 but not CD95 is the sole receptor required for caspase-8 activity and further processing of effector caspases (Figure 3.2).

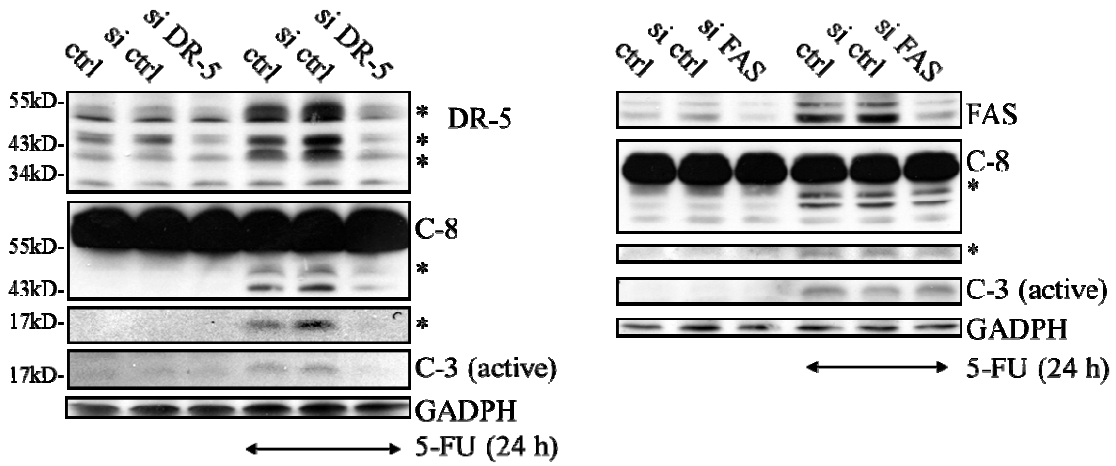

Figure 3.2. Role of death receptor on extrinsic pathway triggered caspase cleavages (Processed caspase fragments are indicated with asterisks)

3.1.3. 5-FU Induction Leads to DISC Formation

By isolating membrane proteins from controls and induced cells, an accumulation of DR5 but also of DISC components FADD and caspase-8 was detected in membrane fractions in response to 5-FU-treatment (Figure 3.3).

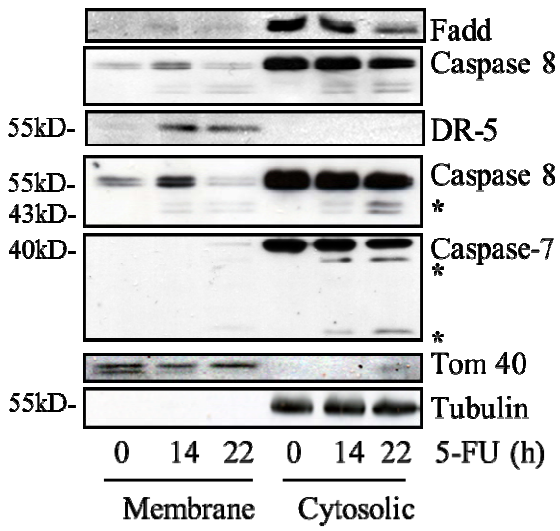

Figure 3.3. 5-FU induced DISC formation (Processed caspase fragments are indicated with asterisks)

DISC non-associated caspase-7, on the other hand, remained in the cytosolic fractions irrespectively of treatment. Since we used a protocol in which the total membrane protein pool where isolated, TOM 40 (Translocase of the Outer Mitochondrial membrane) served as a marker for fractionation efficiency. To rule out the existence of DISC components in cellular membranes other than the plasma membrane, result where confirmed using immunostaining with specific antibodies targeting DR5 and FADD (data not shown).

3.2. Analysis of Potential Regulatory Factors Upstream of DR5-DISC Formation

In sharp contrast to classical extrinsic death pathways, 5-FU-induced apoptosis most certainly emerge from either DNA or RNA damage. Thereby, the question relating to how initial triggering points are transduced to DISC formation and caspase-8 activity arises. p53 is obviously an important factor for the process but a detailed description of signaling events originating from 5-FU-induced cell-stress leading to p53 activity and subsequent DR5 oligomerization is still lacking (O'Connor et al. 1997; Olsson et al. 2009). Therefore, a panel of inhibitors including Ca2+-chelator BAPTA, RIP1-kinase inhibitor NEC1, the antioxidant Trolox, pepstatin A, an inhibitor of acid proteases and cathepsin B inhibitor CA-074 was added in combination with 5-FU to HCT116 cells in order to target potential upstream controlling conduits (Figure 3.4).

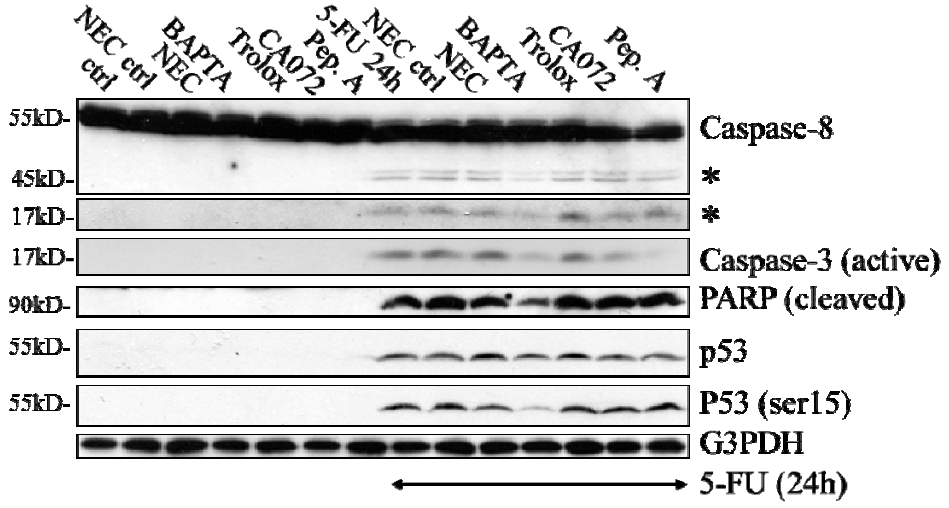

Figure 3.4. Inhibitor screening of 5-FU induced cell death on colon carcinoma (Processed caspase fragments are indicated with asterisks)

Of the inhibitors used, three effectively abrogated effector caspase-3 processing. Two of these, Pepstatin A and CA-074 are silencing lysosomal protease activity (Figure 3.4). However, neither of them had any effect on the most apical caspase-8 activity. Hence, we concluded that lysosomal proteases indeed play a role in 5-FU-induced apoptosis but appear to function as an enhancer of effector caspase activity, downstream of DISC formation. This is well in agreement with a recent report showing that lysosomal membrane permeability and the cytosolic release of cathepsins B, L and D indirectly depends on Bax/Bak and components of the apoptosome (Oberle et al. 2010). In comparison, BAPTA had a profound effect also on caspase-8 processing indicating Ca^{2+} as a messenger acting upstream of the caspase cascade. Moreover, while 5-FU induced p53 levels remained unaffected in presence of BAPTA phosphorylation of ser15 was reduced considerably, thus positioning the effect of Ca^{2+} in advance of p53 posttranslational modifications. With prolonged 5-FU treatment it has not escaped our notice that also HCT116 $p53^{-/-}$ cells undergo a DR5 and caspase-8-dependent cell death. In fact, DR5 is also upregulated in these cells but to a lesser extent compared to the parental cell line (data not shown). Since neither BAPTA nor any of the other inhibitors tested obstructed the weak caspase-3 activity detected in HCT116 $p53^{-/-}$ cells after 48 h of 5-FU treatment we concluded that Ca^{2+} primarily exerts its effect on p53 activity as a response to stress induced by 5-FU (Figure 3.5) .

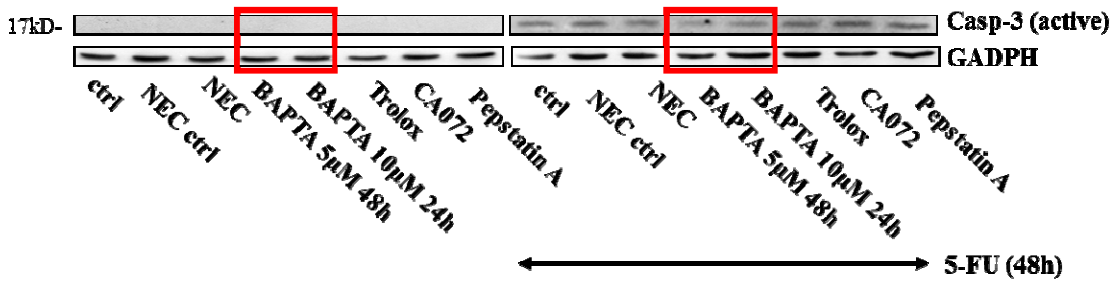

Figure 3.5. Inhibitor screening of 5-FU induced cell death in p53 deficient colon carcinoma cells (HCT116)

3.2.1. Chelation of Ca^{+2} Does not Interfere with 5-FU Induced Transactivation or Oligomerization of DR5

BAPTA interferes with 5-FU induced p53 activation and processing of caspases-3 and -8 in a concentration dependent manner. HCT116 wt cells were left untreated or induced with 5-FU, either alone or in combination with 15 μM of BAPTA or 5 μM Calmidazoline. Interestingly, transactivation and dimerization of the DR5, analyzed by standard and non-denaturing SDS-PAGE, respectively, occurring in response to 5-FU was neither affected by BAPTA nor calmidazolium chloride, indicating that p53 support caspase-8 processing by mechanisms separated from these events (Figure 3.6)

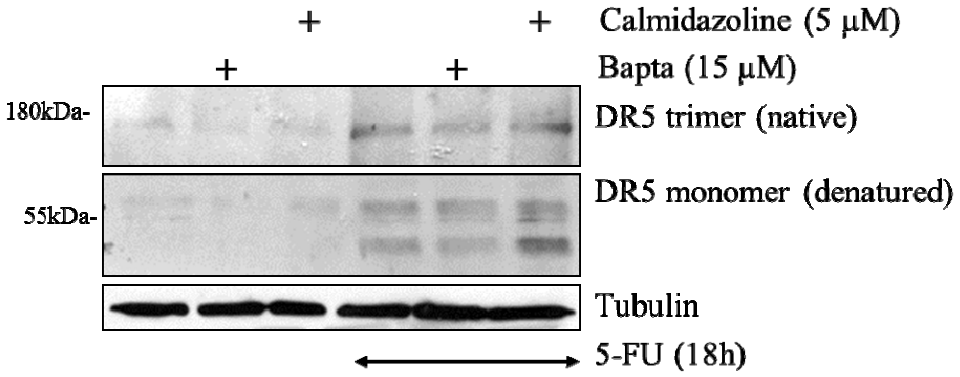

Figure 3.6. Neither 5-FU induced DR5 transactivation nor oligamerization events are affected by inhibition of the Ca+2 pathway

3.2.2. Influx of Extracellular Ca^{+2} is Directing 5-FU Induced p53 Activity

To determine the source of Ca^{2+} required for apoptotic signaling in 5-FU treated HCT116 cells, the following experiments where performed. To begin with, cells where cultured and treated in Ca^{2+}-free media and then analyzed with respect to p53 phosphorylation and apoptotic markers including caspase processing and PARP cleavage (Figure 3.7).

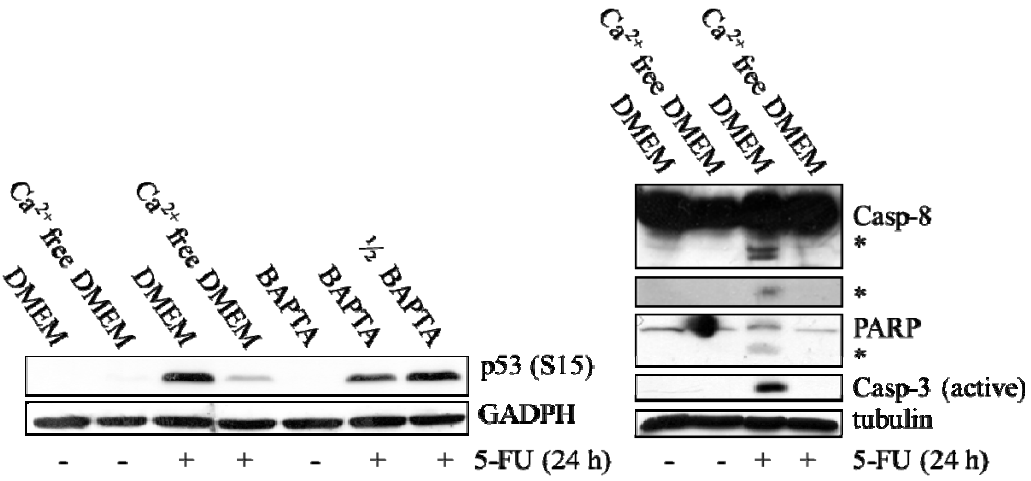

Figure 3.7. Source of Ca^{2+} which accumulates in cytoplasm after 5-fu treatment (Processed caspase fragments are indicated with asterisks)

Since the lack of environmental Ca^{2+} had a clear effect on all parameters tested, reducing phospho-p53 activation even more efficiently than BAPTA and decreasing caspases -3 and -8 processing as well as PARP cleavage to background levels, we concluded that extracellular Ca^{2+} is the original source required for apoptotic proceedings in 5-FU treated HCT116 cells.

3.2.3. Timing of Ca^{+2} Elevation and p53 Serine Phosphorylation

Changes in intracellular Ca^{2+} levels as a response to 5-FU treatment in HCT116 cells were monitored by using the Fluo-4 AM fluorescent indicator. By FACS we detected an increase in intracellular Ca^{2+} at 4 h and a further enhancement at 5 h post-treatment (Figure 3.9). After 5 h, increased levels of Ca2+ remained up to 13 h which is the time point where initiation of caspase processing can be detected by SDS-PAGE (Olsson et al. 2009). Examination of cellular Ca^{2+} by time-laps confocal microscopy using a CO2 chamber was then performed and influx commencement noticed as early as 1.5 h after addition of 5-FU (Figure 3.8). This is well in advance of p53 ser15 phosphorylation which could be detected 5 h post induction by means of western blotting (Figure 3.10). Thus, these data support our findings indicating Ca2+ as a regulatory factor acting upstream of p53 activity in response to 5-FU.

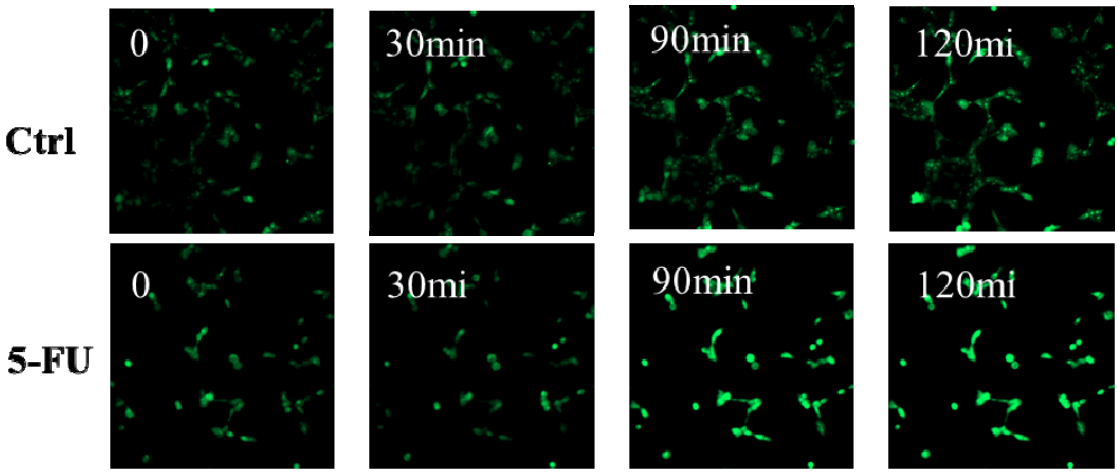

Figure 3.8. Time lapse (short-term) intra-cellular Ca^{+2} measurement

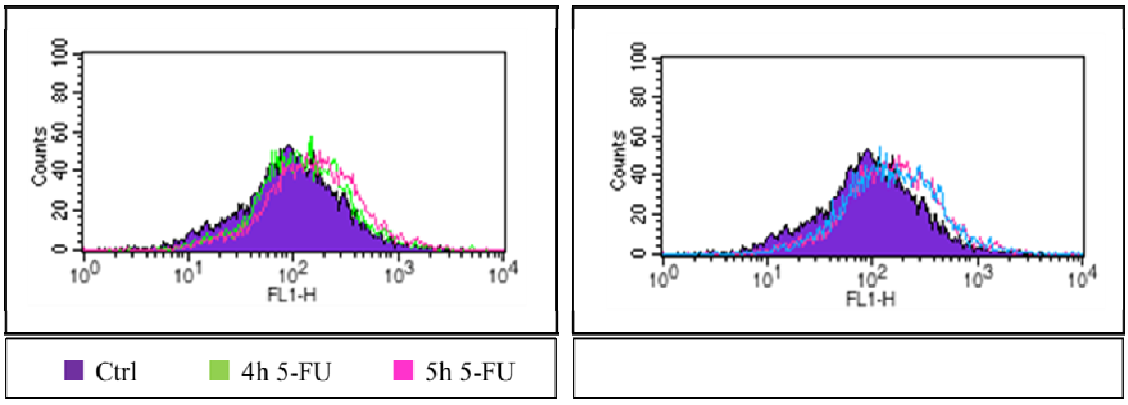

Figure 3.9. Time lapse (long-term) intra-cellular Ca^{+2} measurement

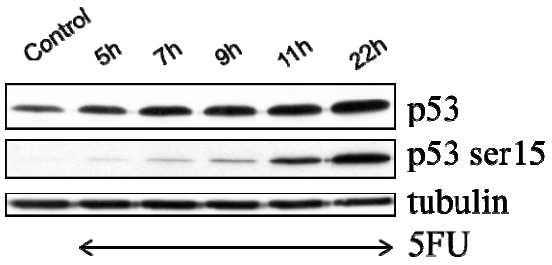

Figure 3.10. Time lapse analysis of p53 ser15 phosphorylation

There are, however, some parameters that have to be considered in this respect. Firstly, western blotting can be a sensitive or insensitive technique depending on the antibody used. Thus, activation of p53 by means of ser15 phosphorylation may occur earlier than what our results predict. Secondly, most likely a critical threshold concentration of Ca2+ must be reached to trigger subsequent p53 activity. By our measurements it is impossible to specify this threshold limit but a qualified guess would be that it's reached between 1.5 and 4 h post induction. Still, irrespectively of these uncertainties elevation of Ca2+ and p53 activity as determined by ser15 phosphorylation remains coordinated sequence of events.

3.3. Identification of Downstream Regulatory Pathway of Ca+2

3.3.1. Apoptosis Regulated by Ca^{+2} Calmodulin Complex After 5-FU Treatment

To maintain normal cellular control and tissue integrity, p53 is regulated at the post-translational level by protein-protein interactions and covalent modifications, including phosphorylation at over twenty phosphor-acceptor sites (MacLaine and Hupp 2011). The reports examining the role of kinases able to modulate p53 activity has led to much controversy within the field but the general view seems to be that one or several kinases may act on the same residue in a cell or stimuli specific manner. Indeed, several acceptor sites of p53 are phosphorylated in response to 5-FU and most likely, majority of them contribute in one way or the other to treatment outcome. However, our focus was to decipher the Ca^{2+}-dependent pathway described, and to analyze its importance for DR5 mediated cell death. Of all different pospho-p53 activity pathways described, few are controlled by Ca^{2+} signaling, in fact only two. One them involves serine/threonine kinase members included in a subgroup of the protein kinase C (PKC) family termed the classical group encompassing PKCs -α, -βI, -βII and –γ (Coutinho et al. 2009; Lavin and Gueven 2006; Pospisilova et al. 2004). The other one is facilitated by the ubiquitous Ca^{2+} sensing protein calmodulin (CaM) and occurs through activation of at least two downstream targets, Death-Associated Protein kinase 1 (DAPk1) and AMP-activated protein kinase (AMPK), enzymes contained the superfamily of CaM-dependent kinases (Raveh et al. 2001; Craig et al. 2007; Jones et al. 2005). Since a

specific inhibitor of PKC (PKC412) not attenuated p53 ser15 phosphorylation in any of the concentrations tested, we concluded that this kinase did not contribute to the 5-FU-induced and Ca^{2+}-dependent events leading to p53 activity described. Interestingly, addition of PKC412 to our experimental system inhibited processing of caspases -3 and -8, but obviously in a manner independent of the p53 ser15 residue (data not shown). In sharp contrast, we observed abrogation of p53 ser15 and ser33 phosphorylations in parallel with decreased processing of caspases occurring in a concentration dependent manner when two different CaM inhibitors, Calmidazolium chloride or Fluphenazine-N-2-chloroethane, was added to HCT116 cells in combination with 5-FU (Figure 3.11). A decrease in p53 ser46 was also noted but only in cells pretreated with Calmidazolium chloride and not Fluphenazine-N-2-chloroethane. Ser37 phosphorylation was indeed blocked using both inhibitors but in a pattern dissimilar to reduction of caspase processing and phospho-activation of ser15 and 33.

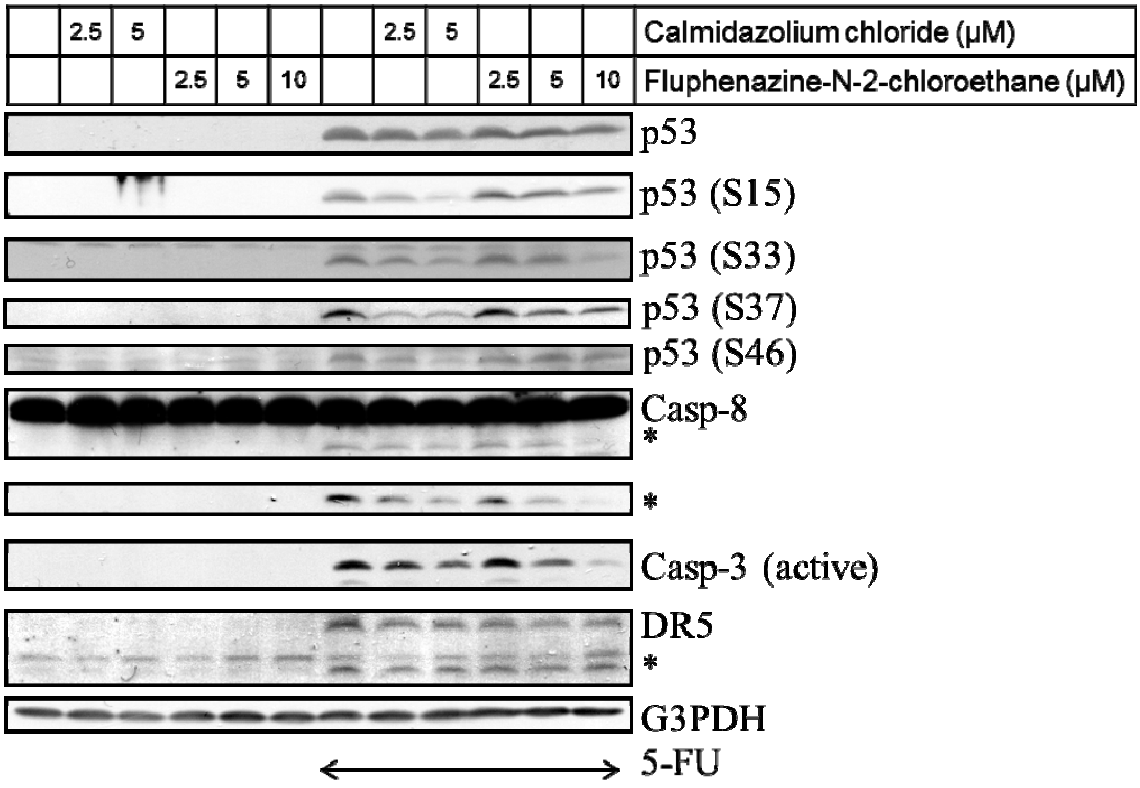

Figure 3.11. Effects of Calmodulin complex inhibition on p53 signaling

3.3.1.1. Calmodulin Dependent Protein Atf3 Might Have Role in p53 Phosphorylation and Apoptosis

Activating transcription factor 3 (ATF3), a 181-amino-acid protein, is a member of the ATF/CREB family of transcription factors that, like p53, is maintained at a low level in quiescent cells. While consequences of ATF3 induction are unclear, it is often assumed that ATF3 functions as a transcription factor to regulate gene expression thereby contributing to cellular responses to oncogenic stresses. ATF3 binds to p53 via this domain, and as a consequence, p53 ubiquitination catalyzed by MDM2, the major ubiquitin ligase in HPV-negative cells, is blocked, leading to up-regulation of the p53 tumor suppressor activity independent of the ATF3 transcriptional activity. It has also been reported that the stress response gene ATF3 acts as a transcriptional activator of DR5 expression by camptothecin in human colorectal cancer cells, and is an essential co-transcription factor for p53 to activate the DR5 gene promoter (Taketani et al. 2011). Therefore, we hypothesized that ATF3 might provide a functional link between calmodulin and p53 by mechanisms separated from its transcriptional activities. Indeed, ATF3 is drastically transactivated in response to 5-FU treatment in HCT116 cells (Figure 3.12).

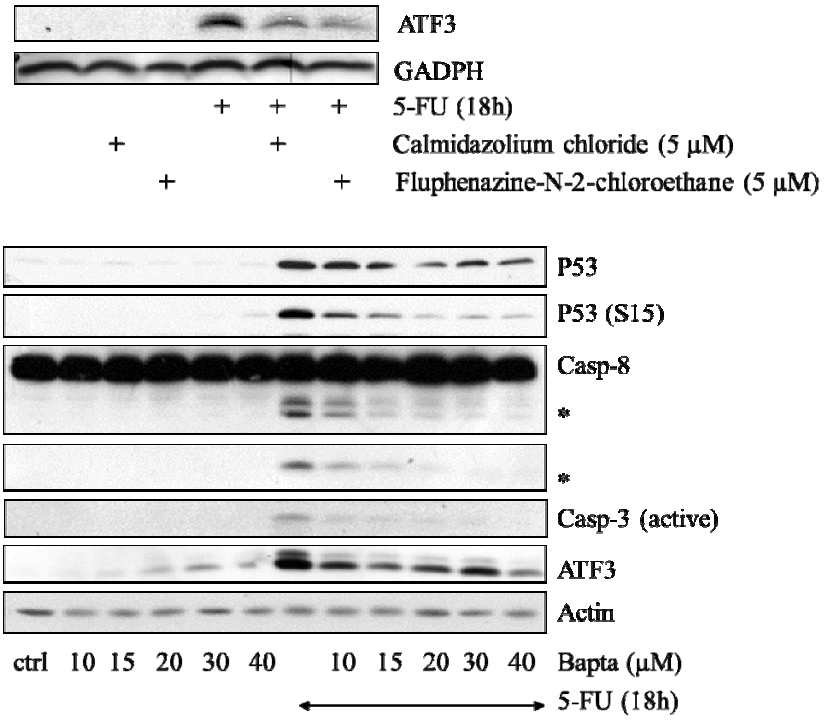

Figure 3.12. ATF3 might have role on Ca^{+2} triggered p53 ser15 phosphorylation

Moreover, BAPTA as well as two inhibitors to calmodulin (Calmidazolium chloride and Fluphenazine-N-2-chloroethane) suppressed ATF3, indicating its involvement in the calcium-p53 pathway described. In an ongoing study, suppression of ATF3 by specific siRNAs aims to determine the role of this protein for activation of p53 and DR5 regulation.

CHAPTER 4

CONCLUSION

In the present report we uncover a new Ca^{2+} dependent cell death mechanism which occur in response to 5-FU and is mediated through CaM and p53 activities.5-FU has been the mainstay of colorectal cancer treatment for over 40 years. However, response rates for 5-FU in advanced colorectal cancer are modest and although combinatorial treatment with the newer chemotherapeutic agents' such as oxaliplatin and irinotecan has improved survival rates, there is a need for new therapeutic strategies. By investigating the 5-FU induced Ca^{2+}-CaM–p53 axis and its downstream apoptotic triggering points, new molecular mechanism by which tumors become resistant to 5-FU can eventually be revealed. In addition, although calcium previously has been implicated in various cell death pathways the novelty of our preliminary data indicating that Ca^{2+}-CaM signaling is required for apoptosis triggered by 5-FU in certain cancer cell lines types must be emphasized. The fact that a widely used therapeutic drug is signaling by these means could provide new therapeutic intervention points, or specify new combinatorial treatment regimes. The association between alterations in intracellular Ca^{2+} homeostasis and various stages of the apoptotic signaling cascade is indisputable (Pinton et al. 2008). Recent findings have also indicated that dietary calcium can modulate and inhibit colon carcinogenesis. Supporting evidence has been obtained from a wide variety of preclinical experimental studies, epidemiological findings and a few human clinical trials (Lamprecht and Lipkin 2003). Together, these data supported a debate over calcium's potential to fight colon cancer. Maybe more interesting for the present study is the fact that adjuvant chemotherapy has been shown to alter the natural history of resected colon cancer. Two regimens (5-FU plus calcium folinate and 5-FU plus levamisole) have been found to prolong disease-free survival and overall survival in affected patients. Previous comparisons of these two regimens indicate that 5-FU plus calcium folinate may offer a small disease-free survival and overall survival advantage (Kumar and Goldberg 2001).
Experiments using verapamil was indicating high-voltage-gated calcium channels (HVGCCs) of the L (Long Lasting)-type as the entry point for extracellular Ca2+ influx

in response to 5-FU (data not shown). This is well in agreement with the fact that elevated Ca2+ levels occurred as an immediate reaction to treatment and then remained until initiation of cell death. The α1 subunits which contains the voltage-sensing machinery and the drug/toxin-binding sites forms the Ca2+ selective pore and are the primary factor operating in HVGCCs. Out of ten α1 subunits described in humans, four are specific for L-type channels and current work aims to identify whether one or several α1 subunits are required for the process described. We are also interested in defining the link between 5-FU and L-type HVGCCs. Here, two possibilities exist. Either 5-FU specific DNA or RNA damage induces a still unidentified signaling cascade activating one or several L-type pores, alternatively, 5-FU or its metabolites acts directly on these pores. In line with our study and supporting that at least the CaM-directed p53 ser15 phosphorylation is important for 5-FU-induced apoptosis are findings coming from expression of p53 mutants at physiological levels in p53 knockout HCT116 cells. Compared with cells expressing exogenous wild type p53, the apoptotic response to 5-FU was >50% reduced in cells expressing S15A or S20A mutant p53, and even more reduced by combined mutation of serines 6, 9,15, 20, 33, and 37 (N6A) (Kaeser et al. 2004).

Since TNF-related apoptosis-inducing ligand (TRAIL) can induce apoptotic cell death in a variety of tumor cells by engaging specific death receptors, DR4 and DR5, while having low toxicity towards normal cells, it has been postulated as a future therapeutic option(Abdulghani and El-Deiry 2010). Interestingly, our present data are indicating that 5-FU induced cell death also involves DR5. Disclosing 5-FU induced death pathways might therefore conform to the highly interesting research field of TRAIL in tumor treatment and the processes involved in the development of TRAIL resistance.

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