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SCHOOL OF BIOLOGICAL SCIENCES

MSc. DEGREE IN MOLECULAR MEDICINE

Analysis of a novel cytotoxic drug induced TRAIL-R2 signalling complex using a new FACS-based Smac/DIABLO release assay

By

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1.List of Abbreviations

Abbreviation	Full forms
5-FU	5-Flurouracil
AP-1	Activator Protein-1
Apaf-1	Adaptor Protein Apoptotic Protease Activating Factor-1
Ask-1	Apoptosis Signal Regulating Kinase-1
Bak	Bcl-2 homologous antagonist
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma-2
Bcl-xl	B cell lymphoma-extra large
BH3	Bcl-2 Homology Domain
BID	BH3 interacting domain death agonist
BIR	Baculovirus IAP repeat
CD95	Cluster of Differentiation 95
C-IAP	Baculovirus IAP repeat containing protein-2
DIOC(6)3	3-3' dihexyloxacarbcyanine iodide
DISC	Death Inducing Signalling Complex
DR5	Death Receptor 5
ETC	Electron transport chain
FACS	Fluorescence Activated Cell Sorting
FADD	Fas-Associated Death Domain

FITC	Fluorescein isothiocynate
IAP	Inhibitor of Apoptosis Protein
JNK	c-Jun N-terminal kinases
MAPK	Mitogen Activated Protein Kinase
MKK7	Mitogen Activated Protein Kinase Kinase
NAIP	Baculovirus IAP repeat containing protein-1
NF- kB	Nuclear factor kappa B
PBS	Phosphate Buffer Saline
RIP-1	Receptor Interacting Protein-1
Smac	Second Mitochondrial Derived Activator of Caspase
t-BID	truncated BID
TNF	Tumour Necrosis Factor
TRADD	TNFR-Associated Death Domain
TRAIL	TNF Related Apoptosis Inducing Complex
WIP-1	Wild type p53 induced phosphatase-1
XIAP	X-linked Inhibitor of Apoptosis Protein
Zvad	benzyloxycarbonil-Val-Ala-DL-Asp-fluoromethylketone

2. Abstract

The induction of programmed cell death (apoptosis) in tumour cells is an essential cellular and molecular process in response to cancer treatment and important for its success. Classically two forms of apoptosis have been distinguished, the extrinsic and the intrinsic apoptosis. Recently novel mechanisms have been proposed that blur the lines between the two apoptosis pathways. One of these novel mechanisms has been proposed by the Mohr/Zwacka laboratory. It involves a new death receptor-independent caspase-8 inducing complex and modulation by the TRAIL-R2 (DR5). The DR5 modulation appears to be mediated by JNK activation, up regulation of a phosphatase and subsequent dephosphorylation of Bid. Dephosphorylated Bid can be cleaved by active caspase-8 and can then convey the apoptotic signal onto mitochondria, which respond with the release of proapoptotic factors such as cytochrome c and Smac/DIABLO into the cytosol. As the release of Smac/DIABLO is important in apoptosis and possibly impaired in DR5-silenced cells, we developed a FACS based Smac/DIABLO release assay in 5-Flurouracil (5-FU) stimulated HCT116 cancer cells. Using this assay we found that DR5 knock-down cells show a markedly reduced Smac/DIABLO release, which corresponds to decreased mitochondrial membrane depolarisation in these cells. These mechanistic differences can explain the significantly reduced apoptosis in 5-FU treated DR5 silenced HCT116 cells. Moreover, this FACS method is a useful tool to understand apoptosis signalling in cancer cells.

3.Introduction

The ability to modulate the life or death of a cell is called Apoptosis. It helps to monitor mitochondrial and cytoplasmic homeostasis, genomic DNA integrity and development of cells. It detects potentially dangerous cells such as virally infected cells or genetically damaged cells and is associated with typical morphological features like cell shrinkage, chromatin condensation, cytoplasmic membrane blebbing, DNA fragmentation and externalization of phosphatidylserine on the cell membrane (Thornberry and Lezabnik, 1998).

Apoptosis can also be deregulated in variety of diseases. Uncontrolled apoptosis can lead to immune response against body's own cells and tissues leading to autoimmune diseases or it can lead to excessive cell proliferation causing cancer. In order to understand how apoptosis regulates the cell function in normal conditions and in diseased state, the mechanism of apoptosis in mammalian cells was discovered and two apoptotic pathways were elucidated namely the extrinsic pathway and the intrinsic pathway. The extrinsic pathway involves death receptor mediated interactions, in which the receptors are members of Tumour Necrosis Factor (TNF) receptor gene superfamily including TNF receptor (TNFR-1), CD95 (Fas or APO-1), Death Receptor 3 (DR3), TNF-related apoptosis inducing ligand receptor-1 (TRAIL-R1 or DR4) and TRAIL-R2 (DR5) (Falschelehner et al, 2006). The death receptors carry a common cysteine rich extracellular domain and a conserved stretch of 80 amino acids called death domain in the intracellular region which is essential for apoptosis (Ozoren and El-Deiry, 2003). Upon ligand binding, recruitment of cytoplasmic adaptor proteins such as Fas associated death domain (FADD) or TNFR associated death domain (TRADD) takes place, which in turn bind

to pro-caspase 8 thereby forming a death inducing signalling complex (DISC). The auto-catalytic activation of caspase 8 triggers the execution phase of apoptosis leading to downstream activation of caspase 3, 6 and 7 and programmed cell death as shown in figure 1. Caspases are group of endonucleases which are produced as inactive pro-caspases called as zymogens. Many models have been proposed for initiator caspase activation. Caspases can be activated by 'induced proximity model' (Salvesen and Dixit, 1999) in which pro-caspases are self-activated when they are brought into close proximity of each other at the death receptor. For example, the recruitment of caspase 8 molecules to the DISC complex at the death receptor by FADD molecule leads to their activation. A model called as 'proximity induced dimerisation' (Shi et al, 2004) on the other hand states that initiator caspases are activated by dimerisation. Chao et al (2005) has also proposed a model to describe initiator caspases activation. According to this model, initiator caspase interact with adaptor protein such as when caspase 9 interact with apoptosome complex consisting of cytochrome c and Apaf-1 which causes conformational changes in the active site of the caspase leading to its activation.

Effector caspases on the other hand are activated by cleavage mediated by initiator caspases which leads to conformational changes creating a functionally mature protein (Reidl and Shi, 2004). The activated effector caspases lead to DNA damage, membrane blebbing and all the characteristic features of apoptosis. Activation of effector caspases can also be cell type dependent. In type 1 cells, effector caspases are directly activated by caspase 8, whereas in type 2 cells, the intrinsic pathway of apoptosis is additionally triggered, which then leads to activation of effector caspases and programmed cell death (Mcllwain et al, 2013). The intrinsic pathway is normally initiated by non-receptor mediated stimuli that causes cellular stress and

directly target the cells leading to mitochondria mediated apoptosis. The stimuli include growth factors, cytokines, toxins, viral infection, free radicals and cytotoxic drugs. These stimuli causes' changes in the inner mitochondrial membrane resulting in mitochondrial membrane permeabilisation, loss of mitochondrial transmembrane potential and release of mitochondrial proteins from the inner membrane (Elmore, 2007) such as cytochrome c, Smac/DIABLO (Second mitochondrial derived activator of caspases) etc. Cytochrome c causes apoptosis by interacting with Apaf-1 and caspase 9 to form the apoptosome complex, which leads to programmed cell death.

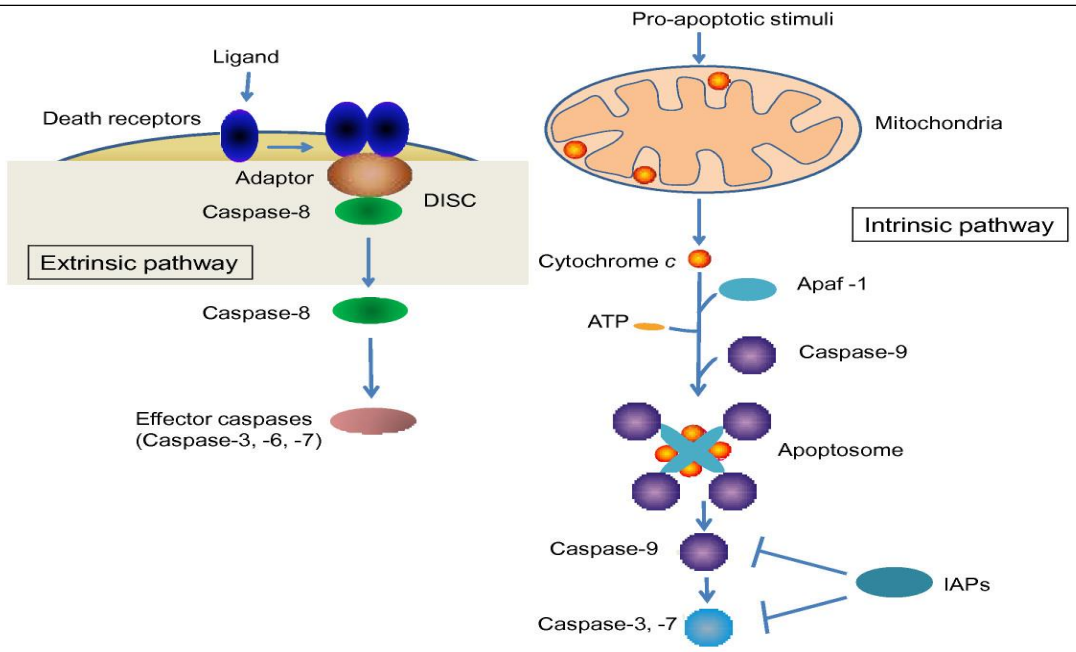

Figure 1-**Schematic of extrinsic and intrinsic pathways of apoptosis.** The extrinsic pathway causes programmed cell death by caspase 8 activation whereas the intrinsic pathway involves mitochondrial membrane permeabilisation and release of mitochondrial proteins from inner membrane and formation of apoptosome complex to mediate apoptosis (Li and Sheng, 2012).

These mitochondrial apoptotic events are regulated by members of the Bcl-2 family of proteins that govern mitochondrial membrane permeability. Some of the family

members are anti-apoptotic such as Bcl-2 and Bcl-xl that preserve mitochondrial integrity (Kluck et al, 1997; Yang et al, 1997), while others are pro-apoptotic like Bax and Bak that move from the cytosol into the mitochondria in response to apoptotic stimuli and promote mitochondrial protein release by forming pores in the outer mitochondrial membrane (Gross et al, 1999).

Recently, a new mechanistic model has been proposed by the Zwacka/Mohr laboratory that is based on their novel findings in HCT116 colon cancer cell lines. This model links the extrinsic and intrinsic pathways of apoptosis in response to cytotoxic drug 5-Flurouracil (5-FU). The drug triggers death receptor independent caspase 8 activation by the FADDosome complex consisting of FADD, RIP-1, caspase 8 and caspase 10 (Personal communication). On activation, caspase 8 then cleaves BH3 protein, BID to its truncated form t-BID, which can then translocate to the mitochondria and activate pro-apoptotic members of Bcl-2 family of proteins, Bax and Bak leading to mitochondrial membrane permeabilisation and release of proapoptotic proteins from mitochondria. Cleavage of BID to t-BID is modulated by TRAIL-R2 (DR5) receptor which is activated independent of its ligand and stimulates JNK signalling through JIST complex leading to activation of the WIP-1 phosphatase. WIP-1 dephosphorylates BID so that caspase 8 mediated cleavage of BID can take place as shown in figure 2.

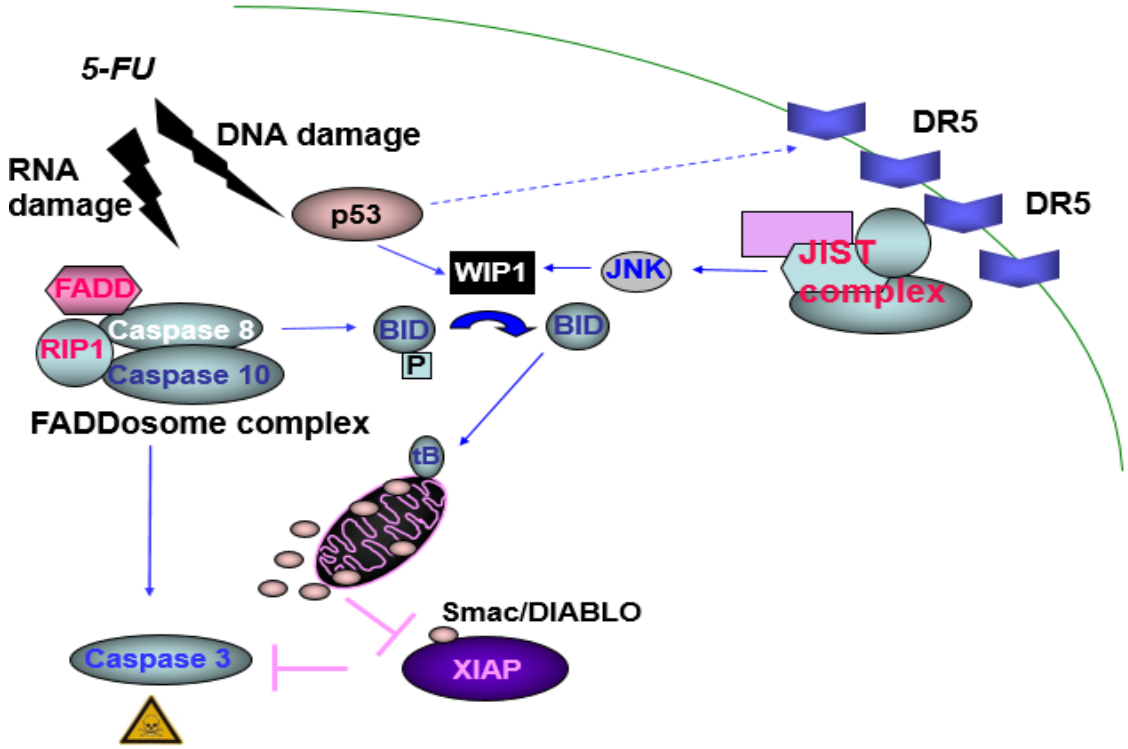

Figure 2- **The mechanistic 5-FU induced DR5 regulated apoptotic model.** The model depicts the cytotoxic drug induced caspase 8 activation and JNK signalling by DR5 receptor leading to apoptosis by promoting BID cleavage and downstream release of Smac/DIABLO which functions to inhibit XIAP causing effector caspases mediated programmed cell death (Personal communication).

DR5 is a membrane protein that has high homology to members of TNF family and induces apoptosis selectively in human tumours but not in normal cells as normal cells are protected from TRAIL induced apoptosis by TRAIL-R3 and TRAIL-R4 which are decoy receptors that lack cytoplasmic death domains (Wu et al, 1997; Degli-Esposti et al, 1997) and are unable to induce apoptosis. TRAIL-R3 which is a 299 amino acid long protein contains only two out of four pseudo repeats which are characteristic of extracellular domain of TNF receptor family due to which it diverts the death receptor mediated signalling thereby providing TRAIL selectivity. TRAIL-R4 has been shown to not just prevent apoptosis but also induce cell survival. It can

activate NF-kB which induces transcription of anti-apoptotic genes in the cell thereby preventing apoptosis (Degli-Esposti et al, 1997).

Downstream signalling commenced by 5-FU and modulated by DR5 lead to release of Smac/DIABLO from mitochondria which plays an more important role in the novel apoptotic model by promoting caspase activation and cell death. Smac/DIABLO is synthesised as a 239 amino acid long amphipathic protein. It forms a mature Smac/DIABLO after cleavage of its mitochondrial targeting signal at the N-terminus following its import into the mitochondria (Du et al, 2000). After dimerisation with other Smac monomers, an arch shaped Smac/DIABLO dimer is formed with amino terminal on the top and carboxyl terminal at the feet of the arch (Chai et al, 2000). The function of Smac/DIABLO is similar to Reaper, Hid and Grim which are drosophila proteins that mediate apoptosis. The primary sequence of Smac/DIABLO is completely different from the drosophila homologs even though they have similar function. This may be due to independent evolution of these proteins which is termed as convergent evolution (Du et al, 2000).

Smac/DIABLO functions to activate caspase 9 and effector caspases by neutralising the inhibitory effect of family of proteins labelled as Inhibitors of Apoptosis Proteins (IAPs) (Du et al, 2000) which are 57 kDa proteins that inhibit programmed cell death. 700 IAPs have been discovered including X chromosome linked IAP (XIAP), C-IAP1, C-IAP2, NAIP, Survivin, ML-IAP (Huang et al, 2001). These IAPs directly supress the caspases or regulate cell-cycle, protein degradation and even regulate caspase-independent signal transduction cascade. They have two main motifs namely the conserved Baculovirus IAP repeat called as BIR domain and RING domain which is found at the C-terminus of the protein (Takahashi et al, 1998).

XIAP inhibits apoptosis by binding to the N-terminus of the linker peptide of active caspase 9 which is associated with Apaf-1 and cytochrome c forming an apoptosome complex. The binding takes place with the BIR3 domain of XIAP and prevents caspase 9 function (Srinivasula et al, 2001). XIAP can also associate with effector caspases such as caspase 3 and caspase 7 and supress their activity. But the site of interaction is near the BIR2 domain rather than BIR3 domain as seen with caspase 9. The XIAP protein binds to the caspases in opposite orientation to the original substrate of caspases and thereby completely block the active site (Salvesen et al, 2002).

Smac/DIABLO inhibits the activity of XIAP by binding to the BIR3 domain. The residues 56-59 of Smac/DIABLO are analogous to the caspase 9 motif that interacts with BIR3 domain of XIAP and thereby prevents caspase 9 binding by competitive inhibition. At the same time, Smac/DIABLO also interacts strongly with the BIR2 domain of XIAP which facilitates Smac-XIAP complex formation that takes place by a network of hydrogen bonding and extensive van der Waal networks. Interaction of amino acid, alanine in Smac/DIABLO with the hydrophobic pocket in the surface groove of XIAP also promotes the complex formation. The Smac-XIAP complex prevents the suppression of caspases thereby leading to apoptosis (Srinivasula et al, 2001).

As Smac/DIABLO has been shown to play an important role in 5-FU induced apoptosis, various techniques can be used to study Smac/DIABLO release from the mitochondria into the cytosol including subcellular fractionation and western blotting as well as immunocytochemistry which can determine Smac/DIABLO release at a single cell level. In the present project we plan to develop a novel FACS based Smac/DIABLO release assay using para-formaldehyde as fixative and detergents

to create pores for anti-Smac/DIABLO antibody to traverse through the cell membrane and bind to Smac/DIABLO. Using this assay we will compare Smac/DIABLO release in 5-FU stimulated HCT116 cells and HCT116shDR5 cells, which have silenced DR5 receptor to confirm the importance of DR5 as a modulator of apoptosis according to the mechanistic model proposed by the Mohr/Zwacka laboratory.

4. Materials and Methods

4.1. Cell culture

The HCT116 colon cancer cell line (ATCC) and its derivate cell line HCT116shDR5 (provided by Dr. Andrea Mohr) were cultured in McCoy's medium (Lonza) supplemented with 10% Foetal calf serum (Life Technologies) and 1% Penicillin-Streptomycin (Sigma) at 37˚C in humified air containing 5% CO_2.

4.2. Regents and antibodies

Nicoletti buffer was prepared by adding 1% Sodium Citrate (Sigma) and Triton X100 (Sigma) to distilled water and mixing Propidium Iodide in the ratio 1:25 on ice. 1X Phosphate Buffer Saline (PBS) was prepared by adding 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 in 800 ml of distilled water and adjusting the pH to 7.4. The 4% Paraformaldehyde (PFA) was prepared by adding 4 g of PFA (Sigma) in 100 ml of PBS (Sigma) and heat stirring until a clear solution was formed. 0.01% PFA and 0.12% PFA were then prepared from the 4% PFA stock solution by dilution in PBS (Sigma). The 0.1% Saponin was made from a 5% stock solution containing 0.25 g of Saponin (Sigma) in 5 ml of PBS (Sigma). The working concentration of 200 µM of 5-Flurouracil (5-FU, Sigma) was achieved by diluting the 10 mM 5-FU stock in medium that was then used to treat the cells. 40 nM DIOC(6)3 (Life Technologies) was made from a 100 mM DIOC(6)3 stock solution by diluting it with warm PBS (37˚C, Sigma). Antibodies used in the study were IgG blocking antibody (Sigma), mouse anti-Smac/DIABLO antibody (Abcam), FITC labelled mouse IgG antibody (Southern Biotechnology) and IgG2b Isotype antibody (Biolegend).

4.3. Measurement of Apoptosis

Unstimulated and 5-FU (200 µM) stimulated HCT116 and HCT116shDR5 cells were cultured in 24 well plates for 24 hours, 36 hours and 48 hours and apoptosis was measured according to the protocol by Nicoletti et al (1991). 1000 events per sample were measured by FACS Accuri and the sub-G1 cells were counted as apoptotic.

4.4. Trypan Blue Exclusion method

Unstimulated HCT116 cells fixed with different concentrations of PFA and permeabilised with varying concentrations of Saponin were stained with 0.1% Trypan blue dye (Sigma) to count the number of intact and permeabilised cells for optimisation of the conditions for the Smac/DIABLO release assay. Intact cells have an undamaged cell membrane and they do not take up the dye whereas permeabilised cells readily take up the Trypan blue dye and are stained blue.

4.5. Analysis of Smac/DIABLO release

Unstimulated and 5-FU (200 µM, for 24 hours, 36 hours and 48 hours) stimulated HCT116 and HCT116shDR5cells, cultured in 25-cm^2 flasks (Sarstedt) were harvested and washed with PBS (Sigma) followed by 20 minutes of pre-fixation with PFA (0.01%, Sigma) on ice. At each step the reagents were mixed by flicking the tubes 5 times with no harsh pipetting done. The cells were then fixed with PFA (0.12%, Sigma) for 20 minutes and permeabilised with 0.1% Saponin for 5 minutes on ice. For detergent mediated permeabilisation step, the cells stimulated with 5-FU were flicked until the pellet was dislodged from the wall of the tube and then the same number of flicks were applied on the other samples. Unspecific binding of anti-

Smac/DIABLO antibody was blocked with 5 µg of mouse IgG antibody (Sigma). 1 mg/ml anti-Smac/DIABLO antibody (Abcam) was added for additional 20 minutes at 4˚C and IgG2b Isotype antibody (Biolegend) was used as control. The cells were incubated with FITC labelled mouse IgG secondary antibody (Southern Biotechnology) for 20 minutes on ice and in the dark. Samples were then analysed by FACS Accuri and 10,000 events per sample were acquired and analysed.

4.6. Measurement of mitochondrial membrane polarity and apoptosis inhibition by Zvad

Unstimulated and 5-FU (200 µM, for 24 hours and 48 hours) stimulated HCT116 cells, cultured in 25-cm^2 flasks (Sarstedt) were harvested and washed with warm PBS (Sigma). 40 nM DIOC(6)3 (Life Technologies) was added and the cells were incubated in a water bath at 37˚C for 15 minutes. 10,000 events per sample were measured immediately by FACS Accuri. HCT116 cells were also treated with Zvad, a pan caspase inhibitor with and without 5-FU (200 µM, Sigma) for 48 hours to examine Smac/DIABLO release and apoptosis was also measured according to the protocol by Nicoletti et al (1991) by FACS Accuri.

4.7. Statistical Analysis

Experiments were performed in triplicates and the experimental values were expressed as mean value +/- standard deviation (S.D.). For significant analyses, ANOVA was used with $p < 0.05$ considered as significant and $p < 0.001$ as highly significant.

5. Results

5.1. Measurement of apoptosis

According to the novel 5-FU induced and DR5 modulated apoptotic model proposed by the Mohr/Zwacka laboratory when 5-FU induces apoptosis, Smac/DIABLO release takes place from mitochondria. We determined the level of apoptosis induced by 5-FU using a DNA hypodiploidy assay, also known as Nicoletti assay, described by Nicoletti et al (1991) in 5-Flurouracil (5-FU) stimulated HCT116 cells and observed an increase in apoptosis with increasing time points of 5-FU stimulation. We measured apoptosis levels of 35% at 48 hours of treatment with just above 5% apoptosis at 24 hours and 15% at 36 hours of treatment with 5-FU respectively (Figure 3).

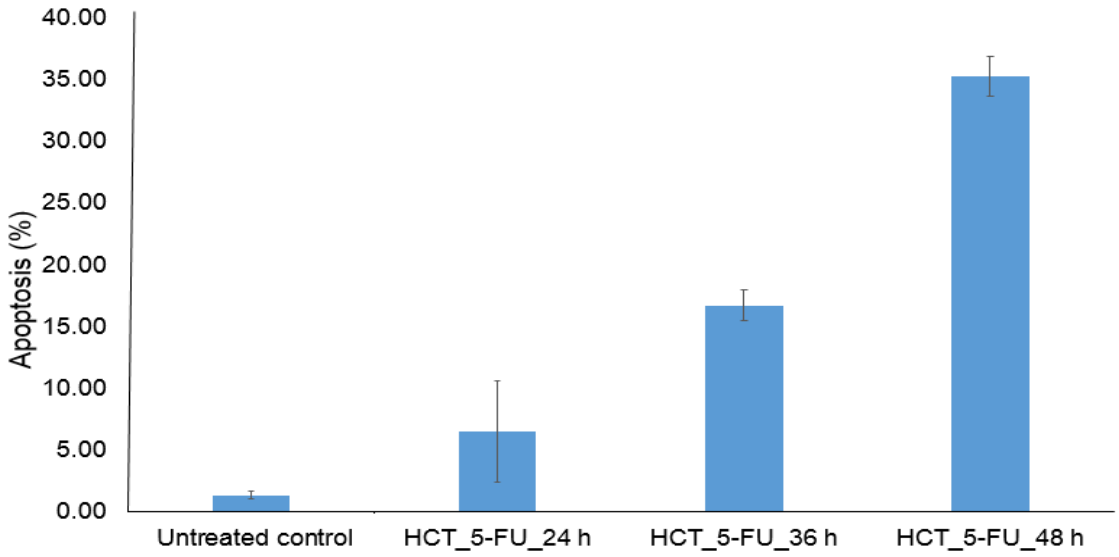

Figure 3-**Measurement of apoptosis in HCT116 cells with DNA hypodiploidy assay using Propidium Iodide.** Difference in percentage of apoptosis in HCT116 cells induced with 5-FU (200 µM) for 24 hours, 36 hours and 48 hours was analysed as compared to untreated control using method described by Nicoletti et al (1991). Error bars denote the standard deviation of mean of triplicate samples.

The exact molecular mechanism of 5-FU induced apoptosis mediated by the Mohr/Zwacka apoptotic model involving death receptor independent caspase 8 inducing complex is still not clear. But the important role of DR5 in regulating this mechanistic apoptotic model has been illustrated by the apoptosis assay performed on DR5 knockdown cells (HCT116shDR5 cells). The percentage of apoptosis was compared with HCT116 wild type cells by treatment with 5-FU for 24 hours, 36 hours and 48 hours and significant differences in apoptosis were observed between these cell lines (Figure 4). A highly significant difference was seen especially at 48 hours of stimulation with 35% apoptosis in HCT116 wild type cells in comparison to 18% in DR5 knockdown cells.

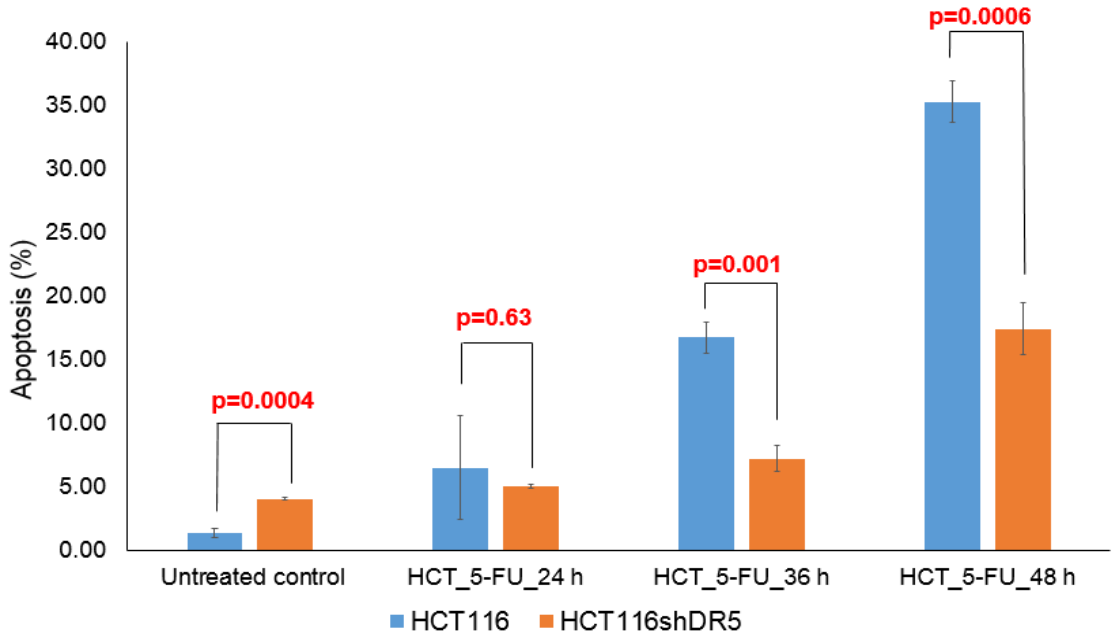

Figure 4- **Comparison of apoptosis in HCT116 and HCT116shDR5 cells after stimulation with 5-FU at different time points**. HCT116 and HCT116shDR5 cells were treated with 200 µM 5-FU for 24 hours, 36 hours and 48 hours and percentage of apoptosis was measured by Nicoletti assay as compared to untreated controls. Blue bars represent HCT116 cells and orange bars represent HCT116shDR5 cells with error bars denoting the standard deviation of mean of triplicate samples. The difference in percentage of apoptosis between HCT116 and HCT116shDR5 cells is significant.

Du et al (2000) has already shown the important role played by the mitochondrial protein Smac/DIABLO in apoptosis by preventing the inhibitory action of XIAP on caspases thereby leading to caspase activation and programmed cell death. But it is necessary to fully understand the relationship between the release of Smac/DIABLO and apoptosis induced by drug 5-FU in the context of the novel apoptotic model. Therefore, we sought to develop and establish an easy and quick Smac/DIABLO release assay.

5.2. Establishment of a FACS based Smac/DIABLO release assay

5.2.1. Examination of different concentrations of Saponin at different time points for membrane permeabilisation

To analyse the Smac/DIABLO release induced by 5-FU, a FACS based Smac/DIABLO release assay was developed. The method involved a Smac/DIABLO specific antibody to traverse the plasma membrane of the cells, after a detergent created pores large enough for the Smac/DIABLO antibody to pass through. Saponin was used as the detergent to permeabilise the cell membrane and different concentrations of Saponin such as 0.1%, 0.2%, 0.5%, 1% and 5% were used together with 4% Paraformaldehyde (PFA) for fixation for different time points ranging from 3 minutes to 30 minutes. Permeabilised cells were counted using the Trypan Blue Exclusion method. In response to these experiments 0.5%, 1% and 5% Saponin concentrations were discarded as cell breakdown was observed at all the time points. Better results were observed for 0.1% and 0.2% of Saponin without signs of breakdown and loss of cells. Furthermore, the cells had to endure four rounds of washing with PBS according to protocols for intracellular staining. We found that it was only with 0.1% Saponin and a 5 minute incubation period that cells

were observed after repeated washing, so it was chosen for later experiments (Figure 5).

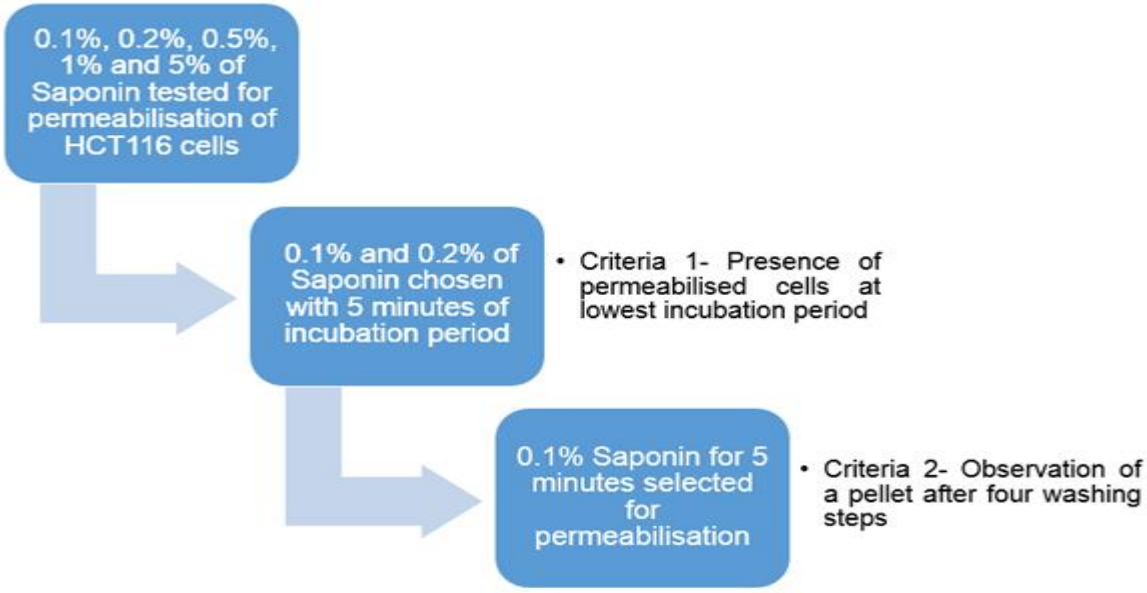

Figure 5- **Schematic diagram to illustrate the steps for examining different concentrations of Saponin for permeabilisation.** 0.1% Saponin was selected from different concentrations of Saponin such as 0.2%, 0.5%, 1% and 5% for permeabilisation with incubation period of 5 minutes using two main criterion namely presence of permeabilised cells at lowest incubation period and observation of a pellet of cells after four washing steps. These criterions were used to determine the correct concentration of Saponin to use for permeabilisation in FACS based determination of Smac/DIABLO release assay.

5.2.2. Assessment of different concentrations of PFA for cell fixation

The combination of 0.1% Saponin for 5 minutes and 4% PFA was tested on HCT116 cells stimulated with 5-FU for 48 hours for fixation and permeabilisation. But stimulated cells were sensitive and a significant proportion of cells were lost after the fourth and final washing step and consequently no signal could be detected in the flow cytometer. A lower concentration of Saponin i.e. 0.05% was also tested but the same result was observed. So it was concluded that over fixation as opposed to higher detergent concentration was leading to the loss of cells and signal. Thus, the

concentration of PFA was decreased to 2%, 1% and 0.5%. The total number of permeabilised cells observed after Trypan Blue staining in the haemocytometer at these concentrations of PFA were the same. But a cell pellet was present only with 0.5% PFA after four rounds of washing as outlined in Table 1.

Table 1- **Analysis of different concentrations of PFA for fixation of HCT116 cells on the basis of two main criterion.**

Concentrations of PFA used	2%	1%	0.5%
Criteria 1- Presence of permeabilised cells	Permeabilised cells	Permeabilised cells	Permeabilised cells
Criteria 2- Observation of a pellet after four washing steps	No pellet	No pellet	Decent pellet

When Smac/DIABLO release was measured after fixation with 0.5% PFA using FACS, a shift in signal was observed with 5-FU treated samples as compared to control (Figure 6) suggesting that the antibody detected Smac/DIABLO in the cytosol which is released from the mitochondria to induce apoptosis in response to 5-FU. This release was seen as a shift in the signal towards higher fluorescence intensity. With a modest signal detected in the pilot experiment, the concentration of PFA was further decreased to improve the method and 0.12% PFA was chosen as at this concentration we could increase the number of cells that survived the washing steps and use for the FACS analysis. When a pre-fixation with 0.01% PFA was added before the fixation step and 0.1% Saponin for 5 minutes was used for permeabilisation, a larger pellet was observed.

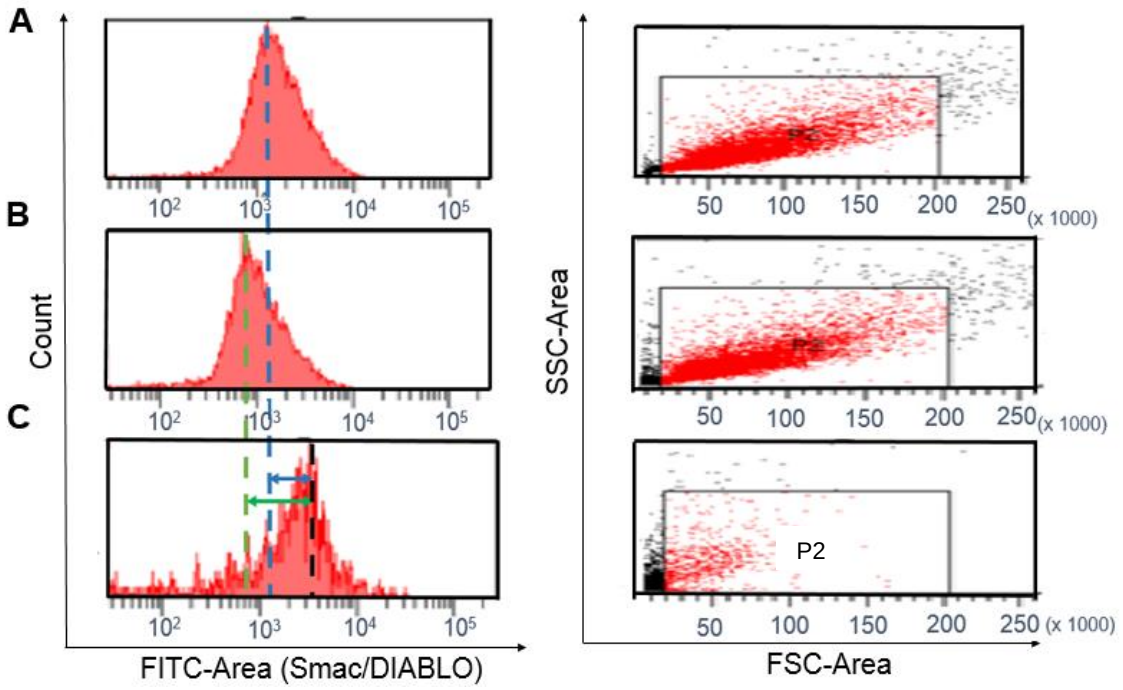

Figure 6- **Analysis of Smac/DIABLO release in HCT116 cells.** Untreated HCT116 cells incubated with Smac/DIABLO antibody (A), stained with Isotype control antibody (B) and treated with 5-FU (200 µM) for 48 hours (C) were analysed for Smac/DIABLO release using 0.5% PFA as a fixative and 0.1% Saponin for 5 minutes for permeabilisation of cells. The shift in the fluorescence signal between A and C is represented by blue dotted line and blue double arrow whereas the shift in the fluorescence signal between B and C is depicted by green dotted line and green double arrow. The shift in fluorescent signal in C as compared to A and B indicates Smac/DIABLO release. P2 represents the percentage of morphologically unchanged cells as gated in the Forward/Side-scatter plot of the FACS analysis.

With a large number of cells and a significant shift in the signal, the protocol for FACS based Smac/DIABLO release was established to involve pre-fixation of the cells with 0.01% PFA, fixation with 0.12% PFA and permeabilisation with 0.1% Saponin for 5 minutes leading to creation of pores in the plasma membrane for Smac/DIABLO antibody to enter the cells and bind to its target protein. The Smac/DIABLO release was then measured by FACS and the efflux of Smac/DIABLO from the mitochondria was detected only in 5-FU stimulated cells as

compared to untreated control (Figure 7). The presence of Smac/DIABLO in the cytosol was observed as a shift in the fluorescent signal in 5-FU treated cells as compared to untreated control (Figure 8). Using this assay, Smac/DIABLO release was also measured in DR5 knockdown cells to understand the role played by DR5 receptor according to the novel apoptotic model as proposed by the Mohr/Zwacka laboratory.

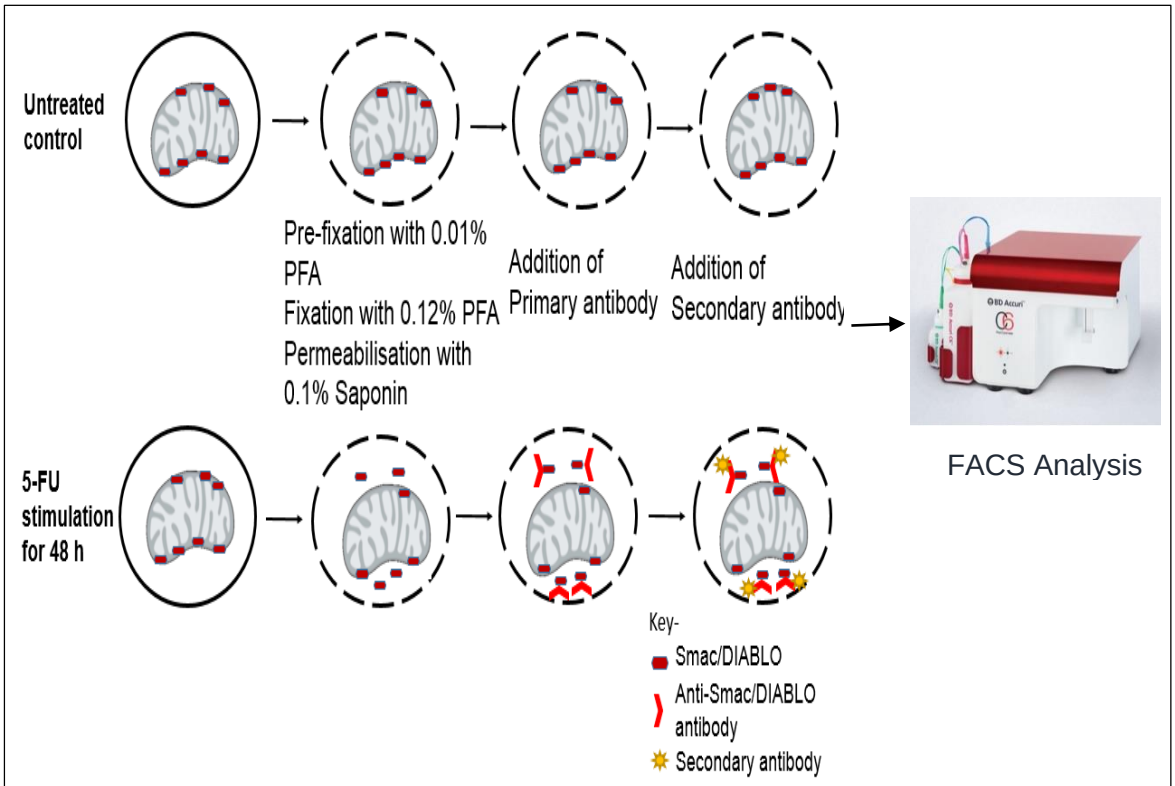

Figure 7- **Schematics of Saponin permeabilised HCT116 cells to detect Smac/DIABLO release in the cytosol by flow cytometry.** Untreated and 5-FU treated cells were pre-fixed with 0.01% PFA, fixed with 0.12% PFA and permeabilised with 0.1% Saponin to create pores in the cells for antibody specific for Smac/DIABLO to travel through the plasma membrane. Primary antibody and FITC labelled secondary antibody was added and Smac/DIABLO release was measured by FACS. In untreated cells, there was no apoptosis due to which no Smac/DIABLO was released in the cytosol for antibody binding. In 5-FU stimulated cells, Smac/DIABLO was released from the mitochondria due to induction of apoptosis so that antibody could bind Smac/DIABLO in the cytosol and the signal was detected using FACS.

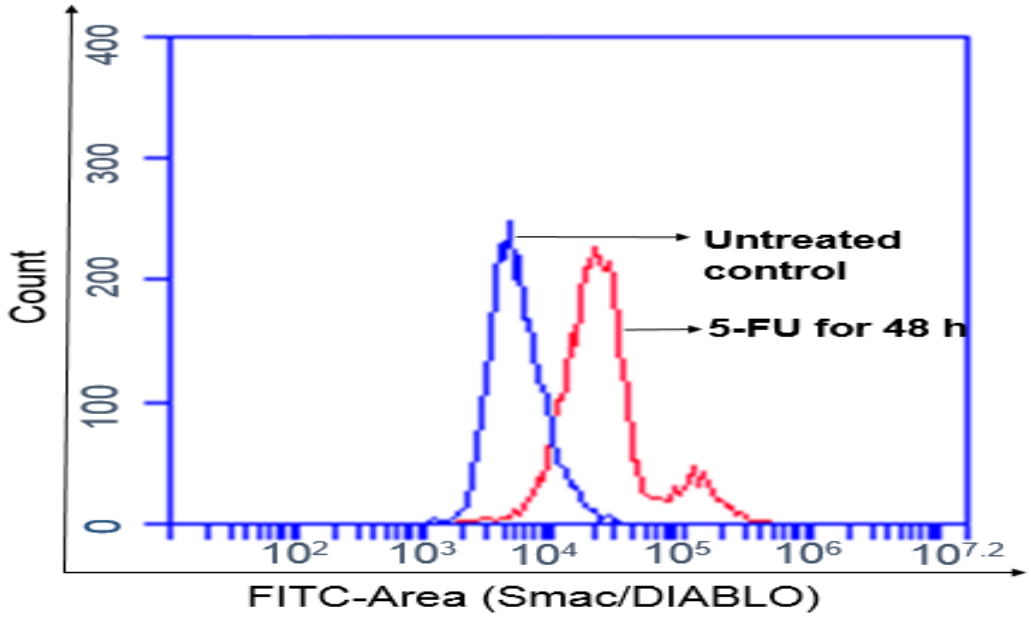

Figure 8- **Examination of cytosolic Smac/DIABLO release in 5-FU stimulated HCT116 cells using FACS.** HCT116 cells were stimulated with 200 µM 5-FU for 48 hours as compared to untreated cells and Smac/DIABLO release was analysed after morphologically unchanged cells were identified in Forward/Side-scatter plot. A shift in peak was observed for cells induced with 5-FU as compared to untreated control depicting Smac/DIABLO release from mitochondria.

5.3. Examining Smac/DIABLO release according to the novel apoptotic model

Specificity of the assay was determined by comparing the Smac/DIABLO release in cells treated with 5-FU to untreated cells and cells stained with Isotype antibody. It was observed that untreated control and Isotype control had a similar fluorescent signal (Figure 9). Whereas, in 5-FU stimulated cells there was a shift in the signal as compared to the controls. Keeping the fluorescent signal of untreated control and Isotype control as threshold, the shift in the peak observed in 5-FU treated cells signified Smac/DIABLO release. The major advantage of this FACS based assay is that it allowed us to measure Smac/DIABLO release at the single cell level and when Smac/DIABLO release was analysed after gating the morphologically unchanged cells by Forward/Side-scatter plot, it was observed that 89.7% of

HCT116 cells stimulated with 5-FU for 48 hours released Smac/DIABLO as compared to untreated controls (Figure 10).

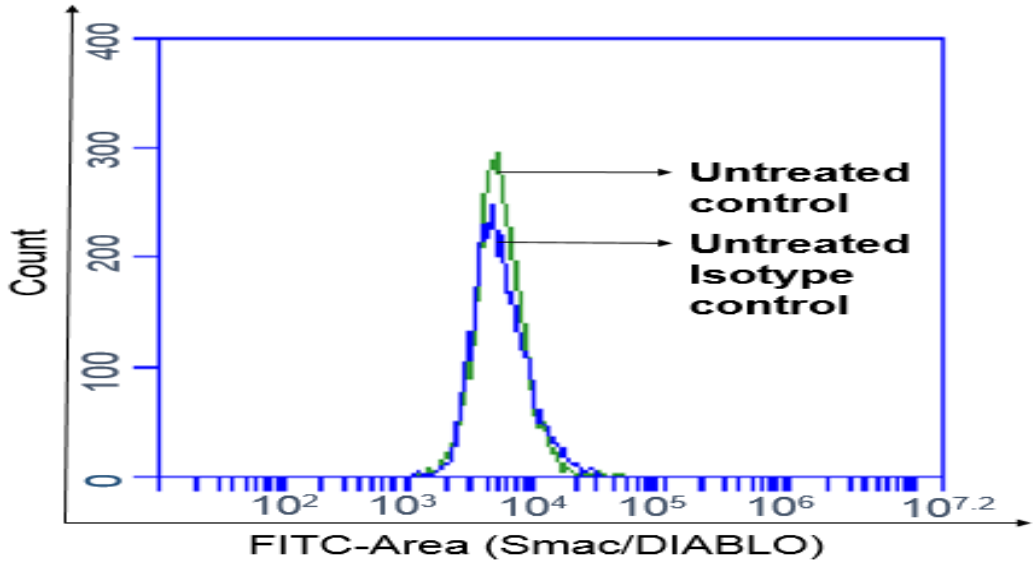

Figure 9- **Comparison between the controls used in Smac/DIABLO release assay**. HCT116 cells were incubated with Smac/DIABLO antibody and cells were also stained with Isotype control antibody forming the untreated control and untreated Isotype control respectively. Smac/DIABLO release was analysed in these samples as described in Materials and Method and overlapping fluorescence signals were observed.

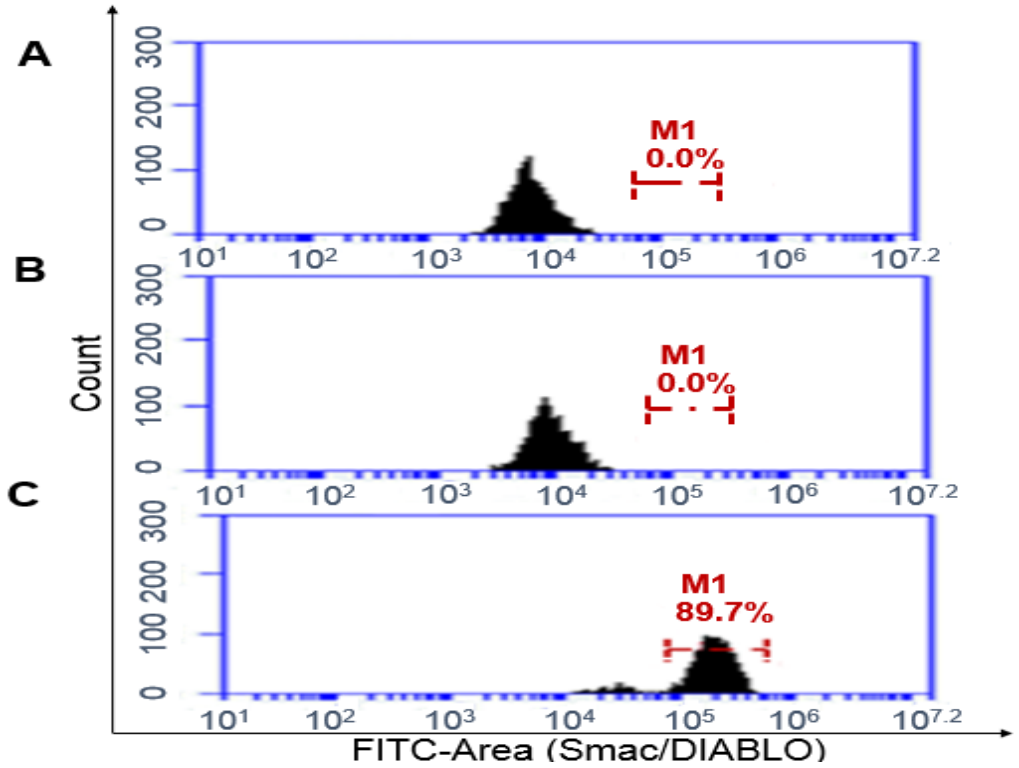

Figure 10- **Analysis of Smac/DIABLO release at a single cell level after treatment of cells with 5- FU (200 µM).** Smac/DIABLO release was examined in untreated HCT116 cells incubated with Smac/DIABLO antibody (A), stained with Isotype control antibody (B), cells treated with 5-FU for 48 hours and incubated with Smac/DIABLO antibody (D). M1 represents the percentage of cytosolic Smac/DIABLO release from the cells. Maximum Smac/DIABLO release was observed in 5-FU stimulated cells incubated with Smac/DIABLO antibody with M1 equal to 89.7%.

We have determined Smac/DIABLO release from the mitochondria at 48 hours of treatment with 5-FU. In order to examine if the 48 hours of 5-FU stimulation is the lowest time point of apoptosis induction, Smac/DIABLO release was compared in treated cells at different time points namely 24 hours, 36 hours and 48 hours. It was observed that the signal in 24 hours of treatment was similar to untreated control and Isotype control (Figure 11) demonstrating that no Smac/DIABLO release has taken place at this time point as there was no shift in the signal. Similarly, the fluorescent signal at 36 hours of 5-FU induction overlapped with the untreated control (Figure 12) thus confirming that Smac/DIABLO release which is

characterised by shift in the signal takes place only at 48 hours of 5-FU induction. These results are in accordance with the results obtained by the Nicoletti assay as shown in Figure 3 in which maximum apoptosis was observed at 48 hours of 5-FU treatment as compared to 24 hours and 36 hours indicating that Smac/DIABLO release leads to increased apoptosis in the cells.

These results were quantified by determining the Mean Fluorescence Intensity Ratio (MFI Ratio) of the above samples in which maximum fluorescence intensity was observed at 48 hours of 5-FU induction as compared to 24 hours and 36 hours of treatment (Figure 13).

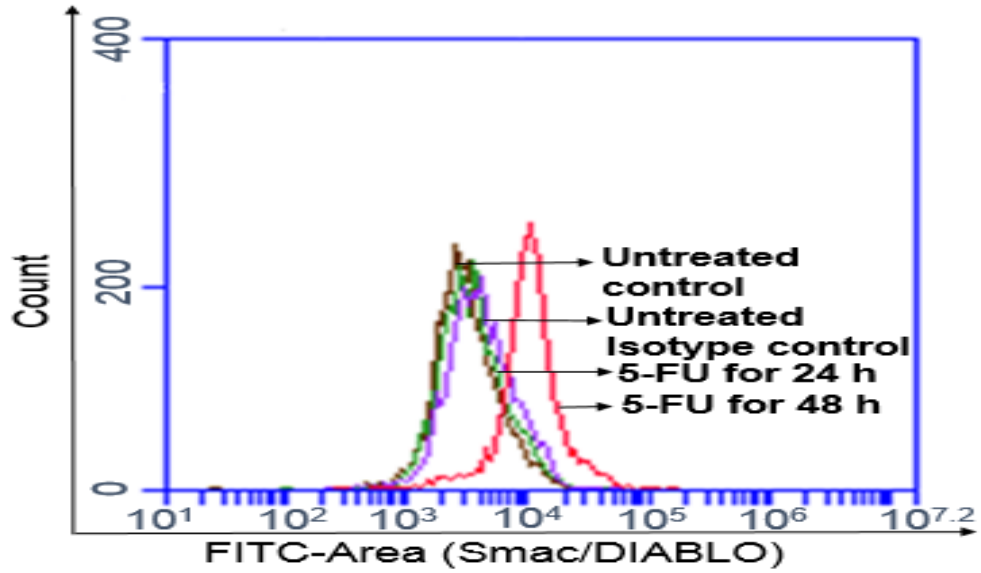

Figure 11- **Examination of Smac/DIABLO release in HCT116 cells at different time points of 5-FU treatment.** HCT116 cells were treated with 5-FU for 24 hours and 48 hours and Smac/DIABLO release was measured according to Smac/DIABLO release assay described in Materials and Methods. Smac/DIABLO release signal after 24 hours of 5-FU stimulation was observed to be similar to untreated control and untreated Isotype control in which untreated cells were incubated with Smac/DIABLO antibody or stained with Isotype antibody respectively. The shift in signal was only seen after 48 hours which is characteristic of Smac/DIABLO release.

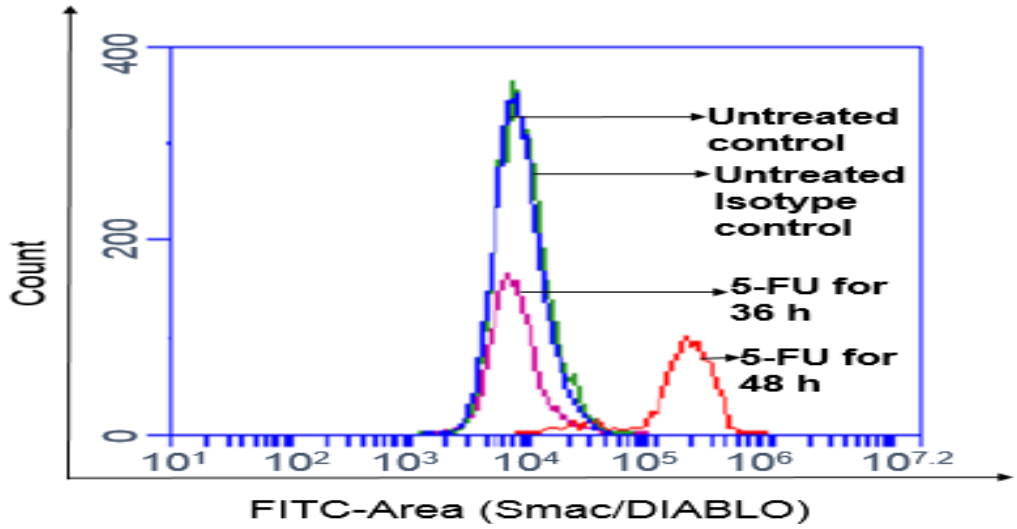

Figure 12- **Examination of cytosolic Smac/DIABLO release after stimulation with 5-FU for 36 hours as compared to 48 hours of stimulation.** HCT116 cells were treated with 5-FU for 36 hours and 48 hours and Smac/DIABLO release was analysed as compared to untreated cells. The fluorescent signal of cells treated with 5-FU for 36 hours was similar to untreated control and untreated Isotype control in which untreated cells were incubated with Smac/DIABLO antibody or stained with Isotype antibody respectively. The shift in signal, which is characteristic of Smac/DIABLO release was observed at 48 hours of 5-FU stimulation only.

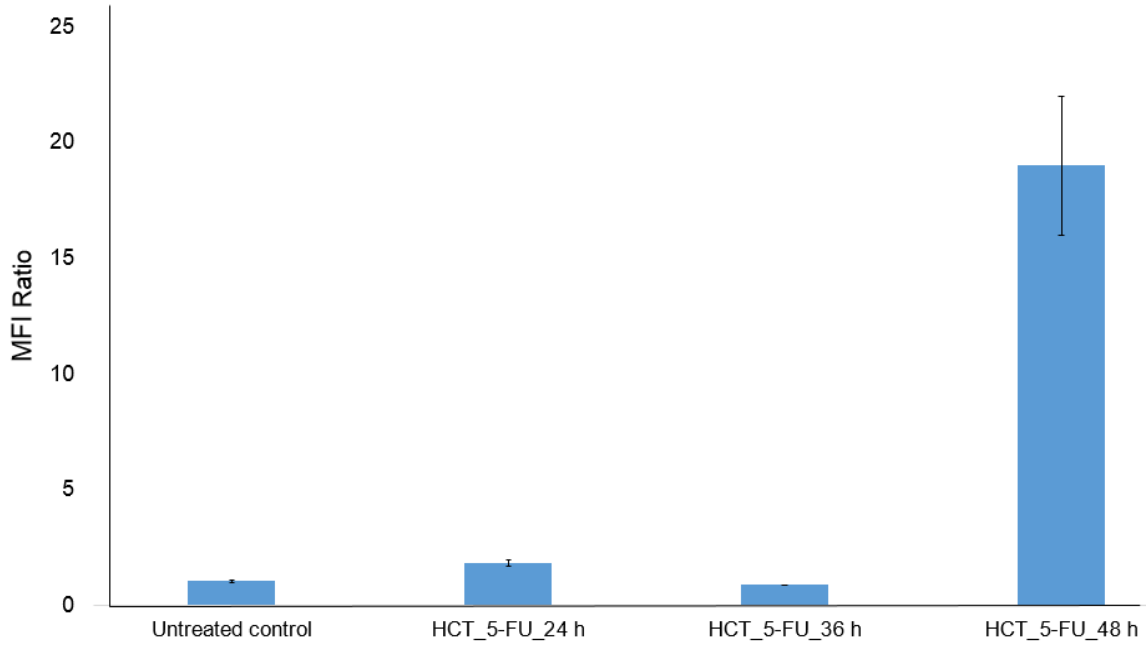

Figure 13- **Quantification of FACS based Smac/DIABLO release.** MFI ratio was calculated for HCT116 cells treated with 5-FU for 24 hours, 36 hours and 48 hours in comparison to untreated control and highest intensity of fluorescent signal was observed at 48 hours of stimulation. Error bars denote the standard deviation of mean of triplicate samples.

Smac/DIABLO release was also accessed in HCT116shDR5 cells (DR5 silenced cells) as compared to HCT116 wild type cells and it was seen that Smac/DIABLO release in the cytosol takes place only in HCT116 cells which is characterised by a shift in the peak as compared to DR5 knockdown cells (Figure 14). This indicates that by silencing DR5, the Smac/DIABLO release is inhibited. These results are in consensus with level of apoptosis measured in HCT116shDR5 cells (Figure 4) in which level of apoptosis dropped from 35% to 15% after knocking down DR5 receptor in cells treated with 5-FU for 48 hours. Thus manifesting the importance of DR5 in modulating Smac/DIABLO release and apoptosis according to the Mohr/Zwacka apoptotic model as it was observed that by silencing DR5, the Smac/DIABLO release did not take place and consequently complete apoptosis did not take occur.

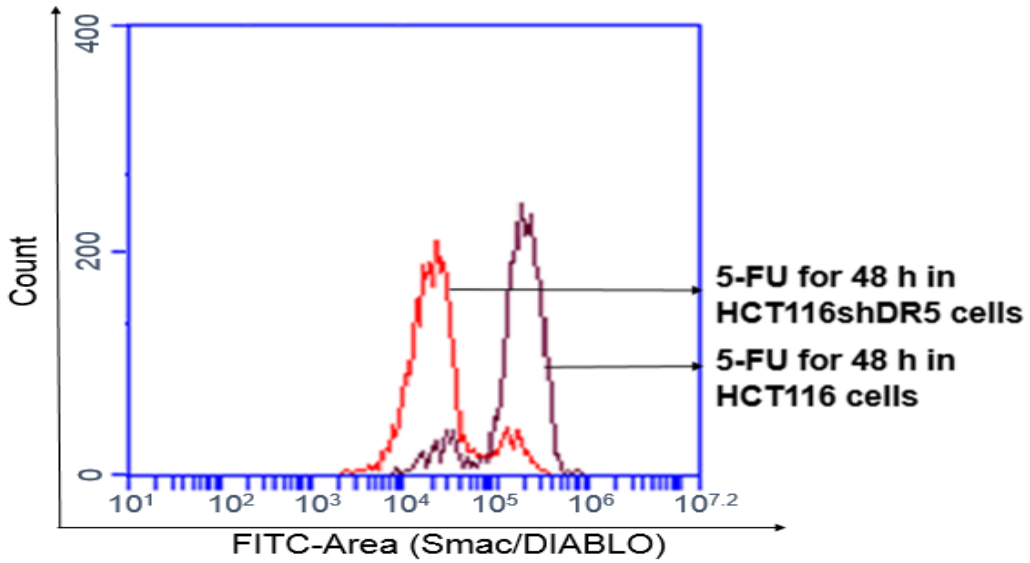

Figure 14- **Examination of Smac/DIABLO release in HCT116 and HCT116shDR5 cells.** HCT116 cells and HCT116shDR5 cells were stimulated with 200 µM of 5-FU for 48 hours and Smac/DIABLO release was analysed by Smac/DIABLO release assay as described in Materials and Methods. Shift in the peak was observed only in HCT116 cells depicting Smac/DIABLO release and not in HCT116shDR5 cells.

These results were quantified by measurement of MFI ratio with maximum signal intensity observed in HCT116 cells as compared to HCT116shDR5 cells. Comparison of fluorescence intensity was also done in these cells at different time points of 5-FU stimulation. The results show that MFI ratio was decreased in DR5 silenced cells at all the time points of 5-FU treatment namely 24 hours, 36 hours and 48 hours as compared to wild type HCT116 cells. The reduction in signal intensity in DR5 knockdown cells at 48 hours of 5-FU induction was quite significant as compared to HCT116 cells (Figure 15).

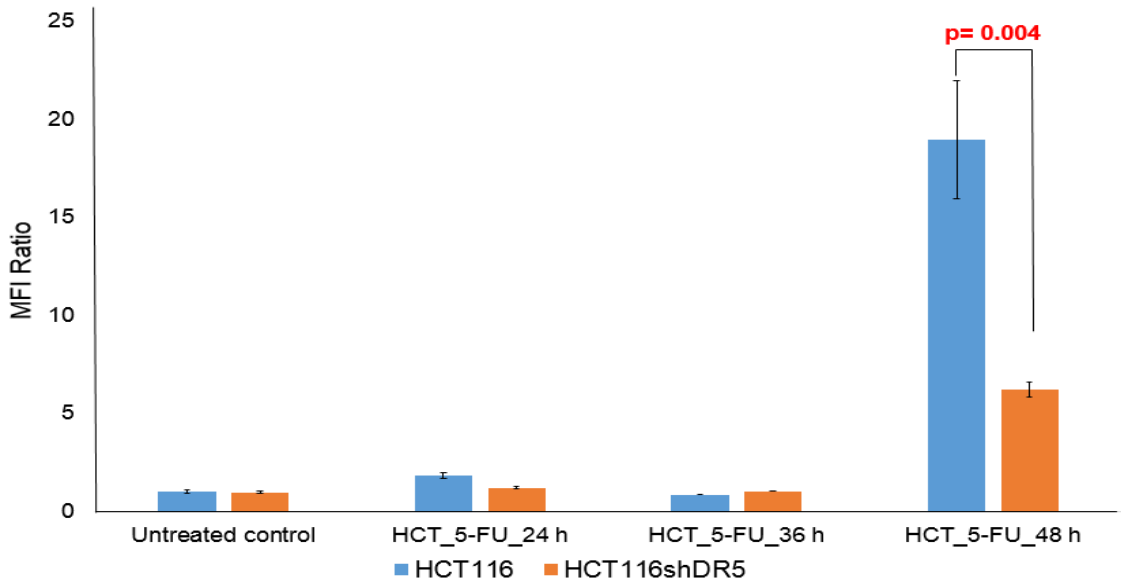

Figure 15- **Quantification of FACS based release of Smac/DIABLO release by MFI ratio.** Smac/DIABLO release in HCT116 and HCT116shDR5 cells was quantified by determining the Mean Fluorescence Intensity Ratio of cells stimulated with 5-FU for 24 hours, 36 hours and 48 hours in comparison to untreated control. Error bars denote the standard deviation of mean of triplicate samples. The difference in MFI ratio between HCT116 and HCT116shDR5 cells when treated with 5-FU for 48 hours is highly significant with p value equal to 0.004.

Using FACS based Smac/DIABLO release assay we have confirmed the involvement of 5-FU and DR5 in cytosolic release of Smac/DIABLO which is in accordance to the novel apoptotic model that is based on work by the Mohr/Zwacka

laboratory. Along with 5-FU and DR5, the model proposes that mitochondrion relies on caspases for Smac/DIABLO release. This was validated by performing the FACS assay after treating the cells with Zvad, a pan caspase inhibitor and analysing Smac/DIABLO release after inhibition of all the caspases. Zvad prevents caspase activation by blocking pro-caspases which are inactive forms of caspases from activating into active caspases by binding to well conserved allosteric sites in caspases to mediate its inhibition (Scheer et al, 2006). Smac/DIABLO release into cytosol is usually observed by shift in the fluorescent signal as compared to untreated controls but the signal in cells treated with Zvad was similar to the untreated control indicating that no Smac/DIABLO release from the mitochondria took place. Cells treated with both Zvad and 5-FU were also analysed for Smac/DIABLO release and no shift in signal was observed indicating that even in the presence of 5-FU which is a cytotoxic drug that induces apoptosis, Zvad could inhibit Smac/DIABLO release (Figure 16). Thus, confirming an important role of caspases in Smac/DIABLO release from the mitochondria.

Level of apoptosis was also measured by Nicoletti assay in presence of Zvad which was observed to be only 3% as compared to 43% in cells stimulated with 5-FU (Figure 17) indicating that by suppression of caspases and therefore Smac/DIABLO release into cytosol, apoptosis was also blocked.

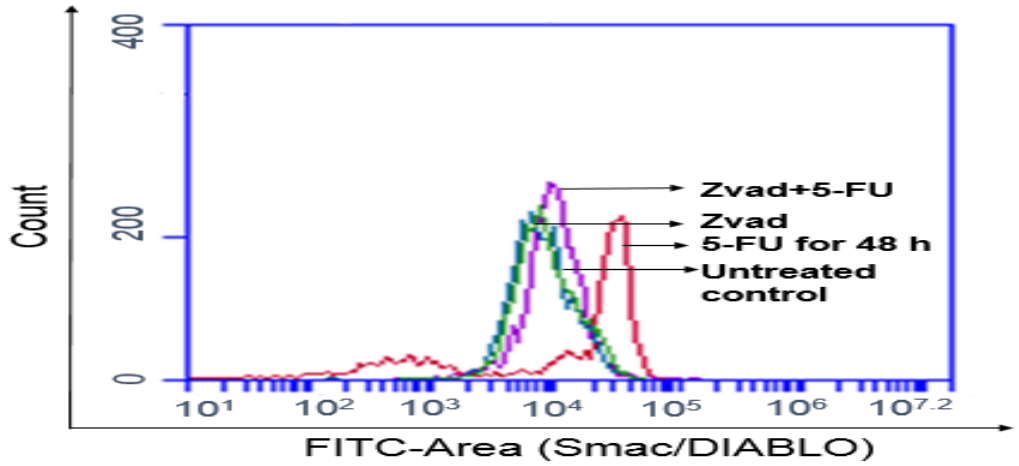

Figure 16- **Examination of Smac/DIABLO release in presence of Zvad, a pan-caspase inhibitor.** Smac/DIABLO release was analysed in HCT116 cells treated with Zvad and 5-FU in comparison to untreated cells treated with Zvad alone. Smac/DIABLO release in these samples were then analysed with cells stimulated with 5-FU for 48 hours and untreated control. The analyses were done after morphologically unchanged cells were gated in Forward/Side-scatter plot. Shift in the peak was observed only in cells stimulated with 5-FU for 48 hours. The fluorescent signals of cells stimulated with Zvad and 5-FU and with Zvad only were similar to untreated control depicting no Smac/DIABLO release.

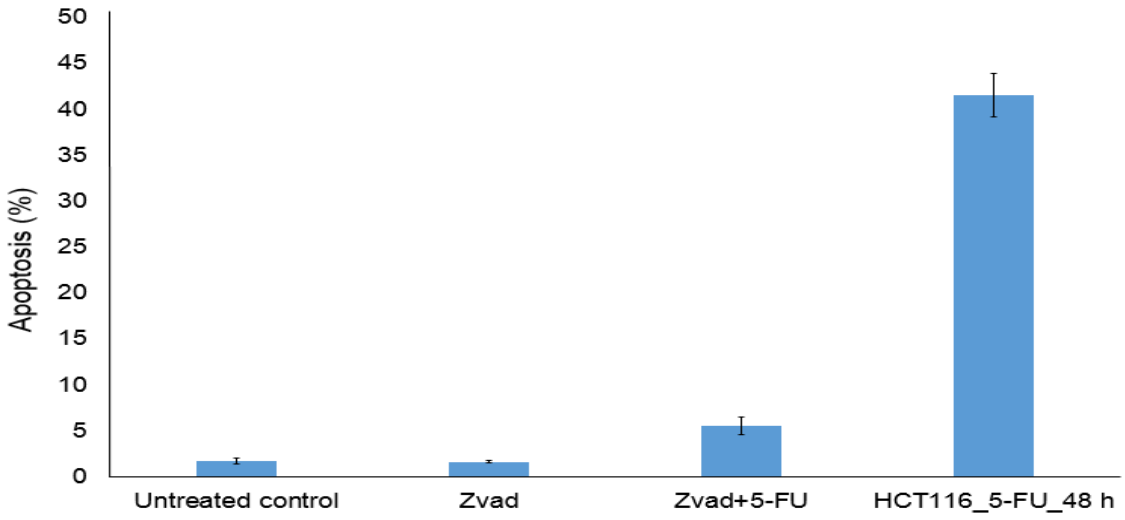

Figure 17- **Measurement of apoptosis in HCT116 cells in presence of Zvad, a pan caspase inhibitor.** Level of apoptosis was examined by Nicoletti assay in HCT116 cells when they were induced with 200 µM 5-FU for 48 hours in comparison to cells treated with Zvad and 5-FU. These samples were also compared with unstimulated cells treated with Zvad only and untreated control. Error bars denote the standard deviation of mean of triplicates samples.

5.4. Connecting the dots

With the finding that Smac/DIABLO release and apoptosis takes place only after 48 hours of 5-FU stimulation as compared to 24 hours and 36 hours of 5-FU treatment, the final piece of the puzzle was to determine the reason behind this observation. Membrane polarity was determined by 3-3' dihexyloxacarbcyanine iodide (DIOC(6)3) which is a hydrophilic fluorescent dye that stains mitochondrial membrane. Using DIOC(6)3 stain, it was observed that membrane depolarisation which is one of the characterising feature of programmed cell death and occurs early in apoptosis takes place only after 48 hours of 5-FU stimulation in comparison to 24 hours of treatment with 5-FU (Figure 18). Depolarisation of the membrane was observed as a shift in the fluorescent signal as compared to untreated control. At 24 hours of treatment, there was only a slight shift in the signal indicating that some amount of membrane depolarisation has taken place but at 48 hours of 5-FU induction there was a greater shift in the signal as compared to untreated control and 24 hours of 5-FU treatment. This result was in accordance with Figure 3 and Figure 11 representing that maximum amount of Smac/DIABLO release and highest percentage of apoptosis takes place at 48 hours of 5-FU stimulation may be because complete depolarisation of the mitochondrial membrane to facilitate Smac/DIABLO release into cytosol and later apoptosis only takes place at this time point.

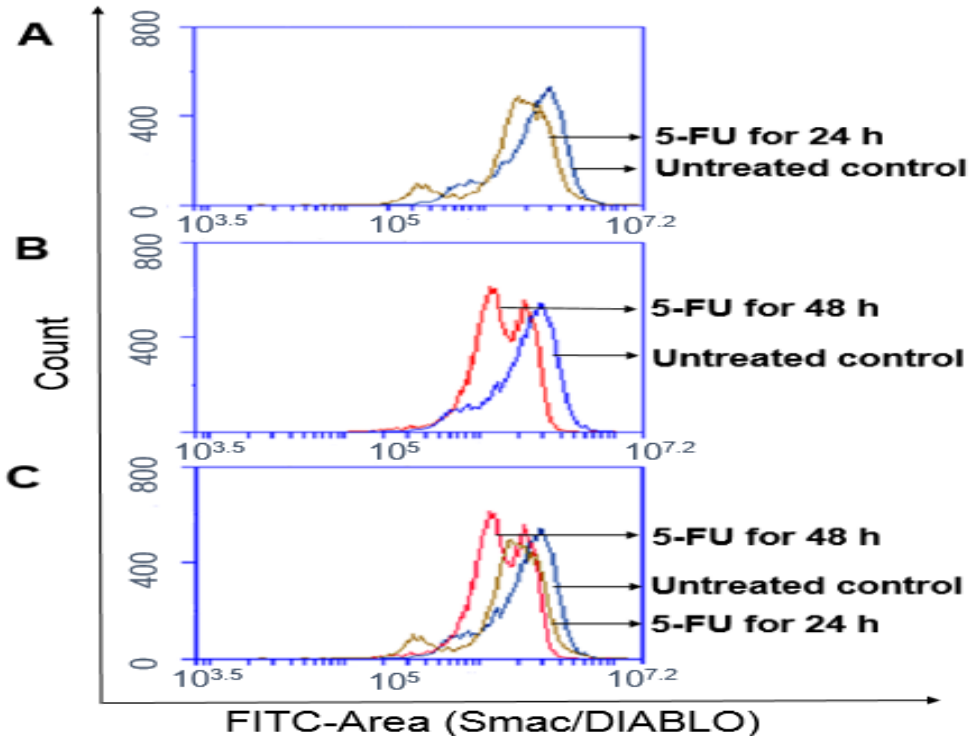

Figure 18- **Measurement of mitochondrial membrane depolarisation by DIOC(6)3 staining.** Membrane depolarisation was measured by staining with DIOC(6)3 in cells stimulated with 5-FU for 24 hours (A) and for 48 hours (B). Membrane polarity was compared in the treated cells as compared to untreated control (C). Membrane depolarisation in 5-FU induced cells was more than the untreated control as seen by a shift in the fluorescence signal but complete depolarisation which was observed by a larger shift in the fluorescent signal was observed only at 48 hours of 5-FU induction as compared to untreated control.

6. Discussion

Our research demonstrates several important findings that increase our understanding of the sequence of events and relationships between death receptor and intracellular stress mediating cytotoxic drug activated apoptosis. The apoptosis mediated by the intracellular stress and death receptor leads to release of inner mitochondrial membrane proteins such as cytochrome c and Smac/DIABLO. We have developed a FACS based Smac/DIABLO release assay in HCT116 cells undergoing apoptosis induced by the drug 5-Flurouracil (5-FU) to better understand the link between the release of proapoptotic factors from mitochondria into the cytosol and the different programmed cell death pathways. The FACS based method is quick, inexpensive and easy to perform as compared to the laborious biochemical method involving subcellular fractionation and western blotting using specific antibodies on mitochondrial and cytosolic fractions. Our FACS method can measure Smac/DIABLO release at single cell level as compared to western blotting which analyses the intracellular protein release in a whole cell population making it difficult to determine if Smac/DIABLO release has taken place in all the cells or if it is a partial redistribution in a small percentage of cells (Waterhouse and Trapani, 2003). Potentially, the FACS method can selectively analyse cancer cells in primary tumour material using specific surface markers along with anti-Smac/DIABLO antibody whereas in order to achieve the same by western blotting the cells need to be sorted first for identification followed by characterisation of tumour cells, which is time consuming and expensive.

The FACS based assay involves the use of Paraformaldehyde (PFA) for pre-fixation which preserves the integrity of the cells preventing their loss in subsequent steps

and use of Saponin for permeabilisation which creates pores in the membrane for antibody to traverse through and bind to Smac/DIABLO. The assay detects release of Smac/DIABLO on induction of apoptosis by formation of channels through the action of Bcl-2 family of proteins which can be pro-apoptotic such as the BH3 only protein, BID and multi domain proteins Bax and Bak or anti-apoptotic such as Bcl-2 and Bcl-xl (Kuwana et al, 2002). These proteins when oligomerise on the outer mitochondrial membrane form pores as they have structures similar to pore forming domain of diphtheria toxin and bacterial colicin leading to escape of large macromolecules such as Smac/DIABLO (Martinou and Green, 2001). It has been suggested by Adrian et al (2001) that when cytochrome c appears in the cytosol it forms the apoptosome complex leading to caspase activation which causes swelling of the mitochondrial matrix and formation of mega pores in the inner and outer mitochondrial membranes leading to Smac/DIABLO release (Martinou and Green, 2001). This caspase mediated feedback loop is important as caspase activity can be inhibited by XIAP which is counter measured by Smac/DIABLO release that binds to XIAP and neutralises its activity thereby leading to apoptosis (Adrian et al, 2001) as shown in Figure 19.

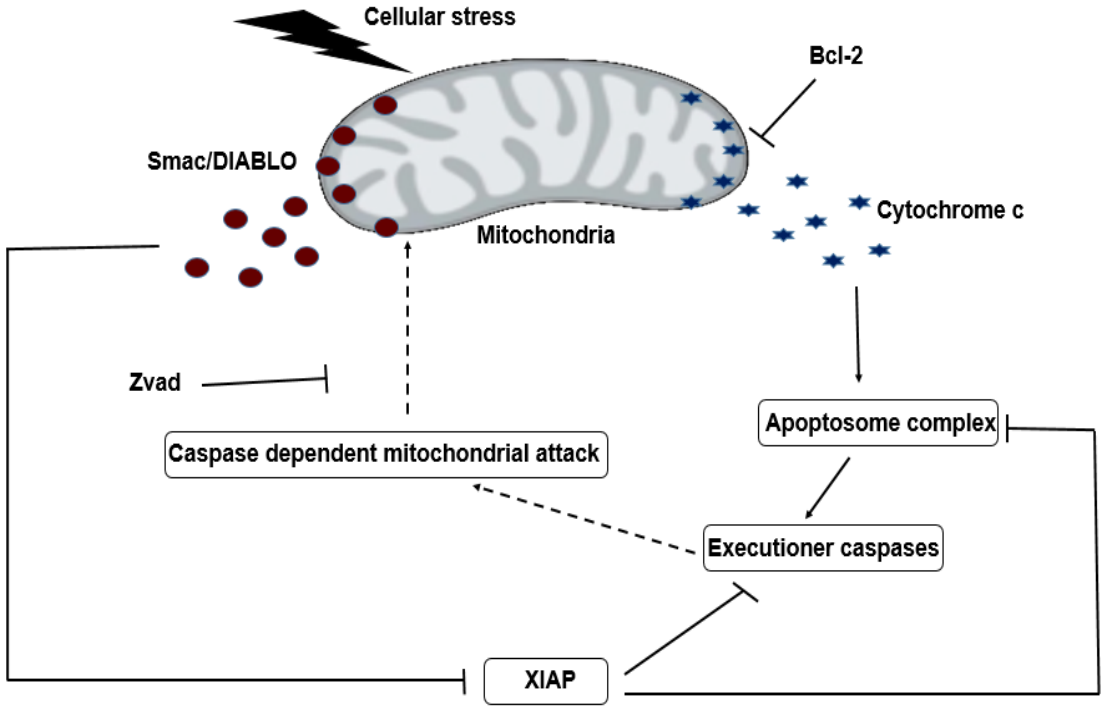

Figure 19- **Model illustrating the dependence of Smac/DIABLO release on executioner caspases**. According to this model, Bcl-2 regulates cytochrome c release from the mitochondria and on its release, cytochrome c interacts with protein Apaf-1 and caspase 9 forming apoptosome complex which activates executioner caspases leading to programmed cell death. Activity of the caspases can be blocked by inhibitory protein XIAP. As a counter measure these caspases mediate Smac/DIABLO release from the mitochondria by attacking the outer membrane and leading to formation of mitochondrial pores for Smac/DIABLO release. Smac/DIABLO in turn inhibits the activity of XIAP thereby promoting caspase mediated apoptosis (Adrian et al, 2001).

In contrast to the mechanism proposed by Adrian et al (2001), we tested a novel model according to which caspases can act upstream of mitochondria in stress induced apoptosis and not just downstream. This mechanistic model is proposed by the Mohr/Zwacka laboratory describing the role of caspase 8 in cleaving the BH3 only protein, BID to its truncated form t-BID which in turn promotes mitochondrial protein release. This was confirmed by measuring Smac/DIABLO release in cells treated with Zvad, a pan caspase inhibitor and it was observed that Smac/DIABLO release was impeded (Figure 16 and Figure 17) due to inhibition of caspases acting

upstream of mitochondria. The most prominent candidate in such a role is caspase 8.

Caspase 8 which has been proposed to act upstream of mitochondria is a part of a complex called as FADDosome consisting of FADD, RIP-1 , caspase 8 and caspase 10 in 5-FU induced apoptosis (Personal communication). In this complex pro-caspase 8 is activated by homotypic interactions (Hoffman et al, 2009) with other members of the FADDosome complex subsequently leading to cleavage of BID to t-BID which then activate Bax and Bak that inserts into the outer mitochondrial membrane to form channels (Kuwana et al, 2002).

This model also links the intrinsic pathway of apoptosis which is initiated by cytotoxic drug 5-FU to the extrinsic apoptotic pathway involving a death receptor to mediate programmed cell death. Usually caspase 8 activation is induced by death receptors via FADD mediated recruitment of caspase 8 to the DISC complex. But 5-FU can activate caspase 8 independent of a death receptor through the FADDosome complex. According to the model, TRAIL-R2 (DR5) a death receptor, modulates apoptosis not by caspase-8 activation but by JNK signalling. DR5 belongs to the family of Tumour Necrosis factor (TNF) superfamily of receptors which also includes TNFR-1, Fas (CD95), TRAIL-R1 (DR4), DR3 and DR6 that contains a death domain to modulate the apoptotic program (Shirley et al, 2011). 5-FU up regulates DR5 that in turn activates c-Jun-N-terminal protein kinase (JNK) that belongs to Mitogen activated protein kinase (MAPK) superfamily. JNK itself is activated by sequential phosphorylation by MAP4Ks and MAP3Ks such as Ask-1 followed by tyrosine and threonine phosphorylation catalysed by members of MAP2K family (Cowan et al, 2003; Dhanasekaran and Reddy, 2008) such as MKK4 and MKK7 which activate JNK and lead to activation of the Activator Protein 1 (AP-1) transcription factor. AP-

1 is a protein complex and consists of c-Jun, members of Fos family and activating transcription factor family, respectively (Liu et al, 2000). Activated AP-1 leads to transcriptional up-regulation of the WIP-1 phosphatase (Song et al, 2010), which regulates BID dephosphorylation and subsequent cleavage. We have observed that following DR5 knockdown by RNA interference, Smac/DIABLO release and apoptosis is blocked as shown in Figure 14 and Figure 4 respectively due to inhibition of signalling normally emanating from DR5 and mediated through JNK. These observations suggest that DR5 may be a critical factor in Smac/DIABLO release and lack of DR5 might serve as a predictive marker for 5-FU responses (Figure 20).

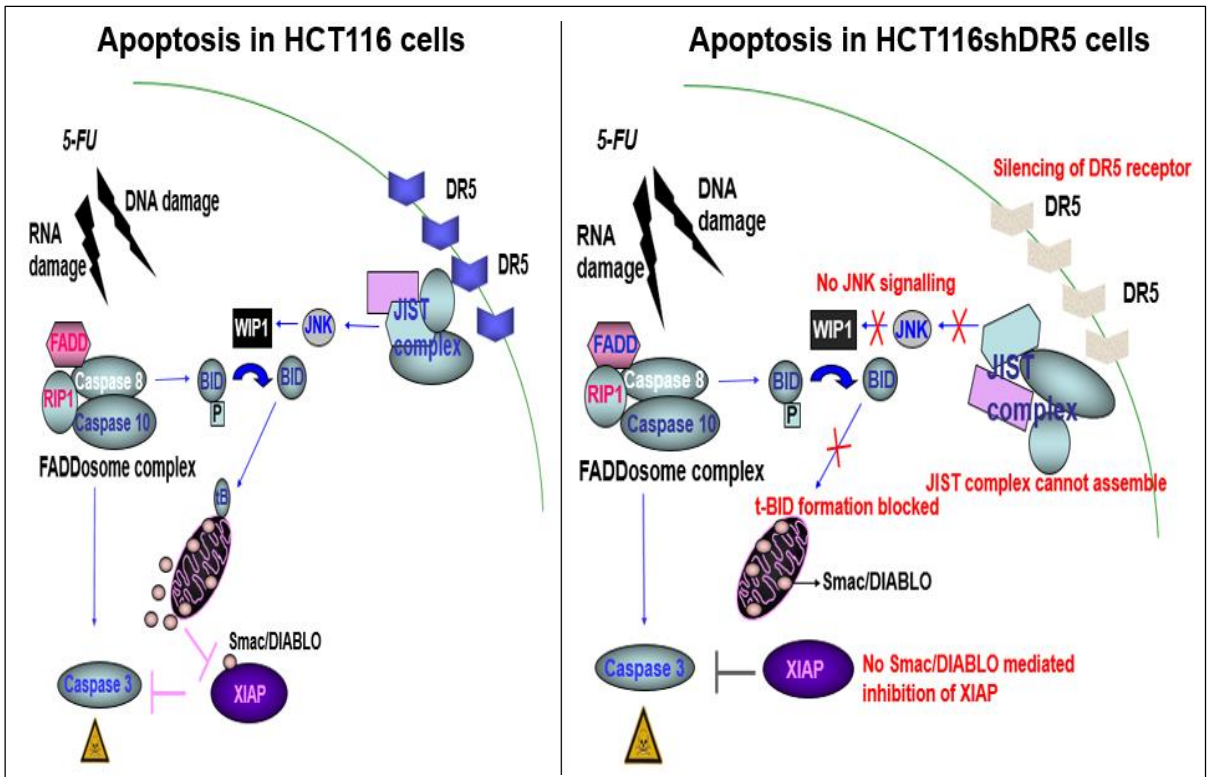

Figure 20 - **Comparison in apoptosis between HCT116 cells and HCT116shDR5 cells.** According to work by Mohr/Zwacka and the resulting model of apoptosis, silencing of DR5 receptor blocks Smac/DIABLO release by inhibition of JNK signalling pathway. JNK activates WIP-1 phosphatase that dephosphorylates BID so that caspase 8 mediated cleavage to t-BID can take place. But due to DR5 knockdown, t-BID formation is blocked thereby preventing Smac/DIABLO mediated inhibition of XIAP protein causing obstruction of caspase activation and inhibition of apoptosis.

The monoclonal antibody that detects Smac/DIABLO release recognises it in the cytosol which is observed as a shift in the Smac/DIABLO signal in apoptotic cells as compared to controls (Figure 8). Whereas, when cytochrome c is detected by FACS in mitochondria a reduction in signal is observed (Stahnke et al, 2004) rather than a upward shift in the signal indicating that antibody detects cytochrome c in mitochondria and on induction of apoptosis when cytochrome c is released from the mitochondria into the cytosol the signal then decreases, which is most likely due to a corresponding conformational change in cytochrome c and failure of the antibody to recognise cytochrome in this altered state. This led us to ask what limits and facilitates the interaction of antibody to Smac/DIABLO in the cytosol. Three theories are available to explain it. Firstly, it is possible that our Smac/DIABLO antibody cannot enter the mitochondria unlike 7H8.2C12, the cytochrome c antibody which detects cytochrome c inside the mitochondria (Stahnke et al, 2004) and that the Smac/DIABLO antibody can only detect the protein when it is released into the cytosol during apoptosis.

Secondly, according to Srinivasula et al (2001) when Smac/DIABLO is released from the mitochondria into the cytosol it performs a major function of binding to XIAP protein. XIAP belongs to a family of Inhibitors of Apoptosis Proteins (IAP) that regulates apoptosis downstream of mitochondria. XIAP specifically interacts with effector caspases, inhibits them and thereby prevents apoptosis. Smac/DIABLO binds to the third Baculovirus IAP repeat (BIR) domain of XIAP so that XIAP mediated caspase inhibition is released leading to apoptosis. Smac/DIABLO undergoes hydrogen bonding and van der Waal interactions with the BIR3 domain forming a Smac-XIAP complex with the N-terminus of Smac/DIABLO binding to the

surface groove of the BIR3 domain thereby preventing the inhibiting contact of XIAP with caspases. The formation of the Smac-XIAP complex could affect the binding of the Smac/DIABLO antibody to the Smac/DIABLO protein as the binding energy between antibodies and their protein antigens are affected by changes in hydrogen bonding, van der Waal contacts and hydrophobic interactions in the protein antigen (Goshorn et al, 1991). Results that points in this direction were observed by Jemmerson et al (1990) who showed that on its release in T-hybridoma cells, cytochrome c undergoes conformational changes may be due to its interaction with Apaf-1 and caspase 9 leading to formation of the apoptosome complex. The resulting conformational changes causes destabilisation of the tertiary structure of cytochrome c leading to opening up of a haeme crevice which is readily detected by a monoclonal antibody that binds to the omega loop region around residue 44 located near the opened haeme crevice. The binding site of our Smac/DIABLO antibody has not been identified but based on our findings we expect that conformational changes that takes place by formation of Smac-XIAP complex would influence the antibody binding in a positive way.

Thirdly, Smac/DIABLO undergoes maturation in mitochondria by cleavage of its signal peptide to gain its apoptotic activity (Du et al, 2000). It is possible that this maturation may affect antibody binding to Smac/DIABLO by exposing an epitope for antibody interaction. When mature Smac/DIABLO is released into the cytosol during apoptosis it can facilitate antibody interaction at the exposed antibody binding region which could be detected by FACS as a shift in the fluorescent signal.

One of the characteristic feature of apoptosis is dissipation of mitochondrial membrane potential which regulates the opening up of channels in inner mitochondrial membrane for pro-apoptotic protein release and their detection by

specific antibody. But in normal cells, membrane polarity is established by transfer of electrons through the electron transport chain (ETC) in the inner mitochondrial membrane and the movement of protons across the inner membrane from the matrix to the inter membrane space. The electron transport takes place by mitochondrial respiratory complexes I, II, III and IV as well as lipid soluble electron carrier, ubiquinone which is present between complexes I/II and III and water soluble electron carrier, cytochrome c which is present between complexes III and IV. The transfer of electrons and movement of protons generates ATP by ATP synthase which is also called as Complex V and the coupling between electron transport and ATP synthesis is called as oxidative phosphorylation. Cytochrome c plays an important role in Electron Transport Chain. It receives electrons from complex II and complex III and transfer them to oxygen in complex IV (Sun et al, 2013). During normal conditions when cytochrome c is involved in ATP generation, it is sequestered inside the mitochondria and mitochondrial membrane is also in resting stage. But on induction of apoptosis, membrane gets depolarised and cytochrome c is also released. Besides cytochrome c, our experiments have indicated a direct relationship between Smac/DIABLO release and membrane depolarisation also. As we have observed that both membrane depolarisation and Smac/DIABLO release takes place at the same time point which is at 48 hours of 5-FU stimulation (Figure 18). Thus indicating that Smac/DIABLO release in cytosol also depends on the membrane polarity to mediate apoptosis.

Thus, the flow cytometric procedure described in this report is useful to understand apoptosis by mitochondrial protein release in cancer cells and can help to elucidate how apoptosis is regulated at the mitochondrial level in anti-cancer therapy. Furthermore, the assay can be used in primary cancer cells to determine their

response to chemotherapeutic drugs at a molecular level. Due to cellular, genetic and biochemical heterogeneity of the tumour, we can determine if the cancer cells are resistant to apoptosis induced by the chemotherapeutic drug using the Smac/DIABLO release assay. This information can then be used to modify the drug for better therapeutic options and anti-cancer treatment.