The Death Receptor 5 Regulates 5-FU-Induced Apoptosis via the p38 SAPK Pathway

Aleyna Güney

A thesis submitted for the degree of

MASTER OF SCIENCE (by Dissertation)

Molecular Medicine

School of Life Sciences

University of Essex

2025

Contents

Table of Figures

List of Abbreviations

5-FU - 5-Fluorouracil

APC - Adenomatous Polyposis Coli

ATR - Ataxia Telangiectasia and Rad3-related protein

ATM - Ataxia Telangiectasia Mutated protein

Bcl-2 - B-cell lymphoma 2

CARD - Caspase-recruitment domain

Caspase - Cysteine-aspartic proteases

cFLIP - Cellular FLICE-like Inhibitory Protein

cIAP - Cellular inhibitor of apoptosis protein

CRC - Colorectal Cancer

DcR1 (TRAIL-R3) - Decoy Receptor 1 for TNF-Related Apoptosis-Inducing Ligand

DD -Death Domain

DED- Death Effector Domain

DISC - Death-Inducing Signaling Complex

DR - Death Receptor

ERK - Extracellular Signal-Regulated Kinase

FdUMP - 5-fluoro-2'-deoxyuridine monophosphate

FdUTP - Fluorodeoxyuridine Triphosphate

FADD - Fas-Associated protein with Death Domain

Fas-L - Fas Ligand

IKK - IκB Kinase

JNK - c-Jun N-terminal Kinase

MAPK - Mitogen-Activated Protein Kinase

MSCs - Mesenchymal Stem Cells

NF-κB - Nuclear Factor kappa-light-chain-enhancer of activated B cells

NEMO - NF-κB Essential Modulator

RAS - Rat sarcoma viral oncogene homolog

RIPK1 - Receptor-interacting serine/threonine-protein kinase 1

RFC - Reduced Folate Carrier

RTX - Raltitrexed

TACE - Tumor Necrosis Factor Converting Enzyme

TNF - Tumor Necrosis Factor

TNFR1 - Tumor Necrosis Factor Receptor 1

TRADD - TNFR1-associated death domain protein

TRAF - TNF Receptor-Associated Factor

TRAIL - TNF-Related Apoptosis-Inducing Ligand

TRAIL-R - TRAIL receptor

TS - Thymidylate Synthase

TTP - Thymidine triphosphate

XIAP - X-linked inhibitor of apoptosis

ABSTRACT

Apoptosis is an important process that helps to eliminate damaged or unwanted cells, including cancer cells during chemotherapy treatment. Previous work identified a novel apoptosis-inducing complex and pathway that is triggered by the chemotherapeutic drug 5FU. 5FU causes DNA damage that gives rise to the activation of ATR and subsequent upregulation of caspase-10, which in turn recruits FADD, caspase-8, TRAF2 and RIP1 to a complex termed FADDosome. Within the FADDosome caspase-8 is activated leading to downstream apoptosis signalling via Bid and mitochondria. Interestingly, 5FU induced apoptosis is lower in p53 knock-out cells without affecting the FADDosome-mediated caspase-8 activation. Therefore, this study investigates the role of p53 and the molecular pathways it controls in 5-FU-induced apoptosis in colorectal cancer cells. I found that 5-FU regulates FADDosome-induced apoptosis through p53-dependent upregulation of TRAIL-receptor 2 (TRAIL-R2) also known as Death Receptor 5 (DR5), leading to a partial activation of the receptor independent of its ligand TRAIL. This activation triggers non-canonical signalling including the p38 MAPK pathway, which is absent or substantially reduced in p53-null and DR5 knock-down cells. Co-treatment of cells with 5-FU and p38 inhibitors diminished apoptosis levels by about 50%. These results demonstrate that the role of p53 in chemotyherapy-induced apoptosis is more complex and potentially more multi-facetted than expected. TRAIL-R2 activated p38 MAPK might promote the activation of Bid by active caspase-8 or other molecular mechanisms that lead to mitochondrial outer membrane permeabilization (MOMP). As MOMP is needed for the release of Smac/Diablo from the mitochondria to inhibit the anti-apoptotic protein XIAP, this mechanisms is essential for the full execution of apoptosis, and can explain the relative resistance in p53-null cells.

INTRODUCTION

1. Colorectal Cancer

Colorectal cancer (CRC), which includes colon and/or rectal cancer, is a major public health concern as it is the world's third most diagnosed and second most lethal cancer (Hossain et al., 2022). Approximately 75% of CRC cases occur sporadically, unrelated to genetic predisposition or family history. However, inherited disorders like Lynch syndrome and familial adenomatous polyposis can also lead to CRC (Yamagishi et al., 2016). The disease originates from a loss-of-function mutation in tumour suppressor genes, followed by the activation of transcription factors that promote uncontrolled proliferation of cancer cells (Tariq and Ghias, 2016). The accumulation of genetic alterations, such as mutations in adenomatous polyposis coli (APC), rat sarcoma viral oncogene homolog (RAS), or tumour protein 53 (TP53), cause the normal colonic mucosa to form adenomatous polyps that gradually advance to dysplasia and, eventually, adenocarcinoma during a 5-15 year period (Yamagishi et al., 2016). Diagnostic methods can only detect 40% CRC cases in early stages (I-II), and the treatment at these stages is possible with surgery. CRC cells are hyperproliferative, and therefore they can quickly become invasive and metastasise to other organs through blood and lacteals. At the metastatic stages (III-IV) the treatment strategy involves surgery, chemotherapy, radiation, and targeted therapy (Hosain et. al., 2022).

2. Mechanisms of Widely Used CRC Chemotherapeutics

Treatment approaches for CRC patients differ based on the stage of the disease and the specific molecular changes driving the cancer. A standardized approach for CRC treatment combines surgery with chemotherapeutics like 5-Fluorouracil (5-FU), and Irinotecan. These agents inhibit enzymes involved in DNA replication or nucleotide base synthesis, leading to DNA damage and tumor cell death (An et al., 2007, Carlsen et al., 2021, Gallois et al., 2022). Today, 5-FU-based regimens, especially when combined with leucovorin (LV), stand out as the most successful first-line chemotherapy for CRC.

5-FU is a synthetic uracil analogue with a fluorine atom at the C-5 position in place of hydrogen, that is used to treat >2 million cancer patients each year worldwide (Gmeiner and Okechukwu, 2023). It has been a key drug in the treatment of colorectal cancer for more than 60 years. However, tumour cell resistance remains a significant limitation to the clinical use of 5-FU, therefore, understanding the underlying signalling mechanisms induced by this agent is crucial for enhancing drug efficacy and determining effective co-treatments for better cancer targeting (Zhang et al., 2008). The canonical molecular mechanism of 5-FU involves disrupting the folate–homocysteine cycle and inhibiting pyrimidine synthesis by targeting Thymidylate Synthase (TS) (Thorn et al., 2011). TS catalyses the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (TMP), a precursor for thymidine triphosphate (TTP), which is an essential component in DNA synthesis. (Peters et al., 2002). By interfering with this process 5-FU causes DNA damage and induces programmed cell death (apoptosis).

The 5-FU metabolic pathway starts with with its conversion to fluorodeoxyuridine via thymidylate phosphorylase (TYMP), and then thymidine kinase transforms fluorodeoxyuridine into FdUMP (Thorn et al., 2011). FdUMP is a competitive inhibitor with a Ki in the low nanomolar range that has a high binding affinity to TS. It forms a ternary complex with TS and CH2-THF, which is the reduced folate co-factor that act as methyl donor in the catalysis of 2'-dUMP to dTMP (thymine) (Peters et al., 2002, Rivory, 2002). The disruption of dTMP synthesis with 5-FU results in reduced deoxythymidine triphosphate (dTTP), indirectly affecting the levels of other nucleotides (dATP, dGTP and dCTP) hence creating an imbalance, leading to severe DNA damage resulting in stalled replication forks and initiation of apoptosis (Zhang et al., 2008).

The disruption of dTTP synthesis via TS inhibition plays a key role in inhibiting cancer cell growth and is fundamental to the therapeutic action of not only 5-FU, but other chemotherapeutics such as raltitrexed (RTX/TOMUDEX®) (Peters et al., 2002). Raltitrexed, a folate cofactor analogue, resembles water-soluble vitamin B. Specifically designed to mimic 5-10-methylene tetrahydrofolate (CH2-THF), Raltitrexed enters cells via the reduced folate carrier (RFC) (Rivory, 2002). Once within, the enzyme folylpolyglutamate synthase (FPGS) rapidly polyglutamates RTX.

This polyglutamation prevents cellular efflux of RTX and causes enhanced and extended inhibition of TS, leading to inadequate dTTP synthesis and subsequent DNA damage and apoptosis (Blackledge, 1998).

Another widely used chemotherapeutic for CRC is Irinotecan, a semisynthetic and water-soluble derivative of camptothecin. It targets S (DNA synthesis) and G2 (pre-mitotic) phases of the cell cycle, which results in cell cycle arrest (Reyhanoglu and Smith, 2023). Upon activation by carboxylesterases, Irinotecan transforms into its biologically active metabolite SN38. This metabolite inhibits Topoisomerase I (TOPO1), a nuclear enzyme crucial for relaxing the DNA strand by creating single-strand breaks, preventing DNA supercoiling during replication (Ozawa et al., 2021). Irinotecan-induced inhibition of TOPO1 promotes the supercoiling of the DNA strand and causes torsional stress. This results in the accumulation of DNA strand breaks, leading to disrupted DNA replication and apoptosis (Yakkala et al., 2023).

3. Regulated Cell Death

Regulated cell death (RCD) is a genetically controlled process designed to maintain cellular homeostasis. It is classed as lytic or non-lytic cell death. Lytic cell death, such as necroptosis and ferroptosis, results in the discharge of cellular contents into the environment. In contrast, non-lytic cell death, such as apoptosis, involves enclosing cellular fragments in apoptotic bodies and discarding them to avoid an inflammatory reaction (Yang et al., 2021).

Apoptosis is considered to be the main cell death-inducing mechanism in cancer therapy. It is an energy-dependent defence mechanism by which cells undergo self-suicide to control cell proliferation or to eliminate cells with DNA damage (Azzwali and Azab, 2019). The dysregulation or inhibition of apoptosis disrupts the growth balance and leads to the uncontrolled proliferation of cells, which is often associated with the development of cancer (Kim et al., 2002)

There are 3 distinct phases of apoptosis: induction, execution, and degradation (Figure 2). In the induction phase, a change in the cellular environment, such as

stress induced by chemotherapeutics that cause DNA damage in cancer treatment, activates apoptosis-related genes like p53 or pro-apoptotic Bcl-2 family proteins (Bax, Bad, Bak, Bid, Bcl-XS) (Hilario et al., 2010, Wilson, 1998). Cancer cells often develop mechanisms to escape from this phase and gain drug resistance. This phase is followed by the execution phase of apoptosis, considered the point of no return, where the cell dictates the direction of apoptotic pathways by activating either death receptors or mitochondrial outer membrane permabilisation (MOMP), thereby triggering a stress response. In both responses, a family of aspartate-specific cysteine proteases (caspases) serve as key initiators and executioners of the phase of apoptosis (Zimmermann and Green, 2001). These caspases initially exist as inactive zymogens and are classified as initiators (caspase -8 and -9) and executioners (caspase -3, -6, and -7). Initiator caspases are activated through induced proximity, while executioner caspases are dimers in their inactive form, and undergo activation via proteolytic cleavage by upstream caspases (Gu et al., 2011).

The execution phase of apoptosis is followed by the degradation phase, which involves the DNA degradation, dismantling, and engulfment of the cell (Hilario et al., 2010). Cells in this phase lose cell-to-cell contact with neighbouring cells and exhibit morphological changes such as shrinkage, condensed chromatins, and membrane blebbing. Apoptotic bodies containing nuclear fragments are formed and subsequently swallowed by the phagocytes nearby, preventing an inflammatory reaction (Yang et al., 2021).

4. Revisiting Conventional Apoptosis Pathways: Initial Findings

Traditionally, apoptosis has been categorized into two distinct pathways: the intrinsic pathway, activated by cellular stress or mitochondrial signals, and the extrinsic pathway, initiated by death receptor activation. However, it is crucial to acknowledge that these rigid pathways may not always fully capture the complex nature of apoptotic process. This section will provide a comprehensive examination of the

traditional understanding of these pathways, as well as examine cases where alternative mechanisms are involved.

4.1 The Mechanism of the Intrinsic Pathway of Apoptosis

Since the discovery of p53 protein in the late 1970s the intrinsic pathway (stress activated pathway, mitochondrial pathway) has been a hot topic in cancer research and is heavily studied. In this pathway, cell death depends on p53, a tumour suppressor protein with numerous transcriptional target genes (~500). P53 regulates cellular response to a wide range of stress signals including DNA damage induced by chemotherapeutic agents, growth factor deprivation, ER stress, and UV radiation (Green and Llambi, 2015). The target of the p53 in the intrinsic apoptosis is interacting with the anti- and pro-apoptotic Bcl-2 homology (BH) domain containing protein family (Aubrey et al., 2018). Within the pro-apoptotic arm of the Bcl-2 family, two distinct subclasses emerge: the "ultimate MOMP effectors," Bax and Bak, known for their ability to induce mitochondrial outer membrane permeabilization (MOMP), and the BH3-only class, comprising both "activators" such as Bid and Bim, which are capable of directly activating Bak and Bax, and "derepressors" including PUMA, NOXA, and Bad, which counteract the anti-apoptotic Bcl-2 proteins to release the pro-apoptotic Bax and Bak (Aubrey et al., 2018, Vaseva and Moll, 2009). Conversely, the anti-apoptotic counterparts of the Bcl-2 family, namely Bcl-2, Bcl-xL, and Mcl-1, bind to pro-apoptotic Bax and Bak, thereby maintaining their inactivation (Figure 1).

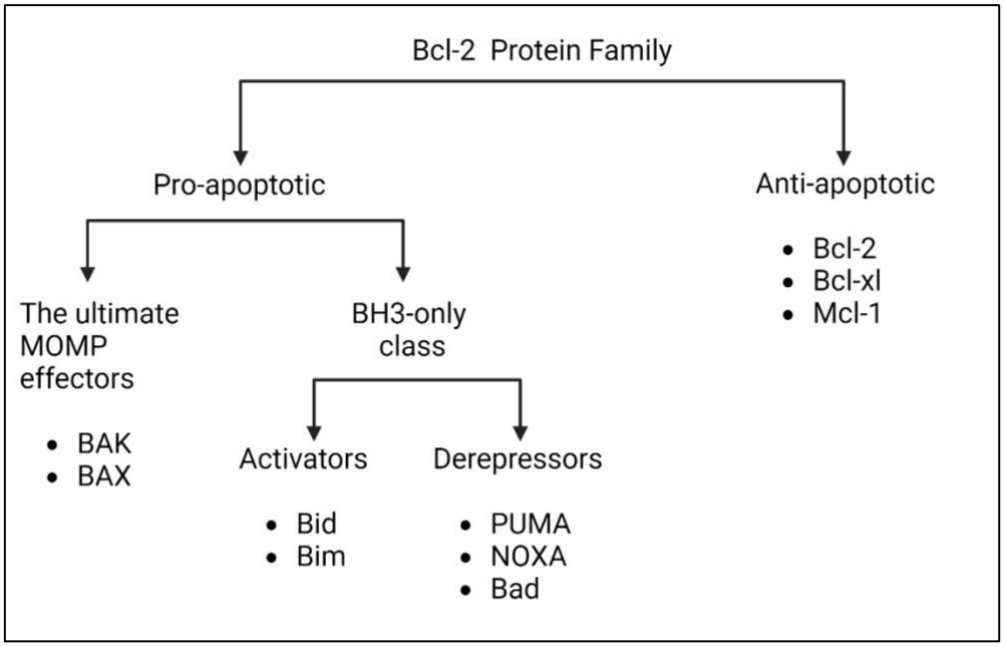


***Figure 1**. Classification of Bcl-2 Family Members.*

The figure demonstrates Bcl-2 family members classified as pro-apoptotic members (ultimate MOMP effectors: Bax, Bak; BH3-only activators: Bid, Bim; derepressors: Bad, Noxa) and anti-apoptotic members (Bcl-2, Bcl-xL). Pro-apoptotic members are subdivided into the ultimate MOMP effectors BAX and BAK, which directly permeabilize the mitochondrial outer membrane, and BH3-only proteins, which include activators (e.g., Bid, Bim) that directly engage BAX/BAK, and derepressors (e.g., Bad, Noxa) that neutralize anti-apoptotic members. Anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL, Mcl-1) sequester BH3-only proteins or inhibit BAX/BAK to block MOMP and promote cell survival. The interplay between these groups determines cell fate in response to stress or survival signals.

Under normal physiological conditions, cellular levels of p53 remain low, nearly undetectable, as it is targeted for proteasomal degradation by the E2 ligase MDM2. However, upon receiving a stress signal the cell upregulates ataxia telangiectasia mutated protein (ATM), or ataxia telangiectasia and Rad3-related protein (ATR), which in turn facilitate the inhibition of MDM2. Consequently, p53 evades ubiquitination, leading to an increase in its cellular abundance and therefore its activation (Aubrey et al., 2018). Activated p53 proceeds to interact with Bcl-2 proteins, either by repressing anti-apoptotic members (Bcl-xL, Mcl-1, Bcl-2) to activate ultimate MOMP effectors Bax/Bak, or by inducing BH3-only proteins (PUMA, NOXA, BIM, BAD) to disrupt the inhibitory effects of anti-apoptotic Bcl-2 family proteins, therefore liberating Bax/Bak (Aubrey et al., 2018). Following, the ultimate MOMP effectors integrate into the mitochondrial membrane, oligomerize, and induce

MOMP, forming dynamic lipid pores that release cytochrome c and second mitochondria-derived activator of caspase (Smac/Diablo) into the cytosol (Ghobrial et al., 2005, Vaseva and Moll, 2009). Here Smac neutralizes X-linked inhibitor of apoptosis (XIAP) while cytochrome-c interacts with apoptotic protease-activating factor 1 (APAF1) and triggers hydrolysis of the Apaf1 cofactor dATP to dADP (Green and Llambi, 2015). Following the exchange of dADP with exogenous dATP, seven APAF1–dATP–cytochrome-c units undergo oligomerization, creating an active apoptosome. Within the apoptosome core, the caspase-recruitment domain (CARD) on APAF1, binds to the CARD of the initiator caspase-9, promoting the activation and autoprocessing of previously inactive caspase-9 monomers (Shakeri et al., 2017). Caspase-9 then cleaves and activates downstream effector caspases caspase-3 or -7, which then cleave cellular substrates and thus apoptosis is induced (Figure 2) (Jan and Chaudhry, 2019).

4.2 The Mechanism of the Extrinsic Pathway of Apoptosis

The extrinsic pathway of apoptosis is characterised by death receptor (DR)-mediated signalling initiated by specific cell surface or soluble proteins, the so called death ligands. So far, three main death ligands have been identified: tumour necrosis factor (TNF-α, Fas ligand (Fas-L), and TNF-related apoptosis-inducing ligand (TRAIL). These ligands bind to the extracellular domain of their cognate death receptors, which include a cytoplasmic death domain. A single ligand can bind to several death receptors. At present, six members of the TNF receptor family have been identified: TNF-R1 (CD120a), Fas (APO-1/ CD95), DR3 (APO-3, LARD, TRAMP, WSL1), DR4 (TRAIL-R1, APO-2), DR5 (TRAIL-R2, KILLER, TRICK2), and DR6 (Jan and Chaudhry, 2019).

The binding of DRs with their ligands or the overexpression of the DRs is the signal that triggers cytotoxicity via the extrinsic pathway (Oh et al., 2015). Since death domains have a propensity to bind together, ligand-bound death receptors pull their cytoplasmic domains into close proximity and transmit the cytotoxic signal, by attracting downstream adaptor proteins that bear a death domain (DD) and a death

effector domain (DED) (Ashkenazi and Dixit, 1998). The death receptors then bind to the adapter proteins (such as Fas-associated protein with death domain (FADD) and TNFR1-associated death domain (TRADD) though their DD (Green and Llambi, 2015) and this interaction recruit initiator caspases -8 or -10, forming a complex named “Death Inducing Signalling Complex” (DISC) (Figure 2) (Gu et al., 2011). From here, the DISC either signals for apoptosis by auto-cleaving of the caspases and activation of the executioner caspases, or gene expression and cell proliferation mediated by nuclear factor-kappa B (NF-κB) and mitogen activated protein kinases (MAPKs) (Gu et al., 2011, Guicciardi and Gores, 2009). The latter signalling pathways are known as non-canonical death receptor signalling.

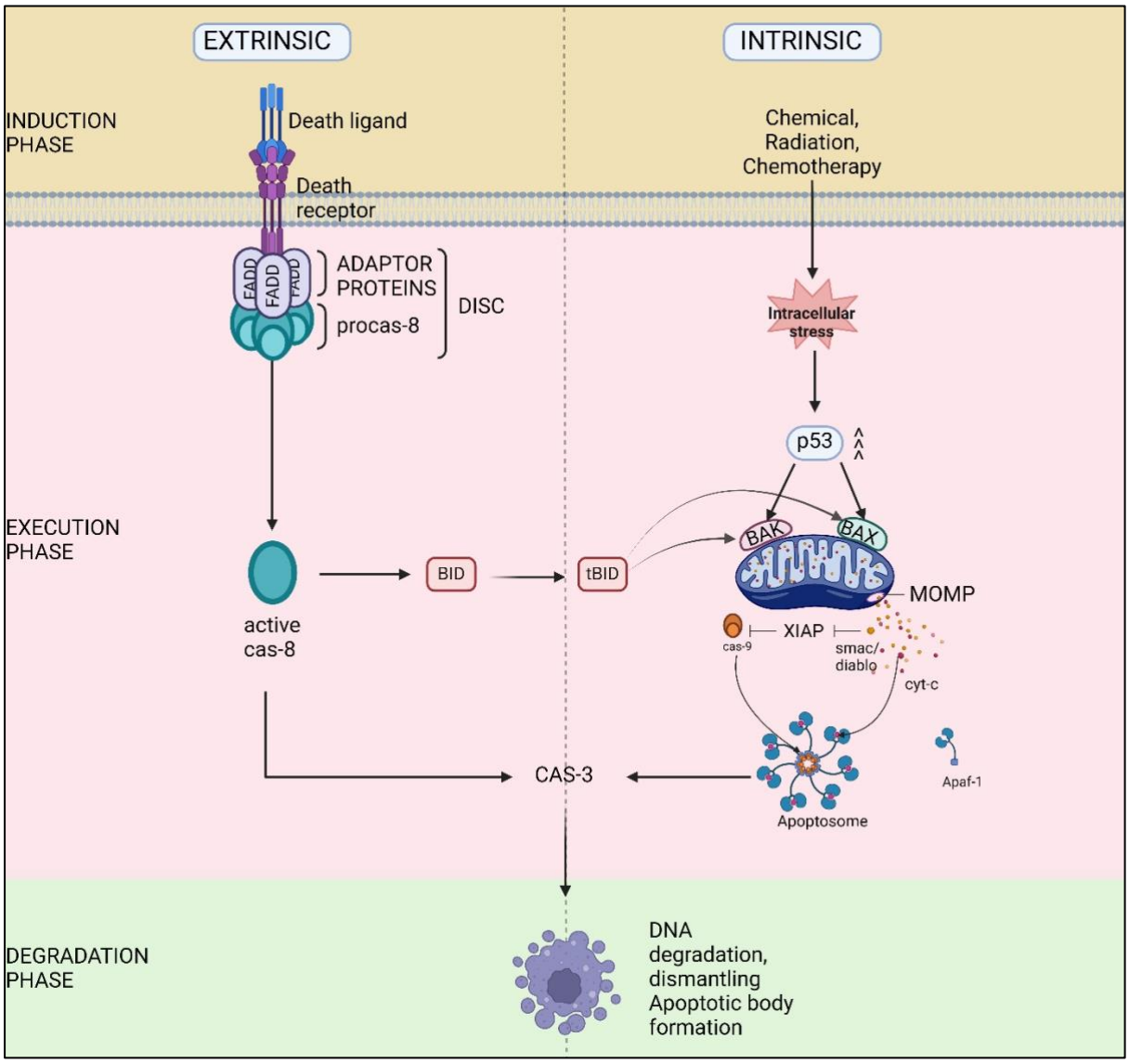


Figure 2. Stages and Pathways of Apoptosis.

This diagram shows the molecular pathways and the phases of apoptosis. The left and right portions shows the two principal routes that trigger apoptosis, extrinsic and intrinsic, respectively. The Extrinsic Pathway (Left) is triggered when extracellular death ligands (e.g.,TRAIL) bind to their death receptors on the cell membrane, resulting in the creation of DISC. DISC is made up of adaptor proteins like FADD and procaspase-8. When activated, procaspase-8 is split into active caspase-8, which can directly activate executioner caspase-3. Furthermore, caspase-8 cleaves the BH3-only protein BID into truncated BID (tBID), which connects the extrinsic and intrinsic pathways by enhancing mitochondrial outer membrane permeabilization (MOMP).Intrinsic Pathway (Right): This pathway is activated by intracellular stress signals, such as DNA damage from radiation, or chemotherapy. These stress signals often activate the tumour suppressor protein p53, which then increases the production and activation of the pro-apoptotic Bcl-2 family members BAX and BAK. These proteins oligomerize and rupture the mitochondrial membrane, resulting in MOMP and the release of cytochrome c (cyt-c) and Smac/DIABLO. Cytochrome C binds to Apaf-1 and procaspase-9 to form the apoptosome, which activates caspase 9. Caspase-9 then activates the downstream executioner caspase-3. Smac/DIABLO inhibits XIAP, a caspase inhibitor, to ensure complete caspase activation.

4.2.1. Fas Mediated Signalling of the Extrinsic Pathway

Fas is a transmembrane receptor that belongs to the TNF-receptor family. It is activated upon binding to its ligand Fas-L, which trimerizes the receptor, setting off the assembly of the Death-Inducing Signalling Complex (DISC) (Figure 3). Within this complex, FADD engages with the death domain of the Fas receptor (Wajant, 2002). Simultaneously, the "death effector domain" of FADD interacts with a repeated tandem domain within the zymogen form of caspase-8 (Ashkenazi and Dixit, 1998). Fas-L-induced clustering triggers autoproteolytic processing of the caspase-8 via induced proximity, liberating active proteases. From here, the amount of active caspase-8 determines the downstream apoptotic cascade. In cells exhibiting high caspase-8 activity, termed type I cells (Ozören and El-Deiry, 2002), direct induction of apoptosis occurs through the activation of effector caspases-3, 6 or -7, independently of mitochondria. Conversely, in type II cells the activation of effector caspases rely on an amplification loop which involves caspase-8 cleaving the Bcl-2 family member Bid (Guicciardi and Gores, 2009, Ozören and El-Deiry, 2002). Truncated Bid (tBid) then translocates to mitochondria, where it initiates the intrinsic apoptosis pathway by activating Bax and Bak to promote mitochondrial outer membrane permeabilization (MOMP). As a result, the IAP antagonists Smac/Diablo

and Omi are released and act to neutralize XIAP, and cytochrome-c is released which contributes to the activation of caspase-9 via apoptosome formation (Green and Llambi, 2015). Active caspase-9 then triggers caspase-3 activation completing the intrinsic cascade (Wajant, 2002). This amplification loop initiated by death receptors that is later regulated by mitochondria serves as a cross talk of both the extrinsic and the intrinsic pathway.

4.2.2. TNF-Receptor Mediated Signalling Cascade

The tumour necrosis factor (TNF) is a type II transmembrane protein containing a TNF homology domain, and is expressed as a trimer at the plasma membrane (Webster and Vucic, 2020). In the canonical TNF pathway TNF ligands initiate the signalling cascade by binding to their cognate receptors. These ligands are pleiotropic cytokines which are ultimately produced via cleavage at the membrane by tumour necrosis factor converting enzyme (TACE), and they transmit signals through two receptors: TNFR1 and TNFR2 (Gough and Myles, 2020).

TNFR1 and TNFR2 differ at the structural level. The intracellular region of the TNFR1 possesses a DD which allows homo- and hetero-typic interactions with other DD-containing proteins such as FADD, TRADD (Grethe et al., 2004, Webster and Vucic, 2020). TNFR2, on the other hand, lacks the DD and instead has a TNF Receptor Associated Factor (TRAF) binding site and therefore is not regarded as a death receptor (Webster and Vucic, 2020).
The signalling pathways triggered by TNFR1 primarily promote proliferation; hence, TNFR1-induced apoptosis is considered to occur indirectly (Nair et al., 2014). Typical TNFR1-mediated signalling begins with TNF ligand binding to the receptor, which trimerizes TNFR1 and initiates intracellular signalling via its death domain (Grethe et al., 2004). The adaptor protein TNFR1-associated death domain protein (TRADD) is then recruited to the receptor's cytoplasmic death domain and it serves as a scaffold for recruiting additional adaptor proteins to the receptor and forms “complex-I”, which is composed of the receptor-interacting serine threonine kinase 1 (RIPK1), FADD, TRAF2, TRAF5, cIAP1, cIAP2, and linear ubiquitin chain assembly complex

(LUBAC) (Baud and Karin, 2001, Tenev et al., 2011). With cIAPs (inhibitors of apoptosis) and LUBAC present, RIPK1 of the complex I undergoes ubiquitylation and recruits the IKK complex to the complex-I. IKK is a complex made of a regulatory component NEMO and the two kinases: IKKα and IKKβ, and it regulates the activation of NF-κB. The recruitment of IKK to the complex I activates NF-κB ultimately leading to proliferation (Tenev et al., 2011, Henry and Martin, 2017).

TNFR-1 can also signal for apoptosis or necroptosis when NF-κB-mediated gene expression is inhibited. This inhibition triggers complex I to detach from TNFR1, leading to the formation of another complexes known as complex-IIA, and complex-IIB.

The complex-IIA is composed of FADD, and caspase-8, and signals apoptosis via FADD mediated activation of caspase-8 (**Figure 3**) (Demarco et al., 2020). The complex-IIB forms upon cIAP depletion or inhibition in the cells and signals for necroptosis. This complex is composed of FADD, RIP1, and caspase-8, and RIP1 activity is the main signal for caspase-8 activation and subsequent apoptosis (Tenev et al., 2011, Demarco et al., 2020). Complex IIB is sometimes called the Ripoptosome, however the Ripoptosome can form independent of the TNF ligand whereas complex-IIB is described as dependent on TNF-TNFR signalling.

4.2.3. TRAIL-R (DR4/DR5) Mediated Signalling Cascade

The DR4/5 mediated signalling pathway has been researched extensively as it has a direct access to the apoptotic machinery and ability to induce apoptosis in cancer cells without affecting normal cells. Previous clinical trials using recombinant TRAIL or death receptor agonists have not been successful due to the short half-lives of these proteins and difficulties in administering them in vivo (Stuckey and Shah, 2013). On the other hand, later research demonstrated MSCs carrying TRAIL were able to move into the lung cancer sites and trigger apoptosis (Mohr et al., 2008). This implies that DR4/5 signalling is still a viable target for research and treatment advancement.

In the canonical DR signalling cascade, 4 receptors are identified so far, and of these 4 receptors, Decoy Receptor 1 (TRAIL-R3/DcR1) and Decoy Receptor 2 (TRAIL-R4/DcR2), bear no death domain and therefore cannot signal for apoptosis, whereas DR4/TRAIL-R1 and DR5/TRAIL-R2 bear a death domain and are able to induce apoptosis (**Figure 3**), but DR5 is regarded as the main apoptosis-inducing receptor.

DR5 signalling initiates upon binding with their ligand, TRAIL leading to receptor trimerization and recruitment of FADD via DD interaction. FADD also contains a death effector domain (DED) at the N-terminus (Bellail et al., 2009).Through this DED domain it binds to the pro-domain of caspase-8, bringing caspase-8 monomers into close proximity (Green and Llambi, 2015). The downstream apoptotic cascade mirror those of Fas receptor signalling with cell type specific responses. While the mechanisms of both receptors are similar, DR5 mediated apoptosis is of therapeutic interest due to its selective toxicity towards cancer cells.

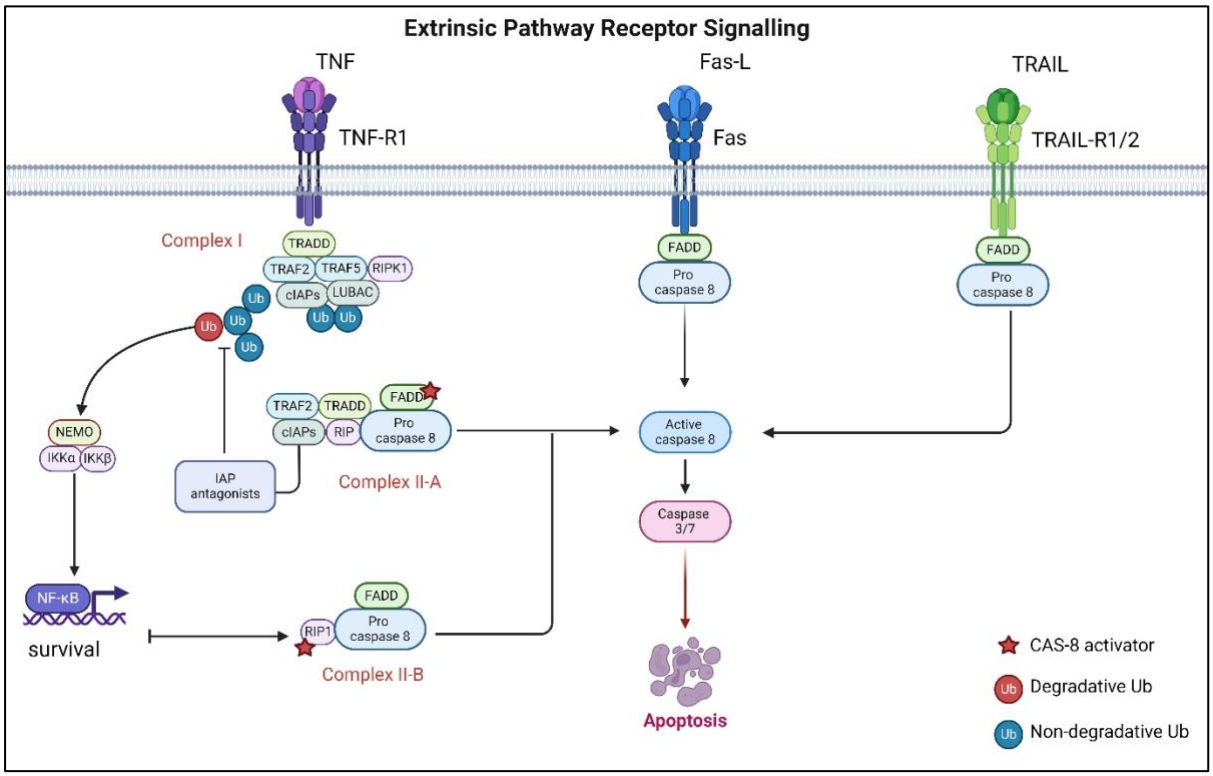


Figure 3. *Extrinsic Pathway Receptor Signalling Pathways.*

This figure illustrates the key components and pathways involved in extrinsic death receptor signalling. It includes TNFR1 with Complex I and II-a/b, Fas receptor, and TRAIL-R signalling. 1) Binding of TNF to TNF-R1 triggers the formation of Complex I,

which includes TRADD, TRAF2/5, RIPK1, cIAPs, and LUBAC. Ubiquitination of RIPK1 leads to NF-κB activation and promotes cell survival. In the presence of IAP antagonists, the pathway shifts to form Complex II-A (TRADD, TRAF2, RIP, FADD, and pro-caspase-8) which leads to apoptosis or Complex II-B (RIP1, FADD, and pro-caspase-8), leading to caspase-8 activation and apoptosis. 2) FasL and TRAIL activate their respective receptors (Fas and TRAIL-R1/2), recruiting FADD and pro-caspase-8 to form the death-inducing signalling complex (DISC). Activated caspase-8 then initiates the executioner caspases 3/7, resulting in apoptosis.

5. Novel Apoptosis Pathways Induced by Chemotherapy

The effects of genotoxic stress trigger a response in cancer cells, resulting in self-destruction by apoptosis. The standard signalling of apoptosis is framed by the intrinsic and extrinsic pathways, which provide an easily accessible framework for understanding cell death. While these pathways provide a structured signalling scaffold, the actual process of apoptosis involves a more complex interaction of proteins and pathways that lack such a strict signalling configuration. Particularly in chemotherapy-induced apoptosis, the apoptotic pathways, and proteins involved in this process can change depending on factors such as cell type, drug type, and molecular context/make-up.

5.1. Ripoptosome: A novel death inducing complex in the TNF-R signalling

The traditional description of the TNF-R pathway was centred around complex I that lead to cell proliferation and complex-II that triggers apoptosis, respectively. Proliferation occurs through complex-I-mediated NF-κB signaling, while apoptosis happens via complex-IIA when NF-κB signaling is suppressed, and through complex-IIB when cIAPs are depleted (Demarco et al., 2020). A study in 2011 revealed the formation of an additional death-inducing platform consisting of molecular factors of the TNF cascade, challenging the conventional understanding of stress-induced apoptosis. The study demonstrated that treatment with Etoposide, a Topoisomerase-II inhibitor that causes double-stranded DNA breaks, and/or SMAC mimetics (SM), which are specific inhibitors of apoptosis proteins (IAPs), resulted in

the formation of a unique complex made up of RIP1, FADD, and caspase-8, independent of death ligands like TNF, TRAIL, and FasL, as well as the upstream mitochondrial pathway (Tenev et al., 2011). As this complex could not be classified as complex-II due to its independent formation from the death ligands, it was termed the "Ripoptosome", a novel cell death platform that converted proinflammatory cytokine signals into pro-death signals. This finding suggests that etoposide, previously known to induce the caspase-9 pathway, can also activate alternative apoptosis pathways independent of caspase-9. Later studies have explored Ripoptosome formation in other chemotherapy induced apoptosis signalling. Cisplatin is a commonly used colorectal cancer treatment that hinders DNA replication by forming intra-strand platinum-DNA adducts, which triggers apoptosis (Rebillard et al., 2010). In a 2015 study, cisplatin in combination with high concentrations of Chal-24, a potential chemotherapy agent, enhanced ERK (a member of the MAPK family) induced proteasomal degradation of c-IAPs and the formation of the Ripoptosome complex as well as enhanced caspase-8 recruitment to the Ripoptosome in lung cancer cells (Shi et al., 2015). These findings provide insight into the previously unknown mechanisms underlying chemotherapy-induced apoptosis. Furthermore, these data opens up the possibility that other apoptosis pathways beyond the classical caspase-9 pathway may exist in chemotherapy induced apoptosis.

5.2 FADDosome: A novel death inducing complex induced by 5-FU mediated caspase-10 activation

The understanding of how 5-FU induces cell death has been a topic of ongoing debate for the last 40 years, and it has been described as an inducer of the classic intrinsic apoptosis pathway. However, research conducted by our group has revealed a novel apoptosis-inducing complex triggered by 5-FU, termed the FADDosome (Mohr et al, 2018).

The FADDosome is formed in response to 5-FU-induced DNA damage, which is detected by the ATR kinase. Activated ATR upregulates caspase-10 independently of p53, setting the stage for FADDosome formation. The key components of the FADDosome are caspase-10, FADD, caspase-8, RIP1, and TRAF2. Caspase-10

and RIP1 recruit TRAF2 to the FADDosome complex, which then ubiquitinates cFLIP-L, an inhibitor of caspase-8 activation. Ubiquitination and subsequent degradation of cFLIP-L frees caspase-8, which acts as the initiator caspase. Additionally, although this complex includes extrinsic pathway components like caspase-8 and FADD, it forms independently of the extrinsic pathway ligands and receptors such as FasL, TNF, and TRAIL and its cognate receptors (Mohr et al., 2018).

5.3 Mitogen Activated Protein Kinases in Apoptosis

Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases involved in various cellular processes, including cell proliferation, differentiation, survival, senescence, stress responses, and apoptosis (Taylor et al., 2013). The MAPK family members are the extracellular-signal-related kinases (ERKs), the p38 MAPK, and the c-Jun N-terminal kinase (JNK). In general, the ERK MAPK is associated with the regulation of cellular proliferation, while the JNK and p38 pathways are activated by inflammatory cytokines, heat shock, and ultraviolet radiation and more associated with apoptosis (Chowchaikong et al., 2018). Consequently, they are termed as stress activated protein kinases (SAPK) and therefore, this study focused on SAPKs, specifically p38 MAPK. So far, 4 isoforms named α, β, γ, and δ of the p38 SAPK have been identified, with p38α and p38β being the most abundant in tissues. Nearly all isoforms are expressed in intestinal tissue, however, p38α seems to be the most abundant (Grossi et al., 2014, Phan et al., 2023).

Like the other MAPKs, p38 SAPKs are activated through dual phosphorylation at a Thr-X-Tyr motif within the kinase activation loop (Yue and López, 2020). This activation is mediated by other upstream serine-threonine kinases through a three-tiered phosphorylation cascade: MAPKKKs, such as ASK1, TPL2, and MEKK3, phosphorylate and activate the MAPKKs (MKK3/MKK6), which activate p38 SAPK isoforms (Pranteda et al., 2020).

The role of SAPK in cellular processes is context-dependent, influenced by cell type, stimulus, and signalling environment. This variability allows these kinases to function differently under various conditions, making them a key focus of research. As expected, studies on p38 SAPK in apoptosis show both pro- and anti-apoptotic roles.

For instance, many (Han et al., 2022, Yue and López, 2020, Phan et al., 2023) reported the cancer enhancing abilities of p38 in the chemotherapy induced cell death pathways. For instance, Stramucci et al. (2019) found that activated p38δ MAPK promotes pro-survival signalling and reduces sensitivity to 5-FU in CRC cells. However, a follow-up study by the same group showed that 5-FU-induced apoptosis also activates the p38α isoform, leading to an anti-cancer effect (Pranteda et al., 2020). Similarly, a 2018 study found that inhibiting p38α with SB203580 reduced cleaved caspase-3 levels and, consequently, apoptosis in LoVo colorectal cancer cells (Chowchaikong et al., 2018). Another study reported that p38 SAPK is activated by cisplatin in various cell lines, and inhibiting p38 led to cisplatin resistance (Losa et al., 2003). This supports that the role of p38 MAPK depends on drug, as well as the specific isoform involved plays a crucial role in cellular and therapy responses.

Apart from the role of p38 MAPK in the drug induced apoptosis, its role in death receptor pathways is not clearly elucidated. Some research revealed the involvement of the p38 MAPK in the death receptor signalling pathways such as its essential role for the expression of Fas and FasL upon T-Cell receptor therapy engagement, as the inhibition of p38 MAPK with SB203580 reduced both surface Fas expression and Fas mRNA levels (Hsu et al., 1999). Additionally P38 was found to mediate TNF-induced apoptosis of endothelial cells via phosphorylation and downregulation of Bcl-2 family members (Grethe et al., 2004). Moreover, activation of p38 MAPK has been observed to facilitate apoptosis triggered by damnacanthal, a specific inhibitor of tyrosine kinase via transcription of DR5, TNF-R1, and Bax. This activates caspase-8, cleaves Bid, and releases cytochrome C, which then activates caspase-3 .

While p38 MAPK is mostly known for activating transcription factors, it can also directly influence mitochondria apoptosis thorough the regulation of Bcl-2 members. A study using arsenite-treated PC12 cells showed that p38 can directly phosphorylate Bim_{EL}, a pro-apoptotic Bcl-2 family protein. This phosphorylation boosts Bim_{EL} ability to promote apoptosis. They also showed that when a mutant with p38 phosphorylation site removed, Bim_{EL} regulated apoptosis was significantly

reduced. Therefore, this suggests that p38 does not just act in concert with the death receptor signalling, but can also directly modify Bcl-2 family proteins to induce mitochondrial damage and drive the cell into apoptosis (Cai et al., 2006).

6. Project Overview: Rationale, Aims, and Objectives

The ongoing debate on the apoptotic pathways activated by chemotherapeutic agents revolves around whether these agents predominantly initiate the death receptor pathway or the mitochondrial pathway. As previously stated, these pathways provide an easy structure to understand apoptosis, when it comes to chemotherapy induced apoptosis but the actual pathways are more complex and the rigid structure does not apply to all cell types and contexts. Numerous apoptosis-inducing agents, including 5-FU, cisplatin, and etoposide, which were initially classified as inducers of the intrinsic pathway (Wu and Ding, 2002) have since been found to activate death receptors or induce alternative death-inducing platforms independently of the canonical caspase-9 pathway (Can et al., 2013, Mohr et al., 2018, Rebillard et al., 2010, Tenev et al., 2011)

Previous research carried out by our group has demonstrated 5FU-induced apoptosis is mediated by a caspase-9-independent process that is initiated by caspase-8 (Mohr et al., 2018). Further analysis of caspase-8 activation has shown that the activation was independent of p53, because the levels of activated caspase-8 stayed the same in p53-null cells, although the level of apoptosis was reduced; showing p53 plays a role in 5-FU mediated apoptosis but is not required for caspase-8 activation. The 5-FU induced caspase-8 activation was also found to be death receptor independent, but similar to p53, silencing of DR5 by RNAi led to a reduction of apoptosis. Therefore, we hypothesised that p53-mediated DR5 might induce an apoptosis-regulating pathway that controls FADDosome-triggered signalling. Surprisingly, the apoptosis levels remained constant when TRAIL (the ligand of DR4 and DR5) was neutralised with an antibody, therefore we concluded TRAIL does not take part in this process and that the upregulation of DR5 on the surface of cells might be sufficient. As caspase-8 activation was not affected by the DR5 status, we

focused on DR5 non-canonical signalling and MAPK p38 in particular as a role for JNK was ruled out through earlier work (**Figure 4**).

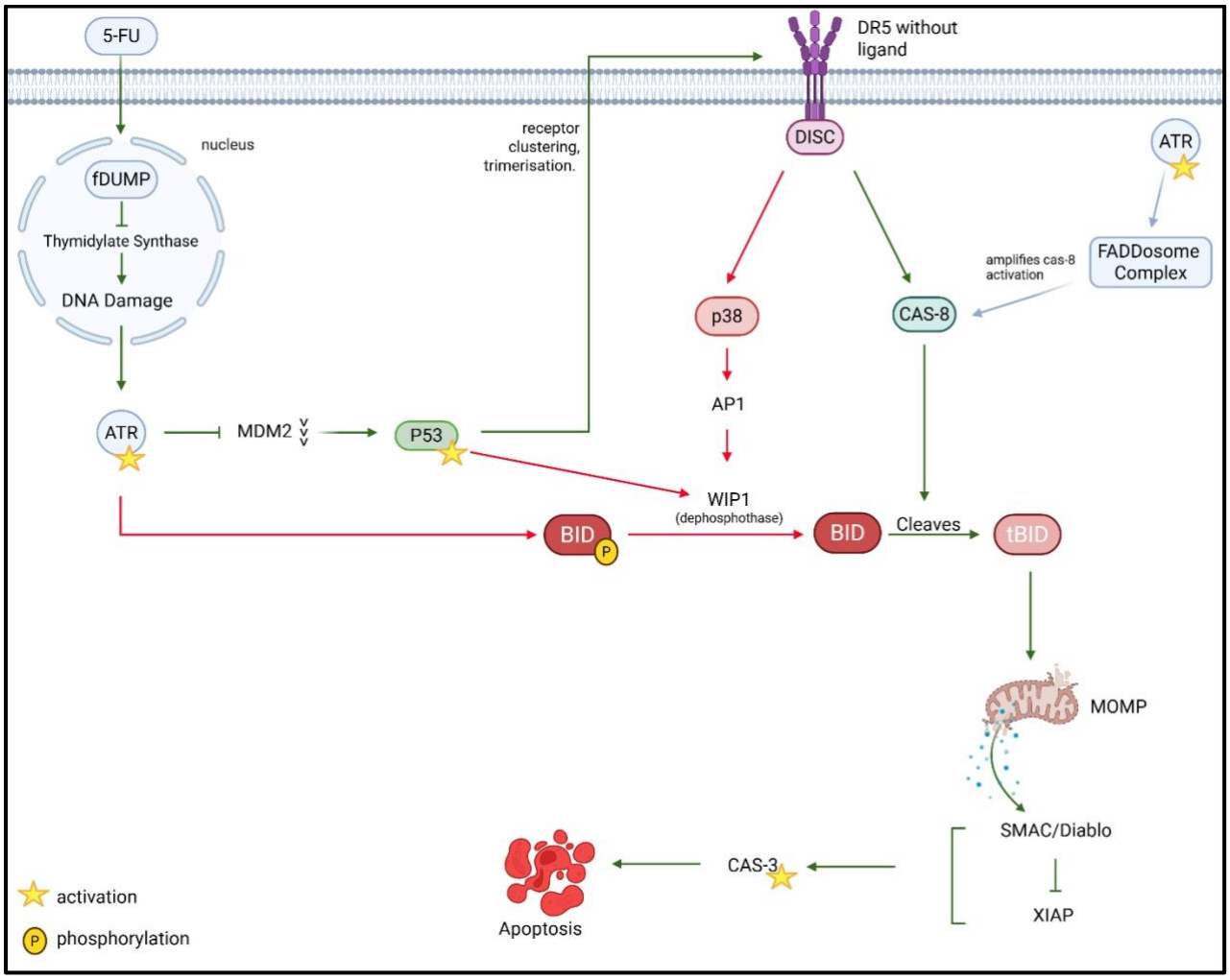


***Figure 4**. Mechanisms of 5-FU Induced Apoptosis via DR5 pathway*

5-FU induced apoptosis starts with its metabolite fDUMP inducing DNA damage. ATR senses the DNA damage, and activated p53 by inhibiting MDM2. ATR also phosphorylates Bid, which makes it harder to cleave by caspase-8. DR5 upregulation by p53 triggers a partial DISC activation without TRAIL ligand, leading to p38 MAPK activation. This activates transcription factors, which dephosphorylates Bid. Caspase 8 truncates Bid, which activates Bak/Bax, and causes MOMP. As a result of MOMP, Smac/Diablo is released and inhibits XIAP, freeing caspase-3 activity. Simultaneously, the FADDosome enhances caspase-8 activation, converging to induce apoptosis.

METHODS

1. Materials

The following materials were used in the experiments: McCoy's Medium (Lonza), Trypsin-EDTA (Lonza), PE anti-human CD261 (DR4, TRAIL-R1) antibody (BioLegend), PE anti-human CD262 (DR5, TRAIL-R2) antibody (BioLegend), PE anti-mouse IgG kappa (BioLegend). For p38 MAPK inhibitor experiments doramapimod (BIRB 796), and pamapimod inhibitors were used.

2. Cell Culture

The HCT116, HCTp53-/-, RKO, RKO-E6, and LoVo cell lines were grown in McCoy's medium (Lonza) containing 10% fetal calf serum and penicillin–streptomycin. The cells were cultivated in a 5% CO_2 atmosphere at 37°C.

3. Apoptosis Assay

Cells were grown to the desired density in 24-well plates. The initial media was removed and stored. Cells were washed with 200 µL of PBS per well, ensuring the PBS covered the entire surface. The PBS was collected into respective tubes as it may contain cells. Next, 200 µL of trypsin was added to each well and incubated for 1 minute to dislodge the cells, which was confirmed under a microscope. 500 µL of medium was then added back into the wells and mixed to deactivate the trypsin. The cells were transferred into tubes and pelleted by centrifugation at 2000 rpm for 5 minutes. The supernatant was removed, and the cells were resuspended in 300 µL of propidium iodide buffer (prepared by adding 1 mL of propidium iodide to 50 mL of Nicoletti buffer), vortexed, and kept on ice until measurement.

4. Fluorescence-Activated Cell Sorting Staining

Cells were seeded at a density of 3×10^5 cells per well. After 24 hours, one set of cells was treated with 5-FU and incubated for 48 hours, while another set was treated with 5-FU the following day and incubated for 24 hours. After the respective incubation periods, cells were harvested by removing the initial media from the flask, retaining 500 µL for the next steps. To wash the cells, 200 µL of PBS was added, followed by retaining the PBS back to the respective tubes. Following, 200 µL of trypsin was added, and the cells were incubated for 3 minutes. Successful trypsin activity was confirmed under a microscope, after which the retained 500 µL of initial media was added to deactivate the trypsin. The cells were then pelleted by centrifugation at 5000 rpm for 1 minute, and the supernatant was removed. The harvested cells were resuspended in 300 µL of PBS and divided equally into three tubes. Following by the addition of the antibody, the cells were incubated on ice for 20 minutes. After incubation, each sample was washed with 1 mL of PBS. The cells were then pelleted again and resuspended in 200 µL of 4% paraformaldehyde (PFA) before analysed by flow cytometry.

5. Crystal Violet Staining

In a 24-well plate, 200 µL of 4% paraformaldehyde was added to each well, and the plate was incubated at room temperature for 60 minutes. After incubation, the wells were washed with distilled water three times. The residual water was removed by inverting the plate and allowing it to dry completely. Once dried, 200 µL of crystal violet solution was added to each well, and the plate was incubated at room temperature for 60 minutes. The cells were then washed with distilled water three times and allowed to dry completely. To solubilize the crystal violet, 400 µL of methanol was added, and the plate was placed on a shaker for 60 minutes. The optical density of each well was measured at 570 nm (OD570), with the OD570 of non-stimulated cells set to 100 as the baseline.

6. Western Blotting

Whole-cell extracts (30 µg of total protein) were separated on a denaturing 12.5% SDS-PAGE gel and transferred to a PVDF membrane (Fisher) via electroblotting for 1 hour. The membrane was then blocked with 4% non-fat dry milk in PBS containing 0.3% Tween-20 (PBST). This blocking solution was also used for all subsequent antibody incubations and washing steps. Primary antibodies were incubated overnight at 4 °C. Afterward, the membrane was washed four times with the blocking solution. Secondary antibody incubation was performed for 1 hour at room temperature, followed by four washes in blocking buffer and two final washes in PBS. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (Fisher).

7. Statistical Analysis

Data obtained from flow cytometry measurements were analysed using Excel pivot table and charts. Calculations of the averages and standard deviation were carried out. Experimental values were expressed as the mean value ± standard deviation. The significance of the variance between groups was calculated using a standard t-test, with p-values of ≤0.05 considered significant (*), p-values of ≤0.01 considered very significant (**), and p-values of ≤0.001 considered highly significant (***).

8. Cell Counting Analysis

Cells were seeded at a density of 1×10^5 cells per well and incubated for 24 hours. Cells were then treated with 50 µM 5-FU, either alone or in combination with 5, 10, or 20 µM pamapimod. Control treatments included pamapimod alone. The same procedure was repeated for doramapimod. Cells were imaged using an IncuCyte at 0, 24, and 48 hours post-treatment. Image segmentation was performed using

Cellpose, and the segmented images were processed in Fiji (ImageJ). To quantify total cells vs dead cells, images were thresholded, and the “Analyze Particles” function was used with the roundness filter to distinguish dead cells from live ones. Roundness parameters were set to 0.8–1.0 based on apoptotic morphology.

RESULTS

1. 5-FU induces caspase dependent apoptosis in the colorectal cancer cell lines.

In this study, we investigated the apoptotic pathways activated by 5-FU in colorectal cancer cells. We utilised RKO and HCT116 cells and treated these cells with 5-FU. To determine 5-FU induces cell death through apoptosis, we performed a flow cytometry-based apoptosis assay with the caspase inhibitor zVAD-fmk to confirm the apoptotic nature of the cell death **(Figure 5)**. Additionally, we assessed cell viability using crystal violet staining to complement our findings **(Figure 6)**. In both cell lines 5-FU induced a dose-dependent cell death. RKO cells were more sensitive to 5-FU than HCT116 with up to 80% cell death at the highest concentration. The addition of zVAD-fmk together with 5-FU (200 μM) significantly reduced cell death to levels of 10%, compared to 90% for 200 μM 5-FU treatment without zVAD-fmk, confirming that the cell death mechanism is caspase-dependent apoptosis. Similarly, HCT116 cells, though more resistant, displayed increased apoptosis with higher concentrations of 5-FU, reaching up to 40% at the highest dose. Again, the combination treatment with 5-FU and zVAD-fmk reduced cell death to 10%. These results indicate that 5-FU induces caspase-dependent apoptosis in both RKO and HCT116 cells **(Figure 5)**.

Crystal violet assay results were consistent with the flow cytometry-based apoptosis assay results. 5-FU induced a dose dependent loss of cell viability, and the treatment with zVAD-fmk was able to restore cell viability by approximately 25%-points in RKO cells **(Figure 6)**. These findings show that apoptosis is part of to the anti-cancer (i.e. loss/reduced number of cells) effect of 5FU. However, as the crystal violet staining

compared to the flow cytometric apoptosis assay could not distinguish between 5FU-induced cell cycle effects and cell death , we opted focus on the apoptosis assay in subsequent experiments.

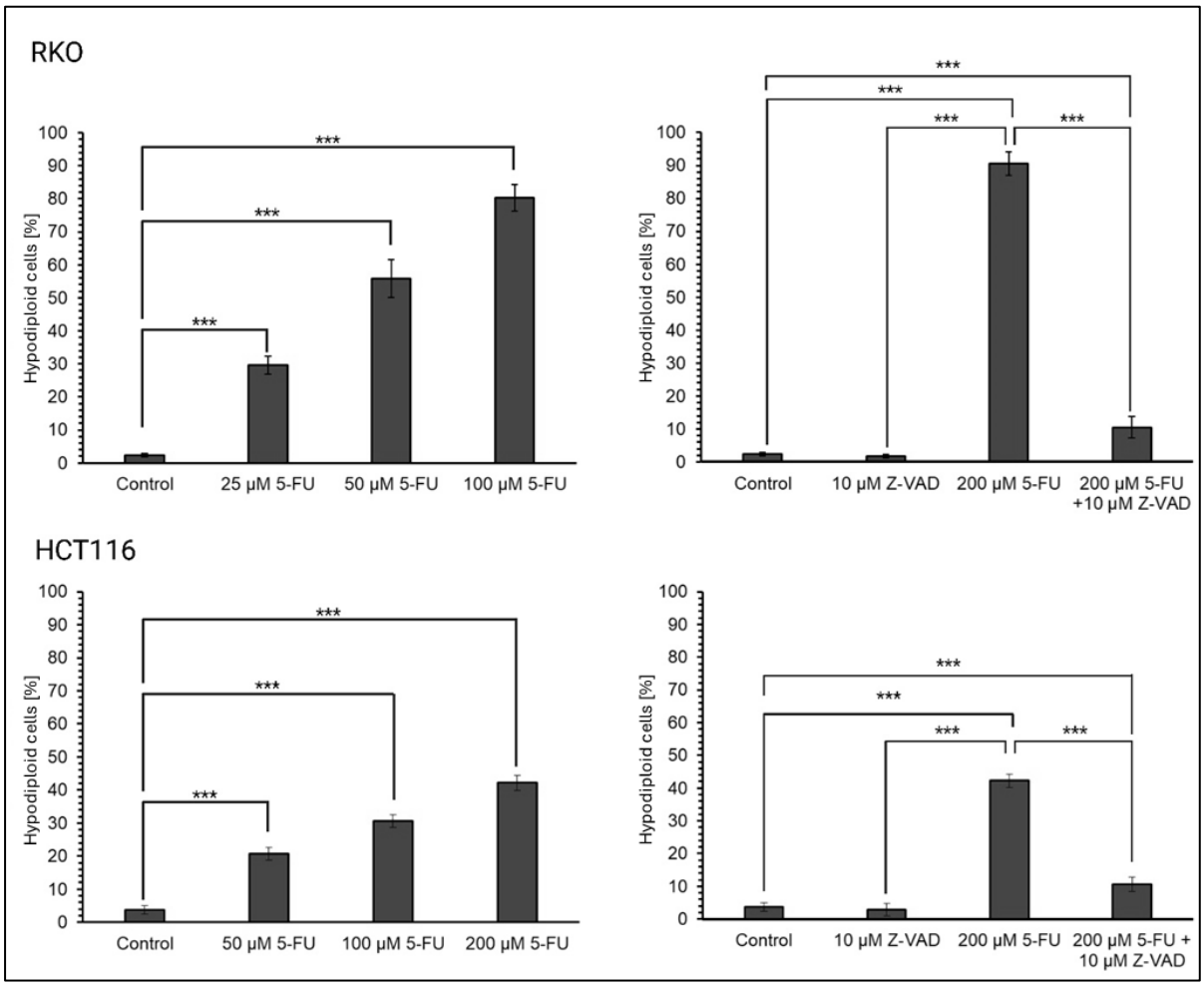


Figure 5. *Dose-dependent effects of 5-FU on apoptosis in colorectal cancer cells.*

*Cells treated with varying concentrations of 5-FU and incubated for 48 hours. After treatment, cells were stained with propidium iodide and analysed for apoptosis using flow cytometry. The results show a dose dependent increase in cell death for both RKO and HCT116 cells. The addition of the caspase inhibitor zVAD-fmk significantly reduced cell death, indicating caspase dependency. Error bars represent standard deviations, and p-values were calculated to determine statistical significance. (P<0.0001= ***).*

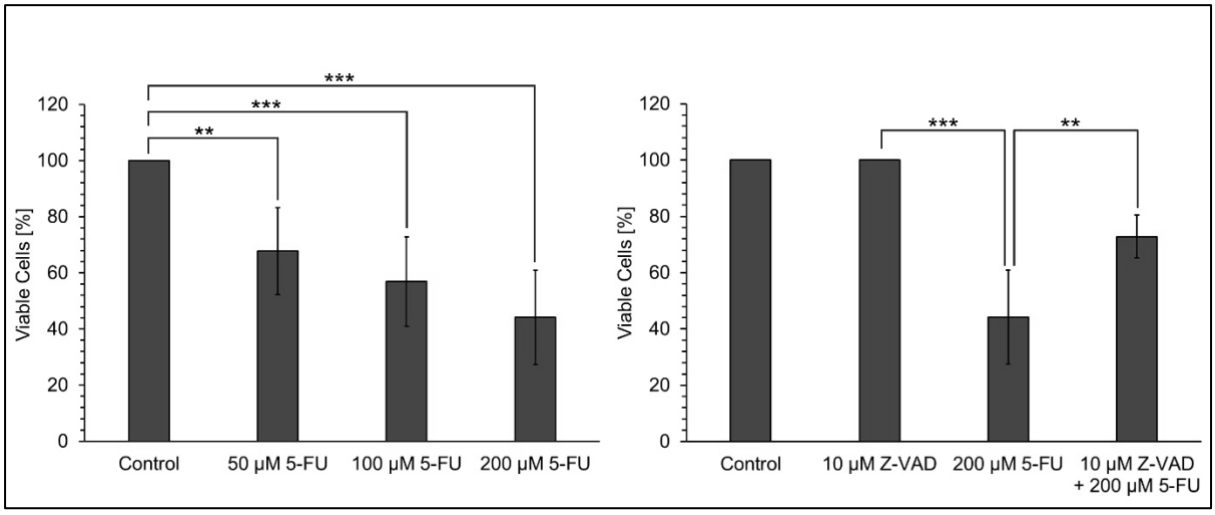


***Figure 6**. Crystal Violet based cell viability assay of RKO cells in response to 5-FU treatment.*

The left graph shows viable cell percent of cells treated with increasing concentrations of 5-FU, whereas the right shows the z-VAD treatment alone and in combination with 5-FU. Crystal violet was used to stain live cells, % of untreated was calculated to determine 5-FU-induced apoptosis. (p-value<0.05=, <0.01=**, 0.001=***)*

2. 5-FU induced apoptosis is p53 dependent in the colorectal cancer cell lines.

Once we confirmed that 5-FU induces apoptosis, we sought to elucidate the specific apoptotic pathway involved. Given the known role of p53 in mediating apoptosis (Akpinar et al., 2015) we conducted an apoptosis assay on p53 deficient RKO-E6 and HCT116 p53-/- cells to investigate the dependency of 5-FU-induced cell death on p53, and compared the apoptosis levels to the parental cell lines (**Figure 7**).

The results showed that 5-FU-mediated apoptosis was significantly lower in p53-deficient cells, with apoptosis levels reduced by more than half compared to their respective parental cell lines. Overall, approximately a 60% reduction in apoptosis was observed across all concentrations in the p53-deficient cell lines compared to their parental counterparts **(Figure 7)**. These findings 5-FU-induced apoptosis is dependent on p53, as only a small residual level of apoptosis observed in cells lacking functional p53.

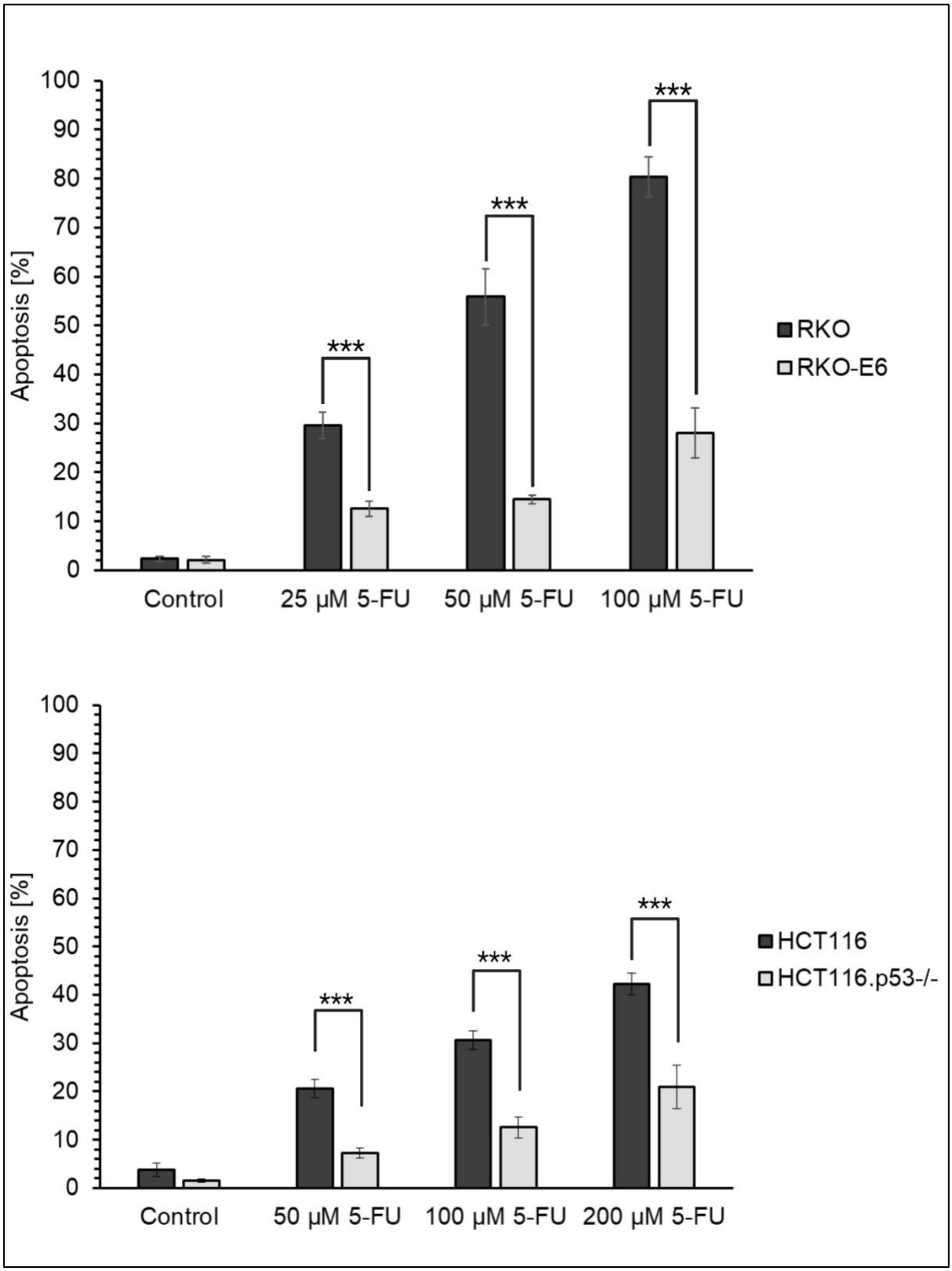


***Figure 7**. 5-FU-Induced Apoptosis in Parental vs. p53-Deficient Cancer Cells*

Comparison of 5-FU-induced apoptosis in parental colorectal cancer cell lines versus their p53-deficient counterparts (HCT116 p53−/− and RKO-E6). Cells were seeded

at a density of 1×10^5 cells per well, treated with increasing concentrations of 5-FU, and incubated for 48 hours. Apoptosis was measured by flow cytometry after staining with propidium iodide (PI).Statistical significance was determined by performing t-tests, and standard deviations were calculated from the percentage of apoptotic cells.

3. DR5 but not DR4 is upregulated in response to 5-FU treatment.

Previous experiments with cells lacking DR5 showed that DR5 is an important part of 5-FU-mediated apoptosis (see **Supplementary Figure 1**) (Akpinar et al., 2015, Yu et al., 2013) . Given these receptors are p53 targets, once it was confirmed 5-FU induces p53 dependent apoptosis, we investigated if the TRAIL receptors (DR4, DR5) are upregulated by 5-FU treatment. To do this, we conducted experiments with parental HCT116 and RKO cell lines (**Figure 8**).

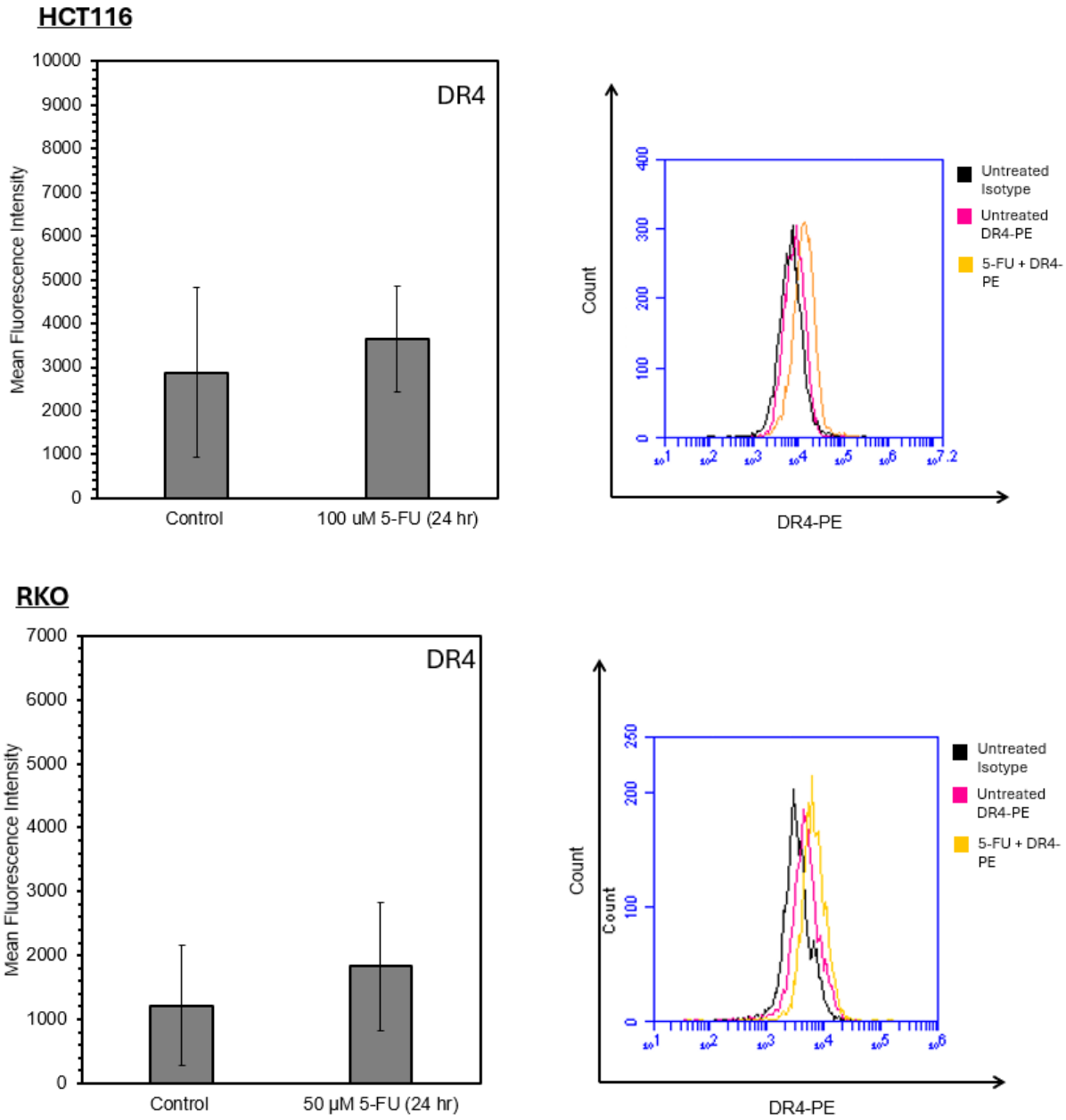


Figure 8. *DR4 expression of HCT116 and RKO cells upon 5-FU treatment.*

The graphs show the expression level DR4 in HCT116 treated with 100 µM 5-FU and RKO cells treated with 50 µM 5-FU, and incubated for 24 hours. Immunostaining was performed using DR4 antibody with respective isotype control. Mean fluorescence intensity (MFI) was calculated by subtracting the isotype control values from the antibody-stained values. Histogram shows the fluorescence intensity for DR4 24 hours post-5-FU treatment, with untreated isotype control (black), untreated cells stained with DR4-PE (pink), and 5-FU-treated cells stained with DR4-PE (orange). Error bars represent standard deviation of three independent experiments.

The DR4 FACS histogram showed low levels of basal DR4 expression and a small shift following 5-FU treatment of the HCT116 cells. However this shift was not

statistically significant when quantified. Similarly, there was no significant upregulation of the DR4 expression on RKO cells when quantified. Consistent results across triplicate experiments indicate that DR4 expression remains unchanged, therefore DR4 is not up-regulated by 5FU and unlikely to play a role in the apoptotic pathway activated by 5-FU in both cell lines. Next, DR5 upregulation was investigated.

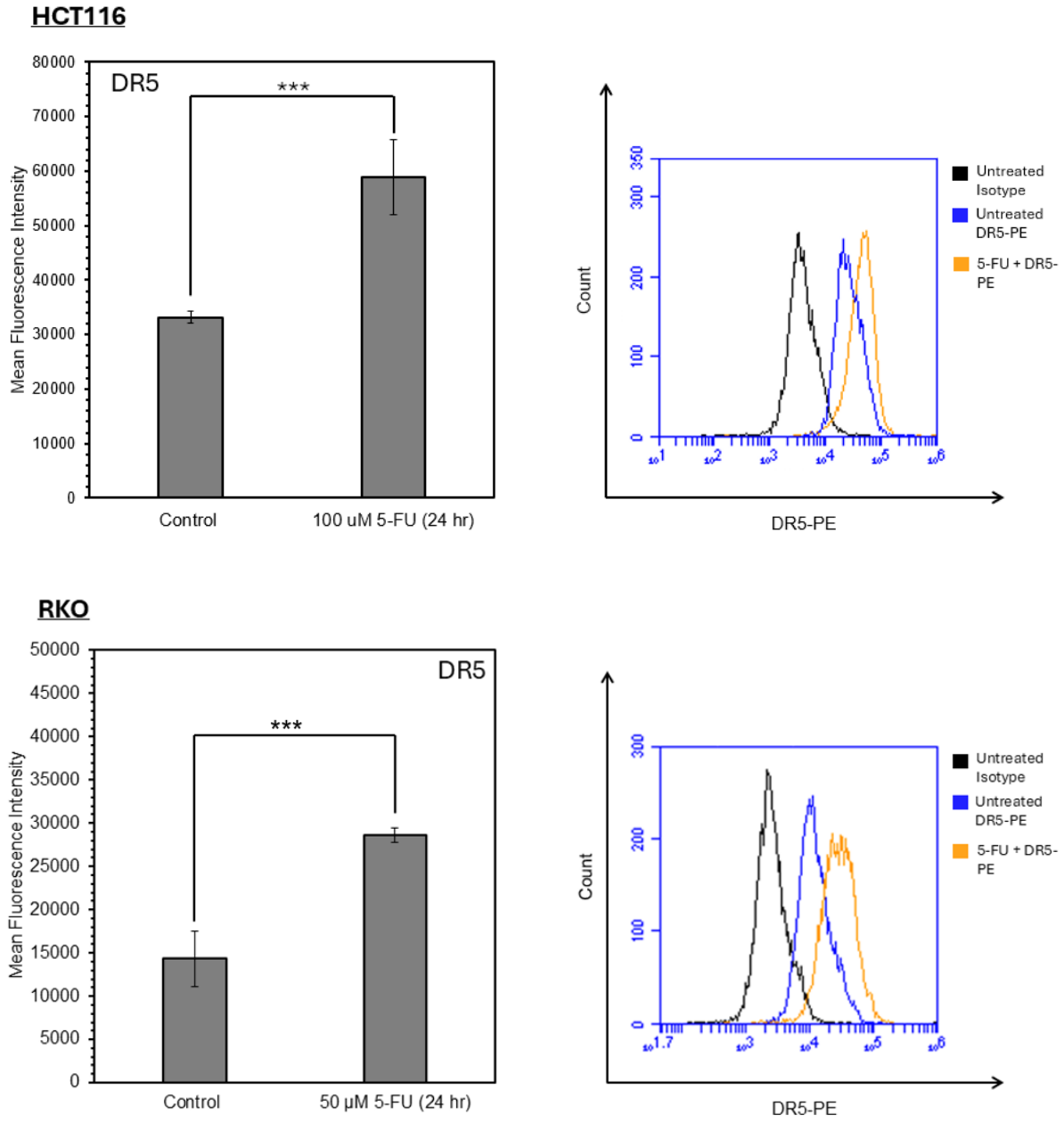


Figure 9. *The DR5 expression of HCT116 and RKO cells.*

*The graphs present the expression levels of DR5 in HCT116 and RKO cells treated with 100 µM 5-FU and 50 µM 5-FU respectively before being incubated for 24 hours. Immunostaining was performed using specific antibody against DR5, with corresponding isotype. Mean fluorescence intensity was calculated by subtracting the fluorescence intensity of the isotype control from that of the DR5 antibody-stained samples. The histograms show fluorescence intensity distributions for DR5 staining at 24 hours: untreated isotype control (black), untreated cells stained with DR5 (blue), and 5-FU-treated cells stained with DR5 (yellow). p-value < 0.05 (*), p < 0.01 (**), and *p < 0.001 (***). Results are expressed as means ± standard error of the mean.*

The results of the DR5 antibody staining experiment showed a clear shift in DR5 expression after 5FU treatment for both HCT116 and RKO cells. When quantified, the average MFI of DR5 in untreated HCT116 cells was around 30,000 after subtracting the isotype control. Upon treatment with 100 μM 5-FU for 24 hours, the MFI of DR5 increased twofold on both RKO and HCT116 cells. This suggests that 5-FU treatment upregulates DR5 expression, which may be associated with the regulation of apoptotic pathways and enhanced sensitivity of cells to apoptosis.

Consistent with findings from previous experiments, where we observed that silencing DR5 in HCT116 cells conferred resistance to 5-FU, our current findings support that DR5 might have an important role in mediating the apoptotic pathway triggered by 5-FU. In contrast, the constant levels of DR4 expression suggest that DR4 is not involved in this apoptotic pathway.

4. P53 Induces DR5 upregulation in response to 5-FU

Given that p53-null or depleted cells show reduced response to 5-FU and that DR5 is a known p53 target, we next investigated whether the upregulation of DR5 in response to 5-FU was dependent on p53. To do this, we first conducted experiments with HCT116 P53-/- and RKO-E6 cell lines and assessed both DR4 and DR5 upregulation (**Figure 10**, **Figure 11**).

Similarly to p53 wild-type cells DR4 was not significantly upregulated in either the HCT116 p53-/- or the RKO-E6 cells following 5-FU treatment .

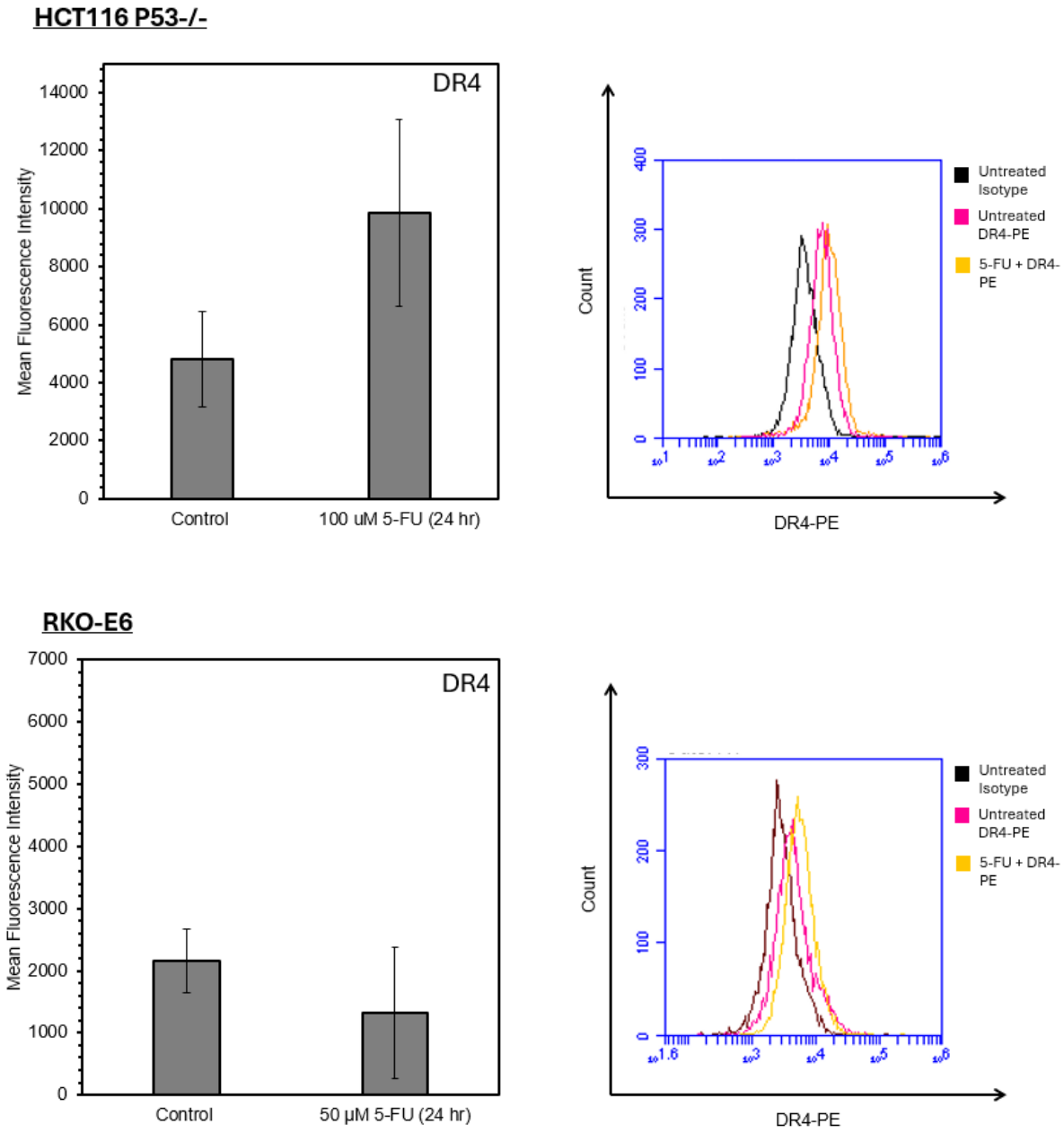


Figure 10. *DR4 upregulation of HCT116 p53-/- and RKO-E6 cells.*

Immunostaining experiment with DR4 antibody with respective isotype control performed on HCT116.p53-/- and RKO-E6 cells which were treated with 100 µM 5-FU and 50 µM 5-FU respectively, and incubated for 24 hrs. Fluorescence intensity (MFI) was calculated by subtracting the isotype control values from the antibody-stained values.

.

Following 5FU treatment of HCT116 p53-/- and RKO-E6 no DR5 upregulation could be observed (**Figure 11**). The results also showed no significant upregulation of DR5 when MFI values were quantified. As previously shown, parental HCT116 cells were

able to upregulate DR5 expression by twofold. This shows that DR5 expression in the 5-FU-treated cells is regulated by p53 providing a possible link between p53, DR5 and the levels of 5-FU-induced apoptosis. In cells lacking p53, DR5 could not be upregulated and apoptosis might not be executed or its levels be diminished.

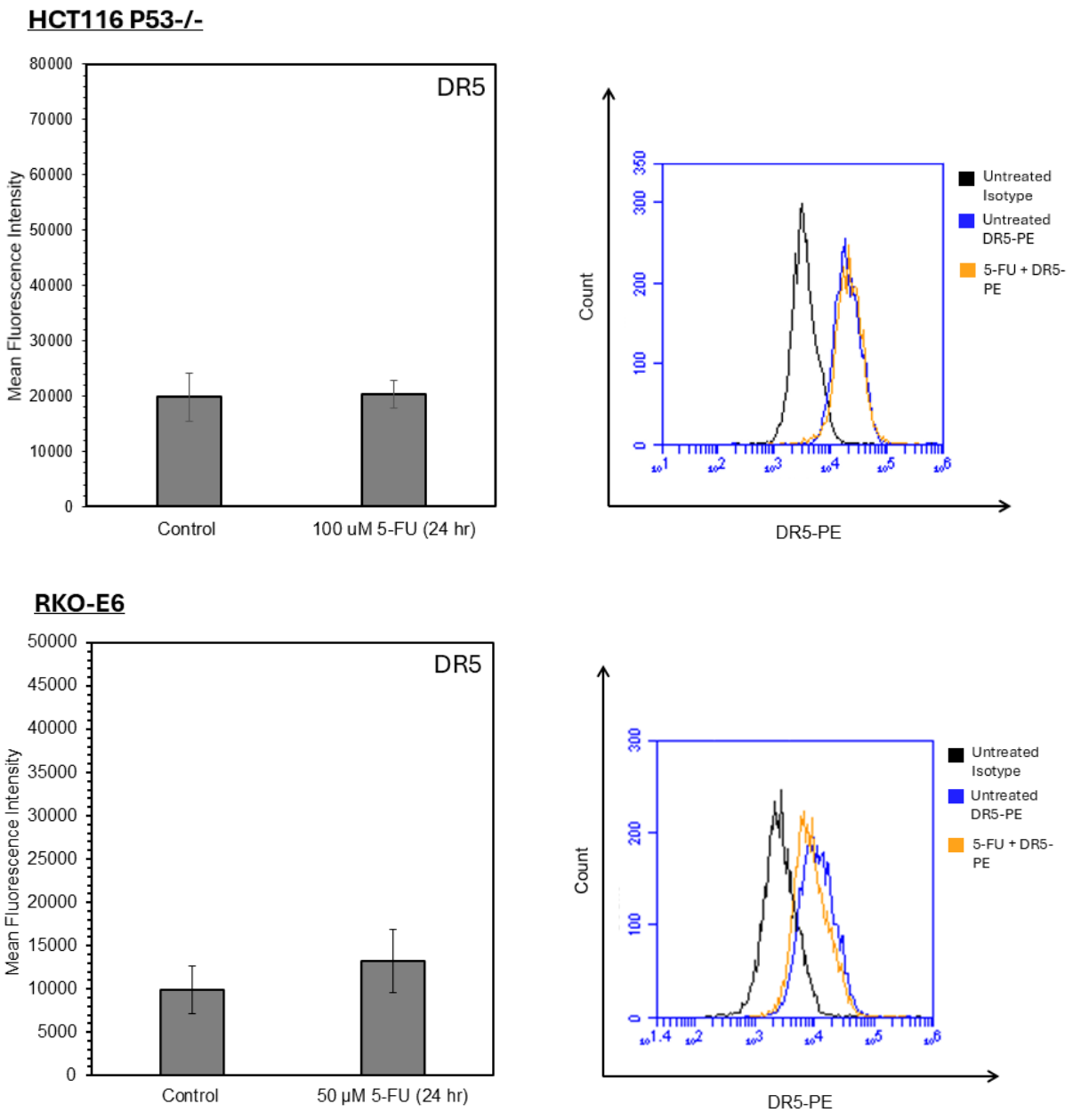


***Figure 11**. DR5 upregulation of the HCT116 p53-/- and RKO-E6 cells.*

Immunostaining experiment with DR5 antibody with respective isotype control performed on HCT116 p53-/- and RKO-E6 cells which were treated with 100 µM 5-FU and 50 µM 5-FU respectively, and incubated for 24 hours. Mean fluorescence

intensity (MFI) was calculated by subtracting the isotype control values from the antibody-stained values.

5. 5-FU induces p38 phosphorylation.

As is was shown earlier that FADDosome-mediated activation of caspase-8 following 5-FU treatment was independent of p53 and death receptors like DR5, I investigated whether non-canonical (i.e. non-caspase) DR5 signalling such as p38 MAPK signalling might be involved in apoptosis after 5-FU stimulation. Therefore, we first examined whether p38 MAPK was activated in response to 5-FU. To this end, we performed western blots with phospho-specific antibodies on protein lysates from RKO and HCT116 cells (**Figure 12**). The results showed that in untreated cells, p38 MAPK was expressed but not phosphorylated, indicating an inactive state. However, upon treatment with 5-FU, there was a marked increase in p38 phosphorylation, suggesting that 5-FU induces p38 MAPK activation (**Figure 12**).

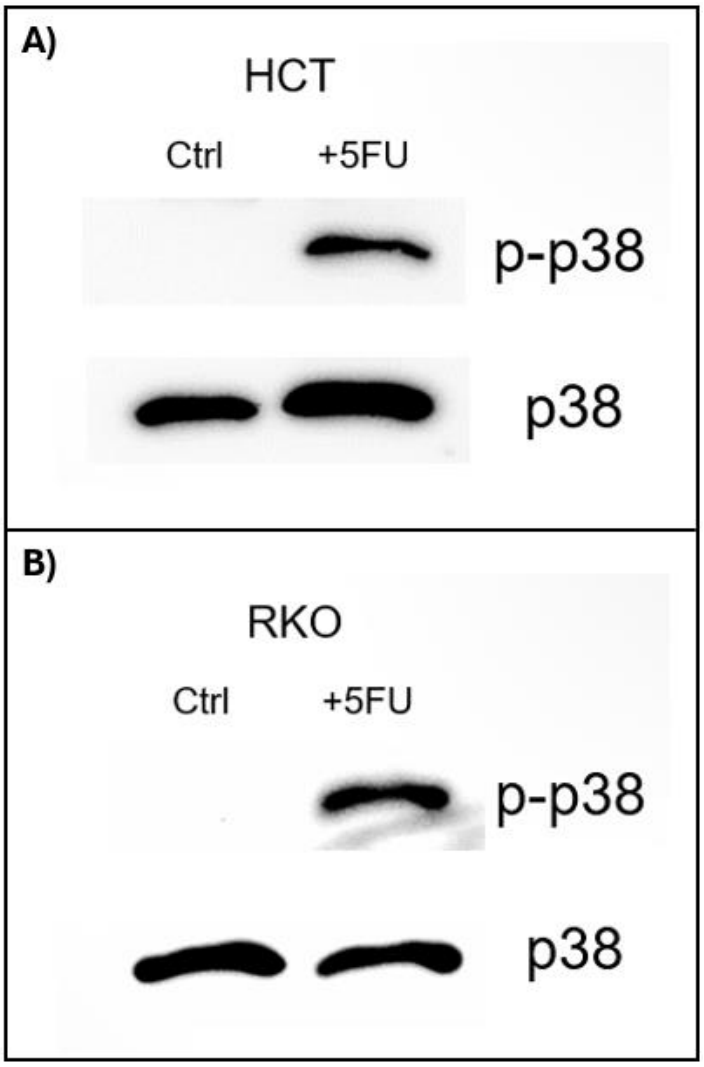


Figure 12. *P38 phosphorylation in response to 5-FU in HCT116 and RKO cells.*

The data shows western blots of HCT116 (A) and RKO (B) cells untreated and treated with 5-FU which were then investigated for their phosphorylated p38 (p-p38) and total p38.

To assess whether this activation is dependent on p53, we conducted experiments using HCT116 p53-/- and RKO-E6 cell lines and compared them to their parental counterparts (**Figure 13**).

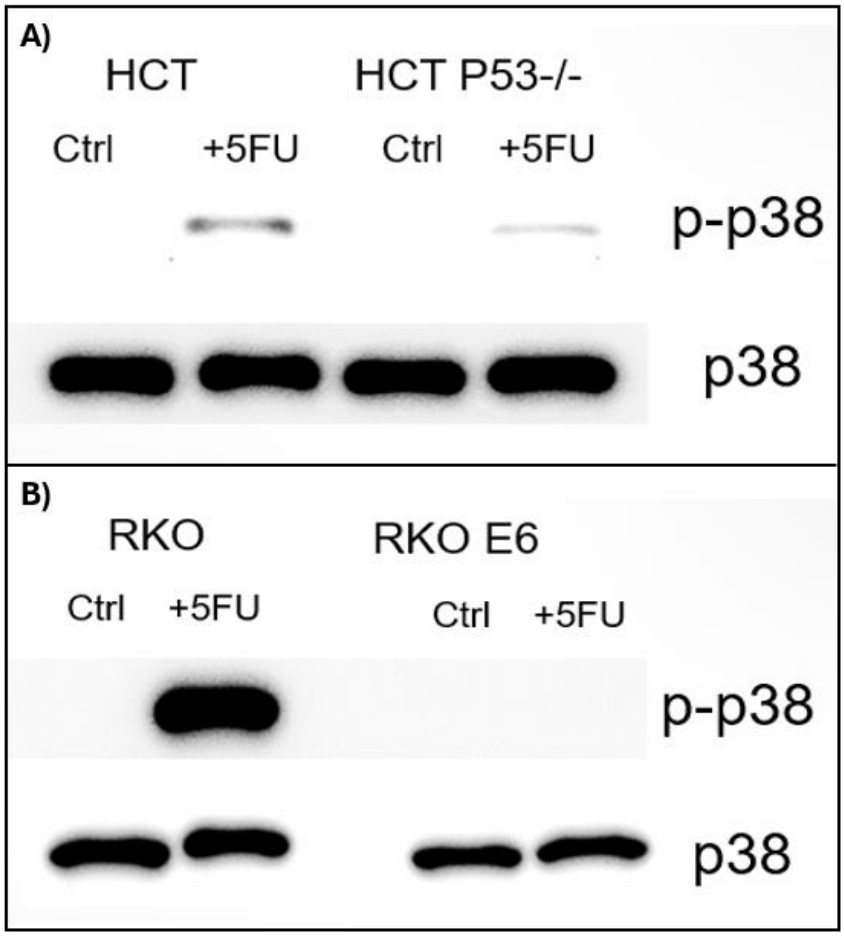


Figure 13. *The effect of p53 status on p38 phosphorylation.*

The Figure depicts HCT116 (A) and RKO (B) cells with their p53 null counterparts. The cells were treated with 5-FU and then investigated for their phosphorylated p38 (p-p38) and total p38.

The results revealed that p38 activation is indeed p53-dependent. In HCT116 p53-/- cells, phosphorylated p38 levels were significantly reduced compared to the parental HCT116 cells. In RKO-E6 cells, the absence of functional p53 resulted in a complete loss of p38 phosphorylation. This led us to hypothesise that 5-FU induces p53 activation, which subsequently upregulates DR5, leading to its (partial) activation and ultimately triggering p38 phosphorylation/activation. To test this hypothesis and determine whether p53 and DR5 directly contribute to p38 phosphorylation, we performed experiments using DR5-knockdown cells generated from HCT116 cells (HCT116.shDR5) (**Figure 14**).

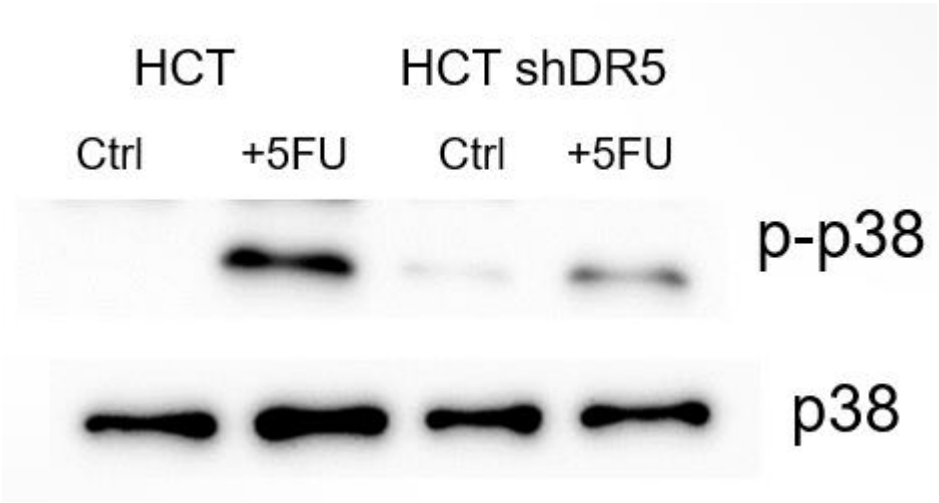


***Figure 14**. DR5 knockdown reduces p38 phosphorylation in response to 5-FU treatment.*

Phosphorylated p38 (p-p38) and total p38 were detected in parental HCT116 cells and DR5-knockdown HCT116.shDR5 cells under untreated (Ctrl) and 5-FU-treated (+5FU) conditions. Total p38 serves as a loading control.

The Western blot analysis of HCT116 and HCT116.shDR5 cells showed clear differences in p38 phosphorylation after 5-FU treatment. In the parental HCT116 cells, p38 was strongly phosphorylated following 5-FU exposure, confirming the activation of the p38 MAPK pathway. In contrast, HCT116.shDR5 cells showed significantly reduced p38 phosphorylation. Untreated HCT116.shDR5 cells exhibited only a faint p-p38 band, indicating minimal basal activation of p38, and while 5-FU treatment slightly increased p38 phosphorylation, the band remained faint compared to the parental cells.

These results indicate that DR5 contributes to p38 phosphorylation/activation in response to 5-FU. However, the faint p-p38 band observed in HCT116.shDR5 cells treated with 5-FU suggests that other pathways or mechanisms may partially mediate p38 activation, too. This supports our hypothesis that DR5 acts as a key link

in the signalling cascade from p53 activation to p38 phosphorylation during 5-FU-induced apoptosis.

6. P38 MAPK is an important part of the 5-FU mediated apoptosis.

We then aimed to investigate whether p38 MAPK is indeed involved in regulating 5-FU-induced apoptosis. To establish the role of p38 MAPK in 5-FU-mediated apoptosis, we performed experiments using specific p38 MAPK inhibitors, namely doramapimod and pamapimod on RKO cells. Using the IncuCyte system, images were captured over a 48-hour period at three time points (0, 24, and 48 hours). A single image from each time point was divided into four segments for quantitative analysis. Each segment was analysed for total and dead cell counts and the data was used to calculate the percentage of dead cells (**Figure 15** , **Figure 16**).

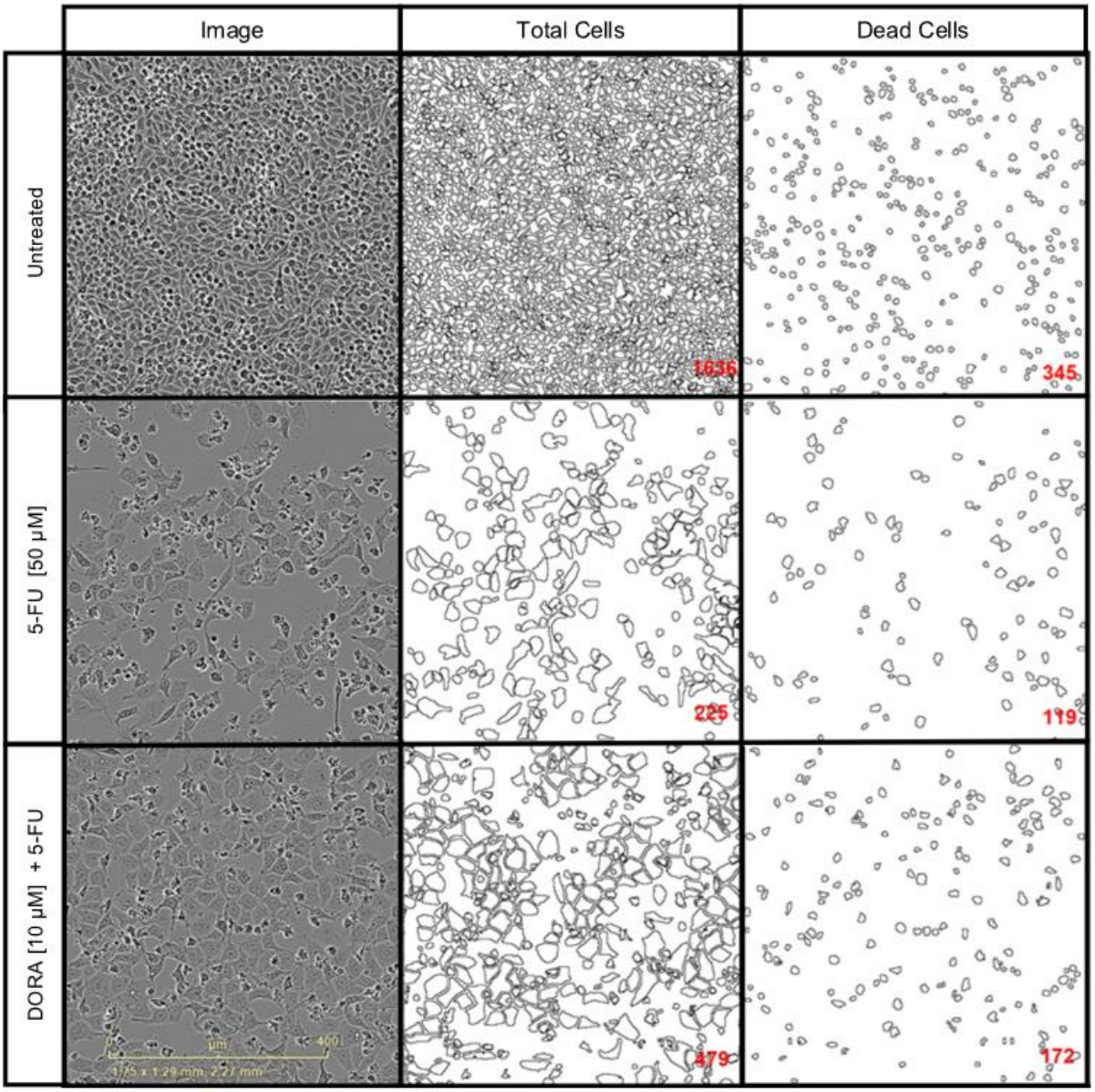


Figure 15. *Segmented Images of cell death analysis- doramapimod.*

The figure shows the analysis of IncuCyte cell death assay image segments as well as the quantification of cell death in untreated and treated conditions at 48 hours post treatment. The first column shows raw images, the second column represents segmented images of total cells, and the third column shows thresholded images highlighting dead cells. Treatment conditions include untreated cells, 50 µM 5-FU, and 50 µM 5-FU combined with 10 µM doramapimod.

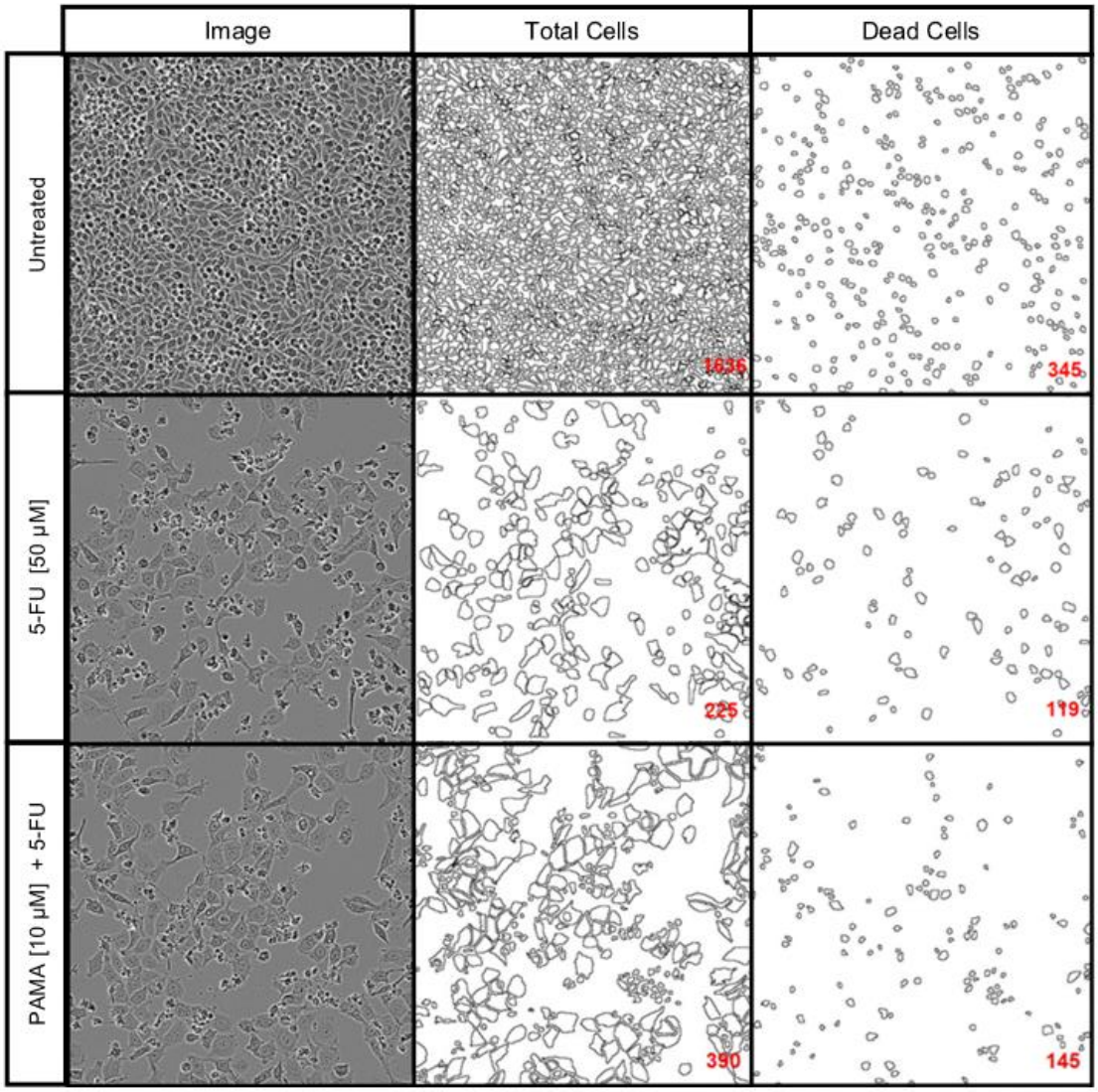
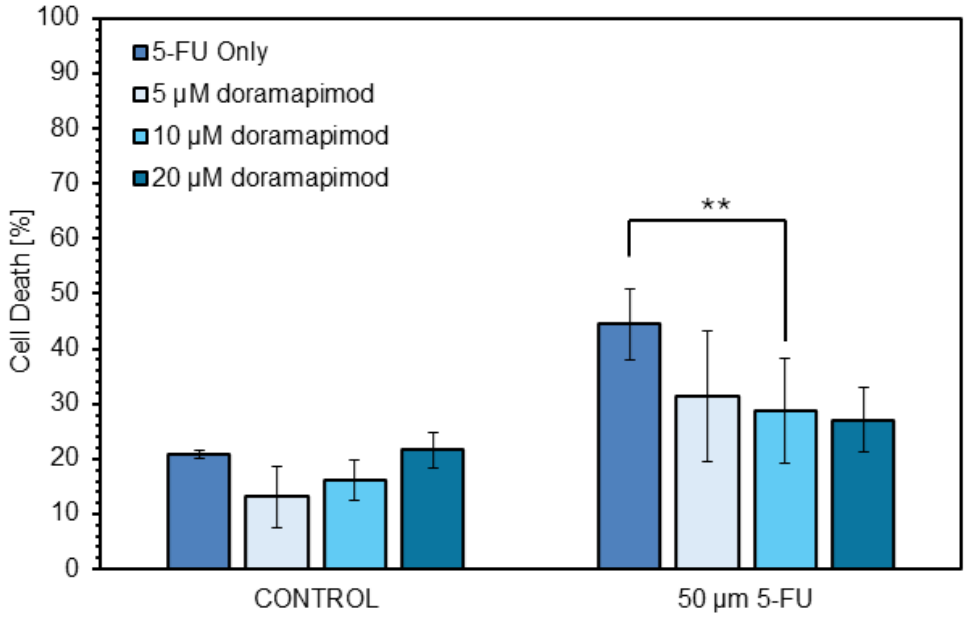


***Figure 16**. Segmented Images of cell death analysis- pamapimod.*

The figure shows the analysis of IncuCyte cell death assay image segments and highlight the dose-dependent effect of pamapimod on 5-FU-induced cell death at 48 hours post treatment. The images illustrate the effects of various treatment conditions on cell populations, including untreated cells, cells treated with 50 µM 5-FU alone, and cells treated with 50 µM 5-FU in combination with 10 µM pamapimod. Each treatment is represented in three columns: the first column shows the raw images, the second column displays the segmented images highlighting total cells, and the third column shows dead cells.

Treatment with 5-FU combined with either doramapimod or pamapimod showed similar effects on cell death. At 5 µM, doramapimod resulted in 29% dead cells, while pamapimod led to 21% dead cells. At 20 µM, doramapimod had 32% dead cells, and

pamapimod showed 22% dead cells. Both inhibitors reduced cell death compared to the 5-FU-only treatment, indicating a protective effect against 5-FU-induced cell death. To quantify the observed effects, the data from the image analysis were compiled and are presented in the following graphs, which show the percentage of dead cells across different treatment conditions at 48 hours (**Figure 17**).



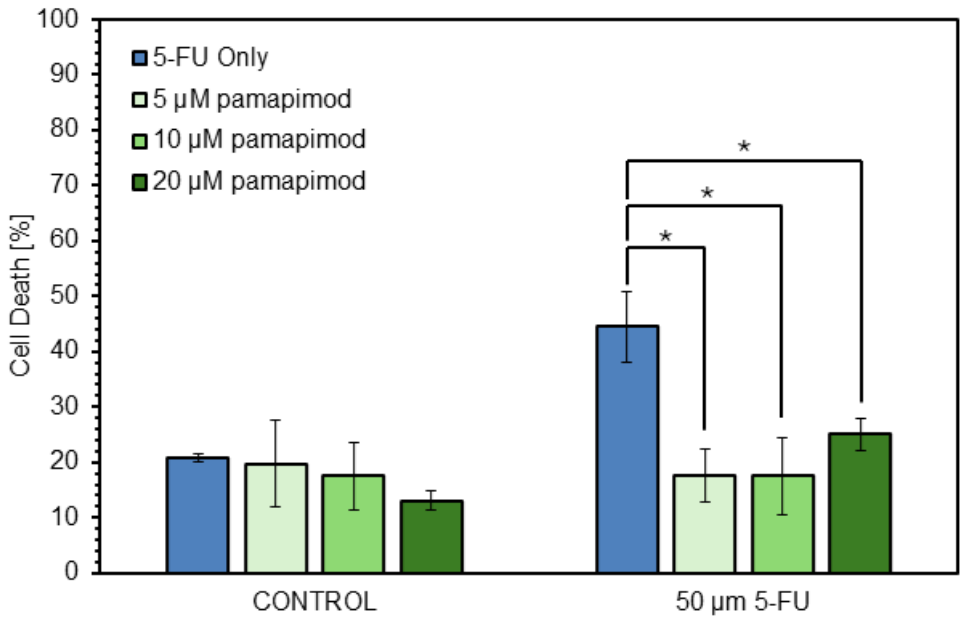


Figure 17. *Effect of p38 inhibitors on 5-FU-induced cell death.*

*The graph shows the percentage of cell death in control and 5-FU-treated cells with and without p38 inhibitors (doramapimod or pamapimod, top and bottom respectively). Left- control groups (untreated, 5 µM, 10 µM, and 20 µM p38i alone) show baseline levels of cell death. Right- 5-FU alone, 5-FU + 5 µM p38i, 5-FU + 10 µM p38i, 5-FU + 20 µM p38i respectively. p-value < 0.05 (*), p < 0.01 (**), and *p < 0.001 (***). Results are expressed as means ± standard error of the mean.*

Treatment of RKO cells with 50 µM 5-FU resulted in significant cell death. The co-treatment with 5-FU and doramapimod led to approximately a 30% decrease in cell death. On the other hand, pamapimod showed a more pronounced effect, around 50% reduction in apoptosis compared to 5-FU alone across all concentrations, suggesting a potential role of p38 MAPK in 5-FU-induced cell death.

In addition to the cell death analysis, an apoptosis assay was conducted (**Figure 18**). Treatment of RKO cells with 50 µM 5-FU resulted in significant apoptosis, around 40%. The co-treatment of RKO cells with 50 µM 5-FU and doramapimod resulted in a significant reduction in apoptosis compared to 5-FU alone. At 5 µM and 10 µM doramapimod, apoptosis was reduced by 37.5%, while at 20 µM, the reduction was more pronounced at 50%. In contrast, co-treatment with pamapimod and 5-FU resulted in a smaller reduction in apoptosis, with a 25% decrease at both 5 µM and 10 µM concentrations, and a 37.5% reduction at 20 µM **(Figure 18)**.

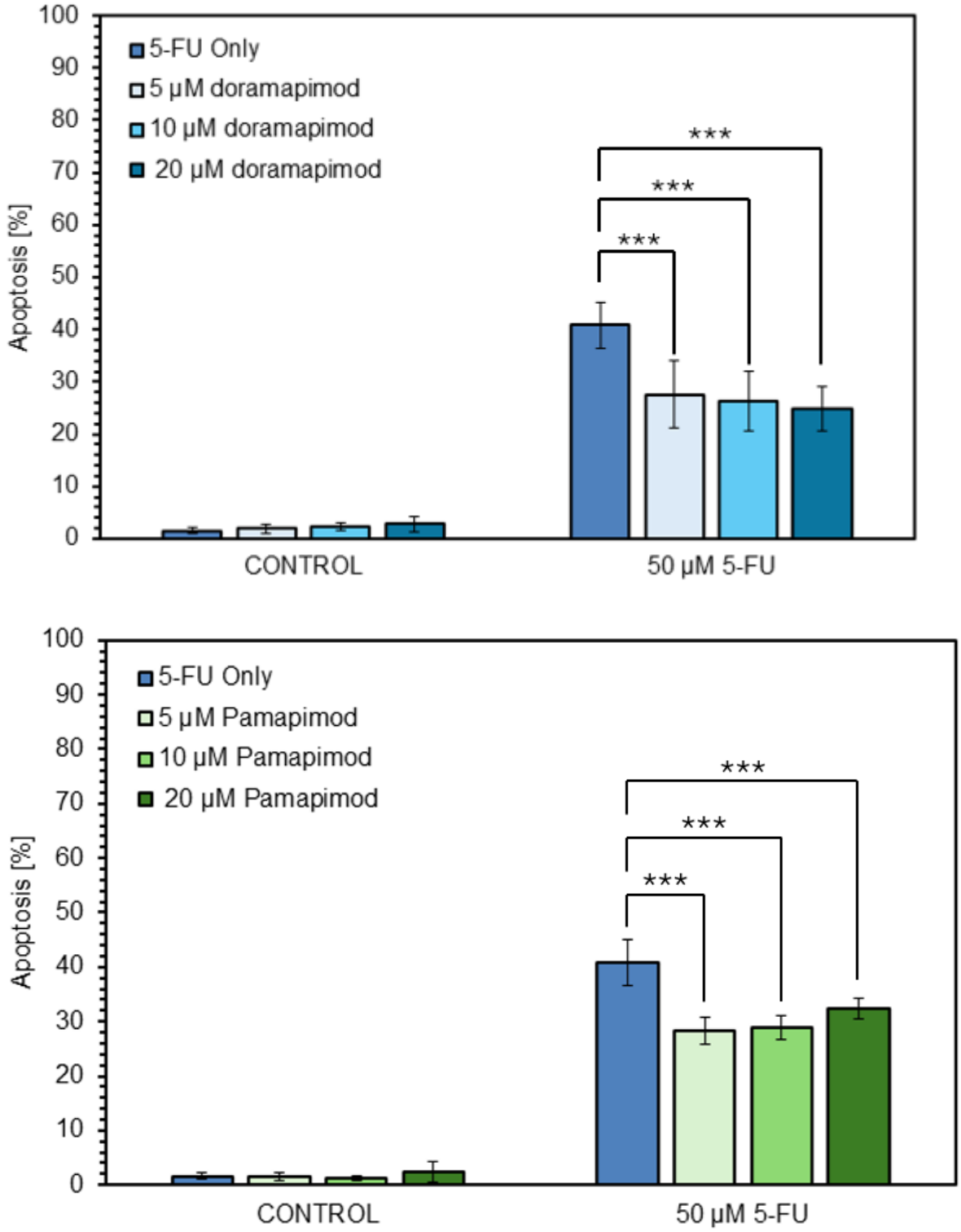


***Figure 18**. Effect of p38 inhibitors on 5-FU-induced apoptosis.*

*The graphs demonstrate the percentage of apoptosis in control and 5-FU-treated cells with and without p38 inhibitors (doramapimod or pamapimod, top and bottom respectively). Left- control groups (untreated, 5 µM, 10 µM, and 20 µM p38i alone) show baseline levels of cell death. Right- 5-FU alone, 5-FU + 5 µM p38i, 5-FU + 10 µM p38i, 5-FU + 20 µM p38i respectively. p-value < 0.05 (*), p < 0.01 (**), and *p <*

0.001 (***). Results are expressed as means ± standard error of the mean.as means ± standard error of the mean.

Taken together, these findings demonstrate that p38 is involved in apoptosis induced by 5-FU, as inhibition of p38 MAPK results in a decrease of apoptosis levels.

DISCUSSION

1. 5-FU mediated cell death is caspase and p53 dependent.

5-FU is a chemotherapeutic agent that interferes with thymidylate synthase and DNA synthesis, causing DNA strand breaks and misincorporation of dNTPs into DNA and subsequent replication fork stalls (Zhang et al., 2008). This experiment has once again showed that 5-FU treatment induces significant apoptosis in colorectal cancer cells. The dose curve experiments with HCT116 and RKO cells has shown dose dependent cell death. When the cells were pre-treated with zVAD-fmk before adding the highest concentration of 5-FU, the cell death levels were reduced to the control levels. This confirms the 5-FU mediated cell death is caspase-dependent apoptosis.

Most chemotherapeutic drugs are dependent on p53 to induced apoptosis. This is because DNA damage is sensed by p53 and it activates transcriptional targets that mediate cell cycle arrest and apoptosis, including the pro-apoptotic proteins from Bcl-2 protein family and extrinsic pathway proteins such as DR5 (Aubrey et al., 2018). This experiment once again confirmed pro-apoptotic role of p53 in 5-FU-induced apoptosis using HCT116 p53-/- and RKO-E6 cells (**above**). HCT116 p53-/- is a double knockout cell line, whereas RKO-E6 cell line contains HPV16 E6 oncoprotein, which degrades p53. Both cell lines were more resistant to 5-FU treatment compared to their parental cell lines. The absence of p53 resulted in a significant reduction in apoptosis in all concentrations. These findings are in line with the current understanding that p53 is a crucial mediator of the DNA damage

response, and that it takes part in regulating the apoptotic pathways in colorectal cancer (Akpinar et al., 2015).

Even though the cells are less capable of initiating apoptosis when p53 is impaired, cell death levels were not completely diminished. It is possible that in the absence of p53 5-FU is inducing cell death through another pathway. Previously, a similar research done by Akpinar et al. (2015) has shown 5-FU treated p53 deficient cells displayed delayed caspase activation, resulting in mitochondrial ROS, necrotic-like features, and suboptimal apoptosis.

2. 5-FU induced apoptosis is dependent on DR5 but not DR4 activation.

This study revealed that 5-FU treatment significantly upregulated DR5 expression, while DR4 expression remained unchanged in both HCT116 and RKO cell lines. Although the DR4 histogram shifted after treatment, this shift was not statistically significant when quantified. This suggests that DR4 is not involved in the apoptotic pathway activated by 5-FU in both cell lines.

On the other hand, we observed a clear two-fold upregulation of DR5 in both HCT116 and RKO cells after 5-FU treatment. In previous experiments we could also show that DR5 knockout HCT116 cells were unresponsive to 5-FU therapy (Aleyna Guney, undergraduate thesis, 2023). This suggest that DR5 is the sole death receptor of the 5-FU mediated apoptosis pathway. Previous studies have also reported DR5-dependent apoptosis in response to 5-FU. For example, studies on HCT116 cells demonstrated that 5-FU administration resulted in the accumulation of DR5 in the plasma membrane, as well as the development of the DR5-associated DISC complex. This activation was found to be p53-dependent (Akpinar et al., 2015, Can et al., 2013).

3. In 5-FU-mediated apoptosis, DR5 activation is p53-dependent and occurs independently of its ligand, TRAIL.

In the generic death receptor pathway, DR5 binds to its ligand TRAIL and activates the caspase cascade which results in apoptosis (Green and Llambi, 2015). However our previous experiments revealed that DR5 activation in 5-FU mediated apoptosis is independent of its ligand, as when the ligand was neutralised with an antagonist the apoptosis levels were not affected (Mohr et al., 2018). Our data also indicate that this upregulation of DR5 is p53-dependent, as cells lacking p53 exhibited no increase in DR5 expression following 5-FU treatment. This supports the hypothesis that p53 activation following 5-FU-induced DNA damage drives the transcriptional upregulation of DR5, enhancing the apoptotic response. Consequently, increased levels of DR5 protein displayed on the cellular surface leads to receptor clustering and activation, as demonstrated in previous studies (Rebillard et al., 2010).

4. P38 SAPK in involved in 5-FU mediated apoptosis.

4.1. The activation of p38 in 5-FU mediated apoptosis is regulated by DR5 and p53

Our study revealed the role of p38 SAPK in regulating 5-FU-induced apoptosis. First, we confirmed p38 phosphorylation and therefore activation upon 5-FU treatment with western blots. Further experiments with p53 deficient cells showed that p53 is the main regulator of p38 activation. We also conducted experiments with DR5 knockdown cells. These experiments showed that DR5 also mediates p38 activation partially. Then, by using antagonists, we blocked p38 and treated RKO and HCT116 cell lines with varying concentrations of 5-FU. The protective effect that arose with p38 SAPK inhibitor were clearly visible, and measured and quantified by the IncuCyte. In this experimental setup we treated RKO cells with 5-FU and p38

inhibitors and then observed the effect of treatments every 4 hours. The p38 inhibitor treated cells were seen to undergo apoptosis much later.

Literature suggests that p38 can exert both pro- and anti-apoptotic effects depending on cell type and stimuli (Lee et al., 2006). We hypothesize that in our system, p38 promotes apoptosis by activating transcription factors such as AP-1, which drive the de-phosphorylation of Bid.

4.2. Mechanisms of 5-FU-Induced Apoptosis: A Proposed Cascade

Our findings suggest that 5-FU induces apoptosis through a complex interplay of signalling pathways. Based on existing literature and our own data, we propose a model where 5-FU activates ATR, which in turn phosphorylates BID (Liu et al., 2011, Zinkel et al., 2007). Phosphorylated BID has been shown to resist caspase-8 cleavage, delaying its conversion to tBID and therefore mitochondrial apoptosis. In line with this, our work (Mohr et al., 2018) confirmed BID's critical role in 5-FU-induced apoptosis as BID-silenced cells showed reduced caspase-3 activation and disrupted mitochondrial signalling with normal caspase-8 activity. This highlights BID as a key regulator, particularly at the mitochondrial checkpoint.

We propose a dual-pathway mechanism for 5-FU-induced apoptosis, integrating both death receptor and mitochondrial signalling. On the receptor side, 5-FU upregulates DR5 in a p53-dependent manner, forming a non-canonical DISC-like complex that activates caspase-8 independently of TRAIL. This form of death receptor activation is consistent with earlier reports (Rebillard et al., 2010).

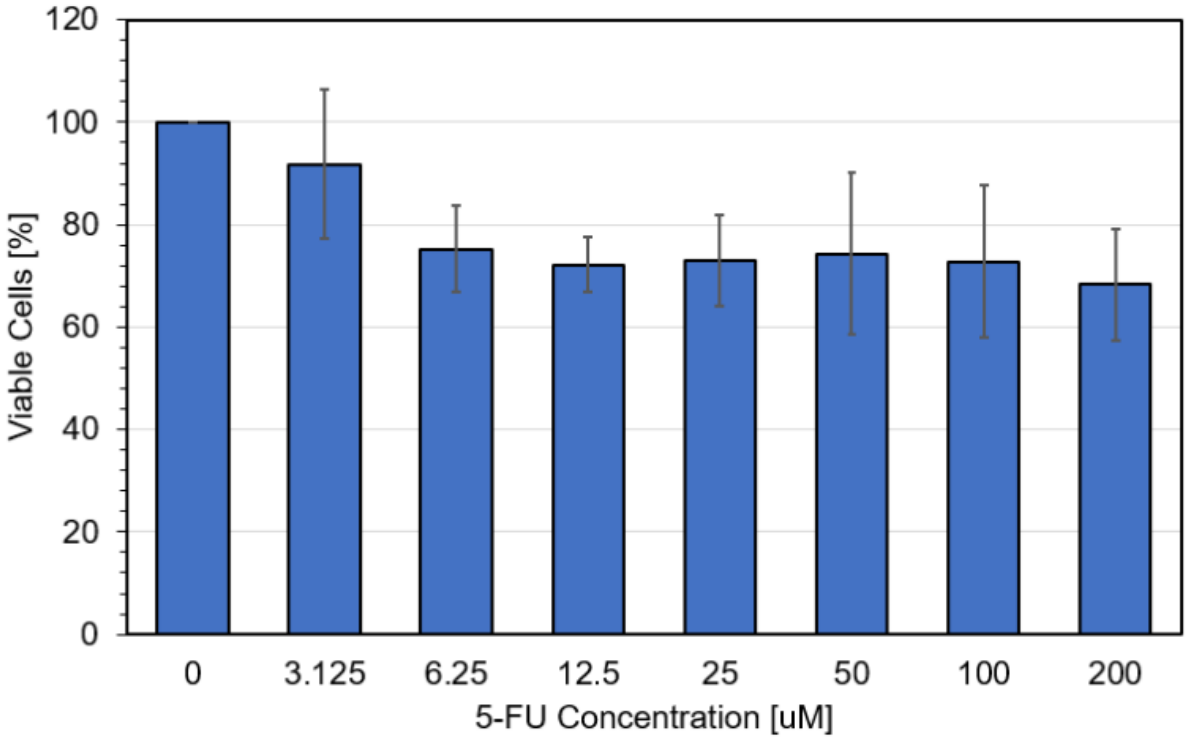
On the mitochondrial side, 5-FU activates ATR, which activates p53 by suppressing MDM2. ATR also phosphorylates BID (Ser61, Ser64, Ser78) (Liu et al., 2011, Zinkel et al., 2007), which renders it resistant to caspase-8 cleavage (Desagher et al., 2001). This phosphorylation delays MOMP and caspase-3 activation, and puts a brake on apoptosis. To overcome this, cells require BID dephosphorylation, a process we believe involves p38.

This study indicates that p38 is activated by 5-FU, partly through p53 and DR5. I propose that this p38 activation drives AP1 activation, and therefore Wip1 expression; a p53-regulated phosphatase known to dephosphorylate ATR/ATM targets, including BID (Tanos et al., 2005). Here, p53 and p38 converge on Wip1, which then dephosphorylates BID, restoring its cleavage by caspase-8 and enabling tBID formation. This step is critical for BAX/BAK activation, MOMP, and Smac/Diablo release, ultimately amplifying the apoptotic cascade. Additionally, we speculate that the non-canonical FADDosome platform we previously identified may work in parallel to enhance caspase-8 activation and reinforce the apoptotic signal.

CONCLUSION

This study has shown that p38 MAPK is activated and regulated partially by DR5 and p53. Our study demonstrates that 5-FU induces apoptosis through a complex interplay of DR5 signalling that includes p53, mitochondria, FADDosome, and transcription factors. We show that p38 is involved in 5-FU mediated apoptosis, and we propose that p38 in conjunction with p53, regulates the expression of Wip1, which dephosphorylates BID, enabling its cleavage and facilitating MOMP, which results in caspase 3 activation and apoptosis.

Supplementary Figures



Supplementary Figure 1. The graph displays the response of DR5 knockout cells to various concentrations of 5-FU over a 7-day period. After treatment, the absorbance of Crystal Violet-stained cells was measured using a spectrometer, and the percentage of viable cells was calculated from the untreated cells. The data indicate that HCT116.shDR5 cells are resistant to 5-FU mediated apoptosis. Standard deviations of the percentages were included.

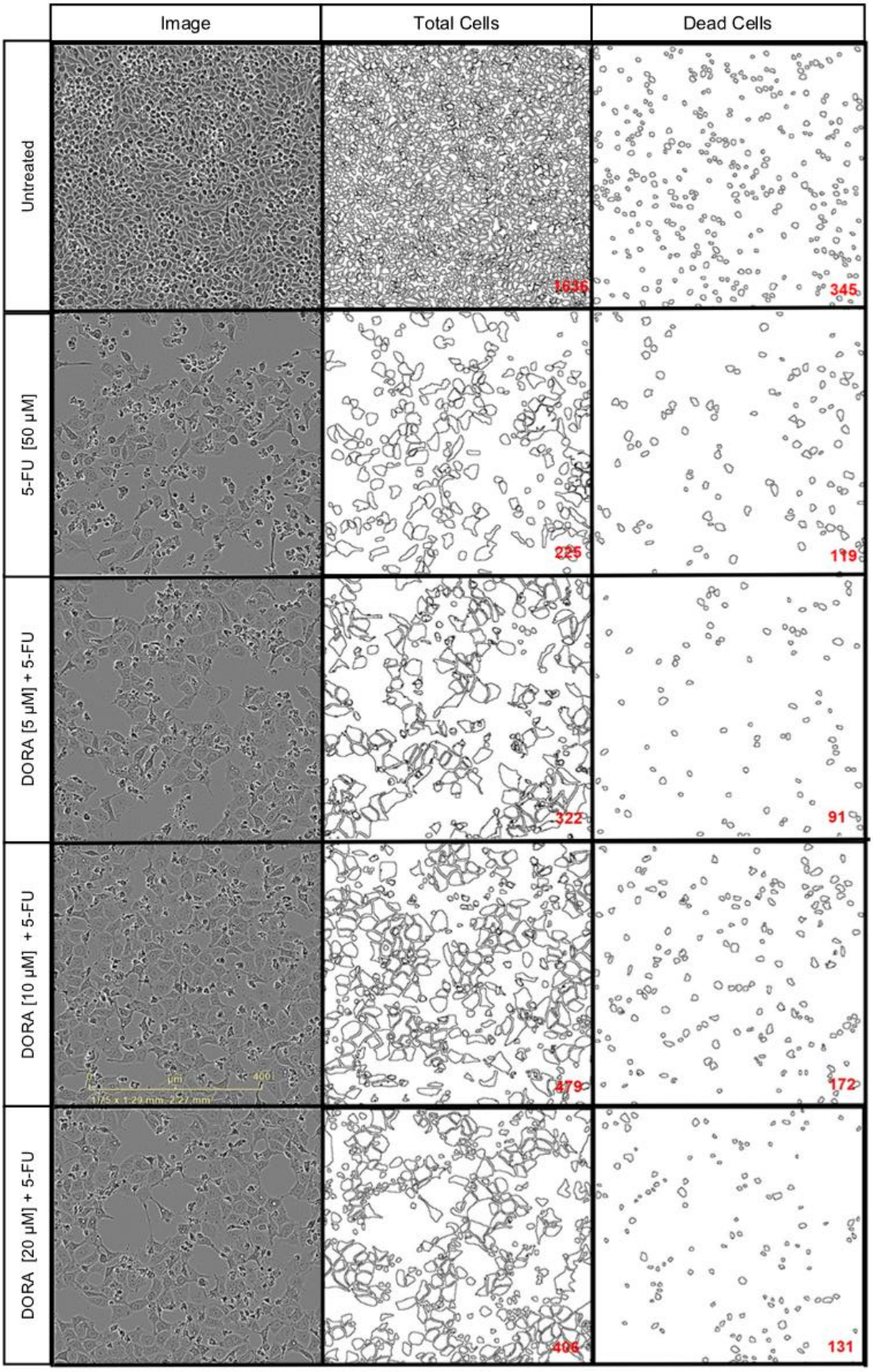


Image
Total Cells
Dead Cells
Untreated
1636
345
5-FU [50 µM]
225
119
DORA [5 µM] + 5-FU
322
91
DORA [10 µM] + 5-FU
479
172
DORA [20 µM] + 5-FU
406
131

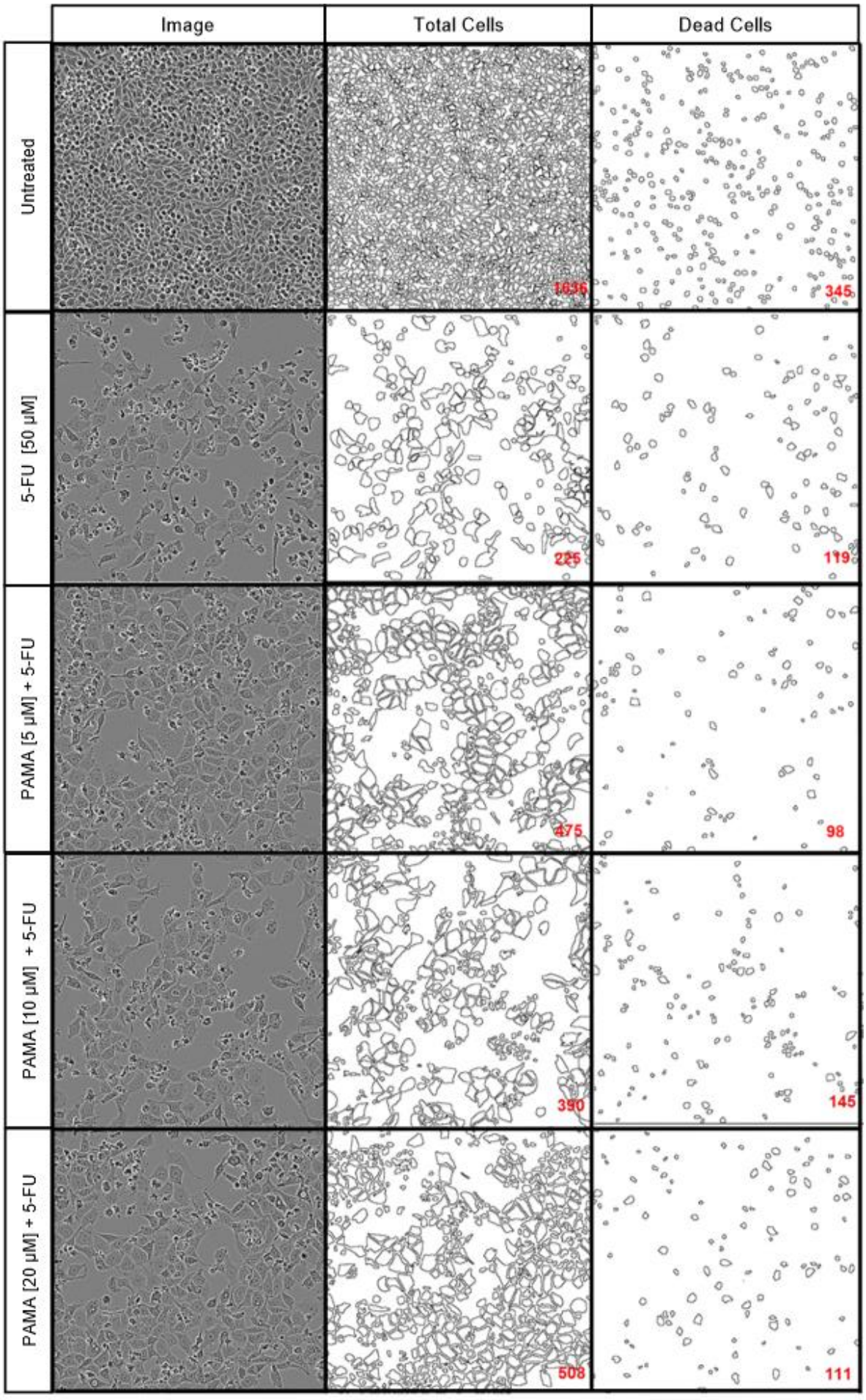


Image
Total Cells
Dead Cells
Untreated
1636
345
5-FU [50 µM]
225
119
PAMA [5 µM] + 5-FU
475
98
PAMA [10 µM] + 5-FU
390
145
PAMA [20 µM] + 5-FU
508
111

Supplementary Figure 2. Representative IncuCyte images are shown for each treatment condition, along with total cell counts obtained using ImageJ and the number of filtered dead cells. The figure includes control cells, cells treated with 5-FU, and cells co-treated with 5-FU and increasing concentrations of the p38 inhibitor.

REFERENCES

AKPINAR, B., BRACHT, E. V., REIJNDERS, D., SAFARIKOVA, B., JELINKOVA, I., GRANDIEN, A., VACULOVA, A. H., ZHIVOTOVSKY, B. & OLSSON, M. 2015. 5-Fluorouracil-induced RNA stress engages a TRAIL-DISC-dependent apoptosis axis facilitated by p53. *Oncotarget,* 6**,** 43679-97.

AN, Q., ROBINS, P., LINDAHL, T. & BARNES, D. E. 2007. 5-Fluorouracil Incorporated into DNA Is Excised by the Smug1 DNA Glycosylase to Reduce Drug Cytotoxicity. *Cancer Research,* 67**,** 940-945.

ASHKENAZI, A. & DIXIT, V. M. 1998. Death Receptors: Signaling and Modulation. *Science,* 281**,** 1305-1308.

AUBREY, B. J., KELLY, G. L., JANIC, A., HEROLD, M. J. & STRASSER, A. 2018. How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? *Cell Death & Differentiation,* 25**,** 104-113.

BAUD, V. & KARIN, M. 2001. Signal transduction by tumor necrosis factor and its relatives. *Trends in Cell Biology,* 11**,** 372-377.

BELLAIL, A., QI, L., MULLIGAN, P., CHHABRA, V. & HAO, C. 2009. TRAIL Agonists on Clinical Trials for Cancer Therapy: The Promises and the Challenges. *Reviews on recent clinical trials,* 4**,** 34-41.

BLACKLEDGE, G. 1998. New developments in cancer treatment with the novel thymidylate synthase inhibitor raltitrexed ('Tomudex'). *British Journal of Cancer,* 77**,** 29-37.

CAI, B., CHANG, S. H., BECKER, E. B. E., BONNI, A. & XIA, Z. 2006. p38 MAP Kinase Mediates Apoptosis through Phosphorylation of Bim_{EL} at Ser-65 *. *Journal of Biological Chemistry,* 281**,** 25215-25222.

CAN, G., AKPINAR, B., BARAN, Y., ZHIVOTOVSKY, B. & OLSSON, M. 2013. 5-Fluorouracil signaling through a calcium–calmodulin-dependent pathway is required for p53 activation and apoptosis in colon carcinoma cells. *Oncogene,* 32**,** 4529-4538.

CARLSEN, L., SCHORL, C., HUNTINGTON, K., HERNANDEZ-BORRERO, L., JHAVERI, A., ZHANG, S., ZHOU, L. & EL-DEIRY, W. S. 2021. Pan-drug and drug-specific mechanisms of 5-FU, irinotecan (CPT-11), oxaliplatin, and cisplatin identified by comparison of transcriptomic and cytokine responses of colorectal cancer cells. *Oncotarget,* 12**,** 2006.

CHOWCHAIKONG, N., NILWARANGKOON, S., LAPHOOKHIEO, S., TANUNYUTTHAWONGSE, C. & WATANAPOKASIN, R. 2018. p38 inhibitor inhibits the apoptosis of cowanin-treated human colorectal adenocarcinoma cells. *Int J Oncol,* 52**,** 2031-2040.

DEMARCO, B., GRAYCZYK, J., BJANES, E., ROY, L., TONNUS, W., ASSENMACHER, C.-A., RADAELLI, E., FETTRELET, T., MACK, V., LINKERMANN, A., ROGER, T., BRODSKY, I., CHEN, K. & BROZ, P. 2020. Caspase-8-dependent gasdermin D cleavage promotes antimicrobial defense but confers susceptibility to TNF-induced lethality. *Science Advances,* 6.

DESAGHER, S., OSEN-SAND, A., MONTESSUIT, S., MAGNENAT, E., VILBOIS, F., HOCHMANN, A., JOURNOT, L., ANTONSSON, B. & MARTINOU, J. C. 2001.

Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. *Mol Cell,* 8**,** 601-11.
GALLOIS, C., HAFLIGER, E., AUCLIN, E., PERRET, A., COUTZAC, C., TURPIN, A., PELLAT, A., RANDRIAN, V., BASILE, D., FAROUX, R., PERNOT, S., LOCHER, C., HAUTEFEUILLE, V., DUBREUIL, O., PALMIERI, L.-J., DIOR, M., ARTRU, P. & TAIEB, J. 2022. First-line chemotherapy with raltitrexed in metastatic colorectal cancer: an Association des Gastro-entérologues Oncologues (AGEO) multicentre study. *Digestive and Liver Disease,* 54**,** 684-691.
GHOBRIAL, I. M., WITZIG, T. E. & ADJEI, A. A. 2005. Targeting Apoptosis Pathways in Cancer Therapy. *CA: A Cancer Journal for Clinicians,* 55**,** 178-194.
GMEINER, W. H. & OKECHUKWU, C. C. 2023. Review of 5-FU resistance mechanisms in colorectal cancer: clinical significance of attenuated on-target effects. *Cancer Drug Resist,* 6**,** 257-272.
GOUGH, P. & MYLES, I. A. 2020. Tumor Necrosis Factor Receptors: Pleiotropic Signaling Complexes and Their Differential Effects. *Front Immunol,* 11**,** 585880.
GREEN, D. R. & LLAMBI, F. 2015. Cell Death Signaling. *Cold Spring Harb Perspect Biol,* 7.
GRETHE, S., ARES, M., ANDERSSON, T. & PÖRN-ARES, M. 2004. p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-x(L). *Experimental cell research,* 298**,** 632-42.
GROSSI, V., PESERICO, A., TEZIL, T. & SIMONE, C. 2014. p38α MAPK pathway: a key factor in colorectal cancer therapy and chemoresistance. *World J Gastroenterol,* 20**,** 9744-58.
GU, C., ZHANG, J., CHEN, Y. & LEI, J. 2011. A trigger model of apoptosis induced by tumor necrosis factor signaling. *BMC Systems Biology,* 5**,** S13.
GUICCIARDI, M. E. & GORES, G. J. 2009. Life and death by death receptors. *Faseb j,* 23**,** 1625-37.
HAN, Z., MENG, L., HUANG, X., TAN, J., LIU, W., CHEN, W., ZOU, Y., CAI, Y., HUANG, S., CHEN, A., ZHAN, T., HUANG, M., CHEN, X., TIAN, X. & ZHU, Q. 2022. Inhibition of p38 MAPK increases the sensitivity of 5-fluorouracil-resistant SW480 human colon cancer cells to noscapine. *Oncol Lett,* 23**,** 52.
HENRY, C. M. & MARTIN, S. J. 2017. Caspase-8 Acts in a Non-enzymatic Role as a Scaffold for Assembly of a Pro-inflammatory "FADDosome" Complex upon TRAIL Stimulation. *Mol Cell,* 65**,** 715-729.e5.
HILARIO, E., CAÑAVATE, M., LACALLE, J., ALONSO-ALCONADA, D., LARA, I., ALVAREZ-GRANDA, L. & ALVAREZ, A. 2010. Cell death. A comprehensive approximation. Delayed cell death.
HOSSAIN, M. S., KARUNIAWATI, H., JAIROUN, A. A., URBI, Z., OOI, J., JOHN, A., LIM, Y. C., KIBRIA, K. M. K., MOHIUDDIN, A. K. M., MING, L. C., GOH, K. W. & HADI, M. A. 2022. Colorectal Cancer: A Review of Carcinogenesis, Global Epidemiology, Current Challenges, Risk Factors, Preventive and Treatment Strategies. *Cancers (Basel),* 14.
HSU, S. C., GAVRILIN, M. A., TSAI, M. H., HAN, J. & LAI, M. Z. 1999. p38 mitogen-activated protein kinase is involved in Fas ligand expression. *J Biol Chem,* 274**,** 25769-76.
JAN, R. & CHAUDHRY, G. E. 2019. Understanding Apoptosis and Apoptotic Pathways Targeted Cancer Therapeutics. *Adv Pharm Bull,* 9**,** 205-218.
LIU, Y., BERTRAM, C. C., SHI, Q. & ZINKEL, S. S. 2011. Proapoptotic Bid mediates the Atr-directed DNA damage response to replicative stress. *Cell Death Differ,* 18**,** 841-52.
LOSA, J. H., COBO, C. P., VINIEGRA, J. G., LOBO, V. J. S.-A., CAJAL, S. R. Y. & SÁNCHEZ-PRIETO, R. 2003. Role of the p38 MAPK pathway in cisplatin-based therapy. *Oncogene,* 22**,** 3998-4006.
MOHR, A., DEEDIGAN, L., JENCZ, S., MEHRABADI, Y., HOULDEN, L., ALBARENQUE, S.-M. & ZWACKA, R. M. 2018. Caspase-10: a molecular switch from cell-autonomous apoptosis to communal cell death in response to chemotherapeutic drug treatment. *Cell Death & Differentiation,* 25**,** 340-352.

MOHR, A., LYONS, M., DEEDIGAN, L., HARTE, T., SHAW, G., HOWARD, L., BARRY, F., O'BRIEN, T. & ZWACKA, R. 2008. Mesenchymal stem cells expressing TRAIL lead to tumour growth inhibition in an experimental lung cancer model. *Journal of Cellular and Molecular Medicine,* 12**,** 2628-2643.

OH, Y. T., YUE, P., WANG, D., TONG, J. S., CHEN, Z. G., KHURI, F. R. & SUN, S. Y. 2015. Suppression of death receptor 5 enhances cancer cell invasion and metastasis through activation of caspase-8/TRAF2-mediated signaling. *Oncotarget,* 6**,** 41324-38.

OZAWA, S., MIURA, T., TERASHIMA, J. & HABANO, W. 2021. Cellular irinotecan resistance in colorectal cancer and overcoming irinotecan refractoriness through various combination trials including DNA methyltransferase inhibitors: a review. *Cancer Drug Resist,* 4**,** 946-964.

OZÖREN, N. & EL-DEIRY, W. S. 2002. Defining characteristics of Types I and II apoptotic cells in response to TRAIL. *Neoplasia,* 4**,** 551-7.

PETERS, G. J., BACKUS, H. H., FREEMANTLE, S., VAN TRIEST, B., CODACCI-PISANELLI, G., VAN DER WILT, C. L., SMID, K., LUNEC, J., CALVERT, A. H., MARSH, S., MCLEOD, H. L., BLOEMENA, E., MEIJER, S., JANSEN, G., VAN GROENINGEN, C. J. & PINEDO, H. M. 2002. Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. *Biochim Biophys Acta,* 1587**,** 194-205.

PHAN, T., ZHANG, X. H., ROSEN, S. & MELSTROM, L. G. 2023. P38 kinase in gastrointestinal cancers. *Cancer Gene Therapy,* 30**,** 1181-1189.

PRANTEDA, A., PIASTRA, V., STRAMUCCI, L., FRATANTONIO, D. & BOSSI, G. 2020. The p38 MAPK signaling activation in colorectal cancer upon therapeutic treatments. *International Journal of Molecular Sciences,* 21**,** 2773.

REBILLARD, A., JOUAN-LANHOUET, S., JOUAN, E., LEGEMBRE, P., PIZON, M., SERGENT, O., GILOT, D., TEKPLI, X., LAGADIC-GOSSMANN, D. & DIMANCHE-BOITREL, M.-T. 2010. Cisplatin-induced apoptosis involves a Fas-ROCK-ezrin-dependent actin remodelling in human colon cancer cells. *European Journal of Cancer,* 46**,** 1445-1455.

REYHANOGLU, G. & SMITH, T. 2023. Irinotecan. *StatPearls.* Treasure Island (FL): StatPearls Publishing

RIVORY, L. P. 2002. New drugs for colorectal cancer-mechanisms of action. *Australian Prescriber,* 25.

SHAKERI, R., KHEIROLLAHI, A. & DAVOODI, J. 2017. Apaf-1: Regulation and function in cell death. *Biochimie,* 135**,** 111-125.

SHI, S., WANG, Q., XU, J., JANG, J. H., PADILLA, M. T., NYUNOYA, T., XING, C., ZHANG, L. & LIN, Y. 2015. Synergistic anticancer effect of cisplatin and Chal-24 combination through IAP and c-FLIPL degradation, Ripoptosome formation and autophagy-mediated apoptosis. *Oncotarget,* 6**,** 1640-51.

STRAMUCCI, L., PRANTEDA, A., STRAVATO, A., AMOREO, C. A., PENNETTI, A., DIODORO, M. G., BARTOLAZZI, A., MILELLA, M. & BOSSI, G. 2019. MKK3 sustains cell proliferation and survival through p38DELTA MAPK activation in colorectal cancer. *Cell Death & Disease,* 10**,** 842.

STUCKEY, D. W. & SHAH, K. 2013. TRAIL on trial: preclinical advances in cancer therapy. *Trends Mol Med,* 19**,** 685-94.

TANOS, T., MARINISSEN, M. J., LESKOW, F. C., HOCHBAUM, D., MARTINETTO, H., GUTKIND, J. S. & COSO, O. A. 2005. Phosphorylation of c-Fos by Members of the p38 MAPK Family: ROLE IN THE AP-1 RESPONSE TO UV LIGHT *. *Journal of Biological Chemistry,* 280**,** 18842-18852.

TARIQ, K. & GHIAS, K. 2016. Colorectal cancer carcinogenesis: a review of mechanisms. *Cancer Biol Med,* 13**,** 120-35.

TAYLOR, C. A., ZHENG, Q., LIU, Z. & THOMPSON, J. E. 2013. Role of p38 and JNK MAPK signaling pathways and tumor suppressor p53 on induction of apoptosis in response to Ad-eIF5A1 in A549 lung cancer cells. *Molecular Cancer,* 12**,** 35.

TENEV, T., BIANCHI, K., DARDING, M., BROEMER, M., LANGLAIS, C., WALLBERG, F., ZACHARIOU, A., LOPEZ, J., MACFARLANE, M., CAIN, K. & MEIER, P. 2011. The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Mol Cell,* 43**,** 432-48.

THORN, C. F., MARSH, S., CARRILLO, M. W., MCLEOD, H. L., KLEIN, T. E. & ALTMAN, R. B. 2011. PharmGKB summary: fluoropyrimidine pathways. *Pharmacogenet Genomics,* 21**,** 237-42.

VASEVA, A. V. & MOLL, U. M. 2009. The mitochondrial p53 pathway. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1787**,** 414-420.

WAJANT, H. 2002. The Fas Signaling Pathway: More Than a Paradigm. *Science,* 296**,** 1635-1636.

WEBSTER, J. D. & VUCIC, D. 2020. The Balance of TNF Mediated Pathways Regulates Inflammatory Cell Death Signaling in Healthy and Diseased Tissues. *Frontiers in Cell and Developmental Biology,* 8.

WILSON, M. R. 1998. Apoptosis: unmasking the executioner. *Cell Death & Differentiation,* 5**,** 646-652.

WU, G. S. & DING, Z. 2002. Caspase 9 is required for p53-dependent apoptosis and chemosensitivity in a human ovarian cancer cell line. *Oncogene,* 21**,** 1-8.

YAKKALA, P. A., PENUMALLU, N. R., SHAFI, S. & KAMAL, A. 2023. Prospects of Topoisomerase Inhibitors as Promising Anti-Cancer Agents. *Pharmaceuticals,* 16**,** 1456.

YAMAGISHI, H., KURODA, H., IMAI, Y. & HIRAISHI, H. 2016. Molecular pathogenesis of sporadic colorectal cancers. *Chin J Cancer,* 35**,** 4.

YANG, J., HU, S., BIAN, Y., YAO, J., WANG, D., LIU, X., GUO, Z., ZHANG, S. & PENG, L. 2021. Targeting Cell Death: Pyroptosis, Ferroptosis, Apoptosis and Necroptosis in Osteoarthritis. *Front Cell Dev Biol,* 9**,** 789948.

YU, R., DEEDIGAN, L., ALBARENQUE, S. M., MOHR, A. & ZWACKA, R. M. 2013. Delivery of sTRAIL variants by MSCs in combination with cytotoxic drug treatment leads to p53-independent enhanced antitumor effects. *Cell Death Dis,* 4**,** e503.

YUE, J. & LÓPEZ, J. M. 2020. Understanding MAPK Signaling Pathways in Apoptosis. *Int J Mol Sci,* 21.

ZHANG, N., YIN, Y., XU, S. J. & CHEN, W. S. 2008. 5-Fluorouracil: mechanisms of resistance and reversal strategies. *Molecules,* 13**,** 1551-69.

ZIMMERMANN, K. C. & GREEN, D. R. 2001. How cells die: Apoptosis pathways. *Journal of Allergy and Clinical Immunology,* 108**,** S99-S103.

ZINKEL, S. S., HUROV, K. E. & GROSS, A. 2007. Bid Plays a Role in the DNA Damage Response. *Cell,* 130**,** 9-10.