Effect of Wnt Inhibition Drug Treatment on Chemoresistant Colorectal Cancer

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Supervisor: Dr. Byron Baron

Daniel Paul Sant
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Abstract

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide, in addition to being responsible for a significant portion of cancer-related deaths. It arises as a result of mutations to genes such as the Adenomatous Polyposis Coli (APC), which aberrantly activate the canonical and non-canonical Wnt signaling pathways, resulting in promoted cell growth. In addition to this, chemoresistance is an additional factor which affects cellular growth by making the cells unresponsive to treatment, allowing them to continue proliferating. Thus, there is a need to investigate potential treatments which can counteract these adverse effects. In this study, two PORCN inhibitors, LGK974 and ETC-159, at concentrations of 10μM and 0.2μM, respectively, were used to treat parental and 5-FU resistant CRC cell lines bearing either wild-type APC or an APC truncation mutation. Presto Blue viability assays were conducted over 72 hours to assess cell viability in response to treatment. Scratch migration and transwell invasion assays were performed to study the drugs' effects on migration and invasion. Western blotting was then carried out for β-catenin, CDC42, EGFR, and mono- and tri-methyl lysine. Lastly, an ELISA was used to quantify total and phosphorylated EGFR in response to treatment. Results indicate that daily treatment with the Wnt inhibitors can reduce CRC cell viability, irrespective of APC mutation or chemoresistance status. Results from the migration and invasion assays continue to reveal the complexity of the canonical and non-canonical Wnt pathways with decreases and increases in migration and invasion rate observed. While Western blotting did not reveal any significant differences in the protein expression of β-catenin, CDC42 and EGFR, results for mono- and tri-methyl lysine were more interesting and suggest that APC truncation and chemoresistance status affect these modifications to some extent. This revealed lysine methylation as a potential diagnostic or prognostic biomarker for CRC treatment using LGK974 and ETC-159, although further studies are warranted to properly reveal the underlying mechanisms which link these characteristics. APC truncation status and chemoresistance were also revealed to potentially be playing some role in the expression of total and phosphorylated EGFR from the results obtained by the ELISA.

Key words: CRC, APC, Wnt, PORCN Inhibitors, Drug Resistance

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LIST OF ABBREVIATIONS

The following abbreviations have been used within this dissertation:

5-FU	5-Fluorouracil
5-FUDR	5-fluoro-2-deoxyuridine
ABC	ATP-binding casette
ACS	Adenoma-Carcinoma Sequence
APAF1	Apoptotic Peptidase Activating Factor 1
APC	Adenomatous Polyposis Coli
ASEF	APC-stimulated guanine nucleotide exchange factor
CA	Carbohydrate Antigen
CAMKII	Calcium/calmodulin-dependent kinase II
CDC42	Cell Division Control Protein 42
CEA	Carcinoembryonic Antigen
CH_2THF	5, 10-methylene tetrahydrofolate
CH_3THF	5-methylenetetrahydrofolate
CIMP	CpG Island Methylator Phenotype
CIMP-H	CIMP-High
CIMP-L	CIMP-Low
CIN	Chromosomal Instability
circRNA	Circular RNA
CK1	Casein Kinase 1
CRC	Colorectal Cancer
CT	Computed Tomography
CtBP1	C-terminal Binding Protein 1
Daam1	Dishevelled associated activator of morphogenesis 1
DAG	Diacyl Glycerol
DLG	Discs-Large
DPD	Dihydropyrimidine Dehydrogenase
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate

Dvl	Dishevelled
EGFR	Epithelial Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
ERCC1	Excision Repair Cross-Complementation group 1
FAP	Familial Adenomatous Polyposis
FdUMP	Fluorodeoxyuridine Monophosphate
FdUR	Fluorodeoxyuridine
FdUTP	Fluorodeoxyuridine Triphosphate
FIT	Fecal Immunochemical Test
FOBT	Fecal Occult Blood Test
FUMP	Fluorouridine Monophosphate
FUTP	Fluorouridine Triphosphate
Fz	Frizzled
GAP	GTPase Activating Protein
gFOBT	Guaiac-based FOBT
GSK3	Glycogen Synthase 3
GTP	Guanine Triphosphate
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IP3	Inositol-1,4,5-triphosphate
IQGAP1	IQ-motid-containing GTPase Activation Protein 1
JNK	c-JUN-N terminal kinase
lncRNA	Long non-coding RNA
LOH	Loss of Heterozygosity
LRP5/6	Low density Lipoprotein Receptor related Proteins 5/6
LV	Leucovorin
M2-PK	M2 Isoform of Pyruvate Kinase
MAP	MUTYH-Associated Polyposis
MCR	Mutation Cluster Region
mCRC	Metastatic CRC
miRNA	Micro RNA
MMP9	Metalloproteinase 9
MMR	Mismatch Repair

MRP8	Multi-drug Resistance Protein 8
MSI	Microsatellite Instability
MSI-H	MSI-H
MSS	Microsatellite Stable
MTHFR	Methylene Tetrahydrofolate Reductase
mTOR	Mechanistic Target of Rapamycin
ncRNA	Non-coding RNA
NFAT	Nuclear Factor of Activated T Cell
NLK	Nemo-like Kinase
Nrf2	NF-E2-related factor 2
OPRT	Orotate Phosphoribosyl Transferase
PCP	Planar Cell Polarity
PDK1	Phosphotidyl inositol-dependent kinase 1
PI3K	Phosphotidylinositol-3-kinase
PJS	Peutz-Jeghers Syndrome
PKC	Protein Kinase C
PLC	Phospholipase C
PORCN	Porcupine
PTK7	Protein Tyrosine Kinase 7
RAC1	Ras-related C3 botulinum toxin substrate 1
RHOA	Ras-homology gene family member A
ROR2	Receptor tyrosine kinase-like Orphan Receptor 2
SAMP	Serine-Alanine-Methionine-Proline
SHMT2	Serine Hydroxymethyltransferase-2
SLC	Solute Carrier
SNP	Single Nucleotide Polymorphism
TAK1	TGFβ Activated Kinase 1
TCF/LEF	T-Cell Factor/Lymphoid Enhancer-binding Factor
TIMP1	Tissue Inhibitor of Matrix Metalloproteinase 1
TK	Thymidine Kinase
TP	Thymidine Phosphorylase
TS	Thymidylate Synthase

TSC2	Tuberous Sclerosis Complex 2
TWIST1	Twist family BHLH Transcription factor 1
UGT1A1	UDP glucuronosyltransferase family 1 member A1
UTP	Uridine Triphosphate
VEGF	Vascular Endothelial Growth Factor
ZEB1	Zinc finger E-box Binding homeobox 1
β-TrCP	β-Transducin repeat-Containing Protein

1 Literature Review

1.1 Colorectal Cancer

1.1.1 Colorectal Cancer Incidence and Mortality

Currently, colorectal cancer (CRC) is the third most commonly diagnosed cancer globally, with around 10% of all cancer diagnoses being CRC. In addition, it is also responsible for a high mortality rate as around 9.4% of all cancer deaths are attributed to CRC (GLOBOCAN, 2020). The statistics for Malta are also quite similar, with CRC being the third most commonly diagnosed cancer, and the second most common cause of cancer death (GLOBOCAN, 2020; Malta National Cancer Registry, 2017).

The 5-year survival rate for CRC varies significantly based on whether the cancer is localised or has already spread (metastasised). Current statistics show that for CRC which is localised the overall 5-year survival rate is around 90%. When the CRC becomes regional, meaning that it has spread to nearby lymph nodes, the survival rate drops to around 72%. The 5-year survival rate then drops significantly to around 14% when the cancer has metastasised ("Cancer Stat Facts", 2021).

1.1.2 Risk Factors Contributing to CRC

The major contributing risk factors for CRC fall into two main categories, these being lifestyle and environmental factors. Among these, some of the most common controllable

risk factors include smoking, the excessive consumption of alcohol, being overweight or obese, and the consumption of high amounts of red and processed meat (Brenner and Chen, 2018). Reducing these factors while also increasing physical activity and increasing the intake of dietary fibre are some ways which can thus reduce the risk of developing CRC. However, apart from these controllable risk factors, familial history of CRC, hereditary conditions, and having an age higher than 50 are other factors which increase the risk of CRC (Brenner and Chen, 2018).

1.1.3 CRC Types

CRC is a type of cancer which can originate from the colon or the rectum, with colon cancer being more prevalent (Centelles, 2012). CRC usually develops from benign polyps growing in the intestinal mucosa, which can eventually become cancerous by acquiring the ability to invade and metastasise via mutations (Simon, 2016). CRC can be classified into two major categories, these being sporadic and inherited.

1.1.3.1 Sporadic CRC

Sporadic CRC is the most common, accounting for approximately 70% to 80% of CRC cases and is mainly connected to the previously mentioned lifestyle and environmental factors which result in the accumulation of mutations in key regulatory genes (Mármol *et al.*, 2017; Aran *et al.*, 2016; Fischer *et al.*, 2019). Sporadic CRC mainly arises through three main genetic pathways: chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) pathways (Aran *et al.*, 2016).

CIN pathway: The CIN pathway accounts for approximately 65-75% of all sporadic CRC cases with some of its main characteristics being the loss of heterozygosity and aneuploidy

(Fischer *et al.*, 2019; Pino and Chung, 2010). The most common mutations which are observed in the CIN pathway occur in the Adenomatous Polyposis Coli tumour suppressor (*APC)*, Kirsten rat sarcoma viral oncogene homolog (*KRAS)*, and tumour protein P53 (*p53)* genes (Fischer *et al.*, 2019; Aran *et al.*, 2016). As a result of this, the CIN pathway has significant consequences in other regulatory pathways such as the Raf-MEK-ERK and the Wnt signaling pathways, resulting in aberrant cell growth and increased likelihood of cancer occurrence (Pino and Chung, 2010; Fischer *et al.*, 2019; Müller, Ibrahim, and Arends, 2016).

MSI pathway: The MSI pathway is found in around 15% of CRCs (Sinicrope and Sargent, 2012; Aran *et al.*, 2016). MSI arises when microsatellites present in the genome of tumours acquire a large quantity of mutations as a result of faulty DNA mismatch repair (MMR) genes (Fischer *et al.*, 2019). Inactivating mutations of MMR genes can mainly occur via two ways, either by the hypermethylation of MMR genes such MutL protein homolog 1 (*MLH1)*, or by germline mutations to the *MMR* genes which is the basis of Hereditary Non-Polyposis Colorectal Cancer (HNPCC), also known as Lynch syndrome (Sinicrope and Sargent, 2012). As a result of the higher frequency of mutations due to a faulty MMR mechanism, microsatellites often exhibit varying lengths, an effect which is termed as MSI at high frequency (MSI-H) (Müller *et al.*, 2016). In addition to this, mutations to v-Raf murine sarcoma viral oncogene homolog B (*BRAF)* and *KRAS* oncogenes have also been seen, with an important observation being made that *BRAF* mutations are present when the *MLH1* gene is hypermethylated (Fischer *et al.*, 2019; Aran *et al,*, 2016). This observation can aid diagnosing as patients having *BRAF* mutations along with *MLH1* hypermethylation should be investigated for possible HNPCC (Fischer *et al.*, 2019; Müller *et al.*, 2016).

CIMP: CIMP occurs in around 20% of CRC cases and is characterised by the hypermethylation of regions in gene promoters called the CpG islands involved in epigenetic regulation (Mojarad, Kuppen, Aghdaei, and Zali, 2013). The result of this hypermethylation is

the dysfunction of tumour suppressors as well as of DNA repair mechanisms. In fact, an example of this has already been seen in MSI where hypermethylation of *MLH1* results in a faulty MMR mechanism, an overlapping feature of CIMP and MSI (Fischer *et al.*, 2019). CIMP itself is commonly subdivided into CIMP-Low (CIMP-L) and CIMP-High (CIMP-H), depending on the number of loci at which hypermethylation occurs (Federova *et al.*, 2019). Furthermore, it was also observed that *KRAS* and *BRAF* mutations frequently occurred in CIMP-H tumours in comparison to CIMP-L, with *BRAF* mutations being more frequent when the tumour was also MSI-H (Aran *et al.*, 2016; Fischer *et al.*, 2019; Ang *et al.*, 2010).

1.1.3.2 Inherited CRC

Inherited CRC accounts for around 20-30% of CRC cases and is characterised by familial history of, or genetic predisposition to, CRC (Stigliano, Sanchez-Mete, Martayan, and Anti, 2014). These types of inherited cancers are further subdivided into polyposis and non-polyposis variants.

Polyposis CRC: The polyposis variants include Familial Adenomatous Polyposis (FAP), MUTYH-associated polyposis (MAP), and Peutz-Jeghers syndrome (PJS). FAP is primarily characterised by the presence of germline mutations in the Adenomatous Polyposis Coli (*APC*) gene, a tumour suppressor crucial in the regulation of the Wnt/β-catenin pathway (Plawski *et al.*, 2013). MAP is characterised by mutations to the *MUTYH* gene, involved in the base excision repair mechanism, while PJS is characterised by mutations to the *LBK1* tumour suppressor gene (Plawski *et al.*, 2013; Korsse *et al.*, 2013).

Non-polyposis CRC: The non-polyposis CRC variants mainly include Hereditary Non-Polyposis Colorectal Cancer (HNPCC or Lynch syndrome), caused by mutations in genes

involved in DNA mismatch repair such as *MLH1*, *MSH2*, and *PMS* (Mármol *et al.*, 2017; Aran *et al.*, 2016).

1.1.4 CRC Screening

Currently, there are various screening tests and procedures which are available to patients. The most commonly used tests can be split into two categories, endoscopy screening and stool testing. Examples of endoscopic screening procedures include colonoscopy, computed tomography (CT) colonography, and sigmoidoscopy, while examples of stool testing include the fecal occult blood test (FOBT), and fecal immunochemical tests (FIT) (Mármol *et al.*, 2017; Brenner and Chen, 2018). In addition to these, the use of biomarkers for diagnosing CRC is increasingly becoming more common.

1.1.4.1 Endoscopic Screening

Colonoscopy involves a screening of the entirety of the colon and is generally performed for patients who are above 50 years of age and who are deemed to have an average risk of CRC (Simon, 2016). Sigmoidoscopy is similar, with the main difference being that it only screens the rectum and the distal portion of the colon. Use of colonoscopy and sigmoidoscopy has been shown to be effective in reducing incidence and mortality of CRC, especially since a major advantage of these techniques is that they allow the clinician to operate on and remove any potentially cancerous lesions detected during the procedure (Simon, 2016; Brenner and Chen, 2018; Lauby-Secretan *et al.*, 2018). However, these tests are not without their limitations and risks which most notably include their invasiveness, complex preparatory procedures, the use of sedatives, and the risks of bowel perforation and bleeding (Ladabaum, Dominitz, Kahi, and Schoen, 2020; Cooper, Kou, and Rex, 2013; Simon,

2016). Another screening procedure that may be employed is CT colonography. This is mainly used for patients for whom a standard colonoscopy is not recommended due to other complications (Ladabaum *et al.*, 2020; Simon, 2016). However, CT colonography has a major limitation in that radiation has to be used (Lauby-Secretan *et al.*, 2018).

1.1.4.2 Stool Tests

FOBT, which is also known as guaiac-based FOBT (gFOBT), and FIT are stool-based tests, performed annually or biennially, which detect the presence of haemoglobin in stool. While FIT is more specific to human haemoglobin owing to its use of an antibody specific to it, both tests have been found to be effective in reducing mortality and incidence (Brenner and Chen, 2018; Lauby-Secretan *et al.*, 2018; Laudabaum *et al.*, 2020; Zorzi *et al.*, 2014). Limitations to these tests include the fact that these tend to generate false positives and negatives (Simon, 2016).

1.1.5 Screening and Diagnostic Biomarkers

The use of biomarkers for diagnosing CRC is increasingly becoming more common, especially as tests improve both in their sensitivity and specificity. These biomarkers can either be genetic or protein markers, and they can be obtained either from stool or blood samples.

1.1.5.1 Diagnostic Protein Biomarkers

One of the most common diagnostic protein biomarker, which has already been briefly mentioned in Section 1.1.4.2, is haemoglobin which can be detected in the stool for a potential CRC diagnosis. Apart from haemoglobin, the M2 isoform of pyruvate kinase (M2-

PK), tissue inhibitor of matrix metalloproteinase 1 (TIMP1), and metalloproteinase 9 (MMP9) are other protein biomarkers which can all be detected in the stool (Lech *et al.*, 2016). These proteins show promise for use in CRC screening however more large scale studies would need to be conducted for their adoption into clinical use (Loktionov, 2020). With regards to blood-based diagnostic biomarkers, some of the most frequently used include the carcinoembryonic antigen (CEA), as well as various carbohydrate antigens (CA) such as CA 19-9, 72-4, and 125. High CEA levels have been found in CRC cases however, studies have also found that such elevated levels of CEA occur in other conditions such as in gastric, pancreatic and ovarian cancers, as well as in inflammatory conditions (Lech *et al.*, 2016). In addition to this, another limitation of CEA is that it is not able to confirm whether any confirmed polyps are benign or malignant. Thus, when such a limitation is coupled with CEA's non-specificity to CRC, CEA is not considered as an ideal screening biomarker (Gao *et al.*, 2018; Lech *et al.*, 2016). However, one of the major uses of CEA lies in its ability to detect the recurrence of CRC after surgical resection of the tumour, especially if combined with other serum protein markers, hence allowing CEA to adopt a crucial monitoring role for patients (Wang *et al.*, 2014; Tan *et al.*, 2008; Lech *et al.*, 2016). Similar to CEA, the carbohydrate antigens are also not specific for CRC and they also show relatively low sensitivity. However, there is some evidence to suggest that using CEA in conjunction with carbohydrate antigens could result in improved sensitivity (Gao *et al.*, 2018).

1.1.5.2 Diagnostic Genetic Biomarkers

As CRC often develops as a result of mutations in certain key driver genes such as *KRAS*, *TP53*, and *APC*, the presence of mutations in these genes could be an indication of CRC, hence such genes are commonly used as genetic biomarkers (Loktionov, 2020). In addition to

this, epigenetic changes, such as DNA promoter methylation, as well as MSI markers are often investigated (Lech *et al.*, 2016). In the previous section it was described how stool tests are employed for detecting protein biomarkers. However, such tests are also being used for genetic biomarker testing. In fact, stool tests are also evolving to incorporate the testing of multiple biomarkers with the aim of increasing both sensitivity and specificity. One such test uses *KRAS* mutation status, promoter methylation status, β-actin measurements, and haemoglobin testing via FIT, which results in good overall sensitivity and specificity, however this is quite expensive (Ahlquist *et al.*, 2012; Loktionov, 2020). Apart from measuring DNA in stool, mRNA is also being employed for the possible detection of CRC, with recent studies also aiming to develop a multitarget mRNA test for the identification of patients having CRC (Koga *et al.*, 2008; Herring *et al.*, 2021). Finally, given the role microRNAs (miRNAs) play in various regulatory processes of gene expression, especially cancer, their detection is also being used as a potential diagnostic biomarker (Romano *et al.*, 2017). Among them, miR-21, miR-106a, miR-223, and miR-451 are some miRNAs which have the most potential for use as CRC diagnostic biomarkers, although more studies are needed both for validation and for optimising the tests (Link *et al.*, 2010; Phua *et al.*, 2014; Loktionov, 2020).

1.1.6 CRC Treatment

For all stages of CRC, especially for the early stages, surgical resection of the tumour is usually the best course of action (Costas-Chavarri *et al.*, 2019; Van Cutsem *et al.*, 2016). Following surgery, patients with stage II and stage III CRC receive adjuvant chemotherapy with the fluoropyrimidines, 5-Fluorouracil (5-FU) and capecitabine. In addition, folinic acid (also known as leucovorin (LV)) may also be used in conjunction with 5-FU (Varghese, 2015). In the case of high risk stage II CRC, as well as stage III, cytotoxic doublet chemotherapy is

generally employed consisting of a combination of fluoropyrimidines, either 5-FU or capecitabine, with leucovorin and/or the platinum compound, oxaliplatin. These treatments are commonly referred to as FOLFOX (LV/5-FU/oxaliplatin) and CAPOX (capecitabine/oxaliplatin) (Varghese, 2015; Taieb and Gallois, 2020). A topoisomerase I inhibitor, irinotecan, may also be used in combination therapy with 5-FU and leucovorin, a treatment which is also known as FOLFIRI (Aran *et al.*, 2016; Mármol *et al.*, 2017).

For Stage IV CRC, which is also referred to as metastatic CRC (mCRC), first-line treatment generally involves therapy with the cytotoxic doublets FOLFOX, CAPOX, or FOLFIRI, and in some cases cytotoxic triplet therapy with FOLFOXIRI (LV/5-FU/oxaliplatin/irinotecan) may also be utilised (Van Cutsem *et al.*, 2016). In addition to these, anti-vascular endothelial growth factor (VEGF) and anti-epithelial growth factor receptor (EGFR) monoclonal antibodies (bavacizumab and cetuximab, respectively) are also commonly used, especially in combination with the cytotoxic doublets (Centelles, 2012; Mármol *et al.*, 2017; Van Cutsem *et al.*, 2016). Second-line and third-line treatment for mCRC includes the use of more monoclonal antibodies against VEGF and EGFR such as the anti-VEGF antibody aflibercept, and the anti-EGFR antibody panitumumab (Aran *et al.*, 2016; Van Cutsem *et al.*, 2016).

1.1.7 Prognostic and Predictive Biomarkers

Apart from the diagnostic biomarkers mentioned in Sections 1.1.5.1 and 1.1.5.2 which aid the clinician in screening and identifying whether a patient potentially has CRC, biomarkers may also be used to give information about the likely outcome of a disease which aid in the choice of the best course of treatment. The presence of such biomarkers can not only give an indication to the likely outcome of the disease but also the effectiveness of a particular treatment, hence aiding in administering the most optimal treatment while avoiding the use

of ineffective, unnecessary, and costly medication for the patient. The three most commonly used biomarkers in this regard are MSI, BRAF, and KRAS.

The presence of microsatellite instability markers has been linked to the overall survival of a patient, where patients having MSI-H tumours in Stage II or Stage III CRC have a higher survival rate than patients with MSI-L tumours, or patients whose tumours have no MSI features, a characteristic described as microsatellite stable (MSS) (Gonzalez-Pons and Cruz-Correa, 2015; Lech *et al.*, 2016). For patients who have Stage II CRC with MSI-H tumours it is recommended that they not be treated with 5-FU adjuvant chemotherapy due to it having little to no benefit in such patients. However, patients with MSS tumours have been found to benefit from such treatment (Boussios *et al.*, 2019; Sargent *et al.*, 2014).

The *BRAF* gene has been found to be commonly mutated, with these mutations being associated with a poor prognosis, particularly in patients having MSI-L or MSS tumours (Gonzalez-Pons and Cruz-Correa, 2015; Lech *et al.*, 2016; Venderbosch *et al.*, 2014). Moreover, it is recommended that for patients having *BRAF* mutations, anti-EGFR therapy should not be given as it has been found that this treatment has limited benefit for these patients (Ursem, Etreya, and Van Loon, 2018; Boussios *et al.*, 2019; Van Cutsem *et al.*, 2016).

KRAS mutations have been found to be associated with poorer survival, especially for patients having MSI-L tumours, in addition to being associated with a higher risk of recurrence after surgery (Ogunwobi, Mahmood, and Akingboye, 2020; Boussios *et al.*, 2019). Furthermore, similar to *BRAF* mutations, anti-EGFR therapy is not recommended for patients with *KRAS* mutations owing to a poor response (Van Cutsem *et al.*, 2016; Ogunwobi *et al.*, 2020).

Apart from these mentioned biomarkers, other biomarkers are also being used for predictive purposes. One such biomarker is the *PI3KCA* gene, mutations in which result in a CRC which is resistant to anti-EGFR therapy, however allowing for the potential use of aspirin

to increase a patient's survival time (Lech *et al.*, 2016). Biomarkers can also give an indication as to possible toxicity from treatment. Three such biomarkers are dihydropyrimidine dehydrogenase (DPD), UDP glucuronosyltransferase family 1 member A1 (UGT1A1), and excision repair cross-complementation group 1 (ERCC1). A lack of DPD has been linked to increased toxicity from 5-FU or capecitabine treatment, polymorphisms in UGT1A1 are associated with increased toxicity to irinotecan, and high expression of ERCC1 is an indication that treatment based on oxaliplatin will not be effective (Van Cutsem *et al.*, 2016; Ogunwobi *et al.*, 2020).

1.2 5-FU

As has been described in Section 1.1.6, 5-FU is commonly used as part of adjuvant therapy. 5-FU is a fluoropyrimidine classed as an anti-metabolite drug owing to its ability to target and inhibit processes which are essential for biosynthesis, and for its ability to disrupt DNA and RNA by being incorporated into their structures (Longley, Harkin, and Johnston, 2003). These are mainly achieved by the anabolism of 5-FU into three main products, these being fluorodeoxyuridine monophosphate (FdUMP), fluorouridine triphosphate (FUTP), and fluorodeoxyuridine triphosphate (FdUTP). FdUMP can form a complex with thymidylate synthase (TS), a key enzyme in the synthesis of DNA precursors, and 5, 10-methylene tetrahydrofolate (CH_2THF) which results in the inhibition of TS. This enzyme is able to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) which is, in turn, phosphorylated to deoxythymidine triphosphate (dTTP), used for DNA synthesis (Rose, Farrell, and Schmitz, 2002). Hence, as a result of TS inhibition via FdUMP, the levels of dTTP decrease thus disrupting the synthesis of DNA and its repair, leading to the death of the tumour cells (Vodenkova *et al.*, 2020). In addition to this, 5-FU is also converted to two other

products, FUTP and FdUTP. These products are incorporated into DNA and RNA in lieu of uridine triphosphate (UTP) and dTTP, respectively. Furthermore, the accumulation of dUMP due to TS inhibition by FdUMP results in it becoming phosphorylated to dUTP, which is also incorporated into the DNA. The consequence of all this is that the RNA and DNA end up fragmenting, which also leads to the death of the tumour cells (Vodenkova *et al.*, 2020). The same processes also occur with other fluoropyrimidines such as capecitabine, with one difference being that these are first converted to 5-FU before eliciting their effects (Centelles, 2012).

1.2.1 5-FU Resistance Mechanisms

A major issue which can arise during treatment with chemotherapeutic drugs is the presence or development of chemoresistant tumour cells, which substantially reduces the overall effectiveness of a given treatment and increases mortality. In fact, it is estimated that over 90% of patients with metastatic cancers are affected by chemoresistant tumours, resulting in ineffective treatment (Longley and Johnston, 2005). Chemoresistance may be classed as either innate, where the genetic changes responsible for chemoresistance are already present prior to treatment, or acquired, where such genetic changes arise during treatment (Vodenkova *et al.*, 2020). Understanding the mechanisms behind chemoresistance as well as its potential downstream effects is crucial for the development of strategies to counteract it.

1.2.1.1 5-FU Metabolism Enzymes

Alterations in the gene and protein levels of such enzymes has been suggested as one possible mechanism by which resistance to 5-FU therapy occurs (Blondy *et al.*, 2020). One of

the central enzymes involved in 5-FU metabolism is TS, as has been described in Section 1.2, which is encoded by the *TYMS* gene (Vodenkova *et al.*, 2020). Owing to the role TS plays in DNA synthesis and its inhibition by FdUMP, a product of 5-FU anabolism, it is thought that an increased expression of TS would result in reduced overall responsiveness to 5-FU. In fact, studies have shown such an inverse relationship, where patients and tumours having low TS expression levels were more responsive to 5-FU treatment, and vice versa (Salonga *et al.*, 2000; Qiu *et al.*, 2008; Popat, Matakidou, and Houlston, 2004). Alterations in TS levels can occur in several ways including due to polymorphisms in the *TYMS* gene, as well as the presence of mutations and amplification of the gene itself (Marsh *et al.*, 2001; Zhang *et al.*, 2008). In addition to this, it is also known that a negative feedback loop is involved in modulating TS levels. This is due to unbound TS being able to bind to its own mRNA with the result that its translation is inhibited. However, once TS binds to FdUMP, a stable association is formed and TS disassociates from its mRNA, allowing for the translation and synthesis of TS, which raises its levels (Chu *et al.*, 1994).

While TS is involved in the anabolism of 5-FU, another enzyme which has been implicated in 5-FU resistance is DPD, which plays a role in the catabolism of this fluoropyrimidine. A low expression of this enzyme means that 5-FU is not broken down in a timely manner, increasing the toxicity of this drug and hence, as has been briefly described in Section 1.1.7, DPD can be used as a predictive biomarker for 5-FU toxicity. Conversely, increased expression of DPD has been found to be linked to 5-FU chemoresistance (Panczyk, 2014; Takebe *et al.*, 2001; Longley and Johnston, 2005). Furthermore, increased expression of DPD mRNA has also been shown to be linked to 5-FU resistance (Salonga *et al.*, 2000; Vodenkova *et al.*, 2020).

TS and DPD are two of the most well documented enzymes known to be involved in 5-FU resistance. Although other enzymes are involved in the metabolism of 5-FU, further in-depth

research is needed to definitively confirm whether they have a role in resistance. An example of such an enzyme is methylene tetrahydrofolate reductase (MTHFR) which catalyses the formation of 5-methylenetetrahydrofolate (CH_3THF) from CH_2THF (Hammond, Swaika, and Mody, 2016). The latter forms a complex with FdUMP which binds to TS, inhibiting it. Single nucleotide polymorphisms (SNPs) of the *MTHFR* gene are known to exist which reduce the enzyme's activity, resulting in the accumulation of CH_2THF which, in turn, increases the amount and stability of complexes it can form with FdUMP. The overall consequence of this is the inhibition of TS and the increased effectiveness of 5-FU (Panzyck, 2014; Etienne-Grimaldi *et al.*, 2010). In contrast, increased enzymatic activity of MTHFR should reduce the amount of CH_2THF which, in turn, should reduce the amount of TS-inhibitory complexes formed, resulting in resistance. However, studies have not shown such an effect so far (Hammond *et al.*, 2016; Vodenkova *et al.*, 2020). Similarly, thymidine phosphorylase (TP) is another enzyme involved in the 5-FU metabolism pathway which catalyses the conversion of 5-FU into 5-fluoro-2-deoxyuridine (5-FUDR), which can then be converted in subsequent steps into FdUMP (Hammond *et al.*, 2016). Although studies have shown that low TP expression is linked to a better response to 5-FU, no comprehensive studies have been conducted thus far to confirm the relationship between 5-FU resistance and TP levels (Salonga *et al.*, 2000; Lindskog *et al.*, 2014). Orotate phosphoribosyl transferase (OPRT), which converts 5-FU into fluorouridine monophosphate (FUMP), was linked to 5-FU resistance as one study found that its decreased expression is linked to resistance (Muhale *et al.*, 2011). And finally, thymidine kinase (TK) which converts fluorodeoxyuridine (FdUR) to FdUMP has also been shown in a few studies to be linked to 5-FU resistance when it is overexpressed (Chung *et al.*, 2000; Fanciullino *et al.*, 2006).

1.2.1.2 Apoptosis and Autophagy

As has been described previously, a major mechanism of how 5-FU operates is by damaging the genetic material of tumour cells, which consequently leads to their destruction via apoptosis. One of the central proteins involved in apoptosis is the tumour suppressor protein, p53, which directs cells with damaged genetic material to be destroyed (Panzyck, 2014). On the other hand, autophagy has been shown to be beneficial to the survival of the cancer cells by recycling macromolecules and organelles during periods of stress, which can result in increased tumour growth and chemoresistance (Zheng, 2017; Vodenkova *et al.*, 2020). In fact, studies have shown that when autophagy is inhibited, apoptosis in the presence of 5-FU was increased (Sasaki *et al.*, 2012; Sasaki *et al.*, 2010). This relationship between autophagy and apoptosis has also been observed in another study where the degradation of p53 was prevented when it was bound to serine hydroxymethlytransferase-2 (SHMT2), hence apoptosis increased. Treatment with 5-FU was then shown to reduce the levels of SHMT2 which inhibited apoptosis while also promoting autophagy, a mechanism which has been suggested to mediate chemoresistance (Chen *et al.*, 2021). In addition, mutations to p53 may also be responsible for inducing chemoresistance and oncogenicity such as by the expression of enzymes which disrupt the functionality of 5-FU (Van Oijen and Slootweg, 2000; Pugacheva *et al.*, 2002).

Apart from p53, another two major pathways which have been implicated in chemoresistance are the p38MAPK and the mTOR signaling pathways. The p38MAPK pathway is known to have important roles in autophagy and its inhibition has been discovered to lead to reduced 5-FU-mediated apoptosis, as well as increased chemoresistance in cell lines including CRC cell lines (de la Cruz-Morcillo *et al.*, 2012). 5-FU treatment was also found to increase the expression of p53 as well as of AMPK, which

resulted in the inhibition of the mechanistic target of rapamycin (mTOR), leading to the induction of autophagy (Li *et al.*, 2010).

Lv *et al.*, (2016) found that an isoform of the CD44 antigen, CD44v6, was overexpressed in CRC cells which were treated with 5-FU. This was linked to increased chemoresistance, arising via the promotion of autophagy. Moreover, the authors also found that overexpression of this isoform correlated with resistance to apoptosis as well as an increase in the MAPK and PI3K signaling pathways, which further contribute to chemoresistance.

1.2.1.3 Membrane Transporters

Membrane transporters are crucial proteins required for the transport of molecules, including drugs, though the plasma membrane, into and out of the cell. In chemoresistance, a major issue with such transporters is that their upregulation can lead to a higher rate of drug efflux, consequently resulting in reduced drug effectiveness. Such an effect can also be the basis of multi-drug resistance (Hu *et al.*, 2016; Blondy *et al.*, 2020). The two superfamilies of transporters are the ATP-binding casette (ABC) and the solute carrier (SLC) transporters (Vodenkova *et al.*, 2020). The majority of transporter-related 5-FU resistance seems to be linked to the ABC transporters and a number of studies have found correlations between members of this transporter family and chemoresistance to 5-FU. Of note, ABCB1, ABCB5, ABCC10, and ABCC11 have been found to be upregulated in response to 5-FU treatment and have been linked to chemoresistance (Wang *et al.*, 2015; Wilson *et al.*, 2011; Hlavata *et al.*, 2012; Oguri *et al.*, 2007; Xie *et al.*, 2017). In addition, multi-drug resistance protein 8 (MRP8), which is also part of the ABC superfamily, has also been found to be involved in 5-FU resistance (Oguri *et al.*, 2007).

1.2.1.4 Epithelial-Mesenchymal Transition (EMT)

EMT is a process in which epithelial cells transition to mesenchymal cells and thus, in the process, acquire features associated with this cell type. As a result, the cells have increased motility and invasiveness. While such a process occurs naturally during the development of the embryo and during tissue regeneration, it has also been observed to occur in cancers and, moreover, has also been associated with chemoresistance (Ribatti, Tamma, and Annese, 2020; Skarkova *et al.*, 2019; Kim *et al.*, 2015). The transforming growth factor beta (TGFβ) pathway is known to be involved in EMT and studies have found that when chemoresistant CRC cells were treated with 5-FU, activation of the TGFβ pathway occurred which is suggested to result in protection against 5-FU toxicity (Romano *et al.*, 2016). In addition to TGFβ, Twist Family BHLH Transcription Factor 1 (TWIST1) may also play roles in 5-FU resistance given its link to EMT, and studies found that inhibition of TWIST1 resulted in sensitisation of CRC cells to 5-FU therapy (Sakowicz-Burkiewicz, 2016). In a separate study by Kim *et al.* (2015), increased expression of TWIST1 was found to be increased in CRC cells resistant to 5-FU, in addition to elevated levels of other EMT promoters such as Zinc Finger E-box Binding Homeobox 1 (ZEB1) and ZEB2. The hedgehog pathway which also has roles within EMT has also been implicated in 5-FU resistance through the induction of elevated GL1 hence further showing the role EMT plays in chemoresistance (Zhang *et al.*, 2017).

1.2.1.5 Non-coding RNAs (ncRNAs)

NcRNAs are known to have crucial roles in cell function including in the transcription process, proliferation, and apoptosis. In addition, it is known that they are involved in tumour progression and in mechanisms of chemoresistance (Micallef and Baron, 2021; Geng *et al.*, 2020; Wei *et al.*, 2019). Within the family of ncRNAs, microRNAs (miRNAs) and long

non-coding mRNAs (lncRNAs) are two subgroups which have been investigated most extensively. However, recently, circular RNAs (circRNAs) have piqued the interest of several research groups owing to their discovered role in cancer progression, and possible role in chemoresistance (Geng *et al.*, 2020). Studies investigating the interaction between ncRNAs and ABC transporters have discovered how the miRNAs miR-21 and miR-361, and the lnc RNA, PVT1, indirectly regulate the expression of ABC transporters, increasing their expression and hence resulting in chemoresistance (Wu *et al.*, 2015; Sun *et al.*, 2020; Fan, Zhu, and Yao, 2018). Furthermore, ncRNAs have also been discovered to interact with signaling pathways and the EMT to increase chemoresistance (Zhang, Talmon, and Wang, 2016; Chen *et al.,* 2020; Li *et al.*, 2016). The inhibition of apoptosis has previously been shown to contribute to chemoresistance in Section 1.2.1.2. Apart from the mechanisms discussed in that section, ncRNAs have also been shown to inhibit apoptosis, especially through the indirect regulation of apoptotic factors such as the apoptotic peptidase activating factor 1 (APAF1) and BCL2 (Shang *et al.*, 2014; Chai *et al.*, 2011; Bian *et al.*, 2016; Xiong *et al.*, 2017). Similarly, the upregulation of autophagy by ncRNAs is another factor contributing to chemoresistance (Yu *et al.*, 2017; Liu *et al.*, 2020; Wang *et al.*, 2019).

1.2.1.6 Epigenetic Modifications

It has been shown that the methylation and demethylation of DNA via epigenetic modifications can also contribute to the development of chemoresistance. Of note, hypermethylation has been shown in studies to downregulate genes involved in processes such as apoptosis signaling in response to 5-FU treatment. As a result, apoptosis is reduced and leads to chemoresistance (Shen *et al.*, 2018; Baharudin *et al.*, 2017). Another example of the effect of hypermethylation on chemoresistance can be seen in MSI. As was discussed

previously in Section 1.1.7, patients who are positive for MSI should not be administered 5-FU therapy. The reason for this is that hypermethylation of the *MLH1* gene results in a dysfunctional MMR mechanism. As a result, the misincorporation of the 5-FU product, FdUTP, into DNA is not recognised by the MMR mechanism, leading to no response and hence, to resistance to 5-FU (Blondy *et al.*, 2020). Similar to hypermethylation, demethylation is another epigenetic modification possibly leading to chemoresistance. Studies have found that reactive oxygen species are produced in response to 5-FU treatment, which alters DNA methylation patterns. In fact, it was observed that 5-FU resistant cells had a demethylated promoter region of the NF-E2-Related Factor 2 (*Nrf2*) gene, which is involved in cellular protective mechanisms, which resulted in the upregulation of this protective gene (Zhao *et al.*, 2015).

1.3 CRC Driver Genes

In CRC, the most commonly mutated genes found to be responsible for tumour development are *KRAS*, *BRAF*, *PIK3CA*, *SMAD4*, *TP53*, and *APC*. These are called the adenoma-carcinoma sequence (ACS) as mutation of these genes is a primary reason for CRC development (Smit *et al.*, 2020; Huang *et al.*, 2018). Such mutations deregulate regulatory processes and cell signaling pathways, hence allowing for the development of tumours. Mutations of *APC*, *KRAS*, and *BRAF* genes are early stage events, which result in the transition of normal cells to adenoma cells. The transition of the adenoma to the carcinoma is a late stage event as a result of mutations to the *PIK3CA*, *SMAD4*, and *TP53* genes (Huang *et al.*, 2018).

1.3.1 *KRAS* and *BRAF*

KRAS encodes a 21kDa protein which activates upon binding to guanine triphosphate (GTP) and regulates cell proliferation and differentiation through pathways such as the PI3K/AKT and RAS/RAF/MAPK signaling pathways (Meng *et al.*, 2021). The KRAS' GTPase and GTPase activating protein (GAP) activity will then return KRAS to its inactive state. However, KRAS mutations impact its GTPase activity by impairing its function, hence the protein stays activated and the pathways it regulates are aberrantly activated. In addition to this, it is known that mutations to *KRAS* may promote angiogenesis, which further increases cell growth and the possibility of cancer development (Meng *et al.*, 2021; Figueras *et al.*, 2013).

BRAF regulates the RAS/RAF/MEK/ERK pathway and is also involved in the regulation of the MAPK pathway (Huang *et al.*, 2018). Mutations to *BRAF* result in the constitutive activation of these signaling pathways, hence promoting aberrant cell growth and survival (Barras, 2015).

1.3.2 *PIK3CA*

PIK3CA encodes for the p110 catalytic subunit of the phosphotidylinositol 3-kinase (PI3K) which has crucial roles within the PI3K/AKT/mTOR pathway (Cathomas, 2014). Studies have shown that CRC tumours may possess activating mutations in the *PIK3CA* gene (Ogino *et al.*, 2014). Such mutations result in the constitutive activation of the p110 catalytic subunit thus aberrantly activating AKT and the downstream mTOR complexes which promote increased cell growth and inhibit cell degradation (Mao *et al.*, 2012; Saxton and Sabatini, 2017).

1.3.3 *SMAD4*

SMAD4 is a known tumour suppressor which encodes for a Smad protein that regulates the TGFβ signal transduction pathway (Fang *et al.*, 2021; Huang *et al.*, 2018). This pathway has roles in the promotion of proliferation, migration, as well as cancer initiation. It is also known that it can cross-talk with other signaling pathways (Zhao, Mishra, and Deng, 2018). In CRC, mutations to *SMAD4* result in its inactivation and non-function, and are correlated with metastasis. In addition, it was also shown how loss of *SMAD4* promotes angiogenesis and increases invasiveness of colon cancer cells (Papageorgis *et al.*, 2011).

1.3.4 *TP53*

TP53 encodes for the p53 tumour suppressor protein which is responsible for the regulation of apoptosis and cell cycle arrest. Mutations to *TP53* are one of the primary causes of the transition of adenoma to carcinoma cells (Li *et al.*, 2015; Huang *et al.*, 2018). The inactivation of p53 by mutations results in the promotion of tumour growth as potentially cancerous cells are not destroyed (Michel *et al.*, 2021).

1.4 The Adenomatous Polyposis Coli (APC) Gene and Protein

APC is one of the most well studied driver gene for the development of CRC, it being involved in important regulatory pathways, with its dysfunction via mutations paving the way for the transition of epithelial cells to adenoma cells, hence setting the stage for the potential development of polyps and CRC (Huang *et al.*, 2018). Owing to its central role in such processes, it is a significant gene of interest in several studies investigating CRC, as a better understanding of its effects within CRC could shed more light on the mechanisms

underlying this cancer and could lead to the development of new tests and treatments for detecting and managing CRC.

1.4.1 *APC* Gene, Protein, and Mutations

The multifunctional tumour suppressor gene of *APC* is located on chromosome 5q21-q22, is composed of 21 exons and is 8535 nucleotides long (Zhang and Shay, 2017). It should also be noted that mammalian cells contain two APC genes, *APC* and *APC2*. *APC2* is similar to *APC* in structure, with some of the main differences including the fact that *APC2* is found on chromosome 19p13.3, and that APC2 is mainly found in the brain and is involved in the development of the central nervous system (Aghabozorgi *et al.*, 2019; van Es *et al.*, 1999). Of the 21 exons of the *APC* gene, exon 15 is one of most interest as the majority of the coding sequence is located here and also because most mutations commonly occur in this region. Such mutations can be either germline or somatic, where germline mutations result in FAP, while somatic mutations can result in the loss of heterozygosity (LOH) of chromosome 5q (Zhang and Shay, 2017).

The *APC* gene encodes for the APC protein which is 2843 amino acids long and 310 to 312kDa in size (Hankey *et al.*, 2018; Zhang and Shay, 2017). The APC protein is comprised of several domains including an oligomerisation domain, an armadillo repeat-domain, a 15- or 20- residue repeat domain, serine-alanine-methionine-proline (SAMP) repeats, a basic domain, and C-terminal domains, as can be seen in **Figure 1** (Aoki and Taketo, 2007; Zhang and Shay, 2017).

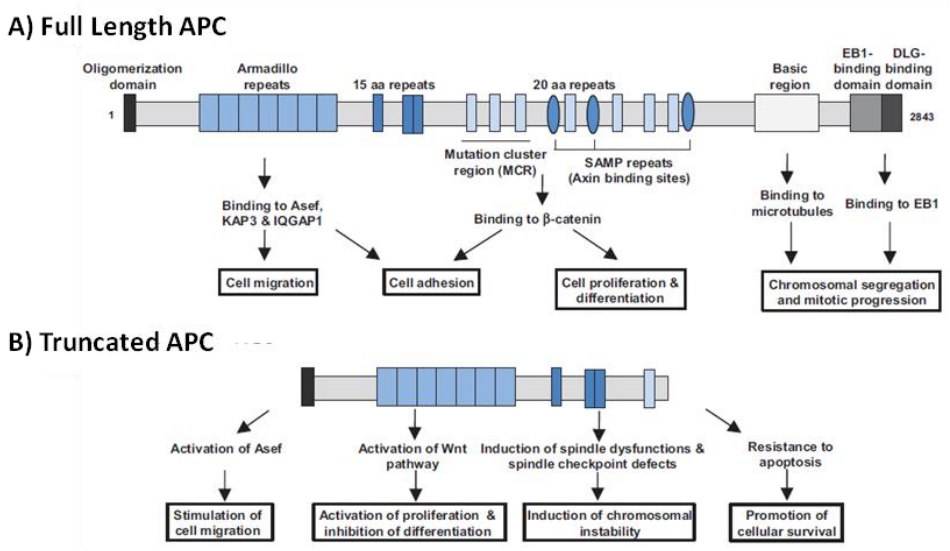

Figure 1: The full-length and truncated APC protein. ***Reproduced from Zhang and Shay (2017).*** **(A) Full length APC; (B) Truncated APC**

The armadillo repeats are able to bind to Asef, PP2A, and IQ-motif-containing GTPase activation protein 1 (IQGAP1) to stimulate cell migration and adhesion (Watanabe *et al.*, 2004; Zhang and Shay, 2017). The 15- or 20- residue repeats bind β-catenin for its degradation. In addition, the 15 amino acid repeat can also bind to C-terminal binding protein 1 (CtBP1) and CtBP2 to induce the oligomerisation of APC (Minde *et al.*, 2011; Schneikert, Brauburger, and Behrens, 2011). It should be noted that, unlike APC, APC2 does not have these 15 amino acid repeats (Aghabozorgi *et al.*, 2019). The SAMP repeats bind Axin which is necessary for the modulation of Wnt signaling (Minde *et al.*, 2011; Song, Wang, and Li, 2014). The basic domain binds to microtubules while the C-terminal domains can bind to EB1 and DLG, all of which have roles in the stabilisation of microtubules and for chromosomal segregation (Zhang and Shay, 2017; Aoki and Taketo, 2007). The vast majority of mutations in the APC protein can be found in a region approximately at the centre of the protein which is called the mutation cluster region (MCR), spanning from about residue 1000 to residue 1600 (Hankey *et al.*, 2018; Aghabozorgi *et al.*, 2019; Minde *et al.*, 2011). These

mutations are predominantly frameshift or non-sense mutations. As a result, stop codons are prematurely generated leading to the formation of stable, truncated APC products which often lack, due to truncation, the C-terminal region (Armaghany *et al.*, 2012; Aoki and Taketo, 2007).

1.4.2 APC Functions and Interactions

The significance of truncated APC products can be understood once the functions of the wild-type APC are described. One of the most significant functions of APC is its role in the negative regulation of the canonical Wnt signaling pathway. However, apart from this, APC carries out additional important functions such as those relating to the control of the cell cycle, migration and adhesion, as well as apoptosis and survival. As a result of this central role APC plays in various processes, it can be seen how APC has complex interactions with a plethora of proteins, a fact which is of particular interest for researchers studying aspects of cancer (Nelson and Näthke, 2013).

1.4.2.1 Interactions with Canonical Wnt

Within the canonical Wnt pathway, APC plays a negative regulatory, i.e. inhibitory, role. The canonical Wnt pathway, which is discussed in further detail in Section 1.5.1, is responsible for growth and developmental processes, with the primary transcriptional activator involved being β-catenin (Nusse, 2012). In the cytoplasm, APC can interact with β-catenin, through a destruction complex, in order to stimulate its phosphorylation, and subsequent ubiquitination followed by proteolytic degradation, which inhibits the pathway (Hankey *et al.*, 2018; Stamos and Weis, 2013). Axin, a component of this destruction complex, is stabilised by APC through interaction with GSK3 which increases the efficiency of

this complex (Pronobis, Rusan, and Peifer, 2015). As described previously, the SAMP repeats in the APC protein are responsible for binding Axin, while the 15- and 20- amino acid repeats bind β-catenin for its degradation. These structures are all found in the C-terminal region of APC hence, as a result of C-terminal truncations, these structures are lost. The result of this is that β-catenin is stabilised, leading to the Wnt pathway becoming constitutively activated, increasing cell proliferation (Nelson and Näthke, 2013; Hankey *et al.*, 2018).

In addition to APC's function in the cytoplasm, it also operates in the nucleus. Here, APC has two major functions which are the export of β-catenin from the nucleus to the cytoplasm, and its interaction with CtBP (Hankey *et al.*, 2018). β-catenin needs to be present in the nucleus in order to activate transcription factors necessary for cell growth (Nelson and Näthke, 2013). Thus, one function of APC in the nucleus is to export β-catenin from the nucleus to the cytoplasm, hence reducing proliferation (Rosin-Arbesfeld, *et al.*, 2003). In fact, a study by Zeineldin *et al.* (2012) found that reduced nuclear presence of APC resulted in increased Wnt signaling in addition to increased proliferation of epithelial cells in the small intestine and colon. APC can also interact with the transcriptional co-repressor CtBP, through its 15 amino acid repeats, to repress TCF-mediated transcription. This is accomplished by the sequestration of β-catenin to CtBP by APC which reduces the amount of nuclear β-catenin available for binding to TCF. Such a mechanism is another method by which the presence of β-catenin in the nucleus is further reduced (Hamada and Bienz, 2004). As the 15 amino acid repeat and the CtBP binding domain are both found at the C-terminal region, loss of this region via truncation results in the loss of the aforementioned functions, driving tumour formation.

1.4.2.2 APC and the Control of the Cell Cycle

Wild type APC also functions to regulate the cell cycle by inhibiting the progression of the G1 to the S phase. This has been demonstrated in CRC cell lines to occur through the regulation of TCF-mediated transcription components such as cyclin D1 and c-Myc (Heinen *et al.*, 2002). In addition, APC also forms a complex with Discs large (DLG) protein, with binding sites for DLG found on the C-terminus of APC, for suppressing cell cycle progression (Ishidate *et al.*, 2000). Thus mutations to APC will impair these functions, resulting in increased cell proliferation.

1.4.2.3 APC in Cell Migration and Adhesion

APC also functions in the control of cell adhesion and migration. One of the ways it can accomplish this is by regulating the distribution of β-catenin and E-cadherin in the cytoplasm and cell membrane by interacting with β-catenin and linking E-cadherin to the actin cytoskeleton (Zhang and Shay, 2017). Furthermore, APC may also interact with β-catenin at cell junctions which allows them to connect cytoskeletal components to cadherins, mediating cell adhesion (Hankey *et al.*, 2018). Truncation mutations which impair β-catenin binding will weaken adhesion and increase tumourigenicity. This was shown in a study where full length APC was introduced in a CRC cell line possessing truncated APC, and the authors found that the levels of nuclear β-catenin, proliferation, and tumourigenicity were all reduced (Faux *et al.*, 2003). In addition to these, APC has important regulatory roles in cell migration. It has been shown that it can regulate the organisation and assembly of actin through several proteins including Rac1, Cdc42, and mDia1, to promote cell migration (Sudhaharan *et al.*, 2011; Okada *et al.*, 2010; Juanes *et al.*, 2017). APC's interaction with Asef in the migration of CRC cells as well as in cell adhesion has also been shown in truncated

APC-knockdown studies (Kawasaki, Sato, and Akiyama, 2003), and the C-terminal region of APC which binds EB1 also influences migration through EB1's role in localising APC to microtubules (Hankey *et al.*, 2018). Hence, it can be seen how C-terminal truncation mutations will impact not just cell-cell adhesion, but cell migration as well.

1.4.2.4 APC in the Regulation of Apoptosis and Cell Survival

The regulation of apoptosis is another important function which APC regulates (Stiegerwald *et al.*, 2005). Truncated APC is able to increase the survival of cells through inhibition of apoptosis which may occur through two main proteins, these being Bcl-2 and survivin. A study found that truncated APC is able to be localised to the mitochondria where it can interact with Bcl-2 by forming a complex with it, with the result that Bcl-2 is stabilised, allowing it to promote its anti-apoptotic functions (Brocardo *et al.*, 2008). In another study, wild-type APC was confirmed to suppress the expression of survivin in normal colonic epithelial cells through TCF/β-catenin signaling and hence, mutated APC could promote cell survival through the constitutive activation of this anti-apoptotic protein (Zhang *et al.*, 2001).

1.5 The Wnt Signaling Pathways

The Wnt signaling pathways can be classed into two major groups, these being the canonical and non-canonical pathways. The canonical Wnt signaling pathway makes use of the transcriptional activator β-catenin which stimulates the transcription of Wnt related genes controlling cellular growth and differentiation upon its entry into the nucleus. In contrast, the non-canonical Wnt pathways do not involve β-catenin, with the two most well studied non-canonical pathways being the planar cell polarity (PCP) pathway which controls the reorganisation of the cytoskeleton, and the Wnt-Calcium signaling pathway, involved in

the regulation of intracellular calcium (Nusse, 2012; Harb, Lin, and Hao, 2019). A graphical overview of these pathways can be seen in **Figure 2** on the next page.

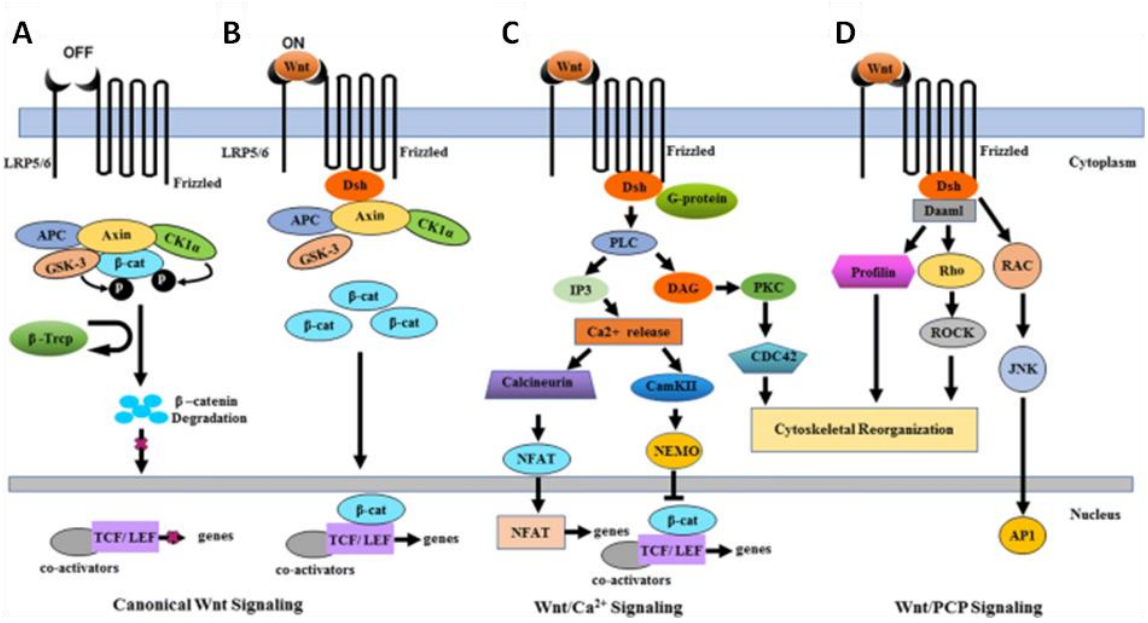

Figure 2: An overview of the canonical and non-canonical Wnt pathways. ***Reproduced from Harb, Lin, and Hao (2019).*** **(A) Canonical Wnt Signaling Pathway in the OFF position; (B) Canonical Wnt Signaling Pathway in the ON position; (C) Non-canonical Wnt/Calcium Signaling Pathway; (D) Non-canonical Wnt/PCP Signaling Pathway**

1.5.1 The Canonical Wnt Signaling Pathway

When there is no Wnt protein present, the levels of β-catenin are maintained at a low amount by its interaction with an Axin destruction complex comprised of four major components, the protein kinases glycogen synthase 3 (GSK3) and casein kinase 1 (CK1), the APC tumour suppressor, and the Axin scaffolding protein (Stamos and Weis, 2013; Nusse, 2012). The protein kinases phosphorylate β-catenin at specific serine and threonine residues, creating a binding site for β-transducin repeat-containing protein (β-TrCP) which is a subunit of E3 ubiquitin ligase (MacDonald, Tamai, and He, 2009; Harb, Lin, and Hao, 2019). This triggers the proteosomal degradation of β-catenin through the ubiquitin pathway (Nusse, 2012). APC also plays a role in this process through its binding with β-catenin where it

protects it from being dephosphorylated by PP2A (MacDonald, Tamai, and He, 2009). It can be seen how the presence of β-catenin mutations to the serine and threonine residues which are normally phosphorylated, or to the region surrounding them, can result in β-catenin becoming resistant to phosphorylation and hence, more resistant to degradation. Such types of mutations are commonly found in cancers (MacDonald, Tamai, and He, 2009).

When Wnt proteins are present, these bind to Frizzled (Fz) receptors and their transmembrane co-receptors low density lipoprotein receptor related protein 5/6 (LRP5/6) (Nusse, 2012). The co-receptors are phosphorylated on their cytoplasmic end by CK1, which allows them to bind Axin and activating the Dishevelled (Dvl) protein, which binds to the Fz receptor (Harb, Lin, and Hao, 2019; MacDonald, Tamai, and He, 2009). This results in a complex consisting of Dvl, Axin, and GSK3. Due to GSK3 being sequestered, it cannot phosphorylate β-catenin and is thus, as a consequence, released from the Axin destruction complex, allowing for the cytoplasmic accumulation of β-catenin (Nusse, 2012). This stable β-catenin then enters the nucleus where it interacts with the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factor proteins, displacing the co-repressor TLE1 and recruiting additional transcriptional co-activators for the expression of Wnt target genes (MacDonald, Tamai, and He, 2009; Nusse, 2012).

1.5.2 The Non-Canonical Wnt Signaling Pathways

One of the most well characterised non-canonical Wnt pathway is the PCP pathway. This pathway is activated when Wnt ligands bind to the Fz receptor, along with its co-receptors which are the protein tyrosine kinase 7 (PTK7) and receptor tyrosine kinase-like orphan receptor (ROR2) (Martinez *et al.*, 2015). Dsh is recruited to the cell membrane, forming a complex with Dishevelled Associated Activator of Morphogenesis 1 (Daam1) (Harb, Lin, and

Hao, 2019). Consequently, downstream signaling by the Ras-homology gene family member A (RHOA), Ras-related C3 botulinum toxin substrate 1 (RAC1), and c-JUN-N terminal kinase (JNK) is activated (Gómez-Orte *et al.*, 2013). This results in various cytoskeletal rearrangement effects which are necessary for directional cell migration, polarisation, division, and developing cellular morphology (Harb, Lin, and Hao, 2019; von Maltzahn *et al.*, 2012).

The Wnt/Ca^{2+} pathway is another non-canonical signaling pathway which has been studied and found to have roles in the regulation of calcium levels, and can control cell adhesion and movement (Harb, Lin, and Hao, 2019; von Maltzahn *et al.*, 2012). Activation of this pathway occurs by the binding of Wnt ligands to the Fz receptor which recruits and activates Dsh via G-proteins. This results in the activation of phospholipase C (PLC), in turn releasing intracellular calcium through the increase in the levels of inositol-1,4,5,-triphosphate (IP3) and diacyl glycerol (DAG) (von Maltzahn *et al.*, 2012; Wang *et al.*, 2014). Such elevated levels of calcium are able to regulate the activation of downstream proteins including calcineurin, calcium/calmodulin-dependent kinase II (CAMKII), and protein kinase C (PKC) (De, 2011). In addition, elevated levels of calcium has further consequences including the inhibition of β-catenin/TCF signaling via TGFβ activated kinase 1 (TAK1) and Nemo-like kinase (NLK), the activation of calcineurin phosphatase, which itself activates the nuclear factor of activated T cell (NFAT), and can also polarise the cells through the activation of the GTPase cell division control protein 42 (CDC42) (Harb, Lin, and Hao, 2019; von Maltzahn *et al.*, 2012).

Apart from these non-canonical pathways, another pathway which has been found to play a role in such non-canonical signaling is the PI3K/AKT/mTOR pathway. Studies have found that Wnt is able to interact and activate this pathway. For instance, in one study, it was suggested that the binding of Wnt to Fz activates PI3K, leading to the activation of

phosphotidyl inositol-dependent kinase 1 (PDK1), in turn phosphorylating and activating AKT. AKT then activates the downstream mTOR complexes. In fact, the study found elevated levels of phosphorylated AKT upon treating cells with Wnt (von Maltzahn, Bentzinger, and Rudnicki, 2011). Other studies have also found links between Wnt and AKT/mTOR pathway through GSK3. This kinase is known to inhibit the mTOR pathway by its role in phosphorylating and activating the tuberous sclerosis complex 2 (TSC2), a regulatory complex which inhibits mTOR (Ma *et al.*, 2011; Shimobayashi and Hall, 2014). However, Wnt inhibits GSK3 and, as a result, the TSC2 is not activated leading to the activation of the mTOR pathway, stimulating cell growth (Inoki *et al.*, 2006).

1.5.3 The Role of APC within Wnt Signaling

As it has already been previously described, the main role of APC within canonical Wnt/β-catenin signaling has to do with controlling the levels of β-catenin. It accomplishes this by ensuring its destruction through the assembly of a destruction complex which targets the β-catenin for phosphorylation and its subsequent degradation (Gao, Xiao, and Hu, 2014; Nelson and Näthke, 2013). In addition, APC is known to protect β-catenin from the phosphatase PP2A thereby preventing its dephosphorylation, and it also functions in sequestering β-catenin in the cytoplasm, preventing its entry into the nucleus where it would associate with TCF/LEF and drive transcription (Stamos and Weis, 2013).

However, APC's role in non-canonical signaling is not as clear owing to the fact that it interacts with several proteins and indirectly regulates various processes (Nelson and Näthke, 2013). However, two proteins which have been found to be indirectly regulated by APC in a β-catenin-independent manner, and which are of interest in this study, are CDC42 and EGFR. CDC42, which has been briefly mentioned in Section 1.5.2, has important roles in

polarisation and cell movement (Watanabe *et al.*, 2004). However, the overexpression of CDC42 and its aberrant activation is known to be oncogenic (Qadir, Parveen, and Ali, 2015). The interaction of APC with CDC42 occurs through a protein called APC-stimulated guanine nucleotide exhange factor (ASEF). ASEF negatively regulates CDC42, preventing its aberrant activation (Yang *et al.*, 2021). However, ASEF binds to truncated APC via the armadillo domain, which prevents its negative regulation of CDC42, aberrantly activating it and increasing cell migration and invasion (Qadir, Parveen, and Ali, 2015; Kawasaki, Sato, and Akiyama, 2003). Furthermore, the activation of CDC42 results in the upregulation of matrix metalloprotease 9 (MMP9) which also promotes cell migration (Yang *et al.*, 2021). Interestingly, the binding of truncated APC to ASEF has another effect on top of the activation of CDC42, which is the fragmentation of golgi bodies that disrupts cell polarity and is another factor which promotes the initiation of CRC and its progression (Rodríguez-Cruz *et al.*, 2018; Yang *et al.*, 2021).

EGFR is a receptor tyrosine kinase, which is known to activate cell growth pathways such as the PI3K/AKT/mTOR pathway (Courtney, Corcoran, and Engelman, 2010). It was also found to be indirectly regulated by APC due to it being downstream of CDC42, where overexpression of CDC42 results in a decrease in the rate of degradation of EGFR (Qadir, Parveen, and Ali, 2015). This occurs through one of the downstream effectors of CDC42 which is called Cool-1/β-pix. This protein can interact with a ubiquitin ligase called c-Cbl, which is responsible for ubiquitinating and degrading EGFR. Thus, as a result of overexpression of CDC42, there is increased expression of Cool-1/β-pix which, in turn, binds with the ubiquitin ligase, reducing EGFR degradation (Wu, Tu, and Cerione, 2003; Qadir, Parveen, and Ali, 2015). However, it should also be noted that EGFR regulation may occur canonically through β-catenin, as it is a transcriptional target of this pathway (Lee *et al.*, 2018; Guturi *et al.*, 2012). However, studies have found that the presence of mutant APC

results in increased EGFR expression, as well as its downstream protein, AKT, suggesting that aberrant activation of the AKT/mTOR pathway occurs, which results in the promotion of cell survival and growth (Moran *et al.*, 2004; Zhang *et al.*, 2014).

1.6 Wnt Pathway Inhibitors

Owing to the central role the Wnt pathway plays in cancer, several inhibitors have been developed which target members of this signaling pathway, with the aim of reducing cancer cell proliferation. An overview of such inhibitors showing their mechanistic targets can be seen in **Figure 3** at the end of this section.

One such class of inhibitors are called Porcupine inhibitors. As it has already been described in previous sections, the binding of Wnt to the Fz receptors is necessary to initiate this signaling pathway. However for this to occur, Wnt needs to undergo post-translational acylation in the endoplasmic reticulum mediated by the Porcupine (PORCN) protein. This acylation then allows Wnt to be transported from within the cell to the cell surface, and allows it to bind more efficiently to the Fz receptors (Herr, Hausmann, and Basler, 2012; Torres, Godoy, and Inestrosa, 2019; Liu *et al.*, 2013). Hence, PORCN inhibitors will impair the function of the Porcupine protein, thereby inhibiting the post-translational acylation and subsequent secretion of Wnt, effectively inhibiting the pathway (Jung and Park, 2020; Zhang and Wang, 2020). Several PORCN inhibitors have been developed such as LGK974 (or WNT974), ETC-1922159 (or ETC-159), CGX1321, and RXC004 (Jung and Park, 2020; Liu *et al.*, 2013; Madan *et al.*, 2015). In addition, owing to the potential of such inhibitors for use in cancer management and treatment, many of these compounds are being investigated in clinical trials. For LGK974, two studies have been completed thus far, one in 2017 (NCT02278133), and another one in 2020 (NCT01351103; Rodon *et al.*, 2021). ETC-159 has

so far been involved in one study (NCT02521844; Ng *et al.*, 2017) while CGX1321 and RXC004 are each currently involved in two clinical trials (CGX1321: NCT02675946 and NCT03507998; RXC004: NCT03447470 and NCT04907851).

Another class of inhibitors are called Wnt and Fz antagonists owing to their ability to bind either to the Wnt ligand or to the Fz receptors which impairs their ability to function properly (Zhang and Wang, 2020). These types of inhibitors are effective due to the fact that many tumours have elevated levels of Wnt thus, inhibitors which compete with this ligand or its receptor can reduce the activation of the Wnt pathway (Harb, Lin, and Hao, 2019). Two examples of such inhibitors are Ipafricept (also called OMP-54F28) which is a truncated Fz receptor able to bind to Wnt ligands, hence reducing binding of Wnt to Fz receptors (Moore *et al.*, 2019; Le, McDermott, and Jimeno, 2015), and Vantictumab (OMP-18R5) which is a monoclonal antibody targeting several Fz receptors (Gurney *et al.*, 2012; Zhang and Wang, 2020). Both of these inhibitors have also been investigated in clinical trials for several cancers such as hepatocellular, pancreatic, and breast cancer.

Inhibitors have also been developed which target members of complexes required for activating the Wnt pathway. An example of such a component is Dvl, which itself is activated by the co-receptors LRP5/6 upon Wnt ligand binding, forming a complex with Axin and GSK3, preventing the phosphorylation and degradation of β-catenin thereby promoting the activation of the Wnt pathway (Nusse, 2012). Inhibitors which target Dvl include NSC668036 and FJ9 (Fujii *et al.*, 2007; Zhang and Wang, 2020).

As described previously, Axin is a scaffolding protein which is required for the formation of the destruction complex to degrade β-catenin hence drugs have been developed which maintain Axin's function. One such class of inhibitors are the tankyrase inhibitors. The protein tankyrase can interact with Axin and promote its degradation, consequently disrupting the destruction complex along with its β-catenin destruction function (Mariotti,

Pollock, and Guettler, 2017; Jung and Park, 2020). Thus, inhibiting tankyrase will reduce Axin degradation, thereby increasing β-catenin removal. Several such inhibitors have been developed such as G007-LK, XAV939, IWR-1, and JW74 (Lau *et al.*, 2013; Tian *et al.*, 2013; Wessel Stratford *et al.*, 2014; Zhang and Wang, 2020).

Another attractive target for inhibitors is β-catenin itself owing to its central role in the canonical signaling pathway. Inhibitors such as PRI-724 and ICG001 are able to block the interaction between β-catenin and CREB-binding protein (CBP), a transcriptional co-activator, thus reducing β-catenin-dependent transcription (Yu *et al.*, 2017; Che *et al.* 2020; Lenz and Kahn, 2014; Zhang and Wang, 2020). CWP291 is another inhibitor which targets the transcriptional products of the canonical Wnt pathway to make cancer cells more susceptible to apoptosis through downregulating genes such as survivin and cyclin D1 (Harb, Lin, and Hao, 2019).

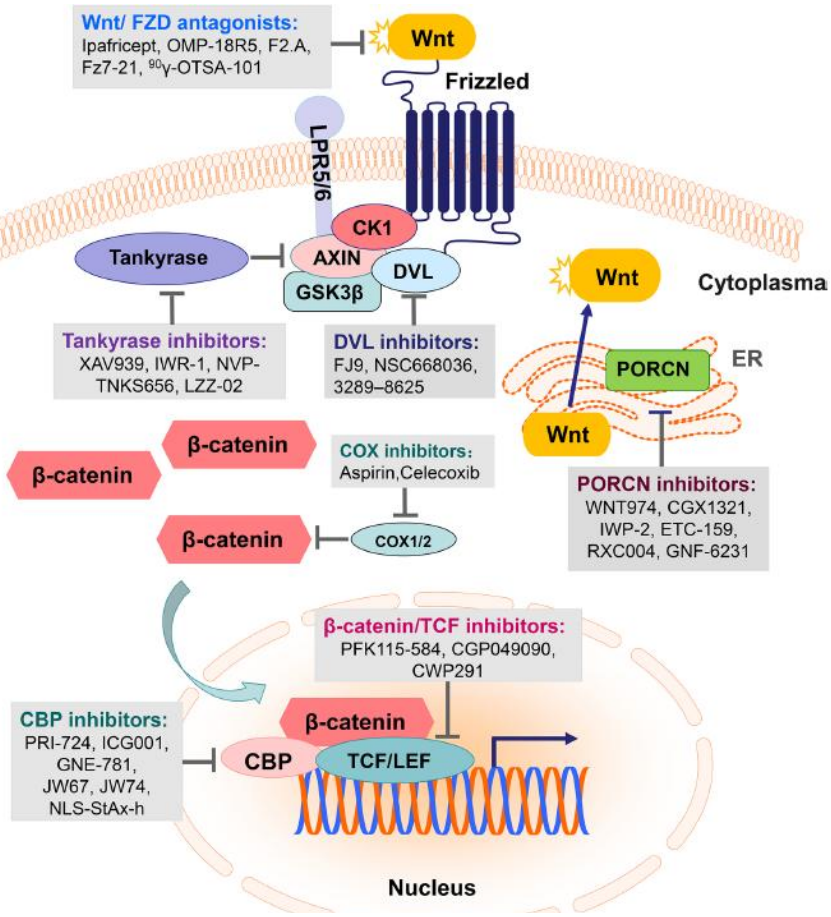

Figure 3: Overview of Wnt inhibitors showing their mechanistic targets. ***Reproduced from Zhang and Wang (2020).***

1.7 Aim and Objectives

1.7.1 Aim

As has been described, the Wnt pathways and APC play central roles in the development of cancer, especially in CRC. In addition, the mechanisms by which chemoresistance arises and its consequences on treatment are areas in which significant research is being conducted in an attempt to elucidate and develop ways how new treatments can mitigate or bypass such mechanisms. In this regard, the roles which APC has both within the Wnt pathways and within CRC in general are subjects of interest, with numerous studies having been published exploring these areas. However, there are still gaps in our knowledge when it comes to properly defining APC-dependent cellular function, more so when Wnt-inhibited chemoresistant CRC is added to the equation. Hence, the primary aim of this study is to investigate chemoresistant CRC for the presence, magnitude, and diagnostic or prognostic value of APC-related proteins following Wnt inhibition.

1.7.2 Objectives

To achieve the aim, the study will make use of CRC cell lines having different APC statuses (wild-type and mutant/truncated). For this reason, available cell lines will be sequenced via Sanger sequencing to find 2 or 3 suitable cell lines possessing mutations of interest.

The chosen cell lines will be cultured in 2D, followed by a period of 5-FU treatment in order to confer resistance. The cells will then be treated with the Wnt pathway inhibitors LGK974 and ETC-159 at concentrations of 10μM and 0.2μM, respectively. These inhibitors were chosen not only because previous studies have shown that they have potential for use in CRC treatment, but they are also currently being tested in clinical trials as mentioned in Section 1.6.

Presto Blue viability assays will be conducted over a period of 72 hours to determine the effect of the inhibitors on cells having different APC statuses. Moreover scratch migration and transwell invasion assays will be performed to investigate the migratory and invasion potential of the inhibitor-treated cells.

Western blotting will be conducted for the APC-related proteins β-catenin, CDC42, EGFR, as well as mono- and tri-methyl lysine to investigate the effects of the inhibitors on canonical and non-canonical signaling. In addition, an enzyme-linked immunosorbent assay (ELISA) will be performed to determine the ratio of phosphorylated to total EGFR in response to Wnt pathway inhibitor treatment.

2 Methodology

2.1 Materials

Table 1: List of materials used including their brand and catalogue number

Name	Brand	Catalogue Number
DNeasy Blood and Tissue Kit	Qiagen	69504
ReddyMix	ThermoScientific	AB-0575/DC/LD
1000bp DNA marker	Solis BioDyne	07-12-000S
4X Laemmli Sample Buffer	Bio-Rad	1610747
Quick Start™ Bradford Protein Assay	Bio-Rad	5000006
Thick Filter Papers (2.45mm, 8.6x13.5)	Capitol Scientific	10426981
Immobilon-FL PVDF Transfer Membrane	Merck	IPFL00010
SeeBlue plus2 Pre-stained Standard protein marker	Bio-Rad	1610374
LGK974	Cayman Chemical	14072
ETC-159	Cayman Chemical	24104
HCT116 cells	ATCC	CCL-247
DLD1 cells	ATCC	CCL-221
Caco2 cells	ATCC	HTB-37
SW837 cells	ATCC	CCL-235
Rabbit anti-β-catenin antibody	Cell Signaling Technology	8480
Rabbit anti-CDC42 antibody	Cell Signaling Technology	2466
Rabbit anti-EGFR antibody	Cell Signaling Technology	4267

Mouse anti-Actin antibody	Cell Signaling Technology	3700
Rabbit anti-Tri Methyl Lysine antibody	Cell Signaling Technology	14680
Rabbit anti-Mono Methyl Lysine antibody	Cell Signaling Technology	14697
5FU	Sigma-Aldrich	F6627
BSA	Cell Signaling Technology	9998
Human phospho-EGFR (Y1045) and Total EGFR ELISA kit	RayBiotech	PEL-EGFR-Y1045-T-1
PrestoBlue™ Cell Viability Reagent	Invitrogen	A13261
2-well silicone inserts	Ibidi	80242
96-well plates Nunclon™ Delta Surface	ThermoScientific	167425
24-well plates Nunclon™ Delta Surface	ThermoScientific	142485
6.5mm Transwell plate with 8μm pores	Corning	3422

2.2 Methods

2.2.1 2D Cell Culture

2.2.1.1 Preparation of Medium

A volume of 50mL of complete DMEM/F12 with 10% serum was prepared with 500μL Penicillin-Streptomycin, 500μL Amphotericin B, 50μL of heparin, 312μL of platelet lysate (stock: 0.2mg/mL), 5mL of human serum, and made up to 50mL with basal DMEM/F12, thus the final complete medium consisted of 10% serum, 1% Penicillin-Streptomycin, and 1% Amphotericin B.

2.2.1.2 Sub-Culturing of Cells

CRC cell lines (HCT116, SW837, Caco2 and DLD1) were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 with 10% human serum, 0.2mg/mL human platelet lysate, 1% PenStrep, and 1% Amphotericin B. Cells were incubated at 37^{o}C and 5% CO_2 with 95% relative humidity.

Whenever they needed to be collected for further experiments, or for maintaining an acceptable level of confluency in the flasks, the medium was removed and 1mL of 1mM EDTA solution in PBS was added. The flask was incubated 5 minutes at 37^{o}C to dislodge the cells. The cells were then collected and pelleted by centrifugation for 5 minutes at 500 x g. The pellet was resuspended in an adequate volume of complete medium and split into flasks or wells.

2.2.1.3 Preparation of APC Inhibitor Drug Solutions

A stock concentration of 1mg/mL was prepared for each of the inhibitors, LGK974 and ETC-159, by dissolving 1mg of each drug in 1mL of DMSO. For drug treatment, new stock solutions were created from the original 1mg/mL solution – for LGK974, a concentration of 50µM was created by diluting 19.8µL of the original 1mg/mL stock concentration in 1mL of basal DMEM/F12, while for ETC-159, a concentration of 1µM was created by diluting 0.4µL of the original 1mg/mL stock in 1mL of basal medium. During the course of the experiments, as the concentration of the drugs given was increased, it was also necessary to create additional stock solutions of 500µM for LGK974 and 10µM for ETC-159. The former was created by diluting 198µL of the 1mg/mL LGK974 stock in 1mL of basal DMEM/F12 media, while the latter was prepared by adding 4µL of the 1mg/mL ETC-159 stock in 1mL of basal medium.

These concentrations were also prepared with DMSO by diluting the aforementioned volumes from the 1mg/mL stock in 1mL DMSO.

2.2.1.4 Conferring Resistance to 5-FU

Cells were treated with an initial concentration of 2.5mM of 5FU (stock solution 1mg/mL) when confluency was observed to be high (over 70%) for three consecutive cycles and eventually, a concentration of 5mM of 5FU for three consecutive cycles when the number of cells had increased following the initial treatment. A cycle was taken as three days with treatment, followed by an additional three days without. If the confluency remained low, cells were not treated with 5FU to allow them a period of recovery. Additionally, the cells were not treated with 5FU when the cells were expected to be treated with Wnt inhibitors. This treatment regimen was maintained for several weeks until it was observed that the majority of cells were not stressed due to 5FU treatment, and until morphological changes were seen. Resistance to 5FU was further confirmed by carrying out a Presto Blue viability assay over a period of 72 hours (refer to Section 2.2.4). 5FU treatments were then periodically administered to maintain chemoresistance.

2.2.1.5 APC Inhibitor Drug Treatment

To investigate the effects of LGK974 and ETC-159, parental (5-FU sensitive) and 5-FU resistant cells were seeded into 96 well plates (for viability assays) or 24 well plates (for Western blotting). These cells were then treated with a range of drug concentrations – for LGK974 the concentrations used initially were 0, 0.5, 1, 2.5, and 5µM which were prepared from the 50µM stock solution, and for ETC-159 the concentrations were 0, 0.02, 0.05, 0.07, and 0.1µM, prepared from the 1µM stock solution. DMSO was added to the untreated cells

as a control. Three days after drug treatment, images were taken to assess any differences in cell growth. It was then decided to substitute the lowest drug concentration for each inhibitor (0.5µM for LGK974, and 0.02µM for ETC-159) with higher concentrations – 10µM and 0.2µM for LGK974 and ETC-159, respectively.

2.2.2 Microscopy

A Nikon Eclipse Ti inverted microscope was used throughout the study, both for checking cell condition and taking images. Images were taken to record changes to cell number and condition in response to 5-FU and Wnt pathway inhibitor treatments.

2.2.3 Sequencing

DNA extraction from cells was performed according to the protocol provided by the kit (Qiagen Dneasy Blood and Tissue Kit, Cat. No.: 69504). This was then followed by DNA quantification.

Two sets of primers (see below) were used such that the region of interest in the APC gene which is commonly mutated (from around nucleotide 2500 to nucleotide 4600) was covered. The first set of primers was used mainly to sequence HT29 and LoVo cell lines for the presence of mutations in this first region, while the second set of primers was used to sequence the SW480, HCT116, Caco-2, DLD1, LoVo, SW837, HT29, and COLO201 cell lines for mutations. End-point PCR was then performed for the aforementioned cell lines using ReddyMix master mix, and an annealing temperature of 54^{o}C. The products were confirmed on a 1% agarose gel, and visualised by ethidium bromide staining using a UV illuminator. Sanger sequencing was performed by MLS BioDNA Ltd. The data was then analysed by

BioEdit, comparing the sequencing data with the wild-type APC sequence to determine whether any mutations were present.

Table 2: Forward and Reverse Primer Sequences used for PCR and their Melting Temperatures

APC Region	Primer Sequence 5' to 3'	Melting Temperature, T_m in ^oC
A-b Forward	TACTGGCAACATGACTGTCC	54.7
A-b Reverse	(5'-CTGCTTGAAGACATATGTTCG-3') CGAACATATGTCTTCAAGCAG	51.5
C-d Forward	TGAGAATACGTCCACACCTTC	54.2
C-d Reverse	(5'-GATGACTTTGTTGGCATGGC-3') GCCATGCCAACAAAGTCATC	54.9

2.2.4 Viability Assays

Presto Blue viability assays were conducted to ensure that cells were made resistant to 5-FU, and to investigate the effects of the Wnt pathway inhibitors. To check that the cells are resistant to 5-FU, parental and 5-FU resistant cells were seeded in a 96 well plate in triplicate. Control cells were treated with DMSO while the other cells were treated with 5mM of 5-FU, as described in Section 2.2.1.4. Then, 24 hours after seeding, the media was discarded and replaced with fresh media containing 10% Presto Blue. Absorbance measurements at 570nm were then taken at 30 minutes, 1 hour, and 2 hours. This was repeated for the 48 and 72 hour post-treatment time-points.

Similarly, to investigate the effects of the Wnt pathway inhibitors, parental and 5-FU resistant cells were seeded in a 96 well plate with 2% serum media, also in triplicate, and treated with either 10μM of LGK974 or 0.2μM of ETC-159 as described in Section 2.2.1.5, with the control cells being treated with DMSO. At the 24 hour time-point, the media was replaced with fresh media containing 10% Presto Blue and absorbance measurements taken

at 30 minutes, 1 hour, and 2 hours at 570nm. This was repeated for the other time-points at 48 and 72 hours post-treatment.

2.2.5 Western Blotting

2.2.5.1 Cell Lysis

24 well plates were seeded with parental (5-FU sensitive) and 5FU resistant cells and the cells were treated with the Wnt inhibitors as described in Section 2.2.1.5. Three days after treatment, the cells were first visualised under the microscope to check their conditions (as described in Section 2.2.2), detached using EDTA (as described in Section 2.2.1.2), collected and lysed with 100µL RIPA lysis buffer followed by vortexing and sonication for a few seconds. The samples were centrifuged at 14,000 rpm for 5 minutes and the supernatant was transferred to a new labelled microcentrifuge tube.

2.2.5.2 Bradford Assay

A Bradford assay was conducted to quantify the amount of protein in the samples, using a 1:5 dilution (Bradford reagent : sterile H_2O), and using 3 BSA controls at concentrations of 0, 2, and 4µg/µL.

2.2.5.3 Sample Preparation

Samples were then prepared for immunoblotting by preparing 5µL Laemmli sample buffer in microcentrifuge tubes and adding to this a volume of water and protein sample to make up to 25µg of protein in a total volume of 20µL in each tube. These samples were then transferred to a heating block at 95^{o}C for 5 minutes, followed by a further 5 minutes in ice.

2.2.5.4 Poly Acrylamide Gel Electrophoresis (PAGE)

A 10% SDS-PAGE resolving gel and a 6% stacking gel were prepared as shown below:

Table 3: Components of 10% SDS-PAGE resolving gel and the 6% stacking gel

10% Resolving Gel		6% Stacking Gel	
Reagent	**Volume**	**Reagent**	**Volume**
Deionised water	2.9 mL	Deionised water	3.51 mL
1.0 M Tris-HCl pH 8.8	3.0 mL	1 M Tris-HCl pH 6.8	0.75 mL
40% (w/v) acrylamide	2 mL	40% (w/v) acrylamide	0.63 mL
10% (w/v) SDS	80 μL	10% (w/v) SDS	50 μL
10% (w/v) APS	80 μL	10% (w/v) APS	50 μL
TEMED	10 μL	TEMED	10 μL

Following this, the samples prepared in Section 2.2.5.3, along with a molecular weight marker, were loaded into the wells. The gel was run for a duration of approximately 1 hour at 250V.

2.2.5.5 Immunoblotting

PVDF membrane was cut and rinsed with methanol. The gel and PVDF membrane were then immersed in transfer buffer (prepared with 80mL H_2O, 10mL concentrate, and 10mL methanol), and the transfer stack was prepared for transfer at 100mA for 1 hour. The membrane was then stained with Ponceau S. Blocking buffer (5%) was prepared with 40mL TBS and 2g sodium caseinate. The membrane was blocked with this prepared blocking buffer for 30 minutes. The membrane was cut into four parts. Antibodies against Actin, β-catenin, CDC42, EGFR, mono- and tri-methyl lysine were diluted at 1:1000 or 1:500 in 5% sodium

caseinate in TBS and added to their respective membrane part. These were then incubated overnight with shaking at 4^{o}C. The following day the membranes were washed three times with TBS for 3 minutes each. The anti-mouse and anti-rabbit IRDye 800 secondary antibodies were diluted 1:10,000 in 5% sodium caseinate in TBS. The membranes were incubated for 1 hour with shaking at room temperature. The membranes were then washed three times with TBS for 3 minutes each. Visualisation of the bands was then carried out using the LiCor Odyssey Infrared Imaging System 9120.

2.2.6 Scratch Migration Assay

24-well plates were seeded with 5-FU sensitive and 5-FU resistant cells using silicone inserts. Each half of the inserts was seeded with 50μL of cell suspension, with an additional 50μL of complete medium added as well. The cells were then allowed to grow until a confluency of 80% was reached and then the media was replaced with fresh media containing 2% serum. The cells were then treated either with 10μM of LGK974 or 0.2μM of ETC-159. An equal volume of DMSO was added to the control cells. Images of the vertical scratch were then taken with the Nikon Eclipse Ti Inverted microscope and the cells were incubated at 37^{o}C and 5% CO_2 with 95% relative humidity overnight. The following day, images of the scratch were taken again to compare migration. Images were taken over 48 hours and Tscratch software was used to calculate the percentage of area filled with cells. For each time point, the average of three images was used in order to calculate the percentage of area that was filled in by the cells with or without treatment.

2.2.7 Transwell Invasion Assay

Transwell plates with a pore size of 8μm (Corning, Product no.: 3422) were used for this experiment. The wells were filled with 1mL of media with 2% serum followed by the addition of the Wnt inhibitors. $5x10^4$ cells were then seeded in the inserts. The cells were incubated for three days and images were taken of the inserts and their corresponding wells. The cells were incubated for a further three days and images were taken again of the inserts and the wells.

2.2.8 ELISA

The ELISA was carried out according to the manufacturer's instructions. The samples were first prepared by lysing the cells with the provided cell lysis buffer to collect the lysate. A Bradford assay was carried out as described previously in Section 2.2.5.2 to determine the concentration of proteins in each of the prepared lysates. Positive controls were prepared by serially diluting the provided positive control sample with the assay diluent. This assay diluent also served as the blank. The anti-phospho EGFR and anti-pan EGFR antibodies were prepared by diluting them with 100μL of the provided 1X assay diluent. A volume of 100μL of the positive controls and samples were pipetted into their respective wells and incubated for a period of 2.5 hours at room temperature. After discarding the solution, the wells were washed 4 times each with wash solution, after which 100μL of the anti-phospho EGFR antibody was pipetted into the appropriate wells. Similarly, a volume of 100μL of the anti-pan EGFR antibody was pipetted into their respective wells. The plate was then incubated for 1.5 hours at room temperature. The solution was discarded and the wells washed 4 times each as described previously. 100μL of the HRP solution was added to the wells which were to detect the phospho-EGFR, while the other wells which were to detect the pan-EGFR were

filled with 100μL of HRP-streptavidin solution. The plate was incubated for 1 hour at room temperature. The solution was again discarded and the wells washed as described before. A volume of 100μL of the provided TMB substrate reagent was added to each well and incubated for 30 minutes at room temperature in the dark. Finally, 50μl of the Stop solution were added to each well and readings were taken at a wavelength of 450nm.

2.2.9 Statistical Analysis

Statistical analysis was performed to confirm whether changes observed were statistically significant or not. Independent samples t-tests were conducted to check if resistance to 5-FU was successfully conferred to the cells by comparing the data obtained for cell viability of the parental and chemoresistant cells at the 72 hour time-point. This type of statistical analysis was also performed for cells which were treated with the Wnt inhibitors, also at 72 hours post-treatment, to investigate if the change in viability observed with inhibitor treatment was significantly different from the untreated control. Additionally, this test was employed for the scratch and migration assays. For the independent samples t-test, normality testing was first carried out using the Shapiro-Wilk normality test. A null hypothesis (H_0) and alternative hypothesis (H_1) were constructed with the former stating that the variable being tested is normally distributed, while latter stating that normality is not present. A p-value greater than a significance value of 0.05 renders H_0 to be true, while a p-value less than 0.05 means that H_1 holds true. Following the determination of normality, the Levene's test was performed to check whether the equality of variances can be assumed. H_0 and H_1 were also constructed with the null hypothesis stating that equality of variances is assumed, while the alternative hypothesis states that equality of variances is not assumed. A significance value of 0.05 was used for this test as well. Finally, the independent samples t-test could be

performed using a significance value of 0.05 with an H_0 stating that the means of the two populations are equal and H_1 stating that the means are not equal.

In addition, a two-way ANOVA with post-hoc analysis was conducted for the results of the Presto Blue viability assays where 'time point' and 'drug treatment' were taken as the independent variables, while 'cell viability' was taken as the dependent variable. This was done to test whether there is an interaction between the time points and drug treatment and thus determine whether the drugs were more effective at 24 hours or 72 hours post-treatment. A p-value of 0.05 was used to determine which of the results were statistically significant.

This statistical analysis was done using Statistical Package for the Social Sciences (SPSS) software (IBM®, Version 27).

3 Results

3.1 Sequencing Data

Table 4: Sequenced cell lines and mutations present

Cell Line	Mutation/s
DLD1	c.2930 G>T; c.4199 delC
LoVo	c.4430 G>A
SW837	c.4299 C>T; c.4430 G>A
COLO201	c.4430 C>A
HCT116	None detected
SW480	None detected
HT29	None detected

End-point PCR and Sanger sequencing was performed for the APC gene for seven CRC cell lines, revealing truncation mutations in three of them, these being DLD1, SW837, and COLO201. From these, DLD1 was chosen to represent the APC-mutated subtype, while HCT116 would represent the wild-type variant. DLD1 is classified as a Duke's Type C adenocarcinoma with an epithelial morphology which can be seen in detail in Section 3.2.2.

3.2 Microscopy

3.2.1 Parental and 5-FU Resistant HCT116 cells

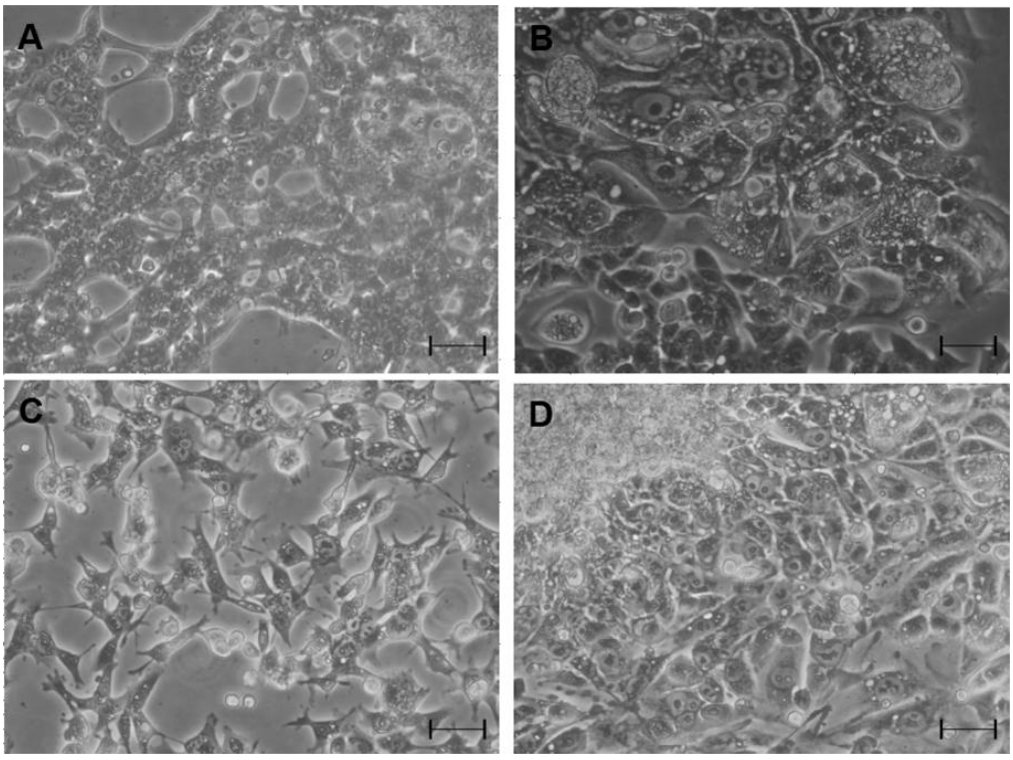

Figure 4: Parental and 5-FU Resistant HCT116 cells, magnification x100, scale 100μm. ***(A, C)*** **Parental cells;** ***(B, D)*** **5-FU Resistant cells. These cells have an epithelial morphology. Resistant HCT116 cells were larger than their parental counterparts and had fewer vacuoles.**

HCT116 cells have an epithelial morphology which can be seen in **Figure 4**. In comparison to parental cells, HCT116 cells which have been made resistant to 5-FU show an altered morphology where such cells appear larger (**Figure 4B**). Such a change was one way used to determine that 5-FU resistance had been conferred. In **Figure 4D**, a colony-forming unit can be clearly seen.

3.2.2 Parental and 5-FU Resistant DLD1 cells

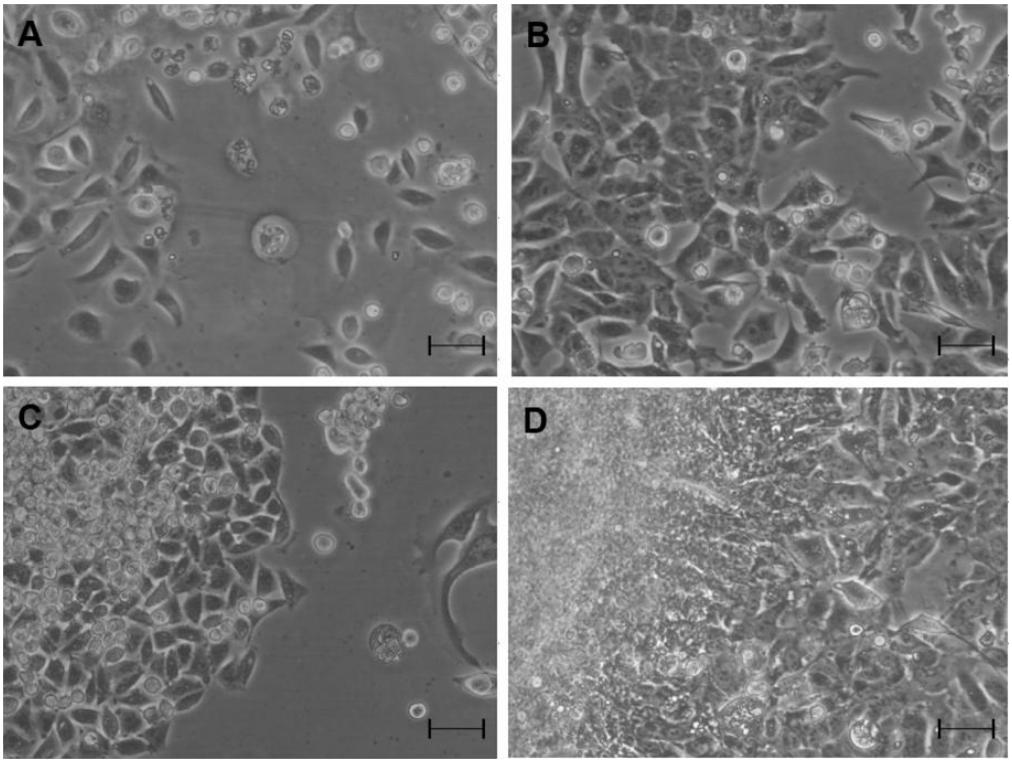

Figure 5: Parental and 5-FU Resistant DLD1 cells, magnification x100, scale 100μm. ***(A, C)*** **Parental cells;** ***(B, D)*** **5-FU Resistant cells. DLD1 cells have an epithelial cell morphology with resistant cells being larger than the parental cells.**

As mentioned in Section 3.1, DLD1 cells are a Duke's Type C adenocarcinoma with epithelial cell morphology. The 5-FU resistant cells also show an altered morphology compared to the parental cells (**Figure 5B**). A colony-forming unit can be seen in **Figure 5D**.

3.2.3 Treatment with Wnt Inhibitors LGK974 and ETC-159

3.2.3.1 Treatment of HCT116 cells with the Wnt Inhibitors

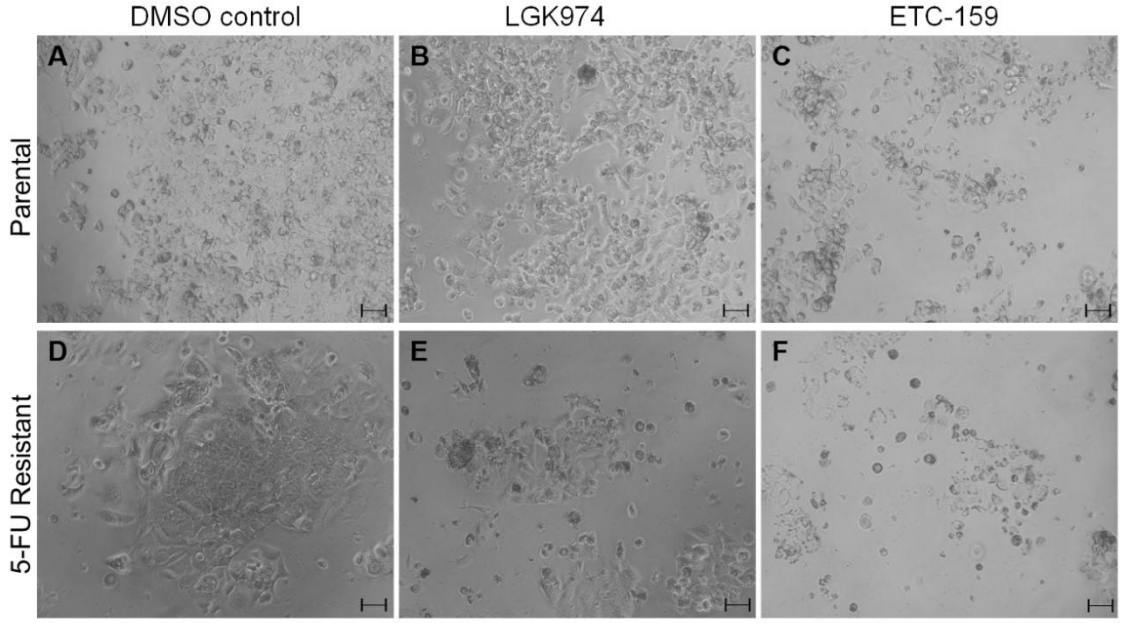

Figure 6: Parental and 5-FU Resistant HCT116 cells treated with 10μM LGK974, or 0.2μM ETC-159 compared to a DMSO-treated control, magnification x100, scale 100μm. *(A)* Parental control; *(B)* Parental with LGK974; *(C)* Parental with ETC-159; *(D)* Resistant control; *(E)* Resistant with LGK974; *(F)* Resistant with ETC-159. LGK974 had little effect on parental HCT116 cells while ETC-159 was more effective at 24 hours post-treatment for both the parental and resistant cells. The Wnt inhibitors were more effective in the chemoresistant variants rather than in the parental cells.

Parental and 5-FU resistant cells were treated with either LGK974 or ETC-159, or with an equal volume of DMSO. **Figure 6** shows the effects of treatment after 24 hours. From the images taken it appeared that LGK974 had little effect on parental HCT116 cells while ETC-159 appeared to have a more pronounced effect after 24 hours (**Figure 6 A-C**). The effect of the Wnt inhibitors appeared to be greater in 5-FU resistant HCT116 cells (**Figure 6 D-F**). Cell viability with the Wnt inhibitors was also quantified with a Presto Blue viability assay and the results can be seen in Section 3.3.2.

3.2.3.2 Treatment of DLD1 cells with the Wnt Inhibitors

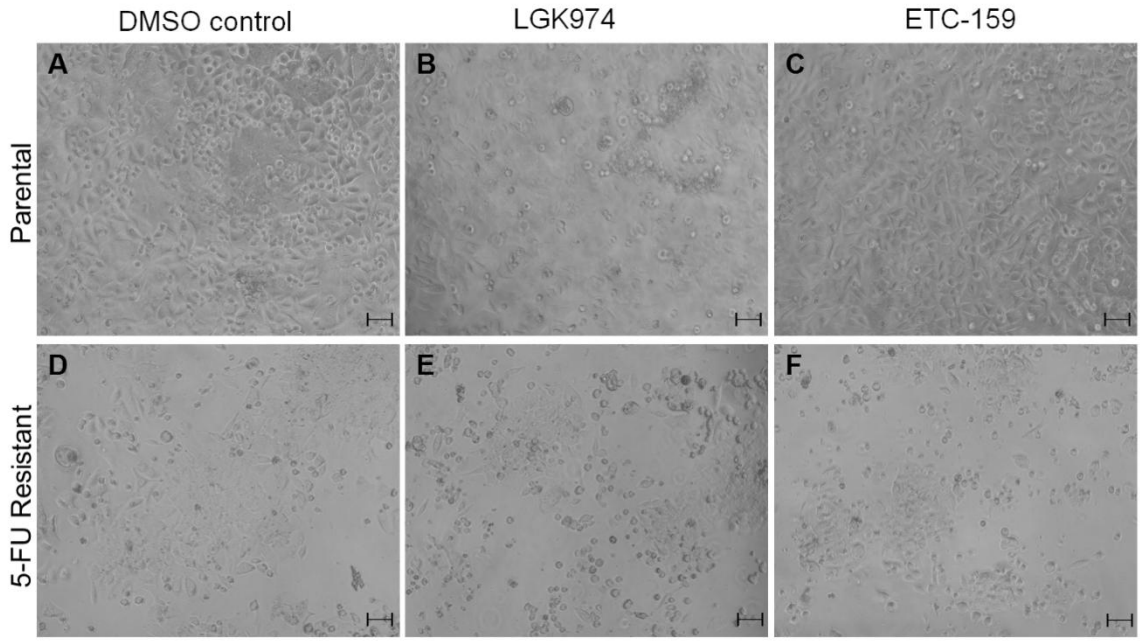

Figure 7: Parental and 5-FU resistant DLD1 cells treated with 10μM LGK974, or 0.2μM ETC-159 compared to a DMSO-treated control, magnification x100, scale 100μm. *(A)* Parental control; *(B)* Parental with LGK974; *(C)* Parental with ETC-159; *(D)* Resistant control; *(E)* Resistant with LGK974; *(F)* Resistant with ETC-159. The Wnt inhibitors had little effect on both the parental and chemoresistant cells at 24 hours post-treatment

Figure 7 shows the effects of the Wnt inhibitors on DLD1 cells after 24 hours. Overall, it was observed that Wnt inhibitors had little effect on both the parental and 5-FU resistant cells after 24 hours. Results with a Presto Blue viability assay can be seen in Section 3.3.2.

3.3 Viability Assays

3.3.1 Conferring 5-FU Resistance

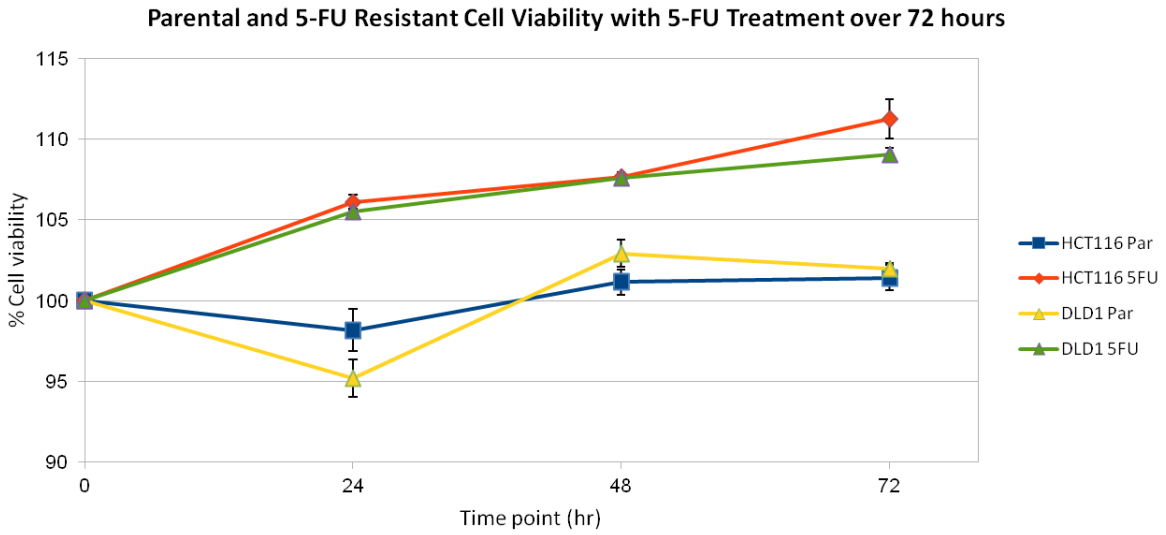

Figure 8: Cell viability of parental and 5-FU resistant HCT116 and DLD1 cells with 5-FU over 72 hours. The most significant difference was seen at 24 hours post-treatment where the viability of both HCT116 and DLD1 parental cells was below 100% whereas that of the resistant cells was approximately 105%. This difference in viability was also seen at 72 hours with both resistant cells having a viability of around 110% while for the parental cells it was around 100%. This confirms that the cells were effectively made resistant to 5-FU.

Figure 8 shows the differences in cell viabilities between parental cells and cells which have been made resistant to 5-FU by repeated treatment with the drug. The most noticeable difference can be observed 24 hours after treatment with 5-FU where the viability of the parental cells of both HCT116 and DLD1 was below 100% whereas that of the chemoresistant cells was above 105%. Although the cell viability of the parental cells went slightly above 100% after 72 hours, the chemoresistant cells still maintained their resistance to 5-FU, as evidenced by the fact that their viability at this time-point was around 110%. This difference in viability between the parental and 5-FU resistant cells at 72 hours post-treatment was also investigated with an independent samples t-test, with which a significant difference between

these viabilities was found ($P < 0.05$), proving that HCT116 and DLD1 cells had successfully been made resistant to 5-FU (**Appendix II**).

3.3.2 Cell viability with Wnt Inhibitor Treatment

3.3.2.1 Effect of Wnt inhibition on parental HCT116 cells

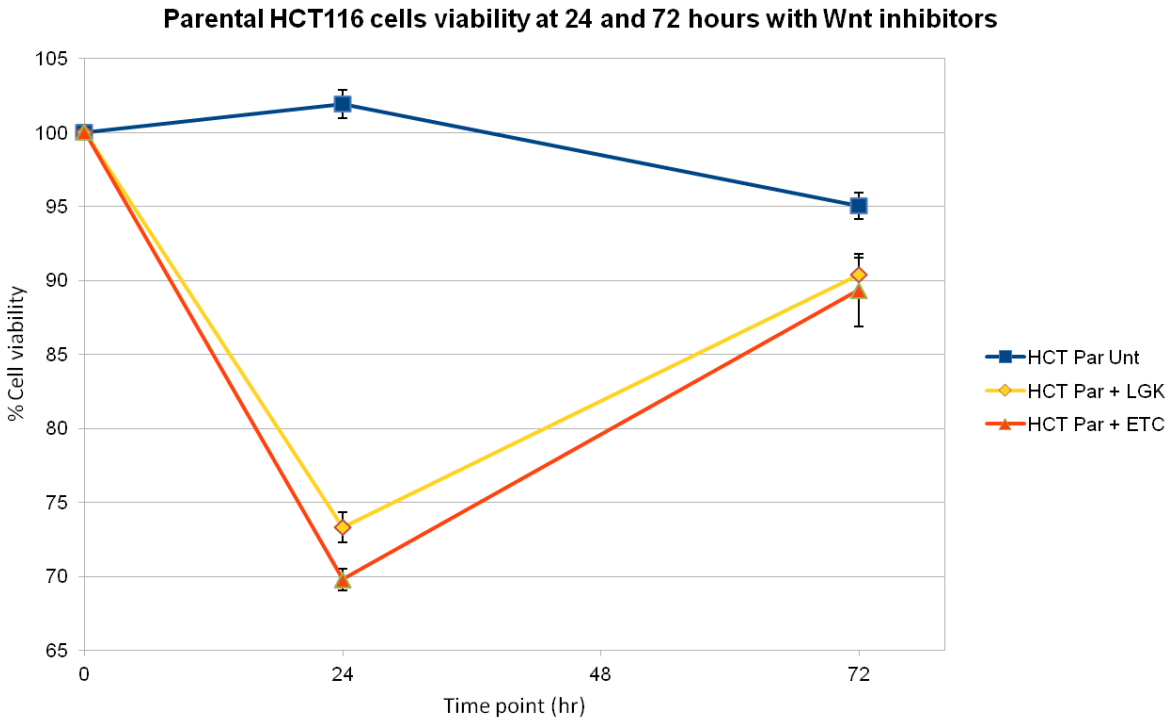

Figure 9: The effect of 10μM LGK974 and 0.2μM ETC-159 on cell viability of parental HCT116 cells 24 and 72 hours after treatment. Both Wnt inhibitors were effective at reducing the viability of these parental cells at 24 hours after treatment, with a t-test conducted using a p-value of 0.05 confirming that this change was statistically significant. At 72 hours, only the decrease in viability with LGK974 was statistically significant.

A Presto Blue viability assay was performed for parental HCT116 cells where the effects of the Wnt inhibitors were investigated at the 24 and 72 hour time-points. This was done as a prior viability assay showed a marked effect at 24 hours post-treatment and thus we wanted to confirm whether the drugs were being broken down within 24 hours after treatment resulting in the cells recovering at 72 hours post-treatment (**Figure 9**).

From these new viability assay results it can be seen how 24 hours after treatment, both LGK974 and ETC-159 seemed to be effective at reducing cell viability, with ETC-159 appearing to be slightly more effective than LGK974. 72 hours after treatment, the effectiveness of the Wnt inhibitors dropped, however both appeared to reduce viability below that of the untreated cells. Performing independent samples t-tests for these results, it was observed that at 24 hours, the decrease in cell viability by the Wnt inhibitors was statistically significant ($P < 0.05$) meaning that the drugs were effective at inhibiting cell growth. At 72 hours, however, only the t-test for the results of LGK974 appeared to be statistically significant whereas the ones for ETC-159 were not. This suggests that, at 72 hours post-treatment, LGK974 is more effective than ETC-159 at reducing cell viability of parental HCT116 cells than ETC-159. Comparing these results to the first viability assay performed (**Appendix II Section 7.2.3**) it was determined that LGK974 was more effective at 72 hours for reducing cell viability. Conversely, it was observed that ETC-159 was more effective at 24 hours post-treatment.

3.3.2.2 Effect of Wnt inhibition on 5-FU resistant HCT116 cells

Next, a Presto Blue viability assay was performed for 5-FU resistant HCT116 cells using the same inhibitor concentrations where the 24 and 72 hour time-points were investigated (**Figure 10**).

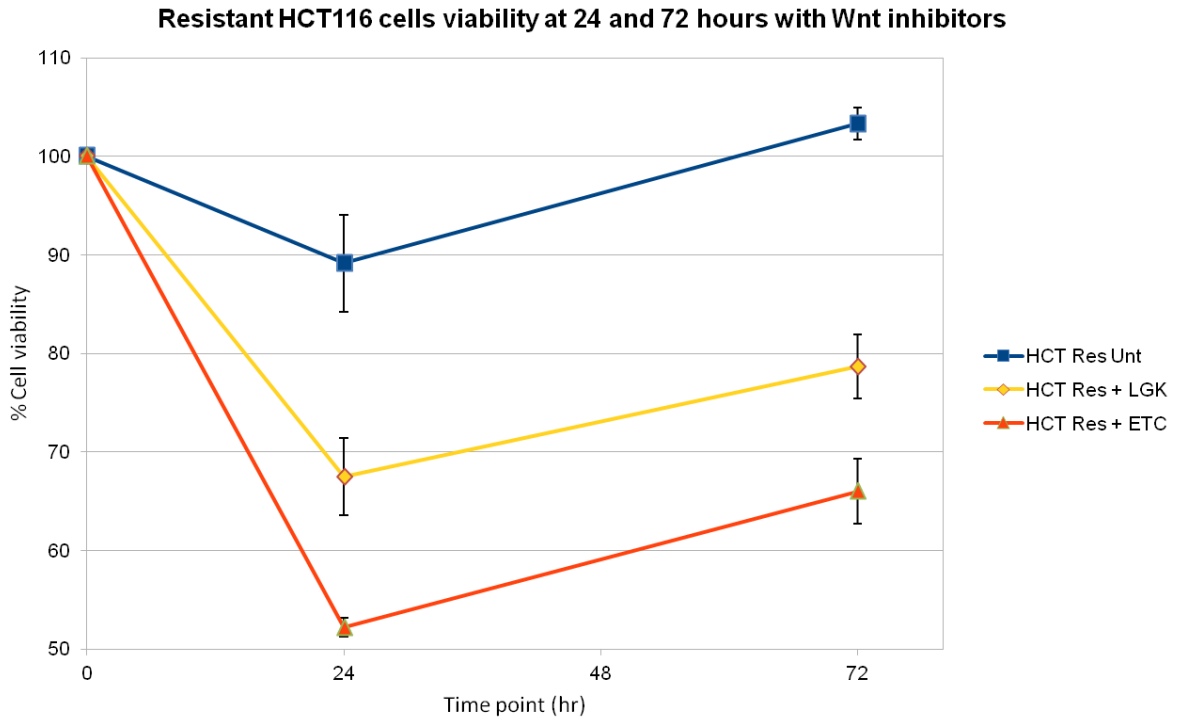

Figure 10: The effect of 10μM LGK974 and 0.2μM ETC-159 on cell viability of resistant HCT116 cells 24 and 72 hours after treatment. ETC-159 was effective at reducing the viability of resistant HCT116 cells at both time points while LGK974 was only effective at 72 hours post-treatment.

For the resistant cells, the results obtained this time were significantly different from the initial assay (**Appendix II Section 7.2.3**). At 24 and 72 hours post-treatment, both the Wnt inhibitors were shown to reduce cell viability when compared to the untreated control. This is in contrast to previous results where the use of these Wnt inhibitors was seen to increase cell viability at these time-points. Statistical analysis performed for these results, however, show that at a time-point of 24 hours, the change in cell viability seen with LGK974 did not significantly differ from the control ($P > 0.05$), whereas for ETC-159, the results were statistically significant ($P < 0.05$). At 72 hours post-treatment, both the Wnt inhibitors' effects on cell viability were significant when tested with an independent samples t-test. These results suggest that for resistant HCT116, ETC-159 was more effective than LGK974 at 24 and 72 hours post-treatment. LGK974 appeared to be most effective 72 hours after treatment while having little to no effect at 24 hours post-treatment.

3.3.2.3 Effect of Wnt inhibition on parental DLD1 cells

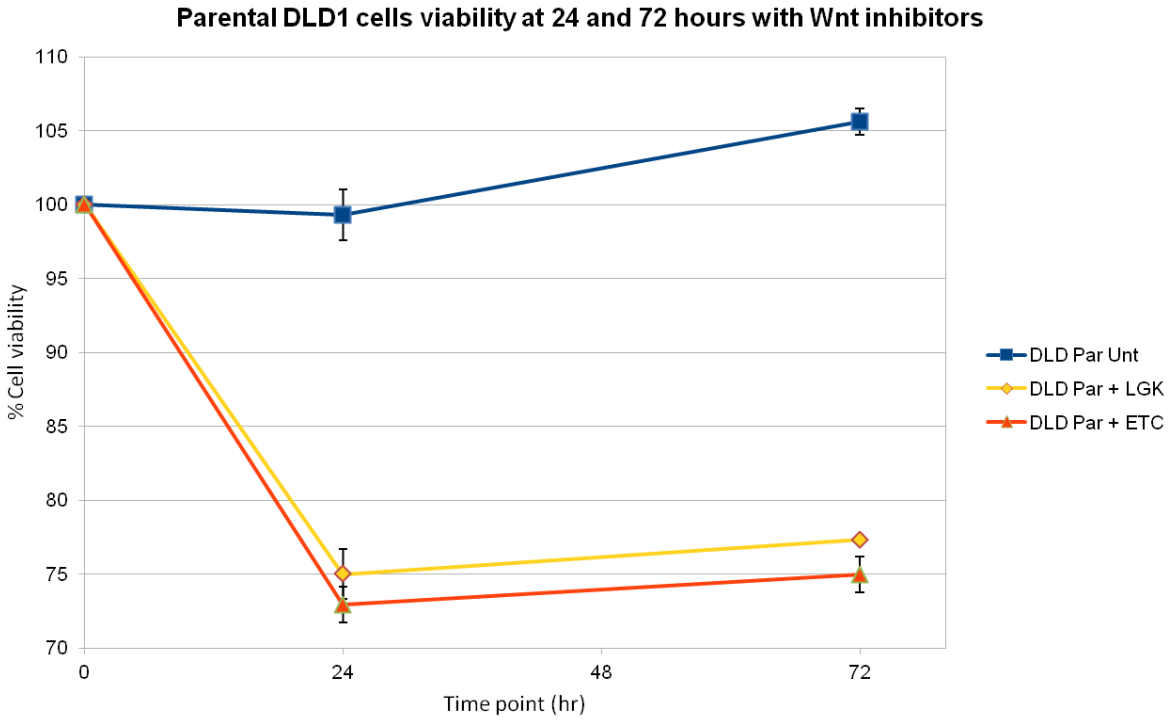

Figure 11: The effect of 10μM LGK974 and 0.2μM ETC-159 on cell viability of parental DLD1 cells 24 and 72 hours after treatment. These results show that both Wnt inhibitors were effective at reducing the viability of parental DLD1 cells, at both the 24 hour and 72 hour time-points.

The results obtained for parental DLD1 cells were similar to the results obtained for the parental HCT116 cells with both Wnt inhibitors appearing to be effective at reducing cell viability at the two time-points, with ETC-159 being slightly more effective than LGK974. Statistical analyses using a p-value of 0.05 also confirmed that the results obtained were significantly different from the untreated controls.

3.3.2.4 Effect of Wnt inhibition on 5-FU resistant DLD1 cells

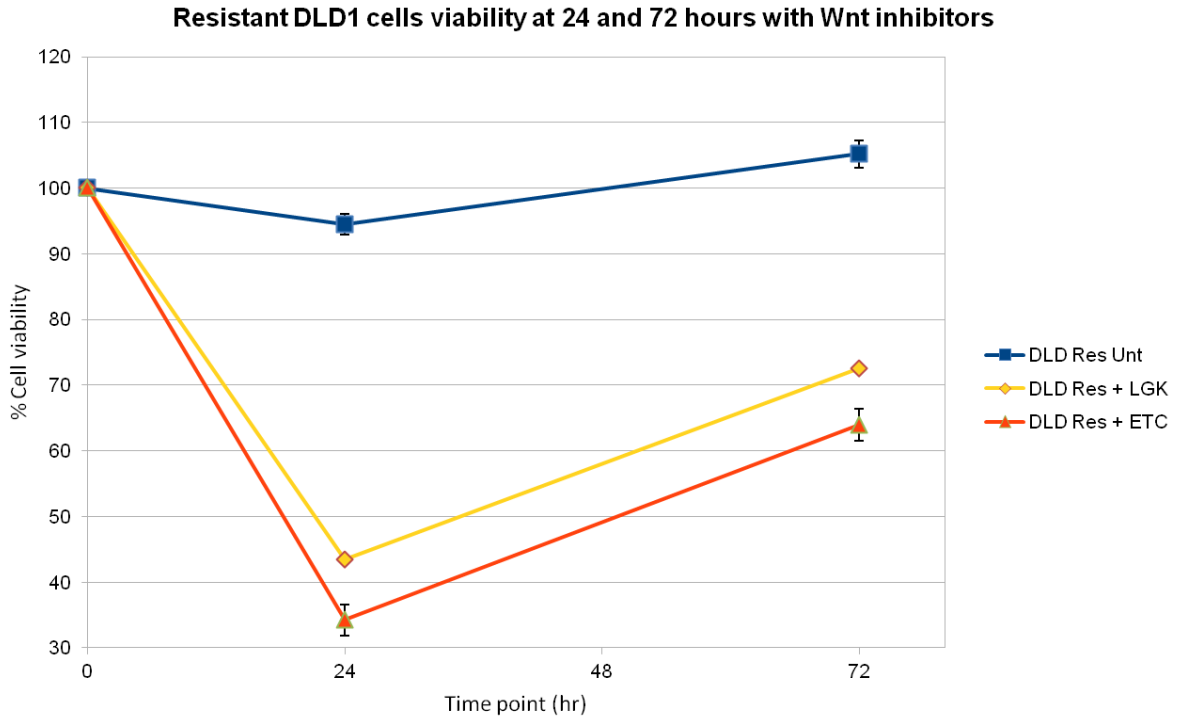

Figure 12: The effect of 10μM LGK974 and 0.2μM ETC-159 on cell viability of resistant DLD1 cells 24 and 72 hours after treatment. Resistant DLD1 cells' viability was successfully reduced with both Wnt inhibitors at both time points.

The resistant DLD1 cells showed similar results to their parental counterparts with the two Wnt inhibitors decreasing cell viability at both time-points. Also similar to parental DLD1 cells, ETC-159 appeared to be slightly more effective at inhibiting cell growth. Statistical analysis confirmed that the results obtained were statistically significant when compared to the untreated control ($P < 0.05$).

3.4 Scratch Migration Assay

3.4.1 Effect of Wnt inhibition on the migration rate of parental HCT116 cells

Table 5: Percent reduction in open image area of parental HCT116 cells over 48 hours when treated with the Wnt inhibitors. Both Wnt inhibitors effectively reduced migration rate at both time points with this reduction confirmed to be statistically significant using a p value of 0.05

	% reduction in open image area after 24 hours	% reduction in open image area after 48 hours
Untreated	4.11	4.4
LGK974	0.66	7.92
ETC-159	3.01	4.86

The results in table 5 show that, in general, both Wnt inhibitors were effective at reducing the migration rate of parental HCT116 cells after 24 hours. However, LGK974 was less effective after 24 hours as the decrease in migration was significantly less than that of the untreated control. At 48 hours post-treatment, both Wnt inhibitors were also effective at reducing the migration. Statistical analysis using a p-value of 0.05 confirmed that the reduction in migration rate with the Wnt inhibitors compared to the control was statistically significant.

3.4.2 Effect of Wnt inhibition on the migration rate of resistant HCT116 cells

Table 6: Percent reduction in open image area of resistant HCT116 cells over 48 hours when treated with the Wnt inhibitors. The Wnt inhibitors were effective at both time points for reducing the migration rate compared to the untreated control, which was also tested statistically using a p value of 0.05

	% reduction in open image area after 24 hours	% reduction in open image area after 48 hours
Untreated	2.46	5.39
LGK974	22.96	22.31
ETC-159	27.15	8.25

As was the case with parental HCT116 cells, the migration rate of resistant HCT116 cells was also reduced with Wnt inhibitor treatment at both time points. The Wnt inhibitors were most effective at 24 hours post-treatment with a significant reduction in migration compared to the untreated control. At 48 hours post-treatment, LGK974 also reduced the migration rate considerably compared to the control, while ETC-159 reduced the migration rate to a lesser extent. Statistical analysis with a t-test using a p-value of 0.05 confirmed that the reduction in migration for both inhibitors at the two time points was statistically significant.

3.4.3 Effect of Wnt inhibition on the migration rate of parental DLD1 cells

Table 7: Percent reduction in open image area of parental DLD1 cells over 48 hours when treated with the Wnt inhibitors. Statistical analysis using a p value of 0.05 showed that LGK974 reduced the migration rate at both time points, whereas ETC-159 did not

	% reduction in open image area after 24 hours	% reduction in open image area after 48 hours
Untreated	3.9	4.96
LGK974	9.45	2.12
ETC-159	4.2	4.5

In general, both Wnt inhibitors reduced the migration of parental DLD1 cells, with LGK974 significantly reducing migration at 24 hours post-treatment. Statistical analysis with a p-value of 0.05, in fact, confirmed that this decrease in migration compared to the control was statistically significant, for both the 24 hour and 48 hour time points. However, no statistically significant change to migration was seen with ETC-159, for both time points.

3.4.4 Effect of Wnt inhibition on the migration rate of resistant DLD1 cells

Table 8: Percent reduction in open image area of resistant DLD1 cells over 48 hours when treated with the Wnt inhibitors. Only the change in migration after 24 hours was found to be statistically significant ($P < 0.05$)

	% reduction in open image area after 24 hours	% reduction in open image area after 48 hours
Untreated	0.62	0.41
LGK974	0.19	0.24
ETC-159	1.23	0.57

For resistant DLD1 cells, a t-test using a p value of 0.05 determined that the only significant reduction to the migration rate occurred at 24 hours post-treatment for both inhibitors. The decrease in migration rate at 48 hours was not found to be significant.

3.5 Transwell Invasion Assay

3.5.1 Effect of Wnt inhibition on parental and resistant HCT116 cells

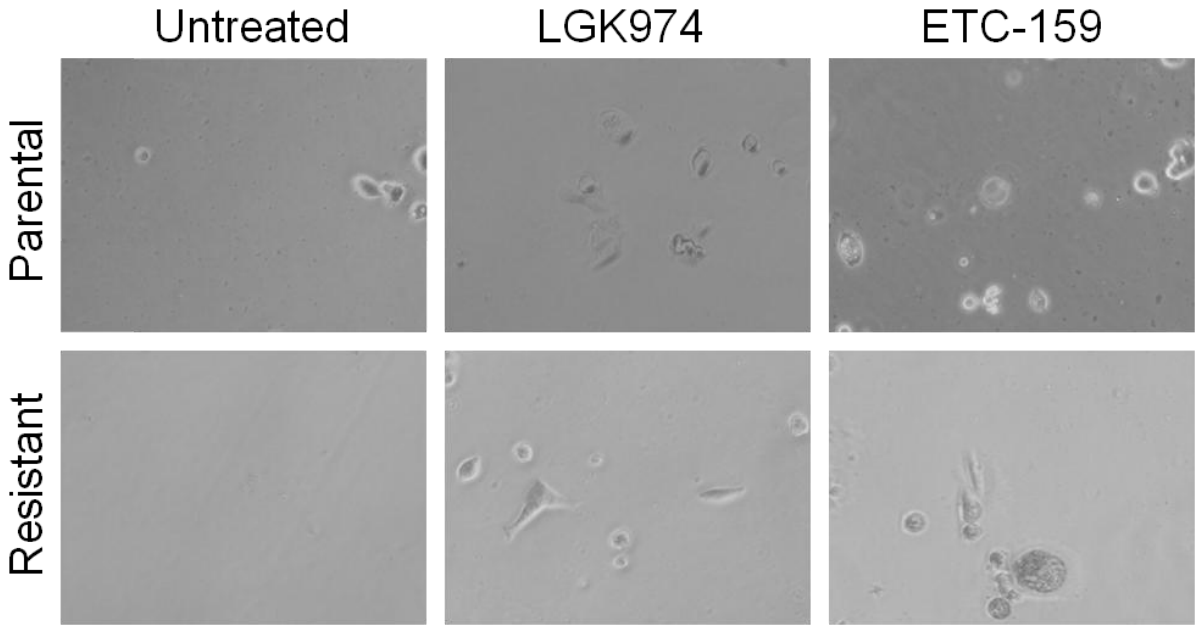

Figure 13: Comparison of transwell invasion of parental and resistant HCT116 cells under different conditions 72 hours post-treatment. Parental cells did not show any significant changes to invasion rate from the untreated control. Resistant cells however, when treated with either inhibitor, had an increased invasion rate which was found to be statistically significant from the untreated control ($P < 0.05$).

Table 9: Average number of invaded HCT116 cells per quadrant of field of view 3 days after treatment

	Untreated	LGK974	ETC-159
Parental	**4**	**4**	**9**
Resistant	**0**	**6**	**4**

It was observed that 3 days after treatment, parental HCT116 cells treated with LGK974 showed no difference in invasion from the untreated control. However, parental cells treated with ETC-159 showed an elevated rate of invasion, with more cells detected 3 days after treatment for the untreated control (**Table 9**). Despite this, an independent samples t-test using a p-value of 0.05 conducted on these populations revealed no significant differences between the number of cells per quadrant of field of view for the Wnt inhibitor-treated wells and the untreated controls (**Appendix IV**). Resistant HCT116 cells, however, when treated with the Wnt inhibitors, showed an increased rate of invasion given that the number of cells which invaded were much higher than the untreated control. An independent samples t-test also confirmed that there was a significant statistical difference between the number of cells which invaded for both LGK974 and ETC-159 when compared to the control ($P < 0.05$).

3.5.2 Effect of Wnt inhibition on parental and resistant DLD1 cells

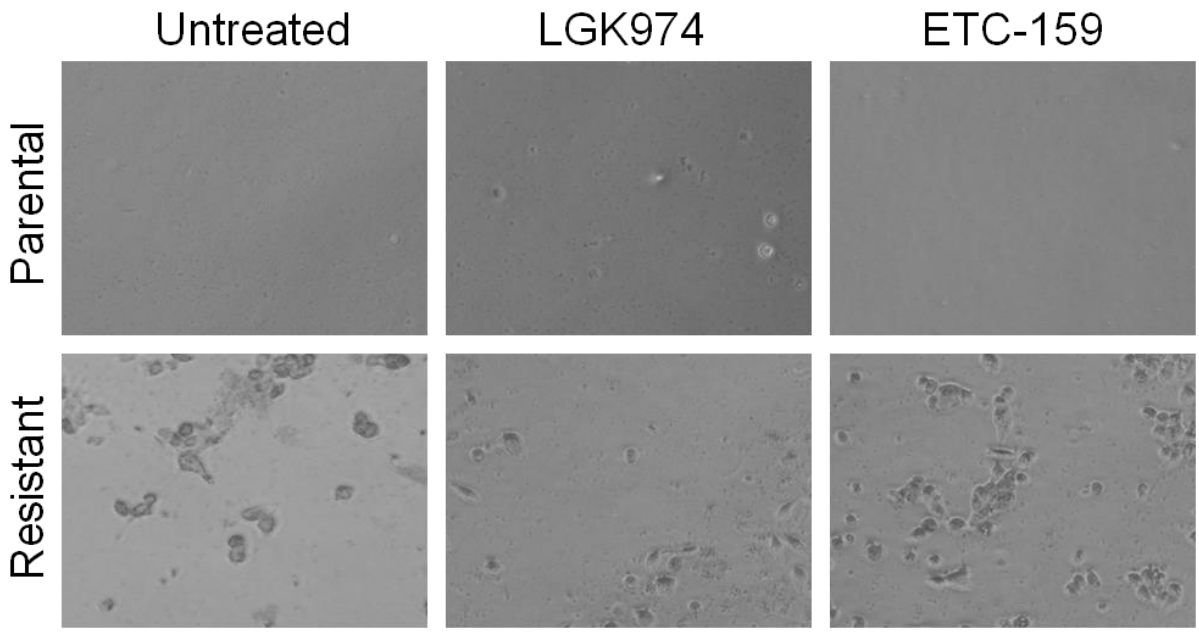

Figure 14: Comparison of transwell invasion of parental and resistant DLD1 cells under different conditions 72 hours post-treatment. The invasion rate of both parental and resistant DLD1 cells was not significantly affected by either inhibitor.

Table 10: Average number of invaded DLD1 cells per quadrant of field of view 3 days after treatment

	Untreated	LGK974	ETC-159
Parental	**0**	**1**	**0**
Resistant	**12**	**15**	**33**

Three days after treatment, it was observed that the invasion potential of parental DLD1 cells was not affected in a significant manner given that the number of cells which invaded was not significantly different from the untreated control, a fact which was also confirmed with an independent samples t-test (**Appendix IV**). The invasion potential of resistant DLD1 cells did not appear to be decreased with Wnt inhibitor treatment; on the contrary, it was observed to have increased, with ETC159-treated cells having the highest invasion rate (**Table 10**). However, statistical analysis showed no significant differences between the

number of cells per quadrant for the treated and untreated cells ($P > 0.05$). In contrast to HCT116, it seems that resistant DLD1 cells had a significantly higher invasion rate than their parental counterparts. On the other hand, similar to HCT116 cells, it was observed that the Wnt inhibitors at concentrations of 10 μM for LGK974 and 0.2 μM for ETC-159 were not effective in reducing the invasion potential of the DLD1 cells. Furthermore, resistant DLD1 cells, akin to resistant HCT116, were observed to have an increased invasion rate when treated with the inhibitors.

3.6 Western Blotting

3.6.1 Effect of Wnt inhibition on protein expression of β-catenin, EGFR, and CDC42

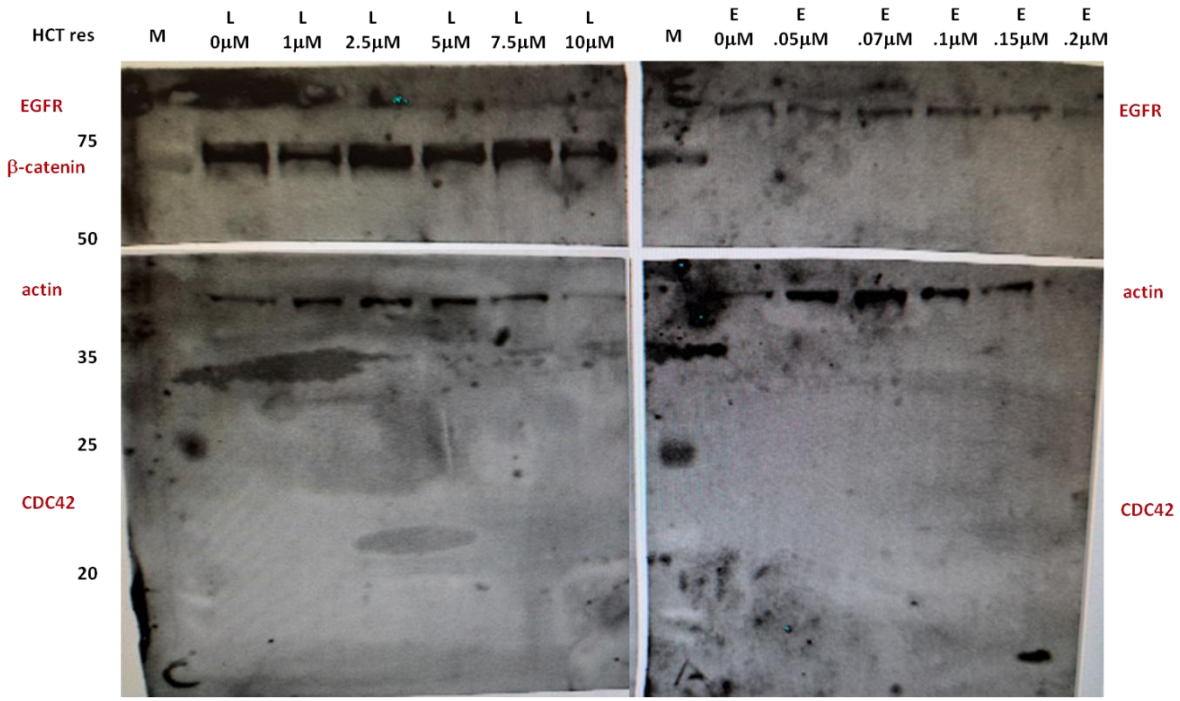

Figure 15: WB using 10% resolving gel and running resistant HCT116 cells treated with a range of Wnt inhibitor concentrations, determining that a concentration of 10μM for LGK974 and 0.2μM for ETC-159 yielded the best results.

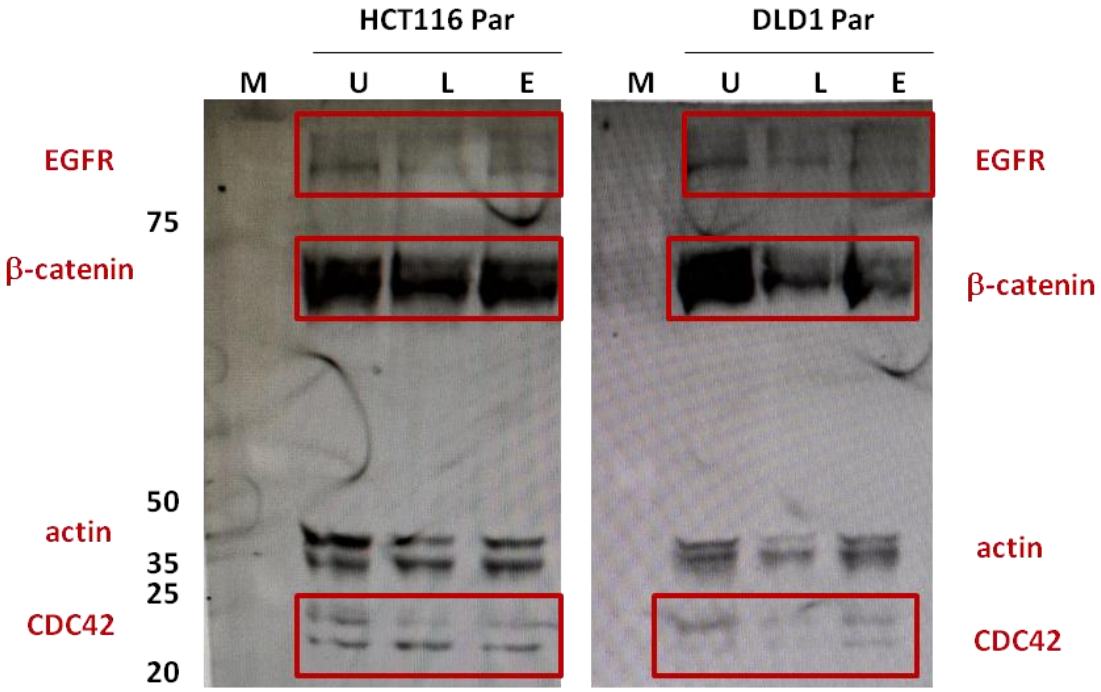

Figure 16: WB for parental HCT116 and DLD1 using 10μM LGK974 and 0.2μM ETC-159 for EGFR and β-catenin. Overall, the results do not show any major differences in the intensity of the bands obtained with either Wnt inhibitor.

From previous immuno-blotting troubleshooting, it was determined that using a 10% resolving gel for Western Blotting runs, in addition to treating cells cultured in 2% serum medium with either 10μM LGK974 or 0.2μM ETC-159 would yield the best results (**Appendix V**; **Figure 15**).

Overall, it was observed that for resistant HCT116 cells, the intensity of the EGFR and β-catenin bands was consistent for all concentrations used (**Figure 15**), suggesting that the protein expression of both EGFR and β-catenin might not be significantly affected by the Wnt inhibitors used, at these concentration. In addition, for both LGK974 and ETC-159, CDC42 bands were not detected.

Another Western blot was then run for parental and resistant HCT116 and DLD1 cells which were treated with 10μM LGK974, 0.2μM ETC-159, or left untreated as a control and the expression of β-catenin, EGFR, and CDC42 was tested for (**Figure 16**). For the parental

cells of both HCT116 and DLD1, there were no significant differences in the intensity of the bands obtained for the Wnt inhibitor-treated cells from the untreated cells. Although the band obtained for parental DLD1 cells treated with LGK974 appears to be less intense, when taking into consideration the corresponding actin band which was also less intense, it can be concluded that there was no overall change to the expression of CDC42 with LGK974. Although the resistant variants of HCT116 and DLD1 cells were also run, unfortunately, only the band for the untreated resistant HCT116 cells appeared (Appendix V **Figure 54**). In this case, given that the actin loading control bands also did not appear in the blot for the resistant cells, the lack of bands cannot be ascribed to effects due to Wnt inhibition.

3.6.2 Effect of Wnt inhibition on lysine tri-methylation

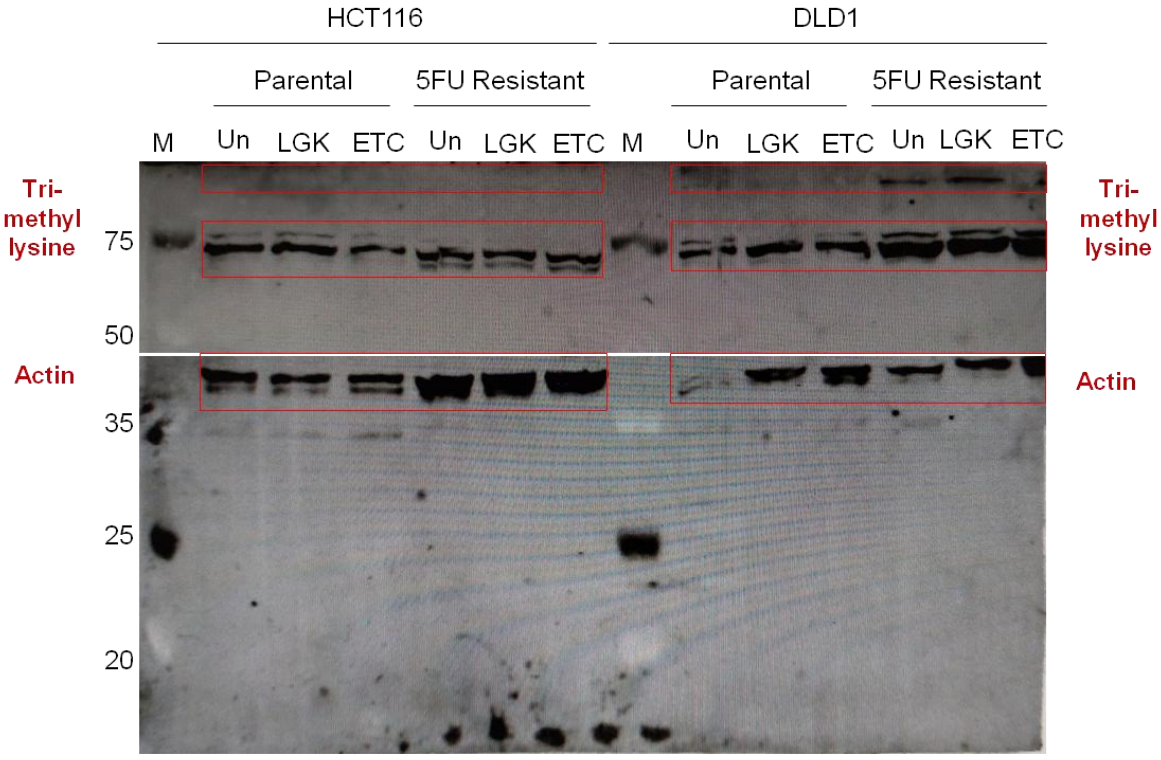

Figure 17: WB for tri-methyl lysine running parental and resistant HCT116 and DLD1 cells treated with 10μM LGK974 or 0.2μM ETC-159. An additional set of bands appears the top of the gel for resistant DLD1 cells which do not appear for HCT116, or parental DLD1 cells. In addition, treatment with ETC-159 reduced the intensity of this top band.

In addition to the effects of the Wnt inhibitors on β-catenin, EGFR and CDC42 expression, the effects of LGK974 and ETC-159 were also investigated with regards to lysine tri-methylation (**Figure 17**).

Considering the bands obtained for the HCT116 cells first, it was observed that, overall, the middle band had a consistent intensity for all the conditions tested. Two additional bands also appeared above and below this middle band. For the parental HCT cells, the top band was observed to have a slightly higher intensity than the ones for the resistant cells. In addition for this top band, the intensity of the band for the ETC-treated condition was observed to be slightly weaker than the other conditions. For the resistant HCT cells, the bottom bands had a much higher intensity than the corresponding bands for the parental cells although their intensity did not appear to be impacted by the treatment status. Furthermore, although the top bands were visible, their intensity was significantly weaker when compared to the corresponding bands for the parental cells.

For the DLD1 cells, it was observed that multiple bands appeared with different intensities. For both the parental and resistant cells, the middle band was observed to have the highest intensity, with resistant DLD1 cells observed to produce the strongest intensity bands. Similar to the HCT116 cells, another two bands were visible above and below this middle band. The top band had a consistent intensity for the resistant DLD1 cells, irrespective of treatment status whereas for the parental cells, treatment with LGK was observed to reduce the intensity of this top band, especially when compared to the ETC-treated band. The untreated band was observed to have a lower intensity however, given that the corresponding actin bands were also of low intensity, it is likely that the normalised intensity of the band should be much higher. The bottom band was relatively faint for both parental and resistant cells and also did not appear to be significantly affected by treatment status. However, in addition to these bands, DLD1 cells were observed to present further

bands near the top of the gel, with the bands for resistant untreated and LGK-treated cells being the most visible.

3.6.3 Effect of Wnt inhibition on lysine mono-methylation

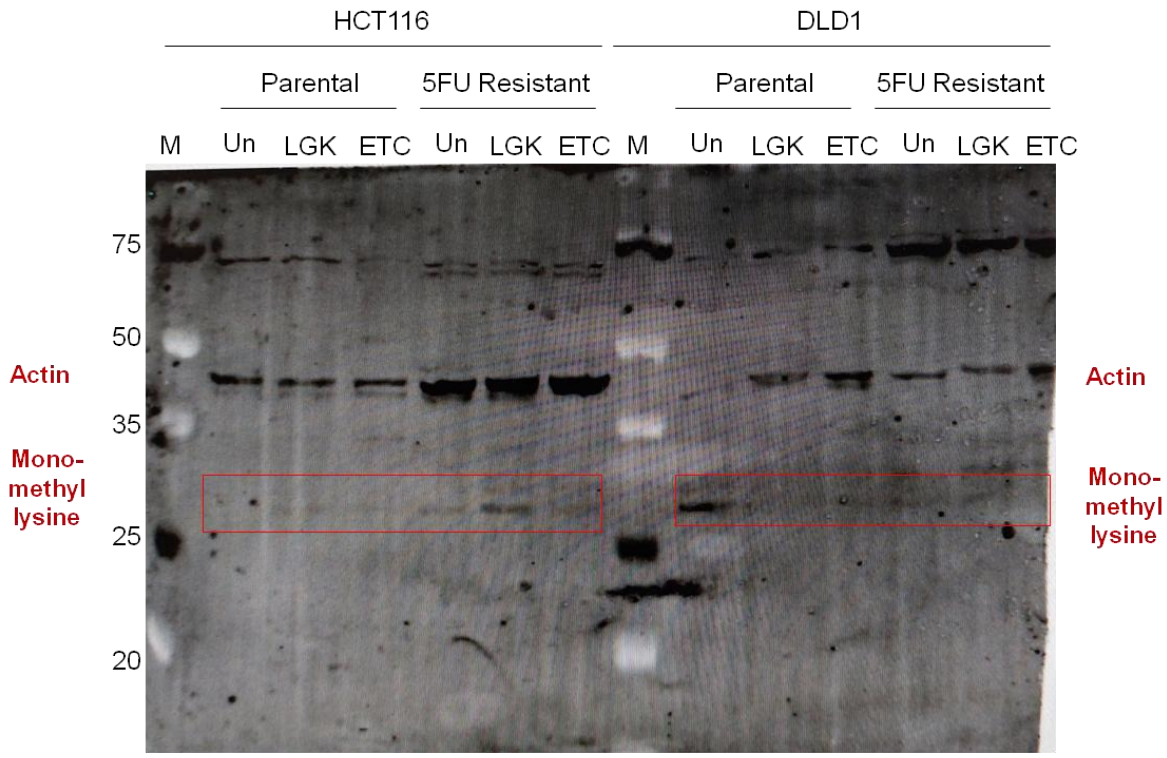

Figure 18: WB for the mono-methyl lysine running parental and resistant HCT116 and DLD1 cells treated with 10μM LGK974 or 0.2μM ETC-159. The bands obtained for parental DLD1 cells show a reduction in intensity with Wnt inhibitor treatment compared to the untreated condition. In addition, the band obtained for resistant HCT116 treated with LGK974 had a higher intensity than the corresponding bands for the untreated or ETC159-treated conditions.

The effects of Wnt inhibition on the mono-methylation of lysine was also investigated as can be seen from **Figure 18**. From these results, it can be seen that the bands obtained for parental HCT116 had a low intensity with little variation in this intensity between the tested conditions. Resistant HCT116 cells were observed to produce slightly more intense bands when compared to the parental cells, with the band obtained for the LGK-treated cells having the most intense bands amongst the three conditions. For the parental DLD1 cells, it

is clear that the untreated condition produced a band with a higher intensity than the Wnt inhibitor-treated ones. However, for the resistant DLD1 variant, all the bands obtained had a low intensity which was observed to be consistent for all the conditions.

3.7 ELISA

3.7.1 Effect of Wnt inhibition on total EGFR expression

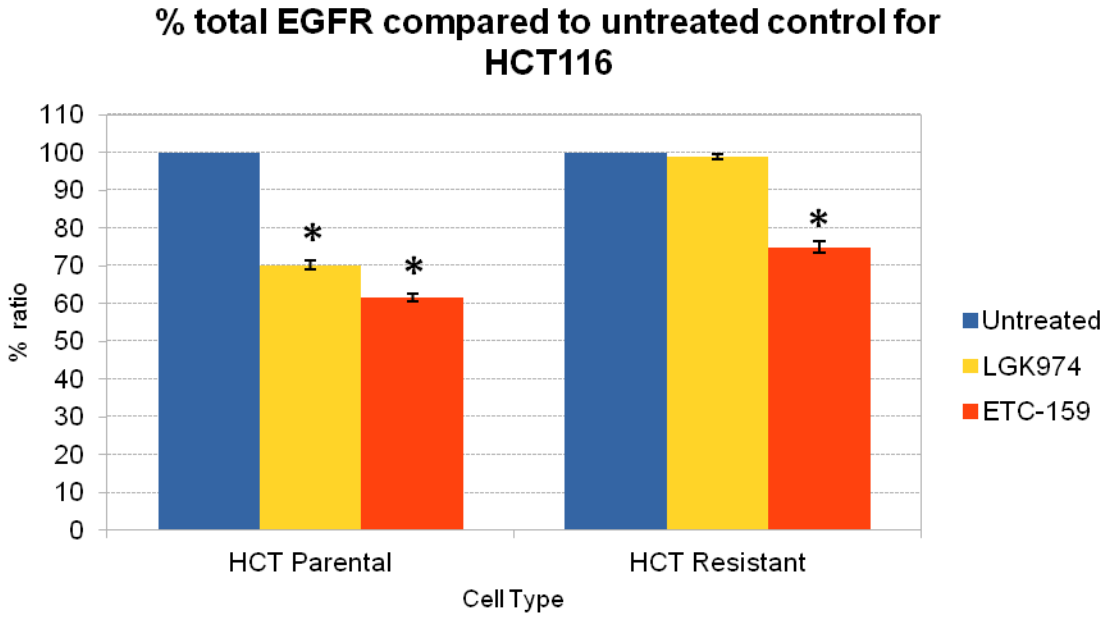

Figure 19: % total EGFR expression of parental and chemoresistant HCT116 cells with Wnt inhibition compared to untreated control. *P < 0.05 vs. untreated control. Treatment with both inhibitors significantly reduced the expression of total EGFR compared to the untreated control. For the chemoresistant cells, only ETC-159 had a significant effect, reducing total EGFR expression.

The results for parental HCT116 cells (**Figure 19**) show that treatment with either of the Wnt inhibitors significantly reduced the expression of total EGFR when compared to the untreated control. For the chemoresistant cells, only ETC-159 had an effect, with total EGFR decreasing when compared to the control.

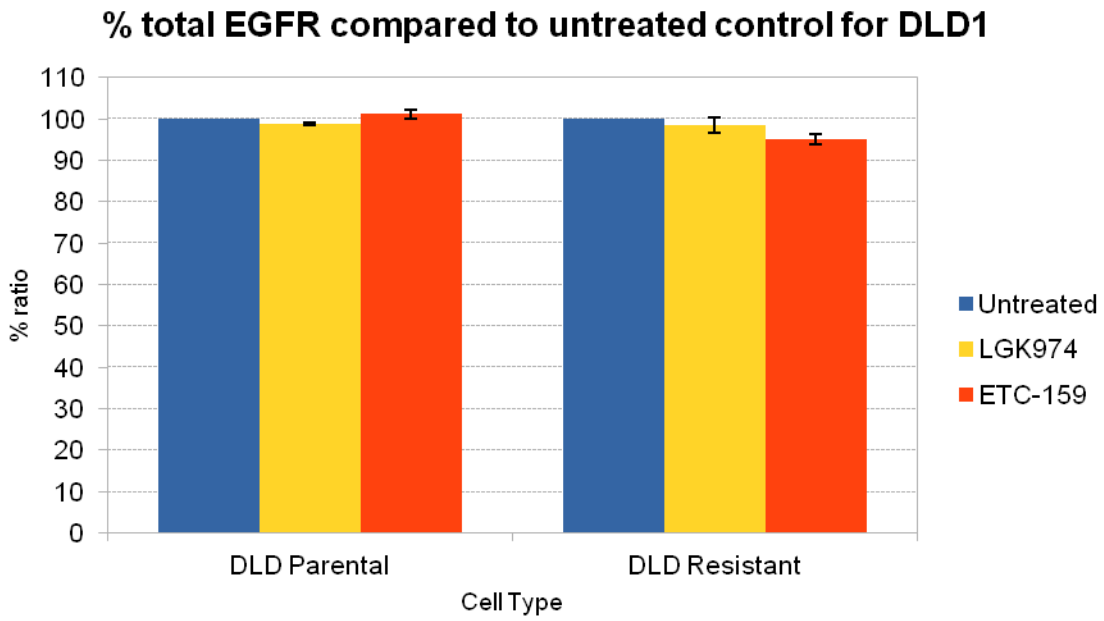

Figure 20: % total EGFR expression of parental and chemoresistant DLD1 cells with Wnt inhibition compared to untreated control. The Wnt inhibitors did not significantly affect the expression of total EGFR for either the parental or resistant DLD1 cells.

For both the parental and chemoresistant DLD1 cells, neither of the Wnt inhibitors had any significant effect on the expression of total EGFR.

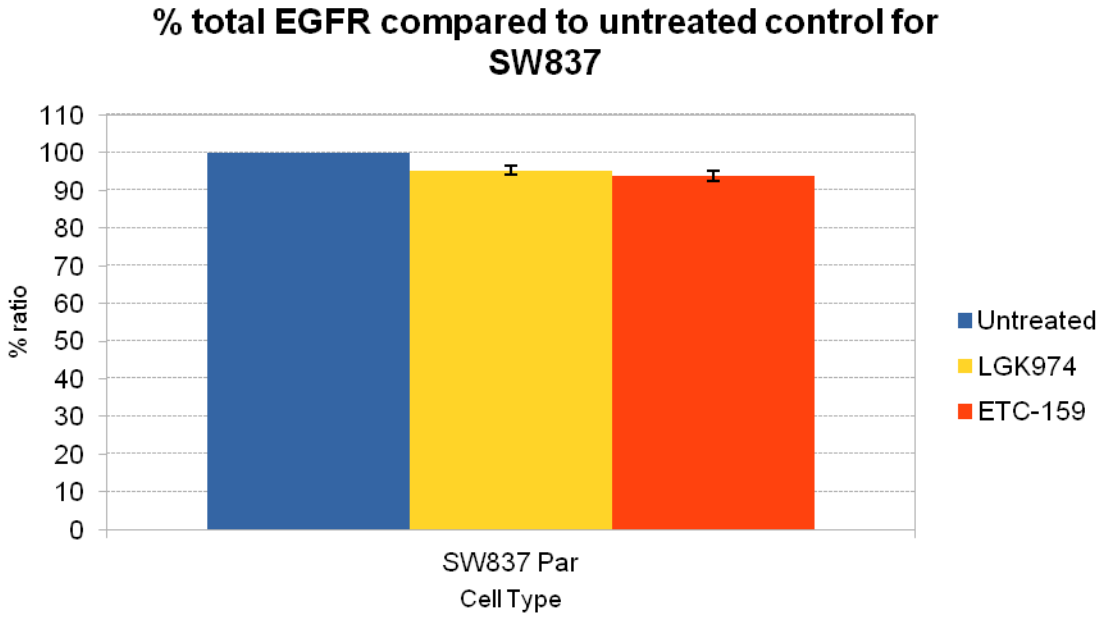

Figure 21: % total EGFR expression of parental SW837 cells with Wnt inhibition compared to untreated control. Wnt inhibition had no significant effect on total EGFR expression for parental SW837.

The results of parental SW837 also show that Wnt inhibition did not have any significant effects on the expression of total EGFR.

3.7.2 Effect of Wnt inhibition on phosphorylated EGFR expression

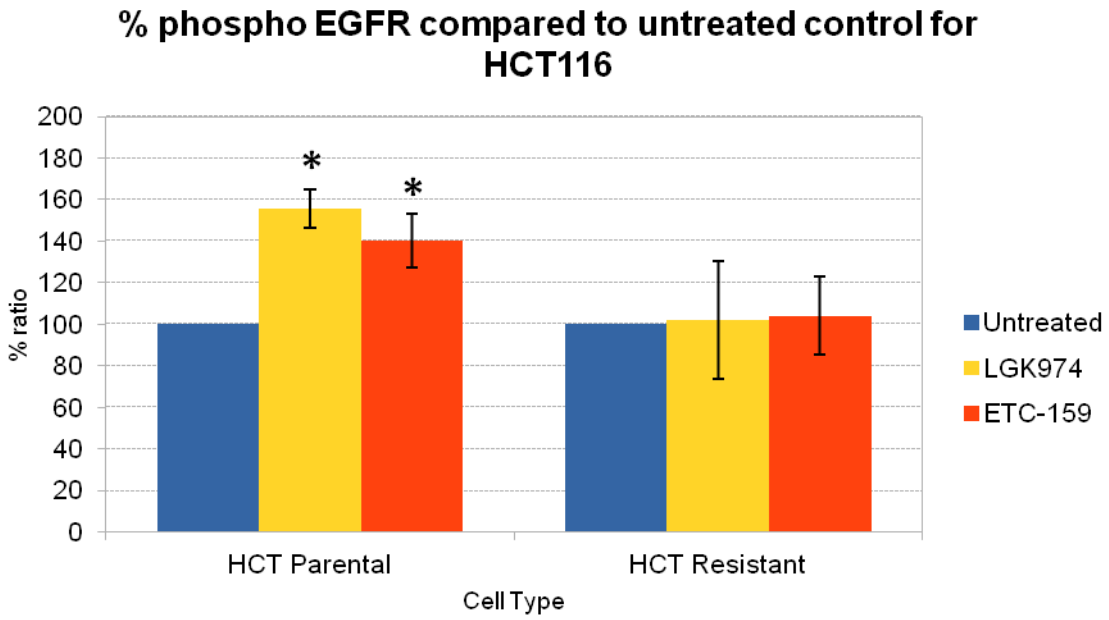

Figure 22: % phosphorylated EGFR expression of parental and chemoresistant HCT116 cells with Wnt inhibition compared to untreated control. *P < 0.05 vs. untreated control. The expression of phosphorylated EGFR was significantly increased in parental HCT116 cells treated with either inhibitor. In contrast, no effect on phosphorylated EGFR was seen for the resistant cells with either inhibitor.

The results in **Figure 22** show that for parental HCT116 cells, both Wnt inhibitors significantly the expression of phosphorylated EGFR when compared to the untreated control. However, in the chemoresistant cells, the Wnt inhibitors had no significant effects on phospho-EGFR.

3.7.3 Effect of Wnt inhibition on ratio of phosphorylated to total EGFR

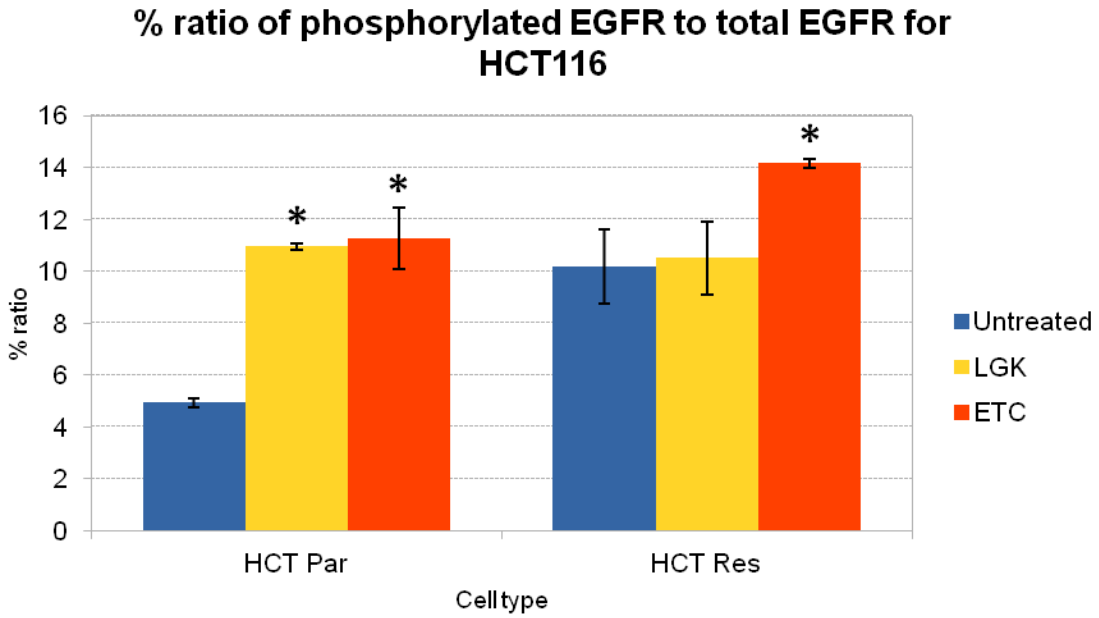

Figure 23: % ratio of phosphorylated to total EGFR for parental and chemoresistant HCT116 cells treated with the Wnt inhibitors compared to the control. *P < 0.05 vs. untreated control. The Wnt inhibitors significantly increased the proportion of phosphorylated to total EGFR in parental HCT116 cells. In the resistant cells, only ETC-159 increased this proportion, with LGK974 having no significant effect.

For parental HCT116 cells, treatment with either of the Wnt inhibitors significantly increased the proportion of phosphorylated to total EGFR, going from 5% proportion of phosphorylated EGFR in the untreated control to approximately 11% with the Wnt inhibitors. For the chemoresistant HCT116 cells, LGK974 did not significantly affect the proportion of phosphorylated to total EGFR compared to the control, whereas treatment with ETC-159 increased this proportion significantly.

4 Discussion

4.1 Introduction

The main objective of this study was to investigate the effects of Wnt pathway inhibition via two small molecule inhibitors on parental and 5-FU resistant CRC cells possessing either wild-type APC or a truncated APC variant. More specifically, we wanted to study various APC-related proteins and their potential for use as biomarkers, either diagnostic or prognostic. As APC is a critically important protein in various cellular regulatory processes, particularly in the canonical Wnt pathway, shedding light on its interactions and influences on other proteins, which will help in elucidating and better explaining mechanisms which underlie not only CRC carcinogenesis, but potentially other cancers that rely on similar pathways. Studying APC within this context is made more important when less studied pathways are involved, such as is the case with the non-canonical Wnt pathways, or when conditions such as chemoresistance are in effect, both of which may significantly affect a patient's outcome and treatment strategies. Through research similar to the one conducted for this study, it is possible to identify candidates which can be effectively targeted within CRC, thereby countering the effects of de-regulation and potentially leading to favourable treatment outcomes.

4.2 Wnt Inhibitor Troubleshooting

In this study, two PORCN inhibitors, LGK974 and ETC-159, have been used. First, we sought to determine the ideal concentration that should be used for each of these inhibitors. A search through the literature for studies in which these inhibitors had been utilised showed that, for LGK974, concentrations between 0.5µM and 20µM were frequently used, while for ETC-159, concentrations between 0.02µM and 0.1µM were commonly used, to good effect (Wellenstein *et al.*, 2019; Guillermin *et al.*, 2021; Kahlert *et al.*, 2015; Li *et al.*, 2020; Madan *et al.*, 2016; Madan, Ke, Le *et al.*, 2016). Thus, we created two stock solutions of these inhibitors using basal DMEM of concentrations of 50µM for LGK974, and 1µM for ETC-159. HCT116 and Caco-2 parental cells, both of which were determined to have no APC truncation mutations, were then seeded in 96-well plates and treated with five different concentrations from each inhibitor – for LGK974 the concentrations used were 0, 0.5, 1, 2.5, and 5µM, while for ETC-159 the concentrations were 0, 0.02, 0.05, 0.07, and 0.1µM. The cells which were not treated with inhibitor were instead treated with a volume of DMSO. After three days, the cells were checked under a microscope and, surprisingly, it was observed that the number of cells which had been treated with the inhibitor was higher than the control with just DMSO. It was then decided to substitute the lowest Wnt inhibitor drug concentration in the plate with a higher concentration to see whether this new higher concentration will decrease cell growth. Thus, the LGK974 concentration of 0.5µM was substituted with 10µM, and the ETC-159 concentration of 0.02µM was replaced with 0.2µM. The cells were then left to grow for an additional three days. However, these new concentrations still did not result in any reduction in cell growth.

Despite this, a Western blot was still carried out for the HCT116 parental and chemoresistant cells to investigate the protein expression of three main APC-related

proteins, β-catenin, CDC42, and EGFR, under the effect of Wnt inhibition. For this, an LGK974 concentration of 10μM and ETC-159 concentration of 0.2μM were used. From the results (**Appendix V Figure 49**), it was seen that LGK974 was having some effect on β-catenin and EGFR protein expression of both the parental and chemoresistant HCT116 cells, as cells treated with this inhibitor resulted in a band intensity which was lower than the untreated control. These results seem to agree with two previous studies using LGK974 which also found a reduction in the expression of β-catenin following treatment with this inhibitor (Bagheri *et al.* 2020; Li *et al.*, 2020).

Following up on these results, it was decided to increase the concentration of the Wnt inhibitors used; LGK974 was increased to 20μM and ETC-159 was increased to 0.5μM. Then, another Western blot was run just for the chemoresistant HCT116 cells. However, the results did not show any decrease in the intensity of the bands for β-catenin and CDC42, while for EGFR, the band did not resolve clearly due to the 12% resolving gel that was used (**Appendix V Figure 50**).

Given that we were having little success with the inhibitor concentrations used thus far, it was decided to seed a fresh 24 well plate and treat chemoresistant HCT116 cells with a higher range of inhibitor concentrations. Since in some studies LGK974 was sometimes used up to a concentration of 100μM and ETC-159 used up to 1μM (Liu *et al.*, 2013; Bagheri *et al.*, 2020; Madan, Ke, Le *et al.*, 2016), we treated the cells with six concentrations for each inhibitor – 0, 20, 40, 60, 80, and 100μM for LGK974, and 0, 0.5, 1, 1.5, 2, and 2.5μM for ETC-159. A volume of DMSO equal to the volume of drug added in the wells with the highest inhibitor concentration was added to the controls. After three days, the cells were examined and, similar to previous treatment results, it was observed how the cells which had been treated with the inhibitors had grown more than the controls with just DMSO. For the Western blot, owing to the fact that a 12% resolving gel was not proving to be adequate for

resolving EGFR, a hybrid stacking gel with 12% and 8% gels was prepared for this Western blot. The results for this experiment also showed no major differences in the intensity of the bands produced, except for the band at 80µM of LGK974 which had an increased intensity when compared to the control and to the rest of the concentrations (**Appendix V Figure 51, 52**). The EGFR was also much more clearly resolved however there was still room for improvement.

In trying to understand why cells which were treated with the Wnt inhibitors were growing more than the untreated cells, it was noticed that the untreated/control cells were being administered a higher volume of DMSO than the treated cells. This is due to the fact that, up to this point, the Wnt inhibitor concentrations for treatment were being prepared by diluting a volume of the original inhibitor stock, which was prepared with DMSO, with basal DMEM. Thus, only a small proportion of that concentration was in fact DMSO as the majority of the volume was DMEM. Hence, when the controls were being given a volume of DMSO equal to the volume of inhibitor added to the cells with the highest drug concentration, that DMSO volume added was far more than the actual volume of DMSO which was present in the other wells. If it was the high volume of DMSO which resulted in a reduction in cell number, then that would give the impression that the cells which had been treated with Wnt inhibitors had a higher growth rate as they would not have as much DMSO as the controls.

To test this, new Wnt inhibitor stock concentrations of 500µM (LGK974) and 10µM (ETC-159) were prepared with DMSO and six different concentrations were used to treat HCT116 cells – 0, 1, 2.5, 5, 10, and 20µM for LGK974, and 0, 0.05, 0.07, 0.1, 0.2, and 0.5µM for ETC-159. The control cells were given a volume of DMSO equal to the volume of drug added to the well with the highest drug concentration. After three days, the cells were checked and it was observed that both the control wells and the wells treated with the highest drug concentrations, for both drugs, had reduced growth rate when compared to the rest of the

wells. This suggests that it was the DMSO which was reducing the cell growth. Interestingly, it was reported in one study by Liu *et al.* (2013) that LGK974 started exhibiting toxicity in cells at a concentration of 20μM, similar to what was seen in our study. Given that in the aforementioned study the authors also used DMSO to prepare the inhibitor concentration dilutions, it might further prove that it is the DMSO at high volumes which results in cytotoxic effects, and not the Wnt inhibitor.

A Western blot was also run for these concentrations. This time, a 10% resolving gel was prepared to try and resolve the EGFR bands more clearly than with a hybrid gel approach. As expected, the bands for the control and the highest inhibitor concentrations had a significantly lower intensity than the bands for the other concentrations (**Appendix V Figure 53**). Apart from these differences, no other significant differences in the band intensities were observed between the other concentrations. As can also be seen, a 10% resolving gel resulted in clear resolution between the bands, especially for EGFR, hence it was decided to use this in all future runs for optimal results.

Given that, so far, we appeared to have little success in properly inhibiting the Wnt pathway with these inhibitors, we tried reducing the percentage of serum used in case it was interfering with the drugs. Thus, instead of 10% serum as we had been using for the former experiments, we tried seeding cells with 2% serum and treated them with six inhibitor concentrations – for LGK974 concentrations of 0, 1, 2.5, 5, 7.5, and 10μM were used, and for ETC-159 we used 0, 0.05, 0.07, 0.1, 0.15, and 0.2μM. The results obtained (**Section 3.6 Figure 15**) showed a clear improvement compared to the results obtained with 10% serum. Thus it was decided that Wnt inhibitor concentrations of 10μM for LGK974, and 0.2μM for ETC-159 would be used going forward. In addition, cells would be cultured in 2% serum whenever treatment with the Wnt inhibitors was to be performed.

4.3 Effect of Wnt pathway inhibition on cell viability

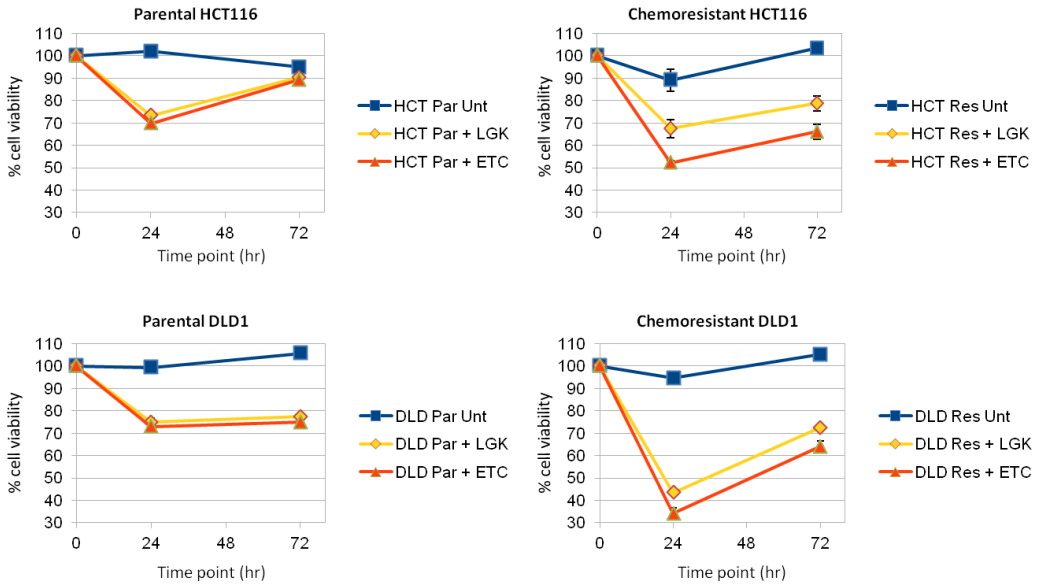

Figure 24: Comparison of the effectiveness of 10 µM LGK974 and 0.2 µM ETC-159 on parental and resistant HCT116 and DLD1 cells at 24 and 72 hours post-treatment. (A) Parental HCT116; (B) 5-FU Resistant HCT116; (C) Parental DLD1; (D) 5-FU Resistant DLD1. The greatest reduction in cell viability was seen for resistant DLD1 cells treated with either inhibitor. However, in general, both inhibitors were effective at CRC cell viability reduction at both time points, with some exceptions for parental and resistant HCT116 cells which showed no significant changes to viability with LGK974 after 24 hours.

Viability assays at 24 hours and 72 hours post-treatment were conducted for parental and resistant HCT116 and DLD1 cells which were treated with either 10 µM of LGK974 or 0.2 µM of ETC-159. In total, two runs of the Presto Blue viability assays were conducted for each cell condition to determine whether the reason the drugs were observed to be less effective 72 hours post-treatment was due to the drugs being broken down 24 hours after treatment. The individual results have been discussed in the Results section however, when comparing the results as a whole (**Figure 24**), some intriguing observations can be made.

It can be seen that ETC-159 was more effective at inhibiting cell growth than LGK974. This was most evident at the 24 hours time-point for both parental and resistant cell types. In general, it was also seen that the Wnt inhibitors were more effective in the resistant cell type than the parental cells, a fact which was, again, most evident at the 24 hour time-point. For

example, for parental HCT116 and DLD1 cells, ETC-159 reduced the viability of the cells at the 24 hour time-point to around 70%. In contrast, for resistant HCT116, the cell viability at this time-point was reduced to almost 50%, and in resistant DLD1, the viability was further reduced to around 30%. LGK974 showed similar results, although it was observed to be less effective than ETC-159. It seemed to be most effective in resistant DLD1 cells at the 24 hour time-point, where it reduced viability to around 40%. At 72 hours post-treatment, both inhibitors seemed to lose effectiveness although, in general, the resistant cells seemed to be more affected than the parental cells. However, a two way ANOVA was also conducted for these results and the results show that for parental HCT116 cells, there were no significant statistical differences ($P > 0.05$) between the effects of LGK974 and ETC-159 on cell viability. In addition, the results also showed that the effects of LGK974 on cell viability at both time points were equal whereas for ETC-159 there was a statistical difference ($P < 0.05$), with this inhibitor being more effective at 24 hours post-treatment. For resistant HCT116 and parental DLD1 cells, no significant statistical differences were found between the two inhibitors' effectiveness on cell viability and no statistical differences between the 24 and 48 hour time points. As expected, for resistant DLD1 cells, the two way ANOVA showed a significant difference between all conditions and between both time points, confirming that for these cells, ETC-159 was more effective for cell viability reduction.

These results are also interesting when compared to the results obtained from the previous Presto Blue viability assay (**Appendix II Section 7.2.3**), especially for the resistant cell types. In that assay, the results showed that the Wnt inhibitors increased cell viability when compared to the untreated control. However, the results obtained in the follow-up assay showed the opposite with both Wnt inhibitors successfully and effectively reducing cell viability. Apart from this, another difference in the results can be seen with regards to the fact that in the previous viability assay, LGK974 appeared to be more effective than ETC-159

in both parental and resistant cells and at almost all time-points. This observation was not seen in this viability assay where it was ETC-159 which was determined to be more effective at both the 24 and 72 hour time-points.

These results could suggest that CRC cells which are dependent on the Wnt pathway for their growth have additional mechanisms in place to resist disruptions to this pathway. These can include strategies such as activating other biochemical pathways or the upregulation of pathways which would not normally be highly active and which work in conjunction with Wnt. Such mechanisms might occur via feedback loops with the goal of maintaining cancer cell growth. Furthermore, the evidence from these results suggests that treatment with these Wnt inhibitors should ideally be done daily as a continuous dosing regimen, as opposed to using an intermittent dosing schedule given that the effectiveness of the drug is significantly decreased after 72 hours. This observation suggests that the drugs start being broken down by the cells within the first 24 hours and thus, by 72 hours post-treatment, the concentration of the drugs are significantly reduced to the point of eliciting little to no effect. In cases such as in resistant DLD1 where the cell viability 24 hours post-treatment was significantly reduced below 50%, it might also be possible to reduce the concentration of the drug used to reduce toxicity. While a continuous dosing schedule might be intriguing for these drugs, it might also present some long-term issues pertaining to drug accumulation and resistance. In this case, it would be worth investigating the use of lower concentrations of the drugs, or using a combination of inhibitors to potentially counteract this issue.

4.4 Effect of Wnt pathway inhibition on migration and invasion rates

It is known that the Wnt pathway is also involved in cell migration and invasion processes, thus the potential effects of Wnt inhibition on these functions for chemoresistant APC-

mutant CRC cells was investigated. In general, a reduction in migration was seen in all cases, for the untreated control as well for the inhibitor-treated conditions. In addition, the reduction in migration was observed to occur for both the 24 and 48 hour time points. In some cases, such as for parental DLD1 cells, the decrease in migration was significant from the control at 24 hours, while at 48 hours, the effectiveness of the inhibitors drops. The inhibitors also had different effects on migration depending on the cell type and condition. For example, for parental HCT116 cells, the inhibitors were more effective at reducing migration after 48 hours and were less effective at 24 hours post-treatment. On the other hand, for resistant HCT116 cells, LGK974 was effective at reducing migration for both time points, while ETC-159 was most effective at 24 hours post-treatment. These are interesting results when put into context of the previous viability assay data where ETC-159 resulted in significant decreases to cell viability 24 hours post-treatment thus one might expect the migration rate to also be decreased. However the results of the migration assay do not always reflect this which might suggest that Wnt inhibition affects other pathways related to migration, resulting in a change in the production of proteins regulating functions such as cell polarity, or structures such as microtubules. Furthermore, since this change in migration was seen both in an APC wild-type cell line (HCT116) and APC-truncated cell line (DLD1), for both the parental and chemoresistant conditions, APC status and 5-FU resistance might not be determining factors influencing the changes in migration observed. It remains to be seen which pathways and their associated proteins are being influenced in these cell lines in response to Wnt inhibition and whether mutations to other CRC-related genes might be affecting migration. It would be interesting to investigate other proteins known to be involved in migration processes such as the Rho-family of GTPases to potentially shed a better light on proteins which could be influenced in this regard. However, overall, the results of this experiment indicate that although treatment with Wnt inhibitors reduced the

migration rate of parental and resistant CRC cells to some extent at either 24 hours and 48 hours post-treatment, it is inconclusive whether APC mutation status or chemoresistance status play a role in such a process.

Apart from migration, the Wnt pathway is also involved in invasion mechanisms and so this was investigated as well. The results for the invasion assay seem to suggest that, overall, treatment with Wnt inhibitors had little to no effect on the invasion rate. The exception to this was chemoresistant HCT116 cells where Wnt inhibitor treatment was observed to increase the invasion rate compared to the untreated control which was confirmed to be statistically significant. However, unlike the migration assay, parental DLD1 cells did not show any significant differences in the invasion rate with either of the inhibitors. Interestingly, it was also observed that untreated parental HCT116 cells had a higher rate of invasion than untreated resistant HCT116 cells. It is possible that the same mechanisms which are activated or upregulated as a result of Wnt inhibition to stimulate migration also affect invasion, with neither APC mutation status nor chemoresistance having any major influence on these processes.

Comparing these results to other studies, Bagheri *et al*. (2020) investigated the effects of LGK974 on the invasion of two CRC cell lines, SW480 and SW742. The authors found that 48 hours after treating the cells with LGK974's IC50, the invasion rate was reduced when compared to the control group. This is in contrast to our study where either no change to the invasion rate was observed or, as was the case with chemoresistant HCT116, an increase in invasion rate was observed. In another study investigating the effects of LGK974 not on CRC but on renal cell carcinoma, Li *et al.* (2020) determined that an inhibitor concentration of 10 μM reduced both the migration and invasion of the cells at 24 hours post-treatment. While the results from these studies were different from the ones observed in our investigation, this could be explained by the fact that in those studies, different cell lines from the ones

investigated here were used. Such studies could further reinforce the idea of administering these Wnt inhibitors on a daily basis when their effects are strongest.

4.5 Effect of Wnt pathway inhibition on the expression of APC-related proteins

In order to investigate any potential effects the Wnt inhibitors may elicit on the expression of proteins known to be involved not only in the Wnt pathways, but with APC as well, drug-treated cells were studied for the expression of three main proteins, these being β-catenin, EGFR, and CDC42. As was seen from the results, surprisingly, the Wnt inhibitors used were not observed to effectively inhibit the Wnt pathway given that the expression of β-catenin of the inhibitor-treated cells did not significantly differ from the untreated controls. This observation was consistent for both the APC wild-type and APC truncated cell lines. As the expression and stability of β-catenin is mainly regulated through the Wnt signaling protein, these results suggest that the PORCN inhibitors used were not effective in inhibiting the post-translational acylation of Wnt, thereby allowing it to bind to the Fz receptor and initiating the Wnt pathway. Alternatively, it may also be the case that the PORCN inhibitors were successfully inhibiting the post-translation acylation of Wnt but the presence of other pathways which cross-signal with the Wnt pathway were still active, or perhaps even upregulated, when the concentration of active Wnt was detected to be low. Our findings, however, are in contrast to previous studies where it was determined that treatment with LGK974 and ETC-159 successfully decreased the expression of Wnt target genes, including β-catenin, in CRC cell lines and renal cancer cell lines (Bagheri *et al.*, 2020; Madan *et al.*, 2016; Li *et al.*, 2020). Despite this, it is possible that the differences can be attributed to the fact that different cell lines were utilised and the possibility of the presence of other mutations.

Apart from from investigating β-catenin which is involved in canonical Wnt signaling, EGFR and CDC42 were also studied as these are potential APC-related proteins involved in the non-canonical Wnt signaling pathways. However, similar to the results seen for β-catenin, no significant changes to the expression of these two proteins were observed with inhibitor treatment. Given that these proteins are known to be involved in other signaling pathways, particularly EGFR which is also involved in the PI3K/AKT/mTOR pathway, it is possible that if they were being down-regulated through the non-canonical pathways, signaling pathways elsewhere might compensate for this and up-regulate them, giving the impression that no overall change was occurring. For this reason, it would be interesting to further investigate this by running Western blots for the expression of proteins involved in related pathways, such as the aforementioned PI3K/AKT/mTOR signaling pathway, to determine whether such a phenomenon was occurring. Unfortunately, although the resistant variants of HCT116 and DLD1 cells were run, only the bands for the untreated resistant HCT116 cells appeared. However, since the loading control bands also did not appear, it is likely that there was a problem with the loading or there was not enough protein for sufficient detection. Thus, the results for the resistant cells could not be used although they can be viewed in Appendix V in **Figure 54**.

4.6 Effect of Wnt inhibition on lysine mono- and tri-methylation

It is known that lysine methylation is a post-translational modification which is often found in cancer, being responsible for gene activation or gene silencing, with recent research viewing the targeting of epigenetic methylation as a potential therapeutic possibility for CRC (Chen *et al.*, 2017; Coppedè, 2014; Huang *et al.*, 2017). With this in mind, we sought to investigate whether the inhibition of the Wnt pathway through PORCN inhibition has any

effect on the mono- and tri-methylation of lysine. In the case of lysine mono-methylation, the results suggest that Wnt inhibition may be having an effect on parental DLD1 cells as the band intensity for the untreated condition was significantly more intense than the corresponding bands for the treated conditions. With regards to HCT116 cells, it was observed that the chemoresistant variants treated with LGK974 produced a slightly more intense band than the untreated or ETC159-treated cells. These results suggest that APC truncation status as well as chemoresistance status may be playing a role in the effectiveness of the Wnt inhibitors on lysine mono-methylation - in the case of chemoresistant APC wild-type status, Wnt inhibition via LGK974 was shown to increase lysine mono-methylation, whereas in the case of parental APC truncated status, Wnt inhibition via the PORCN inhibitors was shown to effectively reduce lysine mono-methylation. It still remains to be seen what underlying mechanisms are present to explain these differences in the results. It would be interesting to follow up on these results by determining which lysines are being mono-methylated and investigate mechanisms which they are involved in to elucidate potential pathways through which such changes could be taking place, especially if it is determined that APC mutation and chemoresistant status affect post-translational methylation.

In the case of lysine tri-methylation, in general, it was observed that Wnt inhibition did not have any significant effects on band intensity between the parental and chemoresistant cells. However, it is interesting to note that for the chemoresistant DLD1 cells, a set of bands appears near the top of the gel which does not appear for either the HCT116 cells or the parental DLD1 cells. What is also interesting is that treatment with ETC-159 significantly reduced the intensity of this top band for chemoresistant DLD1 compared to the untreated and LGK974-treated cells. The fact that these bands appear for the resistant DLD1 and not for their parental counterparts suggests that development of chemoresistance is also

accompanied by more extensive lysine tri-methylation, and that inhibiting the Wnt pathway through ETC-159 has the potential of countering some of these effects. Furthermore, it can also be noted that these top bands appear for chemoresistant cells which possess an APC truncation mutation. In chemoresistant HCT116 cells, which do not possess this mutation status, these top bands do not appear which could suggest that mutations to APC might also affect lysine tri-methylation. Taking these results as a whole, there is basis to conclude that APC truncation status as well as chemoresistance do to some extent affect lysine methylation. Similar to what was suggested previously, it would be interesting to investigate these findings further by determining the identity of these tri-methylated lysines and whether their methylation status has any significance on the progression of CRC. In this way, knowing how APC status and chemoresistance could affect Wnt inhibition, treatments could be suggested which would maximise the benefits and lead to a favourable prognosis.

4.7 Effect of Wnt pathway inhibition on total and phosphorylated EGFR

An ELISA was conducted for total and tyrosine 1045 (Y1045) phosphorylated EGFR in order to further investigate the effects that Wnt pathway inhibition has on an APC-related protein involved in non-canonical Wnt signaling. It is known that EGFR is activated via phosphorylation and has several important functions related to the regulation of epithelial tissue development, cell proliferation, differentiation, and autophagy (Baumdick *et al.* 2015; Sigismund, Avanzato, and Lanzetti, 2018). In addition, studies have shown that EGFR is frequently overexpressed in several cancers (Capuani *et al.*, 2015; Maiti *et al.*, 2013).

From the results obtained, some interesting observations can be made. It was seen how both DLD1 and SW837 cells were not significantly affected by either of the Wnt inhibitors,

resulting in no changes to the expression of total EGFR compared to the control. In contrast, for HCT116 cells it was observed how treatment with the Wnt inhibitors resulted in significant changes to the expression of total and phosphorylated EGFR. In the former case, the majority of results showed a decrease in expression (**Figure 19**) while in the latter, the parental cells showed an increase in expression (**Figure 22**). The fact that total EGFR was reduced with Wnt inhibitor treatment is interesting given that the expression of EGFR is usually increased in cancers. Furthermore, since this decrease in total EGFR was observed to only occur for a cell line having wild type APC, and not for cell lines with truncated APC, it can be suggested that LGK974 and ETC-159 might be able to counteract increased total EGFR expression in CRC cells possessing wild type APC through Wnt signaling inhibition. The increase in Y1045 phosphorylated EGFR could be giving a further indication as to why the expression of total EGFR was decreased. This is because studies have shown that phosphorylation of this tyrosine residue can stimulate the ubiquitination of EGFR (Sigismund *et al.*, 2013; Baumdick *et al.*, 2015), leading to EGFR degradation. Hence, these results show that the Wnt inhibitors used are able to affect the phosphorylation of this specific tyrosine residue in CRC cells with wild type APC to some extent, potentially resulting in reduced total EGFR levels.

In contrast to HCT116, phosphorylated Y1045 was not detected in either the DLD1 or SW847. This suggests that EGFR was not being ubiquitinated to the same extent as it was in HCT116 cells, therefore maintaining its elevated expression, although as it was discussed previously, the results for total EGFR expression did not show any significant changes from the untreated control for these cells. In fact, a previous study had shown that hypophosphorylation of Y1045 reduced EGFR ubiquitination and resulted in increased proliferation of breast cancer cells (Han *et al.*, 2006). Such results could further suggest that

APC truncation mutations affect Y1045 phosphorylation, although further investigations would be required to determine the exact mechanism/s through which this occurs.

From the results of HCT116, it was also observed how parental cells were affected by both Wnt inhibitors whereas the chemoresistant cells were, for the majority of cases, unaffected by the Wnt inhibitors. The exception to this include the decrease observed in total EGFR expression when treated with ETC-159 (**Figure 19**), consequently resulting in an increase in proportion of phosphorylated to total EGFR with ETC-159 (**Figure 23**). These results could suggest that chemoresistance makes the cells more resistant to changes to EGFR expression, however further studies would have to be done to determine whether the changes to the expression of total and phosphorylated EGFR seen have any significant effects on the progression of CRC.

Given the importance that EGFR has in several developmental processes, with its expression being elevated in several tumours, results obtained in such investigations would be important to determine what specific characteristics CRC cells may possess which could affect treatment effectiveness. Thus, taking these results together, it could be suggested that both APC truncation mutations status and chemoresistance might affect the effectiveness of LGK974 and ETC-159 on the expression of total and phosphorylated EGFR.

4.8 Efficacy of PORCN inhibition when downstream APC truncation is present

To a certain extent, the fact that PORCN inhibition did not effectively inhibit the Wnt pathway is not entirely unexpected. Previous studies investigating the interactions within the Wnt pathway posit that inhibitors which act upstream of Wnt, such as PORCN inhibitors, may be ineffective in cancer cells possessing mutations in downstream components such as APC

due to the fact that these often activate the pathway independent of the Wnt ligand (Zhong and Virshup, 2020; Flanagan *et al.*, 2022). As a result, whether or not Wnt is made unavailable through inhibition of post-translational acylation via targeting of PORCN, pathways with downstream Wnt ligand-independent mutations would not be expected to be downregulated. However, some studies investigating CRC possessing such downstream activating mutations have found that, in some cases, inhibiting the Wnt ligand through PORCN inhibition was still an effective method of reducing cell proliferation, suggesting that there are cases where dependence on the Wnt ligand can still be observed (Voloshanenko *et al.*, 2013; Li *et al.*, 2019). On the other hand, Huels *et al.* (2018) observed that using LGK974 to reduce the secretion of the Wnt ligand in intestinal stem cells did not affect the cell growth of cells with APC mutation, suggesting that they are Wnt-independent. Instead, the authors found that tumorigenesis was accelerated. Given all this, using PORCN inhibition on CRC patients possessing downstream mutations may prove beneficial if it is determined that these downstream activating mechanisms are dependent on Wnt. Otherwise, inhibiting Porcupine may either have no effect or may even increase the rate of tumorigenesis. With this knowledge, the results obtained from this study seem to suggest that our cell lines with APC mutations might have had some dependency on Wnt given the results seen in the viability assay. However, given the results of the migration assay, where an increase in migration was observed for both parental and APC-mutated cell lines, and the results of the Western blot where no overall change in expression of APC-related proteins was observed, all of which could indicate a non-dependence on Wnt, it is clear that additional underlying mechanisms are present which are affecting the treatment outcome. Thus, further research is essential to determine exactly which mutation sub-types of APC and other downstream proteins are still dependent on Wnt, as well as the possible mechanisms at play, to be able to choose the most ideal treatment for the best outcome (Flanagan *et al.*, 2022).

4.9 Limitations and Improvements

In this study, the two main cell lines used were HCT116 and DLD1 in order to represent a wild-type variant and a mutated/truncated variant respectively. Initially, there were plans to include at least another APC-truncated CRC cell line from those which had been sequenced at the beginning of the study, such as SW837. This would have been beneficial for the study to investigate whether the results observed with one mutated cell line were reproducible in another mutated cell line. In addition, the results obtained would have been further interesting considering that DLD1 is classified as a Dukes' type C (Stage 3) adenocarcinoma whereas SW837 is a Duke's type D (Stage 4) adenocarcinoma. This would have enabled us to investigate whether the Wnt inhibitors used acted differently depending on the severity of the cancer. However, attempts to culture this cell line proved challenging owing to the slow growth rate, the cells' tendency to cluster, in addition to fungal infections. As a result, this cell line could not be included in any of the experiments conducted in this study. Future studies could attempt to include SW837 cells in at least some of the experiments to generate more interesting results in relation to the effects of Wnt inhibition on different stages of CRC.

Although an ELISA was done for total and phosphorylated EGFR, only the ratio of the two could be investigated. Due to the fact that the concentration of the standard in the kit was not provided, quantification could not be done. In addition, due to time limitations, we did not have time to create chemoresistant variants of SW837 and thus this could not be investigated in the ELISA. In the future it would be ideal to include such cells to further complement the results already obtained and to use a standard with which quantification can be done.

Despite using the most commonly cited concentrations from the literature for the Wnt inhibitors, in our investigation it appeared that these did not achieve the same effects as mentioned in such studies. Consequently, the IC50 of these Wnt inhibitors for the cell types used was not found. This meant that positive or negative controls for the inhibitors could not be used, which could potentially reduce the reliability of the obtained results. Hence, finding the IC50 and using appropriate controls are measures that would certainly improve the confidence in the results.

This study's main focus was on the effects of Wnt inhibitors on APC-mutant CRC subtypes thus interactions with APC and its truncated variant were the highlight of the investigation. Sequencing was, therefore, conducted only for APC to check for its mutation status. However, as it was briefly discussed in the literature review, it is known that APC truncations are far from the only mutations present in CRC, where a plethora of mutations in other genes contribute to carcinogenesis. As a result, the effects of these other mutations on APC, drug interactions, and the Wnt pathway in general cannot be discounted. Thus, a limitation of this study is that sequencing was only done for APC, without taking into account other genes in which mutations might be present and which could be affecting the Wnt pathway in other ways. In the future, a study such as this one could be improved by performing comprehensive sequencing for other CRC driver genes in the chosen cell lines in order to obtain a better picture of the possible effects of such mutations on the Wnt pathway in chemoresistant cells when inhibitors are used.

Another limitation was that the passage number of the cells throughout the chemoresistance process was not recorded. Apart from the fact that parental cells need multiple treatment cycles and thus passages in order to become chemoresistant, in a number of instances chemoresistant cell clusters were spread within the same flask, which could be considered a passage. This means that parental and chemoresistant cells with

different passage numbers were used for the same experiment which could reduce the reproducibility of the results. Although this was not expected to be a major issue as the mutation of interest to the APC would still be present and would likely be unaffected, in the future, it would be a good idea to keep track of this to ensure reproducibility and reliability.

5 Conclusions

5.1 Conclusions and outcomes

In this research, the effects of two Wnt inhibitors, LGK974 and ETC-159, was investigated on parental and 5-FU resistant CRC cell lines possessing either wild-type APC, or an APC-truncated variant. The results reveal that inhibition of the Wnt pathway via the targeting of PORCN may be an effective method by which the cell viability of both parental and chemoresistant CRC cells, irrespective of APC mutation status, can be reduced. Furthermore, it was shown how a daily continuous dosing regimen with these Wnt inhibitors may provide the best results for CRC cell viability reduction. This study also sheds further light on the complexity of the canonical and non-canonical Wnt pathways, especially in terms of cross-talk with supporting pathways. This was evident given the results obtained in the scratch migration and transwell invasion assays where inconsistent decreases and increases in migration and invasion rates were seen, suggesting that chemoresistance and APC mutation status are not determining factors influencing such pathways. This complexity was further seen in the results of Western Blotting of APC-related proteins where no significant difference in their expression was observed in parental cells treated with either of the Wnt inhibitors. However, results from Western blotting of lysine mono- and tri-methylation reveal the interesting possibility that lysine methylation is affected to some extent by APC truncation, as well as chemoresistance status, raising the intriguing possibility that this may have potential for use as a biomarker. Finally, evidence from other studies reveals that

upstream Wnt pathway inhibitors may be ineffective, or may even cause adverse effects, if downstream pathway mutations which are independent of the Wnt ligand are present, an effect which was observed to some extent in this study. This further highlights the need for additional research to determine which downstream Wnt pathway mutations are conducive to better treatment outcomes.

5.2 Future work

As mentioned previously, future work can include an ELISA with a standard which can be used to quantify the amount of total and phosphorylated EGFR expressed in the cells with different treatments. This would allow for better analysis on the changes occurring to this protein.

The inclusion of other CRC cell lines, especially cell lines representing different stages of cancer, would be an interesting addition to such a study as it would elucidate whether the effects of Wnt inhibition are constant across all stages, or if a particular stage is more susceptible to drug treatment. This could then be used to inform treatment strategies for optimal outcomes.

Given that CRC carcinogenesis does not arise via one mutation but is the result of sequential mutations to key regulatory genes, future work can also include other CRC driver mutations. It would be interesting to investigate mutations to *KRAS* and *BRAF* which are similar to *APC* in that mutations of these genes aberrantly activate the Wnt pathway and initiate CRC by transforming normal cells to adenoma cells. However, other important mutations such as mutations of *TP53* and *PIK3CA* are also worth investigating in conjunction with *APC*. Such investigation could study potential inhibitors of pathways such as the

RAS/MEK and MAPK regulatory pathways which are known to be regulated via these genes, and how chemoresistance affects these mechanisms.

In current clinical trials involving such Wnt inhibitors, it is often the case that combinations of drugs are used. These commonly include the use of monoclonal antibodies such as cetuximab and pembrolizumab. Future studies can also include drug cocktail treatments targeting different parts of the Wnt pathway. This has the possibility of reducing overall toxicity as lower concentrations of each drug can be administered.

Mass spectrometry is a technique which can compliment such a study as more in-depth quantitative investigations of dysregulated proteins and post-translational modifications can be carried out. Given the results from this study where lysine methylation was revealed as a potential diagnostic or prognostic biomarker of interest, a comprehensive investigation utilising mass spectrometry is warranted.

Finally, this investigation was carried out using 2D cell culture which is not truly representative of an *in vivo* environment. Thus, there is the possibility of conducting a study using 3D cell cultures. Furthermore, it is also possible to extend this investigation to using patient biopsies which would further represent real world conditions.

6 References

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7 Appendix

7.1 Appendix I

Figure 25: APC sequencing of segment 1 showing region where mutations were found

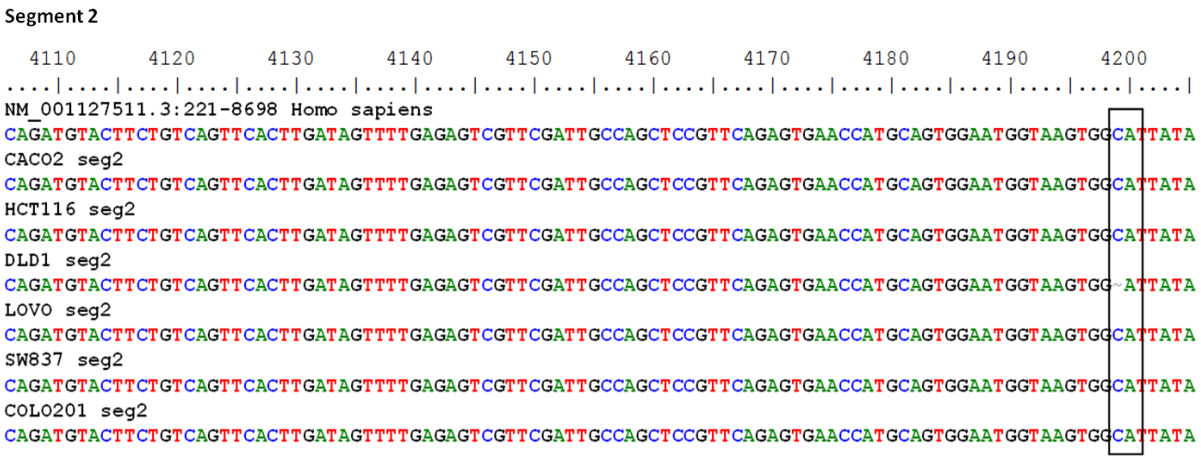

Figure 26: APC sequencing of segment 2 showing first region where mutations were found

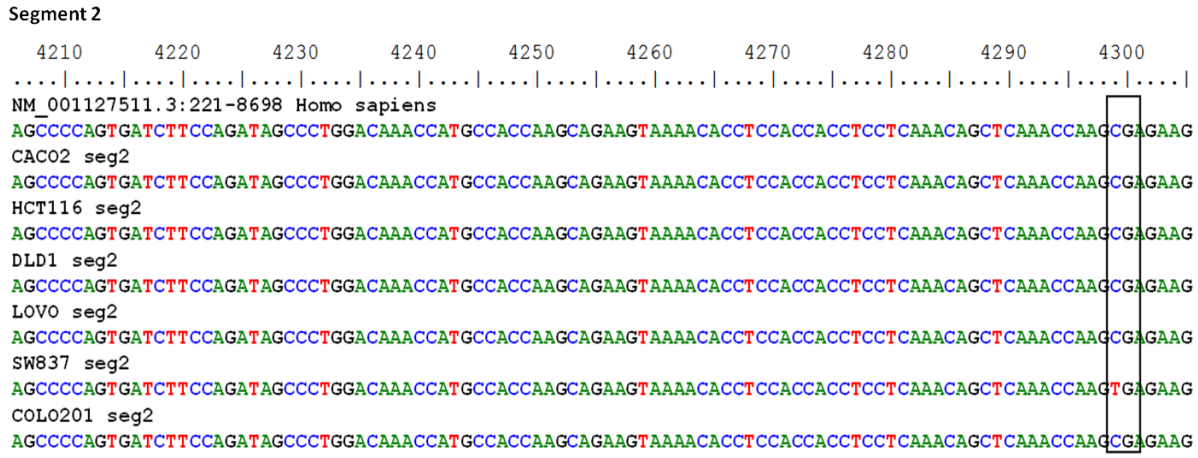

Figure 27: APC sequencing of segment 2 showing second region where mutations were found

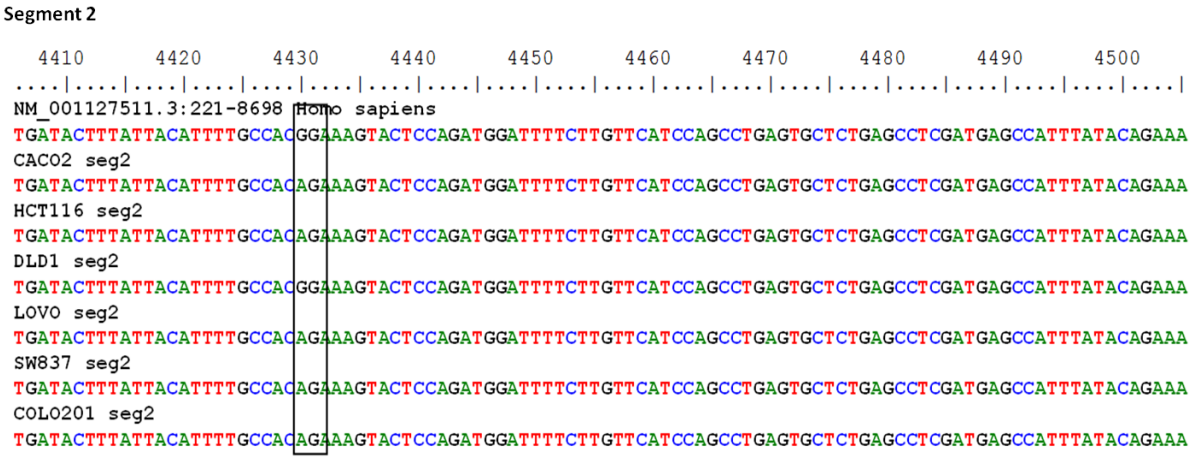

Figure 28: APC sequencing of segment 2 showing third region where mutations were found

7.2 Appendix II

7.2.1 Statistical analysis confirming resistance to 5-FU of HCT116 cells at 72 hours post-treatment

Table 11: Normality test for parental and resistant HCT116 cells at 72 hours

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
cell viability	HCT Parental	.183	3	.	.999	3	.934
	HCT 5FU res	.185	3	.	.998	3	.924

a. Lilliefors Significance Correction

Table 12: Independent samples t-test for parental and resistant HCT116 cells at 72 hours

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
cell viability	Equal variances assumed	.377	.572	-7.047	4	.002	-9.84667	1.39737	-13.72640	-5.96693
	Equal variances not assumed			-7.047	3.459	.004	-9.84667	1.39737	-13.97766	-5.71568

7.2.2 Statistical analysis confirming resistant to 5-FU of DLD1 cells at 72 hours post-treatment

Table 13: Normality test for parental and resistant DLD1 cells at 72 hours

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
cell viability	DLD Parental	.230	3	.	.981	3	.737
	DLD 5FU res	.292	3	.	.923	3	.463

a. Lilliefors Significance Correction

Table 14: Independent samples t-test for parental and resistant DLD1 cells at 72 hours

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
cell viability	Equal variances assumed	.318	.603	-12.228	4	.000	-6.94000	.56753	-8.51571	-5.36429
	Equal variances not assumed			-12.228	3.803	.000	-6.94000	.56753	-8.54852	-5.33148

7.2.3 Presto Blue viability assay 1 results for parental and resistant HCT116 and DLD1

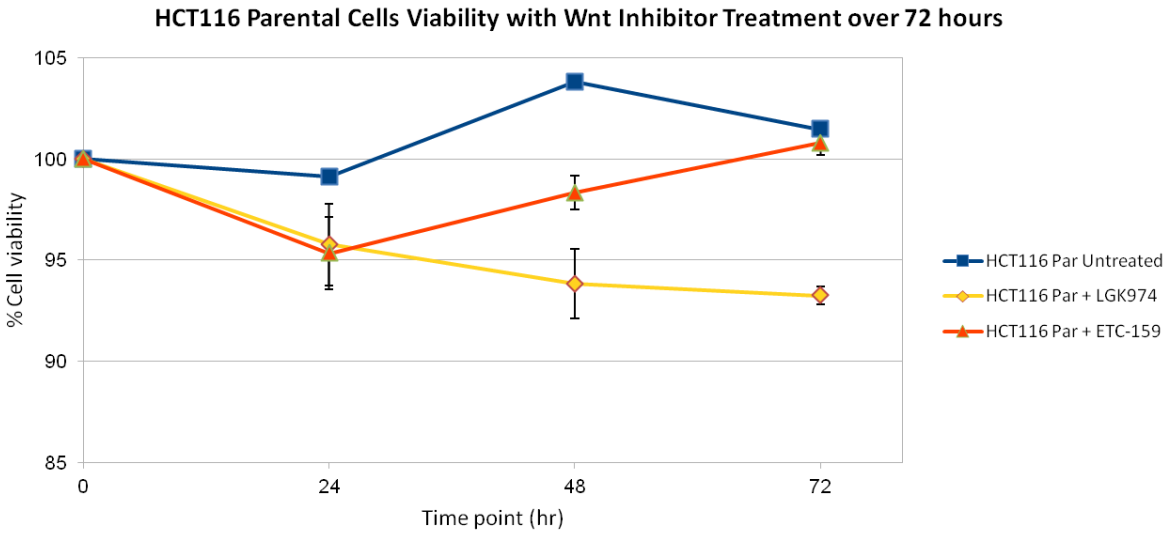

Figure 29: Cell viability of parental HCT116 when treated with 10µM LGK974 or 0.2µM ETC-159 over 72 hours

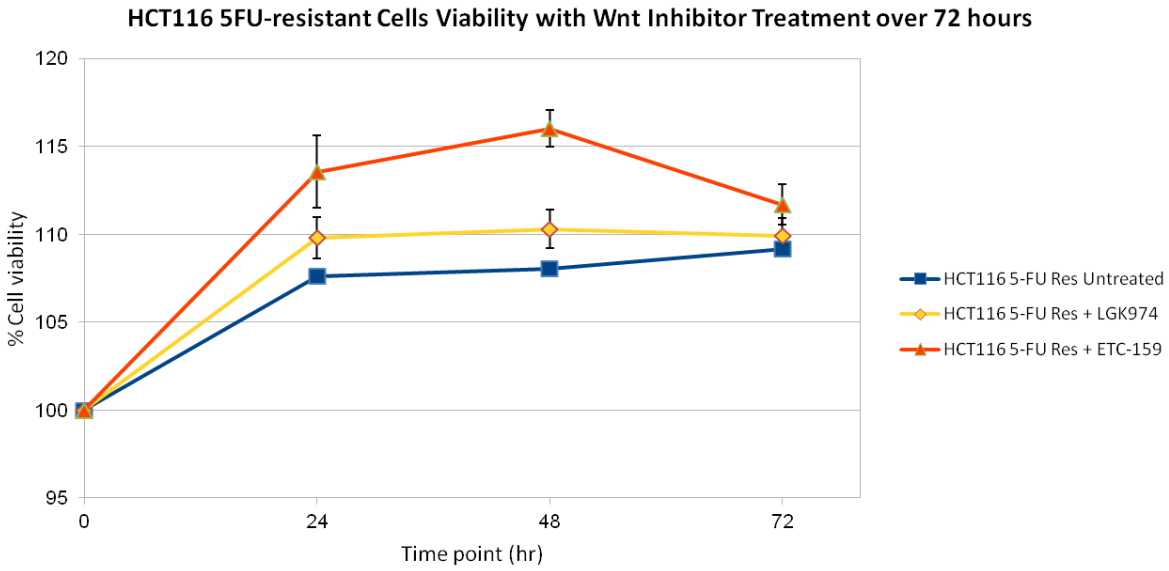

Figure 30: Cell viability of 5-FU resistant HCT116 when treated with 10µM LGK974 or 0.2µM ETC-159 over 72 hours

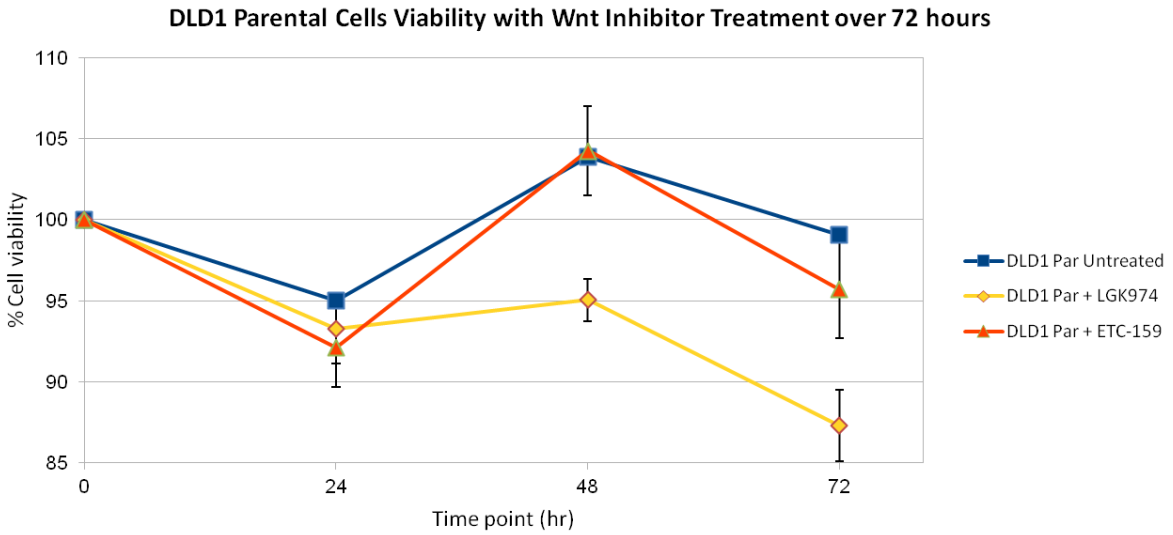

Figure 31: Cell viability of parental DLD1 cells when treated with 10µM LGK974 or 0.2µM ETC-159 over 72 hours

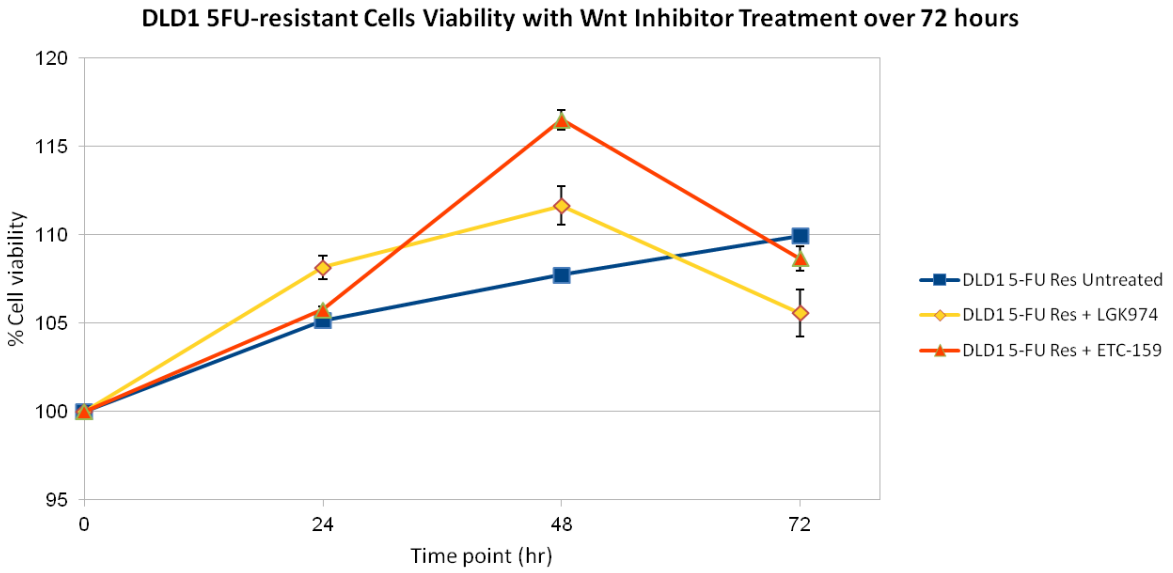

Figure 32: Cell viability of 5-FU resistant DLD1 cells when treated with 10µM LGK974 or 0.2µM ETC-159 over 72 hours

7.2.4 Statistical analysis of HCT116 cells' viability at 72 hours post-treatment with the Wnt inhibitors (Viability assay 1)

7.2.4.1 Untreated parental HCT116 cells vs LGK974-treated parental HCT116 cells

Table 15: Normality test for untreated and LGK974-treated parental HCT116 cells at 72 hours

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Parental Untreated	.250	3	.	.967	3	.649
	HCT Parental LGK	.304	3	.	.907	3	.408

a. Lilliefors Significance Correction

Table 16: Independent samples t-test for untreated and LGK974-treated parental HCT116 cells at 72 hours

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	5.180	.085	2.452	4	.070	6.739333	2.748893	-.892817	14.371484
	Equal variances not assumed			2.452	2.099	.128	6.739333	2.748893	-4.568497	18.047164

7.2.4.2 Untreated parental HCT116 cells vs ETC159-treated parental HCT116 cells

Table 17: Normality test for untreated and ETC159-treated parental HCT116 cells at 72 hours

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Parental Untreated	.358	3	.	.813	3	.147
	HCT Parental ETC	.328	3	.	.871	3	.298

a. Lilliefors Significance Correction

Table 18: Independent samples t-test for untreated and ETC159-treated parental HCT116 cells at 72 hours

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	8.108	.047	.280	4	.794	.679000	2.428856	-6.064586	7.422586
	Equal variances not assumed			.280	2.243	.804	.679000	2.428856	-8.759311	10.117311

7.2.4.3 Untreated resistant HCT116 cells vs LGK974-treated resistant HCT116 cells

Table 19: Normality test for untreated and LGK974-treated resistant HCT116 cells at 72 hours

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Res Untreated	.340	3	.	.848	3	.235
	HCT Res LGK	.340	3	.	.848	3	.236

a. Lilliefors Significance Correction

Table 20: Independent samples t-test for untreated and LGK974-treated resistant HCT116 cells at 72 hours

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.576	.490	-.243	4	.820	-.538333	2.210952	-6.676920	5.600254
	Equal variances not assumed			-.243	3.703	.821	-.538333	2.210952	-6.876300	5.799633

7.2.4.4 Untreated resistant HCT116 cells vs ETC159-treated resistant HCT116 cells

Table 21: Normality test for untreated and ETC159-treated resistant HCT116 cells at 72 hours

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Res Untreated	.340	3	.	.848	3	.235
	HCT Res ETC	.302	3	.	.910	3	.417

a. Lilliefors Significance Correction

Table 22: Independent samples t-test for untreated and ETC159-treated resistant HCT116 cells at 72 hours

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.802	.421	-1.157	4	.312	-2.511000	2.170056	-8.536041	3.514041
	Equal variances not assumed			-1.157	3.602	.318	-2.511000	2.170056	-8.807553	3.785553

7.2.5 Statistical analysis of DLD1 cells' viability at 72 hours post-treatment with the Wnt inhibitors (Viability assay 1)

7.2.5.1 Untreated parental DLD1 cells vs LGK974-treated parental DLD1 cells

Table 23: Normality test for untreated and LGK974-treated parental DLD1 cells at 72 hours

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Par Untreated	.330	3	.	.866	3	.285
	DLD Par LGK	.356	3	.	.816	3	.153

a. Lilliefors Significance Correction

Table 24: Independent samples t-test for untreated and LGK974-treated parental DLD1 cells at 72 hours

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	2.466	.191	4.751	4	.009	11.786000	2.480851	4.898054	18.673946
	Equal variances not assumed			4.751	3.073	.017	11.786000	2.480851	3.995431	19.576569

7.2.5.2 Untreated parental DLD1 cells vs ETC159-treated parental DLD1 cells

Table 25: Normality test for untreated and ETC159-treated parental DLD1 cells at 72

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Par Untreated	.330	3	.	.866	3	.285
	DLD Par ETC	.325	3	.	.874	3	.308

a. Lilliefors Significance Correction

Table 26: Independent samples t-test for untreated and ETC159-treated parental DLD1 cells at 72 hours

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	4.130	.112	1.052	4	.352	3.387667	3.221112	-5.555574	12.330907
	Equal variances not assumed			1.052	2.603	.381	3.387667	3.221112	-7.807870	14.583203

7.2.5.3 Untreated resistant DLD1 cells vs LGK974-treated resistant DLD1 cells

Table 27: Normality test for untreated and LGK974-treated resistant DLD1 cells at 72 hours

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Res Untreated	.358	3	.	.812	3	.143
	DLD Res LGK	.196	3	.	.996	3	.878

a. Lilliefors Significance Correction

Table 28: Independent samples t-test for untreated and LGK974-treated resistant DLD1 cells at 72 hours

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.930	.390	2.221	4	.091	3.560000	1.603055	-.890795	8.010795
	Equal variances not assumed			2.221	2.916	.116	3.560000	1.603055	-1.626273	8.746273

7.2.5.4 Untreated resistant DLD1 cells vs ETC159-treated DLD1 cells

Table 29: Normality test for untreated and ETC159-treated resistant DLD1 cells at 72 hours

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Res Untreated	.358	3	.	.812	3	.143
	DLD Res ETC	.241	3	.	.973	3	.688

a. Lilliefors Significance Correction

Table 30: Independent samples t-test for untreated and ETC159-treated resistant DLD1 cells at 72 hours

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.000	1.000	.474	4	.660	.490667	1.034705	-2.382136	3.363470
	Equal variances not assumed			.474	3.984	.660	.490667	1.034705	-2.386726	3.368060

7.2.6 Presto Blue Linearisation for parental and resistant HCT116 and DLD1

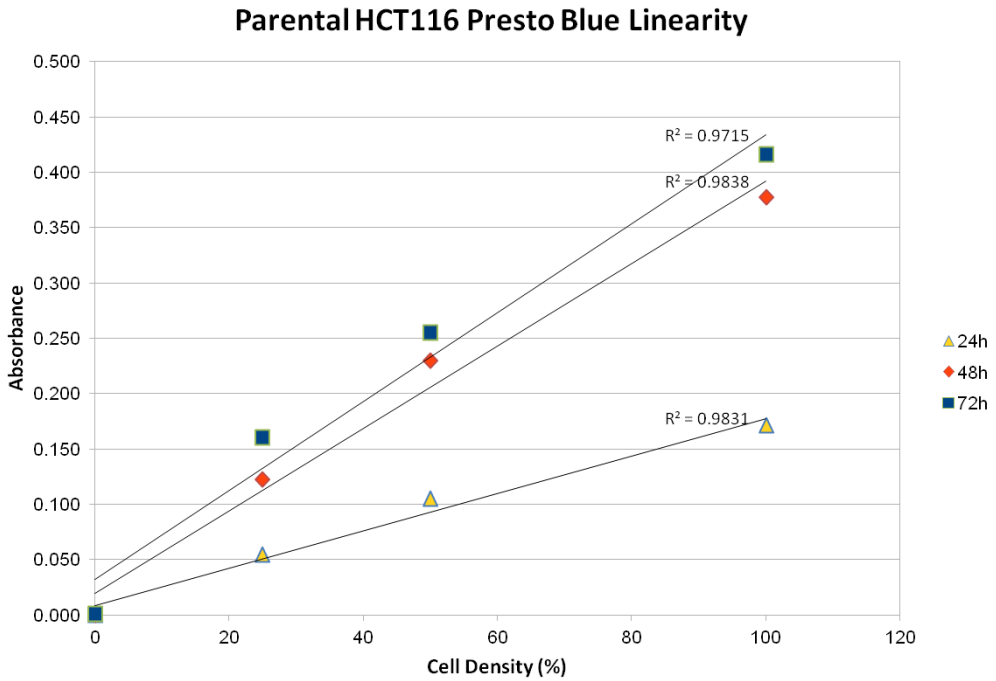

Figure 33: Parental HCT116 Presto Blue linearity over 72 hours

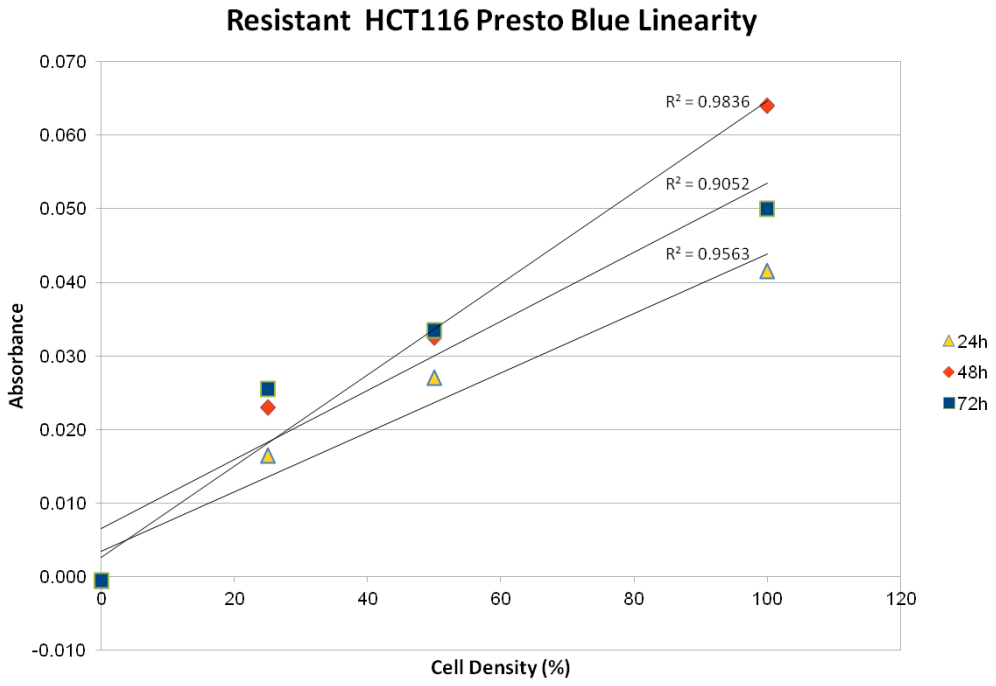

Figure 34: Resistant HCT116 Presto Blue linearity over 72 hours

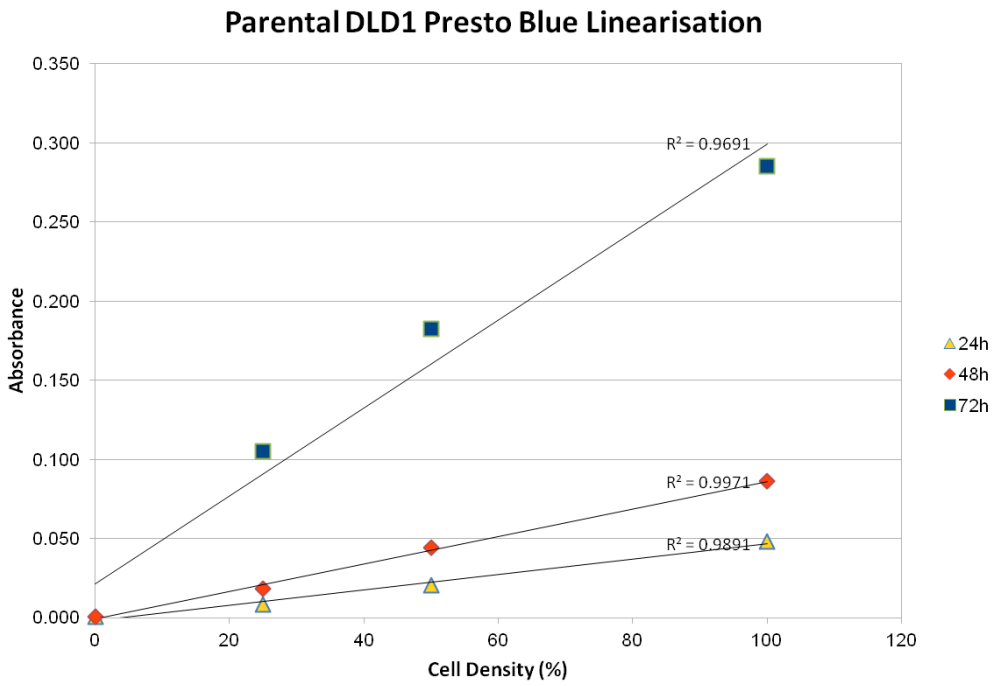

Figure 35: Parental DLD1 Presto Blue linearity over 72 hours

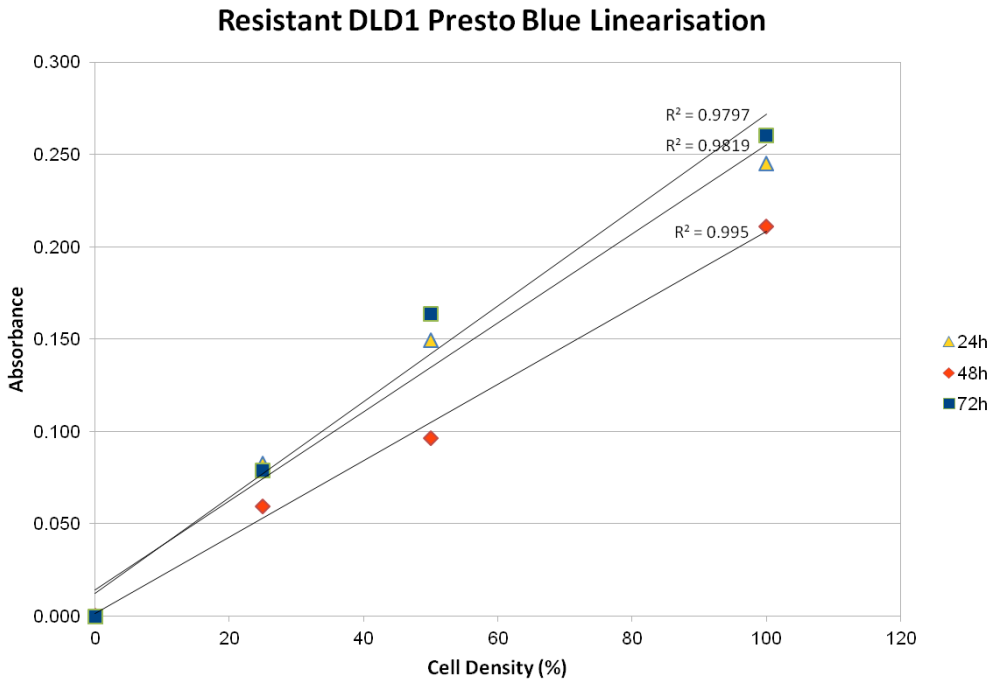

Figure 36: Resistant DLD1 Presto Blue linearity over 72 hours

7.2.7 Statistical analysis for parental and resistant HCT116 at 24 hours post-treatment (Viability assay 2)

7.2.7.1 Untreated parental HCT116 cells vs LGK974-treated HCT116 cells

Table 31: Normality test for untreated and LGK-treated parental HCT116

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Par Untreated	.367	3	.	.793	3	.097
	HCT Par LGK	.253	3	.	.964	3	.637

a. Lilliefors Significance Correction

Table 32: Independent samples t-test for untreated and LGK-treated parental HCT116

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	11.218	.029	4.239	4	.013	33.988333	8.017194	11.729034	56.247633
	Equal variances not assumed			4.239	2.067	.048	33.988333	8.017194	.547438	67.429229

7.2.7.2 Untreated parental HCT116 cells vs ETC159-treated HCT116 cells

Table 33: Normality test for untreated and ETC-treated parental HCT116

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Par Untreated	.367	3	.	.793	3	.097
	HCT Par ETC	.341	3	.	.846	3	.230

a. Lilliefors Significance Correction

Table 34: Independent samples t-test for untreated and ETC-treated parental HCT116

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	5.300	.083	2.838	4	.047	34.163667	12.039703	.736093	67.591240
	Equal variances not assumed			2.838	2.550	.080	34.163667	12.039703	-8.278000	76.605334

7.2.7.3 Untreated resistant HCT116 cells vs LGK974-treated HCT116 cells

Table 35: Normality test for untreated and LGK-treated resistant HCT116

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Res Untreated	.286	3	.	.930	3	.490
	HCT Res LGK	.276	3	.	.942	3	.537

a. Lilliefors Significance Correction

Table 36: Independent samples t-test for untreated and LGK-treated resistant HCT116

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.468	.532	1.277	4	.271	21.687000	16.981796	-25.462026	68.836026
	Equal variances not assumed			1.277	3.666	.276	21.687000	16.981796	-27.207486	70.581486

7.2.7.4 Untreated resistant HCT116 cells vs ETC159-treated HCT116 cells

Table 37: Normality test for untreated and ETC-treated resistant HCT116

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Res Untreated	.286	3	.	.930	3	.490
	HCT Res ETC	.349	3	.	.832	3	.194

a. Lilliefors Significance Correction

Table 38: Independent samples t-test for untreated and ETC-treated resistant HCT116

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.831	.413	3.027	4	.039	39.615000	13.086754	3.280345	75.949655
	Equal variances not assumed			3.027	3.326	.049	39.615000	13.086754	.186095	79.043905

7.2.8 Statistical analysis for parental and resistant DLD1 at 24 hours post-treatment (Viability assay 2)

7.2.8.1 Untreated parental DLD1 cells vs LGK974-treated DLD1 cells

Table 39: Normality test for untreated and LGK-treated parental DLD1

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Par Untreated	.321	3	.	.881	3	.328
	DLD Par LGK	.276	3	.	.942	3	.537

a. Lilliefors Significance Correction

Table 40: Independent samples t-test for untreated and LGK-treated parental DLD1

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	1.347	.310	2.937	4	.043	24.305667	8.275069	1.330391	47.280943
	Equal variances not assumed			2.937	3.325	.053	24.305667	8.275069	-.632690	49.244023

7.2.8.2 Untreated parental DLD1 cells vs ETC159-treated DLD1 cells

Table 41: Normality test for untreated and ETC-treated parental DLD1

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Par Untreated	.321	3	.	.881	3	.328
	DLD Par ETC	.175	3	.	1.000	3	1.000

a. Lilliefors Significance Correction

Table 42: Independent samples t-test for untreated and ETC-treated parental DLD1

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	7.727	.050	4.160	4	.014	27.055667	6.504028	8.997589	45.113745
	Equal variances not assumed			4.160	2.141	.047	27.055667	6.504028	.770379	53.340955

7.2.8.3 Untreated resistant DLD1 cells vs LGK974-treated DLD1 cells

Table 43: Normality test for untreated and LGK-treated resistant DLD1

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Res Untreated	.227	3	.	.983	3	.747
	DLD Res LGK	.366	3	.	.796	3	.104

a. Lilliefors Significance Correction

Table 44: Independent samples t-test for untreated and LGK-treated resistant DLD1

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	8.438	.044	7.688	4	.002	51.052333	6.640495	32.615364	69.489303
	Equal variances not assumed			7.688	2.227	.012	51.052333	6.640495	25.100197	77.004470

7.2.8.4 Untreated resistant DLD1 cells vs ETC159-treated DLD1 cells

Table 45: Normality test for untreated and ETC-treated resistant DLD1

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Res Untreated	.227	3	.	.983	3	.747
	DLD Res ETC	.230	3	.	.981	3	.734

a. Lilliefors Significance Correction

Table 46: Independent samples t-test for untreated and ETC-treated resistant DLD1

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	2.432	.194	12.947	4	.000	60.285000	4.656349	47.356903	73.213097
	Equal variances not assumed			12.947	2.485	.002	60.285000	4.656349	43.569775	77.000225

7.2.9 Statistical analysis for parental and resistant HCT116 at 72 hours post-treatment (Viability assay 2)

7.2.9.1 Untreated parental HCT116 cells vs LGK-treated HCT116 cells

Table 47: Normality test for untreated and LGK-treated parental HCT116

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Par Untreated	.327	3	.	.871	3	.298
	HCT Par LGK	.263	3	.	.955	3	.593

a. Lilliefors Significance Correction

Table 48: Independent samples t-test for untreated and LGK-treated parental HCT116

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.200	.678	3.217	4	.032	4.647333	1.444550	.636619	8.658048
	Equal variances not assumed			3.217	3.788	.035	4.647333	1.444550	.546368	8.748298

7.2.9.2 Untreated parental HCT116 cells vs ETC159-treated HCT116 cells

Table 49: Normality test for untreated and ETC-treated parental HCT116

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Par Untreated	.327	3	.	.871	3	.298
	HCT Par ETC	.210	3	.	.991	3	.821

a. Lilliefors Significance Correction

Table 50: Independent samples t-test for untreated and ETC-treated parental HCT116

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	2.424	.194	1.819	4	.143	5.689000	3.127214	-2.993538	14.371538
	Equal variances not assumed			1.819	2.352	.191	5.689000	3.127214	-6.010098	17.388098

7.2.9.3 Untreated resistant HCT116 cells vs LGK974-treated HCT116 cells

Table 51: Normality test for untreated and LGK-treated resistant HCT116

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Res Untreated	.253	3	.	.964	3	.637
	HCT Res LGK	.196	3	.	.996	3	.878

a. Lilliefors Significance Correction

Table 52: Independent samples t-test for untreated and LGK-treated resistant HCT116

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.306	.609	3.922	4	.017	24.667	6.289	7.205	42.129
	Equal variances not assumed			3.922	3.517	.022	24.667	6.289	6.216	43.117

7.2.9.4 Untreated resistant HCT116 cells vs ETC159-treated HCT116 cells

Table 53: Normality test for untreated and ETC-treated resistant HCT116

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	HCT Res Untreated	.253	3	.	.964	3	.637
	HCT Res ETC	.276	3	.	.942	3	.537

a. Lilliefors Significance Correction

Table 54: Independent samples t-test for untreated and ETC-treated resistant HCT116

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	2.579	.184	4.128	4	.015	37.333	9.043	12.226	62.441
	Equal variances not assumed			4.128	2.696	.032	37.333	9.043	6.625	68.042

7.2.10 Statistical analysis for parental and resistant DLD1 at 72 hours post-treatment (Viability assay 2)

7.2.10.1 Untreated parental DLD1 cells vs LGK974-treated DLD1 cells

Table 55: Normality test for untreated and LGK-treated parental DLD1

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Par Untreated	.343	3	.	.842	3	.220
	DLD Par LGK	.206	3	.	.993	3	.836

a. Lilliefors Significance Correction

Table 56: Independent samples t-test for untreated and LGK-treated parental DLD1

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.706	.448	25.829	4	.000	28.337667	1.097127	25.291553	31.383780
	Equal variances not assumed			25.829	3.679	.000	28.337667	1.097127	25.183731	31.491602

7.2.10.2 Untreated parental DLD1 cells vs ETC159-treated DLD1 cells

Table 57: Normality test for untreated and ETC-treated parental DLD1

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Par Untreated	.343	3	.	.842	3	.220
	DLD Par ETC	.321	3	.	.881	3	.328

a. Lilliefors Significance Correction

Table 58: Independent samples t-test for untreated and ETC-treated parental DLD1

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.526	.509	20.716	4	.000	30.643667	1.479221	26.536690	34.750643
	Equal variances not assumed			20.716	3.695	.000	30.643667	1.479221	26.399444	34.887889

7.2.10.3 Untreated resistant DLD1 cells vs LGK974-treated DLD1 cells

Table 59: Normality test for untreated and LGK-treated resistant DLD1

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Res Untreated	.208	3	.	.992	3	.826
	DLD Res LGK	.209	3	.	.991	3	.823

a. Lilliefors Significance Correction

Table 60: Independent samples t-test for untreated and LGK-treated resistant DLD1

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	2.241	.209	-3.164	4	.034	-7.037667	2.224149	-13.212894	-.862439
	Equal variances not assumed			-3.164	2.423	.068	-7.037667	2.224149	-15.172593	1.097260

7.2.10.4 Untreated resistant DLD1 cells vs ETC159-treated DLD1 cells

Table 61: Normality test for untreated and ETC-treated resistant DLD1

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cell Viability	DLD Res Untreated	.208	3	.	.992	3	.826
	DLD Res ETC	.318	3	.	.887	3	.345

a. Lilliefors Significance Correction

Table 62: Independent samples t-test for untreated and ETC-treated resistant DLD1

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cell Viability	Equal variances assumed	.228	.658	12.669	4	.000	41.202667	3.252247	32.172981	50.232352
	Equal variances not assumed			12.669	3.906	.000	41.202667	3.252247	32.086840	50.318493

7.2.11 Two way ANOVA

7.2.11.1 HCT116 two way ANOVA

Table 63: Two way ANOVA for parental HCT116 comparing treatments

Pairwise Comparisons

Dependent Variable: cellviability

(I) drugtreatment	(J) drugtreatment	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
untreated	LGK	16.650*	5.862	.015	3.877	29.423
	ETC	18.917*	5.862	.007	6.143	31.690
LGK	untreated	-16.650*	5.862	.015	-29.423	-3.877
	ETC	2.267	5.862	.706	-10.507	15.040
ETC	untreated	-18.917*	5.862	.007	-31.690	-6.143
	LGK	-2.267	5.862	.706	-15.040	10.507

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 64: Two way ANOVA for parental HCT116 comparing treatments with time point

Pairwise Comparisons

Dependent Variable: cellviability

drugtreatment	(I) timepoint	(J) timepoint	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
untreated	24hr	72hr	6.867	8.291	.424	-11.197	24.931
	72hr	24hr	-6.867	8.291	.424	-24.931	11.197
LGK	24hr	72hr	-17.100	8.291	.061	-35.164	.964
	72hr	24hr	17.100	8.291	.061	-.964	35.164
ETC	24hr	72hr	-19.633*	8.291	.036	-37.697	-1.569
	72hr	24hr	19.633*	8.291	.036	1.569	37.697

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 65: Two way ANOVA for resistant HCT116 comparing treatments

Pairwise Comparisons

Dependent Variable: cellviability

(I) drugtreatment	(J) drugtreatment	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
untreated	LGK	23.183*	8.595	.019	4.457	41.910
	ETC	37.150*	8.595	.001	18.424	55.876
LGK	untreated	-23.183*	8.595	.019	-41.910	-4.457
	ETC	13.967	8.595	.130	-4.760	32.693
ETC	untreated	-37.150*	8.595	.001	-55.876	-18.424
	LGK	-13.967	8.595	.130	-32.693	4.760

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 66: Two way ANOVA for resistant HCT116 comparing treatments with time point

Pairwise Comparisons

Dependent Variable: cellviability

drugtreatment	(I) timepoint	(J) timepoint	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
						Lower Bound	Upper Bound
untreated	24hr	72hr	-14.167	12.155	.266	-40.650	12.316
	72hr	24hr	14.167	12.155	.266	-12.316	40.650
LGK	24hr	72hr	-11.200	12.155	.375	-37.683	15.283
	72hr	24hr	11.200	12.155	.375	-15.283	37.683
ETC	24hr	72hr	-13.800	12.155	.278	-40.283	12.683
	72hr	24hr	13.800	12.155	.278	-12.683	40.283

Based on estimated marginal means

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

7.2.11.2 DLD1 two way ANOVA

Table 67: Two way ANOVA for parental DLD1 comparing treatments

Pairwise Comparisons

Dependent Variable: cellviability

(I) drugtreatment	(J) drugtreatment	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
untreated	LGK	26.317*	3.464	.000	18.768	33.865
	ETC	28.533*	3.464	.000	20.985	36.082
LGK	untreated	-26.317*	3.464	.000	-33.865	-18.768
	ETC	2.217	3.464	.534	-5.332	9.765
ETC	untreated	-28.533*	3.464	.000	-36.082	-20.985
	LGK	-2.217	3.464	.534	-9.765	5.332

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 68: Two way ANOVA for parental DLD1 comparing treatments with time point

Pairwise Comparisons

Dependent Variable: cellviability

drugtreatment	(I) timepoint	(J) timepoint	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
						Lower Bound	Upper Bound
untreated	24hr	72hr	-6.333	4.899	.220	-17.008	4.342
	72hr	24hr	6.333	4.899	.220	-4.342	17.008
LGK	24hr	72hr	-2.300	4.899	.647	-12.975	8.375
	72hr	24hr	2.300	4.899	.647	-8.375	12.975
ETC	24hr	72hr	-2.067	4.899	.681	-12.742	8.608
	72hr	24hr	2.067	4.899	.681	-8.608	12.742

Based on estimated marginal means

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 69: Two way ANOVA for resistant DLD1 comparing treatments

Pairwise Comparisons

Dependent Variable: cellviability

(I) drugtreatment	(J) drugtreatment	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
untreated	LGK	22.017*	3.508	.000	14.374	29.660
	ETC	50.733*	3.508	.000	43.090	58.376
LGK	untreated	-22.017*	3.508	.000	-29.660	-14.374
	ETC	28.717*	3.508	.000	21.074	36.360
ETC	untreated	-50.733*	3.508	.000	-58.376	-43.090
	LGK	-28.717*	3.508	.000	-36.360	-21.074

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 70: Two way ANOVA for resistant DLD1 comparing treatments with time point

Pairwise Comparisons

Dependent Variable: cellviability

drugtreatment	(I) timepoint	(J) timepoint	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
untreated	24hr	72hr	-10.667	4.961	.053	-21.476	.142
	72hr	24hr	10.667	4.961	.053	-.142	21.476
LGK	24hr	72hr	-68.700*	4.961	.000	-79.509	-57.891
	72hr	24hr	68.700*	4.961	.000	57.891	79.509
ETC	24hr	72hr	-29.733*	4.961	.000	-40.542	-18.924
	72hr	24hr	29.733*	4.961	.000	18.924	40.542

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

7.3 Appendix III

7.3.1 Scratch assay images for the HCT116 cells

7.3.1.1 Parental HCT116 cells scratch assays

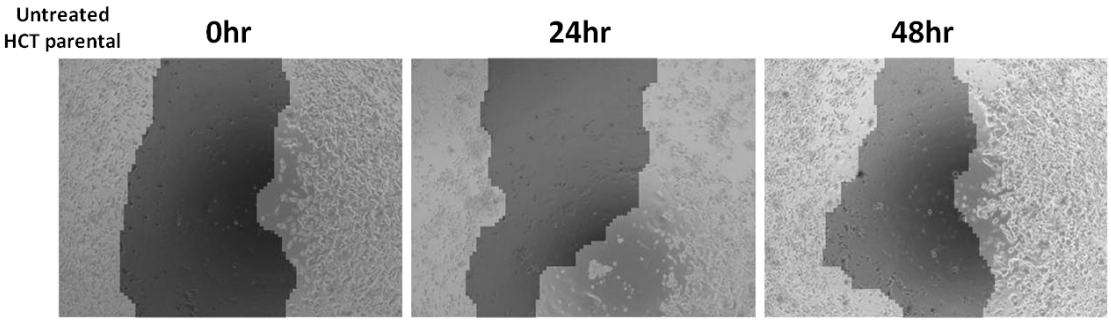

Figure 37: Scratch images of untreated parental HCT116 cells over 48 hours

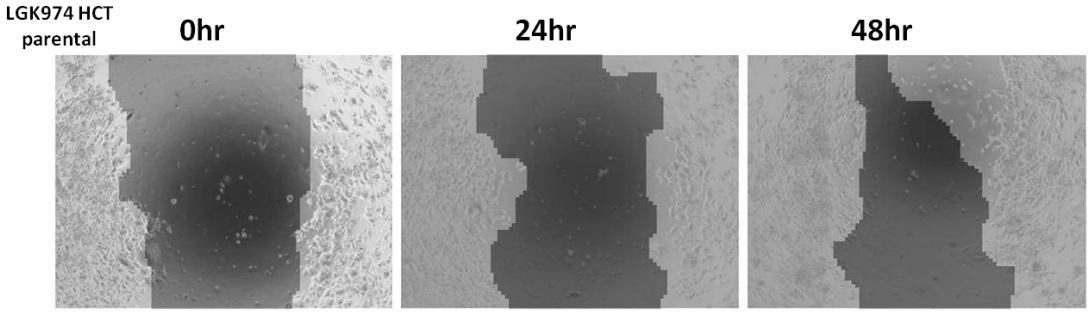

Figure 38: Scratch images of LGK974-treated parental HCT116 cells over 48 hours

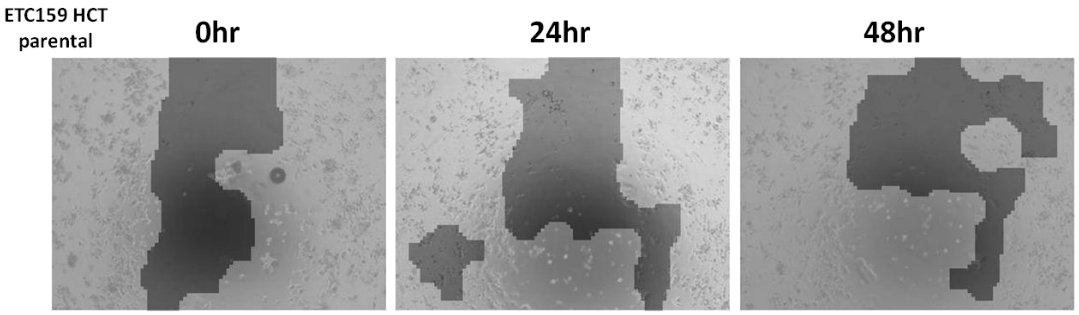

Figure 39: Scratch images of ETC159-treated parental HCT116 cells over 48 hours

7.3.1.2 Resistant HCT116 cells scratch assays

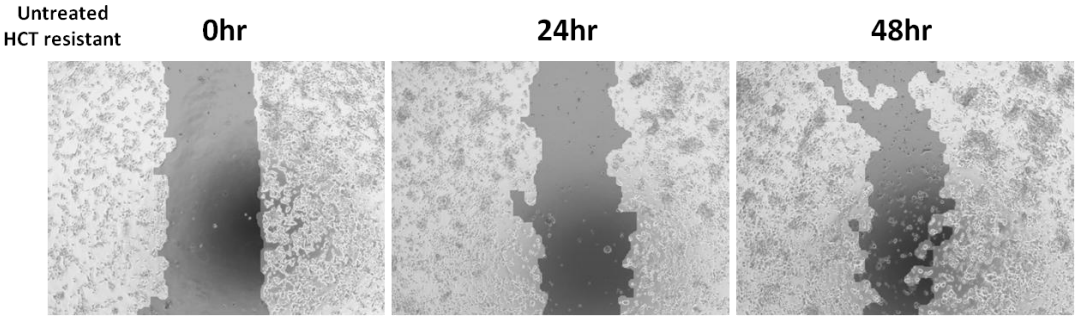

Figure 40: Scratch images of untreated resistant HCT116 cells over 48 hours

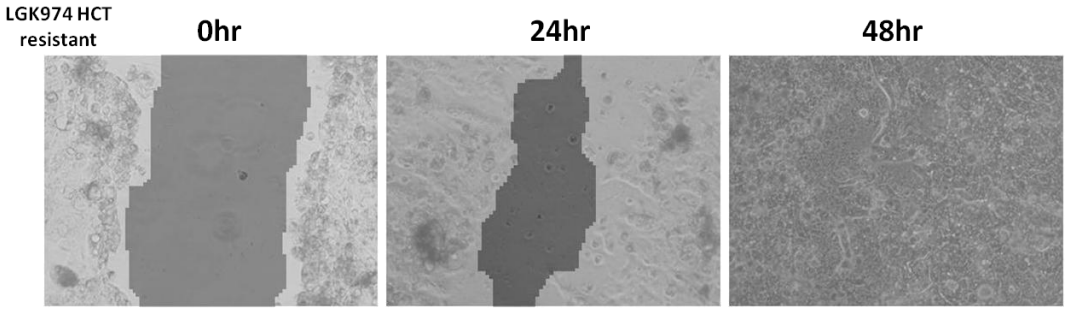

Figure 41: Scratch images of LGK974-treated resistant HCT116 cells over 48 hours

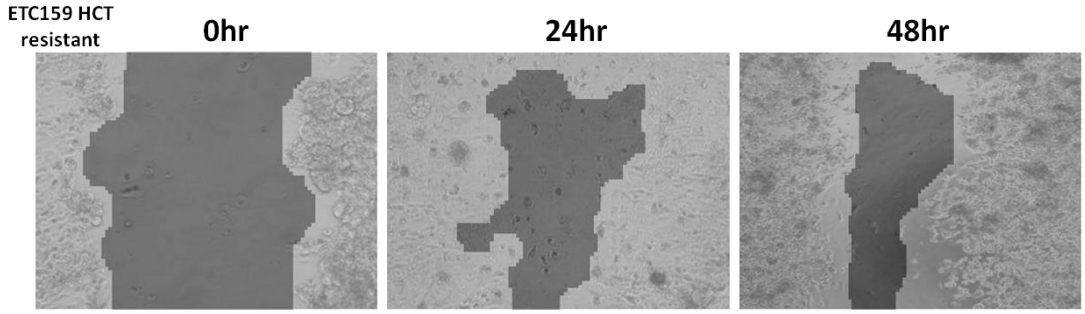

Figure 42: Scratch images of ETC159-treated resistant HCT116 cells over 48 hours

7.3.2 Scratch assay images for the DLD1 cells

7.3.2.1 Parental DLD1 cells scratch assays

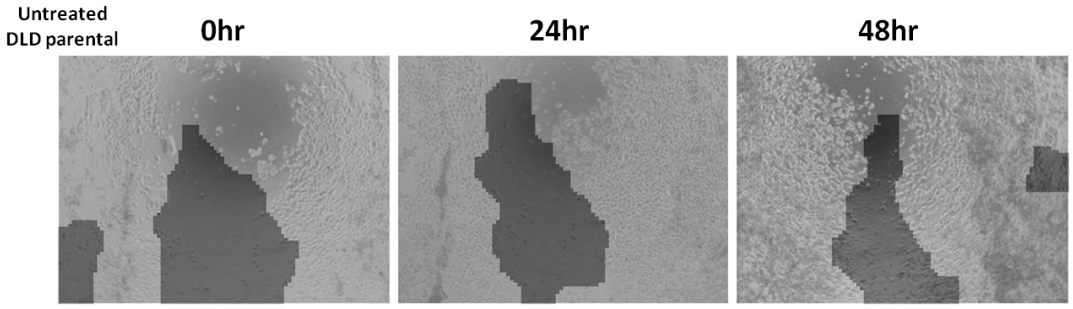

Figure 43: Scratch images of untreated parental DLD1 cells over 48 hours

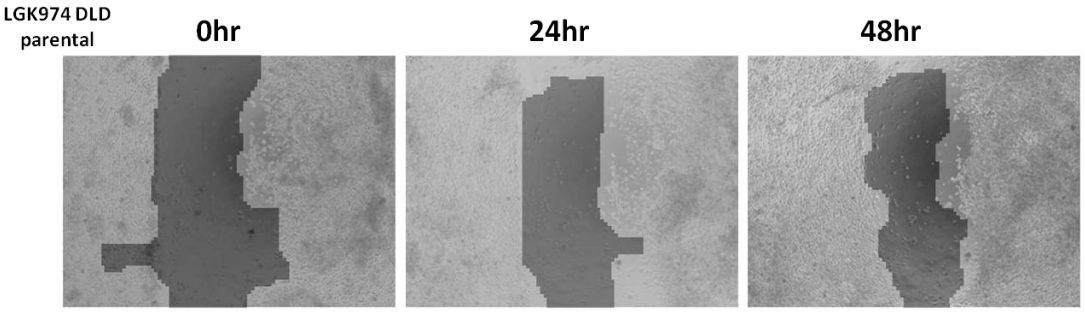

Figure 44: Scratch images of LGK974-treated parental DLD1 cells over 48 hours

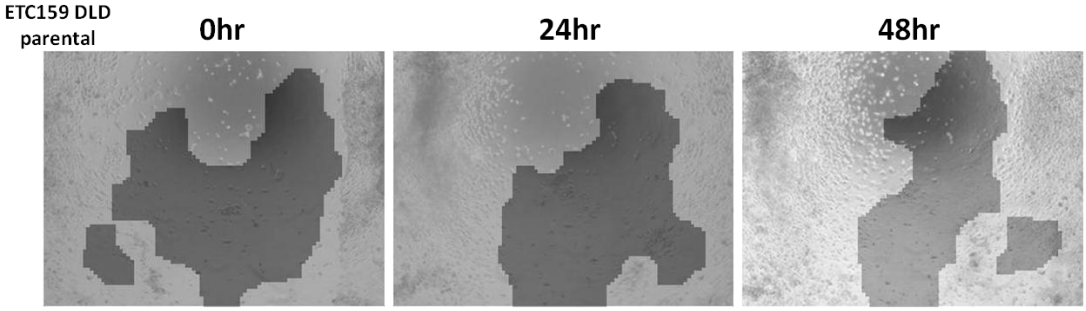

Figure 45: Scratch images of ETC159-treated parental DLD1 cells over 48 hours

7.3.2.2 Resistant DLD1 cells scratch assays

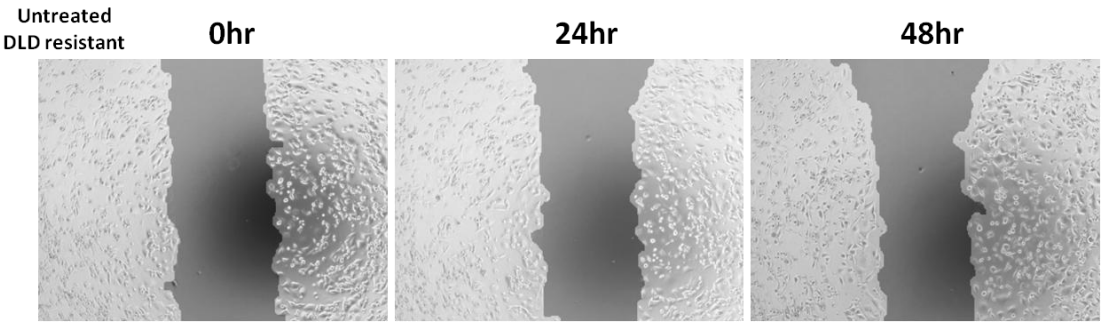

Figure 46: Scratch images of untreated resistant DLD1 cells over 48 hours

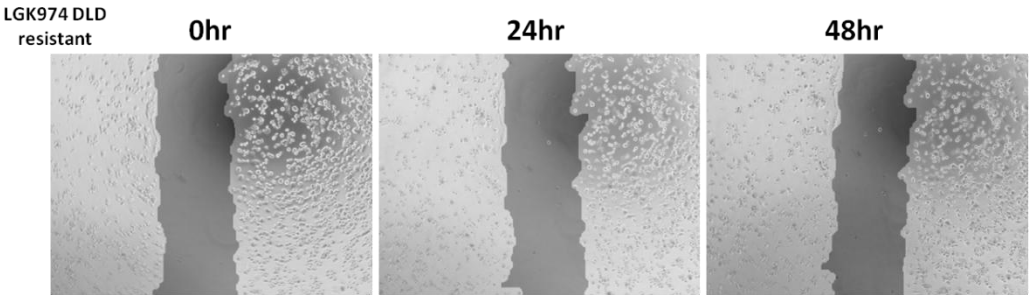

Figure 47: Scratch images of LGK974-treated resistant DLD1 cells over 48 hours

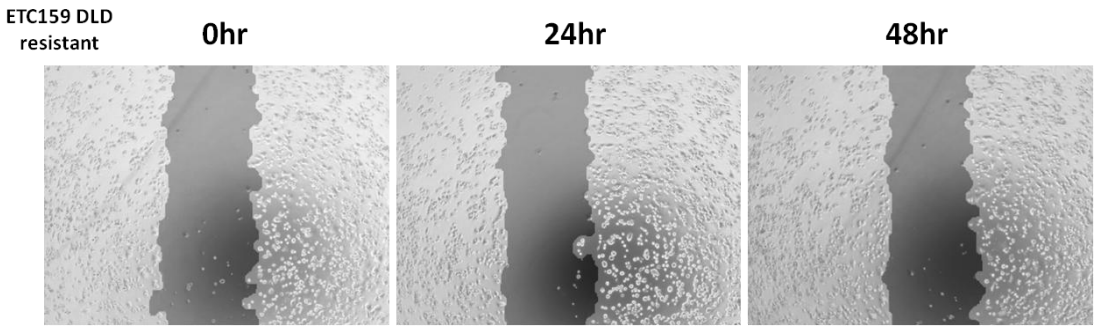

Figure 48: Scratch images of ETC159-treated resistant DLD1 cells over 48 hours

7.3.3 Statistical analysis of HCT116 scratch migration assays

7.3.3.1 Untreated parental HCT116 cells vs LGK974-treated parental HCT116 cells

Table 71: Normality test for untreated and LGK974-treated parental HCT116 cells at the 24 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	HCTpar untreated	.193	3	.	.997	3	.890
	HCTpar LGK	.184	3	.	.999	3	.927

a. Lilliefors Significance Correction

Table 72: Independent samples t-test for untreated and LGK974-treated parental HCT116 cells at the 24 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	.341	.591	33.113	4	.000	3.45000	.10419	3.16072	3.73928
	Equal variances not assumed			33.113	3.486	.000	3.45000	.10419	3.14311	3.75689

Table 73: Normality test for untreated and LGK974-treated parental HCT116 cells at the 48 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	HCTpar untreated	.175	3	.	1.000	3	1.000
	HCTpar LGK	.219	3	.	.987	3	.780

a. Lilliefors Significance Correction

Table 74: Independent samples t-test for untreated and LGK974-treated parental HCT116 cells at the 48 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	.010	.925	-42.805	4	.000	-3.50667	.08192	-3.73412	-3.27922
	Equal variances not assumed			-42.805	4.000	.000	-3.50667	.08192	-3.73412	-3.27921

7.3.3.2 Untreated parental HCT116 cells vs ETC159-treated parental HCT116 cells

Table 75: Normality test for untreated and ETC159-treated parental HCT116 cells at the 24 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	HCTpar untreated	.193	3	.	.997	3	.890
	HCTpar ETC	.276	3	.	.942	3	.537

a. Lilliefors Significance Correction

Table 76: Independent samples t-test for untreated and ETC159-treated parental HCT116 cells at the 24 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	.074	.799	12.493	4	.000	1.06333	.08511	.82702	1.29965
	Equal variances not assumed			12.493	3.977	.000	1.06333	.08511	.82647	1.30020

Table 77: Normality test for untreated and ETC159-treated parental HCT116 cells at the 48 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	HCTpar untreated	.175	3	.	1.000	3	1.000
	HCTpar ETC	.245	3	.	.971	3	.672

a. Lilliefors Significance Correction

Table 78: Independent samples t-test for untreated and ETC159-treated parental HCT116 cells at the 48 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	1.565	.279	-3.215	4	.032	-.42000	.13064	-.78271	-.05729
	Equal variances not assumed			-3.215	2.917	.051	-.42000	.13064	-.84254	.00254

7.3.3.3 Untreated resistant HCT116 cells vs LGK974-treated resistant HCT116 cells

Table 79: Normality test for untreated and LGK974-treated resistant HCT116 cells at the 24 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	HCTres untreated	.219	3	.	.987	3	.780
	HCTres LGK	.178	3	.	.999	3	.954

a. Lilliefors Significance Correction

Table 80: Independent samples t-test for untreated and LGK974-treated resistant HCT116 cells at the 24 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	3.417	.138	-71.874	4	.000	-20.03333	.27873	-20.80720	-19.25946
	Equal variances not assumed			-71.874	2.044	.000	-20.03333	.27873	-21.20822	-18.85844

Table 81: Normality test for untreated and LGK974-treated resistant HCT116 cells at the 48 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	HCTres untreated	.305	3	.	.905	3	.403
	HCTres LGK	.230	3	.	.981	3	.737

a. Lilliefors Significance Correction

Table 82: Independent samples t-test for untreated and LGK974-treated resistant HCT116 cells at the 48 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	.037	.856	-80.693	4	.000	-16.97333	.21034	-17.55734	-16.38933
	Equal variances not assumed			-80.693	3.994	.000	-16.97333	.21034	-17.55771	-16.38895

7.3.3.4 Untreated resistant HCT116 cells vs ETC159-treated resistant HCT116 cells

Table 83: Normality test for untreated and ETC159-treated resistant HCT116 cells at the 24 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	HCTres untreated	.219	3	.	.987	3	.780
	HCTres ETC	.269	3	.	.949	3	.567

a. Lilliefors Significance Correction

Table 84: Independent samples t-test for untreated and ETC159-treated resistant HCT116 cells at the 24 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	5.446	.080	-164.039	4	.000	-24.76333	.15096	-25.18247	-24.34420
	Equal variances not assumed			-164.039	2.154	.000	-24.76333	.15096	-25.37040	-24.15626

Table 85: Normality test for untreated and ETC159-treated resistant HCT116 cells at the 48 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	HCTres untreated	.305	3	.	.905	3	.403
	HCTres ETC	.175	3	.	1.000	3	1.000

a. Lilliefors Significance Correction

Table 86: Independent samples t-test for untreated and ETC159-treated resistant HCT116 cells at the 48 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	.093	.776	-14.104	4	.000	-2.95333	.20939	-3.53469	-2.37197
	Equal variances not assumed			-14.104	3.990	.000	-2.95333	.20939	-3.53526	-2.37141

7.3.4 Statistical analysis of DLD1 scratch migration assays

7.3.4.1 Untreated parental DLD1 cells vs LGK974-treated parental DLD1 cells

Table 87: Normality test for untreated and LGK974-treated resistant DLD1 cells at the 24 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	DLDpar untreated	.196	3	.	.996	3	.878
	DLDpar LGK	.175	3	.	1.000	3	1.000

a. Lilliefors Significance Correction

Table 88: Independent samples t-test for untreated and LGK974-treated resistant DLD1 cells at the 24 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	.792	.424	-93.334	4	.000	-5.55667	.05954	-5.72196	-5.39137
	Equal variances not assumed			-93.334	3.123	.000	-5.55667	.05954	-5.74197	-5.37136

Table 89: Normality test for untreated and LGK974-treated resistant DLD1 cells at the 48 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	DLDpar untreated	.219	3	.	.987	3	.780
	DLDpar LGK	.314	3	.	.893	3	.363

a. Lilliefors Significance Correction

Table 90: Independent samples t-test for untreated and LGK974-treated resistant DLD1 cells at the 48 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	.057	.823	66.724	4	.000	2.81333	.04216	2.69627	2.93040
	Equal variances not assumed			66.724	3.990	.000	2.81333	.04216	2.69615	2.93051

7.3.4.2 Untreated parental DLD1 cells vs ETC159-treated parental DLD1 cells

Table 91: Normality test for untreated and ETC159-treated resistant DLD1 cells at the 24 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	DLDpar untreated	.196	3	.	.996	3	.878
	DLDpar ETC	.385	3	.	.750	3	.000

a. Lilliefors Significance Correction

Table 92: Independent samples t-test for untreated and ETC159-treated resistant DLD1 cells at the 24 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	4.994	.089	-2.049	4	.110	-.10667	.05207	-.25123	.03790
	Equal variances not assumed			-2.049	2.000	.177	-.10667	.05207	-.33070	.11737

Table 93: Normality test for untreated and ETC159-treated resistant DLD1 cells at the 48 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	DLDpar untreated	.219	3	.	.987	3	.780
	DLDpar ETC	.253	3	.	.964	3	.637

a. Lilliefors Significance Correction

Table 94: Independent samples t-test for untreated and ETC159-treated resistant DLD1 cells at the 48 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	5.953	.071	1.835	4	.140	.05333	.02906	-.02735	.13401
	Equal variances not assumed			1.835	2.000	.208	.05333	.02906	-.07170	.17837

7.3.4.3 Untreated resistant DLD1 cells vs LGK974-treated resistant DLD1 cells

Table 95: Normality test for untreated and LGK974-treated resistant DLD1 cells at the 24 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	DLDres untreated	.219	3	.	.987	3	.780
	DLDres LGK	.328	3	.	.871	3	.298

a. Lilliefors Significance Correction

Table 96: Independent samples t-test for untreated and LGK974-treated resistant DLD1 cells at the 24 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	.450	.539	17.395	4	.000	.41000	.02357	.34456	.47544
	Equal variances not assumed			17.395	3.782	.000	.41000	.02357	.34304	.47696

Table 97: Normality test for untreated and LGK974-treated resistant DLD1 cells at the 48 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	DLDres untreated	.314	3	.	.893	3	.363
	DLDres LGK	.385	3	.	.750	3	.000

a. Lilliefors Significance Correction

Table 98: Independent samples t-test for untreated and LGK974-treated resistant DLD1 cells at the 48 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	16.000	.016	2.750	4	.051	.03667	.01333	-.00035	.07369
	Equal variances not assumed			2.750	2.000	.111	.03667	.01333	-.02070	.09404

7.3.4.4 Untreated resistant DLD1 cells vs ETC159-treated resistant DLD1 cells

Table 99: Normality test for untreated and ETC159-treated resistant DLD1 cells at the 24 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	DLDres untreated	.219	3	.	.987	3	.780
	DLDres ETC	.269	3	.	.949	3	.567

a. Lilliefors Significance Correction

Table 100: Independent samples t-test for untreated and ETC159-treated resistant DLD1 cells at the 24 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	1.841	.246	-18.789	4	.000	-.62000	.03300	-.71162	-.52838
	Equal variances not assumed			-18.789	2.909	.000	-.62000	.03300	-.72689	-.51311

Table 101: Normality test for untreated and ETC159-treated resistant DLD1 cells at the 48 hour time-point

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
ReductionOpenArea	DLDres untreated	.314	3	.	.893	3	.363
	DLDres ETC	.269	3	.	.949	3	.567

a. Lilliefors Significance Correction

Table 102: Independent samples t-test for untreated and ETC159-treated resistant DLD1 cells at the 48 hour time-point

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ReductionOpenArea	Equal variances assumed	.966	.381	-1.393	4	.236	-.05667	.04069	-.16964	.05630
	Equal variances not assumed			-1.393	3.581	.244	-.05667	.04069	-.17505	.06172

7.4 Appendix IV

7.4.1 Statistical analysis of HCT116 transwell invasion assay

7.4.1.1 Untreated parental HCT116 cells vs LGK974-treated parental HCT116 cells

Table 103: Normality test for untreated and LGK974-treated parental HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cells per quadrant	HCT Par Untreated	.298	4	.	.849	4	.224
	HCT Par LGK	.260	4	.	.827	4	.161

a. Lilliefors Significance Correction

Table 104: Independent samples t-test for untreated and LGK974-treated parental HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cells per quadrant	Equal variances assumed	.300	.604	.243	6	.816	.250	1.031	-2.272	2.772
	Equal variances not assumed			.243	5.979	.816	.250	1.031	-2.274	2.774

7.4.1.2 Untreated parental HCT116 cells vs ETC159-treated parental HCT116 cells

Table 105: Normality test for untreated and ETC159-treated parental HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cells per quadrant	HCT Par Untreated	.298	4	.	.849	4	.224
	HCT Par ETC	.250	4	.	.963	4	.797

a. Lilliefors Significance Correction

Table 106: Independent samples t-test for untreated and ETC159-treated parental HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cells per quadrant	Equal variances assumed	1.387	.284	-1.984	6	.094	-4.750	2.394	-10.607	1.107
	Equal variances not assumed			-1.984	3.646	.125	-4.750	2.394	-11.658	2.158

7.4.1.3 Untreated resistant HCT116 cells vs LGK974-treated resistant HCT116 cells

Table 107: Normality test for untreated and LGK974-treated resistant HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cells per quadrant	HCT Res Untreated	.	4	.	.	4	.
	HCT Res LGK	.283	4	.	.863	4	.272

a. Lilliefors Significance Correction

Table 108: Independent samples t-test for untreated and LGK974-treated resistant HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cells per quadrant	Equal variances assumed	13.500	.010	-4.352	6	.005	-6.250	1.436	-9.764	-2.736
	Equal variances not assumed			-4.352	3.000	.022	-6.250	1.436	-10.820	-1.680

7.4.1.4 Untreated resistant HCT116 cells vs ETC159-treated resistant HCT116 cells

Table 109: Normality test for untreated and ETC159-treated resistant HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cells per quadrant	HCT Res Untreated	.	4	.	.	4	.
	HCT Res ETC	.283	4	.	.863	4	.272

a. Lilliefors Significance Correction

Table 110: Independent samples t-test for untreated and ETC159-treated resistant HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cells per quadrant	Equal variances assumed	13.500	.010	-7.833	6	.000	-3.750	.479	-4.921	-2.579
	Equal variances not assumed			-7.833	3.000	.004	-3.750	.479	-5.273	-2.227

7.4.2 Statistical analysis for DLD1 transwell invasion assay

7.4.2.1 Untreated parental DLD1 cells vs LGK974-treated parental DLD1 cells

Table 111: Normality test for untreated and LGK974-treated parental DLD1 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cells per quadrant	DLD Par Untreated	.441	4	.	.630	4	.001
	DLD Par LGK	.441	4	.	.630	4	.001

a. Lilliefors Significance Correction

Table 112: Independent samples t-test for untreated and LGK974-treated parental DLD1 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cells per quadrant	Equal variances assumed	1.800	.228	-.447	6	.670	-.250	.559	-1.618	1.118
	Equal variances not assumed			-.447	4.412	.676	-.250	.559	-1.747	1.247

7.4.2.2 Untreated parental DLD1 cells vs ETC159-treated parental DLD1 cells

Table 113: Normality test for untreated and ETC159-treated parental DLD1 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cells per quadrant	DLD Par Untreated	.441	4	.	.630	4	.001
	DLD Par ETC	.	4	.	.	4	.

a. Lilliefors Significance Correction

Table 114: Independent samples t-test for untreated and ETC159-treated parental DLD1 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cells per quadrant	Equal variances assumed	9.000	.024	1.000	6	.356	.250	.250	-.362	.862
	Equal variances not assumed			1.000	3.000	.391	.250	.250	-.546	1.046

7.4.2.3 Untreated resistant DLD1 cells vs LGK974-treated resistant DLD1 cells

Table 115: Normality test for untreated and LGK974-treated resistant DLD1 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cells per quadrant	DLD Res Untreated	.141	4	.	.997	4	.991
	DLD Res LGK	.237	4	.	.926	4	.569

a. Lilliefors Significance Correction

Table 116: Independent samples t-test for untreated and LGK974-treated resistant DLD1 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cells per quadrant	Equal variances assumed	.035	.857	-.513	6	.627	-2.500	4.878	-14.435	9.435
	Equal variances not assumed			-.513	5.861	.627	-2.500	4.878	-14.504	9.504

7.4.2.4 Untreated resistant DLD1 cells vs ETC159-treated resistant DLD1 cells

Table 117: Normality test for untreated and ETC159-treated resistant DLD1 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Cells per quadrant	DLD Res Untreated	.141	4	.	.997	4	.991
	DLD Res ETC	.259	4	.	.857	4	.248

a. Lilliefors Significance Correction

Table 118: Independent samples t-test for untreated and ETC159-treated resistant DLD1 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Cells per quadrant	Equal variances assumed	2.718	.150	-2.130	6	.077	-20.500	9.624	-44.050	3.050
	Equal variances not assumed			-2.130	3.721	.105	-20.500	9.624	-48.031	7.031

7.5 Appendix V

7.5.1 Wnt inhibitor troubleshooting

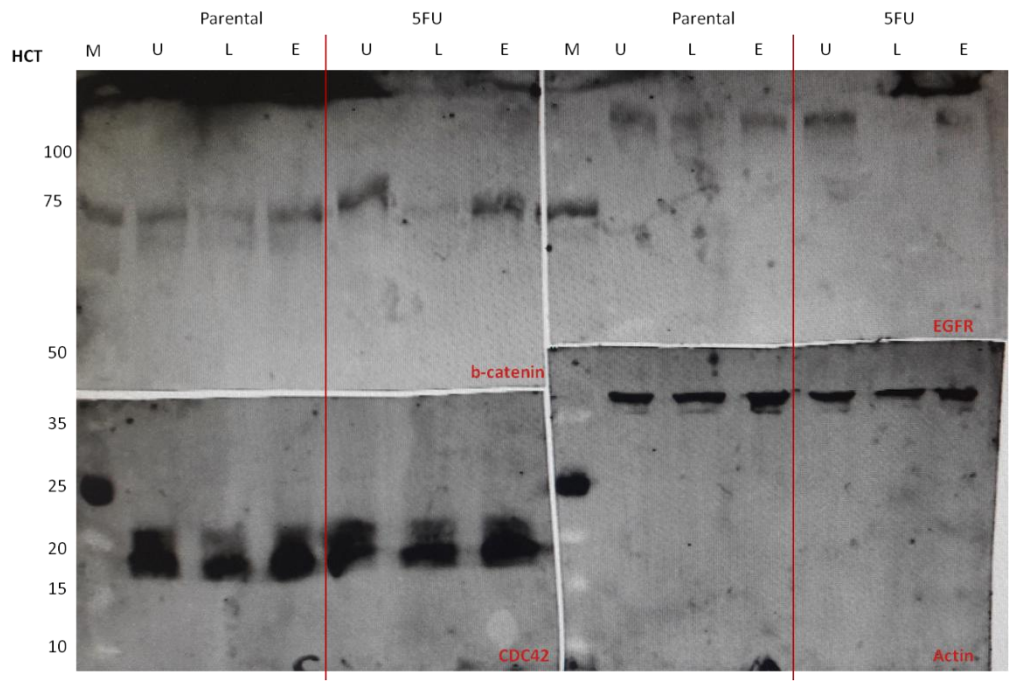

Figure 49: WB for parental and resistant HCT116 with 10% serum for β-catenin, CDC42, and EGFR using 10µM LGK974 and 0.2µM ETC-159 diluted with DMEM

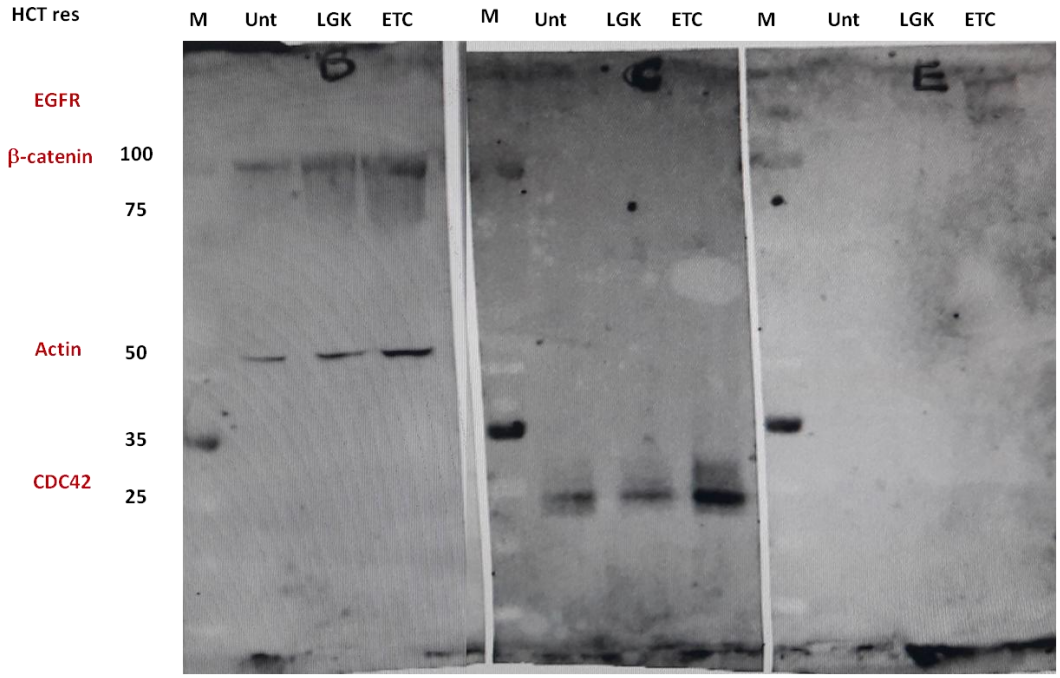

Figure 50: WB for resistant HCT116 with 10% serum for β-catenin, CDC42, and EGFR using 20µM LGK974 and 0.5µM ETC-159 diluted with DMEM

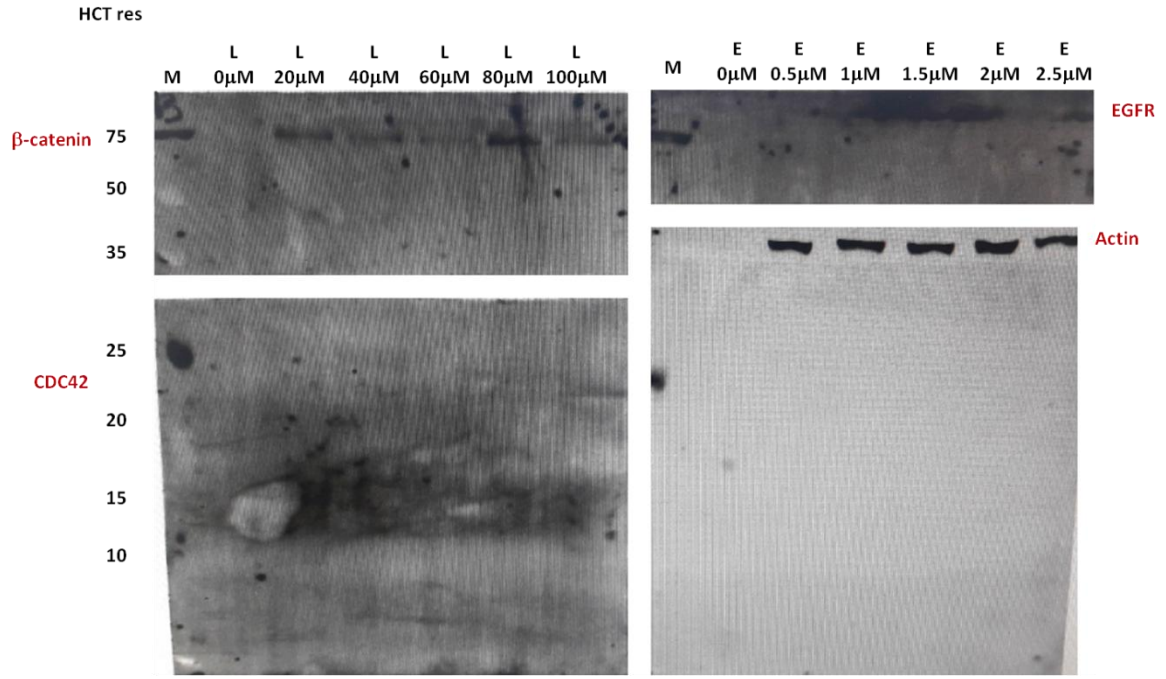

Figure 51: WB for resistant HCT116 with 10% serum for β-catenin, CDC42, and EGFR using a range of 0-100μM for LGK974 and a range of 0-2.5μM for ETC-159 diluted with DMEM

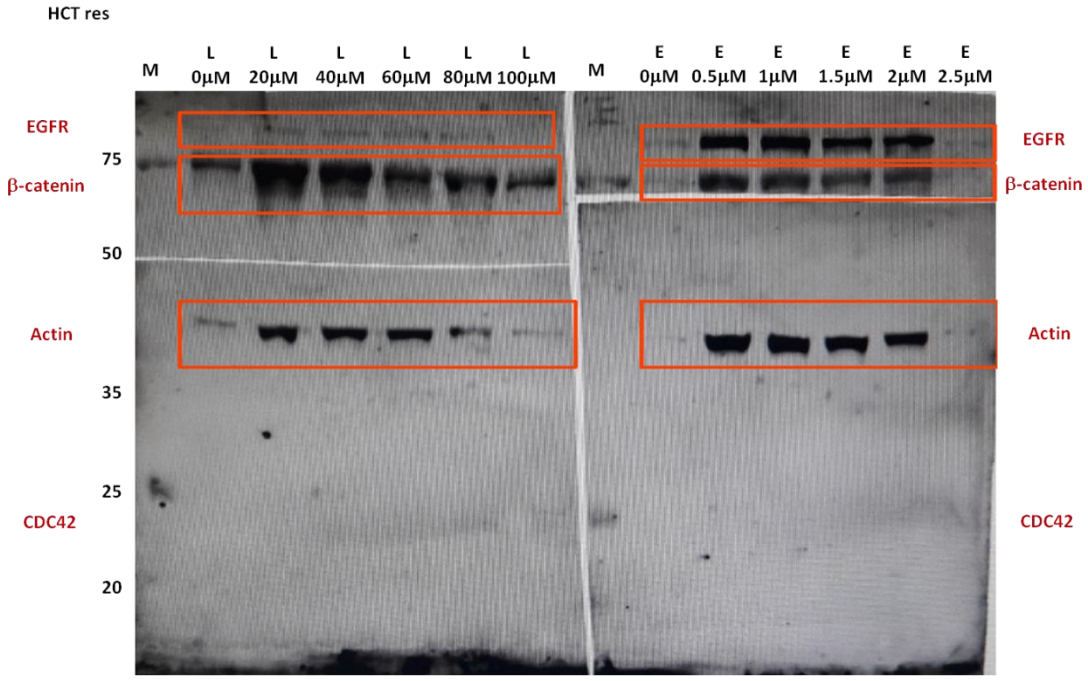

Figure 52: WB for resistant HCT116 with 10% serum for β-catenin, CDC42, and EGFR using a range of 0-100μM for LGK974 and a range of 0-2.5μM for ETC-159 diluted with DMSO

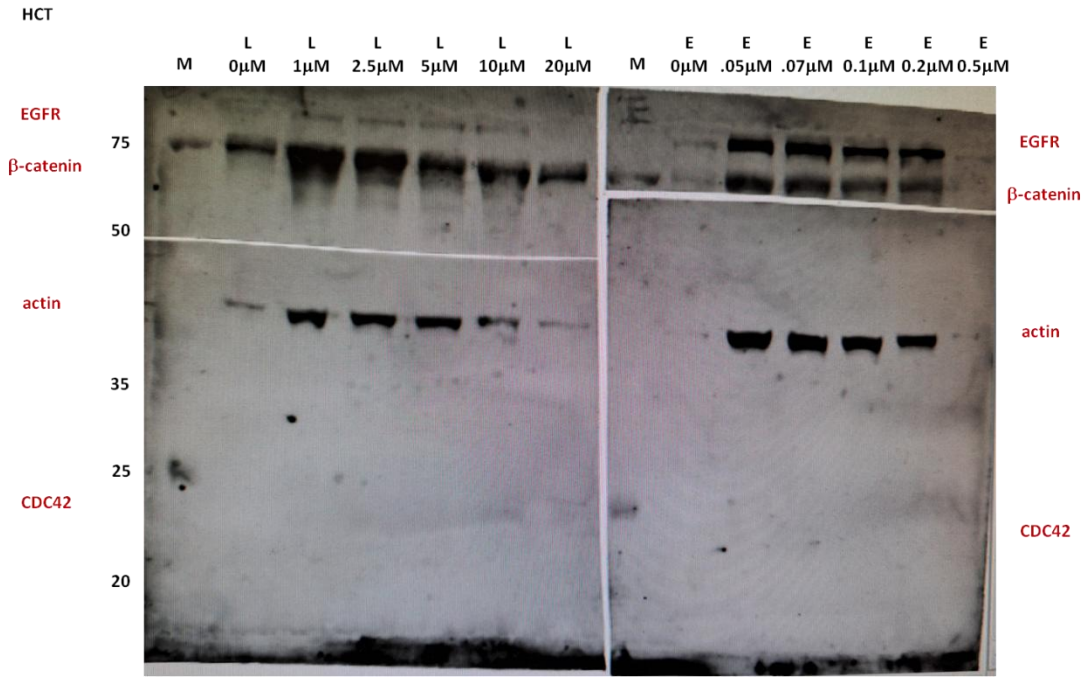

Figure 53: WB for resistant HCT116 with 10% serum for β-catenin, CDC42, and EGFR using a range of 0-20μM for LGK974 and a range of 0-0.5μM for ETC-159 diluted with DMSO and using 10% gel

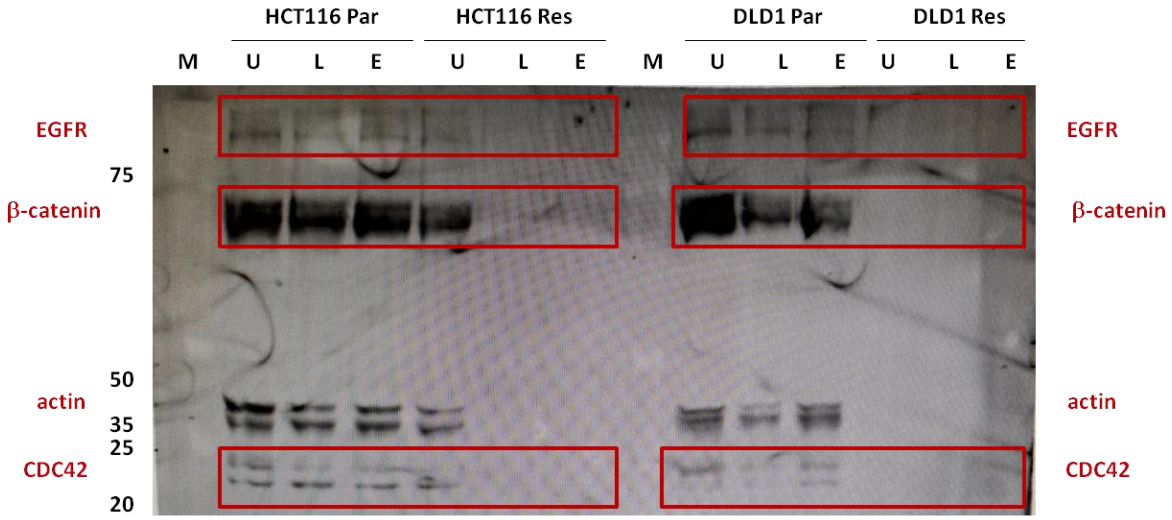

Figure 54: WB for parental and chemoresistant HCT116 and DLD1 cells for β-catenin, EGFR, and CDC42 using either untreated control cells, or cells treated with 10μM LGK974 or 0.2μM ETC-159.

7.6 Appendix VI

7.6.1 ELISA calibration curve

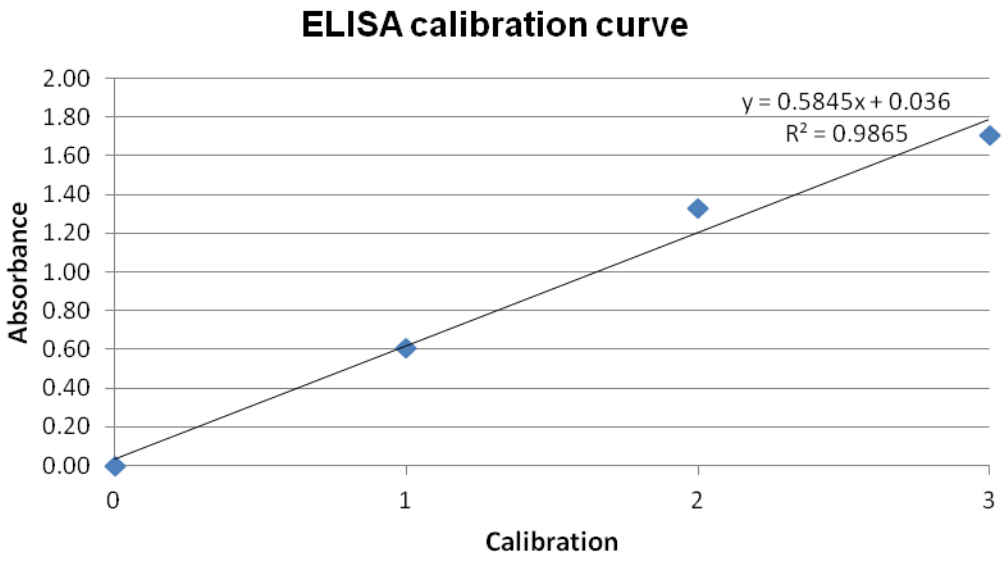

Figure 55: ELISA calibration curve using a serially diluted standard

7.6.2 Total EGFR statistics

7.6.2.1 HCT116 total EGFR

Table 119: Normality test for untreated and LGK974-treated parental HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	HCT Par Untreated	.333	3	.	.763	3	.051
	HCT Par LGK	.236	3	.	.911	3	.488

a. Lilliefors Significance Correction

Table 121: Independent samples t-test for untreated and LGK974-treated parental HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	2.466	.191	4.751	4	.002	29.9000	1.4000	23.8763	35.9237
	Equal variances not assumed			4.751	1.000	.030	29.9000	1.4000	12.1113	47.6887

Table 120: Normality test for untreated and ETC159-treated parental HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	HCT Par Untreated	.333	3	.	.763	3	.051
	HCT Par ETC	.250	3	.	.927	3	.577

a. Lilliefors Significance Correction

Table 122: Independent samples t-test for untreated and ETC159-treated parental HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	4.130	.112	1.052	4	.001	38.4000	1.3000	32.8066	43.9934
	Equal variances not assumed			1.052	1.000	.022	38.4000	1.3000	21.8819	54.9181

Table 123: Normality test for untreated and LGK974-treated resistant HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	HCT Res Untreated	.283	3	.	.863	3	.272
	HCT Res LGK	.302	3	.	.827	3	.161

a. Lilliefors Significance Correction

Table 124: Independent samples t-test for untreated and LGK974-treated resistant HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	.576	.490	-.243	4	.219	1.1500	.6500	-1.6467	3.9467
	Equal variances not assumed			-.243	1.000	.328	1.1500	.6500	-7.1090	9.4090

Table 125: Normality test for untreated and ETC-159-treated resistant HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	HCT Res Untreated	.302	3	.	.827	3	.161
	HCT Res ETC	.302	3	.	.827	3	.161

a. Lilliefors Significance Correction

Table 126: Independent samples t-test for untreated and ETC159-treated resistant HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	.318	.603	-12.228	4	.006	25.1000	1.9000	16.9250	33.2750
	Equal variances not assumed			-12.228	1.000	.048	25.1000	1.9000	.9582	49.2418

7.6.2.2 DLD1 total EGFR

Table 127: Normality test for untreated and LGK974-treated parental DLD1 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	DLD Par Untreated	.283	3	.	.863	3	.272
	DLD Par LGK	.260	3	.	.827	3	.161

a. Lilliefors Significance Correction

Table 128: Independent samples t-test for untreated and LGK974-treated parental DLD1 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	.377	.572	-7.047	4	.111	1.1000	.4000	-.6211	2.8211
	Equal variances not assumed			-7.047	1.000	.222	1.1000	.4000	-3.9825	6.1825

Table 129: Normality test for untreated and ETC159-treated parental DLD1 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	DLD Par Untreated	.283	3	.	.863	3	.272
	DLD Par ETC	.303	3	.	.791	3	.086

a. Lilliefors Significance Correction

Table 130: Independent samples t-test for untreated and ETC159-treated parental DLD1 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	4.130	.112	1.052	4	.452	-1.2500	1.3500	-7.0586	4.5586
	Equal variances not assumed			1.052	1.000	.524	-1.2500	1.3500	-18.4034	15.9034

Table 131: Normality test for untreated and LGK974-treated resistant DLD1 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	DLD Res Untreated	.394	3	.	.773	3	.062
	DLD Res LGK	.229	3	.	.895	3	.404

a. Lilliefors Significance Correction

Table 132: Independent samples t-test for untreated and LGK974-treated resistant DLD1 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	.802	.421	.660	4	.577	1.5500	2.3500	-8.5612	11.6612
	Equal variances not assumed			.660	1.000	.629	1.5500	2.3500	-28.3096	31.4096

Table 133: Normality test for untreated and ETC159-treated resistant DLD1 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	DLD Res Untreated	.260	3	.	.827	3	.161
	DLD Res ETC	.283	3	.	.863	3	.272

a. Lilliefors Significance Correction

Table 134: Independent samples t-test for untreated and ETC159-treated resistant DLD1 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	.318	.603	-12.228	4	.085	4.8000	1.5000	-1.6540	11.2540
	Equal variances not assumed			-12.228	1.000	.193	4.8000	1.5000	-14.2593	23.8593

7.6.2.3 SW837 total EGFR

Table 135: Normality test for untreated and LGK974-treated parental SW837 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	SWpar Untreated	.298	3	.	.849	3	.224
	SWpar LGK	.260	3	.	.827	3	.161

a. Lilliefors Significance Correction

Table 136: Independent samples t-test for untreated and LGK974-treated parental SW837 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	.377	.572	-7.047	4	.085	4.8000	1.5000	-1.6540	11.2540
	Equal variances not assumed			-7.047	1.000	.193	4.8000	1.5000	-14.2593	23.8593

Table 137: Normality test for untreated and ETC159-treated parental SW837 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Condition	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	SWpar Untreated	.298	3	.	.849	3	.224
	SWpar ETC	.250	3	.	.963	3	.797

a. Lilliefors Significance Correction

Table 138: Independent samples t-test for untreated and ETC159-treated parental SW837 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	.576	.490	-.243	4	.061	6.2000	1.6000	-.6842	13.0842
	Equal variances not assumed			-.243	1.000	.161	6.2000	1.6000	-14.1299	26.5299

7.6.3 Phosphorylated EGFR statistics

7.6.3.1 HCT116 phosphorylated EGFR

Table 139: Normality test for untreated and LGK974-treated parental HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	HCT Parental Untreated	.250	3	.	.967	3	.649
	HCT Parental LGK	.304	3	.	.907	3	.408

a. Lilliefors Significance Correction

Table 140: Independent samples t-test for untreated and LGK974-treated parental HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	2.466	.191	4.751	4	.038	-56.2500	11.3500	-105.0851	-7.4149
	Equal variances not assumed			4.751	1.000	.127	-56.2500	11.3500	-200.4654	87.9654

Table 141: Normality test for untreated and ETC159-treated parental HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	HCT Parental Untreated	.358	3	.	.813	3	.147
	HCT Parental ETC	.328	3	.	.871	3	.298

a. Lilliefors Significance Correction

Table 142: Independent samples t-test for untreated and ETC159-treated parental HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	.576	.490	-.243	4	.042	-45.5000	9.6000	-86.8055	-4.1945
	Equal variances not assumed			-.243	1.000	.132	-45.5000	9.6000	-167.4796	76.4796

Table 143: Normality test for untreated and LGK974-treated resistant HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	HCT Res Untreated	.340	3	.	.848	3	.235
	HCT Res LGK	.340	3	.	.848	3	.236

a. Lilliefors Significance Correction

Table 144: Independent samples t-test for untreated and LGK974-treated resistant HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	4.130	.112	1.052	4	.839	-7.9500	34.5500	-156.6067	140.7067
	Equal variances not assumed			1.052	1.000	.856	-7.9500	34.5500	-446.9494	431.0494

Table 145: Normality test for untreated and ETC159-treated resistant HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Cell type	Statistic	df	Sig.	Statistic	df	Sig.
Percentage	HCT Res Untreated	.340	3	.	.848	3	.235
	HCT Res ETC	.302	3	.	.910	3	.417

a. Lilliefors Significance Correction

Table 146: Independent samples t-test for untreated and ETC159-treated resistant HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percentage	Equal variances assumed	.377	.572	-7.047	4	.759	-8.0000	22.8000	-106.1005	90.1005
	Equal variances not assumed			-7.047	1.000	.785	-8.0000	22.8000	-297.7015	281.7015

7.6.4 Phosphorylated to total EGFR proportion statistics

7.6.4.1 HCT116 phosphorylated to total EGFR proportion

Table 147: Normality test for untreated and LGK974-treated parental HCT116 cells

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
PercentRatio	HCT Parental Untreated	.250	3	.	.967	3	.649
	HCT Parental LGK	.304	3	.	.907	3	.408

a. Lilliefors Significance Correction

Table 148: Independent samples t-test for untreated and LGK974-treated parental HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
PercentRatio	Equal variances assumed	.377	.572	-7.047	4	.002	-6.02500	.24602	-7.08353	-4.96647
	Equal variances not assumed			-7.047	1.877	.002	-6.02500	.24602	-7.15316	-4.89684

Table 149: Normality test for untreated and ETC159-treated parental HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
PercentRatio	HCT Parental Untreated	.358	3	.	.813	3	.147
	HCT Parental ETC	.328	3	.	.871	3	.298

a. Lilliefors Significance Correction

Table 150: Independent samples t-test for untreated and ETC159-treated parental HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
PercentRatio	Equal variances assumed	.318	.603	-12.228	4	.050	-6.27500	1.46305	-12.57001	.02001
	Equal variances not assumed			-12.228	1.036	.139	-6.27500	1.46305	-23.39976	10.84976

Table 151: Normality test for untreated and LGK974-treated resistant HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
PercentRatio	HCT Res Untreated	.340	3	.	.848	3	.235
	HCT Res LGK	.340	3	.	.848	3	.236

a. Lilliefors Significance Correction

Table 152: Independent samples t-test for untreated and LGK974-treated resistant HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
PercentRatio	Equal variances assumed	.576	.490	-.243	4	.903	-.34000	2.46074	-10.92772	10.24772
	Equal variances not assumed			-.243	2.000	.903	-.34000	2.46074	-10.92805	10.24805

Table 153: Normality test for untreated and ETC159-treated resistant HCT116 cells

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	CellType	Statistic	df	Sig.	Statistic	df	Sig.
PercentRatio	HCT Res Untreated	.340	3	.	.848	3	.235
	HCT Res ETC	.302	3	.	.910	3	.417

a. Lilliefors Significance Correction

Table 154: Independent samples t-test for untreated and ETC159-treated resistant HCT116 cells

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
PercentRatio	Equal variances assumed	.802	.421	-1.157	4	.001	-9.23000	.30537	-10.54390	-7.91610
	Equal variances not assumed			-1.157	1.934	.001	-9.23000	.30537	-10.58768	-7.87232