ANTIPROLIFERATIVE AND ANTICANCEROGENIC EFFECTS OF 5-ASA AND ITS NOVEL SYNTHESIZED OLIGOMER

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ABSTRACT

ANTIPROLIFERATIVE AND ANTICANCEROGENIC EFFECTS OF 5-ASA AND ITS NOVEL SYNTHESIZED OLIGOMER

In the world, colorectal cancer (CRC) is third common malignant diseases and fourth leading cause of cancer-related death. More recently, epidemiological studies have suggested that regular intake of 5-aminosalicylic acid (5-ASA), the drug used in treatment of inflammatory bowel disease (IBD), reduces the risk of CRC developing in patients with ulcerative colitis. Several action mechanisms of 5-ASA are proposed which are independent from its weak cyclooxygenase (COX) inhibitory property, found overexpressed in many cancer types. Lately, polymeric prodrugs developed which contain bioactive unit and have prolonged activity by sustained release, consequently reduced toxicity. However, large molecular weight and long chain can be challenging in penetrating into the cell membrane. Thus, oligomers with shorter chain of monomer units and lower molecular weight can be preferred choice. In the present study we evaluated antiprolifertaive and anticancerogenic effect of novel synthesized 5-ASA based oligomer compared to its active monomer, 5-ASA in Caco-2, DLD-1, HeLa and CCD-18Co cells. According to MTT, apoptotic rate, cell cycle phase distribution and scratch assay analysis the oligomer showed higher activity compared to its monomer, 5-ASA at lower doses. The oligomer induced cell death and cell cycle arrest in colorectal cancer cells and in HeLa cells. However, no significant induced cell death and cell cycle arrest observed in normal human colon cells, CCD-18Co when exposed to 5-ASA and the oligomer. Overall results indicate that the oligomer can be promising candidate for prodrugs in treatment and prevention of colorectal cancer.

ÖZET

5-ASA VE YENİ SENTEZLENMİŞ OLİGOMERİNİN ANTİPROLİFERATİF VE ANTİKANSEROJENİK ETKİLERİ

En yaygın ölümcül hastalıklardan biri olan kolorektal kanser dünya çapında kanserle ilgili ölümlerde dördücü sırada yer almaktadır. Son yapılan epidemolojik çalışmalara göre inflamatuar bağırsak hastalıkları tedavisinde kullanılan 5-aminosalisilik asit (5-ASA) ilacının düzenli alımı, ülseratif kolit hastalarında oluşan kolorektal kanser riskini azalttığı ileri sürülmüştür. Birçok kanser tipinde eksprese olan siklooksijinaz (COX) enzimlerini inhibe etme özelliğinden bağımsız olan çeşitli etki mekanizması öne sürülmektedir. Son yıllarda biyoaktif birimi içeren, devamlı salınım özelliği ile uzun süreli etkinliğe sahip olan ve bunun sonucunda daha az toksisite göstermesi gibi özelliklere sahip polimerik ön ilaçlar geliştirme çalışmaları yürütülmektedir. Bununla birlikte, bu polimlerlerin büyük moleküler ağırlığa ve uzun monomer zincirlerine sahip olması, hücre zarı içine nüfuz etmede engel olabilir. Bu nedenle, daha kısa monomer birimi ve daha düşük moleküler ağırlıklı oligomerler öncelikli tercih olabilir. Bu çalışmada yeni sentezlenmiş 5-ASA bazlı oligomerin 5-ASA'ya kıyasla Caco-2, DLD-1, HeLa ve CCD-18Co hücrelerindeki antiproliferatif ve antikanserojenik etkileri değerlendirilmiştir. MTT, apoptotik indeks, hücre döngüsü faz dağılımı ve yara tamiri testleri sonucunda, oligomer 5-ASA monomerine göre düşük dozlarda daha yüksek aktivite gösterdiği görülmüştür. Oligomer, kolorektal kanser hücrelerinde ve HeLa hücrelerinde, hücre ölümünü ve hücre döngüsünde faz birikimini indükte etmiştir. 5-ASA ve oligomere maruz kalan normal insan kolon hücrelerinde, CCD-18Co'nun kontrol grubuna kıyası sonucunda apoptotik hücre sayısında ve hücre döngüsü faz dağılımında anlamlı fark görülmemiştir. Genel olarak sonuçlar, oligomerin kolorektal kanserin tedavisinde ve önlenmesinde polimerik ön ilaçlara bir aday olabileceğine işaret etmektedir.

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LIST OF ABBREVIATIONS

CRC	Colorectal Cancer
COX	Cyclooxygenase
PG	Prostaglandin
PGE2	Prostaglandin E2
VEGF	Vascular Endothelial Growth Factor
FAP	Familial Adenomatous Polyposis
HNPCC	Hereditary Non-Polyposis Colorectal Cancer Syndrome
IBD	Inflammatory Bowel Disease
CR	Crohn's Disease
UC	Ulcerative Colitis
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
5-ASA	5-Aminosalicylic Acid
EGFR	Epidermal Growth Factor Receptor
NF-kB	Nuclear Factor kappa B
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal Bovine Serum
RPMI-1640	Roswell Park Memorial Institute-1640
PBS	Phosphate Buffering Saline
DMSO	Dimethyl Sulfoxide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NADH	Nicotinamide adenine dinucleotide
PS	Phosphatidylserine
PI	Propidium Iodide
IC50	Half Maximal Inhibitory Concentration

CHAPTER 1

INTRODUCTION

1.1. Cancer

Cancer is a leading health issue in worldwide causing 8.2 million deaths in 2012 (Torre et al. 2015) and this number is expected to increase more in the future. There are more than 200 diverse cancer types have been defined that can be categorized into four main forms. Carcinomas are most common form of cancer (90%) that originates from epithelial cells. Other forms described as Sarcomas, Leukemias and Lympomas.

Opposed to normal cells which divide and die in a programmed way, cancerous cells divide without stopping which is initiated due to genetic changes in the cell. This mass of abnormal cells that accumulated within the tissue leads to inflammation and damage in the body. In addition, cancerous cells acquire some properties that differentiate from normal cells which are known as hallmarks of cancer. Hanahan and Weinberg summarized them as autonomy in growths signals, insensitivity to growth-inhibitory signals, avoidance of programmed cell death, unlimited replicative potential, continuous angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000)

1.1.1. Cervical Cancer

Cervical cancer is the fourth commonly diagnosed cancer worldwide for females causing around 265,700 deaths (Torre et al. 2015). It is characterized with abnormal cells growth on the cervix out of control. Human papillomavirus (HPV) is accepted as a major risk factor cervical carcinogenesis.

There are researches reported that expression of COX-2 enzyme associated with cervical cancer development and progression (Kim et al. 2013, Kulkarni et al. 2001). The role of COX-2 in cervical neoplasm is suggested as chronic infection in cervical

epithelium increases PGE2 by COX-2 which inhibits E-cadherin, increase cell proliferation and expression of VEGF (Kim et al. 2013).

1.1.2. Colorectal Cancer

In the world, colorectal cancer (CRC) is third common malignant diseases coming after lung and breast. According to global cancer statistics, it is the fourth leading cause of cancer-related death throughout the world, almost 693,900 deaths in 2012 (Torre et al. 2015) which covers the range between large intestine and rectum**.**

Development of CRC usually starts from a "polyp", a non-cancerous growth on the inner surface of colon but in some cases it can turn into a cancer (Haggar and Boushey 2009). It may also develop from areas of abnormal cells in the lining of the colon or rectum which is called dysplasia (Figure 1.1.). Dysplasia is common in people with inflammatory bowel diseases such as Crohn's disease and ulcerative colitis. Risk factors can be genetic predisposition, environmental factors, and lifestyle choices. CRC carcinogenesis is complex and multistep process associated with molecular and cellular changes within the tissue. However exact mechanisms that promote and sustain colon carcinogenesis are not yet known.

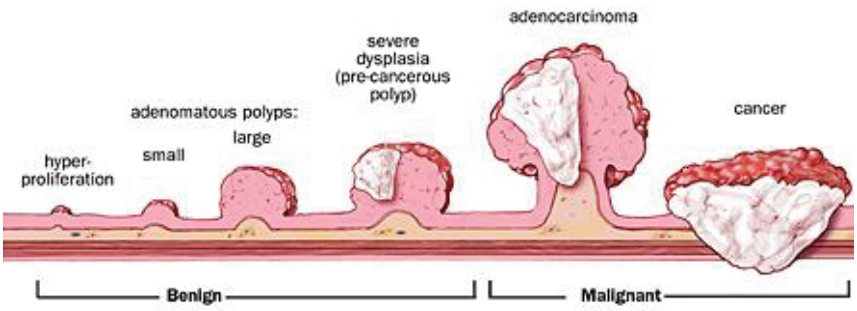

Figure 1.1. Progression of colorectal cancer (CRC)
(Source: Quantified Health, 2013)

1.2. Inflammatory Bowel Diseases

Inflammatory bowel disease (IBD) is an intestinal disorder characterized by abdominal pain, anemia, bleeding, diarrhea, and weight loss. IBD one of the major risk factor for CRC after familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer syndrome (HNPCC) (Haggar and Boushey 2009, Kim and Chang 2014b). The two major types of inflammatory bowel disease are ulcerative colitis (UC) and Crohn's disease (CD). UC a relapsing-remitting condition characterized by chronic inflammation in colon which damages inside the lesions of large intestine. CD involves any segment of the gastrointestinal tract and affects all layers of large intestine (Figure 1.2.).

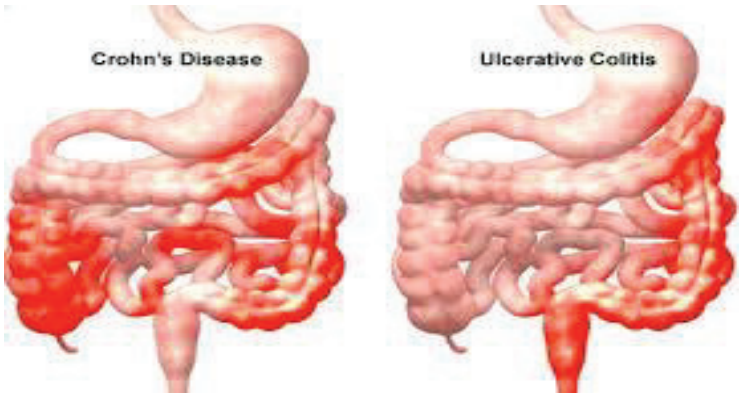

Figure 1.2. Ulcerative colitis and Crohn's disease
(Source: Daller 2016)

According to results meta-analysis 20% of patients with ulcerative colitis will develop CRC over 30 years (Eaden, Abrams, and Mayberry 2001, Janout and Kollárová 2001). Moreover, large-scale nationwide population based studies reported that patients with UC associated CRC have poorer survival than sporadic CRC especially in higher stages (Choi and Zelig 1994, Kim and Chang 2014b). Although, adenoma-carcinoma sequence of colorectal cancer in IBD patients is thought to follow different steps than sporadic CRC, most of the genetic changes associated with development of sporadic CRC also play roles in colitis-associated CRC (Munding et al. 2012, Fearon and Vogelstein 1990, Kim and Chang 2014b). However, the frequency and sequence shows difference in these cancers.

Current cancer prevention in IBD patients with dysplasia uses surveillance colonoscopy (removal of adenomas) and proctocolectomy (removal of part of the colon) (Kim et al. 2009). Because of the some limitations and expense this is not ideal approach to colorectal cancer control.

1.3. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs have pain-relieving and anti-inflammatory activity used for treatment of pain, remitting conditions and tissue damage caused by inflammatory diseases. Kim and Chang report that, large population based studies indicates that using non-steroidal anti-inflammatory drugs (NSAIDs) decrease the risk of CRC in IBD patients up to 40%-50%(Kim and Chang 2014b). Chemopreventive effects of NSAIDs in CRC revealed by more than 200 well-conducted, randomized, placebo-controlled animal studies in which NSAIDs decreased tumors numbers in significant level (Half and Arber 2009). The association between NSAIDs and CRC is a complex phenomenon. The common mechanism of action of NSAIDs is inhibition of cyclooxygenase activity, especially COX-2 activity which is induced by inflammation. Previous studies have shown that expression of COX-2 is upregulated almost in 50% of colorectal adenomas and in 80-90% of carcinomas (Tsujii, Kawano, and DuBois 1997, Stolfi, Fina, Caruso, Caprioli, Sarra, et al. 2008). Thus, protective effect of these drugs in CRC is assumed as down regulation of COX-2 enzyme. However some in vitro studies indicates that NSAIDs can inhibit proliferation and induce apoptosis in COX-deficient CRC cells. This points out that anticancer activity of NSAIDs relatively dependent on COX-2 pathway (Stolfi et al. 2013).

NSAID therapy is limited due to side effects in gastrointestinal tract (GI), cause ulceration and bleeding in GI (Fina et al. 2006). These side effects caused by inhibition of COX-1, which is essential for physological functions like protection of gastric mucosa, platelet aggregation, and regulation of renal function(Stolfi et al. 2013). Recently, 5-aminosalicylic acid (5-ASA) studied extensively as chemopreventive drug in IBD related CRC, which has few side effects compared to other NSAIDs and systemic resorption (Munding et al. 2012).

1.4. 5-Aminosalicylic Acid (5-ASA)

5-Aminosalicylic acid (also known as mesalamine, mesalazine) is the main metabolite of sulphasalazine, balsalazide, and olsalazine (Figure 1.3.). It is an anti-inflammatory drug, mostly well-tolerated that can prevent intestinal inflammation and induce mucosal healing which is mostly prescribed to patients with ulcerative colitis. The acidic properties of salicylates enable them to have high affinity toward sites of inflammation (Koelink, Mieremet-Ooms, et al. 2010, Kim et al. 2009). Recent epidemiological and experimental studies, in vitro and vivo reported that 5ASA can reduce risk of CRC development in colitis patients (Koelink, Hawinkels, et al. 2010, Eaden et al. 2000, Rubin et al. 2006, Stolfi et al. 2013). Meta-analysis of three cohort and six case-control studies that included 1932 UC patients reported that use of 5-ASA in UC was associated with a reduced risk of CRC, and the benefit observed with regular use of at least 1.2 g/day of 5-ASA (Velayos, Terdiman, and Walsh 2005, Kim and Chang 2014a).

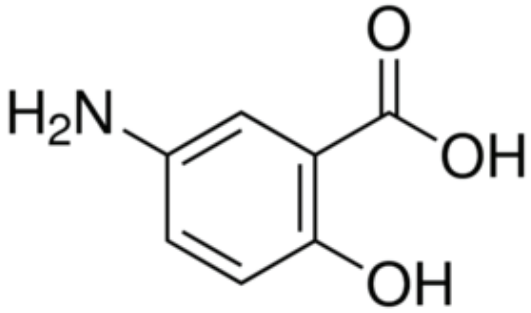

Figure 1.3. Molecular structure of 5-aminosalicylic acid
(Source: Sigma, 2017)

At the cellular level, several mechanisms of 5-ASA investigated but exact relation with cancer development is not clear yet. Thus, beside IBD related CRC, it can also target CRC cells and inhibit pathways that are involved in sporadic CRC cell growth and survival (Stolfi, Pallone, and Monteleone 2012). Therefore, mechanism of 5-ASA in CRC can be classified as COX-dependent and COX- independent pathways.

1.4.1. COX- Dependent and Independent Mechanism of Action of 5-ASA

Cyclooxygenase (COX) enzymes involved in the conversion of arachidonic acid into prostaglandin (PG) G2 which is subsequently converted to various physiologically active PGs (PGE2, PGD2, PGF2, PGI2 and thromboxane A2) (Figure 1.4.). There are two isoforms of COX have been identified so far. COX-1 is expressed constitutively in many tissues and have role in synthesis of PGs. While COX-2 is induced by inflammatory cytokines, growth factors and tumor promoters and identified in most inflammation and neoplastic sites (Kim et al. 2013).

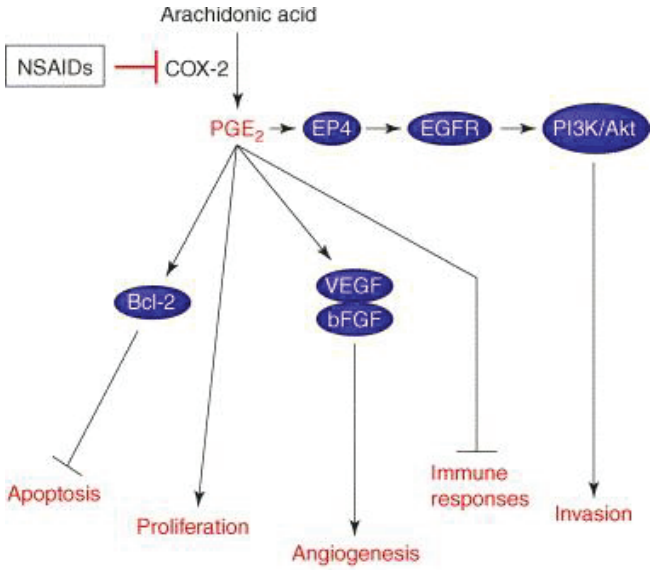

Figure 1.4. The relationship between COX-2 pathway and cancer progression (Source: Riss et al. 2004)

The mechanism of 5ASA in IBD associated CRC patients is may be related to inhibition of pro-inflammatory pathways by downregulating the COX-2/ PGE2 axis, which is assumed as basic mechanism of 5-ASA in preventing UC related CRC. However, mesalamine showed anti-proliferative effects in COX-2 deficient cells which raises possibility of additional mechanisms is involved. (Stolfi, Fina, Caruso, Caprioli, Sarra, et al. 2008).

Epidermal growth factor receptors (EGFRs) are a large family of receptor tyrosine kinases (TK) expressed in several types of cancer, including IBD-associated colorectal cancer (Svrcek et al. 2007). Activated EGFR has shown to trigger mitogenic

and pro-survival signals (Wells 1999). Study by Monteleono et al. showed that mesalamine exposure inhibits EGFR activation in CRC cell lines (Monteleone et al. 2006).

Nuclear Factor kappa B (NF-κB) is a DNA transcriptional factor found in most mammalian cells. Normally NF-κB is inactive with its inhibitory protein. Proinflammatory cytokines, such as TNF a, result phosphorylation of this inhibitory protein and release of NF-κB for nuclear translocation. NF-κB activation is lead to suppress expression of genes that are participate in the development and progression of cancer and enhancing transcription of proinflammatory cytokines and adhesion molecules (Karin, Yamamoto, and Wang 2004, Kim et al. 2009). Recently, it is reported that 5-ASA decreases transcriptional activity of NF-κB (Kaiser, Yan, and Polk 1999, Kim et al. 2009, Saber et al. 2016, Yan and Polk 1999).

One of the key regulating pathways in adenoma initiation and progression is the Wnt/ß-catenin signaling pathway. Another proposed COX-independent mechanism is stimulating B-catenin degradation by interfering Wnt-B/catenin pathway, which is constitutively activated in most of CRC cells (Parenti et al. 2010, Khare et al. 2013, Munding et al. 2012).

Proliferative-activated receptor gamma is key transcriptional factor (PPAR-γ) in tumor cells and activation of PPAR-γ stimulates several pathways including cellular proliferation, differentiation, apoptosis and cell migration. PPARγ is highly expressed in colonic epithelium (Tachibana et al. 2008, Brockman, Gupta, and Dubois 1998) and 5-ASA has been identified to act as an agonist of PPARy. The study by Rousseaux et al. states that 5-ASA increases PPARγ expression and its activation in colorectal cancer cells by inducing its translocation to the nucleus thus therapeutic action of 5-ASA can be related to activation of PPAR-γ (Rousseaux et al. 2005). Thus, inhibition of cancerous cell growth is partly due to the induction of apoptosis via PPARγ activation (Schwab et al. 2008).

Activating checkpoint responses is one of the major mechanisms that regulate cellular proliferation which is activated in case of DNA damage and incomplete DNA replication. Recent lines of research suggested that ASA inhibits cell cycle progression in CRC cells through activation of a replication checkpoint (Reinacher-Schick et al. 2003, Luciani et al. 2007b, Koelink, Mieremet-Ooms, et al. 2010). Lucian et al. reported that 5-ASA reduces the mutation rate and replication fidelity is by acting on the replication machinery (Luciani et al. 2007a).

All these effects of 5-ASA is independent from its weak cyclooxygenase (COX) inhibitory property.

1.5. Cancer Treatment and Chemotherapy

Cancer therapy is highly dependent on stage of the disease at diagnosis. The most common treatments for cancer are surgery, chemotherapy, and radiation. Surgery can be used for solid tumors in which some or all of the part of body is removed. However it is not useful for all type of cancer such as leukemia.

Chemotherapy is the use of natural or synthetic substances to reduce the risk of developing cancer or to reduce the risk of cancer recurrence (Ritland et al. 1999).This therapy is more efficient for cancer that has spread. In clinic, chemotherapy for cancers has been used for nearly half a century.

The ideal chemotherapeutic agent is one that has proven to be effective, has a convenient dosing, has low toxicity in high-risk populations, and should be inexpensive.

1.5.1. Polymeric Drugs

. The aim in new drug designs is to enhance the drug specificity and to increase the duration of its action. In recent years, many polymeric and conjugated pro-drugs have been developed in which lowered toxicity, volume of distribution and prolong the blood concentrations comparable to the free drug given alone (Liso et al. 1996). This phenomenon proposes that prodrugs have ability to release the drug in a controlled way, allow for site-specific drug targeting and reduced side effects which may be occurred if the drug is released immediately (Khandare and Minko 2006). In most cases, drug is conjugated to a polymer with a linker which limits the drug loading. Lately, polymeric prodrugs developed which have biologically active groups in the polymer main chain. They have higher percentage of deliverable drug that is obtained as the polymer degrades. 5-Aminosalicylic acid based poly(anhydride-esters) can be give as an example of this kind of prodrugs that yield salicylic acid and its derivatives as it undergoes hydrolytic degradation (Erdmann and Uhrich 2000). However, large chain molecules have less ability to penetrate the cell membrane compared to small

molecules. In this case, oligomers which have relatively smaller sizes compared to polymers can be advantageous due to smaller molecular weight and higher ability to penetrate cell membrane through membrane proteins. Studies indicated that oligomeric form of phenolic compounds presented strong bioactivity compared to monomers and polymers (Dorenkott et al. 2014, Ito et al. 2003, Hamada et al. 2015).

1.6. Aim of the Study

Colorectal cancer has a natural history of transition from an adenomatous polyp to cancer that takes nearly 10 -20 years which gives opportunity for intervention and prevention. Although wide number of new pharmacological agents introduced in clinical for cancer therapy, there is need for discovery of new natural agents with anticancer properties that can selectively induce apoptosis in the cancer cells. Therefore, the aim of this study to investigate antiproliferative and anticancerogenic effects of novel synthesized 5-ASA based oligomer on Caco-2, DLD-, HeLa and CCD-18Co cells. Also, to investigate activity of the oligomer compared to monomer, 5-ASA.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Medias

All medias used in this study listed in Appendix A

2.1.2. Cell Lines

Human colorectal carcinoma cell lines Caco-2, DLD-1 and normal human colon myofibroblasts CCD-18Co were provided by Hülya Ayar-Kayalı laboratory were maintained and grown at Izmir International Biomedicine and Genome Institute. Cervical cancer cell line, HeLa was obtained from Biotechnology and Bioengineering Research Center, İzmir Institute of Technology.

2.1.2.1. Caco-2 Cell Line

Caco-2 (Cancer coli-2) was established from a human colorectal adenocarcinoma by Jorgen Fogh at the Sloan-Kettering Cancer Research Institute (Fogh, Fogh, and Orfeo 1977). As most CRC cell lines, Caco-2 cells grow in monolayers in vitro (Figure 2.1.). Although derived from a colon carcinoma they can show enterocyte-like properties such as polarized morphology, express tight junctions, microvilli, and small intestinal hydrolase enzymes (Sambuy et al. 2005). The Caco-2 cell line is highly heterogeneous in culture and contains subpopulations with different morphologies. Consequently, results obtained under similar experimental conditions in different laboratories may not be directly comparable (Sambuy et al. 2005).

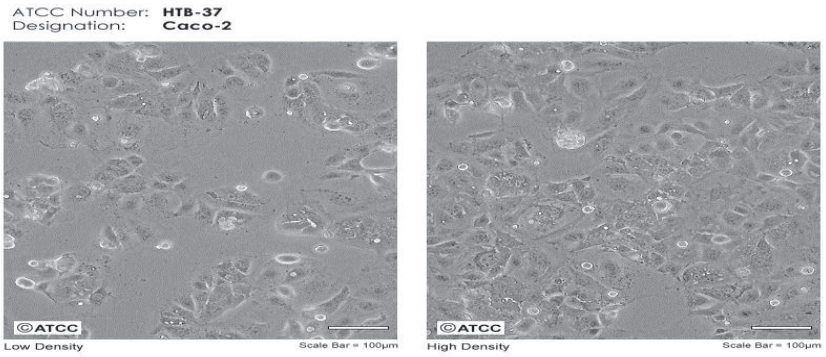

Figure 2.1. Morphology of Caco-2 monolayer. Adapted from ATCC

2.1.2.2. DLD-1 Cell Line

DLD-1cell line established by D. L. Dexter from colon carcinoma tissue of a 45 year old white man (Dexter et al. 1981) . It is an epithelial cell type and has adherent culture property (Figure 2.2.). According genomic researches, DLD-1 found similar genetic characteristics with HCT-15, HCT-8 and HRT-18 cells (Chen et al. 1995, Vermeulen et al. 1998).

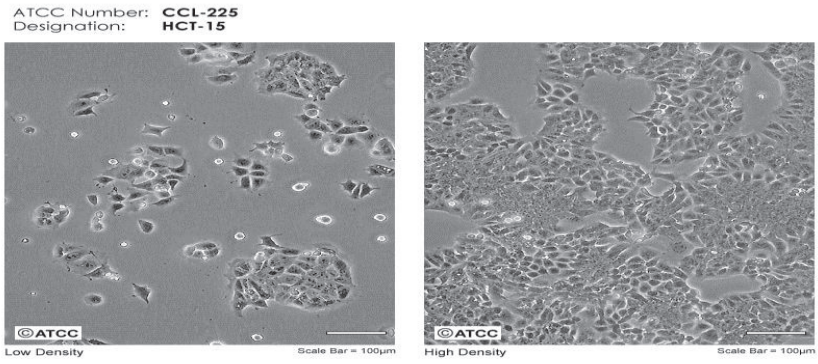

Figure 2.2. Morphology of DLD-1 monolayer. Adapted from ATCC

2.1.2.3. CCD-18Co Cell Line

CCD-18Co is non-diseased, human normal colon myofibrolasts. The cell line isolated from the colon of a female homosapien. The cell line is considered to have fibroblast morphology and its culture growth properties are adherent (Figure 2.3.).

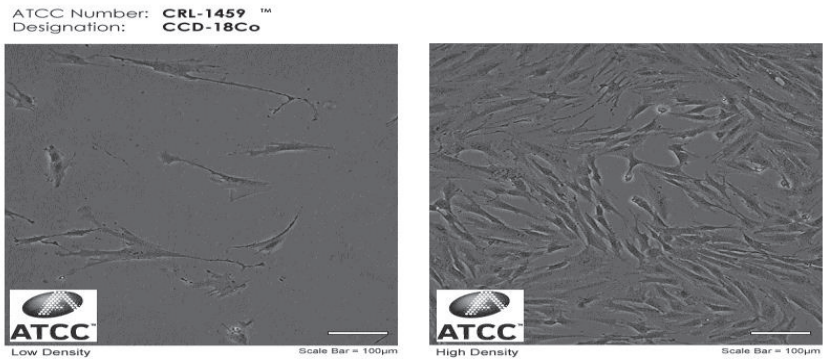

Figure 2.3. Morphology of CCD-18Co Cells. Adapted from ATCC

2.1.2.4. HeLa Cell Line

A HeLa cell line is the first human cell line established in a culture. It is most common used cell line in scientific researches, derived from cervical cancer cells from Henrietta Lacks, a patient who died from cervical cancer(Lucey, Nelson-Rees, and Hutchins 2009). 'HeLa', the first two letters of the patient's first and last name; this became the name of the cell line. It has epithelial morphology and adherent growth property (Figure 2.4.).

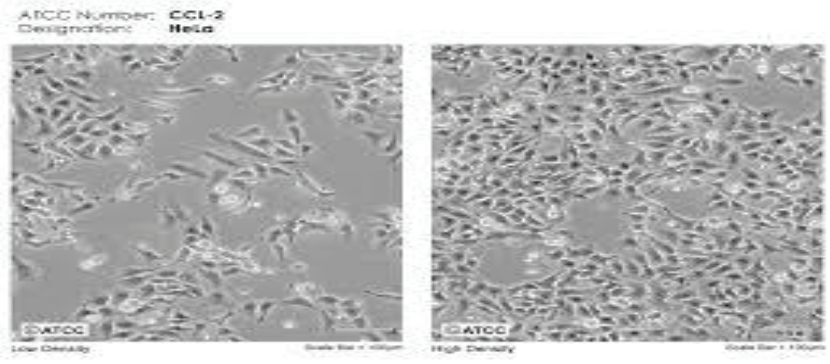

Figure 2.4. Morphology of HeLa Cells. Adapted from ATCC

2.1.3. Drugs

5-Aminosalicylic Acid (5-ASA) and its oligomer provided by Assoc. Prof. Dr. Ali BİLİCİ from Çanakkale Onsekiz Mart University, Faculty of Science, Chemistry Department. The oligomer was synthesized by oxidation polymerization of 5-ASA by using special techniques. Drugs were prepared fresh in culture medium for each study and all experiments carried out protected from direct light.

2.1.4. Reagents and Solutions

Reagents and solutions were presented in Appendix B.

2.2. Methods

2.2.1. Cell culture

Caco-2, CCD-18Co and HeLa cells were grown in Dulbecco's modified Eagle's(DMEM) medium supplemented with %10 fetal bovine serum (FBS), 100IU/ml penicillin G, 100g/ml streptomycin and 2mM glutamine. DLD-1 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) growth medium containing 10% fetal bovine serum (FBS) and 1% antibiotic solution (100IU/ml penicillin G, 100g/ml streptomycin). All cells were incubated in humidified 37°C, 5% CO_2 incubator.

2.2.2. Passaging of Cell Lines

Cultures examined daily to ensure they are healthy and absence of contamination by microscope. Confluence should be kept around 70-80% (log phase of growth). Medium was renewed 2-3 times per week and sub-cultured once confluent. In order to sub-culture first fresh culture medium, trypsin and PBS warmed at 37°C. Old medium was carefully discarded from tissue culture flask and the surface of monolayer was washed with of Phosphate Buffering Saline (PBS) to get rid of any FBS residual in

the flask, using approximately half volume of culture medium. After that Trypsin-EDTA was added to cover bottom surface of the flask (1 ml per 25cm^2 surface area) and incubated in 37°C incubator until cell layer detached. In order to avoid over-trypsinization which can damage cells, they were checked every few minutes. When all cells detached fresh medium was added to inactivate the trypsin. Cell suspension collected into the 15 ml sterile falcon tube and centrifuged at 800 rpm for 5 minutes at room temperature. After centrifugation, supernatant was removed from the falcon and the pellet was re-suspended with 2 mL of growth medium, RPMI-1640 (10% FBS and 1% antibiotic solution) for DLD-1 and DMEM (10% FBS, 1% antibiotic solution and 2mM glutamine) for Caco-2, HeLa and CCD-18Co. It was transferred into a sterile filtered tissue culture flask in 1:2 to 1:10 sub-cultivation ratio and growth medium pre-warmed at 37^{0}C added (Table 2.1.). Then it was incubated in humidified incubator 5% CO2 at 37°C.

Table 2.1. Generally used amount of media and Trypsin/EDTA in cell sub-culturing

	Trypsin/EDTA	**Growth Medium**
25 cm^2 flask	1 ml	5 ml
75cm^2 flask	3 ml	15 ml
150cm^2 flask	5 ml	25 ml

2.2.3. Thawing the Frozen Cells

It is important to properly thaw cell to maintain viability and recover of the culture. Some cryoprotectants (i.e. DMSO) can be toxic above +4^{0}C. Thus, cells (1ml) in cryogenic vial were removed from frozen liquid nitrogen storage at -80°C and quickly (1-2 min) thawed with agitation in a water bath at 37°C. When small amount of ice crystals left, outside the tube wiped with 70% ethanol and transferred immediately into sterile falcon tube(15ml) diluted with culture media in a 10-fold volume, diluting cell suspension in order to decrease toxicity of dimethyl sulfoxide (DMSO) which is added as cryoprotectant. Then, cell suspension centrifuged at 800 rpm for 5 minutes at +4°C. The supernatant was removed from the tube and cells were re-suspended in 1-2 ml of fresh medium. All content was transferred into a sterile filtered tissue culture

flask (25cm2) containing 5 ml of RPMI-1640 growth medium or DMEM and incubated at 37°C in 5% CO2. After incubation for 24h, cells were passaged as mentioned before.

2.2.4. Freezing the Cells

All cell lines in continuous cultures are under contamination risk. Therefore it is important that the cells are stored by frozen down to prevent the contamination risk and enable to keep for long-term storage. For freezing the cells, firstly check confluence and absence of contamination. Culture media pour off and cell layer was washed with PBS. Then, trypsinized and centrifuged at 800 rpm for 5 minutes at room temperature. After centrifugation, the supernatant removed and the pellet re-suspended with freezing medium (Table 2.2). Then, gentle pipetting was applied and the 1ml cell suspension was transferred to the cryogenic vials (2 ml) by labeling. At the following step, these cryogenic vials were incubated at for 1 hour at +4°C then at -20°C and finally transferred to -80°C N_2 freezer for long-term storage.

Table 2.2. Generally used freezing medium

Growth medium (DMEM/RPMI)	7 ml
Fetal Bovine Serum (FBS)	2 ml
Dimethyl Sulfoxide (DMSO)	1 ml

2.2.5. Determination Cell Number and Viability with Trypan Blue Dye

Determining number of cell in culture is vital for standardization of culture conditions and for carrying accurate quantitation experiments. First cell suspension prepared by detaching cells from flask surface by trypsinization, centrifuging and re-suspending in fresh medium. Then cell suspension was diluted with trypan blue dye solution (0,4%w/v in PBS) in 10 fold basically 900 µl of trypan blue solution added to 100 µl of cell suspension. When cells were treated with trypan blue dye, viable cells can be observed under microscope as round and unstained. Nonviable cells would take up the dye due to breakdown in membrane integrity thus they would be observed as blue

stained cells under a microscope. Cell suspension diluted in trypan blue is transferred to hemacytometer counting chamber and viable cell counted under microscope.

Number of cell /ml = average counted cell number x dilution factor (10) x 10^4

% viable cell = (number of unstained cell/ total number of cell) x 100

2.2.6. MTT Cell Viability Assay

Calorimetric essays are convenient and high throughput screening essays for determining effect of tested molecule on cell proliferation and cytotoxicity effects. There are several types of tetrazolium compounds have been used to detect viable cells such as MTT, MTS, XTT and WST-1 (Riss et al. 2004). The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first calorimetric cell viability assay developed by Mosmann for a 96-well format(Mosmann 1983). Viable cells with active metabolism convert MTT into a colored formazan product which is directly proportional to number of viable cells. Absorbance recorded at 570 nm by plate reading spectrophotometer is used to estimate number of live cells. The exact cellular mechanism of MTT reduction to formazan is not known but it is hypothesized as involving mitochondrial reductase NADH molecule (Figure 2.5.).

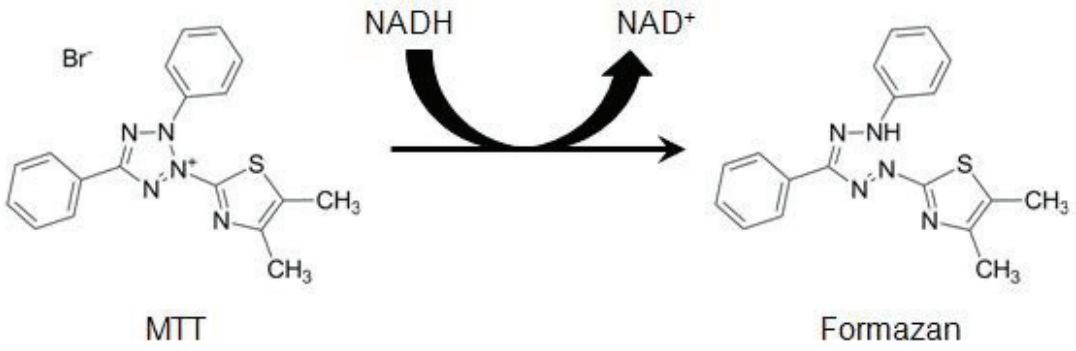

Figure 2.5. Structures of MTT and colored formazan product
(Source: Riss et al. 2004)

To perform MTT assay, $3x10^3$ of HeLa cells, $1x10^4$ of DLD-1cells, $5x10^3$ of Caco-2 and CCD-18Co cells per well were seeded into 96-well flat bottom cell culture plates(Greiner Bio) at and allowed to adhere for 24 h. The next, cells incubated with 150 µl medium with and without 5-ASA (50-13000µM) and the oligomer (50-1000µM) for 24, 48 and 72 hours. At the end of each incubation period medium with drugs was removed carefully, replaced with MTT-containing medium (final concentration is 0.5mg/ml) and incubated further for 3-4 hours at 37 °C. Then plate was centrifuged at 1800 rpm for 10 minutes at room temperature. The medium discarded and precipitated blue formazan crystals at the bottom of the flask solubilized with DMSO in a shaker (150rpm, room temperature) for 10-15 min. The quantity of formazan is measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer (Varioscan Flash). Wells containing only medium used as blank and absorbance of cells without drugs were taken as 100 % cell viability (i.e. the control). The experiment repeated three times and each concentration was used in triplicate. IC50 values, the concentration of drug that inhibits 50% of cell proliferation were calculated by using GraphPad Prism 6.01 computer program (GraphPad Software Inc., USA).

2.2.7. Detection and Quantification of Apoptosis

Every day billions of cells divide and die in programmed way which is controlled by strict mechanisms. Apoptosis is one of the hypothesized cell deaths which is potential target for cancer therapy during the past 30 years. It is characterized by DNA fragmentation, cell shrinkage, blabbing of the plasma membrane, formation of apoptotic bodies and exposure of the phospholipid phosphatidylserine (PS) to the outer cell membrane (Kroemer et al. 2009, Lauber et al. 2004).

Autophagic cell death, another type of cell death, is characterized by a massive accumulation of double-membrane containing vacuoles known as autophagosomes, which subsequently fuse with lysosome vacuoles. Type III cell death, better known as necrosis, is usually considered to be an uncontrolled and accidental cell death which, unlike apoptosis, is not energy-dependent (Kroemer et al. 2009).

One of the acquired characteristics of cancerous cells is evasion of programmed cell death mechanism and continuing for division (Hanahan and

Weinberg 2000). Designing molecules which targets activation of apoptotic pathways could result efficient therapy in cancer treatment.

Annexin-V, a calcium-dependent phospholipid-binding protein, has a high affinity for PS(Lizarbe et al. 2013). Although it will not bind to normal living cells, Annexin-V will bind to the PS exposed on the surface of apoptotic which can be analyzed with flow cytometer. Annexin-V for detecting apoptotic cells has advantages such as sensitivity and rapidity; the disadvantage is that necrotic cells also be labeled due to breakdown of their plasma membrane. Therefore, propidium iodide (PI) which is membrane-impermeable DNA dye is used as control. Thus, healthy cells are negative to both annexin-V and propidium iodide, whereas cells in the early phases of apoptosis are annexin-V-positive but propidium iodide-negative, and necrotic/late apoptotic cells are doubly positive to annexin-V and propidium iodide (Figure 2.6.).

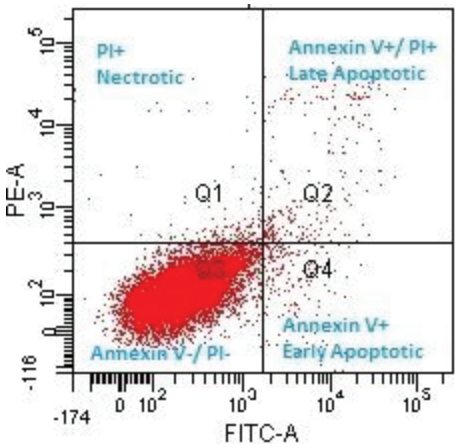

Figure 2.6. FITC Annexin V/ PI staining and analysis in flow cytometer.

Detection and quantification of apoptosis induced by 5-ASA and its oligomer on HeLa, DLD-1, Caco-2, CCD-18Co cells Annexin V- FITC detection kit used (BioLegend). Cells seeded at density $2x10^5$-$3x10^5$ cells/well in a 6-well plate and incubated for 24h for cells adhere. After incubation 5-ASA and the oligomer dissolved in medium and added at concentrations of above and below the IC50 and incubated at 37°C in 5% CO2 for 48 hours. Untreated cells were used as a control group. At the end of the incubation period the cells in the wells collected to 15 ml falcon tubes by trypsinization and centrifuged at 800 rpm for 5 minutes. Then supernatant discarded carefully and the pellet was dissolved in 5 ml of PBS and centrifuged again to wash out. PBS discarded and pellet was re-suspended in 100 µL of Annexin V Binding Buffer and transferred to 5 ml tubes. 5 µl of FITC Annexin V and 10 µl Propidium

Iodide (PI) solution was added according to the suggested instruction of manufacturer of the kit. Cells gently vortexed and incubated for 15 minutes at room temperature in the dark. Finally, 400 µL of Annexin V binding buffer added to each tube and analyzed by flow cytometer (FACSCanto, BD).

2.2.8. Analysis of Cell- Cycle Phase Distribution

DNA content of cells in pre-replicative phase (G0/1), DNA replicative phase (S phase) and post replicative plus mitotic (G2 + M) phase determines cell cycle distribution of cells (Darzynkiewicz 2011). It is generally identified by direct quantitative measurement of the DNA with flow cytometer based on staining of DNA with fluorescence dye such as propidium iodide (PI) (Krishan 1975).

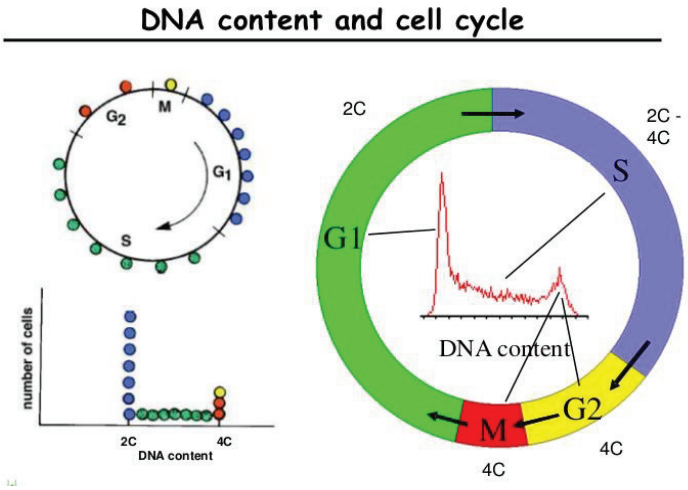

Figure 2.7. DNA content analysis by flow cytometer
(Source : SlideShare, 2011)

The cell-cycle distribution for control, 5-ASA and its oligomer treated cells were determined after 48 hours of treatment. Cells were seeded into 6-well plates at density of $2x10^5$-$3x10^5$ cells/well and incubated for 24 hours for cells adhere. After incubation 5-ASA and the oligomer dissolved in medium and added at concentrations of above and below the IC50 and incubated at 37°C in 5% CO2 for 48 hours. Untreated cells were used as a control group. At the end of the incubation period the cells in the wells harvested by trypsinization and centrifuged at 1200 rpm for 10 minutes. Then ethanol fixation and PI staining applied according to the protocol by Çakmak Ö. (Çakmak 2011). Supernatant discarded carefully and the pellet was pellet re-suspended in 1 ml of

cold PBS. Tubes were kept on the top of the ice and ethanol fixation carried on by adding 4 ml of ethanol at -20°C slowly on low speed vortex. Ethanol fixed cells transferred to -20°C freezer and allowed at least one night before the analyzing.

Cells in falcon tubes at -20°C centrifuged at 1200 rpm for 10 minutes at 4°C. The pellet was re-suspended in 1 ml cold PBS (4°C) and completed to 5ml by adding 4 ml more PBS. Cell suspension centrifuged again and PBS discarded. The pellet was re-suspended in 200 µl 0.1 % Triton X-100 in PBS. 20µl RNase A (200 µg/ml) was added to cell suspension and incubated in 37°C in for 30 min. At the end of the incubation period 20 µl of PI solution (1mg/ml) was added and incubated at room temperature for another15 min. Subsequently, DNA content was measured by flow cytometer, and cell cycle distribution was calculated using ModFit software for Windows. A minimum 10,000 events were measured for each sample.

2.2.9. Analysis of Migration of Cells by in Vitro Scratch Assay

Metastasis is characterized as one of the hallmarks of the cancerous cells, which is defined as invasion of tumor cells into surrounding tissues(Hanahan and Weinberg 2000). Metastasis of tumor accounts nearly 90% of cancer related deaths. It involves several steps such as loss of adhesion between cells, which results in the separation of the cell from the primary tumor and alterations in cell-matrix interaction which results increased invasive and motility (Jiang et al. 2015). This process is continues by activation secretion of enzymes to degrade the basement membrane, proteolysis and extracellular matrix and also the expression/ suppression of proteins involved in the control of motility and migration (Tracey A. Martin 2013).

One of the members of regulators of cell adhesion and invasion is β-catenin, also involved in of cell signaling and transcription regulation. It was reported that COX2 inhibitors decrease MMP-2 and −9 expression (Saber et al. 2016). Like NSAIDs, 5-ASA inhibits the Wnt/β-catenin pathway which can be associated with inhibition of migration of colorectal cancer cells by the drug (Stolfi et al. 2013).

The scratch wound healing assay widely used to study the effects of molecule tested on cell migration and proliferation. In a typical scratch wound healing assay, a “wound gap” in a cell monolayer is created by scratching, and the “healing” of this gap by cell migration and growth towards the center of the gap is monitored and often

quantitated (Yarrow et al. 2004). Increased or decreased rate of "healing" of the gap can be associated to effects of tested molecule on migration of cells. This assay is simple, inexpensive, and experimental conditions can be easily adjusted for different purposes.

HeLa, Caco-2, DLD-1 and CCD-18Co cells seeded into 6 –well tissue culture plates at density 10^6 /ml. After 24 h of growth, they reached ~70-80% confluence. The monolayer gently and slowly scratched in one direction with a new 1 ml pipette tip across the center of the well. Then wells carefully washed twice with medium to remove the detached cells. Fresh medium with 5-ASA and the oligomer added, for controls only medium. Plate was placed under the fluorescence microscope (Zeiss Z1 Inverted Fluorescence Microscope) and monitored for 48 hours. Photos of monolayer taken on a microscope and quantitatively evaluated using software ImageJ.

2.3. Statistical Analysis

All date obtained from MTT, apoptotic rate, cell cycle phase distribution and wound healing assay were analyzed statistically in GraphPad Prism 6.1 (GraphPad Software Inc., USA). In MTT assays, 24, 48 and 72 hours 5-ASA and the oligomer treated groups were compared with control group. In addition, 24, 48 and 72 hours drug treated groups were compared with each other for both cell lines by using non-linear regression dose response analysis using GraphPad Prism 6.1 (GraphPad Software Inc., USA). P (calculated probability) value less than or equal to 0.05 was considered as statistically significant (**** indicates $p \leq 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, *indicates $p < 0.05$). Differences between the means were analyzed for significance using two-way ANOVA test of GraphPad Prism 6.1 (GraphPad Software Inc., USA) with Dunnett's multiple comparison test which were used to assess the differences between independent groups. The results are expressed as mean ± SEM and the mean values.

CHAPTER 3

RESULTS

3.1. Cytotoxic Activity of 5-ASA and Its Oligomer on Caco-2 Cells

The cytotoxic effects at various concentrations of 5-ASA (200-13000μM) and its oligomer (50-1000μM) at different incubation periods (24, 48 and 72 hours) were evaluated. Using GraphPad Prism 6.1 (GraphPad Software Inc., USA) Figure 3.1. was obtained. Significant cytotoxicity effect of 5-ASA on the cells started at 500 μM (at 72 hours) while this number is much lower in the oligomer (50 μM, at 72 hours). According to MTT results, fraction of surviving cells is decreased with longer incubation time and with increasing concentration. The IC50 concentrations-concentration that inhibits 50% of growth of the cells, were calculated using sigmoidal dose response curve-fitting models (GraphPad Software Inc., USA). Thus, lowest IC50 value is observed at 72 hours incubation period for 5-ASA and its oligomer as shown in Table 3.1. In addition, inhibition concentrations of 5-ASA on Caco-2 cells are in the range of previous findings (Koelink, Mieremet-Ooms, et al. 2010, Saber et al. 2016). Comparing IC50 values, the oligomer was more cytotoxic at lower doses than monomer, 5-ASA at all time periods.

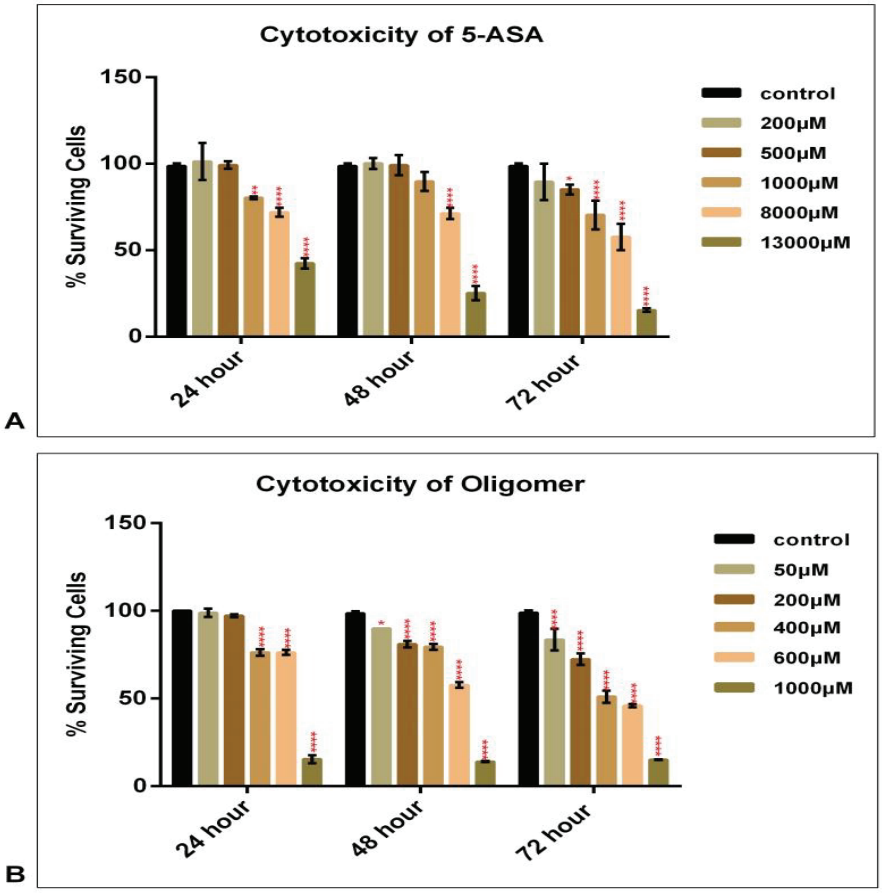

Figure 3.1. Fraction of surviving Caco-2 cells after exposure to 5-ASA and its oligomer (A and B). Caco-2 cells were treated with 200-13000µM of 5-ASA and 50-1000µM of the oligomer for 24, 48 and 72 hours and after incubation, cell viability was assessed using MTT assay. Results are expressed as means ±SEM. Three independent experiments performed at least in triplicate. (**** indicates $p \leq 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).

Table 3.1. Calculated IC50 values at µM for 5-ASA and its oligomer with respect to proliferation in Caco-2 cells

DRUGS	24 hours	48 hours	72 hours
Oligomer	763.8 ± 17.9 (R^2= 0.99)	609.9 ± 50.4 (R^2= 0.90)	366.0 ± 53.4 (R^2= 0.94)
5-ASA	11631 ± 803 (R^2= 0.96)	9969 ± 609 (R^2= 0.95)	2369 ± 179 (R^2= 0.98)

3.2. Cytotoxic Activity of 5-ASA and Its Oligomer on DLD-1 Cells

The cytotoxic effects at various concentrations of 5-ASA (200-13000µM) and its oligomer (50-1000µM) at different incubation periods (24, 48 and 72 hours) were investigated in DLD-1 cells. Figure 3.2. was obtained from GraphPad Prism analysis. Longer incubation time and increasing concentration showed more cytotoxic affects for both 5-ASA and its oligomer. 5-ASA showed lower activity compared to Caco-2 cells while the oligomer inhibited surviving DLD-1 significantly at lower concentrations. Overall, IC50 values calculated for each time period (Table 3.2.) indıcates that the oligomer has higher cytotoxic effects than monomer, 5-ASA at all time periods.

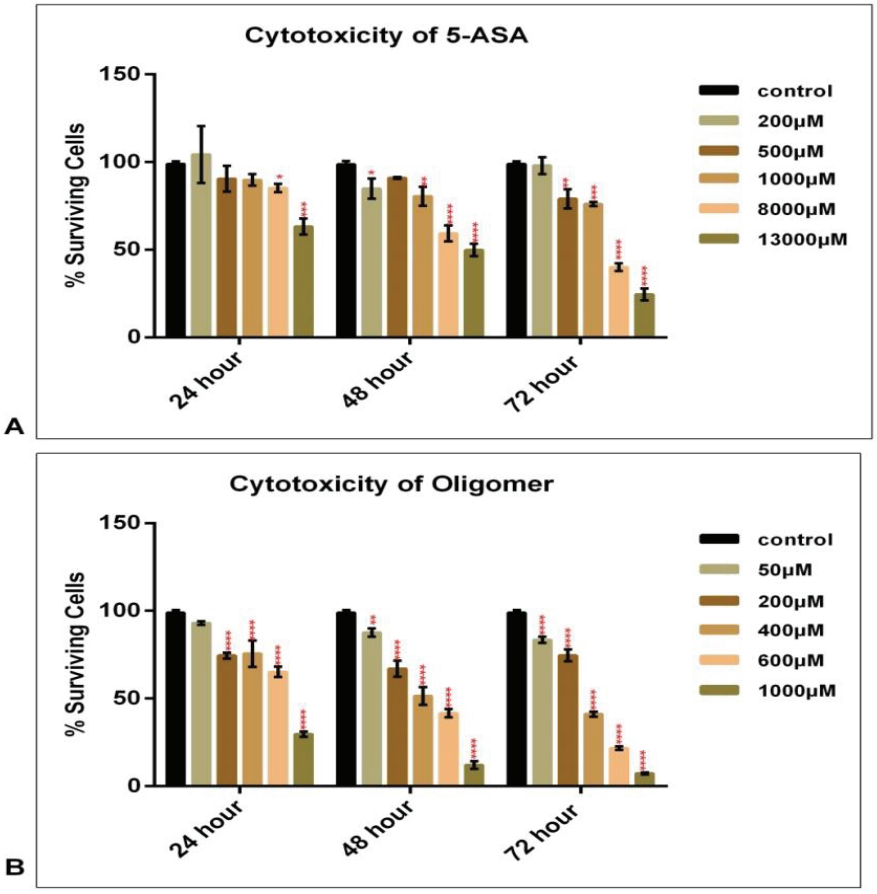

Figure 3.2. Fraction of surviving DLD-1 cells after exposure to 5-ASA and its oligomer (A and B). DLD-1 cells were treated with 200-13000µM of 5-ASA and 50-1000µM of oligomer for 24, 48 and 72 hours and after incubation, cell viability was assessed using MTT assay. Results are expressed as means ±SEM. Three independent experiments performed at least in triplicate. (**** indicates p≤ 0.0001, *** indicates p< 0.001, ** indicates p< 0.01, * indicates p<0.05).

Table 3.2. Calculated IC50 values at μM for 5-ASA and its oligomer with respect to proliferation in DLD-1 cells

DRUGS	24 hours	48 hours	72 hours
Oligomer	343.2 ± 35.8 (R^2= 0.97)	321.4 ± 28.2 (R^2= 0.97)	328.0 ± 13.6 (R^2= 0.99)
5-ASA	17837 ± 1785(R^2= 0.95)	12116 ± 1685(R^2= 0.97)	4713 ± 785 (R^2= 0.93)

3.3. Cytotoxic Activity of 5-ASA and Its Oligomer on HeLa Cells

MTT assay analysis on HeLa cell when treated with 5-ASA (500-25000μM) and its oligomer (50-2000μM) at different incubation periods (24, 48 and 72 hours) was performed. Figure 3.3. and Table 3.3. indicate cytotoxic effects and IC50 concentrations of tested molecules. According to the results, HeLa cells show more resistance to 5-ASA and its oligomer compared to previous two colon cancer cells. However, the oligomer again shows higher inhibition activity compared to monomer.

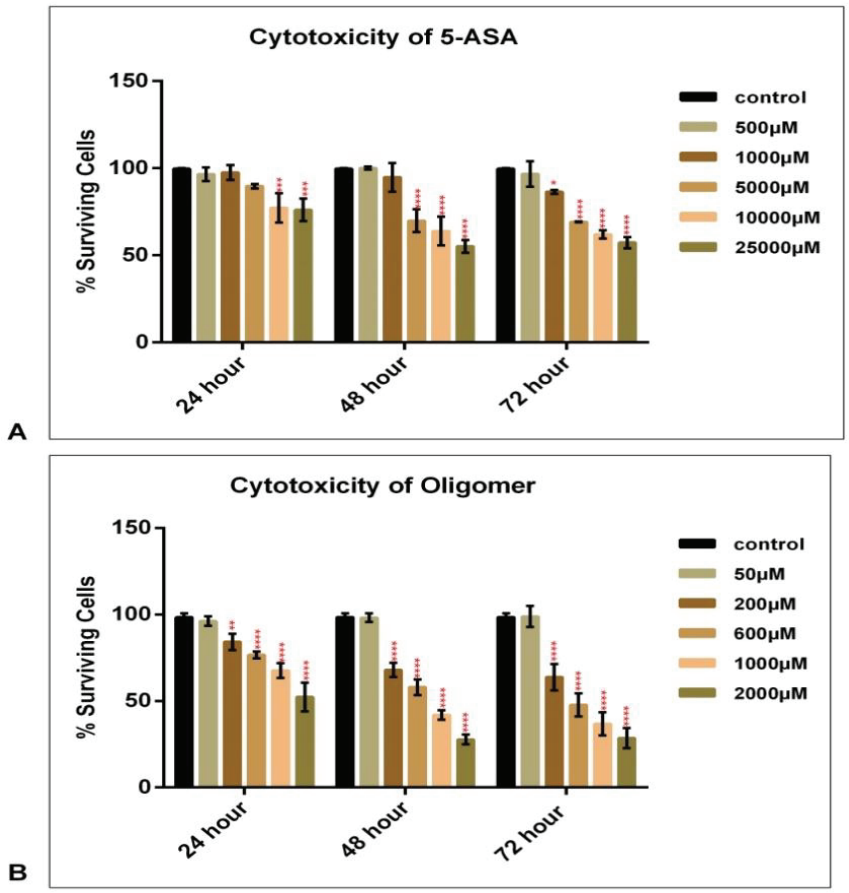

Figure 3.3. Fraction of surviving HeLa cells after exposure to 5-ASA and its oligomer (A and B). HeLa cells were treated with 500-25000µM of 5-ASA and 50-2000µM of oligomer for 24, 48 and 72 hours and after incubation, cell viability was assessed using MTT assay. Results are expressed as means ±SEM. Three independent experiments performed at least in triplicate. (**** indicates $p \leq 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).

Table 3.3. Calculated IC50 values at µM for 5-ASA and its oligomer with respect to proliferation in HeLa cells

DRUGS	24 hours	48 hours	72 hours
Oligomer	1941 ± 319 (R^2= 0.93)	677.1 ± 100 (R^2= 0.96)	608.4 ± 70.8 (R^2= 0.93)
5-ASA	17250 ± 4287(R^2= 0.89)	19571 ± 4533(R^2=0.94)	6599 ± 1278 (R^2= 0.93)

3.4. Cytotoxic Activity of 5-ASA and Its Oligomer on CCD-18Co Cells

The cytotoxic effects of various concentrations of 5-ASA (500-15000µM) and its oligomer (50-1000µM) on normal human myofibroblasts at different incubation periods (24, 48 and 72 hours) were investigated. GraphPad Prism 6.1 generated Figure 3.4. and IC50 values at Table 3.4. Fraction of surviving cells is decreasing with longer incubation time and increasing concentration compared to control group. IC50 values for both molecules (5-ASA and its oligomer) are comparable with DLD-1 MTT assay analysis.

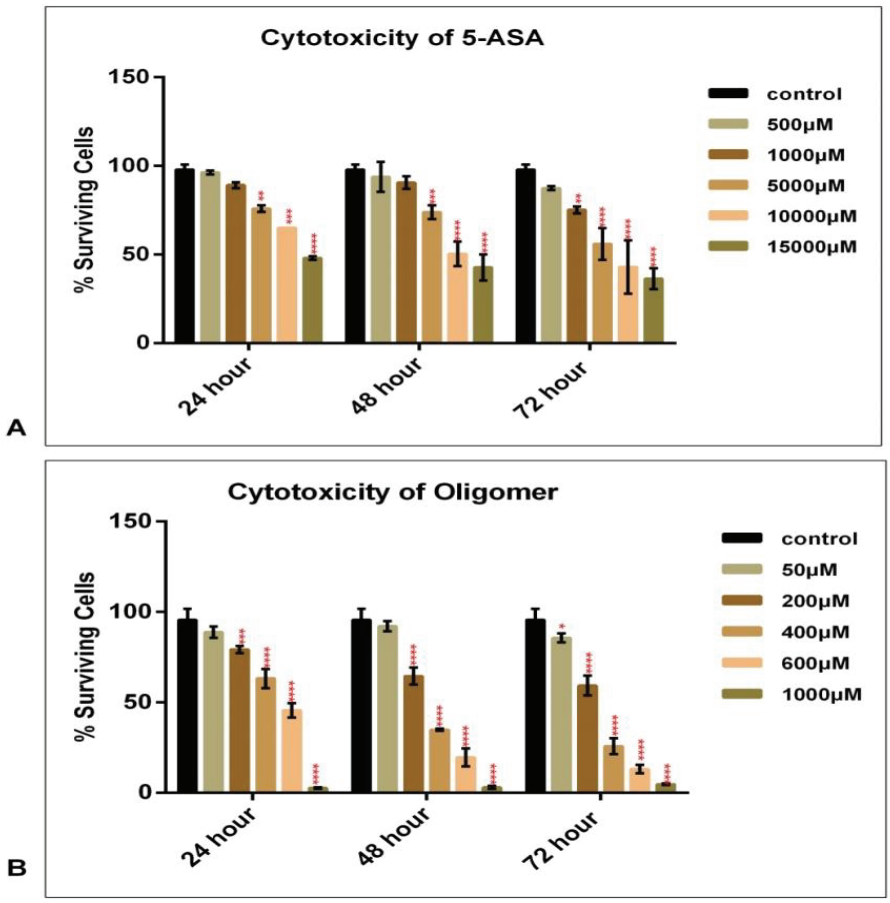

Figure 3.4. Different dose of 5-ASA and its oligomer inhibit cell growth in CCD-18Co cells (A and B). CCD-18Co cells were treated with 500-15000µM of 5-ASA and 50-2000µM of oligomer for 24, 48 and 72 hours and after incubation, cell viability was assessed using MTT assay. Results are expressed as means ±SEM. Two independent experiments performed in triplicate. (**** indicates $p \leq 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).

Table 3.4. Calculated IC50 values at μM for 5-ASA and its oligomer with respect to proliferation in CCD-18Co cells

DRUGS	24 hours	48 hours	72 hours
Oligomer	540.6 ± 106 (R^2= 0.90)	276.6 ± 50 (R^2= 0.91)	213.8 ± 43.2 (R^2= 0.90)
5-ASA	14924 ± 1820(R^2= 0.97)	10782 ± 1448(R^2=0.96)	5335 ± 869 (R^2= 0.94)

3.5. Determination of Apoptotic Index in Caco-2 cells

In order to investigate if 5-ASA and its oligomer induce cell death modes, Flow Cytometry analysis were performed as indicated in section 2.2.7. Caco-2 cells were treated with 5-ASA and the oligomer at IC50 (10000 and 600 μM) for 48 hours. A minimum 10,000 events were measured for each sample and number of dead cells was given as ratio of total events. As demonstrated in Figure 3.5A, compared to 5-ASA, the oligomer was found more potent for its apoptotic efficiency. Thus, the oligomer was further investigated for dose-dependent effects. Increasing the concentration significantly increased percent of cell death in Caco-2 cells compared to untreated group as shown in Figure 3.5B. These results explain the reason of decrease in percent of surviving cells in MTT assay results; 5-ASA and its oligomer induce cell death in Caco-2 cells.

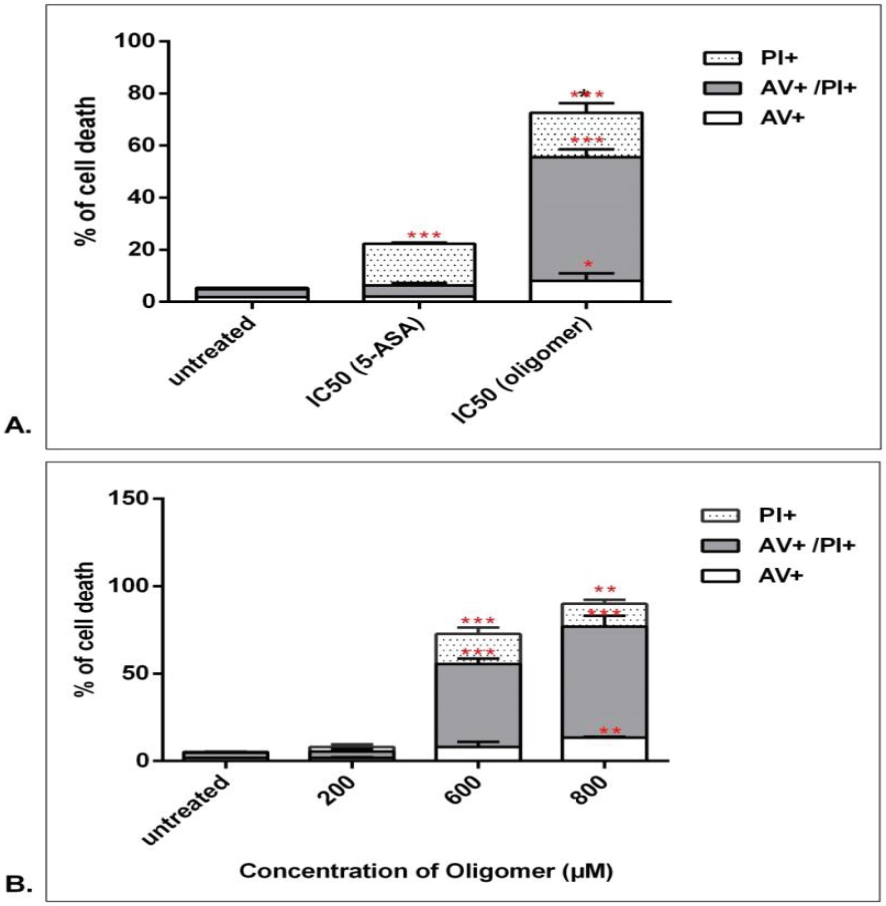

Figure 3.5. Apoptosis induction analysis of Caco-2 cells treated with 5-ASA and its oligomer for 48 hours. Cell death analysis was performed by Flow Cytometry - AnnexinV labeling. **A)** Caco-2 cells were treated with 5-ASA (IC50) and its oligomer (IC50) for 48 hours. **B)** Caco-2 cell treated with the oligomer at 200 µM (below IC50), 600 µM (IC50) and 800µM (above IC50) concentrations for 48 hours. Results are expressed as means ±SD representative of at least two replicates. (**** indicates $p \leq 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).

3.6. Determination of Apoptotic Index in DLD-1 cells

DLD-1 cells were treated with 5-ASA and its oligomer for 48 hours and determination of induced cell death carried on as explained in section 2.2.7. A minimum 8,000 events were measured for each sample and number of dead cells was given as ratio of total events. As demonstrated in Figure 3.6. any significant cell death occurred in 5-ASA treated cells at IC50. However, the oligomer induced dose-dependent apoptosis in DLD-1 cells. These results reconfirm and explain MTT assay results, in which 5-ASA decreased percent of surviving cells at higher doses compared to Caco-2 cells and oligomer showed cytotoxicity in DLD-1 cells at lower doses than Caco-2 cells.

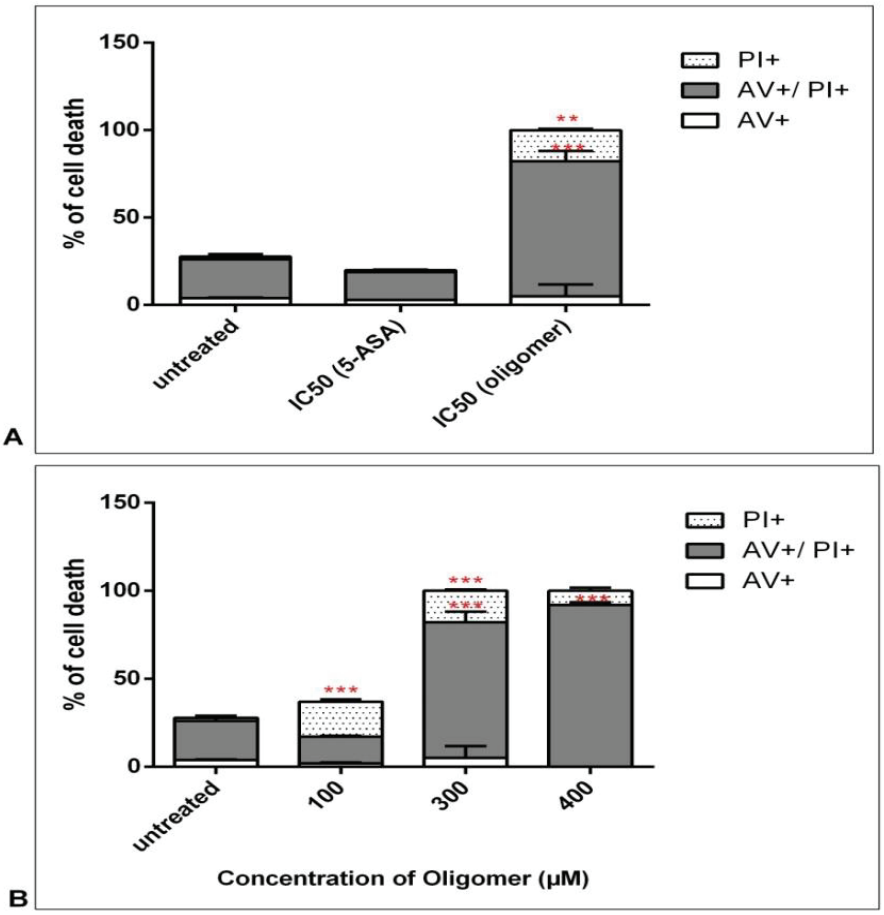

Figure 3.6. Apoptosis induction analysis of DLD-1 cells treated with 5-ASA and its oligomer for 48 hours. Cell death analysis was performed by Flow Cytometry - AnnexinV labeling. **A)** DLD-1 cells were treated with 5-ASA (IC50) and its oligomer (IC50) for 48 hours. **B)** DLD-1cell treated with the oligomer at 100µM (below IC50), 300µM (IC50) and 400µM (above IC50) concentrations for 48 hours. Results are expressed as means ±SD representative of at least two replicates. (**** indicates $p \leq 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).

3.7. Determination of Apoptotic Index in HeLa cells

HeLa cells were exposed to 5-ASA and its oligomer at their IC50 and incubated for 48 hours. A minimum 10,000 events were measured for each sample by using flow cytometry and number of dead cells measured as ratio of total events. As demonstrated in Figure 3.7A, neither 5-ASA nor its oligomer changed percent of cell death significantly compared to untreated group. Only at 800 µM (above IC50) the oligomer significantly showed apoptotic (AV+) and necrotic (PI+) effects.

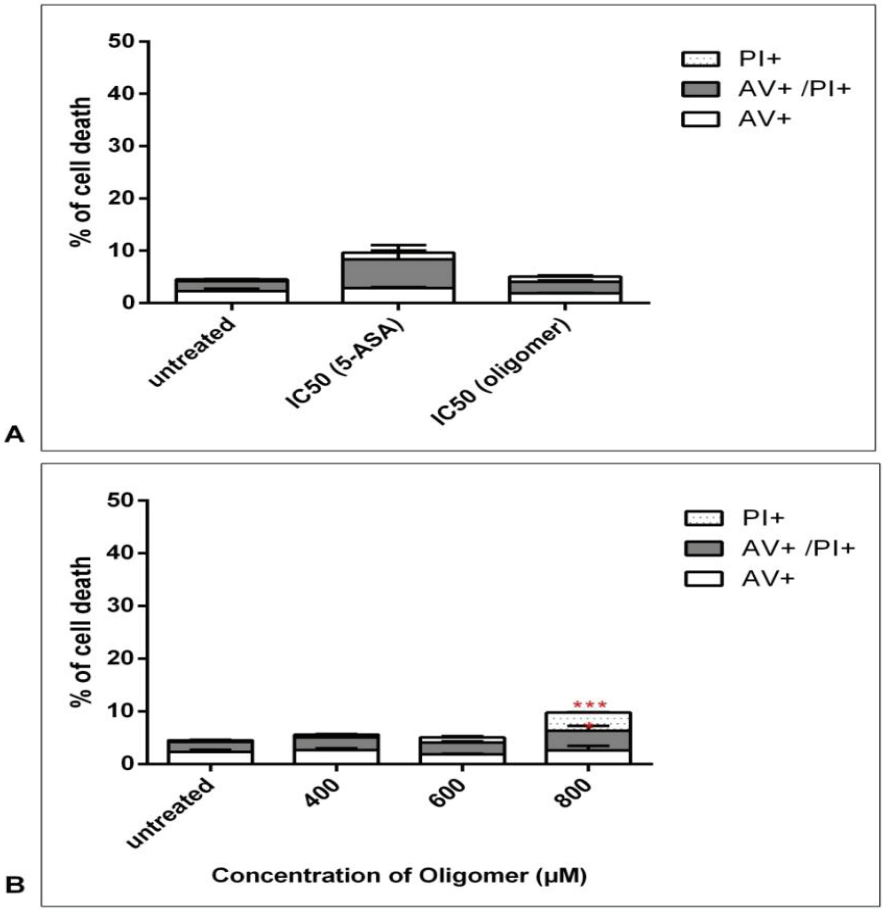

Figure 3.7. Apoptosis induction analysis of HeLa cells treated with 5-ASA and its oligomer for 48 hours. Cell death analysis was performed by Flow Cytometry - AnnexinV labeling. **A)** HeLa cells were treated with 5-ASA (IC50) and its oligomer (IC50) for 48 hours. **B)** HeLa cell treated with the oligomer at 400 µM (below IC50), 600µM (IC50) and 800µM (above IC50) concentrations for 48 hours. Results are expressed as means ±SD representative of at least two replicates. (**** indicates $p \leq 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).

3.8. Determination of Apoptotic Index in CCD-18Co cells

Flow Cytometry analysis were performed on normal human myofibroblast cells as indicated in section 2.2.7. A minimum 9,000 events were measured for each sample. At IC50 both 5-ASA (10000µM) and its oligomer (250µM) did not induced cell death significantly compared to untreated group (Figure 3.8A.). Interestingly, increasing concentration of the oligomer to 400µM did not affected apoptotic index of CCD-18Co cells as shown in Figure 3.8B.

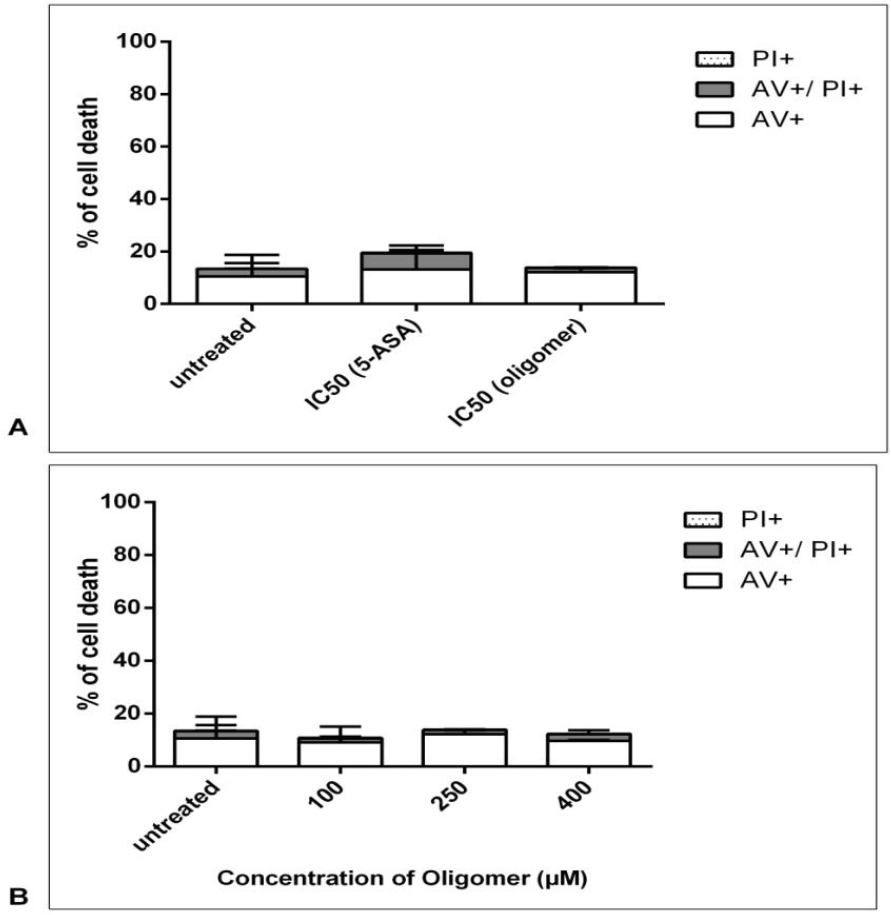

Figure 3.8. Apoptosis induction analysis of CCD-18Co cells treated with 5-ASA and its oligomer for 48 hours. Cell death analysis was performed by Flow Cytometry - AnnexinV labeling. **A)** CCD-18Co cells were treated with 5-ASA (IC50) and its oligomer (IC50) for 48 hours. **B)** CCD-18Co cell treated with the oligomer at 100µM (below IC50), 250µM (IC50) and 400µM (above IC50) concentrations for 48 hours. Results are expressed as means ±SD representative of at least two replicates. (**** indicates $p \leq 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$).

3.9. Determination DNA content of Caco-2 cells by Flow Cytometry

Quantitative measurement of the DNA with flow cytometry gave distribution of cell phases in Caco-2 cells. 5-ASA treated group at IC50 showed significant increase in average percent of cells in G1 phase as represented in Figure 3.9., whereas the oligomer treated groups at concentration range 600-800µM did not show significant change in cell phase distribution compared to control and 5-ASA treated groups. Long arrest of the cells in G1 phase may lead to necrosis which is also observed in our study. 5-ASA

caused arrest in G1 phase and necrosis in Caco-2 cells. Table 3.5 summarizes average percent of cells in each cell cycle phases.

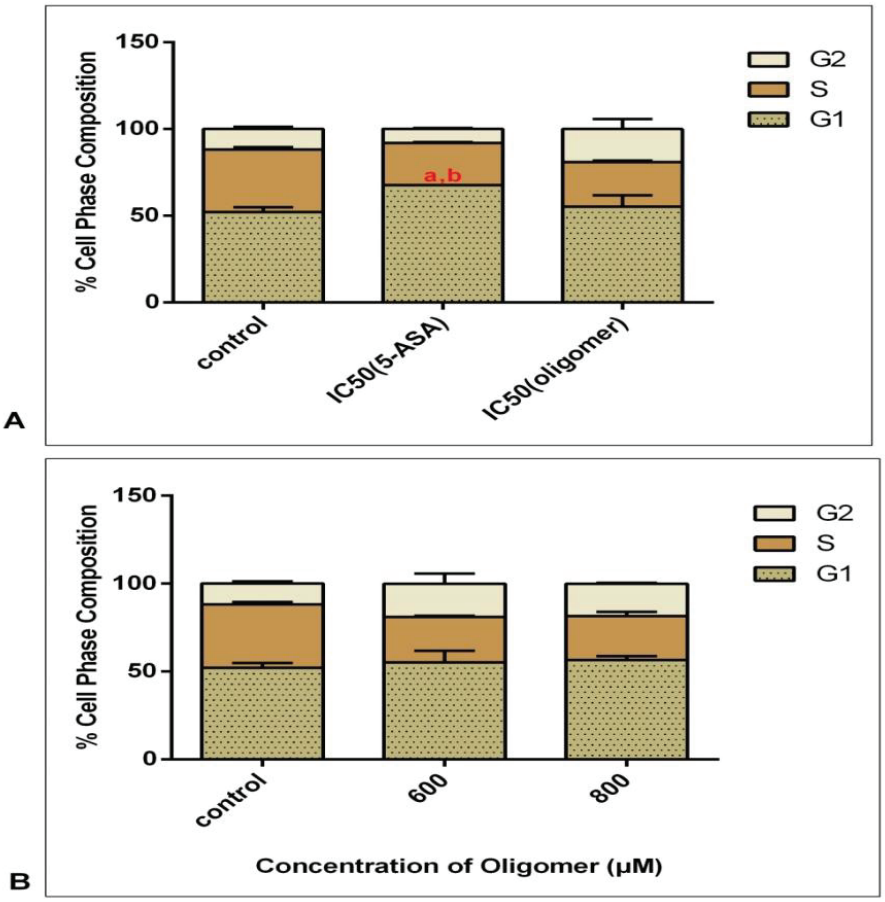

Figure 3.9. Percent cell phase distribution of Caco-2 cells in G1, S and G2 phases after treatment with 5-ASA and its oligomer for 48 hours. **A)** Caco-2 cells were treated with 5-ASA (IC50) and its oligomer (IC50) for 48 hours. **B)** Caco-2 cell treated with the oligomer at 600 µM (IC50) and 800µM (above IC50) concentrations for 48 hours. Results are expressed as means ±SD representative of at least two replicates.
a: Significantly different than control group ($p \leq 0.05$).
b: Significantly different 5-ASA/ Oligomer treated group ($p \leq 0.05$)

Table 3.5. % Cell phase distribution of Caco-2 cells in control, 5-ASA and its oligomer treated groups at IC50 concentration after 48 hour incubation period

	G0/G1	S	G2/M
Control	52.10 ± 2.76	35.92 ± 1.59	11.99 ± 1.17
IC50 (5-ASA)	67.60 ± 0.02	24.30 ± 0.54	8.11 ± 0.52
IC50 (oligomer)	55.19 ± 6.49	25.70 ± 0.77	19.11 ± 5.73

3.10. Determination DNA content of DLD-1 cells by Flow Cytometry

DLD-1 cells treated with IC50 concentrations of 5-ASA and its oligomer for 48 hours and cell cycle analysis performed on flow cytometry instrument and analyzed with ModFit LT software. Obtained average percent of cells at G1, S and G2 is demonstrated in Table 3.6. 5-ASA did not significantly effected cell cycle phase distribution of DLD-1 cells compared to control group which shows similar trend with cell death analysis in 5-ASA treated groupy6. However in the oligomer treated group cells accumulated in S phase and significance level is $p< 0.0001$. Increase in concentration of the oligomer increased cells in G2 and S phases as shown in Figure 3.10.

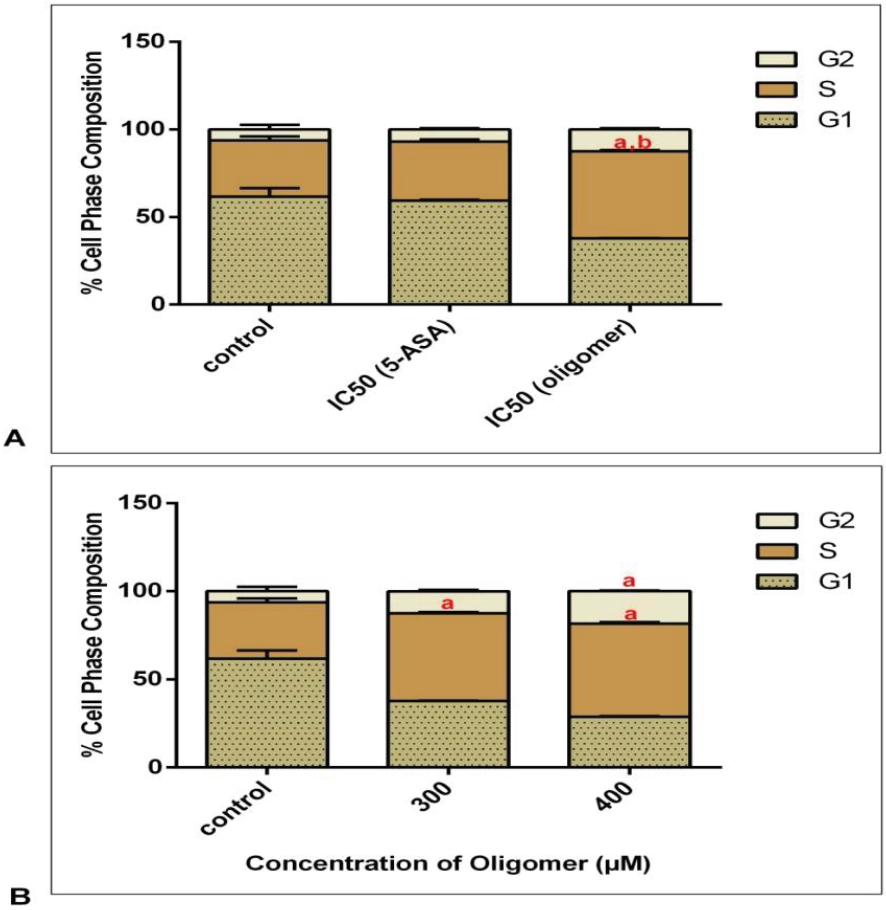

Figure 3.10. Percent Cell phase distribution of DLD-1 cells in G1, S and G2 phases after treatment with 5-ASA and its oligomer for 48 hours. **A)** DLD-1 cells were treated with 5-ASA (IC50) and its oligomer (IC50) for 48 hours. **B)** DLD-1cell treated with the oligomer at 300µM (IC50) and 400µM (above IC50) concentrations for 48 hours. Results are expressed as means ±SD representative of at least two replicates.

a: Significantly different than control group ($p \leq 0.05$).
b: Significantly different 5-ASA/ Oligomer treated group ($p \leq 0.05$)

Table 3.6. % Cell phase distribution of DLD-1 cells in control, 5-ASA and its oligomer treated groups at IC50 concentration after 48 hours incubation period.

	G0/G1	S	G2/M
Control	61.63 ±4.78	32.06 ±2.29	6.32 ±2.50
IC50 (5-ASA)	59.3 ±0.51	33.72 ±1.26	6.98 ±0.75
IC50 (oligomer)	37.84 ±0.10	49.55 ±0.78	12.62 ±0.69

3.11. Determination DNA content of HeLa cells by Flow Cytometry

Flow Cytomeric analysis of HeLa obtained after treatment with IC50 concentrations of 5-ASA and its oligomer for 48 hours. Obtained average percent of cells at G1, S and G2 is demonstrated in Table 3.6. 5-ASA did not interfere in progression of cell cycle in HeLa. However in the oligomer treated group cells accumulated in G2 phase ($p < 0.05$). Increase in concentration of the oligomer (800µM) followed same trend, cells arrested in G2 phase (Figure 3.11.).

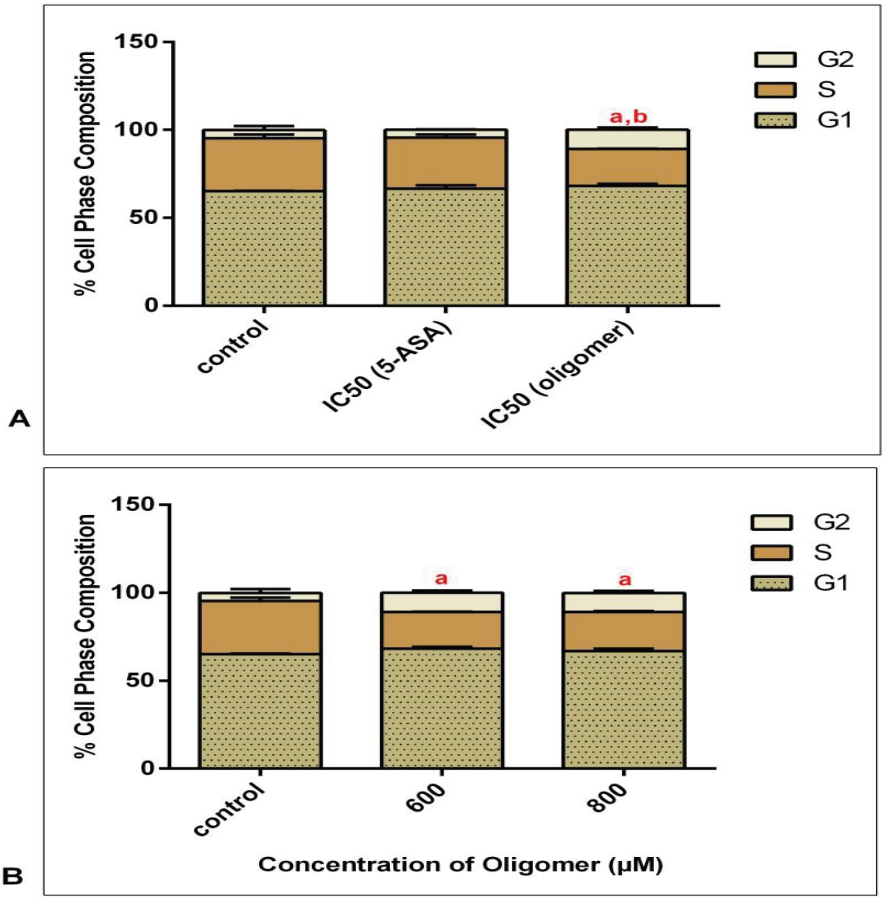

Figure 3.11. Percent cell phase distribution of HeLa cells in G1, S and G2 phases after treatment with 5-ASA and its oligomer for 48 hours. **A)** HeLa cells were treated with 5-ASA (IC50) and its oligomer (IC50) for 48 hours. **B)** HeLa treated with the oligomer at 600µM (IC50) and 800µM (above IC50) concentrations for 48 hours. Results are expressed as means ±SD representative of at least two replicates.

a: Significantly different than control group ($P \leq 0.05$).

Table 3.7. % Cell phase distribution of HeLa cells in control, 5-ASA and its oligomer treated groups at IC50 concentration after 48 hours incubation period

	G0/G1	S	G2/M
Control	65.19 ±0.25	30.15 ±1.94	4.66 ±2.19
IC50 (5-ASA)	66.50 ±2.11	29.22 ±1.73	4.29 ±0.37
IC50 (oligomer)	68.19 ±1.13	20.97 ±0.14	10.841.27

3.12. Determination DNA content of CCD-18Co cells by Flow Cytometry

CCD-18Co cells treated with IC50 concentrations of 5-ASA and its oligomer for 48 hours and cell cycle analysis performed by using flow cytometry instrument and analyzed with ModFit LT software. Obtained percent of cells at G1, S and G2 is demonstrated in Table 3.8. Cell cycle analysis showed similar effects in normal human colon myofibroblasts, neither 5-ASA nor its oligomer treated group showed significant difference in cell phase distribution compared to control group (Figure 3.12).

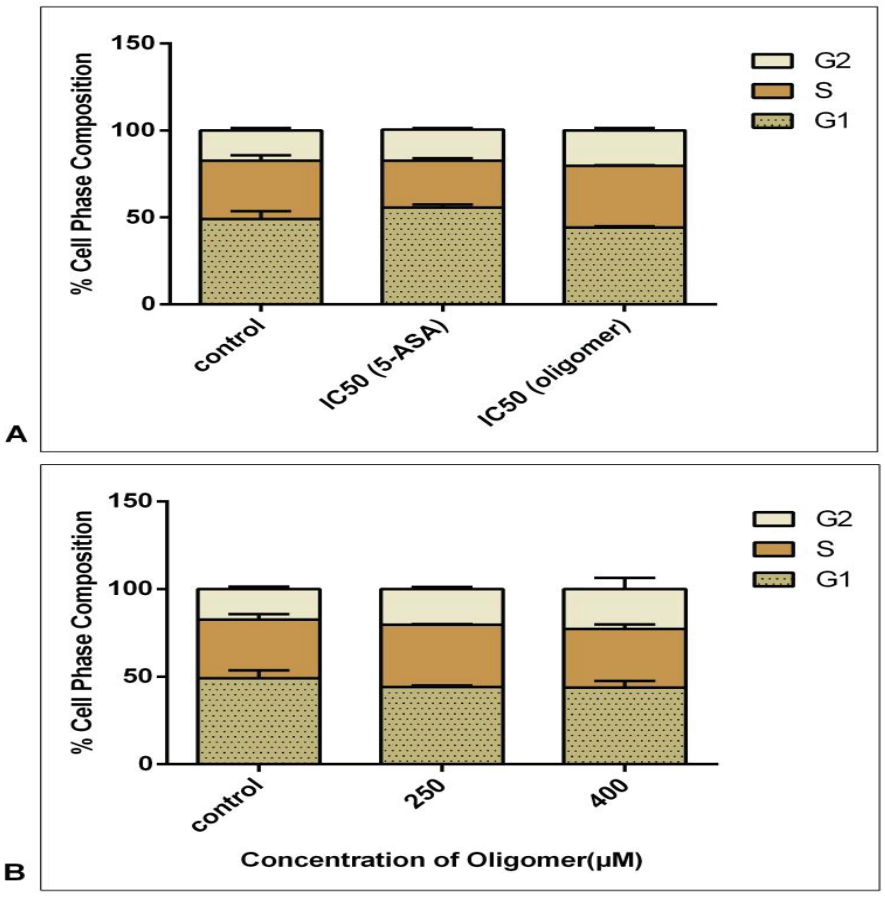

Figure 3.12. Percent cell phase distribution of CCD-18Co cells in G1, S and G2 phases after treatment with 5-ASA and its oligomer for 48 hours. **A)** CCD-18Co cells were treated with 5-ASA (IC50) and its oligomer (IC50) for 48 hours. **B)** CCD-18Co treated with the oligomer at 250μM (IC50) and 400μM (above IC50) concentrations for 48 hours. Results are expressed as means ±SD representative of at least two replicates.

Table 3.8. % Cell phase distribution of CCD-18Co cells in control, 5-ASA and its oligomer treated groups at IC50 concentration after 48 hours incubation period

	G0/G1	S	G2/M
Control	49.07 ±4.60	33.52 ±3.10	17.41 ±1.50
IC50 (5-ASA)	55.66 ±1.80	26.92 ±1.53	17.93 ±0.98
IC50 (oligomer)	44.13 ±0.86	35.53 ±0.45	20.34 ±1.32

3.13. Scratch Wound Healing Essay Results in Caco-2 Cells

Gap created by scratching the monolayer of cell and treated with 5-ASA and its oligomer at IC50 concentrations. Healing of the gap or filling gap by proliferation and migration of the cells monitored. Images of the wells were recorded in every 4 hours for 48 hours period (Figure 3.13.). Area of gap analyzed and calculated by ImageJ software. After 48 hours there was not significant migration of Caco-2 cells in control and the oligomer treated group, whereas cells in 5-ASA treated group filled the gap by around 6% as shown in Figure 3.14.

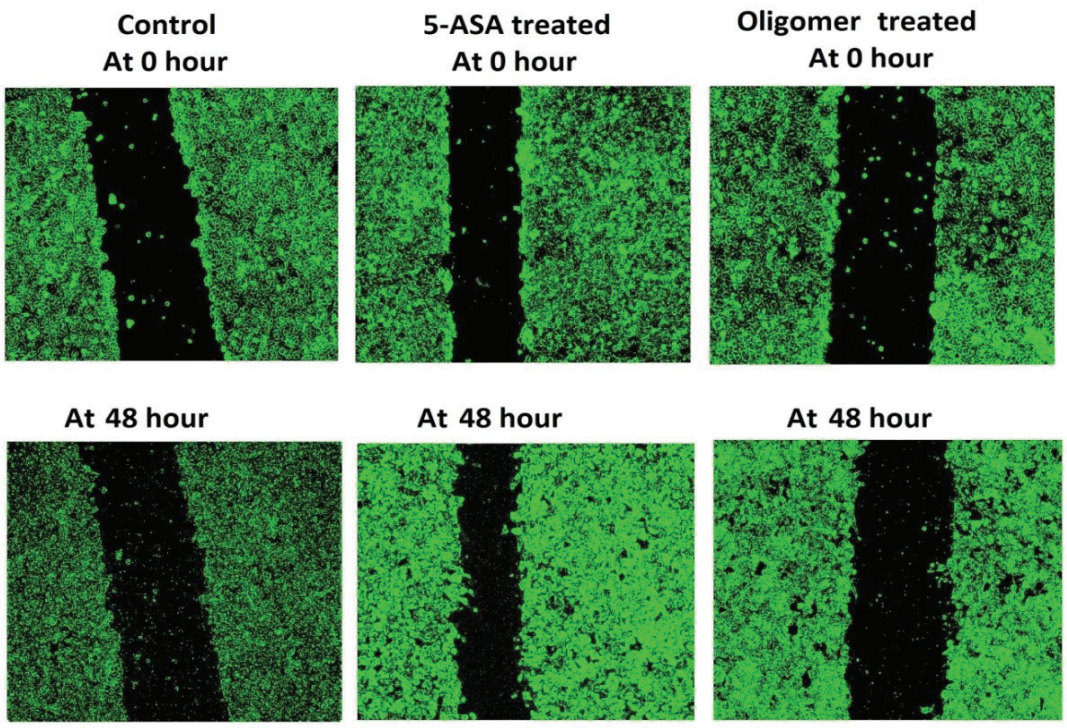

Figure 3.13. Photos of Caco-2 monolayer in control, 5-ASA or its oligomer treated groups at 0 and 48 hours. Caco-2 monolayer scratched and treated with 5-ASA (IC50) and its oligomer (IC50). Photo of wells were taken for 0- 48 hours by florescence microscopy.

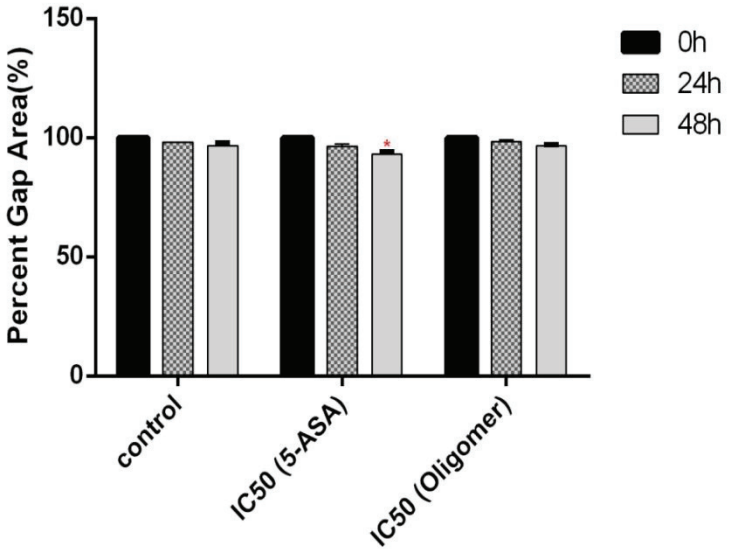

Figure 3.14. Graphical representation of filled gap in Caco-2 monolayer in control, 5-ASA or its oligomer treated groups. Results are expressed as means ±SD representative of at least two replicates. (* indicates $p<0.0001$).

3.14. Scratch Wound Healing Essay Results in DLD-1 Cells

Healing of the gap by proliferation and migration of the cells monitored for 48 hours after treatment with drugs. Images of the wells recorded at 0 and 48 hour are shown in Figure 3.15. Area of gap analyzed and calculated by ImageJ software. At IC50 concentrations the oligomer significantly inhibited migration of DLD-cells compared to control and 5-ASA treated groups as represented in Figure 3.16.

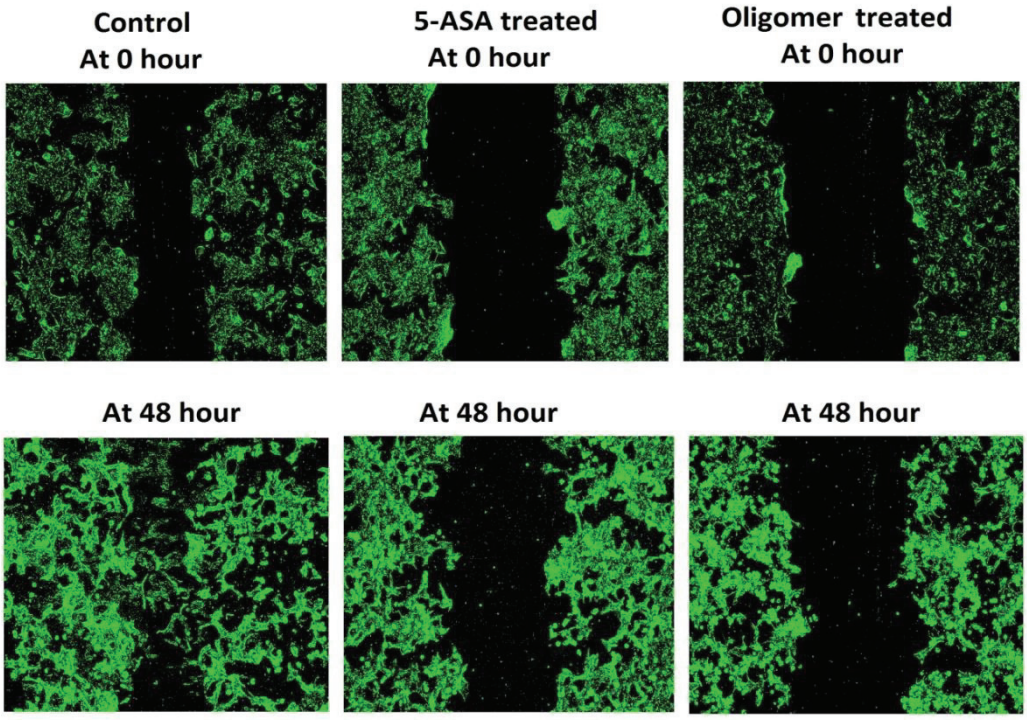

Figure 3.15. Photos of DLD-1 monolayer in control, 5-ASA or its oligomer treated groups at 0 and 48 housr. DLD-1monolayer scratched and treated with 5-ASA (IC50) and its oligomer (IC50). Photo of wells were taken for 0- 48 hours by florescence microscopy.

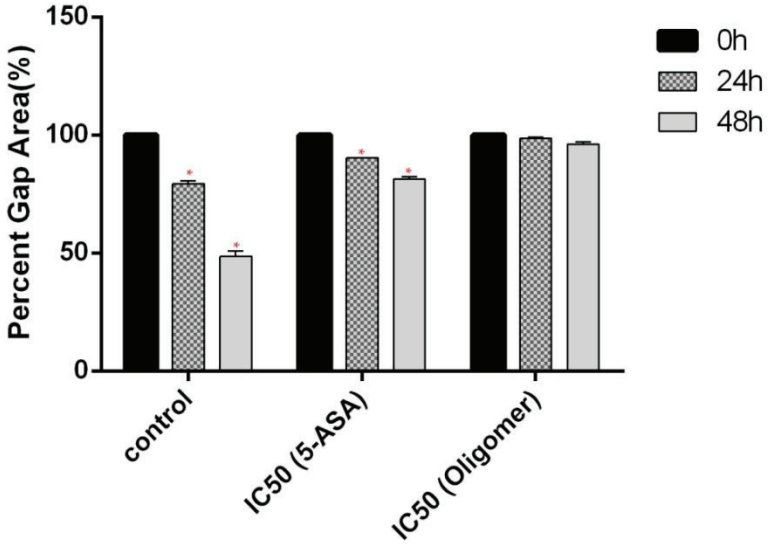

Figure 3.16. Graphical representation of filled gap in DLD-1 monolayer in control, 5-ASA or its oligomer treated groups. Results are expressed as means ±SD representative of at least two replicates. (*indicates $p<0.0001$).

3.15. Scratch Wound Healing Essay Results in HeLa Cells

HeLa cells treated with 5-ASA and its oligomer at IC50 concentrations. Images of the wells were recorded in every 4 hour for 48 hours period (Figure 3.17.). Images were analyzed by ImageJ software and area of gap filled calculated. The results of the scratch wound healing assay were different for 5-ASA and the oligomer treated groups. After 48 hours the 40% of the gap was filled by cells in control whereas 20 % in 5-ASA treated well and 7% in the oligomer treated well as shown in Figure 3.18

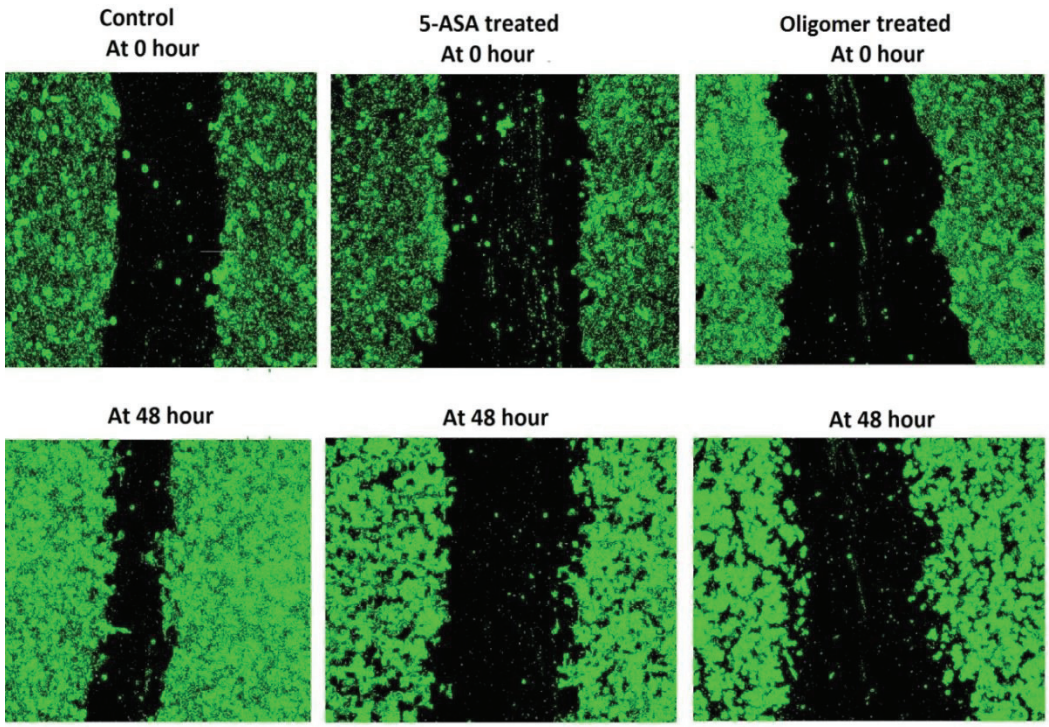

Figure 3.17. Photos of HeLa monolayer in control, 5-ASA or its oligomer treated groups for 48 hours. HeLa were treated with 5-ASA (IC50) and its oligomer (IC50). Photo of wells were taken at 0- 48 hours by florescence microscopy.

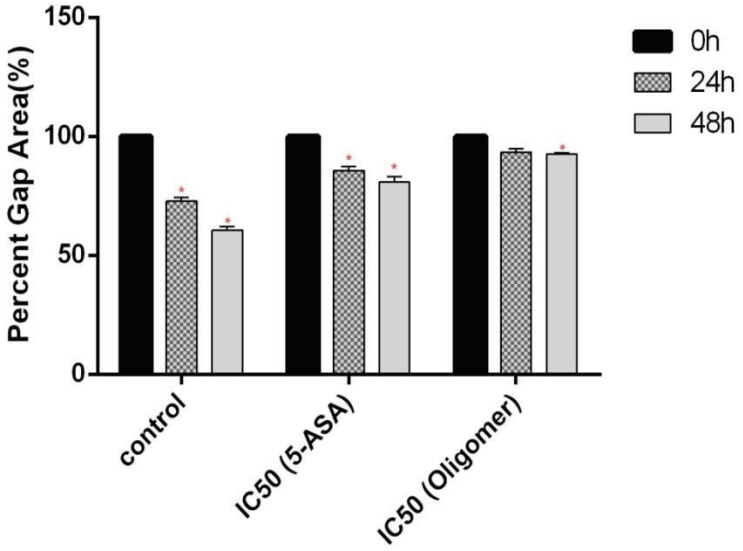

Figure 3.18. Graphical representation of filled gap in HeLa monolayer in control, 5-ASA or its oligomer treated groups. Results are expressed as means ±SD representative of at least two replicates. (* indicates $p<0.0001$).

3.16. Scratch Wound Healing Essay Results in CCD-18Co Cells

Wound formed in CCD-18Co monolayer by scratching and cells treated with 5-ASA and its oligomer for 48 hours. Healing of the gap monitored by florescence mıcroscopy and images recorded in each 4 hours. Images of the wells recorded at 0 and 48 hours are shown in Figure 3.19. Area of gap analyzed and calculated by ImageJ software. At 48 hours, migration of cells toward the center of the gap observed in control and 5-ASA treated groups. At IC50 concentration, in the oligomer treated group no significant migration of myofibroblast cells observed as represented in Figure 3.20.

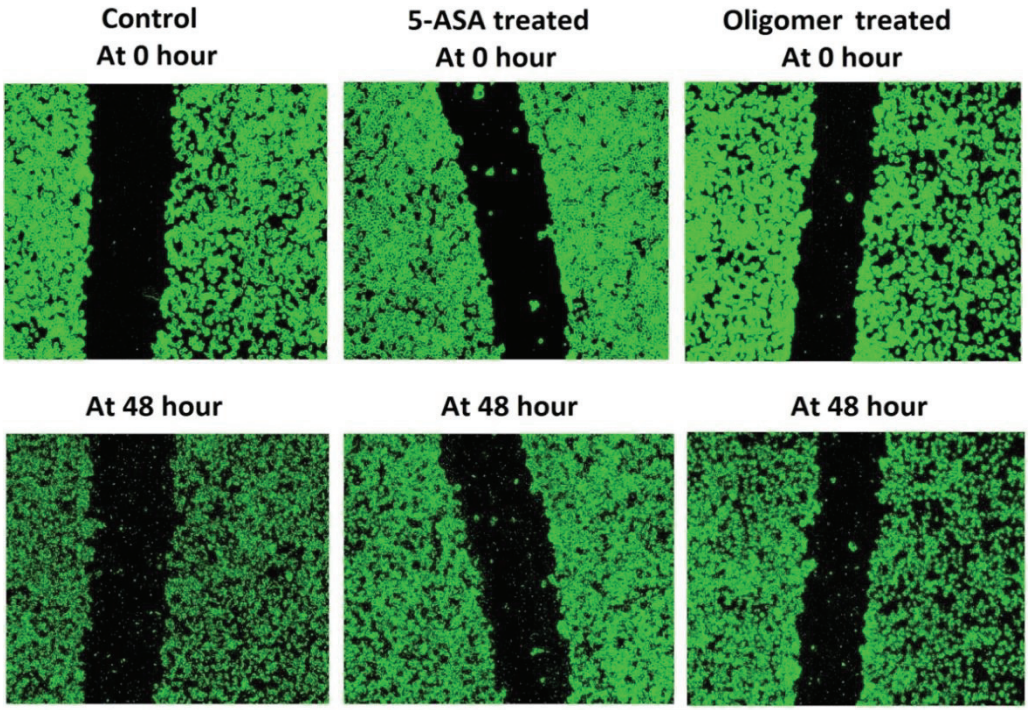

Figure 3.19. Photos of CCD-18 Co monolayer in control, 5-ASA or its oligomer treated groups at 0 and 48 hours. CCD-18 Co cells were treated with 5-ASA (IC50) and its oligomer (IC50). Photo of wells were taken at 0- 48 hours by florescence microscopy.

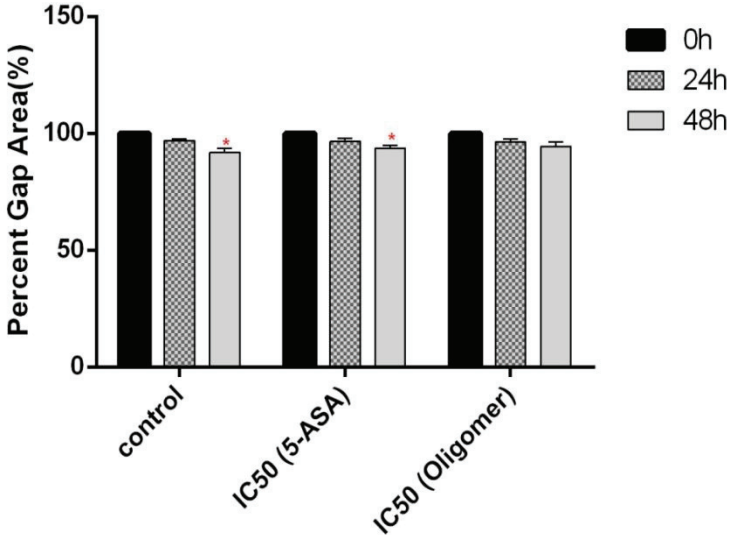

Figure 3.20. Graphical representation of filled gap in CCD-18Co monolayer in control, 5-ASA or its oligomer treated groups. Results are expressed as means ±SD representative of at least two replicates. (* indicates $p < 0.0001$).

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Colorectal cancer is fourth most common cancer type covers almost 8% of cancer related death. The 5-year relative survival rate for people with stage I colon cancer is about 90% while this number decreases to 12% in stage IV colon cancer (Siegel, DeSantis, and Jemal 2014) . Although many treatment options available for people with this stage I, cancer spread to other parts of the body are often harder to treat. In the line of this of this information, chemotherapy could be best choice for cancers at higher stages. Despite there are various chemotherapeutic agents used in clinic, researches on developing drugs with lower toxicity, longer therapeutic activity, target specific are ongoing. 5-ASA is proposed in treatment and prevention of colorectal cancer associated with IBD (Eaden et al. 2000, Half and Arber 2009, Kim and Chang 2014b, Terdiman et al. 2007, Xu et al. 2015). Main mechanism that 5-ASA follows in inhibiting cancerous cell proliferation is assumed as blocking COX-2 enzyme which is highly expressed in many cancer types such as colorectal and cervical cancers.

Polymerized form of active monomers could show different activity than monomer, thus activity of the longer chain of drug could be greater than that of the monomeric drug. Accordingly, in the present study we evaluated antiproliferative and anticancerogenic effect of novel synthesized oligomer compared to its active monomer, 5-ASA on Caco-2, DLD-1, HeLa and CCD-18co cells.

Cytotoxic effects of 5-ASA and its oligomer on Caco-2, DLD-1, HeLa and CCD-18Co cells were dose and time dependent thus higher doses and longer incubation period were more cytotoxic in all cell lines. In agreement with our hypothesis the oligomer showed higher activity in which treatment with very low drug concentrations of the oligomer compared to 5-ASA concentration showed a significant decrease in the surviving fraction of cells. Both monomer (5-ASA) and the oligomer showed lowest cytotoxicity in HeLa cells. Previously it was reported that 5-ASA negatively regulates COX-2/PGE2 axis in IBD patients which is assumed main pathway in preventing ulcerative colitis related colorectal cancer (Sharon et al. 1978). Later studies showed that 5-ASA can also inhibit COX-2 deficient colorectal cancer cells which interferes pathways involved in sporadic CRC cell growth and survival, raising possibility that

there are other inhibition mechanism that 5-ASA involved in. This can explain that why we observed more inhibition activity of both 5-ASA and its oligomer in colon cancer cell (Caco-2 and DLD-1) cells compared to cervical cancer cell, HeLa cells. Stolfi et al. summarized COX-2- independent action mechanisms of 5-ASA in their article, such as inhibition of Nuclear Factor κappa B (NF-κB) activation, inhibition of epidermal growth factor receptors (EGFRs) activation, activation of peroxisome proliferator-activated receptor gamma (PPAR-γ), and interfering Wnt-B/catenin pathway. All these pathways are known to regulate cellular proliferation, differentiation and apoptosis (Stolfi et al. 2013).

. Studies reported that 5-ASA dose-dependently induced cell death in CRC cells (Fina et al. 2006, Koelink, Mieremet-Ooms, et al. 2010, Reinacher-Schick et al. 2003, Stolfi, Fina, Caruso, Caprioli, Sarra, et al. 2008). Annexin V labeling assay reconfirmed previous studies that growth suppression of Caco-2 and DLD-1cells according MTT assay was found to be due to induced cell death by 5-ASA and the oligomer, except that 5-ASA did not induced significant cell death on DLD-1 cells when treated at IC50. This can be due to low doses (IC50) used compared to previous studies. Only higher doses of the oligomer induced cell death in HeLa cells which is also observed in MTT assay results. Interestingly, both 5-ASA and its oligomer did not trigger cell death modes in CCD-18Co cells.

There are various "checkpoints" throughout the cell cycle, and if a cell has not completed what is necessary to continue its cycle, it arrests itself there, tries to fix the problem, and continue. Studies by Koelink et al. and Reinacher-Schick et al reported that inhibition of CRC cell growth by 5-ASA can associated with the modulation of different replication checkpoints (Koelink, Mieremet-Ooms, et al. 2010, Reinacher-Schick et al. 2003). As a cell approaches the end of the G1 phase, vital checkpoint is activated, where the cell determines whether or not to replicate its DNA and checks for DNA damage. Cells with damaged DNA that cannot be repaired are arrested and "commit suicide" through apoptosis, or programmed cell death. 5-ASA may be activated these checkpoint and induce cell death in Caco-2 cells, thus increse in necrotic cell number in Caco-2 cell culture can be due to induced long term arrest in G1 phase. Stolfi et. al. reported that 5-ASA cause accumulation of CRC cells in S phase via negatively regulating CDC25A protein expression that is known to regulate the G1/S transition and S phase progression through the modulation of different cyclin/cyclin-dependent kinase (CDK) complexes (Stolfi, Fina, Caruso, Caprioli, Fantini, et al. 2008).

The oligomer, being the polymeric form of 5-ASA caused a progressive accumulation of CRC, DLD-1 cells in the S phase. Study by Reinacher-Schick et al. reported that mesalamine (5-ASA) cause G2/M arrest in colon cancer cells. In present study we did not observe mitotic arrest in Caco-2 and DLD-1 cells when treated with 5-ASA or its oligomer, except higher doses of oligomer induced G2 arres in DLD-1 cells. However, according to PI dyed DNA content analysis in flow cytometry there is significant accumulation in G2 phase of HeLa cells when treated with the oligomer. We propose that the effect of the oligomer on growth of HeLa is maybe due to a block in mitosis. In accordance to our results, several kind of cell cycle arrests induced by 5-ASA have been described in the literature (Luciani et al. 2007a, Schwab et al. 2008, Reinacher-Schick et al. 2003) which might be related to differences in the culture system adopted, including the dose and time of exposure to the drug dosages used. Similarly with apoptosis analysis, nor 5-ASA neither its oligomer did not exerted significant influence in cell phase distribution of normal human intestinal cells (CCD-18Co cells) compared to control group.

Tumour invasion is a multistage process and degradation of the extracellular matrix (ECM) seems to be a critical step which involves metalloproteinases (MMPs) and the plasminogen activator (uPA). One of the major functions of the NF-kB transcription factors is control the expression of these proteins. The studies suggest that 5-ASA interfere the cancer progression by downregulation of metalloproteinase expression by induction of activation NF-kB pathway(Kim et al. 2009). In addition several studies indicated that 5-ASA activates PPARγ (Rousseaux et al. 2005). Activated PPAR gamma trigger pathways that are involved in tumor growth and metastasis, increase cell adhesion and decrease cell migration(Reka et al. 2010, Schwab et al. 2008, Tachibana et al. 2008). Cell migration is one of the initial steps of metastasis which can be assayed by simple wound healing assay. Migration rate of DLD-1 and HeLa cells is significantly inhibited by 5-ASA and its oligomer compared to control group according to calculated gap area filled by migration or proliferation of cells. Inhibition of cell migration by oligomer is also observed in Caco-2 and CCD-18Co cell lines.

Taken together, results of MTT, Apoptosis and Cell Cycle analysis indicates that oligomer have more antiproliferative and anticancerogenic effects at much more lower doses on CRC and HeLa cells compared to its monomer, 5-ASA. Polymers that contain bioactive unit prepared for several drugs. Biological activity of this type of polymers is

related to that cleavage of active monomer in biological environment. In this respect these polymers are type of drug release systems. Some polymers do not readily cleave but still show biological activity. In these cases polymer is transformed into the cell by endocytosis. However, large molecular weight and long chain is still challenging in penetrating into the cell membrane. Oligomers with shorter chain of monomer units and lower molecular weight but still with higher bioactivity compared to monomer can be preferred choice.

Previous studies reported that 5-ASA can interfere with CRC cell growth and survival through COX-dependent and independent mechanisms. It is hypothesized that oligomer may also follows similar pathways in inhibiting growth of cancerous cells but we need further molecular studies to confirm this hypothesis. Additionally, the oligomer did not induced death in normal human colon cells, which is desired property in design of new drugs in cancer therapy. This results are promising that the oligomer could be potential drug in cancer treatment, particularly in colorectal cancer.

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APPENDIX A

MEDIAS

A.1. DMEM and RPMI-1640 Growth Medium

Roswell Park Memorial Institute – 1640 (RPMI 1640) growth medium, fetal bovine serum (FBS) and antibiotic solution were obtained from Gibco, BRL.

Dulbecco's modified Eagle'S medium (DMEM) growth medium obtained from Sigma, fetal bovine serum (FBS), antibiotic solution and L-glutamine solution were from Gibco, BRL.

Table A.1. Growth media contents

DMEM/RPMI with L-glutamine - 50 ml removed from 500 ml bottle then added the other constituents	450 ml
10% FBS	50 ml
100 U penicillin/ 0.1 mg/ml streptomycin	5 ml

APPENDIX B

CHEMICALS, SOLUTIONS AND REAGENTS

B.1. 5- Aminosalicylic Acid and Oligomer

5- Aminosalicylic Acid and oligomer provided by Assist. Prof. Ali BİLİCİ from Çanakkale Onsekiz Mart University, Faculty of Science, Chemistry Department. They were prepared fresh in culture medium for each study.

B.2. Sterile Phosphate buffered saline(1x PBS) Solution

To prepare PBS stock solution; 0.34 g of KH_2PO_4, 1.58 g of K_2HPO_4 and 8.0 g of NaCl added to distilled water. pH is adjusted to 7.04 and total volume brought to 1 liter. Sterilization performed by autoclaving and stored at $+4^0$C.

B.3. Trypan Blue Solution in PBS

To prepare 0.4% Trypan blue stock solution, 4 mg of Trypan blue powder weighed and dissolved in 1x PBS. Then, filter sterilized by 0.2 μm filters.

B.4. MTT Reaction Solution

MTT stock solution was prepared in phosphate buffered saline (PBS) in concentration of 5mg/ml, filter-sterilized through a 0.2 μM filter, transferred into light protected falcon and stored at +4°C. For 96-well plate MTT diluted in 1:10 ratio with culture media(DMEM or RPMI).

Table B.1. Chemicals and reagents used in experiments

NO	CHEMICALS	COMPANY
1	Dimethyl Sulfoxide (DMSO)	CARLO ERBA
2	Trypan Blue Dye	SIGMA-ALDRICH
3	MTT	AMRESCO
4	Annexin-V Apoptosis Detection Kit I	BioLegend
5	Absolute Ethanol	EMSURE
6	Trypsin	SIGMA
7	Triton X-100	AMRESCO
8	RNase	AppliChem