MOLECULAR CHARACTERIZATION OF THE EFFECTS OF VALDECOXIB ON COLON CANCER CELL LINES (HT29 & SW620) USING ATR-FTIR SPECTROSCOPY

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AYSUN İNAN GENÇ

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submitted by AYSUN İNAN GENÇ in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry Department, Middle East Technical University by,

Prof. Dr. Canan ÖZGEN
Dean, Graduate School of Natural and Applied Sciences

Prof. Dr. Orhan ADALI
Head of Department, Biochemistry

Prof. Dr. Feride SEVERCAN
Supervisor, Biology Dept., METU

Examiing Committee Members:

Assoc. Prof. Dr. Çagdaş SON
Biology Dept., METU

Prof. Dr. Feride SEVERCAN
Biology Dept., METU

Assoc. Prof. Dr. Sreeparna BANERJEE
Biology Dept., METU

Assoc. Prof. Dr. Ergin M. ALTUNER
Biology Dept., Kastamonu University

Assist. Prof. Dr. Özlem BOZKURT
Biophysics Dept., Adnan Menderes University

Date: 05.02.2014
I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Aysun İNAN GENÇ
Signature : 
Colorectal cancer is the third most common malignancy and the fourth most frequent cause of cancer deaths worldwide, with 945,000 estimated new cases and 492,000 deaths per year in industrialized countries. Chronic inflammation of the bowel is associated with increased levels of pro-inflammatory cytokines and the increased signaling, which plays important roles in the development of carcinoma. During chronic inflammation COX-2 levels are dramatically increased which is the main enzyme in the arachidonic acid pathway. It is observed from the previous studies that COX-2 inhibitors have chemopreventive as well as therapeutic effects on different cancer types. Therefore, the effect of COX-2 inhibitors on cancer therapy is an area of high interest. In this study Valdecoxib (VLX) is chosen as a member of COX-2 inhibitors. There are lots of studies related to the effect of COX-2 inhibitors on colon cancer at molecular level, but there is a limited number of studies at structural level.

In this study, it was aimed to investigate the alterations in the macromolecules of HT 29 and SW 620 colon cancer cell lines after VLX treatment using ATR-FTIR spectroscopic techniques. Spectral analysis at C-H region (3030-2800 cm\(^{-1}\)) revealed that lipid concentration, order and membrane fluidity of two colon cancer cell lines were highly affected due to VLX treatment.
Furthermore, spectral analysis at fingerprint region (1800-900 cm\(^{-1}\)) showed that protein concentration parameters were also affected due to VLX treatment. In addition, in this study it was also observed that the above results were COX-2 independent. After additional research, VLX may have a chance as an adjuvant therapy in the treatment of colon cancer.

**Keywords:** Colon cancer, COX-2 inhibitors, VLX, HT 29, SW 620, ATR-FTIR spectroscopy
ÖZ

VALDECOXİB’İN KOLON KANŞER HÜÇRELERİ (HT 29 ve SW 620) ÜZERİNE OLAN ETKİLERİNİN ATR-FTIR SPEKTROSКОПİSİ İLE MOLEKÜLER KARAKTERİZASYONU

İNAN GENÇ, Aysun
Yüksek Lisans, Biyokimya Bölümü
Tez Yöneticisi: Prof. Dr. Feride SEVERCAN
Ortak Tez Yöneticisi: Doç. Dr. Sreeparna BANERJEE

Şubat 2014, 110 sayfa

Kolorektal kanser, malignant türler arasında en yaygın üçüncü türdür ve dünya genelinde kanser ölümleri arasında dördüncü sıradadır. Gelişmiş ülkelerde her yıl 945,000 yeni vaka ve 492,000 ölüm olması tahmin edilmektedir. Bağırsaklardaki kronik inflamasyonun; yüksek uyarılma sonucu öncü inflamasyon sitokinlerinin salgılanmasında artışa neden olduğu gösterilmiştir. Bu durum kanser gelişiminde önemli bir rol oynamaktadır. Arakidonik asit yolunun en önemli enzimlerinden olan COX-2 seviyesi kronik inflamasyon sırasında ciddi şekilde artış göstermektedir.


Bu çalışmada, Valdecoxib’ın (VLX) HT 29 ve SW 620 kolon kanseri hücre hatlarında makromoleküler düzeyde yapacağı etkilerin ATR-FTIR spektroskopisi tekniği kullanılarak araştırılması hedeflenmiştir. 3030-2800 cm⁻¹ (C-H bandı) bölgesi spestral analizleri, her iki hücre hattında da lipit konsantrasyonu, düzennılığı ve membran akışkanlığında VLX uygulamasının önemli değişikliklere neden olduğunu göstermiştir.

**Anahtar Kelimeler:** Kolon kanser, COX-2 inhibitoryorları, VLX, HT 29, SW 620, ATR-FTIR Spektroskopi
Dedicated to the memory of my aunt,

Türkan GÖNÜLAL
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<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ATR</td>
<td>Attenuated Total Reflectance</td>
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<tr>
<td>CLX</td>
<td>Celecoxib</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<td>CRC</td>
<td>Colorectal Carcinoma</td>
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<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
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<tr>
<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared spectroscopy</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration that caused %50 inhibition of the growth</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IR</td>
<td>Infrared</td>
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<tr>
<td>LOX</td>
<td>Lipoxygenase</td>
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<tr>
<td>LPS</td>
<td>Lipop polysaccharide</td>
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<tr>
<td>NSAIDS</td>
<td>Nonsteroidal Anti-inflammatory drugs</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty Acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>S/N</td>
<td>Signal to noise ratio</td>
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<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>TNM</td>
<td>Staging system of the malignant tumors</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>VLX</td>
<td>Valdecoxib</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>XTT</td>
<td>Cell Proliferation Assay</td>
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CHAPTER 1

INTRODUCTION

1.1 Cancer

Chronic diseases cause increasing numbers of deaths worldwide. Cancer caused for 7.1 million deaths in 2012 and the number of new cases is expected up to rise 15 million by 2020. According to American Cancer Society among all cancer types lung, colon, breast, pancreas and prostate cancers have the highest death incidence.

Biostructure disorders are called cancer. Cancer starts uncontrolled cell division, invasive cells growth into the adjacent tissue and finally spread of other body sites (metastasis). It can arise in different sites of the organs or tissues and behaves in your own way depending on its origin. They grow and disturb normal biological functions. About 200 different types of cancer have been described, whose properties and treatments are different. There are four main forms. The most common ones are Carcinomas (90%) which are solid cancers that arise from the epithelial cells. The second ones are Sarcomas that are also solid cancers developed from the cells produced muscle, nerves, or blood vessels. Leukemias are cancers of white blood cells and Lymphomas are cancers that developed from cells in lymph glands (Pardee, 2006). Each cancer type has own characteristic behaviour for example, grow faster or slower, respond differently to same treatment, produce different symptoms and has high or low tendency of metastasis according to their originated cell type. Group of cancerous cells are called tumor. The original site of a tumor is called primary tumors when these tumors spread of the other parts of the body then it is called secondary tumor (Debasis, 2005).
DNA carries the genetic information of the cells. Cancer arise from the mutations in DNA structure that change genetic information. Some cancers could originate from agents such as toxic chemicals, radiation. Some others produced by virus infection; for example, human papilloma virus causes cervical cancers. If there is any mutation on the DNA, molecular mechanisms detect the damage and cell cycle is stopped. Then enzymes for repair are activated, and the cell might recover if the damage is not too severe. The inability to repair damaged DNA may also result in cancer.

When it cannot be repaired cancer-related alterations occur in the exchange of signals between the cell nucleus and cytoplasm, which are critical for control of cell regulatory machinery. Effects of mutation limit growth and differentiation into specialized cells, causing apoptosis, metastasis and angiogenesis (Morgan, 2004).

1.1.1 Colorectal Cancer

Colorectal cancer is the third most common malignancy and the fourth most frequent cause of cancer deaths worldwide, with 945,000 estimated new cases and 492,000 deaths per year in industrialized countries. After prostate and lung cancer, it is at the third place among most common in men and also in women it is on the third place, after breast cancer and gynecological cancers (Zora, 2012). According to National Cancer Institute, it is estimated that 142,820 men and women will be diagnosed with and 50,830 men and women will die of cancer of the colon and rectum in 2013.

Colorectal cancer represents more than 70% of colon cancer and less than 30% cancer of rectum. Difference in risk factors for proximal and distal colorectal cancers may influence incidence rates (Wu, 2006). The black/white incidence ratio decreased, and the male/female incidence ratio increased from the proximal to the distal colon (Devesa, 1993); (Troisi, 1999). It is still unclear, why colorectal cancer location differs by sex. Differences in sex hormones, which may influence fecal transit time and composition and bile acid metabolism may be partly responsible (Manton, 2009).
Development of colorectal cancer is reported to be associated with multiple factors, including inherited genetic susceptibility, environmental factors, and lifestyle choices. There are three specific patterns in which colon cancer is generally observed: sporadic, inherited, and familial. Although the majority of cases of colon cancer are sporadic in nature, as many as 10% of cases are thought to be hereditary. The two most common forms of hereditary colon cancer are the familial adenomatous polyposis and the hereditary nonpolyposis colorectal cancer, both of which result from a specific germline mutation (Naveen, 2011).

It has been demonstrated that there is a relationship between inflammatory bowel disease (IBD) and colorectal cancer. IBD is a group of syndromes where the gastrointestinal track undergoes chronic or relapsing idiopathic immune activation and inflammation. Crohn’s disease and ulcerative colitis are the two major forms of IBD. In ulcerative colitis, there is about a 20-30-fold higher risk of developing colorectal carcinoma than in the general population. When Crohn’s disease involves the colon, the risk of Colorectal Carcinoma (CRC) appears to be similar to that of ulcerative colitis. The risk of cancer in IBD patients is also related to the duration and extent of disease (Morgan, 2004).

There are also different mechanisms that may be responsible for the development of carcinoma in IBD patients, one being the production of reactive oxygen species (ROS). ROS can induce damage to DNA which influenced to gene mutations, and thus can contribute to carcinogenesis. Some of the pathways included in ROS induced carcinogenesis includes MAP kinase and phosphoinositide 3-kinase (PI3K) pathways that are induced during the development of carcinogenesis and promotes cell survival (Saba, 2012).

As a consequence, the progress of colorectal cancer is a typical multistep process: several mutations occur in key oncogenes, such as the adenomatous polyposis coli (apc), ki-ras or the p53 tumor suppressor gene, triggering cell alteration and uncontrolled proliferation. Moreover these mutations cause an increase of
cyclooxygenase-2 (COX-2) expression as much as 80% of colon carcinomas compared with normal intestinal mucosa (Sano, 1995) (Eberhart, 1994).

1.1.2 Cancer and Inflammation

Cancer is a disease that has strong links to chronic inflammation. There are many cancers that develop in the background of chronic inflammation. Epidemiological data indicate that over 25% of all human cancer cases are associated with chronic inflammation (Hussain, 2007).

Inflammation is essentially a protective response to infection or injury that normally resolves with the restoration of normal tissue structure and function. It is a multi-step process that begins with the recruitment and activation of inflammatory cells at the site of tissue injury, infection or stress. In response to chemoattractants; leukocytes, dendritic cells (DCs), and lymphocytes migrate from the bloodstream and lymphatics and enter affected tissues by extravasating through vessel walls into the Extracellular matrix (ECM). At the site of tissue damage, inflammatory cells apply their effector function by releasing preformed and newly synthesized inflammatory mediators including cytokines, proteases, membrane perforating agents, by direct or specific killing of abnormal cells (Morgan, 2004).

When inflammation persists it can cause tissue damage and loss of function. Chronic inflammation may occur due to the persistence of infection or antigen, recurring tissue injury, or a failure of endogenous anti-inflammatory mechanisms that drive the resolution of inflammation (Figure 1). Importantly, inflammation is a critical part of innate immunity and has a role in priming the adaptive immune system but chronic inflammation can be associated with immune-suppression (Vita, 2010).
Figure 1. The crosstalk of inflammation (Nature.com, 2008)

It is hypothesized that reactive oxygen and nitrogen species produced by inflammatory cells cause DNA damage and cell mutation and finally leading to the malignant transformation of normal cells into cancer cells. Pro-inflammatory cytokines are frequently detected in tumor tissue, and tumors are consistently permeated by inflammatory cells (Figure 2). The outgrowth of neoplastic cells is then driven by the cytokines, chemokines and angiogenic factors some of which have functions as regulators of tumor growth, tumor metastasis and angiogenesis (Marusawa, 2013).
Studies of human cancer show a relationship between cancer progression with the expression of inflammatory markers and macrophage infiltration (Figure 3). It was also shown that blocking the expression of inflammatory mediators or infiltration of inflammatory cells inhibits growth of tumors (Bingle, 2012). Therefore chronic inflammation provides an appropriate environment for cancer to form and grow (Morgan, 2004).
It is a remarkable note that chronic inflammation is especially involved in the tumorigenesis of gastrointestinal and hepatobiliary organs (Figure 4). The reasons why chronic inflammation and the resultant tumorigenesis are so evident in gastrointestinal tissues are unknown. One possibility is that the gastrointestinal tract and hepatobiliary organs are physiologically exposed to a plethora of dietary and environmental factors that contain pro-inflammatory agents such as microorganisms (Chiba, 2012).

**Figure 3.** Illustration of pro- and anti-tumor roles for inflammation in cancer (Vita, 2010).
Chronic inflammation of the bowel is associated with increased levels of pro-inflammatory cytokines and the increased signaling, which plays important roles in the development of carcinoma (Saba, 2012). At the molecular level one of the most well-known transcription factors involved in the processes of both inflammation associated tumor promotion and tumor progression is the nuclear factor (NF)-κB. This inflammatory and oncogenic factor is activated by various pro-inflammatory cytokines such as tumor necrosis factor (TNFα) and interleukin (IL-1b), (IL-6) leading to the expression of a set of genes which influence oncogenic biological processes, such as epithelial cell proliferation, survival/anti-apoptosis and migration, as well as angiogenesis and epithelial-to-mesenchymal transition (Di Donato, 2012) (Figure 5).
In the view of such information it can be concluded that; inflammation plays a significant role in the neoplastic progression of many types of cancer. Inflammatory cells, growth factors and genotoxic agents work in concert to support neoplastic progression so it provides an ideal site for carcinogenesis.

1.1.3 Cancer Therapy

Cancer therapy is a complex and challenging approach. The main objective of cancer therapy is improving the survival and qualities of life. To success in the treatment of these diseases efforts to combine multiple fields are required.

Over the past three decades major progress has been made in the treatment of malignant diseases. Five major approaches are used to treat cancer patients: (1) surgery, (2) radiation, (3) drug therapy (4) integrative medicine, and (5) palliative care. The choice of treatment approaches depends on a number of factors: (1) the stage of cancer determined by the TNM system (2) the organ or tissue in which the cancer originated.

Figure 5. Macrophages activation switch during the course of tumour progression (Porta, 2009)
(3) the type of cancer (e.g., adenocarcinoma, germ cell cancer) and (4) specific biological factors or biomarkers. Clinical trials and continued interest of basic biological mechanisms of cancer development form the basis of future progress in cancer therapy (Pardee, 2006).

To describe the size of tumor and whether it metastasize or not researchers use the staging system. For appropriate treatment knowing the stage is very important. There are many different type of grading system. In general, lower grades mean a less aggressive behavior, and higher grades mean a relatively more aggressive cancer. Stage refers to the degree and size of the cancer. Each cancer type has its specific staging system.

Stage 0: Very early cancer. The abnormal cells are found only in the first layer of cells of the primary site and do not invade the deeper tissues.
Stage I: Cancer involves the primary site, but has not spread to nearby tissues.
  Stage IA: A very small amount of cancer that is visible under a microscope and is found deeper in the tissues.
  Stage IB: A larger amount of cancer is found in the tissues.
Stage II: Cancer has spread to nearby areas but is still inside the primary site.
  Stage IIA: Cancer has spread beyond the primary site.
  Stage IIB: Cancer has spread to other tissue around the primary site.
Stage III: Cancer has spread throughout the nearby area.
Stage IV: Cancer has spread to other parts of the body.
  Stage IVA: Cancer has spread to organs close to the pelvic area.
  Stage IVB: Cancer has spread to distant organs, such as the lungs.
Recurrent: After a period of time cancer has come back. The recurrence may be in the same location as the original tumor or in a different location (Debasis, 2005).

At the primary tumor level (stage 0, stage I and stage II) local control of tumor cells is the main case, therefore surgery and radiation therapies are commonly used concepts. On the other hand at metastatic level (stage III and IV) drug therapy is the main
approach, which include chemotherapy, immunotherapy, cell therapy, genetic therapy, hormone therapy, targeted therapy and adjuvant therapy.

In cancer treatment, inhibition of tumour promotion is key aspect. Angiogenesis, which plays a key role in carcinogenesis, is the formation of new blood vessels it is critical for cancer progression. Furthermore, new tumour vessel growth often corresponds with tumour metastasis. There is a number of signaling molecules have a role to initiate and induce tumour promotion and progression process. Therefore, by targeting initiators, co-carcinogens and tumour promoters, tumour progression could potentially be inhibited. Unfortunately, the identification of such markers can be difficult (Hyde, 2009). Many of these markers are of tissue remodeling and inflammation associated with the neoplastic state (Wang T., 2002).

New findings about the interactions of natural killer (NK) cells and dendritic cells (DCs) in the initiation of the inflammatory response as well as secondary responses suggest their critical role in immunity and potential utility as immunotherapies or the use of cytokine gene therapy. In order to identify suitable biomarkers and surrogates of tumor during this period is one of the great aim so that novel agents might be applied (Unwin, 1999) (Lotze, 2002) (Omenn, 2002) (Espina, 2004) (Dow, 1996).

Furthermore inflammation causes upregulation of the enzyme cyclooxygenase type 2 (COX-2). COX-2 upregulation plays an important role in neoplastic progression. It was shown that, inhibition of the enzymes COX-2, LOX and CYP450 lead to inhibition of cell proliferation and neo-angiogenesis of cancer cells (Morgan, 2004). It is observed from the studies that long-term use of aspirin and other non-steroidal anti-inflammatory drugs particularly COX-2 inhibitors have reduced cancer risk. The effect of Nonsteroidal Anti-inflammatory drugs (NSAIDs) on cancer therapy and chemoprevention is investigated in respect of new therapeutical approaches. According to experimental data it is clear that these drugs have chemopreventive effects as well as therapeutic effect on different cancer types, such as esophageal cancer, breast cancer, lung cancer, gastric cancer, bladder cancer and colorectal cancer. It is proved that Aspirin reduces the number of adenomatous polyps in patients with
histories of both colorectal polyps and colorectal cancer. Celecoxib, (selective COX-2 inhibitor), for example, has significantly reduced colorectal polyp burden in familial adenomatous polyposis. It is approved by Food and Drug Administration (FDA) for the adjuvant therapy on familial adenomatous polyposis patients (Morgan, 2004).

1.2 COXs and Cancer

1.2.1 COX-2 Pathway as a Molecular Target of Cancer

Arachidonic acid (AA) is one of the body's essential fatty acids required to maintain normal biological functions of the majority of mammals. It is a 20-carbon polyunsaturated fatty acid and the vital eicosanoid precursor in mammalian cells. Arachidonic acid synthesis is triggered by inflammatory signals, cytokines, growth factors and hormones. It is derived from membrane phospholipids by the activation of phospholipase A2 (PLA2) enzyme.

Arachidonic acid converted into biologically active eicosanoids via enzymatically or non-enzymatically. Cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450) enzymes convert arachidonic acid into eicosanoids metabolites (Figure 6). Eicosanoids embrace a number of lipid signaling mediators that play a central role in cellular signalling cascades of physiological and pathophysiological processes (Hyde, 2009); (Cathcart, 2012).
The cyclooxygenases catalyze the first step in the synthesis of prostaglandins from arachidonic acid. Prostaglandins are lipid mediators made by most cells in the body except for red blood cells. They involve in a wide spectrum of biological actions such as modulation of vascular tone, gastric cytoprotection and the inflammatory response (Figure 7). Prostaglandins have also been associated with such diseases which are arthritis, cardiovascular diseases, autoimmune diseases, pulmonary diseases and cancer (Cathcart, 2012).

There are three different forms of COX enzymes which are COX-1, COX-2 and COX-3. In terms of the therapeutic approaches COX-1 especially COX-2 is most interested among these. Although COX-1 and COX-2 have the same three-dimensional protein folds, they differ only in one amino acid at their COX catalytic sites. Both are membrane bounded enzymes (Luong, 1996).
Actually the most profound difference between the two isoforms relates to their expression (Smith W., 2001). COX-1 is constitutively expressed in most tissues throughout the body (Pairet, 1996). In contrast, COX-2 expression is restricted under normal physiological conditions, but its expression is increased at inflammatory states (Esser, 2005); (Botting, 2006).

![Diagram of the COX pathway](image)

**Figure 7.** Diagram of the COX pathway (Cebola, 2012).

Molecular studies show that over-expression of COX-2 is an important aspect of almost every form of cancer (Figure 8). High levels of expression are observed in premalignant lesions, invasive cancer, and metastatic disease (Table 1).
Figure 8. Effectors of the COX pathway. Genes are shown in red boxes are hypermethylated and linked to the cancer types (Cebola, 2012).

Table 1. COX-2 expression in neoplasms of the different cancer types (Harris R. E., 2009)
High levels of COX-2 expression are evident throughout tumorigenesis in every cell and tissue site that has been examined. Figure 9 shows the mean frequency of lesions over-expressing COX-2 in the progression of tumorigenesis for different cancer types.

![COX-2 in Cancer Progression](image)

**Figure 9.** COX-2 expression level at different cancer types (Hull, 2005)

It was observed that COX-2 overexpression is sufficient to convert normal cells into malignant. (Harris R., 2007). It is also important that mutations of oncogenes and tumor suppressor genes are usually absent in premalignant lesions. (Soto, 2004).

It is noteworthy that the induction of constitutive COX-2 expression and prostaglandin (PGD2, PEG2, PGF2, PGI2 and TXA2) biosynthesis are sufficient to stimulate the hallmarks of carcinogenesis including mutagenesis, mitogenesis, angiogenesis, metastasis, inhibition of apoptosis and immunosupression with reduced antineoplastic activity of T and B lymphocytes which is shown in Table 2 (Harris R., 2007).
Table 2. Reference the studies which have examined the roles of the prostanoids in the hallmarks of G.I. cancers. (Cathcart, 2012)

<table>
<thead>
<tr>
<th>Prostanoid</th>
<th>Cancer type</th>
<th>Cancer hallmark</th>
<th>Result</th>
<th>Author</th>
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</thead>
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<td>Proliferation</td>
<td>-</td>
<td>Takashima et al.</td>
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<td></td>
<td></td>
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<td>+</td>
<td>Takashima et al.</td>
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<td></td>
<td>Proliferation</td>
<td>-</td>
<td>Takashima et al.</td>
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<tr>
<td>Gastric</td>
<td></td>
<td>Apoptosis</td>
<td>+</td>
<td>Chen et al.</td>
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<td></td>
<td>Proliferation</td>
<td>-</td>
<td>Leung et al.</td>
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<td>Angiogenesis</td>
<td>-</td>
<td>Fu et al.</td>
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<td>Proliferation</td>
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<td>Ma et al.</td>
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<td>+</td>
<td>Lin et al.</td>
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<td></td>
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<td></td>
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1.2.2 NSAIDs and COX-2 Inhibitors

NSAIDs are among the oldest and most successful drugs known to modern medicine. The progenitor of the NSAIDs is acetyl salicylic acid. It is also the world’s first synthetic drug, was patented in 1899 and was called Aspirin: A from acetyl, spir from *Spiraea ulmaria*, and in as a then-typical name ending for medicines (Harald, 2010). Then in 1946 phenylbutazone, successor of the Aspirin was developed (by JR Geigy, Basel, Switzerland). In 1959, ibuprofen was synthesized with its analgesic, antipyretic, and anti-inflammatory properties similar to aspirin (John Nicholson from the Boots Company with Stuart Adams). Ibuprofen, which has high safety profile at dose ranges, was the first non-steroidal anti-inflammatory drug. After this invention, lots of pharmaceutical companies carried out the discovery and development of a wide range of NSAIDs. These newly discovered NSADs were the precursors of the prostaglandin inhibitors (Harris R., 2007).

At present, about 50 different NSAIDs are existing and they are among the most commonly suggested drugs worldwide (Harald, 2010). NSAIDs are classified in terms of their chemical structure. These classification include; salicylates (aspirin), arylalkanoic acids (diclofenac, indomethacin, nabumetone, sulindac), 2-arylpropionic acids or profens (ibuprofen, flurbiprofen, ketoprofen, naproxen), N-arylanthranilic acids or fenamic acids (mefenamic acid, meclofenamic acid), pyrazolidine derivates (phenylbutazone), oxicams (piroxicam, meloxicam), sulfonanilides (nimesulide) and diaryl-substituted pyrazoles or furanones (coxibs) (Harald, 2010). General chemical categorization NSAIDs classes are shown in the Figure 10.
**Figure 10.** The general chemical categorization NSAIDs classes are shown (Lemke, 2012).

In general, NSAIDs structurally consist of an acidic moiety (carboxylic acid, enols) attached to a planar, aromatic functionality (Figure 11).
NSAIDs have antipyretic, analgesic and anti-inflammatory activity. They are widely used for the treatment of minor pain, edema, fever and tissue damage resulting from inflammatory diseases. These drugs inhibit either COX-1, COX-2 enzymes or both. Major indications for NSAIDs therapy includes: rheumatoid arthritis (RA), osteoarthritis (OA), and acute gouty arthritis, ankylosing spondylitis and dysmenorrhea. Actually their main therapeutic effect is inhibition of the pain from different origin. NSAIDs show their anti-inflammatory activity in different ways such as, the reduction of superoxide radicals, induction of apoptosis, alteration of cellular membrane functions, decrease of proinflammatory cytokine levels and modification of lymphocyte activity (De Ruiter, 2002); (Botting, 2006).

Generally, their therapeutic actions occur inhibition of the both COX-1 and COX-2 enzymes (Figure 12). The COX-2 isoenzyme plays a significant role in pain and inflammation.
Most NSAIDs such as, aspirin, ketoprofen, indomethacin, piroxicam and sulindac are mainly COX-1 selective but others such as ibuprofen, naproxen and diclofenac are considered slightly selective for COX-1. There is also one other group considered slightly selective for COX-2 (eg, etodolac, nabumetone, and meloxicam). Coxibs (celecoxib, rofecoxib and valdecoxib) are the only group those primarily selective inhibitors of COX-2; there is any inhibition on the COX-1 isoenzyme (De Ruiter, 2002) (Figure 13).

![Figure 12. Inhibition of COX by NSAIDs (De Ruiter, 2002)](image-url)
All coxibs are diaryl-5-membered heterocycles. Figure 14 shows typical chemical structure of precursor COX-2 inhibitors. In place of the carboxyl group of their chemical structure, celecoxib and valdecoxib include sulfonamide group and that is methylsulfonide of rofecoxib. Celecoxib has a central pyrazole ring and two adjacent phenyl substituents, one containing a methyl group and the other a polar sulfonamide moiety; rofecoxib has a central furanone ring and two adjacent phenyl substituents, one containing a methylsulfone group, unlike celecoxib. Valdecoxib has a central oxazole ring and one phenyl ring with a polar sulfonamide like celecoxib (De Ruiter, 2002). The sulphur-containing phenyl rings of these drugs bind into the side pocket of the COX catalytic channel of COX-2 but interact weakly with the active site of COX-1 (Kurumbail, 1996). Thus, they are potent inhibitors of COX-2 and weak inhibitors of COX-1 (Botting, 2006).
Despite their wide range of therapeutical effects and widespread consumption; NSAIDs have certain side effects to a number of organ systems, particularly the gastrointestinal tract, where it has been estimated that chronic use of these drugs is associated with gastrointestinal ulceration and bleeding (Lichtenberger L., 2012). The side effects of NSAIDs are caused by inhibition of the constitutive enzyme, COX-1, that is responsible for the essential physiological functions such as platelet aggregation, inhibition of thrombogenesis, regulation of renal function and protection of the gastric mucosa. The therapeutic effects of NSAIDs are due to inhibition of COX-2 inducible enzyme (Nijkamp, 2005).

The apparent high risk of myocardial infarctions and the exacerbation of symptoms of hypertension and elevation of blood pressure led to the worldwide dramatic withdrawal of rofecoxib in 2004. Celecoxib and valdecoxib (Pfizer Inc.) were also withdrawn from the US market because of the same adverse effect. Selective COX-2 inhibitors and traditional NSAIDs are required to carry a warning of the risk of cardiovascular side effects recently. FDA approved the celecoxib as an adjuvant treatment of Familial Adenoma Polyposis (FAP) patients in 1999 and it has been still used.

**Figure 14.** Structure of COX-2 inhibitors (Non-Steroidal Anti-inflammatory Drugs), (De Ruiter, 2002)
A key factor that has emerged from the analysis of reasons why rofecoxib, valdecoxib, and celecoxib may have led to development of the cardio-renal syndrome is that myocardial infarction and hypertension are apparent with high dose levels and long period of use of these drugs (Harris R., 2007).

1.2.3 Effects of COX-2 Inhibitors in Colorectal Cancer

In the normal gastrointestinal tract, very little COX-2 or undetectable level of COX-2 is expressed. It is found that COX-2 expression is increase approximately 50% of adenomas and 85% of adenocarcinomas (Cathcart, 2012). Cohesive scientific evidence from molecular, animal, and human investigations supports the hypothesis that aberrant induction of COX-2 and up-regulation of the prostaglandin cascade play a significant role in colon cancer initiation and progression (Figure 15) (Kau, 2012). There are many studies try to explain the relationship between CRC and COX-2 expression into colorectal polyps, adenoma, and adenocarcinoma.

![COX-2 Expression](image)

Figure 15. COX-2 overexpression levels in colon carcinoma (Hilmi, 2006)
Evidence from epidemiological and preclinical observations, indicating that regular and prolonged treatment with anti-inflammatory drugs can reduce the incidence and recurrence of several human cancers by up to 50% (Asano, 2004). Aspirin and NSAIDs may inhibit colorectal carcinogenesis by increasing the rate of apoptosis in colon cancer cells, inhibiting tumor angiogenesis, cell proliferation and tumor growth, and decreasing metastatic potential (Figure 16) (Hyde, 2009); (Harald, 2010).

**Figure 16.** The relationship between arachidonic acid pathway and cancer progression (Mann, 2004)

In a study with 77 FAP patients were treated with celecoxib 100 mg BID, celecoxib 400 mg BID, or placebo. At 6 months, patients receiving celecoxib 400 mg BID demonstrated a significant reduction in polyp burden compared with placebo (30.7% vs 4.9%).
It led to approval of celecoxib for cancer prevention in familial adenomatosis polyposis patients. Actually cancer prevention is a viable clinical preference thus, COX-2 inhibitors have outstanding potential as chemopreventive agents in cancer (Table 3 & Table 4) (Kau, 2012).

**Table 3.** The most clinically applied and studied COX inhibitors (Hyde, 2009).
It is demonstrated that Vascular Endothelial Growth Factor (VEGF) and other growth factors may further increase COX-2 expression. Inhibition of this cycle by celecoxib has been found to limit angiogenesis and arrest the progression and metastatic spread of tumors in animals. Induction and up-regulation of COX-2 in cancer tissue is tightly correlated with increased activity of matrix metalloproteinases (MMP). These enzymes degrade cell membranes and basement membrane and are thus associated with tumor invasiveness, metastasis, and poor survival. Particularly, NSAIDs that inhibit COX-2 have been demonstrated to reduce MMP levels thereby decreasing the metastasis of colon cancer in animals (Harris R., 2007).

Compounds that inhibit COX-2 and PGE-2 appear to enhance both intrinsic and extrinsic apoptosis, and as a consequence, COX-2 inhibitors used in combination with radiation show beneficial synergism in the elimination of cancer cells in solid tumors. Nonsteroidal anti-inflammatory drugs also increase apoptosis by increasing bioavailable arachidonic acid pools necessary for conversion of sphingomyelin to ceramide since ceramide accumulation in the cell triggers apoptosis (Harris R., 2007). In conclusion, COX-2 inhibitors contribute to tumor genesis and the malignant phenotype of tumor cells at least five mechanisms: enhancing of apoptosis, inhibition of angiogenesis, and prevention of invasiveness, modulation of inflammation and immune-suppression, and conversion of carcinogens (Tuynman B., 2004).
NSAIDs have considerable importance for potential use in cancer treatment and chemoprevention. NSAIDs, either alone or in combination with other therapies, in reduces cancer risk, as well as improves survival rate in histologically different types of cancer. The underlying mechanisms of these chemopreventive effects have yet to be fully described. One of the favored hypothesis relates to suppression of prostaglandin E2-mediated angiogenesis. However, the focus of the hypothesis is an intriguing and less well described aspect of the anti-tumour activity of NSAIDs, namely the reversal of tumour-induced immune suppression. Evidence has accumulated to support the suggestion that NSAIDs modulate the effector mechanisms of anti-tumour immunity, both through COX-dependent and COX-independent ways. Such actions may constitute an important side of the effectiveness of anti-cancer immunotherapy (Hussaina, 2012). There is also prominent therapeutic effects of COX-2 inhibitor. Preclinical data suggests that, selective and non-selective COX-2 inhibitors also have a COX independent effect which leads anti-tumor effect of these agents. Moreover, data from cell lines and animal models have shown that NSAIDs in combination with chemotherapy enhances efficacy or can even avoid drug resistance. Similar findings have been also described for NSAIDs in combination with novel molecular targeted therapeutics (De Groot, 2007); (Kau, 2012).

1.3 Basis of spectroscopy

The interaction between electromagnetic radiation and matter is the main research interest of spectroscopy. This interaction leads specific peaks of different types of matter which is called spectrum. A spectrum is plotted absorbance versus the wavelength of the energy. Spectra are used to identify the components of a sample. It can be also used either to measure of the material or to detect the amount of material in a sample. According to the frequency of its wave electromagnetic radiation is classified into seven groups; radio waves, microwaves, infrared radiation, visible light, ultraviolet radiation, X-rays and gamma rays (Figure 17).
Figure 17. The electromagnetic spectrum.

When electromagnetic radiation interacts with matter, its behavior changes qualitatively with the frequency changes. The interaction between electromagnetic radiation and matter can cause transition between energy levels of the electrons or atoms. This transition between energy levels needs energy to move electron from one energy state to another. Possible electronic transition occurs between the ground state and the fourth vibrational level of the first excited state. (Freifelder, 1982). Typical energy level diagram are shown in Figure 18.
There is a simple theory which explains the absorption of light by molecules. When energy is absorbed by the matter this stimulated the transition of the molecules in the matter between different energy states. Light (wave) consists of interrelated perpendicular electric and magnetic fields. The absorbed energy of a typical wave can be explained the equations indicated below,

\[ \Delta E = h\nu \]

Where \( \Delta E \) is the separation between the energy states of interest, \( \nu \) is the frequency of the applied radiation and \( h \) is Planck’s constant (\( h = 6.6x10^{-34} \) joule second).

\[ c = \lambda \nu \]

Where \( c \) is the speed of light in vacuum (\( 3.0x10^8 \) ms\(^{-1} \)) and \( \lambda \) is the wavelength of radiation. The wavenumber is the spatial frequency of wave. In spectroscopy the wavenumber of electromagnetic radiation is defined as;
\[ \bar{\nu} = \text{wavenumber} = \left( \frac{1}{\lambda} \right) \]

Thus, \( E = h \nu = h c \bar{\nu} \),

From these calculations, it can be concluded that both wavenumber and frequency are directly related to energy.

### 1.3.1 Infrared (IR) spectroscopy

Infrared vibrational spectroscopy is a technique which examines absorption and transmission of the molecules in the infrared region (IR), based on their modes of vibration. Transition between ground and excited states of a molecule is caused by the absorption of light in the infrared region. At this level IR spectra are produced by the characteristic vibrations between chemical bond of specific functional groups (e.g., N-H, CH\(_2\), C=O) of the molecules. These characteristic vibrations cause as a bond asymmetric/stretching, bond bending, and etc. motions of the functional groups of the molecules.

The vibrational frequencies of most molecules correspond to the frequencies of infrared light. The infrared portion of the electromagnetic spectrum is usually divided into three regions; the near-, mid- and far- infrared, named for their relation to the visible spectrum (Smith, 1999) (Table 5).

<table>
<thead>
<tr>
<th>Region</th>
<th>Wavenumber Range (cm(^{-1}))</th>
<th>Wavelength (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Near-IR</td>
<td>14000 -4000</td>
<td>0.8-2.5</td>
</tr>
<tr>
<td>Middle-IR</td>
<td>4000 -400</td>
<td>2.5-25</td>
</tr>
<tr>
<td>Far-IR</td>
<td>400-4</td>
<td>25-1000</td>
</tr>
</tbody>
</table>

Table 5. Infrared Spectral regions (Smith, 1999)
The vibrational spectra of a molecule corresponds to the number and type of the bonds of this molecule. This specific type and number of the bonds also affect the number of the spectrum in the IR spectra region. The atoms in a complex molecule can vibrate in six different ways; symmetric and antisymmetric stretching, scissoring, rocking, wagging and twisting, as shown in Figure 19.

![Vibrational Modes](image)

**Figure 19.** The vibrational modes associated to a molecular dipole moment change detectable in an IR absorption spectrum (Marcelli, 2012)

### 1.3.2 Fourier Transform Infrared (FTIR) spectroscopy

Fourier transform infrared (FTIR) spectroscopy, is an infrared vibrational spectroscopy technique which attains broadband near-IR region to IR spectra. FTIR
spectrometer is the instrument of FTIR spectroscopy. An FTIR spectrometer collects and digitizes the interferogram, performs the FT function, and displays the spectrum.

An FTIR spectrometer obtain infrared spectra by first collecting an interferogram of a sample signal using a Michelson interferometer, and then performing a Fourier Transform on the interferogram to obtain the spectrum. The interferometer employs a beamsplitter which takes the incoming infrared beam and divides it into two optical beams. One beam goes to a flat mirror which is fixed in place. The other beam goes to a moving flat mirror which moves a very short distance away from the beamsplitter. The two beams come back from their respective mirrors and are recombined when they meet back at the beamsplitter.

The signal which exits the interferometer is the result of these two beams interfering with each other. The resulting signal is called an interferogram (Gerwert, 2010). In an interferogram, the signals that comes from the source has information about every infrared frequency. Thus by this way all frequencies are also being measured at the same time. For data analysis frequencies of the spectrum are used. A mathematical function called Fourier transform allows us to convert an interferogram into an intensity-versus-frequency spectrum. This transformation is performed by the spectrometer computer and a plot of intensity against wavelength (cm), or more usually frequency cm$^{-1}$ is presented for further analysis (Figure 20).

A background spectrum is taken for relative scaling of absorption intensity. Then this background spectrum is compared with the spectrum of the sample of interest to determine the percent transmittance and to remove the instrumental characteristics and finally to get pure spectrum where all the signals are due to the sample.
1.3.3 Advantages of the FTIR spectroscopy

FTIR spectroscopy has some advantages comparing to other spectroscopic techniques which are:

- The first advantage of FTIR is known as Felgett Advantage (Diem, 1999). Spectral measurements can be done simultaneously, most measurements by FTIR are made in a matter of seconds rather than several minutes.
- The second advantage of FTIR is known as Jacquinot Advantage. This means throughput advantage. Spectrometer has greater optical throughput and has a higher signal to noise ratio (S/N). It means the detectors are much more sensitive, and the fast scans enable the coaddition of several scans in order to reduce the random measurement noise to any desired level. The sensitivity benefits enable to get high quality infrared spectra from sample amounts as low as few micrograms (Stuart, 1997).
• The third advantage is known as Connes Advantage. This advantage is related to frequency scale of the spectrum. In FTIR spectroscopy frequency scale of the spectrum is very accurately. This allow the collection of many spectra and increase to signal to noise S/N ratio.

• Different types of samples (e.g.; solids, liquids, powder etc.) can be examined. Furthermore, it is a non-disturbing technique which provides structural and functional information about the sample (Liu K., 1996); (Dogan, 2007).

• Finally, FTIR spectrometer employ a HeNe laser as an internal wavelength calibration standard. It has an accurate wavelength calibration. Thus, it is self calibrating and never need to be calibrated by the user.

These advantages, along with several others, make FTIR spectroscopy extremely precise and transferable. It is a valuable technique due to its high sensitivity in detecting changes in the functional groups of the specific biological samples, such as lipids, proteins, carbohydrates and nucleic acids (Dogan, 2007). Analysis of the spectral parameters such as frequency shifts, band locations, bandwidths and band intensities or areas of the spectral bands give valuable structural and functional information, which may have diagnostic importance (Liu K., 1996).

1.3.4 Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy

ATR-FTIR spectroscopy is the most widely used FTIR sampling technique. This spectroscopic technique is used for the qualitative and quantitative analysis of samples with little or no sample preparation, which makes it a rapid method.

The working principles of an attenuated total reflection is based on measuring the changes that occur in a totally internally reflected infrared beam when the beam contacts with a sample. An infrared beam is directed onto an optically dense crystal which has a high refractive index, at a certain angle. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal into the sample that is held in contact with the crystal.
This evanescent wave projects only a few microns (0.5 μ - 5 μ) beyond the crystal surface and into the sample. When the sample absorbs energy in the target spectrum region, the evanescent wave will be attenuated. The attenuated energy from each evanescent wave is passed back to the IR beam, which then exits the opposite end of the crystal and is passed to the detector in the IR spectrometer (Figure 21). The system then forms an infrared spectrum.

The most important requirements for the technique are; the sample must be in direct contact with the ATR crystal and the refractive index of the crystal must be significantly greater than that of the sample. If the latter requirement is not met, the light will be transmitted rather than internally reflected in the crystal. Among many kinds of materials used for ATR crystal including diamond, amorphous material transmitting IR (AMTIR), germanium and silicon, zinc selenide (ZnSe) is the most common one (Kazarian, 2006).

![Figure 21. Schematic representation of ATR attachment top plate (Ellis, 2006)](image)

The main benefit of ATR sampling comes from the very thin sampling pathlength and depth of penetration of the IR beam into the sample. This is in contrast to traditional
FTIR sampling by transmission where the sample must be diluted with IR transparent salt, pressed into a pellet or pressed to a thin film.

When the sample is in a liquid or solid form, the intensity of the spectral features is determined by the thickness of the sample. Thus, a constant pathlength is highly desirable when performing quantitative analyses. ATR technique provides a constant pathlength for the samples since the wave can protrude a limited distance beyond the surface of the crystal into the sample (Cocciardi, 2005). While the analysis of samples by ATR is easy, there is also important experimental factors they affect the final spectrum of the sample. These are the followings:

- Refractive indices of the ATR crystal and the sample
- Angle of incidence of the IR beam
- Critical angle
- Depth of penetration
- Wavelength of the IR beam
- Effective pathlength
- Number of reflections
- Quality of the sample contact with ATR crystal
- ATR crystal characteristics

1.3.5 Applications of the FTIR spectroscopy

FTIR spectroscopy is an extremely reliable and well recognized fingerprinting method. Many substances can be characterized, identified and also quantified by this technique. Any changes in molecular compositions and structures of the samples can be detected by rapid and small sample amount. It has a wide range of application area. Such as for medical purposes it could detect biochemical signatures of tissues that associated with generation and progression of different type of diseases.
In a FTIR spectrum, important spectral parameters give valuable structural and functional information about the biomolecules, which may have diagnostic and therapeutic importance.

Any group or bond in a molecule gives rise to characteristic band(s) in the infrared spectra. Thus, these characteristic spectral features can simply be assigned to the particular groups or bonds in the corresponding molecules. This approach in vibrational spectroscopy is referred to as group frequencies (Diem, 1999). Hence, spectra can be examined in several groups depending on the type group frequency.

The main near-infrared spectral assigned bands are: the methyl C-H stretching vibrations, methylene C-H stretching vibrations, aromatic C-H stretching vibrations, N-H from primary amides, secondary amides (both alkyl, and aryl group associations), N-H from primary, secondary, and tertiary amines, and N-H from amine salts and O-H stretching vibrations. Minor but still important spectral assigned bands are: methoxy C-H stretching, carbonyl associated C-H stretching.

FTIR spectroscopy has been widely used in the molecular characterization and diagnosis of many diseases extending from cancer and metabolic diseases to infectious diseases (Dogan, 2007); (Rigas, 1990). During abnormal biological conditions changes in the chemical composition or structure of biofluids and other biological components occur at the molecular level (Mantsch H. , 1996), at this level FTIR spectroscopy identifies this kind of changes as a signatures of specific biomolecules. These biomolecules (lipids, carbohydrates, proteins, RNA and DNA) are introduced by distinct vibrational absorption characteristics in spectral analysis. Therefore, FTIR spectroscopy could serve as a diagnostic instrument for identifying and characterising different diseases or the therapeutic effects of drugs on the diseases (Maziak, 2007).

FTIR spectroscopy is also useful for different type of biological samples including cell lines, biological fluids and freshly dissected tissues. Serum IR spectroscopy now has been readily used in analyzing and differentiating clinical diseases, such as examining bovine spongiform encephalopathy serum (Menze, 2007) distinguishing Alzheimer
disease (Griebe, 2007), assigning myocardial infarction (Haas, 2010), and is also used as a prognostic tool for examining the severity of acute pancreatitis (Petrov, 2007). FTIR spectroscopic technique has a promising classification efficacy on freshly dissected tissues of thyroid nodules and metastatic lymph nodes (Liu K., 1996). In different cancer types, different tissue types have been used and the changes between the healthy and cancerous tissues have been investigated at the molecular level (Rigas, 1990).

Since IR spectroscopy techniques elucidate the biochemical composition of cells, tissues and fluids, together with advanced statistical and chemometric analysis methods, they have been commonly and successfully used to detect and discriminate different types of cancer cells such as leukemia, colon, urinary bladder, breast, liver, and cervix based on spectral differences. Moreover, this technique has been used in the discrimination of drug-resistant and drug-sensitive cancer cells, for the determination of apoptosis in cancer cells with anti-cancer drug treatment (Gaigneaux, 2006).

Macromolecular content changes and/or altered membrane dynamics, provided by FTIR known to be indicators of changes in important biological pathways and processes in cancer cells such as glucose consumption, lipid biosynthesis, lipid fluidity and order, proliferation, invasion and metastasis (Mourant J., 2003).

In summary FTIR spectroscopy has a wide application range in medical purposes so it seems to be a promising and powerful tool for therapeutic and diagnostic approaches.

1.4 Aim of the Study

CRC is the second prominent death cause of malignancy in the western countries, with 945,000 estimated new cases and 492,000 deaths per year in industrialized countries. After prostate and lung cancer it is at the third place among most common in men and also in women is on the third place, after breast cancer and gynecological cancers.
Treatment and chemoprevention of the colorectal cancer is currently one of the most investigated topic. Actually there are multiple therapeutical approaches in this area; conventional cancer therapies have some limitations such as the absence of suitable biomarkers which limit the application of anti-cancer therapies at the early stage of the disease (Wang T., 2002).

Low efficiency (resistance of the drug) and side effects are other complications of conventional cancer therapeutical agents. NSAIDs especially COX-2 inhibitors appear as a novel therapy for colorectal cancer chemoprevention and/or treatment. Celecoxib is one of the most investigated COX-2 inhibitors in this area and approved as a chemopreventive agent for FAP patients by FDA. CLX has anti-carcinogenic effects on CRC hallmarks such as induction of apoptosis, inhibition of angiogenesis, prevention of invasiveness, modulation of inflammation and immune-suppression. VLX which is successor of celecoxib is used as a therapeutical agent.

The previous molecular studies reported in the literature attract the attention more this area. However, there is a need for new studies in terms of structural level. Therefore, this current study aims to investigate the effects of valdecoxib on COX-2 positive and COX-2 negative colon cancer cell lines on the biomolecules in terms of compositional and structural level. The main aim is to reveal the effect of VLX on these cell lines and to explain whether these effects are COX dependent or not.

ATR spectroscopy was used to analyze cells for structural and functional alterations in their biomolecular components. ATR-FTIR provides rapid, sensitive and powerful monitoring of different functional groups of biomolecules in the systems. Other advantages, such as small sample size and easiness of sample preparation, make this technique a good candidate for biological studies. The samples can directly be investigated without any preparation processes by placing them on the ATR-crystals. In addition, ATR-FTIR studies are not affected by the sample thickness.

For this purpose, ATR-FTIR spectroscopy was used to characterize the effect of VLX on COX-2 expressing and COX-2 non-expressing colon cancer cell lines.
spectroscopy can provide information on the concentration of macromolecules (DNA, RNA, lipid, protein etc.) based on spectral band areas or band intensities, and on membrane dynamics from lipid bandwidth analysis such as CH₂ symmetric and asymmetric stretchings.
2.1 Cell Culture Experiments

2.1.1 Cell Line and Culture Conditions

The human colon cancer cell lines HT29 (HTB-38) (Figure 22) and SW620 (CCL-227) (Figure 23) were obtained from Banerjee Lab and grown in McCoy's 5a (Biochrome) Modified Medium supplemented with 1.5 mM L-glutamine, %10 FBS, 1% penicillin/streptomycin and in Leibovitz's L-15 Medium (Biochrome) supplemented with 2 mM L-glutamine, %10 FBS, 1% penicillin/streptomycin and %7.5 NaHCO₃ respectively. The cells were grown in a 5% CO₂ atmosphere at 37°C. Before the experiments, cells were seeded at a density of 3×10⁴ cells/cm² and cultured for 24 hours to allow them to adhere to the substratum and then cells were treated with either test compounds or Dimethyl Sulfoxide (DMSO).
Figure 22. HT29 colon carcinoma: HT29 cells isolated from primary adenocarcinoma of colon grade 1

Figure 23. SW620 colon carcinoma cell: SW620 cell line isolated from metastasis of primary adenocarcinoma of colon into lymph node, cells have fully developed invasive and metastatic potential.
2.1.2 Materials and Reagents

VLX was purchased from Cayman Chem. (Ann Arbor, Michigan, USA) via Algen Diagnostic (Distributor of Turkey). VLX was dissolved overnight in molecular biology grade DMSO (Sigma Chemical) and freshly diluted in culture medium before each experiment. Working concentration of DMSO in all treatments was adjusted to be less than 0.1%. The final DMSO concentration never exceeded 0.1% and this condition was used as control in each experiment.

VLX belongs to the aryl sulfonamide groups. It has poor water solubility (hydrophobic). Aryl sulfonamides are weak acids. The acidic nature results from the ability of the SO$_2$ moiety to stabilize the nitrogen anion through resonance. In the presence of a strong base, such as sodium or potassium hydroxide, aryl sulfonamides will react to form a salt (Figure 24). The sodium or potassium salt of a sulfonamide will readily dissociate in water, leading to highly water-soluble products, the result of ion-dipole bonding (Lemke, 2012).

![Figure 24. Salt formation of benzene sulfonamide](image)

Because of high hydrophobicity of the VLX during dilution, precipitation was observed. In order to avoid this problem, 0.1 M NaOH was added to the medium, so that solubility of VLX was increased. The selected molarity of NaOH was not toxic for the cell lines.
2.1.3 XTT (Cell Viability) Assay

SW680 and HT29 cells were seeded in 96-well plastic plates at a density of 1x10^3 cells/well. They were plated in a final volume of 100 µL in complete cell line specific medium and incubated in a humidified atmosphere of 5% CO₂ at 37°C for 24, 48 and 72 hours respectively. After incubation period, treatment with various concentrations of VLX (20, 30, 40, 50, 60, 70, 80, 90, 100, 125 and 150µM) for 24, 48 and 72 hours were applied. For cell viability assay XTT (Cell Proliferation Kit-II, Roche applied science) method was selected. Before this step, medium was replaced with 100 µL RPMI (Biochrome, w/o phenol red) medium. Firstly, XTT reagents (XTT labeling and e- coupling reagents) were thawed. Then, XTT assay was done for 96 well-plate. 5 ml XTT labeling reagent was mixed with 0.1 ml e- coupling reagents. McCoy's 5a (Biochrome) Modified Medium for HT29 cell line and Leibovitz's L-15 Medium for SW620 cell line were added in the control group. After 24 hours first XXT treatment was applied, XTT solution (50 µl/well) was added to each well and cells were incubated for an additional 24 hours at 37°C. The absorbance at 570 nm was measured on an ELISA reader with DMSO as a blank control. Each XTT assays were conducted for three times. The absorbance values were presented as relative viable cell number. The growth inhibition was calculated according to the following formula: Growth inhibition rate (IR %) = (1 - absorbance values of treated samples / absorbance values of untreated samples) x 100.

XTT assay results showed that VLX treatment for 72 hours has toxic effect on the cell lines, so it was not used in the experimental study. According to XTT assay results, IC₅₀ values (concentration that caused 50% inhibition of growth) were calculated using Graphpad software. Then targeted VLX doses were obtained by IC₅₀ calculations. IC₅₀ dose for HT29 was obtained as 77.5 µM for 24 hour treatment, and 72 µM for 48 hour treatment. For SW620 cell line, on the other hand, these doses were obtained as 71 µM for 24 hours and 74 µM for 48 hour treatments respectively.
Figure 25 and Figure 26 show the graphical representation of IC$_{50}$ results before and after NaOH application respectively.

**Figure 25.** Graphical representation of IC$_{50}$ results before NaOH application.
2.1.4 Experimental Groups and Cell Collection

HT 29 and SW 620 cells were seeded in 75 cm² plates. After 24 hours, cells were properly treated with VLX and their proliferation period started. For HT29, IC₅₀ dose was obtained as 77.5 µM for 24 hours and 72 µM for 48 hours respectively. On the other hand, for SW620, it was obtained as 71 µM for 24 hours and 74 µM for 48 hours respectively. After 24 and 48 hour treatment periods, cells were treated with trypsin/EDTA to detach them from the flask surface. Activity of trypsin/EDTA was immediately stopped by growth medium addition.
After that, cells were centrifuged at 100g for 5 min and supernatant was discarded. Live cell samples were counted by a hemocytometer using the light microscope. Two million cells/10 mL PBS solutions were prepared with 3 replicates for each sample. All 3 replicates of each independent replica (n=6) of both cell lines were counted for 4 times.

2.2 ATR-FTIR spectroscopy Measurements

2.2.1 Sample Preparation and ATR-FTIR Spectra Acquisition

FTIR spectra of tissues were recorded with an ATR unit (Perkin Elmer) combined with a Perkin-Elmer Spectrum 100 FTIR spectrometer. The spectra were recorded in the 4000–650 cm\(^{-1}\) region at room temperature. A total of 100 scans were taken for each interferogram at 4 cm\(^{-1}\) resolution. Collections of spectra and data manipulations were carried out using Spectrum 100 software (Perkin-Elmer). Each sample was scanned as three different replicates and the average spectra of these replicates were then used in the detailed analysis.

The 6 independent ATR-FTIR spectra for each cell lines (SW620 and HT29) were collected and used in the spectral analysis. Two million cells/10 mL PBS solutions were prepared and 2 µl sample from this stock solution was directly placed on to the ATR-Diamond/ZnSe (Di/ZnSe) crystal. Mild drying with N\(_2\) flux was applied for 5 minutes in order to remove the water in the PBS solution. This step was repeated for 5 times. A total of 10 µl sample was placed on to the ATR-Diamond/ZnSe (Di/ZnSe) crystal for each independent (n=6) replica (Gaigneaux, 2006).

2.2.2 ATR-FTIR spectroscopy and Spectral Analysis

IR spectra of VLX treated samples were obtained using a PerkinElmer Spectrum 100 FTIR spectrometer equipped with a Universal ATR accessory. The interfering spectrum of air was recorded as background and subtracted automatically by using
appropriate software (Spectrum 100 software). Using the same software, the spectra were first smoothed with nine-point Savitsky–Golay smooth function was used to remove the noise.

Classification and demonstration of the spectral data often requires a standardization step, so that information extracted from biologically equivalent signals can be quantified for comparison between classes. In this case, normalization allows for an effective comparison across a heterogeneous set of samples (Randolph, 2006). With normalization, information about the intensity of the spectrum is completely eliminated. Since this pretreatment is too tough for general data, it can be said that the use of normalization is limited to qualitative analysis (Kramer, 1998). Besides, baseline correction can also be preferably performed before the analysis of infrared spectra. It is frequently used to remove a sloping or curving baseline, which is essentially a wavelength-dependent intercept that can be unique for each sample spectrum (Franke, 2001). While all quantitative analysis were performed on non-normalized but pre-processed average spectra, for visual demonstration of the variations, the baseline corrected and normalized data were taken into consideration.

Baseline-corrected and normalized average spectra of the specific spectral bands were analyzed. Three main parameters were calculated in infrared spectra which are wavenumber of the spectral bands, band area values and bandwidth values, all of which provide information about the effect of VLX on macromolecules in the cell lines.

Firstly, the wavenumber value corresponding to the center of weight was assigned as the band positions in the well resolved bands. For the other case, peak position is determined from the second derivative of the spectra. Determination of the band positions is important to assign the peak of the bands. The wavenumber shifts in these band positions provides structural information about the biomolecules. Secondly, the band areas were calculated from smoothed and baseline-corrected spectra using Spectrum software. The ratios of the band areas were also examined. By analyzing the alterations in various ratios calculated, information about the relative concentrations
of the respective biomolecules in the system can be obtained. In addition to these two parameters bandwidth values, calculated as the width at 0.80 x height of the signal in terms of cm$^{-1}$ were also analyzed. Bandwidth analysis give dynamic information about the biomolecule of interest. For example, for the lipid (C-H) region, alterations in the bandwidth values give information about the changes in the membrane fluidity, while for the protein region, the alterations in this parameter give information about the mobility of the protein overall.

### 2.2.3 Cluster Analysis

For the observation of the spectral differentiation among experimental groups, cluster analysis was performed by using OPUS 5.5 software (Bruker Optics, GmbH). For cluster analysis, second derivative of each sample was taken in the 4000-650 cm$^{-1}$ region and subsequently vector normalization was applied over the investigated frequency range. For this analysis, spectral distances were calculated between pairs of spectra as Pearson’s correlation coefficients (Helm, 1991). Cluster analysis for the separation of control versus other groups was based on the Euclidean distances. Ward’s algorithm was used to construct dendrograms (Severcan F., 2010); (Lasch, 2004); (Helm, 1991).

Via the hierarchical cluster algorithm, two spectra showing the highest similarity, such as the spectra with the smallest spectral distance, were merged into a cluster, and the distance between the cluster constructed and all the remaining spectra were calculated. After that, the two spectra with the smallest distance merged into another new cluster again and again. Then, the distance between the newly formed cluster and the remaining spectra were calculated once more. These two sequential events of forming a cluster with the spectra having the smallest distance and calculating the distance between the clusters constructed and the remaining spectra were repeated till single cluster was obtained.
2.2.4 Statistical Analysis

In XTT assays, 24 and 48 hour drug treated groups were compared with control group. In addition, 24 and 48 hour drug treated groups were compared with each other for both cell lines. The data were analyzed statistically using non-linear regression dose response analysis using GraphPad Prism 6 software. In ATR-FTIR data analysis, the values were expressed as mean value ± standard error of mean (SEM).

P (calculated probability) value less than or equal to 0.05 was considered as statistically significant. The degree of significance was denoted as p<0.05 #, *, p<0.01 ##, **, p<0.001 ###, ***. Differences between the means were analyzed for significance using one-way ANOVA test with Tukey multiple comparisons which were used to assess the differences between independent groups.
CHAPTER 3

RESULTS

3.1 General FTIR Spectrum and Band Assignment of Colon Cancer Cell Lines

This study was conducted with 6 (six) experimental groups of colon cancer cell lines. Three of them were HT 29 colon cancer cell lines and these are control (n=5), 24 hour VLX treated (n=5), and 48 hour VLX treated (n=5) groups. Other three of them were SW 620 colon cancer cell lines and these are control (n=5), 24 hour VLX treated (n=5), and 48 hour VLX treated (n=5) groups. The main aim is to reveal the effect of VLX on two different COX-2 expressing (HT 29) and COX-2 non-expressing (SW 620) cell lines and to explain whether these effects are COX dependent or not. Moreover it is aimed to show whether VLX can be used as an anticarcinogenic and/or chemopreventive agent on the colon cancer induced damages.

In this study, ATR-FTIR spectroscopy was used as a basic informative method. Through the use of this spectroscopic method the structural and compositional alterations such as protein and lipid concentration, lipid/protein ratio, lipid peroxidation, acyl chain flexibility and membrane dynamics were compared among control, 24 hour and 48 hour VLX treated groups of both cell lines.

The existence of some infrared absorption bands suggests the presence of specific groups of atoms in the system (Steele, 1971). Therefore it is possible to assign specific wavelength of the infrared absorption bands to specific functional groups. Moreover, any alterations in different modes of vibrations correspond to the alterations in the conformation and structure. Through this approach, changes in the macromolecular content and composition could be revealed.
Figure 27 demonstrates the representative infrared spectrum of SW 620 and HT 29 colon cancer cell lines in the 4000-650 cm$^{-1}$ spectral region respectively. The main bands of the infrared spectra were labeled based on previous studies. The spectral band assignments of HT 29 and SW 620 colon cancer cell lines were presented in Table 6 and Table 7 respectively.

Figure 27. Representative infrared spectrum of HT 29 and SW 620 cell lines in the 4000-650 cm$^{-1}$ spectral region
### Table 6. ATR-FTIR band assignment of HT 29 colon cancer cell line

<table>
<thead>
<tr>
<th>Band</th>
<th>Wavenumber (cm⁻¹)</th>
<th>Band Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3287</td>
<td>Mainly N-H stretching (Amide A) of proteins with contribution from O-H stretching of polysaccharides (Garip, 2009)</td>
</tr>
<tr>
<td>2</td>
<td>3008</td>
<td>Olefinic HC=CH: unsaturated lipids (Liu K. B., 2002); (Severcan &amp; Korkmaz, 2005)</td>
</tr>
<tr>
<td>3</td>
<td>2955</td>
<td>CH₃ anti-symmetric stretching: equal contribution from lipids, protein side chains (Szalontai B., 2009); (Severcan F., 2010)</td>
</tr>
<tr>
<td>4</td>
<td>2923</td>
<td>CH₂ anti-symmetric stretching: mainly lipids (Ozek N., 2010)</td>
</tr>
<tr>
<td>5</td>
<td>2872</td>
<td>CH₃ symmetric stretching: mainly proteins, with the little contribution from lipids, carbohydrates, nucleic acids (Ozek N., 2010)</td>
</tr>
<tr>
<td>6</td>
<td>2853</td>
<td>CH₂ symmetric stretching: mainly lipids (Ozek N., 2010)</td>
</tr>
<tr>
<td>7</td>
<td>1742</td>
<td>Carbonyl C=O stretching: cholesterol esters (Ozek N., 2010)</td>
</tr>
<tr>
<td>8</td>
<td>1642</td>
<td>Amide I: protein C=O stretching β sheet structure (Haris &amp; Severcan, 1999); (Krafft, 2004)</td>
</tr>
<tr>
<td>9</td>
<td>1539</td>
<td>Amide II: protein N-Hbend, C-N stretch α helical structure (Kneipp, 2000); (Krafft, 2004)</td>
</tr>
<tr>
<td>10</td>
<td>1454</td>
<td>CH₂ bending: lipids (Bozkurt, 2010)</td>
</tr>
<tr>
<td>11</td>
<td>1397</td>
<td>COO symmetric stretching: fatty acids (Ozek N., 2010)</td>
</tr>
<tr>
<td>12</td>
<td>1234</td>
<td>PO₂ anti-symmetric stretching: mainly nucleic acids with the little contribution from phospholipids (Kneipp, 2000); (Akkas, 2007)</td>
</tr>
<tr>
<td>14</td>
<td>1074</td>
<td>PO₂ symmetric stretching: nucleic acids and phospholipids C-O stretch: glycogen, polysaccharides, glycolipids (Kneipp, 2000); (Akkas, 2007)</td>
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<tr>
<td>15</td>
<td>971</td>
<td>C-N²-C stretching: RNA (Garip, 2009)</td>
</tr>
<tr>
<td>16</td>
<td>919</td>
<td>Ribose ring vibrations: RNA/DNA (Ozek N., 2010)</td>
</tr>
</tbody>
</table>
Table 7. ATR-FTIR band assignment of SW 620 colon cancer cell line

<table>
<thead>
<tr>
<th>Band</th>
<th>Wavenumber (cm⁻¹)</th>
<th>Band Assignment</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>3284</td>
<td>mainly N-H stretching (Amide A) of proteins with contribution from O-H stretching of polysaccharides (Garip, 2009)</td>
</tr>
<tr>
<td>2</td>
<td>3009</td>
<td>Olefinic HC=CH: unsaturated lipids (Liu K. B., 2002); (Severcan &amp; Korkmaz, 2005)</td>
</tr>
<tr>
<td>3</td>
<td>2957</td>
<td>CH₂ anti-symmetric stretching: equal contribution from lipids, protein side chains (Szalontai B., 2009); (Severcan F., 2010)</td>
</tr>
<tr>
<td>4</td>
<td>2924</td>
<td>CH₂ anti-symmetric stretching: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids (Ozek N., 2010)</td>
</tr>
<tr>
<td>5</td>
<td>2872</td>
<td>CH₃ symmetric stretching: mainly proteins, with the little contribution from lipids, carbohydrates, nucleic acids (Ozek N., 2010)</td>
</tr>
<tr>
<td>6</td>
<td>2855</td>
<td>CH₂ symmetric stretching: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids (Ozek N., 2010)</td>
</tr>
<tr>
<td>7</td>
<td>1742</td>
<td>Carbonyl C=O stretching: cholesterol esters (Ozek N., 2010)</td>
</tr>
<tr>
<td>8</td>
<td>1640</td>
<td>Amide I: protein C=O stretching β sheet structure (Haris &amp; Severcan, 1999); (Krafft, 2004)</td>
</tr>
<tr>
<td>9</td>
<td>1537</td>
<td>Amide II: protein N-H bend, C-N stretch α helical structure (Kneipp, 2000); (Krafft, 2004)</td>
</tr>
<tr>
<td>10</td>
<td>1451</td>
<td>CH₂ bending: lipids (Bozkurt, 2010)</td>
</tr>
<tr>
<td>11</td>
<td>1396</td>
<td>COO⁻ symmetric stretching: fatty acids (Ozek N., 2010)</td>
</tr>
<tr>
<td>12</td>
<td>1235</td>
<td>PO₂⁻ anti-symmetric stretching: mainly nucleic acids with the little contribution from phospholipids (Kneipp, 2000); (Akkas, 2007)</td>
</tr>
<tr>
<td>14</td>
<td>1073</td>
<td>PO₂⁺ symmetric stretching: nucleic acids and phospholipids C-O stretch: glycogen, polysaccharides, glycolipids (Kneipp, 2000); (Akkas, 2007)</td>
</tr>
<tr>
<td>15</td>
<td>971</td>
<td>C-N⁻⁻⁻C stretching: RNA (Garip, 2009)</td>
</tr>
<tr>
<td>16</td>
<td>919</td>
<td>Ribose ring vibrations: RNA/DNA (Ozek N., 2010)</td>
</tr>
</tbody>
</table>

3.2 Effects of VLX on HT 29 and SW 620 Colon Cancer Cell Lines

ATR-FTIR spectral data were collected in the range of 4000-650 cm⁻¹. In order to be clearer, in the imagining of the alterations between the control and VLX treated spectra, the analysis were performed in two spectral ranges. The first range was 3030-2800 cm⁻¹ which corresponds to mainly lipid region and the second range was 1800-900 cm⁻¹, which corresponds to fingerprint region.
The figures below show the spectral alterations between the experimental groups in representative spectra, which are different from each other.

### 3.2.1. Effects of VLX in the C-H region (3030-2800 cm\(^{-1}\)) on HT 29 Colon Cancer Cell Line

Figure 28 shows the representative infrared spectrum of the control and 24, 48 hour VLX treated HT 29 colon cancer cell line in the 3030-2800 cm\(^{-1}\) region. The spectra were normalized with respect to the CH\(_2\) anti-symmetric stretching band.

**Figure 28.** The representative infrared spectrum of the control and 24, 48 hour VLX treated HT 29 colon cancer cell line in the 3030-2800 cm\(^{-1}\) region.
3.2.2. Effects of VLX in the C-H Region (3030-2800 cm\(^{-1}\)) on SW 620 Colon Cancer Cell Line

Figure 29 shows the representative infrared spectrum of the control and 24, 48 hour VLX treated SW 620 colon cancer cell line in the 3030-2800 cm\(^{-1}\) region. The spectra were normalized with respect to the CH\(_2\) anti-symmetric stretching band as before.

![Infrared Spectrum](image)

**Figure 29.** The representative infrared spectrum of the control and 24, 48 hour VLX treated SW 620 colon cancer cell line in the 3030-2800 cm\(^{-1}\) region.

3.2.3 Effects of VLX in the Fingerprint Region (1800-900 cm\(^{-1}\)) on HT 29 Colon Cancer Cell Line

Figure 30 shows the representative infrared spectrum of the control and 24,48 hour VLX treated HT 29 colon cancer cell line in the 1800-900 cm\(^{-1}\) region. The spectra were normalized with respect to the Amide I band.
Figure 30. the representative infrared spectrum of the control and 24,48 hour VLX treated HT 29 colon cancer cell line in the 1800-900 cm$^{-1}$ region.

3.2.4 Effects of VLX in the Fingerprint Region (1800-900 cm$^{-1}$) on SW 620 Colon Cancer Cell Line

Figure 31 shows the representative infrared spectrum of the control and 24, 48 hour VLX treated SW 620 colon cancer cell line in the 1800-900 cm$^{-1}$ region. The spectra were normalized with respect to the Amide I band.
Figure 31. The representative infrared spectrum of the control and 24, 48 hour VLX treated SW 620 colon cancer cell line in the 1800-900 cm$^{-1}$ region.

3.3 Numerical Comparisons of The Bands of Control, 24 and 48 hour VLX treated HT 29 and SW 620 Colon Cancer Cell Lines

Determining the position of a band and specifying the shifts in the band positions enable us to identify the bands and to obtain structural information about the biomolecules, respectively. To consider the possible spectral differences between the experimental groups, the mean and the SEM values of the wavenumber values of the bands, and the direction of the shifts were calculated. Control group was compared with respect to 24 and 48 hour VLX treated group. On the other hand, 24 hour treatment group was compared with respect to the 48 hour treatment group. All experimental groups were compared with the aid of ANOVA, and then Tukey test, as a post test, and the significance values were calculated. The results of significant changes in the wavenumber values of main bands of HT 29 and SW 620 were given in Table 8 and Table 9 respectively.
Table 8. Numerical summary of the detailed differences in the band frequencies of the HT 29 control, 24 and 48 hour VLX treated groups. The values are the means± SEM for each group. Tukey test was used to compare the groups. The control group was compared with respect to 24 and 48 hour VLX treated group.

<table>
<thead>
<tr>
<th>Band</th>
<th>Band Assignment</th>
<th>Control (n=5)</th>
<th>24 hours (n=5)</th>
<th>48 hours (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mainly N-H stretching (Garip, 2009)</td>
<td>3287.36±0.34</td>
<td>3287.31±0.95</td>
<td>3289.62±0.62</td>
</tr>
<tr>
<td>2</td>
<td>Olefinic HC=CH (Liu K. B., 2002); (Severcan &amp; Korkmaz, 2005)</td>
<td>3008.86±0.30</td>
<td>3008.55±0.32</td>
<td>3007.91±0.26</td>
</tr>
<tr>
<td>3</td>
<td>CH$_3$ anti-symmetric stretching (Szalontai B., 2009); (Severcan F., 2010)</td>
<td>2955.57±0.06</td>
<td>2956.51±0.19</td>
<td>2957.72±0.12</td>
</tr>
<tr>
<td>4</td>
<td>CH$_2$ anti-symmetric stretching (Ozek N., 2010)</td>
<td>2923.14±0.13</td>
<td>2923.55±0.05</td>
<td>2923.80±0.06</td>
</tr>
<tr>
<td>5</td>
<td>CH$_3$ symmetric stretching (Ozek N., 2010)</td>
<td>2872.80±0.05</td>
<td>2872.65±0.03</td>
<td>2872.67±0.04</td>
</tr>
<tr>
<td>6</td>
<td>CH$_2$ symmetric stretching (Ozek N., 2010)</td>
<td>2853.28±0.11</td>
<td>2853.40±0.03</td>
<td>2853.41±0.03</td>
</tr>
<tr>
<td>7</td>
<td>Carbonyl C=O stretching (Ozek N., 2010)</td>
<td>1742.69±0.07</td>
<td>1742.77±0.08</td>
<td>1742.80±0.03</td>
</tr>
<tr>
<td>8</td>
<td>Amide I (Haris &amp; Severcan, 1999) (Krafft, 2004)</td>
<td>1642.32±0.21</td>
<td>1641.30±0.12</td>
<td>1640.70±0.13</td>
</tr>
<tr>
<td>9</td>
<td>Amide II (Kneipp, 2000); (Krafft, 2004)</td>
<td>1539.64±0.31</td>
<td>1539.82±0.13</td>
<td>1540.56±0.19</td>
</tr>
<tr>
<td>10</td>
<td>CH$_2$ bending (Bozkurt, 2010)</td>
<td>1454.08±0.37</td>
<td>1454.38±0.07</td>
<td>1455.05±0.14</td>
</tr>
<tr>
<td>11</td>
<td>COO$^-$ symmetric stretching (Ozek N., 2010)</td>
<td>1397.05±0.11</td>
<td>1397.54±0.28</td>
<td>1398.11±0.10</td>
</tr>
</tbody>
</table>
Table 8 (continued)

<table>
<thead>
<tr>
<th>Band</th>
<th>Band Assignment</th>
<th>Control (n=5)</th>
<th>24 hours (n=5)</th>
<th>48 hours (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>PO$_2$ anti-symmetric stretching (Kneipp, 2000) (Akkas, 2007)</td>
<td>1234.79±0.2</td>
<td>1235.02±0.22</td>
<td>1234.30±0.15</td>
</tr>
<tr>
<td>13</td>
<td>CO–O–C anti-symmetric stretching (Ozek N., 2010)</td>
<td>1166.81±1.42</td>
<td>1165.26±1.01</td>
<td>1167.99±0.31</td>
</tr>
<tr>
<td>14</td>
<td>PO$_2$ symmetric stretching (Kneipp, 2000); (Akkas, 2007)</td>
<td>1074.23±1.42</td>
<td>1073.83±0.4</td>
<td>1074.78±0.40</td>
</tr>
<tr>
<td>15</td>
<td>C-N$^+$-C stretching (Garip, 2009)</td>
<td>971.98±0.64</td>
<td>974.87±1.2</td>
<td>972.29±0.56</td>
</tr>
<tr>
<td>16</td>
<td>Ribose ring vibrations (Ozek N., 2010)</td>
<td>919.86±0.87</td>
<td>920.90±0.97</td>
<td>916.96±0.36</td>
</tr>
</tbody>
</table>
Table 9. Numerical summary of the detailed differences in the band frequencies of the SW 620 control, 24 and 48 hour VLX treated groups. The values are the means ± SEM for each group. Tukey test was used to compare the groups. The control group was compared with respect to 24h and 48 hour VLX treated group.

<table>
<thead>
<tr>
<th>Band</th>
<th>Band Assignment</th>
<th>Control (n=5)</th>
<th>24 hours (n=5)</th>
<th>48 hours (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mainly N-H stretching (Garip, 2009)</td>
<td>3284.39±0.14</td>
<td>3303.47±4.36</td>
<td>3304.18±1.61</td>
</tr>
<tr>
<td>2</td>
<td>Olefinic HC=CH (Liu K. B., 2002); (Severcan &amp; Korkmaz, 2005)</td>
<td>3009.60±1.41</td>
<td>3008.63±0.82</td>
<td>3007.35±0.25</td>
</tr>
<tr>
<td>3</td>
<td>CH₃ anti-symmetric stretching (Szalontai B., 2009); (Severcan F., 2010)</td>
<td>2957.31±0.23</td>
<td>2958.41±0.10</td>
<td>2961.05±0.74</td>
</tr>
<tr>
<td>4</td>
<td>CH₂ anti-symmetric stretching (Ozek N., 2010)</td>
<td>2924.85±0.23</td>
<td>2925.54±0.11</td>
<td>2929.17±0.90</td>
</tr>
<tr>
<td>5</td>
<td>CH₃ symmetric stretching (Ozek N., 2010)</td>
<td>2872.63±0.05</td>
<td>2872.42±0.05</td>
<td>2872.35±0.08</td>
</tr>
<tr>
<td>6</td>
<td>CH₂ symmetric stretching (Ozek N., 2010)</td>
<td>2854.79±0.06</td>
<td>2854.92±0.43</td>
<td>2856.22±0.03</td>
</tr>
<tr>
<td>7</td>
<td>Carbonyl C=O stretching (Ozek N., 2010)</td>
<td>1742.40±0.34</td>
<td>1742.72±0.05</td>
<td>1742.73±0.03</td>
</tr>
<tr>
<td>8</td>
<td>Amide I (Haris &amp; Severcan, 1999) (Krafft, 2004)</td>
<td>1641.05±0.36</td>
<td>1639.83±0.13</td>
<td>1639.43±0.20</td>
</tr>
<tr>
<td>9</td>
<td>Amide II (Kneipp, 2000); (Krafft, 2004)</td>
<td>1537.86±0.17</td>
<td>1539.18±0.26</td>
<td>1542.28±0.73</td>
</tr>
<tr>
<td>10</td>
<td>CH₂ bending (Bozkurt, 2010)</td>
<td>1451.08±0.05</td>
<td>1451.77±0.07</td>
<td>1452.00±0.23</td>
</tr>
<tr>
<td>11</td>
<td>COO symmetric stretching (Ozek N., 2010)</td>
<td>1396.79±0.19</td>
<td>1398.08±0.16</td>
<td>1399.13±0.31</td>
</tr>
</tbody>
</table>
Table 9 (continued)

<table>
<thead>
<tr>
<th>Band</th>
<th>Band Assignment</th>
<th>Control (n=5)</th>
<th>24 hours (n=5)</th>
<th>48 hours (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>PO\textsubscript{2} anti-symmetric stretching (Kneipp, 2000) (Akkas, 2007)</td>
<td>1235.43±0.21</td>
<td>1234.60±0.07</td>
<td>1234.81±0.23</td>
</tr>
<tr>
<td>13</td>
<td>CO–O–C anti-symmetric stretching (Ozek N., 2010)</td>
<td>1164.97±1.98</td>
<td>1166.58±1.03</td>
<td>1157.70±1.51</td>
</tr>
<tr>
<td>14</td>
<td>PO\textsubscript{2} symmetric stretching (Kneipp, 2000); (Akkas, 2007)</td>
<td>1073.75±0.32</td>
<td>1074.74±0.76</td>
<td>1079.08±0.76</td>
</tr>
<tr>
<td>15</td>
<td>C-N\textsuperscript{+}-C stretching (Garip, 2009)</td>
<td>971.29±0.61</td>
<td>973.13±0.26</td>
<td>983.68±1.58</td>
</tr>
<tr>
<td>16</td>
<td>Ribose ring vibrations (Ozek N., 2010)</td>
<td>919.03±2.39</td>
<td>915.97±0.17</td>
<td>904.00±2.75</td>
</tr>
</tbody>
</table>

By analyzing the alterations in the band area values, information about the concentrations of the biomolecules in the experimental groups can be obtained. To consider the possible spectral differences which could be observed between the experimental groups, the mean and SEM values of the band area values and trend of the alterations in the areas were calculated. Control group was compared with respect to 24 and 48 hour VLX treated group, on the other hand 24 hour treatment group was compared with respect to the 48 hour treatment group. All experimental groups were compared with the aid of ANOVA, and then Tukey test, as a post test, and the significance values were calculated. The results of significant changes in the area values of main bands of HT 29 and SW 620 were given in Table 10 and Table 11 respectively.
Table 10. Numerical summary of the detailed differences in the band areas of the HT 29 control, 24 and 48 hour VLX treated group. The values are the means± SEM for each group. Tukey test was used to compare the groups. The control group was compared with respect to 24 and 48 hour VLX treated group.

<table>
<thead>
<tr>
<th>Band</th>
<th>Band assignment</th>
<th>Control (n=5)</th>
<th>24 hours (n=5)</th>
<th>48 hours (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mainly N-H stretching (Garip, 2009)</td>
<td>57.39±0.93</td>
<td>67.48±2.15</td>
<td>72.58±2.23</td>
</tr>
<tr>
<td>2</td>
<td>Olefinic HC=CH (Liu K. B., 2002); (Severcan &amp; Korkmaz, 2005)</td>
<td>57.69±0.90</td>
<td>68.23±2.15</td>
<td>72.87±2.25</td>
</tr>
<tr>
<td>3</td>
<td>CH3 anti-symmetric stretching (Szalontai B., 2009); (Severcan F., 2010)</td>
<td>2.71±0.07</td>
<td>2.65±0.03</td>
<td>2.18±0.02</td>
</tr>
<tr>
<td>4</td>
<td>CH2 anti-symmetric stretching (Ozek N., 2010)</td>
<td>4.53±0.11</td>
<td>4.03±0.06</td>
<td>4.02±0.07</td>
</tr>
<tr>
<td>5</td>
<td>CH3 symmetric stretching (Ozek N., 2010)</td>
<td>66.37±1.03</td>
<td>77.48±2.04</td>
<td>81.47±2.21</td>
</tr>
<tr>
<td>6</td>
<td>CH2 symmetric stretching (Ozek N., 2010)</td>
<td>3.05±0.06</td>
<td>2.65±0.06</td>
<td>2.58±0.05</td>
</tr>
<tr>
<td>7</td>
<td>Carboxyl C=O stretching (Ozek N., 2010)</td>
<td>72.21±1.11</td>
<td>83.65±2.00</td>
<td>87.41±2.22</td>
</tr>
<tr>
<td>8</td>
<td>Amide I (Haris &amp; Severcan, 1999); (Krafft, 2004)</td>
<td>22.63±0.44</td>
<td>21.55±0.38</td>
<td>20.87±0.24</td>
</tr>
<tr>
<td>9</td>
<td>Amide II (Kneipp, 2000); (Krafft, 2004)</td>
<td>16.40±0.52</td>
<td>15.04±0.37</td>
<td>14.39±0.27</td>
</tr>
<tr>
<td>10</td>
<td>CH3 bending (Bozkurt, 2010)</td>
<td>4.34±0.16</td>
<td>4.27±0.12</td>
<td>3.77±0.08</td>
</tr>
<tr>
<td>11</td>
<td>COO' symmetric stretching (Ozek N., 2010)</td>
<td>4.75±0.16</td>
<td>5.41±0.13</td>
<td>5.59±0.23</td>
</tr>
<tr>
<td>12</td>
<td>PO2 anti-symmetric stretching (Kneipp, 2000) (Akkas, 2007)</td>
<td>6.44±0.34</td>
<td>6.26±0.21</td>
<td>5.43±0.16</td>
</tr>
<tr>
<td>13</td>
<td>PO2 symmetric stretching (Kneipp, 2000); (Akkas, 2007)</td>
<td>11.57±0.36</td>
<td>10.82±0.26</td>
<td>9.97±0.27</td>
</tr>
<tr>
<td>14</td>
<td>C-N'C stretching (Garip, 2009)</td>
<td>1.83±0.07</td>
<td>1.54±0.06</td>
<td>1.11±0.05</td>
</tr>
</tbody>
</table>
Table 11. Numerical summary of the detailed differences in the band areas of the SW 620 control, 24 and 48 hour VLX treated group. The values are the means ± SEM for each group. Tukey test was used to compare the groups. The control group was compared with respect to 24 and 48 hour VLX treated group.

<table>
<thead>
<tr>
<th>Band</th>
<th>Band Assignment</th>
<th>Control (n=5)</th>
<th>24 hours (n=5)</th>
<th>48 hours (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mainly N-H stretching (Garip, 2009)</td>
<td>58.42±0.96</td>
<td>72.67±2.73</td>
<td>97.30±4.27</td>
</tr>
<tr>
<td>2</td>
<td>Olefinic HC=CH (Liu K. B., 2002); (Severcan &amp; Korkmaz, 2005)</td>
<td>60.95±0.98</td>
<td>76.95±2.95</td>
<td>97.37±4.39</td>
</tr>
<tr>
<td>3</td>
<td>CH₃ anti-symmetric stretching (Szalontai B., 2009); (Severcan F., 2010)</td>
<td>2.64±0.05</td>
<td>3.11±0.06</td>
<td>3.31±0.22</td>
</tr>
<tr>
<td>4</td>
<td>CH₂ anti-symmetric stretching (Ozek N., 2010)</td>
<td>4.47±0.07</td>
<td>3.85±0.10</td>
<td>3.29±0.31</td>
</tr>
<tr>
<td>5</td>
<td>CH₃ symmetric stretching (Ozek N., 2010)</td>
<td>71.03±1.09</td>
<td>85.90±2.75</td>
<td>104.62±3.80</td>
</tr>
<tr>
<td>6</td>
<td>CH₂ symmetric stretching (Ozek N., 2010)</td>
<td>3.64±0.13</td>
<td>3.30±0.06</td>
<td>0.81±0.09</td>
</tr>
<tr>
<td>7</td>
<td>Carbonyl C=O stretching (Ozek N., 2010)</td>
<td>77.44±1.27</td>
<td>92.30±2.63</td>
<td>110.42±3.63</td>
</tr>
<tr>
<td>8</td>
<td>Amide I (Haris &amp; Severcan, 1999) (Krafft, 2004)</td>
<td>26.03±0.61</td>
<td>23.06±0.24</td>
<td>21.24±0.98</td>
</tr>
<tr>
<td>9</td>
<td>Amide II (Kneipp, 2000); (Krafft, 2004)</td>
<td>18.97±0.62</td>
<td>16.13±0.48</td>
<td>11.44±1.22</td>
</tr>
<tr>
<td>10</td>
<td>CH₂ bending (Bozkurt, 2010)</td>
<td>4.19±0.15</td>
<td>3.97±0.17</td>
<td>2.61±0.31</td>
</tr>
<tr>
<td>11</td>
<td>COO⁻ symmetric stretching (Ozek N., 2010)</td>
<td>5.92±0.27</td>
<td>5.34±0.31</td>
<td>3.66±0.48</td>
</tr>
</tbody>
</table>
Table 11 (continued)

<table>
<thead>
<tr>
<th>Band</th>
<th>Band Assignment</th>
<th>Control (n=5)</th>
<th>24 hours (n=5)</th>
<th>48 hours (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>PO$_2$ anti-symmetric stretching (Kneipp, 2000)</td>
<td>7.20±0.27</td>
<td>5.90±0.27</td>
<td>3.32±0.51</td>
</tr>
<tr>
<td></td>
<td>(Akkas, 2007)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>PO$_2$ symmetric stretching (Kneipp, 2000); (Akkas,</td>
<td>13.03±0.34</td>
<td>11.73±0.50</td>
<td>7.66±1.33</td>
</tr>
<tr>
<td></td>
<td>2007)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>C-N$^+$-C stretching (Garip, 2009)</td>
<td>1.80±0.13</td>
<td>1.64±0.11</td>
<td>1.05±0.27</td>
</tr>
</tbody>
</table>

Bandwidth analysis give dynamic information about the interested biomolecule. For example, for the lipid (C-H) region, alterations in the bandwidth values give information about the changes in the membrane fluidity, while for the protein region the alterations in this parameter give information about the mobility of the protein overall. Table 12 and Table 13 give the numerical summary of the detailed differences in the bandwidths of SW 620 and HT 29 cell lines for control, 24 and 48 hour VLX treated groups.

Dynamics of the lipid membranes can be monitored by the information from the alterations in the bandwidth values of the CH$_2$ anti-symmetric stretching, and the CH$_2$ symmetric stretching bands (Bozkurt, 2010). In these bands located at C-H region, there were significant changes in SW 620 and HT 29 cell lines for control, 24 and 48 hour VLX treated groups. There were also significant increase in the bandwidth values of the Amide I band in SW 620 and HT 29 cell lines for control, 24 and 48 hour VLX treated groups. The change in the bandwidth of Amide I band indicated that there might be alterations in the mobility of proteins in both cell lines (Naumann, 1991).
Table 12. Numerical summary of the detailed differences in the bandwidths of SW 620 control, 24 and 48 hour VLX treated groups. The values are the means ± SEM for each group. Tukey test was used to compare the groups. The control group was compared with respect to 24 and 48 hour VLX treated groups.

<table>
<thead>
<tr>
<th>Band</th>
<th>Band Assignment</th>
<th>Control (n=5)</th>
<th>24 hours (n=5)</th>
<th>48 hours (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>CH\textsubscript{2} anti-symmetric stretching (Ozek N., 2010)</td>
<td>11,00±0,12</td>
<td>9,98±0,08</td>
<td>9,31±0,29</td>
</tr>
<tr>
<td>6</td>
<td>CH\textsubscript{2} symmetric stretching (Ozek N., 2010)</td>
<td>4,77±0,16</td>
<td>4,47±0,10</td>
<td>3,81±0,20</td>
</tr>
<tr>
<td>8</td>
<td>Amide I (Haris &amp; Severcan, 1999) (Krafft, 2004)</td>
<td>33,56±0,17</td>
<td>34,21±0,06</td>
<td>35,47±0,33</td>
</tr>
</tbody>
</table>

Table 13. Numerical summary of the detailed differences in the bandwidths of HT29 control, 24 and 48 hour VLX treated groups. The values are the means ± SEM for each group. Tukey test was used to compare the groups. The control group was compared with respect to 24 and 48 hour VLX treated groups.

<table>
<thead>
<tr>
<th>Band</th>
<th>Band Assignment</th>
<th>Control (n=5)</th>
<th>24 hours (n=5)</th>
<th>48 hours (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>CH\textsubscript{2} anti-symmetric stretching (Ozek N., 2010)</td>
<td>11,28±0,09</td>
<td>10,96±0,06</td>
<td>10,63±0,24</td>
</tr>
<tr>
<td>6</td>
<td>CH\textsubscript{2} symmetric stretching (Ozek N., 2010)</td>
<td>6,10±0,13</td>
<td>5,03±0,20</td>
<td>4,99±0,13</td>
</tr>
<tr>
<td>8</td>
<td>Amide I (Haris &amp; Severcan, 1999) (Krafft, 2004)</td>
<td>31,82±0,12</td>
<td>33,37±0,21</td>
<td>33,96±0,17</td>
</tr>
</tbody>
</table>
3.4 Hierarchical Clustering of the HT 29 and SW 620 Colon Cancer Cell Lines in Specific Spectral Ranges

Regarding the spectral differences between SW 620 control and HT 29 control cancer cell lines, these groups were differentiated using cluster analysis with high accuracy. The result is demonstrated in Figure 32.

The heterogeneity value obtained from the cluster analysis in the 4000–650 cm$^{-1}$ region indicated that there were profound differences in macromolecules between HT 29 and SW 620 colon cancer cell lines. As seen from Figure 32, these two groups were successfully differentiated from each other (6/6 for HT 29, 5/5 for SW 620) such that the higher heterogeneity value showed the power of ATR-FTIR spectroscopy in the successful discrimination of HT 29 and SW 620 colon cancer cell lines.

![Figure 32. Hierarchical clustering of the HT 29 and SW 620 colon cancer cell lines. Clustering was performed using Ward’s algorithm and second derivative vector normalized spectra in the spectral range of 4000 –650 cm$^{-1}$](image)
The heterogeneity value obtained from the cluster analysis in the 3030–2800 cm\(^{-1}\) region indicated that there were again profound differences in macromolecules between HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines. As seen from Figure 33 these three groups were successfully differentiated from each other (5/5 for control, 5/5 for 24 hour and 5/5 for 48 hour VLX treated). The higher heterogeneity value showed that ATR-FTIR spectroscopy was successful at the discrimination of HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines.

**Figure 33.** Hierarchical clustering of the HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines. Clustering was performed using Ward’s algorithm and second derivative vector normalized spectra in the spectral range of 3030-2800 cm\(^{-1}\) C-H region

The heterogeneity value obtained from the cluster analysis in the 1800–900 cm\(^{-1}\) region indicated that there were also profound differences in macromolecules between SW 620 control, 24 and 48 hour VLX treated colon cancer cell lines.
As seen from Figure 34, these three groups were successfully differentiated from each other (5/5 for control, 5/5 for 24 hour and 5/5 for 48 hour VLX treated). The higher heterogeneity value again showed that ATR-FTIR spectroscopy was successful in the discrimination of HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines.

**Figure 34.** Hierarchical clustering of the SW 620 control, 24 and 48 hour VLX treated colon cancer cell lines. Clustering was performed using Ward’s algorithm and second derivative vector normalized spectra in the spectral range of 1800-900 cm\(^{-1}\) fingerprint region.

The heterogeneity value obtained from the cluster analysis in the 4000–650 cm\(^{-1}\) region indicated that there were profound differences in macromolecules between SW 620 control, 24 and 48 hour VLX treated colon cancer cell lines and HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines. As seen from Figure 35, these six groups were again successfully differentiated from each other (for SW 620: 5/5 for control, 5/5 24 hour and 5/5 48 hour VLX treated; for HT 29: 5/5 for control, 3/5 for 24 hour and 5/5 for 48 hour VLX treated). The higher heterogeneity value again showed the
power of ATR-FTIR spectroscopy in the successful discrimination of SW 620 control, 24 and 48 hour VLX treated colon cancer cell lines and HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines respectively.

Figure 35. Hierarchical clustering of the HT 29 and SW 620 control, 24 and 48 hour VLX treated colon cancer cell lines. Clustering was performed using Ward’s algorithm and second derivative vector normalized spectra in the spectral range of 4000–650 cm⁻¹.
3.5 Detailed Spectral Analysis

3.5.1 Effects of VLX on SW 620 and HT 29 Colon Cancer Cell lines in The C-H region (3030-2800 cm\(^{-1}\))

The band positioned around 3011 cm\(^{-1}\) represents H-C=H vibrational stretchings of lipid molecules. The bands centered at 2962 cm\(^{-1}\), 2923 cm\(^{-1}\), 2873 cm\(^{-1}\) and 2853 cm\(^{-1}\) monitor CH\(_3\) asymmetric, CH\(_2\) asymmetric, CH\(_3\) symmetric and CH\(_2\) symmetric stretching vibrations respectively (Cakmak G. T., 2003; Cakmak G., 2006). Phospholipids acyl chain unsaturation can be monitored by evaluation of the intensity value of 3011 cm\(^{-1}\) band.

By analyzing the alterations in the band area values, information about the concentrations of the biomolecules in both cell lines can be obtained. The area of olefinic band was increased significantly in both cell lines for 24 and 48 hour VLX treatment (Figure 36).

![Figure 36](image.png)

**Figure 36.** The comparison of the olefinic band area between all groups. The degree of significance was denoted as *, # P < 0.05; **, ## P < 0.01 and ### P < 0.001. The VLX treated groups were compared with respect to control group. (*) means significance with respect to control, # means significance with in treated groups.)
The degree of conformational modifications is closely related to the frequency shifts of the CH$_2$ stretching vibrational modes. For this purpose, the CH$_2$ stretching vibrations in the system can assist to obtain trans/gauche isomerization in the lipid acyl chain (Mantsch H., 1996). An increase in the frequency of the CH$_2$ stretching band implies an increase in the number of gauche conformers in the fatty acyl chains (Cakmak G. T., 2003); (Cakmak G., 2006). The CH$_3$ asymmetric, CH$_2$ asymmetric and CH$_2$ symmetric stretching bands arise from the vibration of the related functional groups in lipid molecules. However, CH$_3$ symmetric stretching band monitors proteins (Mantsch H., 1996); (Cakmak G., 2006); (Cakmak G. T., 2003). The area under the CH$_3$ symmetric and CH$_2$ asymmetric bands increased significantly for both cell lines for 24 and 48 hour VLX treatments (Figure 37 and Figure 38).

![Figure 37](image)

**Figure 37.** The comparison of the CH$_3$ bending band area between all groups. The degree of significance was denoted as *, # $P < 0.05$; **, ## $P < 0.01$ and ### $P < 0.001$. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups.)
Bandwidth analysis can give different information for different regions of the spectrum. For the lipid (C-H) region, alterations in the bandwidth values give information about the changes in the membrane dynamics parameters such as membrane fluidity, while for the protein region the alterations in this parameter give information about changes in the protein conformation. The bandwidth value of the CH$_2$ asymmetric and symmetric bands decreased in both cell lines significantly for 24 and 48 hour VLX treatments (Figure 39 and Figure 40).

Figure 38. The comparison of the CH$_2$ band area between all groups. The degree of significance was denoted as *, # P < 0.05; **, ### P < 0.01 and ***, #### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups.)
Figure 39. The comparison of the CH$_2$ Asym. bandwidth between all groups. The degree of significance was denoted as *, # P < 0.05; **, ### P < 0.01 and ***, #### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups.)

Figure 40. The comparison of the CH$_2$ Sym. bandwidth between all groups. The degree of significance was denoted as *, # P < 0.05; **, ### P < 0.01 and ***, #### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups.)
3.5.2 Effects of VLX on SW 620 and HT 29 Colon Cancer Cell Lines in the Fingerprint Region (1800-900 cm\(^{-1}\))

The band centered at 1642 cm\(^{-1}\) was assigned as Amide I which is the main protein band. Significant decreases were observed in the band areas of both cell lines (Figure 41). The band at 1539 cm\(^{-1}\) was assigned as Amide II. Peak area of this band also decreased significantly for both cell lines (Figure 42).

**Figure 41.** The comparison of the Amide I band area between all groups. The degree of significance was denoted as *, # P < 0.05 ; **, ## P < 0.01 and ***, ### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups.)
The band at 1742 cm\(^{-1}\) is due to the C=O stretching vibrations of esters of phospholipids and cholesterol. Significant increase was observed in the band area of this band for both cell lines (Figure 43). The band located at 971 cm\(^{-1}\) was assigned to symmetric stretchings of dianionic phosphate monoesters of C-N\(^+\)-C stretching group (Cakmak G. T., 2003) and ribose-phosphate main chain vibrations of the RNA backbone. There was significant decrease in the band area values for both cell lines for this band (Figure 44).
Figure 43. The comparison of the C=O band area between all groups. The degree of significance was denoted as *, # P < 0.05; **, ## P < 0.01 and ***, ### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups).

Figure 44. The comparison of the C-N-C band area between all groups. The degree of significance was denoted as *, # P < 0.05; **, ## P < 0.01 and ***, ### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups).
The band located at 1233 cm$^{-1}$ was considered as hydrogen-bonded PO$_2^-$ asymmetric stretching groups of phospholipids (Wong, 1991); (Rigas, 1990). There was significant decrease in the band area of this band for both cell lines. The band centered at 1074 cm$^{-1}$ was assigned to PO$_2^-$ symmetric stretching vibrations of phospholipids (Ozek N.S., 2010). There were also significant decreases in the band areas of this band for both cell lines (Figure 45).

**Figure 45.** The comparison of the PO$_2$ bending band area between all groups. The degree of significance was denoted as *, # P < 0.05; **, ## P < 0.01 and ***, ### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups.)

### 3.5.3 The Effects of VLX for 24 and 48 hour On Different Band Area Ratios of Various Functional Groups in HT 29 and SW 620 Colon Cancer Cell Lines

The ratios of the band areas were also examined in order to get information about the relative concentrations of the respective biomolecules in the system. The mean and the SEM values of the band ratios of some specific bands, and direction of the alterations in these ratios were calculated. Control group was compared with respect to 24 and 48 hour VLX treated group whereas 24 hour treatment group was compared with respect to the 48 hour treatment group.
All experimental groups were compared with the aid of ANOVA, and then Tukey test, as a post test, and the significance values were calculated. The assignments of the band ratio values calculated, and the results of changes in the band ratio values were given in the following figures.

One of the important factors that alters the membrane structure and dynamics is the concentration of proteins and lipids of the membranes (Szalontai B. N., 2000). A lipid to protein ratios can be calculated precisely by measuring the ratio of the band areas emerging from proteins and lipids. The ratio of the olefinic to total lipid increased significantly in both cell lines. The ratio of the total CH$_2$ to total lipid increased significantly only in SW 620 (Figure 46 and Figure 47). The assignments of the band ratio values were given in Table 14 below. CH$_2$ Asym to total lipid ratio was significantly increased for SW 620. Olefinic to total CH$_2$ ratio also increased in the same cell line (Figure 48 and Figure 49).

Table 14. FTIR band ratios and their assignments

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Olefinic/Total Lipid*</td>
<td>Unsaturation level of the system</td>
</tr>
<tr>
<td>Olefinic/Total CH$_2$</td>
<td>Unsaturation level of the system</td>
</tr>
<tr>
<td>CH$_2$ sym + CH$_2$ asym / Total Lipid*</td>
<td>Saturation level of the system</td>
</tr>
<tr>
<td>CH$_2$ asym / Total Lipid*</td>
<td>Chain length of lipid</td>
</tr>
</tbody>
</table>

*Total Lipid = CH$_2$ asym + CH$_2$ sym + CH$_2$ bending + Ester + Olefinic
Figure 46. The comparison of the Olefinic/Total lipid band area ratio between all groups. The degree of significance was denoted as *, # P < 0.05; ** , ## P < 0.01 and ***, ### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups.)

Figure 47. The comparison of the Total CH2/Total lipid band area ratio between all groups. The degree of significance was denoted as *, # P < 0.05; ** , ## P < 0.01 and ***, ### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups)
Figure 48. The comparison of the CH$_2$ Asym/Total lipid band area ratio between all groups. The degree of significance was denoted as *, # P < 0.05; **, ## P < 0.01 and ***, ### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups).

Figure 49. The comparison of the Olefinic/Total CH$_2$ band area ratio between all groups. The degree of significance was denoted as *, # P < 0.05; **, ## P < 0.01 and ***, ### P < 0.001. The VLX treated groups were compared with respect to control group (* means significance with respect to control, # means significance within treated groups.)
CHAPTER 4

DISCUSSION

Colorectal cancer is the third most common cancer globally and has the fourth highest mortality rate, accounting for 7.6% of cancer-related deaths worldwide. Colorectal cancer may be described as a heterogeneous disease, with at least three major forms identified: hereditary, sporadic, and colitis-associated CRC (WHO, 2013).

The development of colon cancer is a typical multistep process: several mutations occur in key oncogenes, such as ki-ras, the adenomatous polyposis coli (apc), or the p53 tumor suppressor gene, causing cell degeneration and uncontrolled proliferation. Besides these mutations, as much as 80% of colon carcinomas show an enhanced cyclooxygenase-2 (COX-2) expression compared with normal intestinal mucosa (Cathcart, 2012). The COX enzymes catalyze the first step in the synthesis of prostanoids from arachidonic acid. COX was shown to exist as two distinct isoforms. These included the constitutively expressed COX-1 and the inducible form of COX-2, associated with inflammation (Warner, 2004). Actually, the arachidonic acid pathway is responsible for the generation of a wide variety of bioactive metabolites. On the other side, these metabolites have been shown to be involved in many different pathologies, including inflammation and cancer (Greene, 2011).

There has been a significant interest in COX-2 and its role in the development and progression of cancer (Antonarakis, 2009). Enhanced COX-2 expression leads in a dysregulation of arachidonic acid metabolism and excessive production of prostaglandins. Cells expressing high levels of COX-2, develop alterations in their adhesion to extracellular matrix and are resistant to apoptosis-inducing stimuli. Both of these phenotypic changes are consistent with an increased tumorigenic potential (Grosch S. T., 2001). Evidence for the role of the prostanoids in carcinogenesis
emerges from the numerous epidemiological studies. It was shown that chronic intake of non-steroidal anti-inflammatory drugs (NSAIDs) prevents development of the different types of cancer (Thun, 2002). Several potential mechanistic pathways have been implicated in the anti-tumor effects of NSAIDs. One of the most studied mechanism is their inhibitory effect on cyclooxygenase COX-2 mediated synthesis of prostanoids (Rahme, 2003); (Rao, 2002); (Tuynman J. , 2004); (Schrör, 2011).

The relationship between colorectal cancer (CRC) and COX-2 expression is the investigated research area of colorectal polyps, adenoma, and cancer. A recent study in patients with familial adenomatous polyposis (FAP), the use of both NSAIDs and selective COX-2 inhibitors has been shown to reduce both the size and number of intestinal adenomas (Eisinger, 2007). Although many of the anti-tumor effects of NSAIDs may be explained by their inhibitory effects on COX synthesis, several COX-independent mechanisms have also been identified. One of which is the NSAIDs membrane interaction mechanism (Grosch S. T., 2001); (Escriba, 2008).

NSAIDs are hydrophobic drugs, which have high penetration in inflamed tissues where pH is lower. It has monotopic properties, and it is able to penetrate on the cell membrane (Simmons, 2004); (Smith W. , 2011). NSAIDs effect on membranes at different physical states (Simmons, 2004); (Smith W. , 2011); (Nunes, 2011). The nature of the chemical modifications occur on lipid membrane, resulting from their interaction (Seddon, 2009). The antioxidant activity of NSAIDs has also been studied using membrane models and the overall results suggest that the antioxidant properties of NSAIDs are not only a consequence of their scavenging activity, but also of their ability to interact with membranes which leads to a more efficient contact between antioxidant molecules and free radicals (Santos, 2008); (Zhou, 2010); (Lucio, 2008). With respect to NSAIDs and cancer, COX-2 is constitutively expressed in several human solid tumors and hematological malignancies. Although the anticancer activity of NSAIDs may be related to the inhibition of COX-2 (Rizzo, 2011); (Ghosh, 2010); (Khan, 2011), alteration of membrane properties by anti-inflammatory agents may be an additional mechanism for their anticancer activity, apart from the inhibition of COX (Tavolari, 2012); (Lobo, 1994).
The previous molecular studies in the literature have focused more onto this area. However, there is a need for new studies in terms of structural level on the cell lines and cell membrane-NSAIDs interactions. Therefore, this current study aims to investigate the effects of VLX (as a member of NSAIDs) on COX-2 positive (HT29) and COX-2 negative (SW620) colon cancer cell lines on the lipid and protein molecules in terms of the compositional and structural level. Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) was used to analyze these cells for structural and functional alterations in their biomolecular components. ATR-FTIR provides rapid, sensitive and simultaneous monitoring of different functional groups of molecules in the biological systems, small sample size and easiness of sample preparation, make this technique a good candidate for biological studies. For this purpose, ATR-FTIR spectroscopy was used to characterize the effects of VLX on COX-2 expressing and COX-2 non-expressing colon cancer cell lines. FTIR spectroscopy can provide information about the biomolecules based on spectral band areas or band intensities, and on membrane dynamics from lipid bandwidth analysis. In infrared spectroscopic analysis, assignment of the band positions and the shifts in the positions of the bands enable to provide structural information about the biomolecules. Furthermore, determination of the alterations in the band area values give information about the concentrations of the corresponding biomolecules in the system. Moreover, through the calculation of various band area ratios, information about the relative concentrations of the respective biomolecules in the system can be obtained. Additionally, bandwidth of the spectral bands can also be examined to gather information about the changes in the membrane fluidity for the lipid (C-H) region, and to gather information about the alterations in the protein conformation, for the protein region of the spectra (Ozek N., 2010); (Ozek N.S., 2010).

In the current study, firstly, the 3030-2800 cm⁻¹ region of the infrared spectra were analyzed. This region is mainly composed of the bands originated from the lipids. The first band in this lipid region is the olefinic band which gives information about the unsaturation state of acyl chains in the system (Takahashi, 1991); (Severcan & Korkmaz, 2005); (Liu K. B., 2002); (Severcan F., 2003). In the literature, it has been reported that the shift in the band position of olefinic band to lower values is an
The shifts in the frequencies of the CH$_2$ stretching vibrations can be used as markers for the detection of changes in acyl chain flexibility (order/disorder state of lipids) (Ozek N., 2010). For this purpose, the CH$_2$ stretching vibrations in the system can assist to obtain trans/gauche isomerization in the lipid acyl chain (Mantsch H., 1996). An increase in the frequency of the CH$_2$ stretching band implies an increase in the number of gauche conformers (disordered state) in the fatty acyl chains (Cakmak G., 2003); (Cakmak and Severcan, 2006). The CH$_3$ asymmetric, CH$_2$ asymmetric and CH$_2$ symmetric stretching bands arise from the vibration of the related functional groups in lipid molecules. However, CH$_3$ symmetric stretching band monitors proteins (Mantsch H., 1996); (Cakmak and Severcan, 2006); (Cakmak G., 2003). In this study, the peak positions of these bands shifted slightly to higher values in HT29 cell line and significantly in SW620. This indicated an increase in the number of gauche conformers of lipid molecules.
In addition, this trend indicated an increase in lipid disorder, an increase in acyl chain flexibility (Severcan F., 1997); (Moore, 1995). These results can be explained by the mechanisms of NSAIDs, which is the interaction with cellular membranes and altering their biophysical properties. Therefore, it has been reported that NSAIDs can induce changes in the fluidity, permeability and biomechanical properties of cell membranes (Lichtenberger L. Z., 2012).

The shifts in the frequencies of the C=O carbonyl stretching gives information about the strength of H bonding (Mendelsohn, 2003). After VLX treatment, it was observed that these band shifts increased slightly. This means a decrease in the strength of the H bonds. Moreover, it leads to dehydration of both cell lines.

The band shifts derived from COO⁻ symmetric stretching vibrations of fatty acids side chains (Ozek N.S., 2010); (Cakmak G., 2003); (Cakmak and Severcan, 2006) have significant increases for both cell lines. It was shown that the system turns to more ordered state after VLX treatment. There was a molecular interaction between the NSAID and the membrane phospholipid. This association occurred between the polar carboxyl group of the NSAID and the polar head group of PC. In addition, multiple interactions were detected between the constituent atoms present in the aromatic rings of the NSAID and PC, most likely representing the acyl side-chains of the phospholipid. These interactions caused changes of membrane structure and lipid raft (Lichtenberger L. Z., 2012).

To detect any alterations in the protein content of HT29 nad SW620 cell lines, the shifts of the Amide I and Amide II bands were analyzed (Cakmak and Severcan, 2006); (Dogan, 2007); (Haris & Severcan, 1999). The Amide I band is a broad band that consists of several unresolved sub-structures of the protein secondary structural elements whose locations in an infrared spectrum are well defined (Liu K., 1996). After 24 and 48 hour VLX treatment on the cell lines, decrease was observed for Amide I band frequency. This shows a conformational change of protein secondary structure of the cells. On the other side, there is a dramatic increase for Amide II band for both cells, which is an indicator of the decrease of random coil structure (Spassov,
The pharmacological actions of drugs may be a consequence of their direct interaction with proteins and these results can be related to the NSAIDs direct interaction with proteins which may function as receptors, transporters or enzymes. Moreover, this interaction alters the protein biophysical properties, localization and function thus modifying protein conformation of the colon cancer cells in a positive way (Leite, 2013).

By analyzing the alterations in the band area or band intensity values, information about the concentrations of the corresponding biomolecules in the system can be obtained. Actually lipid peroxidation occurs primarily at double bond sites of polyunsaturated acyl chains and this results in loss of double bonds and leads a decrease in the band area due to lipid peroxidation (Cakmak G., 2011). However, in our case the area of olefinic band increased significantly in both cell lines for 24 and 48 hour VLX treatment. The increase in the olefinic band area was previously shown to monitor an increase in lipid peroxidation end products in biological systems (Severcan & Korkmaz, 2005); (Kinder, 1997); (Leskovjan, 2010); (Krishnakumar, 2009), (Severcan & Korkmaz, 2005). In cancer cells lipid peroxidation occurs at very high level, because of the increased level of inflammation and reactive oxygen species formations (ROS). As a result of free radical damage in long chain fatty acids (lipid peroxidation), fatty acids breakdown into smaller fragments. It is usually accompanied by the formation of a wide variety of degradation products, including alkanes and carbonyl compounds (Zwart, 1999); (Ozek N., 2010). COX-2 inhibition of VLX might be considered as the possible reason of this result, however both cell lines showed the same trend therefore COX-independent mechanism was the actual reason. NSAIDs have antioxidant properties which are related to the decreased lipid peroxidation. The effects of NSAIDs on membranes seem to be related to other in vivo beneficial actions of these drugs, such as their antioxidant properties and antitumoral activities. NSAIDs have free radical scavenging properties, which contribute for their anti-inflammatory effects since reactive oxygen species are considered as inflammatory mediators and have major roles in the pathological processes of chronic inflammatory disorders such as lipid peroxidation (Fernandes, 2004); (Chiurchiu, 2011).
The effects of VLX treatment on saturated lipid content and structure were investigated by analyzing the CH\textsubscript{2} asymmetric and symmetric stretching bands originating from lipid acyl chains (Cakmak and Severcan, 2006). The band area values of both bands in the HT29 and SW620 cells decreased. This indicated an decrease in the content of saturated lipids in the both cells. The decrease in the saturated lipid content observed in HT 29 and SW 620 cells also implies a slow cellular growth behavior. Lipid synthesis rate is known to be higher in dividing cells. As cancer cells have very high proliferation rate, the decrease in the saturated lipid content observed in the VLX treated cells supports the concept that VLX has antiproliferative effect on colon cancer cell lines by COX-independent manner (Maier, 2004). Furthermore, cancer cells have resistance on apoptosis. The above results may also indicate an increase in the apoptotic potential of the cells. These observations therefore support the hypothesis that VLX has apoptotic activity inducing ability by COX-independent mechanism (Grosch S. M., 2006).

The cholesterol esters and triacylglycerols were investigated by analyzing the C=O stretching band, whose area increased in the both cell lines. This increase suggested a high abundance of ester groups of triacylglycerols and cholesterol (Cakmak and Severcan, 2006); (Nara, 2002). Peroxidation of lipids which causes a breakdown of long chains into smaller fragments is usually accompanied by the formation of a wide variety of degradation products including alkanes and carbonyl compounds. However the results are not in a correlation with other acyl chain and fatty acid chain bands (CH\textsubscript{2} assymmetric and symmetric stretching bands). This situation might be related to the therapeutic action of VLX. Each membrane has a stable and specific lipid composition, which will only change under physiological or pathological situations such as cancer (Escriba, 2008). Since NSAIDs are weak acids, they are negatively charged at pH 7.4 and thus they interact preferentially with the head group region of the phosphatidylcholines (PC). In contrast, at pH 5.0, these NSAIDs are able to penetrate within the acyl chains of DPPC which has an ordering effect on the monolayer. This diverse interaction of NSAIDs with monolayers at physiologic and acidic conditions may have implications on the therapeutic actions of these drugs in vivo (Nunes, 2011). As cancer cells have acidic environment, NSAIDs-acyl chain interaction is strongly
related to this special condition of cancer cells and this leads to a recovery effect on the membrane lipid composition on colon cancer cell lines.

By evaluating the bands $\text{PO}_2^-$ antisymmetric and $\text{PO}_2^-$ symmetric stretching, the alterations in the nucleic acid contents were determined (Cakmak and Severcan, 2006); (Ozek N., 2010); (Wang J. J., 1997). The band area values of the nucleic acids bands of the HT29 and SW620 cells decreased. The information about RNA content was obtained from the $\text{C-N}^+\text{-C}$ streching band (Banyay, 2003). The band area values of both of these bands were found to decrease significantly in both cell lines. These reductions indicated a decrease in the amount of RNA in both cells. This reduction might be due to the decreased proliferation rate of cells and this could be related to the antiproliferative effect of VLX on colon cancer cell lines (Mourant J. Y., 2003), (Grosch S. T., 2001).

It was investigated and reported in multiple culture experiments with CRC cell lines that NSAIDs and coxibs cause a significant reduction of growth and viability of the cells. Interestingly, these are not always dependent on expression of COX-2. Waskewich tested the effects of celecoxib and rofecoxib in COX-2-positive and negative cell lines and he proved the anti-proliferative effect of these drugs. And this effect was independent of COX-2 status (Waskewich, 2002).

The band area of the Amide I and II vibrations give information about the proteins concentration. The significant decreases in the area values of these bands indicate a decrease in the protein content in VLX treated colon cancer cell lines. As previously mentioned VLX treatment showed a recovery effect on cancer-induced alterations in protein structure according to the results of the band shift analysis of amide bands. However, the decrease of the amide band’s area was related to the antiproliferative effects of VLX. Tuynman et all. showed that growth inhibition by NSAIDs may be mediated by distinct cellular processes including induction of apoptosis and the induction of cell-cycle arrest.
NSAIDs have shown to decrease the expression of cyclins. Furthermore, an increased expression of the cell-cycle-inhibitory proteins was observed. These resulted in a cell-cycle arrest independent of COX-2, since these effects were also observed in COX-2-negative CRC cell lines (Tuynman J. , 2004). As a result, the decrease in protein concentration supports the hypothesis that VLX has an antiproliferative effect.

In addition to band area and frequency shift parameters; bandwidth values were also analyzed. The alterations in the bandwidth values of the (C-H) region give information about the membrane dynamics. In addition, membrane fluidity is an important membrane dynamic parameter. According to CH2 symmetric and asymmetric stretching bandwidth results, there are significant decreases in both cell lines. These bands give the information about membrane dynamics and in both cell lines membrane fluidity parameters decrease after VLX treatment. In fact, each membrane has a stable and specific lipid composition which will only change under physiological or pathological situations (Escriba, 2008). Since metastatic tumor cells have higher membrane fluidity compared to non-metastatic cells (Sade, 2010), decrease of the membrane fluidity can be interpreted as therapeutic effect of VLX. Evidence from the studies have also supported the concept that the interaction of NSAIDs with PC lipids and the ability of the drugs to partition into the membranes can induce marked changes in the permeability, fluidity and biomechanical properties of these membranes (Moreno, 2009). Other NSAIDs like aspirin and etoricoxib that are known to have chemopreventive potential were found to restore the increased fluidity of colonic brush border membranes in colon carcinogenesis model in rats (Kanwar, 2007). In addition, several studies on cancer metastasis, have revealed that increased membrane fluidity is associated with the metastatic properties like motility and invasion (Nakazawa I., 1989) which could thus be counteracted by the ability of VLX to decrease membrane fluidity. Therefore, studies on membrane fluidity have been considered as a promising approach for cancer therapy.

The alterations in the unsaturation and the saturation levels of the lipids were also shown by calculating the ratios of olefinic/total lipid band area ratios and olefinic/total CH2 (CH2 asym + CH2 sym) band area ratios for indicating the unsaturation level and
CH$_2$ asym + CH$_2$ sym/total lipid band area ratio for indicating the saturation level. The olefinic/total lipid and olefinic/total CH$_2$ band area ratio increased significantly in SW 620 and HT 29 cell lines. These results also proved that, the degree of unsaturation increased dramatically in VLX treated group, compared to their controls. Besides, these results correlated well with the results obtained by the area analysis of the CH$_2$ bands, which means that VLX has a therapeutic effect against the inflammation-induced alterations in lipid un/saturation due to elevated lipid peroxidation levels.

To reveal the spectroscopic changes in the content of lipids, the ratios of the some specific lipid bands to the total lipid which are directly related to variation in lipid synthesis were evaluated. For example, CH$_2$/lipid ratio was used to examine the chain length of lipids, carbonyl ester/lipid ratio was used to examine carbonyl content of the system, olefinic = CH/lipid ratio was used to test unsaturation level of the system and finally CH$_3$/lipid was used to examine methyl concentration in the system (Cakmak G., 2011); (Acerbo, 2009); (Antoine, 2010).

Another ratio, CH$_2$ asym/total lipid band area, makes it possible to gather information about the alterations in the acyl chain lengths of lipids present in the system. The structural phospholipids, such as in the membrane structure and in the mitochondria, contain relatively large amount of polyunsaturated fatty acid in their phospholipid structure. These unsaturated lipid contents are susceptible to be attacked by free radicals in the case of elevated lipid peroxidation levels, and in turn it results with the breakdown of longer chains into smaller lipid acyl chains (Bozkurt, 2010). This process usually accompanied by the formation of a wide variety of degradation products, including alkanes and carbonyl compounds (Cakmak G., 2012). In pathological states, acyl chain lengths of lipids were found to decrease dramatically whereas the treatment of VLX was shown to reverse these alterations for both cell lines. The effect of VLX might be due to their free radical scavenging properties, which contributes for their anti-inflammatory effects, since reactive oxygen species are considered inflammatory mediators and have major roles in the pathological processes of chronic inflammatory disorders such as lipid peroxidation (Fernandes, 2004).
Finally, to test the power and sensitivity of ATR-FTIR spectroscopy cluster analysis on the VLX treated and control cell lines was also performed. Using the ATR-FTIR analysis, variety of alterations in the spectral parameters, like frequency, signal intensity/area and bandwidth were obtained in VLX treated colon cancer cells compared to the control samples. Based on the spectral variations, successful differentiation between 24 and 48 hour VLX treated colon cancer cells and control groups was obtained in different spectral regions using the cluster analysis. The results of the current study further showed the power and sensitivity of ATR-FTIR spectroscopy to detect the biomolecular alterations. Regarding the spectral differences between SW 620 and HT 29 control cancer cell lines, these groups were also successfully differentiated using cluster analysis. This was demonstrated in Figure 32. The heterogeneity value obtained from the cluster analysis in the 4000–650 cm$^{-1}$ region indicated that there were profound differences in macromolecules between HT 29 and SW 620 colon cancer cell lines. As seen from Figure 32, these two groups were successfully differentiated from each other (6/6 for HT 29, 5/5 for SW 620). The high heterogeneity value showed the power of ATR-FTIR spectroscopy in the successful discrimination of HT 29 and SW 620 colon cancer cell lines. Moreover, the heterogeneity value obtained from the cluster analysis in the 3030–2800 cm$^{-1}$ region indicated that there were profound differences in macromolecules between HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines. As seen from Figure 33, these three groups were successfully differentiated from each other (5/5 for control, 5/5 for 24 hour and 5/5 for 48 hour VLX treated). The higher heterogeneity value showed the power of ATR-FTIR spectroscopy in the successful discrimination of HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines.

The heterogeneity value obtained from the cluster analysis in the 1800–900 cm$^{-1}$ region showed that there were profound differences in macromolecules between SW 620 control, 24 and 48 hour VLX treated colon cancer cell lines. Again, as seen from Figure 34, these three groups were successfully differentiated from each other (5/5 for control, 5/5 for 24 hour and 5/5 for 48 hour VLX treated). Once more the higher heterogeneity value showed that ATR-FTIR spectroscopy is an accurate technique for
the successful discrimination of HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines.

Finally, the heterogeneity value obtained from the cluster analysis in the 4000–650 cm$^{-1}$ region suggested that there were profound differences in macromolecules between SW 620 controls, 24 and 48 hour VLX treated colon cancer cell lines and HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines. As seen from Figure 35, these six groups were successfully differentiated from each other (for SW 620: 5/5 for control, 5/5 24 hour and 5/5 48 hour VLX treated; for HT 29: 5/5 for control, 3/5 for 24 hour and 5/5 for 48 hour VLX treated). The higher heterogeneity value showed that ATR-FTIR spectroscopy technique again was successful in the discrimination of SW 620 control, 24 and 48 hour VLX treated colon cancer cell lines and HT 29 control, 24 and 48 hour VLX treated colon cancer cell lines.

Although many studies are present in the literature about the effects of COX- inhibitors (NSAIDs) on the colon cancer, the effects of VLX treatment on the macromolecular structure, composition and function of colon cancer cells have not been demonstrated yet. In this manner, the current study fills the missing part of the literature about the therapeutic effects of VLX treatment on the COX-2 expressing (HT29) and nonexpressing (SW620) colon cancer cell lines at macromolecular level. Furthermore, another aim of this study was to explain that these possible therapeutic effects of VLX are COX-dependent or not.
CHAPTER 5

CONCLUSION

The results of this study show that VLX treatment of the COX-2 expressing HT29 and COX-2 non-expressing SW620 colon cancer cell lines show remarkable effects on the membrane fluidity, the acyl chain order, dynamics and the phosphate head groups of both cell lines in a COX-2 independent manner.

The detailed spectral analysis of ATR-FTIR spectroscopy showed the following remarks: The saturation level of the lipids in the corresponding biological system altered due to the increased level of inflammation in colon cancer. The unsaturated lipid content was shown to decrease in colon cancer state due to increased levels of inflammation and free radical formation through elevated lipid peroxidation levels. As a result of the chronic inflammation, the COX-2 expression increased in cancer cells. There is also an imbalance between the production and scavenging of free radicals and inflammatory cytokines. This situation forms the basis of the development of cancer. VLX treatment inhibits the increased level of COX-2 expression which is COX-dependent mechanism of VLX. On the other hand, VLX can able to interact with cell membrane and it can change the membrane structure which is the COX-2 independent mechanism of VLX. Through these ways, VLX treatment can effectively recover the damages and protect the colon cancer cells and the biomembranes against peroxidative damage. The orderness of the system was also found to be changed due to the increased level of inflammation. Chronic inflammation is the reason of lipid peroxidation. Besides the alterations in the unsaturation/saturation levels of lipids, contents of lipids and proteins also changed, that is, the lipid contents decreased in the cancer cells. These reductions in lipid contents were caused by the elevated levels of inflammation and lipid peroxidation. However after VLX treatment the lipid contents were increased in both cell lines.
The results indicated that VLX, as a COX-2 inhibitor, has a therapeutic effect on inflammation-induced alterations in colon cancer cell lines. However, protein contents decreased after VLX treatment, which might be caused by the antiproliferative effects of VLX on the colon cancer cells. As cancer cells have high cell proliferation rate, it can be concluded that VLX treatment reversed this pathologic condition by the COX-independent way.

In addition to the changes in the protein content, the protein conformation also altered in cancer cells. Through the band shift and area analyses, the aggregation and denaturation of proteins were observed in the case of cancer. VLX treatment showed a therapeutic effect on chronic inflammation-induced alterations in protein structure, by decreasing the aggregated and denatured protein contents. Moreover, fatty acid levels, phospholipid amounts in the membrane structure and also nucleic acids content were also shown to decrease in cancer cells as consistent with the previous findings mentioned above. However, VLX treatment can reverse these inflammation-induced alterations in the colon cancer cell lines. Fatty acid levels, phospholipid amounts in the membrane structure and also nucleic acids content were all shown to be recovered after VLX treatment.

Throughout their in vivo pathway, NSAIDs inevitably interact with membranes to be absorbed, distributed, metabolized and eliminated and also reach their targets and exert their therapeutic actions. Therefore, the NSAIDs-membrane interaction has notable implications for their pharmacokinetics and their therapeutic effects. In this regard, the study of the NSAID effects on membrane level may uncover valuable details about their biological actions, which may constitute the rational basis for the development of new drugs with better efficacy and/or safety profiles (Leite, 2013). The therapeutic actions of NSAIDs are possibly associated with their capacity to inhibit the inflammatory cascade in vivo, not only by directly inhibiting enzymes, but also by inducing changes in membrane properties (Leite, 2013). Actually, the modification of membrane lipid composition and structure seems to be related with the development of numerous diseases, indicating that membrane-lipid therapy might have a potential role in the treatment of several conditions (Escriba, 2008).
By the help of the findings obtained from this study, new therapeutic approaches for the treatment of colon cancer using VLX can be considered in order to treat inflammation induced changes for colon cancer cell lines. It can be also concluded that this effect is COX- independent. Moreover, a different approach from the main clinical strategies, adjuvant therapy as well as chemoprevention, can also be suggested as a complementary therapeutic strategy for colon cancer patients since VLX treatment for this particular case, can effectively have a therapeutic effects on the biomolecular and membrane structures of the colon cancer cells against peroxidative damage due to the increased level of inflammation. Chemopreventive as well as adjuvant therapy has a great deal of importance for all cancer types in order to have a chance to treat the patients more effectively. As a conclusion, this current study provides a new insight on the therapeutic aspects of colon cancer.
REFERENCES


