RESEARCH ARTICLE

Anticancer Effect of COX-2 Inhibitor DuP-697 Alone and in Combination with Tyrosine Kinase Inhibitor (E7080) on Colon Cancer Cell Lines

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Abstract

Colorectal cancer remains one of the most common types of cancer and a leading cause of cancer death worldwide. In this study, we aimed to investigate effects of DuP-697, an irreversible selective inhibitor of COX-2 on colorectal cancer cells alone and in combination with a promising new multi-targeted kinase inhibitor E7080. The HT29 colorectal cancer cell line was used. Real time cell analysis (xCELLigence system) was conducted to determine effects on colorectal cell proliferation, angiogenesis was assessed with a chorioallantoic membrane model and apoptosis was determined with annexin V staining. We found that DuP-697 alone exerted antiproliferative, antiangiogenic and apoptotic effects on HT29 colorectal cancer cells. For the antiproliferative effect the half maximum inhibition concentration (IC_{50}) was 4.28×10^{-8} mol/L. Antiangiogenic scores were 1.2, 0.8 and 0.5 for 100, 10 and 1 nmol/L DuP-697 concentrations, respectively. We detected apoptosis in 52% of HT29 colorectal cancer cells after administration of 100 nmol/L DuP-697. Also in combination with the thyrosine kinase inhibitor E7080 strong antiproliferative, antiangiogenic and apoptotic effects on HT29 colorectal cancer cells were observed. This study indicates that DuP-697 may be a promising agent in the treatment of colorectal cancer. Additionally the increased effects observed in the combination with thyrosine kinase inhibitor give the possibility to use lower doses of DuP-697 and E7080 which can avoid and/or minimize side effects.

Keywords: Colon cancer - DuP-697 - COX-2 inhibition - tyrosine kinase inhibitor

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Introduction

Colorectal cancer (CRC) remains one of the most common types of cancer and leading cause of cancer worldwide. In the United States, CRC is the third cause of cancer related death (Siegel et al., 2011). Capecitabine monotherapy or combination chemotherapies such as infusional 5-fluorouracil/leucovorin with oxaliplatin and infusional 5-fluorouracil/leucovorin with irinotecan are considered standard regimens for CRC. However, the positive response rate is low and these drugs carry substantial toxicity (Bokemeyer et al., 2009). Therefore, better systemic therapies are needed to assess increased efficacy and improve the clinical outcomes of patients.

Cyclooxygenase (COX) is a very important enzyme in the production of prostaglandins and many other biologically active molecules from arachidonic acid. It has two isoforms COX-1 and COX-2. Cyclooxygenase-1 found in all cells constantly releases, is not affected of stimulation and its levels does not fluctuate (Koki and Masferrer, 2002). COX-2 is an inducible enzyme and it can be affected by mitogens, growth factors and hormones. COX-2 is of great importance in tumorogenesis. It has been shown that COX-2 induces the production of vascular endothelial growth factor (VEGF), thus contributes to angiogenesis. COX-2 also increases the metalloproteins which improves the invasion of tumor vessels and reduces the production of antiangiogenic cytokine interleukin-12. It has also been shown that COX-2 increases resistance to apoptosis (Pereg and Lishner, 2005; Pandurangan and Esa, 2013). Initially it was reasoned that because of the cardiotoxicity associated with COX-2 inhibition, the COX-2 independent antitumorigenic effects of coxibs are needed to be pursued further (Grösch et al., 2006). Therefore, targeting COX-2 pathway may be a reasonable approach for cancer therapy. 5-Bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl) phenyl]-thiophene (DuP-697), is a selective COX-2 inhibitor. Some studies suggest that the mechanism of cytotoxic effect of DuP-697 may be via induction of apoptosis and that this was related with the upregulation of caspases 3, 8 and 9 (Churchman et al., 2007; Peng et al., 2008).

Vascular endothelial growth factor and its corresponding tyrosine kinase (TK) receptors play a critical role in physiological and pathological angiogenesis, a process of forming new capillaries from existing blood vessels. Angiogenesis is essential for solid tumor growth and metastasis. VEGF is involved in tumor angiogenesis by inducing endothelial cell proliferation, migration and survival (Darakhshan et al., 2013). Its importance in CRC

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growth and development has been documented (Stocmann et al., 2008).

There are currently two groups of targeted agents to VEGF, monoclonal antibodies and small-molecule TK inhibitors which both inhibit the signal transduction pathways through TK receptors. Monoclonal antibodies bind to the ligand or the extracellular domain of the receptor while TK inhibitors target the intracellular part of the receptor (Cohen et al., 2005). Tyrosine kinases play critical role in modulation of growth factor signaling. Activated forms of these enzymes can cause increases in tumor cell proliferation and growth, induce antiapoptotic effects, and promote angiogenesis and metastasis (Blume-Jensen and Hunter, 2001).

E7080 (lenvatinib) is a new multi-targeted kinase inhibitor that is currently in clinical development. The main targets of E7080 include vascular endothelial growth factor receptors (VEGFR), fibroblast growth factor receptor (FGFR) and platelet derived growth factor receptors (PDGFR) and stem cell factor (SCF) receptors. It has been shown to inhibit tumor angiogenesis by targeting endothelial cells (Glen et al., 2011). Currently, E7080 is being tested in several phase I and phase II studies and has shown promising signs of anti-cancer activity (Dubbleman et al., 2012). Promising results in different types of cancer have been demonstrated for E7080 (Matsui et al., 2008; Ogino et al., 2011) and we also recently reported that E7080 has antiproliferative, antiangiogenic and apoptotic effects on HT29 colorectal cancer cells (Altun et al., 2013).

In many types of cancer upregulation of COX-2 and the epidermal growth factor receptor (EGFR) have been detected together (Fildaro, 2002; Heuckmann, 2012). It is also known that activated EGFR upregulates COX-2 expression through an EGFR- mitogen activated protein kinase (MAPK)-COX2 cascade (Xu et al., 2009). In a study recently conducted it was shown that Rutin, a well known flavonoid, inhibits B[a] PDE-induced COX-2 expression by suppressing the Raf/MEK/ERK and Akt signaling pathways (Choi et al., 2013).

The objective of the present study was to investigate the antitumor effect of a selective COX-2 inhibitor DuP-697 on CRC as monotherapy and in combination with a TK inhibitor E7080 with a hypothesis that combined treatment would be improvable for antitumor activity than that of single agent treatment and usage of lower doses may avoid and/or minimize the side effects.

Materials and Methods

DuP-697 was purchased from Tocris Bioscience, E7080 was purchased from Selleck Chemicals LLC (Boston, USA). All medium, solution and enzymes for cell culture were purchased from Sigma Aldrich.

Cell culture

Human colon adenocancer cells HT29 were purchased from the Sap Institute (The Ministry of Food, Agriculture and Livestock, Ankara, Turkey). Cells were multiplied in three passages, frozen in aliquots and stored in liquid nitrogen. The cells were maintained in DMEM with phenol red and $NaHCO_3$. The culture medium was supplemented with 10% heat inactivated FBS, 1% penicillin and streptomycin. Cells were grown in T-75 cm^2 culture flasks in a humidified atmosphere containing 5% CO_2 at 37°C.

Instrumentation

xCELLigence system: the xCELLigence system was used according to the instructions of the supplier (Roche Applied Science and ACEA Biosciences, 2008). The xCELLigence system consists of four main components: the RTCA analyzer, the RTCA DP station, the RTCA computer with integrated software, and disposable E-plate 16. The RTCA DP station fits inside a standard tissue-culture incubator, while an analyzer and laptop computer with software will be on the outside. The core of the xCELLigence system is the E-plate 16: this is a single use, disposable device used for performing cell-based assays on the RTCA DP instrument, which has similar application like commonly used 96-well micro titer plate. However the E-plate 16 differs from standard 96-well micro titer plates vastly with its incorporated gold cell sensor arrays in the bottom, which contributes cells in-side each well to be monitored and assayed. The E-plate 16 has a low evaporation lid design (the bottom diameter of each well is 5.0mm±0.05mm; with a total volume of 210±5 μL, approximately 80% of the bottom areas of each well is covered by the circle-on-line electrodes, which is designed to be used in an environment of +15 to +40°C, relative humidity 98% maximum without condensation. The electronic impedance of sensor electrodes is measured to allow monitoring and detection of physiological changes of the cells on the electrodes. The voltage applied to the electrodes during RTCA measurement is about 20mV (RMS). The impedance measured between electrodes in an individual well depends on electrode geometry, ion concentration in the well and whether or not cells are attached to the electrodes. In the absence of cells, electrode impedance is mainly determined by the ion environment both at the electrode/solution interface and in the bulk solution. In the presence of cells, cells attached to the electrode sensor surfaces will act as insulators and thereby alter the local ion environment at the electrode/solution interface, leading to an increase in impedance. Thus, when more cells are growing on the electrodes, the larger is the value of electrode impedance. The RTCA associated software allows users to obtain parameters such as: average value, maximum and minimum values, standard deviation (SD), half maximum effect of concentration (EC50), half maximum inhibition of concentration (IC_{50}), cell index (CI), and in addition graphics. The data expressed in CI unit scan is exported to Excel for any type of mathematical analysis (Roche Applied Science and ACEA Biosciences, 2008).

Cell growth and proliferation assay using xCELLigence system: HT29 cells were grown and expanded in tissue-culture flasks. After reaching 75% confluence, the HT29s (passage 6) were washed with PBS, afterwards detached from the flasks by a brief treatment with trypsin/EDTA. Subsequently, 100 μL of cell culture media at room temperature was added into each well of E-plate16. After this the Eplate16 was connected to the system and checked in the cell culture incubator for proper electrical contacts

and the background impedance was measured. Meanwhile, the cells were re-suspended in cell culture medium and adjusted to 40.000 cells/mL. 100 μL of cell suspension was added to the 100 KL medium containing wells on E-plate16. After 30 min incubation at room temperature, E-plate16 was placed into the cell culture incubator. Finally, proliferation of the cells was monitored every hour for a period of up to 72h via the incorporated sensor electrode arrays of the E-Plate16. The electrical impedance was measured by the RTCA-integrated software of the xCELLigence system as a dimensionless parameter termed CI.

Cytotoxicity assay using xCELLigence system: first, the optimal seeding concentration for proliferation experiments of the HT29 was determined. After seeding the respective number of cells in 100 μL medium to each well of the E-plate16, the proliferation of the cells was monitored every 30 min by the xCELLigence system. Approximately 18 h after seeding, when the cells were in the log growth phase, the cells were exposed to 10 μL of medium containing DuP-697 (100, 50, 25, 12.5 and 6.25 nM/well) both alone and in combination with E7080 (50, and 25 nM/well). Controls received either medium only, or medium+DuP-697 or medium+E7080. All experiments were run for 72h.

Angiogenesis assay using chorioallantoic membrane (CAM) assay

Preparation of the pellets: DuP-697 and E7080 were prepared and mixed with agarose in order to form pellet. The agarose (Merck, Damstadt, Germany) is added to distilled water to obtain a 2.5% (w/v) solution. This solution is put into the autoclave in 121°C and under 1 atmospheric pressure to provide dissolution and sterilization. Then, it is let to be cooled in a sterile container up to 37°C. The drug used in the study is added at this stage. Appropriate volumes of solutions were used to achieve three different concentrations of DuP-697 and E7080 (100 nM, 10 nM and 1 nM per 10 µl pellet). Approximately one hundred pellets for each study set are used. Thus, approximately 1 ml of combined agar and drug solution (10 µl×100=1 ml) was prepared initially for DuP-697 and E7080. The drug solutions with 10 nM and 1 nM concentrations were prepared by diluting these initial mixtures ten folds with the agarose solution again. Using a micropipette, 10 µl drops of this mixed solution were placed on previously sterilized, vertical, cylindrical stainless steel rods which were 5 mm in diameters to obtain circular pellets with the same diameter. Then the pellets were let to be solidified at room temperature in a sterile setting.

Chicken chorioallantoic membrane (CAM) assay: ross 308 strain fertilized hens' eggs were obtained from Yemsel Poultry Company (Kayseri, Turkey). The work described has been carried out in accordance with EU Directive 2010/63/EU for animal experiments and the study protocol was approved by the Cumhuriyet University Animal Ethics Committee. The fertilized hens' eggs were incubated in horizontal position with environmental conditions of 37.5°C temperature and 80% relative humidity. On the fifth day of the incubation period, 5 ml of albumen was taken through the eggshell with a syringe (Figure 1A) and a shell piece of 2-3 cm in diameter was removed from the contrary side of the eggs. Normal development of the CAM was verified (Figure 1B) and malformed or dead embryos were excluded. The windows on the egg shells were sealed with gelatin and thereafter, the eggs were incubated for 72 more hours to have CAM reaching 2 cm in diameter. Subsequently (on day 8), the seal was removed and the pellets were placed on the chorioallantoic membrane of each egg (Figure 1C). The seal was placed again and the eggs were then incubated for 24 hours. The angiogenesis level was evaluated after that period. For each concentration of drugs, twenty eggs were used. As the negative control group, pellets containing just agar were utilized. As the positive control group, pellets containing bevacizumab, FDA approved antiangiogenic agent, was used. All the tests were duplicated. The eggs in which the pellets caused inflammation and embryo toxicity were excluded.

Angiogenesis scoring: the inhibitory effects of the drugs were determined with a stereoscopic microscope according to the scoring system used in a number of studies (Bürgermeister, 2002; Demirci, 2003). In this system, the change in the density of the capillaries around the pellet and the extent of the effect are assessed (Figure 1D). For each subject initial scoring was evaluated as follows: *i*) Score 0: indicated the absence of any demonstrable antiangiogenic effect (normal embryo and no difference in surrounding capillaries); *ii*) Score 0.5: represented a very weak antiangiogenic effect (no capillary-free area but an area with reduced density of capillaries which is not larger than the pellet area); *iii*) Score 1: a weak moderate antiangiogenic effect (a small capillary-free area or a small area with significantly decreased density of capillaries; less than double the size of the pellet is involved); and *vi*) Score 2: a strong antiangiogenic effect (a capillary free area around the pellet which is equal to or more than double the size of the pellet itself).

The equation used for the determination of the average score was as follows: ***Average score= (Number of eggs (Score 2) ×2 + Egg number (Score 1) ×1]/[Total number of eggs (Score***

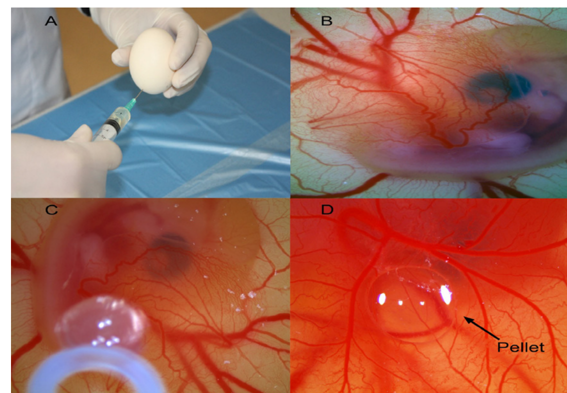

Figure 1. A) Albumen is Removed with a Syringe; B) The Appearance of the Chorioallantoic Membrane (CAM) Through a Window on the Egg Shell (×8); C) The Placement of the Pellet on the CAM (×8); D) Inhibiton of the Capillaries on the CAM (score:1) by the Drug (×8). 247×247mm (300×300 DPI)

0, 1, and 2)]. According to this scoring system, a score of <0.5 meant that there was no antiangiogenic effect; a score of 0.5 to 1 indicated a weak antiangiogenic effect, and a score of >1 implied a strong antiangiogenic effect.

Detection of apoptosis by annexin V method

A flouresin isothiocynate (FITC)-conjugated Annexin V/PI assay kit by flow cytometry was used for measuring apoptotic cell death. Briefly, 5×10^5 cells were washed with icecold PBS, resuspended in 100 ml binding buffer, and stained with 5 ml of FITC conjugated Annexin V (10 mg/ml) and 10 ml of PI (50 mg/ml). The cells were incubated for 15 min at room temperature in the dark, 400 ml of binding buffer was added, and the cells were analyzed (FACScan, Becton-Dickinson, USA). The HT29 cells were gated separately according to their granularity and size on forward scatter (FSC) versus Side Scatter (SSC) plots. Early and late apoptosis was evaluated on fluorescence 2 (FL2 for propidium iodide) versus fluorescence 1 (FL1 for Annexin) plots. Cells stained with only annexin V were evaluated as being in early apoptosis; cells stained with both annexin V and propidium iodide were evaluated as being in late apoptosis or in a necrotic stage.

Statistical analysis

The scores of angiogenesis were compared with Kruskal-Wallis ANOVA test and Mann-Whitney U test. A p value of less than 0.05 was considered as statistically significant.

Results

Monitoring dynamic cell proliferation and attachment in real-time using xCELLigence system

At first, we determined the optimal concentration for cell proliferation and viability measurements. For this purpose 100,000, 50,000, 25,000, 12,500, 6,250, 3,125 and 1,562 cells/well were seeded in the E-Plate 16 and the impedance was determined. According to the results of this experiment, we concluded that 50,000 cells/well experiments reflect cell cycle effects best. After this part of the study all wells were seeded with 50,000 cells/well.

Monitoring of cytotoxicity in real-time using xCELLigence system

We used the 50,000 cells/well concentration in the xCELLigence assay to examine the anti-proliferative effects revealed by DuP-697. The 50,000 cells/well concentration has an optimal treatment window between 16-24 hours. DuP-697 treated HT29 cells exhibited decreasing CI values in a concentration dependent manner. While 100 nM has shown complete cytotoxic effect and decreased CI to 0.050, 50 nM decreased CI to 0.102 and showed statistically significant cytotoxic effect when compared to the control (p<0.05) (Figure 2A). Twenty-four hours after treatment with DuP-697, an IC_{50} value of (IC_{50}) 4.28×10^{-8} M was achieved. In order to determine interaction between DuP-697 and E7080, 25nM and 12.5 nM DuP-697 were combined with 50 and 25 nM E7080 which have shown more cytotoxic effect than treated alone DuP-697 on HT29 cells. The cytotoxic dose 50 and non-cytotoxic 25nM doses of E7080 were chosen according the results in our recently reported study (Altun et al., 2013). DuP-697 25+E7080 50nM caused higher cytotoxic effect on HT29 cells when compared to DuP-697 25 alone (p<0.05) (Figure 2B). The CI of DuP-697 25+E7080 50nM was significantly low when compared to the DuP-697 25 nM alone (p<0.05). When non-cytotoxic DuP-697 12.5nM and non cytotoxic E7080 25nM were combined there was also a higher cytotoxic effect compared to the effective DuP-697 25nM. Also the DuP-697 12.5nM and E7080 25nM combination had significant cytotoxic effect on HT29 cells compared to non-cytotoxic DuP-697 12.5nM (p=0.017). The CI of DuP-697 12.5 nM+E7080 nM was significantly low when compared to DuP-697 12.5 nM alone (p<0.05) (Figure 2B).

Determining antiangiogenic effects of DuP-697 alone and in combination with E7080

The eggs on which a 10 µl-agarose pellet with no drug was installed demonstrated no significant antiangiogenic effect (average antiangiogenic score=0.2). All the study drugs demonstrated some antiangiogenic effect compared to the negative control (p<0.05). Each tested drug is evaluated separately and the results with different solutions were compared. Fig 3 shows the antiangiogenic scores of DuP-697, E7080 and DuP-697+E7080 in 100, 10 and 1 nM concentrations. DuP-697 in 100, 10 and 1 nM concentrations caused antiangiogenic effect. Antiangiogenic scores of DuP-697 were 1.2, 0.8 and 0.5, respectively. These scores show that DuP-697 caused concentration dependent antiangiogenic effect on CAM. As shown in the scatter graph, the antiangiogenic score with the 100 nM of DuP-697 was significantly higher than 1 nM (p<0.05) (Figure 3). Antiangiogenic scores of E7080 were 1.2, 1.0 and 0.6, respectively. These scores show that E7080 caused concentration dependent antiangiogenic effect on CAM. As shown in the scatter graph, the antiangiogenic score with the 100 nM of E7080 was significantly higher than 1 nM (p<0.05) (Figure 3).

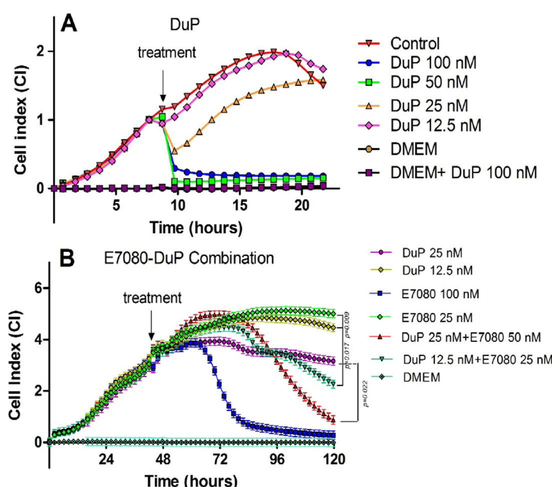

Figure 2. Real-time Monitoring of A) Cytotoxic Effect of DuP-697 and B) Cytotoxic Effect of DuP-697-E7080 combination, on HT29 Colorectal Cancer Cells using RTCA

In order to determine interaction between DuP-697 and E7080, the antiangiogenic scores of DuP-697 100 nM, E7080 100 nM and DuP-697+E7080 50+50 nM were 1.2, 1.2 and 1.4, respectively. There was no significant difference when compared to each other (p>0.05). Similar results were observed when lower concentrations of DuP-697 and E7080 were combined.

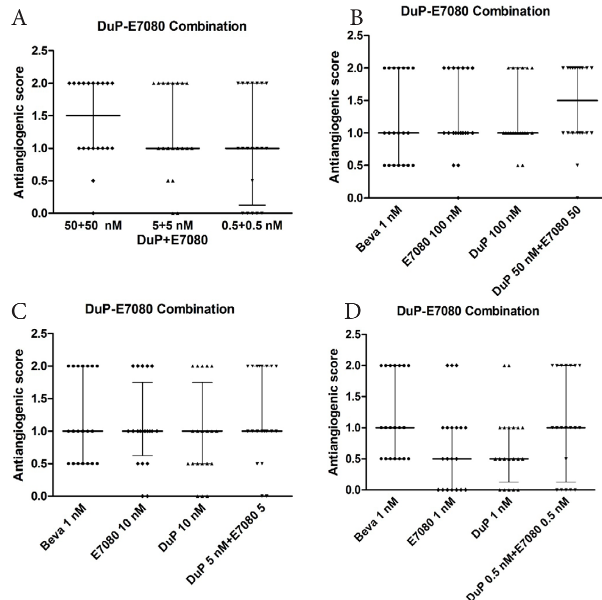

Figure 3. The Antiangiogenic Scores of DuP-697 and E7080 Alone and in Combination. A) DuP-697+ E7080 50+50, 5+5, 0.5+0.5 nM; **B)** DuP-697 100, E7080 100, DuP-697+ E7080 50+50 nM; **C)** DuP-697 10, E7080 10, DuP-697+ E7080 5+5 nM; **D)** DuP-697 1, E7080 1, DuP-697+ E7080 0.5+0.5 nM, Beva (bevacizumab) is a positive control as stated in method

Determining apoptotic effects of DuP-697 alone and in combination with E7080

The apoptosis induction was assessed by Annexin V-FITC assay. In the dot plot of flow cytometric analysis (Figure 4), the lower-right (LR) area was the Annexin V positive/PI negative portion which represented the preapoptotic fraction, the upper-right (UR) area was the Annexin V positive/PI positive portion which represented the apoptotic fraction. DuP-697 caused concentration dependent apoptosis in HT29 cells. The percentage of UR (apoptosis portion) area increased gradually according to the concentration of DuP-697 from 7% in control group to 52% in 100 nM DuP-697. The agents DuP-697 +E7080 combinations at 50+50, 25+25 and 12.5+12.5 nM showed strong concentration dependent apoptotic effect. The percentage of UR (apoptosis portion) area was 78.4%, 67.3% and 33.4% respectively. While DuP-697 50 nM produced 36% apoptosis, DuP- 697+E7080 50+50 nM produced 78.4% apoptosis. The ratio was significantly high when compared to using alone (p<0.05). Similar effects were observed when DuP-697+E7080 25+25 and 12.5+12.5 nM concentrations were compared with DuP-697 applications alone (Figure 5).

Discussion

Colorectal cancer is one of the leading causes of cancer related death worldwide. The American Cancer

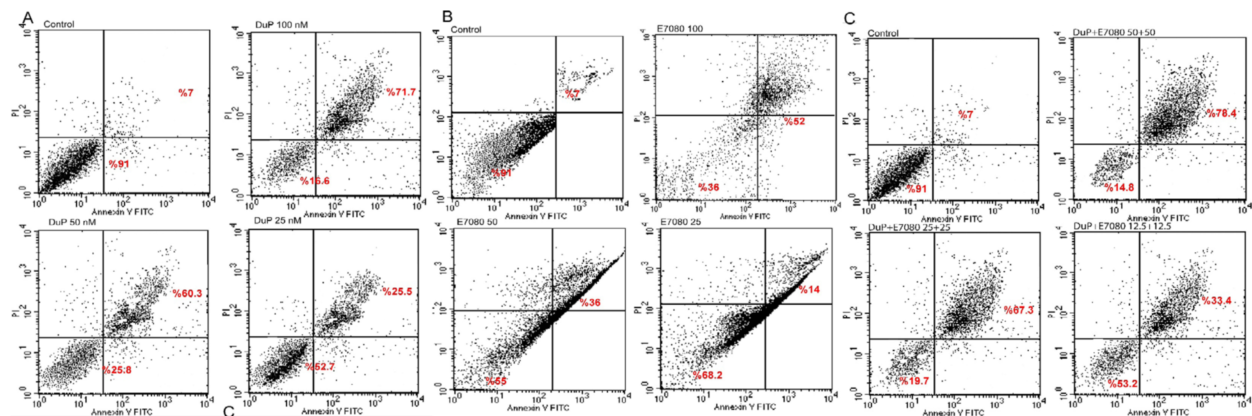

Figure 4. A) Dot Plot Distribution of Live, Preapoptotic and Apoptotic Cells after Administration of A) DuP-697 Alone; B) E7080 Alone; C) DuP-697 and E7080 Combination

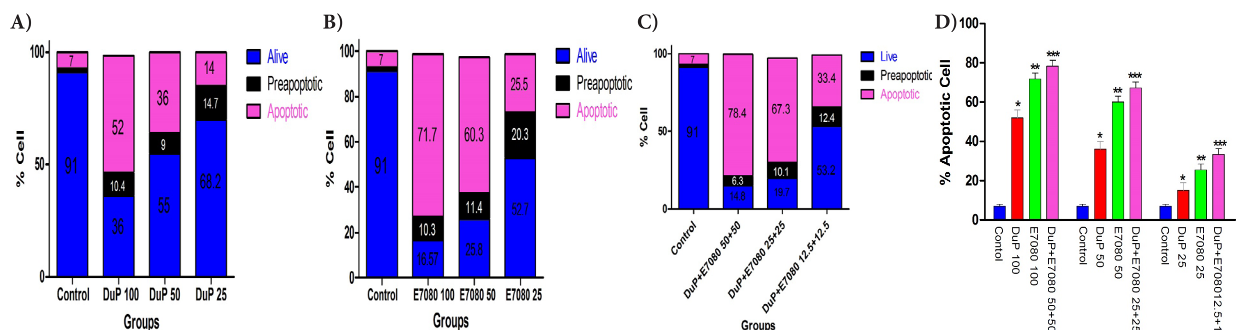

Figure 5. A) Distribution of Live, Preapoptotic and Apoptotic Cells after Administration of A) DuP-697 Alone; B) E7080 Alone; C) DuP-697 and E7080 Combination; and D) Comparison of Apoptotic Cell Percentage of Alone and Combination Administrations

Society predicts for about 102,480 new cases for colon cancer and 40,340 new cases for rectal cancer in United States for 2013. Although remarkable progress has been made in the management of CRC, over the past decade, surgical resection of the primary tumor is only curative for localized disease. Approximately 20% of CRC patients have locally advanced or metastatic forms of CRC at the time of the diagnosis, which essentially precludes a cure by surgical treatment alone. Also, among patients who undergo apparently surgical resection, many still develop recurrent CRC which then requires systemic treatment. This has leaded researchers to install active drug treatment strategies (Segal and Saltz, 2009).

The current conventional cytotoxic drugs such as oxaliplatin, irinotecan and 5-fluoroacil (5-FU) have caused significant progress in the treatment of CRC. 5-FU is a nucleotide analog which was developed in 1957. İrinotecan is a topoisomerase I inhibitor; and oxaliplatin is a platinum-based compound and bulky DNA adduct (Wolpin and Mater, 2008). Standard treatment has evolved from 5-FU, with a median overall survival of 10-12 months and an overall response rate of 10%, to combinations of oxaliplatin and irinotecan that have dramatically improved survival to 14-16 months (Best et al., 2000; Jiang et al., 2012). When compared to surgery alone, it is clear that conventional chemotherapies have a relatively modest effect on CRC (Saltz, 2010).

Cytotoxic drugs remain the backbone of current treatment, but they are limited by a narrow therapeutic index, significant toxicities and frequently acquired resistance. An improved understanding of cancer biology has given rise to new treatment options, including targeted agents and cancer immunotherapy. Targeted approaches aim to inhibit molecular pathways that are crucial for tumor growth and maintenance (Vannman and Drannoff, 2012). Following the dramatic success of imatinib the first small- molecule -targeted agent for the treatment of chronic myeloid leukemia, which demonstrated the potential impact of targeted therapy, great effort has been put into developing therapies that are directed against specific molecules and pathways that are altered in malignant cells. Bevacizumab is a successful example of this approach. It inhibits angiogenesis by binding to VEGF A, as well as cetuximab and panitumumab, which are monoclonal antibodies that block, EGFR (Van Loon and Venook, 2012). With the introduction of targeted therapy, the overall survival of metastasized CRC patients has been further prolonged (Jiang et al., 2012).

Tumor cells secrete a number of pro-angiogenic factors that stimulate the proliferation and migration of endothelial cells, resulting in the outgrowth of new capillaries into the tumor. Vascular endothelial growth factor is a key regulator of physiologic angiogenesis, and plays a major role in the pathobiology of cancer. Recognizing the prominent role for VEGF in cancer led to develop antiangiogenic therapies targeting this pathway (Ellis and Hicklin, 2008; Feng et al., 2014). Truly, these therapies have become the standard of care for several malignancies, including metastatic CRC (Hurwitz et al., 2005). The VEGF receptor (VEGFR) family includes three related receptor TKs, known as VEGFR-1, -2, and -3, which mediate the angiogenic effect of VEGF ligands. VEGFR-2 is considered to be the dominant signaling receptor for endothelial cell permeability, proliferation, and differentiation (Olsson et al., 2006). Binding of VEGF to its cognate receptor VEGFR-2, stimulates one of the most important angiogenic signaling pathways, thus the blockade of this interaction is a rational anticancer approach (Ellis and Hicklin, 2008).

There is accumulating evidence that nonsteroidal anti-inflammatory drugs (NSAIDs) have promise as anticancer drugs (Thun et al., 2002; Ruder et al., 2011). Two groups of COX inhibitors are known. The non-selective COX inhibitors (e.g. aspirin, ibuprofen, indomethacin), which inhibit the activity of both enzyme isoforms and selective COX-2 inhibitors (Koki and Masferer, 2002). The new selective COX-2 inhibitors are better tolerated in therapeutical doses since they do not inhibit the COX-1 isoenzyme. It has been shown that NSAIDs, particularly the highly selective COX-2 inhibitors help to suppress malignant transformation and tumor growth by stimulating apoptosis and inhibiting the production of VEGF, thus inhibiting angiogenesis (Toomey et al., 2009; Ma et al., 2013). Many epidemiologic studies have found that long term users of aspirin or other NSAIDs have a lower risk of CRC (Ruder et al., 2011; Coghill et al., 2012; Huang et al., 2013). Studies showing the higher PGE2 levels in CRC brought up the hypothesis that NSAIDs might prevent the occurrence or severity of CRC (Toomey et al., 2009; Wang and Dubois, 2010; Ma et al., 2013). Another issue that has been raised is whether the preventive effects of the NSAIDs are related to the inhibition of PG synthesis or are due to off-target effects. Some studies suggest that COX-independent mechanisms may contribute or be fully responsible for their anticancer effects. In a study done recently it has been shown that the expression of CD133-positive cancer stem cells in colon cancer which are resistant to conventional chemotherapy were downregulated by celecoxib through inhibition of the Wnt signaling pathway (Deng et al., 2013). Although there is continuing uncertainty about the molecular pathways of NSAIDs that may inhibit colorectal neoplasia, there is linking evidence that tumor inhibition may be mediated by at least two cellular processes involving the ability to restore apoptosis and inhibition of angiogenesis (Tanwar et al., 2010; Zhou et al., 2012). Identifying alternative targets included in NSAİD activity may lead to develop more effective and safer drugs without the gastrointestinal, cardiovascular and renal side effects associated with NSAIDs and COX-2 inhibitors. Previously non-COX-inhibitory derivatives of celecoxib and sulindac have been developed that have antitumor activity without inhibiting COX enzymes (Schönthal, 2006; Piazza et al., 2009; Whitt et al., 2012).

In our study, we determined the effects of DuP-697, which is a slow, time-dependent irreversible highly selective inhibitor of COX-2, on HT29 cell proliferation, angiogenesis and apoptotic effect. Similar to other popular selective agents, celecoxib and rofecoxib, DuP-697 is a member of the vicinal diaryl heterocycles, which have a completely different structure from classic COX inhibitors. DuP-697 is not clinically used because of its

very long plasma half life, 242 h in humans and secondary to its enterohepatic recirculation. It is used in laboratory practice for COX-2 inhibition modeling. However, DuP-697 has been also tested for long-term cancer prevention and treatment (Dannhardt and Kiefer, 2001). In our study DuP-697 demonstrated a potent action in inhibiting HT29 cell growth and inducing apoptosis and antiangiogenic effect.

Tyrosine kinase inhibitors are small molecules that are able to pass through the cell membrane and are able to block the activation of various downstream signaling pathways intracellularly. Many anti-angiogenic TK inhibitors target a number of different kinases, which are involved in several signaling pathways (Gotink and Verheul, 2010). Resistance is the major threat for the tyrosine kinase treatment. Most tumors treated with tyrosine kinases become resistant to treatment in a short time (Engelman and Settleman, 2008). This resistance is often because of the constitutive activation of downstream signal transducers (Sartore- Biachi et al., 2009). In this case combining tyrosine kinase inhibitors with an agent that has a different mechanism seems more rational.

E7080 is an investigational orally active multi-targeted kinase inhibitor whose targets include VEGFR, fibroblast growth factor receptor (FGFR) and platelet derived growth factor (PDGRF) and SCF receptors (Glen et al., 2011). E7080 shows anti-tumor activity in xenograft models of various human cancer cell lines and this activity has been attributed to its ability to inhibit angiogenesis through effects on VEGFR-2 inhibiton but also through inhibition of KIT and FGFR-1(Matsui et al., 2008; Bruheim et al., 2011).

It has been shown that E7080 has antitumour activity against a panel of xenografts derived from different histotypes of human sarcomas, gone with reduction in microvessel densities (Bruheim et al., 2011). Also Matsui J et al showed that E7080 inhibited proliferation of human small cell lung cancer (SCLC), H526 cells which expressed KIT, at concentrations needed for the inhibition of KIT kinase (Matsui et al., 2008). In a study, Glen H. et al showed that the usage of E7080 (both at 1μM and 10 μM) led to a significant inhibition of cell migration of both DX3 melanoma and U2OS osteosarcoma cells. Also in the same study it has been showed that E7080 inhibited proliferation of cell lines including DUI145 prostate cancer and E375 melanoma cells (Glen et al., 2011). All these studies are consistent with our previous study (Altun et al., 2013) in which we reported that E7080 caused inhibition on the microvessels developing on chorioallantonic membrane *in vivo* CAM model and it is clear that E7080 has a strong antiangiogenic effect most likely mediated by VEGF2R. We also showed that E7080 has a strong apoptotic effect on HT29 colorectal cancer cells. In the strong antitumor effect of E7080 this apoptotic effect probably takes part along with its antiangiogenic properties.

In this study we combined DuP-697 with low concentrations of E7080 which have separate antitumor functions in order to discover if such application produces a collaborative antitumor effect. A therapeutic union may possibly enhance the effect of chemotherapy and reduce the dose and adverse reaction, of using a single drug. There are studies showing the effectiveness of combining selective COX-2 inhibitors with chemotherapy yet the exact mechanism of the anti-tumor effect produced by these treatments is unclear.Here we report that combined treatment with DuP-697 and E7080 increases the antiproliferative, antiangiogenic and apoptotic effects of DuP-697 *in vitro* in HT29 colorectal cancer cells. This may be due to the combined inhibitory effects of both DuP-697 and E7080 on cancer cell production of pro-angiogenic factors, such as VEGF, which may result in a significant reduction in endothelial cell migration and growth. In the biopsies of different human cancers, including colon, COX-2 overexpression has been shown (Masunaga et al., 2000) and it has been reported that newly formed blood vessels in tumors express COX-2 (Masferrer et al., 2000). Also, COX-2-dependent promotion of neoangiogenesis has been associated with induction of VEGF (Masferrer et al., 2000) and it has been demonstrated that the activation of receptor TKs may induce COX-2 expression and prostaglandin production (Turini and DuBois, 2002). Similar studies, a cooperative antitumor activity has been reported by the combination of trastuzumab with the selective COX-2 inhibitor celecoxib (Mann et al., 2000) and the antitumor activity of ZD6474, a VEGF-2 and EGFR small molecule TK inhibitor, in combination with SC-236, a COX-2 inhibitor has been reported (Tuccillo et al, 2005). Also it has been shown that the combined treatment of three different signal transduction inhibitors targeting EGFR, type 1 cyclic AMP- dependent protein kinase, and COX-2 results in a significant antitumor activity in a human colon cancer xenograft model (Tortora et al., 2003). There is need for more detailed studies on tissue pharmacokinetices and tumor uptake and as in many studies in literature, this study was performed using human cancer cell lines and *in vivo* significance is yet to be determined for these studies.

In conclusion, we found that the DuP-697, a selective COX-2 inhibitor has antiproliferative, antiangiogenic and apoptotic effects on HT29 colorectal cancer cells. The combination of DuP-697 with E7080, a multityrosine kinase inhibitor showed stronger antiproliferative, antiangiogenic and apoptotic effects than DuP-697 alone. The combined use of two drugs had synergistic antitumor effects. This study shows that combination of COX-2 inhibitors with TK inhibitors may let using COX-2 inhibitor and TK inhibitor in lower concentrations which may prevent resistance to these drugs and reduce side effects. We believe that further studies are required to establish the mechanism of action.

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