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Original Article

Antitumor activity of sorafenib on colorectal cancer

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ABSTRACT

In spite of progress in treatment on colorectal cancer, it is not still convincing. Because of that, there is a pursuit to find a better treatment option. Our goal of this study is to research antiproliferative effect and mechanism of action of sorafenib on colorectal cancer cell line. Antiproliferative effects of sorafenib have been evaluated by using colorectal DLD-1 cell line. Materials and Methods: For determining the effects of sorafenib on colorectal cell, Real time analysis was performed. Chorioallantoic membrane model (CAM) was used for assessing angiogenesis. Different concentrations of sorafenib (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95, 0.97 and 0.48 nM/well) applied on DLD-1 colorectal cancer cells, caused strong antiproliferative effect in a concentration manner. IC50 value of sorafenib was 1.26 × 10-9 M. Sorafenib (100, 10 and 1 nM) also showed strong angiogenic effect in CAM model with scores 1.05, 0.75 and 0.55. The present study indicates that sorafenib has strong antitumor and antiangiogenic affect. These important features make it valuable treatment agent for colorectal cancer which is strongly connected to angiogenic factors. In order to verify and enlighten antitumor mechanism of sorafenib, there is still need for further investigations.

1. Introduction

Colorectal cancer (CRC) is one of the most frequently occurring malignancies in the world and the second leading cause of cancer-related death in worldwide, affecting both men and women equally.[1–4] While approximately 25% of CRC cases are diagnosed in the metastatic setting, 25–35% of cases will develop metastases during the course of their disease.[5] The 5-year overall survival rate is 10–20% for metastatic disease. For 2 decades, major progresses have been made in treatment of CRC. The median survival has reached from less than 6 to almost 24 months after newer chemotherapeutic and targeted biological agents have been introduced.[6]

Angiogenesis and signaling pathways play critical role in of tumor's aggressive behavior. Therefore, the treatment based on inhibiting angionesis treatment and signaling pathways became more important.[6] Drugs that target vascular endothelial growth factor (VEGF) and receptors (VEGFR) have been developed. Recent studies have shown that targeted therapy has prolonged the survival of CRC paitiensts.[7–9]

Sorafenib (Nexavar; Bayer Pharmaceuticals, West Haven, CT) inhibits tumor growth, tumor cell proliferation, angiogenesis and induces tumor cell apoptosis via targeting serine/threonine Raf kinases, MAPK signaling pathway and inhibiting VEGFRs and palteled-derived growth factor receptor (PDGFR) authophosphorylation.[4,10–12] Approving by the FDA for unresectable hepatocellular carcinoma and advanced renal cell cancer in adults, it has been used.

Recently, after additting targeted therapy such as bevacizumab, cetuximab and panitumumab to cytotoxic chemotherapy, survival of patients with CRChas been prolonged. In this study, we evaluated antitumoral and antiangiogenic effects of sorafenib in colorectal cancer cells.

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2. Materials and methods

2.1. Cell culture

DLD-1 cells have been purchased in 2011 from the Sap Institute (Ankara, Turkey). Human cancer cells frozen in cryo tubes, stored in nitrogen and multiplied in different passages. Cancer cells were seeded in DMEM with 1 g/L glucose and L-glutamine.

The culture medium were enriched with 10% heat inactivated FBS, %1 penicilin and streptomicin. Cells has been multiplied in T¬75 (120 ml, 75 cm2) cell culture containers in a conditioned atmosphere containing 5% CO2 at 37 °C.

2.2. xCELLigence assay

xCELLing system which is consisted of rhe RCTA analyze, ehe RTCA DP station, the RTCA computerized with software, and disposable E-plate 16 were used to assay (Roche Applied Science and ACEA Biosciences) (13). The other important thing is the golden electrodes which were placed at the bottom. For determining the physiological changes of cells, the electronic impedance probe/sensor were used and 20 mV voltage was given.[13] Valuable parametres such as average value, maximum and or minimum values, standard deviation (SD), half maximum effect of concentration (EC50), half maximum inhibition of concentration (IC50), cell index (CI) and graphics were obtained by using software.[13]

2.3. Real time cell analyzer for monitoring cell proliferation

DLD-1 cells were multiplied in tissue-culture flasks. After reaching 75% confluence, medium ingredient has been washed out by using PBS, afterwards DLD1s have detached from the flasks by a transient treatment with trypsin/EDTA. Subsequently, 100 μL of medium mixture (DMEM+%10 FBS + Pen-strp) has been added into each well of E-plate16.

After multiplying the DLD-1 cells, 100 μL of medium mixture (DMEM+%10 FBS + Pen-strp) were added into each well of E-plate16 which was placed in to system. Then baseline impedance was recorded in order to abstract from final impedance. Meanwhile, the cells were re-suspended in cell culture medium and adjusted to 400.000 cells/mL. 100 μL of cell suspension was added to the 100 μL medium containing wells on E-plate16. After 30 min incubation at room temperature under the hood, 40,000 cells/200 μL/well E-plate16 was put into the DP station which is placed in to the CO2 incubator. Finally, cell proliferation was monitored every hour for duration of up to 72 h. Then, electrical impedance was assessed.

2.4. Xcelligence system for assaying cytotoxicity

Fistly, the ideal number of DLD-1 cells was identified. After adding to adequate number of cells in 100 μL to E-plate, the expansion of them followed every 1 h via xCELLing system. Logarithmic growth phase was reached after 18 h of seeding. Then, the cells were administered to 10 μL of medium containing the Nexavar® (Bayer Schering, Germany) (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95, 0.97 and 0.48 nM/well). Positive and negative controls have been used. DLD1 cells in control wells have been administered either medium only or medium + sorafenib with a definitive concentration of 500 nM. All tests were performed for 72 h.

2.5. Chorioallantoic membrane (CAM) assay for angiogenesis

2.5.1. Pellets' preparation

In the present study, the effect of sorafenib on angiogenesis has been studied. Sorafenib was in its commercially avaible form as 200 mg tablet. Sorafenib has been dissolved. Tablet dissolution was performed using the UPS Aparatus 2 (paddle) operated at 50 rpm. The dissolution medium was phosphate buffer saline. After preparing the solution of sorafenib, it was mixed agarose to give shape to “o”. For providing dissolution and sterilization, it was put into autoclave in 121 °C and under 1 atmospheric pressure. The Sorafenib was added at this stage and three different concentrations of sorafenib (100 nM, 10 nM and 1 nM per 10 μL pellet) were obtained. One ml of combined agar and drug solution (10 μL × 100 = 1 ml) was prepared initially for sorafenib to create 100 nM sorafenib concentration. Then the other concentrations (10 nM and 1 nM concentrations) have been made diluting initial mixture ten folds with the agarose solution again.

2.5.2. Chicken chorioallantoic membrane (CAM) assay

Yemsel poultry Corporation (Kayseri, Turkey) supplied Ross 308 strain fertilized hens'egss. These eggs were incubated to environment if which temperature was 37.5 °C and of which humidity was approximately 80%. After 5 days of incubation, albumen was removed via syringe (Fig. 1A). The part of egg shell 2–3 cm in diameter was removed. Confirmation of normal growth was seen (Fig. 1B). The eggs confirmed no normal growth were exclude. After covering the eggs shell window with a gelatin, these eggs were incubated for 72 h again to reach CAM with 2 cm diameter. After this, gelatinous shell was taken out and pellets were placed on the CAM (Fig. 1C). After placing gelatinous shell, they were incubated for 24 h. Evaluating the angiogenesis was done after this. Twenty four eggs were used for each doses of sorafenib. Simply agarous pellets were used as a control group. Bevacizumab, FDA approved antiangiogenic agent, containing pellets has been used to verify the way we perform CAM assays is able to show antiangiogenic effect of test agent (positive control). All the tests were performed twice. The eggs in which the pellets created inflammation and embryo toxicity were excluded.

2.5.3. Angiogenesis scoring

For assaying the angiogenetic effect of sorafenib, CAM was used and evaluated via stereoscopic microscope based on the rating technique according to previous studies.[14,15] The alterations were determined (Fig. 1D). The scores were stratified 0 to2 (no antiangiogenetic effect to strong antiangogenetic effect). Average score = [Number of eggs (Score 2) × 2 + Egg number (Score 1) × 1]/[Total amount of eggs (Score 0, 1, 2)].

2.6. Statistical analysis

Kruskal–Wallis test and Mann–Whitney U test were used to compare the scores of abgiogenesis and Significant statistical value (p value) was determined less than 0,005.

3. Results

3.1. xCELLigence system for monitoring the viability and proliferation cell capacity

Firstly, to measure the viability and proliferation cell capacity ideal cell count was determined. Therefore, 100,000, 50,000, 25,000, 12,500, 6250, 3125 and 1562 cells/well were placed into the E-lapte 16. And then, impedance was monitorized. We came to the conclusion that ideal cell concentration which will let us to observe

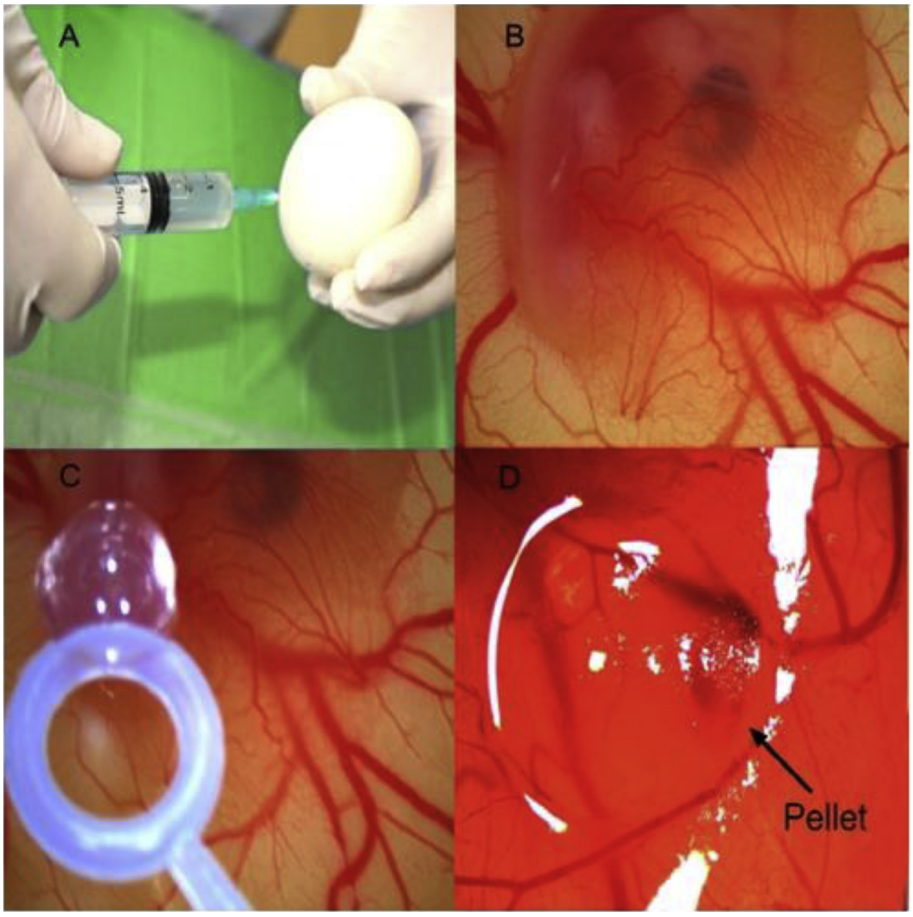

Fig. 1. A. Albumen is removed with a syringe. **B**. The appearance of the chorioallantoic membrane (CAM) through a window on the egg shell (×10). **C**. The placement of the pellet on the CAM (×10). **D**. Inhibition of the capillaries on the CAM (score: 1) by the drug (×10)

the effects of sorafenib on cell proliferation in 72 h period is 40,000 cells/well which were seeded into all wells.

3.2. xCELLigence system for monitoring cytotoxity

Next, for analyzing the antiproliferative effect of different doses of sorafenib, prepared cell/well were used in the xCELLing assay (Fig. 2). It showed us decreasing cell index values depended on concentration (Fig. 3). Although the dose which was equal to 31,5 and high had a remarkable cytotoxic effect on cells, there was no remarkable difference between them. 15.6 and 7.8 nM sorafenib

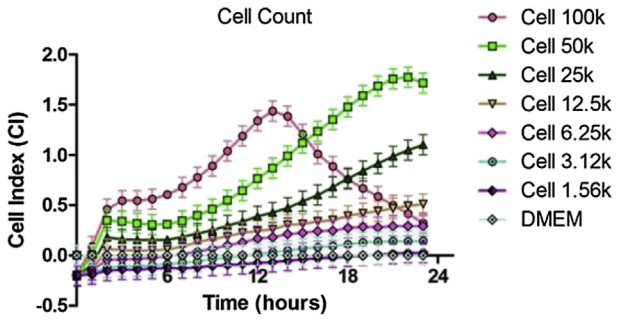

Fig. 2. Analyzing the antiproliferative effect of different doses of sorafenib via xCELLing analysig system. . DLD1s at a density of 100.000, 50.000, 25.000, 12.500, 6.250, 3.125 and 1.562 cell per well in E-Plates 16 were observed during 24 h

also caused statistically significant cytotoxic effect, but this effect was significantly low when compared to the 500, 250, 125, 62.5 and 31.5 nM sorafenib. The change in cell index between these groups was statistically different. IC50 value of sorafenib reached 1.24 × 10-9 after 24 h adding sorafenib (Fig 4).

3.3. Determining antiangiogenic effects of sorafenib

In order to show agarose has no effect on angiogenesis, we used agarose pellet which has no drug in it (Negative control). All tree applied concentrations of the sorafenib showed antiangiogenic effect ($p < 0.05$). Concentrations of sorafenib and the results of it were evaluated and compared between each other. Fig. 5 demonstrates the antiangiogenic scores of sorafenib in different concentrations. The scores of 100, 10 and 1 nM of sorafenib concentration were 1.05, 0.75 and 0.55, respectively. This was related to doses of sorafenib. Fig. 5 demonstrated usa score of 100 nM of sorafenib was significantly high.

4. Discussion

Cytotoxic chemotherapy agents including 5-fluorouracil, folinic acid, irinotecan, capesitabine, and oxaliplatin are mainstay of CRC treatment in adjuvant and metastatic setting. Recently, additting the targeted therapy such as bevacizumab, cetuximab and panitumomab to the cytotoxic therapy has provided survival advantage in patients with advanced stage CRC.[7,16] Despite of improved outcomes with combination of cytotoxic chemotherapy and targeted

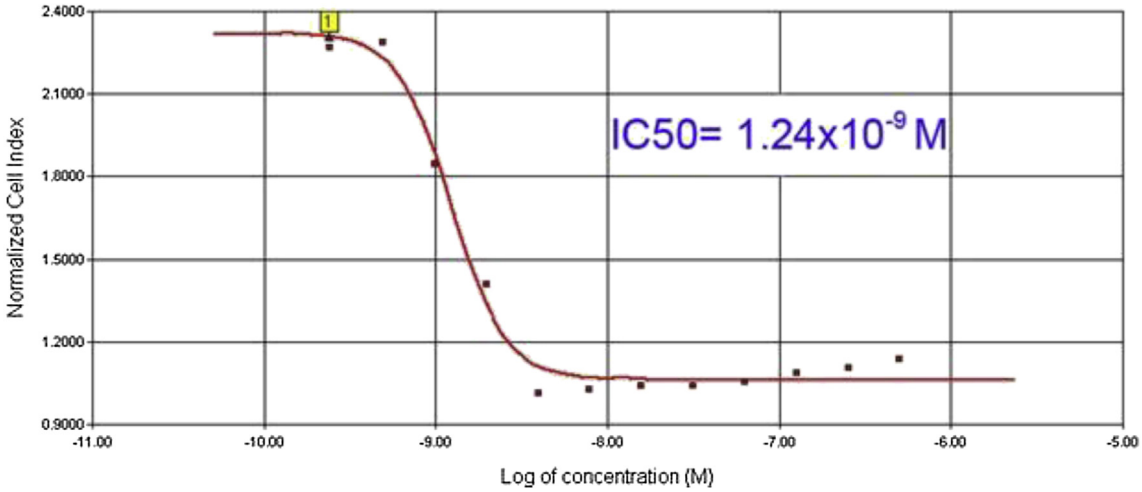

Fig. 3. IC_{50} of Sorafenib ($IC_{50} = 1.24 \times 10^{-9}$ M, square = 0.989)

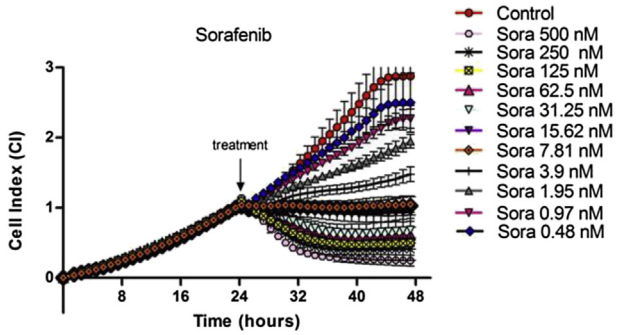

Fig. 4. Real-time monitoring Sorafenib's cytotoxic effect on human colorectal cancer cells

therapy agents, the prognosis for advanced stage disease has not still desirable. Thus, newer treatment options have needed.

Angiogenesis and signaling cascades are important for tumor growth, survival, invasion and metastasis.[17,18] Activation or inhibition of VEGF, PDGFR-mediated, the PI3K/Akt pathway, MAPK/ERK pathways are crucial role in tumor pathogenesis Thus, inhibiting the these pathways could be useful treatment in angiogenetc tumors.[17,19] Bevacizumab as first line for metastatic CRC in combination with intraveous 5FU based chemotherapy were highlighted in antiangioenic treatment.[17,20] Inhibiting these pathways with SU11248 and TKI-31 has showed that multi-targeted TKIs have anti-angiogenic and anjiostatic property.[21]

Sorafenib inhibits tumor growth, tumor cell proliferation, angiogenesis and induces tumor cell apoptosis via targeting serine/threonine Raf kinases, MAPK signaling pathway and inhibiting VEGFRs and palteled-derived growth factor receptor (PDGFR) authophosphorylation. However, the number of preclinic and clinic studies on activity of sorafenib is limited.[22,23]

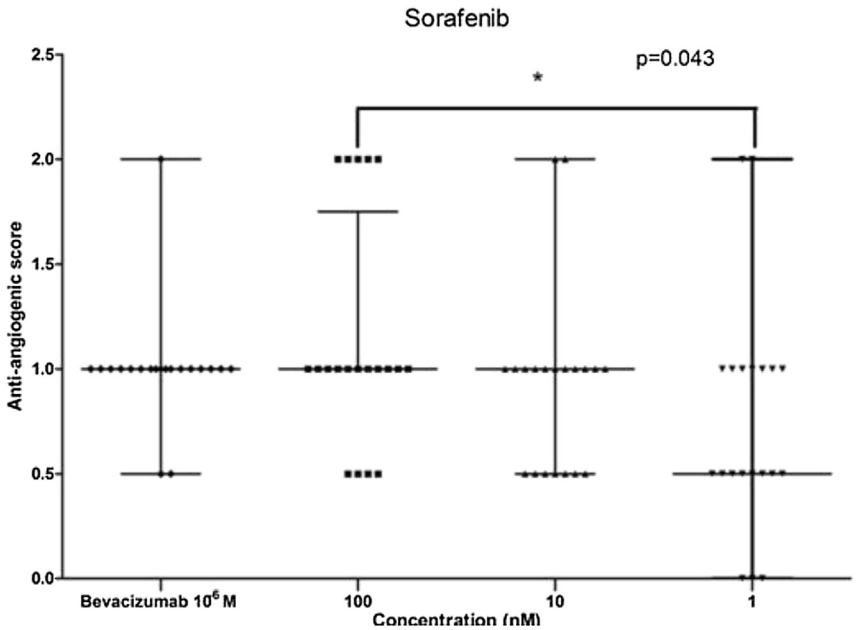

Fig. 5. The antiangiogenic scores of Sorafenib (100, 10 and 1 nM concentrations)

The effect of sorafenib on angiogenesis was evaluated preclinical model. A significant reduction in tumor vascularite was shown.[24] Gunningham et al showed that VEGFR and angiogenesis play central rol on renal cell carcinoma (RCC). And also showed clinical benefits.[25] In addition, sorafenib has shown advantages to placebo in patients with refractory metastatic RCC.[26]

In another preclinical study performed by Lui et al., showed that sorafenib could increase tumor cell apoptosis and decrease tumor vascularization in PLC/PRF/5 hepatocellular cell cancer (HCC) xenograft model.[9,27] In one study, performed by Martinelli E et al., showed that sorafenib prolonged the survival of the patients with HCC.[27]

In the HT-29 colon tumors and Colo-205 tumor models indicated the antiangiogenic and proliferative effect as a monotherapy.[10,28]

Combination with sorafenib, erlotinib cetuximab exhibited antitumor activity in human colorectal and lung cancer cells.[29] The antitumor activity of these drugs was also synergistic antitumor effects. Furthermore, it was evaluated combination with radiation of sorafenib in 2 different studies.[30,31] In the first study, Plastaras et al observed to inhibit cell cycle progression of sorafenib at irradiated cells. Also, this study exhibited that sorafenib caused a reversible G1 delay and that radiotherapy followed sequentially by sorafenib delayed tumor growth. In the other study, however, Suen and colleague showed that the combination of radiation and sorafenib inhibited tumor proliferation and angiogenesis on human colorectal cancer cell lines. Also, this combination was synergistic. Sorafenib was also combinated with platinum derivates such as cisplatin and oxaliplatin in vitro colorectal cell lines.[32] Sorafenib may interact with platinum transporter, and thus, it reduced cellular uptake of these compounds. When sorafenib is combined with platinum derivate, it causes markedly an antagonistic effect and the cell cycle arrests in the G1 or G2 phases.

In our study, we observed that sorafenib was broad activity in viability assays in human colorectal cell lines. The cytotoxic effect of sorafenib gradually decreased with its decreasing concentrations on colorectal cancer cells. We also suggested that sorafenib have antiangiogenic effect on human CRC cell lines and that this effect positively correlated its concentration.

In conclusion, sorafenib's antitumor and antiangiogenic effects on CRC cell line depend on doses But, the outcomes of our study should be confirmed with clinical trials used combination with cytotoxic chemotherapy or targeted therapy agents.

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