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Article

Inhibition of angiotensin II type I pathway reduced tumor growth and ameliorates fibrosis/inflammation associated with colorectal cancer

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Abstract: Dysregulation of the angiotensin-II Type-I receptor (AT1R) and its pathway was reported to associate with poor-prognosis in several malignancies, including colorectal-cancer (CRC). We have explored the therapeutic-potential of targeting AT1R using valsartan, and its pharmacological-interaction with Fluorouracil (5-FU) in CRC. Anti-proliferative function was evaluated in 2-/3-dimensional cells and in vivo models. Anti-proliferative, anti-migratory, apoptotic function and effect on cell-cycle was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), wound-healing test, and Fluorescence-activated cell sorting (FACS), respectively, while gene-expression was determined at mRNA/protein levels. By histogical analysis and measuring of oxidative/antioxidant markers, we evaluated the anti-inflammatory properties of valsartan. Valsartan suppressed cell-growth and impacted the anti-tumor-activities of 5-FU by apoptosis-induction. Valsartan inhibited the cells migration by perturbation of Matrix metalloproteinase

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(MMP1). Furthermore, valsartan inhibited tumor-growth and metastasis, and this was more notable in valsartan/5-FU combination-treated-group. The mechanism was plausible to be via the induction of Reactive-oxygen-species (ROS) and down-regulation of Superoxide-dismutase (SOD), thiol/catalase (CAT) as well as Vascular endothelial growth factor (VEGF) and Transforming growth factor beta (TGF-β). Valsartan may protect cells against intestinal fibrosis by modulation of pro-fibrotic and pro-inflammatory components include fibronectin, Interleukin) IL-1β (, Tumor necrosis factor alpha) TNF-α (, Interferon gamma) INF-γ (, and Monocyte Chemotactic Protein 1 (MCP-1). Our findings demonstrated that targeting the AT1R receptor may inhibit tumor-growth and ameliorate fibrosis and inflammation associated with CRC via modulation of AT1 and TGF-β pathways.

Keywords: Angiotensin-II Type-I receptor; renin-angiotensin system; valsartan; colorectal cancer

1. Introduction

Colorectal cancer (CRC) is a common cancer for death globally, and some cases develop lungs metastasis [1]. The renin-angiotensin system (RAS) has an major role in blood pressure regulation and fluid body homeostasis [2]. However, there is increasing evidence that RAS may also influence cellular apoptosis, proliferation, cell adhesion and inflammation [3]. Moreover angiotensin II (AT II), was proposed to improve cancer cell development and metastasis [4]. RAS pathway activity depends on the action of ATII, which is related to Angiotensin-I-converting enzyme (ACE), the action of which AT1R antagonists and ACE inhibitors can be blocked. Renin-angiotensin system inhibitors (RASIs) are well known for their potential function in suppressing tumor progression in CRC [5], and there are suggestions that they play a significant role in tumor cell lines inhibiting growth and angiogenesis [6, 7]. Although several epidemiological reports have reported that RASIs suppress the growth and metastasis of various cancers [4]. It has been shown in the model of gastric cancer that valsartan can inhibit the tumor growth and suppress the tumor angiogenesis [8]. In addition, Wang study was designed to identify the effects of valsartan with γ-rays on the expression of vascular endothelial growth factor (VEGF), radiation sensitivity, invasive potential and proliferative activity of nasopharyngeal carcinoma (CNE-2) in vitro. Wang's research showed that valsartan prevented the proliferation and invasion by CNE-2 cells of the nasopharyngeal carcinoma line and increased its radiation sensitivity [9]. Clinical studies have shown that use of valsartan compared to other ARBs in patients with hypertension significantly decreases inflammatory markers [10-12] .In addition, the results of the meta-analysis showed an increase in neoplastic diseases using candesartan, losartan and telemizartan, which was not significant, while the incidence of neoplastic diseases using valsartan showed a significant decrease [13] .The potential of RASIs as chemopreventive agents against CRC is a subject of interest. To evaluate this association, we have investigated the therapeutic potential and the molecular mechanisms of actions of valsartan (one of the family members of the AT1R antagonist) in CRC progression and metastasis

2. Results

2.1. Inhibition of cell proliferation, migration and induction of apoptosis

To evaluate the anti-cancer results of valsartan and valsartan + 5-FU, the viability of CT26 cells was tested using MTT assay. As shown in Fig. 1A, valsartan, and 5-FU dose-dependently inhibited cell growth. Results showed that co-treatment of valsartan and 5-FU decreased the IC50 value of 5-FU (IC50 valsartan = 1mM and IC50 5-FU= 10 μM). To assess the potential inhibitory effect of valsartan on the migration of CRC cells, the effect of valsartan on the expression of matrix metalloproteases-1 (MMP-1) was evaluated. The potential migratory behavior of valsartan-treated CRC cells was significantly inhibited as comparison with the untreated group ($P<0.05$) (Fig. 1B, C). As well as, the administration of valsartan decreased expression the enzymatic activities of MMP-1 as visualized by

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qPCR (Fig. 1 D). Also we found that valsartan increased in both early (annexin V positive/ PI negative) and late (annexin V and PI positive) apoptosis (Fig. 1E, F).

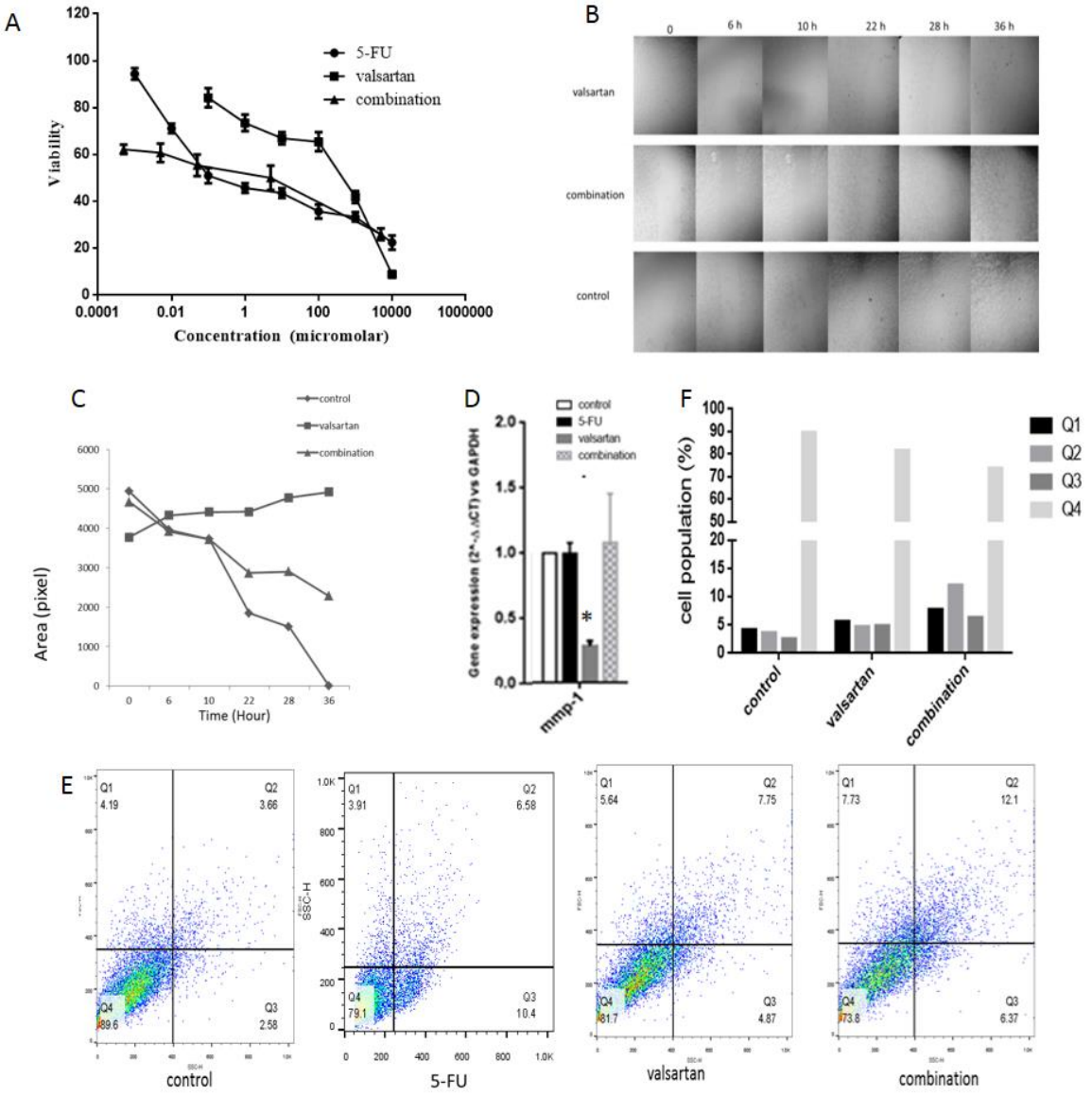

Figure 1. Valsartan inhibits cell migration and induces apoptosis of CRC cells. (A) Growth inhibitory effects of valsartan (1mM) after 24,48 and 72 hr exposure CT-26 cells. (B, C) Effect of valsartan on the migration of the CT-26 cells. (D) Expression level MMP-1 in CRC cells treated with valsartan as detected by real-time RT-PCR. (E, F) CT-26 cells were treated with valsartan for 24 h and apoptosis was explored by flow cytometry using annexin V/PI staining. The values of the lower right and the upper right area indicate the percentage of the cells in early and late apoptosis, respectively.

2.2. Inhibition of tumor growth and lung metastatic

Our data showed that the administration of valsartan reduced tumor size compared to control and 5-FU group (Fig. 2A-B). Fig 2B shows a reduction in the size of tumor significantly between combination and 5-FU group. Histopathological changes of the tumor are shown in the sections stained with H&E are shown in Fig. 2C. Administration of valsartan and 5-FU enhance tumor necrosis and tumor density but in valsartan-treated group tumor necrosis and tumor density higher than that 5-FU group as the standard chemotherapeutic regiment in CRC (Fig. 2C). Administration of valsartan decreased tumor density compared to the control group. Using Trichrome stain shows

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that treatment with valsartan decreased vascular density (Fig. 2D), fibrotic tissue and collagen deposition in tumor tissue compared to the control group. Additionally, valsartan in combination with 5-FU decreased the expression level of VEGF, IL-1, MCP-1, fibronectin, collagen type1 genes (Fig. 2E). Interestingly, our finding illustrated that valsartan was able to inhibit lung metastasis and improve body weight (Figure 3A-H), compared to the control and 5-Fu group. Also this effect was more pronounce in the mice which were received valsartan plus 5-FU (Fig. 3) in both macroscopic and microscopic tumor nodules examinations (Fig 3).

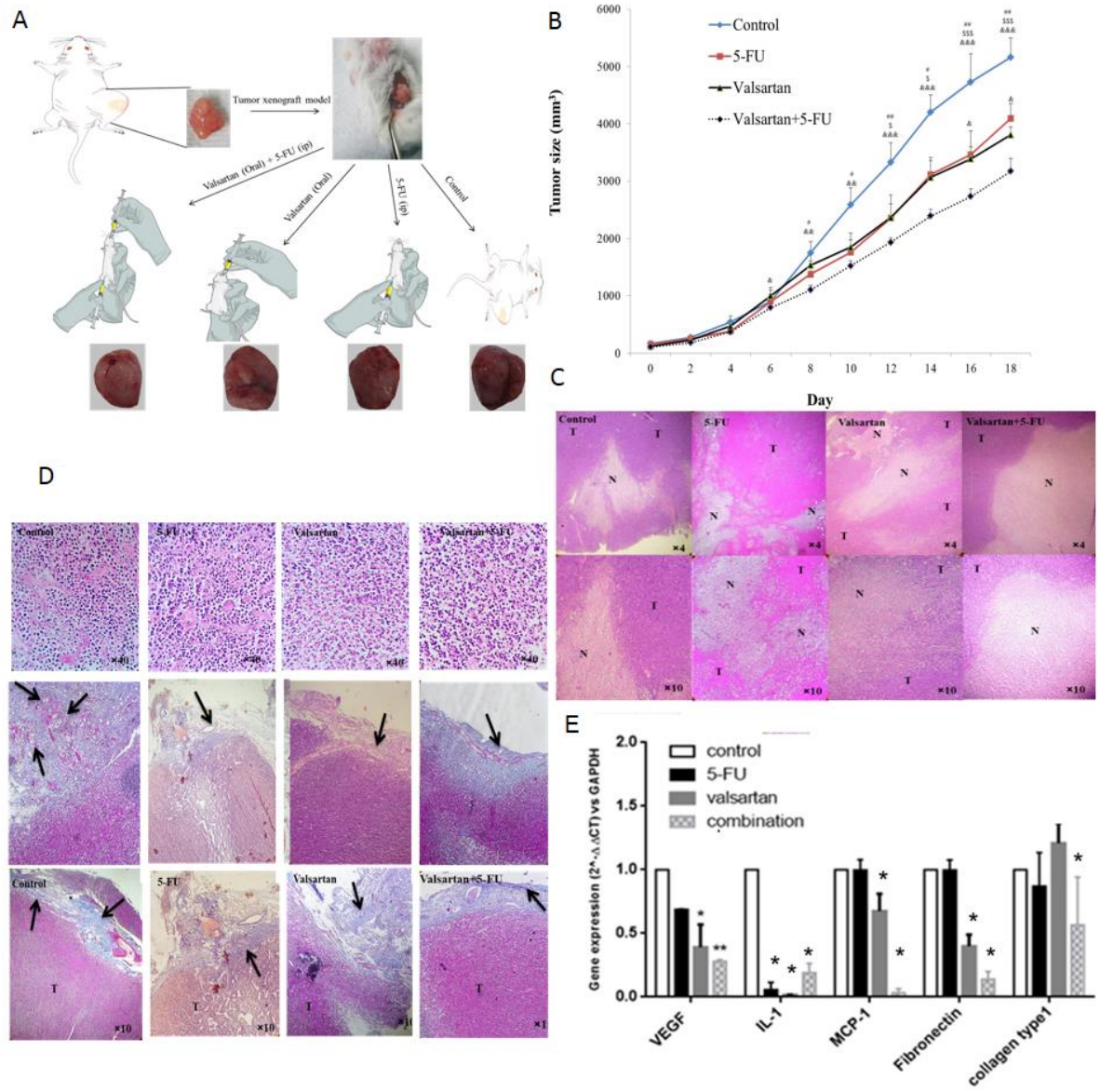

Figure 2. Valsartan suppresses tumor growth. (A) The treatment schedules and development of colorectal cancer models. Schematic of the Mice-derived tumor xenograft model. Tumor tissues were retranslated into Balb/c mice (n = 24). After the tumor volume reached to 80-100mm3, the treatment was initiated for 18 days. Relative variations of tumor volumes in different groups. (B) Tumor volumes were monitored every other day using a digital caliper. Representative H&E staining showed that tumor necrosis indicated by the N (necrosis) and T (tumor) in each group. magnification was mention under each figure. (C) Representative H&E staining showed that Tumor density and (D) images of Masson's trichrome staining were showed that Vascular density and Tumor fibrosis in each group. magnification was mention under each figure. (E) The expression level of VEGF, IL-1, MCP-1, fibronectin, collagen type1 genes in CT26 cells treated with valsartan as detected by real-time RT-PCR.

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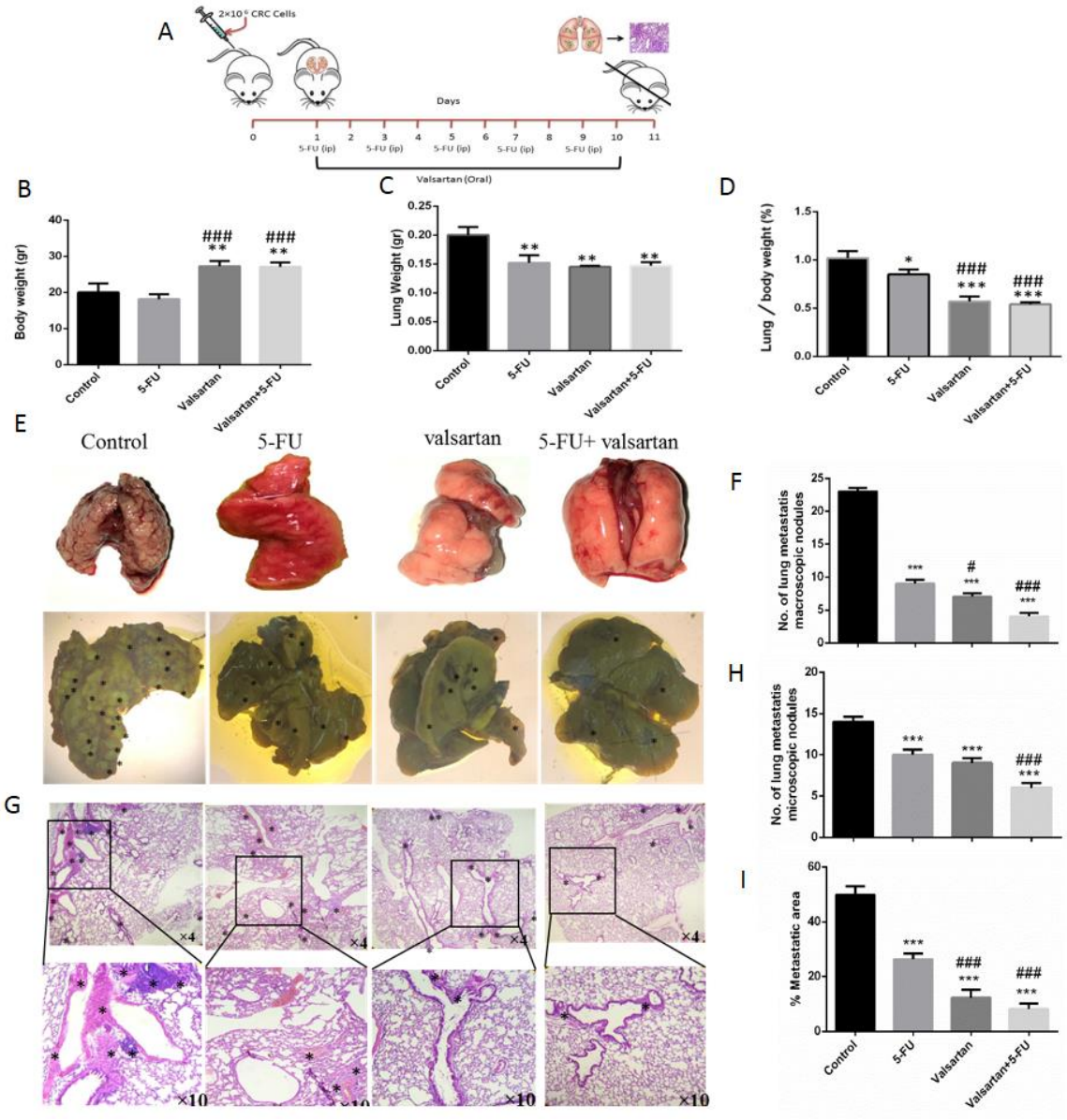

Figure 3: Effect of valsartan on the lung metastatic. (A)The treatment schedules and schematic representation of experimental protocol of lung metastasis derived CT-26 model. Twenty-four male inbred BALB / c mice were received 2x10^6 CT26 cells by tail intravenous injection and treated 24 hours after the cell injection for 10 days. Control group received normal saline. 5-FU group received 5mg/kg/every other day; intraperitoneal. Valsartan group received 40mg/kg/day; oral gavage. Combination group received 5-FU (5mg/kg/every other day; ip) plus (valsartan 40mg/kg/day; oral gavage); (B)The analysis showed that valsartan treatment significantly increased body weight (C) and decreased lung weight (D) and the lung/body weight ratio, compared with the control group (*P< 0.05, **P< 0.01 and ***P< 0.001 compare with control group and ###P<0.001 compare with 5-FU group). (E, F) Observational analysis showed that valsartan administration decreased the number of macroscopic metastatic lung nodules. Furthermore, the evaluation of tumor formation with Bouin's solution showed that valsartan treatment significantly reduced the number of macroscopic metastatic lung nodules. (G) HE staining demonstrated valsartan treated groups decreased higher number of (H) microfoci and (I) percent of metastatic area in lung significantly (The asterisk represent lung microfoci). Magnification was mentioned under each figure (G).

2.3. Valsartan induced oxidative stress

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The valsartan and combination groups had a higher MDA level compare to the control group (P<0.001 for both). A significant increase in MDA concentration was observed in the valsartan and valsartan-5-FU group compare to the 5-FU group (P<0.01 and P<0.001 respectively) (Fig. 4A). Administration of the valsartan decreased the total thiol, CAT and SOD (Fig4B-D). The NO metabolites of tumor tissues of valsartan-treated group and valsartan and combination groups were higher than the control group (P < 0.05, Fig. 4E). Moreover, our data showed that valsartan increased ROS generation in CRC cells, as detected by measured by DCFH-DA staining (Fig. 4G-H). These results clearly suggest that valsartan disrupts the antioxidant/oxidant balance, leading to more ROS production.

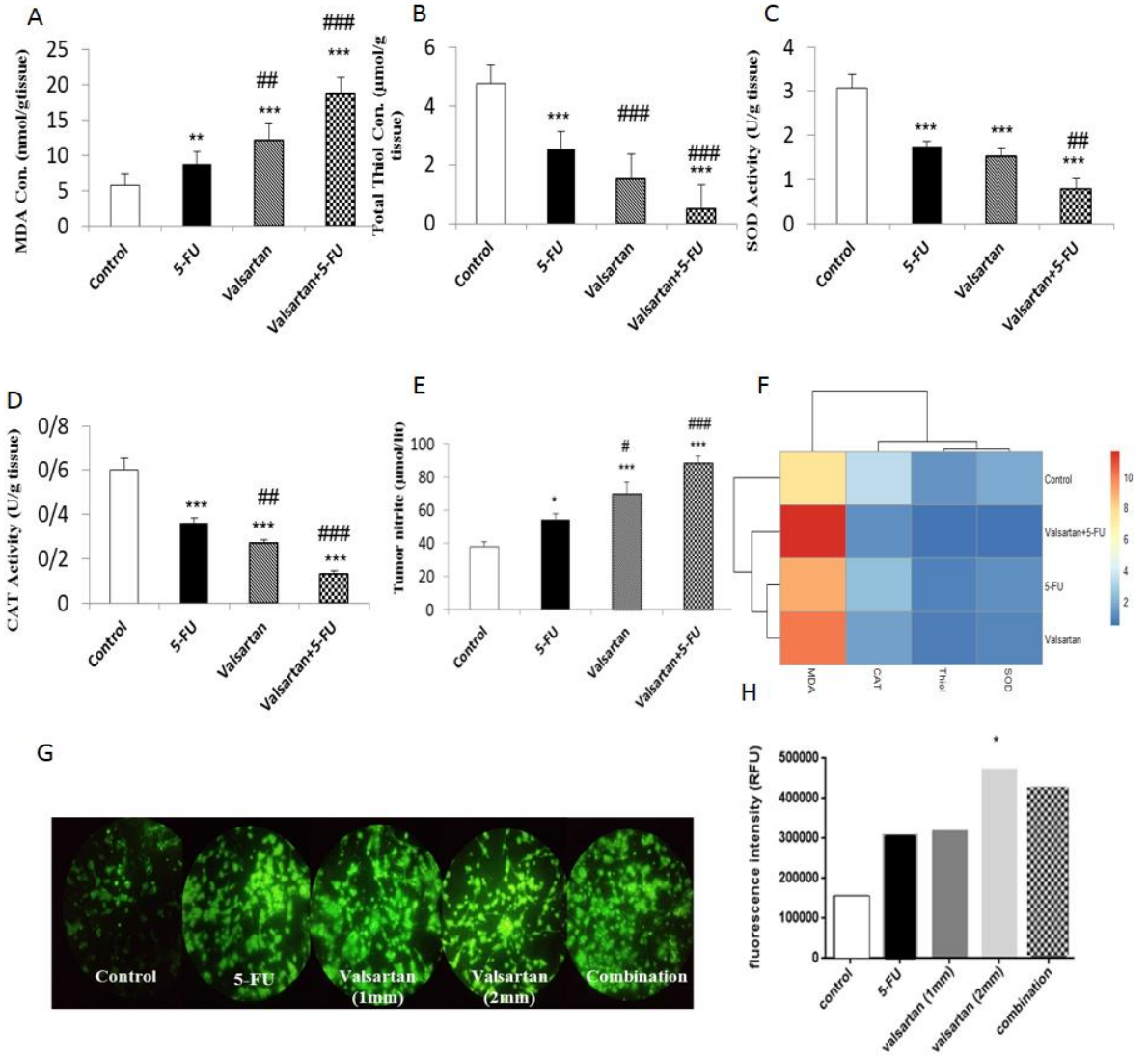

Figure 4: Valsartan induces cell senescence by expression oxidative stress in homogenized colon samples (A) The tumoral MDA concentrations, (B) total thiol content, (C) SOD, (D) CAT and (E) tumoral NO metabolites, (F) Heat map of oxidative stress markers, (G, F) ROS generation. Data are shown as Mean ± SEM (n = 6 in each group). *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control group. # P < 0.05, ## P < 0.01 and # # # P < 0.001 compared to 5-FU group. Data are shown as Mean ± SEM (n = 6 in each group). (F) Drug response for MDA concentrations, total thiol content, SOD, CAT. (G) Qualitative characterization of ROS generation using fluorescence microscopy. The stimulatory effect of valsartan on production of cellular ROS was investigated in CT-26 cells treated with two concentrations (1 and 2 mM) of valsartan.

2.4. The interaction of valsartan with AT1R, TGFβ, and inflammatory/anti-inflammatory cytokines

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We first performed in silico and binding energy analyses for valuation of the interaction of valsartan with AT1R, IFNγ, TGFβ, TNFα, MCP-1 and IL-1β (Fig5A-F). Analysis of the amount of hydrogen and hydrophobic bonds between inhibitors and enzymes reveals that the complex has many intermolecular hydrogen bonds, suggesting a higher affinity with AT1R. Our study suggested that the drug interaction would be the better on linkage to hydrogen if functional groups had been having ability in the most interactions. The lowest energy obtained in docking simulations AT1R, IFN-γ, TGFβ, TNFα, MCP-1and Ilβ respectively. Based on these observations, angiotensin 2 type 1 receptor is the most potent ligand among the other ligands which binds with valsartan and shows the best binding affinity with AT1R protein structure. Among the six different structures of AT1R, IFN-γ, TGFβ, TNFα, MCP-1and Ilβ is found that valsartan with binding affinity -11.5 shows the best docking result. The structure attained from the docking results is shown in Fig. 5. Together, the heat map of the data show in the batch cluster indicates oxidative stress markers and IL-6, VEGF and VEGFR-1 that the clustering algorithm discerned batch to batch variation as the most important source of variation within this data set (Fig. 5). We then evaluated the effect of valsartan on inflammatory cytokines expression at protein level. We found that valsartan administration decreased the expression levels of INF-γ, TGF-β, IL-6 and TNF-α in CRC significantly (Fig. 6A-D). Also the expression of VEGF and VEGFR-1 in valsartan or valsartan plus 5-FU group was reduced compared to the control or 5-FU groups (Fig. 6E-G).

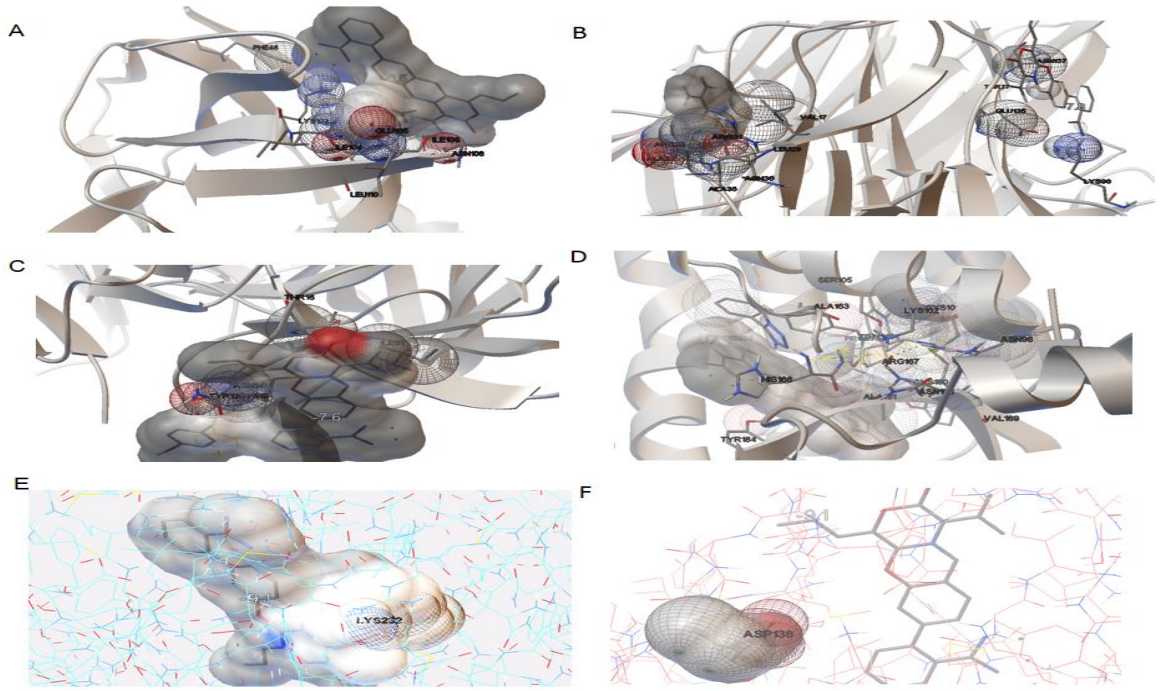

Figure 5: In silico analysis of valsartan response signature. The plots generated by autodock and LigPlot+ software shows interaction between Valsartan and IL-β (A), TNFα (B), MCP-1 (C), AT1R (D), TGF-β (E).

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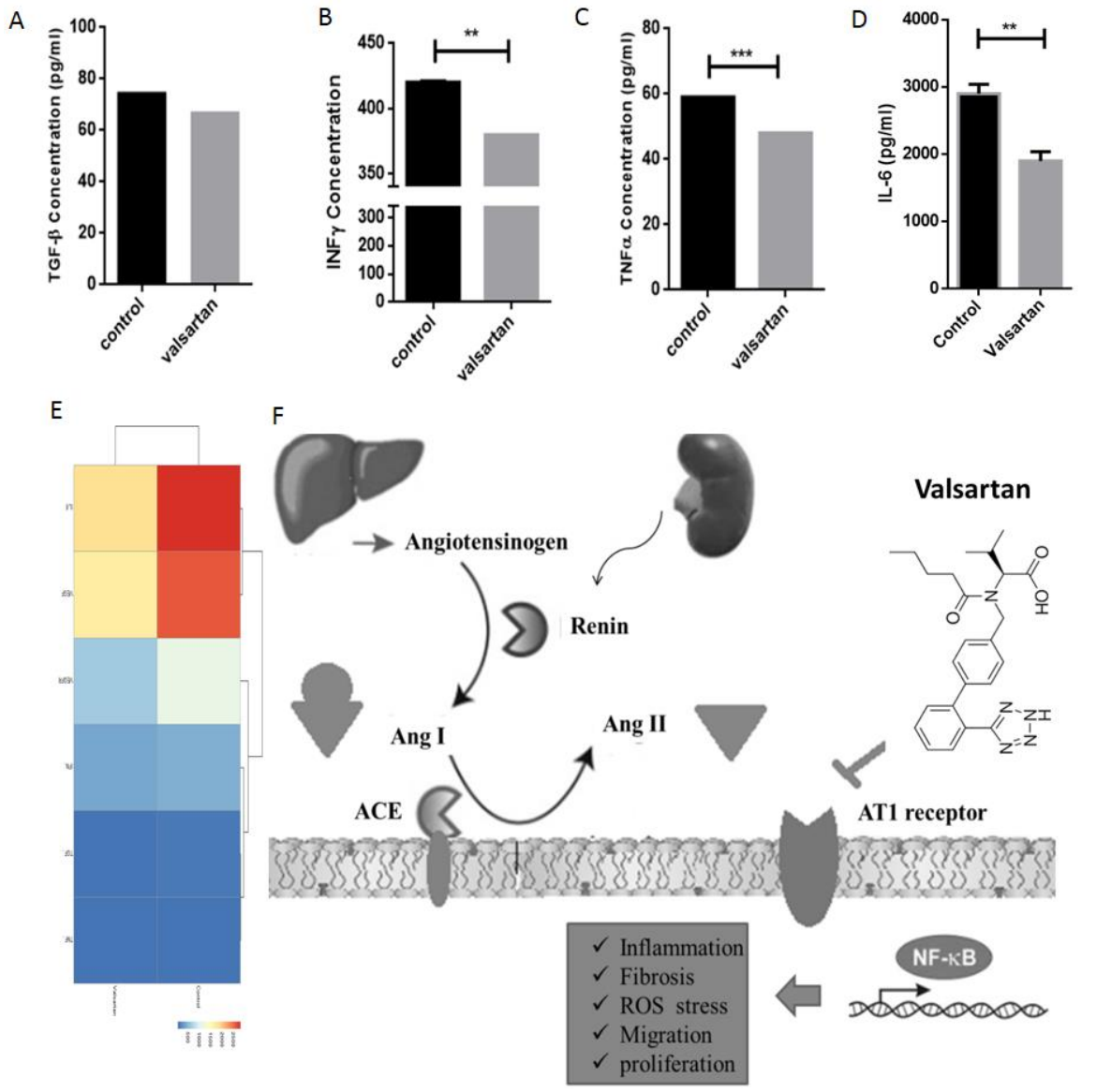

Figure 6: Valsartan decrease inflammatory cytokine expression and angiogenesis. Reduction of IFN-γ (A), TGF-β (B), TNF-α (C), IL-6 (D), with valsartan (**P < 0.01 and ***P < 0.001 compared to control group). (E) Drug response for VEGF, VEGF-R and IL-6; (F) Schematic representation summarizing the molecular mechanisms of anti-tumor activities of valsartan in colorectal cancer.

3. Discussion

To the best of our knowledge, we firstly demonstrated valsartan's antiproliferative behavior and its association with 5-FU in CRC. Our data indicated that targeting the angiotensin II type I pathway may inhibit tumor growth and ameliorate fibrosis and inflammation through transforming growth factor beta pathway in colorectal cancer. There widespread evidence indicating that AT-II may perform an vital role in many types of cancers by modulating adhesion, angiogenesis, tumor growth, migration, invasion, and proliferation [14]. It has been shown in the Osumi et al study that the use of ARBs was associated with longer overall survival (OS) and progression-free survival (PFS) in metastatic CRC patients undergoing first-line bevacizumab-based chemotherapy. This suggests that RAS suppression may inhibit tumor growth and enhance survival [15]. Conclusions The effect of ACEIs or ARBs on disease progression or survival in cancer patients has been proven [16]. There has been some evidence to indicate that the use of these inhibitors may be related to improvement in cancer outcomes [16]; however, to further investigate this relationship, broader epidemiological

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studies with adequate information on drug dosage, frequency, and length are needed. Additionally, Patients with advanced CRC with ACEI / ARB and β-Blockers have been shown to have increased survival and reduced tumor growth and hospitalization levels [17]. Several clinical trials have also shown that RASIs can have therapeutic effects in a wide variety of cancers [18]. The survival advantage is tumor and stage dependent and ranges from 3 months (advanced non-small cell lung cancer (NSCLC)) to more than 25 months (metastatic renal cell carcinoma (RCC)) in retrospective studies. Moreover, the RASI medication response can not only differ with the type of tumor but can also rely on characteristics of certain tumors, treatment of cancer, type and dosage of RASI. More specifically, RCC, hepatocellular carcinoma (HCC), pancreatic ductal adenocarcinoma (PDAC), glioblastoma, cancer of the urinary tract, and non-small cell lung cancer tend to relate to the groups of sensitive tumors, whereas breast cancer does not respond to RASI. There are several studies about the activity of RAS inhibitors in NSCLC, gastric cancer, and colorectal cancer who received platinum-based chemotherapy and those with anti-VEGF therapy RCC, HCC, and CRC (e.g., sunitinib) [18].

The AT1R activity mediated via AT II, has been shown in numerous malignant tissues [19]. With regard to the role of ATII in proliferation and migration of cell, it is suggested that certain stages of tumor genesis and tumor progression play a role [20, 21]. Blockade with AT1R antagonist resulted in a decrease in tumor growth [16]. The main actions of ARBs are mediated by inhibiting AT1R, and they have an enhanced inhibitory effect on tumor growth [20]. However, some researchers have shown continuing tumor growth despite AT1R B treatment [21]. ARBs are widely used in clinical practice every day because of their proven good tolerability, low side effects profile and well-known effectiveness [22]. The differences in structures between ARBs including variations in distribution, lipid solubility, biotransformation, bioavailability, plasma half-life and elimination [23]. The finding of the present study was to elucidate that tumor growth-induced CRC was suppressed by treatment valsartan in mice.

The AT1Rs can induce several signaling pathways such as PI3K/Ras/mTOR/AKT which involved in regulation of metastasis, apoptosis and cellular proliferation [24, 25]. Recent studies reported that PI3K/mTOR/AKT pathway is responsible for human colorectal cancers [26, 27]. Jaclyn et al, have shown that captopril and irbesartan (as RAS blocker) decreased tumor growth in CRC liver metastases that correlated with the reduction of central microvascular density significantly [28]. Another study showed that treatment with candesartan as an AT1RB decreased tumor growth in an experimental breast cancer model [29]. According to our results, Miyajima et al. demonstrated that the administration of candesartan reduced the development of lung metastases in a murine model of metastatic renal cell carcinoma [30]. Irbesartan enhanced Kupffer cell anti-tumor activity and reduced the severity of liver metastasis in people with CRC [28, 31]. Some studies have shown in vivo enhancement of tumor vascularization by ARBs and direct stimulation of AT2R [7, 32]. Cancer cell metastatic properties are determined by their migration and invasion. Therefore, a primary strategy in cancer treatment is impeding migration and cancer cell invasion that AT1RB such as valsartan is involved in this process [14]. Although other mechanisms may act synergistically in the development of CRC, in recent years it has been explained that the inflammation and oxidative stress indicators play an important part in this process [33]. Our finding investigated the anti-tumor mechanisms of valsartan in vivo and in vitro in a tumor xenograft model. We showed that valsartan elicited its anti-tumor properties by reducing tumor weight and size, inducing cell senescence, apoptosis, oxidative stress and inflammatory responses inhibiting cell cycle progression, in both CRC cells and mouse model CRC.

ROS play a vital role in apoptosis induction and diminish of cell viability in the early stage of cancer [4, 34]. AT1RB have a critical role in inhibition of tumor growth by apoptosis induction, cellular proliferation and vascular invasion [35]. In prostate cancer cells, telmisartan inhibited proliferation but this effect was not observed with valsartan, losartan, irbesartan or candesartan [36]. However, a recent report in human prostate cancer cells indicated that losartan may increase apoptosis [35]. The affect cell viability [37, 38] or proliferation [7] did not show with candesartan. There is an impact on

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the invasive potential of tumor cells when they have treated with ARBs [39]. According to our results, demonstrated that valsartan increases cellular senescence by up-regulation of inflammatory mediators, inducing oxidative stress, increasing ROS generation in CRC cells

Moreover, our data showed that tumor-related NO can develop and suppress cancer growth depending on context [40]. It was the suggestion that AT2Rs are important in NO production [41]. Nguyen et al demonstrated that AT2R inhibition reduces iNOS levels while AT1R blockade was likely to increase iNOS levels due to a proportional rise in AT2R activity [40]. Taken together, our findings have shown that co-administration of valsartan and 5-FU increased oxidant marker and reduced antioxidant indicators and tumor proliferation CRC in this experimental model. This results showed that the shift in oxidant-antioxidant status could be one of the underlying mechanisms that led to valsartan action against CRC. Lung metastasis involves the invasion and movement of tumor cells into the lungs through the bloodstream [42]. Several studies have been reported that ATII to accelerate lung metastasis of cancer cells [43]. Recent evidence suggests that AT1R B, valsartan (40 mg/kg/day), suppressed the effect of AT-II [44]. The results of this study indicate that valsartan improve lung metastasis of colorectal cancer via downregulation of inflammatory markers such as IL-6 and the level of VEGF and VEGFR-1. The present finding also supports Deshayes et al study which concluded that treatment administration of ATIIR1 blockers (ARBs) have been associated with a lower occurrence of metastases and lower VEGF levels [45]. The finding is consistent with the finding of Ager et al, which administration with candesartan and irbesartan restrained growth of tumor, angiogenesis, and metastasis in an animal model of CRC metastasis to the lung [2]. Based on the results, valsartan through induction of ROS can be obstructed lung metastasis. This is supported by Piskounova study which reveals that enhancement of oxidative stress in metastasis derive of human melanoma cells suppresses distant metastasis [46].

In conclusion, the current study indicated that valsartan inhibited CRC growth by inhibition of cell proliferation, apoptosis, migration, and enhancement pro-inflammatory responses.

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4. Materials and Methods

4.1. Chemicals and drugs

Valsartan was obtained from Cayman Co, Michigan which dissolved in ethanol and diluted in sterile water, while Fluorouracil (5-FU) was a gift from the Mashhad University of Medical Science and diluted in sterile water. The Netherlands Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL, Life Technologies Inc. (Gaithersburg, MD, USA).

4.2. Cell culture

The CT26 cell line was cultured in DMEM/F12 medium and subsequently incubated with 10% heat-inactivated FBS and 1% streptomycin at 5% CO2 at 37 ° C. In its exponential log step, the CT26 cells were harvested using trypsin-EDTA.

4.3. MTT assay

The anti-cancer properties of valsartan, 5-FU, and the both of them on CRC cells were assessed using the MTT test before and after cell treatment within 24 hours as described previously [47].

4.4. Apoptosis analyses

Annexin-V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) assay kits (Cayman chemical Co, Michigan) were used to assess apoptotic cell death [48]. Briefly, the cells of CRC were distributed at a density of 2×106 cells per well in 6-well plates, and treated with Valsartan (1mM) for 24 hr. The cell extracts were suspended in 200 µL 1X Annexin V binding buffer. After centrifuging cells (for 5 minutes at 400 g), the cell pellets were re-suspended in Annexin V-FITC/PI staining solution and have been incubated at room temperature for 15 minutes. Subsequently, the rate of viable, secondary necrotic cells, early and late apoptotic were quantified using FACSCalibur flow cytometer (BD Biosciences-US) and FlowJo software [49].

4.5. In vitro migration assays

Valsartan 's ability to inhibit the migratory activity of CT-26 cells was investigated using an in vitro migration assay, as followed mentioned. At their 5 ×IC50s, the cells were exposed to the drugs. At the beginning of the exposure, images were taken with those taken after 6, 10, 22, 28 and 36 hours.

4.6. In vivo model for CRC

Twenty-four Inbred BALB/c mice (average old 7 weeks) were purchased from the Pasteur Institute (Tehran, Iran). They have been held in normal conditions approved by the Institute animal ethics committee (temperature 23 ± 2 °C, humidity of 53 ± 3% and 12 h Period light / dark). Approximately two weeks after the tumor cells implant, when tumors reached a volume of 80-100mm3 [50], mice were separated into four groups randomly as below: (1) Control (Untreated mice), (2) 5-FU (treated with 5mg/kg every other day, intraperitoneally) [51], (3) Valsartan (treated with 40 mg/kg/day, orally) [52], (4) combination (treated with 5mg/kg 5-FU every other day i.p. and Valsartan 40 mg/kg/day, orally) (Fig 1). The size of tumor was assessed every other day with the digital caliper and the tumor volume was determined according to the formula: Tumor volume = (tumor length) × (tumor width) 2/ 2. At the end of the fourteen day, tumor tissue was harvested for assessment of histological and biochemical dimensions.

4.7. In vivo model for developing lung metastasis

Twenty-four Inbred BALB/c animals were separated into 4 groups (n=6): a control group (no treated), a 5-FU-treated group (5mg/kg every other day (ip)), a valsartan-treated group (40 mg/kg/day (oral gavage)), and a combination-treated group (5mg/kg 5-FU every other day (ip) plus valsartan 40

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mg/kg/day (oral gavage)). Lung metastases in the animals were produced by injecting $2x10^6$ CT26 cells in 100 mL of PBS intravenous via the lateral tail vein. Treatment was started 24 hours after injection and continued for 10 days (Fig 7) [53]. The mice were sacrificed at 11 days, and the lungs were separated. The lung weights were measured, and tissues fixed in Bouin's solution for counting tumor colonies.

4.8. Histological evaluation

The tumors (CRC model) and lung (CRC lung metastasis model) were isolated and then put in 10% formalin overnight. After dehydration, the tumors were coated in paraffin and chopped with a microtome. The sections were stained with Hematoxylin-Eosin (H&E) and Masson's trichrome stains and studied with light microscopy (×40 magnification).

4.9. qRT-PCR

RNAs was quantified in the CRC tissues after treatment with valsartan and 5-FU and complementary DNAs (cDNA) synthesized according to the manufacturers' protocol. The real-time PCR analysis was carry out using specific primers Macrogene (Macrogene Co, Seoul, Korea) by light cycler real-time Polymerase chain reaction (PCR) (Roche Diagnostics, Mannheim, Germany). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control gene as described [54].

4.10. Oxidant and anti-oxidant assessments

The tumor and lung specimen were homogenized with a PBS (phosphate buffer solution with pH 7.4). The homogenates for 15 min were centrifuged and malondialdehyde (MDA), total thiol, superoxide dismutase (SOD) [44], catalase (CAT) [28] were measured.

4.11. Malondialdehyde

The biomarker of lipid peroxidation is MDA. The supernatant of tumor and lung homogenate was affixed to thiobarbituric acid. Then the absorbance was measured at 535nm wavelength using spectrophotometer against a blank and the MDA content was determined [13].

4.12. Total thiol group

Measurement of total thiol groups using dithionitrobenzoic acid (DTNB). In Tris-EDTA buffer (pH=8.6), the tumor and the lung supernatants were incubated with DTNB. The composition was then kept for ten min at room temperature, and the wavelength of the absorbance was 412 nm. The total thiol content was calculated using below formula [55]. Total thiol concentration (mM) = (A2-A1-B) × 1.07/ (0.05 × 13.6)

4.13. Superoxide dismutase

Measurement of SOD activity was based on the Pyrogallol auto-oxidation and suppression of conversion of MTT to formazan. The formazan was dissolved in DMSO and its absorption was read at a wavelength of 570 nm [56].

4.14. Catalase

The CAT behavior was calculated using spectrophotometer with a wavelength of 240 nm based on the decomposition of hydrogen peroxide (H2O2) [57].

4.15. Reactive oxygen species (ROS) evaluation by DCFH-DA method

For evaluation of intracellular ROS production used from the change of redox-sensitive dye 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to a green fluorescent product, dichlorofluorescein

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(DCF). Briefly, the CT-26 cells were treated with valsartan, 5-FU, and the combination of valsartan and 5-FU for 6 hours, in complete medium. Then the cells were incubated in fresh culture medium plus DCFH-DA for 30 min. After washing Per PBS three times, the fluorescence values were measured using a fluorimeter and cells were imaged with an inverted fluorescence microscope [34].

4.16. Nitric oxide (NO) metabolites

The tumor NO metabolites were determined using the Griess reaction using colorimetric assay (Promega Corp. USA) according to the standard protocol [58].

Evaluation of Interleukin-6 (IL-6), Interferon gamma) IFN-γ (, TGF-β, tumor necrosis factor alpha (TNF-α) and angiogenic markers

Quantification of IL-6, IFN-γ, TGF-β, and TNF-α in colon cancer cells was performed using cytokine detection ELISA kits (eBioscience). Also the level of VEGF, and VEGF receptor-1 (VEGFR1) was measured using ELISA kits (Zellbio) according to the manufacturers' protocol.

4.17. In silico and heat map analysis of valsartan response signature

The Interaction of valsartan with IL-β, TNFα, MCP-1, AT1R, TGF-β, oxidant/antioxidant factors, IL-6, and VEGF/VEGFR1 markers was evaluated by autodock and LigPlot+ software or by R software.

4.18. Statistical analysis

Data are presented as mean±SEM and analyzed by One-way ANOVA followed by post LSD comparison tests. Data analysis was undertaken using SPSS v.20 statistical software (IBM, Chicago). Differences for a $P < 0.05$ were considered statistical significant.

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