IN VITRO STUDY OF THE EFFECT OF GOLD NANOPARTICLES ON SW 620 AND HCT 116 COLON CANCER CELL LINES

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A Thesis Submitted in Partial Fulfillment of the Requirements
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การศึกษาอิทธิพลของอนุภาคทองคำระดับนาโนเมตรต่อเซลล์ sw620 และเซลล์มะเร็ง HCT 116

นายกนิชกา พุชภิธา มหา ธนันธิฤทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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กนิชกา พุชภิธา มหา ธนันธิฤทธิ์ : การศึกษาอิทธิพลของอนุภาคทองคำระดับนาโนเมตรต่อเซลล์ sw620 และเซลล์มะเร็ง HCT 116 (*IN VITRO* STUDY OF THE EFFECT OF GOLD NANOPARTICLES ON SW 620 AND HCT 116 COLON CANCER CELL LINES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นพ.อมรพันธุ์ เสรีมาศพันธุ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.โรจน์ฤทธิ์ โรจนธเนศ, 4 หน้า.

มะเร็งลำไส้ใหญ่เป็นมะเร็งที่เป็นสาเหตุของการเสียชีวิตเป็นอันดับที่สามจากมะเร็งทั้งหมด เนื่องจากเซลล์มะเร็งชนิดนี้มักเกิดภาวะดื้อยาจากการทำเคมีบำบัดและการรักษาด้วยรังสี อีกทั้งไม่สามารถใช้วิธีการผ่าตัดในการรักษามะเร็งในระยะลุกลามได้ ดังนั้นจึงมีความจำเป็นต้องศึกษาหาวิธีการรักษาที่มีความจำเพาะกับเซลล์เป้าหมาย หนึ่งในปัจจัยที่มีความสำคัญต่อการเกิดมะเร็งระยะลุกลามคือการสร้างเส้นเลือดแดงเส้นใหม่ ซึ่งในมะเร็งลำไส้ใหญ่พบว่ามีวาสคูลาร์ เอ็นโดทีเลียล โกรทแฟกเตอร์ซึ่งเป็นสารกระตุ้นในการสร้างเส้นเลือดมากกว่าเมื่อเทียบกับเซลล์เยื่อบุของลำไส้ใหญ่ ทั้งนี้งานวิจัยที่ผ่านมาได้มีการพัฒนายาบีวาซีซูแมบ ซึ่งเป็นโมโนโคนอลแอนติบอดีที่สามารถจับกับ วาสคูลาร์ เอ็นโดทีเลียล โกรทแฟกเตอร์ชนิดเอได้ อย่างไรก็ตามพบว่าผู้ป่วยที่ทำการรักษาด้วยยาบีวาซีซูแมบ มีการพัฒนาของเซลล์มะเร็งเป็นระยะลุกลามหรือเกิดการดื้อยาได้ อนุภาคทองคำระดับนาโนเมตรถูกพบว่าเป็นตัวเลือกที่ดีสำหรับนำมาประยุกต์ใช้เพื่อการรักษาในทางการแพทย์ และอาจเป็นทางเลือกที่ดีสำหรับการรักษามะเร็งลำไส้ใหญ่ เนื่องจากอนุภาคทองคำระดับนาโนเมตรมีคุณสมบัติสามารถจับกับวาสคูลาร์ เอ็นโดทีเลียล โกรทแฟกเตอร์ได้ งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อประเมินผลของอนุภาคทองคำระดับนาโนเมตรต่อเซลล์มะเร็งลำไส้ใหญ่ (SW620 และ HCT116) โดยทำการศึกษาเซลล์มะเร็งลำไส้ที่ได้รับอนุภาคทองคำระดับนาโนเมตรที่มีขนาดต่างกัน และสารที่ทำให้คงตัวต่างกัน ผลการศึกษาพบว่า อนุภาคทองคำระดับนาโนเมตรที่มีโซเดียมบอเรตเป็นสารที่ทำให้คงตัวทำให้เซลล์มะเร็งตาย ลดการเกิดการเคลื่อนที่ของเซลล์ และชักนำให้เกิดการตายแบบเนโครซิส นอกจากนี้ยังช่วยลดการแสดงออกของวาสคูลาร์ เอ็นโดทีเลียล โกรทแฟกเตอร์ในเซลล์มะเร็งทั้งสองชนิด ในทางกลับกันอนุภาคทองคำระดับนาโนเมตรที่มีโซเดียมบอเรต มีผลต่อการแสดงออกของไฮพ็อกเซียอินดิวซิเบิลแฟคเตอร์ในเซลล์มะเร็ง HCT 116 เท่านั้น ซึ่งจากผลการศึกษาสรุปได้ว่า อนุภาคทองคำระดับนาโนเมตรมีผลต่อเซลล์มะเร็งลำไส้ใหญ่แตกต่างกันขึ้นอยู่กับชนิดของสารที่ทำให้คงตัว และอาจใช้เป็นหนึ่งในทางเลือกสำหรับการรักษามะเร็งลำไส้ใหญ่ได้

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KANISHKA PUSHPITHA MAHA THANANTHIRIGE: *IN VITRO* STUDY OF THE EFFECT OF GOLD NANOPARTICLES ON SW 620 AND HCT 116 COLON CANCER CELL LINES. ADVISOR: ASST. PROF. DR.AMORNPUN SEREEMASPUN, M.D., Ph.D., CO-ADVISOR: ASST. PROF. DR.ROJRIT ROJANATHANES, Ph.D., 4 pp.

Colon cancer acts as a third most leading role in causing death among all the cancer types. Because cancer cells developed resistance against the chemotherapy, radiotherapy, and inability of using surgical treatment methods for advanced metastasis colon cancer, there is a higher demand of more targeted treatment method. Parameter plays a role in cancer metastasis as angiogenesis; vascular endothelial growth factor (VEGF) is as a pro-angiogenic factor for angiogenesis. Colorectal cancer cells have VEGF in very high concentration when compared to normal colon epithelial cells. Based on those findings, scientists developed Bevacizumab, humanized monoclonal antibody, which is capable of binds to all the isomers of VEGF-A. Even though patients treat with targeted therapies, cancer cells show advanced metastasis either by developing new pathways for metastasis or by developing resistance against the treatment. Gold Nanoparticles are a better candidate in various medical applications such as treating cancer as a treatment, drug or gene delivery system, and molecular detection system. Because of its ability to bind with VEGF, gold nanoparticles become the better candidate for cancer treatment. With all these understandings, this study aimed to determine the effect of gold nanoparticles (GNPs) on SW620 and HCT116 colon cancer cell lines. Using SW620 and HCT116 cell lines, treatment with various sizes of Sodium borate (SB) and Sodium citrate (SC) stabilized GNPs for 24h. In our findings, SB stabilized GNPs decreased the cell viability, cell migration and induce cell necrosis. Moreover, SB stabilized GNPs decreased VEGF expression levels in both the cancer cell lines. But, only HCT116 showed decreased HIF-1-alpha expression levels when treated with SB stabilized GNPs. Our results conclude that effect of GNPs on colon cancer cell lines by stabilizing agent dependent pattern. These findings suggest that GNP-SB can be a promising alternative treatment for colorectal malignancy in medicine.

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Student's Signature .....
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CONTENTS

Page

LIST OF FIGURES

CHAPTER 1

Introduction

Colon cancer turns out to be very common among both western and eastern societies. Numerous cancer cell types were developed resistance against the orthodox treatments and progress until metastasis level. Both tumour development and metastasis depend on angiogenesis [1]. Colorectal cancer cells have vascular endothelial growth factor (VEGF) in very higher concentrations than normal colon epithelial cells [2]. VEGF family members play a vital role in angiogenesis as a pro-angiogenic factor [1,2]. Bevacizumab, humanised monoclonal antibody which is capable of binding to all isomers of VEGF-A, which is a standout amongst the most effective targeted therapies for colorectal malignancy [3]. However, many colorectal cancer patients have shown relapse with lack or no response to Bevacizumab [2]. The monoclonal antibodies of VEGF has promote, advance metastasis, which lead to the formation of secondary tumors by increasing HIF-1α [4]. Therefore, still there is a more demand for developing new treatment regime against colorectal cancer.

Nanoparticles are the best candidate in various medical applications such as treating cancer as a treatment, drug or gene delivery system and molecular detection system [5]. There are various nanoparticles with distinctive sizes used in nanomedicine. Yet, high toxicity was reported as the fundamental drawback of nanoparticle applications in the field of nanomedicine. Gold nano particles (GNPs) turn into a preferable candidate against colorectal cancer because of its less toxicity, higher surface area to volume ratio, ability to show anti angiogenic properties by binding with heparin binding proteins such as VEGF 165 and basic Fibroblast Growth Factor among all the types of nano particles [5,6,7].

With all these understandings, this study was aimed to determine the anti-angiogenic effect of GNPs on colon cancer, by studying its impact on VEGF expression. As a one of the pro angiogenic factors and its higher expression in colon cancer cells, VEGF is the best candidate to study anti angiogenic effect of GNPs. Besides, this study focused to figure out the effect of GNPs on cancer metastasis, by studying their impact on HIF-1α expression. As a tumour metastasis promoter, HIF-

1α is a better candidate to study the effect of GNPs on cancer metastasis. Moreover, in this research, we were focused to study the effect of key determinant factors of GNPs on colon cancer cell lines. In light of previous research works we have hypothesised that, GNPs will bind with VEGF and inhibit the angiogenesis in colorectal cancer as an anti angiogenic factor. Furthermore, GNPs will inhibit the expression of HIF-1α and hinder the advance metastasis. Finally, the key determinants will enhance the impact of GNPs on colon cancer cell lines.

We have led our experiments by using two colorectal cancer cell lines. They were SW620 (homozygous KRAS mutation), HCT116 (heterozygous KRAS mutation) and CRL1790 (normal colon epithelial cell line). In this research, we have pointed out how GNPs effect on colon cancer cell lines based on their concentration, particle size and stabilising agents. In addition, we explain anti angiogenic and anti HIF-1α properties of GNPs on colon cancer cells.

CHAPTER 2

Literature review

Angiogenesis and Targeted Therapy

Angiogenesis is a process that forms new blood vessels from preexisting blood vessels [8]. Angiogenesis control by using both pro angiogenic factors and anti angiogenic factors [9]. During tumor development and metastasis, angiogenesis plays an important role [9]. It is a known fact, that without angiogenesis tumors will not grow more than 2-3mm^3 [8,10,11]. Furthermore, without an angiogenesis tumor may become necrotic or apoptotic [8]. In tumor cells, concentration of pro angiogenic factors is higher than anti angiogenic factors [8].

Among all pro angiogenic factors, high concentration of Vascular Endothelial Growth Factor (VEGF) present in tumor cells. Due to alternative splicing there are five members in VEGF family. They are VEGF-A, B, C, D and Placental Growth Factor (PLGF) [8]. However, present in large quantities in growing tumor cells, VEGF pathway induces the production of sprouting blood vessels and VEGFR expressed at high

levels in tumor cells than normal cells, VEGF is become a better candidate for the targeted therapies [5].

In 1993 Kim et al., Showed injection of the mouse monoclonal antibody used against the human VEGF isoforms suppress the tumor growth in mice in vivo [12]. Bevacizumab is such a humanized monoclonal antibody which uses to treat advanced colorectal carcinoma. It blocks the interaction of VEGF with VEGFR2 [8]. Even though, those targeted therapies increase the liveliness of the colon cancer patients, they have limitations and drawbacks.

In 2009 Eric Van Cutsem et al., Showed first line treatment of Cetuximab with FOLFIRI (irinotecan, fluorouracil, and leucovorin) reduced the risk of progression of metastatic colorectal cancer only in patients with *KRAS* wild-type tumors [13]. Moreover, in 2009 Jolien Tol et al., Showed the addition of Cetuximab and Bevacizumab in to capecitabine, oxaplatin treatments resulted in shorter progression free survival [14]. Panitumumab with FOLFOX-4 (oxaliplatin, fluorouracil, and leucovorin) treatment indicate lack of response in patients with RAS mutation [11]. According to another

study Bevacizumab as an adjunct therapy did not increase the disease free survival [15]. Moreover, researchers proposed that Bevacizumab should not be used in the adjunct treatment of patients with curatively resected stage 3 colon cancer [15].

There are several hypotheses about developing advanced metastasis against targeted therapies such as Bevacizumab;

1. Developing resistance against the targeted therapy
2. Developing different pathways for the metastasis

In 2013 Naoko Yamagishi et al., Proved that chronic inhibition of VEGF by an anti-VEGF monoclonal antibodies increase the VEGF concentrations and induce the resistance to hypoxia induced apoptosis [4]. Also in 2013 Christopher H Lieu et al., showed that increase in PLGF and VEGF-D after progression on chemotherapy with bevacizumab and they hypothesize that those ligands may be associated with resistance to Bevacizumab containing chemotherapy in metastatic colorectal carcinoma [16].

HIF-1α and metastasis

In year 2000 Christine Blancher et al., found that breast cancer cell lines show higher levels of HIF-1α expression [17]. Debbie Liao et al., showed that HIF-1α is a significant positive regulator of tumor progression and metastasis in colon cancer [18]. Also in 2009 Dan Cao et al., mentioned that HIF-1α expression significantly associated with tumor stage, lymph node and liver metastasis [19]. In the same year Takaaki Imamura et al., found that HIF-1α deficient colon cancer cells display lower rate of proliferation and migration. Also, they found that deficiency of HIF-1α can inhibit the overall tumor growth [20]. In 2014 Lin Zhang et al., showed that in esophageal squamous cell carcinoma, the HIF-1a expression is significantly associated with tumor stage and lymph node metastasis. Also, they reported esophageal squamous cell carcinoma patients with higher HIF-1α expression show worse survival rate [21].

Nano medicine and Gold nano particles

"Intentional design, characterization, production and application of materials, structures, devices and system by controlling their size and shape in the nano scale range" called as nano technology [21]. There are various applications of nanotechnology in various fields including medicine. In the field of nano medicine, properties and physical characteristics of nano materials use in the diagnosis and treatments of disease conditions like cancer in a molecular level [22]. Due to their high ratio of surface area to volume and optical, electrical, magnetic, biological properties, nano materials are playing an important role in nano medicine [23, 24]. There are different nano materials which consist of metallic, organic and semi conducting particles [22]. In nano medicine, there are different types of nano particles in use such as gold nano particles, silver nano particles, carbon nano particles and silicon nano particles. Nano particles can be used as a transport agent through biological barriers, molecular detectors and drug delivery agents. In 2014 Wei Rao et al., showed that the chemotherapy encapsulated nanoparticle system which

is used as a drug delivery system eliminates cancer stem like cells [25]. Due to high surface area to volume ratio permits high surface loading of therapeutic agents to nano particles. Also, nano particles can minimize the uptake of medicines by healthy cells [26, 27].

Due to less toxicity, size- and shape-dependent optical and electronic features, high surface area to volume ratio and surfaces that can be readily modified with ligands containing functional groups there are lots of applications of gold nano particles in nano medicine [5].

Aurimmune is a 27nm gold nano particle solution which is coated with recombinant human tumor necrotic factor alpha (TNF-α) and polyethylene glycol. Aurimmune is in phase 2 clinical trials for the treatment for cancer patients who doesn't show any respond to conventional treatments with an advance metastasis level. Histopathological studies showed that those nano gold particles localized within or around their tumor with less uptake into healthy organs [28,29]. In 2005 Priyabrata Mukherjee et al., showed that gold nano particles posses anti VEGF properties [6]. In

that study, they found that gold nano particles inhibit the properties of VEGF-165 and bFGF. Furthermore, they showed that gold nano particles significantly inhibit the VEGFR-2 phosphorylation, intracellular calcium release and cell migration. In 2011 Rochelle R Arvizo et al., explained the mechanism of anti angiogenic property of gold nano particles [30]. In that study, they used different surface charges and different sizes of gold nano particles. They found that gold nano particles bind and alter the confirmation only in heparin binding growth factors like VEGF-165, bFGF.

Also in 2014 Yunlong Pan et al., showed inhibition effects of gold nano particles on proliferation, migration in hepatic carcinoma [31].

Effect of gold nano particles on cancer cells depends on various factors like size of nano particles, surface charges and stabilizing agent. [29,32,33]. Sodium Borate is one of the stabilizing agents which will be used in this research, and there are some evidences that show anti cancerous properties of borate compounds. In 2006 W.T. Barranco et al., showed that pharmacologically relevant doses of Boric acid can reduce the migration, invade matrigel properties of DU-145 prostate cancer cell by

inducing cellular changes [34]. Also in R. Scorei et al., conducted a set of experiments to investigate the effect of calcium fructoborate (CF) and Boric acid (BA) on activating apoptotic pathway in MDA-MB-231 human breast cancer cells [35,36,37]. They showed that an exposure to boric acid and calcium fructoborate inhibit the proliferation of breast cancer cells in a dose dependent manner. Also, they reported that even though both boric acid and calcium fructoborate inhibit the apoptosis only calcium fructoborate induce the apoptosis in MDA-MB-231 breast cancer cell lines. Most importantly in 2012 Suxia Sun et al., showed that Sodium butyrate triggers colon cancer cell apoptosis in a Store Calcium Entry pathway dependant manner [38,39]. In this research they showed that Sodium butyrate induce Ca^{2+} release from the endoplasmic reticulum, which cause extracellular Ca^{2+} influx.

CHAPTER 3

Material and methods

1. Colon cell lines

Both colon cancer (SW620 and HCT 116) and normal colon epithelium (CRL 1790) cell lines have been carefully maintained in a humidified tissue culture incubator at 37°C in 5% CO_2. Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, USA) was used for both SW620 and CRL1790 cell lines. Roswell Park Memorial Institute Medium (RPMI) (Gibco® by life technologies™, USA) was used for HCT116 cell line. All the media contained 10% Fetal Bovine Serum (FBS) and 1% antibiotics (Penicillin, Streptomycin).

2. Synthesis of Gold nano particles and characterization

Gold nano particles were synthesized by using both sodium citrate and sodium borate based methods as mention below. Characterization was done by using UV-Vis absorption spectroscopy and transmission electron microscopy (TEM).

2.1 Citrate method;

0.5ml of 1% $HAuCl_4$ was mixed with 24.5ml of MilliQ water. The solution was stirred at 180^0C for 5min. 38.8mM of Sodium Citrate was added and stir at 120^0C for 15min. When the colour of the solution change to wine red lowered the temperature and stir over night.

2.2 Borate method;

0.5ml of 1% $HAuCl_4$ was mixed with 24.5ml of MilliQ water and add 4.5ml of freshly prepared 10.5mM Sodium borate solution and stir over night.

3. Cell Morphology

Cells were seeded into 24-well plate (500μl/well with5×10^4 cells) and incubate for 12h under 37^0C with 5% of CO_2. Cells were treated with GNPs and re incubates for 24h under at 37^0C with 5% CO_2. Observed under phase contrast microscope (Nikon®, Japan).

4. Cell Viability

The cell viability test was used to measure the ability of mitochondrial dehydrogenase to reduce blue colour Resazurin to pink colour Resofurin. Cells were seeded in 96-well plates (45ul/well and contain 5000cells/ well) and incubated 12h under 37^0C with 5% CO_2. After incubation, cells were treated (45μl/ well) and again incubated for 24h at 37^0C with 5% CO_2. All the experimental conditions and controls were triplicated. 10 μl PrestoBlue™ reagent (Molecular Probes, Invitrogen), resazurin-based solution was added to each well and incubated for 30min under dark conditions. Fluorescence detection was done at 560 nm and 590 nm wavelengths by using a microplate reader and calculated the % cell viability by using the following equation.

% cell viability = (Fluorescence treatment / Fluorescence Control) × 100

5. VEGF and HIF-1α Concentration levels

The concentration of VEGF protein in the medium of control and treated cells were measured by using commercially available sandwich enzyme-linked immunoassay

(ELISA) kit according to the manufacturers' instructions (Human VEGF ELISA kit, Thermo Scientific®, USA). HIF-1α protein levels in treated and control cells were measured by using an ELISA kit (Path Scan® Total HIF-1α sandwich ELISA Kit, Cell Signalling Technology®, USA). Briefly, cells were cultured in 24-well plates and treated with GNPs. After 24 h the media were collected, centrifuged for a short period and the supernatant was tested by immunoassay kit.

6. Cell migration Assay

The migration ability of colon cancer cells and normal epithelial cells with the GNP treatments were investigated by using Corning® Trnswell® chambers (Corning® Incorporated Life Sciences, USA) according to the manufacturer's procedure. The protocol used for this experiment mention below.

- Cells were trypsinized and seeded at a density of 5×10^4 cells in 0.2ml of serum free medium with different treatments on to the upper chambers,bottom wells were treated by medium with 5% FBS and incubated at 37^0C for 48h.

- Supernatents were removed out from both the chambers and cells were fixed and stained by 3.7% w/v formaldehyde and 0.1% crystal violet solution respectively. Non invaded cells were scraped off from the top of the trans well by using a cotton swab
- Invaded cells were counted in 5 random fields under the light microscope.

7. ROS generation

Cells were seeded in 96 black well plates at a density of 5×10^3 cells/well and incubated for 24h under 37^0C. Cells were treated with GNPs and H_2O_2. Again cells were incubated for 24h at 37^0C. Cells were washed with Phosphate Buffer Saline (PBS) twice and treated with 0.1μM of 2',7' – dichlofluoreceine-diacetate (H_2DCF-DA) (Molecular Probes™, USA) (100μL/well). Cells were re incubated for 30min. at 37^0C. Later H_2DCF-DA was removed and washed with PBS. Cells were treated with PBS (100μL/well). Fluorescence was measured under the 520nm using microplate reader (Varioskan Flash™, Thermo®, UK).

8. Cell Apoptosis Assay

500μl of cell suspension (50000cells/well) was transferred onto 24-well plate and incubated at 37^0C and 5% CO_2 for 12h. GNPs and controls were introduced to the wells (500μl/well) and incubate at 37^0C and 5% CO_2 for 24h. Cells were harvested and washed with PBS. Cells were centrifuged at 2000rpm and re suspended on 100μl of 1X Annexin buffer. 5μl of FITC Annexin V and 1μl of the 100μl/ml PI were added to each cell suspension (Invitrogen®, USA). Cells were incubated for 15min at room temperature and analyse the stained cells by flow cytometry (Becton Dickenson™, USA) under 530nm and 575nm.

9. Data Analysis

Statistical analysis was performed using the Graph Pad Prism® (version 5.04). P values below 0.05 will be considered as statistically significant.

CHAPTER 4

Results and discussion

Results

1. Characterization of gold nano particles.

During this research we used four types of gold nanoparticles. They were 5nm Sodium citrate based gold nano particles (5nm GNP-SC), 5nm Sodium borate base gold nano particles (5nm GNP-SB), 30nm Sodium citrate based gold nano particles (30nm GNP-SC) and 30nm Sodium borate based gold nano particles (30nm GNP-SB). Sizes of those GNPs were measured by UV – visible spectrophotometric methods, zeta potential and transmission electron microscopy (TEM). According to the UV – visible spectrophotometric data all the GNPs showed maximum peak between 500nm to 600nm wavelength range (Figure 1A.). Moreover, the stability of the GNPs were measured by zeta sizer and it showed that all the GNPs are negatively charged (Figure 1B.). TEM results showed that all the nano particles have spherical shape with approximately 5nm and 30nm in size (Figure 1C.).

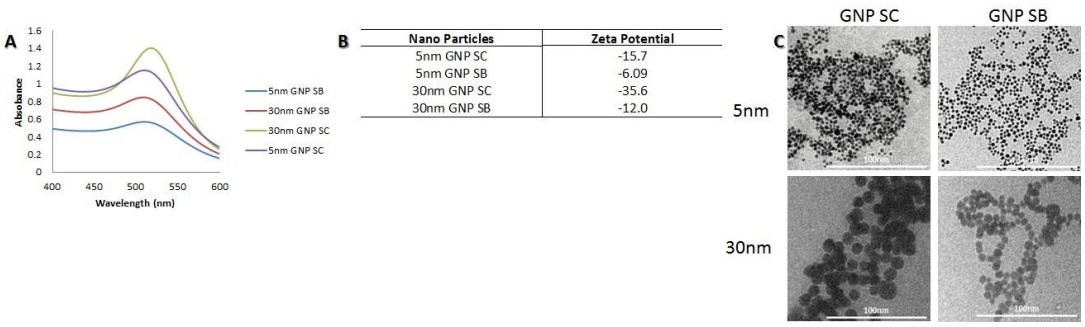

Figure 1 ***Characterization of GNPs. (A).*** UV - visible spectrum of GNPs. ***(B).*** Zeta potential of GNPs to measure the stability and surface charge***. (C).*** Transmission electron microscopic images of GNPs.

2. Viability of cells due to effect of the GNPs

To measure the effect of gold nano particles to the viability of the colon cancer cell lines we performed resazurine based cell viability assay (Figure 2). In this set of experiment we compared the effect of GNPs between normal colon epithelial cells and cancer cells (Figure 2). GNPs did not decrease the viability of CRL 1790 cells (Figure 2A). But it decreases the viability of both SW 620 and HCT 116 colon cancer cells (Figure 2B, 2C). Moreover, 50ppm GNP treated cancer cells showed decreased cell viability (figure 2B, 2C).

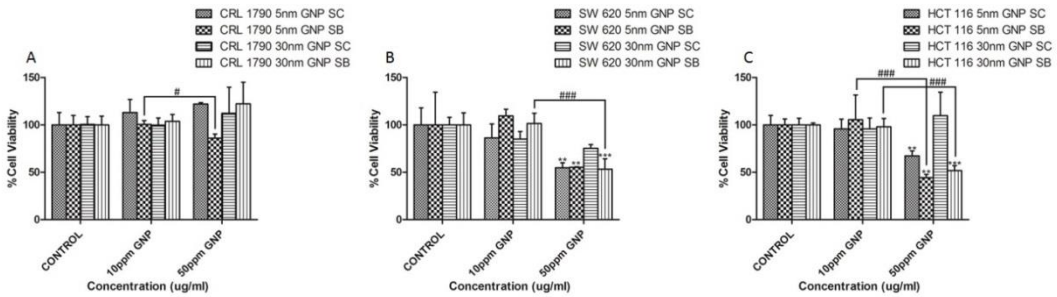

Figure 2 ***Cell viability after 24h incubation with all the four types of GNPs.*** ***(A).*** Comparison of percentage cell viability of CRL 1790 cells after treatment of GNPs***. (B).*** Comparison of percentage cell viability of SW 620 cells after treatment of GNPs. ***(C).*** Comparison of percentage cell viability of CRL 1790 cells after treatment of GNPs.

* - significant variations between control and treatments, # - Significant variation between low concentration treatment and high concentration treatment, + - significant variations within the same group. *P* – Values are depicted as follows: *, #, + *P*≤0.05, **, ##, ++ *P*≤0.01, ***, ###, +++*P*≤0.001, ****, ####, ++++*P*≤0.0001.

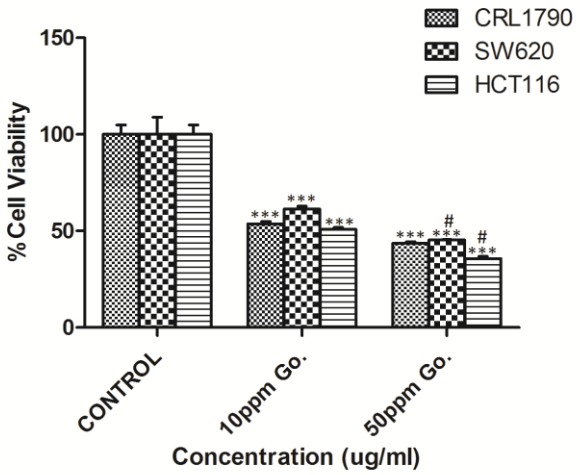

Figure 3***. Cell viability after 24h incubation with Go.***

* - significant variations between control and treatments, # - Significant variation between low concentration treatment and high concentration treatment, + - significant variations within the same group. *P* – Values are depicted as follows: *, #, + *P*≤0.05, **, ##, ++ *P*≤0.01, ***, ###, +++*P*≤0.001, ****, ####, ++++*P*≤0.0001.

Solution of Gold (Go) treated cells showed significantly decreased cell viability in all the three colon cell lines (Figure 3). 50ppm Go treated cancer cells showed significantly decreased cell viability compare to 10ppm Go treated cells. But, we did not observe any significant cell viability changes between 10ppm Go treated CRL1790 and 50ppm Go treated CRL1790 cells.

50ppm SB treated HCT116 cells showed significantly decrease cell viability compare to the 50ppm SC treated HCT116. But, SW620 cells did not show any significant cell viability changes due to SC or SB (Figure 4B). Both the 50ppm GNP-SB treated colon cancer cells were shown significantly decreased cell viability compare to the CRL1790 cells (Figure 4C, 4D). But, we did not find a significant difference in cell viability between 5nm and 30nm GNP-SB treated colon cancer cells (Figure 4E, 4F). Moreover GNP-SB treated colon cancer cells showed similar cell viability changes (Figure 4G, 4H). 10ppm GNP-SB treated cells did not show any significant cell viability changes when compare to the control.

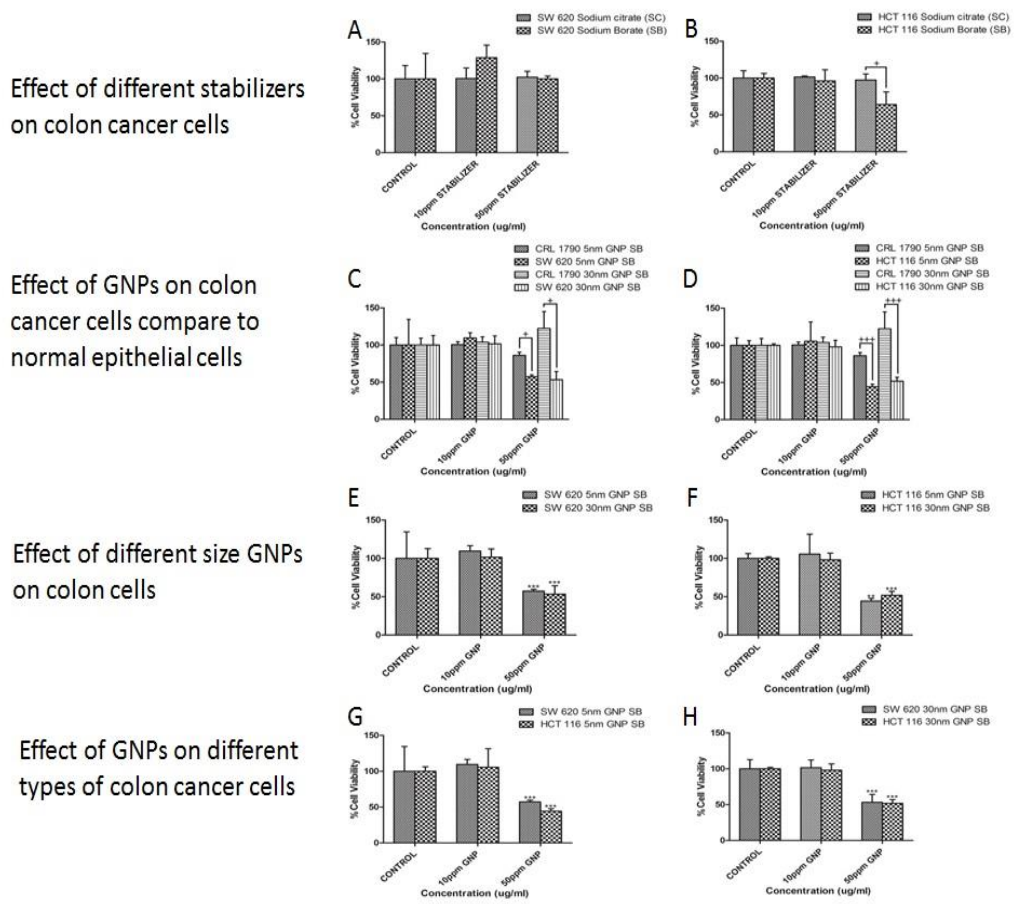

Figure 4*. Cell viability after 24h incubation with GNP-SB.*

(A). Percentage cell viability of SW 620 cells, which are treated with stabilizing agents. ***(B)***. Percentage cell viability of HCT 116 cells, which are treated with stabilizing agents. ***(C)***. Effect of GNP SB on SW 620 when compare to CRL 1790. ***(D)***. Effect of GNP SB on HCT 116 when compare to CRL 1790. ***(E)***. Percentage cell viability of SW 620 cells, which are treated with 5nm and 30nm SB based GNPs. ***(F).*** Percentage cell viability of HCT 116 cells, which are treated with 5nm and 30nm SB based GNPs. ***(G).*** Percentage cell viability of SW 620 and HCT 116 cells, which are treated with 5nm GNPs. ***(H).*** Percentage cell viability of SW 620 and HCT 116 cells, which are treated with 30 nm GNP .

* - significant variations between control and treatments, # - Significant variation between low concentration treatment and high concentration treatment, + - significant variations within the same group. *P* – Values are depicted as follows: *, #, + $P \leq 0.05$, **, ##, ++ $P \leq 0.01$, ***, ###, +++$P \leq 0.001$, ****, ####, ++++$P \leq 0.0001$.

3. Effect of GNPs on morphological changes of the cells

In addition to the cell viability experiment we observed the morphological changes of the cell lines after treatments of the GNPs. According to the data we found that both the SW 620 and HCT 116 cells were shown morphological changes after treating with 50ppm GNP-SB (Figure 5C, 5E, 5H, 5J). 50ppm GNP-SB treated cancer cells were shrunken and had lower number of cells compared to the control. But normal colon epithelial cells did not show any significant morphological changes after GNP treatments (Figure 5L, 5M, 5N, 5O).

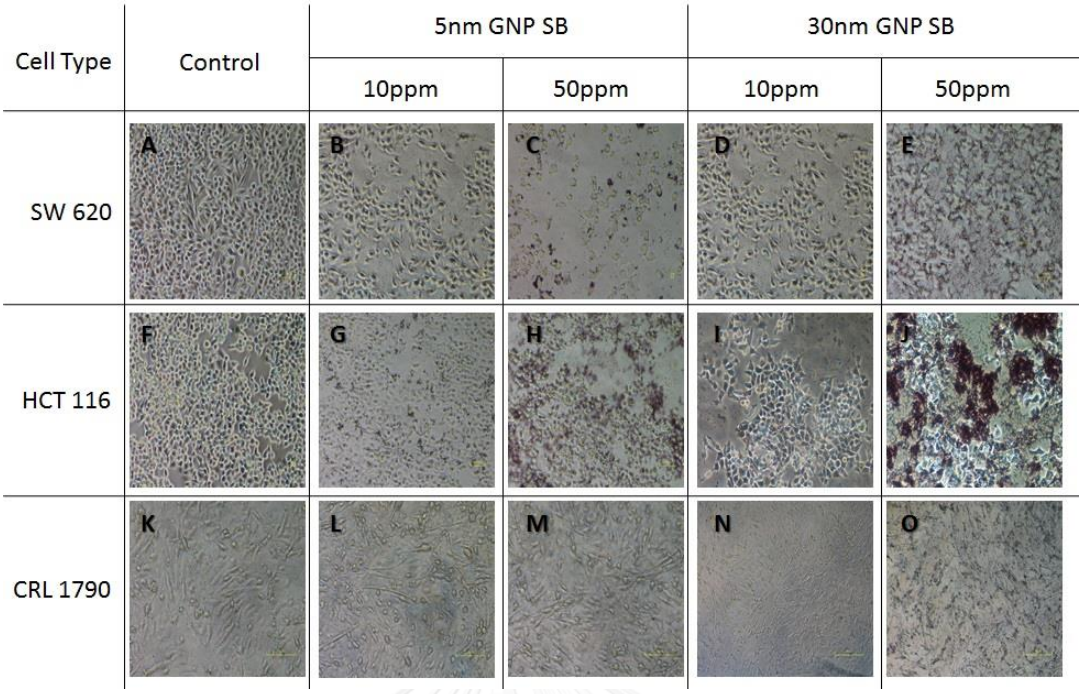

Figure 5*. Cell morphology after 24h incubation with GNPs.*

(A). Treated with FBS free DMEM. ***(B).*** Treated with 5nm 10PPM SB based GNP. ***(C).*** Treated with 5nm 50PPM SB based GNP. **(D)** Treated with 30nm 10PPM SB based GNP. **(E)** Treated with 30nm 50PPM SB based GNP. **(F)** Treated with FBS free RPMI. **(G)** Treated with 5nm 10PPM SB based GNP. **(H)** Treated with 5nm 50PPM SB based GNP. **(I)** Treated with 30nm 10PPM SB based GNP. **(J)** Treated with 30nm 50PPM SB based GNP. **(K** Treated with FBS free DMEM. **(L)** Treated with 5nm 10PPM SB based GNP. **(M)** Treated with 5nm 50PPM SB based GNP **(N)** Treated with 30nm 10PPM SB based GNP. **(O)** Treated with 30nm 50PPM SB based GNP.

* Magnification – 200X

4. Scratch wound assay and migration assay

To evaluate the ability of cell migration after GNP treatment we performed scratch wound assay and migration assay (Figure 6, 7). In the scratch wound assay both SW 620 and HCT 116 showed low wound healing rate when compare to the control. 50ppm GNP-SB treated cells showed lower rates of wound healing compare to the control (Figure 6F, 6J, 6H, 6L). In cell migration assay, we observed that lower migration rates of both the GNP treated cell lines when compared to the control (Figure 7). In HCT 116 cells, we observed that 50ppm GNP-SB treated cells showed lower percentage cell migration when compared to the control as well as Bevacizumab treated cells (Figure 7L).

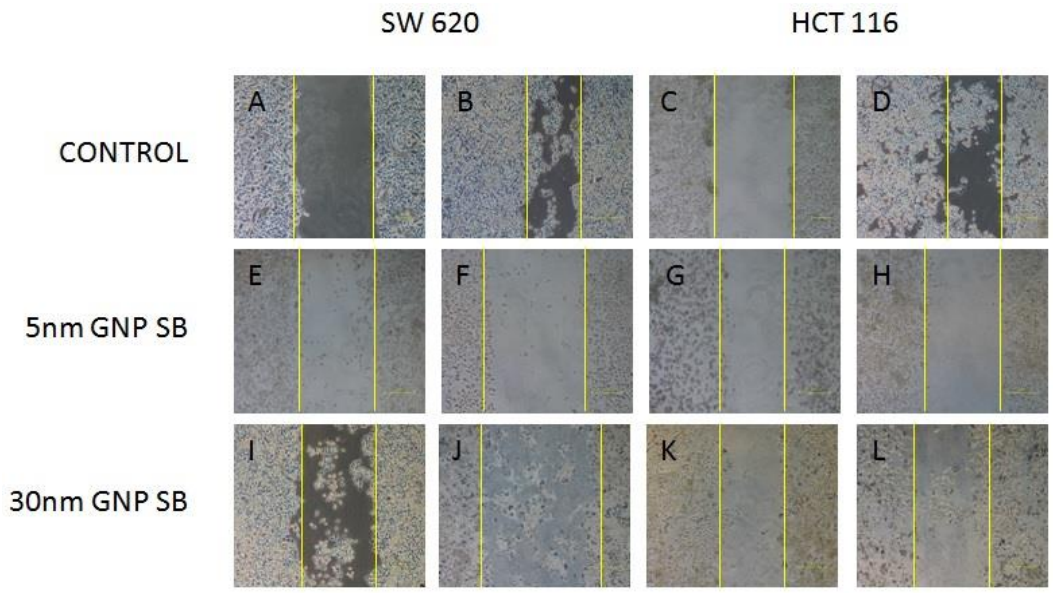

Figure 6*. Scratch wound assay after 24h incubation with GNPs.*

(A). 0h scratch on SW 620 cell line. ***(B).*** Treated with FBS free DMEM. ***(C).*** 0h scratch on HCT 116 cell line. ***(D).*** Treated with FBS free RPMI. **(E)** Treated with 5nm 10PPM SC based GNP. **(F**) Treated with 5nm 50PPM SC based GNP. **(G)** Treated with 5nm 10PPM SC based GNP. **(H)** Treated with 5nm 50PPM SC based GNP. **(I)** Treated with 5nm 10PPM SB based GNP. **(J)** Treated with 5nm 50PPM SB based GNP. **(K)** Treated with 5nm 10PPM SB based GNP. **(L)** Treated with 5nm 50PPM SB based GNP.

* Magnification – 200X

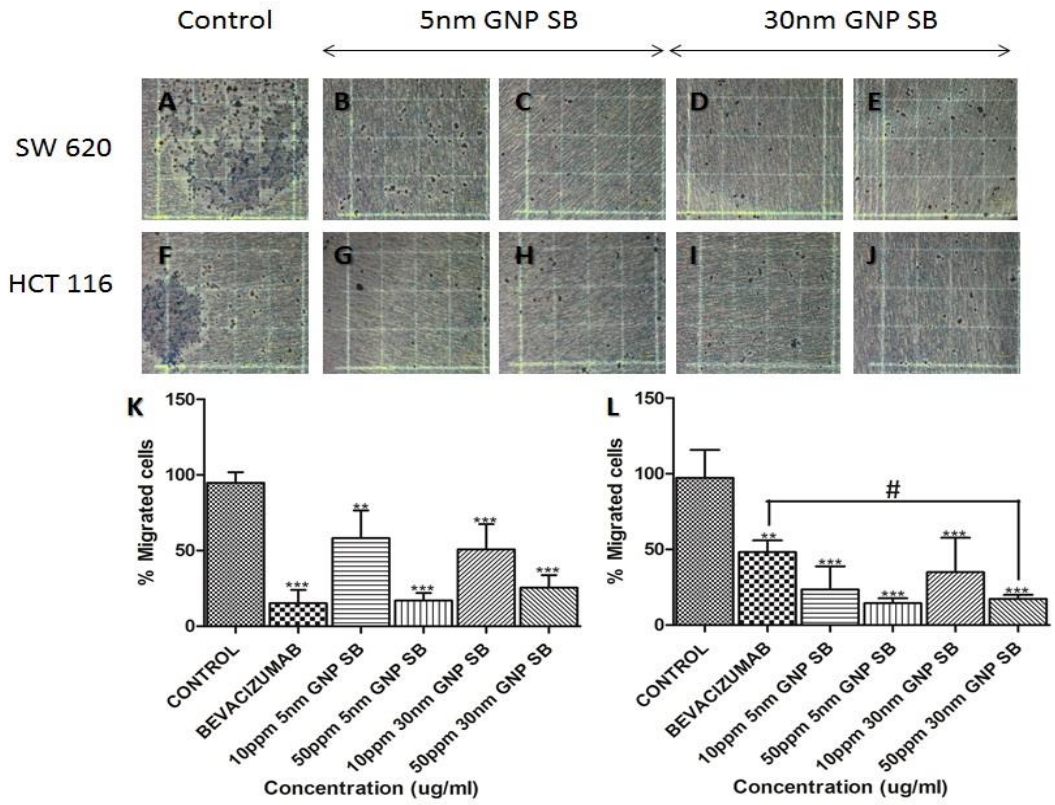

Figure 7*. **Cell migration after 24h incubation with GNPs.***

(A). Treated with FBS free DMEM. ***(B).*** Treated with 5nm 10PPM SB based GNP. **(C).** Treated with 5nm 50PPM SB based GNP. **(D).** Treated with 30nm 10PPM SB based GNP. **(E)** Treated with 30nm 50PPM SB based GNP. **(F**) Treated with FBS free RPMI. **(G)** Treated with 5nm 10PPM SB based GNP. **(H)** Treated with 5nm 50PPM SB based GNP. **(I**) Treated with 30nm 10PPM SB based GNP. **(J)** Treated with 30nm 50PPM SB based GNP. **(K)** Quantitation of the amount of migrated GNP SB treated SW620 cells. **(L**) Quantitation of the amount of migrated GNP SB treated HCT 116 cells.

* - significant variations between control and treatments, # - Significant variation between low concentration treatment and high concentration treatment, + - significant variations within the same group. *P* – Values are depicted as follows: *, #, + *P*≤0.05, **, ##, ++ *P*≤0.01, ***, ###, +++*P*≤0.001, ****, ####, ++++*P*≤0.0001. Magnification – 200X.

5. Cell apoptosis assay

In cell apoptosis assay, we observed that increased cell necrosis than apoptosis in both SW 620 and HCT 116 cell lines (Figure 8). Even though 5nm GNP-SB treated SW620 cells showed increased percentage apoptosis, yet increased percentage necrosis was significantly higher than percentage apoptosis levels (Figure 8A, 8B). Moreover, our GNP-SB treated HCT116 cells showed only significantly increased necrosis (Figure 8C, 8D).

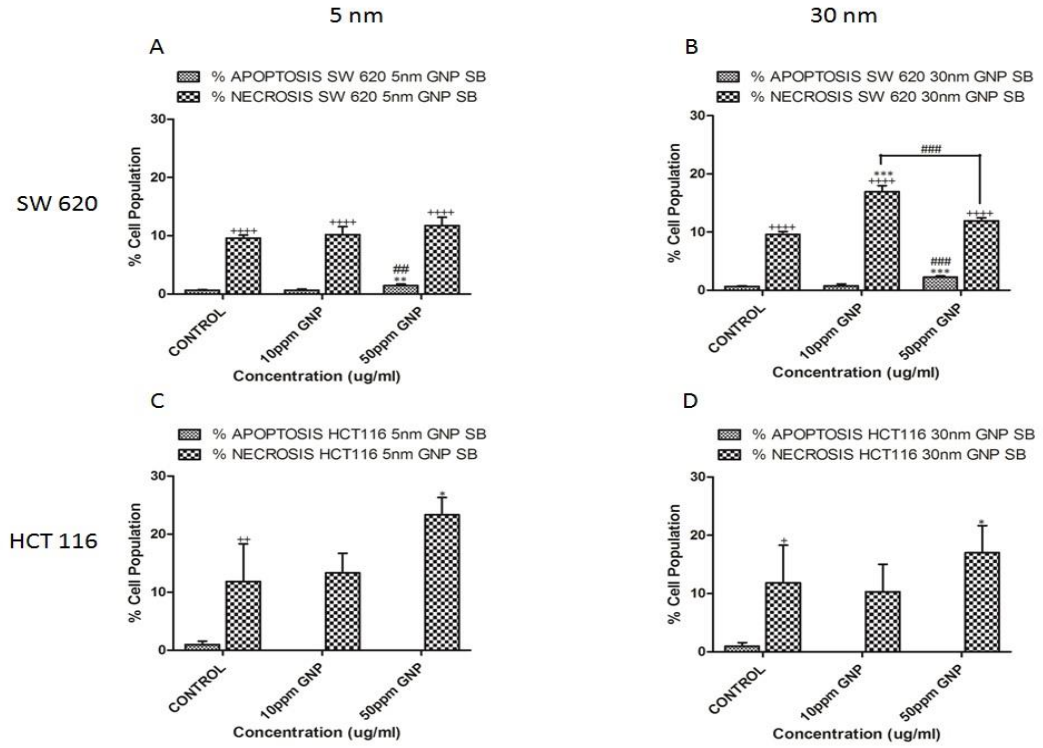

Figure 8*. Annexin V/PI after 24h incubation with GNPs.*

(A). SW 620 cells treated with 5nm GNP SB. ***(B).*** SW 620 cells treated with 30nm GNP SB. (**C).** HCT 116 cells treated with 5nm GNP SB. . **(D** HCT 116 cells treated with 30nm GNP SB.

* - significant variations between control and treatments, # - Significant variation between low concentration treatment and high concentration treatment, + - significant variations within the same group. *P* – Values are depicted as follows: *, #, + *P*≤0.05, **, ##, ++ *P*≤0.01, ***, ###, +++*P*≤0.001, ****, ####, ++++*P*≤0.0001. Magnification – 200X.

6. ROS generation

Using DCFH-DA techniques, the effect of GNPs on reactive oxygen species generation was evaluated. All the cells were incubated for 24h with GNPs. Even

though, 5nm 50ppm GNP treated CRL1790 cells showed increased ROS production, yet 30nm 50ppm GNP treated CRL1790 cells did not show significant ROS changes compare to the control (Figure 9A). 5nm 50ppm GNP-SB treated cancer cells show lower ROS production (Figure 9B, 9C). But, 30nm GNP SB treated cancer cells showed increased ROS production (Figure 9B, 9C). Moreover, 10ppm GNP treated cells did not show any significant ROS production compared to the control.

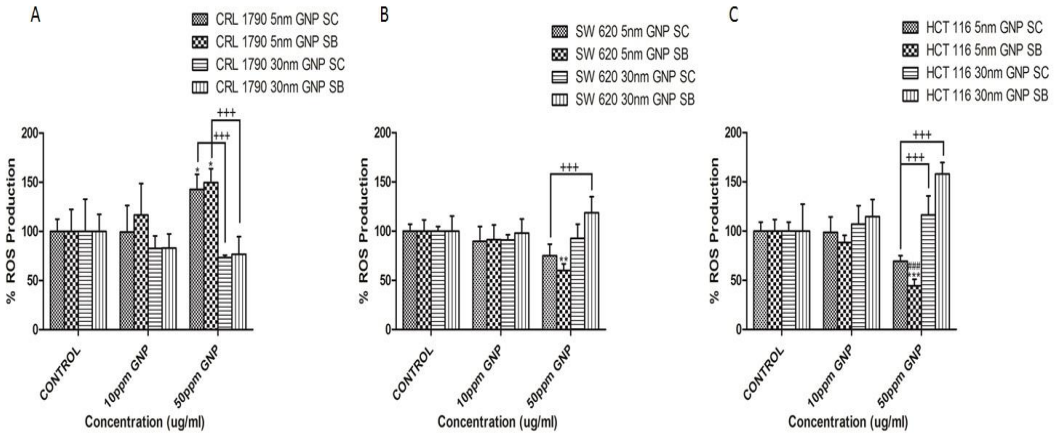

Figure 9*. **ROS production after 24h incubation with all the four types of GNPs.*** ***(A).*** Comparison of percentage ROS production of CRL 1790 cells after treatment of GNPs*.* ***(B).*** Comparison of percentage ROS production of SW 620 cells after treatment of GNPs. ***(C).*** Comparison of percentage ROS production of CRL 1790 cells after treatment of GNPs.

* - significant variations between control and treatments, # - Significant variation between low concentration treatment and high concentration treatment, + - significant variations within the same group. *P* – Values are depicted as follows: *, #, + $P \leq 0.05$, **, ##, ++ $P \leq 0.01$, ***, ###, +++$P \leq 0.001$, ****, ####, ++++$P \leq 0.0001$.

50ppm SB treated SW620 and HCT116 cells were shown significantly lower ROS production compared to the SC treated HCT116 cells (Figure 10A, 10B). 50ppm 5nm GNP-SB treated cancer cells were shown decreased ROS production compared to the CRL1790 cells (Figure 10C, 10D). Moreover 50ppm 30nm GNP-SB treated cells were showing increased ROS than 50ppm 5nm GNP-SB treated cells (Figure 10E, 10F). But 50ppm 30nm GNP-SB treated HCT116 cells showed a significant increase of ROS comparable to the 50ppm 30nm GNP-SB SW620 cells (Figure 10H)

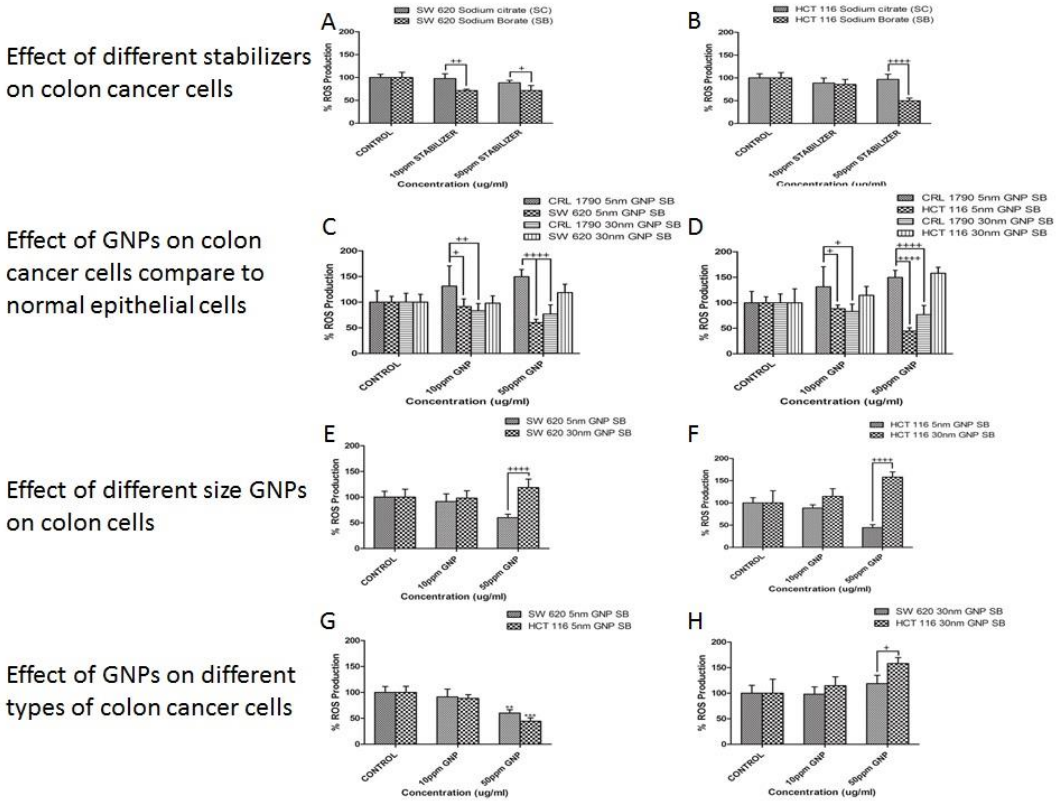

Figure 10*. **ROS production after 24h incubation with four types of GNP-SB.***

(A). Percentage ROS production of SW 620 cells which are treated with stabilizing agents. ***(B)***. Percentage ROS production of HCT 116 cells which are treated with stabilizing agents. ***(C)***. Effect of GNP SB on SW 620 when compare to CRL 1790. ***(D)***. Effect of GNP SB on HCT 116 when compare to CRL 1790. ***(E)***.Percentage ROS production of SW 620 cells which are treated with 5nm and 30nm SB based GNPs. ***(F).*** Percentage ROS production of HCT 116 cells which are treated with 5nm and 30nm SB based GNPs. ***(G).*** Percentage ROS production of SW 620 and HCT 116 cells, which is treated with 5nm GNPs. ***(H).*** Percentage ROS production of SW 620 and HCT 116 cells which are treated with 30 nm GNP .* - significant variations between control and treatments, # - Significant variation between low concentration treatment and high concentration treatment, + - significant variations with in the same group. *P* – Values are depicted as follows: *, #, + $P \leq 0.05$, **, ##, ++ $P \leq 0.01$, ***, ###, +++$P \leq 0.001$, ****, ####, ++++$P \leq 0.0001$.

7. Effect of GNPs on VEGF and HIF-1α expression levels

To evaluate the VEGF expression levels after treatment of GNPs we performed a sandwich ELISA experiment. Both the SW620 and HCT116 cells were showed lowest VEGF expression levels when they treated with Bevacizumab. (Figure 11A, 11B). Also, it showed significantly lower VEGF levels when cells were treated with 50ppm GNP-SB (Figure 11A, 11B). Even though GNP treated SW620 cells showed lower level of VEGF expression yet GNP were unable to effect on HIF-1α expression levels (Figure 11C). But both the Bevacizumab and GNP treated HCT116 cells showed significantly lower HIF-1α levels comparable to the control (Figure 11D)

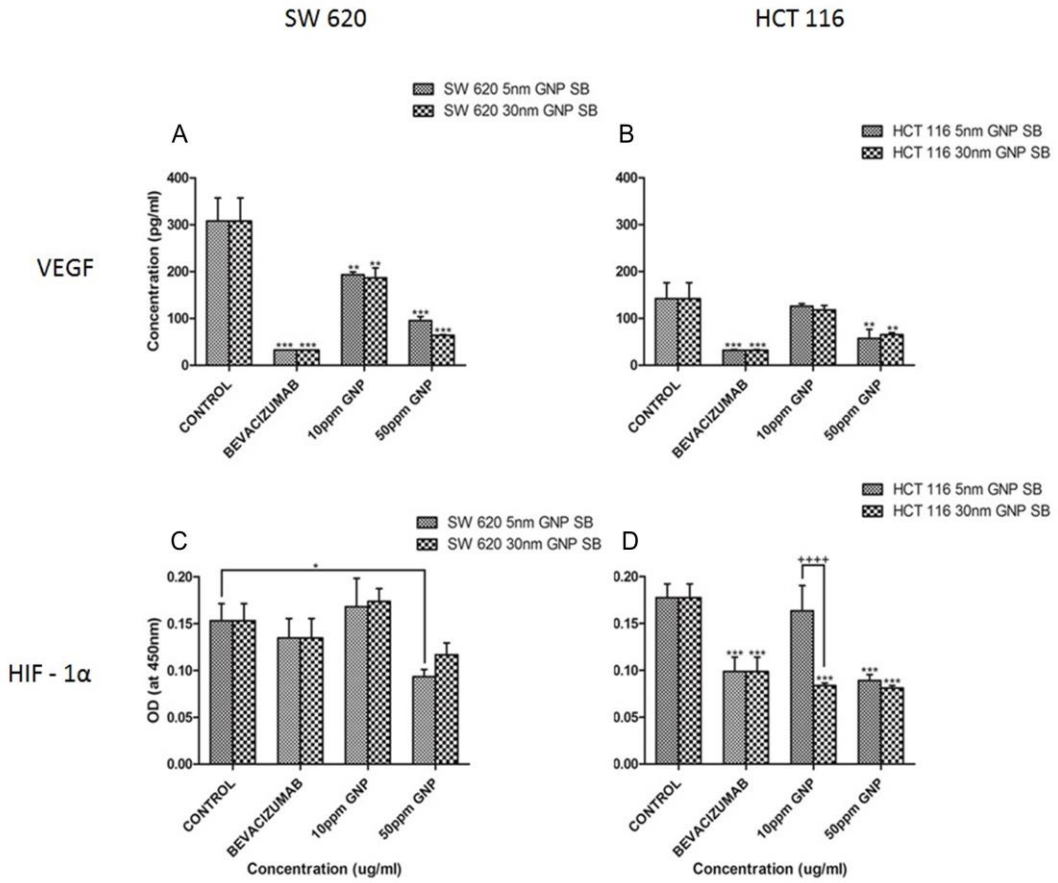

Figure 11. ***VEGF and HIF 1α expression levels after 24h incubation with GNPs.*** ***(A).*** VEGF of SW 620 cells, which are treated with SB GNPs. ***(B).*** VEGF of HCT 116 cells, which are treated with SB GNPs. ***(C).*** HIF 1α of SW 620 cells which are treated with SB GNPs. ***(D).*** HIF 1α of HCT 116 cells which are treated with SB GNPs.

* - significant variations between control and treatments, # - Significant variation between low concentration treatment and high concentration treatment, + - significant variations with in the same group. *P* – Values are depicted as follows: *, #, + *P*≤0.05, **, ##, ++ *P*≤0.01, ***, ###, +++*P*≤0.001, ****, ####, ++++*P*≤0.0001.

Discussion

Colorectal cancer is one of the deadliest cancer on the planet. Despite the fact that there are distinctive medications for the colon malignancy, yet it grows still to its metastasis level. In this study, our results showed the effect of GNPs based on their particle size, concentration and stabilizing agents. In Addition, we found ability of GNP-SB to decrease the cell viability than GNP-SC. The GNP-SB also decreased the cell migration, VEGF and HIF-1α expression levels. However, GNPs did not effect on CRL1790 cells.

In this study one of our main objectives was to determine the anti angiogenic effect of GNPs on colon cancer cells. As per our results GNPs treated colon cancer cells were showed low cell viability [Figure 4]. But, GNPs did not decrease the cell viability of CRL1790 cells. VEGF is abundantly vicinity in colon cancer cells than normal colon epithelial cells [6]. We have hypothesized that GNPs binds to VEGF and diminished the cell viability. VEGF has the ability to induce endothelial cell proliferation, promotes cell migration, and inhibits cell apoptosis [4,5,36]. VEGF is a

major regulator of vasculogenesis and angiogenesis during embryo development. Further, the role of VEGF in the etiology of diseases is characterized by deregulated angiogenesis [16]. GNPs can act as an anti angiogenic factor by binding with VEGF and cause structural changes [6,7]. Therefore, when GNPs binds to VEGF, it prompts the hypoxic conditions by diminishing angiogenesis and cause cell necrosis, which in turn leads to the decreased cell viability. Our cell morphology and ELISA studies bolstered this hypothesis as we observed a low number of cells, changes in cellular morphology and decreased [Figure 5]. Moreover, in our study have shown significant cell viability decrease in colon cancer cells when they were treated with GNP-SB compare to the GNP-SC [Figure 4]. When cancer cells were treated with SB and SC we have observed low cell viability in SB treated cells [Figure 4B]. In 2012 Sun et al showed the ability of borate compounds to trigger colon cancer cell apoptosis on store operated Ca^{2+} signaling pathway (SOCE) dependent manner [34,35]. However, Ca^{2+} plays an essential role in cell growth, muscle contractions and neurosecretion. But, uncontrolled Ca^{2+} influx leads to the cell apoptosis [37]. Therefore, ability of botare compounds to trigger cell apoptosis in cancer cells might be the reason for

the decreased cell viability in SB treated cells compare to the SC treated cancer cells. Our result was indicated HCT116 cells have lower cell viability in contrast with SW620 cells with same GNP treatment. SW620 cells were isolated from the lymph node metastasis of colorectal malignancy and HCT116 cells from the primary colon cancer . In addition, SW620 cells have a homozygous KRAS mutation in codon 12 and HCT116 cells possess a heterozygous KRAS mutation in codon 13. Which are the most common types of KRAS mutations among colon cancer patients [14]. Metastasis colon cancer cells show more resistance for the treatments by several mechanisms. Such as, activating intracrine pathways to protect antibody blockade and down regulation of target of the tumor micro environment [14]. Furthermore, metastasis cancer cells have the ability of translocation of VEGF to the nucleus during unfavorable conditions [14]. That might be the reason of decreased cell viability in HCT 116 cells compared to the SW 620 cells.

VEGF assumes a critical part in cell migration [6,7]. Our study showed decreased cell migration when colon cancer cells treated with GNPs [Figure 7]. Moreover, our

ROS results indicated that colon cancer cells were in stress conditions when they were treated with GNPs. During unfavorable conditions VEGF has prone to translocate to the nucleus [14]. There for, translocation of VEGF might led to decrease the cancer cell migration. Furthermore, we have hyothesysed that there may be a probability of GNPs to down regulate the SNAIL and CDH1 genes. Which are involved in *epithelial–mesenchymal transition* pathway (EMT).

Our ROS results showed increased ROS in 50ppm 5nm GNP treated CRL 1790 cells, yet decreased ROS in 50ppm 5nm GNP-SB treated colon cancer cells. As indicated by Amruta Manke et al ROS production in cells due to nanoparticles relies upon their particle size and surface properties [6]. On account of the high surface area to volume ratio and high concentration, 5nm GNP-SB was able to bind to more VEGF than 30nm GNP-SB. In this manner 50ppm 5nm GNP-SB treatment has decreased the viability in more colon cancer cells. Consequently, 50ppm 5nm GNP-SB treated colon cancer cells have decreased ROS compare to the 50ppm 30nm GNP-SB treated colon cancer cells [Figure 10E, 10F]. In previous study, smaller nano

particles impelled greater ROS production compare to larger particles [25]. In our study, we have incubated GNP treated colon cancer cells for 24hours. May be long period of incubation ROS can cause apoptosis in colon cancer cells.

Our Annexin V/PI experiment demonstrated increased necrosis over apoptosis in GNP-SB treated colon cancer cells [Figure 8]. When colon cancer patients were treated with Bevacizumab, cancer cells were led to the necrosis due to an anti angiogenic effect of the treatment. Moreover, GNPs have the ability to bind with heparin binding proteins like VEGF and bFGF. Hence, we have hypothesized GNP also cause necrosis in colon cancer cells.

ELISA results showed decreased VEGF in GNP treated colon cancer cells. GNP treated SW 620 cells did not show any significant vacillations in HIF-1α levels, but GNP-SB treated HCT116 cells indicated decreased VEGF and HIF-1a levels [Figure 11C, 11D]. Codon 12 KRAS mutated cancer cells have shown HIF-1α independent VEGF expression. In contrast, codon 13 KRAS mutated cancer cells have shown HIF-1α dependent VEGF expression [47,48]. Effect of two different mutations in KRAS might

be the reason for decreased HIF-1α expression in HCT116 cells when they were treated with GNP-SB [Figure 11D].

CHAPTER 5

Conclusion

In conclusion, we have reported the impact of GNPs on colon cancer cells as an anti VEGF factor. However, SB treated colon cells have decreased viability. But, GNP-SB treated CRL1790 cells did not decrease the cell viability. Also, we have found that the effect of the GNP-SB, showed an anti VEGF effect on colon cancer cells in a concentration dependent manner. Moreover, our study indicates the effect of the GNP-SB on both metastasis colon cancer and the primary tumor as an anti VEGF as well as anti HIF-1α treatments. Based on our findings GNP-SB can be a promising alternative treatment for colorectal malignancy in medicine.

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APPENDIX

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
GNPs	Gold nanoparticles
CO2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
BA	Boric acid
bFGF	basic Fibroblast Growth Factor
CF	Calcium fructoborate
ELISA	Enzyme-linked immunesorbant assay
FOLFIRI	irinotecan, fluorouracil, leucovorin
FOLFOX-4	oxaliplatin, fluorouracil, leucovorin
FITC	Fluorescein isothocyanate
Go.	Gold Soution
HIF-1α	Hypoxia inducible factor 1α
H_2DCF-DA	2', 7' –dichlofluorescein-diacetate

KRAS	Kirsten rat sarcoma viral oncogene homolog
PBS	Phosphate buffered saline
PI	Propidium iodide
PLGF	Placental growth factor
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute medium
SC	Sodium citrate
SB	Sodium borate
TEM	Transmission Electron Microscopy
VEGF	Vescular endothelial growth factor
VEGFR	Vescular endothelial growth factor receptor
HAuCl4.3H2O	Chloroauric solution

EQUIPMENT AND CHEMICALS

1. Autoclave (Hirayama, Japan)

2. Biohazard Laminar Flow (Gibco, USA)

3. CO2 incubator (Esco, Singapore)

4. Laboratory balance (Denver instrument, Germany)

5. Malvern Zetasizer Nano Series (Malvern Instrument, England)

6. Microcentrifuge (Hettich, Germany)

7. pH meter (Denver instrument, Germany)

8. Phase contrast inverted microscope (Nikon, Japan)

9. Shaker incubator (Heidolph, Germany)

10. Transmission Electron Microscope (Hitachi, Japan)

11. Varioskan Flash microplate reader (Thermo, England)

12. Vortex mixer (Scientific industries, USA)

13. StepOnePlus Real-Time PCR System (ABI Applied Biosystems)

14. Water bath (Memmert, Germany)

15. 24-well plate (Corning, USA)

16. 96-well plate (Corning, USA)

17. Cell culture flask (SPL, Korea)

18. Centrifuge tube 1.5 mL (Corning, USA)

19. Centrifuge tube 15 mL (Corning, USA)

20. Centrifuge tube 50 mL (Corning, USA)

21. Filter Tip (Corning, USA)

22. Dulbecco's Modified Eagle's Medium (Sigma, USA)

23. Fetal Bovine Serum (Gibco, USA)

24. Penicillin/Streptomycin (Gibco, USA)

25. Gold nanoparticles 5 nm (Sigma, USA)

26. H2DCFDA (Invitrogen, USA)

27. Roswell Park Memorial Institute Media (Sigma, USA)

28. PrestoBlue™ Cell viability Reagent (Invitrogen, USA)

CHEMICAL PREPARATIONS

1. Phosphate buffer saline

KCl 0.2 g

KH2PO4 0.2 g

NaCl 8.0 g

Na2HPO4 1.15 g

Mix all of chemical component and add DI water to 1,000 mL, then adjust pH to 7.4 with HCl

2. Dulbecco's Modified Eagle's Medium (DMEM)

1) Dissolve 13.4 g of DMEM with 900 mL DI water

2) Add 3.7 g of Na2HCO3

3) Adjust pH to 7.2 with HCl

4) Add DI water to 1,000 mL

5) Filtrate by 0.2 μM filter and keep as a stock medium

6) For working medium preparation, add 100 mL of heat Fetal Bovine Serum, 5 mL of antibiotic (Pen-Strep).

3. Roswell Park Memorial Institute Medium (RPMI)

1) Dissolve 13.4 g of RPMI with 900 mL DI water

2) Add 2 g of Na2HCO3

3) Adjust pH to 7.2 with HCl

4) Add DI water to 1,000 mL

5) Filtrate by 0.2 μM filter and keep as a stock medium

6) For working medium preparation, add 100 mL of heat Fetal Bovine Serum, 10 mL of antibiotic (Pen-Strep).

Cell Viability Assay Protocol (PrestoBlueTM, Invitrogen, USA, Catalog number A13261)

1. Cell culture

Materials

1. 96-well plate

2. Cell Culture Media

3. Micropipetters

4. CO2 incubator

Method

1. Cells are seed in 96-well plates at a density of 1-10 X 103 cells/well in 45 μL.

2. Incubate at 37°C and 5% CO2 for 12 h.

2. Cell viability assay

Materials

1. Unknown sample for toxicity test

2. DMSO (positive control)

Method

1. Add 45 μL of culture medium for negative control.

2. Add 45 μL of DMSO for positive control.

3. Treat with 45 μL of unknown samples.

4. Incubate at 37°C and 5% CO2 for 24 or 48 h.

5. Add 10 μL PrestoBlue™ reagents and incubate for 30 min.

6. Measure fluorescent product by using a microplate reader at 560 and 590 nm.

Reference

-Product information sheet: PrestoBlue™ Cell Viability Reagent Protocol from Invitrogen™

Reactive Oxygen Species (ROS) Generation Protocol (H2DCFDA, Invitrogen, USA, Catalog number D399)

1. Cell culture

Materials

1. 96-black well plate

2. Cell Culture Media

3. Micropipetters

4. CO2 incubator

Method

1. Cells are seed in 96-black well plate at a density of 1-10 X 103 cells/ well in 100 μL.

2. Incubate at 37°C and 5% CO2 for 12 h.

2. DCFH-DA assay

Materials

1. Unknown sample for ROS generation test

2. H2O2 (positive control)

3. Vitamin C (negative control)

4. Phosphate Buffer Saline (PBS)

Method

1. Wash the cells 2 times with PBS

2. Add 100 μL of 0.1M H2DCFDA

3. Incubate at 37°C and 5% CO2 for 30 min.

4. Wash 2 times with PBS

5. Add 100 μL of culture medium for control.

6. Add 100 μL of 0.5% H2O2 for positive control.

7. Add 100 μL of 5 mg/mL vitamin C for negative control.

8. Treat with 100 μL of unknown sampless.

9. Measure fluorescence excitation and emission at 485 and 528 respectively by using microplate reader.

Reference

- Product Information Sheet : H2DCFDA from InvitrogenTM

Cell Apoptosis Assay Protocol (FITC Annexin V, Invitrogen, USA, Catalog number V13242)

1. Cell culture

Materials

1. 24-well plate

2. Cell Culture Media

3. Micropipetters

4. CO2 incubator

Method

1. Cells are seed in 24-well plates at a density of 5 X 10^5 cells/well in 500 μL.

2. Incubate at 37°C and 5% CO2 for 12 h.

2. Annexin V and PI Assay

Materials

1. Unknown sample for toxicity test

2. 15ml tube

3. Tube for flow cytometer

Method

1. Add 500 μL of culture medium for negative control.

2. Treat with 500μL of unknown sample.

3. Incubate at 37°C and 5% CO2 for 24h.

4. Harvest the cells and wash in cold PBS.

5. Centrifuge at 2200 rpm, discard the supernatant, and re-suspend the cells with 100μL of 1X annexin buffer.

6. Add 5μL of FITC annexin V and 1μL of the 100μg/ml PI to each cell suspension.

7. Incubate the cells at room temperature for 15 min.

8. Analyze the stained cells by flow cytometry, measuring the fluorescence emission at 530nm and 575nm.

Chemical Preperations

1. Dilute 1X annexin buffer from 5X annexin buffer.

2. Prepare a 100μg/ml working solution of PI by diluting 5μL of 1mg/ml PI stock in 45μL 1X annexin buffer

Reference

-Product information sheet: FITC Annexin V/ Dead Cell Apoptosis Kit with FITC annexin V and PI, for Flow Cytometry from Invitrogen™

ELISA Protocol VEGF-A (Thermo, Cat. EH2VEGF)

Date...../...../.....

Step	Reagent/dilution	Condition
1. Add diluted standards and sample **Stock conc. 4000 pg/ml** Top standard 1000 pg/ml = 125 µl + 375 µl diluent Standard 1000, 500, 250, 125, 62.5, 31.25 pg/ml = two-fold serial dilutions of 250 µl + 250 µl	50 µl/well	Room temp (20-25°C) for 2 hrs
2. Wash: 1X Washing Buffer **(stock = 30X)**	300 µl/well	3 times
3. Add biotinylated detection Ab	100 µl/well	Room temp (20- 25°C) for 1 hr
4. Wash: 1X Washing Buffer **(stock = 30X)**	300 µl/well	3 times
5. Add Streptavidin-HRP **Dilute 1:400** (0.25 µl/well)	..... µl + HRP diluent ..... µl 100 µl/well	Room temp (20-25°C) for 30 min
6. Wash: 1X Washing Buffer **(stock = 30X)**	300 µl/well	3 times
7. TMB substrate	100 µl/well	Room temp (20- 25°C) for 30 min **in the dark**
8. Stop reaction: Stop Sol_	100 µl/well	
9. Read at OD 450 nm within 30 min		

Reference

-Product information sheet: Human VEGFA ELISA Kit, Thermo Scientific™.

ELISA Protocol HIF-1α (Cell Signaling Technology®, Cat. #13127C)

Date...../...../.....

Step	Reagent/dilution	Condition
1. Add diluted sample **Stock conc. 4000 pg/ml**	50 µl/well	Room temp (20-25°C) for 2 hrs
2. Wash: 1X Washing Buffer **(stock = 20X)**	300 µl/well	3 times
3. Add biotinylated detection Ab	100 µl/well	Room temp (20-25°C) for 1 hr
4. Wash: 1X Washing Buffer **(stock = 20X)**	300 µl/well	3 times
5. Add Streptavidin-HRP **Dilute 1:400** (0.25 µl/well)	..... µl + HRP diluent..... µl 100 µl/well	Room temp (20-25°C) for 30 min
6. Wash: 1X Washing Buffer **(Stock = 20X)**	300 µl/well	3 times
7. TMB substrate	100 µl/well	Room temp (20-25°C) for 30 min **in the dark**
8. Stop reaction: Stop Sol_	100 µl/well	
9. Read at OD 450 nm within 30 min		

Reference

-Product information sheet: PathScan® Sandwich ELISA Protocol, Cell Signalling Technology®, Inc

VITA

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