Development of Sudan III based lipid staining method and pharmacological evaluation of lipid droplets role in cancer cells adaptation to serum starvation

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اسم البحث باللغة العربية

تطوير مادة سودان 3 لصب قطرات الدهن الخليوية وتقييم القيمة الدوائية لهذه العناصر في تكيف خلايا السرطان مع نقص التغذية بالمصل

ملخص الدراسة:

سودان 3 هو كاشف أحمأ اللون لديه القدرة على الارتباط بالدهون وصيغها، وبالتالي يستخدم في تحديد مستوى الدهون في النباتات والغذاء والبراز. قمنا في دراستنا هذه بتطوير طريقة لاستخدام سودان 3 في صب قطرات الدهون في خلايا سرطان القولون بطريقة انخفاضية. كذلك قمنا -باستخدام هذه الطريقة المتطورة- بدراسة دور قطرات الدهون الخليوية في قدرة خلايا السرطان على التكيف عند نقص التغذية بالمصل. تتيح فسافيليا أي 2 باستخدام العلاج بتامينازون أدى إلى انخفاض ملموس على مقدار تشكل قطرات الدهون وكذلك تسبب في تثبيط نمو الخلايا منزوعة المصل. تعد هذه
النتيجة تأكيداً إضافياً على أهمية قطرات الدهون و إنزيم فسفوليباز أي 2 في التكيف الخلوي للخلايا السرطانية على نقص التغذية.
**English Abstract:**

Sudan III is a red color reagent that has the ability to bind lipids and thus is used to determine the fat (lipid) level in plants, food and feces. In our study we develop a method to use Sudan III as a selective stain for lipid droplets in colorectal cancer cells. Using the optimized Sudan III method we have also investigated the role of lipid droplets in cancer cell adaptation to serum starvation. Inhibiting phospholipase A2 by betamethasone has decreased the formation of lipid droplets as well as caused growth inhibition in serum-starved cell and not nutrient supplemented cells. This further emphasis on the importance of lipid droplets and phospholipase A2 enzyme in cellular adaptation of cancer cells to starvation.
1. Introduction:
Cancer refer to a group of diseases where one or more of the body’s cells begin to grow and become reproductive in an uncontrolled pattern. These cells can then invade and destroy surrounding healthy cells, to develop a whole tissue and eventually to become at organ level.

Genetics and other external factor such as the way and quality of what we eat, physical inactivity, and chemical exposure around us pose a serious challenge in cancer development and treatment. Treatment for cancer depends on many factors, such as early detection, the stage of cancer, location, and previously mentioned factors. Cancer treatment usually include one or a combination of chemotherapy, surgery, and/or radiotherapy.

Over the past century, cancer treatment developed in tremendous way starting from radiotherapy on localized tumor and then combination therapy which considered the greatest achievement in field on oncology. For instance, Cisplatin is a chemotherapy used as mono-therapy posing a breakthrough results in treating testicular cancer in which considered the first solid tumor treated with chemotherapy. Surgery also play a major role in treatment of breast cancer. Not to mention hormonal therapy in which how androgen deprivation therapy helps in slowing the progression of prostate cancer. Screening tests in field of oncology has a beneficial effect in detecting tumor modalities and these will help us to treat cancer. Pap smear as test to detect for pre-cancer in which considered as first screening test and then the development of other screening test such mammography and colon cancer screening test.
Oncology is a field of observation and innovation as consideration of each part of cancer cell and its role in developing cancer treatment. One of these parts is lipid droplets as a new role player in cancer [1]. There is an evidence that cancer cells show specific changing in different aspects of lipid metabolism. These changes can affect the existence of structural lipids for the synthesis of membranes, the synthesis and degradation of lipids that contribute to energy homeostasis and the abundance of lipids with signaling functions. Changes in lipid metabolism can affect multitude of cellular processes, including cell growth, proliferation, differentiation and motility [2]. Thus understanding the role of lipid droplets in cancer biology may open a window of opportunity to find new targets that can be used to target cancer specifically. Therefore the aim of this project is to develop a method to detect the existence of lipid droplets in colon cancer cells and to explore some of the factors that may influence the formation of these droplets.
2. Literature review

New cancer incident cases reported to the Saudi Cancer Registry (SCR) in year 2012 was 14,846. Overall cancer was more among women than men as reports shows 7,048 (47.5%) cancer cases among males compared to 7,798 (52.5%) cases for females. Total of 11,333 cases were reported among Saudis, and 3,393 among Non-Saudis and 120 of Unknown Nationality [3]. Colorectal cancer considered the second most common cancer in Saudi Arabia (11.1%) and as the most common cancer type among males in Saudi Arabia (13.3% male, 9.3% female) [3].

Different options found to treat cancer ranging from surgical removal of the cancer lesion, radiotherapy, chemotherapy, hormone therapy, biological therapies, bisphosphonate, to bone marrow and stem cell transplant. With the regards to Colorectal cancer treatment below is a brief description of current treatment options:

2.1. Current treatment options of cancer:

A) Surgery

Surgery is the main option for most colorectal cases. For instance, localized polyp lesions of colorectal cancers can be removed completely during a colonoscopy. Larger cancer lesion can be resected by Endoscopic mucosal resection. Laparoscopic surgery which is a new technique in the field of cancer surgery used to remove Polyps that not removed during colonoscopy in form of small incision rather than larger one making patient recover faster with notable reduction in pain after operation. Another technique is robotic
surgery where a surgeon sits at a control panel and operates robotic arms to perform the surgery.

In case of invasive colon cancer, numerous of surgery option will used including Partial colectomy in which the surgeon removes the part of colon affected with cancer, beside a part of normal tissue on either side of the cancer and then connecting the healthy part of colon again with rectum. If not feasible to connect, then it requires to have permanent or temporary colostomy in addition to create a way inside abdomen to take bowel waste into a special bag. In case of advanced cancer, surgery role here is to reduce signs and symptoms such as rectal bleeding and pain but is not viable for cure.

B) Radiotherapy

By using powerful energy sources, such as X-rays, to kill cancer cells that might remain after surgery, or to shrink large tumors before an operation so that they can be removed easily, or to reduce symptoms of colon and rectal cancer.

C) Chemotherapy

Chemotherapy is mainly aims to eliminate micro-metastatic lesion in order to reduce the risk of tumor recurrence and/or spreading. Drugs such as 5 fluorouracil (5-FU), leucovorin and capecitabine are usually used after surgery to prevent recurrence and if lymph node included in term of cancer spreading or before operation in order reduce the size of it. Other beneficial effect of chemotherapy is reducing symptoms that may spread to other sites of the body. Combination of chemotherapy and radiotherapy before and after
surgery considered as typical regimen for colorectal cancer patient. Currently, targeted therapeutic agents are introduced to treat advanced colorectal cancers. Targeted therapies are aimed to selectively kill the cancer cells in a way to leave healthy cells untouched. The development of such selective therapies require a better understanding of the differences between the biology of healthy and cancer. Recently a growing research interest is directed to explore the energy resources and metabolic events in cancer cells. For example,

2.2. Lipid metabolism and cancer:

Whilst carbohydrates are known to be the main sources of energy in cancer cells, lipids can be considered as a reserved recourse of energy for these cells [4]. Lipids in cells can be stored in the cellular membrane or inside the cytoplasm as lipid droplets. Lipid droplets are major cellular organelles for the storage of neutral lipids. Excessive lipid storage in Lipid droplets is linked to the pathogenesis of common metabolic diseases, such as diabetes, atherosclerosis and obesity. Lipid droplets have been recognized as multifunctional organelles in different cell's modality and organism. The main function of Lipid droplets in general is unknown but their activity and existence is increased in many malignant tumors including colon cancer. Colon cancer cells seems to contain high numbers of Lipid droplets compared with healthy colon. This was observed in colon adenocarcinomas polyps that formed inside the colon. The accumulation of lipid droplets believed to be associated with the development of colorectal cancer [1]. Likewise, the accumulation of lipid droplets as a result of increased lipogenesis is
considered a sign for poor prognosis in breast, prostate cancers. Lipid droplets in colon polyps may serve as a reservoir of energy to cell cluster to maintain them survived under tumor microenvironment such as hypoxia [5]. Alteration that occur in cancer cell to the enzymes that modulate lipid metabolism as well as metabolic regulator considered a potential target for anticancer. Regulation of lipid droplets still unclear, however, by looking to their content of protein adipose differentiation-related protein (ADRP) and caveolin, lipid metabolic enzymes and proteins of Rab family, eicosanoid-forming enzymes, and protein kinases, such as phosphoinositide 3-kinase (PI3K), mitogen activated protein kinase, and protein kinase C, it is believed that lipid droplets are functionally important for cancer cells [6]. Lipid droplets also have major roles in regulating arachidonic acid metabolism. Arachidonic acid has signaling functions and considered as precursor of prostaglandins and leukotrienes in which are also stored in its esterified form at lipid droplets. Arachidonic acid metabolism plays an important role in colon carcinogenesis. Unesterified arachidonic acids a signal for apoptosis of colon cancer cells, and thus, esterification of arachidonic acid into lipid pools depletes may lead to diminish the proapoptotic signal and promote cancer development [6]. Around 80% to 90% of colon cancer show an enhanced cyclooxygenase-2 (COX-2) expression compared with normal intestinal mucosa. COX-2 is the enzyme that convert arachidonic acid into prostaglandins. High levels of prostaglandins, particularly prostaglandin E2 (PGE2), were observed in colorectal cancers [6].
3. Materials and Methods:

3.1. Materials:

DLD1 colorectal cancer cells were a kind gift from Dr. Ahmad Al-Jada'a (King Saud bin Abdulaziz University for Health Sciences, Riyadh, KSA). Trypsin/EDTA solution, RPMI 1640 medium and its' components were obtained from invitrogen, USA. All other chemicals were obtained from Sigma-Aldrich (UK) unless stated otherwise. All plastics were obtained from Corning (USA).

3.2. Maintenance and sub-culturing of cell line:

DLD-1 cells were kept frozen at -80°C for long storage. When required cells were briefly warmed to 37°C before transfer to pre-warmed 10 ml RPMI 1640 medium consisted of 1mM Sodium pyruvate, 2 mM L-glutamine and 10% fetal Bovine Serum and centrifuged for 5 min at 1700 RPM (250 g). Carefully only the medium was disposed leaving cell palette unbroken. Then, cells were re-suspended in 15 ml complete medium. DLD-1 cells were then placed into 75ml flask left to adhere
overnight in humidified incubator at 37°C. During the project DLD1 cells were maintained routinely as monolayer in complete RPMI 1640 medium in 5% CO₂ humidified incubator at 37 °C. Cells were left to grow in sterile T75 cell culture flasks until reached approximately 80% confluence. When required, supernatant was removed and monolayer cells were washed twice in Hanks’ Balanced Salt Solution (HBSS) before adding 5 ml of trypsin/EDTA solution for 10 minutes. After all cells are detached from surface forming a single cell suspension, 10 ml of medium was added to neutralize the trypsin. Cell suspension was then centrifuged at 250 g for 5 minutes. Resultant cell pallet was re-suspended in 10 ml complete RPMI medium and counted under inverted microscope using heamocytometer.

3.3. Determination of viable cell number in a cell suspension:

Trypan Blue is a stain used in dye exclusion procedures for viable cell counting. This method is based on the principle that Trypan Blue does not enter viable cells (remain clear), whereas it enters and stain the dead cells (stained blue). Briefly, 100 µl of cell suspension was transferred into a microcentrifuge tube, followed by 100 µl of 0.4% Trypan Blue solution (w/v), and mixed well. After that, 10 µl of the suspension mixture was transferred to each chamber of a haemocytometer. Viable (non-stained) and non-viable cells (stained blue) in each 1 mm square were counted and at least of 10 counts were performed. The number of cells per ml of solution was then determined using the following calculations:

\[
\text{Cells per ml} = \text{average no. cells} \times \text{dilution factor} \times 10^4
\]
Total cells = cells per ml x original cell suspension volume
% cell viability = (number viable cells / number of total cells) X 100%

3.4. Trypan Blue Staining of adherent cells:

DLD1 cells were detached by trypsin and seeded on clean cover slides at concentration of 2 x 10^5 cells/ml and left to adhere overnight. On top of each coverslip 0.5 ml of 0.2% of trypan blue (w/v) solution was added for 2 minutes then fixed for 10 minutes with 4% paraformaldehyde (PFA, pH 6.9) inside fume hood. Each cover slip was washed three times with phosphate buffered saline (PBS) in order to remove the remaining PFA. Eventually, coverslips were mounted onto glass slides with glycerol and visualized under inverted microscope.

3.5. Sudan III Staining of lipid droplets in adherent cells:

The Sudan III staining method was developed in house based on information collected from literature and experts opinions. The method development is described later in results and discussion section. Here is the description of the optimized method; the cells on cover slips were fixed with ice-cold methanol for 10 min at -20 °C. After that, cover slips were washed twice with PBS and dipped 5 times in Hematoxyline solution. Fixed cells were then washed 5 times in PBS before the cells were stained with Sudan III 0.3% in 70% ethanol for 30 min. After the time course, cells were washed with 50% ethanol 3 times to remove excess stain. Finally, cells were mounted on slides using glycerol and visualized under microscope.

3.6. Induction of Autophagy by serum starvation:
The method of autophagy induction by serum starvation was adapted from that published by Sanchez et al. [7]. Briefly, DLD-1 cells were seeded as $10^5$ cells/cover slip and incubated for 3 hours. After that, cells were gently washed twice with HBSS and the complete medium was replaced with serum-free RPMI 1640 medium (containing no Sodium pyruvate nor L-glutamine) and incubated humidified incubator at $37°C$ overnight to become autophagic. Cells were then stained with either trypan blue or Sudan III as described above.

### 3.7. Evaluation of phospholipase A2 inhibitors on the formation of lipid droplets in DLD1 cells:

DLD-1 cells were seeded as $10^5$ cells/cover slip and incubated for 3 hours. After that, the complete medium was replaced with complete RPMI 1640 medium containing beclomethasone or hydrocortisone (150 µg/ml or 300 µg/ml) respectively. The concentrations of drugs were selected based on therapeutic serum levels in human. And incubated in humidified incubator at $37°C$ overnight to become autophagy. Cells were then stained with trypan blue as described above before the existence of necrotic cell death was investigated under light microscope.

### 3.8. Evaluation of phospholipase A2 inhibitors on the formation of lipid droplets in serum-starved DLD1 cells:
DLD-1 cells were seeded as $10^5$ cells/cover slip and incubated for 3 hours. After that, the complete medium was replaced with serum-free RPMI 1640 medium containing beclomethasone or hydrocortisone (150 µg/ml or 300 µg/ml) respectively. The concentrations of drugs were selected based on therapeutic serum levels in human. And incubated humidified incubator at 37°C overnight to become autophagy. Cells were then stained with Sudan III as described above before the existence of lipid droplets was investigated under light microscope.

3.9. The MTT assay:

MTT assay to be performed as described in literature [8] 20 µl of MTT solution (5 mg/ml) to be added into each well and incubated in the dark at 37°C for 4 hours. The supernatant was removed and formazan crystals were dissolved in DMSO (150 µl). The absorbance was read immediately at 540 nm in multiwell plate spectrophotometer (BioTek, USA). The average absorbance in the control wells was taken as 100% survival, and the IC$_{50}$ values were defined as the drug concentrations that inhibited the cell growth by 50% after 96 h drug exposure.

3.10. Evaluation of phospholipase A2 inhibitors on cell survival:

DLD-1 cells were seeded as $3 \times 10^3$ cells/well 96 well-plates and left to adhere overnight in humidified incubator at 37°C. Medium was then replaced with fresh one containing a suitable concentration range of beclomethasone or hydrocortisone. Cells were then incubated in humidified incubator at 37°C for 96 hours before the MTT assay was performed as described above.
3.11. Evaluation of phospholipase A2 inhibitors on serum-starved cell survival:

DLD-1 cells were seeded as $3 \times 10^3$ cells/well 96 well-plates and left to adhere for 3 hours in humidified incubator at 37°C. Cells were then washed very gently with HBSS and 200 µl of serum-free medium was dispensed to each well. Cells were then overnight in humidified incubator at 37°C. Next day, Medium was replaced with fresh one (serum-free) containing a suitable concentration range of beclomethasone or hydrocortisone. Each plate included the groups of Blank (wells with no cells), control A (non-starved non-treated cells), control B (starved non-treated cells) and test group (starved treated cells). Cells were then incubated in humidified incubator at 37°C for 96 hours before the MTT assay was performed as described above.

4. Results and Discussion:

Although the role of lipid droplets has been investigated by many research groups, its function in cancer remain unclear [1, 2, 4, 9, 10]. Understanding more about lipid droplets importance in cancer biology may provide a window of opportunity to design drugs that target cancer selectively. Lipid droplets are known to accumulate in many types of healthy and cancer cells. However, its accumulation in cancer cells such as in colon cancer is excessive which indicates important role in such cells. Due to the rapid proliferation of tumor cells, many of cancers cells grow away from blood stream. This results in limited tissue perfusion and imbalance between the supply and consumption of oxygen and nutrients [11, 12]. In other words, a tumor microenvironment is created. The main physiological characteristics of the
tumor microenvironment are hypoxia, high lactate levels, extracellular acidosis, glucose and energy deprivation [12]. Under such conditions, cells start to consume the intracellular reserved energy resources such as lipids in order to maintain its survival [13]. This phenomena is called autophagy. Incubating cancer cells under similar condition (serum free media) has been show to force these cells to become autophagic. In the view of investigating the role of lipid droplets as an energy source in autophagic cells we have developed a method to stain intracellular lipid droplets by using Sudan III dye. Sudan III is a lipid soluble stain that only stain liquid form of lipids. When we incubated DLD1 cells in the presence of 0.3% Sudan III (dissolved in 0.1% DMSO) for 30 minute, Sudan III failed to stain cell membranes which is known to contain solid layers of phospholipids (data not shown). Interestingly, no stained lipid droplets were observed, indicating that Sudan III dye may have limited penetration into intact cells. In order to confirm that, cells were made necrotic by 50% DMSO treatment for 2 minutes. The necrosis was confirmed with trypan blue (figure 1,a). Sudan III have also failed to stain the cell membrane but lipid droplets were slightly stained. This indicates that membrane permeabilization enhances the Sudan staining. For this purpose cells were stained with 0.3% Sudan III in the presence of 70% ethanol room temperature for 30 minutes. Lipid droplets were good stained (figure 1,b and c).
Figure (1): (a) DMSO induced necrosis stained with trypan blue (b and c) DLD1 cells stained with 0.3% Sudan III in the presence of 70% ethanol room temperature for 30 minutes.
In order to improve the visualization by contrasting the nucleus, the cell nuclei cells were stained with hematoxylin by for 15 minutes prior to Sudan III staining. However, staining living cells with hematoxylin did not give good contrast in addition to that cells were coming off the slides indicating that pre-fixation of the cells may be beneficial. Therefore, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature before it is stained with hematoxylin for 10 minutes and Sudan III for 30 minutes. This method is similar to that published by Hampel et al. [14]. To further enhance the nuclei stain with hematoxylin, the cells were fixed with paraformaldehyde then permeabilized by methanol for 5 minutes at room temperature. We have noticed that permeabilization resulted in a more dense and fast stain of the nuclei (figure 2, a). Due to the fact that methanol can be used to fix the cells, the two steps of fixation and permeabilization was merged in one step by incubating the cells in ice-cold methanol for 10 minutes at - 20 °C. This way did not alter the results and good stain of nuclei was obtained (figure 2, b). It may be worth mentioning that staining lipid droplets with Sudan III (0.3 % dissolved in 70% ethanol) before the hematoxylin stain may reduce the time of experiment as 70% ethanol can fix and permeabilize the cells. However, although good nuclei stain can be obtained by this method, the lipid droplet stain quality was diminished significantly as the Sudan III stain was bleached during the hematoxylin staining step.
Figure (2): (a) Pre-fixed and permeabilized DLD1 nuclei stained with hematoxylin (b) Pre-fixed and permeabilized DLD1 cells doubled stained with hematoxyline and 0.3% Sudan III.

In order to investigate the existence of lipid droplets in serum starved cells, DLD1 cells were incubated in serum free medium for 24 hours and then stained with Sudan III. Interestingly, lipid droplets were disappeared (Figure 3) indicated that cells have been using lipid droplets as a source of energy when they are starved. This result adhere with the findings of Hampel et al. [14] as they have shown that HMGE cells were absence of lipid droplets when cultivated in serum-free medium.
Guijas et al. [15] have demonstrated that lipid droplets is initially synthesized from the cell membrane bilayer and its formation is regulated phospholipase A2 enzyme. This bring the question of what if the phospholipase enzyme is inhibited when the cancer cells are under the starvation condition? Does this lead to its death due to failure to adaptation? Interestingly, lipid droplets were extremely inhibited in the presence of phospholipase A2 inhibitors (betamethasone and hydrocortisone) treated cells (figure 4). More interestingly, starved DLD1 proliferation was affected by this treatment with betamethasone compared to cells incubated in the presence of serum (figure 5).
Figure (4): Non-starved DLD1 treated with (a) betamethasone 150 mg/ml and (b) hydrocortisone 300 mg/ml.
5. Conclusions:
Sudan III staining method that we developed in house may serve as a good qualitative tool to investigate the lipid droplet existence. It is sensitive, easy and relatively fast performed method. Visualizing the pictures using specific softwares may help in quantifying the density and distribution of lipid droplets and make it serve as semi-quantifying method. Using this method we have demonstrated that lipid droplets may play an important role in cancer cell adaptation to nutrient starvation. Our finding also suggest that phospholipase A2 is playing a central role in both lipid droplet formation and cellular adaptation. Targeting such enzyme by regular therapeutic dose of betamethasone may add more beneficial treatment to colorectal cancer patients. Not failing the fact that this study is preliminary and further experimental and clinical work is warranted to prove this conclusion.

6. References:


7. Appendix:

a. Abbreviation:

b. Preparations:

* Preparation of 1M NaOH

4g of NaOH were added to 100ml of distilled water.

* Preparation of 1M of 35%HCl
9 ml of conc. HCl were added to 91 ml distilled water.

• **Preparation of 4% Paraformaldehyde**

100 ml of PBS (phosphate buffered saline) was added to 4 mg of paraformaldehyde. Then, heated and stirred to 60°C. 1M of NaOH was added to increase solubility of PFA (by increasing pH). After dissolving PFA pH was adjusted to reach 6.9 using 1M of 35% HCl. Finally, the solution was filtered (all of these steps were conducted inside a fume hood).

• **Preparation of 0.2% Trypan Blue Stain**

5 ml of HBSS (Hanks' balanced salt solution) was added to 5 ml of 0.4% trypan blue stain.

• **Preparation of 0.3% Sudan III in 70% Ethanol**

We weighed 150mg of Sudan III and added them to 35ml of 100% ethanol (100% ethanol helps to dissolve the Sudan III dye better). The solution was completed to 50ml with distilled water (to give 70% ethanol). Then the solution was filtered.

• **Preparation of (150ng/ml) Betamethasone using a (7mg/ml)**

**Betamethasone**

10µg/ml of betamethasone were added to 45.6µg of DMSO to give a concentration of 150µg/ml. From the new concentration we took 1µl and added it to 1ml of RPMI medium to give the desired concentration of 150ng/ml.

• **Preparation of 300ng/ml Hydrocortisone**
3mg of hydrocortisone were weighed and added to 100µl of DMSO to give a concentration of 30mg/ml. From the new concentration we took 10µl and added them to 1ml of serum free RPMI medium to give a concentration of 300µg/ml. From the last new concentration we took 1µl and added it to 1ml of RPMI medium to give the desired concentration of 300ng/ml.