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Comparative experimental study between valproic acid and sodium dichloroacetate effect upon lung adenocarcinoma cell line A549 in vitro

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Abstract

Introduction: Lung cancer is the most lethal cancer worldwide. Adenocarcinoma is the most common form of lung cancer. Adenocarcinoma is also the most frequently diagnosed subtype in women, as well as in non-smokers. **Materials and methods :** In this experimental study, we assessed valproic acid versus sodium dichloroacetate effect upon lung adenocarcinoma cell line A549 in vitro via microscopic examination ,flow cytometry and gene expressions by using real-time PCR technique .**Results :** Each substance has an apoptotic effect on A549 cells in vitro. The apoptotic effect of valproic acid on A549 cells in vitro is mediated via up-regulation of NOTCH-1 gene while

that apoptotic effect of sodium dichloroacetate is mediated via down-regulation of PDKII gene. Up-regulation of STAT3 gene is associated with each substance . **Conclusion:** The apoptotic effect of valproic acid is more potent than that apoptotic effect of sodium dichloroactate on A549 cell line in vitro .

*** Introduction**

Pulmonary malignancy is a serious international problem. Globally, lung cancer is accountable for the high rates of cancer mortality. Understanding of mechanisms involved in pulmonary malignancy has an important role in discovering new effective treatments **(Dutta et al., 2014)**. Worldwide, lung cancer is the most common cancer among men in terms of both mortality and incidence. Among women, it has the third highest incidence and the second after breast cancer in mortality. In 2012, there were 1.82 million new cases globally, and 1.56 million deaths due to lung cancer, representing 19.4% of all cancer deaths **(Stewart and Wild, 2014)**.

Lung cancer is considered the most fatal cancer. As stated by reports of the World Health Organization (WHO) in 2013, it causes 1, 37 million deaths every year **(Dutta et al., 2014)**. It includes different pathological types, broadly divided into small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) **(Herbst et al., 2008).**

NSCLC makes up about 85% of lung cancers and comprises mainly squamous cell carcinoma, adenocarcinoma, and large-cell lung cancer **(Dela et al., 2011).** Lung adenocarcinoma is the most common form of lung cancer **(Subramanian and Govindan, 2007)**.

Lung adenocarcinoma is also the most commonly diagnosed subtype in women, as well as in non-smokers, although most patients with adenocarcinoma are smokers **(Travis and Harris, 2004)** Surgery, chemotherapy and radiation are common treatments for NSCLC, and palliative care can be combined with treatments to deal with the symptoms and signs of NSCLC **(Moghissi, 2004; Hanna, 2010; Schild et al., 2010; Howington et al., 2013 and West et al., 2013).**

A large number of lung cancer gene therapy strategies has been tried **(Vachani et al., 2010)**. A549 is a human alveolar adenocarcinoma cell line upon which many studies about

lung adenocarcinoma can be performed in vitro **(Saczako et al., 2007).**

Some studies described Notch-1 activation and apoptosis induction by valproic acid (VPA) in several tumor cell lines **(Greenblatt et al., 2007).** It has been reported that over-expression of activated Notch-1(NOTCH-1) suppressed the growth of lung adenocarcinoma A549 cells in vitro **(Zheng et al., 2007).** Notch-1 over-expression and apoptosis induction by VPA in A549 cell line have also been studied **(Wael et al., 2014).**

Sodium dichloroacetate (DCA) is a recent drug and there is an important evidence that it might be beneficial in human malignancy in vivo and in vitro models **(Cairns et al., 2007; Cao et al., 2008 and Wong et al., 2008).** In vitro, a part of mechanism of action of sodium dichloroacetate on cancer cells could be explained by that sodium dichloroacetate activates pyruvate dehydrogenase complex (PDH) by inhibition of pyruvate dehydrogenase kinase (PDK). The isozyme with the highest sensitivity to sodium dichloroacetae and constitutively expressed in many tissues is pyruvate dehydrogenase kinase II (PDKII) **(Bonnet et al., 2007).**

An important role of Signal Transducer and Activator of Transcription 3(STAT3) activity in lung cancer was suggested by constitutive activation of STAT3 in some lung cancer cell lines including A 549 cell line **(Song et al., 2003 and Schütz et al., 2015)** as well as in lung cancer tissue **(Yukioseki et al., 2004).**

*** Materials and methods**

1- Cell line: A549 cell line, lung adenocarcinoma (obtained from Medical Technology Center, Alexandria University).

2- Chemicals: Valproic acid (2-propylpentanoic acid: Sigma-Aldrich, PHR1061-1G) and sodium dichloroacetate (Sodium dichloroacetate 97%: Alfa Aesar, B21827)

3- Culture Media

Dulbecco's modified Eagle's medium (DMEM) containing 0.2%penicillin-streptomycin, 10%feotal bovine serum.

4- Others

Trypsin/ethylenediaminetetraacetic acid 0.25% (Trypsin/EDTA 0.25%) (200mg/L EDTA, 170, 000U Trypsin/L) (Lonza, Belgium), Methyl thiazolyl diphenyl tetrazolium bromide

assay (MTT assay) materials including 96-well plate (Sigma-Aldrich, USA), Trypan blue 0.4% solution (Lonza, USA), Annexin V-FITC Apoptosis Detection Kit (ab 14085) including Annexin-V, Propidium Iodide (PI) and buffers (Abcam, United Kingdom [UK]), GF-1 Total RNA Extraction Kit (Vivantis, Malaysia), Thermo Scientific RevertAid First Standard cDNA Synthesis Kit (Thermo Fisher scientific, USA), SensiFAST SYBER ®No-ROX Kit (Bioline, USA), 75 cm^2 cell culture flasks (Abcam, UK), Falcon tubes(Greiner Bio one, Germany), Polymerase chain reaction tubes (PCR tubes) and real -time PCR plates (Axygen BioScience, Inc., USA), Pipettes and Pipette tips (Viper Pipette Service, USA), Nitrile Powder Free Examination Gloves (Super Care, Thailand), Distilled water (Sigma-Aldrich, USA), Ethyl Alcohol 70% for sterilization (Natco Pharma Limited, India), Dimethyl sulfoxide (DMSO) solvent (Sigma-Aldrich, USA), phosphate buffer saline 1X (PBS) (Hyclone, USA) and primers of genes of interest (Vivantis, Malaysia).

Gene	Primer sequence	PCR product size (base pair)	Accession number
GAPDH	F: 5'- CTC TGC TCC TCC TGT TCG AC - 3' R: 5'- GCG CCC AAT ACG ACC AAA TC -3'	121	NM_002046.3
STAT3	F: 5'-GAG CCA GGA GCA TCC TGA AG-3' R: 5'-GGT CGT TGG TGT CACACA GAT -3'	82	NM_139276.2
NOTCH-1	F: 5'-ACC AAT ACA ACC CTC TGC GG -3' R: 5'-GGC CCT GGT AGC TCA TCA TC -3'	141	NM_017617.3
PDK 2	F: 5'-CGT ATA GGC TTG CAC CCT GG -3' R: 5'-AGG GTC CAG CTC CTT CTA TCC -3'	115	NM_001199898.1

F: forward primer (sense primer), R: reverse primer (anti-sense primer).

After appropriate culturing of A549 cell line (lung adenocarcinoma) microscopic examination was performed before adding the drugs to be able to recognize the normal morphologies of A549 cells. By using cell viability assay (mtt assay), the half maximal inhibitory concentration (IC50) of both of valproic acid and sodium dichloroacetate was assessed. Some A549 cells samples line were treated with the effective dose (IC50) of valproic acid while other samples were treated with the effective dose(IC50) of sodium dichloroacetate

for a period of 48 hours. Thus, we obtained cell line samples (control), cell line samples treated with valproic acid (VPA) and cell line samples treated with sodium dichloroacetate (DCA).By trypsinization cells were harvested to get the samples in falcon tubes in a form of suspension of medium and A549 floating cells, then centrifugation and cells counting were done. Flow cytometry was used to confirm and assess apoptosis and necrosis in control samples of A549 cells or samples treated either by VPA or DCA via Annexin-v and Promodium Iodide (PI).Afterwards, pure RNA extracted from cells of control, VPA and DCA samples was converted to complementary DNA(cDNA) to study gene expression of some genes. Via quantitative real time PCR technique and by using the appropriate primers we could study gene expression of the following genes : notch-1 (Notch-1), pyruvate dehydrogenase kinase II (PDKII) and signal transducer and activator of transcription3 (STAT3). In quantitative real time PCR technique, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene and a mathematical delta–delta method to make comparison between treatments of relative expression results in real-time PCR, which is developed by Perkin Elmer Applied Biosystems (PE Applied Biosystems) ,was also used via the following equation:

$$\text{Ratio} = 2^{-[\,\Delta CP \text{ sample} - \Delta CP \text{ control}\,]}$$

$$\text{Ratio} = 2^{-\Delta\Delta CP}$$

*** Results:**

1- Results of control A549 cells

Control A549 cells gathered in sheets and clumps with ill-defined cellular borders. They have a characteristic spindle shaped morphology. There were unclear points of contact between A549cells in the control flasks, supporting that tumor cell growth was not inhibited. They showed 0.14%positive for Annexin-V, 0.08% positive for PI, 0.61% positive for both stains and 99.17% viability (negative for both stains) assessed by Annexin-v as an apoptotic marker and Propidium Iodide (PI).

2- Results of treated A549 cells

A- Apoptotic effect on treated cells

A549 cells treated with valproic acid **(1.014mM)** and sodium dichloroacetate **(5.811 mM)** had well-defined cellular margins and became retracted and round- shaped under light microscopic examination. The absence

of contact between A549 cells can be noticed in some treated cells.Valproic acid treated cells showed 0.72% positive for Annexin-V, 46.44% positive for PI, 4.63% positive for both stains and 48.21% viability (negative for both stains) While sodium dichloroacetate treated cells showed 0.86% positive for Annexin-V, 30.44% positive for PI, 3.79% positive for both stains and 64.91% viability (negative for both stains).

B- Mechanistic effect on treated cells

While valproic acid treated cells are associated with Notch-1 gene activation (over-expression), A549 cells treated with sodium dichloroacete are associated with PDK2 gene inhibition (down-expression).Association of STAT3 gene over-expression is with valproic acid treated cells as well as A549 cells treated with sodium dichloroacetate (STAT3 over-expression is higher with valproic acid treated cells than sodium dichloroacetate treated ones).

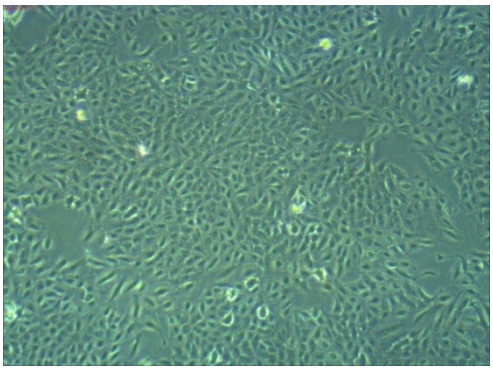
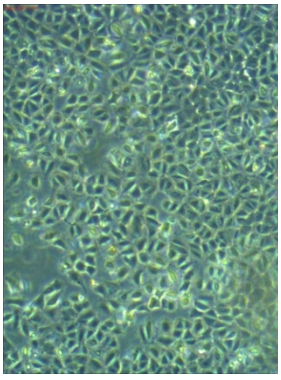
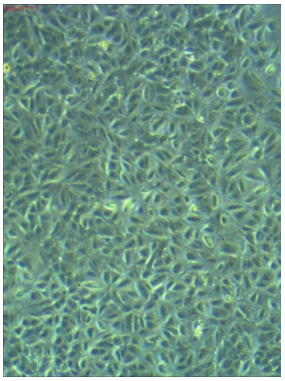


Figure (1): Contact between control A549 cells.

Examination of A 549 cells under light microscope shows that control A549cells gathered in sheets and clumps with ill-defined cellular borders and with a characteristic spindle shaped morphology. There were unclear points of contact between A549 cells in the control flasks, supporting that tumor cell growth was not inhibited.

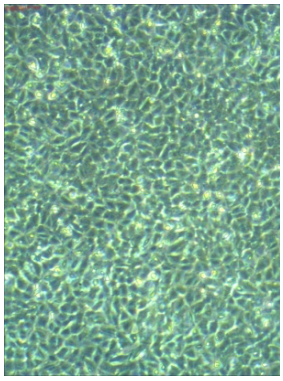




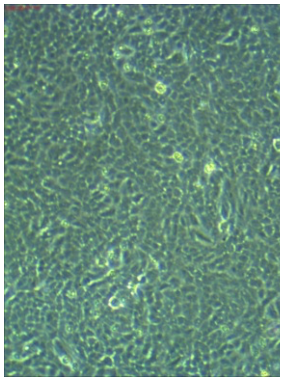
(Figure 2) **(Figure 3)**

Figures (2 and 3): **The lack of contact between treated A549 cells**.

The lack of contact between A549 cells(gaps size between the one confluent A549cells increased) can be noticed in some treated samples either with valproic acid or sodium dichloroacetate.



(Figure 4)



(Figure 5)

Figures (4 and 5): Morphological changes of treated A549 cells.

Cells treated with valproic acid and sodium dichloroacetate, separately, showed marked morphologic changes. Cells had clearly defined cellular margins and became retracted and round-shaped.

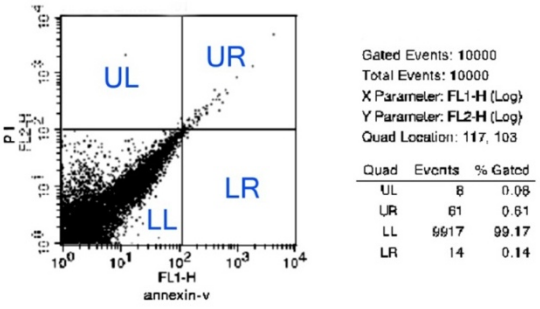


Figure (6): Flow cytometry result of control A549cells

Control cells showed 0.14%positive for Annexin-V, 0.08% positive for PI, 0.61% positive for both stains and 99.17% negative for both stains. Note the followings:-

1- X parameter (FL1-H) represents Annexin-v stain while Y parameter (FL2-H) represents PI stain.

2- We have in each flowcytometry figure 4 quadrants:-

A- Lower left quadrant (LL) which represent negativity for both stains (viable cells).

B- Lower right quadrant (LR) which represents positivity for Annexin-v stain (early apoptosis).

C- Upper right quadrant (UR) which represents positivity for both Annexin-v and PI stains (late apoptosis).

D- Upper left quadrant (UL) which represents positivity for PI stain (necrosis).

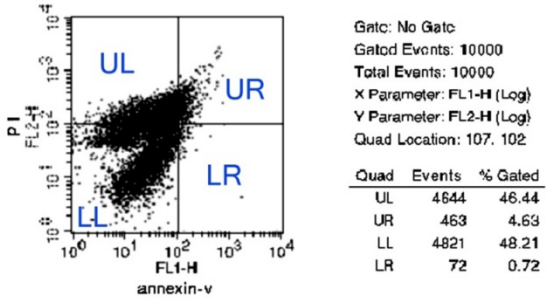


Figure (7): Flow cytometry result of valproic acid treated A549cells

Valproic acid treated cells showed. 0.72% positive for Annexin-V, 46.44% positive for PI, 4.63% positive for both stains and 48.21% negative for both stains.

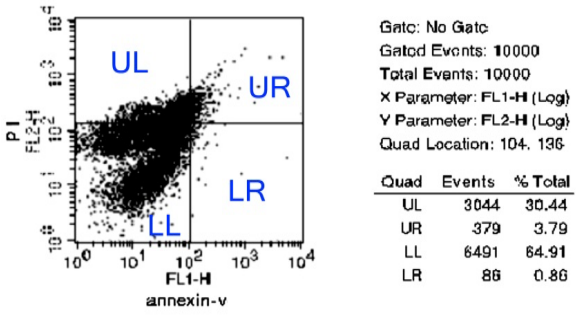


Figure (8): Flow cytometry result of sodium dichloroacetate treated A549cells

Sodium dichloroacetate treated cells showed 0.86% positive for Annexin-V, 30.44% positive for PI, 3.79% positive for both stains and 64.91% negative for both stains.

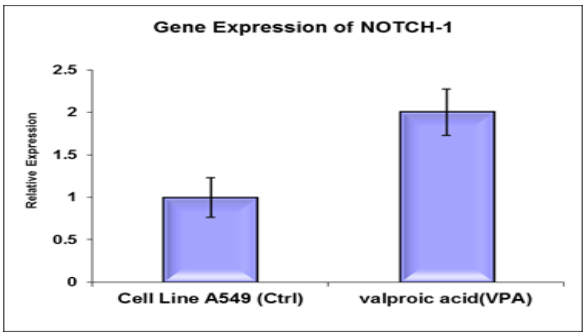


Figure (9): Gene expression of NOTCH-1

A549 cells treated with valproic acid are associated with Notch-1 gene activation (over-expression).

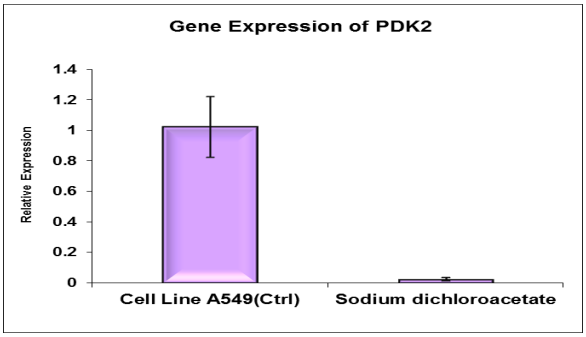


Figure (10): Gene expression of PDK2

A549 cells treated with sodium dichloroacetate are associated with PDK2 gene inhibition (down-expression).

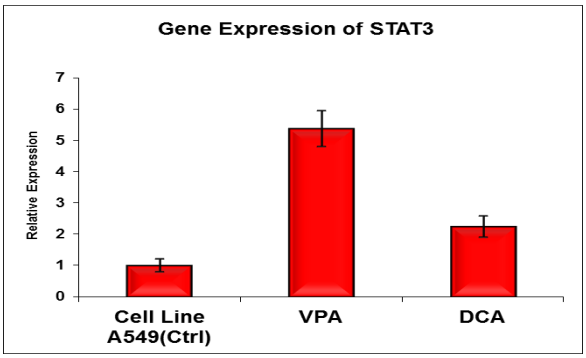


Figure (11): Gene expression of STAT3

Association of over-expression of STAT3 gene with A549 cells treated with valproic acid (VPA) as well as A549 cells treated with sodium dichloroacetate (DCA) (STAT3 over-expression is higher with valproic acid treated cells than sodium dichloroacetate treated ones).

*** Discussion**

This study has been done to compare valproic acid effect with sodium dichloroactate effect on A549

cell line (lung adenocarcinoma) in vitro.

According to microscopic examination results in our study, Control A549 cells gathered in sheets and clumps with ill-defined cellular borders. They have a characteristic spindle shaped morphology. There were indefinite points of contact between A549cells in the control samples **(Figure 1)**, supporting that tumor cell growth was not inhibited. While, the marked morphological changes which include the lack of contact between A549 cells treated either by valproic acid or sodium dichloroacetate **(Figures 2 and 3)** and A549 treated cells themselves became retracted and round-shaped with well-defined cellular margins under light microscopic examination **(Figures 4 and 5)** suggest strongly the inhibitory effect of both substances (VPA and DCA) on A 549 cell line, separately. The findings of Singh and his colleagues who reported that treatment of A549 cells with valproic acid resulted in a significant reduction in cell viability **(Singh et al., 2013)** were in agreement with our result which revealed that valproic acid has an inhibitory effect on A549 cell line in vitro causing reduction in cell viability and cell death. Similarly, the findings of Allen and his colleagues who reported that dichloroacetate alters Warburg metabolism, inhibits cell growth, and increases the X-ray sensitivity of A549 cells **(Allen et al., 2015)** were in agreement with our result which revealed that sodium dichloroacetate has an inhibitory effect on A549 cell line in vitro causing reduction in cell viability and cell death.

IC50 is the concentration of a substance that causes 50% cytotoxicity (50% inhibition of the cell line). In an experiment VPA inhibited the viability of all ESCC (oesophageal squamous cell carcinoma) cells in a dose-dependent manner. The 50% inhibitory concentration (IC50) value of VPA in each cell line was between 1.02-2.15 mM **(Shoji et al., 2012)**. It has been also reported that IC50 for VPA in A549 =48506 ±87.95 (µg/m) (about 2mM) **(Gumbarewicz et al., 2016)**. Valproic acid, however, acts through a distinct pathway that involves direct inhibition of histone deacetylase (IC50 for HDAC1 = 0.4 mM) **(Phiel et al., 2001)**. In our study, by using mtt assay and IC50 assessment we assessed

value of IC50 for valproic acid on A549 cells in vitro which was approximately 1.014 millimole (mM). As for IC50 for sodium dichloroacetate in A549 cells, A study showed that sodium dichloroacetate (DCA) can induce cancer cell apoptosis and inhibit tumor growth, but its cytotoxic activity is low (IC_{50} > 1000 μM for A549) **(Yang et al., 2010)**. Another study reported that IC50 for sodium dichloroacetate =4286 ± 375 μM in A549 **(Li et al., 2012)**. These findings were in agreement with our result which revealed that IC50 for sodium dichloroacetate on A549 cells in vitro was approximately 5.811 mM. The inhibitory (apoptotic) effect of VPA on A 549 cells was confirmed and assessed by flow cytometry results as valproic acid treated cells showed 0.72% positive for Annexin-V(an apoptotic marker), 46.44% positive for PI (a marker for necrosis), 4.63%positive for both stains and 48.21% viability (negative for both stains) **(Figure 7)**, and the inhibitory (apoptotic) effect of DCA on A 549 cells was also confirmed and assessed by flow cytometry results as DCA treated cells showed 0.86% positive for Annexin-V, 30.44% positive for PI, 3.79% positive for both stains and 64.91% viability (negative for both stains) **(Figure 8)**. According to IC50 and flow cytometry results, we could conclude that valproic acid has more potent inhibitory (apoptotic) effect than that inhibitory (apoptotic) effect of sodium dichloroactate on A549 cell line in vitro.

Studies have described the activation of Notch-1 signaling and induction of cell apoptosis by VPA in several tumor cell lines **(Greenblatt et al., 2007 and Platta et al., 2008)**. Notch-1 over-expression and induction of cell apoptosis by VAP in A549 cell line have also been studied **(Wael et al., 2014)**. These studies were in agreement with our result which revealed that the inhibitory effect of valproic acid on A549 cells is associated with Notch-1 gene activation **(Figure 9)**. This was achieved by gene expression using quantitative real-time PCR technique in our study. Association of PDK2 gene inhibition and induction of cell apoptosis by DCA in NSCLC have been studied and subsequent injection of DCA-treated NSCLC and control NSCLC into nude rats flank has been done. Those rats were imaged with a rodent PET-CT. Fluoro-deoxyglucose-

glucose PET imaging (FDG-Glucose PET imaging) showed that the size of the tumor and the glucose uptake in the tumor are decreased by DCA therapy **(Michelakis et al., 2008)**. This was also in agreement with our result that showed that the inhibitory effect of sodium dichloroacetate on A549 cells is associated with PDK2 gene inhibition **(Figure 10)**. This could be also achieved by gene expression using quantitative real-time PCR technique.

However, an important role of STAT3 activity in lung malignancy was suggested by constitutive STAT3 activation in a number of lung cancer cell lines including A 549 cell line **(Song et al., 2003 and Schütz et al., 2015)** as well as in lung cancer tissue **(Yukioseki et al., 2004)**, STAT3 has been recently reported to have a function as a tumor suppressor, depending on the stage of the malignancy or the mutational context **(Couto et al., 2012)**. Moreover, in some situations, cancer progression could be promoted by inhibiting STAT3. For instance, STAT3 conditional deletion of STAT3 increases carcinogen or oncogenic K-Ras-induced tumorigenesis in mouse lung epithelial cells**(Zhou et al., 2014)** and in thyroid cancer cell lines, knocking down STAT3 increases growth of the tumor as a xenograft**(Couto et al., 2012)**. According to a study on P19 embryonal carcinoma cells VPA exposure also resulted in increased STAT3 protein expression. However, STAT3 acetylation and phosphorylation, which both contribute to STAT3 activation, were significantly decreased as a result of VPA exposure **(Bricker, 2016)**. In the light of the previous information, Our results concerning association of over-expression of STAT3 gene with the inhibitory effect of valproic acid as well as with that inhibitory effect of sodium dichloroacetate on A549 cells growth (notice that STAT3 over-expression is higher with valproic acid than sodium dichloroacetate) **(Figure 11)** are acceptable.

*** Conclusion**

This experimental study was established to compare between valproic acid effect with sodium dichloroacetate effect upon lung adenocarcinoma cell line A549 in vitro. According to this study, it has been shown that the apoptotic (inhibitory) effect of valproic acid is more potent than that apoptotic

(inhibitory) effect of sodium dichloroactate on A549 cell line in vitro and this inhibitory effect of valproic acid on A549 cells is associated with Notch-1 gene activation (over-expression) while the inhibitory effect of sodium dichloroacetate on A549 cells is associated with PDK2 gene inhibition (down-expression). Association of over-expression of STAT3 gene with the inhibitory effect of valproic acid as well as with that inhibitory effect of sodium dichloroacetate on A549 cells growth in vitro could be also concluded (We notice that STAT3 over-expression is higher with valproic acid than sodium dichloroacetate).

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