

Original Article

Running Title: Nic-ALA Derivative Increased Apoptosis of A375 Melanoma Cell Line

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**Anti-Cancer Properties of Nicotinic Cid-Alpha Linolenic Acid Derivative on A375 Melanoma Cell Line:
Assessment of Apoptosis and WNT Signaling Pathways**

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Abstract

Background: Malignant melanoma is aggressive skin cancer whose survival rate is extremely low. Commencing apoptosis is believed to be a significant issue in cancer treatment and targeting the apoptosis and WNT signaling pathways, which is probably a potentially successful strategy to overcome tumor plasticity in melanoma.

Method: We conducted the present in vitro study to investigate anti-proliferative and apoptotic effects of Nic-ALA, as a new compound, on A375 melanoma cell line using MTT assay and flow cytometry, respectively. The gene expression profiles of the cancer cells were obtained for Bcl-2 and Bax as the main genes of the apoptosis signaling pathway and WIF1 and beta-catenin genes from the WNT signaling pathway with qRT-PCR.

Results: Nic-ALA's cytotoxicity on A375 melanoma cell line from MTT assay was obtained with IC50 166.7, 144.2, and 146.1 μ M. This novel derivative induced 11.3, 46.1, and 85.7% of apoptosis in 24, 48, and 72h time points, respectively. In the treated cells, the expression of Bax, beta-catenin, and WIF1 genes increased while the expression of Bcl-2 decreased significantly at 200 μ M concentration and the treated times of 48 and 72h.

Conclusion: The anti-proliferation of Nic-ALA at a lower value than what we found in nicotinic acid alone represented the higher bioavailability and transport efficiency of this novel derivative through A375 melanoma cell line. Its antipoeitic effects were obtained by increasing the apoptosis rate and expression of the Bax gene and reducing Bcl-2 gene expression. Up-regulation of WIF1 and beta-catenin in the WNT signaling pathway emphasized Nic-ALA's anti-cancer effect on A375 melanoma cell line.

Keywords: Nicotinic acid-alpha linolenic acid, WNT signaling pathway, WIF1, beta-catenin, Apoptosis, Melanoma

Introduction

Melanoma is aggressive skin cancer with a high mortality rate, whose frequency has increased over the past two decades.^{1,2} This cancer is the fifth and seventh most popular cancer among men and women, respectively.³ The most prevalent risk factors for malignant melanoma growth are known to be exposure to ultraviolet radiation, fair skin, dysplastic nevi syndrome, age, and family history.⁴ Although the early diagnosis of melanoma leads to a good prognosis, the average survival of patients with metastatic malignant melanoma is 6–9 months.^{5,6} The therapeutic interventions that are used for metastatic melanoma, including surgery, radiation therapy, and chemotherapy are not enough, and so far, only minor improvement in survival has been achieved in the published reports.⁷ Therefore, understanding the biology of malignant melanoma initiation and development is vital.

Unsaturated fatty acids have an antioxidant activity that results into anticancer effects in the human body.⁸⁻¹⁰ According to the double bonds, they are divided into two groups: polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids,^{11, 12} both of which have critical features.^{13, 14} PUFAs consist of omega 3 and omega 6 families and have anticancer activity *in vitro* and *in vivo*.^{8, 10, 15}

Nicotinic acid and its amide, nicotinamide, are the most common forms of B-vitamin niacin (vitamin B3) that can control the intracellular calcium levels in a concentration and exposure time-dependent manner.^{16, 17} Moreover, a relatively high concentration of nicotinic acid triggers cytoskeleton disassembly through inducing Beta-tubulin degradation. Since cytoskeleton plays a vital role in cell migration, this disassembly of cytoskeleton by nicotinic acid might interrupt the metastasis process of tumor development. This demonstrates that nicotinic acid has an excellent clinical therapy potential for the treatment of various diseases in the future.^{18, 19}

Apoptosis as a programmed cell death strategy is a defense mechanism that occurs when cells age to balance cell populations. The apoptotic pathway is one of the critical signaling pathways with various gene and protein families that respond to numerous apoptotic agents, such as

lack of growth factors, Fas ligand binding, and chemotherapy.^{20, 21} In the apoptotic pathway, anti and pro-apoptotic members, including Bcl-2 family (B-cell lymphoma 2; Bcl-2, Bcl-XL, Mcl-1, Bcl-W, Bfl-1, A1), play a crucial role as anti-apoptotic in the control of apoptosis and the pro-apoptotic members (*BAX*, *BAK*, *BOK*).^{22, 23} The other crucial signaling pathway involved in cell proliferation and cell fate is Wnt-related integration site (Wnt) signaling pathway. It has been reported that an unusual regulation of WNT signaling pathway causes several types of human cancers, such as lung, prostate, and breast cancer.²⁴⁻²⁶ Wnt inhibitory factor-1 (*WIF1*) gene encodes a secreted protein that binds to WNT proteins, and the *beta-catenin* are the major critical proteins preventing Wnt/*beta-catenin* signaling pathway.²⁷⁻²⁹

Thus, even though nicotinic acid has shown useful biochemical characteristic, the assessment of its clinical applications are restricted due to its limited bioavailability and lower permeability coefficient during the absorption. This study aimed to evaluate a newly designed complex of nicotinic acid-omega 3 (Nic-ALA) (Figure 1), investigate its effects on A375 melanoma cell proliferation and apoptosis, and clarify the role of apoptosis and WNT as the major molecular pathways of cell death by measuring Bcl-2, Bax (Bcl-2 Associated X-protein), *WIF1*, and *beta-catenin* gene expressions.

Material and Methods

Cell culture

In this experimental study, A375 melanoma cell line was purchased from Pasteur Institute of Iran and cultured in monolayers using RPMI 1640 Glutamax (Biosera, France) supplemented with 10% fetal bovine serum and 1% antibiotic (100 U/ml of penicillin, 100 µg/ml of streptomycin) at 37°, 90 % humidity, and 5% CO₂. The cells were seeded in 96 well cell culture plates, each containing 2500 cells for all the concentrations and the control. Following the treatment of the cells with different concentrations of Nic-ALA 25, 50, 100, 150, and 200 at intervals of 24, 48, and 72 h, the cells were harvested and separated from the medium.

MTT assay

MTT was utilized in an attempt to test cell viability. A375 melanoma cells were seeded at a

concentration of cells at each well and incubated for 24 h. Subsequently, the cells were exposed to 200 µl of different Nic-ALA concentrations (50, 100, 150, 200 pg/ml) for 24, 48, and 72 h. After 24 h, the medium was cleared, and 80 µl of the new medium was added. When the treatment period finished, the solution was eliminated and 20µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma, Germany) was added to each well. The wells were then incubated for 4 h at 37°C in the dark. Afterwards, the MTT was removed, replaced with 100µl DMSO, and incubated for 10 minutes in a shaking incubator. 25 µl Glycine buffer was added and finally, the optical absorbance was evaluated at 570 and 630 nm with an ELISA (the enzyme-linked immunosorbent assay) plate reader. All the examinations were carried out in triplicate.

Apoptotic assay via flow cytometry

The annexin-V-FLUOS staining kit (Roche Life Science, 11 858 777 001) was employed to detect apoptotic and necrotic cells. This kit uses a dual-staining protocol in which the apoptotic cells are stained with Annexin-V-FLUOS (green fluorescence). The necrotic cells are stained with propidium iodide (PI; red fluorescence). A375 melanoma cancer cells were seeded in 6-well plates at a density of 3×10^5 cells/well and allowed to attach overnight. The medium was replaced with fresh RPMI-1640 medium containing Nic-ALA derivative at µM concentration and treated 24, 48, and 72 h. The cells were trypsinized, washed with PBS, and processed for labeling with Annexin-V-Fluorescein. They were then used according to the manufacturer's protocol. The labeled cells were analyzed in a flow cytometer (Cyflow, Partec).

Measurement of Bax, Bcl-2 and, Beta-catenin gene expression

Reverse Transcription-PCR (RT-PCR)

Total cellular RNA was extracted from the cells homogenized in 1 mL of RiboEx reagent (Gene all). After the addition of chloroform (0.2 volumes), the homogenate was separated into aqueous and organic phases via centrifugation (15 minutes at 12000g and 4 °C). Subsequently, the superior aqueous RNA-rich phase was isolated. The kit steps were carried out in sequence and the sample was then applied

to the RNase-free mini spin column; RNA was eluted in 30 mL of RNase-free water. Final RNA condensations were assessed with a Nanodrop ND-1000 Spectrophotometer and OD260/OD280 nm absorption ratio >1.95.

Optimization of RT-PCR

Table 1 represents all the Real-Time PCR primers sequences. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression was considered as the control. Due to the Taq DNA polymerase, dNTP concentrations, forward and reverse primers, cDNA, MgCl₂ concentrations, and different annealing temperatures (53–60°C), the conditions for all the PCRs were optimized in a gradient cyclor. We separated the RT-PCR amplification products on a 2% agarose gel electrophoresis.

Quantitative Real-Time RT-PCR

Up to 2 µg of the total RNA was used to synthesize cDNA utilizing the excellent volume cDNA Kit based on the manufacturer's guidelines (Gene All). PCR analysis of each of the target genes was accomplished in the following PCR mixtures: 2 µl of Diluted cDNA, 12/5 µl of SYBR Green PCR Master Mix, 0.5 µl of 10mM forward primer, 0.5 µl of reverse primer, and 9/5 µl of distilled water. Quantitative RT-PCR was performed in triplicate.

North Tehran branch of Islamic Azad University and Qazvin University of Medical Sciences approved all the protocols and procedures. Ethics Committee approved the present work under the reference number IR.QUMS.REC.1397.329.

Statistical Analysis

All the data were triplicated and expressed as the mean ± SD. To compare the differences between the treatment groups, Graph pad prism statistical software was used to analyze the variances. The treatments were considered to be significantly different at $p < 0.05$.

Results

Viability assay by MTT

Human A375 melanoma cell line treated with designed Nic-ALA derivative resulted in inhibiting cell growth in a dose and time-dependent manner. Figure 2 illustrates MTT results, in which the treated concentrations of 25 and 50 µM did not affect the cell viability. Still,

the percentage of cell viability started to decrease, particularly in concentration higher than 100 μM . The magnitudes of 50% of the Nic-ALA inhibition concentration (IC50) were obtained to be 166.7, 144.2, and 146.1 μM for 24, 48, and 72 hours, respectively. The morphological changes in A375 melanoma cell line before and after the treatment with different concentrations of Nic-ALA derivative could also be observed in microscopic images (Figure 3A-F).

Apoptotic effect by flow cytometry analysis

The apoptotic effect of the Nic-ALA derivative on A375 melanoma cell line was evaluated using the flow cytometry technique. As depicted in figures 4 and 5, the treated cells were stained with Annexin-V-FLUOS staining kit and the percentages of apoptotic cells were 11.3, 46.1, and 85.7% respectively in 24, 48, and 72 h time points in comparison with their control values of 5.4, 5.3, and 6.7, respectively. The averages of LL (normal live cells) and UL (necrotic cells) were higher (92.66 and 2.98 %, respectively) in the control A375 melanoma cells compared to the treated cells with Nic-ALA derivative (Table 2) however, in the case of UR (late apoptotic cells) and LR (early apoptotic cells), the treated cells were found to have higher values (3.14 and 7.04 %, respectively; Table 2)

Changes of Bax, Bcl-2, Beta-catenin and, WIF1 gene expression by qRT-PCR

The expression of *GAPDH*, as an internal control gene, was constant. The expression of Bcl-2 and Bax genes, as the key elements of apoptosis pathways, was measured relative to the reference gene (*GAPDH*). The results of qRT-PCR indicated that Bax gene expression increased significantly ($P < 0.001$) at the concentration of 200 μM Nic-ALA derivative and time scales of 48 and 72 h (Figure 6A). Bcl-2, the other member of the apoptosis signaling pathway, decreased significantly ($P < 0.001$) at the concentration of 200 μM Nic-ALA and time scales of 48 and 72 h in A375 melanoma cells (Figure 6B). Evaluation of WNT signaling pathway was performed through beta-catenin and *WIF1* gene expression analysis. Furthermore, in Figure 6C, we observed that 200 μM Nic-ALA induced a significant increase ($P < 0.001$) in the nuclear beta-catenin in A375 melanoma cells. Furthermore, the *WIF1* gene

expression reached its highest expression rate at this concentration at the time intervals of 48 and 72 h ($P < 0.05$; Figure 6D).

Discussion

The present work demonstrated the antiproliferative and apoptotic effects of a novel Nic-ALA derivative on A375 melanoma cancer cell line. Additionally, it changed the expression level of anti-apoptotic Bcl-2 and pro-apoptotic BAX genes of apoptosis and *WIF1* and beta-catenin genes from WNT signaling pathways. Despite previous findings in cancer therapy, toxicity of normal cells and development of drug resistance due to the modulation of the expression of different genes and their molecular products involved in cell death, proliferation, and survival, persuaded researchers to design more efficient anti-cancer treatments and combinational therapies for targeting different molecular signals involved in cancer cell death. Saturated, monounsaturated, and trans fatty acids enhance the risk of cancer incidence by jeopardizing cellular immunity if a high amount is taken.³⁰ In contrast, omega-3 PUFAs improve cellular immune function and lessen the tumor incident, increase the expression of TNF- α , IL-1b, IL-6, and IL-8, suppress the level of reactive oxygen species, and affect the metabolism of nutrient in cancer cells.^{30, 31} To date, numerous studies have demonstrated that omega-3 induces cell death through apoptosis³² and nicotinic acid prevents angiogenesis via cytoskeleton remodeling,¹⁸ suggesting that ALA synthesis, as omega-3, and nicotinic acid, as a new derivative, may be safe chemo-preventive and therapeutic agents for certain types of cancer. Our previous study focused on the synthesis of polyunsaturated acid derivatives and the effect of chrysin- omega-3 and -6 derivatives that are the inhibitors of tyrosinase enzyme which is overexpressed in melanoma.³³ In addition, our other study investigated the inhibitory effects of benzoic acids and pyridine compounds as the potential therapeutic inhibitors on tyrosinase enzyme and A375 melanoma cell line. The concentrations at which the inhibitors reduced the cell density up to 50% (IC50) were obtained at 3.61 and 2.42 mM for nicotinic and picolinic acids, respectively.³⁴

Combining the anti-cancer agents to augment or eliminate the drawbacks of the agents is a well-established approach to cancer chemotherapy.³⁵ As mentioned in the results of the MTT assay in Figure 2, the IC50s of Nic-ALA on A375 melanoma cell line were 166.7, 144.2, and 146.1 μ M for the time scales of 24, 48, and 72 h, respectively, which emphasizes further anti-proliferative effect of this new derivative compared with the nicotinic acid and ALA alone. In a similar study, He et al. generated a derivative of curcumin by adding two niacin molecules which improved bioavailability and exhibited a notable anti-proliferative function against cancer cell lines of the colon (HCT116), breast (MCF-7), and nasopharyngeal (CNE2, 5-8F, and 6-10B).³⁶ Moreover, induction of apoptosis in addition to arresting the cell cycle at the G2/M phase with a p53-mediated mechanism was found to be the main approach of this derivative while maintaining a high level of selectivity.

It has been reported that quercetin and its ester derivative were not transported during paracellular pathways. It was also poorly absorbed by active transport whereas its ester derivative was mainly absorbed by passive diffusion.³⁷ Therefore, it could be suggested that improving the lipophilicity of nicotinic acid by conjugation with an aliphatic carboxylic acid could increase its transport across the cell membrane as reported in figure 2 and 4. According to these figures, the apoptosis percentage of A375 melanoma cell line increased dramatically in comparison with the control groups in time steps of 48 and 72 h. We synthesized this derivative of nicotinic acid to improve its bioavailability and transport efficiency; this molecular structural modification probably augments its intestinal absorption. Nicotinic acid and nicotinamide adenine dinucleotide (NAD⁺) are the two precursors of vitamin B3 or niacin. These two compounds act as a substrate of NAD⁺-degrading enzymes, such as poly (ADP-ribose) polymerases (PARPs), CD38, and sirtuins. Additionally, 1 NAD⁺ degradation by PARP1/2 allows PAR production and DNA repair and through DNA damage, PARP1/2 activity leads to cell demise by apoptosis.¹⁷ Furthermore, previous reports concerning nicotinic acid receptors in human

skin keratinocytes have indicated the protective role of niacin as a potential skin cancer prevention. Thus, this receptor is a potential target for skin cancer prevention agents.³⁸

The gene expression data exhibited in Figures 6A- and -B showed the up-regulation of Bax and down-regulation of Bcl-2 in A375 melanoma cell line treated with Nic-ALA. The control and induction of apoptosis are related to the ratio of anti- and pro-apoptotic protein expression, such as Bcl-2/Bax as the main mitochondria-related apoptotic factors. The consequence is the release of cytochrome c from the mitochondria into the cytosol, leading to caspase-9 activation and another cascade of caspases, thereby adducting the intrinsic cell death apoptosis pathway.³⁹ Based on the significant expression rate of the mentioned genes in 200 μ M concentration of Nic-ALA compared with the *GAPDH* as an internal control gene, the lower ratio of Bcl-2 to Bax was due to the elevation of Bax and the decrease in Bcl-2; this proved the significant apoptotic effect of the new derivative in comparison with the control group. In accordance with this study, the universal involvement of Bcl-2/Bax in cancer cells and the positive effect of its down-regulation on apoptosis has been frequently observed;^{22, 40-43} the vital role of Bcl-2/Bax was vividly presented by Sheng and Wei who reported that the knockdown of *CASC15* prevented Wnt/ β -catenin signaling pathway and inhibited β -catenin expression, which ultimately enhanced apoptosis of melanoma cells through reducing the expression of Survivin and Bcl-2 and upregulating Bax in A375 and SK-MEL-28 cells lines.⁴¹ Curcumin has been known to inhibit cell proliferation while inducing apoptosis and increasing caspase-3 activity in cancer cells. Mou et al. found that curcumin's apoptotic effect is through down-regulation of Bcl-2 and PI3K protein and up-regulation of the phospho (p)-Akt protein expression of human laryngeal cancer cells.⁴⁰

In other studies on normal cells, niacin pretreatment has been shown to protect the skin keratinocytes (HaCaT cells) against UV-induced cell death and apoptosis by enhancing the pro-survival pathways AKT, mTOR, and S6. mTOR promotes cell survival and stimulating cell growth and eIF4E, as an essential downstream

effector of mTOR, induces its control activity on cell survival through 4E-BP1.⁴⁴ A few primary anti-apoptotic agents, such as XIAP, c-IAP1, Bcl-XL, and Bcl-2, are mTORC1 and cap-dependent.⁴⁵

Wnt/ β -catenin pathway has been reported to be a predisposing factor in melanoma contributing to its progression.⁴⁶⁻⁴⁸ Thus, inhibiting this pathway through the inhibitors, overexpression of the *WIF1* gene would be of paramount significance. In our previous studies, we observed that Omega-3 increased *WIF1* gene expression in a time-dependent manner and reduced WT1 mRNA in a time and concentration-dependent manner, leading to a decrease in cell viability in pancreatic cancer.⁴⁹ A study showed that a nuclear protein (Nop14) inhibits melanoma proliferation and invasion via this pathway.⁵⁰ In a research conducted in 2016, endogenously synthesized n-3 fatty acids were observed to stop melanoma development through increasing E-cadherin expression and inhibiting AKT/ β -catenin signaling pathway.⁵¹ β -catenin has a connection with WNT signaling pathway by activating the transcription of genes responsible for developing cells. It also acts in cell adhesion at the plasma membrane. Either of these functions is dysregulated in human malignancies.⁵² In a study, it was demonstrated that downregulation of β -catenin results in malignant transformation.⁵³ However, it has been reported that the overexpression of *WIF1*, as a tumor suppressor gene, results in the inhibition of cell development in various cancer cell lines.⁵⁴ In line with these studies, as illustrated in Figures 6C and 6D, the significant increase of β -catenin and *WIF1* expression levels in the treated cells with 200 μ M concentrations of Nic-ALA proved the anti-cancer effect of this novel derivative. We hypothesized that the stimulatory effect of Nic-ALA on *WIF1* could be associated with modifications induced by this derivative in the molecular pathway of β -catenin. Various studies have shed light on the importance of Wnt/ β -catenin signaling pathway in metabolism of energy and cellular development; thus, this pathway holds a therapeutic potential for different cancers.⁵⁵ In agreement with this study, where enhancement of β -catenin suggested a positive involvement in Wnt/ β -

catenin signaling pathway in encouraging apoptosis in melanoma cancer cells, several other researches have confirmed the same findings.⁵⁶⁻⁵⁹ Most recently, Uka et al. revealed that even temporal activation of Wnt/ *BETA-CATENIN* signaling pathway can prevent the expression of a critical transcription factor, Sox10, in melanoma growth and proliferation.⁴⁸ On the other hand, a large body of literature still favors the oncogenic nature of this signaling pathway; for instance, Sarma et al. indicated that by losing AD6B, a ubiquitin-conjugating enzyme, led to disruption of the expression of melanoma phenotype as result of inhibiting Wnt/ β -catenin signaling pathway.⁶⁰ Moreover, Sheng and Wei observed an increase in the relative expression of CASC15, as a carcinogen, and β -catenin. In their study, knocking down the former led to downregulation of Wnt/ β -catenin signaling pathway. Accordingly, they concluded that the proliferation, migration, and invasion of CASC15 are associated with the activation of this pathway.⁴¹ Despite significant endeavors, the exact role of Wnt/ β -catenin signaling pathway still remains controversial.

Conclusion

Overall, the newly designed Nic-ALA induced its anti-proliferative and apoptotic effects on A375 melanoma cell line in a time and concentration-dependent manner, its lower IC50 compared to nicotinic acid alone. ALA proved that this molecular modification induced further bioavailability for the treated cells. The induction of apoptosis by Nic-ALA was achieved by down-regulation of Bcl-2 and up-regulation of *BAX* and increasing the expression level of β -catenin and *WIF1* as prominent members of WNT signaling pathways, which proved the anti-cancer effect of this nicotinic acid omega3 fatty acid derivative. Therefore, this new derivative, such as niacin and nicotinamide, could be proposed for further studies as a chemo-preventive supplement candidate for skin cancer and disorders.

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Conflict of Interest

None declared.

References

1. Wan Q, Jin L, Wang Z. Comprehensive analysis of cancer hallmarks in cutaneous melanoma and identification of a novel unfolded protein response as a prognostic signature. *Aging (Albany NY)*. 2020;12(20):20684-701. doi: 10.18632/aging.103974.
2. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, et al. Cancer statistics in China, 2015. *CA Cancer J Clin*. 2016;66(2):115-32. doi: 10.3322/caac.21338.
3. Trotter SC, Sroa N, Winkelmann RR, Olencki T, Bechtel M. A global review of melanoma follow-up guidelines. *J Clin Aesthet Dermatol*. 2013;6(9):18-26.
4. Erdmann F, Lortet-Tieulent J, Schüz J, Zeeb H, Greinert R, Breitbart EW, et al. International trends in the incidence of malignant melanoma 1953-2008--are recent generations at higher or lower risk? *Int J Cancer*. 2013;132(2):385-400. doi: 10.1002/ijc.27616.
5. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011;364(26):2507-16. doi: 10.1056/NEJMoa1103782.
6. Gogas HJ, Kirkwood JM, Sondak VK. Chemotherapy for metastatic melanoma. *Cancer*. 2007;109(3):455-64. doi: 10.1002/cncr.22427.
7. Berrocal A, Cabañas L, Espinosa E, Fernández-de-Misa R, Martín-Algarra S, Martínez-Cedres JC, et al. Melanoma: Diagnosis, staging, and treatment. Consensus group recommendations. *Adv Ther*. 2014;31(9):945-60. doi: 10.1007/s12325-014-0148-2.
8. Arendse, Lyle. The modulating effect of conjugated linoleic acid (CLA) on cancer cell survival in vitro. [dissertation] Cape Town: University of the Western Cape; 2014. 158 p.
9. Asghari H, Chegini KG, Amini A, Gheibi N. Effect of poly and mono-unsaturated fatty acids on stability and structure of recombinant S100A8/A9. *Int J Biol Macromol*. 2016;84(2):35-42. doi: 10.1016/j.ijbiomac.2015.11.065.
10. Zajdel A, Wilczok A, Tarkowski M. Toxic effects of n-3 polyunsaturated fatty acids in human lung A549 cells. *Toxicol In Vitro*. 2015;30(1):486-91. doi: 10.1016/j.tiv.2015.09.013.
11. Das UN. Essential fatty acids: biochemistry, physiology and pathology. *Biotechnol J*. 2006;1(4):420-39. doi: 10.1002/biot.200600012.
12. Hussain G, Schmitt F, Loeffler JP, Gonzalez De Aguilar J. Fattening the brain: a brief of recent research. *Front Cell Neurosci*. 2013;7:144. doi: 10.3389/fncel.2013.00144.
13. Giordano C, Plastina P, Barone I, Catalano S, Bonfiglio D. n-3 polyunsaturated fatty acid amides: new avenues in the prevention and treatment of breast cancer. *Int J Mol Sci*. 2020;21(7):2279. doi: 10.3390/ijms21072279.
14. Ljungblad LM, Johnsen JI, Wickström M, Kogner P, Gleissman H. Abstract 3275: A novel approach to treat medulloblastoma: The omega-3 fatty acids DHA and EPA reduce medulloblastoma tumor growth in vitro and in vivo. *Cancer Res*. 2015;75(15):3275. doi:10.1158/1538-7445.AM2015-3275.
15. Das UJ. Tumoricidal action of cis-unsaturated fatty acids and their relationship to free radicals and lipid peroxidation. *Cancer Lett*. 1991;56(3):235-43. doi: 10.1016/0304-3835(91)90008-6.

16. Moniri NH, Farah Q. Short-chain free-fatty acid G protein-coupled receptors in colon cancer. *Bioch Pharmacol.* 2021;186:114483. doi: 10.1016/j.bcp.2021.114483.
17. Bogan KL, Brenner C. Nicotinic acid, nicotinamide, and nicotinamide riboside: a molecular evaluation of NAD⁺ precursor vitamins in human nutrition. *Annu Rev Nutr.* 2008;28(3):115-30. doi: 10.1146/annurev.nutr.28.061807.155443.
18. Pan L, Yu G, Chen X, Li X. Nicotinic acid inhibits angiogenesis likely through cytoskeleton remodeling. *Organogenesis.* 2017;13(4):183-91. doi: 10.1080/15476278.2017.1364829.
19. Dubrall D, Pflock R, Kosinska J, Schmid M, Bleich M, Himmerkus N, et al. Do dimethyl fumarate and nicotinic acid elicit common, potentially HCA₂ -mediated adverse reactions? A combined epidemiological-experimental approach. *Br J Clin Pharmacol.* 2021. doi: 10.1111/bcp.14787.
20. Norbury CJ, Hickson ID. Cellular Responses to DNA Damage. *Annu Rev Pharmacol Toxicol.* 2001;41(1):367-401. doi: 10.1146/annurev.pharmtox.41.1.367.
21. Berke GJC. The CTL's kiss of death. *Cell.* 1995;81(1):9-12. doi: 10.1016/0092-8674(95)90365-8.
22. Trisciuglio D, Del Bufalo D. New insights into the roles of antiapoptotic members of the Bcl-2 family in melanoma progression and therapy. *Drug Discov Today.* 2021;S1359-6446(21)00059-3. doi: 10.1016/j.drudis.2021.01.027.
23. Tessoulin B, Papin A, Gomez-Bougie P, Bellanger C, Amiot M, Pellat-Deceunynck C, et al. BCL2-family dysregulation in B-cell malignancies: From gene expression regulation to a targeted therapy biomarker. *Front Oncol.* 2019;8:645. doi: 10.3389/fonc.2018.00645.
24. Liu YL, Yang HP, Gong L, Tang CL, Wang HJ. Hypomethylation effects of curcumin, demethoxycurcumin and bisdemethoxycurcumin on WIF-1 promoter in non-small cell lung cancer cell lines. *Mol Med Rep.* 2011;4(4):675-9. doi:10.3892/mmr.2011.473.
25. Paul S, Dey AJN. Wnt signaling and cancer development: therapeutic implication Minireview. *Neoplasma.* 2008;55(3):165-76.
26. Xiong X, Xu W, Gong J, Wang L, Dai M, Chen G, et al. miR-937-5p targets SOX17 to modulate breast cancer cell cycle and cell proliferation through the Wnt signaling pathway. *Cell Signal.* 2021;77:109818. doi:10.1016/j.cellsig.2020.109818.
27. Hsieh JC, Kodjabachian L, Rebert ML, Rattner A, Smallwood PM, Samos CH, et al. A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature.* 1999;398(6726):431-6. doi:10.1038/18899.
28. Mazieres J, He B, You L, Xu Z, Lee AY, Mikami I, et al. Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer. *Cancer Res.* 2004;64(14):4717-20. doi: 10.1158/0008-5472.CAN-04-1389.
29. Jalilvand A, Soltanpour MS. Promoter hypermethylation of Wnt/ β -catenin signaling pathway inhibitor WIF-1 gene and its association with MTHFR C677T polymorphism in patients with colorectal cancer. *Oman Med J.* 2020;35(3):e131. doi: 10.5001/omj.2020.49.
30. Freitas RD, Campos MM. Protective effects of omega-3 fatty acids in cancer-related complications. *Nutrients.* 2019;11(5):945. doi:10.3390/nu11050945.
31. Ma Y, Wang J, Li Q, Cao B. The Effect of omega-3 polyunsaturated fatty acid supplementations on anti-tumor drugs in triple negative breast cancer. *Nut. Cancer.* 2021;73(2):196-205. doi:10.1080/01635581.2020.1743873.

32. Jing K, Song KS, Shin S, Kim N, Jeong S, Oh HR, et al. Docosahexaenoic acid induces autophagy through p53/AMPK/mTOR signaling and promotes apoptosis in human cancer cells harboring wild-type p53. *Autophagy*. 2011;7(11):1348-58. doi:10.1080/01635581.2020.1743873.
33. Jamali Z, Rezaei Behbehani G, Zare K, Gheibi N. Effect of chrysin omega-3 and 6 fatty acid esters on mushroom tyrosinase activity, stability, and structure. *J Food Biochem*. 2019;43(3):e12728. doi:10.1111/jfbc.12728.
34. Gheibi N, Taherkhani N, Ahmadi A, Haghbeen K, Ilghari D. Characterization of inhibitory effects of the potential therapeutic inhibitors, benzoic acid and pyridine derivatives, on the monophenolase and diphenolase activities of tyrosinase. *Iran J Basic Med Sci*. 2015;18(2):122-9.
35. Kiesewetter B, Mayerhoefer ME, Lukas J, Zielinski CC, Müllauer L, Raderer M. Rituximab plus bendamustine is active in pretreated patients with extragastric marginal zone B cell lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma). *Ann Hematol*. 2014;93(2):249-53. doi:10.1007/s00277-013-1865-3.
36. He YC, He L, Khoshaba R, Lu FG, Cai C, Zhou FL, et al. Curcumin nicotinate selectively induces cancer cell apoptosis and cycle arrest through a P53-mediated mechanism. *Molecules*. 2019;24(22):4179. doi:10.3390/molecules24224179.
37. Hu Jn, Zou Xg, He Y, Chen F, Deng ZY. Esterification of quercetin increases its transport across human Caco-2 cells. *J Food Sci*. 2016;81(7):H1825-H1832. doi:10.1111/1750-3841.13366.
38. Benavente CA, Jacobson MK, Jacobson EL. NAD in skin: therapeutic approaches for niacin. *Curr Pharm Des*. 2009;15(1):29-38. doi:10.2174/138161209787185760.
39. Luo H, Rankin GO, Li Z, DePriest L, Chen YC. Kaempferol induces apoptosis in ovarian cancer cells through activating p53 in the intrinsic pathway. *Food Chem*. 2011;128(2):513-9. doi:10.1016/j.foodchem.2011.03.073.
40. Mou S, Zhou Z, He Y, Liu F, Gong L. Curcumin inhibits cell proliferation and promotes apoptosis of laryngeal cancer cells through Bcl-2 and PI3K/Akt, and by upregulating miR-15a. *Oncol Lett*. 2017;14(4):4937-42. doi:10.3892/ol.2017.6739.
41. Sheng L, Wei R. Long non-coding RNA-CASC15 promotes cell proliferation, migration, and invasion by activating Wnt/ β -Catenin signaling pathway in melanoma. *Pathobiology*. 2020;87(1):20-9. doi:10.1159/000502803.
42. Fakhrabadi HG, Rabbani-Chadegani A, Ghadam P, Amiri S. Protective effect of bleomycin on 5-azacitidine induced cytotoxicity and apoptosis in mice hematopoietic stem cells via Bcl-2/Bax and HMGB1 signaling pathway. *Toxicol Appl Pharmacol*. 2020;396:114996. doi:10.1016/j.taap.2020.114996.
43. Qin XY, Wang YN, Liu HF, Luo ZH, Zhang PL, Li-Fang H, et al. Anti-cancer activities of metal-based complexes by regulating the VEGF/VEGFR2 signaling pathway and apoptosis-related factors Bcl-2, Bax, and caspase-9 to inhibit angiogenesis and induce apoptosis. *Metallomics*. 2020;12(1):92-103. doi:10.1039/c9mt00248k.
44. Mungamuri SK, Yang X, Thor AD, Somasundaram K. Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. *Cancer Res*. 2006;66(9):4715-24. doi:10.1158/0008-5472.CAN-05-3830.
45. Proud C. Role of mTOR signalling in the control of translation initiation and elongation by nutrients. In: Thomas G, Sabatini D.M., Hall MN, editors. *TOR. Current topics in microbiology and immunology*. vol 279. Springer: Berlin, Heidelberg; 2014. doi:10.1007/978-3-642-18930-2_13.
46. James RG, Bosch KA, Kulikauskas RM, Yang PT, Robin NC, Toroni RA,

- et al. Protein kinase PKN1 represses Wnt/ β -catenin signaling in human melanoma cells. *J Biol Chem.* 2013;288(48):34658-70. doi: 10.1074/jbc.M113.500314.
47. Kaur A, Webster MR, Weeraratna AT. In the Wnt-er of life: Wnt signalling in melanoma and ageing. *Br J Cancer.* 2016;115(11):1273-9. doi: 10.1038/bjc.2016.332.
 48. Uka R, Britschgi C, Krättli A, Matter C, Mihic D, Okoniewski MJ, et al. Temporal activation of WNT/ β -catenin signaling is sufficient to inhibit SOX10 expression and block melanoma growth. *Oncogene.* 2020;39(20):4132-54. doi: 10.1038/s41388-020-1267-7.
 49. Rahmani B, Asl DH, Farivar TN, Azad M, Sahmani M, Gheibi N. The effects of omega-3 PUFA (ALA) on WT1 gene expression in pancreatic cancer cell line (MIA PaCa-2). *Middle East J Fam Med.* 2018;99(5832):1-5. doi: 10.3892/mmr.2016.5639.
 50. Li J, Fang R, Wang J, Deng L. NOP14 inhibits melanoma proliferation and metastasis by regulating Wnt/ β -catenin signaling pathway. *Braz J Med Biol Res.* 2019;52(1):e7952. doi:10.1590/1414-431x20187952.
 51. Yin X, Yu XW, Zhu P, Zhang YM, Zhang XH, Wang F, et al. Endogenously synthesized n-3 fatty acids in fat-1 transgenic mice prevent melanoma progression by increasing E-cadherin expression and inhibiting β -catenin signaling. *Mol Med Rep.* 2016;14(4):3476-84. doi: 10.3892/mmr.2016.5639.
 52. Brembeck FH, Rosário M, Birchmeier W. Balancing cell adhesion and Wnt signaling, the key role of β -catenin. *Curr Opin Genet Dev.* 2006;16(1):51-9. doi: 10.1016/j.gde.2005.12.007.
 53. Takayama T, Shiozaki H, Shibamoto S, Oka H, Kimura Y, Tamura S, et al. Beta-catenin expression in human cancers. *Am J Pathol.* 1996;148(1):39-46.
 54. Kim J, You L, Xu Z, Kuchenbecker K, Raz D, He B, et al. Wnt inhibitory factor inhibits lung cancer cell growth. *J Thorac Cardiovasc Surg.* 2007;133(3):733-7. doi: 10.1016/j.jtcvs.2006.09.053.
 55. Kovacs D, Migliano E, Muscardin L, Silipo V, Catricalà C, Picardo M, et al. The role of Wnt/ β -catenin signaling pathway in melanoma epithelial-to-mesenchymal-like switching: evidences from patients-derived cell lines. *Oncotarget.* 2016;7(28):43295. doi: 10.18632/oncotarget.9232.
 56. Zimmerman ZF, Kulikauskas RM, Bomsztyk K, Moon RT, Chien AJ. Activation of Wnt/ β -catenin signaling increases apoptosis in melanoma cells treated with trail. *PLoS One.* 2013;8(7):e69593. doi: 10.1371/journal.pone.0069593.
 57. Biechele TL, Kulikauskas RM, Toroni RA, Lucero OM, Swift RD, James RG, et al. Wnt/ β -catenin signaling and AXIN1 regulate apoptosis triggered by inhibition of the mutant kinase BRAFV600E in human melanoma. *Sci Signal.* 2012;5(206):ra3-ra3. doi: 10.1126/scisignal.2002274.
 58. Atkinson JM, Rank KB, Zeng Y, Capen A, Yadav V, Manro JR, et al. Activating the Wnt/ β -catenin pathway for the treatment of melanoma—application of LY2090314, a novel selective inhibitor of glycogen synthase kinase-3. *PLoS One.* 2015;10(4):e0125028. doi: 10.1371/journal.pone.0125028.
 59. Chien AJ, Moore EC, Lonsdorf AS, Kulikauskas RM, Rothberg BG, Berger AJ, et al. Activated Wnt/ β -catenin signaling in melanoma is associated with decreased proliferation in patient tumors and a murine melanoma model. *Proc Nat Acad Sci.* 2009;106(4):1193-8. doi: 10.1073/pnas.0811902106.
 60. Sarma A, Gajan A, Kim S, Gurdziel K, Mao G, Nangia-Makker P, et al. RAD6B loss disrupts expression of melanoma phenotype in part by inhibiting WNT/ β -catenin signaling. *Am J Pathol.* 2021;191(2):368-84. doi: 10.1016/j.ajpath.2020.10.015.

Table 1. Forward and reverse primer sequences of *Wif1*, *Bcl-2*, *Bax*, *Beta-catenin*, and *GAPDH* genes

| Gene | Primer |
|------------------|--|
| Wif-1 | F: 5'- CCGAAATGGAGGCTTTTGTGTA -3' R: 5'- TGGTTGAGCAGTTTGCTTTG -3' |
| Bcl-2 | F: 5'- TGGGAAGTTTCAAATCAGC -3' R: 5'- GCATTCTTGGACGAGGG -3' |
| Bax | F: 5'- TGGCAGCTGACATGTTTTCTGAC -3' R: 5'- TCACCCAACCACCTGGTCTT -3' |
| β -catenin | F: 5'-CTCTGATAAAGGCTACTGTTGGATTGATTC-3' R: 5'-TTGCTGCTGTGTCCCACCCA-3' |
| GAPDH | F: 5'-CATGGCCTCCAAGGAGTAAG-3' R: 5'-GGTCTGGGATGGAATTGTGA-3' |

Table 2. Mean \pm SD among LL (normal live cells), UL (necrotic cells), UR (late apoptotic cells), and LR (early apoptotic cells) in untreated and treated groups with Nic-ALA derivative in A375 melanoma cell line

| | LL | UL | UR | LR |
|---------|------------------|-----------------|-----------------|-----------------|
| Control | 92.66 \pm 0.87 | 2.98 \pm 0.95 | 2.07 \pm 0.75 | 2.28 \pm 0.27 |
| Treated | 87.93 \pm 1.93 | 1.87 \pm 0.25 | 3.14 \pm 0.88 | 7.04 \pm 1.74 |

LL: lower left; UL: upper left; UR: upper right; LR: lower right

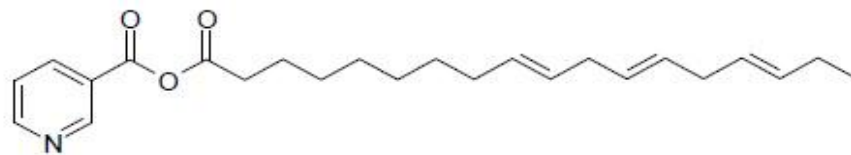


Figure 1. This figure shows the chemical structure of nicotinic acid -Alpha Linoleic Acid (Nic-ALA).

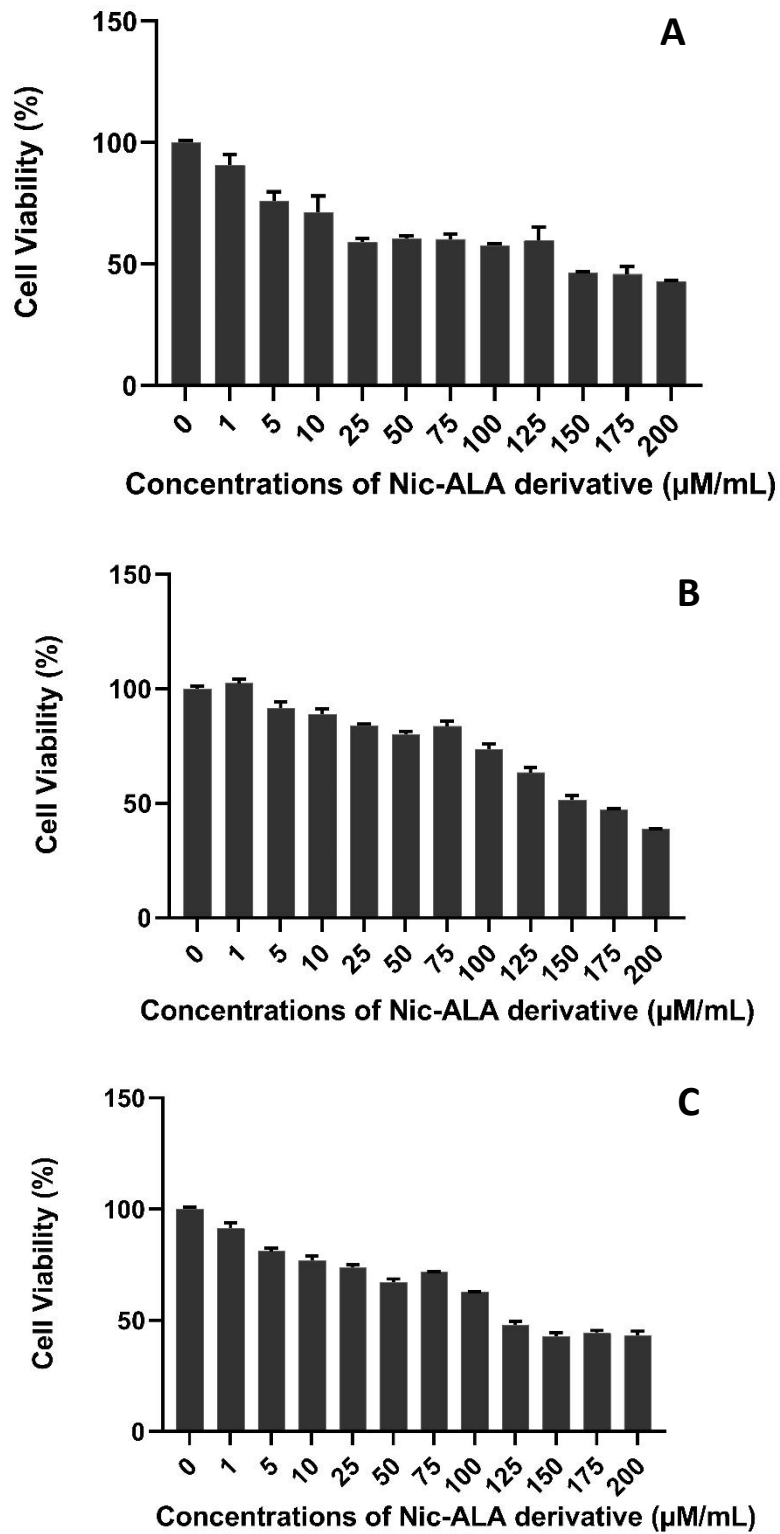


Figure 2. This figure shows the treatment of A375 melanoma cells with viability cell analysis after A. 24h, B. 48h, and C. 72 h following the treatment with Nic ALA derivative.

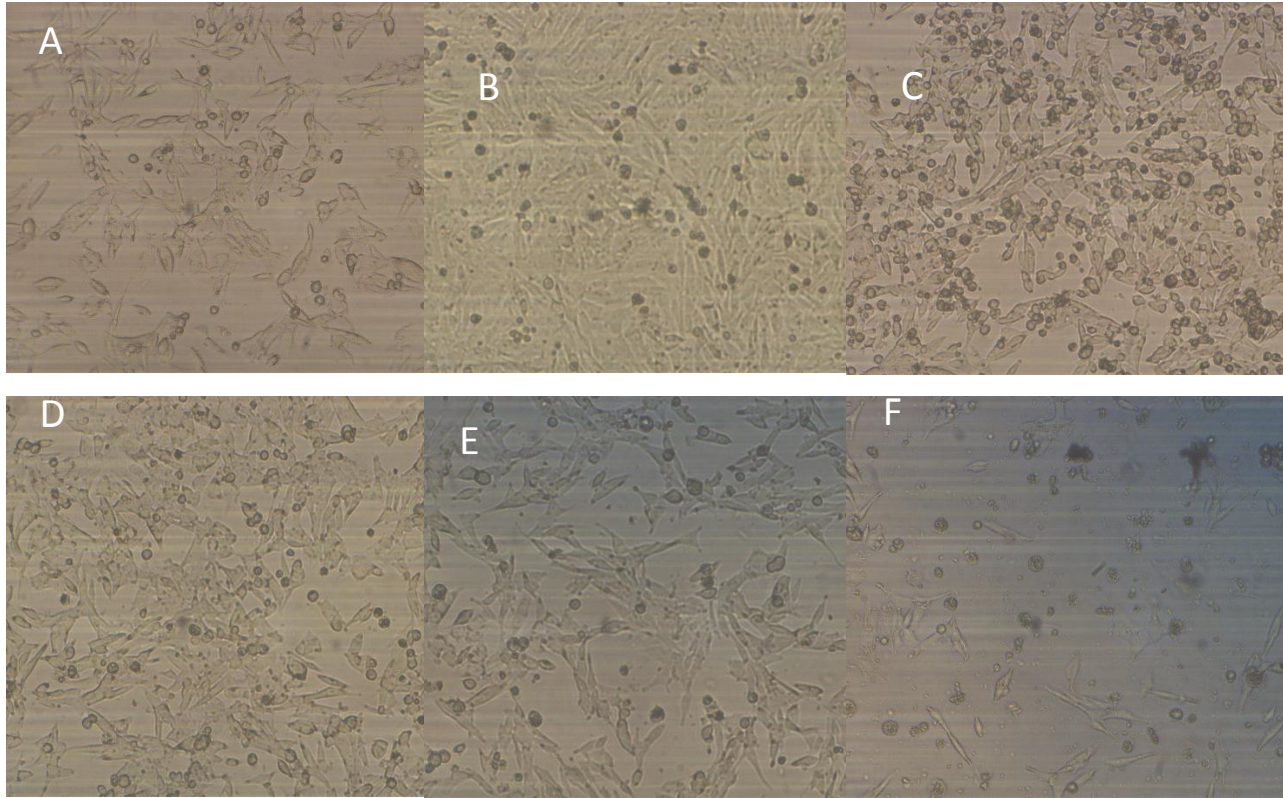


Figure 3. The above images show the morphological changes in A375 melanoma cells under different cell treatment 25 (B), 50 (C), 100 (D), 150 (E), and 200 (F) μ M nicotinic acid complexed with omega-3.

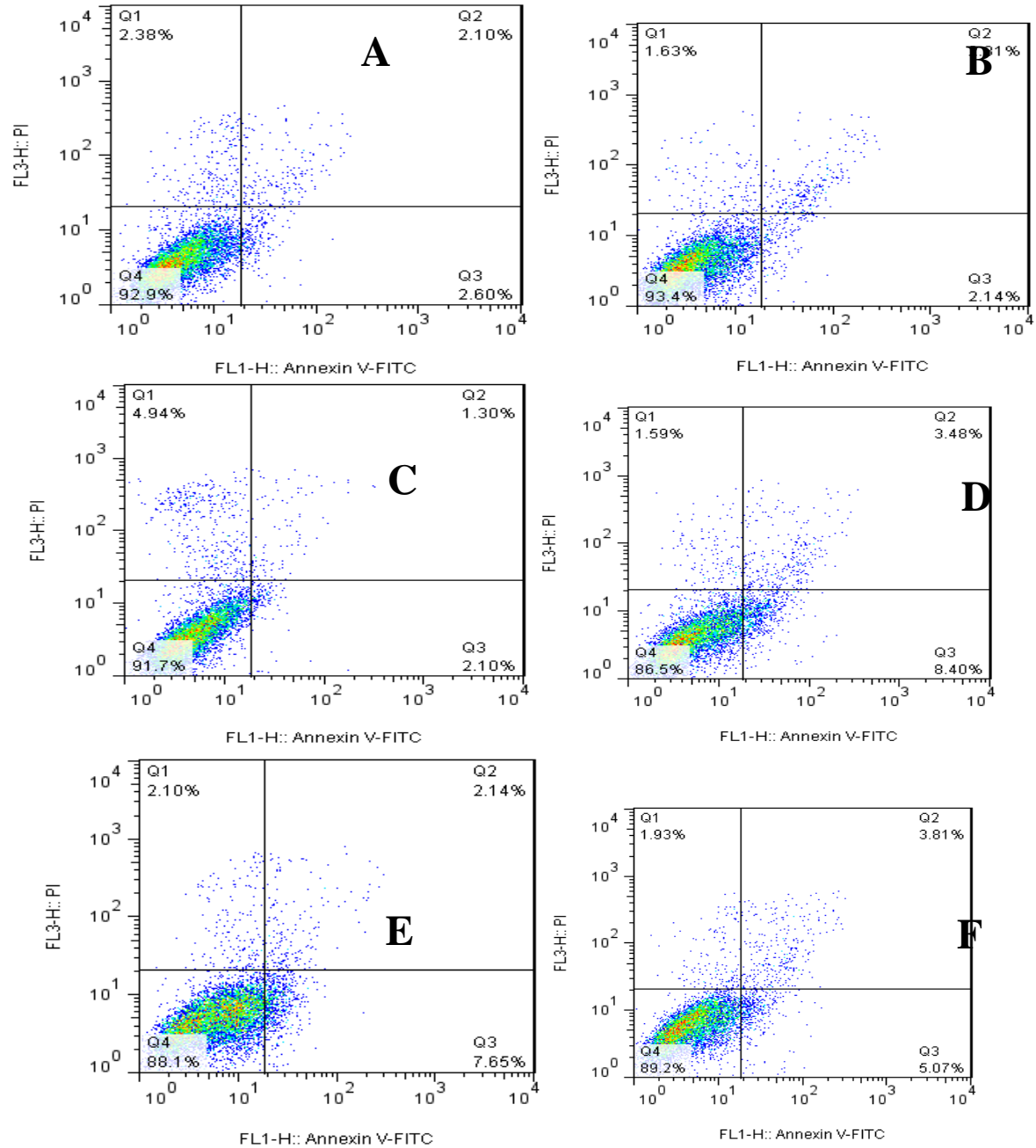


Figure 4. The scatterplot of Annexin/PI staining test where A375 melanoma cells were exposed to different concentrations and time treatments as follows **A:** 24 h control, **B:** 48 h control, **C:** 72 h control, **D:** Treated with 166.7 μ M nicotinic acid complexed with omega-3 after 24 h, **E:** Treated with 144.2 μ M nicotinic acid complexed with omega-3 after 48 h, **F:** Treated with 146.1 μ M nicotinic acid complexed with omega-3 after 72 h.

PI: propidium iodide

FITC: fluorescein 5-isothiocyanate

FL1-H: flow cytometry (x-axis)

FL2-H: flow cytometry (y-axis)

Q1, Q2, Q3 and Q4: quarter

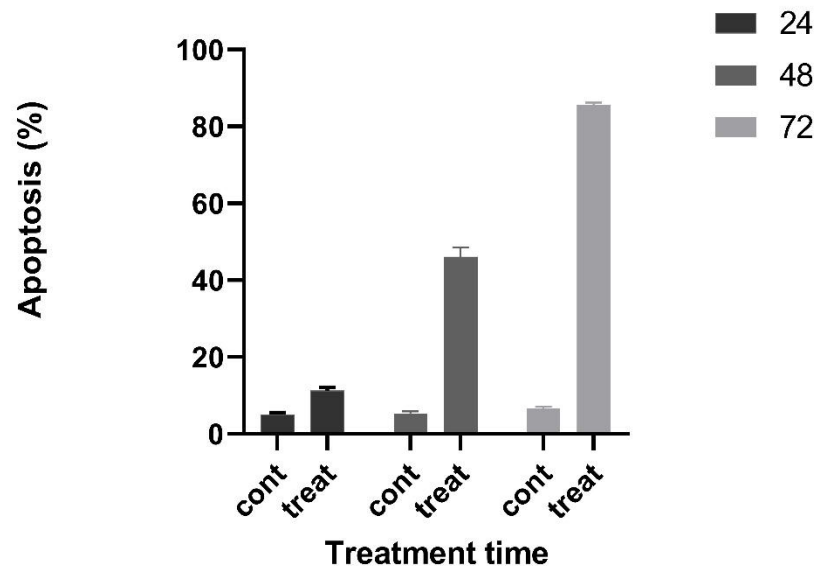


Figure 5. This figure shows the analysis of flow cytometry results after 24 h, 48 h, and 72 h indicated notable differences between cont (control) and treat (treated) cell lines.

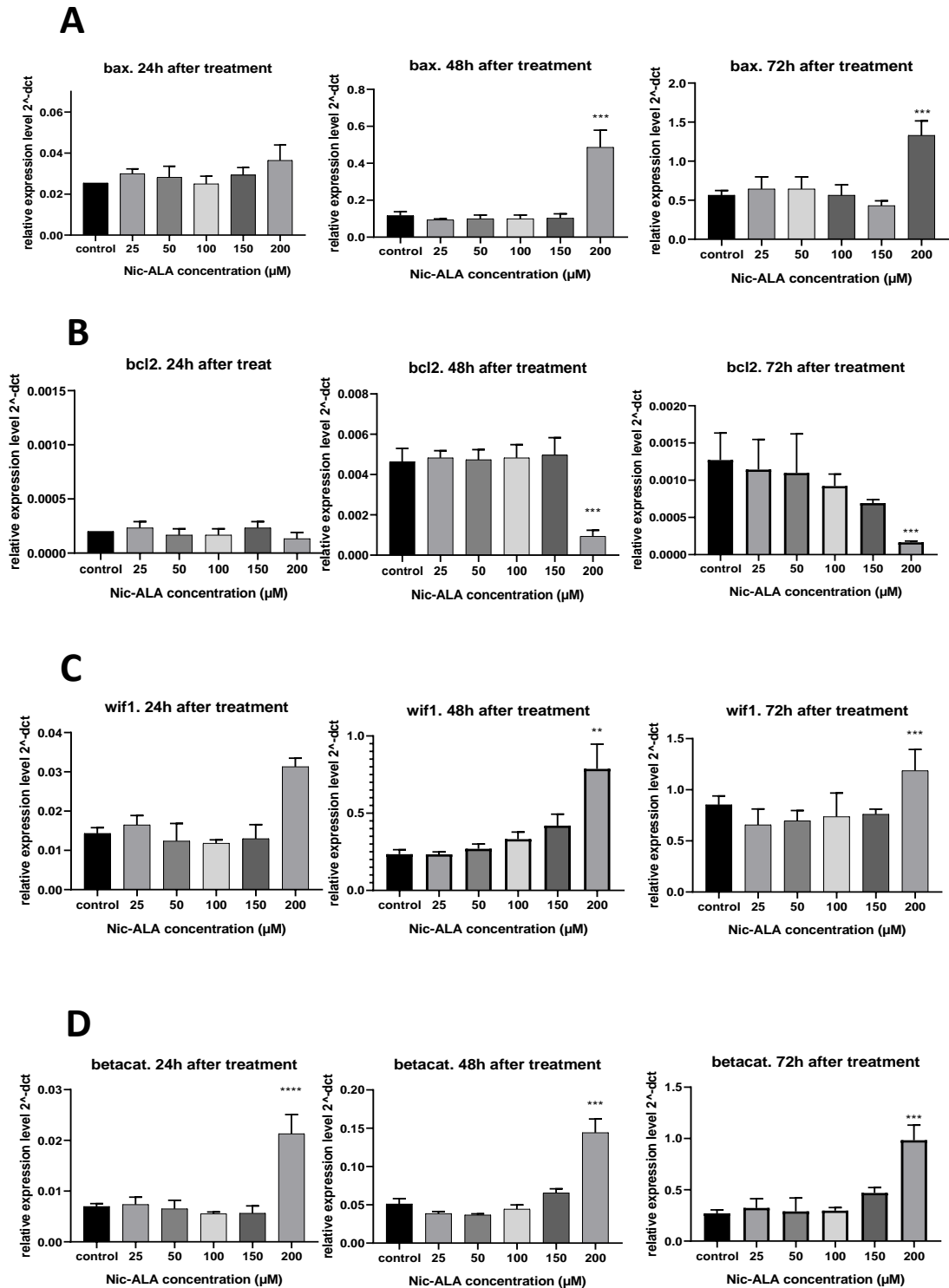


Figure 6. This figure shows the effect of different concentrations of omega-3,omega-6, and nicotinic acid on the expression level of **A. Bax**, **B. Bcl-2**, **C. Beta-catenin**, and **D. WIF1** after 24,48, and 72h.

Bax,: Bcl-2 Associated X-protein

Bcl-2: B-cell lymphoma 2

WIF1: Wnt Inhibitory Factor 1