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Assessment of the Regulatory Effect of Novel Indole-Core-Based Compound on Apoptosis and Cell Survival of Acute Myeloid Leukemia Cell Line

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Abstract

Background: Heterocyclic compounds are generally introduced as important valuable sources of pharmacologically active compounds. Amongst these compounds, the indole is largely distributed within the bioactive molecules, containing antitumor agents. Due to their unique physiochemical and biological properties, indole and its derivatives have been used as privileged scaffolds for designing antitumor agents.
Objectives: The current experimental study aimed to evaluate the anti-cancer effects of a novel compound with indole-core-base on acute myeloid leukemia (AML) cells.
Methods: Following being cultured, AML cells that had been multiplicities were treated by the demonstrated concentration of novel indole compounds (at doses of 100-300µg/mL) for 24 h. The percentage of living and dead cells was subsequently determined by trypan bluedye (MERK, Germany). The survival rate of treated cells was also examined by MTT assay. The calculated fold changes of the studied genes expression against *β-actin* were determined by the real-time polymerase chain reaction (PCR) technique. The collected data were statistically analyzed by student t-test and repeated measure test.
Results: Results showed that the intended novel indole-core-based derivative (C18H10N2F6O) followed both dose-dependent cytotoxic and anti-proliferative patterns on the AML cell line. The compound was able to induce apoptosis in 50% of the cells at the dose of 250µg/mL. Real-time PCR analysis indicated that in compound-treated cells the gene expression level of Bcl-2 has been downregulated, while *Bax* was upregulated, compared to untreated control cells.
Conclusion: Despite the lack of knowledge in this regard, in this study, results of this leading mechanism(s) that may run by the compound indicated that indole3carbaldehyde derivative has cytotoxic effects on AML cells in a dose-dependent fashion.

Keywords: AML, Apoptosis, C18H10N2F6O, Indole, Leukemia

1. Background

Leukemia is characterized as the cancer of hematopoietic tissues, such as bone marrow and the lymphatic system. There exist several equivocations about the diagnosis and treatment of these disorders (1). Leukemia is clinically divided into two categories, namely chronic and acute. They are classified into two main subclasses based on their cellular origins; accordingly, the hematological malignancy is of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) (2). The AML is a heterogeneous hematological disorder that immortally implicates myeloid precursors and is the most frequent leukemia in the early months of life (3,4).

Acute promyelocytic leukemia (APL) is one of the AML subgroups which occurs at the age of 40-50 years and comprises 10-15% of total AMLs. Regularly, APL is caused by a myeloid lineage maturation defect that leads to the association of immature cells called promyelocyte. Nowadays, several types of therapeutic strategies, such as surgery, radiation, and chemotherapy, are introduced for AML. Since chemotherapy is associated with drug resistance and recurrence and is accompanied by toxicity effects on normal cells (5), this study aimed to investigate the efficacy of other compounds, like indole derivatives on AML cell lines (APL cells).

Heterocycles are noticeable for their biological effects and redundancy in natural products (6). Indole is one of these compounds with anticancer effects. Indoles are among the strongest elements of plants that affect cancers and other disorders (7,8). It is well fitted into the category of aromatic heterocyclic organic compounds waving a bicyclic structure, containing a six-membered ring fusing with a five-membered nitrogen-containing pyrrole ring. Epidemiological reviews alongside animal-based studies have reported the beneficial and valuable effects of some novel indole-core-based reagents against tumorigenesis (9).

Indole derivatives have been reported to be able

to limit the proliferation of several cancer cell lines, including breast (10-13), colon (14,15), prostate (16,17), and endometrium (10) (18-22).

2. Objectives

Therefore, the present study aimed to examine pro- and anti-proliferative or apoptotic effects of indole-3-carboxaldehyde, as a newly synthesized indole-derivative (Figure 1.), on AML cells to investigate its efficacy on cellular apoptosis of these cells.

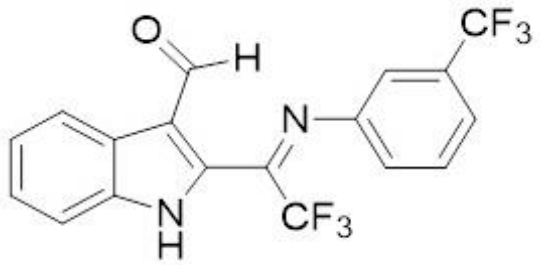


Figure 1. Chemical structure of C18H10N2F6O.

3. Methods

This case study was conducted in the laboratory of Kerman University of Medical Science, Kerman, Iran. The research and its tool were approved by the Research Ethics Committee of Kerman University of Medical Sciences, and ethical considerations were observed during the research.

3.1. Cell culture

The NB4 cell line as an AML-originated and lined cell was purchased from the Pasteur Institute of Iran (Tehran, Iran). The cells were simply cultured into RPMI 1640 media (Shellmax, Iran) which was supplemented with 10% fetal bovine serum (Gibco-BRI, USA) and 2% penicillin/streptomycin (Shellmax, Iran) in an atmosphere of CO2 (5%) and O2 (95%) at 37 ºC temperature.

3.2. Compound treatment

Once the cells reached the appropriate confluence (nearby70%), they were treated by the novel indole-core-based derivative (C18H10N2F6O) (Figure. 1) with various concentrations of 100, 150, 200, 250, and 300 µg/mL. The drug stoke was prepared with 100 µL dimethyl sulfoxide (DMSO) (MERK, Germany) as a solvent. Afterward, the working solution was made by the addition of 900 µL RPMI media. For treatment, the cells were cultured in six well plates (SPL, South Korea). It should be mentioned that different doses were applied.

3.3. Assessment of cell viability with trypan blue

After 24 h of treatment, trypan blue was employed for checking the viability of the cells basically via the exclusion test. The dye (50µL) was added to 50 µL of the cell suspension (in 1:1 ratio) and the living cells were counted using Neubauer chamber as they did not absorb trypan blue dye. The number of living cells was divided by the total number of cells and multiplied by 100 to calculate the percentage of total living cells. It should be noted that the experiment was performed in triplicate.

(Living cells/total cells)×100=percentage of the living cells

3.4. MTT assay

The cellular metabolic activity was examined by MTT assay. For this purpose, the plate and cells were treated by intended doses in a 96-well plate, and the MTT powder (Sigma Aldrich, Germany) was added after 24 h. The MTT solution was prepared in 10 mg/mL phosphate-buffer saline concentration. A volume of 100 µL of the resultant MTT solution was added to each well of the plate and the plate was covered properly by aluminum foil.

In the next step, 4 h after the incubation, the treated plates were centrifuged at 4000 rpm for 7 min. The resultant supernatant liquid was drained out and 100 µL of DMSO was further added to each well and pipetted for proper mixture and dilution of formazan crystals. In the next step, the light absorbance was determined by a spectrophotometer at 570 nm wavelength. Therefore, the cytotoxic dose in which the compound was able to destroy 50% of the cells (the IC50 dose) was evaluated. Analyses were performed in triplicate for each dose and the three following types of controls were also set: 1) cells only, 2) cells+DMSO (10%), and 3) cells+DMSO+indole (for only 200, 250, and 300 µg/mL doses). Accordingly, the toxicity of DMSO and indole alone was also investigated (23).

3.5. Assessment of apoptosis by annexin V and PI flow cytometry

Annexin V is a sensitive marker for the assessment of apoptotic cells which indicates the expression of phosphatidylserine on their surface. PI is a marker for the differentiation of apoptotic cells from necrotic ones which colors the nucleus. The next steps were performed using IC50 dose (250) alongside the control specimen. A number of 1×10^6 cells were seeded in 1 mL of phosphate-buffered saline in test tubes. These cells were treated with an IC50 dose (250 µg/ml) for 24 h. It should be noted that the experiments were performed in triplicate.

3.6. Extraction of total RNA and cDNA of generation

The cellular total RNA content of cultured NB4 cells was extracted by Trizol reagent (Invitrogen, USA) based on the guidelines of the manufacturer. The RNA concentrations were determined by measuring the absorbance at 260/280 nm using a Nanodrop spectrophotometer.

3.7. cDNA Synthesis

The cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Thermo #K1622). Following completion of the cDNA synthesis processes, the resultant cDNA was stored at −20 °C until use.

3.8. Quantitative real-time polymerase chain reaction and gene expression

The expression levels of target genes (Table 1) were analyzed by the application of SYBR Green I Master Mix polymerase chain reaction (PCR) (Genet Bio, Korea) using an ABI real-time PCR (RT-PCR) System (Applied Biosystems, USA). The Vector NTI software was also used to design the specific primers for *Bcl-2*, *Bax*, and *β-actin* which were used as an endogenous control.

3.9. Statistical analysis

The collected data were statistically analyzed in SPSS software (version 18). All the experiments were performed three times for each individual sample and all of the obtained results were presented as the mean value of those three experiments. The data were also analyzed using student t-test and repeated measures while a p-value of less than 0.05 was considered statistically significant.

4. Results

4.1. Trypan blue assay results

The results indicated that the cell viability was considerably reduced in a dose-dependent fashion. The results of cell survival after 24 h of treatment by trypan blue are shown in Figure 2.

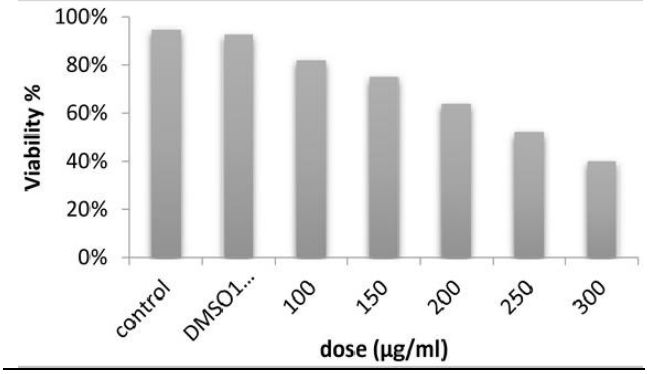


Figure 2. Results of cell survival concentration after 24 h of treatment by trypan blue. The results show that cell viability has reduced depending on dose amplification. All changes observed in the cell viability are significant (P<0.05).

4.2. MTT assay results

Results of the MTT assay indicated that at the dose of 250 µg/ml, the novel indole derivative had cytotoxic effects which killed 51% of the AML cells. Overall, based on the results of the MTT assay, the cell viability was reduced in a dose-dependent fashion after 24 h of treatment (Figure 3).

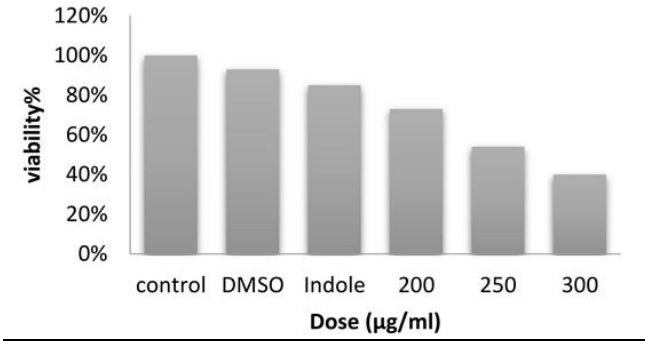
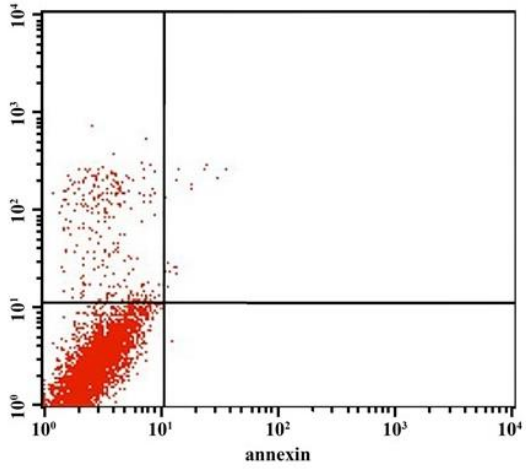


Figure 3. MTT assay results after 24 h. The MTT assay results illustrate that in 250 µg/mL dose, indole derivative had cytotoxic effects which killed 51% of AML cells. The results show that cell viability reduced in a dose-dependent fashion.

4.3. Flow cytometry results

After the statistical analysis of the data, the flow cytometry results revealed that increased apoptosis caused by the effect of the C18H10N2F6O compound was significant after 24 h, compared to the control group (Figure 4).



Gate:G1 Total Events:10000

Quad	Events	%Gated	%Total	X Mean	Y Mean
UL	252	5.54	2.52	3.87	98.36
UR	16	0.35	0.16	17.12	127.43
LL	4276	94.08	42.76	3.05	3.12
LR	1	0.02	0.01	12.52	4.45

Figure 4. Flow cytometric cell viability and apoptosis. Based on the statistical analysis, the flow cytometry data revealed that an increase in apoptosis caused by the effect of the C18H10N2F6O compound was significant at 24 h, compared to the untreated control group. After the treatment, the cells were stained with Annexin V and propidium iodide and then the apoptotic effect of the indole-core-based novel on cells was detected by flow cytometry. Flow cytometry results for IC50 dose (left picture) and the control (right picture). UL: upper-left, UR: upper-right, LR: lower-right, LL: lower-left.

4.4. Amplification curves of cDNA samples of Bcl-2 and β-actin

Amplification and melting curve analysis of quantitative RT-PCR for MICAL2, *Bax*, *Bcl*-2, and *β-actin* genes indicated that the amplification was performed in an appropriate manner (Figure 5).

4.5. Melting curves of real-time polymerase chain reaction

According to the results of the melting curve for each separate primer, they acted as a special shape and also demonstrated any non-especial patches and secondary structures (Figure 6)

4.6. Effect of C18H10N2F6O on the expression of Bax and Bcl-2 genes

The quantitative RT-PCR data revealed that the expression of *Bax* was substantially increased in the AML cells treated with C18H10N2F6O for 24 h, compared to the untreated control cells (P<0.05) (Figure 7a). Inversely, the quantitative RT-PCR data revealed that the expression of *Bcl-2* was substantially decreased in AML cells treated to untreated C18H10N2F6O for 24 h, compared with control cells. This is indicative of the fact that the expressions of both *Bax* and *Bcl-2* follow a time-dependent pattern (P<0.05) (Figure 7b).

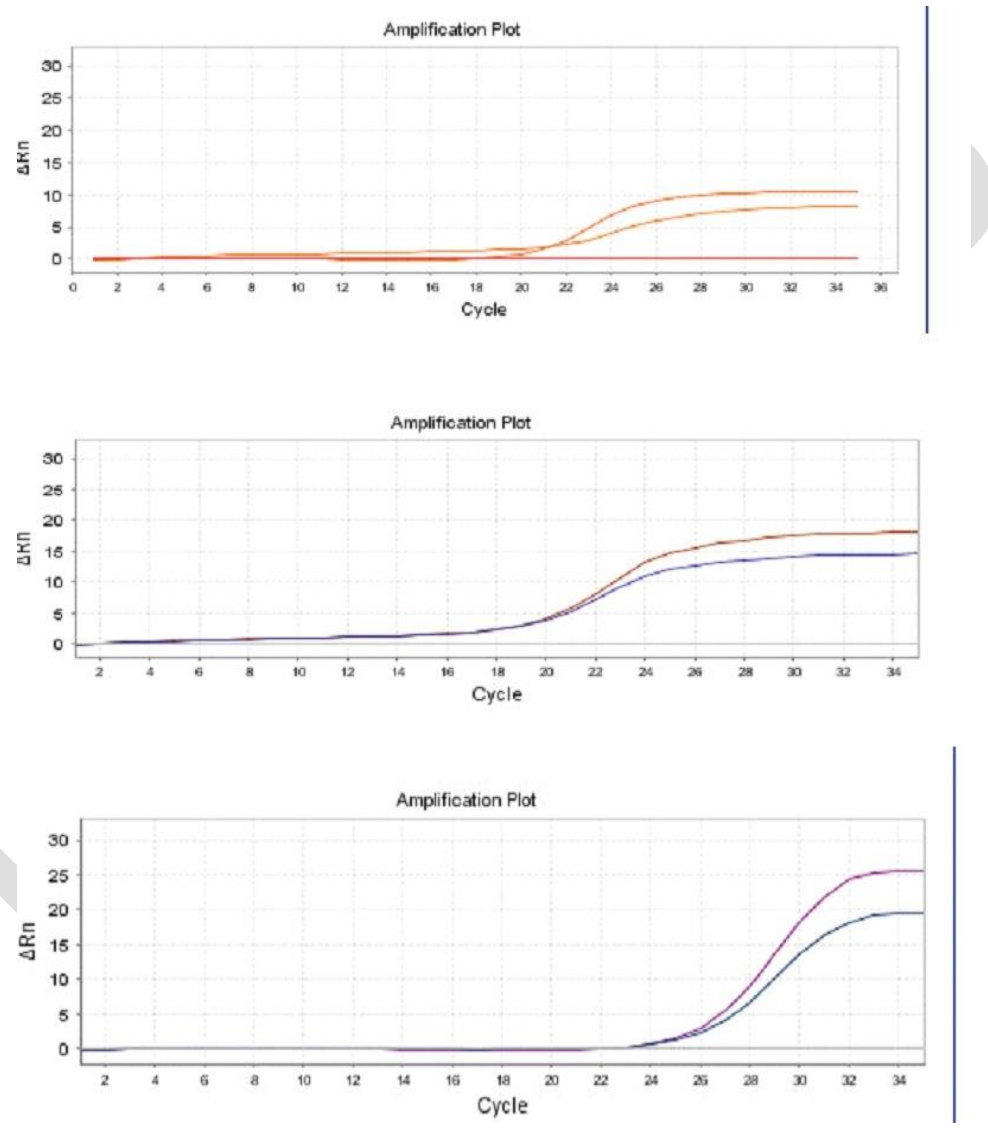


Figure 5. Diagram amplification and melting curve analysis of the real-time polymerase chain reaction analysis for Bax, Bcl-2, and β-actin genes.
a.Diagram of β-actin gene proliferation before and after 24-h treatment with indole.
b. Diagram of Bax gene amplification before and after 24-h treatment with indole
c.Diagram of Bax gene amplification before and after 24-h treatment with indole

Table 1. Sequences of the e mployed primers in this study

Primer	Forward (5'→3')	Reverse (5'→3')
Bcl-2	GGTGAACTGGGGGAGGATTG	CGTACAGTTCCACAAAGGCATC
Bax	AGGATGCGTCCACCAAGAAG	CGGCCCCAGTTGAAGTTGC
β-actin	GGGCATGGGTCAGAAGGATT	CGCAGCTCATTGTAGAAGGT

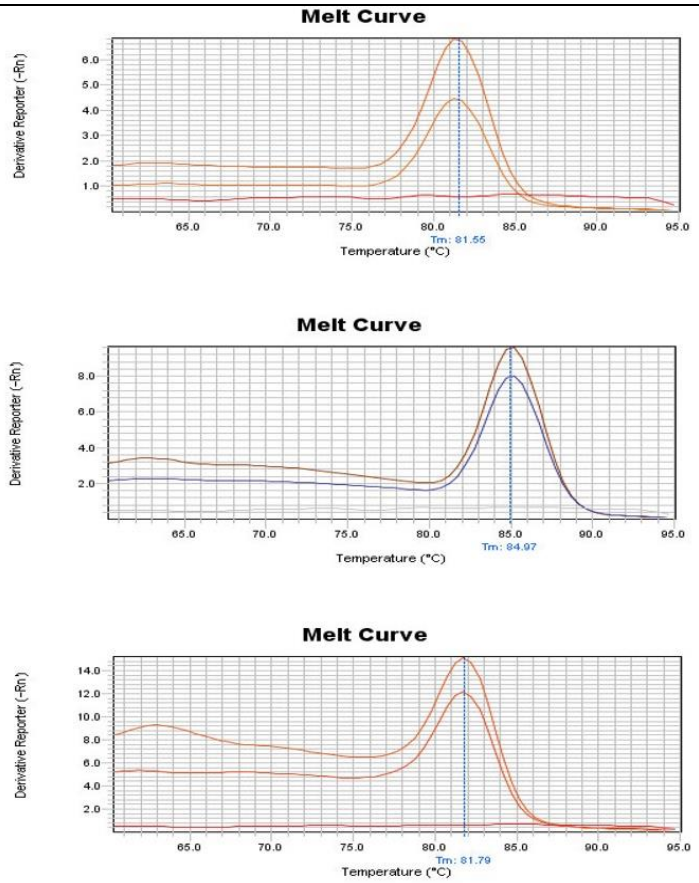


Figure 6. *β-actin* melting curve before and after 24-h treatment with novel indole-core-based compound
a.*Bax* melting curve before and after 24-h treatment with novel indole-core-based compound
b. *Bcl-2* melting curve before and after 24-h treatment with novel indole-core-based compound

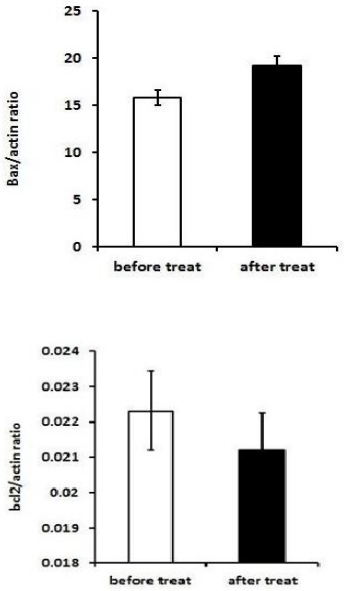


Figure 7. A. Results of *Bax* gene expression, as an anti-apoptotic gene before and after indole derivative treatment for 24 h, compared to control cells at IC50 dose by real-time polymerase chain reaction.
B. Diagram of changes in *Bcl-2* gene expression as an anti-apoptotic gene before and after indole derivative treatment for 24 h, compared to control cells at IC50 dose by real-time polymerase chain reaction.

5. Discussion

As mentioned in the introduction, the blood malignancy of APL is one of the AML subgroups which was primarily named AML-M3 in French-American-British classification (24). Initially, daunorubicin (1973) was used as a chemotherapy reagent for APL patients. Thereafter, anthracycline combination (daunorubicin, idarubicin, and Ara-C) was introduced as the first-line treatment for APL. Moreover, in 1985, all-trans retinoic acid (ATRA) was released to the market as a new treatment for APL (25).

In early 1990, ATO prescription led to more satisfactory therapy of refractory and relapsed patients in addition to patients with primary APL (24). A therapeutic protocol was also established by the combination of ATRA and ATO which has resulted in increased survival of the patients with the primary diagnosis of APL (25). The ATO therapy protocol was successful; however, the further possibility of relapse was reported in some APL cases (26). The arsenic was also applied as a beneficial agent in relapse cases of APL (27); nonetheless, arsenic was not able to induce apoptosis on its own at lower doses, and it is very toxic and harmful for the healthy tissues (28).

In general, indole derivatives fit within the most important heterocyclic compounds in the field of drug-discovery studies. They are well known as a very important category of molecules, playing very important parts in cell biology. These main indole derivatives have been the subject of several research projects in multiple fields varying from the usage of bioactive molecules against microbes and cancer cells to various types of disorders in human studies worldwide. These types of indole inhibitory effects are regulated through the initiation of several intracellular signaling pathways.

Production of novel compounds with anticancer properties is a procedure through which cancer therapy may be achieved (29). Comprehension of the background molecular pathways that are involved and co-operate in the development of leukemia might possibly lead to the development of new therapeutic protocols to improve the survival rate of patients (30). The *BCL-2* has been reported to be overexpressed in the AML subtypes of leukemia which might mean that it plays a fundamental role in both pathogeneses and the development of AML. Accordingly, as *BCL-2* is the most well-known member of the paramount family members of apoptosis, gene group, and recent pieces of evidence have indicated that these family proteins, such as *Bax*, also play key roles in this phenomenon. (30).

The present study examined the effect of C18H10N2F6O on *Bax* and *BCL-2* expression levels in AML cells. The results revealed that in 250 µg/mL dose, the indole derivative exhibited cytotoxic effects which resulted in killing 51% of the AML cells. These findings can confirm that the desired reduction of cellular viability is probably due to the fact that the viability reduction was associated with the compound in a dose-dependent fashion. It should be mentioned that this was also statistically confirmed by the RT-PCR. The RT-PCR revealed that the expression of *Bax* was substantially increased in AML cells treated with C18H10N2F6O, compared to the untreated control cells after 24 h.

These achievements are possibly indicative of the fact that in addition to the dose, it has followed a time-dependent pattern as well (31). The anti-tumor properties of C18H10N2F6O are likely to be linked to its antioxidant activities. It has also been well established that this synthetic compound can prevent the activation of several enzymes and signals varying from cyclooxygenase, hydroperoxidase, protein kinase C, *BCL-2* phosphorylation, Akt, a kinase binding position (focal adhesion kinase) to nuclear factor-kappa B (NF-κB), matrix metalloprotease-9, and cell cycle regulators.

Jazirehi and Bonavida (32) and Wei XN have indicated that I3C has displayed inhibitory effects on protein expression of NF-κB pathway by employing a protein array method (33). In another research program, the researchers found that I3C induced the protein expression of caspase 8. Overall, these results may indicate that the inhibition of the NF-κB pathway along with stimulation caspase 8 might be involved in the pro-apoptotic effect of I3C. In the TNF-α signaling pathway, the RIP1 is at the top of the upstream targets of the NF-κB signal. The K63 ubiquitination state also acts as a cell-death switch that responds to TNF signaling. The RIP1 ubiquitination is achieved by K63 and, in turn, the NF-κB survival pathway, whereas inversely, de-ubiquitinated RIP1 leads to caspase 8 stimulation and further triggers apoptosis (33).

It is now revealed that apoptosis plays a key role in the response to chemotherapy, which suggests a relationship between drug-induced apoptosis and therapeutic efficiency in leukemic patients (34) In this regard, the analysis of anti-apoptotic and pro-apoptotic genes can increase our knowledge of the key prognostic tool for expecting resistance to chemotherapy compounds (35). An alteration was reported in Bcl-2 (as an anti-apoptotic gene) and *Bax* (as a pro-apoptotic) genes expressions (by *Bax* and Bcl-2 ratio) subsequent to the stimulation of programmed death by some of the indole combinations in NB4 cell lines.

Finally, it can be concluded that this indole-core-based novel derivative (C_{18} H_{10} N_2F_6O) may have antiproliferative and cytotoxic effects by inducing the apoptosis of AML cells. These effects are related to the different components of drugs, such as CF_3. Accordingly, we believe that indole-3-carbaldehyde derivative could presumably be considered a multi-targeted agent that appears to serve as a useful tool either as a single agent and/or as a combined formulation alongside conventional chemotherapeutic

reagents to limit the processes of tumor progression in the treatment of human malignancies. However, we also believe that a future in-depth mechanistic *in vitro* research program, homologue to *in vivo* animal models and rationally designed novel clinical trials are highly needed for the better understanding of the value of these "natural products" in the field of treatment of human malignancies.

6. Conclusion

This project was limited due to financial issues and more funding was needed to examine the mechanisms involved in the effects of this novel derivative on apoptosis of AML-NB4 cells. For future studies, the authors are planning to use this derivative in *in vivo* models on animal/rodent models of leukemia.

Acknowledgments

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Footnotes

Conflicts of Interest: The authors declare no conflict of interest, financial or otherwise.
Ethics Approval and Consent to Participate: Not applicable.
Human and Animal Rights: No animals/humans were used for studies that are the basis of this research.
Consent for Publication: Not applicable.
Availability of Data and Materials: Not applicable.
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