Immunology Letters 190 (2017) 7–14

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Evaluating of OCT-4 and NANOG was differentially regulated by a new derivative indole in leukemia cell line

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ARTICLE INFO

Keywords:
NANOG
OCT4
Antitumor
Indole-3-carbaldehyde
Treatment

ABSTRACT

Background: The potential exists to improve treatment through characterization of tumor stem cells and identification of therapeutic targets Using OCT-4 and NANOG genes. Here we have synthesized and investigated the potential of; New Indole-3-carbaldehyde derivative (NI-3-CD) in inhibiting the expression of self-renewal regulatory factors and cancer stem cell gene in a leukemia cell line NB4.

Methodology: The NB4 cells were cultured in RPMI1640 medium contained NI-3-CD and I3F (15.12–1000 μg/mL) for 24, 48 and 72 h. Inhibition of cell proliferation was assessed by trypan blue staining technique and MTT assay. The percentage of apoptotic cells was determined by flow cytometry analysis using Annexin V/PI apoptosis detection kit. The fold changes of NANOG/OCT4 expression against β-actin were determined by real-time-PCR technique. Western blotting analysis was also applied for evaluating the expression of NANOG/OCT4 at protein level. Data were analyzed by student t and repeated measure tests. Differences were considered significant if ($P < 0.01$).

Results: There was a significant difference in cell viability, when various concentrations of NI-3- were used for 24, 48 and 72 h in comparison to I3C regarding the cellular viability. Furthermore, the NI-3-CD, had markedly elevated anticancer activity than I3C (IC50 values for novel I3C in 24, 48 and 72 h were 225.77, 123.13 and 63.72 M respectively while for I3C were 728.05, 407.82 and 277.92 M respectively). Flow cytometry results exhibited an obviously significant augmentation in apoptotic NB4 cells. Real Time- PCR analysis indicated that the expression of NANOG/OCT4 was down regulated in compare to untreated control cells and I3C treated cells ($P < 0.05$). In concert with RT-PCR, western blot analysis showed that the OCT4 expression in NI-3-CD treated cells was also significantly decreased in compare to both untreated control cells and I3C treated cellular populations.

Conclusion: Our results imply that NI-3-CD treatment decreases the sphere-forming ability of NB4 cells. In summary, this study provides valuable information on the presence of stem-cell genes expression in NB4 cells.

1. Introduction

APL is described as an AML, subset accounting for 10–15% of all AML cases [1]. APL is characterized by the selective expansion of immature hematopoietic precursors inhibited at the promyelocytic stage [1]. Cancer stem cells was been reported to have similarities with stem cells, in multiple aspects, such as exhibiting self-renewal, immortality and differentiation. The concept of cancer stem cell concerns the fact that only a subset of cancer cells represent the unique ability of self-renewal its accompanied by unlimited proliferation [2,3]. However, the

Abbreviations: NI-3-CD, new indole-3-carbaldehyde derivative; I3C, indole-3-carbaldehyde; APL, acute promyelocytic leukemia; AML, aacute myeloid leukemia; Oct-4, octamer-binding transcription factor 4; ES, eembryonic stem; HNSCC, head and neck squamous cell carcinoma; NB4, cell line name; DMSO, dimethyl sulfoxide; NI-3-CD, 2-(1((3,5-bis(trifluoromethyl) phenyl)imino)-2,2,2-trifluoroethyl)-1H- indole-3-carbaldehyde; MTT, 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide; mRNA, messenger RNA; PVDF, polyvinylidene fluoride; RIPA, radio immune precipitation; IgG, immunoglobulin G; HRP, horseradish peroxidase; ECL, emitter-coupled logic; UL, up-left; UR, up-right; LR, low-right; LL, low-left

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http://dx.doi.org/10.1016/j.imlet.2017.06.012
Received 3 May 2017; Received in revised form 11 June 2017; Accepted 28 June 2017
Available online 08 July 2017

descriptions of these cells are restricted, due to their small number and lack of appropriate markers. The OCT-4 and NANOG which serve as pluripotent-asocial factors are the key regulators of self-renewal in embryonic and induce pluripotent features of stem cells. The POU (Pit-Oct-Unc) transcription factor family, OCT-4 (also known as OCT-3, OCT-3/4 and POU5F1), is a key regulator of self-renewal and differentiation in embryonic stem cells [4,5]. Expression of OCT-4 is claimed to be restricted to pluripotent cells, and its expression down regulates with the onset of differentiation and loss of pluripotency in these cells [6,7]. The OCT-4 expression was initially reported in early cleavage stage, inner cell mass, primitive ectoderm, primordial germ cells [8] and also in embryonic stem (ES), embryonic germ and embryonic carcinoma cells.6 In 1992, a low level of OCT-4 expression in human adult tissues was observed by Takeda and co-workers [9] but the significance of the findings was to be elucidated until a recent report was released by Tai and colleagues [10]. The latter report demonstrated the expression of OCT-4 at both mRNA and protein levels in several human tissue-specific adult stem cells. NANOG is another key transcription factor which is believed Tobe involved in the maintenance of pluripotency and self-renewaling of undifferentiated ES cells [11]. However, NANOG-mediated molecular mechanisms in HNSCC still remain identified. There are a number of studies showing that the expression of these genes is potentially associated with tumor behavior and therapotic resistance [12–14]. Therefore, the selection of anti-cancer drugs targeting stem-like populations in a heterogeneous cancer cell population is of importance. Targeting of tumor-initiator cells propose that disruption of stemless-related gene expression down-regulates the epithelial – mesenchymal transition, resulting in less invasiveness and increased chemo sensitivity [15,16]. Investigations performed on various cell lines have indicated that the phytochemical I3C has potent antiproliferative activities [17–19], which makes it as a promising potential therapeutic for therapy of human cancers. Current pre- clinical evidences are in favor of the translational advantages of I3C in cancer development as a therapeutic target. To synthesis the I3C derivatives, the final structures were changed so drastically that the final yields more closely resembles self-condensation products of I3C [20], proposing that these biological activities were not only related to the parental compound of I3C. Synthesizing the new compounds for anticancer properties is a methods by which may approach cancer treatment [21]. To achieve this, addition the CF3 groups to the compound elevating its antitumor activity, as well as stabilize the combination and hence is leading to important biological properties. In the present study, we demonstrated that the effects of this NI-3-CD are not limited for inducing apoptosis in cancer cells. Once lower doses, NI-3-CD was shown to induce a decrease in the expression of stem-cell-related markers. These stem cell markers are associated with pluripotency, such as NANOG, OCT-4. Therefore, we aimed to evaluate the effects of NI-3-CD in reducing the NB4 cells population. Evaluation of the anti-leukemic efficacy and safety of NI-3-CD may offer interesting clinical opportunities for AML treatment and possibly other types of malignancies. The expression of related gene and proteins NANOG and OCT4 were also investigated to explore possible molecular mechanistic events.

2. Materials and methods

2.1. Preparation of 2-(1-((3,5-bis(trifluoromethyl)phenyl)imino)-2,2,2-trifluoroethyl)-1H-indole-3-carbaldehyde

The chemical compound was synthesized according the following reported procedure [22]. In an experimental recipe, a dry, two-necked, 50 mL round-bottomed flask was equipped with a nitrogen inlet and charged with 5 mL of dry acetonitrile, 0.145 g (1.0 mmol) of indole-3-carbaldehyde,and 0.24 g (1.0 mmol) of NaH. The yielded solution was then stirred under nitrogen atmosphere at room temperature for 30 min, and subsequently a solution of *N*-(3,5-bis(trifluoromethyl) phenyl)-2,2,2-trifluoroacetimidoyl chloride (1.0 mmol) was dropwise and gently mixed up via syringe. The mixture was stirred at room temperature for a longer duration of 20 h under N_2 atmosphere and was then filtered into another fresh flask. After removing the solvent under reduced pressure the crude product was purified by recrystallized from ethanol (twice) give the resultant product.

This compound was obtained as white solid with following chames tizations:, M.P = 78–80 °C, Yield = 89%, FT-IR (KBr) υ_{max} = 1698, 1673, 1557 cm^{-1}. ^{1}H-NMR (DMSO-d_6 500 MHz) δ = 10.11 (s, 1H), 9.94 (s, 1H), 8.21 (s, 2H), 8.05 (d, 1H), 7.92 (d, 1H), 7.51 (d, 1H), 7.26 (m, 2H) ppm. Anal. Calcd for $C_{19}H_9F_9N_2O$ (452.27): C, 50.46; H, 2.01; N, 6.19%. Found: C, 50.17; H, 1.94; N, 6.24%.

Table 1
Indicates the sequences of the employed primers in this study.

Primer	Forward (5′ → 3′)	Reverse (5′ → 3′)
OCT4	GATCCTCGGACCTGGCTAAG	CCACAGAACTCATACGGCG
NANOG	ATGGATCTGCTTATTCAGGACA	TGGAGGCTGAGGTATTTCTGT
β-actin	GGGCATGGGTCAGAAGGATT	CGCAGCTCATTGTAGAAGGT

Table 2
Exhibits the IC50 values (mM) of NI-3-CD and I3C against NB4 cells after 24, 48 and 72 h of treatment.

Indole derivatives	IC50 (mM)		
	24 h	48 h	72 h
NI-3-CD	225.77	123.131	63.72
I3C	728.05	407.82	277.91

2.2. Cell culture

The NB4 cell line was purchased from the National Cell Bank of Iran (NCBI, Tehran, Iran). Cells were cultured in RPMI-1640 medium supplemented with10% heat-inactivated fetal bovine serum (Bio-Rad, San Diego, CA, USA), and 100 IU/ml penicillin and 100 µg/mL streptomycin at 37 °C with, humidified atmosphere, containing 95% O2- 5% CO_2. Upon approaching appropriate confluence, cultured cells were subjected to passage.

2.3. The cell viability assay, using MTT method

This assay determines the MTT [3- (4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide] reduction (Sigma, USA) (employing a colorimetric method). The MTT technique is based on the mitochondrial dehydrogenase to blue formazan product, which reflects the normal activities of mitochondria and therefore the measurement of upcoming cytotoxicity and cell viability. NB4 cells were seeded in a 96-well plate at a density of 1 × 10^4 cells/well. Different concentrations of new indole compound (15.12, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) along with vehicle control (DMSO) and similar doses of basic indole were added to appropriate as wells. Further incubation for 24 and 48, the MTT reagent (5 mg/L) was added to each well and incubation for further 4 h. The supernatant was replaced by DMSO and the relative absorbance was read at 570 nm using a micro plate scanning spectrophotometer (ELISA reader, Bio Tek EIK 808, USA). The numbers of viable cells were calculated with appropriate controls taken into account. The Mean ± SD values are shown from three independent experiments. The inhibition rates were also calculated according to the following formula: Inhibition rate = [(absorbance value of control group − absorbance value of test group)/absorbance value of control group] × 100%.

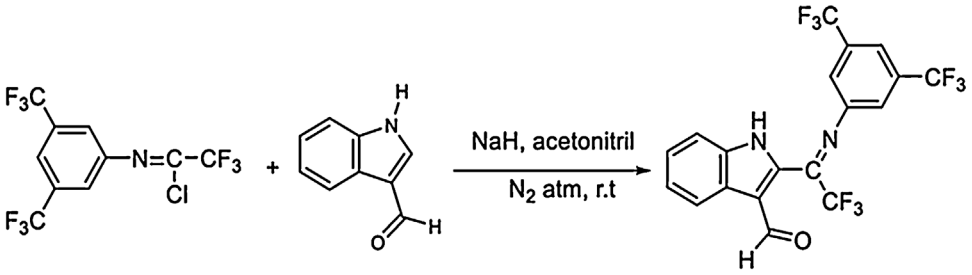


Fig. 1. Chemical structure of NI-3-CD.

2.4. The cell viability assay, using trypan blue method

NB4 cells were seeded onto a 96-well plate at a density of 1×10^4 cells/well. As stated in MTT assay briefly varicose amounts of new indole compound (15.12, 31.25, 62.5, 125, 250, 500 and 1000 μg/mL), similar doses of indole and vehicle control (DMSO) were added to respect wells. Following incubation for 24 and 48 h, the number of alive cells in each well was counted under microscope, using neobar lam. All experiments were repeated three times.

2.5. Flowcytometric based cell analysis of apoptosis

To investigate the event of apoptosis, Annexin V-FITC/PI staining was performed. Following 48 h of incubation with relative concentrations of new indole compound, NB4 cells of different groups were collected and transferred to the 5 ml plastic tubes and, washed twice in cold. The apoptosis was detected using FITC Annexin V/PI Apoptosis Detection Kit (eBioscience, USA) according to the manufacturer's instructions with a flow cytometry machine.

2.6. Total mRNA extraction, cDNA synthetize and real-time PCR

2.6.1. RNA extraction

Total mRNA was extracted from the NB4 cell line using RNeasy Mini Kit (QIAGEN, Hilden, Germany) one step procedure according to manufacturer's instructions (Bioneer, Korea). Following being treated with various concentrations of I3C and novel indole derivative for 24, 48 and 72 h. RNA sample were dissolved in diethylpyrocarbonate (DEPC)-treated water and RNA measures were determined by an APEL PD-303UV spectrophotometer (Japan). The fidelity of extracted RNA was examined by electrophoresis 1% agarose and further staining with DNA Green Viewer™ and three bands with the Gel Doc was seen from each RNA sample (Fig. 5).

2.6.2. cDNA synthetize

Complementary DNA (cDNA) was made using a reverse transcriptase kit (Bioneer, Korea) The cDNA was stored at −20C or immediately used for quantitative real-time polymerase chain reaction. The cDNA was synthesized by Bio-Rad device manufactured by America Hack Company. Subsequently, the converted RNA to cDNA by reverse transcriptase enzyme, it was used as a target in the RT-PCR technique. To do so, we used to 10 μl of DEPC, total RNA, oligo dT, and random hexamer primers. Then, incubated them for 10 min at 70 °C. After this step, 10 μl of RT-mix was added into micro-tube. The mixture was put at 42 °C for 60 for cDNA synthesis. Finally, to inactivate RT-enzymes; the mixture was we left it at 95 °C for 5 min.

2.6.3. Rt-PCR

The gene expression of Bcl-2 and Bax was detected by real time PCR. To achieve this, the deserved of synthesized cDNA was taken at this step and well mixed with master mix contains DNA Taq polymerase. It contains DNA Taq polymerase mixture and allowing let RT-PCR to be permed in 40 cycles to denature the cDNA 96° C for 10 s was used. Relative quantification of gene expression was performed in triplicate. The mRNA expression was determined using. SYBR Premix Ex Taq™ kit (Takara Bio, USA). The primers sequences were demonstrated in Table 1. The β-actin was used as a reference gene and the expression of target genes one relative to its expression. Relative expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ formula.

2.7. Western blotting

As mentioned for other above experiments, subsequent to being treated with relative concentrations of I3C and novel indole derivative obtained for 24, 48 and 72 h, the NB4 cells were lysed in ice-cold radio immune precipitation (RIPA) buffer with protease inhibitors. Briefly, NB4 cells were treated with lysis buffer on ice for 30 min and were centrifuged at 13,000*g* for 30 min at 4 °C. The protein concentration was measured using the bradford method with a Bio-Rad protein assay reagent (Bio-Rad, San Diego, CA, USA). Cellular protein content was electrophoresed on 12% SDS-PAGE and proteins were transferred to Polyvinylidene fluoride (PVDF) membrane. The resultant blots were then blocked with 5% skimmed dry milk in TBST [50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 0.1% Tween-20] for 90 min at room temperature. Membranes were subjected to three times washing with TBST prior to immunoblotting. Primary antibodies: Bax (cat. no 633601; 1:200; monoclonal mouse IgG1 anti-human) (BioLegend; San Diego, USA, β-actin (cat. no. sc-47778; 1:100, monoclonal mouse IgG1 anti-human) (Santa Cruz Biotechnology Inc., USA) were added and the membrane was incubated overnight at 4 °C. Subsequently, the membrane was rinsing with TBST for 30 min, and then incubated with the corresponding secondary antibody [goat anti-mouse immunoglobulin G (IgG)/horseradish peroxidase (HRP); cat. no 405306; 1:1000 (BioLegend; San Diego, USA)] and β-actin [goat anti- Mouse immunoglobulin g (IgG)/horseradish peroxidase (HRP); cat. no sc-516102; 1:1500 (Santa Cruz Biotechnology Inc., USA)] for 1 h. Chemiluminescence was detected with emitter-coupled logic (ECL) Plus (Bio-Rad, San Diego, CA, USA). All experiments were repeated three times.

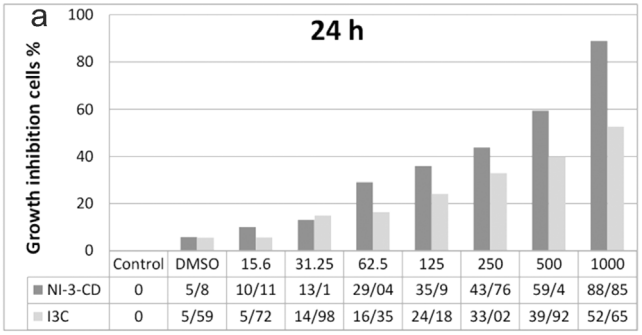
2.8. Statistical analysis

To statistically analysis the data SPSS software (version 21) was applied. All experiments were performed three times in each individual sample and all of obtained results were presented as the mean value of those three. Data were also analyzed by Student *t*-test and repeated measures of a p value less than 0.05 was considered significant (Table 2).

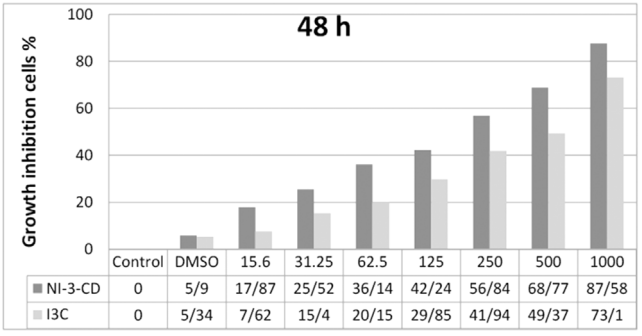
3. Results

3.1. NI-3-CD induced cytotoxicity and anti-proliferative effects of NB4 cells

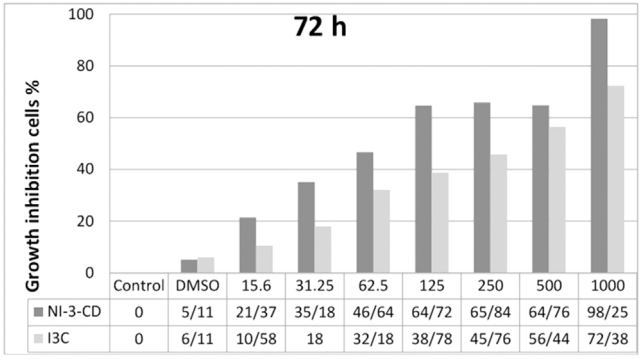
MTT and trypan blue results showed a significant difference among various I3C and NI-3-CD concentrations on increased growth inhibition cells of NB4 cells in 24, 48 and 72 h in a time and concentration dependent fashion ($P < 0.05$) increased growth inhibition cells of NB4 cells in NB4 cells after 24, 48 and 72 h treatment with NI-3-CD compared to I3C were have showed in using MTT method (Fig. 2) and also



	Control	DMSO	15.6	31.25	62.5	125	250	500	1000
NI-3-CD	0	5/8	10/11	13/1	29/04	35/9	43/76	59/4	88/85
I3C	0	5/59	5/72	14/98	16/35	24/18	33/02	39/92	52/65



	Control	DMSO	15.6	31.25	62.5	125	250	500	1000
NI-3-CD	0	5/9	17/87	25/52	36/14	42/24	56/84	68/77	87/58
I3C	0	5/34	7/62	15/4	20/15	29/85	41/94	49/37	73/1



	Control	DMSO	15.6	31.25	62.5	125	250	500	1000
NI-3-CD	0	5/11	21/37	35/18	46/64	64/72	65/84	64/76	98/25
I3C	0	6/11	10/58	18	32/18	38/78	45/76	56/44	72/38

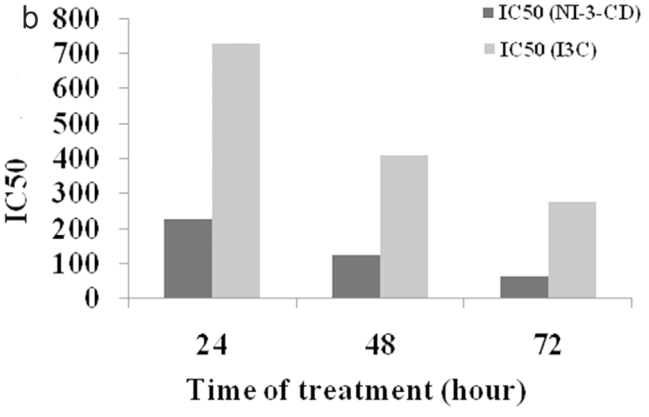
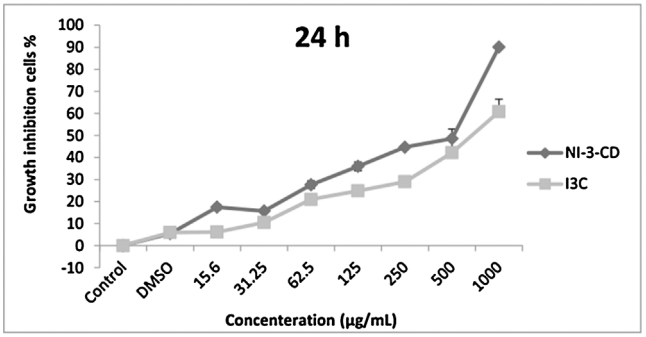
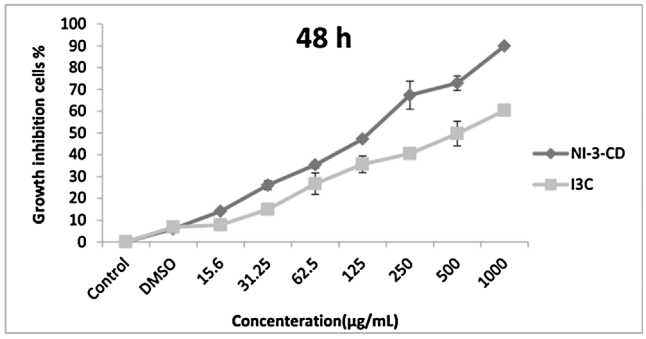


Fig. 2. (a) The growth inhibition in NB4 cells treated with NI-3-CD compared to I3C after 24 h 48 h and 72 h, using MTT method. All changes observed in the cell viability are significant ($P < 0.05$) except those observed DMSO, 62.5 and 1000 μg/mL. (b) Exhibits the IC50 values (mM) of NI-3-CD and I3C against NB4 cells after 24, 48 and 72 h of treatment.

using trypan blue results method (Fig. 3) respectively. The IC50 value evaluated after 24, 48 and 72 h of I3C against NB4 cells was 728.05 μg/mL, 407.82 μg/Ml and 277.91 μg/mL respectively ($p \leq 0.05$). The IC50 value evaluated after 24, 48 and 72 h of NI-3-CD against NB4 cells was 225.77 μg/mL, 123.131 μg/Ml and 63.72 μg/mL respectively ($p \leq 0.05$) (Fig. 2a).





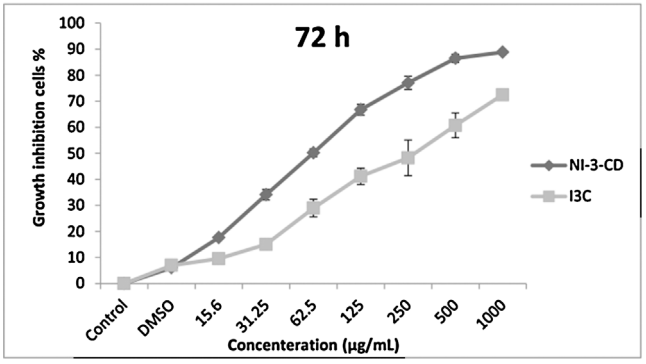


Fig. 3. The growth inhibition in NB4 cells treated with NI-3-CD compared to I3C after 24 h 48 h and 72 h, using trypan blue method. ($P < 0.05$).

3.2. Apoptosis of NB4Cells in response to NI-3-CD and I3C using flow cytometry method

To examine the apoptosis, NB4 cells were treated with I3C and NI-3-CD in IC50 concentrations. further to treatment cells were stained with annexin V and propidium iodide and then the apoptotic effect of I3C and NI-3-CD on NB4 cells was detected by flow cytometry. The Annexin V expression levels of the apoptosis rate after in untreated and treated NB4 cells with I3C and NI-3-CD have been showed in the results of flow cytometry analysis revealed that NI-3-CD induced Apoptosis in treated NB4 cells after 48 h compared to I3C and control (Fig. 4a,b). We have shown the comparison of the mean ± SD between UL (necrotic cells), UR (late apoptotic cells), LL (normal live cells) and LR (early apoptotic cells) in untreated, treated with I3C and treated with NI-3-CD groups using Flow cytometric analysis. Data are the means of three different experiments in Table 3.

3.3. Regulatory effects of I3C and NI-3-CD on OCT4 and NANOG gene expression

Our observations showed that the mRNA expression of both OCT4 and NANOG genes were significantly decreased in NB4 cells treated with NI-3-CD compared to both control cells and cells treated with I3C after 24, 48 h and 72 h (Fig. 6a,b).

Table 3
Demonstrates the comparison of the mean ± SD between UL (necrotic cells), UR (late apoptotic cells), LL (normal live cells) and LR (early apoptotic cells) in untreated, treated with I3C and treated with NI-3-CD groups using Flow cytometric analysis. Data are the means of three different experiments.

Groups		Statistical analysis		
		Mean	Std.Deviation	Result of *T*-Test
(IC50) LL	Control	93.955	1.22	P = 0.007*
	I3C	46.950	0.311	
(IC50) LL	Control	93.955	1.223	P = 0.001*
	2-AITFEI-3-C	46.195	1.166	
(IC50) LL	I3C	46.950	0.311	P = 0.470
	2-AITFEI-3-C	46.195	1.166	
(IC50) LR	Control	0.250	0.056	P = 0.140
	I3C	5.540	1.753	
(IC50) LR	Control	0.250	0.056	P = 0.016*
	2-AITFEI-3-C	34.390	1.202	
(IC50) LR	I3C	5.540	1.753	P = 0.005*
	2-AITFEI-3-C	34.390	1.202	
(IC50) UR	Control	4.600	2.008	P = 0.020*
	I3C	18.155	1.887	
(IC50) UR	Control	4.600	2.008	P = 0.202
	2-AITFEI-3-C	1.880	0.424	
(IC50) UR	I3C	18.155	1.887	P = 0.043*
	2-AITFEI-3-C	1.880	0.424	
(IC50) UL	Control	0.695	0.021	P = 0.002*
	2-AITFEI-3-C	29.355	0.176	P = 0.013*
(IC50) UL	Control	0.695	0.012	
	2-AITFEI-3-C	17.535	2.793	
(IC50) UL	I3C	29.355	0.176	
	2-AITFEI-3-C	17.535	2.793	P = 0.02*

* Significant difference between two groups (P < 0.05).

3.4. Effects of I3C and NI-3-CD on the protein expression of OCT4

To further corroborate the results of this study, the expression levels of OCT4 protein were analyzed by western blotting in NB4 cells treated with NI-3-CD compared to both control (untreated) cells and cells treated with I3C after 24, 48 and 72 h under IC50 concentrations. The results of western blot analysis showed that OCT4 protein expression levels were decreased in NB4 cells treated with NI-3-CD when compared to both control (untreated) cells and cells treated with I3C after 24, 48 and 72 h incubation. Therefore the western blot analysis confirmed our Real Time PCR results and up-regulation of OCT4 with predicted size 20 kDa after treatment with novel indole derivatives. Western blot analysis of β-actin levels was used as control (Fig. 7). The density of the protein band was expressed as the ratio to β-actin protein. Data are the means of three different experiments (Fig. 7).

4. Discussion

More recent studies proposed a role for cancer stem cell self-renewing during tumorigenesis [2,3]. These cells are generated through uncontrolled self-renewal of normal stem or progenitor cells and the examining of the expression and their involvement regulating the genes in carcinogenesis is important. To our best knowledge, this study representing the first report for the potential of NI-3-CD in reducing the cancer cell population expressing multipotency- and pluripotency-associated markers. These results were aimed to highlight the potential utilization of this new derivative of indole for targeting cancer cells with stem-like features for either the prevention or treatment of cancer. Here, in present study we examined the expression of a well-known self-renewal regulatory factor, OCT-4 and NANOG in NB4 and further determined a correlation between the expression of these genes and tumor. In a study Chen and colleagues reported reduced expression of pluripotency genes including OCT4, Sox2, and NANOG in leukemia [23]. Recently, Tai et al., also revealed that OCT-4 is expressed in

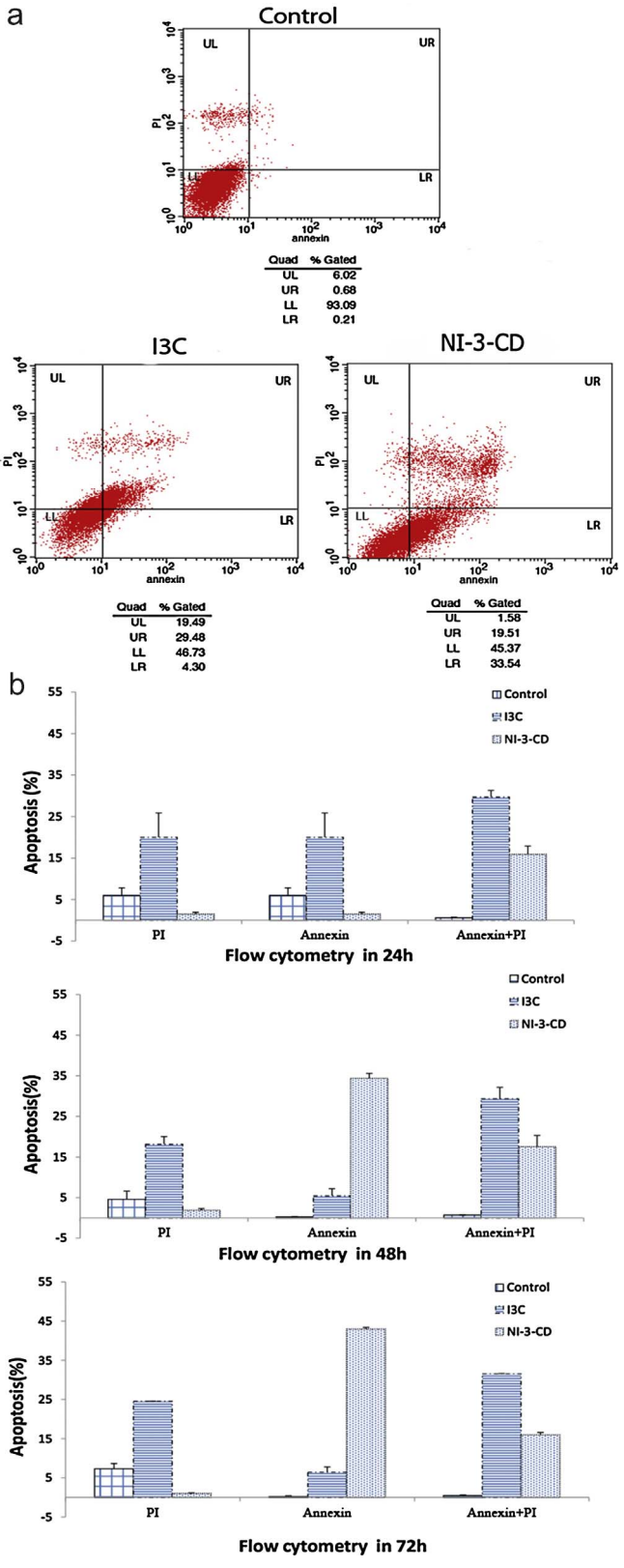


Fig. 4. (a) Induction of apoptosis in NB4 cells after treatment with I3C and NI-3-CD in comparison with untreated cells after 48 and at IC50 concentrations. (b) The comparison of induction of apoptosis in NB4 cells after treatment with I3C and NI-3-CD in comparison with untreated cells after 24, 48 and 72 h at IC50 concentrations.

multiple human adult stem cells [10] (e.g. breast, pancreas and liver stem cells) and Matthai et al., reported OCT-4 expression in normal human endometrium [24]. Jeter, C.R and coworkers showed ectopic

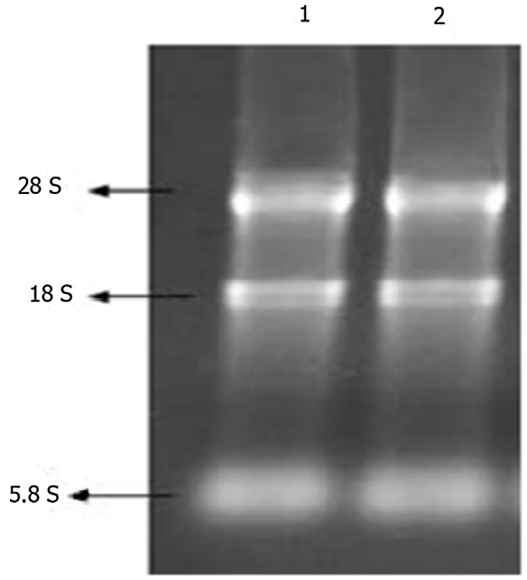


Fig. 5. The fidelity of extracted RNA was examined by electrophoresis 1% agarose and further staining with DNA Green Viewer™ and three bands with the Gel Doc was seen from each RNA sample.

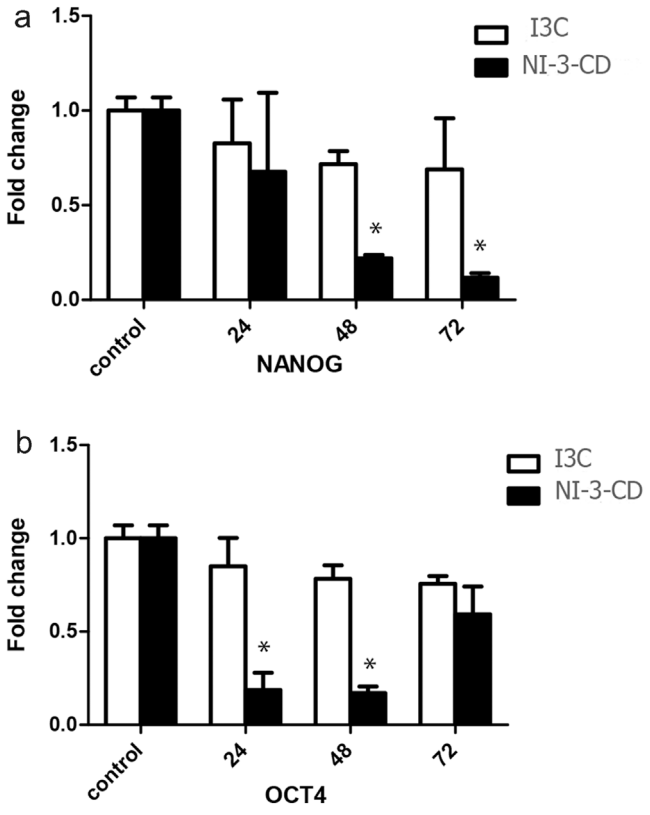


Fig. 6. (a) Expression of OCT4 mRNA, against β-actin as an internal control, in treated NB4 cells by NI-3-CD compared to both control cells and cells treated with I3C. The mRNA expression of OCT4 was detected after treating the NB4 cell lines at IC50 concentrations for 24, 48 and 72 h by real time PCR. Values are the average of triple determinations with the SD. indicated by error bars. *P < 0.05 compared to control. (b) Expression of NANOG mRNA, against β-actin as an internal control, in treated NB4 cells by NI-3-CD compared to both control cells and cells treated with I3C. The mRNA expression of NANOG was detected after treating the NB4 cell lines at IC50 concentrations for 24, 48 and 72 h by real time PCR. Values are the average of triple determinations with the SD. indicated by error bars. *P < 0.05 compared to control.

overexpression of NANOG in prostate cancer cells enhanced clonal growth and tumor regenerative capacity [25], and the activation of the embryonic NANOG gene caused a subpopulation of colorectal cancer cell to adopt a stem-like phenotype [26]. Tsai, following knockdown of NANOG impeded cell proliferation, migration and invasion. Overexpression of NANOG was also shown to be associated with chemoresistance in HNSCCs [27]. Our findings suggest that I3C has potent anticancer activities; hence it would be worthy to be considered as highly promising candidate for drug designing. To synthesis I3C derivatives, the final structures were re-structured, in a form which the final compounds are closely resembling self-condensation products of I3C [21,28–30], this may in turn mean that these biological activities were not as similar as the parental compound of I3C. Accordingly, in several recent studies more potent I3C dimerization products are synthesized, with enhanced potencies for cytoxicity and induction of apoptosis and cell cycle arrest of cancer cells [31]. The in vitro cellular anti proliferative activities of NI-3-CD and the low effective concentration makes this novel I3C derivative a promising candidate for its future development as a therapy for the rest of cancers. Synthesizing the new compounds for anticancer activity is a manner by which may approach cancer treatment [21]. The structure-activity analysis of I3C revealed that substituents linked to nitrogen number in the indole ring that inhibit dehydration and the formation of the reactive indole nine increases potency (which stabilizes the compound and prevents formation of oligomeric self-condensation products), whereas, the C-3 hydroxy methyl substituent on the indole ring is required for maintaining biological activity. Furthermore, increased number of carbons in the N-alkoxy derivatives led to significant elevation in potency, suggesting that the hydrophobic properties of the substituents at this indole ring positions are pivotal for increasing the potency of the generated molecules. We synthesized NI-3-CD that was designed to be stable, with induced hydrophobic character of the substituent, and maintain the C-3 hydroxy methyl substituent. The synthesis of NI-3-CD is described in more detail in methods section (Fig. 1). The elevated stability of carbaldehyde in compare to I3C may depend on the steric effect of bulky addition of the CF3 groups and substituent, which hampers oligomerization of NI-3-CD action formed under protonation. Thus, the biological effects of NI-3-CD are not dependent on its conversation into a like derivative. To compare the efficacies of the anti-proliferative effects of NI-3-CD and the I3C parental compound, NB4 cells were treated with increasing concentrations of indole for 24, 48 and 72 h this result suggests the NI-3-CD is the more potent anti-proliferative I3C derivative characterized. Here, in thus novel investigation we demonstrated for the first time, that NI-3-CD had inhibitory effects on the expression of pluripotency-associated markers like OCT-4 and NANOG in cancer cells. To the best of our knowledge, our result study is the first investigation that was performed and addressed a role for both I3C and NI-3-CD in regulating of the expression of OCT-4 and NANOG, to date. Our data showed that the mRNA expression of OCT-4 and NANOG expression was decreased in NB4 cells, once treated with NI-3-CD compared to both control cells and cells treated with I3C. The effects of NI-3-CD on the protein expression of OCT-4 was also examined in NB4 cancer cells being treated for 24, 48 and 72 h with or without this highly potent I3C derivative. Western blot analysis revealed that NI-3-CD has strongly downregulated the expression of the OCT-4 in NB4 cell lines, where, the NI-3-CD concentration was decreased and NI-3-CD treatment of NB4 cells reduced the expression. Furthermore, the sphere-forming ability of cancer cells was impaired upon NI-3-CD treatment, proposing that NI-3-CD might have been interfered with the tumor-initiating ability of these cells. Although previous reports have proposed an evident link between multipotency- and pluripotency-associated marker expression and the sphere-forming ability of cancer cells [32–35], the mechanism of the decrease in sphere-forming ability in NB4 cells upon NI-3-CD treatment deserves to be further clarified. An emerging body of evidences reported that a subpopulation of a tumor carries a distinct molecular gene and is selectively resistant to

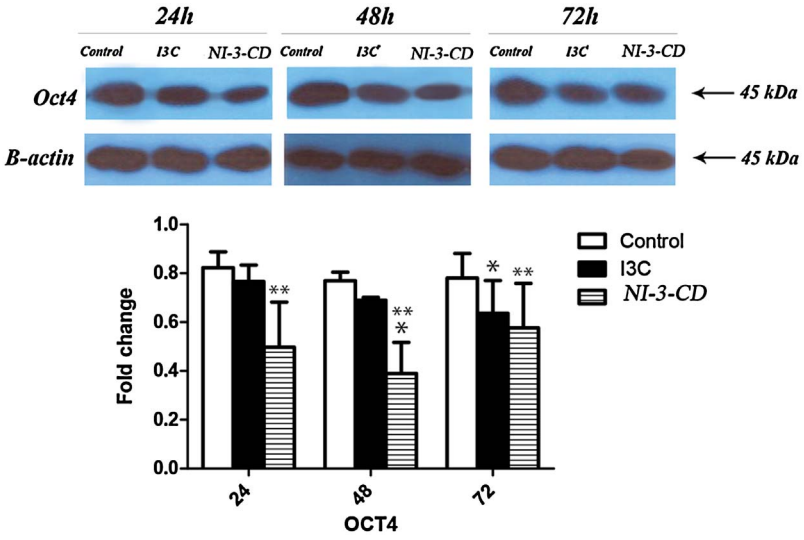


Fig. 7. The expression of OCT4 protein in treated NB4 cells by NI-3-CD compared to both control cells and cells treated with I3C.
The expression level of OCT4 protein was detected after treating the NB4 cells at IC50 concentrations for 24, 48 and 72 h by western blot analysis. Expression level of β-actin protein was used as control. The density of the protein band was expressed as the ratio to β-actin protein. Data are the means of three different experiments (Fig. 5).
* = significant in compared with Con group ($P < 0.05$).
** = Significant in compared with different time points 24 h, 48 h, and 72 h ($P < 0.05$).

chemotherapy. In recent years, many therapeutic methods have been developed to identify and specifically target these subpopulations in different tumor tissues. Increases in the expression of stem-ness markers and gene, including NANOG, OCT were shown to play a critical role in chemoresistance to cisplatin, doxorubicin, docetaxel, 5-fluorouracil and carboplatin [33]. These therapies mostly concerned the inhibiting of self-renewal of cancer stem cells, inducing their differentiation [16,36]. However, therapy strategies with existing anti-cancer agents like cisplatin, oxaliplatin and 5-fluorouracil shown to enhance cell viability and promote the self-renewal and survival of cancer stem cells in different cancer models [37–39]. Therefore, approaching novel agents with capacity suppress the stem-like features of cancer cells and simultaneously inhibiting their proliferation is of importance. Considerable levels of efforts have been made toward replacing chemical anticancer therapy agents with synthesized compounds to eradicate the undesirable effects of the chemicals during treatment and synthetic components successfully inhibited pluripotency-, multipotency- and angiogenesis-associated genes like NANOG, OCT-4 [40,41].

In this study we showed that NI-3-CD could act as a synthetic compound which has the ability to target cancer cells with stem-like characteristics. The next investigation step would then be the determination of the mechanism by which NI-3-CD interrupts pluripotency- and multipotency-associated marker expression in cancer cells, and to evaluate the in vivo efficiency of a new derivative ‘indole in cancer models.

Conflict of interest

None of the authors of present study declared conflict of interest.

Acknowledgment

This project was financially supported by a grant from the University of Medical Sciences.

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