TARGETING LEUKEMIC SIDE POPULATION CELLS BY ISATIN DERIVATIVES OF NICOTINIC ACID AMIDE

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Side population (SP) cells mediate chemoresistance in leukemia. However, chemical inhibition approach to target SP cells has been poorly studied. Herein, we report the discovery of isatin derivatives of nicotinic acid amide as potent side population cell inhibitors. The selected derivatives showed superior potency over the reference drug verapamil. Furthermore, the treatment increased chemosensitivity and inhibited the cell proliferation on three different leukemic cell lines, K562, THP-1 and U937, suggesting that both SP and the bulk of leukemic cells are affected. Moreover, treatment with the most potent compound Nic9 reduced the expression of ABCG2, demonstrating that side population inhibition effect of the target derivatives is at least via ABCG2 inhibition. Importantly, the target derivatives induced erythrocyte/dendritic differentiation to leukemic cells mainly through Musashi/Numb pathway modulation.

Leukemia can be described as abnormal hematopoietic tissue initiated by a few leukemic stem cells (LSCs) that undergo an aberrant and poorly regulated process of organogenesis similar to that of normal hematopoietic stem cells (1). One of the main properties of cancer stem cells (CSCs) is resistance to a given therapy and ability to survive the primary treatment and relapse. A number of factors may govern this phenomenon, including stem cell quiescence, protected niche environment, upregulated expression of xenobiotic efflux pumps, and enhanced anti-apoptotic and DNA repair pathways (2). CSCs can be isolated and enriched by a variety of techniques and methods, including cell surface markers and dye excluded side population (SP). Dye excluded SP method was first described in murine bone marrow and SP cells represented a small subset of cells that were enriched at least 1000-fold in hematopoietic stem cell (HSC) activity (3). The SP cells are identified according to their ability to efflux the Hoechst dye at a higher pace than the remaining tumor cells termed the main population (non-SP). Furthermore, the degree of efflux activity seems to correlate with the maturation state, so that cells displaying the highest efflux activity are

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the most primitive in terms of differentiation potential (4). More importantly, in the patients with B-cell chronic lymphocytic leukemia, SP cells appeared resistant to conventional B cell-chronic lymphocytic leukemia (B-CLL) treatments, such as fludarabine, bendamustin or rituximab (5). Accordingly, in order to block the chemoresistance of leukemic cells, there is an urgent need to develop small molecule inhibitors of side population (SP) cells. Nicotinic acid derivatives and its isomers have anti-bacterial, anti-oxidant, anti-inflammatory, anti-carcinogenic and anti-tubercular activities (6). Isatin constitutes an important class of bioactive compounds exhibiting caspase inhibition and anti-proliferative activity (7-9). One of the main ways to treat leukemia is by induced differentiation. The concept of differentiation therapy of cancer is ~40 years old. Despite many encouraging results obtained in laboratories, in both *in vitro* and *in vivo* studies, the only really successful clinical application of differentiation therapy was all-trans-retinoic acid (ATRA)-based therapy of acute promyelocytic leukemia (APL) (10). One of the main pathways that regulate stem/differentiation state of leukemic cells is Musashi/Numb pathway. The Musashi (Msi) family is a group of RNA-binding proteins characterized by two RNA recognition motifs (RRMs) and is evolutionarily conserved (11). In mammals, the function of Msi has been found to activate Notch signaling through the translational repression of NUMB, which represses an intracellular Notch signaling, by binding to the 3′ untranslated region (UTR) of the Numb mRNA thereby activating self-renewal state (12). In our laboratory, through small screening program, we reported the discovery of many active small molecules that are able to target different subpopulation of CSCs, including SP cells and CD133 positive cells and ALDH positive, cells (13-15). Herein, we report the discovery of isatin derivatives of nicotinic acid amide as potent SP cell inhibitors and differentiation cell inducers via upregulation of the cell fate determinant Numb.

MATERIALS AND METHODS

Chemistry

All the tested compounds were synthesized, described and fully characterized according to previously reported procedures (16).

Biological Evaluation

Cell line and tissue culture

K562, THP-1 and U937 cell lines were purchased from the American Type Culture Collection. Cells were maintained in RPMI 1640 (Sigma), supplemented with 10% FBS (Lonza), 100IU/mL penicillin, 100mg/mL streptomycin and 2mmol/L L-glutamine (Sigma). Cell viability was assessed by trypan blue exclusion analysis. Cell numbers were determined by using a hemacytometer.

Side population staining by DyeCycle™ violet stain

For DCV staining, cells were pelleted and suspended in RPMI cell culture medium at a concentration of $1*10^6$ cells/mL. DCV (Invitrogen Molecular Probes®, Eugene, OR) was added at a final staining concentration of 10 μM, as this concentration gave optimal separation between SP and non-SP cells. PI stained was used to exclude dead cells. To gate only side population cells, a reference blocker Virapamil (100 μM) was used. All analyses were performed on a FACS LSRII (BD Biosciences). Debris and cell clusters were excluded during side-scatter and forward-scatter analyses.

WST-1 cell proliferation assay

Cells were seeded into 96-well plates at $0.4*10^4$/well and incubated overnight. The medium was replaced with a fresh one containing the desired concentrations of the compounds. After 48 h, 10 μL of the WST-1 reagent was added to each well and the plates were re-incubated for 4 h at 37°C. The amount of formazan was quantified using ELISA reader at 450 nm.

Apoptosis assay

Vybrant apoptosis assay kit (Annexin-V, APC conjugate; Molecular Probes™) was used to evaluate cell viability as per the manufacturer's recommendation. Briefly, both adherent and floating cells were collected. DAPI (4′,6-diamidino-2-phenylindole) was used as a viability dye. Fluorescence was analyzed on a total of 10^4 cells per sample using a flow cytometer and cells were considered viable if they were double negative for Annexin-V and DAPI.

Western blot

SDS-PAGE was performed using 12% separating mini gels, the protein load was 30 μ) for each sample. After separation the protein was transferred from the gel onto polyvinylidene difluroide membrane (PVDF). Next, the membrane was incubated overnight with the appropriate antibodies, ABCG2 (Aviva System Biology) Numb and GAPDH (Cell Signaling) then developed using Image Quant LAS-4010.

Immunofluorescence microscopy

K562 cells were cultured in 6-well plate and treated by Ni9 with various doses for 48 hours. The cells of treated and untreated wells were washed, dried and fixed in 4% formaldehyde. Cells were stained overnight with anti-Musashi1,2 and anti-Numb (Cell Signaling) at 1:100. Cells were washed and stained with secondary Abs FITC (Invitrogen). DAPI (Invitrogen) was added for 10 min to counterstain the nuclei. Slides were mounted and immunofluorescence was visualized using BD Pathway 855 system.

Differentiation analysis

The differentiation of K562 cells was followed by the increase in the expression of marker as follows: CD271 for dendritic cells differentiation, glycophorin GYPA for erythrocyte differentiation and CD15 for macrophage differentiation. Anti-CD34 (FITC), anti-CD271 (APC), anti-GYPA (FITC), anti-CD15 (FITC) were purchased from BD Pharmingen™. Cells were freshly stained and incubated for 45 min on ice in phosphate buffered saline with 2% FBS. Side scatter and forward scatter profiles were used to eliminate cell doublets and DAPI was used to eliminate dead cells. Flow cytometry was performed on a BD™ LSR II flow cytometer.

RESULTS

Chemistry

In the present work, originally, the isatin derivatives of nicotinic acid amide were designed based on the suggestion that the coupling of two heterocyclic compounds (nicotinic acid and isatin), *via* certain amino acid as a bridge, could result in producing small molecules with potent biological activity.

Side population inhibition effect of isatin derivatives of nicotinic acid amide

In order to find a series of compounds that showed activity to block the chemo resistance of leukemic cells, we used a dye-excluded side population technique. The percentage of SP cells of untreated and treated K562 cells was measured and the inhibition percentage was given for each derivative shown in Table I. The SP cell analysis showed a potent inhibition effect against leukemic SP cells compared with untreated cells as can be seen in Fig. 1, A and B. The target derivatives showed inhibition effect with more than 50% at 10 μM. Compared with reference SP cell inhibitor drug virapamil, the target derivatives showed a potent effect of at least 5-fold. The ranging effect of verapamil took a place between 50 to 200 μM. All the tested compounds showed > 50% inhibition effects on side population cells at 10 μM, except Nic2.

Cell proliferation inhibition and chemoresistance blocking effect of isatin derivatives of nicotinic acid amide

To test whether these compounds may have activity on the bulk of tumor cells, we performed cell proliferation WST-1 assay by using three different leukemic cell lines, K562, THP-1 and U937. All the tested compounds showed inhibitory effect on these cell lines with IC_{50}> 10 μM, and the most active compound on K562 cells was Nic10 with IC_{50}=20μM, indicating the selectivity of these series toward SP cells, and the effect was cell proliferation and cell toxicity independent. *In vitro* cytotoxic activity of the prepared compounds against cancer cell lines were measured and recorded in Table II.

The cross talk between chemoresistance of leukemic cells and SP is very high, therefore, we hypothesized that the inhibition of side population cells by the target derivatives may increase the sensitivity of leukemic cells to chemotherapy. To test our hypothesis, we used the induction of apoptosis by chemotherapeutic agent Mitoxantrone at 100 nM alone or after one-day treatment by the most active compound Nic9 as test for increased chemo-sensitivity. The treatment by the mentioned compound, one day before the chemotherapy,

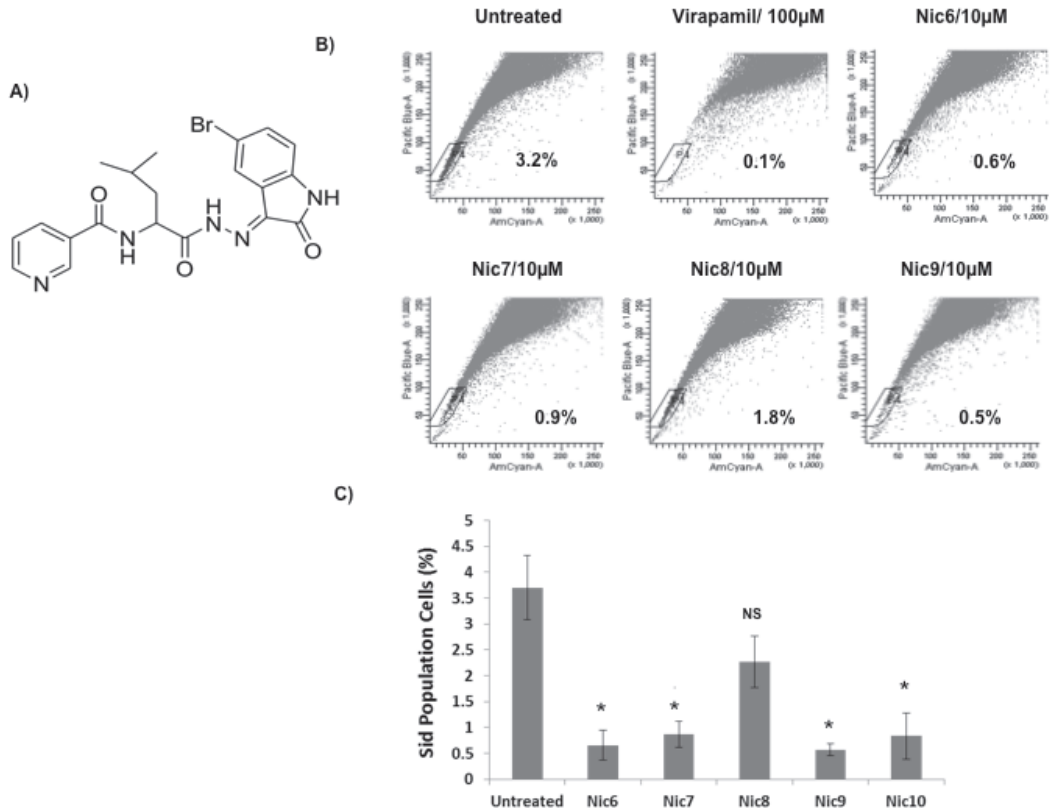

Fig. 1. ***A)*** *The chemical structure of the most potent member Nic9.* ***B)*** *DCV SP staining of K562 cells treated with Isatin derivatives of nicotinic acid amide Nic6-9 10 μM and SP inhibitor reference drug Verapamil 100 μM for 48 hrs.* ***C)*** *Bar graph showing SP% cells after treatment with Nic 6-10. Data represent the average ± SD (error bar) of three independent experiments.*

increased the sensitivity of leukemic cells to apoptosis as shown in Fig. 2, A and B. Mitoxantrone alone at 100 nM increased apoptosis by 25% while the combination therapy increased apoptosis by 50%, showed a synergistic effect in the combination therapy compared with chemotherapy alone.

ABCG2 inhibition effect of isatin derivatives of nicotinic acid amide

ATP-binding cassette sub-family G member 2 (ABCG2) is defined as specific marker for the SP in many tissues. Furthermore, it is highly expressed in SP cells and is responsible for the maintenance of SP phenotype (17). In order to test whether these derivatives inhibit the expression of ABCG2, we used Western blot technique to show the expression of ABCG2 protein in K562 cells treated with different doses of the most active compound Nic9. The treatment showed inhibition in the expression of ABCG2 level in 5 and 10 μM concentrations. However, the treatment with low dose 2.5 μM showed no effect in the expression suggesting that the effective low range of theses derivatives is 5 μM. Importantly, the treatment had no effect on internal protein control GAPDH, as seen in Fig. 3.

Isatin derivatives of nicotinic acid amide induced differentiation to leukemic cells through musashi/ numb pathway modulation

Recently, induced cell differentiation has been gaining major attention since CSCs are one of the major obstacles for cancer treatment. The target derivatives showed a potent inhibitory effect on SP cells, therefore, we hypnotized that the treatment may induce differentiation to leukemia cells given that SP cells are highly enriched by CSCs.

Table I. *Side Population (SP) % of K562 cells treated with compounds (1-10) and the inhibition % compared with untreated cells.*

Compounds	Side Population % [*]at 10 μM	Inhibition % [#]
1	3.23±**0.7**	12.61
2	4.86±**0.7**	-31.53[&]
3	2±**0.1**	45.94
4	3.53±**0.47**	4.50
5	2.4±**0.1**	35.13
6	0.65±**0.28**	82.25
7	0.86±**0.25**	76.57
8	2.26±**0.5**	38.73
9	0.56±**0.1**	84.68
10	0.83±**0.45**	77.47

[*] *Side population % as Mean*±**SD** *of three independent experiments.*
[#] *Inhibition % =100-(SP% of treated cells/SP% of untreated cells)*100.*
[&] *Minus sign indicated that the compound* ***2*** *increased SP%.*

Table II. *In vitro cytotoxic activity of the prepared compounds against cancer cell lines.*

Compounds	IC_{50} [a] (μM)		
	Histiocytic lymphoma U937	Acute monocytic leukemia THP-1	Chronic myelogenous leukemia K562
1	26.18±0.01	In[b]	84.92±0.05
2	47.78±0.28	39.77±0.26	45.68±0.05
3	13.82±0.37	26.40±0.17	22.15±0.07
4	16.81±0.08	17.22±0.36	28.36±0.07
5	27.20±0.29	127.30±0.08	70.70±0.06
6	53.58±0.44	45.40±0.59	58.20±0.02
7	84.89±0.22	57.42±0.38	46.41±0.04
8	11.90±0.09	24.87±0.47	21.65±0.2
9	16.63±0.36	21.19±0.25	24.16±0.04
10	18.70±0.06	18.99±0.17	20.11±0.02

[a] IC_{50}*: concentration of the compound (μM) producing 50% cell growth inhibition after 48 h of compound exposure.*
[b] *Inactive within 300 μM concentration range.*

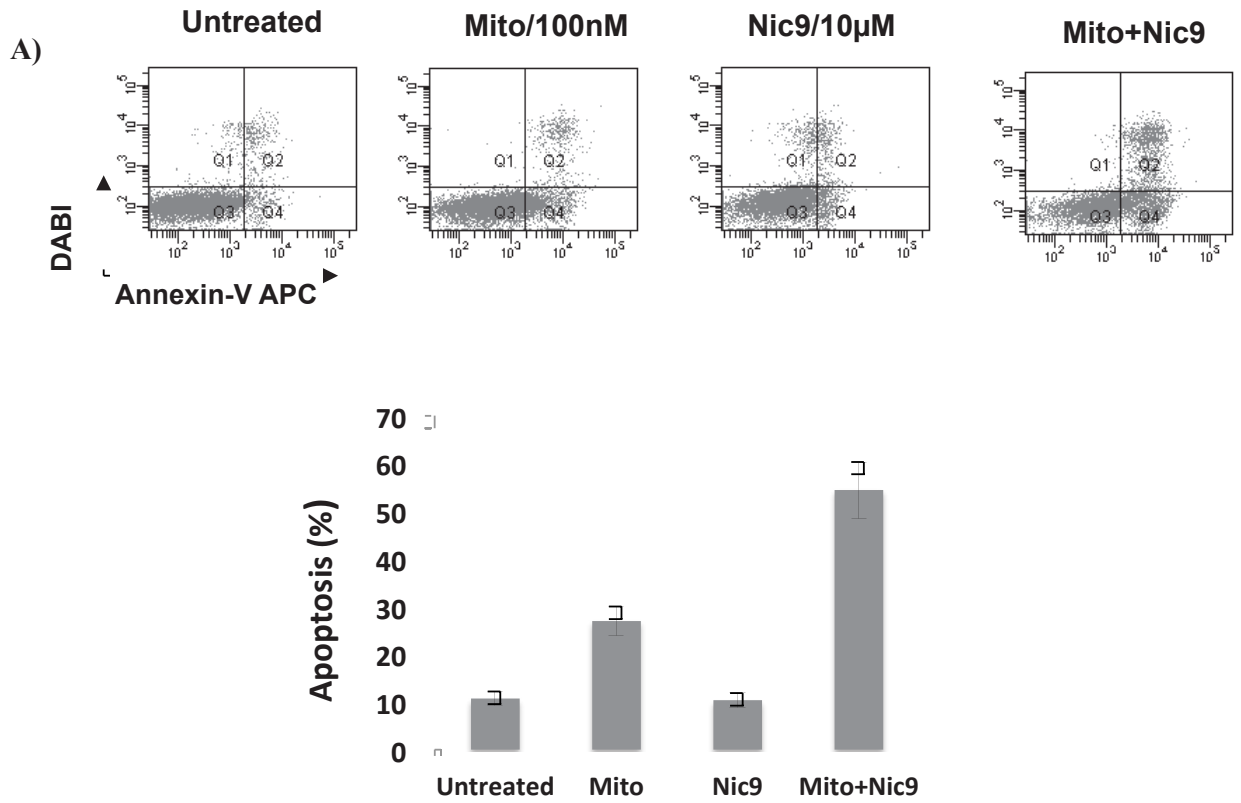

Fig. 2. ***A)*** *Dot plot of Annexin V and DABI staining following treatment of K562 cells with chemotherapeutic agent Mitoxantrone alone and Nic9 alone and the combination for 48 h.* ***B)*** *Bar graphs showing mean ± SD. apoptotic cells of chemotherapy alone and the combination with Nic9. The pretreatment of leukemic cells with Nic9 increased the killing effect of Mitoxantrone by 2-fold. Data represent the average ± SD (error bar) of three independent experiments.*

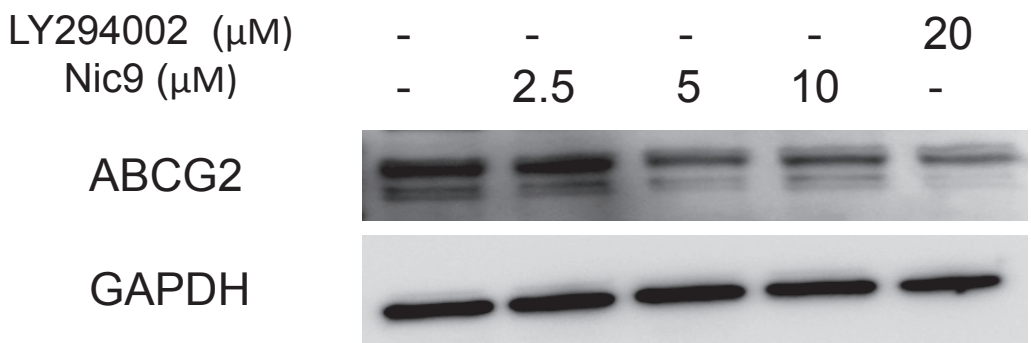

Fig. 3. *Western blot showing the expression levels of ABCG2 in K562 cells following treatment with various doses of Nic9 for 48 h. AKT pathway inhibitor LY294002 was used as positive control for ABCG2 inhibition.*

To examine our hypothesis, a panel of stem and differentiation markers, namely CD34 for leukemic CSCs, CD271 for dendritic cell differentiation, glycophorin GYPA for erythrocyte differentiation and CD15 for macrophage differentiation, were used. Flowcytometric analysis of K562 cells treated with Nic9 showed a decrease in the expression of leukemic cancer stem cell marker CD34. Accompanied with the decrease in the expression of stem cell marker, expression of CD271 increased by about one-fold in 5 and 10 μM concentrations. Increasing the concentration to 20 μM resulted in an approximate 4-fold increase of CD271 positive cells, as shown in Fig. 4, A and B. The increase in CD271 expression was concentration-dependent. The treatment also increased the expression of glycophorin GYPA. The increased in GYPA was in a concentration-independent manner in which the highest induction, about 3-fold, was detected in 5 μM. The lower induction of GYPA was detected in the higher concentration 20 μM. The treatment showed no increase in macrophage marker CD15. The results demonstrated that isatin derivatives of nicotinic acid amide induced erythrocyte and dendritic cell differentiation but not macrophages differentiation.

One of the main pathways that regulate stem/differentiation state of leukemic cells is Musashi/Numb pathway. Therefore, we asked whether the derivatives main effect is through the said pathway or not. In order to evaluate the effect of isatin derivatives of nicotinic acid amide on Musashi/Numb pathway, we used immunefluorescence microscopy to visualize both proteins in the treated and untreated K562 cells. As seen in Fig. 5A, inhibition in the expression of Musashi1 and 2 was observed. In contrast to inhibition of Musashi1and 2, a detectable increase in the expression of cell fate determined Numb protein in K562 cells treated with Nic9 compared with untreated cells, as shown in Fig. 5B. The increase in the expression was dose-dependent in which the highest induction was seen in 20 μM. The induction of expression was in both nucleus and the cytoplasm. For further confirmation, we used Western blot to detect the change on the expression of Numb protein after treatment. Nic9 treatment by the same protocol resulted in the increase in Numb protein expression in a dose-dependent manner, mimicking the results of immunofluorescence, as shown in Fig. 5C.

DISCUSSION

Chemoresistance remains the main reason for relapse and represents the most significant obstacle to successful chemotherapy for cancer and particularly for leukemia. The cross-talk between chemoresistance and cancer stem cells is very high. The SP cells are identified according to their ability to efflux the fluorescence dyes, and are responsible for chemoresistance in leukemia. In this manuscript, we report a novel activity of interested conjugation of nicotinic acid and isatin nucleus through amino bridge. The hybrid derivatives showed a potent inhibitory effect against leukemic side population SP cells. The target derivatives showed >50% inhibition activity at 10 μM, whereas the reference drug Virapamil showed >50% inhibition activity at 100 μM, demonstrating superior potency of these series. In agreement with previous reports demonstrating that SP cells are enriched by cancer stem cells CSCs, we provide proof of selective elimination of SP cell phenotype in K562 cells which may have application to overcome the chemoresistance and induce differentiation to leukemic cells. The pre-treatment sensitized leukemic cells to chemotherapy. Interestingly, the treatment with Nic9 alone, showed no increased in apoptosis suggesting that the target derivatives SP inhibition effect is through a therapeutic response and not through toxicity response, increasing the potential of the preclinical and clinical development of isatin nicotinic acid amides as SP inhibitor for the treatment of leukemia.

The role of ABCG2 in the regulation of SP cell phenotype is well characterized. Furthermore, ABCG2 is sharply down-regulated during hematopoietic stem cell differentiation and is expressed at a low level in mature cells compared with progenitor cells (18). The highly regulated expression of ABCG2 suggests that ABCG2 may play a regulatory role in maintaining stem cells in an undifferentiated state (19). CSCs are also supposed to be responsible for the acquisition of multi-drug

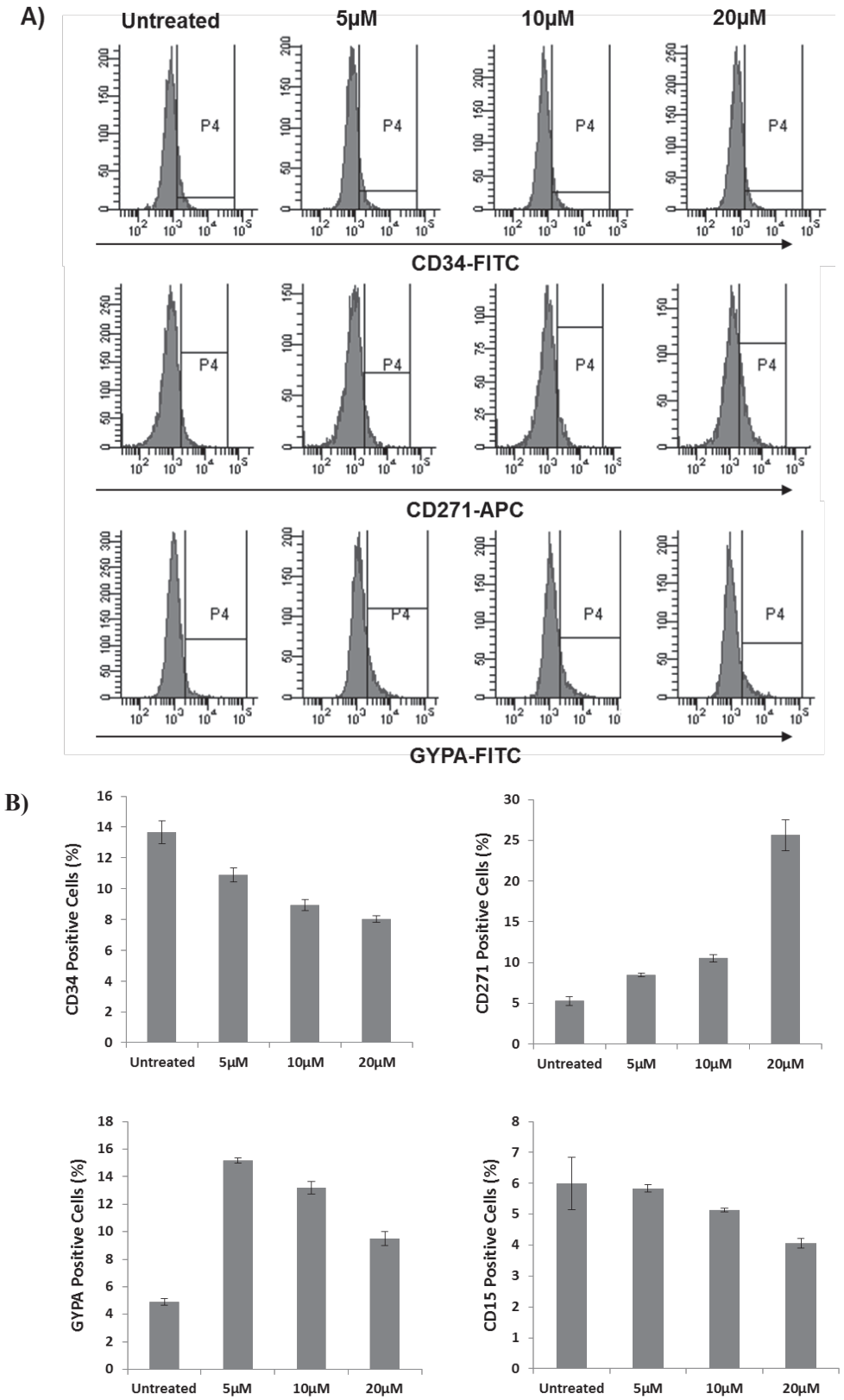

Fig. 4. ***A)*** *Histograms of different CD markers of K562 cells treated with Nic9 10 μM for 48 hrs.* ***B)*** *Bar graph showing positive cell percentage after treatment with Nic9. Data represent the average ± SD (error bar) of three independent experiments.*

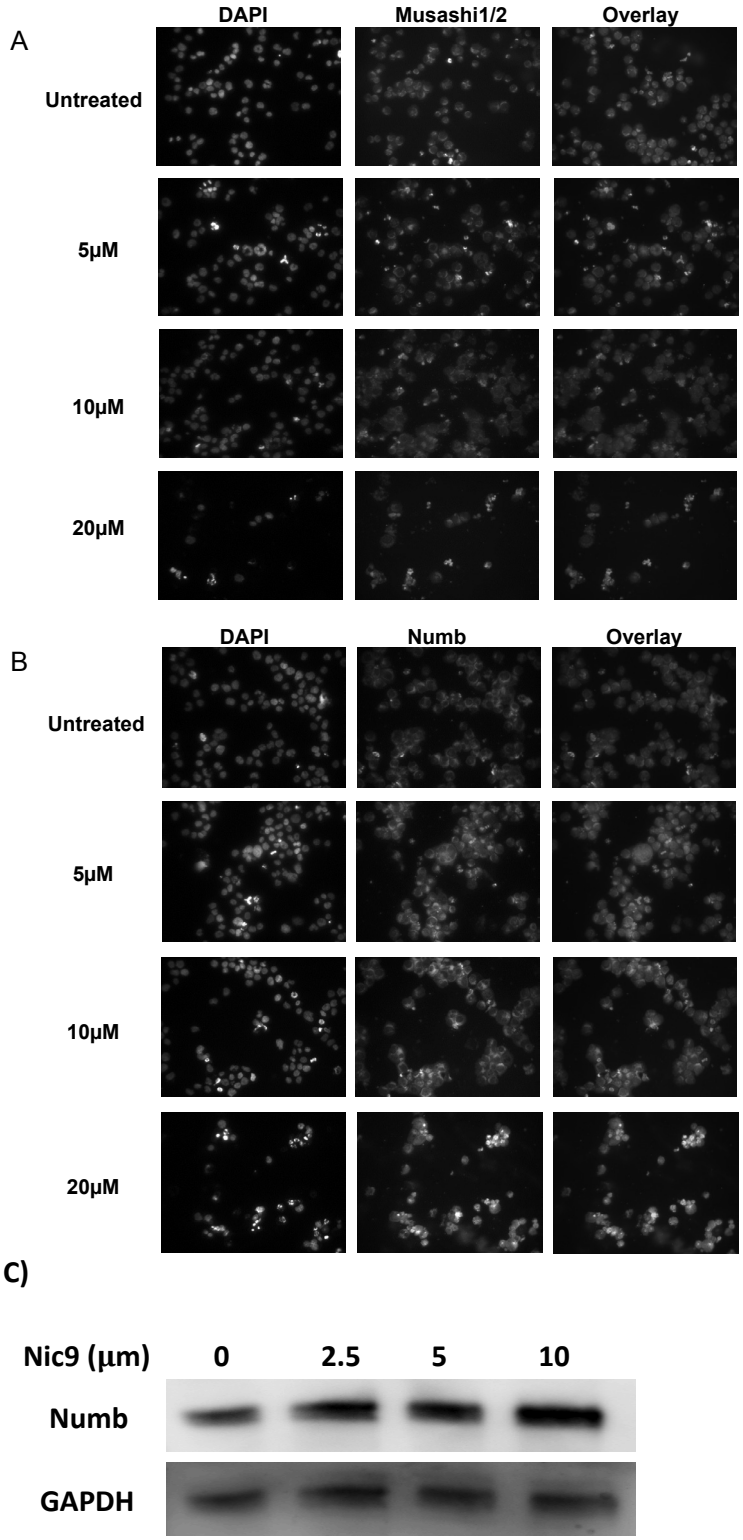

Fig. 5. *Using immunofluorescence staining for Numb and Musashi1,2 on K562 cells treated with different doses of Nic9 for 48 hrs.* ***A****) IF staining sowed decreased in the expression of Musash1,2 in a dose-dependent manner.* ***B****) In contrast of Musashi1,2 inhibition, the treatment by various doses of Nic9 increased the expression of Numb.* ***C****) Western blot showing the expression levels of Numb in K562 cells following treatment with various doses of Nic9 for 48 h.*

chemoresistance and lead to cancer relapse. Side population SP and chemoresistance suggest a close link between ABCG2 and CSCs. Targeting CSCs by the small molecule compound inhibitor of ABCG2 represents an attractive strategy because dual effect which reverses chemoresistance and induces differentiation to cancer cells. Therefore, we suggest that isatin derivatives of nicotinic acid amide had a dual targeted effect on leukemic stem cells via SP cells and ABCG2.

Induced cell differentiation is gaining major attention since CSCs are one of the major obstacles for cancer treatment. We demonstrated induction of leukemic cells to differentiate to erythrocyte and dendritic cells but not to macrophages. One of the main pathways that regulate stem/differentiation state of leukemic cells is Musashi/Numb pathway. The most potent member in this series, Nic9, inhibits the expression of stem cell driver Musashi1 and 2 proteins. The Musashi (Msi) family is a group of RNA-binding proteins characterized by two RNA recognition motifs (RRMs).(13) In mammals, the function of Msi has been found to activate Notch signaling through the translational repression of NUMB, which represses an intracellular Notch signaling by binding to the 3′ untranslated region (UTR) of the Numb mRNA and therefore activating self-renewal state (12). In myeloid leukemia cells, MSI2 is highly expressed, and depletion results in decreased proliferation and increased apoptosis (20); for this reason, we can indicate that isatin derivatives of nicotinic acid amide cell proliferation inhibition and chemotherapy sensitization effects are mainly through Msashi/Numb pathway modulation.

To our knowledge, this is the first report to demonstrate functional targeting of leukemic SP cells by using isatin derivatives of nicotinic acid amide, supporting the preclinical and clinical development to block the chemoresistance and induce differentiation to leukemic cells.

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