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Target β-catenin/CD44/Nanog axis in colon cancer cells by certain *N′*-(2-oxoindolin-3-ylidene)-2-(benzyloxy)benzohydrazides

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ABSTRACT

Cell surface molecule CD44 plays a major role in regulation of cancer stem cells CSCs on both phenotypic and functional level, however chemical inhibition approach of CD44 to targets CSCs is poorly studied. Herein, we report the discovery of certain *N′*-(2-oxoindolin-3-ylidene)-2-(benzyloxy)benzohydrazides as a novel inhibitor of CD44. Molecular docking study showed interference of the scaffold of these compounds with β-catenin/TCF-4 complex, building a direct relationship between CD44 inhibition and observed well-fitted binding domain. Compound **11a**, most potent member elicits inhibition effect on TCF/LEF reporter activity conformed the involvement of Wnt pathway inhibition as a mechanism of action. Furthermore, the treatment by the mentioned compound leads to inhibition of embryonic transcriptional factor Nanog but not Sox2 or Oct-4 suggested specific targeted effect. Moreover, the cytotoxicity and cell cycle effect of this series seems to be dependent on CD44 expression.

Cancer remains one of the most difficult health problems worldwide. The difficulty on our opinion is origin from the development nature of these diseases and weak activity of current available treatments. Cancers may have a hierarchical organization in which subpopulations are arranged into tumorigenic and non-tumorigenic cells.[1] Recently, several studies demonstrated that tumorigenic potential of the tumor cells is driving by small sub population called cancer stem cells (CSCs). The model of CSCs had been applied in leukemia[2] and extended to breast,[3] brain,[4] lung,[5] colon,[6] and other cancers. The CSCs hypothesis is based on tumorigenic potential enabled by self-renewal in which that tumors are maintained by a self-renewing CSC population capable of differentiating into non-self-renewing cell populations that constitute the bulk of the tumor.[7] The self-renew/tumorigenic state of tumor cells is plastic and cells with stem cells properties can be generated by epithelial–mesenchymal transition (EMT).[8] CD44 is a member of a large family of cell adhesion molecule. In addition to its role in cellular adhesion, CD44 play a major role involved in growth and motility of many cancers. The crosstalk between CD44 and CSCs had been demonstrated in both phenotypic and functional level. Phenotypically, CD44 is used as a marker of CSCs in many cancers. On functional level, first CD44 might regulate the CSCs metastasis through adhesion and EMT.[8,9] Second, CD44 might regulate self-renew through Nanog phosphorylation, Hedgehog and Notch pathway.[9–13] Third, CD44 through interaction with hyaluronic acid can stimulate the upregulation of MDR-1 and IAPs. Moreover, knockdown of CD44 resulted in reduce Bcl-2 level in breast CSCs.[14,15] Fourth, Wnt signaling pathways play a major role in embryonic development and carcinogenesis. Functionally, Wnt pathway work in the absence of secreted glycolipoprotein Wnt, cytoplasmic β-catenin protein is constantly degraded by the action of the Axin complex, which is composed of the scaffolding protein Axin, the tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). CK1 and GSK3 sequentially phosphorylate the amino terminal region of β-catenin, resulting in β-catenin recognition by β-Trcp, an E3 ubiquitin ligase subunit, and subsequent β-catenin ubiquitination and proteasomal degradation.[16] Activation of Wnt receptors blocks the GSK3β activity and degradation of β-catenin, which is translocated to the nucleus where it interacts with members of the T cell factor (TCF)/LEF family of HMG-domain transcription factors to activate Wnt target gene transcription.[17] Cell surface molecule CD44 is a downstream target of Wnt/β-catenin

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pathway.[18] Finally, a therapeutic approach using an activating monoclonal antibody directed to the adhesion molecule CD44 had been successfully used to eradicate human acute myeloid leukemic stem cells.[19]

Using the crystal structure information of β-catenin/Tcf4, 6 hot spots or cavities were identified on its surface (Fig. 1). Of all these hot spots, cavity 2 is the largest one as it has two small pockets on both sides, one containing K435 and the other near R469. As a result, cavity 2 contains several clefts that can potentially anchor a small molecular weight inhibitor and was, therefore selected as a hot spot for Tcf4 competitor study.[20,21]

Recently, starting from both biophysical-combined virtual screening and biochemical screening studies, new inhibitors of the β-catenin/Tcf4 interaction were discovered. In the former study, Trosset screened a library of 17,000 compounds and selected 22 compounds for biophysical screening, of which three compounds confirmed as binders and Tcf4 competitors. PNU74654 (Fig. 2) was the most active hit.[20] The latter study was done by Lipourcelet using biochemical screening of a library of 7000 natural compounds. Lipourcelet succeeded to identify 6 compounds (Fig. 2) as antagonists of β-catenin/Tcf4 complex with in vitro IC_{50} values <10 μM.[22]

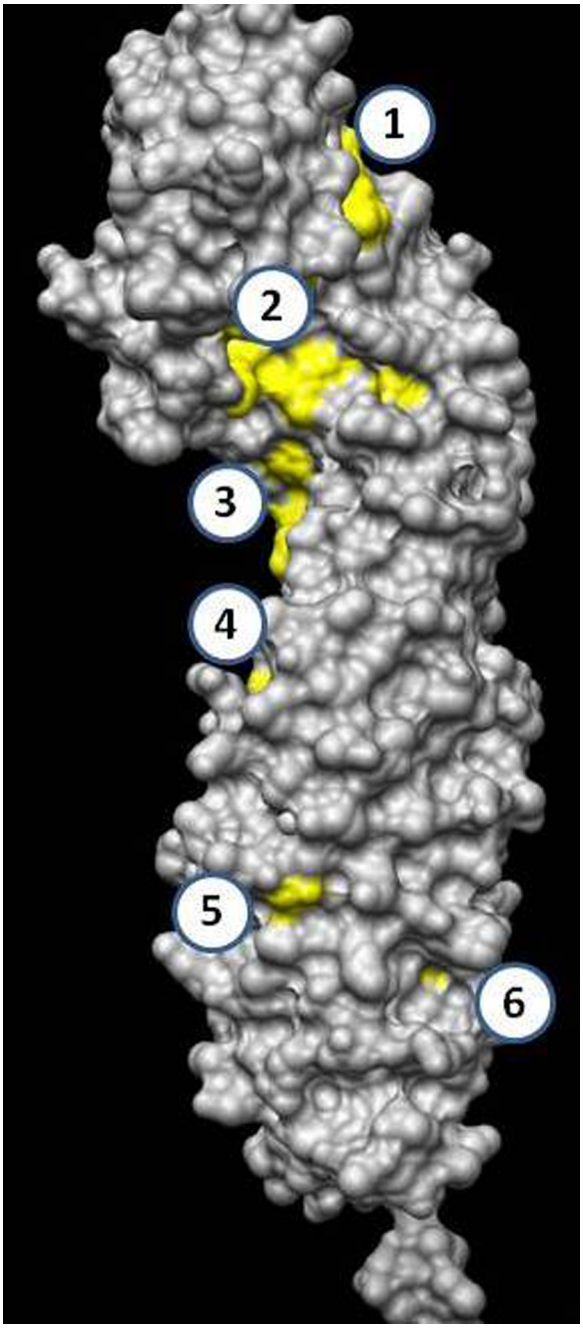


Figure 1. β-Catenin crystal structure.[20] The Tcf4 is deleted and the β-catenin is shown as solid surface. The hotspot areas are shown as solid yellow surface.

Trosset et al predicted the binding mode of PNU74654 in cavity 2 as it binds one small pocket near K435 and another small pocket near R469 on both sides of cavity2 (Fig. 3). On the other hand, the larger Lipourcelet's compounds don't show binding to the two adjacent pockets of K435/R469 hot spot or cavity 2 as deeply as PNU74654. The large central ring systems make more extensive hydrophobic contact with the floor of the hot spot, which may explain their potency (Fig. 3).[20,22]

The present study is aimed to report the discovery of small molecule inhibitor of CD44 and its viability to target cancer stem cells in preclinical setting of colon cancer. The compounds were designed to combine the central large hydrophobic moiety aimed to make hydrophobic contact with the floor of cavity2 as in Lipourcelet's compounds and small moieties that bind to the small pockets or clefts in cavity 2 as with Trosset's compounds (Fig. 3).

A series of related analogs, aimed to combine the two binding modes, were designed, and synthesized in multi-steps reaction as shown in Scheme 1. At first, the 2-benzyloxy/chlorobenzyloxy benzohydrazides were prepared via known two-step synthesis starting with the reaction of methyl salicylate with the appropriate benzyl chloride in alkaline hydromethanolic solution.[23] Hydrazinolysis of the resulting methyl 2-benzyloxy/chlorobenzyloxy benzoates **1a**,**b** resulted in the corresponding benzohydrazides **2a**,**b**.[24] Synthetic entry to the designed compounds **3a**,**b**–**11a**,**b** (Supplementary information) was readily undertaken in a two-step sequence reaction. At first, compounds **3a**,**b** is converted into hydrazide hydrazone upon condensation of **2a**,**b** with isatin followed by installation of alkylaminomethyl or aralkylaminomethyl group via a Mannich reaction resulting in compounds **4a**,**b**–**11a**,**b**.

Recently the main stream of cancer drug discovery shifted to target CSCs, be caused of tumor initiation, chemoresistance and metastasis potential. Cell surface molecule CD44 play a major role in regulation of CSCs in functional level, therefore we used a flow cytometry to evaluate the inhibitory effect of the targeted derivatives **3a**,**b**–**11a**,**b** on CD44 compared with untreated cells (Table 1). The inhibitory results against the cell surface CD44 were reported in Table1 indicate that we have produced some new potent CD44 inhibitors belonging to the 2-oxoindolin-3-ylidene)-2-(benzyloxy)benzohydrazides series **3a**,**b**–**11a**,**b**. The majority of the compounds showed high CD44 inhibition percent (>50%) at 10 μM (Compounds **4a**, **5a**,**b**–**10a**,**b**, **9a**, **11a**,**b**). The most active member in this series is **11a** with 76.18% inhibition effect. Focusing on the results reported in Table 1, we can observe that elimination of *p*-chloro substituent is unfavourable for ligand-binding site interaction (compare **3a**–**11a** and **3b**–**11b**). Also, the elimination of the *N*-Mannich base fragment is unfavourable for CD44 inhibition activity (compare **3a**,**b** and **4a**,**b**–**11a**,**b**). At the same time, increasing the length of the carbon chain of the secondary amine of the Mannich base substituent results in a significant increase in the inhibition activity (compare **4a**,**b**, **5a**,**b**, **6a**,**b** and **7a**,**b**). While, the morpholino and piperidino Mannich bases show comparable CD44 inhibition activity with that of the diethylamino Mannich bases (compare **5a**,**b** with **8a**,**b** and **9a**,**b**). The Mannich base derivatives bearing 2-hydroxy-*N*-methylethylamino or the *N*-methylbenzylamino fragment explored highest CD44 inhibition activity (compare **10a**,**b**, **11a**,**b**). Indicating the bulk effect on potency has a ceiling and the bulkier *p*-chlorobenzyl group such as **3b**–**11b** showed lower activity (Inhibition%, 18.48–59.52) than the corresponding analogs **3a**–**11a** (Inhibition%, 31.25–76.18). This is

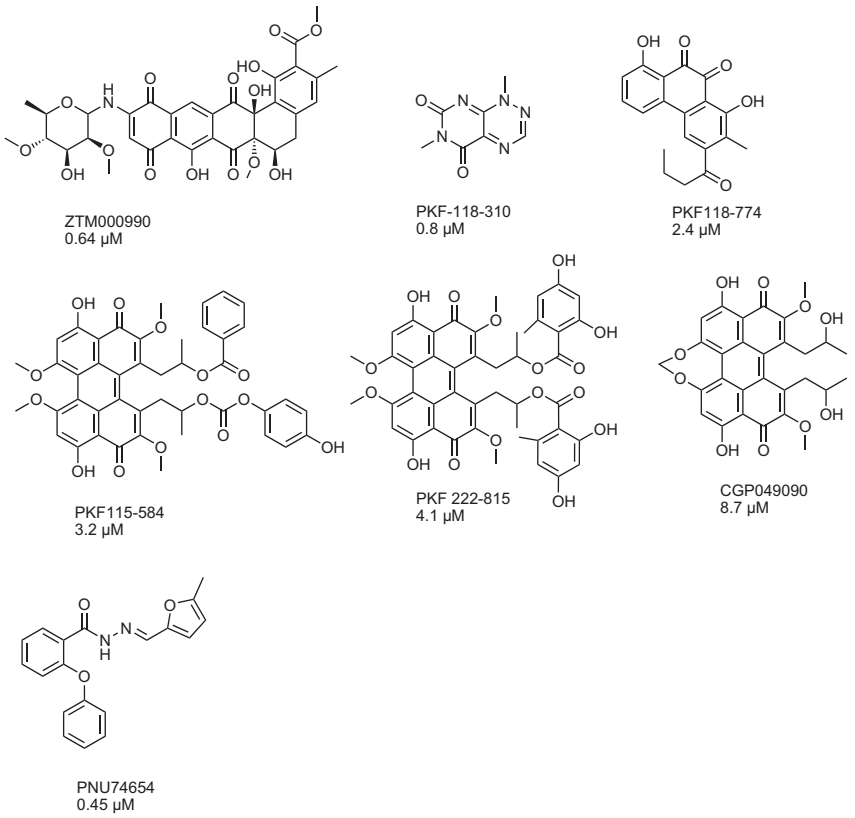


Figure 2. Chemical structures of small- and large-size inhibitors of Tcf4/b-catenin interaction.[18,20]

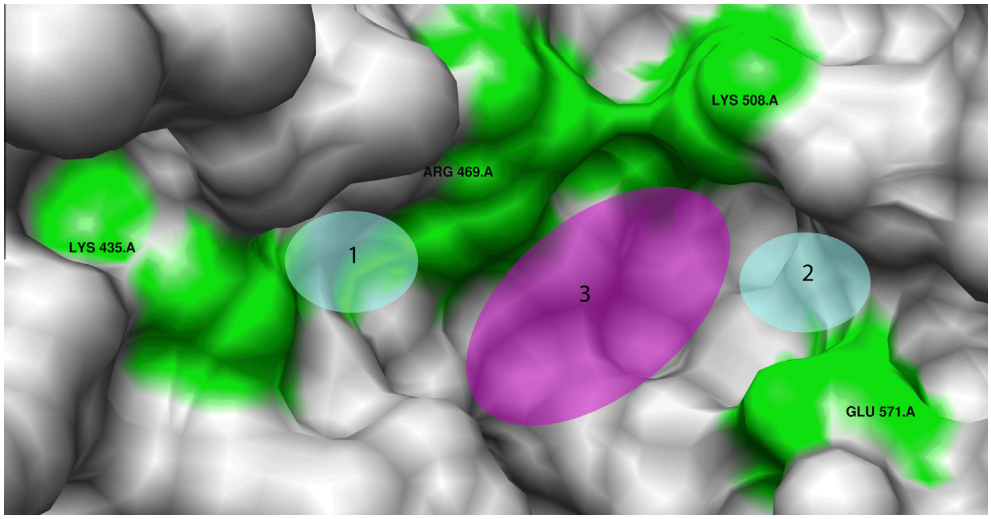
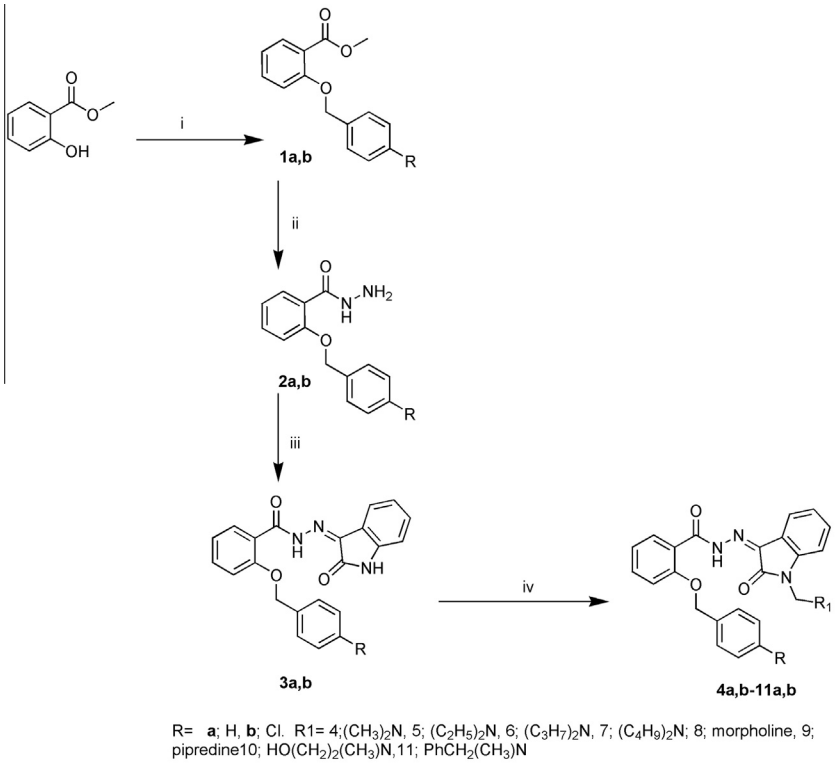


Figure 3. β-Catenin crystal structure.[20] Cavity 2 shows small binding pockets near Lys435/Arg469 referred to number 1, binding cleft between Lys508 and Glu571 referred to number 2; the floor area refers to number 3.

supportive of the notion of binding to a hydrophobic pocket where a size limit of the benzyl side chain is imposed that the chlorobenzyl group is oriented in different position than that of the benzyl group. Here it can be seen that increasing the dialkylamino from dimethylamino (**4a**, **4b** Inhibition% 29.77, 50.65, respectively) to the corresponding dibutylamino (**7a**, **7b** Inhibition% 53.50, 66.34, respectively) leads to an increase of activity. This indicates that hydrophobicity is an important parameter at this position. However, cyclization of the dibutylamino group (**7a**, **7b** Inhibition% 53.50, 66.34, respectively) into the corresponding morpholinyl group (**8a**, **8b** Inhibition% 50.55, 59.54, respectively) or piperidinyl moiety (**9a**, **9b** Inhibition% 45.63, 51.95, respectively) leads to decrease of activity. In addition, the piperidinomethyl derivative **9a**,**b** is less active than the morpholino methyl analog **8a**,**b**. The *N*-methyl benzylamine compounds are the most active (**11a**, **11b** Inhibition% 59.52, 76.18, respectively). This is supportive of the notion of preferred binding of the planar aromatic ring to a hydrophobic pocket.

Based on the fact that cell surface molecule CD44 is a downstream target of Wnt/β-catenin pathway,[18] docking studies were



Scheme 1. Synthesis of target compounds. Reagents and conditions: (i) appropriate benzylchloride, CH_3ONa; (ii) $NH_2NH_2{\cdot}H_2O/C_2H_5OH$, reflux; (iii) isatin/$C_2H_5OH$, reflux; (iv) appropriate secondary amine, HCHO, DMSO, rt.

Table 1
Measured in vitro CD44 inhibition%** and docking results of compounds **3a**,**b**–**11a**,**b**

No.	H-bond donor	H-bond acceptor	H-bond distance (Å)	Score in energy (kcal/mol^{-1})	MFI* ± SD	Inhibition%
	ResName.No.Atom	ResName.No.Atom				
3a	rec ASN.516.NH2	3a 3a.N3	3.282	−36.557	3142 ± 747	31.25
	rec ASN.516.NH2	3a 3a.O3	3.422			
4a	rec ARG.471.NH2	4a 4a.O2	3.269	−42.34	2276.66 ± 893	50.65
	4a 4a.N1	rec ASN.516.OD1	2.897			
5a	rec ARG.474.NH2	5a 5a.O2	3.132	−43.243	2164 ± 226	52.65
	rec ARG.474.NH1	5a 5a.O1	2.927			
6a	rec ARG.474.NH2	6a 6a.O2	3.222	−45.139	1875.33 ± 411	58.96
7a	rec ARG.515.NH1	7a 7a.O2	3.398	−44.916	1538 ± 543	66.34
	rec ARG.474.NH2	7a 7a.O3	3.541			
8a	ec ARG.474.NH1	8a 8a.N2	3.553	−43.087	1849 ± 421	59.54
	rec ARG.515.NH2	8a 8a.O3	3.751			
9a	rec ARG.474.NH1	9a 9a.O1	3.124	−42.307	2195.66 ± 904	51.95
	rec ARG.474.NH2	9a 9a.O2	3.081			
10a	10a 10a.O4	rec LYS.508.O	3.137	−45.825	1578.33 ± 36	65.46
	rec ARG.474.NH2	10a 10a.O2	3.008			
11a	rec ARG.474.NH1	11a 11a.O1	3.055	−46.023	1088.33 ± 47	76.18
	rec ARG.474.NH2	11a 11a.O2	3.017			
3b	rec ARG.515.NH2	3b 3a.N3	3.367	−39.684	3725.66 ± 564	18.48
	rec ASN.516.NH2	3b 3a.O1	3.617			
4b	ASN.516.NH2	4b 4b.O1	4.063	−38.586	3209.66 ± 986.8	29.77
5b	rec ARG.515.NH2	5b 5b.O1	2.897	−42.153	2233.33 ± 171	51.13
6b	rec ARG.474.NH1	6b 6b.N2	3.382	−43.747	2182 ± 226.3	52.25
	rec ARG.474.NH2	6b 6b.O2	2.868			
7b	rec ARG.474.NH1	7b 7b.N2	3.323	−44.792	2125 ± 225.3	53.50
	rec ARG.474.NH2	7b 7b.O2	2.932			
8b	rec ARG.474.NH1	8b 7b.O1	3.482	−41.511	2260 ± 713	50.55
	rec ARG.474.NH2	8b 7b.O2	3.231			
9b	rec ARG.474.NH2	9b 9b.O2	3.215	−40.505	2484.66 ± 183	45.63
10b	rec ARG.474.NH1	10b. 10b.O1	3.086	−45.181	2060 ± 256	54.92
	rec ARG.515.NH2	10b 10b.O3	2.991			
	10b 10b.O4	Rec Glu571.OE1	3.459			
11b	rec ARG.474.NH2	11b 6a.O2	2.992	−46.399	1850 ± 192.6	59.52

* Mean fluorescence intensity.
** The expression of treated cells by the different compounds is normalized to untreated cells.

carried out in order to investigate a possible binding mode of the synthesized compounds, using the available crystal structure of β-catenin, pdb code 1jdh.[25] Compound **11a** was docked into the allosteric site, cavity 2 of β-catenin and possible binding modes were analyzed based on their Gridscore fitness. The docking poses show four key interactions with the receptor: one nitrogen of the hydrazine group, imine nitrogen, act as H-bond acceptor for the Arg515 while the other nitrogen, amide nitrogen, of the hydrazine group acts as H-bond donors for the side chain of a glutamic acid Glu571; the benzene ring of the salicylic acid moiety binds to a small cleft between Lys508 and Glu571; the benzyl group binds to a small pocket adjacent to Lys435 while the *N*-methylbenzylamine moiety binds to a small pocket adjacent to Arg469; the isatin scaffold shows hydrophobic stack with the floor of the binding site. In addition, a π–cation interaction was suggested between a charged lysine and arginine and the aromatic rings of the compound. In addition, the lysine, Lys508 and arginine, Arg571 residues present in the site could show π–cation interactions with the aromatic rings *N*-methylbenzylamine and benzyloxy groups respectively (Fig. 4).

To confirm the docking study on a real setting, the most active compound **11a** was investigated whether it could reduce the activity of Wnt pathway reporter. In this study we used colon cancer cells transfected with TCF/LEF reporter plasmid. The activity is measured by the expression of green fluorescence GFP by flow cytometer. The transfected cells were cultured in 6 well and challenged by 2.5,5 and 10 μM for 48 h. The flow cytometric analysis showed consistence inhibition effect as seen in Figure 5.

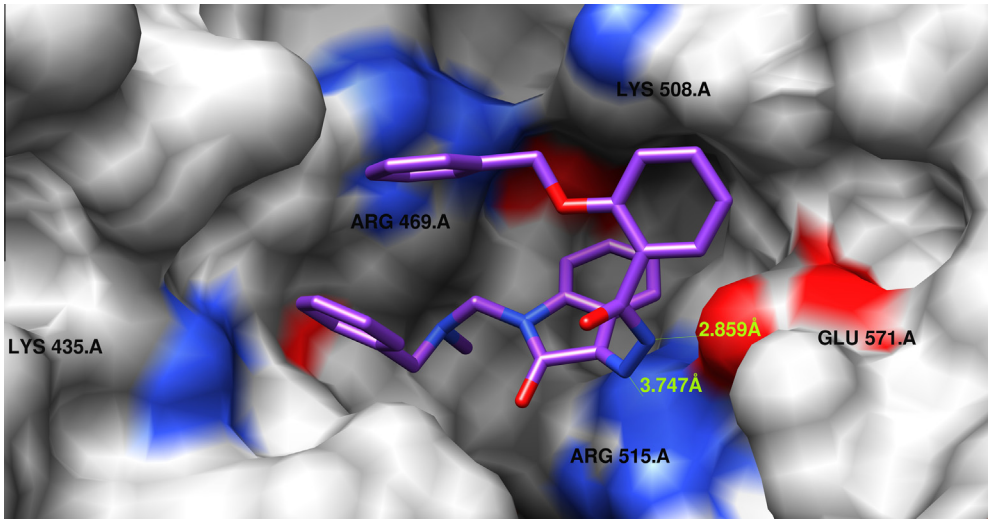


Figure 4. Suggested binding mode of **11a** (magenta) to the cavity 2 of 1jdh.pdb. It binds to both the floor of the cavity and the Lys435/Arg469 hot spot. Hydrogen bonds are colored green.

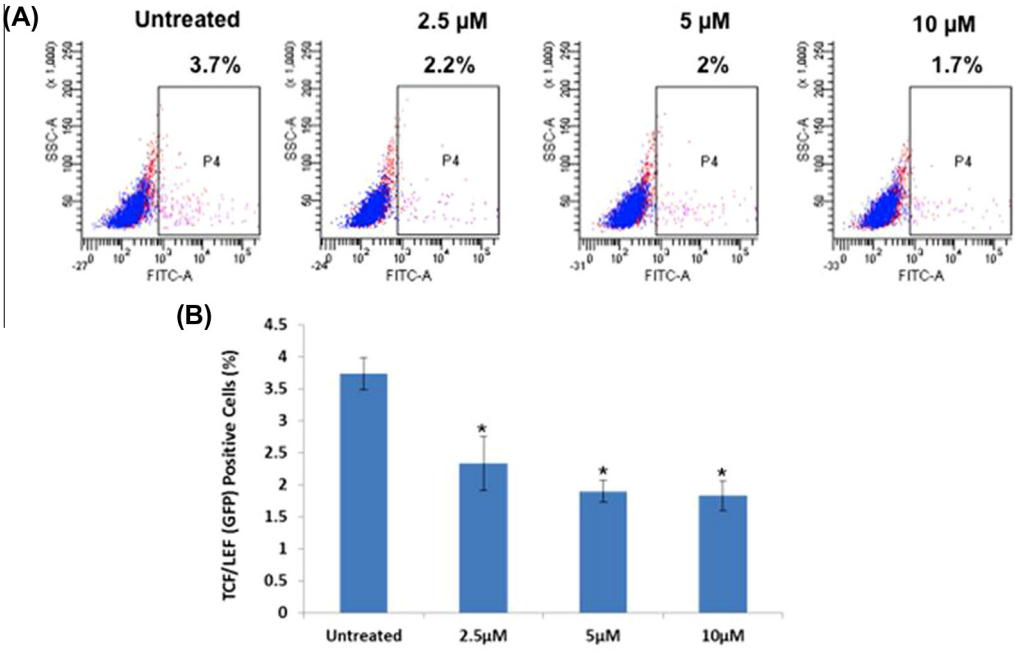


Figure 5. (A) Show representative dot plot of Wnt reporter-GFP of untreated and treated cells with **11a** 2.5, 5 and 10 μM. (B) Bar graphs showing mean ± SD Wnt reporter-GFP. Data represents the average ± SD (error bar) of three independent experiments.

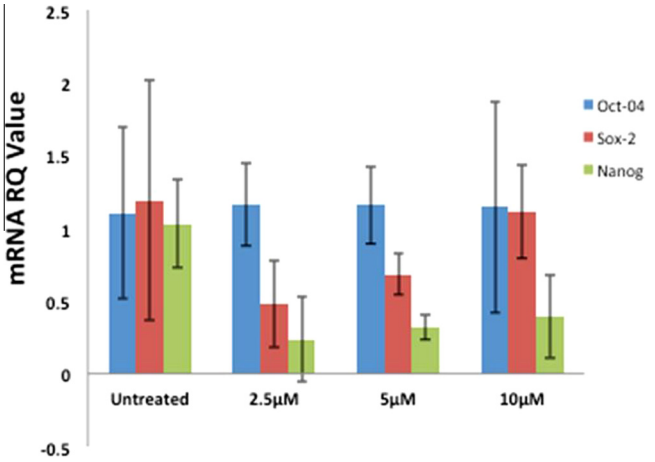


Figure 6. Endogenous levels of embryonic transcriptional factors Oct-4, Sox-2 and Nanog mRNA were examined by real-time RT-PCR. Nanog transcript was notably decreased in 10 μM and no changes were detected in the transcript of Oct-4 or Sox-2.

Table 2
In vitro cytotoxic activity of **4a**–**11a**, **11b** against cancer cell lines

Compound	IC_{50}[a] (μM)	
	CD44 negative HT-29	CD44 high LOVO
4a	48.77 ± 0.01	12.91 ± 0.03
5a	84.78 ± 0.02	14.76 ± 0.26
6a	95.54 ± 0.7	19.77 ± 0.17
7a	202.81 ± 0.4	27.22 ± 0.36
8a	In[b]	16.30 ± 0.08
9a	In	22.40 ± 0.59
10a	In	22.42 ± 0.38
11a	In	24.87 ± 0.47
11b	In	17.19 ± 0.25

[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.

Embryonic transcriptional factors Nanog, Sox2 and Oct-4 play critical roles in maintaining the pluripotence and self-renewal characteristics of CSCs. Therefore, it will be very interested for us to address the effect of *N′*-(2-oxoindolin-3-ylidene)-2-(benzyloxy) benzohydrazide derivatives represented by **11a** on these transcriptional factors. Practically, LOVO colon cancer cells were cultured in 6 well and treated by **11a** in 2.5, 5 and 10 μM for 48 h. The RNA was extracted and the genes expression was measured by RT-PCR. The treatment by different doses showed decreased in the expression of Nanog in dose dependent manner as shown in Figure 6. However, no change in the expression of Sox2 and Oct-4 was detected at 10 μM, suggested specificity in the effect and supported by the fact that CD44 increased the expression of Nanog through the interaction with Hyaluronan (HA).[26]

The endpoint in cancer treatment is killing cancer cells and inhibiting the highly proliferative nature of tumor cells. To test whether targeted derivatives induction of cytotoxicity is

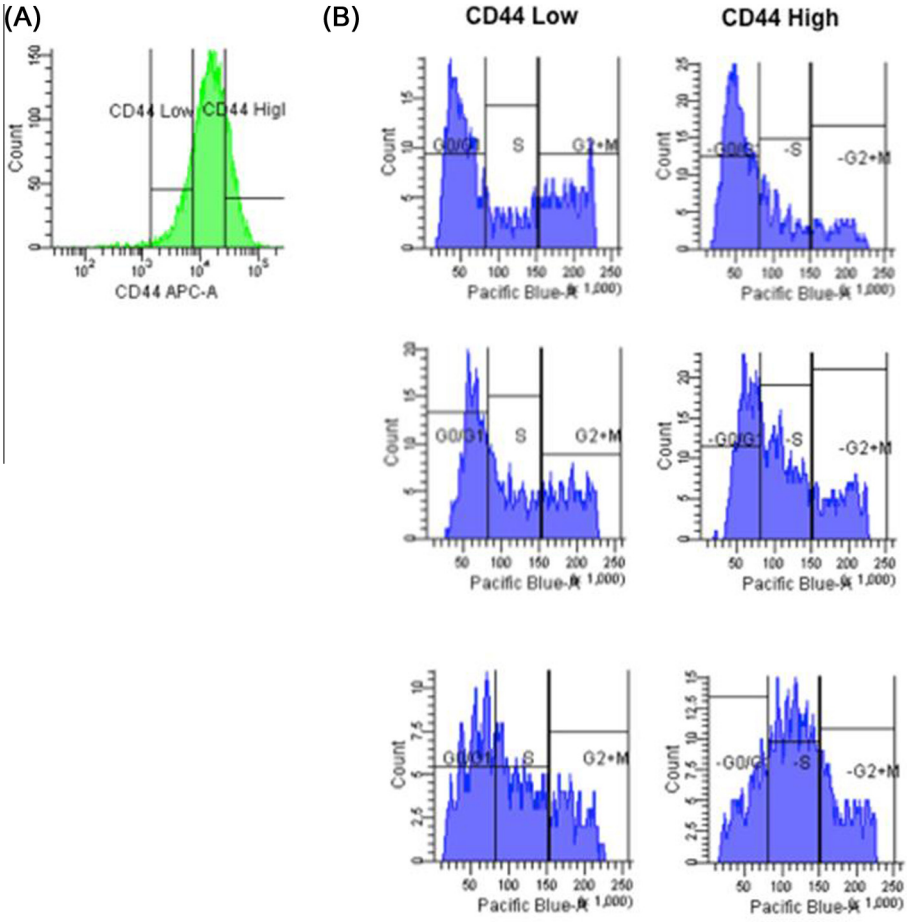


Figure 7. (A) Histogram of CD44 expression showed gated CD44 low and CD44 high subpopulation. (B) DNA cell cycle analysis of CD44 low and CD44 high subpopulation of untreated and treated cells with **11a** in 5 and 10 μM treatment.

dependent on CD44 expression or not, we used highly CD44 expressed colon cancer cell line LOVO and low CD44 expressed colon cancer cell line HT-29. The target derivatives with more than 50% inhibition effect on CD44 were used for the screening. To this end, by the used of WST-1 cytotoxic/cell proliferation assay, a selective cytotoxic effect of these targeted derivatives on LOVO cells but not HT-29 cells were found and IC_{50} for the selected derivatives was shown on Table 2. To confirmed CD44 dependent effect of these derivatives, the DNA cell cycle effect of the most active derivative **11a** based on CD44 expression on the same type of cells in culture was evaluated. By using combination of DCV and CD44-APC antibody, the DNA cell cycle in CD44 high and CD44 low expressed cells on same cells with and without treatment can be analyzed. Interestingly, in untreated cells the majority of CD44 high cells were found to be at G0 checkpoint in non-proliferative phase. On the other hand CD44 low expressed cells from the same culture cells were found to be in proliferative stages at S, G2 checkpoint and M phase. By challenging the HT-15 cells with different doses of **11a** for 48 h, we found that **11a** block the cell cycle inhibitory effect of CD44 and induced cell accumulation of CD44 high cells in cycling phase in S, G2 and M phase as seen in Figure 7. The effect on cell cycle was seen in 5 and 10 πM on CD44 High cells and no effect on DNA cell cycle was seen in CD44 Low cells in 5 μM treatment. In 10 μM treatment, CD44 high cells showed more sensitivity than CD44 Low cells and more accumulation were seen at S phase. One of the mechanisms of how CSCs are resistance to chemotherapy is slow cycling nature of these cells and therefore insensitive to toxic drug. Taken together, it is clear that *N*′-(2-oxoindolin-3-ylidene)-2-(benzyloxy)benzohydrazides blocks CD44 effect on cell cycle and push CD44 high cells toward cycling phases. The general cytotoxic effect seems to be dependent on CD44 expression in which these derivatives showed no toxicity on CD44 negative HT-29 cells as shown in Table 2.

In summary, we focused on the evaluation of *N*′-(2-oxoindolin-3-ylidene)-2-(benzyloxy)benzohydrazide derivatives as potent and novel CD44 inhibitors. The docking and reporter activity demonstrated the involvement of Wnt/β-catenin as a main pathway target of these derivatives. The in vitro antitumor effect of this series including cytotoxicity and cell cycle effect seems to be dependent on CD44 expression.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.02.064.

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