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Identification of compounds that augment the lectin-sensitivity of Chinese Hamster Ovary cells

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ABSTRACT

Glycosylation is now recognized as one of the most important modifications of eukaryotic proteins. In cancer biology, alterations in cell surface glycosylation have been exploited as valuable biomarkers, and the relationship of this modification to the metastatic characteristics of cancer cells has also been well-documented. Chemicals that can alter cell surface glycosylation patterns will therefore become attractive lead compounds for controlling the metastatic characteristics of cancer cells, one of the critical factors in their malignancy and prognosis of the disease. In this study, we established a system for screening compounds that have the potential to alter cell surface glycosylation by taking advantage of the susceptibility of cells toward various lectins. Through our screening of a chemical library, we were able to identify two compounds that augment the sensitivity of Chinese Hamster Ovary (CHO-K1) cells against the L4-PHA lectin. Surprisingly, these compounds did not result in alterations in cell surface glycan structures. Instead, they appeared to render the cells to be more sensitive to various lectins with distinct carbohydrate specificities. These compounds promise to be valuable, not only as tools for providing insights into the intracellular signaling of lectin-mediated growth arrest, but also as potential lead compounds for use as therapeutic, anti-cancer drugs.

1. Introduction

Glycosylation is one of the most common post-translational modifications of eukaryotic proteins, and has been shown to affect their physicochemical/physiological properties [1]. It is also known that malignant transformations in tumors are often associated with alterations in the glycosylation pattern [2–6], and that glycan-based epitopes represent valuable biomarkers for monitoring the occurrence/growth status of tumor cells [7–9]. It has also been proposed that changes in glycan structures on cancer cells can serve as hallmarks for their metastatic potential, affecting multiple steps during this devastating process [4,10,11]. As cancer is one of the leading causes of death for humans, both diagnostic/therapeutic uses of glycans for this mortal disease have attracted considerable interest from the biomedical science community. Drugs that could impact the glycosylation pattern of cancer cells are attractive compounds, especially if it could change the metastatic property of cancer cells [12–15]. Because of this, efforts have been directed to the development of anti-cancer drugs using inhibitors of glycan processing [16,17].

Historically, Chinese Hamster Ovary (CHO-K1) cells have been used for the isolation of various glycosylation mutants (Lec cells) [18]. These mutants were selected for their resistance to the cytotoxicity of plant lectins [19,20], and their resistance is attributed to the reduced cell surface-glycan structures recognized by lectins. The objective of this study was to screen small chemicals that could alter the sensitivity of CHO-K1 cells against the L4-PHA lectin. Through the screening of 456 compounds which represent the 25,000 small chemical compound library (NPDepo), two compounds that render the cells to be more sensitive to the lectin were identified. Interestingly, these compounds did not cause an alteration in the cell surface glycan structures, but instead, caused the cells to be more sensitive to various lectins with distinct carbohydrate-binding specificities, indicating that these compounds cause the cells to be more susceptible to lectin-mediated growth arrest. At least one of the compounds identified in this study exhibited similar characteristics, *i.e.* an enhancement in sensitivity against various lectins, for HeLa cells, implying that the effect of the compound is not specific for CHO-K1 cells but may well be common to a variety of cell-types. These chemical compounds promise to be

Abbreviations: Con A, concanavalin A; RGI, relative growth index.

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useful for not only clarifying the still-enigmatic molecular mechanism of lectin-mediated growth arrest, but also could be attractive lead compounds for anti-cancer drugs in combination with lectins that recognize cancer-specific carbohydrate-antigens.

2. Materials and methods

2.1. Materials

Concanavalin A (Con A), E4-PHA, and L4-PHA lectins were obtained from Seikagaku (Tokyo). Biotinylated L4-PHA and Con A were obtained from Vector Laboratories (Burlingame, CA). Alexafluor 488 conjugated streptavidin was obtained from Invitrogen (Tokyo).

2.2. Cell culture

CHO-K1 cells were maintained in Ham's F-12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo) and antibiotics (100 U/ml penicillin and 100 ng/ml streptomycin; Nacalai Tesque, Kyoto). HeLa cells were maintained in Dulbecco's Modified Eagle Media (Nacalai Tesque), supplemented with 10% FBS and antibiotics. Cells were incubated in a humidified incubator at 37 °C under a 5% CO_2 atmosphere.

2.3. Lectin sensitivity assay

In standard assays, 2.0×10^4 cells were plated in each well of 96-well plates and incubated with 25 μg/ml of chemicals in the presence or absence of the L4-PHA lectin (20 μg/ml). After 48 h, the cells were washed once with PBS (PBS (−); Nacalai Tesque), fixed with 3% paraformaldehyde–PBS at room temperature for 20 min, and washed again with PBS. The nuclei of cells were then stained with 300 nM DAPI (D-1306; Life Technologies, Tokyo) solution in PBS. After 20 min, the DAPI solution was removed and the cells were washed with PBS. The fluorescence of each well was determined by means of an In Cell Analyzer 1000 (GE Healthcare) with a 20× objective. Fifteen images were acquired per well and cell numbers were counted using the In Cell Analyzer analysis software Developer. Relative growth index (RGI) was calculated using the following formula:

$$\mathrm{RGI} = (\text{average cell numbers in an image in the presence of lectin}) / (\text{average cell numbers in an image in the absence of lectin})$$

2.4. Screening of a chemical library for lectin sensitivity assay

Screening of a chemical library for lectin sensitivity was carried out using the RIKEN Natural Product Depository (NPDepo), a library of small chemicals focused primarily on microbial metabolites [21]. An authentic NPDepo library (80 compounds) and an NPDepo pilot library (376 compounds) were used. The former is a collection of compounds whose biological activities have been previously reported. The latter contains representative compounds of NPDepo, whose structures are not similar to each other. Detail of these libraries is available upon request.

2.5. Flow cytometry

CHO-K1 cells were treated with either 14 μM swainsonine or drugs (cepharanthine (2.1 μM), NP463 (4.0 μM) or NPD26 (30 μM)) and were incubated for 48 h. Cells were washed with PBS, incubated with 1 mM EDTA/PBS for 5 min, and then suspended in PBS containing 10 mg/ml BSA to give a concentration of 1.5×10^6 cells/ml. The cells were then stained with biotinylated L4-PHA or Con A (10 μg/ml) and streptavidin-Alexa fluor 488 conjugate (1:400 dilution) and subjected to a flow cytometry analysis using a BD LSR flow cytometer and CellQuest Pro software (BD Biosystems).

2.6. Determination of IC50 concentration of various lectins

Lectin sensitivity assays were carried out under various lectin concentrations, and the RGI was determined as described above. The logarithm of the lectin concentration was plotted against the RGI, and IC50 values were calculated by linear regression around a 50% growth inhibition concentration (*i.e.* RGI = 0.5) of data.

3. Results and discussion

3.1. Establishment of screening conditions for a lectin sensitivity assay

In this study our initial goal was to establish the conditions for the screening of a chemical library leading to the isolation of compounds that could affect cell surface glycan structures by utilizing growth arrest caused by a plant lectin. The L4-PHA lectin was used in the initial screening, because it has been shown to react with the GnT-V product, *i.e.* β1–6 GlcNAc-branching *N*-glycans [22,23] and the presence of β1–6 GlcNAc-branching *N*-glycans with poly-*N*-acetyllactosamine structures has been reported to be correlated with the malignant transformation of cells [24,25]. Chemicals that cause cells to be sensitive toward the L4-PHA lectin, therefore, would be expected to have an impact, directly or indirectly, on the expression level of β1–6 GlcNAc-branching *N*-glycans, and in turn the malignancy and/or metastatic potential of cancer cells.

As shown in Fig. 1A, CHO-K1 cells were shown to exhibit sensitivity against the L4-PHA lectin at concentrations above 5 μg/ml. We therefore used concentrations of 20 μg/ml for the screening conditions, where we observed the relative growth index (RGI: growth in the presence of the lectin relative to growth in the absence of lectin) of 0.60 ± 0.10.

If a chemical were to affect the sensitivity of cells toward this lectin, one would expect that cell growth would either recover (with less lectin binding) or decrease further (with more lectin binding). As a proof-of-concept, swainsonine was used to test whether this compound influenced the sensitivity of CHO-K1 cells against L4-PHA lectins by altering the cell-surface glycan structures. Swainsonine is an inhibitor of Golgi α-mannosidase II and, as such, it would be expected that this compound would inhibit the formation of β1–6-linked GlcNAc structures on *N*-glycans (Fig. 1C) and, as a result, swainsonine-treated cells would become resistant to the L4-PHA lectin. As anticipated, sensitivity against L4-PHA was canceled in the presence of swainsonine (Fig. 1B). These results indicate that our screening conditions have the capability for selecting chemical compounds that can affect the formation of glycan epitopes that are recognized by the L4-PHA lectin.

3.2. The identification of compounds that render CHO-K1 cells more susceptible to the L4-PHA lectin

Having established appropriate screening conditions, we screened chemical compounds using the RIKEN Natural Product Depository (NPDepo) library [21]. Among 25,000 compounds in the depository, 456 were selected, and hit compounds were selected by the screening strategy outlined in Fig. 2A. In the primary screening step, compounds were selected based on the following three criteria; (I) enhanced resistance: compounds showing resistance against L4-PHA, *i.e.* a relative growth index (RGI) >0.8; (II) enhanced sensitivity: compounds exhibiting sensitivity against

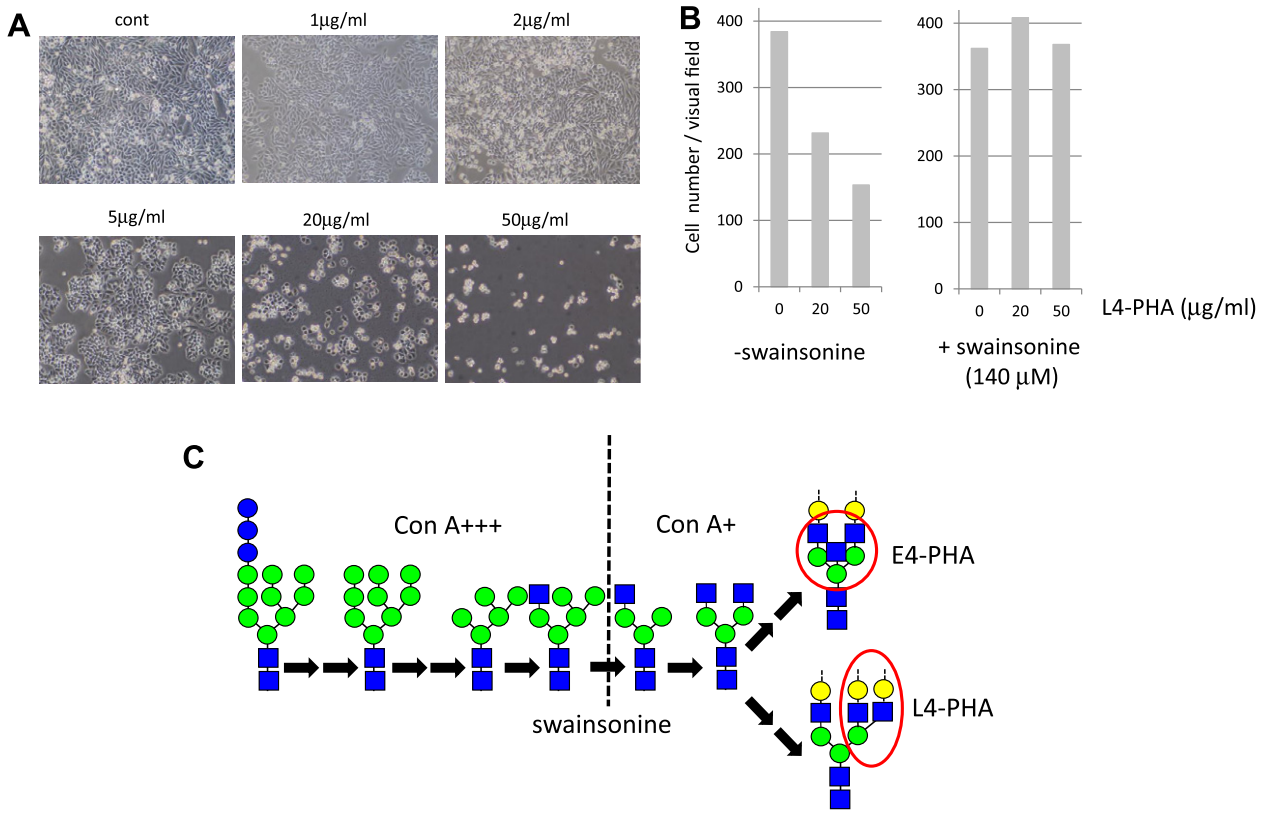


Fig. 1. (A) Phase-contrast images of CHO-K1 cells in the absence or presence of various concentrations of the L4-PHA lectin. (B) Effect of swainsonine on the sensitivity against L4-PHA. (C) Schematic representation of glycan processing for *N*-glycans in mammalian cells and the effect of swainsonine on this pathway. Swainsonine inhibits the activity of Golgi α-mannosidase II and therefore inhibits the formation of GnT-V-mediated β1–6 linked GlcNAc residues. Structures recognized by L4-PHA or E4-PHA were also indicated [22,40].

L4-PHA, *i.e.* RGI <0.3; (III) compounds that are toxic to CHO-K1 cells; exhibiting a <10% growth compared to CHO-K1 cells in the absence of the compounds. After screening 456 compounds, 45 in category (I), 22 in (II) and 16 in (III) were selected for further screening. In the secondary screening, the optimal concentration for the lectin sensitivity assay (maximal concentration where no inhibitory effect on growth was observed) was determined for each compound. After fixing the concentrations for these compounds, further screening was carried out. As a result, 5 compounds from category I and 10 compounds from category II were subjected to further analysis. After a careful dose-dependency analysis of drugs to determine the optimal concentration for the lectin-mediated growth arrest assay, 2 compounds were finally identified from category (II) that showed a significantly enhanced sensitivity against L4-PHA lectin. The structures of the compounds are shown in Fig. 2B.

The isolated compounds did not change the cell surface glycan structures of CHO-K1 cells, but enhanced the sensitivity of the cells against lectin-mediated growth arrest.

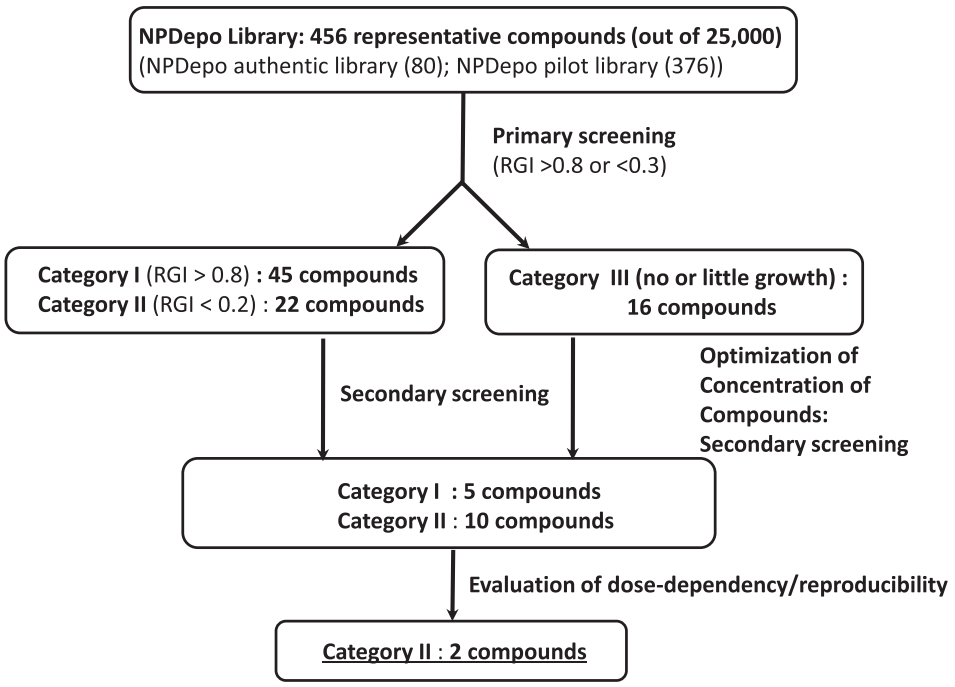
Since the pilot library used for the screening was designed as a representative one for the 25,000 compounds in the NPDepo library, once the hit compounds are identified, structurally-related compounds can be further selected using a chemo-informatical approach. In this screening, 20-related compounds were chosen for NP430 (cepharanthine), designated here as group 1, while 9 compounds were chosen for NPD26, designated as group 2. Through the screening of these structurally-related compounds, 7 compounds (5 from Group 1 and 2 from Group 2) were further identified as potent enhancers (equal to or better than the original compound) for the lectin-mediated growth arrest of CHO-K1 cells (data not shown; see also Fig. 3B). The structures of these compounds are shown in Fig. 3A.

Next we addressed the issue of how they alter the lectin-sensitivity of CHO-K1 cells. For this purpose, the effects of sensitivity against various different lectins were examined. We hypothesized that, if the compounds could affect the cell surface glycan structures, their sensitivity toward different lectins would be altered, as were the cases for various Lec (glycosylation) mutants isolated from CHO-K1 cells [18]. To our surprise, lectin-sensitivity was enhanced for all the lectins examined (Fig. 3B). Swainsonine was examined as a control, and, as expected, distinct effects were observed, *i.e.* sensitivity for Con A and resistance against L4-PHA or E4-PHA (structures recognized by these lectins were shown in Fig. 1C) were enhanced. These results indicated that the isolated compounds might not affect the cell surface glycan structures, but rather they appear to affect the signaling pathway for lectin-mediated growth arrest.

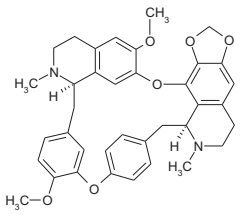
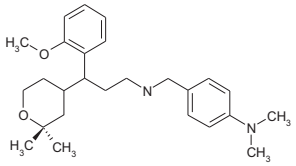
To unequivocally demonstrate that these compounds did indeed enhance the sensitivity against various lectins, the effect of the compounds on lectin sensitivity was examined using Con A, a lectin with a distinct binding specificity from L4-PHA. For this analysis, 3 compounds each were selected from both group 1 and group 2. As shown in Fig. 3C, all compounds resulted in an increased sensitivity against Con A, as were the cases with the original L4-PHA lectin, further supporting the conclusion that these compounds augment the sensitivity of a cell against lectins, irrespective of their binding specificity.

To further confirm that the compounds did not alter the cell surface glycan structures, a FACS analysis was carried out. As shown in Fig. 3D, a drastic reduction in staining with L4-PHA for

A



B

Name	Structure	Formula	MW	Structurally related compounds[*1]
NP430 (Cepharanthine)		$C_{37}H_{38}N_2O_6$	606.73	20
NPD26		$C_{26}H_{38}N_2O_2$	410.60	9

[*1] number of available compounds found in NPDepo library.

Fig. 2. (A) Scheme of screening for small chemicals affecting the sensitivity against L4-PHA lectins. (B) Structures of chemical compounds isolated by the screening.

swainsonine-treated cells (top panel), concomitant with the increase in staining with Con A (bottom panel), was observed. These results indicate that the structures of cell surface glycans on swainsonine-treated cells had been altered. In sharp contrast, no significant changes in lectin staining were observed for cells treated with the compounds (Fig. 3D). These results clearly support the hypothesis that the compounds did not directly affect the cell surface glycan structures of CHO-K1 cells, but rather changed the sensitivity against lectin-mediated growth arrest.

Finally, we examined the issue of whether the isolated compound had similar effect on other cell lines. As shown in Table 1, cepharanthine (NP430) was found to be effective for enhancing the effect of lectin-mediated growth arrest in HeLa cells with all lectins tested. This result most likely indicates that the effect of these compounds on the lectin-mediated growth arrest is a common phenomenon for a wide variety of mammalian cells, but the details of the mechanism for this remains to be determined.

3.3. General discussion

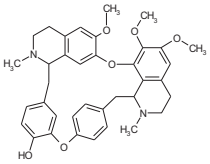
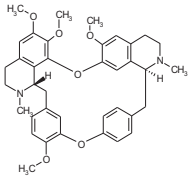
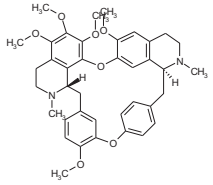
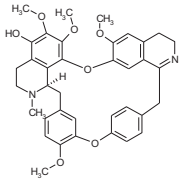
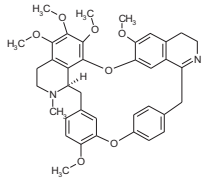
In this study, our initial effort was directed at establishing a screening method for low molecular weight compounds that could affect the structures of cell surface glycans. Our methodology can be applied for screening for potential inhibitor/activators of any glycosyltransferases of interest, as long as a lectin with the desired carbohydrate-binding specificity is available.

In our pilot screening, we chose to use the lectin sensitivity of CHO-K1 cells as the output for the effect of compounds, since this cell line has been successfully used for the isolation of various glycosylation mutants [18]. We chose the L4-PHA lectin to determine if, by altering the sensitivity of this lectin, that the activity of the GnT-V gene would be affected. The GnT-V gene product has been linked to the metastatic property of cancer cells [10], and therefore compounds that could render cells resistant could be attractive lead compounds for the development of anti-metastatic drugs.

Our screening method worked well for swainsonine, an inhibitor of the formation of β1–6 GlcNAc-branching *N*-glycans (Fig. 1C) and which has been tested for a potential anti-cancer drug [16,17,26].

Despite our initial expectations, the compounds identified by our pilot screening did not alter the cell surface glycan structures of CHO-K1 cells. Instead, the CHO-K1 cells showed a hypersensitivity toward various lectins in the presence of these compounds. These results led us to hypothesize that they likely render CHO-K1 cells more sensitive to lectin-mediated growth arrest (we refer to the phenotype "growth arrest" as the case where the effect of a lectin is reversible under our experimental conditions). To the best of our knowledge, the molecular mechanism for cytotoxicity or growth arrest by lectins against certain cells is unclear, with the exception of ricin-type lectins bearing a ribosome-inactivating protein subunit [27,28]. For example, the apoptosis-inducing activity of Con A against various cell lines has been well-documented [29,30]. Under our experimental conditions, however, based on the morphology of the nuclei, no indication of apoptotic reactions for CHO-K1 cells in the presence of the lectins tested was observed. This fact, together with the fact that the effect is reversible, leads us to believe that the lectin-mediated growth arrest observed in this study may not be related to apoptosis. More importantly, the issue of how lectins with different binding specificities can mediate similar growth arrest by binding to the surface of certain cells is

A Group 1

Name	Structure	Formula	MW
NP443 (Rapandine/Oxyacanthine)		$C_{37}H_{40}N_2O_6$	608.74
NP463 (Tetrandrin) (Frachinine)		$C_{38}H_{42}N_2O_6$	622.77
NP257 (Hernandezin)		$C_{39}H_{44}N_2O_7$	652.79
NP77		$C_{37}H_{38}N_2O_7$	622.72
NP43		$C_{38}H_{40}N_2O_7$	636.75

Group 2

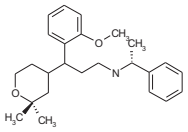
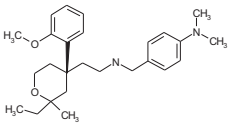
Name	Structure	Formula	MW
NPD85		$C_{25}H_{35}NO_2$	381.56
NPD48		$C_{26}H_{38}N_2O_2$	410.60

Fig. 3. (A) Chemical structures of related compounds that enhance sensitivity toward various lectins. (B) Effect of various lectins on sensitivity of the chemicals. Numbers represent the optimized concentration of compounds (μM) used for this analysis. Original compounds identified by screening were underlined. SW, swainsonine. Lectin concentrations used in this analysis were as follows: Con A, 25 μg/ml; E4-PHA, 30 μg/ml; L4-PHA, 25 μg/ml. (C) Dose-dependency of structurally-related chemicals (3 from group 1 and 3 from group 2) on Con A-sensitivity. (D) FACS analysis of cell-surface glycan structures on swainsonine- and compounds-treated CHO-K1 cells. upper panel: staining with biotinylated L4-PHA; bottom panel: staining with biotinylated Con A.

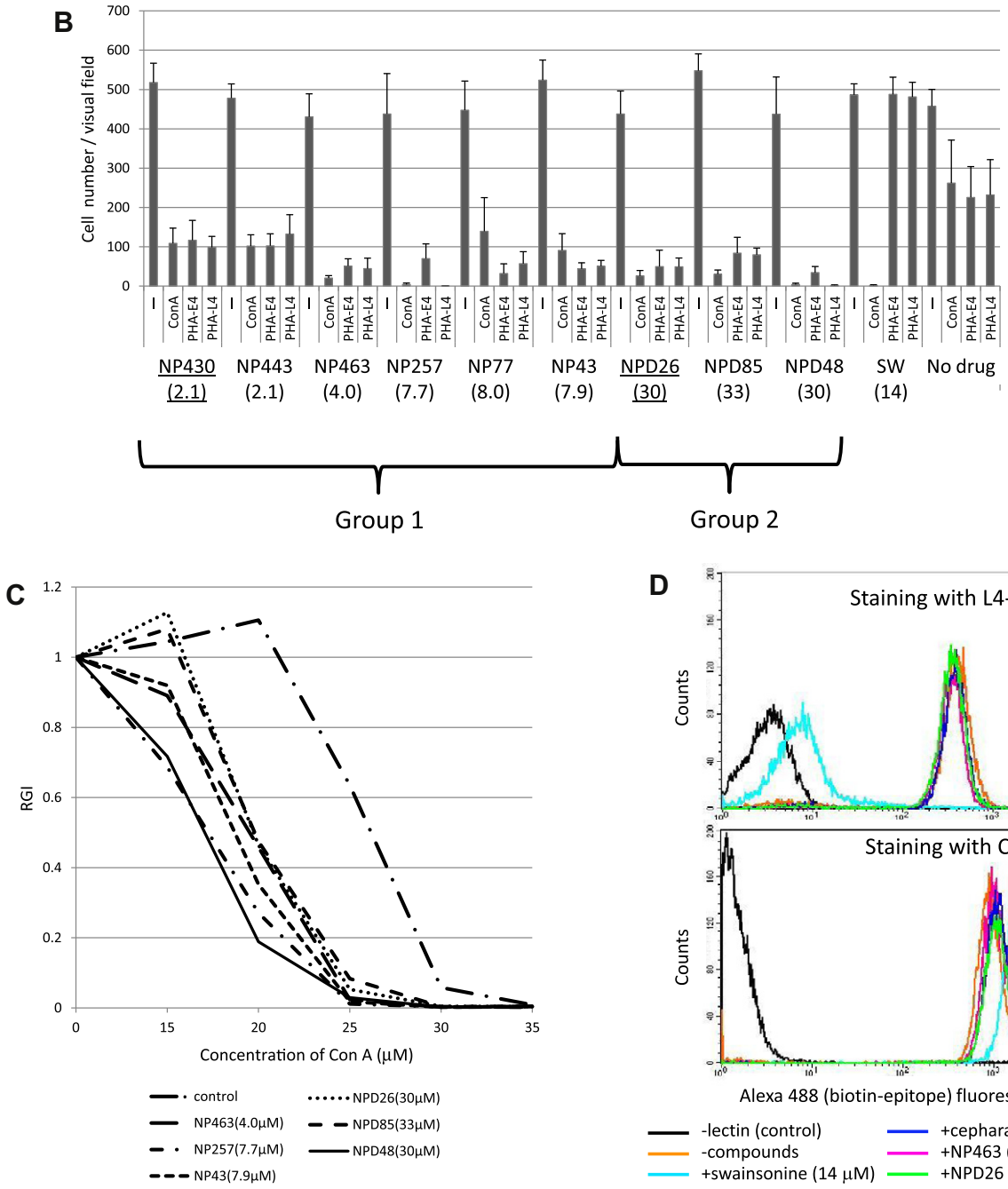


Fig. 3 (*continued*)

not fully understood. The compounds identified in our screening thus could be useful for collecting more detailed information related to the underlying mechanism. In this sense it should be noted that, at least for cepharanthine, such an effect was not specific for CHO-K1 cells but could be observed for HeLa cells, suggesting that the effect for the lectin-mediated growth arrest is a rather general phenomenon.

It is worth noting that the compounds identified in our screening could also serve as promising lead compounds for the development of anti-cancer drugs, since many examples of cancer-specific carbohydrate-antigens have been reported in the literature [7–9]. Cepharanthine is a particularly interesting compound that has already been approved by the Japanese Ministry of Health for the treatment of a wide variety of acute and chronic diseases [31,32]. While cepharanthine exhibits various beneficial pharmacological activities such as reversing multi-drug resistance [33,34] or potentiating chemotherapy [35,36], our study provides yet another

Table 1
IC50 values of various lectins for HeLa cells in the absence and presence of group 1 compounds.

		IC50 (μg/ml)		
		Con A	E4-PHA	L4-PHA
CHO-K1 cells	Control	26	29	33
	+compound[a]	19	21	12
HeLa cells	Control	14	45	>200
	+compound[b]	9.7	30	130

[a] NP463 (4.0 μM).
[b] Cepharanthine (8.2 μM).

intriguing bioactivity for this compound, *i.e.* augmenting lectin-mediated growth arrest. A number of studies have also shown that cepharanthine itself exerts antitumor activity through the induction of apoptosis in various cancer cells [37–39]. It is therefore tempting to speculate that, in combination with a lectin/anti-carbohydrate antibody that recognizes cancer-specific antigens, the antitumor activity of cepharanthine could be further augmented, without damaging the non-cancerous, normal tissues. Validating such an intriguing possibility must await further studies.

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