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# A DNA Topoisomerase II Inhibitor Results in *Ex Vivo* Differentiation of THP-1 Cells and Activation of Dendritic Cells

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**Abstract.** *Background/Aim: Effective ex vivo maturation of dendritic cells (DCs) can increase the efficiency of cancer immunotherapy. We aimed to identify novel chemicals with the potential to differentiate and activate immature DCs (iDCs) to mature DCs (mDCs). Materials and Methods: The expression of surface markers on THP-1 monocytes treated with the screened compounds was analyzed using FACS. Subsequent DC subset analysis and secreted cytokine profiling were also performed. Results: FACS analysis showed that THP-1 cells treated with amsacrine hydrochloride, a DNA topoisomerase II inhibitor, exhibited the typical phenotype of conventional DCs (cDCs). The expression of DC activation markers was also increased after amsacrine treatment. The profile of cytokines produced by THP-1 cells treated with amsacrine was similar to that of mDCs. Conclusion: Amsacrine has an ex vivo capability of differentiating THP-1 monocytes into cDCs. As amsacrine has been used as a stable chemotherapeutic agent in humans, it can be useful for producing mDCs for cancer immunotherapy.*

Dendritic cells (DCs) are the most potent type of antigen-presenting cells (APCs) of the mammalian immune system. Professional DCs break down antigens into peptides through phagocytosis, which are bound to major histocompatibility complex (MHC) glycoproteins that carry the peptides to the surface where they can be presented to lymphocytes. DCs migrate into lymphoid tissue and activate T cells, thus commencing the adaptive immune response (1). Further, they promote immune activation or tolerance by providing immune regulation signals *via* cytokine secretion (2).

DCs are derived from the hematopoietic stem cells in the bone marrow and roughly categorized into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs are derived from common DC precursors and preclassical DCs (3), and categorized into cDC1 and cDC2. Representative markers for cDC1 are BDCA-3 [cluster of differentiation (CD) 141], the chemokine receptor XCR1, C-type lectin Clec9A, and the cell adhesion molecule CADM1 (3-5), whereas cDC2s express CD1c, the signal-regulatory-protein SIRP (CD172a), CD14, and CD5. pDCs are derived from common DC precursors or lymphoid precursors and characterized by the expression of CD123, BDCA-2 (CD303), and BDCA-4 (CD304) (6). Despite their different roles, both pDCs and cDCs migrate from the bloodstream to the lymphoid and non-lymphoid tissues (3) and have complementary effects.

DCs are originally present as immature DCs (iDCs) in the blood, and recognize pattern recognition receptors, including toll-like receptors (TLRs), when exposed to exogenous pathogens. iDCs degrade phagocytosed pathogens, and when matured, present pathogen–MHC complexes to cell surface (1). Further, they upregulate costimulatory molecules such as CD80 (B7.1), CD83 (B7.2), and CD40 on their surface, thereby enhancing T-cell activation. After encountering antigens, mature DCs (mDCs) migrate to lymph nodes *via* the bloodstream and activate naïve T cells and B cells in the secondary lymphoid organs (7).

Cancer immunotherapy is gaining increasing attention, and substantial efforts to expand its application are underway. DCs as professional APCs activate the naïve T cells and consequently play an important role of inducing

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and maintaining antitumor response (8). DCs also mediate antitumor immunity triggered by chemo-radiation therapy-induced immunogenic cell death (ICD) (9). When "eat-me" signal is exposed on the cell surface, dying tumor cells are uptaken by DCs. Simultaneously, ICD leads to the release of damage-associated molecular patterns such as ATP, high mobility group box 1 (HMGB1), and calreticulin from dying tumor cells (10), which can be sensed by DCs through TLRs and promote cross-presentation of tumor-associated antigens (11, 12). Indeed, facilitating *in vivo*/*ex vivo* maturation and activation of DCs could be a way of potentiating efficacy of cancer immunotherapy.

Here, we aimed to study novel chemicals that have potential to differentiate and activate iDCs to mDCs *via* drug screening method. Of note, amsacrine hydrochloride, a DNA topoisomerase II inhibitor, was found to have *ex vivo* capability of differentiating THP-1 cells to DCs even faster than TNF-α and ionomycin. Amsacrine-treated THP-1 cells showed mature-looking dendrites and surface marker expression reflecting DC activation.

## Materials and Methods

*THP-1 cell culture*. THP-1 cells (human monocyte cell line) were purchased from American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Corning, Woodland, VA, USA) and 1% penicillin/streptomycin (P/S, Invitrogen, Eugene, OR, USA) at 37˚C in a 5% $CO_2$ incubator. Cells were sub-cultured when cell concentration reached $8\times10^5$ cells/ml.

*High throughput screening*. To screen for substances that differentiate or activate DCs, $1\times10^4$ THP-1 cells were seeded in a 384-well plate in 40 μl of serum free medium containing 100 ng/ml of recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF, PeproTech, East Windsor, NJ, USA), recombinant human interleukin 4 (IL-4, PeproTech), 0.05 mM β-mercaptoethanol (2-ME, Life Technologies), and P/S. Next, 1,280 pharmacologically active compounds (LOPAC1280 library, Sigma-Aldrich, St. Louis, MO, USA) were tested using JANUS automated workstation (PerkinElmer, Waltham, MA, USA). All compounds were used at concentrations of 0.5 μM and 1 μM. Consequent cell images were obtained through Operetta High content image system (PerkinElmer). For negative control, DMSO (Sigma-Aldrich) was added and for positive control, 10 ng/ml of tumor necrosis factor-α (TNF-α, PeproTech) and 200 ng/ml of ionomycin (Sigma-Aldrich) were added.

*Differentiation of DCs and flow cytometry analysis*. THP-1 cells were resuspended in serum-free medium supplemented with 100 ng/ml GM-CSF, 200 ng/ml IL-4, 1% P/S, and 500 nM 2-ME. The cells were seeded in a 6-well plate at a concentration of $4\times10^5$ cells in 2 ml of medium per well. After 3 h of incubation, the cells were treated with the selected six chemical compounds (Sigma-Aldrich) at 0.1, 0.5, 1, or 10 μM for 3 days. The cells were treated with DMSO for the negative control and with 10 ng/ml TNF-α and 200 ng/ml ionomycin for the positive control. Next, the cells were harvested and washed by fluorescence-activated cell sorting (FACS) buffer [Dulbecco's phosphate-buffered saline (DPBS), Biowest, Nuaillé, France] containing 2% FBS. Fc receptors were blocked using anti-CD16/32 antibodies (Human TrueStain, BioLegend, San Diego, CA, USA) for 5 min at room temperatures and stained with fluorescent labeled antibodies for 30 min at 4˚C in the dark. The cells were then washed and stained with 0.3 μM of 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen, Eugene, OR, USA) to discriminate dead cells. Cells were analyzed by flow cytometry (FACS Canto II, BD Bioscience, San Jose, CA, USA). Antibodies including anti-human CD11c (Bu15, BioLegend), anti-human CD14 (M5E2, BioLegend), anti-human CD 80 (2D10, BioLegend), anti-human CD83 (HB15E, BioLegend), anti-human CD86 (IT2.2, BioLegend), anti-human CD123 (6H6, BioLegend), and anti-human HLA-DR (L243, BioLegend) were used in flow cytometry. The obtained data were analyzed using FlowJo software version 10.6.2 (Tree Star, Ashland, OR, USA).

*Human cytokine arrays and image analysis*. Cytokines produced by DCs were analyzed using human cytokine antibody array kit (Abcam, Cambridge, UK) according to manufacturer's instructions. Briefly, antibody coated membranes were blocked by blocking buffer for 30 min at room temperatures. After removing the blocking buffer, samples were added and incubated overnight at 4˚C. The membranes were washed and stained with biotin-conjugated anti-cytokine for 2 h at room temperature. After washing the membrane, HRP-conjugated Streptavidin was added and stained for 2 h at room temperature. Next, the membranes were developed by detection buffer. The immunoblots were detected and analyzed using a ChemiDoc (Bio-Rad, Hercules, CA, USA).

*Quantification of mRNA expression by qPCR*. RNA was extracted using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. The concentration of RNA was determined using Nanodrop 2000 Spectrophotometer (Thermo Scientific, Madison, WI, USA). For cDNA synthesis, RNA was mixed with OligodT (Bioneer, Daejeon, Republic of Korea) and incubated at 70˚C for 5 min. After chilling on ice, samples were mixed using RT premix (Bioneer), and reverse transcription (RT) reaction (42˚C, 60 min and 94˚C, 5 min) was performed using a Thermal Cycler (Bio-Rad). For qPCR, cDNA was mixed with primers and green dye mix (qPCR Green mix Hi-ROX, Enzo, Farmingdale, NY, USA). Real-time qPCR (95˚C for 10 s, 60˚C for 20 s, and 65˚C for 10 s for 40 cycles) was performed using a Light Cycler 480 II (Roche, Rotkreuz, Switzerland).

*Statistical analysis*. GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. T-test was executed to compare the differences between the two groups.

## Results

*Screening of compounds resulting in differentiation of DCs*. We used a high throughput screening system to identify compounds that differentiate or activate DCs. THP-1 cells were treated with 1,280 pharmacologically active compounds at the concentration of 0.5 and 1 μM, and subsequent morphological changes of the cells were analyzed after 3 days. THP-1 cells treated with GM-CSF and IL-4 alone, which represent iDCs, showed no change in their morphology. In total, six compounds

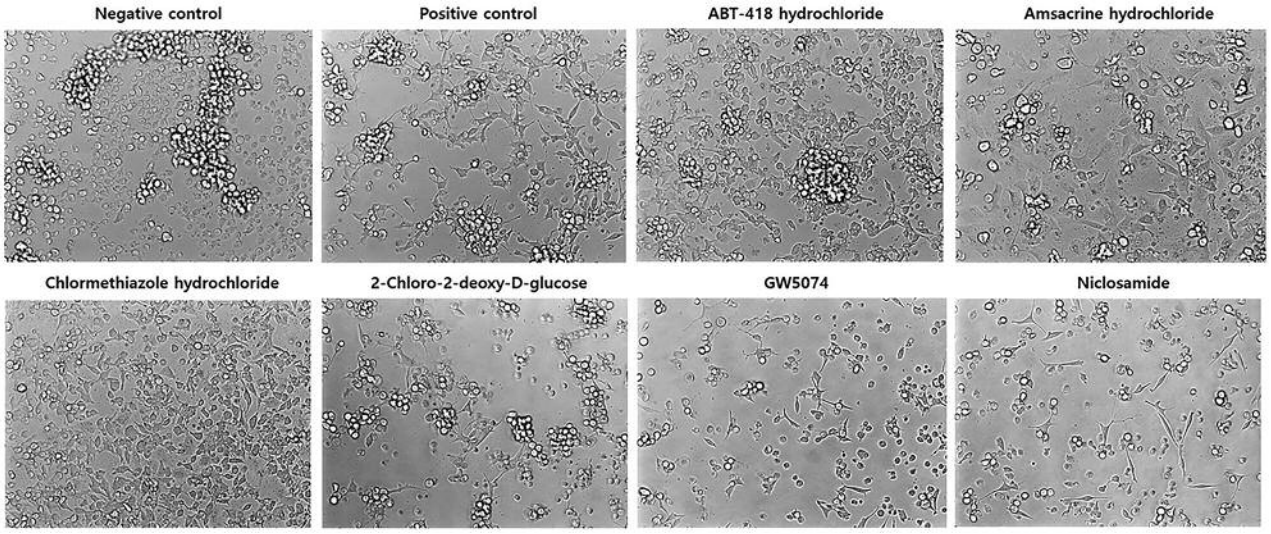


Figure 1. *Morphologies of THP-1 cells treated with pharmacologically active compounds. For screening the molecules that can cause differentiation and activation of DCs, THP-1 cells were seeded in 384-well plate, and a total of 1,280 chemicals were added at 0.5 μM and 1 μM for 3 days. Among them, ABT-418 hydrochloride, amsacrine hydrochloride, chlormethiazole hydrochloride, 2-chloro-2-deoxy-D-glucose, GW5074, and niclosamide were identified as DC modulators that result in morphologic changes in the cells. Cell images were captured through Operetta.*

caused changes in the morphology of THP-1 cells. ABT418, amsacrine hydrochloride, chlormethiazole hydrochloride, 2-chloro-2-deoxy-2-D-glucose, GW5074, and niclosamide changed the morphology of THP-1 cells to dendritic-like cells (Figure 1). Similar changes were shown in positive control cultures treated with TNF-α and ionomycin. These features were considered as representing differentiation of iDCs into mDCs that have well-developed dendrites.

*Differentiation and activation of DCs induced by the screened substances*. To confirm the effect of the selected molecules on DC maturation and activation, THP-1 cells were treated with each of the six screened compounds at 0.1, 0.5, 1.0, or 10 μM for 3 days and the expression of several surface markers was analyzed. CD11c and CD123 were used as DC subset markers, and CD80, CD83, CD86, and HLA-DR were considered as DC activation markers. As a result, ABT418, chlormethiazole hydrochloride, 2-chloro-2-deoxy-2-D-glucose, GW5074, and niclosamide were not found to upregulate the expression of DC subset markers and activation markers. In contrast, treatment of THP-1 cells with amsacrine hydrochloride resulted in up-regulation of CD11c and down-regulation of CD123 expression (Figure 2A). As these phenotypes are typical to conventional DCs (cDCs), amsacrine hydrochloride was suggested to have roles in differentiating iDCs into cDCs. The expression levels of CD80, CD83, and CD86 also were increased in the cells treated with amsacrine hydrochloride, which suggests that amsacrine hydrochloride affects both differentiation and activation of DCs.

Of interest, the positive control did not show an increase in CD11c expression levels on the third day after treatment (Figure 2A). However, its expression levels increased 7 days after treatment (Figure 2B). These results indicate that amsacrine hydrochloride differentiates iDCs into cDCs faster than TNF-α and ionomycin. In addition, we observed the morphology and the levels of the analyzed surface marker on THP-1 cells treated with amsacrine hydrochloride for 7 days. The cell size and surface expression of CD11c, CD80, CD83, and CD86 increased more in the cells treated for 7 days than in those treated for 3 days (Figure 2B).

*Subtype identification of DCs induced by amsacrine hydrochloride*. To identify the subset of DCs induced by amsacrine hydrochloride, we analyzed mRNA expression levels of certain TLRs, transcription factors, and CD molecules, which are known as specific markers for pDCs, cDC1, and cDC2 through qPCR (Figure 3A). The cells treated with amsacrine hydrochloride showed higher expression levels of TLR1, 3, 4, and 8 that are specific markers for cDC and lower expression levels of TLR7 and TLR9 that are specific markers for pDC, suggesting that amsacrine-treated DCs were mainly differentiated into cDCs. TLR3, a marker of cDC1 showed increased mRNA expression in cells treated with amsacrine hydrochloride. Simultaneously, the transcription factor IRF-4, a specific marker for cDC2, was significantly upregulated more than twice in the amsacrine-treated group. In addition, amsacrine hydrochloride induced higher expression of CD11c and

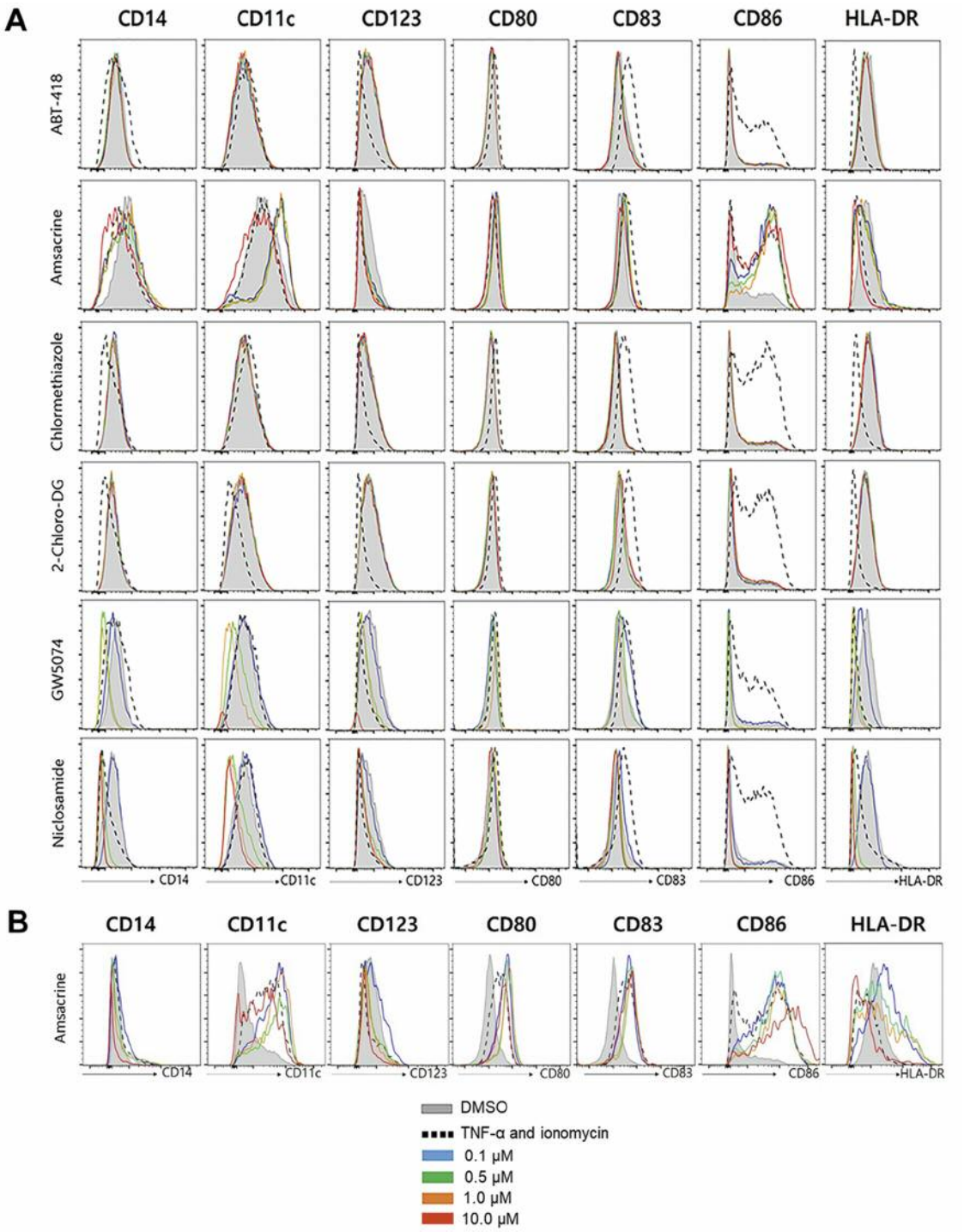


Figure 2. *Expression of cell surface markers by THP-1 cells treated with selected compounds representing subsets and activation of dendritic cells. (A) THP-1 cells ($4\times10^5$) were seeded and treated with each of the selected six chemicals, ABT-418 hydrochloride, amsacrine hydrochloride, chlormethiazole hydrochloride, 2-chloro-2-deoxy-D-glucose, GW5074, and niclosamide at 0.1, 0.5, 1, or 10 μM for 3 days in serum free medium containing 100 ng/ml of GM-CSF and 200 ng/ml of IL-4. The cells were treated with DMSO for the negative control and with 10 ng/ml TNF-α and 200 ng/ml ionomycin for the positive control. Surface expression levels were analyzed using FACS. Only amsacrine hydrochloride resulted in upregulation of CD11c, CD80, CD83, and CD86 and down-regulation of CD123 expression of THP-1 cells. Notably, the positive control did not show an increase in CD11c expression level. 2-Chloro-DG: 2-chloro-2-deoxy-D-glucose. (B) Seven days after treatment, TNF-α and ionomycin resulted in similar surface marker expression of THP-1 cells treated with amsacrine hydrochloride. Amsacrine hydrochloride increased surface expression of CD11c, CD80, CD83, and CD86 on THP-1 cells in 7 days.*

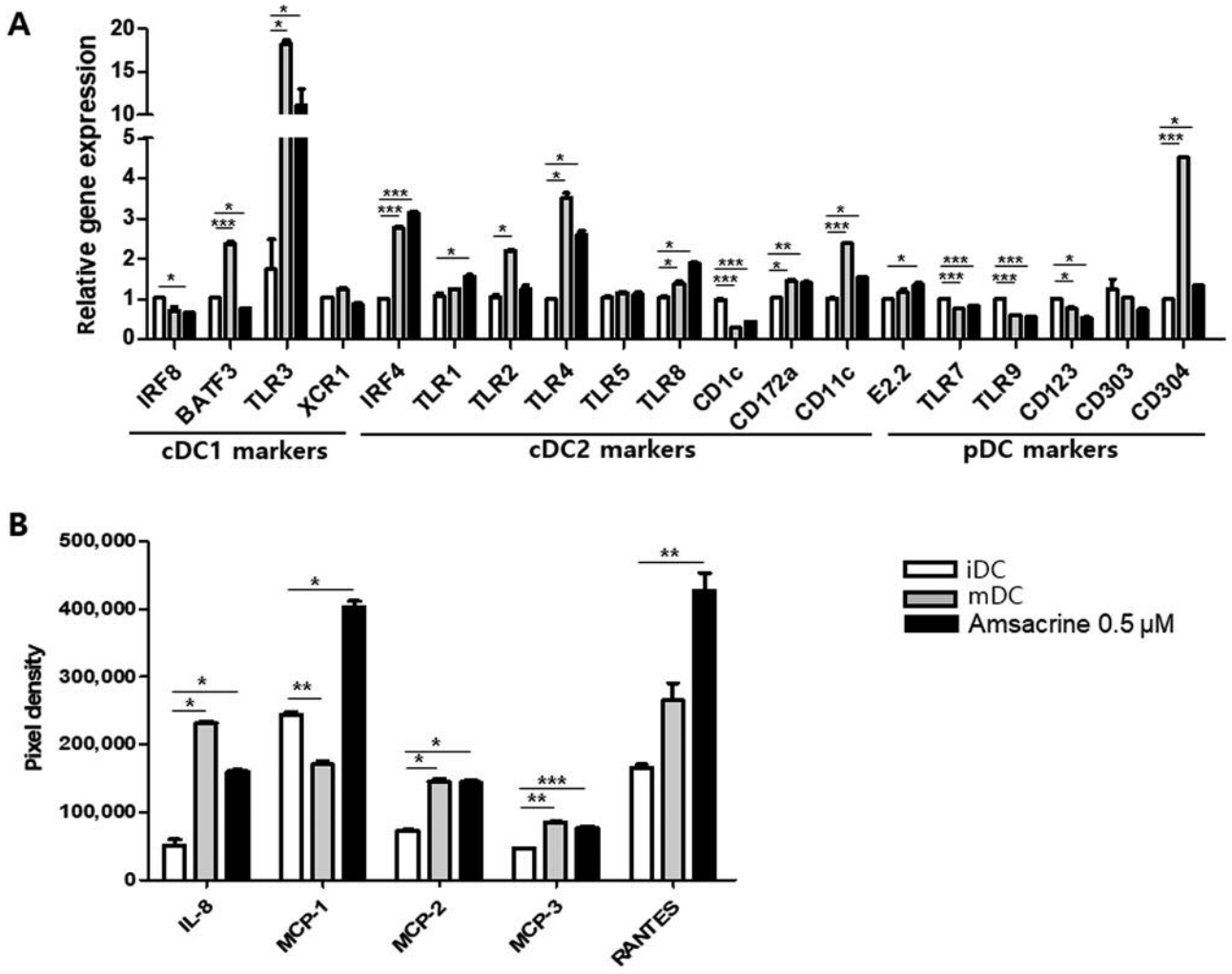


Figure 3. *Expression levels of transcription factors, TLRs and cells surface markers specific for pDC, cDC1 and cDC2 (A) and secreted cytokine profiling of dendritic cells (B) induced by amsacrine hydrochloride. (A) THP-1 cells ($4\times10^5$) were seeded, and treated with 0.5 μM amsacrine or TNF-α and ionomycin as positive control. Real-time quantitative PCR was performed to analyze mRNA expression levels reflecting DC subsets in induced dendritic cells. Amsacrine hydrochloride showed increased expression of specific markers for cDC2. (B) For cytokine analysis, cell cultured medium was harvested after 3 days and the secreted cytokine profiles were measured using a cytokine array kit. DCs that were treated with amsacrine hydrochloride produced higher levels of IL-8, MCP-1, MCP-2, MCP-3, and RANTES (CCL5) than negative control, which was similar pattern with those derived from positive control cultures. TLRs: Toll-like receptors; pDC: plasmacytoid dendritic cell; cDC: conventional dendritic cell. *p<0.05, **p<0.01, ***p<0.001 in t-test.*

CD172 that are also specific markers for cDC2. These findings indicated that amsacrine hydrochloride results in DC differentiation mainly into cDC2 subtype.

*Cytokine production by DCs treated with amsacrine hydrochloride*. Next, we analyzed the cytokines produced by DCs differentiated by amsacrine hydrochloride and compared them with those produced by negative control representing iDCs and positive control representing mDCs. THP-1 cells were treated with 0.5 μM of amsacrine hydrochloride or TNF-α and ionomycin in the presence of GM-CSF and IL-4. After 3 days, the supernatant of cell culture was collected and the cytokines secreted by DCs were analyzed. DCs that were treated with amsacrine hydrochloride produced higher levels of IL-8, monocyte chemoattractant protein (MCP)-1 (also known as CCL2), MCP-2 (CCL8), MCP-3 (CCL7), and Regulated on Activation Normal T Cell Expressed and Secreted (RANTES, CCL5) than negative control cells representing iDC. The levels of secreted IL-8, MCP-2, MCP-3, and RANTES were higher in both mDCs derived from positive control cultures and DCs induced by amsacrine compared with those in iDCs (Figure 3B). Although positive control and DCs induced by amsacrine

showed opposite results regarding MCP-1 secretion, the other cytokines showed a similar profile.

## Discussion

In the present study, we used high throughput screening and identified 6 of 1,280 drugs that had the capability to differentiate THP-1 cells to mDCs. Among them, amsacrine hydrochloride, which belongs to DNA intercalating agents and DNA topoisomerase II inhibitors, induced higher levels of both the DC markers (CD11c, *etc.*) and activated DC markers (CD80, CD83, and CD86) in THP-1 cells, which indicates that amsacrine induces their differentiation into and functional activation of mDCs.

Amsacrine is a well-known part of the chemo-regimens for high-risk acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Fludarabine, amsacrine, and cytarabine (FLAMSA) combined with conventional reduced-intensity conditioning regimens for hematopoietic stem cell transplantation is considered a reasonable and effective treatment regimen for persisting AML and MDS in several countries (13).

In the 1980s, Miller *et al*. found that amsacrine showed unexpected immune-modulatory features, suggesting its potential as an immunoregulator (14). Reportedly, amsacrine also induces apoptosis of primary thymocytes in culture (15). However, its role in the adaptive immune system has not been fully understood. Recently, teniposide, another DNA topoisomerase II inhibitor, was revealed to induce the secretion of HMGB1 and sequential type I interferon (IFN-I) signaling in tumor cells. Given that teniposide mechanistically induced innate immune signaling that resulted in the activation of DCs and T cells (16), amsacrine could also have a similar effect on IFN-I signaling in respect to the activation of mDCs. Elucidation of its precise mechanisms regarding the maturation of iDCs still remains.

cDC1 is associated with cross-presentation and efficient priming of $CD8^+$ T cells (17), which is crucial to both anti-tumoral and anti-viral immunity (2). In humans and mice, intratumoral cDC1 has been reported to stimulate cytotoxic T cells and predict better survival outcome in certain cancer types (18). Of interest, pDCs foster an immune-subversive environment (19) and are potential therapeutic targets by increasing type 1 IFN release and antigen presentation by cDCs (20). Meanwhile, cDC2s are key mediators for the induction of $CD4^+$ T cell immunity (17). Although some human studies showed that cDC1 and cDC2 have overlapping features in their functions (17, 21), the significance of cDC2 in cancer immunology has not been fully understood compared with that of cDC1. When we analyzed the subtypes of DCs matured by amsacrine hydrochloride, both cDC1 and cDC2 were identified. This might be attributed to the THP-1 monocyte cells used as DC precursors.

Monocyte-derived DCs (mo-DCs) are a specific type of DCs found in inflammatory conditions (17); however, some authors raise concern that mo-DCs constitute a highly plastic cell type, the features of which partially overlap with those of DCs (22). Although in vitro mo-DCs cultured with growth factors including GM-CSF and IL-4 were reported to be considerably different from other human DC subsets, (23) those inflammatory DCs share molecular features with $BDCA1^+$ cDCs (24). The clinical implications of mo-DCs in human cancer are still unclear, but most DC vaccination trials have been based on mo-DCs differentiated from $CD14^+$ monocytes from peripheral blood mononuclear cells with GM-CSF and IL-4 in the presence of maturation factors and tumor antigen loading (17, 25). However, this commonly used approach still has the limitations of requiring long time and have high cost (26). Given that amsacrine hydrochloride is a chemotherapeutic agent already in stable use, it might be used for the production of mDCs for cancer immunotherapy.

THP-1 cells treated with amsacrine hydrochloride secreted higher levels of IL-8, MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), and RANTES (CCL5) compared with iDCs. Among them, the secretion of MCP-1 only showed opposite result between mDCs derived from positive control and amsacrine-treated THP-1 cells. CCR2 was found to play an important role in DC maturation through the activation of NF-kB (27). In addition, anthracycline, a well-known DNA topoisomerase II inhibitor, has been reported to induce recruitment and differentiation of $Ly6C^{hi}CD11b^+CD11c^+$ cells through a CCR2/CCL2-dependent mechanism (28, 29). Amsacrine, which belongs to the same category with anthracycline, can be assumed to have a similar effect through the CCR2/CCL2 signaling axis, which might result in a different pattern of MCP-1 secretion.

To the best of our knowledge, this is the first report to delineate the role of amsacrine hydrochloride as a novel compound for maturating and activating DCs. In the field of cancer immunotherapy, DC vaccination is a promising strategy alone or in combination with adoptive T cell therapy. As a clinical trial testing allogenic DC vaccination against AML (NCT01373515) is already underway, it is worthwhile to investigate the effect of amsacrine hydrochloride not only as an anti-leukemic agent but also on monocytic recruitment and DC maturation. Amsacrine hydrochloride can also be used to generate mo-DCs as APCs for adoptive T cell therapeutics. Extending our understanding of its clinical implications is strongly warranted.

## Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

## Authors' Contributions

Conception and design: G.G. and H.J.L. Development of methodology: Y.J.C., H.L., J.K. and G.G. Acquisition of data: Y.J.C and H.L. Analysis and interpretation of data: H.L. and J.K. Writing, review, and/or revision of the article: Y.J.C. and I.A.P. Administrative, technical, or material support: H.J.L. and I.A.P. Study supervision: H.J.L. and I.A.P.

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