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Global and focused transcriptional profiling of small molecule aminopeptidase N inhibitor reveals its mechanism of angiogenesis inhibition

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ABSTRACT

We recently developed a specific small molecule inhibitor of aminopeptidase N (APN), named as HNSA, through a high throughput screening. In the present study, we investigated the major cellular phenotypes of HNSA in comparison with those of APN knock-down in human fibrosarcoma cells and the mechanism of angiogenesis inhibition by the compound using DNA microarray analyses. Global gene expression analyses showed that HNSA signatures are significantly correlated with those of APN knock-down in HT1080 cells, suggesting that APN is a primary target of HNSA in the cells. Using the angiogenesis-focused DNA microarrays, nine of angiogenesis-related genes were identified as crucial mediators of angiogenesis inhibition by HNSA. These data demonstrate that HNSA can be used as a valuable tool to decipher the APN function in angiogenesis.

Aminopeptidase N (APN), also known as CD13, is a membrane-bound zinc-dependent metallopeptidase [1]. It has a conserved zinc binding motif, HEXXH, in its extracellular metalloprotease domain and therefore belongs to the M_1 family of aminopeptidase [2]. Recent evidences have shown that APN plays an important role in angiogenesis and tumor metastasis. The activated endothelial cells by potent angiogenic factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hypoxia highly express APN in their cell surface, whereas the quiescent endothelial cells do not [3]. Moreover, a specific monoclonal antibody against human APN and several pharmacological inhibitors, including bestatin and curcumin, inhibit angiogenesis and tumor growth in xenograft mice [4,5]. These data suggest that APN can be a potential target of inhibiting angiogenesis.

For past years, much effort has been paid to discover specific small molecule inhibitors of APN and we recently developed a hydroxamic acid-containing inhibitor of APN, *N*-hydroxy-2-(naphthalene-2-ylsulfanyl)-acetamide (HNSA), through a fluorescence-based high throughput screening [6]. Although HNSA inhibited the activity of APN at low micromolar ranges, the compound was very specific for APN over other metalloproteases and even other M_1 family of aminopeptidases such as adipocyte-derived leucine aminopeptidase. As expectedly, HNSA potently inhibited bFGF-induced angiogenesis of endothelial cells in vitro model, implying that HNSA can be developed as a new angiogenesis inhibitor targeting APN.

Because small molecules generally have a potential to bind more than one target protein in the cells, it cannot be ruled out that anti-angiogenic activity of HNSA may be combination effects of inhibiting several targets in the cells. In addition, because the role of APN during angiogenesis has not been clearly understood, the anti-angiogenic mechanism of HNSA is also required to be elucidated. To address these questions, we conducted two different DNA microarray analyses using human fibrosarcoma cells which express high amount of APN in their cell surface. As a result, we found that HNSA treatment is significantly correlated with APN knock-down phenotype. In addition, nine of angiogenesis-related genes were identified as key mediators of HNSA-induced angiogenesis inhibition through the analyses.

Materials and methods

Cell culture. Dulbecco's modified Eagle's medium (DMEM) and cell culture supplements were bought from Invitrogen (Carlsbad, CA). HT1080 cells were grown in DMEM containing 10% FBS. The cells were maintained at 37 °C in a humidified incubator adjusted at 5% CO_2.

APN knock-down and RNA isolation. Human HT1080 fibrosarcoma cells were treated with either phorbol-12-myristate-13-acetate (PMA) or HNSA with PMA for 24 h. For APN knock-down, the cells were transfected with siRNA specific for human APN (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen). After 24 h, the cells were treated with PMA and incubated for additional 24 h. Total RNAs were isolated using Trizol Reagent (Invitrogen) and APN knock-down was confirmed through RT-PCR analysis using primer pairs for APN as described previously [7].

DNA microarray analysis. Human 8K twin chip (Digital Genomics, Inc., Seoul, Korea) and angiogenesis cDNA chip (Takara Korea Biomedical, Inc., Seoul, Korea) were used for global and angiogenesis-focused transcription profiling analyses, respectively. RNA from test groups was labeled with Cy5-dUTP by reverse-transcription and RNA from control groups with Cy3-dUTP. Labeled products were set

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onto cDNA chips and incubated at 65 °C for 16 h in a dark water-bath. Following wash, scanning by 428 scanner (Affymetrix, Santa Clara, CA), raw data extraction, and statistical analysis using GeneSight data analysis software ver. 3.5 (BioDiscovery, Los Angeles, CA) were achieved.

Signature correlation coefficient value (ρ). Correlation coefficients between two signatures were calculated using the equation:

$$\rho = \sum_k x_k y_k \Big/ \left(\sum_k x_k^2 \sum_k y_k^2 \right)^{1/2}$$

where x_k is the $\log_{10}$ of the expression ratio for the kth gene in the x signature, and y_k is the $\log_{10}$ of the expression ratio for the kth gene in the y signature [8].

Results and discussion

Knock-down of APN and transcriptional profiling in HT1080 cells

Due to the high expression level of APN on the cell surface and their high metastatic potential, we used the human fibrosarcoma HT1080 cells for transcription profiling of APN-regulated genes [7]. HT1080 cells were also known to express high level of tumor angiogenesis-related genes such as matrix metalloproteases (MMPs) and cyclooxygenase-2 (COX-2) in response to phorbol-12-myristate-13-acetate (PMA) [9,10]. The whole DNA microarray experiments were conducted as shown in the experimental design (Fig. 1) to get signatures of PMA, HNSA, and APN knock-down (APN-KD). Each of gene expression signatures was expressed as either $\log_2$ or $\log_{10}$ of Cy5/Cy3 ratios. The successful knock-down of APN gene by a specific siRNA in the cells was confirmed by RT-PCR analysis (Fig. 2A). To analyze the correlation between HNSA and APN-APN-KD signatures, we first employed hierarchical clustering of all three signatures. The clustering patterns of APN-KD and HNSA signatures were very similar, but those of PMA signatures were quite different from those two signatures (Fig. 2B), suggesting that the phenotypes of APN-KD and HNSA are similar. Next, we calculated signature correlation coefficient values (ρ) between each signature. When the signature correlation coefficient value (ρ) was 0.7 ± 0.1 or over, those two signatures were considered to be significantly correlated. As a result, the signatures of APN-KD and HNSA were significantly correlated as shown by the correlation coefficient value of 0.70 (Fig. 2C). However, APN-KD vs. PMA or HNSA vs. PMA showed the correlation coefficient values lower than 0.5, suggesting that those signatures are not significantly correlated each other (Fig. 2D and E). Knocking-down of adipocyte-derived leucine aminopeptidase (A-LAP), another M1 family aminopeptidase involved in angiogenesis, also did not correlated with APN-KD signatures (Fig. 2F). The fact that the cellular phenotypes induced by APN expression blockade and those by HNSA treatment are very similar suggests that APN is a biologically relevant target of HNSA.

Angiogenesis-focused transcriptional profiling in HT1080 cells

A large number of genes were changed in their expression levels by HNSA, which include genes involved in signal transduction pathways, cell adhesion, transcription regulation, etc. Among those genes, we collected a subset of genes which showed contrastive expression patterns between PMA and HNSA. Twenty-three genes were found to be decreased in PMA but increased in HNSA treatment (Fig. 3A). Two known angiogenesis inhibitory proteins such as serine protease inhibitor clade F (SERPINF2) and calreticulin were included. In contrast, 50 genes were found to be increased in PMA but decreased in HNSA treatment, which include three pro-angiogenic genes such as APN, COX-2 and urokinase-type plasminogen activator (uPA) (Fig. 3B). These data indicate that HNSA inhibits angiogenesis through gene expression regulation of some pro- and anti-angiogenic genes. To explore the regulation of angiogenic gene expression by HNSA in detail, we conducted angiogenesis-focused transcriptional profiling using an angiogenesis-focused cDNA chip which contains total of 153 angiogenesis-related genes spotted in duplicate [11]. After analysis of gene expression, total 36 genes were found to be increased (20 genes) or decreased (16 genes) by HNSA treatment (Fig. 3C and D). Interestingly, majority of those genes showed a contrastive expression pattern between PMA and HNSA treatments, i.e. matrix metalloproteinase-9 (MMP-9) was increased in response to PMA but was decreased by HNSA treatment. In contrast, gelsolin was decreased by PMA but was increased upon the treatment with HNSA. These data demonstrate that anti-angiogenic activity of HNSA is through activation of some tumor suppressive genes and inhibition of the expression of some pro-angiogenic genes.

Genes responsible for anti-angiogenic activity of HNSA

Using a DNA microarray analysis software, we finally collected a subset of genes showing clear contrastive expression patterns between PMA and HNSA treatments. Four genes including tissue inhibitor of matrix metalloprotease-3 (TIMP-3), plakoglobin, gelsolin, and cyclin-dependent kinase inhibitor 2C ($p18^{INK2C}$) were found to be decreased by PMA, but were increased by the treatment with HNSA (Fig. 4A, left panel). Those microarray data were validated through semi-quantitative RT-PCR analysis (Fig. 4A, right panel).

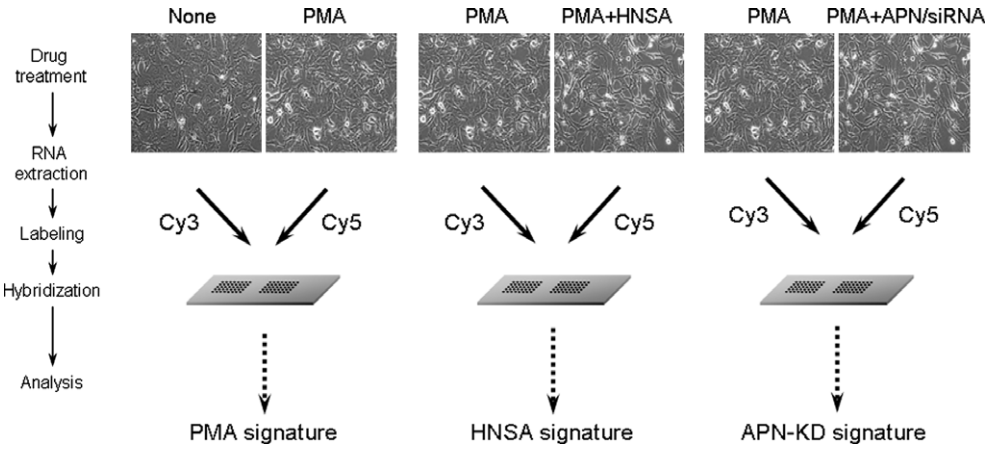

Fig. 1. Experimental design of DNA microarray analysis. Transcription profiles of HT1080 cells treated with or without PMA were designated as 'PMA signature'. Transcription profiles of HT1080 cells treated with or without HNSA in the presence of PMA were designated as 'HNSA signature'. Transcription profiles of the cells transfected with or without APN/siRNA before exposed to PMA were designated as APN-KD signature.

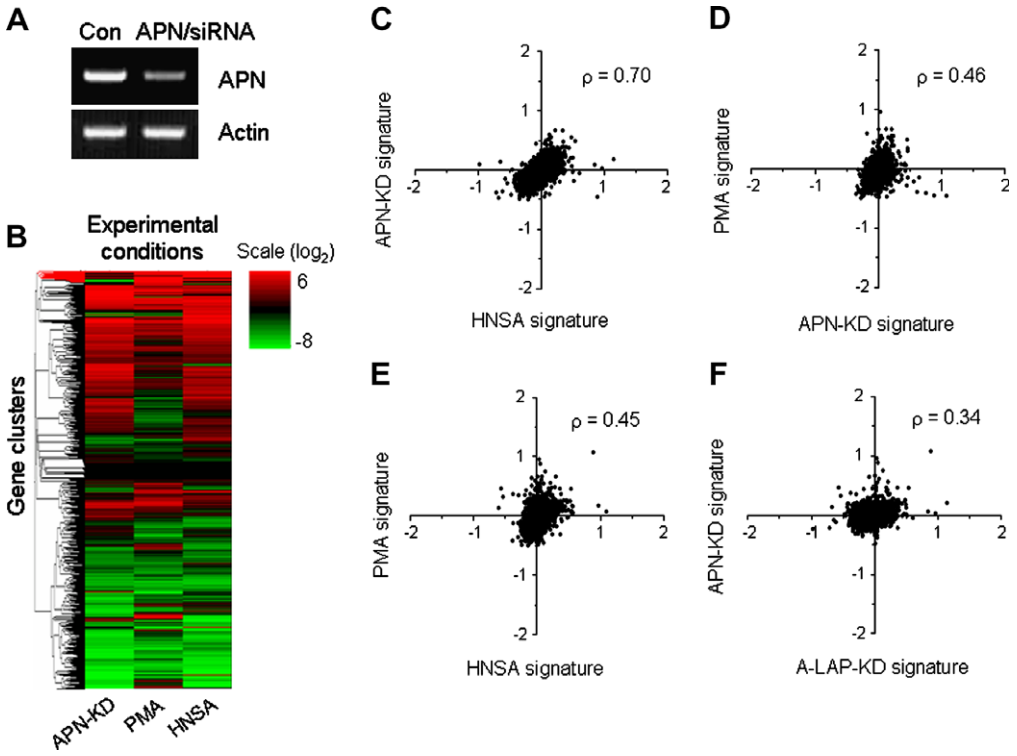

Fig. 2. DNA microarray correlation analysis. (A) RT-PCR analysis of APN in control and APN/siRNA transfected HT1080 cells. Actin expression level was determined as an internal control. (B) Hierarchical clustering of three transcription profiles is shown. Each signature represents $\log_2$ of Cy5/Cy3. Signature correlation coefficient values (ρ) of HNSA vs. APN-KD (C), PMA vs. APN-KD (D), HNSA vs. PMA (E), and APN-KD vs. A-LAP-KD (F) are presented. Each signature represents $\log_{10}$ of Cy5/Cy3.

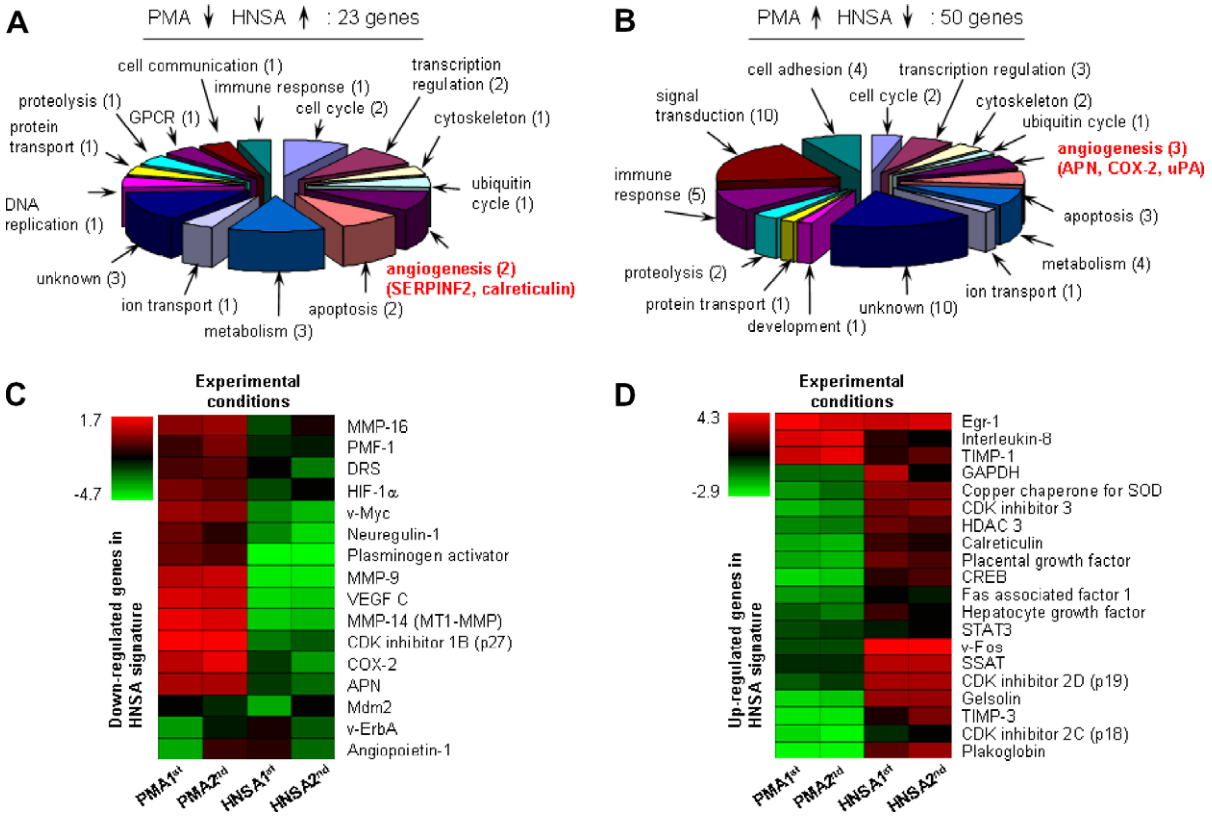

Fig. 3. Global and angiogenesis-focused transcription profiles. (A) Genes down-regulated by PMA, but up-regulated by HNSA from global transcription profiling are shown. Each gene was classified according to its function. (B) Genes up-regulated by PMA, but down-regulated by HNSA from global transcription profiling are shown. (C) Down-regulated genes in response to HNSA from angiogenesis-focused transcription profiling are shown. Each duplicated signatures is shown separately (1st and 2nd). The scale represents $\log_2$ of Cy5/Cy3. (D) Up-regulated genes in response to HNSA from angiogenesis-focused transcription profiling are shown.

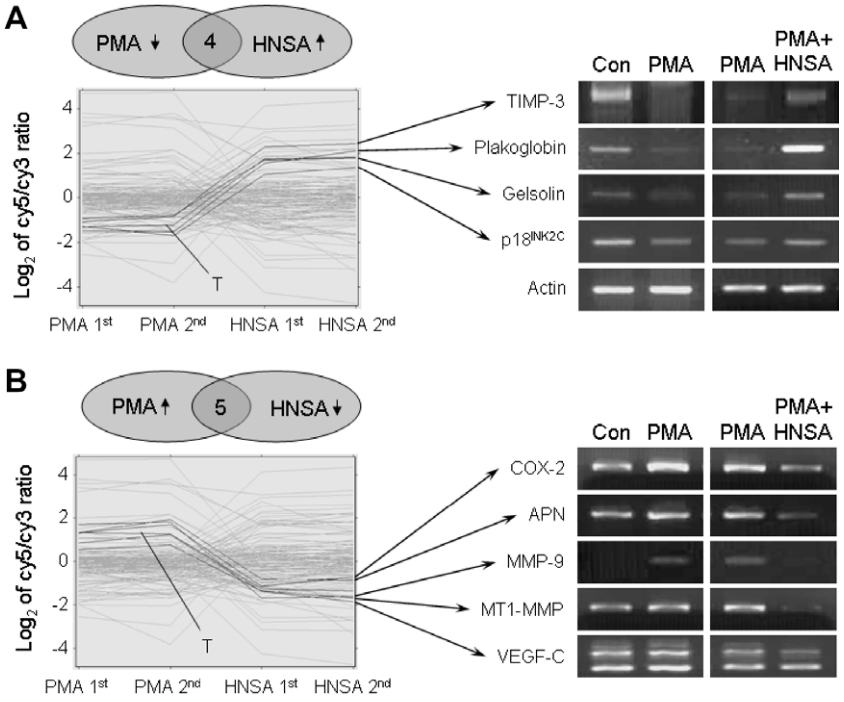

Fig. 4. Collection of genes showing contrastive expression patterns between PMA and HNSA signatures from angiogenesis-focused transcription profiling. (A) A subset of genes down-regulated by PMA, but up-regulated by HNSA is shown. Each gene expression regulation was confirmed through RT-PCR analysis. (B) A subset of genes up-regulated by PMA, but down-regulated by HNSA is shown. T represents the template of analysis.

Expressions of those four genes were significantly down-regulated by PMA treatment, but those PMA effects were almost completely reversed by the treatment with HNSA. All these genes are known to have tumor suppressive function. In particular, TIMP-3 is a well known angiogenesis inhibitor through its ability to inhibit MMPs [12]. Since, TIMP-3 is silenced in a number of human cancers and tumor cell lines due to hypermethylation of its promoter, HNSA's ability to up-regulate TIMP-3 expression can be useful to restore tumor suppressive pathway in those cancer cells [13,14]. On the other hand, five genes including cyclooxygenase-2 (COX-2), APN, MMP-9, membrane type-1 matrix metalloproteinase (MT1-MMP), and vascular endothelial growth factor C (VEGF-C) were up-regulated by PMA and were down-regulated upon the treatment with HNSA (Fig. 4B). As shown in RT-PCR data, all those five genes were up-regulated by PMA and those PMA-induced up-regulations were completely abolished by HNSA treatment. It is interesting that an APN inhibitor, HNSA can inhibit APN expression which is activated by protein kinase C (PKC) agonist, PMA. This suggests a possible existence of a positive feedback loop at the down-stream signaling pathway of APN, which involves PKC signaling. COX-2 and MMP-9 were activated by PMA and the activation was completely blocked by HNSA. This suggests that some transcription factors down-stream of PKC, which are involved in the gene expression regulation of COX-2 and MMP-9 may be regulated by HNSA. We therefore examined one of the strong candidate transcription factors, NF-κB, after treated with HNSA. The result demonstrated that HNSA does not inhibit PMA-induced NF-κB activation in HT1080 cells (data not shown). Ongoing study is focused on the identification of a PKC-regulated transcription factor which is crucially inhibited by HNSA.

Our present study demonstrates that the primary phenotype induced by HNSA is through the inhibition of APN activity in the mammalian cells. Using a global and a focused DNA microarray analyses, we identified a subset of genes which are crucial for anti-angiogenic activity of HNSA. Furthermore, this study suggests a possible linkage between APN and PKC signaling pathways in angiogenesis and HNSA can be a unique tool to elucidate the function of APN in angiogenesis.

Acknowledgments

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