N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA), Histone Deacetylase Inhibitors, Suppresses COX-2 expression in colon cancer

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Directed by Professor Jin Suck Suh

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This certifies that the Master's Thesis of Ji Hyun Park is approved.

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Table of contents

List of Figures

List of Tables

Abstract

N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA), Histone Deacetylase Inhibitors, Suppresses COX-2 expression in colon cancer

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Cyclooxygenase-2 (COX-2) plays important roles in cell adhesion, apoptosis, and angiogenesis, and is essential for tumor angiogenesis. Levels of COX-2 expression are low in normal cells, but very high level in human and animal colorectal tumors.

Histone deacetylase (HDAC) plays a crucial role in carcinogenesis[1] and is over-expressed in several tumor cells[2]. The histone deacetylase inhibitor N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA) is a new anti-angiogenesis drug,

and has been shown to inhibit COX-2 expression in colon cancer. Here, we confirm that HNHA decreases COX-2 expression in colon cancer, similar to COX-2 inhibitors such as Celecoxib and Rofecoxib. Moreover, hypoxia inducible factor-1α (HIF-1α), and tumor necrosis factor-α (TNF-α) are involved in COX-2 expression and their expression is also decreased by HNHA in colon cancer. The effect of HNHA on tumor growth was examined in a human colon cancer tumor model and investigated by western blot, immunohistochemistry, and FACS analysis.

Key Words: Histone deacetylase (HDAC), N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA), (Hypoxia-inducible factor-1α (HIF-1α), Cyclooxygenase-2 (COX-2)

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I. INTRODUCTION

Histone acetylation is regulated by acetylase and deacetylase and plays a key role in gene expression in eukaryotes. Recent studies have shown that histone deacetylase (HDAC) plays a crucial role in carcinogenesis[1] and is over-expressed in several tumor cells[2]. A new HDAC inhibitor N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA) has anti-angiogenic activity[3]. HNHA suppresses the hypo-acetylation of histones, down-regulating the target gene and inhibiting invasion of the cancer cells and tumor growth by repressing enzyme activity[3]. Thus, if HDAC

inhibitors strongly repress cancer cells, they could be used to repress cancer metastasis or growth. Cyclooxygenase (COX) is an enzyme that catalyzes the formation of prostaglandins from arachidonic acid[4]. There are two COX enzyme isoforms: cyclooxygenase-1 (COX-1) is constitutively expressed in normal tissues as a "housekeeper" of mucosal integrity[5], whereas cyclooxygenase-2 (COX-2) is not normally expressed in most tissues, but is induced by a wide spectrum of growth factors and pro-inflammatory cytokines in specific pathophysiological conditions[6]. COX-2 has important functions in cell adhesion and apoptosis, and is essential for tumor angiogenesis. If angiogenesis is blocked, the tumor cannot survive. The level of COX-2 expression is low in normal cells, but is very high in human and animal colorectal tumors.

COX-2 is a highly inducible by pro-inflammatory cytokines such as TNF-α[18], which in turn is regulated by NF-κB. HDAC inhibitor blocks TNF-α activation of COX-2 protein and mRNA synthesis, and suppresses COX-2 activity[7]. TNF-α is involved in promotion and progression of experimental and human cancer through intracellular signaling pathways leading to activation of the NF-κB and AP-1 transcription factor complexes[17]. Moreover, COX-2 up-regulation is associated with induction of hypoxia-inducible factor-1α (HIF-1α)[8] through binding of HIF-1α to a hypoxia-responsive element (HRE) on the COX-2 promoter. COX-2 up-

regulation during hypoxia is accompanied by increased levels of prostaglandin E_2 (PGE_2), which promote tumor cell survival under hypoxic conditions[8].

Hypoxia, oxygen deficiency in tissues, is a universal characteristic of solid tumors and represents a key regulatory factor in tumor growth and survival[9]. The coordinated homeostatic response to hypoxia is largely transcriptional and is mediated primarily through activation of the heterodimeric transcription factor hypoxia-inducible factor (HIF)-1[10]. HIF-1 is composed of two subunits: the oxygen sensitive HIF-1α subunit and the constitutively expressed HIF-1β subunit[10]. Under normoxic conditions, HIF-1α is hydroxylated at key proline residues facilitating von Hippel-Lindau protein binding, which in turn allows ubiquitination and subsequent proteasome-targeted degradation[11]. Under hypoxic conditions, proline hydroxylation is inhibited, thereby stabilizing HIF-1α, which can translocate into the nucleus and bind to constitutively expressed HIF-1β, forming the active HIF-1 complex[12]. HIF-1α is over-expressed in various types of cancer, including colorectal cancer[13], and compelling evidence supports a role for HIF-1 in tumorigenesis[14, 15]. HIF-1α levels are elevated in the cytoplasm and the nucleus of cells in various solid tumors[16]. Moreover, under hypoxic conditions COX-2 protein levels increase in colorectal adenoma and carcinoma cells, indicating that COX-2 up-regulation is associated with HIF-1α induction. Therefore, if HNHA

inhibits COX-2, we predict that expression of HIF-1α would also decrease[8].

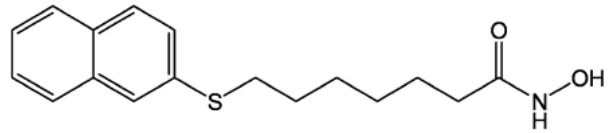

Figure 1. Chemical structure of HDAC inhibitors. N-Hydroxy-7-(2-naphthylthio) heptanomide (HNHA), a novel HDAC inhibitor[3]

II. MATERIALS AND METHODS

1. Cell Culture

HT-29 is a human colorectal carcinoma cell line with a doubling time of 20-24 hours. C1300 cell is a mouse neuroblastoma cell line with a doubling time of 30-33 hours. These two cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mg/mL sodium bicarbonate, 10% penicillin/streptomycin and 5% CO_2 incubator at 37℃. Drug treatment was started the day after seeding using an Inhibiting Concentration 50 (IC_{50}) dose.

2. Tumor Animal Model and Experimental Designs

BALB/C nude mice are used as a general-purpose strain in a variety of research areas including cancer, immunology and inflammation, and cardiovascular biology research. HT-29 colorectal cancer cells were cultured *in vitro*, harvested, and injected (1.0×10^7/mouse) into the dorsal sub-membrane of a 6-week-old mouse. Drug injection was performed once every 2 days, 10-12 times in total, when the tumor size reached 0.6 cm x 0.6 cm. Two different drugs were injected at Lethal Dose 50 (LD_{50}): N-Hydroxy-7-(2-naphthylthio) heptanomide (HNHA, 206 μg/mL), and suberoylanilide hydroxamic acid (SAHA, 138 μg/mL).

3. Immunohistochemistry for COX-2, HIF-1α

Immunostaining was performed on 5 μm sections after deparaffinization in xylene and re-hydration in descending alcohols. In the primary antibody reaction, slides were incubated with monoclonal mouse anti-COX-2 (Santa Cruz Biotechnology) and monoclonal mouse anti-HIF-1α (Novus) for 1 hour. The sections were incubated with biotinylated secondary antibodies (1:500) for 10 minutes, washed with PBS, and an avidin–biotin complex (Strept ABComplex; DAKO, CA) was applied. Finally, the sections were rinsed in PBS, developed with diaminobenzidine tetrahydrochloride substrate for 3 minutes and counterstained with hematoxylin.

4. Western blot analysis for COX-2, HIF-1α and TNF- α

The homogenate was sonicated three times for 20 seconds at RT and centrifuged at 12,000 rpm and -4℃ for 10 minutes. The supernatant was diluted with electrophoretic sample buffer to a final protein concentration of 5.0μg/μL and heated at 100℃ for 7 minutes prior to electrophoresis under denaturing conditions by SDS-PAGE using a discontinuous procedure with 4.5% polyacrylamide stacking gels and 12% polyacrylamide separating gels. Paired mini-gels (Mini-protein II cell, Bio-Rad Laboratories, U.S.A) were loaded with 35.0 μg protein per well.

After electrophoresis, one mini-gel was routinely stained by the Coomassie blue staining method and the other was equilibrated in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v), pH 7.3). The proteins were electrotransferred in transfer buffer to a PVDF transfer membrane (0.2 µm immunoblot TM PVDF membrane for protein blot analysis, Bio-Rad) for 1 hour at RT and 90 volts. To visualize the transferred proteins, the PVDF membrane was stained with ponceau S (Amresco, Solon, Ohio) for 10 minutes and subsequently incubated in TBS-T (TBS with 0.05% Tween-20) with 1% skim milk for 1 hour at RT to block non-specific sites. The blot was rinsed with TBS-T and incubated with primary antibody overnight at 4℃, then with horseradish peroxidase conjugated secondary antibody to IgG for 2 hours at RT. The peroxidase reaction was developed with Amersham ECL reagents (Amersham Biosciences, USA).

5. Cell cycle analysis by flow cytometry

HT-29 cells were seeded in plates (5×10^1 cells/plate) and incubated for 24 hours prior to treatment with the indicated drugs and further incubation for 24 hours in RPMI 1640 with 10% FBS. Cells were harvested, centrifuged, and resuspended in phosphate buffered saline (PBS, pH7.4). RNase (80µg/mL) was added to reduce background staining and DNA was labeled using propidium iodide

(50µg/mL). DNA histograms were determined using a Beckton-Dickenson FACS Vantage flow cytometer system (Beckton-Dickenson, San Jose, CA) and the cell cycle distribution was analyzed using Cell Quest software version 3.2 (Beckton-Dickenson).

III. RESULT

Table 1. IC_{50} concentration of HNHA and SAHA. Using CCK-8[1]

Cell line	Tissue derived	HNHA IC_{50} (μM)	SAHA IC_{50} (μM)
HT-29	Human colorectal	16.98	24.91
C1300	Mouse neuroblastoma	55.63	78.22
MCF-7	Human breast	14.32	19.95

[1] CCK-8 : Cell counting kit-8

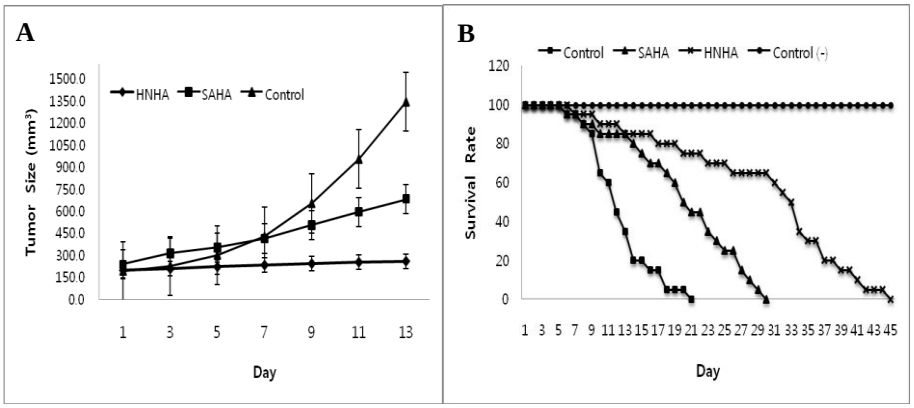

Figure 2. Size of HT-29 tumor and survival rate. A. Size of HT-29 tumor. B. Survival rate. A, tumor size was calculated by (length*width^2)/2. B. Control group was treated with saline ( ), the drug treatment groups were treated with SAHA or HNHA, and the negative control received no treatment ( ).

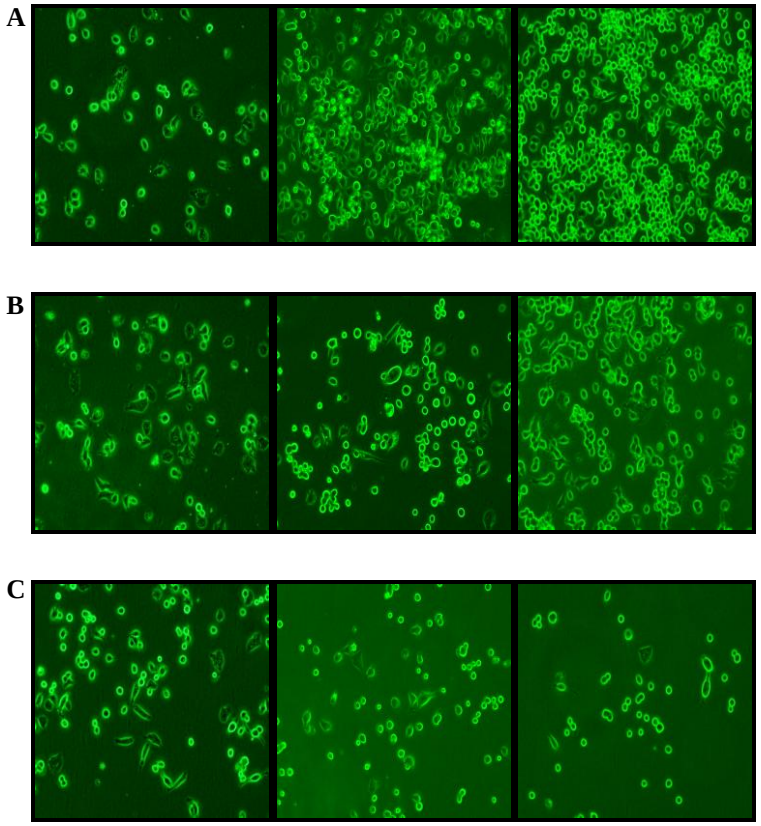

Figure 3. Effect of drug treatment on growth of C1300 neuroblastoma cell line. A. Control, B. SAHA, C. HNHA. C1300 cells were treated with SAHA and HNHA after preculture *in vitro.* The same number of cells (5 x 10^1 cells) was seeded on each plate.

C1300 (mouse neuroblastoma cell line) was treated with HNHA and SAHA to compare the effects of the new drug and the existing drug. The initial cell population was the same because the plates were seeded at the same density, but

the cells treated with the new drug, HNHA, grew slowly compared with the control and SAHA treated-plate; while the cell population increased on control plates treated with saline, the number of cells on HNHA-treated plates was significantly decreased. In addition, the morphology of drug-treated cells was also different. In the control plate, although the cell number increased the cell morphology did not change between the first and last day (Figure 3. A). In contrast, the morphology of cells treated with SAHA and HNHA changed during this time; the shape of cells treated with SAHA differed between the first day and the last day (Figure 3. B), and HNHA-treated cells showed both a decrease in population and altered morphology (Figure 3. C).

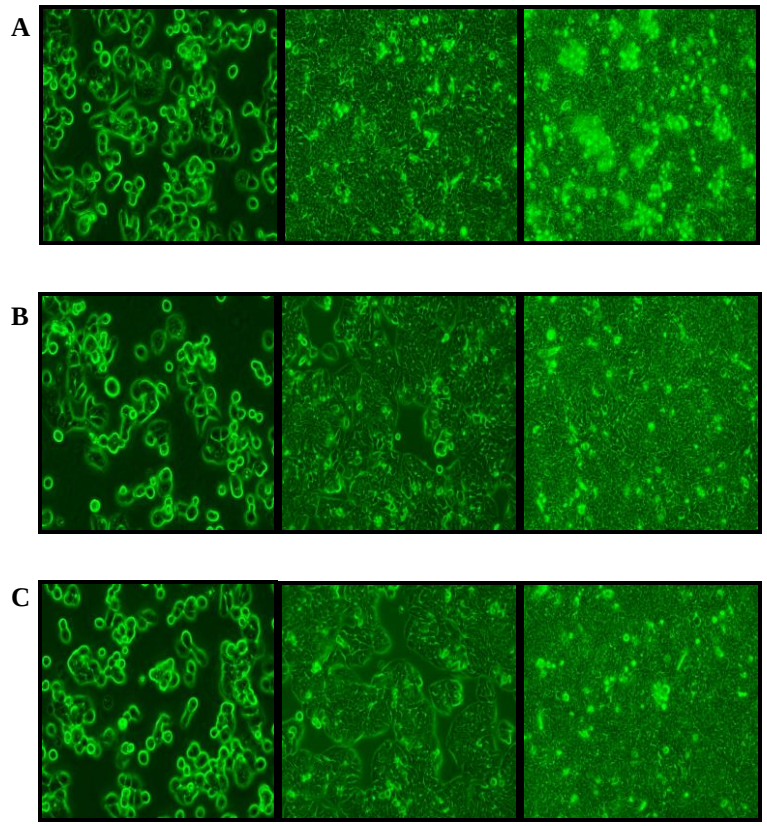

Figure 4. Effect of drug treatment on growth of HT-29 colorectal cancer cell line. A. Control, B. SAHA, C. HNHA. HT-29 cells were treated with SAHA and HNHA after preculture *in vitro*. The same number of cells (5×10^1 cells) was seeded on each plate.

The human colorectal cancer cell line HT-29 was also treated with SAHA and HNHA to compare the effects of the two drugs. As for C1300, the cell population on the first day was the same because we seeded at the same cell density. Whereas the cell population increased in control plates treated with saline, growth

of the cells treated with HNHA was obviously decreased compared with the control and SAHA treatment. Therefore the new drug, HNHA, exhibited a stronger effect than SAHA.

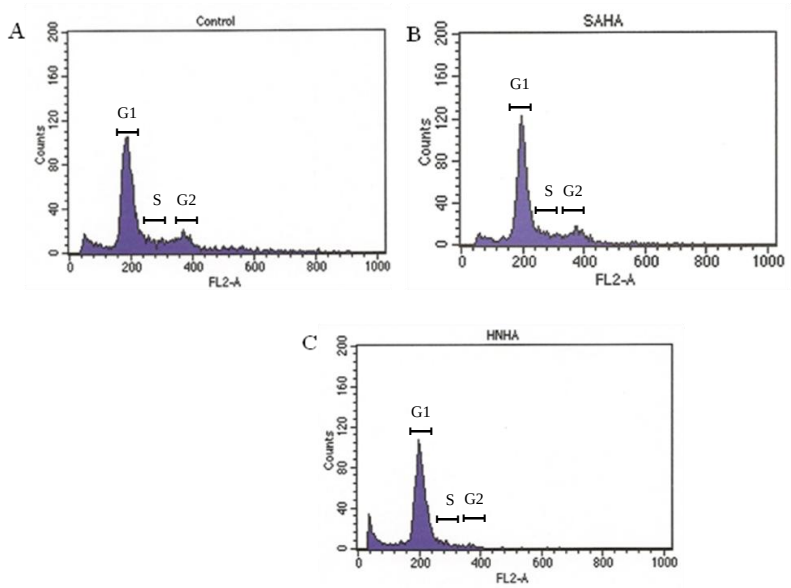

Figure 5. Cell cycle analysis. A. Control, B. SAHA, C. HNHA. The same number of cells (5 x 10^1 cells) was seeded on each plate and treated with each drug at an IC_{50} dose. After 24 hours treatment, the cells were harvested and the effects of the drugs on the cell cycle were analyzed by FACS.

We then used the same cell line, HT-29, to investigate which stage of the cell cycle is affected by HNHA. Our data shows that HNHA induced cell arrest and inhibited replication. Generally, tumor cells have a high proportion of cells in S and G2 phase, before cleavage. However, following treatment with HNHA, the number of S and G2 phase cells decreased and the number of G0-G1 phase cells increased, compared with control and SAHA-treated cells. Thus, inhibition of HDAC activity by HNHA suppressed cell replication during the tumor cell cycle.

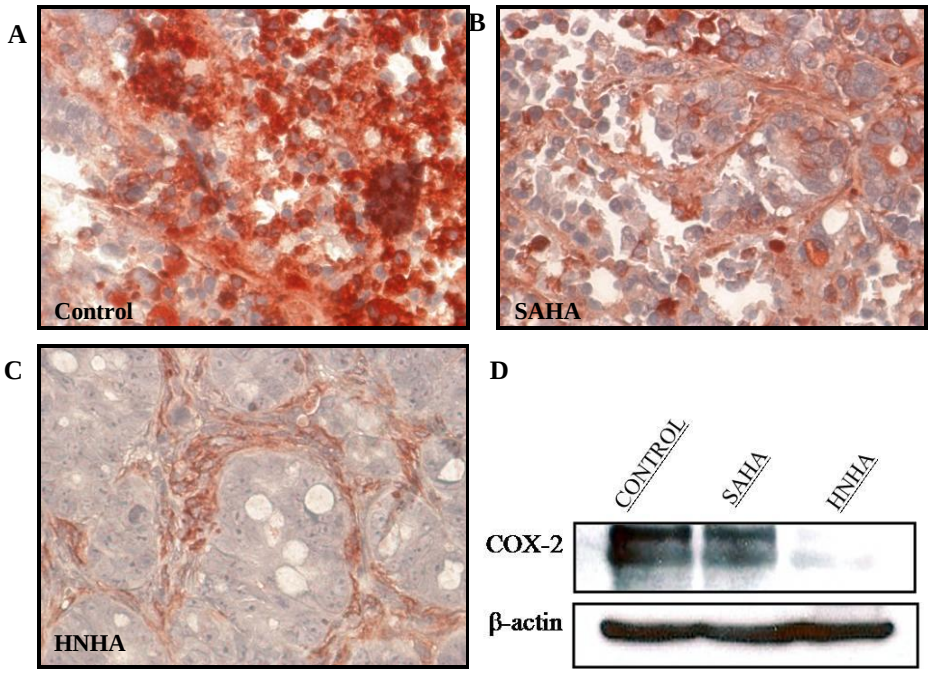

Figure 6. Expression of COX-2. A. Control, B. SAHA, C. HNHA. Cultured cells ($1x10^7$) were injected into the sub-membrane of BALB/C nude mouse. When the tumor reached an appropriate size, drugs were injected at the LD_{50} dose once every 2 days, for a total of 10-12 injections. After drug treatment, the cancer tissue was removed and the level of COX-2 expression was detected by immunohistochemistry and western blot.

We next measured the level of COX-2 expression in tumor tissue of nude mice by immunohistochemistry. COX-2 expression was lower in HNHA and SAHA treated tissue than in controls, and HNHA showed more effective down-regulation of COX-2 expression than SAHA. COX-2 was over-expressed in control cancer tissue (Figure 6. A). COX-2 expression was lower in tissue treated with SAHA than in control tissue (Figure 6. B), and very low in cancer tissue

treated with HNHA (Figure 6. C). Western blot analysis showed decreased levels of COX-2 total protein extract from HNHA-treated tumor tissue, consistent with the immunohistochemistry result (Figure 6. D).

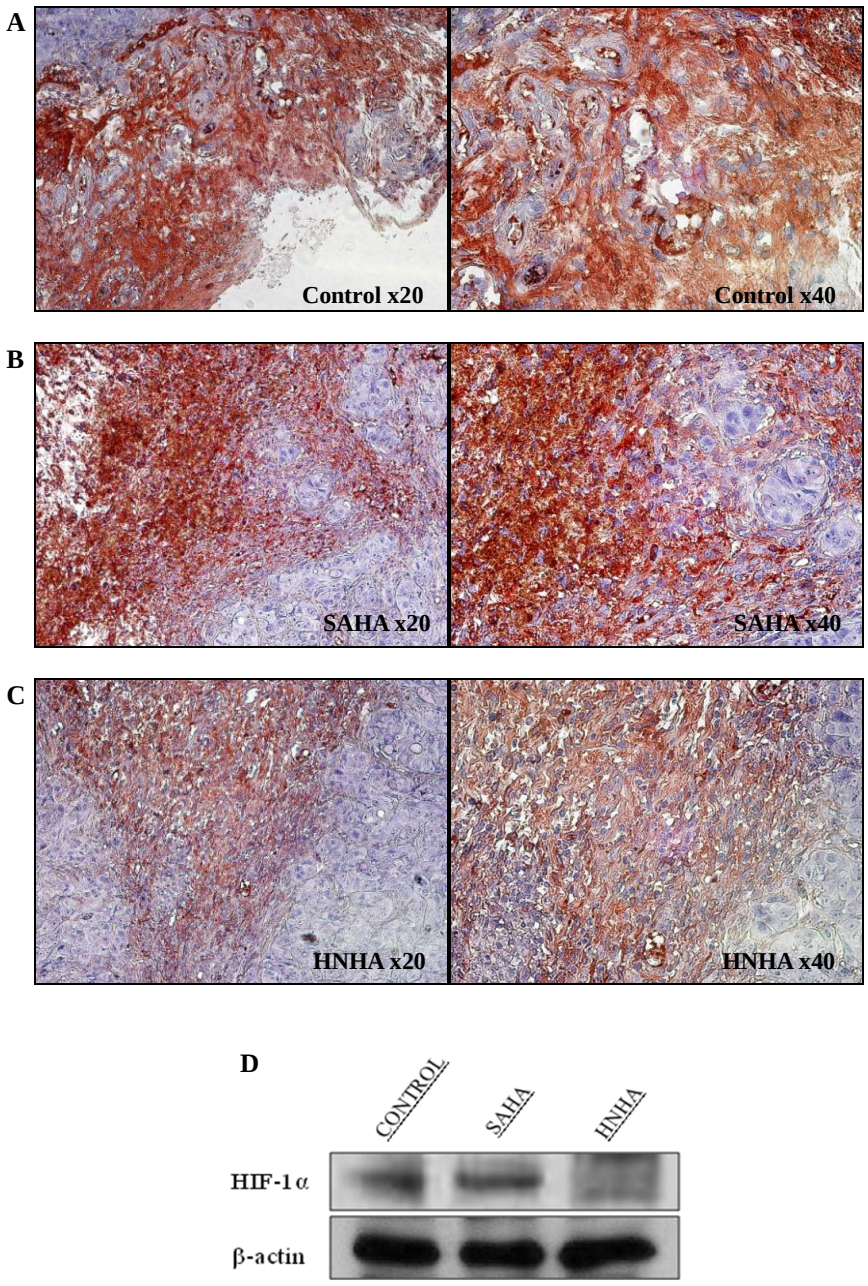

Figure 7. Expression of HIF-1α. A. Control, B. SAHA, C. HNHA, D. detection of HIF-1α by western blotting. Cultured HT-29 cells (1 x 10^7 cells) were injected into the sub-membrane of

BALB/C nude mice. When the tumor size was approximately 0.6 cm x 0.6 cm, the LD_{50} dose of each drug was injected once every 2 days for a total of seven injections. After treatment, the animals were sacrificed and the cancer tissue removed for measurement of HIF-1α expression by immunohistochemistry (A, B, C). Total protein was extracted from the same tissue, and expression of HIF-1α was measured by western blot analysis (D).

HIF-1α was widely expressed in control cells and it was hard to distinguish morphologically between cells that do or do not express HIF-1α (Figure 7. A). HIF-1α was also over-expressed in tissue that was treated with SAHA, but it was easy to identify which cells expressed HIF-1α (Figure 7. B). In HNHA-treated cancer tissue, the level of HIF-1α expression was decreased and cell morphology was almost normal (Figure 7. C). Total protein was extracted from the tissue used in the prior experiment and expression of HIF-1α was measured by western blot. HIF-1α expression was decreased in HNHA-treated tissues compared with control and SAHA-treated tissue (Figure 7. D).

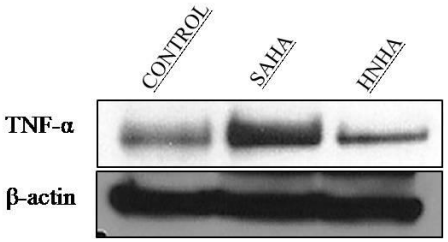

Figure 8. Expression of TNF- α. Lane 1: Control; lane 2: SAHA; lane 3: HNHA. Total protein prepared from tumor tissue as described above was subjected to western blot analysis of TNF-α expression.

Using the same total protein extracts, we also showed decreased levels of TNF-α in HNHA-treated tissue. TNF-α is a cytokine involved in COX-2 expression. Specifically, TNF-α activates several cellular signaling pathways, which mediate the expression of COX-2 by facilitating the recruitment of various transcription factors to the COX-2 promoter[20]. Therefore activation and increased expression of TNF-α result in increased expression of COX-2 expression.

IV. DISCUSSION

Histone acetylation is regulated by acetylases and deacetylases, and plays a key role in eukayotic gene expression. Recent studies have shown that histone deacetylase (HDAC) plays a crucial role in carcinogenesis[1] and is over-expressed in several tumor cells[2]. COX-1 is constitutively expressed but COX-2 is inducible as an early response gene controlled by growth factors, tumor promoters, oncogenes, and carcinogens[19]. More specifically, COX-2 plays an important role in colorectal cancer carcinogenesis[5]. Moreover, through the production of prostaglandins, COX-2 suppresses apoptosis and promotes angiogenesis and tumor invasion[19].

In this study, we investigated the effect of a new HDAC inhibitor, HNHA, on the expression of COX-2 compared with the existing HDAC inhibitor SAHA. The IC_{50} of SAHA is higher than that of HNHA by CCK-8 (Table 1), indicating that HNHA is a more efficient inhibitor than SAHA. Indeed, treatment with HNHA inhibited survival of C1300 (mouse neuroblastoma cell line) and HT-29 (human colorectal cancer cell line) cells more effectively than treatment with SAHA (Figure 3, 4). More specifically, *in vitro* data showed that HNHA reduced the proportion of colorectal cancer cells in S phase of the cell cycle but increased the number of cells in Go-G1 phase (Figure 5). Thus, HNHA arrested cells prior to S phase, inhibiting tumor cell replication. COX-2 expression was decreased in colon cancer

tissue treated with HNHA (Figure 6) because histone acetylation normally relaxes the chromatin, allowing transcription factor binding and RNA polymerase II recruitment. The addition of HDAC inhibitors has a similar net effect to increasing the amount of histone acetylase activity, resulting in enhanced transcription levels[21]. Expression of HIF-1α was decreased in HNHA-treated mouse tissue compared with controls (Figure 7), as was expression of TNF-α (Figure 8).

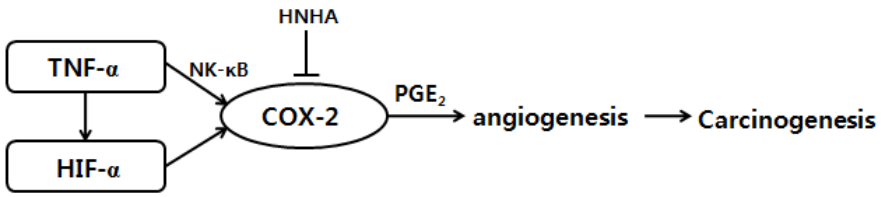

Figure 9. Proposed mechanism of cancer suppression by HNHA

The expression of HIF-1α can be induced by tumor necrosis factor, therefore TNF-α induces HIF-1α expression, which in turn increases transcriptional expression of COX-2 through binding of HIF-1α to a hypoxia-responsive element on the COX-2 promoter[8]. Thus, COX-2 up-regulation during hypoxia is accompanied by promotion of tumor cell survival. Recent studies have shown that

inhibition of COX-2 represses various cancers including colon cancer and cervical cancer [5, 6, 19, 22]. Moreover, prostaglandin formation significantly correlates with COX-2 expression. Our data indicate that the new HDAC inhibitor, HNHA, also functions as a COX-2 inhibitor. A number of signaling pathways are involved in the regulation of COX-2 expression in colorectal carcinoma cells and other cancer cells. In future studies we plan to link the regulation of COX-2 expression with key oncogenic signaling pathways.

V. CONCLUSION

The present study clarifies the effects of HNHA on colon cancer. The results yield the following conclusions:

1. HNHA induces cell cycle arrest of cancer cells, and acts like a HDAC inhibitor and a COX-2 inhibitor.
2. Decreased expression of COX-2 is related to reduced expression of HIF-1α and TNF-α.

In conclusion, these data prove that the new drug HNHA inhibits COX-2 expression in cancer cells and tumor tissues. Further studies are needed to confirm that this effect is at the RNA level and not the protein level.

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ABSTRACT (IN KOREAN)

대장암에서 신약 HNHA(N-hydroxy-7-(2-naphthylthio) heptanomide)의 COX-2 발현억제

박 지 현

연세대학교 대학원 의과학과

(지도교수 서 진 석)

최근의 연구를 통하여 nucleosome 의 구조가 변형하는데 전사인자와 DNA 와의 접근을 촉진시키는 SWI/SNF, RSC, NURF, NRD 등과 같은 염색질 재형성 인자 (chromatin remodeling factor), histone 의 아세틸화 상태를 조절하는 Histone acetyltransferases (HATs)와 Histone deacetylase (HDACs)가 중요한 조절인자로 작용함이 밝혀졌다. 또한, HDAC 는 저산소증,

저포도당, 세포암화 등 열악한 환경조건에서 높이 발현되어 세포증식 억제인자의 발현을 저해함으로써 세포증식을 촉진시키는 역할을 하는 것이 밝혀지면서 세포의 암화 및 분화를 조절하는데 있어 중요한 인자로 인식되고 있다.

COX-2 는 cell adhesion, apoptosis, angiogenesis 에서 중요한 역할을 하여, 정상세포에서는 낮은 레벨로 발현하지만 tumor 에서는 높은 레벨로 발현되는 것으로 알려졌다. 앞서 언급한대로 COX-2 는 angiogenesis 에서 중요한 역할을 하는 것으로도 밝혀져 암 조직에서 HDAC inhibitor 를 이용한 COX-2 의 억제는 암세포의 증식과 생존을 억제한다.

HT-29 라는 human colorectal cancer cell line 과 mouse neuroblastoma cell line 인 C1300 을 *in vitro* 에서 배양하여 기존 상용화 된 유사약물과 비교하여 약을 처리하였다. *In vivo* 실험에서는 HT-29 를 배양하여 실험동물 모델을 만들어 일정한 tumor 크기가 되었을 때 각 종류별 drug 을 피하 주사한다. 약물처리가 완료되면 cancer tissue 를 획득한 후 total protein 을

추출하여 western blot analysis, immunohistochemistry 를 수행하여 약물이 cancer 에 미친 영향을 관찰하였다.

HDAC inhibitor 는 COX-2 의 발현을 억제하며 COX-2 발현 전단계인 TNF-α 발현 또한 억제한다. 또한 정상세포에서는 거의 발현하지 않지만 암세포에서는 많이 발현되는 HIF-1α 의 발현도 HNHA 를 처리한 조직에서는 그 발현이 억제되었다. 이와 같이 COX-2 는 HNHA inhibitor 에 의해서도 억제됨을 확인하였으며, 따라서 암 조직에서 COX-2 의 억제는 암세포의 증식과 생존을 억제할 것이며, 또한 신생혈관 형성작용을 억제함으로써 암 성장을 억제할 수 있을 것이다.

핵심되는 말 : Histone deacetylase (HDAC), N-hydroxy-7-(2-naphthylthio)heptanomide (HNHA), Cyclooxygenase-2 (COX-2), Hypoxia-inducible factor-1α (HIF-1α), Tumor necrosis factor-α (TNF-α)