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Research Article

Synergistic Anti-cancer Effect of Inhibition of Histone Deacetylase and Blockade of the Glycolytic Pathway

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2. Key words

Anaplastic thyroid cancer; Cancer stem-like cell; Anti-apoptosis; Apoptosis; Metabolic stress; Histone deacetylase inhibition

1. Abstract

Anaplastic Thyroid Cancer (ATC) is characterized by a higher percentage of epigenetic changes; these occur more often than genetic mutations. Studies using preclinical models have reported that a combination of N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA) and 2-deoxy-D-glucose (2DG) plays a crucial role in cancer stem cell-like cells in ATC. This study aimed to investigate whether combinatorial therapy with HNHA and 2DG promotes tumor suppression via caspase cleavage and cell cycle arrest in ATC. ATC cell lines 8505C and SNU 80, isolated from the current patient, were treated with HNHA and 2DG alone or in combination, and cell viability was determined via the MTT assay. Synergistic anti-cancer effects of combinatorial therapy on the cell cycle and intracellular signaling pathways were assessed via flow cytometry and immunoblot analyses. An ATC cell line-derived xenograft model was used to examine anti-tumor activity *in vivo*. Combinatorial therapy with HNHA and 2DG synergistically reduced the viability of ATC cells and significantly induced apoptotic cell death, evident from caspase-3 cleavage. Furthermore, combinatorial therapy downregulated anti-apoptotic factors. Thus, combinatorial therapy significantly suppressed tumor volume in ATC cell xenografts, compared to HNHA or 2DG alone.

The present results show that combinatorial therapy with HNHA and 2DG is more effective than treatment with HNHA or 2DG alone in ATC, thereby suggesting a new therapeutic approach for ATC, including cancer stem-like cells.

3. Introduction

The thyroid is a gland in the neck, which usually secretes thyroid hormones; they play crucial roles in regulating normal metabolism. Thyroid cancer comprises 4 major types: papillary, follicular, medullary, and anaplastic [1]. Thyroid cancer accounts for more than 80% of total endocrine-related carcinoma and is the most common endocrine malignancy, with an increasing worldwide incidence [2]. Thyroid cancer can be well-differentiated, poorly-differentiated, and anaplastic based on cell differentiation characteristics and its sustenance of the follicular cell phenotype. Differentiated Thyroid Cancer (DTC) is the most common type, accounting for more than 90% of all thyroid carcinomas. DTC comprises papillary and follicular histological subtypes [3, 4]. However, poorly-differentiated Anaplastic Thyroid Cancer (ATC) has a poor prognosis owing to chemotherapeutic resistance and aggressiveness [5], with a total median survival of several months [6]. Currently, new targeted therapies have successfully increased the longevity of cancer patients. Kinase inhibitors have been suggested for treating radioactive iodine (RAI)-refractory DTC patients with metastatic, rapidly progressive, and/or imminently threatening disease, which cannot be controlled locally using alternative approaches [7]. However, this has not been observed among patients with advanced cancer subtypes. Treatment of progressive metastatic cancer often yields limited benefits; hence, new therapeutic approaches for patients at a high risk of cancer-related mortality are warranted [1]. Recent studies have reported molecules and mechanisms closely associated with poor clinical outcomes in advanced thyroid cancer [8, 9]. Among these mechanisms, the synergistic anti-cancer effect of the glucose catabolism 2-deoxyglucose (2-DG) and histone deacetylase inhibitor (HDACI)-induced suppression of cancer stem cells (CSCs) could be considered one of the probable reasons for poor clinical outcomes [10]. Other than typical mutations, epigenetic silencing of tumor suppressor genes often dysregulate tumorigenic signaling pathways [11]. Histone acetyl-transferase (HAT) and HDACs catalyze the acetylation and deacetylation, respectively, of lysine residues in histone tails, thus regulating the interaction of transcriptional complexes for DNA [12]. Consequently, HAT and HDAC recruitment constitute a key element in dynamic gene regulation in cellular proliferation and differentiation during car-

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cinogenesis [13]. Moreover, a hallmark of rapid tumor growth is the shift from mitochondrial respiration to aerobic glycolysis (also known as the Warburg effect) [14, 15], although aerobic glycolysis is ineffective in fulfilling the energy and biomass requirements associated with rapid tumor growth [16]. The unique metabolic attributes of cancer cells enable therapies targeting metabolic pathways in cancer cells. Pharmacotherapeutic agents targeting glucose catabolism by 2-deoxyglucose (2-DG) have been reported, with varied efficacy, in many subtypes of solid tumors [17-20].

This study aimed to elucidate the mechanism underlying the synergistic anti-cancer effect of inhibition of histone deacetylase and blockade of glycolysis by N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA) and 2DG in anaplastic thyroid cancer stem cells.

4. Materials and Methods

4.1. Patients/Tissue Specimens

Fresh tumors were resected from primary thyroid cancer and metastatic sites in patients with biochemically and histologically proven aggressive RAI-refractory papillary thyroid cancer, who were treated at the Thyroid Cancer Center, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. Further protocol and details are described in our previous articles [21, 22].

4.2. Tumor Cell Isolation and Primary Culture

On the day after resection, the tumors were placed in normal saline with antifungal and antibiotic agents and transported to the laboratory. The protocol and details are showed in our previous articles [21, 22]. The research protocol was approved by the Institutional Review Board of the Thyroid Cancer Center, Gangnam Severance Hospital, Yonsei University College of Medicine (IRB Protocol: 3–2016-0076).

4.3. Cell Culture

ATC cell lines 8505C, SNU-80, and GSA1 were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) or the Korea Cell Line Bank (Seoul National University, Seoul, Korea) or via tumor cell isolation from the patient (at the Thyroid Cancer Center, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea) culturing in RPMI-1640 medium with 10% FBS (Table 1). Mycoplasma contamination was invariably checked with the Lookout Mycoplasma PCR Detection Kit (Sigma-Aldrich; MP0035). Cell lines were authenticated via short tandem repeat profiling/karyotyping/isoenzyme analysis [22].

4.4. Cell Viability Assay

Cell viability was determined using the 3-(4, 5-dimethylthi- azol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Fur- ther details are given in our previous article [22].

4.5. Immunoblot Analysis

Equal amounts of protein (20 μg) were separated electrophoretically on 8–10% SDS-polyacrylamide gels and electro-transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were subsequently blocked and incubated with the following primary antibodies overnight at 4°C: anti-Ki-67 (Abcam), anti-cyclin D1 (Santa Cruz Biotechnology, Dallas, TX, USA), anti-p21 (Santa Cruz Biotechnology), anti-Bcl-2 (Santa Cruz Biotechnology), anti-caspase-3 (Santa Cruz Biotechnology), and anti-β-actin (Santa Cruz Biotechnology). Further protocols and details are described in our previous article [21].

4.6. Flow Cytometry Analysis of the Cell Cycle

Cells were treated with 2DG and HNHA alone or in combination in RPMI-1640 medium containing 10% FBS for 40 h, harvested via trypsinization, and fixed with 70% ethanol. Protocol is given in our previous articles [21, 22].

4.7. Human ATC Cell Xenograft Model

Human ATC cells (2.0×10^6 cells/mouse) were cultured in vitro and then injected subcutaneously into the upper left flank of female BALB/c nude mice. After 7 d, tumor-bearing mice were grouped randomly (n = 10/group) and treated with 25 mg/kg HNHA (intraperitoneally) alone, 500 mg/kg 2DG (p.o.) alone, or a combination of 6.5 mg/kg HNHA and 95 mg/kg 2DG, once every 2 d for 10~12 injections. Tumor size was measured every alternate day, using calipers. Tumor volume was estimated using the following formula: L × S/2 (where L is the longest diameter and S is the shortest diameter). Animals were maintained under specific pathogen-free conditions. All experiments were approved by the Animal Experiment Committee of Yonsei University [21-23].

5. Results

5.1. Synergistic Anti-Cancer Effects of Combinatorial Administration of 2DG and HNHA Were More Effective Than Those of 2DG or HNHA Administration Alone

The synergistic effects of the tumor suppression via inhibition of histone deacetylase and blockade of the glycolytic pathway were more effective using a combination of HNHA and 2DG than using HNHA or 2DG alone in ATC cells. We assayed for cell viability in 8505C, SNU 80, and GSA1 cells upon treatment with either HNHA or 2DG alone and a combination of both, via an MTT assay (Figure *1A, B* and *C*). The combination of 2DG and HNHA suppressed cell proliferation more effectively than either agent used alone (Figure *1A, B,* and *C*).

5.2. Synergistic Effects of 2DG and HNHA on Cancer Cell Proliferation in Ptient-Drived ATCs

To assess the synergistic anticancer effects of a combination of 2DG and HNHA on ATC, we assayed 8505C, GSA1, and SNU 80 cells, and patient-derived ATCs (Figure 2, Information regarding ATC, obtained from the ECACC or the Korea Cell Line Bank or tumor cell isolation from the current patient); cell proliferation was assessed in the presence and absence of these compounds.

IC_{50} was the lowest for the combination of 2DG and HNHA among all treatment groups for 8505C, SNU 80, and GSA1 cells (Table 1).

Combinatorial treatment with 2DG and HNHA most significantly induced the sub-G_0G_1 cells, thereby inducing cell death in 8505C, SNU 80, and GSA1 cells (Figure 3*A, B,* and *C*). The synergistic effect of 2DG and HNHA most potently increased the sub-G_0G_1 population, thus inducing apoptosis, cell cycle arrest, and reduction in the viability of 8505C, SNU 80, and GSA1 cells.

Together, these data indicate that inhibition of histone deacetylase and blockade of the glycolytic pathway upon treatment with a combination of HNHA and 2DG effectively suppresses ATC cells.

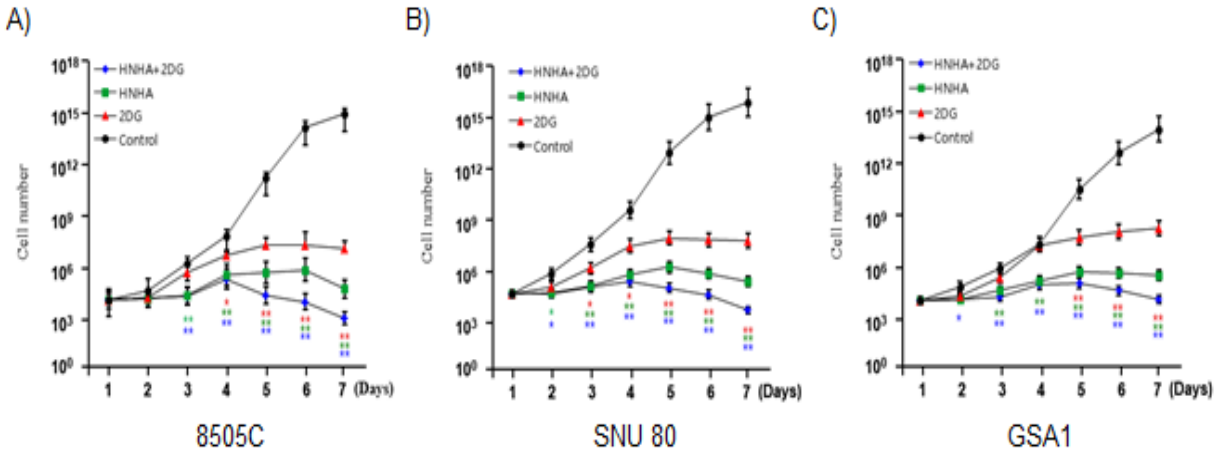


Figure 1: Synergistic anti-cancer effects of 2-deoxy-D-glucose (2DG) and N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA) on anaplastic thyroid cancer (ATC) cells in comparison with the effects of each agent used alone.
Cell proliferation assay upon combinatorial treatment with 2DG and HNHA and each agent treated alone in ATC, 8505C, SNU 80, and GSA1 (patient-derived ATC) cells. Points show mean % of the value observed in the solvent-treated control. All experiments were repeated at least 3 times. Data represent means ± SD. **P* < 0.05 vs. control, ***P* < 0.01 vs. control, ****P* < 0.005 vs. control.

	8505C	SNU-80	GSA1
Primary Disease Site	Thyroid	Thyroid	Thyroid
Stage	IVc	IVc	IVc
Primary Pathology	Anaplastic thyroid cancer	Anaplastic thyroid cancer	Anaplastic thyroid cancer
Classification of specimen used for culture	-	-	Fresh tumor
Obtained from	ECACC	Korea Cell Line Bank	Gangnam Severance Hospital, Seoul, Korea

Figure 2: Cell line characteristics and cell viability after drug treatment of all anaplastic thyroid cancer (ATC) cell lines.
ATC cell lines 8505C, SNU-80, and GSA1 were obtained from the European Collection of Cell Cultures or the Korea Cell Line Bank or from via tumor cell isolation from tumors resected from the patient.

Table 1: Half maximal inhibitory concentration (IC_{50}) determination based on a cell proliferation assay. Combinatorial treatment with N-hydroxy-7-(2-naphthylthio) heptanomide **(**HNHA) and 2-deoxy-D-glucose (2DG) yielded a lower IC_{50} value than treatment with HNHA or 2DG. Each data point represents the mean of 3 independent 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assays for IC_{50}. SD, standard deviation.

Cell Line	Hisopathology	Animal	Cell Proliferation $IC_{50*)}$		
			HNHA (μM)	2DG (mM)	HNHA (μM) + 2DG (mM)
8505C	Thyroid cancer: Anaplastic	Human	17.12 (± 0.2)	22.52 (± 0.3)	4.05 (± 0.2) + 14.15 (± 0.1) *
SNU-80	Thyroid cancer: Anaplastic	Human	10.43 (± 0.5)	19.54 (± 0.2)	5.32 (± 0.3) + 10.22 (± 0.5) *
GSA1	Thyroid cancer: Anaplastic	Human	28.55 (± 0.5)	31.22 (± 0.3)	7.02 (± 0.6) + 18.99 (± 0.4) *

A)

8505C

Status	Sub-G_0G_1	G_0G_1	S	G_2/M
Control	1.9 ± 0.04	33.4 ± 0.02	29.2 ± 0.01	35.5 ± 0.03
2DG only	21.4 ± 0.02	44.5 ± 0.01	19.8 ± 0.03	14.3 ± 0.03
HNHA only	29.2 ± 0.01	46.2 ± 0.05	12.2 ± 0.05	12.4 ± 0.03
HNHA + 2DG	61.3 ± 0.01	29.5 ± 0.03	7.7 ± 0.04	1.5 ± 0.01

B)

SNU 80

Status	Sub-G_0G_1	G_0G_1	S	G_2/M
Control	1.4 ± 0.02	29.1 ± 0.03	28.1 ± 0.05	41.4 ± 0.02
2DG only	18.7 ± 0.05	40.5 ± 0.02	21.3 ± 0.01	19.5 ± 0.04
HNHA only	24.1 ± 0.02	45.5 ± 0.03	20.5 ± 0.04	9.9 ± 0.02
HNHA + 2DG	57.4 ± 0.02	31.3 ± 0.05	5.9 ± 0.05	5.4 ± 0.05

C)

GSA1

Status	Sub-G_0G_1	G_0G_1	S	G_2/M
Control	1.5 ± 0.04	37.3 ± 0.02	35.1 ± 0.02	26.1 ± 0.04
2DG only	23.5 ± 0.02	40.8 ± 0.01	22.7 ± 0.03	13.0 ± 0.01
HNHA only	32.3 ± 0.02	39.4 ± 0.05	21.2 ± 0.03	7.1 ± 0.01
HNHA + 2DG	66.4 ± 0.01	24.5 ± 0.03	5.5 ± 0.01	3.6 ± 0.04

Figure 3: Cell cycle arrest and apoptotic cell death were most markedly induced upon combinatorial treatment with 2-deoxy-D-glucose (2DG) and N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA).
(A, B, and C) Cells were exposed to the indicated inhibitors, harvested, and stained with propidium iodide before flow cytometry and FlowJo v8 analyses.

5.3. Combinatorial Teatment with 2DG and HNHA Iduced Aoptosis and Cell Cycle Arrest in ATC Cells

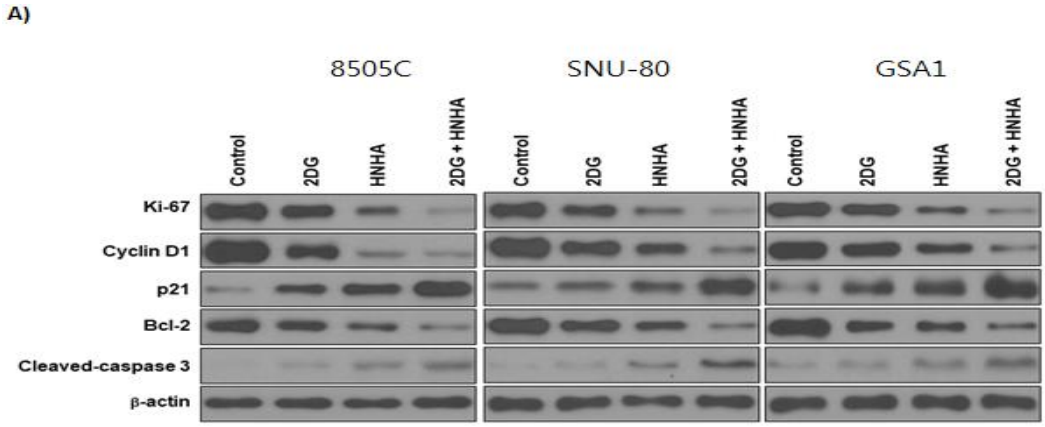
Immunoblot analyses of protein levels in 8505C, SNU 80, and GSA1 cell lines indicated that combinatorial administration of 2DG and HNHA most prominently increased p21 levels, a well-known cell cycle arrest protein, and reduced cyclin D1 levels, which is a positive cell cycle regulator, compared with responses to either agent administered alone (Figure 4A). Notably, proliferation (Ki-67) and anti-apoptotic (Bcl-2) markers were most prominently suppressed upon combinatorial treatment with 2DG and HNHA. Apoptotic markers (cleaved-caspase 3) were most upregulated upon combinatorial treatment with 2DG and HNHA compared with groups treated with either agent alone.

Together, the results suggest that combinatorial administration of 2DG and HNHA effectively suppresses ATC function.

5.4. Combinatorial Administration of HNHA and 2DG Prominently Reduced Tumor Size in a Xenograft Model

To estimate the synergistic anticancer effect of combinatorial administration of 2DG and HNHA *in vivo*, we generated a mouse xenograft tumor model with 8505C, SNU 80, and patient-derived ATC, GSA1 cells (Figure 4B-J). Each agent used alone did not prominently suppress 8505C, SNU 80, and GSA1 cell xenograft tumors; however, combinatorial administration of 2DG and HNHA prominently resulted in tumor suppression (Figure *5B, E,* and *H*). Moreover, there was no evidence for systemic toxicity or treatment-related mortality in any group. Mouse body weight was not significantly influenced by treatment with sorafenib, lenvatinib, or HNHA (Figure *5D, G* and *J*). Combinatorial treatment with 2DG and HNHA significantly decreased tumor volumes in comparison with each agent used alone (Figure *5C, F* and *I*). Accordingly, combinatorial treatment with 2DG and HNHA had potent anticancer effects in ATC and in the ATC xenograft model.

Consequently, these results propose a potential new therapeutic approach to treat patients at a high risk of cancer-related mortality.



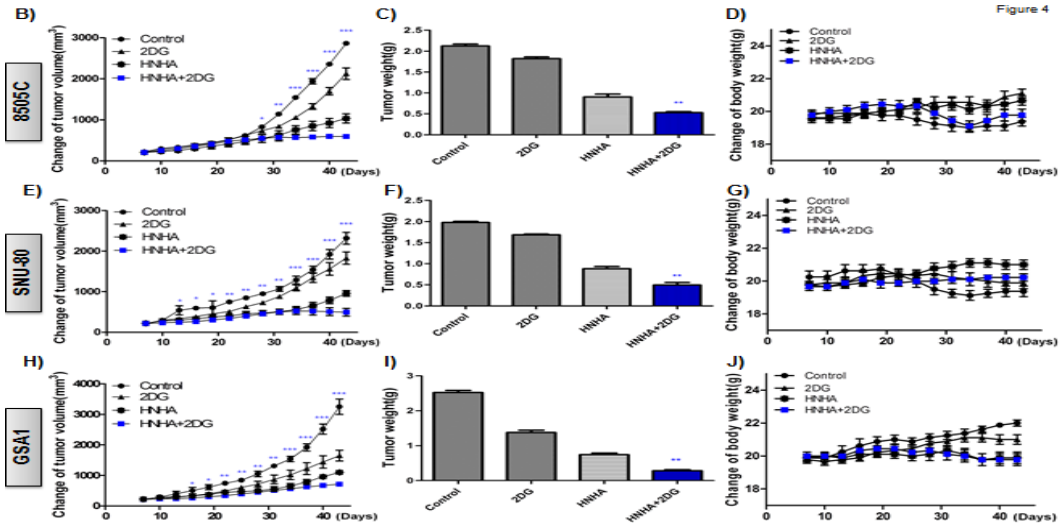


Figure 4: Synergistic anticancer effects of combinatorial treatment with 2-deoxy-D-glucose (2DG) and N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA) in anaplastic thyroid cancer (ATC) cell xenografts *in vivo*.

A, 8505C, SNU 80 and GSA1 cells were exposed to the indicated inhibitors for 24 h prior to the analysis of the expression of Ki-67 (cell proliferation marker), Cyclin D1 (cell cycle marker), p21 (cell cycle arrest marker), cleaved-caspase 3 (apoptosis marker), and Bcl-2 (anti-apoptosis marker) via immunoblot analysis.

Athymic nude mice with established tumors were treated with the indicated inhibitors. Data represent mean tumor volumes. Inhibition of tumor progression by combinatorial treatment of 2DG and HNHA in mice with ATC cell (8505C, *B-D*; SNU 80, *E–G*; and GSA1, *H-J*) xenografts (n = 10 mice/group). Changes in tumor volume *(B, E, and H)*. The compounds had no significant effect on mouse body weight (*D, G, and J*). Weight of dissected tumors (*D, G, and J*). *P < 0.05; **P < 0.01; ***P < 0.005 for the comparison with the control.

6. Discussion

Thyroid cancer is the most common endocrine-related malignancy [24], with an increased worldwide incidence, including Korea [25]. Age-based cancer incidence rates during 2011 were 81.0 per 100,000 (27.9 in men, 134.1 in women) in accordance with the data curated in the Korea National Cancer Incidence Database. The incidence of thyroid cancer increases by 23.3% per year in both men and women, and thyroid cancer has been the most common cancer among women in Korea, since 2009 [26]. High-resolution ultrasonography may have contributed to early detection of asymptomatic small thyroid nodules [27]. Consequently, the size ration of identified thyroid cancers has decreased [28, 29]. The induction in numerous surgically treated cases of thyroid cancer were mainly due to induction in cancers measuring 1 cm or less. Consequently, increased evaluation of thyroid cancer via neck ultrasonography and treatment in the early phase may reduce thyroid cancer-related mortality. Unfortunately, however, ATC is still one of the most treatment-resistant cancers [30]. Therefore, a new clinical approach is warranted for treating ATC. This study suggests a promising new treatment strategy for some intractable diseases in future. Synergistic anticancer effects of combinatorial treatment with drugs inhibiting non-overlapping cancer pathways is a reasonable approach to suppress cancer cell proliferation. Moreover, doses of two drugs could be lower than those of individual drugs and increase the therapeutic efficacy with minimum side effects.

2DG and HDACIs can penetrate the blood-brain barrier, a prerequisite for glioma treatment, and are also well-tolerated by patients [10]. The present results show that HDACIs such as HNHA firmly synergize with 2-DG, resulting in cancer cell death. In addition to HDAC inhibition, drug candidates are expected to block glucose metabolism. Glycosylation of indispensable proteins such as transcription factors could be severely influenced by the absence of glucose [31] or by blocking glycolysis, using 2-DG [31]. In an energy-deficient state, the reduced ATP-to-AMP ratio stimulates the first cellular energy sensor, the tumor suppressor LKB1 [32]. Stimulation of AMP-protein kinase results in a global reduction in translation and in cell size by preventing the phosphorylation of downstream effectors such as rapamycin [33]. 2-DG inactivates the transcriptional activity of Sp1 by affecting its O-GlcNAcylation levels [34]. Since numerous transcription factors are reformed by O-GlcNAc, O-GlcNAcylation of other transcription factors can lead to the regulation of gene expression in response to glucose.

Histone modification constitutes a rather old concept of epigenetic regulation. Among some other types of histone modification, histone deacetylation is deregulated in numerous cancers. A current study reported that that HDAC1, HDAC2, and HDAC3 are upregulated in RCC [35]. Moreover, certain reports suggest that overexpression of class I HDACs, in particular HDAC1, serves as a cancer marker associated with poor prognosis [36]. HDAC inhibitors reverse gene silencing by inhibiting HDAC activity, thereby allowing for histone acetylation. Preclinical studies have proved the potential of HDACI in treating thyroid cancer. HNHA reportedly alters cell cycle-regulating proteins, particularly CDK4, 6, cyclin D1, p53, p21, and related apoptotic proteins and significantly inhibits the growth of thyroid cancer *in vitro* and *in vivo* [23].

This study showed the synergistic anti-cancer effect of 2-DG, an inhibitor of the glycolytic pathway, and HNHA, an inhibitor of HDAC, resulting in the induction of apoptosis in ATC cells. Epigenetic silencing of tumor suppressor genes results in the dysregulation of tumorigenesis. Notably, the present results indicate that

combinatorial treatment with 2DG and HNHA constitutes a potentially effective, new clinical approach for ATC.

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