



OPEN

Synthesis and initial screening of lactate dehydrogenase inhibitor activity of 1,3-benzodioxole derivatives

Dicky Annas^{1,4}, Se-Yun Cheon^{2,4}, Mohammad Yusuf¹, Sung-Jin Bae³, Ki-Tae Ha³✉ & Kang Hyun Park¹✉

Cancer is one of the main causes of mortality in the world. Many cancer cells produce ATP through high-level lactic acid fermentation catalyzed by lactate dehydrogenase (LDH), which converts pyruvic acid to lactic acid. LDH plays a dominant role in the Warburg effect, wherein aerobic glycolysis is favored over oxidative phosphorylation. Due to the high lactic acid production level in cancer cells, LDH-targeting could be a potential cancer treatment strategy. A few approaches, such as drug treatment, reportedly inhibited LDH activity. In this study, we describe new 1,3-benzodioxole derivatives that might be potential small molecule candidates for LDHA inhibition. The synthesis was carried out by trans-esterification between aryl ester and alcohol groups from piperonyl alcohol. Compounds 2 and 10 exhibited a selective LDHA IC₅₀ value of 13.63 μM and 47.2 μM, respectively. Whereas only compound 10 showed significant cytotoxicity in several lines of cancer cells, especially in human pancreatic cancer PANC-1 cells. These synthesized compounds possess 2 aromatic rings and –CF₃ moiety, which expectedly contributes to LDHA inhibition. The presented products have the potential to become a promising LDHA inhibitor drug candidate.

Cancer is one of the biggest health concerns for humans, which takes place at a tissue level^{1–3}. Cancer develops through a series of genetic mutations that result in a change in cell fate. The Warburg effect is a phenomenon wherein cancer cells consume more glucose than healthy cells do to ensure ATP supply for energy production and its catabolites as building blocks simultaneously. In particular, ATP and the precursors of lipid, protein, and nucleotide synthesis are produced through glucose conversion during aerobic glycolysis in cancer cells with lactic acid as the primary end product⁴.

Lactate dehydrogenase (LDH) is an enzyme with a tetrameric structure that catalyzes pyruvate conversion to lactate and vice versa. LDH has two known isoforms. LDHA mainly converts pyruvic acid to lactic acid, while LDHB catalyzes the reverse reaction⁵. Several studies have reported that LDHB is constitutively expressed in various cancer cell types, while LDHA is proposedly important for tumor initiation as it is often overexpressed in cancer. Reduced LDHA levels were related to less cellular transformation and delayed tumor formation^{6,7}.

Chemical approaches are among the common strategies to inhibit LDHA activity in cancer cells. The 1,3-benzodioxole ring was described as a component of many natural compounds with various biological activities. These compounds and their derivatives are widely-used pesticides and herbicides⁸. Certain studies reported that the 1,3-benzodioxole ring possesses antitumor, antiparasitic, antifungal, antioxidant, and antibacterial bioactivities^{9–13}, such as the antioxidant sesamol (Fig. 1)¹⁴. In addition, 1,3-benzodioxole derivatives could act as carcinogenesis-associated histone deacetylase inhibitors during cancer treatment¹⁵.

Several 1,3-benzodioxole ring-containing chemical compounds have been developed with the aim to inhibit LDHA activity. These compounds include 1,3-benzodioxole derivatives, such as Machilin A (Fig. 1), which are efficient competitive inhibitors that function by blocking the nicotinamide adenine dinucleotide (NAD) binding site of LDHA, suppressing lactate production and cancer cell growth¹⁶. Furthermore, benzodioxole ring-containing thiazolyl-pyrazoline derivatives were tested against MCF-7 and B16-F10 tumor cells and showed significant antiproliferative activity *in vitro*¹⁷. In addition, we previously reported that a selenobenzene compound

¹Department of Chemistry, Pusan National University, Busan 46241, Republic of Korea. ²AmcoBio Inc, Seoul 08758, Republic of Korea. ³Healthy Aging Korean Medical Research Center and Department of Korean Medical Science, School of Korean Medicine, Pusan National University, Yangsan 50612, Republic of Korea. ⁴These authors contributed equally: Dicky Annas and Se-Yun Cheon. ✉email: hakis@pusan.ac.kr; chemistry@pusan.ac.kr

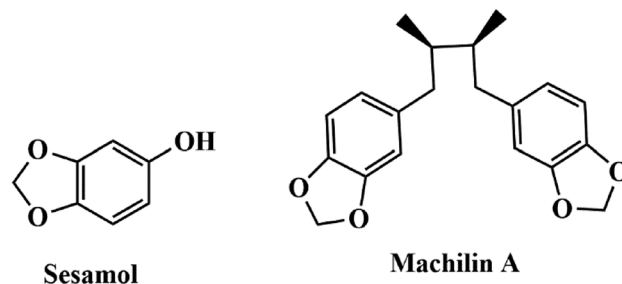
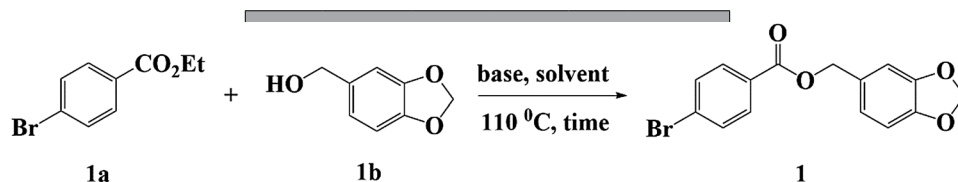


Figure 1. Chemical structure of natural product contained 1,3-benzodioxole rings.



Entry	Base	Solvent	T (°C)	t (h)	Yield ^a (%)
1	Pyridine	Toluene	110	12	39
2	K ₂ CO ₃	Toluene	110	12	32
3	NaOH	Toluene	110	12	45
4	KOH	Toluene	110	12	53
5	Cs ₂ CO ₃	Toluene	110	12	74
6	Cs ₂ CO ₃	DMSO	110	12	40
7	Cs ₂ CO ₃	DCM	40	12	Trace
8	Cs ₂ CO ₃	THF	60	12	20
9	Cs ₂ CO ₃	Toluene	25	12	Trace
10	Cs ₂ CO ₃	Toluene	60	12	29
11	Cs ₂ CO ₃	Toluene	110	18	91
12	Cs ₂ CO ₃	Toluene	110	24	94

Table 1. Optimization of reaction conditions for synthesis of compound 1 to 8. Reaction conditions: ethyl 4-bromobenzoate (1 mmol), piperonyl alcohol (1.1 mmol), Cs₂CO₃ (1 mmol), and toluene (5 mL). ^aIsolated yields.

harboring trifluoromethyl group, 1-(phenylseleno)-4-(trifluoromethyl) benzene, showed an anti-tumor effect thorough suppressing LDHA activity¹⁸. Based on the aforementioned results, we developed new 1,3-benzodioxole and trifluoromethyl derivatives through trans-esterification and evaluated the in vitro LDHA inhibitor activity of the synthesized compounds through decreasing NADH intensity.

Results and discussion

The main chemical reaction in this study was a trans-esterification reaction between the ester group of ethyl 4-bromobenzoate (**1a**) and the alcohol groups of piperonyl alcohol (**1b**). A base was used to abstract the protons from the alcohol groups, creating an anion that could directly abstract the carbonyl in the ester groups, and release the alcohol moiety¹⁹. The reaction between ethyl 4-bromobenzoate and piperonyl alcohol was carried out as presented in Table 1. Initially, the study was carried out using pyridine as a base in toluene at 110 °C under air condition and the reaction mixture was stirred for 12 h. The yield of the product was 39% (Table 1, entry 1). The reaction using K₂CO₃ as a base resulted in a lower yield compared to that using pyridine, which yielded 32% (Table 1, entry 2). By increasing the degree of basicity, the product could be obtained with yields of 45% and 53% in the case of NaOH and KOH, respectively (Table 1, entry 3–4). The highest yield (74%) was obtained when Cs₂CO₃ was used as a base under these reaction conditions (Table 1, entry 5). Therefore, Cs₂CO₃ was selected as a reaction base for further optimization due to its higher activity, yield, and catalytic speed compared to acid catalysts²⁰. The transesterification mechanism using Cs₂CO₃ has proposed²¹. The carbonyl group coordinates with a metal ion to make the carbon center more electrophilic, while the alcohol group is activated by carbonate ion to make a negative charge on the oxygen of the hydroxyl group. This anion directly abstracts the activated carbonyl to form the ester group and release ethanol.

We then screened for the solvent effect in this reaction. When an aprotic polar solvent, such as DMSO, was used, the product yield decreased to 40% (Table 1, entry 6). No reaction could be observed when DCM was

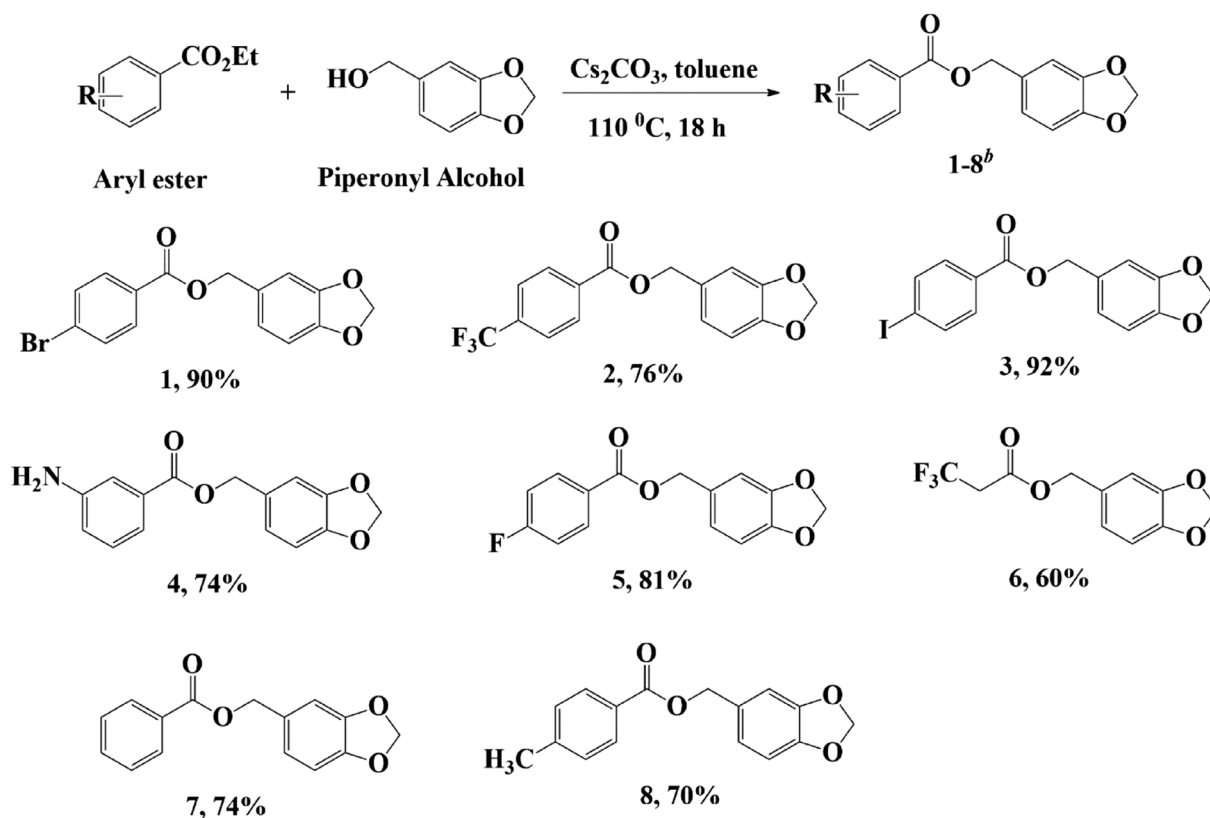


Figure 2. Substrate scope. Reaction conditions: Arylethyl ester (1 mmol), piperonyl alcohol (1.1 mmol), Cs_2CO_3 (1 mmol), and toluene (5 mL). ^bIsolated yields.

used as a solvent (Table 1, entry 7), whereas reaction using THF was less successful (Table 1, entry 8). We could conclude that toluene was the best solvent for this reaction. The solvent effect plays an important role in organic equilibrium reactions, such as tautomerization, electron transfer reaction, isomerization, and acid–base balance²².

Furthermore, the reaction was tested at different conduction temperatures. At room temperature, no product was observed (Table 1, entry 9). We could only detect a yield of 29% at 60 °C (Table 1, entry 10). We also aimed at optimizing the reaction time. When the reaction time was increased to 18 h, an excellent yield (91%) was obtained (Table 1, entry 11). We observed no significant difference when the reaction time was increased to 24 h (Table 1, entry 12). Based on the optimization results, we selected Cs_2CO_3 as a base, toluene as a solvent, 110 °C as reaction temperature, and 18 h as the reaction time for further experiments.

Under these optimized conditions, we synthesized other 1,3-benzodioxole derivatives by changing the R substituent. This method can tolerate some of the substituents in good to excellent yield (Fig. 2). Aryl ester as a substrate with halogen substituents (*p*-Br, *p*-I, and *p*-F) resulted in excellent yield, between approximately 81 and 92% (compound 1, 3, and 5). Furthermore, reactions with trifluoromethyl (*p*-CF₃) and amine (*m*-NH₂) as substituents also proceeded smoothly and resulted in the products 2 and 4, with a yield of 76% and 74%, respectively. The addition of electron-donating groups, such as in the case of compounds 7 and 8, also resulted in good yields of 74% and 70%, respectively. However, the use of –CF₃ as a substituent did not provide a favorable result when it was not attached to the aromatic ring (compound 5). This suggested that the aromatic ring played a role in the reaction efficiency as it could delocalize electrons that would result in more electrophilic carbonyl groups.

Selenide compounds reportedly exhibit various bioactivities, such as antioxidant, antibacterial, and anticancer effects^{23–25}. In our study, we also developed 1,3-benzodioxole-modified selenide compounds as we expected that such modifications would further increase the bioactivity of the compound. The modification of the selenide compound and 1,3-benzodioxole was achieved by reacting aryl iodide with diphenyl diselenide via the cleavage of the Se–Se bond (Fig. 3). Our results showed that compound 3, the product of the trans-esterification from the previous reaction, acted as an aryl iodide compound that reacted with diphenyl diselenide to form an asymmetrical diaryl chalcogenide compound. Using this method, we successfully synthesized target product 9 with a 48% yield.

Apart from the selenide compound modification, we also synthesized an aryl-heteroatom C–S bond, with a heterocyclic group in order to study its bioactivity as an LDHA inhibitor. This compound was used as a comparison for a 1,3-benzodioxole ring and *p*-CF₃ moiety in the structure of compound 2. N and S heterocyclic compounds are well-known for their diverse biological activities and are used in several diseases treatments^{26–28}. In our study, we synthesized such a compound using (trifluoromethyl)phenylboronic acid and 2,2'-dithiobis(benzothiazole) via S–S cleavage, resulting in a C–S bond with an N and S heterocyclic ring 10, with a 68% yield (Fig. 4).

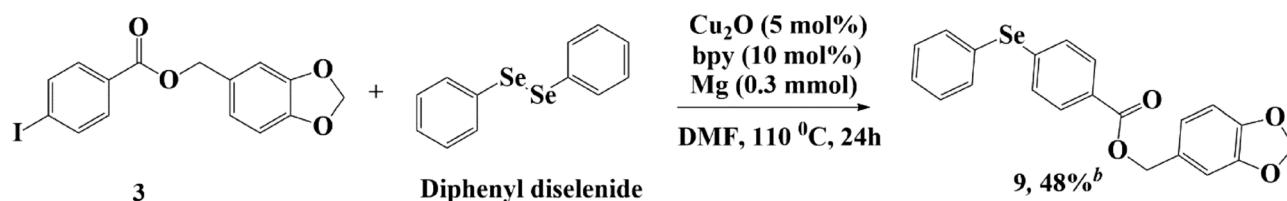


Figure 3. Synthesis of compound 9. Reaction conditions: Compound 3c (0.6 mmol), diphenyl diselenide (0.3 mmol), and DMF (1 mL). ^bIsolated yields.

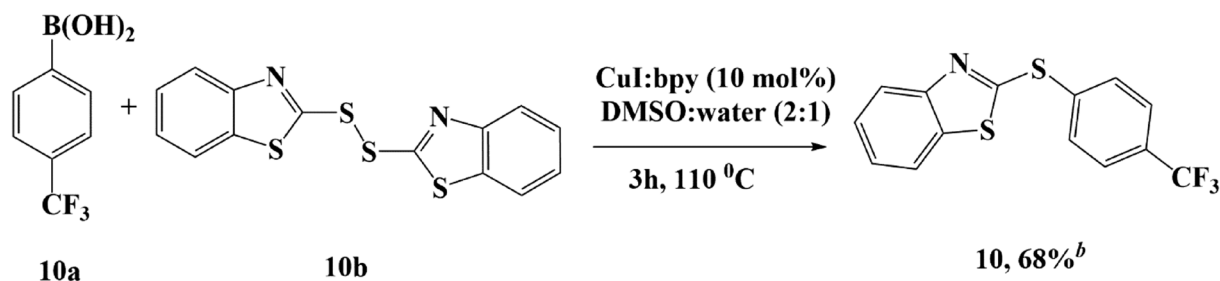


Figure 4. Synthesis of compound 10. Reaction conditions: phenyl boronic acid (1.3 mmol), 2,2'-dithiobis(benzothiazole) (0.6 mmol), and DMSO:water (2:1). ^bIsolated yields.

Entry	Compounds	LDHA IC ₅₀	LDHB IC ₅₀	LDHB/LDHA
1	1	> 1000 μM	79.05 μM	ND
2	2	13.63 μM	395.3 μM	29.00
3	3	> 1000 μM	150.8 μM	ND
4	4	182.5 μM	7.87 μM	0.043
5	5	477.5 μM	> 1000 μM	2.094
6	6	452.5 μM	129.8 μM	0.29
7	7	842.6 μM	> 1000 μM	> 1.18
8	8	> 1000 μM	248 μM	ND
9	9	> 1000 μM	151.1 μM	ND
10	10	47.20 μM	> 1000 μM	> 21.18
11	GSK2837808A	2.6 nM	130.3 nM	50.12
12	GNE140	59.9 nM	151.1 nM	2.52

Table 2. Bioactivity of synthesized compounds in inhibits LDHA and LDHB. ND not determined.

The *in vitro* evaluation of the LDHA inhibitor activities of the synthesized compounds was determined by the NADH intensity decrease through oxidation in a solution of HEPES-K⁺, NADH, and pyruvate at a pH = 7.2. The NADH oxidation fluorescence intensity was measured using a spectrofluorometer at 340 nm excitation and 460 nm emission wavelengths, representing the NADH-specific fluorescence spectrum. Furthermore, the *in vitro* evaluation of the LDHB inhibitor activity was determined as a reverse reaction, which converts lactate to pyruvate by determining the amount of NAD⁺ converted to NADH using the above-mentioned spectrophotometric experimental setting (Table 2). Based on this analysis, the half-maximal inhibitory concentration (IC₅₀) of the synthesized compounds was obtained.

We used GSK2837808A and GNE140 as standard compounds in this measurement that is a routinely used potential LDH inhibitor with an IC₅₀ of 2.6 nM and 59.9 nM, respectively. Since LDHA is often overexpressed in cancer, LDHB was used to evaluate the LDHA-selectivity of the synthesized compounds. We tested various aryl esters, presented in Fig. 2, of which compound 1 (*p*-Br), 3 (*p*-I), and 8 (*p*-CH₃) exhibited the highest LDHA IC₅₀ values of over 1000 μM. However, compound 1 exhibited the lowest LDHB IC₅₀ value of 79.05 μM, indicating that compound 1 is a selective LDHB inhibitor compare to the other 3 compounds. Moreover, compound 7 also showed high LDHA IC₅₀ value (842.6 μM) and LDHB IC₅₀ value (over 1000 μM) values. These values were too high to be compared with the standards; thus, we concluded that these 4 aryl ester compounds were inactive LDHA inhibitors.

Furthermore, compound 5, with 2 aromatic rings and a *p*-F moiety, exhibited a moderate LDHA IC₅₀ value of 477.5 μM and was a selective LDHA inhibitor due to its LDHB IC₅₀ value, which was greater than 1000 μM. Besides compound 5, compound 6, with only one aromatic ring and a -CF₃ moiety, also had a moderate LDHA IC₅₀ value of 452.5 μM. However, compound 6 was not selective for LDHA based on its LDHB IC₅₀ value, which

Entry	Compounds	Cell lines				
		PANC-1 (GI ₅₀) (μM)	A549 (GI ₅₀) (μM)	MCF-7 (GI ₅₀) (μM)	MiaPaCa-2 (GI ₅₀) (μM)	U87 (GI ₅₀) (μM)
1	1	> 1000	> 1000	> 1000	> 1000	> 1000
2	2	> 1000	> 1000	> 1000	> 1000	> 1000
3	3	269.3	> 1000	> 1000	> 1000	> 1000
4	4	203.4	> 1000	729	> 1000	379.5
5	5	643.9	> 1000	> 1000	> 1000	> 1000
6	6	> 1000	> 1000	> 1000	> 1000	> 1000
7	7	243.3	> 1000	> 1000	> 1000	> 1000
8	8	86.46	> 1000	> 1000	> 1000	> 1000
9	9	21.11	924.8	455.1	108	> 1000
10	10	12.19	56.46	61.38	88.88	343.4
	GSK2837808A	11.31	ND	ND	ND	ND
	GNE140	11.93	ND	ND	ND	ND

Table 3. The 50% growth inhibition concentration (GI₅₀) of compounds on various human cancer cell lines. ND not determined.

Compounds	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
CYP isozyme activity (% of control activity)					
Compound 10	36.7	97.4	12.1	95.1	> 100
Ketoconazole (reference)	99.1	97.5	> 100	99.0	25.0
Ketoconazole: CYP3A4 inhibitor (0.1 μM)					
Compounds	Human (%)		Rat (%)		
Human and rat liver microsomal stability (% remaining during 30 min)					
Compound 10	9.5		5.2		
Verapamil (reference)	15.3		–		
Compounds	Human		Rat		
	30 min	120 min	30 min	120 min	
Human and rat plasma stability (% remaining)					
Compound 10	98.1	91.6	93.8	87.5	
Procaine (reference)	1.5 (5 min)	0.4 (10 min)	89.8	52.6	
Enalapril (reference)	95.0	93.7	37.7	1.8	

Table 4. Summary of in vitro ADME for compound 10.

was lower than its LDHA IC₅₀ value. In addition, compound 4, containing an *m*-NH₂ moiety, was not selective for LDHA either, as we measured the lowest LDHB IC₅₀ value (7.87 μM) in the case of this compound. The lowest and selective LDHA inhibitor was compound 2 (13.63 μM) with 2 aromatic rings and *p*-CF₃ moiety. This compound was selective as LDHA inhibitor compares to its LDHB inhibitor activity (LDHB IC₅₀ value of 395.3 μM). These values were still higher compared to the IC₅₀ values of the GSK2837808A and GNE140 standards but lower compared to other established LDHA inhibitors, such as galloflavin, which was also used as a reference compound since it is routinely used in enzymatic LDH assays, with an IC₅₀ value of approximately 110 μM²⁹. This result suggested that it could potentially become LDHA inhibitor drug candidate. Moreover, we also synthesized selenide compounds, containing 1,3-benzodioxole (9) and benzothiazole (10) groups. Compound 9 was not efficient with LDHA and LDHB IC₅₀ values of 1000 μM, while compound 10 exhibited a favorable selectivity as an LDHA inhibitor with an LDHA IC₅₀ value of 47.20 μM and an LDHB IC₅₀ value of over 1000 μM.

To assess the anticancer potency of these compounds in several cancer cells, we determined the cell toxicities of these compounds using an MTT assay. As shown in Table 3, the compound 2 showed was too weak. It was over 1000 μM of the concentration of half-maximal growth inhibition (GI₅₀) value. On the other hand, compound 10 has higher cytotoxicity compared to other compounds in various cancer cell lines. Among these cell lines, compound 10 is most effective in reducing the cell viability in human pancreatic cancer PANC-1 cells (GI₅₀ value of 12.19 μM). In addition, we analyzed the cytotoxicity of two standard compounds, GSK2837808A and GNE140. The calculated GI₅₀ values of GSK2837808A and GNE140 were 11.31 μM and 11.59 μM, respectively. There is a small difference between standard compounds and compound 10 in growth inhibition of PANC-1 cells.

In addition to intracellular anticancer activity, to provide the basic in vitro drug-like data of compound 10, we performed basically in vitro assays, such as human liver microsomal stability, plasma stability, and CYP inhibition (Table 4). The compound 10 was rapidly metabolized by human and rat microsomes; only 9.5% and 5.2% of

compound **10** remained after 30 min of incubation. However, a plasma stability test showed compound **10** was stable in both human and rat plasma. The compound **10** is a moderate perpetrator of drug–drug interactions based on their inhibition of the most abundant CYP450 enzymes, such as 1A2 and 2C19.

The compound **2** exhibits a simple structure with one 1,3-benzodioxole group compared to the previously reported Machilin A¹⁶, containing two 1,3-benzodioxole groups. Furthermore, it has a lower LDHA IC₅₀ value compared to Machilin A (84 μM). Sada et al., also presented the LDHA inhibitory activity of stiripentol analogs at a higher dose (500 μM)³⁰. However, piperonyl alcohol and 1,3-benzodioxole did not exhibit an LDHA inhibitory activity even at concentrations up to 1 mM¹⁶. However, unlike the previously reported compounds harboring 1,3-benzodioxole group, compound **2** did have an anti-cancer effect in several lines of cancer cells. It could be a result of its chemical properties including low stability or poor cellular uptake. Compound **10**, a benzothiazole compound harboring a –CF₃ moiety, could also be considered as a potential LDHA inhibitor. Although higher IC₅₀ in vitro LDHA assay, the GI₅₀ value of compound **10** is lower than that of previously reported selenobenzene compound, 1-(phenylseleno)-4-(trifluoromethyl) benzene¹⁸. The GI₅₀ value of compound **10** in PANC-1 cells is comparable to that of standard compounds, GSK2837808A and GNE140.

From these results, compound **2** and **10** among the synthesized compounds, with the simple structure and comparable activity, could be potentially used as an LDHA inhibitor and should be further investigated. These synthesized compounds possess 2 aromatic rings and –CF₃ moiety, which is expected to contribute to LDHA inhibition. The compounds have the potential to become a promising LDHA inhibitor for the anticancer drug candidate. To improve the in vitro LDHA inhibition and intracellular activity of these compounds, it is needed to conduct an extensive structure–activity relationship study, including substitutions in a different position, bioisosteres replacement, and scaffold hopping.

Conclusions

Here we described 1,3-benzodioxole derivatives, synthesized through trans-esterification between aryl ester and piperonyl alcohol groups. Through this approach, the substrate scope of this reaction was also investigated and could tolerate many substituents, with good to excellent yields. In addition, we also synthesized benzothiazole derivatives and 1,3-benzodioxole-modified selenide compounds. Compound **2**, containing a benzodioxole ring and a –CF₃ moiety, showed a potent inhibitory action in vitro assay, however, failed to show anticancer effect in human cancer cells. Compound **10**, a benzothiazole harboring a –CF₃ group, showed both activities of in vitro LDHA inhibition and intracellular cytotoxicity. These compounds could potentially be used as an LDHA inhibitor due to its optimal activity and selectivity based on the decrease in the NADH intensity and as it has the smallest IC₅₀ among all the compounds. Thus, compound **10** could be considered a potent LDHA inhibitor for further in vivo evaluations.

Methods

General methods. All chemicals and reagents were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO) and used without further purification. Fourier-transform infrared (FT-IR) spectra were recorded on NICOLET 380. ¹H (400 MHz) and ¹³C (100 MHz) NMR of all synthesized compounds were recorded on Bruker Magnet System 400/54 Ascend. Gas Chromatography–Mass Spectrometry spectra were performed on Shimadzu GC-1010 Plus GCMS-QP2010 SE. High-resolution Mass Spectrometry (HR-MS) data were performed on 6530Accurate-Mass Q-TOF LC/MS. Spectrofluorometer spectra were performed on Spectramax M2; Molecular Devices, Sunnyvale, CA, USA.

Synthesis of compound 1–8. Arylethyl ester (1 mmol) was reacted with piperonyl alcohol (1.1 mmol) in 5 mL toluene. Cesium carbonate (1 mmol) was added to the mixture as a base. The reaction mixture was stirred at reflux under air condition for 18 h. The reaction was monitored by TLC and GC–MS for completion reaction. After the completion reaction, the mixture was cooled to room temperature. The mixture was diluted by dichloromethane and evaporate the solvent. The crude product was stored in a refrigerator for 24 h to conduct white solid. The crude product was purified by column chromatography over silica gel.

Synthesis of compound 9. The reactions referred to³¹ with modification. Compound **3** (0.6 mmol) was reacted with diphenyl diselenide (0.3 mmol) in 1 mL DMF. Cu₂O (5 mol%), bpy (10 mol%), and Mg (0.6 mmol) were added to the reaction mixture. The mixture was stirred at 110 °C for 24 h. The reaction was monitored by TLC and GC–MS. After the completion reaction, the mixture was cooled to room temperature. Then, the crude product was separated by an extraction process using dichloromethane and brine solution. The organic layer was evaporated and the crude product was purified by column chromatography over silica gel.

Synthesis of compound 10. The reactions referred to³² with modification. Trifluoromethyl phenyl boronic acid **10a** (1.3 mmol) was reacted with 2,2-dithiobis(benzothiazole) **10b** (0.6 mmol) in DMSO:water (2:1). CuI;bpy (10 mol%) was added to the reaction mixture. The mixture was stirred at 110 °C for 3 h. The reaction was monitored by TLC and GC–MS. After the completion reaction, the mixture was cooled to room temperature. Then, the crude product was separated by an extraction process using dichloromethane and brine solution. The organic layer was evaporated and the crude product was purified by column chromatography over silica gel.

In vitro evaluation on LDHA inhibitor activity. LDHA activity assay was performed in accordance with previous studies^{16,18}. Briefly, the various concentrations of compounds were incubated with reaction buffer containing 20 mM HEPES-K⁺, 20 μM NADH, 2 mM pyruvate, and 100 ng of purified recombinant human LDHA protein (Abcam, Cambridge, UK). The fluorescence of NADH, which has an excitation wavelength of 340 nm and an

emission wavelength of 460 nm, was detected using a microplate spectrofluorometer (Spectramax M2; Molecular Devices, Sunnyvale, CA).

In vitro LDHB Activity Assay. In vitro analysis was determined the amount of NAD⁺ converted to NADH³³. The assay mixture composed of 100 mM Tris–HCl buffer (pH 8.0), 200 mM sodium L-lactate, 2.5 mM NAD⁺, and 100 ng of purified recombinant human LDHB protein (Abcam). The fluorescence intensity of NADH was measured using a spectrofluorometer (Spectramax M2) at 340 nm as excitation wavelength and 460 nm as emission wavelength which is the specific fluorescence of NADH.

Cell culture. The human pancreatic cancer cell lines, PANC-1 and MiaPaCa-2, human lung cancer A549 cells, human breast cancer MCF-7, and human glioma U87 cells were obtained from the Korean cell line bank (Seoul, Korea). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin/streptomycin (ThermoFisher Scientific). The cells were cultured at 37 °C in an atmosphere containing 5% CO₂.

Intracellular LDHA activity assay. To observe the LDH activities from the lysates of cells, we performed in accordance with previous studies^{16,18}. Briefly, Total protein from lysate (1 µg) was mixed with containing 20 mM HEPES-K⁺, 20 µM NADH, 2 mM pyruvate. The fluorescence of NADH, which has an excitation wavelength of 340 nm and an emission wavelength of 460 nm, was detected using a microplate spectrofluorometer (Spectramax M2).

Cell viability. The potential cytotoxicity of compounds at different concentrations was evaluated using the MTT assay. Briefly, cancer cell lines were pre-incubated in 96-well plates with compounds for 48 h. Subsequently, MTT working solution (2 mg/mL in phosphate buffer solution) was added to each well and the plate was incubated for 4 h at 37 °C in an atmosphere containing 5% CO₂. Then, the conditioned media were aspirated, and the formed formazan crystals in living cells were quantified using the microplate reader (Spectramax M2) at 540 nm. The concentrations that produce 50% cell growth inhibition (GI₅₀) were calculated by curves constructed by the plot of cell survival by an assistant of PRISM software (GraphPad, San Diego, CA).

LC–MS/MS analysis. Chromatographic separation was performed on the Shimadzu Nexera XR system (Kyoto, Japan). Detection was performed on a Thermo TSQ Vantage triple quadrupole LC–MS/MS (MA, USA). The analytes were separated on a Phenomenex Kinetex C18 (2.1 × 100 mm, 2.6 µm particle size) column (Torrance, USA). The mobile phase system consisted of water containing 0.1% formic acid (mobile phase A, MPA), and acetonitrile containing 0.1% formic acid (mobile phase B, MPB). Xcalibur 1.6.1 software (Thermo Scientific) was used for data acquisition and processing.

Plasma stability. Human and rat plasma (Sigma-Aldrich) are incubated at 37 °C with test compounds. During the incubation, aliquots are withdrawn at 0, 30, 120 min time points and acetonitrile solution (containing chlorpropamide) is added. After vortexing, the aliquots are centrifuged and the supernatant is withdrawn for analysis by LC–MS/MS.

CYP isozymes activity assay. Pooled Human liver microsomes (Sigma-Aldrich; 0.25 mg/mL), 0.1 M phosphate buffer solution (pH 7.4), the five most commonly used substrate cocktails, such as 50 µM phenacetin (CYP1A2), 10 µM diclofenac (CYP), 100 µM S-mephenytoin (CYP2C19) 5 µM dextromethorphan (CYP2D6), and 2.5 µM midazolam (CYP3A4), and compound 10 are pre-incubated at 37 °C for 5 min, then incubated with NADPH generation system solution for 15 min. To finish the enzymatic reaction, an acetonitrile solution (containing terfenadine) is added. The reaction tubes are centrifuged and the supernatant is withdrawn for analysis by LC–MS/MS.

Microsomal stability. The assay use liver microsomes from two species (human, rat, 0.5 mg/mL), liver microsomes preincubated with 0.1 M PBS (pH 7.4) and 1 µM compound 10 at 37 °C for 5 min, then incubated with NADPH regeneration system solution for 30 min. To finish the reaction, an acetonitrile solution (involved in chlorpropamide) is added. The reaction tubes are centrifuged and the supernatant is withdrawn for analysis by LC–MS/MS.

Statistical analysis. Results were presented as mean ± standard deviation (SD) of triplicate experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test, and *p* values of lesser than 0.05 were considered statistically significant.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information Files).

Received: 9 June 2020; Accepted: 5 November 2020

Published online: 16 November 2020

References

- Hassanpour, S. H. & Dehghani, M. Review of cancer from perspective of molecular. *J. Cancer Res. Pract.* **4**, 127–129 (2017).
- Fisher, R., Puzstai, L. & Swanton, C. Cancer heterogeneity: Implications for targeted therapeutics. *Br. J. Cancer* **108**, 479–485 (2013).
- Meacham, C. E. & Morrison, S. J. Tumour heterogeneity and cancer cell plasticity. *Nature* **501**, 328–337 (2013).
- Doherty, J. R., Cleveland, J. L., Doherty, J. R. & Cleveland, J. L. Targeting lactate metabolism for cancer therapeutics find the latest version: Review series targeting lactate metabolism for cancer therapeutics. *J. Clin. Invest.* **123**, 3685–3692 (2013).
- Goldman, R. D., Kaplan, N. O. & Hall, T. C. Lactic dehydrogenase in human neoplastic tissues. *Cancer Res.* **24**, 389–399 (1964).
- Le, A. *et al.* Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc. Natl. Acad. Sci. USA* **107**, 2037–2042 (2010).
- Xie, H. *et al.* LDH-A inhibition, a therapeutic strategy for treatment of hereditary leiomyomatosis and renal cell cancer. *Mol. Cancer Ther.* **8**, 626–635 (2009).
- Ugolini, L., Della Noce, I., Trincia, P., Borzatta, V. & Palmieri, S. Benzodioxole derivatives as negative effectors of plant proteases. *J. Agric. Food Chem.* **53**, 7494–7501 (2005).
- Wei, P. L. *et al.* The in vivo antitumor effects on human COLO 205 cancer cells of the 4,7-dimethoxy-5-(2-propen-1-yl)-1,3-benzodioxole (apiole) derivative of 5-substituted 4,7-dimethoxy-5-methyl-1,3-benzodioxole (SY-1) isolated from the fruiting body of *Antrodia camphorata*. *J. Cancer Res. Ther.* **8**, 532–536 (2012).
- Leite, A. C. L., Peixoto Da Silva, K., De Souza, I. A., Magali De Araújo, J. & Brondani, D. J. Synthesis, antitumor and antimicrobial activities of new peptidyl derivatives containing the 1,3-benzodioxole system. *Eur. J. Med. Chem.* **39**, 1059–1065 (2004).
- Kamau, E. *et al.* A novel benzodioxole-containing inhibitor of *Toxoplasma gondii* growth alters the parasite cell cycle. *Antimicrob. Agents Chemother.* **55**, 5438–5451 (2011).
- Nelson, F. R. & Hoosseinthrani, B. Effects of benzylphenol and benzyl-1,3-benzodioxole derivatives on fertility and longevity of the yellow fever mosquito (Diptera: Culicidae). *J. Econ. Entomol.* **75**, 877–878 (1982).
- Bakhite, E. A. G. & Radwan, S. M. Synthesis, reactions and biological activity of some new thieno[2,3-f]-1,3-benzodioxoles. *Pharmazie* **54**, 491–498 (1999).
- Shenoy, R. R. *et al.* Normal and delayed wound healing is improved by sesamol, an active constituent of *Sesamum indicum* (L.) in albino rats. *J. Ethnopharmacol.* **133**, 608–612 (2011).
- Kumar, N. *et al.* Preclinical evaluation and molecular docking of 1,3-benzodioxole propargyl ether derivatives as novel inhibitor for combating the histone deacetylase enzyme in cancer. *Artif. Cells Nanomed. Biotechnol.* **46**, 1288–1299 (2018).
- Chung, T. W. *et al.* Machilin inhibits tumor growth and macrophage m2 polarization through the reduction of lactic acid. *Cancers (Basel)* **11**, 1–21 (2019).
- Wang, H. H. *et al.* Synthesis, molecular docking and evaluation of thiazolyl-pyrazoline derivatives containing benzodioxole as potential anticancer agents. *Bioorg. Med. Chem.* **21**, 448–455 (2013).
- Kim, E. Y. *et al.* A novel lactate dehydrogenase inhibitor, 1-(phenylseleno)-4-(trifluoromethyl) benzene, suppresses tumor growth through apoptotic cell death. *Sci. Rep.* **9**, 1–12 (2019).
- Karmee, S. K., Mahesh, P., Ravi, R. & Chadha, A. Kinetic study of the base-catalyzed transesterification of monoglycerides from Pongamia oil. *J. Am. Oil Chem. Soc.* **81**, 425–430 (2004).
- Yusup, S. & Khan, M. A. Base catalyzed transesterification of acid treated vegetable oil blend for biodiesel production. *Biomass Bioenergy* **34**, 1500–1504 (2010).
- Xiong, Y. & Zhang, X. Significant heterogeneous carbonate salt catalyzed acetylation of alcohols via a transesterification process with carbonate salt-activated alcohol 1H NMR evidence. *Chin. J. Chem.* **29**, 1143–1148 (2011).
- Reichardt, C. Solvents and solvent effects: An introduction. *Org. Process Res. Dev.* **11**, 105–113 (2007).
- Sentkowska, A. & Pyrzyńska, K. Investigation of antioxidant activity of selenium compounds and their mixtures with tea polyphenols. *Mol. Biol. Rep.* **46**, 3019–3024 (2019).
- Álvarez-Pérez, M., Ali, W., Marć, M. A., Handzlik, J. & Domínguez-Álvarez, E. Selenides and diselenides: A review of their anti-cancer and chemopreventive activity. *Molecules* **23**, 628–646 (2018).
- Zhao, L. *et al.* Selenium-containing naphthalimides as anticancer agents: Design, synthesis and bioactivity. *Bioorg. Med. Chem.* **20**, 2558–2563 (2012).
- Ardiansah, B. Chalcones bearing N, O, and S-heterocycles: Recent notes on their biological significances. *J. Appl. Pharm. Sci.* **9**, 117–129 (2019).
- Chauvière, G. *et al.* Synthesis and biological activity of nitro heterocycles analogous to megazol, a trypanocidal lead. *J. Med. Chem.* **46**, 427–440 (2003).
- Saleh, S. S., Al-Salihi, S. S. & Mohammed, I. A. Biological activity study for some heterocyclic compounds and their impact on the gram positive and negative bacteria. *Energy Procedia* **157**, 296–306 (2019).
- D'Andrea, F. *et al.* Synthesis and biological evaluation of new glycoconjugated LDH inhibitors as anticancer agents. *Molecules* **24**, 3520 (2019).
- Sada, N., Lee, S., Katsu, T., Otsuki, T. & Inoue, T. Targeting LDH enzymes with a stiripentol analog to treat epilepsy. *Science* **347**, 1362–1368 (2015).
- Taniguchi, N. & Onami, T. Magnesium-induced copper-catalyzed synthesis of unsymmetrical diaryl chalcogenide compounds from aryl iodide via cleavage of the Se–Se or S–S bond. *J. Org. Chem.* **69**, 915–920 (2004).
- Taniguchi, N. Convenient synthesis of unsymmetrical organochalcogenides using organoboron acids with dichalcogenides via cleavage of the S–S, Se–Se, or Te–Te bond by a copper catalyst. *J. Org. Chem.* **72**, 1241–1245 (2007).
- Dave, K. K. & Punekar, N. S. Expression of lactate dehydrogenase in aspergillus Niger for L-lactic acid production. *PLoS ONE* **10**, 1–16 (2015).

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2020R111A3067208 to K.H.P and 2014R1A5A20009936 to K.-T.H).

Author contributions

D.A. performed synthesized, analyze the organic compounds and manuscript writing; M.Y. performed analyze the synthesized compounds; S.Y.C. and S.J.B. performed most of the in vitro experiments and manuscript writing; K.-T.H. and K.H.P. designed the study and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41598-020-77056-4>.

Correspondence and requests for materials should be addressed to K.-T.H. or K.H.P.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2020