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A novel metformin derivative, HL010183, inhibits proliferation and invasion of triple-negative breast cancer cells

Minsoo Koh [a], Jong-Cheol Lee [b], Changhee Min [c], Aree Moon [a,*]

[a] College of Pharmacy, Duksung Women's University, Seoul 132-714, Republic of Korea
[b] Energy Materials Research Center, Korea Research Institute of Chemical Technology, Daejeon, Republic of Korea
[c] HanAll Biopharma Co., Ltd, Central Research Institute, Daejeon, Republic of Korea

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ABSTRACT

Mounting evidence suggests that metformin (*N*,*N*-dimethylbiguanide), a widely prescribed drug for the treatment of type II diabetes, exerts an anti-tumor effect on several cancers including breast cancer. Breast cancer has been estimated as one of the most commonly diagnosed types of cancer among women. In particular, triple-negative breast cancers are associated with poor prognosis and metastatic growth. In the present study, we synthesized a novel metformin derivative **5** (HL010183) and metformin salts, **9a**, **9b**, and **9c** (metformin gamma-aminobutyric acid (GABA) salt, metformin pregabalin salt and metformin gabapentin salt), which exerted more potent inhibitory effects on the proliferation and invasiveness of Hs578T triple-negative breast carcinoma cells than metformin. Importantly, **5** showed approximately 100-fold more potent effects compared to metformin. In a triple-negative breast cancer xenograft model, **5** showed a comparable degree of inhibitory effect on in vivo tumor growth at the 100 mg/kg dose to that of metformin at 500 mg/kg. Our results clearly demonstrate that **5** exerts a potent anti-tumor effect both in vitro and in vivo, paving the way for a strategy for treatment of triple-negative breast cancer.

1. Introduction

The metabolic abnormalities associated with diabetes mellitus type II have been linked to an increased cancer risk. A central energy sensor, AMP-activated protein kinase (AMPK) which inhibits the mammalian target of rapamycin (mTOR), is known as a crucial factor in the interaction between metabolism and cancer.[1] An AMPK activator metformin (*N*,*N*-dimethylbiguanide) is a widely prescribed oral medication used for the treatment of type II diabetes which causes reversal of metabolic disturbances such as hyperglycemia and insulin resistance.[2] Several concordant series of epidemiological studies indicate that metformin has a high potential efficacy as an anti-cancer drug.[3–6] Animal and cellular studies support that metformin has a strong anti-proliferative effect on various cancers.[7–11] The anti-tumor effect of metformin shown in vitro and in vivo, and its low toxicity favor the use of this compound for cancer treatment.

Breast cancer has been estimated as the second most common cause of cancer death among women.[12] Especially, triple-negative breast cancers, which do not express the genes for estrogen and progesterone receptors (ER/PR) and HER2, are associated with poor prognosis and metastatic growth.[13] Major cause of death from breast cancer is the metastatic spread of the cancer.[14] Invasion of breast cells requires a matrix-degrading activity which is exerted by matrix metalloproteinases (MMPs), especially MMP-2 which is shown to be associated with breast cell invasion.[15,16] Metformin suppressed cancer metastasis in vivo as well as the invasion and migration of cancer cells in vitro.[11,17,18] In addition, metformin induced apoptosis of breast cancer cells in vitro and reduced the tumor growth in a breast cancer xenograft mouse model.[8] The anti-tumor effect of metformin on breast cancer has been demonstrated in clinical studies.[1,4,19]

A biguanide analogue, phenformin (phenethyl biguanide) was used as an anti-diabetic drug, but was withdrawn from clinical use due to the association with lactic acidosis.[2] Interestingly, recent reports demonstrated that phenformin exerted more potent anti-tumoral activity than metformin in colon cancer cells and in a triple-negative breast cancer xenografts model,[20,21] suggesting a promising application of this agent as an anti-cancer drug. Gamma-aminobutyric acid (GABA) and its structural analogues, gabapentin and pregabalin, are FDA-approved drugs for the treatment of anxiety and convulsions.[22] GABA has emerged as a regulator of cancer cell proliferation including colon, gastric, ovarian and breast cancers.[23]

Our current study aimed to synthesize a metformin derivative and metformin salts that may have more potent inhibitory effects on the proliferation and invasive phenotype of triple-negative breast cancer cells than metformin. Here, we present a novel triazine compound **5** which exerted a stronger potency to inhibit pro-

* Corresponding author. Tel.: +82 2 901 8394; fax: +82 2 901 8386.
E-mail address: armoon@duksung.ac.kr (A. Moon).

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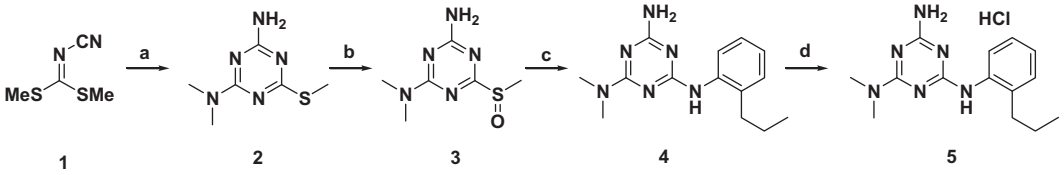
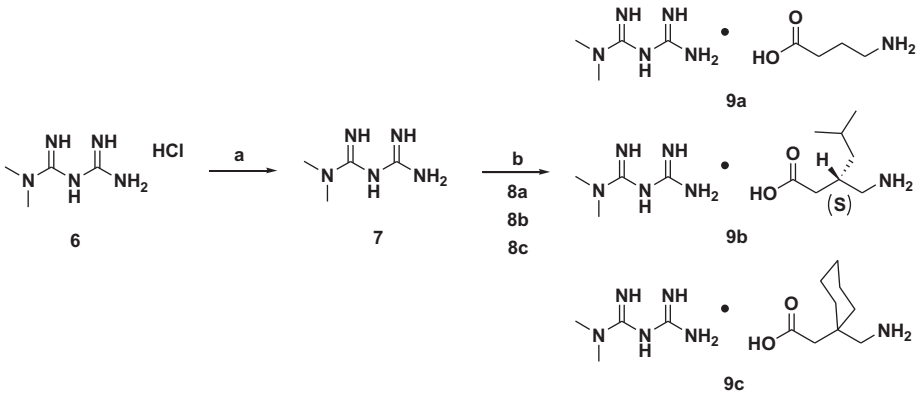
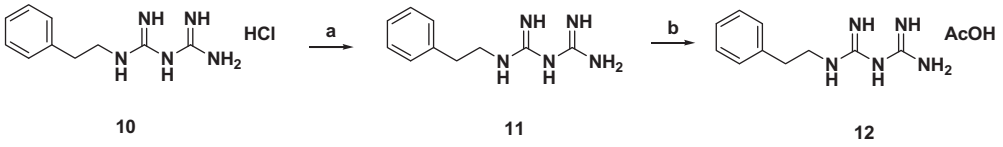


Scheme 1. Synthesis of a metformin derivative, **5**. Reagents and conditions: (a) *N*,*N*-dimethylguanidine sulfate, 40% K_2CO_3 solution, DMSO, 120 °C, 2 h; (b) *m*-CPBA, CH_2Cl_2, 0 °C to room temp, 12 h; (c) 2-propylaniline, 1,4-dioxane, reflux, 15 h; (d) 4 M HCl in dioxane, room temp, 2 h.



Scheme 2. Synthesis of metformin salts, **9a**, **9b**, and **9c**. Reagents and conditions: (a) KOH, *i*-PrOH, 50 °C, 2 h; (b) acid (**8a**: GABA, **8b**: pregabalin, **8c**: gabapentin), *i*-PrOH, 50 °C, 2 h.



Scheme 3. Synthesis of a phenformin acetic acid salt, **12**. Reagents and conditions: (a) KOH, *i*-PrOH, 50 °C, 2 h; (b) acetic acid, *i*-PrOH, 50 °C, 2 h.

liferation and invasion of a triple-negative breast cancer cells than metformin. Metformin salts, **9a**, **9b**, and **9c**, also showed higher anti-proliferative and anti-invasive activities than metformin.

2. Chemistry

2.1. Chemical synthesis of a metformin derivative, 5 (HL010183)

Based on the structures of metformin and phenformin, we synthesized a novel metformin derivative **5** which contains a rigid and symmetrical scaffold as shown in Scheme 1. The triazine formation reaction by treating dimethyl-*N*-cyanodithioiminocarbonate (compound **1**) with *N*,*N*-dimethylguanidine sulfate in the presence of potassium carbonate (K_2CO_3) afforded the triazine compound **2**. Next, the subsequent oxidation of the triazine compound **2** was carried out with *m*-chloroperbenzoic acid (*m*-CPBA) at room temperature to give the sulfinyl compound **3**. Then, treatment of compound **3** with 2-propylaniline in 1,4-dioxane under reflux condition, provided compound **4**. Finally, compound **4** was treated with hydrochloric acid (HCl) to give compound **5**, denominated as HL010183, in a quantitative yield.

2.2. Chemical synthesis of metformin salts, 9a, 9b, and 9c

Because GABA was shown to be involved in the regulation of several cancer cells including breast cancer,[23] we synthesized metformin salts, **9a**, **9b**, and **9c**, as shown in Scheme 2. The transformation of compound **6** to free base (compound **7**) was achieved using potassium hydroxide in isopropyl alcohol. Then, treatment of the free base with organic acids resulted in the production of **9a**, **9b** and **9c**.

2.3. Chemical synthesis of phenformin acetic acid salt, 12

Compound **12** was synthesized as shown in Scheme 3. To a stirred suspension of phenformin hydrochloride (compound **10**) in *i*-PrOH was added potassium hydroxide. The filtrate was concentrated under reduced pressure and dried in vacuo to provide a phenformin free base (compound **11**). Acetic acid was added to the compound **11** in *i*-PrOH to give compound **12**. Structures of metformin, **5** and **12** were depicted in Figure 1.

3. Results and discussion

3.1. Compound 5, 9a, 9b, and 9c showed more potent anti-proliferative effects than metformin on triple-negative breast cancer cells

To examine the effects of **5**, **9a**, **9b**, and **9c** on the growth of breast cancer cells, we performed the MTT assay on Hs578T and MDA-MB-231 human breast carcinoma cells which were derived from triple-negative breast cancer.[24] As shown in Figure 2A, **5**

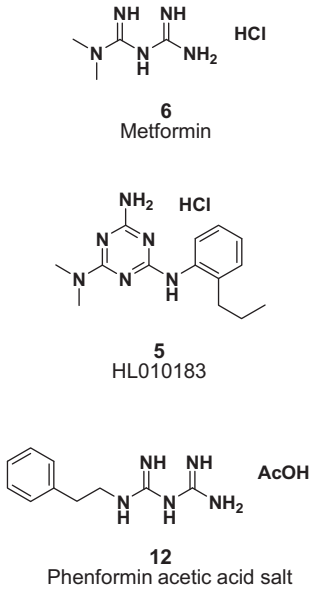


Figure 1. Chemical structures of metformin, **12** and **5**.

and **12** showed stronger cytotoxic activities than metformin in both cell lines. A novel compound **5** exerted approximately 100-fold more potent cytotoxic effect on the growth of Hs578T cells (IC_{50} value of 0.28 mM) compared to metformin (IC_{50} value of 26.8 mM). Compound **5** inhibited the growth of MDA-MB-231 cells with an IC_{50} value of 0.28 mM which was much lower than that by metformin (IC_{50} value of 58.4 mM) (Table 1). Compounds **9a**, **9b**, and **9c** exerted more potent cytotoxic effects against Hs578T with IC_{50} values of 9.1, 7.2 and 4 mM, respectively, than metformin (IC_{50} value of 26.8 mM). Compound **9a**, **9b** and **9c** also inhibited the proliferation of MDA-MB-231 cells with IC_{50} values of 41.5, 17.7 and 16.1 mM, respectively (Fig. 2B and Table 1). Treatment with GABA alone did not markedly affect the growth of these cells. Compound **5** showed stronger cytotoxic activities than **9a**, **9b**, and **9c**, showing the most potent cytotoxic effect on the growth of Hs578T cells.

Next, we examined whether the most potent **5** can induce apoptosis stronger than metformin by flow cytometric analysis. As shown in Figure 3, treatment with 0.1 mM of **5** and 10 mM of metformin showed a similar effect on the induction of apoptosis in MDA-MB-231 cells.

3.2. Compound 5 inhibited invasive phenotype of Hs578T cells

We investigated the effects of **5**, **9a**, **9b**, and **9c** on invasive phenotype of Hs578T cells which are highly invasive.[13,24] As shown in Figure 4A, treatment with 0.2 mM of **5** inhibited the invasiveness of Hs578T cells by 36.6%, which was comparable to the inhibiting effect exerted by 20 mM of Metformin. These data implicate that about 100-fold more potent activity was exhibited by **5** compared to metformin.

Neither metformin nor GABA affected the invasive phenotype at 10 mM concentration (Fig. 4A). Treatment with metformin GABA salts, however, significantly inhibited the invasiveness in a dose-dependent manner. Compounds **9a**, **9b**, and **9c** at 5 mM concentration reduced the invasiveness of Hs578T cells by 43.6%, 64.6%, and 73.6%, respectively. GABA has been shown to reduce norepinephrine-induced migration of colon carcinoma cells and inhibit isopro-

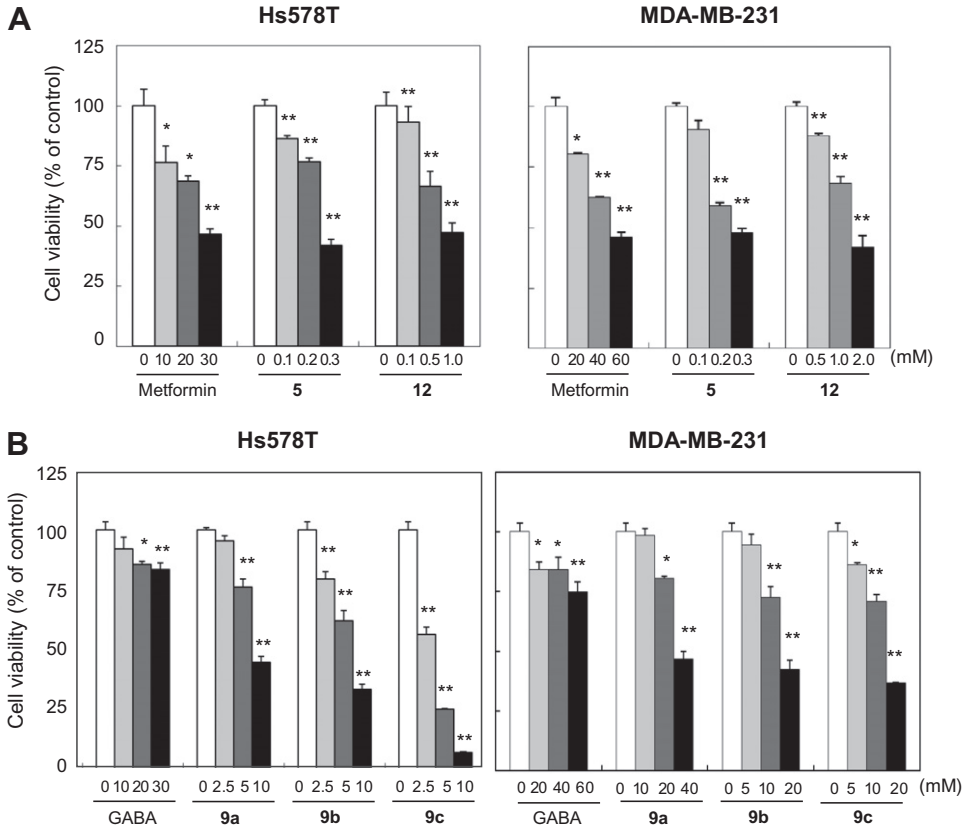


Figure 2. Compound **5** shows a potent anti-proliferative effect on Hs578T and MDA-MB-231 triple-negative breast cancer cells. Cells were treated with various concentrations of compounds for 24 h and subjected to MTT assay. The results represent means ± SE of triplicates. *, ** Statistically different from the control at p <0.05 and p <0.01, respectively.

Table 1
IC_{50} values for metformin, **5**, **9a**, **9b**, **9c** and **12** in Hs578T and MDA-MB-231 cells

Compound	IC_{50} (mM)	
	Hs578T	MDA-MB-231
Metformin	26.8	58.4
5	0.28	0.28
9a	9.1	41.5
9b	7.2	17.7
9c	4.0	16.1
12	0.9	1.68

terenol-induced migration of pancreatic cancer cells.[25,26] Our data showed that GABA exerted neither anti-proliferative nor anti-invasive activities in Hs578T cells. Interestingly, the GABA salts of metformin, **9a**, **9b**, and **9c**, showed strong inhibitory effects on proliferation and invasion, implying that the combinatorial synthesis of metformin with GABA or its analogues exerted synergistic anti-tumoral and anti-invasive activities than the use of metformin or GABA alone.

Among the compounds tested, we selected **5** for subsequent studies since it showed the most potent inhibitory effect on proliferation and invasive phenotype of Hs578T human breast carcinoma cells. We examined whether **5** affects the expression of MMP-2 or MMP-9 in Hs578T cells. The expression level of MMP-2, but not that of MMP-9, was dose-dependently reduced in conditioned media of Hs578T cells treated with metformin and **5** as evidenced by gelatin zymogram assay (Fig. 4B, top) and immunoblot analysis (Fig. 4B, bottom). A comparable degree of inhibition was observed by treatment with 10 mM of metformin and 0.1 mM of **5**. These results demonstrated that a novel metformin derivative **5** exerted approximately 100-fold higher anti-invasive activity compared to metformin, suggesting anti-invasive activity of **5** is associated with down-regulation of MMP-2 in Hs578T triple-negative human breast cancer cells.

Metformin reduced phorbol-12-myristate-13-acetate-induced migration and invasion as well as the expression of MMP-2 and MMP-9 in HT-1080 fibrosarcoma cells at 5 mM concentration.[18] In this study, we showed that 5 mM of metformin did not inhibit the invasion of Hs578T cells, suggesting that the in vitro potency of metformin on cell invasion may depend on cell type. Recent studies showed that phenformin showed a more potent anti-tumoral activity than metformin in vitro and in vivo.[20,21] Consistent with these findings, our data showed that the anti-proliferative and anti-invasive effects of phenformin salts, **12**, on breast cancer cells were higher than those of metformin. Because phenformin has been known to be less suitable for clinical use due to the higher rates of lactic acidosis,[2] further investigations would be needed to evaluate the efficacy and/or safety of **12** in breast cancer therapy.

3.3. Compound 5 showed about 100-fold more potent effect on AMPK activation and mTOR inhibition compared to metformin

Metformin is a known activator of AMPK which inhibits phosphorylation of mTOR.[1] To compare the effects of metformin and **5** on the activation of AMPK, we performed immunoblot analysis. As shown in Figure 5A, 20 mM of metformin and 0.2 mM of **5** exerted a comparable degree of activation of AMPK in Hs578T cells. A similar level of inhibitory effect on mTOR phosphorylation was observed by 20 mM of metformin and 0.2 mM of **5** (Fig. 5B). These data indicate that **5** exerts about 100-fold more potent effect on the activation of AMPK and inhibition of mTOR phosphorylation

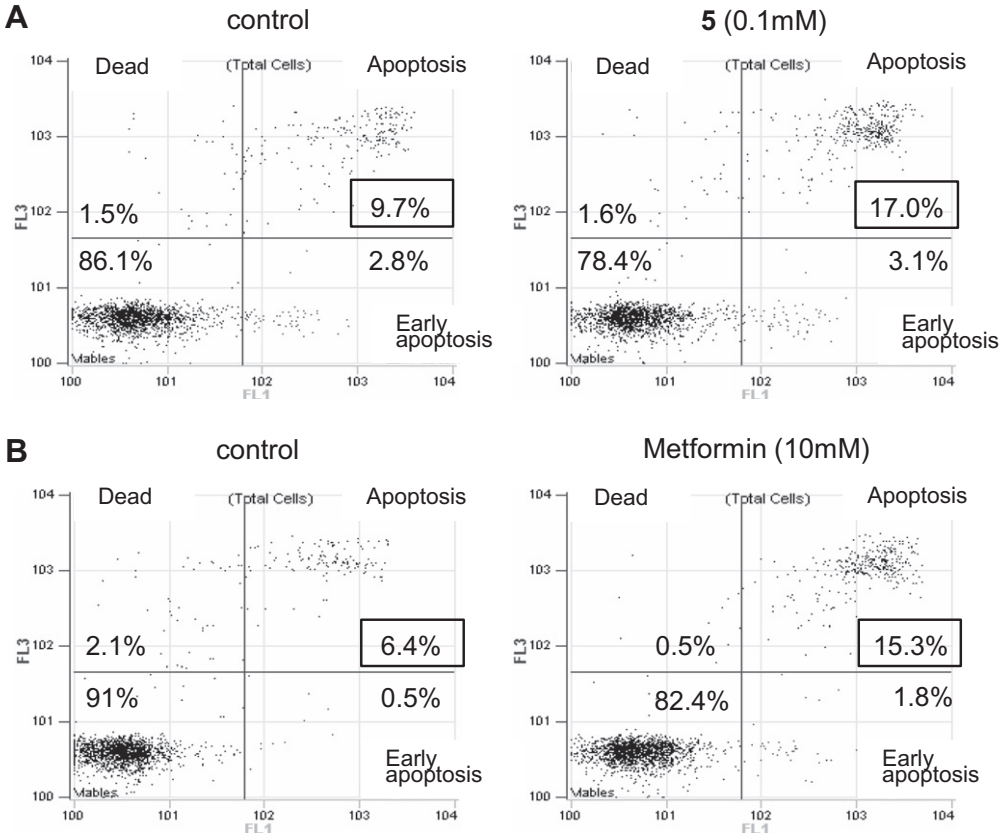


Figure 3. Compound **5** induces apoptosis of MDA-MB-231 triple-negative breast cancer cells. MDA-MB-231 breast cancer cells were treated with metformin (A) and **5** (B) for 24 h. Flow cytometric analysis was performed using annexin V-FITC and PI stain.

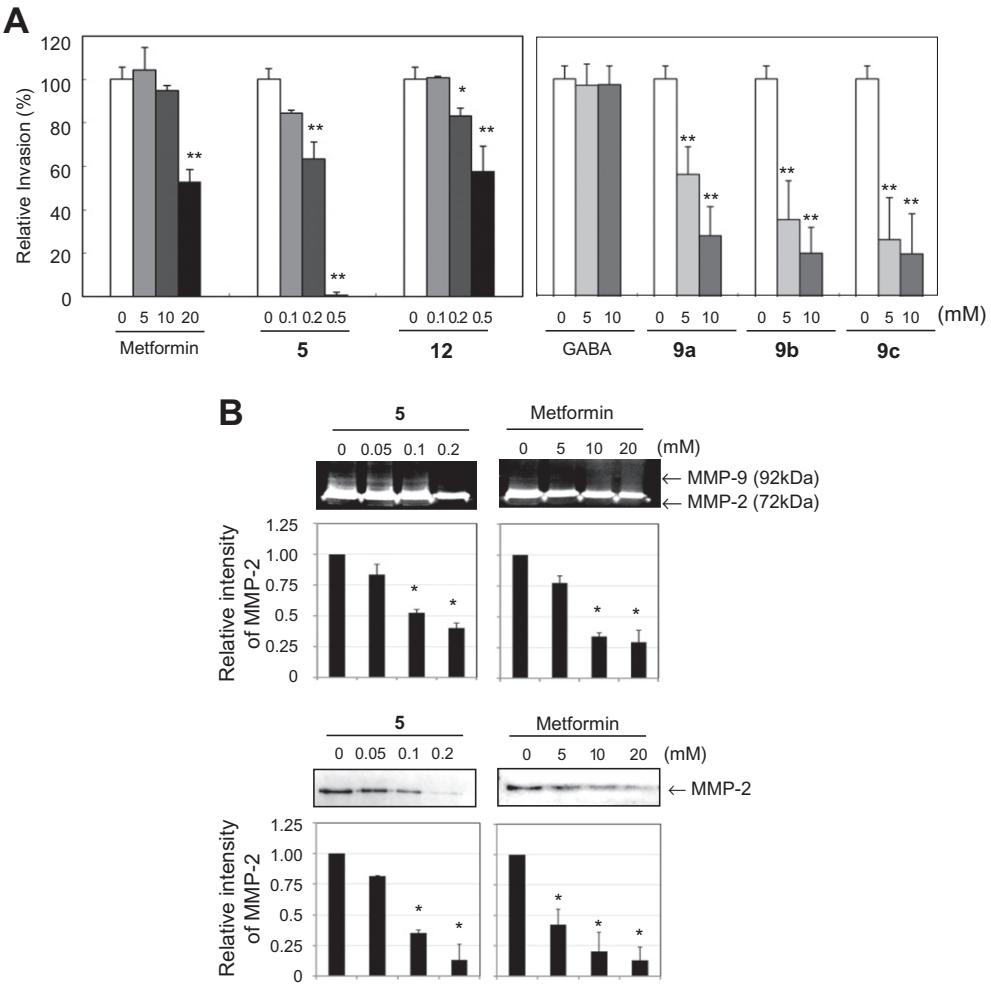


Figure 4. Compound **5** inhibits invasive phenotype and MMP-2 in Hs578T cells. (A) Cells were treated with various concentrations of compounds and subjected to in vitro invasion assay. The number of invaded cells per field was counted (×400) in 13 arbitrary visual fields. (B) The conditioned media were analyzed for MMP-2 by gelatin zymogram assay (top) and immunoblot analysis (bottom). *, ** Statistically different from the control at p <0.05 and p <0.01, respectively.

compared to metformin, suggesting that the rigid and symmetrical scaffold structure of **5** may potentiate the pharmacological activities of metformin.

3.4. Compound 5 showed a more potent anti-tumor effect than metformin in vivo

Next, we investigated the inhibitory effect of **5** on the in vivo tumor growth in a xenograft mouse model using a MDA-MB-231 triple-negative breast cancer cells. The MDA-MB-231 cells were injected subcutaneously into mice. The tumor-bearing mice were orally administered with **5** (25, 50 and 100 mg/kg) and metformin (500 mg/kg) once a day for 35 days. The changes in body weight between the vehicle and the **5** or metformin-treated mice were not remarkably different during the experiment (data not shown). Tumors were excised and tumor volume was measured. Tumor growth was time-dependently slowed in the **5** or metformin-treated mice (Fig. 6). The mean volume of tumor tissues from mice treated with **5** for 35 days at 50 and 100 mg/kg doses was significantly reduced by 17.4% and 27.9%, respectively, compared to vehicle group. The anti-tumor effect of **5** at the dose of 100 mg/kg which caused 27.9% growth inhibition was comparable to that of metformin at the dose of 500 mg/kg (28% growth inhibition) (Table 2). These results demonstrate that **5** inhibits breast cancer cell growth more effectively than metformin in vivo, suggesting that it could be a promising therapeutic drug against triple-negative breast cancer progression.

Liu et al. (2009) reported that metformin treatment (2 mg/ml in drinking water) significantly slowed tumor growth in a xenograft tumor model.[8] In the present study, a similar degree of growth inhibition was observed by **5** at the dose of 100 mg/kg to that by metformin at the dose of 500 mg/kg. Compound **5** showed about fivefold stronger in vivo anti-tumor efficacy than metformin, while **5** exerted approximately 100-fold more potent in vitro inhibitory effects on proliferation and invasion of breast cancer cells. A plausible explanation would be unfavorable in vivo pharmacokinetic properties of **5**.[27] It is important to understand the underlying mechanisms responsible for the differences between in vitro and in vivo efficacies of **5**, which may lead to a successful and rational drug development.

4. Conclusions

In the present study, we synthesized a novel metformin derivative **5** with a rigid and symmetrical scaffold on the basis of biguanide structure. Here, we report that **5** exerts a stronger antiproliferative and anti-invasive activities than a phenformin salt, **12** and metformin against triple-negative breast cancer cells. Since metastasis is the major cause of death from breast cancer, the potent anti-invasive activity of **5** against triple-negative breast cancer

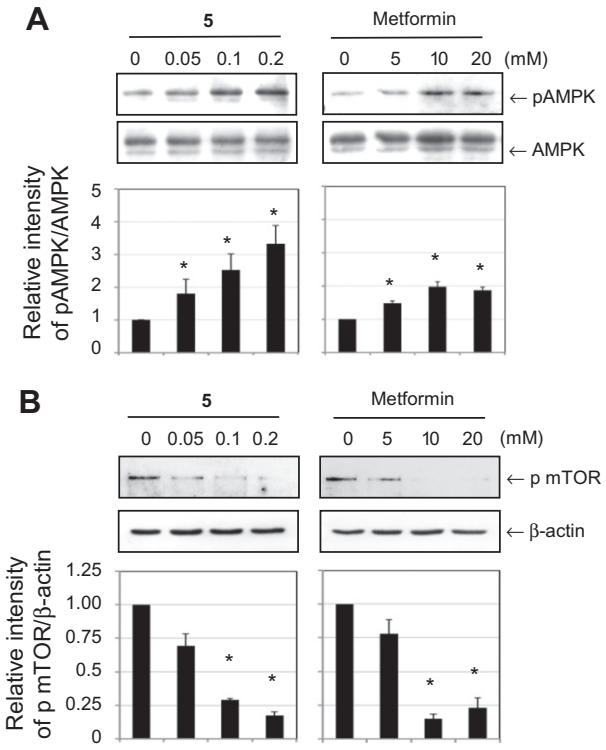


Figure 5. Compound **5** activates AMPK and inhibits phosphorylation of mTOR in Hs578T cells. Hs578T cells were treated with various concentrations of metformin and **5** for 24 h. Immunoblot analysis was performed on cell lysates using anti-phospho AMPK antibody (A) and anti-phospho mTOR antibody (B). * Statistically different from the control at p <0.05.

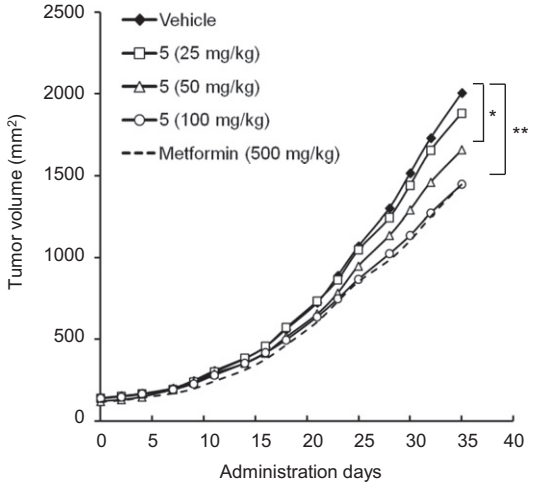


Figure 6. Compound **5** shows an anti-tumor effect in vivo. Metformin and **5** were administrated orally to MDA-MB-231 cell-bearing xenograft mice for 35 days. Tumor volumes (n = 6) were measured three times a week. *, ** Statistically different from the control at p <0.05 and p <0.01, respectively.

cells suggests a promising application of this compound for ameliorating the progression of breast cancer. Compounds **9a**, **9b** and **9c**, GABA salts of metformin, showed strong inhibitory effects on proliferation and invasion, implying that the combinatorial synthesis of metformin with GABA or its analogues exerted synergistic anti-tumoral and anti-invasive activities than the use of metformin or GABA alone. Taken together, a novel metformin derivative synthesized from this study and metformin combination therapy with current drugs may accelerate the successful development for breast cancer therapeutics.

Table 2
The anti-tumor effects of **5** and metformin in a MDA-MB-231 breast cancer xenografts model

Compound	Tumor volume (mm^3)	Tumor growth inhibition (%)
Vehicle		
	2005.7 ± 246.7	0.0
5		
25 mg/kg	1881.2 ± 271.7	6.2
50 mg/kg	1656.5 ± 161.0	17.4
100 mg/kg	1445.4 ± 106.1	27.9
Metformin		
500 mg/kg	1444.1 ± 169.0	28.0

5. Experimental section

5.1. Chemistry

Melting points were determined on an Electrothemal No.9201 and were uncorrected. Proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded on the following instruments: Bruker model Avence-500 (500 MHz) and Varian model Inova-600 (600 MHz). Chemical shifts were reported in ppm with residual undeuterated solvent peaks as internal reference for ^{1}H NMR: $CDCl_3$ (7.24 ppm), DMSO (2.50 ppm), or D_2O (4.80 ppm). Multiplicities were reported as singlet (s), doublet (d), triplet (t), or multiplet (m), and coupling constants (J) are given in Hz. Mass spectra were obtained on Schumadzu GCMSQP 1000 mass spectrometer at 70 eV and recorded herein (relative intensity and assignment). Flash chromatography was carried out using silica gel (F60, 230–400 mesh) and thin layer chromatography (TLC) was conducted on silica gel 60F-254, 0.25 mm pre-coated TLC plates purchased from E. Merck. TLC plates were visualized using UV254 and ninhydrin (1.5 g ninhydrin, 100 mL n-butanol, 3 mL acetic acid) with charring. A purity of more than 95% was determined for all compounds by NMR using the conditions described above. Reagents were purchased from commercial sources.

5.1.1. N^2,N^2-Dimethyl-6-methylthio-1,3,5-triazine-2,4-diamine (compound 2)

To a stirred solution of dimethyl-*N*-cyano-dithioiminocarbonate (compound **1**) (5.0 g, 34.2 mmol) in 40% K_2CO_3 aqueous solution (35 mL) at room temperature was added a solution of *N*,*N*-dimethylguanidine sulfate (4.6 g, 17.1 mmol) in DMSO (40 mL). After stirring for 2 h at 120 °C, the cooled reaction mixture was poured into H_2O. The white precipitate was filtered and then washed with MeOH and H_2O to provide a compound **2**, which was used for next step without further purification. Yield: 67% (2.1 g).

^{1}H NMR (500 MHz, DMSO-d_6) δ 6.75 (s, 2H), 3.03 (br s, 6H), 2.37 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.9, 165.5, 164.1, 35.9, 12.6; EIMS (70 eV) m/z (rel intensity): 185 (M^+, 100), 170 (20), 139 (20).

5.1.2. N^2,N^2-Dimethyl-6-methylsulfinyl-1,3,5-triazine-2,4-diamine (compound 3)

To a solution of compound **2** (2.0 g, 10.8 mmol) in CH_2Cl_2 (50 mL) at 0 °C was added *m*-CPBA (2.2 g, 12.9 mmol), and the reaction mixture was stirred for 12 h at room temperature. The mixture was diluted with CH_2Cl_2 and washed with ice water and 5% $NaHCO_3$ aqueous solution, and the organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under vacuum to afford the crude product. Further purification by column chromatog-

raphy (MeOH/CH_2Cl_2 = 5/95 (v/v)) provided a compound **3** as a white solid. Yield: 77% (1.7 g).

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.28–7.37 (m, 2H), 3.09 (s, 3H), 3.07 (s, 3H), 2.77 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.8, 166.5, 164.6, 36.2; EIMS (70 eV) m/z (rel intensity): 201 (M^+, 60), 156 (30), 138 (68), 96 (100).

5.1.3. N^2,N^2-Dimethyl-N^4-(2-propylphenyl)-1,3,5-triazine-2,4,6-triamine (compound 4)

To a solution of compound **3** (2.4 g, 11.9 mmol) in 1,4-dioxane (30 mL) was added 2-propylaniline (2.5 mL, 17.9 mmol), and the mixture was stirred at reflux for 15 h. The resulting solution was evaporated, diluted with CH_2Cl_2, and washed with 5% $NaHCO_3$ aqueous solution, and the organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by column chromatography (MeOH/CH_2Cl_2 = 10/90 (v/v)) to afford compound **4** as a white solid. Yield: 72% (2.3 g).

Mp: 134.8–137.2 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.90–7.92 (m, 1H), 7.18–7.24 (m, 2H), 7.05–7.10 (m, 1H), 6.87 (br s, 1H), 5.33 (br s, 2H), 3.18 (s, 3H), 3.09 (s, 3H), 2.59 (t, J = 7.4, 2H), 1.63 (q, J = 7.4, 2H), 0.97 (t, J = 7.3, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.9, 165.8, 164.8, 136.4, 134.0, 129.2, 126.0, 124.2, 123.9, 36.0, 33.3, 22.7, 13.9; EIMS (70 eV) m/z (rel intensity): 272 (M^+, 60), 257 (90), 229 (20), 84 (100); HRMS (EI) calcd for $C_{14}H_{20}N_6$: 272.1749, found: 272.1751.

5.1.4. N^2,N^2-Dimethyl-N^4-(2-propylphenyl)-1,3,5-triazine-2,4,6-triamine HCl (compound 5)

To a solution of compound **4** (2.0 g, 0.73 mmol) in $CHCl_3$ (20 mL) was added 4 M HCl in dioxane (0.22 mL, 0.88 mmol), and the mixture was stirred at room temperature for 2 h. The resulting solution was evaporated and dried under vacuum to afford compound **5** as a white solid. Yield: 95% (2.2 g).

Mp: 145–148 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.92 (br s, 1H), 8.06 (br s, 2H), 7.22–7.39 (m, 4H), 3.11 (s, 6H), 2.50 (s, 2H), 1.51 (s, 2H), 0.87 (s, 3H). MS (ESI) m/z 273.7 ([M−Cl]$^+$).

5.1.5. Preparation of metformin free base (compound 7)

A two-necked 500 mL round-bottom flask equipped with a mechanical stirrer was charged with metformin hydrochloride (compound **6**) (49.8 g, 300 mmol) and *i*-PrOH (200 mL). Potassium hydroxide (18 g, 321 mmol) was added to the stirred solution, at 50 °C. The white slurry was stirred at 50 °C for 2 h, and then the reaction mixture was cooled to room temperature. The resulting mixture was filtered and the filter-cake was washed with *i*-PrOH (30 mL) and acetone (2 × 50 mL). The combined filtrates were concentrated under reduced pressure affording a white solid. The solid was again dissolved in acetone (300 mL), the insoluble material was filtered, and the filtrate was concentrate under reduced pressure. The residue was dried in vacuo to provide metformin free base (compound **7**) as a white solid. Yield: 98% (38.2 g).

Mp: 119–120 °C; ^{1}H NMR (600 MHz, D_2O) δ 3.07 (s, 6H); ^{13}C NMR (150 MHz, D_2O) δ 161.1, 158.5, 37.35.

5.1.6. Metformin GABA salt (compound 9a)

To a stirred suspension of metformin free base (compound **7**) (8.00 g, 61.9 mmol) in *i*-PrOH (30 mL) was added GABA (compound **8a**) (7.02 g, 68.1 mmol), and the reaction mixture was heated to 50 °C. After stirring at 50 °C for 2 h, ethyl acetate (200 mL) was added dropwise to the mixture. The resulting precipitate was filtered, and the filter-cake was washed with *i*-PrOH (20 mL) and acetone (50 mL). The solid residue was dried under vacuum to provide metformin GABA salt (compound **9a**) as a white solid. Yield: 76% (10.9 g).

Mp: 127–128 °C; ^{1}H NMR (600 MHz, D_2O) δ 2.99 (s, 6H), 2,55 (t, J = 7.2 Hz, 2H), 2.14 (t, J = 7.8 Hz, 2H), 1.50 (m, 2H); ^{13}C NMR (150 MHz, D_2O) δ 183.15, 160.22, 158.62, 40.72, 37.60, 35.33, 29.07. Anal ($C_8H_{20}N_6O_2$) C, 41.4; H, 8.80; N, 36.5; O, 13.8.

5.1.7. Metformin pregabalin ((*S*)-3-(aminomethyl)-5-methylhexanoic acid) salt (compound 9b)

The title compound was prepared according to the same procedure for compound **9a**, using (*S*)-3-(aminomethyl)-5-methylhexanoic acid (compound **8b**). Yield: 63% (11.3 g). Mp: 147–148 °C; ^{1}H NMR (600 MHz, D_2O) δ 2.85 (s, 6H), 2.34 (m, 2H), 1.92 (m, 2H), 1.70 (m, 1H), 1.43 (m, 1H), 1.03 (m, 1H), 0.89 (m, 1H), 0.70 (d, J = 6.6 Hz, 6H); ^{13}C NMR (150 MHz, D_2O) δ 182.7, 160.2, 158.6, 44.76, 41.55, 41.27, 37.63, 36.03, 24.82, 22.46. Anal ($C_{12}H_{28}N_6O_2$) C, 48.0; H, 9.30; N, 30.2; O, 13.20.

5.1.8. Metformin gabapentin (2-[1-(aminomethyl)cyclohexyl]acetic acid) salt (compound 9c)

The title compound was prepared according to the same procedure for compound **9a**, using 2-[1-(aminomethyl)cyclohexyl]acetic acid (compound **8c**). Yield: 47% (8.81 g). Mp: 122–123 °C; ^{1}H NMR (600 MHz, D_2O) δ 2.90 (s, 6H), 2.45 (s, 2H), 2.03 (s, 2H), 1.32–1.20 (m, 10H); ^{13}C NMR (150 MHz, D_2O) δ 181.68, 160.17, 158.63, 49.02, 48.16, 45.08, 37.61, 36.59, 33.58, 25.97, 21.41. Anal ($C_{13}H_{28}N_6O_2$) C, 51.01; H, 9.27; N, 25.77; O, 12.33.

5.1.9. Phenformin acetic acid salt (compound 12)

To a stirred suspension of phenformin hydrochloride (compound **10**) (10.2 g, 42.1 mmol) in *i*-PrOH (50 mL) was added potassium hydroxide (2.54 g, 42.1 mmol), and the reaction mixture was heated to 50 °C. After stirring at 50 °C for 2 h, the resulting mixture was filtered and the filter-cake was washed with *i*-PrOH (250 mL). The filtrate was concentrated under reduced pressure and dried in vacuo to provide a phenformin free base (compound **11**) as a white solid. Yield: 87%, (7.48 g).

To a stirred suspension of the compound **11** (7.48 g, 36.5 mmol) in *i*-PrOH (50 mL) was added acetic acid (6.25 mL, 109.2 mmol), and the reaction mixture was heated to 50 °C. After stirring at 50 °C for 2 h, ethyl acetate (50 mL) was added dropwise to the mixture. The resulting precipitate was filtered, and the filter-cake was washed with *i*-PrOH (10 mL) and ethyl acetate (50 mL). The solid residue was dried under vacuum to afford phenformin acetic acid salt (compound **12**) as a white solid. Yield: 80%, (7.75 g).

Mp: 130–131 °C; ^{1}H NMR (600 MHz, D_2O) δ 7.34 (m, 2H), 7.26 (m, 3H), 3.45 (t, J = 6.6 Hz, 2H), 2.83 (m, 2H), 1.86 (s, 3H); ^{13}C NMR (150 MHz, D_2O) δ 181.25, 159.84, 159.72, 138.92, 129.08, 128.90, 126.85, 42.80, 34.78, 23.73. Anal ($C_{12}H_{19}N_5O_2$) C, 54.51; H, 7.07; N, 25.65; O, 10.23.

5.2. Cell culture and preparation of reagents

Hs578T breast cancer cells were purchased from the Korean Cell Line Bank (KCLB). MDA-MB-231 breast cancer cells were kindly provided by Dr. Dong Young Noh (Seoul National University, Seoul, Korea). Hs578T and MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS and 100 μg/ml penicillin–streptomycin. Cells were maintained in humidified atmosphere with 95% air and 5% CO_2 at 37 °C.

Compounds **5** and **12** were dissolved in DMSO. Metformin, GABA, **9a**, **9b**, and **9c** were dissolved in distilled water. Preparation of each reagent is listed in Table 3. Stock solutions were stored at −20 °C.

5.3. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Cells (5 × 10^3) cultured in a 96-well plate were treated with the indicated drugs for 24 h. After 24 h of incubation, 25 μl of 0.5 mg/

Table 3
Preparation of reagents used for in vitro and in vivo experiments

Compound	In vitro			In vivo		
	Vehicle	Final conc (M)	Treatment vol (v/v) (%)	Vehicle	Final conc (mg/ml)	Dosage vol (ml/kg)
Metformin	D.W.	1.5	4	20%	100	5
5	DMSO	1	0.1	PEG400, 5% DMSO in D.W.	20	5
GABA	D.W.	1.5	4	—	—	—
9a	D.W.	1	4	—	—	—
9b	D.W	1	4	—	—	—
9c	D.W.	1	4	—	—	—
12	DMSO	2	0.1	—	—	—

ml of MTT was added and incubated for 4 h. The crystals of formazan which are converted from MTT were dissolved with 100 μl of DMSO. The optical density was measured at 540 nm using a micro-ELISA reader (Molecular Devices, Sunnyvale, CA) for quantification of cell viability. Assays were performed triplicate.

5.4. In vitro invasion assay

In vitro invasion assay was performed using 24-well transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA) as previously described.[28] The lower compartment was filled with serum-free media containing 0.1% BSA with the indicated drugs. Cells (5×10^4 cells) were placed in the upper compartment with the indicated drugs. After 17 h of incubation, the invasive phenotypes were determined by counting the cells that had invaded the lower side of the filter using light microscopy (Olympus CKX31, Tokyo, Japan) at 400× optical resolution. Thirteen fields were counted for each filter and each sample was assayed in triplicate.

5.5. Gelatin zymogram assay

Cells were cultured in serum-free media containing metformin and **5** for 24 h. Gelatinolytic activity of the conditioned medium was determined by gelatin zymogram assay as described previously.[28] Relative band intensities were determined by quantitation of each band with a Gel Logic 200 Imaging System (Kodak, Rochester, USA).

5.6. Immunoblot analysis

Immumoblot analysis was performed as previously described.[29] Protein extracts in lysis buffer (0.5% Triton X-100, 0.15 M NaCl, 50 mM Tris–HCl, pH 7.4, 25 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4) containing protease inhibitor cocktail (Roche, Mannheim, Germany) were subjected to 10% SDS–PAGE analysis. Anti-phospho-AMPKα, anti-AMPKα, and anti-phospho-mTOR antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-MMP-2 antibodies were purchased from R&D system Inc. (Minneapolis, MN, USA). The enhanced chemiluminescence system (ECL, Amersham-Pharmacia, UK) was used for detection. Relative band intensities were determined by quantitation of each band with a Gel Logic 200 Imaging System (Kodak, Rochester, USA).

5.7. Flow cytometric analysis for apoptosis

Flow cytometric analysis was performed using FITC annexin V apoptosis detection kit (BD Biosciences, San Diego, CA) as manufacture's protocol. MDA-MB-231 breast cancer cells were exposed to metformin and **5** for 24 h with cultured in serum-free media. The cells (1×10^6 cells/ml) were added 5 μl of FITC annexin V and/or propidium iodide and analyzed by flow cytometry (Beckman Coulter, UK). The results are represented in the form of dot plots divided into four quadrants. Lower left quadrant of the dot plots shows viable cells. Lower right quadrant shows early apoptotic cells with preserved plasma membrane integrity. Upper right/left quadrants show late apoptotic/necrotic cells which have lost their plasma membrane integrity.

5.8. Anti-tumor activity of 5 in vivo a xenograft tumor model

Four-week-old female BALB/c athymic nude mice were purchased from Orient Bio Co. (Seongnam, Korea). All experiments were approved and carried out according to the Guide for Care and Use of Animals (Chungbuk National University Animal Care Committee, Korea). MDA-MB-231 cells were injected subcutaneously (5×10^6 cells in 0.1 mL serum free medium per mouse) into the right-lower flanks of the mice. When the tumors had reached an average volume of 100–130 mm^3, the tumor-bearing nude mice ($n = 6$) were administered orally with **5** (25, 50 and 100 mg/kg) and metformin (500 mg/kg) once a day via oral gavage needle. The preparation of each reagent is listed in Table 3.

The body weights and tumor volumes of mice were monitored three times a week. The tumor volumes were measured with vernier calipers and calculated by the following formula: $(A \times B^2)/2$, where A is the length and B is the width of the two dimension tumor.

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