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Evaluation of T-Type Calcium Channel Blockers against Human Pancreatic MIA PaCa-2 Carcinoma Xenografts

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Two piperazine-containing 3,4-dihyroquinazolines (**BK10007S/8S**) have been synthesized, based on our previous work on the synthesis and antitumoral activity of 3,4-dihyroquinazolines. After evaluating them for T-type calcium channel blocking effect and *in vitro anti*-cancer effect, they were profiled for acute and repeat dose toxicity (40 mg/kg, 2 weeks) to BALB/c mice. **BK10007S/8S** were further *in vivo* evaluated against human pancreatic MIA PaCa-2 carcinoma in BALB/c$^{nu/nu}$ nude mice, which exhibited 54 and 61% tumor growth inhibition through 57-day oral administration of 2 mg/kg of body weight, respectively.

Key Words : T-type calcium channel, 3,4-Dihydroquinazoline, Repeat dose toxicity, MIA PaCa-2 xenografts

Introduction

Calcium is an important activator or inhibitor of numerous intracellular enzymes in the cytosol, organelles and nucleus.[1] Proliferation of tumors and non-tumor cells, is regulated, in part, by the second messenger calcium.[2,3] Among several regulation mechanisms of calcium level, T-type (low voltage activated) calcium channels are well recognized to play a role in regulating calcium signaling during cell proliferation and differentiation in many tissues.[4,5] Recently, the *anti*-cancer effects of a T-type Ca^{2+} channel antagonists (or blockers) including mibefradil and pimozide on tumor cells *in vivo* have been reported by many researcher groups.[6,7] Our group also have reported the identification of 3,4-dihydroquinazoline compound (**KYS05090**), which exhibits both selective/potent T-type calcium channel blocking effect and strong *anti*-cancer effect on A549 cancer cell lines comparable to doxorubicin and paclitaxel *in vivo*.[8-12] As a continuous work, two piperazine-containing 3,4-dihyroquinazolines (**BK10007S/8S**) have been synthesized and biologically evaluated for the growth inhibition effect against human pancreatic MIA PaCa-2 carcinoma xenografts, based on our previous work (Figure 1). The results of these studies are reported herein.

Experimental

General Chemistry. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Unity Plus 400 (400 MHz) spectrometer or a Bruker Avance-DRX 400 (400 MHz) spectrometer, using TMS as the internal standard; the chemical shifts (δ) are reported in parts per million and coupling constants (*J*) values are given in hertz (Hz). Signal multiplicities are represented by: s (singlet), d (doublet), t (triplet), q (quartet), br s (broad singlet), m (multiplet) and dd (double doublet). Low-resolution and High-resolution mass spectra (FABMS, FAB energy: 6 keV, Emission current: 5 mA, Acceleration voltage: 10 kV) were obtained using JMS-700 mass spectrometer (JEOL, JAPAN). The progress of all reactions was monitored using TLC on precoated silica gel plates (Merck Silica Gel 60 F_{254}). The chromatograms were viewed under UV light at 254 nm and 365 nm. For column chromatography, Merck Silica Gel (230-400 mesh ASTM) was used. Chemicals were purchased from Sigma-Aldrich, TCI, Alfa aesar, and Fluka.

Chemical Synthesis.

Methyl *trans*-2-Aminocinnamate (3): To a solution of methyl *trans*-2-nitrocinnamate (500 mg, 1.41 mmol) in MeOH (20 mL) was added activated Zn powder (1.07 g,

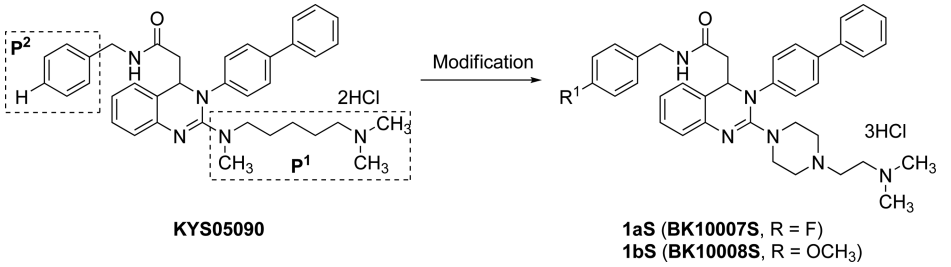


Figure 1. Modification of **KYS05090** into **1aS/1bS** (**BK10007S/8S**).

19.31 mmol) and NH_4Cl (0.29 g, 5.52 mmol) at 0 °C and the solution was stirred at rt for 12 h. The resulting mixture was filtered through celite 545 and evaporated in vacuo to give the crude product. The concentrated product was added into EtOAc (20 mL) and stayed under sonicator for 10 min. EtOAc-insoluble matter was filtered and the filtrate was washed with sat. $NaHCO_3$, dried ($MgSO_4$), evaporated in vacuo to give the desired product **3** in 96% (0.41 g) as an orange color solid. mp 61.1-62.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (1H, d, *J* = 15.6 Hz, -C*H*=CH-CO_2Me), 7.38 (1H, dd, *J* = 8 and 1.6 Hz, Ph), 7.19 (1H, t, *J* = 8 Hz, Ph), 6.77 (1H, t, *J* = 7.6 Hz, Ph), 6.72 (1H, dd, *J* = 8 and 0.8 Hz, Ph), 6.35 (1H, d, *J* = 16.0 Hz, -CH=C*H*-CO_2Me), 3.97 (2H, br s, -NH_2), 3.80 (3H, s, -OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.7, 145.5, 140.3, 131.3, 128.1, 119.9, 119.0, 117.7, 116.8, 51.7; HRMS (FAB+): calcd for $C_{10}H_{12}NO_2$ (M+H) 178.0868, found: 178.0866.

Methyl *trans*-2-(3-Biphenylureido)cinnamate (4): To a solution of biphenyl-4-carboxylic acid (5.00 g, 25.23 mmol) in distilled toluene (50 mL) was added 97% diphenyl phosphorazidate (DPPA) (7.13 mL, 31.70 mmol) and Et_3N (5.89 mL, 53.33 mmol) at rt for Curtius rearrangement. The reaction mixture was stirred at rt for 3 h, further stirred at 100 °C for 3 h, followed by an addition of a solution of **3** (3.70 g, 20.88 mmol) in distilled toluene (100 mL) at rt. The reaction mixture was stirred for 12 h and concentrated under reduced pressure to give a solid, which was washed with MeOH (50 mL) to give desired urea (**4**) in 81% (6.23 g) as a white solid. mp 210-213.3 °C; ^{1}H NMR (400 MHz, DMSO) δ 9.08 (1H, s, -N*H*-CO-), 8.55 (1H, s, -N*H*-CO-), 7.91 (1H, d, *J* = 16.0 Hz, -C*H*=CH-CO_2Me), 7.78 (2H, t, *J* = 6.8 Hz, Ph), 7.63 (4H, t, *J* = 8.2 Hz, Ph), 7.58 (2H, d, *J* = 8.8 Hz, Ph), 7.46-7.39 (3H, m, Ph), 7.32 (1H, t, *J* = 7.6 Hz, Ph), 7.15 (1H, t, *J* = 7.6 Hz, Ph), 6.61 (1H, d, *J* = 16.0 Hz, -CH=C*H*-CO_2Me), 3.75 (3H, s, -OCH_3); ^{13}C NMR (100 MHz, DMSO) δ 166.7, 152.7, 139.8, 139.1, 137.8, 133.6, 130.7, 128.8, 127.1, 127.0, 126.8, 126.1, 123.9, 123.7, 118.8, 118.6, 51.5; HRMS (FAB+): calcd for $C_{23}H_{21}N_2O_3$ (M+H) 373.1552, found: 373.1554.

Methyl *trans*-2-(3-Biphenylcarbodiimido)cinnamate (5): A solution of urea (**4**) (2.2 g, 6.0 mmol) in distilled CH_2Cl_2 (100 mL) and Et_3N (2.5 mL, 18.0 mmol) was added into a solution of dibromotriphenylphosphorane (3.8 g, 9.0 mmol) in distilled CH_2Cl_2 (50 mL) at 0 °C under Ar. The reaction mixture was stirred at the same temperature for 3 h and treated with water (30 mL). The mixture was extracted with CH_2Cl_2 (3 ×), dried ($MgSO_4$), filtered, and evaporated in vacuo to give a yellow-colored soild, which was added with MeOH (50 mL) to give the desired carbodiimide (**5**) in 80% (1.69 g) as a white solid. mp 105.0-105.8 °C; ^{1}H NMR (400 MHz, DMSO) δ 8.03 (1H, d, *J* = 16.0 Hz, -C*H*=CH-CO_2Me), 7.89 (1H, dd, *J* = 7.6 and 1.2 Hz, Ph), 7.73-7.69 (2H, m, Ph), 7.68-7.66 (2H, m, Ph), 7.49-7.42 (4H, m, Ph), 7.39-7.35 (3H, m, Ph), (1H, d, *J* = 16.0 Hz, -CH=C*H*-CO_2Me), 3.74 (3H, s, -OCH_3); ^{13}C NMR (100 MHz, DMSO) δ 166.5, 139.2, 139.1, 137.9, 137.2, 136.3, 133.5, 131.8, 129.0, 128.0, 127.8, 127.7, 127.5, 126.5, 126.2, 125.8, 124.8, 119.4, 51.6; HRMS (FAB+): calcd for $C_{23}H_{19}N_2O_2$ (M+H) 355.1447, found: 355.1450.

3-Biphenyl-4-yl-2-{[2-(*N*,*N*-dimethylamino)ethyl]-1-piperazino}-4-methoxylcarbonylmethyl-3,4-dihydroquinazoline (6): To a solution of carbodiimide (**5**) (2.30 g, 6.49 mmol) in distilled toluene (40 mL) was added a solution of *N*-[2-(dimethylamino)ethyl]piperazine (1.02 g, 6.49 mmol) in distilled toluene (20 mL) at rt, and the mixture solution was stirred for 2 h. The mixture was concentrated in vacuo and subjected to flash column chromatography (CH_2Cl_2: MeOH:NH_4OH = 100:9:1) to provide the desired product (**6**) in 97% (3.32 g). mp 146.3-147.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53-7.51 (2H, m, Ph), 7.50-7.46 (2H, m, Ph), 7.46-7.40 (2H, m, Ph), 7.36-7.30 (1H, m, Ph), 7.26-7.21 (1H, m, Ph), 7.21-7.18 (1H, m, Ph), 7.17-7.13 (2H, m, Ph), 7.01-6.97 (2H, m, Ph), 5.16 (1H, dd, *J* = 10.4 and 4.8 Hz, -CO-CH_2-C*H*-N-), 3.77 (3H, s, -OCH_3), 3.51 (4H, br s, -N-(CH_2-CH_2)$_2$-N-CH_2-), 2.86 (1H, dd, *J* = 15.2 and 10.8 Hz, -CO-CH_2-), 2.55 (1H, dd, *J* = 15.2 and 4.8 Hz, -CO-CH_2-), 2.50-2.42 (4H, m, -N-*CH*$_2$-*CH*$_2$-N-Me$_2$), 2.39 (4H, br s, -N-(CH_2-*CH*$_2$)$_2$-N-CH_2-), 2.26 (6H, s, -N*Me*$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.9, 152.5, 144.9, 143.7, 140.3, 136.8, 128.8, 128.5, 127.9, 127.1, 126.8, 125.8, 124.7, 123.0, 122.6, 122.3, 60.9, 56.9, 56.7, 53.2, 51.9, 45.9, 45.5, 39.6; HRMS (FAB+): calcd for $C_{31}H_{38}N_5O_2$ (M+H) 512.3026, found: 512.3027.

***N*-(4-Fluorobenzyl)-3-biphenyl-4-yl-2-{[2-(*N*,*N*-dimethylamino)ethyl]-1-piperazino}-4-methoxylcarbonylmethyl-3,4-dihydroquinazolin-4-ylacetamide (1aF: BK10007F):** The reaction mixture containing ester (**6**) (0.5 mmol) with 1,5,7-triazabicyclo[4,4,0]dec-5-ene (TBD) as a catalyst and 4-fluorobenzylamine was stirred for 12 h under solvent-free condition. Flash column chromatography (CH_2Cl_2:MeOH: NH_4OH) of mixture gave the desired product (**1aF)** in 61%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55-7.52 (2H, m, Ph), 7.48-7.45 (2H, m, Ph), 7.44-7.39 (2H, m, Ph), 7.34-7.29 (1H, m, Ph), 7.24-7.16 (5H, m, Ph), 7.11 (1H, d, Ph), 6.32 (1H, t, *J* = 5.6 Hz, -CO-N*H*-CH_2-), 5.25 (1H, dd, *J* = 9.6 and 5.6 Hz, -CO-CH_2-C*H*-N-), 4.47-4.36 (2H, m, -NH-CH_2-Ph-), 3.33 (4H, br s, -N-(CH_2-CH_2)$_2$-N-CH_2-), 2.58 (1H, dd, *J* = 14.4 and 9.6 Hz, -CO-CH_2-), 2.43 (1H, dd, *J* = 14.4 and 5.6 Hz, -CO-CH_2-), 2.38-2.30 (4H, m, -N-*CH*$_2$-*CH*$_2$-N-Me$_2$), 2.22 (6H, s, -N*Me*$_2$), 2.18 (4H, br s, -N-(CH_2-*CH*$_2$)$_2$-N-CH_2-); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.0, 153.0, 144.8, 143.4, 140.3, 137.0, 134.0, 130.0, 129.8, 128.8, 128.4, 127.8, 127.2, 126.8, 126.9, 125.0, 122.8, 122.7, 122.7, 115.6, 115.4, 60.9, 56.8, 56.6, 53.0, 45.9, 45.7, 43.0, 41.9; HRMS (FAB+): calcd for $C_{37}H_{42}N_6OF$ (M+H) 605.3404, found: 605.3406.

***N*-(4-Methoxybenzyl)-3-biphenyl-4-yl-2-{[2-(*N*,*N*-dimethylamino)ethyl]-1-piperazino}-4-methoxylcarbonylmethyl-3,4-dihydroquinazolin-4-ylacetamide (1bF: BK10008F):** Prepared by the reaction of ester **6** with 4-methoxybenzylamine in 75% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55-7.52 (2H, m, Ph), 7.48-7.45 (2H, m, Ph), 7.44-7.40 (2H, m, Ph), 7.34-7.29 (1H, m, Ph), 7.22-7.13 (6H, m, Ph), 6.99-6.96 (1H, m, Ph), 6.94-6.89 (1H, m, Ph), 6.81-6.78 (2H, m, Ph), 5.83 (1H, t, *J* = 5.6 Hz, -CO-N*H*-CH_2-), 5.48 (1H, dd, *J* = 9.6 and 5.6 Hz, -CO-CH_2-C*H*-N-), 4.45-4.30 (2H, m, -NH-CH_2-

Ph-), 3.75 (3H, s, -OCH_3), 3.48 (4H, br s, -N-(CH_2-CH$_2$)$_2$-N-CH$_2$-), 2.57 (1H, dd, J = 14.4 and 9.6 Hz, -CO-CH_2-), 2.43 (1H, dd, J = 14.4 and 5.6 Hz, -CO-CH_2-), 2.36 (4H, s, -N-*CH*$_2$-*CH*$_2$-N-Me$_2$), 2.22 (6H, s, -N*Me*$_2$), 2.20 (4H, br s, -N-(CH$_2$-*CH*$_2$)$_2$-N-CH$_2$-); ^{13}C NMR (100 MHz, CDCl$_3$) δ 170.1, 160.9, 153.3, 144.8, 143.0, 140.3, 137.1, 134.5, 130.0, 129.9, 128.8, 128.4, 127.8, 127.2, 126.8, 126.6, 125.1, 122.9, 122.8, 122.2, 115.5, 115.3, 60.7, 56.7, 56.4, 52.8, 45.8, 42.9, 41.6; HRMS (FAB+): calcd for $C_{38}H_{45}N_6O_2$ (M+H) 617.3604, found: 617.3602.

Preparation of 1aS/1bS (BK10007S/8): Each product (**1aF** or **1bF**) was added into a mixture of EtOAc (20 mL) and *conc.* HCl in H_2O (2 mL). The mixture was stirred for 1 h and evaporated in vacuo to give a solid, which was thoroughly washed with diethyl ether (50 mL) and dried in vacuo to give the desired hydrochloride salt compound (**1aS** or **1bS**) as a light cream-colored solid in > 99% yield for each compound. **1aS (BK10007S)** mp 202.9-206.7 °C; HRMS (FAB+): calcd for $C_{37}H_{42}N_6OF$ (M+H) 605.3404, found: 605.3406. **1bS (BK10008S)** mp 198.6-200.2 °C; HRMS (FAB+): calcd for $C_{38}H_{45}N_6O_2$ (M+H) 617.3604, found: 617.3602.

Biological Assays.

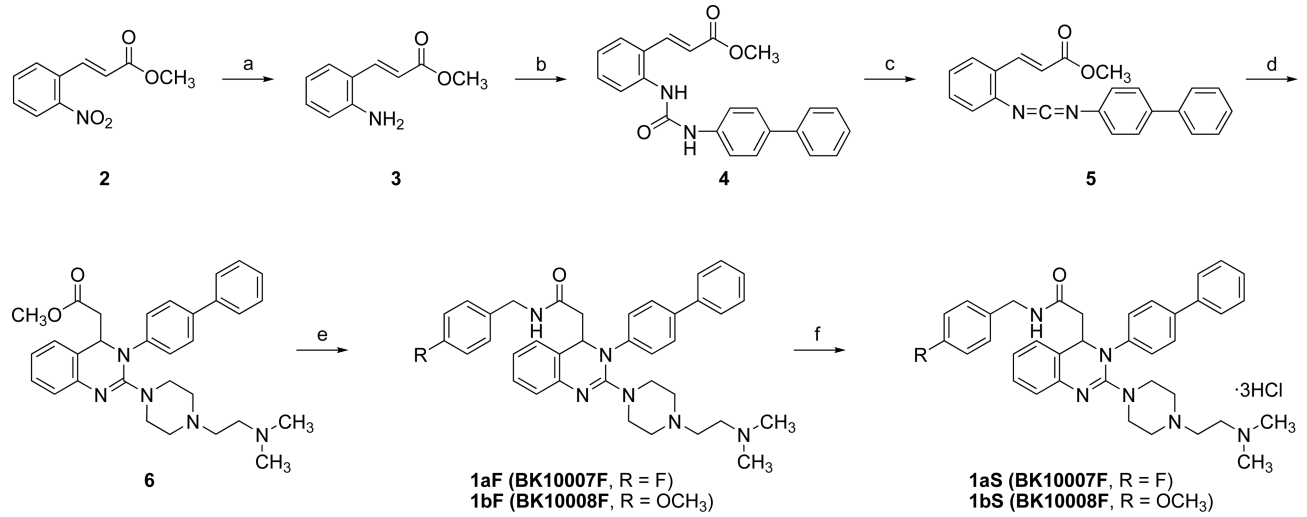
Method for Screening T-type Calcium Channel Blockers: For the recordings of α_{1G} T-type Ca^{2+} currents, the standard whole-cell patch-clamp method was utilized. Briefly, borosilicate glass electrodes with a resistance of 3-4 MΩ were pulled and filled with the internal solution containing (in mM): 130 KCl, 11 EGTA, 5 Mg-ATP, and 10 Hepes (pH 7.4). The external solution contained (in mM): 140 NaCl, 2 $CaCl_2$, 10 Hepes, and 10 glucose (pH 7.4). α_{1G} T-type Ca^{2+} currents were evoked every 15 s by a 50 ms depolarizing voltage step from −100 mV to −30 mV. The molar concentrations of test compounds required to produce 50% inhibition of peak currents (IC_{50}) were determined from fitting raw data into dose-response curves. The current recordings were obtained using an EPC-9 amplifier and Pulse/Pulsefit software program (HEKA, Germany).

Acute Toxicity Study: BALB/c male mice (4 weeks old, 16-18 g) were purchased from NARA B/D (Pyeongtaek, Korea) and were acclimatized for 1 week before the experiment began (5 weeks old, 17.8-18.6 g). During this period and throughout the experiment the mice were housed in stainless cages, and were given access ad lib. to water and pellet chow (Pico Lab Rodent Diet 20, LabDiet, U.S.A.). The mice were kept under controlled conditions of temperature (23.1 ± 0.17 °C) and relative humidity (50.3 ± 1.14%), with a 12 h light/dark cycle. **BK10007S/8S** was suspended in saline(Dahan Pharm Co. Ltd., Korea) and given by gavages to three groups of two mice at doses of 0 (control: sterile distilled water) and 500 mg/kg body weight once daily. The animals were observed during 7 days. After this period of time, mice were weighed, killed and dissected to detect any macroscopic injuries of organs.

Repeat Dose Toxicity Study: BALB/c male mice (5 weeks old, 17-19 g) were purchased from NARA B/D (Pyeongtaek, Korea) and were acclimatized for 1 week before the experiment began (6 weeks old, 19.7-20.6 g). During this period and throughout the experiment the mice were housed in stainless cages, and were given access *ad lib.* to water and pellet chow (Pico Lab Rodent Diet 20, LabDiet, U.S.A.). The mice were kept under controlled conditions of temperature (23.1 ± 0.17 °C) and relative humidity (50.3 ± 1.14%), with a 12 h light/dark cycle. **BK10007S/8S** was dissolved in saline (Dahan Pharm Co. Ltd., Korea) and given by gavages to three groups of five mice at doses of 0 (control: sterile distilled water) and 40 mg/kg body weight for 2 weeks daily. The animals were observed during 15 days. After this period of time, mice were weighed, killed and dissected to detect any macroscopic injuries of hearts.

MTT Assay: The effect of **BK10007S/8S** on cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 3 replicates. Each cancer cells were seeded and incubated in 96-well, flat-bottomed plates for 24 h and exposed to various concentrations of test agents dissolved in DMSO (final concentration, 0.1%) for 48 h. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. The medium was removed, replaced by 200 μL of 0.5 mg/mL MTT in 10% fetal bovine serum containing DMEM, and cells were incubated in the carbon dioxide incubator at 37 °C for 2 h. Supernatants were removed from the wells and the reduced MTT dye was solubilized in 100 μL/well DMSO. Absorbance at 570 nm was determined on a plate reader.

Antitumor Activity Using MIA PaCa-2 Xenograft Nude Mice. BALB/c$^{nu/nu}$ nude mice (6 weeks old) were purchased from Central Lab. Animal Inc. (Seoul, Korea) and were acclimatized for 1 or 2 weeks, before the experiment began. During this period and throughout the experiment the mice were housed in polysulfonate cages, and were given access *ad libitum* to water and pellet chow (TEKLAD CERTIFIED GLOBAL 18% PROTEIN RODENT DIET 2918C, Harlan TEKLAD, U.S.A.). The mice were kept under controlled conditions of temperature (20.7-22.0 °C) and relative humidity (26.7-49.9%), with a 12 h light/dark cycle. The MIA PaCa-2, which had been grown in Ham's F-12 Medium plus 10% FBS, were collected and reconstituted in Dulbecco's Phosphate Buffered Saline (D-PBS, pH 7.4). Approximately, 5-10 × 10^7 cells/mL were injected subcutaneously into the left flank region of the BALB/c$^{nu/nu}$ nude mice and the tumors were allowed to grow. After 2 weeks, tumors resulting after 2 weeks in donor animals were aseptically dissected and mechanically minced. Pieces of tumor tissue (3 mm^3 in size) were transplanted by a trocar needle into nude mice. When tumors reached about 100 mm^3 in size, the mice were randomized into four groups (n = 6 per group). Then the treatments were started, each compound (**BK10007S/8S**) with 2 mg/kg dose in saline was administered once daily through oral administration route for consecutive 57 days and doxorubicin with 2 mg/kg dose in saline was administered through intraperitoneal (ip) injection twice per week during 37 days. Tumor lengths and widths were measured once in 4 days using a caliper and tumor volume was calculated as 1/2 × length × width × height until animal sacrifice.



Scheme 1. (a) Zn, NH_4Cl, MeOH, reflux, 96%; (b) biphenyl-4-carboxylic acid, DPPA, Et_3N, toluene, rt to 100 ºC, 79%; (c) $Ph_3P{\cdot}Br_2$, Et_3N, CH_2Cl_2, 0 ºC, 81%; (d) *N*-[2-(dimethylamino)ethyl]piperazine, toluene, rt, 97%; (e) R-$BnNH_2$, TBD, rt, 61% for **1aF** (**BK10007F**) and 75% **1bF** (**BK10008F**); (f) *conc.* HCl (aq), EtOAc, rt, > 99% for **1aS/1bS** (**BK10007S/8S**).

At the end of experiment, all mice were weighed and sacrificed and their tumors were excised. Tumors were weighed and the mean tumor weight was calculated. The tumor growth inhibition rates (TGIR) were calculated as follows: Tumor growth inhibition rates, TGIR (%) = 100 × (*C*–*T*)/*C*, where *T* is the average tumor weight of the treated and *C* is the average tumor weight of the control.

Results and Discussion

Synthesis. We used a short synthetic route (see Scheme 1) that would allow the rapid and simultaneous optimization of the two variable points (P^1 and P^2) and to produce two novel 3,4-dihydroquinazoline compounds (**1aS/1bS**).[12] The reduction of methyl *trans*-2-nitrocinnamate (**2**) with activated Zn and NH_4Cl in MeOH afforded methyl *trans*-2-aminocinnamate (**3**), followed by an addition of biphenyl isocyanate, which was *in situ* prepared from the reaction of biphenyl-4-carboxylic acid and diphenyl phosphorazidate (DDPA) in the presence of TEA, produced methyl *trans*-2-(3-biphenylureido)cinnamate (**4**).[13] The dehydration of **4** with $Ph_3P{\cdot}Br_2$ and Et_3N provided methyl *trans*-2-(3-biphenylcarbodiimido)cinnamate (**5**) as a key intermediate of this reaction. Subsequently, the coupling of **5** with *N*-[2-(dimethylamino)ethyl]piperazine in toluene afforded the 2-piperazinyl-3,4-dihydroquinazoline ester derivative (**6**) *via* the tandem intramolecular conjugate addition. The treatment of **6** with benzylamine and 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) as a catalyst under solvent-free condition afforded directly the corresponding 3,4-dihydroquinazoline amide derivatives **1aF** and **1bF** (**BK10007F/8F**), respectively.[14] Finally, salt formation to enhance aqueous solubility of compound **1aF** and **1bF** was carried out using 35% HCl in EtOAc to afford **1aS** and **1bS** (**BK10007S/8S**), respectively.

T-Type Channel Blocking Assay and Cytotoxicity Test. The channel blocking activity of **BK10007F/8F** was evaluated against HEK293 cells which stably express both T-type calcium channel $Ca_v3.1$ with α_{1G} subunit and potassium channel Kir2.1.[15] As shown in Table 1, two compounds showed the similar T-type channel blocking activity (IC_{50} value of *ca.* 0.3 μM; 10-fold active than mibefradil) but were slightly less active than **KYS05090** in this work. These compounds were evaluated for cytotoxicity against five cancer cell lines, including human lung carcinoma (A549), human colon carcinoma (HT-29), human prostate carcinoma (DU145), human ovarian carcinoma (SK-O-V3) and human pancreatic carcinoma (MIA PaCa-2) using MTT assay.[16] Cytotoxicity results are reported as IC_{50} values in Table 1. In

Table 1. T-type calcium channel blocking effect and *anti*-cancer activity

Code	Calcium channel blockage [T-type: $Ca_v3.1$ (α_{1G})][a,b]		Cytotoxicity (IC_{50}: μM)				
	% inhibition (1 μM)	(IC_{50}: μM)	A549[c]	HT-29[d]	DU145[e]	SKOV3[f]	MIA PaCa-2[g]
KYS05090	76.6 ± 0.7	0.26 ± 0.01	4.69	1.46	6.01	2.47	1.74
Mibefradil	95.9 ± 1.7[h]	1.34 ± 0.49	31.38	11.74	40.39	20.54	25.20
BK10007F	81.0 ± 2.5	0.32 ± 0.03	4.91	2.67	10.35	3.09	2.88
BK10008F	75.9 ± 2.2	0.31 ± 0.07	4.39	1.53	8.67	2.47	1.83

[a]T-type calcium channel (α_{1G}) expressed on HEX293 cell. [b]Value was determined from dose–response curve and obtained from three independent experiments. [c]A549 is the human lung carcinoma. [d]HT-25 is the human colon carcinoma. [e]DU145 is the human prostate carcinoma. [f]SKOV3is the human ovarian carcinoma. [g]MIA PaCa-2 is human pancreatic carcinoma; % inhibition at 10 μM.

general, both **BK10007F/8F** showed the similar correlation between T-type channel blocking effect and cytotoxic effect, irrespective of a kind of carcinoma, compared to **KYS05090** and mibefradil. In addition, they exhibited stronger cytotoxic activity than mibefradil, as expected based on T-type channel blocking effects. As like **KYS05090**, two compounds exhibited a range of cytotoxicity between 1.53 and 10.35 μM dependent on the tested carcinomas (HT-29 > MIA PaCa-2 > SKOV3 > A549 > DU145) as shown in Table 1. Of them, **BK10008F** was as active as **KYS05090** against five different tumor cell lines.

Toxicity Assay: For the oral administration, **BK10007F/8F** were converted into their HCl salt (**BK10007S/8S**) and profiled for their preliminary acute toxicity to BALB/c mice.[17] Each compound was dissolved in saline (Dahan Pharm Co. Ltd., Korea) and given by gavages to three groups of two mice at doses of 0 (control: only sterile distilled water) and 500 mg/kg body weight once. The animals were observed and weighed every day during 7 days. First of all, it should be noted that all the mice given a single oral injection of **BK10008S** at the dose of 500 mg/kg of body weight showed no clinical signs and the mortality rate was zero. In the case of **BK10007S**, however, symptom such as loss of locomotor activity was observed. One animal was found dead six days after dosing and the mortality rate was 1/2 (50%). Meanwhile, a little of body weight loss was observed but recovered after 3 days on all alive mice against both compounds (data not shown). For repeat dose toxicity study,[18] BALB/c mice were again assigned to three groups (n = 5 per group) and mice in group 1 and 2 were orally given dose of 40 mg/kg of each compound, respectively, for 13 consecutive days. Mice in group 3 were administered vehicle (control: sterile distilled water) only. After this period of time, mice were weighed, killed and dissected to detect any macroscopic injuries of organs. As a result, no animal was found dead during 2-week study. No clinical sign was observed on any of the animals treated by each compound and control. No difference in body weight and relative food or water consumption between treatment groups was found (data not shown). As a result of dissection of all mice, finally, there was neither difference in organ weights between treatment groups nor macroscopic injuries of organs against each compound (Table 2). With respect to calcium channel blockage-induced cardiac toxicity study, in particular, the histopathology of heart in mice (three animals per each group) exhibited no specific abnormal findings as shown in Figure 2. Based on these overall data, **BK10007S/8S** are shown to be relatively less toxic compared to other cancer chemotherapeutic agents.

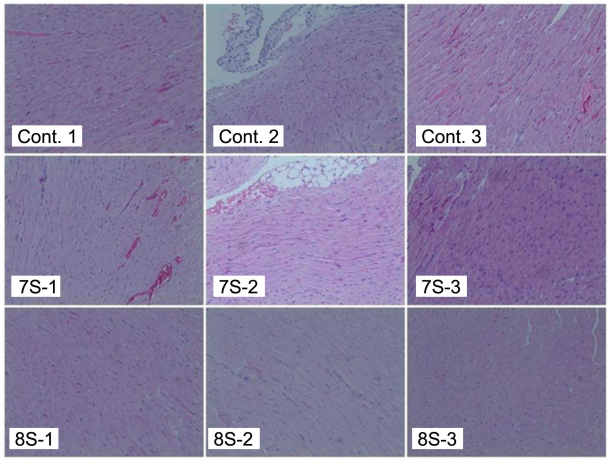


Figure 2. Histopathology of the hearts in the animals. No specific abnormal findings were detected in all the animals examined.

Antitumor Activity Assay.[19-21] Since **BK10007S/8S** was found to be potent cytotoxic against MIA PaCa-2, we evaluated the *in vivo* antitumor activity of **BK10007S/8S** against MIA PaCa-2 (human pancreatic carcinoma) xenografts in BALB/c$^{nu/nu}$ nude mice model in comparison with doxorubicin as positive control. MIA PaCa-2 (5-10 × 10^7 cells/mL) were injected subcutaneously (s.c.) into the left flank region of BALB/c$^{nu/nu}$ nude mice and the tumors were allowed to grow. When tumor nodules were clearly visible (*ca.* 100 mm^3) after 14 days, each compound with 2 mg/kg dose in saline was orally (po) administered once per day for consecutive 57 days and doxorubicin with 2 mg/kg dose in physical normal saline was administered through intraperitoneal (ip) injection twice per week during 37 days because all mice treated with doxorubicin were killed within 37 days after administration. Tumor lengths and widths were measured once in 4 days using a caliper and tumor volume was calculated as 1/2 × length × width × height until animal sacrifice. The results are documented in Figure 3 and Table 3. Figure 3(a) displays the effects of **BK10007S/8S** on tumor

Table 2. A 2-week repeat dose toxicity of compound **BK10007S/8S** on BALB/c mice

Compound	Dose (mg/kg)	AR[a]	No. of animal (sex)	Clinical signs and organ weight[b]	No. of dead animal	Mortality (%)
Control[c] (vehicle)	-	po	5 (male)	✓Change of body weight: normal ✓Organ weight: heart, liver, kidney and spleen are normal	0	0
BK10007S	40	po	5 (male)	✓Change of body weight: normal ✓Organ weight: heart, liver, kidney and spleen are normal compared to vehicle (control)	0	0
BK10008S	40	po	5 (male)	✓Change of body weight: normal ✓Organ weight: heart, liver, kidney and spleen are normal compared to vehicle (control)	0	0

[a]Administration route. [b]After dissection of all mice. [c]Sterile distilled water.

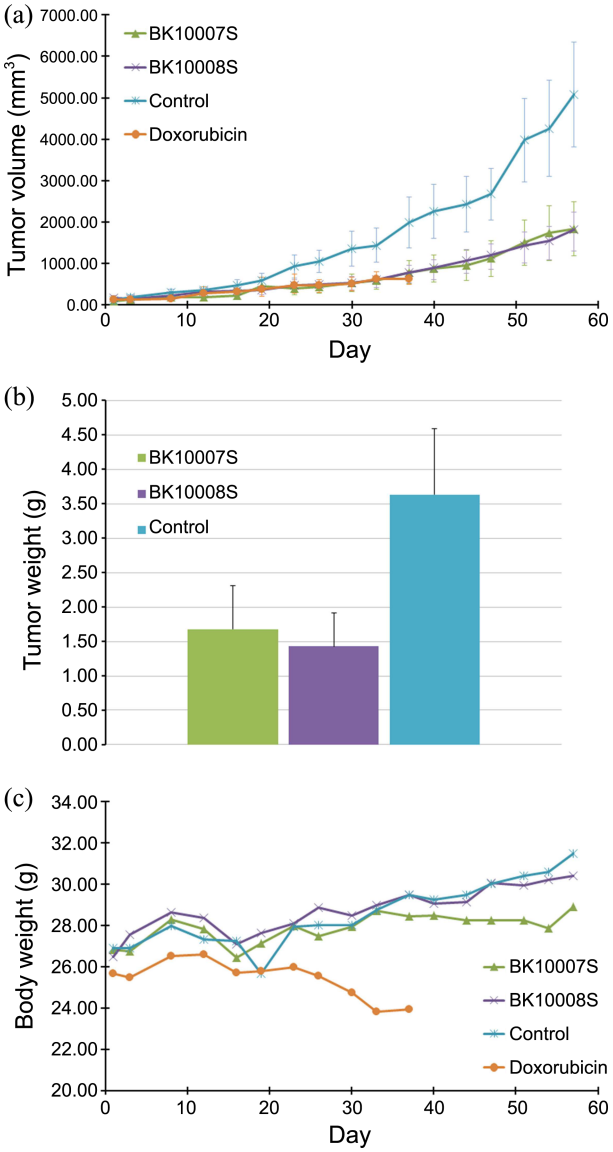


Figure 3. *Anti*-cancer effect of **BK10007S/8S** in mouse xenografts models. MIA PaCa-2 cells (5-10 × 10^7) were injected subcutaneously into the left flank of nude mice. Mice were separated into four groups (n = 6 per each group). After 14 days when tumor nodules were clearly visible, control and test compounds were administered for 57 days. All animals were euthanized after 57 days and the tumors were excised and measured. Data points represent a means ± S.E.; (a) tumor volumes in mice of each group; (b) tumor weights in mice of each group measured on the last day of the experiment; (c) body weights in mice of each group during the experiment.

growth in nude mice presented as tumor volume over time. The rates of tumor volume increment in mouse model with both **BK10007S/8S**-treated groups were remarkably slower than that of control group (vehicle only) and comparable to that of doxorubicin during 33 days. Meanwhile, all mice treated with doxorubicin were killed within 37 days after administration, which showed the high toxicity of doxorubicin. With respect to the tumor volume, **BK10007S/8S** exhibited *ca*. 2.8-fold efficacy than control at 57th day as shown in Figure 3(a). At the end of experiment, all mice were weighed and sacrificed, and their tumors were excised. Tumors were weighed and the mean tumor weight was calculated. The tumor growth inhibition rates (TGIR) were calculated as follows: Tumor growth inhibition rates (TGIR, %) = 100 × (*C*–*T*)/*C*, where *T* is the average tumor weight of the treated and *C* is the average tumor weight of the control (vehicle only). The results presented in Figure 3(b) and Table 3 showed that showed that **BK10007S/8S** through oral administration of 2 mg/kg of body weight exhibited good experimental therapeutic efficacy *in vivo* against MIA PaCa-2 xenograft in mice by 54 and 61%, respectively, compared with control alone. With respect to body weight loss related with its chronic toxicity, there was no statistical difference between average total body weights in mice treated with **BK10008S** and vehicle (control). However, **BK10007S**-treated group exhibited slightly body weight loss compared to **BK10008S**-treated group as shown in Figure 3(c), which implies relatively stronger toxicity of **BK10007S** than **BK10008S**. This body weight result is somewhat consistent with the acute toxicity data of **BK10007S/8S** above. These overall results revealed that **BK10008S** may have a tumor suppressor function in human pancreatic cancer cells and could be a promising treatment in oral pancreatic cancer chemotherapy.

Conclusions

As a continuous part of our research for *anti*-tumoral agents using T-type calcium channel blockers, two novel piperazine-containing 3,4-dihyroquinazolines (**BK10007S/8S**) were selected from the modification of our lead compound **KYS05090** and evaluated for their biological effects such as channel blocking effect, cytotoxicity, toxicity, and growth inhibition against human pancreatic MIA PaCa-2 carcinoma in BALB/c$^{nu/nu}$ nude mice. Based on these comprehensive data, **BK10008S** could be a promising chemotherapeutic

Table 3. *Anti*-cancer efficacy of compound **BK10007S/8S** against MIA PaCa-2 xenografts in nude mice[a]

Compound	Dose (mg/kg)	AR[b]	No. of animal	Sex	Tumor volume (mm^3)[c]	Tumor weight (g)[c]	Tumor growth inhibition rate (%)[d]
Control[e]	-	po	6	male	5078 ± 1275	3.63 ± 0.96	-
BK10007S	2	po	6	male	1841 ± 651	1.68 ± 0.64	54
BK10008S	2	po	6	male	1820 ± 523	1.43 ± 0.48	61
Doxorubicin[f]	2	iv	6	male	ND[g]	ND[g]	ND[g]

[a]During 57 days after administration. [b]Administration route. [c]Data are expressed as mean ± S.E.. [d]TGIR (%) = 100 × (*C*–*T*)/*C*. [e]saline for test compound. [f]10 mL/kg of doxorubicin hydrochloride in saline. [g]ND: not determined because all mice treated with doxorubicin were killed within 37 days after administration.

agent for the pancreatic cancer treatment and thus its intensive preclinical experiments are in progress using high dosage (40 mg/kg body weight) and will be announced in the future.

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