Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 16 (2006) 6001–6005
Bioorganic & Medicinal Chemistry Letters

Synthesis and biological evaluation of 5-arylamino-6-chloro-1*H*-indazole-4,7-diones as inhibitors of protein kinase B/Akt

Jong Hee Ko,[b] Seung Woo Yeon,[b] Jung Su Ryu,[b] Tae-Yong Kim,[b] Eun-Ha Song,[a] Hea-Jung You,[a] Rae-Eun Park[a] and Chung-Kyu Ryu[a,*]

[a]*College of Pharmacy, Ewha Womans University, Seodaemun-ku, Seoul 120-750, Republic of Korea*
[b]*ILDONG Research Laboratories, ILDONG Pharmaceutical Co. Ltd., 260-5, Eonnam-Dong, Kuseong-Gu, Yongin, Kyongki-Do 449-910, Republic of Korea*

Received 7 July 2006; revised 14 August 2006; accepted 30 August 2006
Available online 20 September 2006

Abstract—A series of 5-arylamino-6-chloro-1*H*-indazole-4,7-diones were synthesized and evaluated for their inhibitory activity on protein kinase B/Akt. The compounds exhibited a potent Akt1 inhibitory activity. Further mechanistic study revealed that they might have dual inhibitory effects on both activity and phosphorylation of Akt1 in PC-3 tumor cell line.

Akt (protein kinase B, PKB) is a serine/threonine kinase that promotes cellular proliferation, growth, survival, and tumor formation.[1] Akt is comprised of three mammalian isoforms, namely Akt1, Akt2, and Akt3. Akt as a downstream target of PI-3 kinase can induce a variety of biological responses. Many growth factors such as IGF-1 and PDGF bind to their receptors and lead to activation of PI-3 kinase. PI-3 kinase phosphorylates the Ptdlns to generate Ptdlns-3-phosphates, Ptdlns(3)P, Ptdlns(3, 4)P_2, and Ptndlns(3,4,5)P_3. The Ptdlns-3-phosphates cause the translocation of Akt from the cytoplasm to the plasma membrane.[1,2] Then, Akt is activated when residues Thr308 and Ser473 are phosphorylated by PDK1 and PDK2. Active Akt inhibits apoptosis and stimulates cell cycle progression by phosphorylating numerous targets in various cell types, including cancer cells. Persistent activation of Akt also occurs as a result of a deletion in tumor suppressor gene PTEN, a negative regulator of Akt.[3] Overexpression of Akt as a result of inactivation of PTEN was found in a variety of human tumors.[4] At the genomic level, Akts were amplified in many cancer types.[5] Akt1 was amplified or overexpressed in gastric adenocarcinomas, breast cancer, hepatocarcinoma, and prostate carcinoma and its activation correlates to cancer progression.[6,7] Consequently, inhibitors of the Akt1 activity could be powerful anticancer agents.[2]

We describe herein our preliminary results on synthesis of 1*H*-indazole-4,7-dione series (Fig. 1) and their Akt1 inhibitory activity. In the course of screening of 25,000 compounds offered from Korea Chemical Bank, we identified that 1*H*-indazole-4,7-diones inhibited Akt1 activity in vitro. We further synthesized structurally related derivatives to evaluate their Akt inhibitory activity and cytotoxic potential on cancer cell lines. Additional mechanistic study of Akt1 inhibitory activity by those 1*H*-indazole-4,7-diones was also performed.

A method for synthesis of 5-arylamino-6-chloro-1*H*-indazole-4,7-diones **5a**–**h** (Table 1) from commercially available 7-nitro-1*H*-indazole (**1**) is shown in Scheme 1. 7-Nitro-1*H*-indazol-4-amine (**2**) was synthesized by the amination of the indazole **1** with $HONH_2$ and

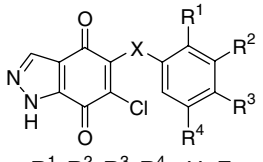
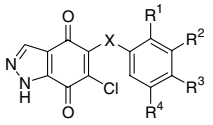


Figure 1. 1*H*-Indazole-4,7-dione derivatives.

Keywords: Akt; Inhibitor; 1*H*-indazole-4,7-dione; Antitumor.
* Corresponding author. Tel.: +82 2 3277 3027; fax: +82 2 3277 3051; e-mail: ckryu@ewha.ac.kr

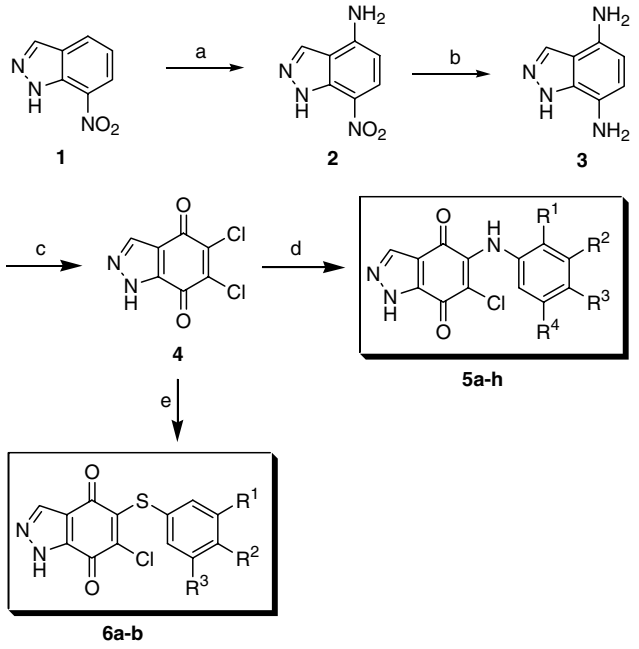
0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.08.120

Table 1. Structures and biological activities of 1*H*-indazole-4,7-diones



Compound	R^1	R^2	R^3	R^4	X	AKT1 activity[a] IC_{50} (μM)	Cytotoxicity[b] IC_{50} (μM)		
							KATOIII[c]	HepG2	PC-3
5a	F	H	H	H	NH	15.8	20.2	15.8	nt[d]
5b	H	F	H	H	NH	11.8	3.9	8.9	6.3
5c	H	H	F	H	NH	>50	61.4	37.2	nt
5d	H	F	H	F	NH	>50	75.8	37.6	nt
5e	F	H	F	H	NH	16	9.9	2.0	nt
5f	H	H	OCH_2CH_3	H	NH	4.9	9.7	11.2	nt
5g	H	H	CF_3	H	NH	12.4	13.8	9.2	13.7
5h	H	Br	H	H	NH	13.9	8.9	6.1	nt
6a	H	CH_3	H	CH_3	S	>50	>100	>100	nt
6b	H	Cl	Cl	H	S	12.3	>100	88.7	nt
3						>50	>100	>100	nt
SW[e]						25.0	21.0	nt	nt

[a] AKT1 inhibitory activity: active Akt1 proteins treated with test compounds were incubated with GSK3 fusion protein and subjected to Western blot analysis, using anti-phospho GSK3α/β antibody.
[b] Cytotoxicity evaluation: MTT colorimetric assay.[11]
[c] Tumor cell lines tested: KATOIII (gastric adenocarcinoma), HepG2 (hepatocarcinoma), and PC-3 (prostate carcinoma).
[d] nt, not tested.
[e] SWU2009: 2-thioxo-[1,3]dithiolo[4,5-β][1,4] dithiine-5,6-dicarboxylic acid dimethyl ester.[9]



Scheme 1. Synthesis of 1*H*-indazole-4,7-diones. Reagents and conditions: (a) $HONH_2$/ EtOH/KOH/5 h/60 °C; (b) H_2/PtO_2/EtOH/ RT/30 psi/4 h; (c) $NaClO_3$/HCl/1 h/65 °C/64–91%; (d) arylamine (1 equiv)/EtOH/$CeCl_3$/reflux/5 h/62–91%; (e) arylthiol (1 equiv)/EtOH/ $CeCl_3$/reflux/5 h/55–81%.

KOH in EtOH in 83% yield. Compound **2** was reduced to 1*H*-indazole-4,7-diamine (**3**) by catalytic hydrogenation. The 5,6-dichloro-1*H*-indazole-4,7-dione (**4**) was synthesized by oxidizing compound **3** with the $NaClO_3$/HCl variation in 62% yield. The 5-arylamino-6-chloro-1*H*-indazole-4,7-diones **5a**–**h** were synthesized by nucleophilic substitution of compound **4** with appropriate arylamines. Most of these substitutions went as expected and had overall high yields of 64–91%.

In similar manner, 5-arylthio-6-chloro-1*H*-indazole-4,7-diones **6a**–**b** were synthesized by reaction of the compound **4** with appropriate arylthiols.

Synthesized 1*H*-indazole-4,7-diones **5a**–**h** and **6a**–**b** were evaluated in vitro for their Akt1 inhibitory activity using a substrate phosphorylation assay. Experimental details for this procedure are cited in Ref. 8. The IC_{50} values were determined and compared to those of the positive control SWU2009 (2-thioxo-[1,3]dithiolo[4,5-β][1,4]dithiine-5,6-dicarboxylic acid dimethyl ester).[9] As shown in Table 1, many of them tested exhibited a good inhibitory activity with IC_{50} values in the low-micromolar range. In particular, compounds **5a**, **5b**, **5e**, **5f**, **5h**, and **6b** revealed potent inhibitory activity and were superior or comparable to that of SWU2009. In contrast, compounds **5c**, **5d**, and **6a** did not show any inhibitory activity up to 50 μM.

Akt1 is activated by the phosphorylation on residues Thr308 and Ser473. Active Akt1 then regulates downstream signal through the phosphorylation of cellular substrates such as GSK3α/β, Bad, p27, p21, and Par-4.[2,10] Active Akt1 proteins treated with representive compound **5b** or **5g** were incubated with a cellular substrate GSK3[10] fusion protein and subjected to Western blot analysis, using anti-phospho (Ser21/9) GSK3α/β antibody. As a result, a remarkable decrease of GSK3 phosphorylation was detected with the treatment of compound **5b** or **5g** (Fig. 2). Compounds **5b** and **5g**

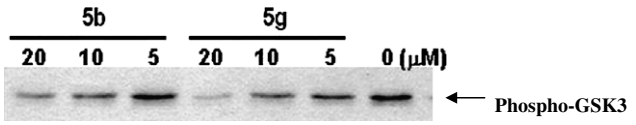


Figure 2. Inhibitory effects of compounds **5b** and **5g** on Akt1 activity in vitro. Active Akt1 proteins treated with compound **5b** or **5g** were incubated with GSK3 fusion protein and subjected to Western blot analysis, using anti-phospho (Ser21/9) GSK3α/β antibody.

inhibited in vitro Akt1 activity in a dose-dependent manner.

The cytotoxicity against tumor cell lines was also investigated because Akt1 is an anti-apoptotic protein kinase and the blockade of its activity leads to cell death. The cytotoxic potential of 1*H*-indazole-4,7-diones **5a**–**h** and **6a**–**b** against human cancer cell lines was determined according to NCI protocols.[11] The following cell lines were used: KATOIII (gastric adenocarcinoma), HepG2 (hepatocarcinoma), and PC-3 (prostate carcinoma), in which Akt1 was amplified or overexpressed.[6,7] Among them tested, compounds **5b**, **5e**, **5f**, **5g**, and **5h** exhibited a good cytotoxicity. They were identified as both Akt1 inhibitor and cytotoxic agents (Table 1). However, compound **6b** inhibited Akt1 activity but did not exhibit cytotoxicity.

Further mechanistic study on the Akt1 inhibitory activity was performed using potent compounds **5b**, **5e**, **5f**, **5g**, and **5h** in cultured PC-3 cell line.[12] We examined whether these five compounds inhibit the phosphorylation of a representative cellular substrate GSK3α/β and Akt1 itself. PC-3 cell line was treated with 10 μM each test compound up to 5 h. These lysates were subjected to Western blot analysis, using anti-phospho-GSK3α/β (Ser21/9) antibody. Among them tested, only two compounds **5b** and **5g** induced time-dependent inhibition of phosphorylation of GSK3α/β (Fig. 3). The compounds **5b** and **5g** inhibited also the phosphorylation of Akt1 at Ser473, which is required for full activation of Akt1. There were no changes of total expression levels of GSK3α/β and Akt1. These results indicate that compounds **5b** and **5g** have dual inhibitory effects on both the activity of Akt1 and the phosphorylation of Akt1. Since Akt1 is phosphorylated on residues Thr308 and Ser473 by PDKs in PI3 kinase pathways[2], activities of PDKs and related kinases may also be regulated by compounds **5b** and **5g**. Further study of 1*H*-indazole-4,7-diones on other related kinases will be designed and clarify the relationship.

Many protein kinase inhibitors act as a competitive inhibitor of ATP binding in catalytic domain. To further determine whether compounds **5b** and **5g** inhibit also Akt1 activity in ATP-dependent manner, the inhibition type was investigated. The assay was carried out by using the Western blot assay with anti-phospho-GSK3α/β antibody and GSK3 fusion protein as a substrate (Figs. 4A and B).[8] The inhibition of compounds **5b** and **5g** with respect to varying ATP concentrations yielded double reciprocal plots converging on the *y*-axis (Fig. 4). Because inhibitors **5b** and **5g** affected the K_m rather than the V_{max} of the reaction, they can be considered ATP-competitive inhibitors of Akt1.

Further evaluation of in vivo efficacy in human tumor xenograft was performed using one representive compound **5g**.[13] Akt1 is constitutively active in PC-3 prostate cancer cells due to the homozygous deletion of PTEN, which is a tumor suppressor gene.[14] Therefore, a human tumor xenograft model, PC-3 prostate cancer, was used to test the effect of Akt1 inhibitor **5g** on tumor growth in vivo, although the cytotoxicity against PC-3 cell line was similar to those against KATOIII and HepG2 cell lines. Mice were injected with 5 mg/kg/day

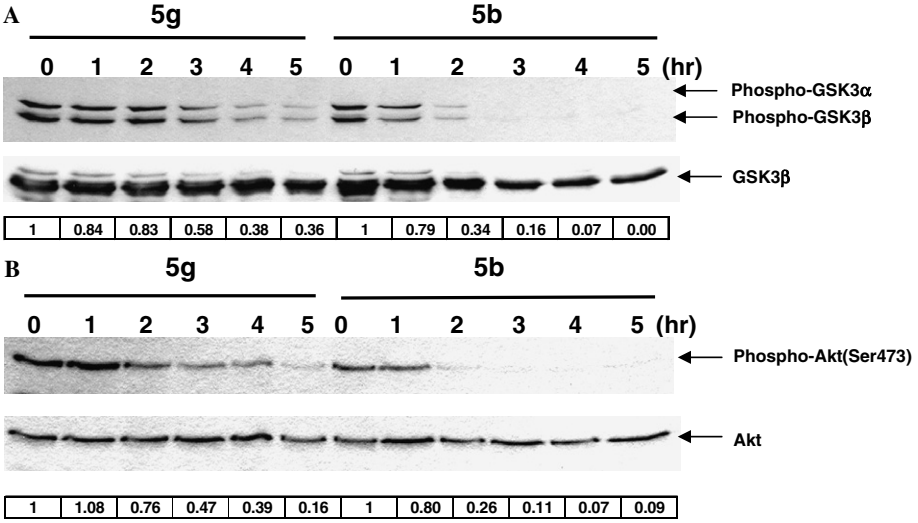


Figure 3. Effects on the phosphorylation of Akt and GSK3α/β in PC-3 cell line. PC-3 cell line was treated with 10 μM compound **5b** or **5g** up to 5 h. These lysates were subjected to Western blot analysis, using phospho-GSK3α/β (Ser21/9). (A) and phospho-Akt (Ser473). (B) Total GSK3β and Akt are shown on the bottom part of A and B, respectively. The values under each blot represent the quantification of phosphorylated-GSK3 or Akt relative to treatment with DMSO.

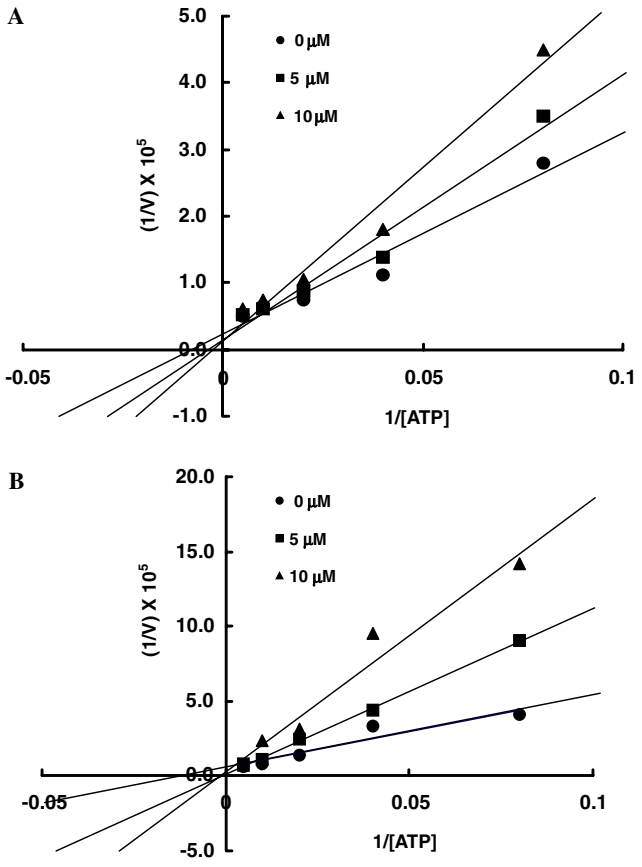


Figure 4. Double reciprocal plots of the rate of Akt1 catalytic activity against the substrate ATP. The kinase activity of Akt1 was assayed at various concentrations of ATP in the presence of increasing concentrations of compound **5g** (A) or **5b** (B). Akt activity was determined and expressed as the mean intensities measured by densitometric analysis. Data points were collected in triplicate, plotted on Lineweaver–Burk double reciprocal plots, and fitted to linear regression.

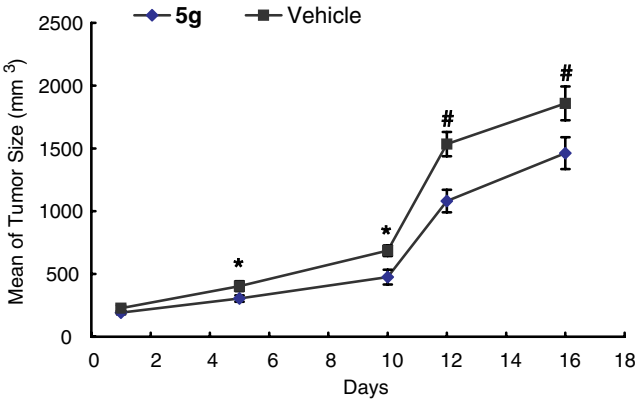


Figure 5. Antitumor efficacy of compound **5g** in sc PC-3 prostate cancer xenograft model. PC-3 cells were sc injected into nude mice. When the tumors reached an average size of about 200 mm^2, mice were treated with either vehicle (control) or 5 mg/kg/day compound **5g**. Each measurement represents an average of 6 tumors. Compound **5g** inhibits PC-3 tumor growth by 27.1% for 16 days. $^{*}p < 0.05$; $^{\#}p < 0.01$.

compound **5g** until day 16. The treatment with compound **5g** (5 mg/kg/day) led to significant antitumor effect, showing 27.1% ($p < 0.01$) inhibition of tumor growth compared to vehicle treatment at day 16 (Fig. 5). There was no difference in body weights between the treated and vehicle group throughout the treatment period. We identified that compound **5g** exhibited the Akt1 inhibitory activity and the antitumor effect.

In conclusion, 5-arylamino-6-chloro-1*H*-indazole-4,7-diones **5b** and **5g** are considered to be new class inhibitors of Akt1, and they might have dual inhibitory effects on both activity and phosphorylation of Akt1 in the PC-3 tumor cell line. Further pharmacological investigations of these 1*H*-indazole-4,7-diones and the structural optimization are in progress in our group.

Acknowledgment

This study was supported by a grant of the Korea Science and Engineering Foundation (KOSEF R06-2002-011-01004-0).

References and notes

1. Scheid, M. P.; Woodgett, J. R. *FEBS Lett.* **2003**, *546*, 108.
2. Cheng, J. Q.; Lindsley, C. W.; Cheng, G. Z.; Yang, H.; Nicosia, S. V. *Oncogene* **2005**, *24*, 7482.
3. Nicholson, K. M.; Anderson, N. G. *Cell. Signalling* **2002**, *14*, 381.
4. Vivanco, I.; Sawyers, C. *Nat. Rev.* **2002**, *2*, 489.
5. Staal, S. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5034.
6. Nakatani, K.; Thompson, D. A.; Barthel, A.; Sakaue, H.; Liu, W.; Weigel, R. J.; Roth, R. A. *J. Biol. Chem.* **1999**, *274*, 21528.
7. Goswami, A.; Ranganathan, P.; Rangnekar, V. M. *Cancer Res.* **2006**, *66*, 2889.
8. *Materials*: Anti-phospho-GSKα/β antibody, anti-phospho-Akt antibody, anti-Akt antibody, anti-GSKβ antibody, Akt1/PKB kinase, and GSK3 fusion protein were purchased from Cell Signaling Technology Inc. (Beverly, MA). KATOIII, HepG2, and PC-3 cell lines were purchased from Korean cell line bank. MTT [1-(4,5-dimethylthiazol-2-yl)-2,4-diphenyl-2*H*-tetrazolium bromide] and other reagents were purchased from Sigma and Aldrich (St. Louis, MO). *Cell culture*: The cell lines were maintained in Dulbecco's modified essential medium containing 10% heat-inactivated (30 min at 56 °C) fetal bovine serum. The cell lines were incubated at 5% CO_2 and 95% humidity in a 37 °C chamber. *Akt kinase assay in vitro*: Akt1/PKB protein kinase (10 U/μL) was incubated at 37 °C for 10 min in reaction buffer (20 mM HEPES, 14.3 mM NaCl, 1 mM EDTA, and 2 mM DTT) containing the test compounds dissolved in DMSO. This mixture was added 2 mM ATP 3 μL and GSK3 fusion protein (1 mg/mL) 1 μL and incubated at 37 °C for 30 min. The reaction was stopped by incubation for 5 min at 70 °C. This sample was separated by 12% SDS–PAGE and transferred to PVDF membranes by Western blotting or Dot blotting. After blocking by incubation in TBST (10 mM Tris–HCl, pH 7.5, 170 mM NaCl, and 0.5% Tween 20) containing 5% (w/v) skim milk for 10 min, membrane was incubated overnight at 4 °C with primary antibody (1:1000). Subsequent to washing with TBST, membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) and then washed.

Membrane was incubated with ECL (Amersham Bio., UK) substrate reagent and exposed to X-ray film. Phosphorylated GSK3 fusion protein was quantified by a densitometric analysis using ImageMaster 2D platinum v5.0 (Amersham Bio., UK). For the investigation of inhibitor type, the initial velocity v was obtained as the intensities of phosphorylated GSK3 substrates (arbitrary unit/30 min). Data points were collected in triplicate, plotted on Lineweaver–Burk double reciprocal plots, and fitted to linear regression.

9. Ko, J. H.; Yeon, S. W.; Lee, H. S.; Kim, T. Y.; Noh, D. Y.; Shin, K. S.; Hong, S. K.; Kang, S. S. *Yakhak Hoeji* **2004**, *48*, 105.
10. Ali, A.; Hoeflich, K. P.; Woodgett, J. R. *Chem. Rev.* **2001**, *101*, 2527.
11. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, T. W.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107.
12. *Western blot analysis for inhibition of Akt1 and GSK3α/β phosphorylation*: PC-3 cells (2×10^6) were incubated in 35-mm culture dishes in DEME with 10% FBS in a 5% CO_2 atmosphere at 37 °C for one day. The cells were incubated with compounds at 10 μM in DMEM up to 5 h. The culture media were aspirated, and the cells were lysed in 50 mM Tris–HCl, pH 7.6, 180 mM NaCl, 2 mM EDTA, 1% Triton X-100, protease inhibitor cocktail, and Na_3VO_4. Seventy micrograms of total cell lysates was loaded on a 12% acrylamide/bisacrylamide gel, separated by electrophoresis, and transferred to PVDF membrane, preincubated in 5% skim milk TBST for 10 min, and incubated with following antibodies against: phospho-GSK3α/β antibody, anti-phospho-Akt antibody, anti-Akt antibody, and anti-GSK3β antibody. Immunoreactive bands were detected and quantified by the same method described for the quantification of phosphorylated GSK3 fusion protein at Akt kinase assay in vitro.
13. *Evaluation of in vivo antitumor effect with human tumor xenograft*: PC-3 prostate cells were inoculated (10^6 cells/site) in the flank of male nude mice, 4 weeks of age, and mice were maintained in a pathogen-free environment. Tumors were measured twice weekly using a digital caliber, and tumor volumes were calculated using the formula $length \times (width)^2/2$. Once tumors reached 200 mm^3, mice were randomly assigned to groups ($n = 6$). Compound **5g** was dissolved in cremopore solution (ethanol 7%, cremopore 7%, and 84% distilled water) and injected iv at the dose of 5 mg/kg per mouse per 0.2 mL daily for 16 days. *Statistical analysis*: The results are shown as means ± SD of the number (n) of experiments. Statistical analysis of difference was determined by a one-way analysis of variation (ANOVA). Differences were accepted as statistically significant at a p value of $^{*}p < 0.05$ and $^{\#}p < 0.01$.
14. McMenamin, M. E.; Soung, P.; Perera, S.; Kaplan, I.; Loda, M.; Sellers, W. R. *Cancer Res.* **1999**, *59*, 4291.