

Identification of a Novel SHP-2 Protein Tyrosine Phosphatase Inhibitor

Anna Ju,[1] Huiyun Seo,[1] Heemun Kim,[1] Byoung Chul Park,[2] Sung Goo Park,[2] Jeong Hoon Kim,[2] Hyung-Kyoon Choi,[1] Kyung Hoon Min,*[1] and Sayeon Cho*[1]

[1]College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea

[2]Medical Proteomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejon 305-333, Korea

Received August 6, 2013; E-mail: sycho@cau.ac.kr, khmin@cau.ac.kr

The Src homology 2 (SH2) domain-containing phosphatase 2 (SHP-2) is a nonreceptor protein tyrosine phosphatase (PTP) involved in extracellular-regulated kinase (ERK) activation. Recent studies have shown that gain-of-function mutations in SHP-2 are associated with several diseases, including LEOPARD syndrome, Noonan syndrome, and juvenile myelomonocytic leukemia. In this study, we identified the novel SHP-2 inhibitor 3-(1-benzimidazolylmethyl)-6-*p*-tolyl-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (MLS-001). SHP-2 activity was inhibited by MLS-001, whereas other types of PTPs, namely ACP1, CDC25A, DUSP3, DUSP14, DUSP18, DUSP22, DUSP23, DUSP26, and SSH3, were not. Furthermore, TCPTP and SHP-1 that are closely related to SHP-2 were not inhibited by the inhibitor. Kinetic studies with MLS-001 and SHP-2 revealed a competitive inhibition. The SHP-2 expressing cells treated with MLS-001 demonstrated reduced SHP-2 phosphatase activity, thereby suggesting that MLS-001 effectively passes through cell membranes. In addition, MLS-001 reduced SHP-2-mediated phosphorylation in the activation loop of ERK in cells. Therefore, MLS-001 could be a lead compound for developing a potent SHP-2 inhibitor.

Most signal transductions in mammalian cells are regulated by protein phosphorylation and dephosphorylation. These reversible reactions can turn on or off biological functions, and therefore, regulate various cellular mechanisms such as growth, proliferation, and differentiation.[1] Protein phosphorylation occurs on serine, threonine, or tyrosine residues of target proteins by the action of protein kinases. Such phosphorylation induces conformational change in target molecules and subsequently regulates its activity. Among phosphorylation-mediated signaling pathways, the mitogen-activated protein kinase (MAPK) pathway is the most well-known signaling pathway that communicates a signal from the outside of cells to the gene in the nucleus.[2] The major step in the inactivation of the MAPK pathway is dephosphorylation of phosphorylated signaling proteins by protein phosphatases that are members of the protein tyrosine phosphatase (PTP) superfamily.[3] The PTP superfamily consists of over 100 enzymes and many studies have shown that modulation of PTP activities is involved in regulating diverse cellular biological functions and diseases.[4–7] Therefore, identification of chemical reagents that regulate PTP activities is important in therapeutic studies for cancer, inflammation, and other diseases.

SHP-2, which is encoded by the *PTPN11* gene, is a nonreceptor PTP that contains 2 SH2 domains.[7] SHP-2 regulates cell signaling mediated by growth factors and cytokines, such as epidermal growth factor (EGF), hepatocyte growth factor, and interleukin-6. SHP-2 is involved in activation of the Ras-Raf-Erk1/2 MAP kinase pathway by EGF.[8] Under unstimulated conditions, SHP-2 is self-inactivated by its N-terminal SH2 domain.[9] SHP-2 attaches to the membrane-bound tyrosine-phosphorylated docking proteins to become activated. In growth factor-stimulated and cytokine-stimulated cells, SHP-2 binds to Grb2-associated binder-1 (Gab1) through SH2 domains. Gab1 and SHP-2 interactions and SHP-2 activity are important in Erk1/2 activation by EGF.[10,11] The Gab1-bound SHP-2 dephosphorylates Gab1 phosphotyrosines that recruit Ras-GAP.[12] SHP-2 is also involved in promoting cell survival by mediating phosphatidylinositol 3-kinase/Akt activation by growth factors.[13]

Most mutations of SHP-2 result in enhanced activity of SHP-2 protein and are linked to childhood developmental disorders such as Noonan syndrome,[14] juvenile myelomonocytic leukemia,[15] diabetes,[16] and several types of human malignancies. Therefore, SHP-2 is an important target for regulating growth factor signaling pathways and a potential target for development of novel therapies for SHP-2-associated diseases. In this study, we identified a novel chemical compound that specifically inhibits SHP-2 phosphatase activity.

Experimental

Reagents and Antibodies. Anti-ERK and anti-phospho-ERK (phospho-Thr202 and phospho-Tyr204) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Epidermal growth factor (EGF) was purchased from Sigma-Aldrich (St. Louis, MO). Lipofectamine used for transfection into HEK 293 cells was purchased from Invitrogen (Grand Island, NY).

Purification of 6x His-Tagged Phosphatase Proteins. PTP expression plasmids were constructed in pET28a (+) (Novagen, Darmstadt, Germany) and transformed into BL21 (DE3)-RIL *E. coli*. Recombinant proteins were induced with 1 mM isopropyl β-D-thiogalactopyranoside at 37 °C, 30 or 22 °C

Published on the web December 25, 2013; doi:10.1246/bcsj.20130221

for 3 to 16 h. Cells were harvested and then lysed by sonication in 50 mM Tris-HCl (pH 8), 300 mM NaCl, 1% Tergitol-type NP-40, 1 mM phenylmethanesulfonyl fluoride (PMSF). The lysates were clarified at 11000 rpm for 30 min at 4 °C. The supernatants were applied by gravity flow to a column of Ni–NTA resin (PEPTRON, Daejon, Korea). The resin was washed with 20 mM Tris-HCl (pH 8), 500 mM NaCl, 50 mM imidazole and eluted with 20 mM Tris-HCl (pH 8), 500 mM NaCl, 200–300 mM imidazole. The eluted proteins were dialyzed overnight against 20 mM Tris-HCl (pH 8), 100 mM NaCl, 30% glycerol, 0.5 mM PMSF before storage at −80 °C.

In Vitro Phosphatase Assays and Kinetic Analysis. Activities of PTPs were measured using the substrate 3-*O*-methylfluorescein phosphate (OMFP; Sigma-Aldrich) in a 96-well microtiter plate assay. MLS-001 and OMFP were solubilized in DMSO. All reactions were performed at a final concentration of 1% DMSO. The final incubation mixtures (100 μL) were optimized for enzyme activity and composed of 30 mM Tris-HCl (pH 7), 75 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.33% bovine serum albumin (BSA), and 100 nM of PTP. Reactions were initiated by addition of 100 μM OMFP and incubated for 30 min at 37 °C. Fluorescence emission from product was measured with a multiwell plate reader (Synergy H1; excitation at 485 nm; emission at 535 nm). The reaction was linear over the time period of the experiment and was directly proportional to both enzyme and substrate concentrations. Half-maximal inhibition constant (IC_{50}) was defined as the concentration of an inhibitor that caused a 50% decrease in the PTP activity. Half-maximal inhibition constants and best curve fit for Lineweaver–Burk plots were determined by using the curve fitting program Prism 3.0 (GraphPad Software). All experiments were performed in triplicate and were repeated at least three times.

Inhibition Study. The inhibition constant (K_i) to SHP-2 for the inhibitor was determined through Michaelis–Menten enzyme analysis by measuring the initial rates at several OMFP concentrations for each fixed concentration of the inhibitor. The data were fitted to the following equation to obtain the inhibition constant of competitive inhibitors. The slopes obtained were replotted against the inhibitor concentrations. The K_i value was obtained from the slopes of these plots.

$$1/V = K_m(1 + [I]/K_i)/V_{max}[S] + 1/V_{max} \quad (1)$$

Synthesis of MLS-001. MLS-001 was synthesized by means of the synthesis pathways shown in S1 of Supporting Information.

Effects of MLS-001 on SHP-2 Phosphatase Activity in Cells. HEK 293 cells were transfected with FLAG-SHP-2 phosphatase expression plasmid. After 48 h of transfection, cells were pretreated with MLS-001 (20 μM) for 3 h. Cells were washed twice with phosphate buffered saline (PBS) buffer and lysed in PTP lysis buffer (0.5% NP-40, 0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% glycerol, 1 mM PMSF, and 1 μg mL^{-1} aprotinin) for 30 min at 4 °C. Cleared cell lysates from centrifugation were mixed with FLAG M2-agarose (Sigma-Aldrich, St.Louis, MO) and incubated for 1 h at 4 °C using a rotation device. After incubation, FLAG M2-agarose was washed three times with PTP lysis buffer and their phosphatase activities were measured.

Effects of MLS-001 on ERK Phosphorylation Mediated by SHP-2 in Cells. Transfected HEK 293 cells were treated with MLS-001 (0 and 5 μM) for 3 h. Cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, and 1 mM NaF. The samples were separated by SDS-PAGE, followed by immunoblotting analysis.

Immunoblotting Analysis. Samples were run in SDS-12% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat skim milk and incubated with an appropriate antibody, followed by incubation with secondary antibody conjugated to horseradish peroxidase. The immunoreactive bands were visualized using an ECL system (Pierce, Rockford, IL).

Results and Discussion

Owing to its role in human diseases such as neurodegeneration, cancer, and diabetes,[17] SHP-2 is a target for drug development.[18] Among the several potent SHP-2 inhibitors that have been developed so far,[19] NSC-87877 is one of the most well-known, and inhibits SHP-2 phosphatase activity with strong potency.[20] However, NSC-87877 lacks inhibition specificity as it also inhibits other phosphatases.[21–26] Therefore, it is necessary to discover novel SHP-2-specific inhibitors. In an effort to identify a novel SHP-2-specific inhibitor, we tested many organic compounds for SHP-2 inhibition by in vitro phosphatase assays using OMFP as a substrate. Of them, MLS-001 (Figure 1) inhibited SHP-2 phosphatase activity in vitro. An inhibition curve was plotted for SHP-2 and SHP-2 was inhibited with an IC_{50} value of 3.54 ± 0.61 μM (Figure 2A).

The PTP inhibitory activity of MLS-001 was then assessed against several other PTPs (ACP1, CDC25A, DUSP3, DUSP14, DUSP18, DUSP22, DUSP23, DUSP26, TCPTP, SHP-1, and SSH3) that belong to different subclasses of PTPs using in vitro phosphatase assays. Recombinant human PTPs were overexpressed in bacteria as 6 × His-tagged proteins and purified by nickel-chelate column chromatography as previously described.[27] As shown in Table 1 and Figure S2, MLS-001 did not show any inhibitory effects on other PTPs. Among those, SHP-1 and TCPTP that are closely related to SHP-2 were not also inhibited by MLS-001. Therefore, the results suggest that MLS-001 selectively inhibits SHP-2.

To study the binding mechanism of SHP-2 and MLS-001, kinetic studies based on the Michaelis–Menten equation were performed with SHP-2 and MLS-001. Kinetic studies using SHP-2 and MLS-001 revealed a competitive inhibition, thereby suggesting that MLS-001 binds to the catalytic cleft of SHP-2 (Figure 2B). The K_m value of SHP-2 phosphatase for OMFP was 179 ± 43 μM and the Lineweaver–Burk plot shows that the K_i was 5.4 ± 1.6 μM.

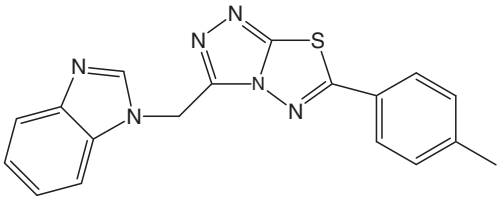

Figure 1. Chemical structure of MLS-001.

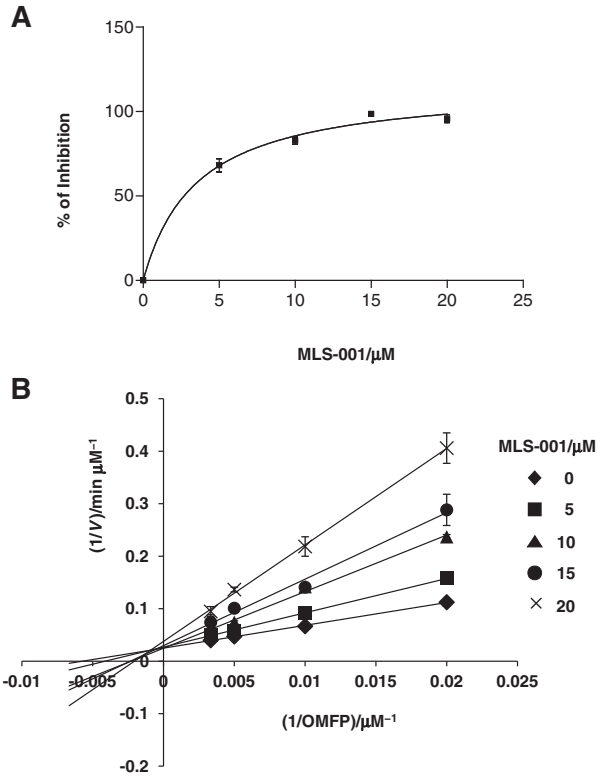

Figure 2. Inhibitory effects of MLS-001 on SHP-2 and kinetic analysis of SHP-2 inhibition by MLS-001. (A) SHP-2 (100 nM) was incubated with various concentrations of MLS-001 (0, 5, 10, 15, and 20 μM) at 37 °C for 30 min. Fluorescence emission from the product was measured using a multiwell plate reader as described in Experimental. The graph represents the percentage of inhibitory effects caused by various concentrations of MLS-001. (B) SHP-2 (30 nM) was mixed with various concentrations of MLS-001 (0, 5, 10, 15, and 20 μM) and OMFP (50, 100, 200, and 300 μM). Fluorescence emission from the product was measured every 5 min for 30 min using a multiwell plate reader as described in Experimental. Lineweaver–Burk plots of SHP-2 were generated from the reciprocal data.

We next examined whether MLS-001 could inhibit SHP-2 in cells. After HEK 293 cells were transfected with a FLAG-tagged SHP-2 expression plasmid, the transfected cells were incubated with or without MLS-001 prior to cell lysis. SHP-2 was immunoprecipitated from the cell lysates using anti-FLAG M2-agarose, and the SHP-2 phosphatase activity was determined using OMFP as a substrate (Figure 3A). The results showed that MLS-001 effectively penetrated the cell membrane and inhibited SHP-2 activity. However, treatment of cells with 20 μM MLS-001 reduced SHP-2 activity by 30%, which indicates that the inhibitory effect of MLS-001 on SHP-2 in cells was somewhat lower than that obtained in vitro. One possible explanation is that the bound MLS-001 dissociates from SHP-2 during immunoprecipitation. Another possibility is that MLS-001 may have inefficient cell permeability. It is also possible that SHP-2 expressed in mammalian cells is less sensitive to MLS-001 because of protein modification. To rule out the possibility that mammalian cell-expressed SHP-2 is less sensitive to MLS-001, SHP-2 was immunoprecipitated from transfected cell lysates and used for in vitro phosphatase assays in the presence of 10 μM MLS-001. In vitro incubation with MLS-001 reduced the SHP-2 activity by 65%, which was similar to that of SHP-2 purified from bacteria (Figure 3B). We further examined whether MLS-001 is able to inhibit SHP-2-mediated ERK activation in cells. To this end, we measured the in vivo phosphorylation levels of ERK in HEK 293 cells treated with 5 μM of MLS-001 for 3 h. Immunoblotting analyses of cell lysates using an anti-phospho-ERK antibody showed that the phosphorylation level of the endogenous ERK was reduced in the presence of MLS-001 (Figure 3C). Taken together, these results suggest that MLS-001 effectively passes through cell membranes and inhibits SHP-2 activity to reduce SHP-2-mediated ERK phosphorylation.

Table 1. Selective Inhibition of PTPs by MLS-001[a)]

PTPs	IC_{50}/μM ($n = 3$)
ACP1	≫25
CDC25A	≫25
DUSP3	≫25
DUSP14	≫25
DUSP18	≫25
DUSP22	≫25
DUSP23	≫25
DUSP26	≫25
SSH3	≫25
TCPTP	≫25
SHP-1	≫25
SHP-2	3.54 ± 0.61

a) Inhibition of enzyme activity by MLS-001 was measured using the PTPs: ACP1, CDC25A, DUSP3, DUSP14, DUSP18, DUSP22, DUSP23, DUSP26, SSH3, TCPTP, SHP-1, and SHP-2. Each experiment was performed in triplicate. The in vitro phosphatase assay was performed as described in Experimental. Data are presented as mean ± standard error of the mean. The IC_{50} value of SHP-2 determined by this experiment was 3.54 ± 0.61 μM ($n = 3$) whereas other PTPs were not significantly inhibited.

Conclusion

The results of our study suggest that MLS-001 is a potent competitive inhibitor of SHP-2. SHP-2 plays important roles in signaling pathways and its mutation is related to several human diseases such as Noonan syndrome, juvenile myelomonocytic leukemia, and several types of human malignancies. Our results showed that MLS-001 selectively inhibits SHP-2 activity in a dose-dependent manner. In vitro PTP assays showed that MLS-001 has strong inhibitory effects on SHP-2, which were significantly greater than that observed using 12 recombinant PTPs from different classes. Although significant progress has been made in the past years, signaling mechanisms are still not completely understood because of lack of selective SHP-2 inhibitors. Better understanding of the role of SHP-2 may provide new insights into the pathogenesis of SHP-2 mutation-associated human diseases. Therefore, MLS-001 could be used as a therapeutic reagent in various SHP-2-related diseases or as a lead compound for developing better SHP-2 inhibitors.

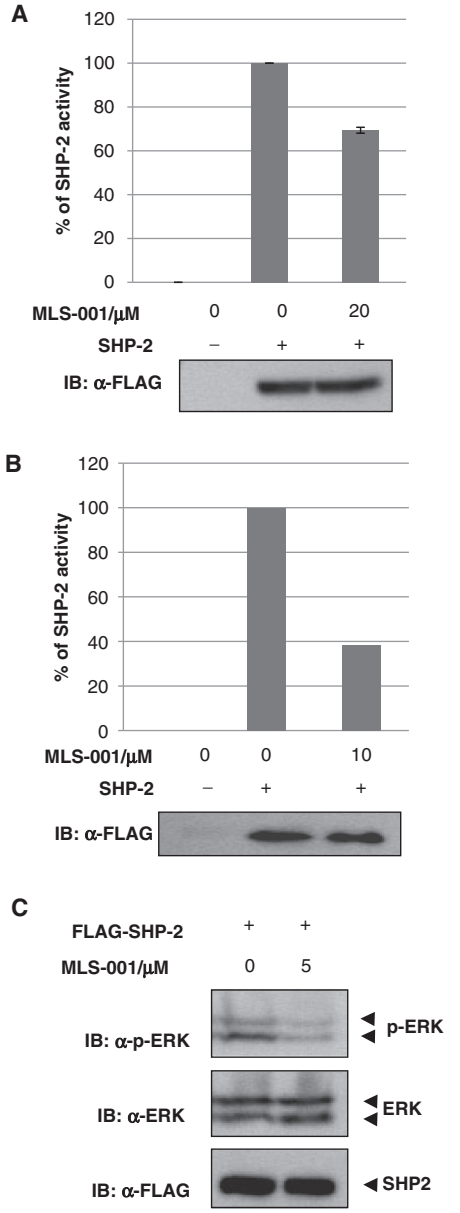

Figure 3. Inhibition of SHP-2 expressed in HEK 293 cells by MLS-001 in vitro and in vivo. (A) HEK 293 cells were transfected with 1 μg of the SHP-2 expression plasmid. After 48 h of transfection, the cells were treated with MLS-001 (0 or 20 μM) for 3 h. The cell lysates were immunoprecipitated with anti-FLAG M2 agarose beads for 1 h at 4 °C. The bound SHP-2 phosphatase activity was measured using the substrate OMFP (30 μM) for 30 min at 37 °C. (B) The SHP-2 expression plasmid (1 μg)-transfected HEK 293 cells were harvested and lysed and the lysates were immunoprecipitated as described above. The bound SHP-2 was incubated with MLS-001 (0 or 10 μM) and the substrate OMFP (30 μM) for 30 min at 37 °C. Fluorescence emissions from the product were measured using a multiwell plate reader as described in Experimental. (C) HEK 293 cells were transfected with 1 μg of SHP-2 expression plasmid. After 48 h of transfection, cells were pretreated with MLS-001 (0 or 5 μM) for 3 h. Cell lysates were analyzed by immunoblotting with appropriate antibodies as described.

This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Science, ICT & Future Planning (Nos. NRF-2011-0030029 and NRF-2012R1A2A2A01047338).

Supporting Information

Figures show the synthesis of MLS-001 and inhibition assay data with other PTPs. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

References

1 M. Camps, A. Nichols, S. Arkinstall, *FASEB J.* **2000**, *14*, 6.

2 G. L. Johnson, R. Lapadat, *Science* **2002**, *298*, 1911.

3 A. Alonso, J. Sasin, N. Bottini, I. Friedberg, I. Friedberg, A. Osterman, A. Godzik, T. Hunter, J. Dixon, T. Mustelin, *Cell* **2004**, *117*, 699.

4 J. N. Andersen, P. G. Jansen, S. M. Echwald, O. H. Mortensen, T. Fukada, R. Del Vecchio, N. K. Tonks, N. P. H. Møller, *FASEB J.* **2004**, *18*, 8.

5 M. Jacobsen, D. Schweer, A. Ziegler, R. Gaber, S. Schock, R. Schwinzer, K. Wonigeit, R.-B. Lindert, O. Kantarci, J. Schaefer-Klein, H. I. Schipper, W. H. Oertel, F. Heidenreich, B. G. Weinshenker, N. Sommer, B. Hemmer, *Nat. Genet.* **2000**, *26*, 495.

6 E. Kawasaki, J. C. Hutton, G. S. Eisenbarth, *Biochem. Biophys. Res. Commun.* **1996**, *227*, 440.

7 M. Adachi, M. Ishino, T. Torigoe, Y. Minami, T. Matozaki, T. Miyazaki, T. Taniguchi, Y. Hinoda, K. Imai, *Oncogene* **1997**, *14*, 1629.

8 T. B. Deb, L. Wong, D. S. Salomon, G. Zhou, J. E. Dixon, J. S. Gutkind, S. A. Thompson, G. R. Johnson, *J. Biol. Chem.* **1998**, *273*, 16643.

9 P. Hof, S. Pluskey, S. Dhe-Paganon, M. J. Eck, S. E. Shoelson, *Cell* **1998**, *92*, 441.

10 J. M. Cunnick, S. Meng, Y. Ren, C. Desponts, H.-G. Wang, J. Y. Djeu, J. Wu, *J. Biol. Chem.* **2002**, *277*, 9498.

11 B. G. Neel, H. Gu, L. Pao, *Trends Biochem. Sci.* **2003**, *28*, 284.

12 A. Montagner, A. Yart, M. Dance, B. Perret, J.-P. Salles, P. Raynal, *J. Biol. Chem.* **2005**, *280*, 5350.

13 C.-J. Wu, D. M. O'Rourke, G.-S. Feng, G. R. Johnson, Q. Wang, M. I. Greene, *Oncogene* **2001**, *20*, 6018.

14 M. Bentires-Alj, J. G. Paez, F. S. David, H. Keilhack, B. Halmos, K. Naoki, J. M. Maris, A. Richardson, A. Bardelli, D. J. Sugarbaker, W. G. Richards, J. Du, L. Girard, J. D. Minna, M. L. Loh, D. E. Fisher, V. E. Velculescu, B. Vogelstein, M. Meyerson, W. R. Sellers, B. G. Neel, *Cancer Res.* **2004**, *64*, 8816.

15 M. Tartaglia, C. M. Niemeyer, K. M. Shannon, M. L. Loh, *Curr. Opin. Hematol.* **2004**, *11*, 44.

16 M. G. Myers, Jr., R. Mendez, P. Shi, J. H. Pierce, R. Rhoads, M. F. White, *J. Biol. Chem.* **1998**, *273*, 26908.

17 Z. Z. Chong, K. Maiese, *Histol. Histopathol.* **2007**, *22*, 1251.

18 K. Hellmuth, S. Grosskopf, C. T. Lum, M. Würtele, N. Röder, J. P. von Kries, M. Rosario, J. Rademann, W. Birchmeier, *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 7275.

19 H. R. Lawrence, R. Pireddu, L. Chen, Y. Luo, S.-S. Sung, A. M. Szymanski, M. L. R. Yip, W. C. Guida, S. M. Sebti, J. Wu, N. J. Lawrence, *J. Med. Chem.* **2008**, *51*, 4948.

20 L. Chen, S.-S. Sung, M. L. R. Yip, H. R. Lawrence, Y. Ren, W. C. Guida, S. M. Sebti, N. J. Lawrence, J. Wu, *Mol. Pharmacol.*

2006, *70*, 562.
21 S.-J. Park, M.-A. Song, S.-Y. Cho, *Bull. Korean Chem. Soc.* **2009**, *30*, 3098.
22 M.-N. Song, S.-Y. Cho, *Bull. Korean Chem. Soc.* **2009**, *30*, 1858.
23 M.-N. Song, S.-Y. Cho, *Bull. Korean Chem. Soc.* **2009**, *30*, 1190.
24 M.-N. Song, S.-Y. Cho, *Bull. Korean Chem. Soc.* **2009**, *30*, 924.
25 M. Song, J. E. Park, S. G. Park, D. H. Lee, H.-K. Choi, B. C. Park, S. E. Ryu, J. H. Kim, S. Cho, *Biochem. Biophys. Res. Commun.* **2009**, *381*, 491.
26 M.-N. Song, S.-J. Park, S.-Y. Cho, *Bull. Korean Chem. Soc.* **2009**, *30*, 236.
27 H.-Y. Seo, H.-M. Kim, S.-Y. Cho, *Bull. Korean Chem. Soc.* **2011**, *32*, 1379.