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Discovery of 3-(2-aminoethyl)-5-(3-phenyl-propylidene)-thiazolidine-2,4-dione as a dual inhibitor of the Raf/MEK/ERK and the PI3K/Akt signaling pathways

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ABSTRACT

A thiazolidine-2,4-dione derivative, 3-(2-aminoethyl)-5-(3-phenyl-propylidene)-thiazolidine-2,4-dione (**2**), was identified as a dual inhibitor of the Raf/MEK/ extracellular signal-regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K)/Akt signaling cascades. The discovered compound inhibited cell proliferation, induced early apoptosis, and arrested cells in G_0/G_1 phase in human leukemia U937 cells. These results indicate its potential as a new lead compound to develop novel dual signaling pathway inhibitors and anticancer agents.

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Dysregulated signaling pathways have been implicated to promote cancer cell survival and growth, in which the Raf/MEK/extracellular signal-regulated kinase (ERK) cascade and the phosphatidylinositol 3-kinase (PI3K)/Akt cascade are the best characterized. The Raf/MEK/ERK pathway is one of the evolutionarily conserved mitogen-activated protein kinase (MAPK) pathways that play critical roles in driving proliferation and preventing apoptosis.[1] Upon activation by growth factors, serum, cytokines and osmotic stresses, ERK can phosphorylate and regulate multiple substrates such as cytoskeletal proteins, kinases and transcription factors within various cellular compartments.[2] These events in turn result in gene expression changes and alteration in cell proliferation, differentiation and survival. This pathway has received particular attention in the past 15 years as substantial evidence has shown that aberrant activation of this pathway at different levels is involved in the oncogenesis of various human cancers, especially in melanoma, breast cancers, ovarian cancers and human leukemias.[3,4] Numerous structurally diverse molecules have been developed by targeting the Raf/MEK/ERK pathway in search for potential medications for various human cancers and have been extensively reviewed in recent articles.[3,5,6]

PI3K/Akt signaling pathway is another signaling cascade that has been implicated to be crucial in cancer development. Genomic aberrations in this pathway are prevalent compared to any other pathway in human cancers with the possible exception of the p53 and retinoblastoma pathway.[7] Upon stimulation by growth factors and cytokines, PI3K is recruited to the plasma membrane and subsequently converts phosphatidylinositol-3,4-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3) that will in turn recruits and activates a serine/threonine kinase Akt together with 3′-phosphoinositide-dependent kinase (PDK). Signals through this cascade regulate many fundamental cellular functions such as cell growth, proliferation, survival, apoptosis, and metabolism through a variety of downstream effectors including proapoptotic and antiapoptotic factors, mTOR, glycogen synthase kinase-3 (GSK-3), and p53, among others.[7–9] Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a negative regulator of PI3K/Akt signaling by specifically dephosphorylating PIP3 has been detected to lose its activity by either genetic or epigenetic modifications in many primary and metastatic human cancers.[9,10] Mutations and/or activation of PI3K and Akt have been detected in various human cancers.[9,11,12] Therefore, it is logical to target this pathway to develop potential treatment agents, and indeed small molecule inhibitors including PI3K inhibitors, Akt inhibitors and mTOR inhibitors have been developed and/or approved as treatment agents for various human cancers.[13]

Notably, these two signaling pathways intimately and cooperatively link with each other, rather than exclusively, to regulate apoptosis and the survival of transformed cells.[4,14] Both signaling pathways can phosphorylate and regulate many common downstream effectors involved in the regulation of cell survival and

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apoptosis such as CREB, Bad, Bim and caspase 9, among others.[4] Accumulating evidence has strongly suggested crosstalk and the possible existence of a feedback regulation loop between these two pathways. For example, most recently, studies have demonstrated the activation of Raf/MEK/ERK cascade upon the treatment with mTOR inhibitor in patients with metastatic cancers as well as in cancer cell lines and prostate cancer animal model, which strongly suggests feedback regulation loops in and crosstalk between the Raf/MEK/ERK and PI3K/Akt cascades.[15] This phenomenon may contribute to drug resistance to inhibitors targeting single cascade. Another elegant study also supported this notion by showing frequent activation of Raf/MEK/ERK and PI3K/Akt cascades in advanced human prostate cancer.[16] More importantly, several elegant studies have demonstrated synergistic effects in triggering cancer cell death by concomitant interruption of these two pathways both in vitro and in vivo,[15–19] which indicates that more clinically beneficial pharmacotherapies may be obtained by co-targeting these two pathways simultaneously. However, to our knowledge, all the combined targeted therapies use a mixture of two individual inhibitors for the Raf/MEK/ERK and PI3K/Akt pathways and no single small molecule has been reported or developed to inhibit these two pathways simultaneously. The use of dual pathway inhibitors may provide certain advantages over single pathway inhibitors in the following aspects: (1) enhanced potency and reduced risk of drug resistance; (2) reduced toxicity and improved patient compliance. Thus, the design and development of such dual inhibitors would provide the cancer research community with novel chemical tools and potential newer anticancer agents.

Molecules containing the thiazolidine-2,4-dione moiety such as the anti-diabetic drug troglitazone have been recently reported to have anticancer activities through inhibition of the Raf/MEK/ERK signal cascade.[20] In our effort to design and discover novel templates targeting the Raf/MEK/ERK signaling cascade, we have embarked on development of the thiazolidine-2,4-dione derivatives as potential substrate-specific ERK inhibitors.[21] From our recent studies, we discovered that the structural extension of benzylidene in compound **1** to alkylidene shifted the biological target from ERK to their upstream activators. Herein, we report the discovery of a new thiazolidine-2,4-dione compound, 3-(2-aminoethyl)-5-(3-phenyl-propylidene)-thiazolidine-2,4-dione **2**, as a novel lead structure for developing dual pathway inhibitors of the Raf/MEK/ERK and PI3K/Akt pathways and anticancer agents (Fig. 1).

Compound **2** contains several structural features that may contribute to its functional activities, such as the phenylpropylidene double bond which can act as a Michael addition reaction acceptor, the primary amine in the ethylamine tail for ionic interactions, and the aromatic ring for hydrophobic interactions. Therefore, compounds **3**, **4**, and **5** were designed along with **2** to shed light on its potential binding interaction features. The synthesis of **2** and its analogs began with the synthesis of thiazolidine-2,4-dione intermediates **7**. As shown in Scheme 1, alkylation of thiazolidine-2,4-dione **6** with Boc protected 2-bromoethaneamine afforded **7**, which on Knoevenagel condensation reaction with propionaldehyde or 3-phenylpropionaldehyde to afford **2** or **3**, respectively.[22] Reduction of **2** under catalytic hydrogenation conditions yielded saturated analog **4** in good yield. Acetylation of **2** with acetic anhydride afforded compound **5**.

The activation of the Raf/MEK/ERK pathway and the PI3K/Akt pathway has been shown to play multiple important roles in the proliferation and apoptosis of hematopoietic cells.[4] Furthermore, it has been shown that interruption of this process by MEK and Akt inhibitors leads to a dramatic increase in mitochondrial damage and apoptosis in human leukemia cells. Additionally, human leukemia cells have been shown to be good models to assay new potential ERK inhibitors in our laboratory.[20] Therefore, human leukemia U937 cells were initially employed to evaluate **2** and its analogs for their inhibitory effects on cell viability with the MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay. The known MEK inhibitor PD184352 was used as positive control.[23] As shown in Figure 2A, **2** exhibited significant inhibition on U937 cell viability at higher concentrations (15, 20, and 25 μM) but not at concentrations less than 10 μM. PD184352 and **2** showed comparable potencies at 15, 20, and 25 μM concentrations. Notably, compounds **3**, **4**, and **5** did not show any inhibitory effects at tested concentrations, which may suggest that the phenylpropylidene, primary amine and aromatic ring are important structural determinants for activities (Fig. 2B). The inhibitory effects of **2** were further confirmed in multiple cancer cell lines including OVCAR5, SKOV3, PC3, DU145, and HT-29 cells (data not shown). The MTS assay only examines the metabolically healthy cells, but cannot distinguish whether the cells are actively dividing or quiescent. In contrast, the [^{3}H]-thymidine incorporation assay assesses actively dividing cells in a sample by detecting DNA synthesis. Therefore, **2** and PD184352 were further evaluated using [^{3}H]-thymidine incorporation assays in U937 cells. As shown in Figure 2C, at 10 μM, **2** significantly inhibited the [^{3}H]-thymidine incorporation, while PD184352 did not. At 30 μM, **2** almost completely inhibited [^{3}H]-thymidine incorporation, and PD184352 also exhibited significant inhibition at this concentration. Taken together, these results may indicate that **2** may induce early apoptosis of U937 cells through inhibiting DNA synthesis at 10 μM but not affecting cell viability.

To get insight into the signaling pathways that are possibly involved in **2**'s functional activities, Western blot analysis was then performed in U937 cells. As shown in Figure 3A, PD184352 significantly inhibited the phosphorylation of both ERK and its downstream substrate Rsk1 at as low as 3 μM concentration. Compound **2** also significantly inhibited the phosphorylation of

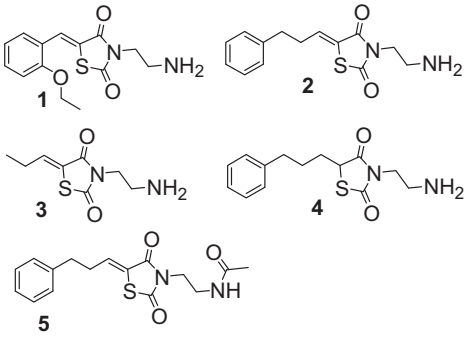
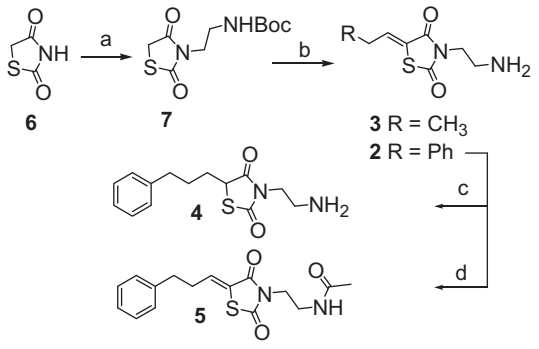


Figure 1. Chemical structures of designed compounds.



Scheme 1. Synthesis of designed compounds. Reagents: (a) $BocNHCH_2CH_2Br$, TBAI, acetone; (b) (i) RCHO, piperidine, EtOH; (ii) HCl/dioxane; (c) H_2, Pd–C, MeOH; (d) Ac_2O, CH_2Cl_2.

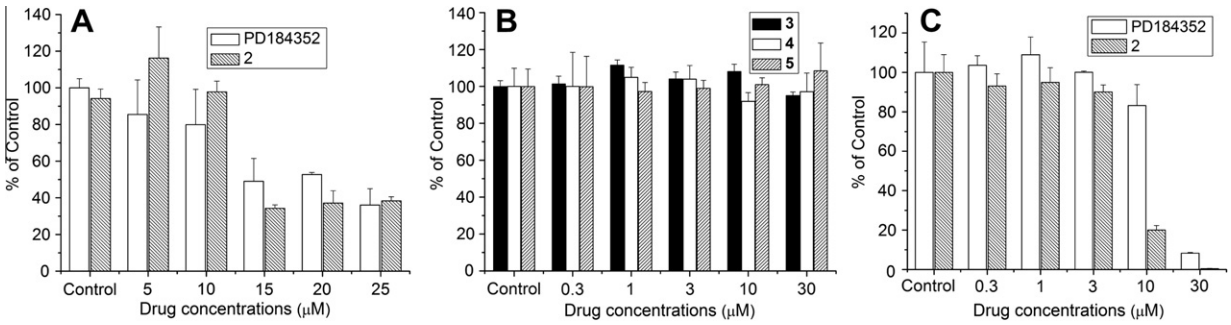


Figure 2. Inhibition of cell proliferation using MTS assay (A and B) and [^{3}H]-thymidine incorporation (C) in human leukemia U937 cells.

ERK and Rsk1 at 10 and 30 μM concentrations, therefore being slightly less potent than PD184352. However, when the p-MEK level was evaluated, it is notable that **2** dose-dependently decreased the p-MEK level in U937 cells while treatment with PD184352 resulted in a dose-dependent increase in the p-MEK levels, which is consistent with the reported negative feedback mechanism in the Raf/MEK/ERK pathway.[24] This might indicate that **2** targets either an upstream activator of MEK in the Raf/MEK/ERK signaling pathway or it inhibits MEK via a different mechanism. When compounds **3**, **4**, and **5** were evaluated, no inhibitory effects on p-MEK and p-ERK were observed (Fig. 3B), which is consistent with their activities in cell viability assays. These limited structure–activity relationship (SAR) data further highlight the importance of the phenylpropylidene, the primary amine, and the aromatic ring for activity.

To further evaluate whether other signaling pathways that are involved in apoptosis and survival regulation are affected by **2**, we next examined the levels of p-Akt, p-p38 and p-JNK upon treatment with PD184352 and **2** in U937 cells. Notably, **2** at 25 μM significantly and consistently suppressed the p-Akt level while the specific MEK inhibitor PD184352 exhibited no inhibitory effects on p-Akt (Fig. 3C). Surprisingly, PD184352 increased the total Akt level at tested concentrations. Compound 2 also increased the total Akt level at tested concentrations except for 25 μM. At this stage, it is not clear at what level (PI3K or PDK) **2** targets the PI3K/Akt cascade to reduce the p-Akt level. Further structural modifications of **2** in our laboratory have demonstrated consistent p-MEK and p-Akt suppression and studies have been undertaken to identify target protein(s), to elucidate the mode of action, and to increase its potencies. Furthermore, both **2** and PD184352 exhibited minimal

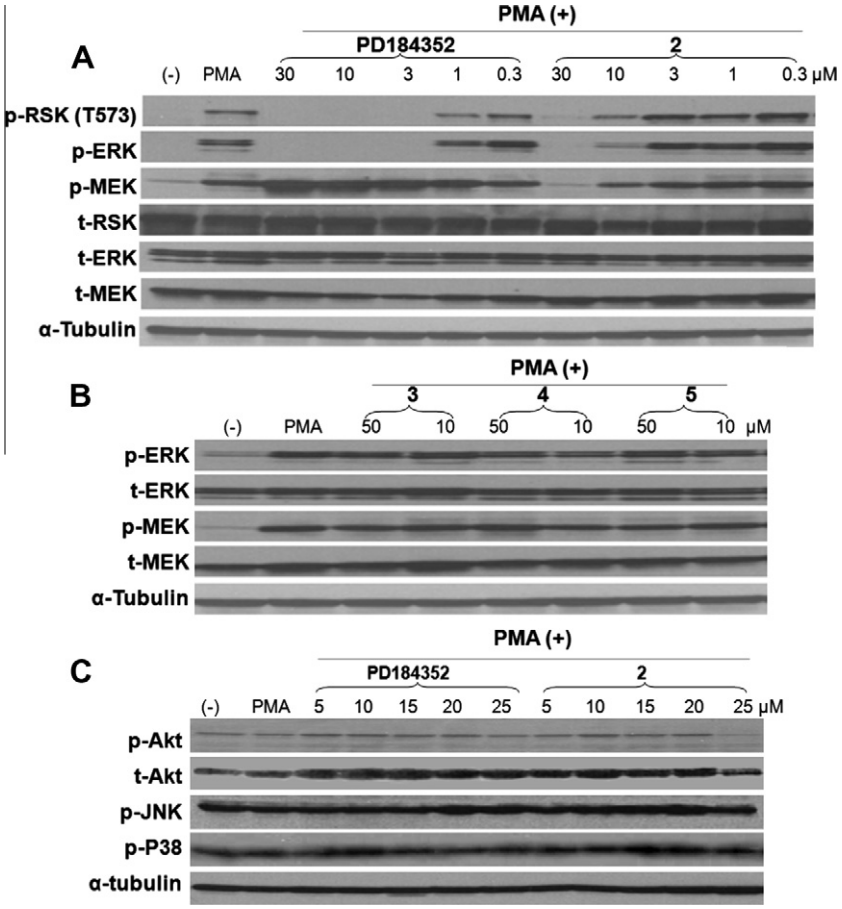


Figure 3. Western blot analysis of compound **2** and its analogs in U937 cells.

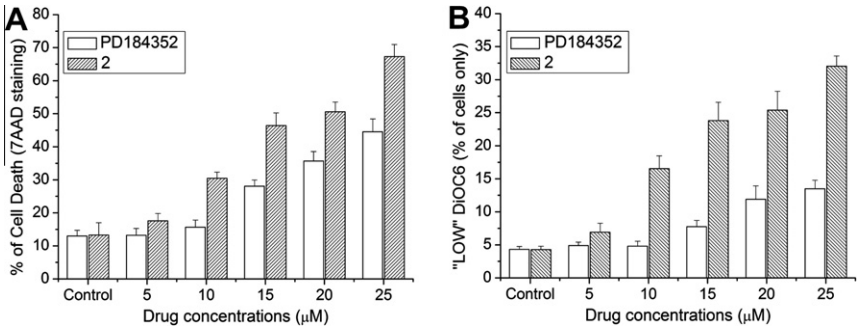


Figure 4. Apoptotic effects of compound **2** and PD184352 on U937 cells.

effects on the level of p-p38 and p-JNK. These results may indicate that **2** has specific dual inhibition towards the Raf/MEK/ERK and the PI3K/Akt signaling pathways. Further studies are warranted to evaluate its target specificity.

The Raf/MEK/ERK cascade and the PI3K/Akt cascade have been demonstrated to play important roles in the regulation of apoptosis.[4] In addition, mitochondria have been shown to play an important role in cell death and the loss of mitochondria membrane potential is an early event in mitochondrially mediated apoptosis.[25] Therefore, we next studied the effects of **2** in inducing apoptosis in U937 cells using 7-aminoactinomycin D (7-AAD) combined with the 3,3′-dihexyloxocarbocyanine iodide ($DiOC_6$) uptake to measure the extent of mitochondria membrane potential change ($\Delta\psi_m$) in U937 cells by flow cytometry.[26,27] As shown in Figure 4A and B, **2** at 10 μM moderately induced U937 cell death (17.2%) and slightly increased the number of U937 cells exhibiting mitochondria membrane potential loss (13%) indicating early apoptosis induction. However, **2** exhibited significant inhibitory effects on DNA synthesis without affecting the cell viability in U937 cells at this concentration (Fig. 2A and C). This may indicate that the lethal effects induced by **2** at this concentration (Fig. 4A) are mainly through early apoptosis while preserving the metabolic functions of U937 cells. At higher concentrations (15, 20 and 25 μM), **2** dose-dependently increased both cell death and the number of cells exhibiting mitochondria membrane potential loss in U937 cells. Together with the results from MTS assay (Fig. 2A), these results indicate that **2** may inhibit U937 cell proliferation through both apoptotic and necrotic pathways. On the other hand, PD184352 only exhibited moderate lethal effects on U937 cells in 7-AAD assay and minimal effects on mitochondria membrane potential loss at higher concentrations (15, 20, 25 μM), which suggests that PD184352 inhibits U937 cell growth mainly through necrotic mechanism. The superior effects of **2** in inducing apoptosis as compared to PD184352 in U937 cell under these experimental conditions might be due to its dual inhibition of the Raf/MEK/ERK and PI3K/Akt pathways compared to PD184352's inhibition of only the Raf/MEK/ERK pathway.

Both Raf/MEK/ERK pathway and PI3K/Akt pathway have been shown to regulate the G_1–S and G_2–M transition in the cell cycle.[28,29] Since **2** demonstrated dual inhibition of these two signal-

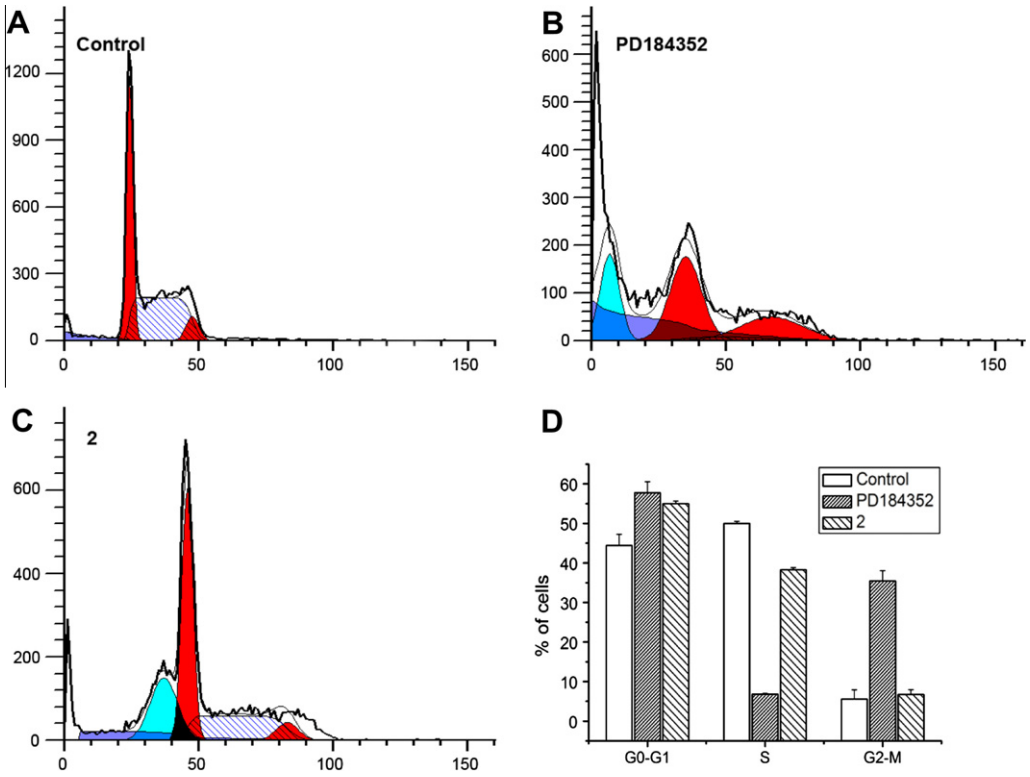


Figure 5. Effects of compound **2** and PD184352 (25 μM) on U937 cell cycle.

ing pathways, growth inhibition of various cancer cells, and the induction of apoptosis in U937 cells, it would be of value to evaluate whether it has effects on the cell cycle of U937 cells. As shown in Figure 5, treatment of U937 cells with PD184352 (25 μM) for 24 h arrested U937 cells at both G_0/G_1 (13.9% increase) and G_2/M (23.1% increase), an event accompanied with significant decrease of the S phase population (37% decrease). However, treatment of U937 cells with **2** (25 μM) only moderately increased G_0/G_1 population (11% increase) and decreased S population (11.8% decrease). There are no effects on G_2/M population. Again, the differential effects exhibited by PD184352 and **2** in cell cycle may be due to their different inhibitory effects on the PI3K/Akt signaling cascade since both of them have similar effects on the Raf/MEK/ERK signaling pathway at this concentration. Further studies such as evaluation of the levels of cyclins, p21 and p27 are warranted to better understand the mechanism.

In summary, compound **2**, a thiazolidine-2,4-dione analog, was identified to inhibit cancer cell proliferation, induce apoptosis, and moderately arrest U937 cells at G_0/G_1 phase. The functional activities of **2** are associated, at least partially, with its dual inhibition of the Raf/MEK/ERK and PI3K/Akt pathways as demonstrated by Western blot analysis. Given the synergistic effects in inducing apoptosis and inhibiting cancer cell growth by the combination of Raf/MEK/ERK and PI3K/Akt signaling pathway inhibitors, the results of **2** suggest its translational potential as a novel lead structure to develop small molecule dual inhibitors of the Raf/MEK/ERK and PI3K/Akt pathways as potential anti-cancer agents. Further studies are being undertaken in our laboratory to identify the potential biological targets of **2** and to understand the mechanism of apoptosis induction and cell cycle interference.

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Supplementary data

Supplementary data (details for synthetic procedures, analytical data, and biological studies for compounds **2**–**5**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.030.

References and notes

1. Schubbert, S.; Shannon, K.; Bollag, G. *Nat. Rev. Cancer* **2007**, *7*, 295.
2. Yoon, S.; Seger, R. *Growth Factors* **2006**, *24*, 21.
3. Roberts, P. J.; Der, C. J. *Oncogene* **2007**, *26*, 3291.
4. Steelman, L. S.; Abrams, S. L.; Whelan, J.; Bertrand, F. E.; Ludwig, D. E.; Basecke, J.; Libra, M.; Stivala, F.; Milella, M.; Tafuri, A.; Lunghi, P.; Bonati, A.; Martelli, A. M.; McCubrey, J. A. *Leukemia* **2008**, *22*, 686.
5. Wong, K. K. *Recent Pat. Anti-Cancer Drug Discovery* **2009**, *4*, 28.
6. Sebolt-Leopold, J. S. *Clin. Cancer Res.* **2008**, *14*, 3651.
7. Hennessy, B. T.; Smith, D. L.; Ram, P. T.; Lu, Y.; Mills, G. B. *Nat. Rev. Drug Disc.* **2005**, *4*, 988.
8. Marone, R.; Cmiljanovic, V.; Giese, B.; Wymann, M. P. *Biochim. Biophys. Acta* **2008**, *1784*, 159.
9. Paz-Ares, L.; Blanco-Aparicio, C.; Garcia-Carbonero, R.; Carnero, A. *Clin. Transl. Oncol.* **2009**, *11*, 572.
10. Parsons, D. W.; Wang, T. L.; Samuels, Y.; Bardelli, A.; Cummins, J. M.; DeLong, L.; Silliman, N.; Ptak, J.; Szabo, S.; Willson, J. K.; Markowitz, S.; Kinzler, K. W.; Vogelstein, B.; Lengauer, C.; Velculescu, V. E. *Nature* **2005**, *436*, 792.
11. Jucker, M.; Sudel, K.; Horn, S.; Sickel, M.; Wegner, W.; Fiedler, W.; Feldman, R. A. *Leukemia* **2002**, *16*, 894.
12. Chin, Y. R.; Toker, A. *Cell. Signalling* **2009**, *21*, 470.
13. Ghayad, S. E.; Cohen, P. A. *Recent Pat. Anti-Cancer Drug Discovery* **2010**, *5*, 29.
14. Gao, H.; Ouyang, X.; Banach-Petrosky, W. A.; Gerald, W. L.; Shen, M. M.; Abate-Shen, C. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 14477.
15. Carracedo, A.; Ma, L.; Teruya-Feldstein, J.; Rojo, F.; Salmena, L.; Alimonti, A.; Egia, A.; Sasaki, A. T.; Thomas, G.; Kozma, S. C.; Papa, A.; Nardella, C.; Cantley, L. C.; Baselga, J.; Pandolfi, P. P. *J. Clin. Invest.* **2008**, *118*, 3065.
16. Kinkade, C. W.; Castillo-Martin, M.; Puzio-Kuter, A.; Yan, J.; Foster, T. H.; Gao, H.; Sun, Y.; Ouyang, X.; Gerald, W. L.; Cordon-Cardo, C.; Abate-Shen, C. *J. Clin. Invest.* **2008**, *118*, 3051.
17. She, Q. B.; Solit, D. B.; Ye, Q.; O'Reilly, K. E.; Lobo, J.; Rosen, N. *Cancer Cell* **2005**, *8*, 287.
18. Lambert, P. J.; Shahrier, A. Z.; Whitman, A. G.; Dyson, O. F.; Reber, A. J.; McCubrey, J. A.; Akula, S. M *Expert Opin. Ther. Targets* **2007**, *11*, 589.
19. Rahmani, M.; Anderson, A.; Habibi, J. R.; Crabtree, T. R.; Mayo, M.; Harada, H.; Ferreira-Gonzalez, A.; Dent, P.; Grant, S. *Blood* **2009**, *114*, 4507.
20. Motomura, W.; Tanno, S.; Takahashi, N.; Nagamine, M.; Fukuda, M.; Kohgo, Y.; Okumura, T. *Biochem. Biophys. Res. Commun.* **2005**, *332*, 89.
21. Li, Q.; Al-Ayoubi, A.; Guo, T.; Zheng, H.; Sarkar, A.; Nguyen, T.; Eblen, S. T.; Grant, S.; Kellogg, G. E.; Zhang, S. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6042.
22. Momose, Y.; Maekawa, T.; Yamano, T.; Kawada, M.; Odaka, H.; Ikeda, H.; Sohda, T. *J. Med. Chem.* **2002**, *45*, 1518.
23. Sebolt-Leopold, J. S.; Dudley, D. T.; Herrera, R.; Van Becelaere, K.; Wiland, A.; Gowan, R. C.; Tecle, H.; Barrett, S. D.; Bridges, A.; Przybranowski, S.; Leopold, W. R.; Saltiel, A. R. *Nat. Med.* **1999**, *5*, 810.
24. Dougherty, M. K.; Muller, J.; Ritt, D. A.; Zhou, M.; Zhou, X. Z.; Copeland, T. D.; Conrads, T. P.; Veenstra, T. D.; Lu, K. P.; Morrison, D. K. *Mol. Cell.* **2005**, *17*, 215.
25. Johnson, B. W.; Boise, L. H. *J. Biol. Chem.* **1999**, *274*, 18552.
26. Nguyen, T. K.; Rahmani, M.; Harada, H.; Dent, P.; Grant, S. *Blood* **2007**, *109*, 4006.
27. Wang, Z.; Van Tuyle, G.; Conrad, D.; Fisher, P. B.; Dent, P.; Grant, S. *Cancer Res.* **1999**, *59*, 1259.
28. Meloche, S.; Pouyssegur, J. *Oncogene* **2007**, *26*, 3227.
29. Chang, F.; Lee, J. T.; Navolanic, P. M.; Steelman, L. S.; Shelton, J. G.; Blalock, W. L.; Franklin, R. A.; McCubrey, J. A. *Leukemia* **2003**, *17*, 590.