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Structure–activity relationship (SAR) studies of 3-(2-amino-ethyl)-5-(4-ethoxy-benzylidene)-thiazolidine-2,4-dione: Development of potential substrate-specific ERK1/2 inhibitors

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ABSTRACT

A series of analogs of 3-(2-amino-ethyl)-5-(4-ethoxy-benzylidene)-thiazolidine-2,4-dione, a putative substrate-specific ERK1/2 inhibitor, were synthesized and biologically characterized in human leukemia U937 cells to define its pharmacophore. It was discovered that shift of ethoxy substitution from the 4- to the 2-position on the phenyl ring significantly improved functional activities of inhibiting cell proliferation and inducing apoptosis. This may provide access to a new lead for developing ERK1/2 substrate-specific inhibitors.

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The Raf/MEK/Extracellular Signal-Regulated Kinase (ERK1/2) pathway is one of the evolutionarily conserved mitogen-activated protein kinase (MAPK) pathways that play critical roles in driving proliferation and preventing apoptosis. Signal transduction through this pathway is regulated by a three-tiered sequential phosphorylation cascade composed of MAPK (ERK1/2), MAPK kinase (MEK1/2) and MAPK Kinase kinase (A-raf, B-raf, or Raf-1).[1] Upon activation, ERK1/2 phosphorylates multiple substrates within various cellular compartments, which in turn results in gene expression changes and alterations in cell proliferation, differentiation and survival.[2] Substantial evidence has validated that deregulation of this pathway is involved in the oncogenesis of various human cancers.[3,4] For example, B-Raf mutations are found in about 66% of malignant melanomas and at lower frequency in many other human malignancies including ovarian cancer.[3–8] Recently, it has also been reported that a high frequency of acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL) display constitutive activation of the Raf/MEK/ERK1/2 pathway.[8–10] Therefore, this pathway represents an attractive target for development of potential anti-cancer drugs. Indeed, numerous structurally diverse small molecule inhibitors, such as Raf and MEK inhibitors, have been developed-with Sorafenib being the most successful.

While small molecule kinase inhibitors have emerged as powerful tools and promising targeted cancer therapeutic approaches, a continuing challenge faced with the majority of current inhibitors is the lack of specificity to their targets.[11] On the other hand, protein–protein interactions have been highlighted as powerful means in coordinating signaling processes.[12] Thus, novel substrate-specific kinase inhibitors that disrupt protein–protein interactions would overcome the lack of specificity available when targeting the highly conserved ATP binding sites. ERK1/2 represents a near optimal target to develop such inhibitors given the following facts: (1) The recent identification of the docking groove composed of a common docking (CD) domain and an ED domain on ERK2 (Fig. 1) and the validation of their critical roles in controlling docking interactions with substrate proteins;[13–16] (2) the important position of ERK1/2 in the Raf/MEK/ERK1/2 pathway that controls the distribution of signals from upstream; and (3) the recent disclosure of the crystal structures of ERK2.[17,18] Recently, Shapiro's group has identified several such inhibitors by computer-aided virtual screening of a small library of compounds (Fig. 2).[19,20]

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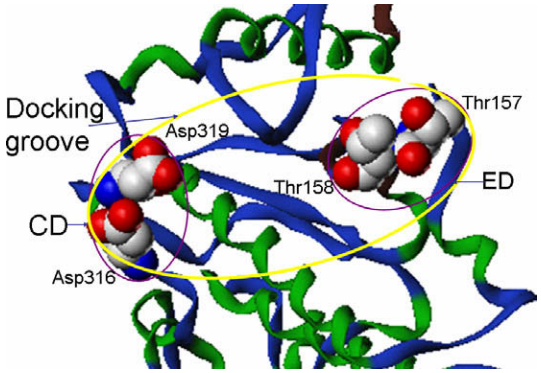
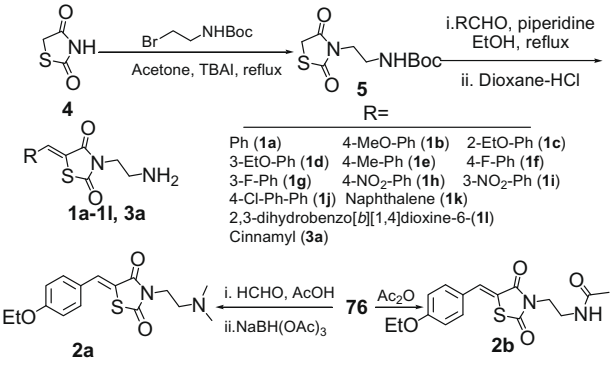


Figure 1. CD and ED domains on ERK2.



Scheme 1. Synthesis of **1a–1l**, **2a, 2b** and **3a**.

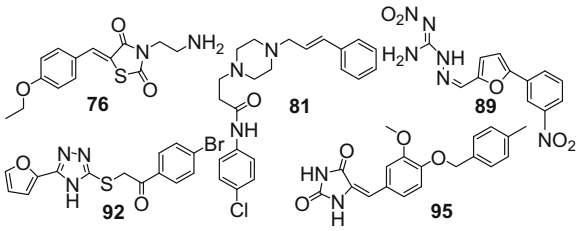


Figure 2. Putative substrate-specific ERK1/2 inhibitors.

Among these structures, compound **76** represents an interesting chemical scaffold. Compound **76** inhibited the phosphorylation of Rsk1 and Elk-1, the downstream substrates of ERK1/2, in HeLa cells with minimal effects on the phosphorylation of ERK1/2. It also inhibited the growth and survival of several cancer cell lines. In addition, small molecules containing the thiazolidine-2,4-dione moiety, such as the anti-diabetic drug troglitazone, have been recently reported to have anti-cancer activities.[21–23] Thus, these results demonstrated the potential of compound **76** as a new chemotype to develop substrate-specific ERK1/2 inhibitors. Because compound **76** was discovered as a lead through screening, little information about the pharmacophore of compound **76** is known and no further effort to modify **76** to improve its bioactivity has been reported. In this study, we initiated preliminary structure–activity relationship (SAR) studies of compound **76** to define its pharmacophore and to search for more potent and selective substrate-specific ERK1/2 inhibitors.

As shown in Figure 3, we mainly modified **76** in three domains: the phenyl ring, the ethylamine tail, and the spacer domain between the phenyl ring and thiazolidine-2,4-dione. In the first case, functional groups of varied lipophilicity, size and electronic properties were introduced in the phenyl ring to evaluate these effects on biological activities (**1a**–**1l**). Next, dimethylated and acetylated ethylamine tails were designed to evaluate whether a basic primary amine is needed for activity (**2a, 2b**). Finally, the aforementioned spacer was extended by two carbon atoms to gain insight into the influence of molecular size on activity (**3a**). Furthermore, we introduced two additional hydroxyl groups on this extended analog to evaluate whether introduction of hydrogen bond interactions at this domain can improve selectivity and potency (**3b, 3c**). The synthetic protocol for **1a**–**1l** is shown in Scheme 1. Briefly, N-alkylation of thiazolidine-2,4-dione (**4**) with Boc-protected 2-bromoethylamine provided **5**, which on Knoevenagel condensation with various aldehydes followed by Boc deprotection afforded **1a–1l** and **3a** in good yields.[24] Reductive amination of **76** with formaldehyde gave the dimethylated analog **2a**. Acetylation of **76** with acetic anhydride yielded **2b**. The synthesis of **3b** and **3c** is shown in Scheme 2. Dihydroxylation of **6** with AD-mix-α and AD-mix-β gave **7** and **8**, respectively. Protection of these two hydroxyl groups followed by diisobutylaluminium hydride (DIBALH) reduction provided **9** and **10**, respectively. Knoevenagel condensation followed by deprotection afforded **3b** and **3c**.

After synthesis, all of the compounds were initially evaluated for their abilities to inhibit phosphorylation of Rsk1 and ERK1/2 by immunoblot analysis in human U937 leukemia cells under the stimulation of phorbol 12-myristate 13-acetate (PMA) (Supplementary data S3). Then, the active compounds were further tested for relative selectivity by measuring their effects on phosphorylation of Rsk1, ERK1/2, Elk-1, MEK and p38 (Fig. 4). The parent compound **76** reduced the phosphorylation of Rsk1 at 50 μM with minimal effects on the phosphorylation of ERK1/2, which is consistent with the reported results.[19,20] Substitution with F, NO_2 at the 4- and 3-positions on the phenyl ring (**1f–1i**) abolished the inhibition of Rsk1 phosphorylation indicating that electron withdrawing substituents are not favored at these two positions. However, 4-Cl substitution (**1j**) exhibited similar effects as **76** (Fig. 4). The results from **1a**, **1b**, **1d** and **1e** clearly indicate that steric effects and an

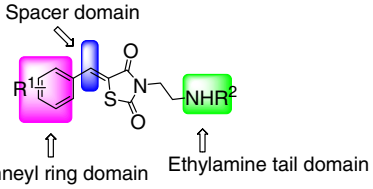
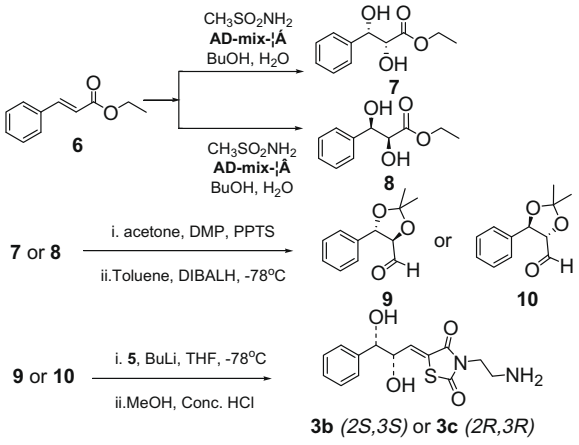


Figure 3. Structural modifications of compound **76**.



Scheme 2. Synthesis of **3b** and **3c**.

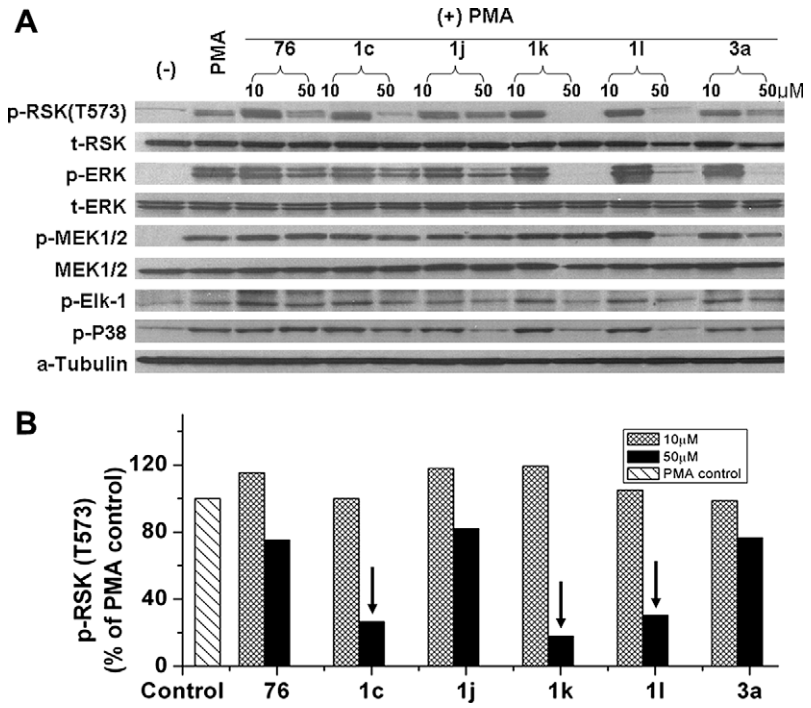


Figure 4. Effects of test compounds on Rsk1, ERK1/2, Elk-1, MEK1/2 and p38 phosphorylation (A: immunoblot results; B: quantification of Rsk1 phosphorylation by densitometry) in U937 cells.

oxygen atom at the 4-position are important factors for activity since none of them showed inhibition activities (Supplementary data S3). N-Dimethylation and N-acetylation (**2a** and **2b**) also abolished the inhibition of Rsk1 phosphorylation indicating that the primary amine is essential to biological activity. The spacer extended analog **3a** significantly inhibited phosphorylation of both Rsk1 and ERK1/2 at 50 μM (Fig. 4). Surprisingly, it also significantly inhibited the phosphorylation of MEK1/2 at this concentration, thus indicating that **3a** may target upstream activating kinases of ERK1/2 such as MEK and Raf-1. These results also demonstrate that the benzylidene moiety of **76** is necessary for its interaction with ERK1/2 and molecular size is an important structural determinant for target specificity. This speculation is further supported by **1k** (a structurally constrained analog of **3a**) and **1l** that both showed similar activity profiles as **3a**. Interestingly, **1k** and **1l** showed better inhibition of Elk-1 phosphorylation at 50 μM than **3a**. In addition, **1k** and **1l** exhibited inhibition for p38 phosphorylation at 50 μM while **3a** did not. This further indicates the importance of molecular size on target specificity. Saturation of the extra double bond of **3a** and introduction of two addition hydroxy groups led to **3b** and **3c**. These structural modifications almost completely abolished activities for Rsk1 and ERK1/2 except for weak inhibition of Rsk1 phosphorylation at 50 μM by **3b** (Supplementary data S3). Thus, conjugation is especially important for the biological activity of **3a**. It is notable that the shift of the ethoxy substitution from the 4- to the 2-position on the phenyl ring (**1c**) exhibited better inhibition of Rsk1 phosphorylation than **76** at both 10 and 50 μM, had minimal effects on the phosphorylation of ERK1/2 and enhanced inhibition on Elk-1 phosphorylation at 50 μM (Fig. 4). This may lead to improved functional activity of **1c** and suggest options for optimizing selectivity. Compounds **76** and **1c** did not show any inhibition for p38, MEK and JNK (data not shown) indicating that they primarily target ERK1/2. Similar effects of phosphorylation of ERK1/2 and its downstream targets were observed after treatment of ovarian cancer cells (OVCAR5 and SKOV3) with the test compounds prior to stimulation with epidermal growth factor (EGF) (data not shown). To further prove that **76** and **1c** do not affect the catalytic activity of ERK1/2, in vitro cell free nonspecific substrate myelin basic protein (MBP) phosphorylation assays were performed using active recombinant ERK2 (Fig. 5). As expected, **1c** and **76** did not affect ERK1/2 catalytic activities. Taken together, these results strongly suggest that **1c** may serve as a new lead structure and that modification at the 2-position and the adjacent heterocycle ring (such as fused ring analogs) may lead to new candidate compounds with improved potency and selectivity profile.

To test whether the immunoblot analysis results correlate with their functional activities, the active compounds were further evaluated for their effects on cell viability and proliferation in U937 cells with the MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay and incorporation of [^{3}H]-thymidine assay, respectively (Fig. 6). MTS assay assesses the metabolic activity as an indicator of healthy

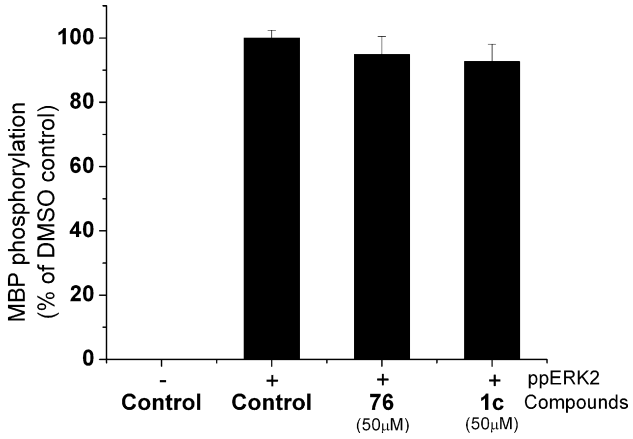


Figure 5. Effects of **76** and **1c** on MBP phosphorylation using active recombinant ERK2.

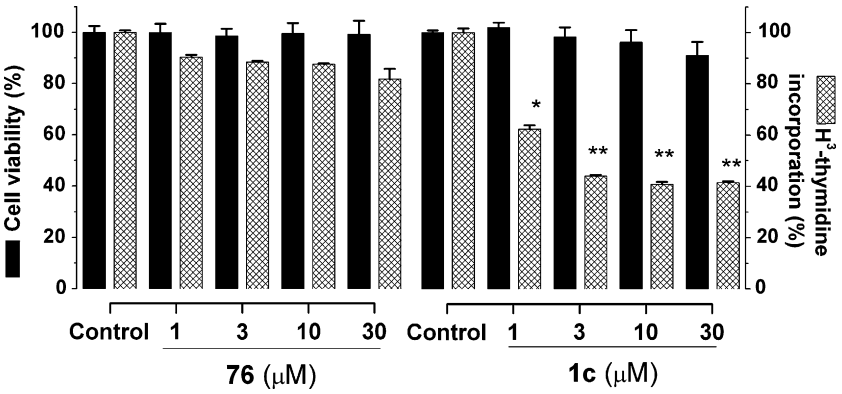


Figure 6. Effects of **76** and **1c** on the viability (MTS, closed bars) and proliferation ([^{3}H]-thymidine incorporation, dense bars) of U937 cells. Each bar represents the mean ± SEM of at least three different experiments. $^{*}P$ <0.05 or $^{**}P$ <0.01 indicates significant differences from control group.

cells. But this assay cannot distinguish whether the cells are actively dividing or quiescent. On the other hand, [^{3}H]-thymidine incorporation assay will assess actively dividing cells in a sample by detecting DNA synthesis. The evaluation of active compounds in these two assays might shed light on the possible mechanism of actions. All compounds tested (measured by MTS assay and up to 30 μM) showed no effects on U937 cell viability. In the original report, **76** dose-dependently inhibited cell growth of various cancer cell lines in a colony formation assay. This discrepancy might be caused by the different assays (MTS) and cancer cells (U937) used in this study. Interestingly, **1c** dose-dependently reduced the incorporation of [^{3}H]-thymidine. These results suggest that **1c** is able to reduce the proliferation rate without significant effects on cell viability possibly due to its enhanced inhibition of Elk-1 and Rsk1 phosphorylation. Again, the potential of **1c** to serve as a new lead structure is evident.

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.[25,26] To investigate whether the active analogs induce early apoptotic effects, the mitochondria membrane potential change ($\Delta\psi_m$) in U937 cells was measured using the 3,3′-dihexyloxocarbocyanine iodide ($DiOC_6$) uptake assay. As shown in Figure 7, only **1c** dose-dependently increased the number of cells exhibiting mitochondria membrane potential loss, which suggests inductive effects of **1c** on early apoptosis of U937 cells. Compounds **1k** and **1l** both exhibited stronger inhibition of ERK1/2 and Rsk1 phosphorylation than **1c**, but they, especially **1k**, exhibited minimum effects on mitochondria membrane potential. This might be due to their inhibitory effects on the phosphorylation of p-38 (Fig. 4) and JNK (data not shown), that may play opposite roles in regulating apoptosis and survival.

Since **1c** retained activity as a putative ERK1/2 substrate-specific inhibitor like **76**, but showed better functional activities, it would be of interest to understand the nature of the interactions of these two compounds with ERK2. **76** and **1c** were docked into the docking groove of active ERK2 (PDB code: 2ERK) as described by Hancock et al.[19] using GOLD 3.0[27] (Fig. 8). The docking results suggest that **1c** retains the ionic interactions with Asp316 and Asp319 and the π-cation interactions with Arg133 are also the same as **76**. An additional hydrogen bond interaction was observed between the 2-ethoxy group and Asn80 that may contribute to its better Elk-1 inhibition and functional activities in cell proliferation and apoptosis assays. Additional studies such as X-ray crystallography are needed to verify these predicted interactions.

In summary, a series of analogs of compound **76** with different substitutions on the phenyl ring, modified ethylamine tail and extended spacer between phenyl ring and thiazolidine-2,4-dione were synthesized to conduct SAR studies and to define the pharmacophore of **76**. The immunoblot analysis assay of ERK1/2 down-

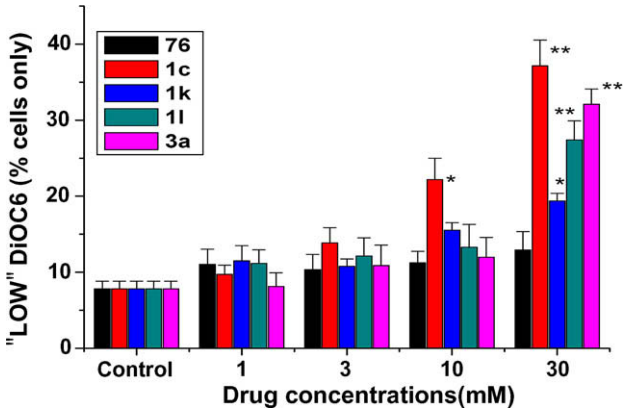


Figure 7. Effects of active compounds on the mitochondria membrane potential loss ($\Delta\psi_m$) of U937 cells. Each bar represents the mean ± SEM of at least three different experiments. $^{*}P$ <0.05 or $^{**}P$ <0.01 indicates significant differences from control group.

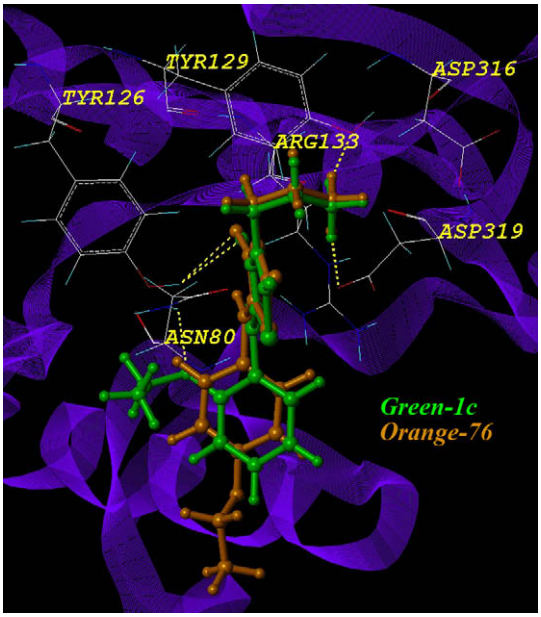


Figure 8. Predicted binding of **76** and **1c** to active ERK2.

stream substrate phosphorylation established that an oxygen atom attached at 4-position of phenyl ring is important for its activity. Steric effects in the phenyl ring domain are important factors in determining target specificity since introduction of bulky moieties or extension of the spacer between the phenyl ring and thiazolidine-2,4-dione changes the targets, possibly upstream of ERK1/2. We also discovered that the shift of 4-ethoxy substitution from the 4-position to the 2-position on the phenyl ring of **76** can enhance Rsk1 and Elk-1 phosphorylation inhibition activity. More importantly, **1c** has no effects on the phosphorylation of ERK1/2 and their catalytic activities. Cell-based in vitro assays further demonstrated that **1c** can reduce the proliferation of U937 cells without effects on cell viability and also induces mitochondria membrane potential loss in a dose-dependent manner. These results strongly encourage further investigation of **1c** and analogs to develop more potent substrate-specific ERK1/2 inhibitors as chemical probes and potential anti-cancer agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.09.057.

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