

Identification of coumarin derivatives as a novel class of allosteric MEK1 inhibitors

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Abstract—A homogenous TR-FRET-based in vitro coupling assay for the MAP3Ks–MEK1–ERK2 kinase cascade was established and was used to screen for inhibitors of the ERK/MAPK pathway. A series of coumarin derivatives were identified from the screen. These compounds potently inhibit the activation of the *unactivated* human MEK1 by upstream MAP3Ks (including BRAF and COT), but do not inhibit the activity of the *activated* MEK1. In addition, the potency of these compounds in inhibiting MEK1 activation is not affected by varying the ATP concentration, suggesting that these inhibitors are not competitive with ATP. As expected, the coumarin compounds potently inhibit LPS-induced TNF α production and ERK phosphorylation in THP-1 cells, with the most potent compound having an IC₅₀ of 90 nM. Molecular modeling studies suggest that these coumarins bind to an allosteric site in the inactive conformation of MEK1. This site has been shown to be utilized by the biarylamine series of MEK inhibitors such as PD318088. Very interestingly, the identified coumarin derivatives are almost identical to a series of inhibitors recently reported that block LPS-induced TNF α production. Our findings have therefore raised the possibility that other naturally occurring or synthetic coumarins with anti-cancer and anti-inflammatory activities might exert their biological function through the inhibition of MEK1. © 2005 Elsevier Ltd. All rights reserved.

The coumarin-based natural products comprise a large class of substances found in a variety of sources, especially in green plants.¹ Natural and synthetic coumarin derivatives have been shown to possess a diverse array of pharmacological and biochemical properties. The anti-coagulation and anti-thrombotic activities of the coumarin derivative such as warfarin are well known.² Other activities such as anti-HIV and lipid-lowering effect have also been reported recently.^{3–6} However, the most widely reported activities for coumarin derivatives are their anti-inflammatory and anti-cancer activities. For example, cloricromene, a semi-synthetic coumarin derivative, has been shown to inhibit TNF α production and protect against collagen-induced arthritis and DNB-colitis in animal models.^{7–9} Other coumarin derivatives such as those isolated from *P. pabularia* and the 7-carbamate-substituted coumarins have also been shown to inhibit TNF α production.^{10,11} Coumarin derivatives with anti-cancer activities include aromatase inhibitors,

carbonic anhydrase inhibitors, and steroid sulfatase inhibitors.^{12–14} Other coumarin-based anti-cancer compounds include the naturally occurring GUT-70 from *C. brasiliense*,¹⁵ 7-isopentenylcoumarin from *H. lanatum*,¹⁶ 5-oxygenated-6,7-methylenedioxy coumarins from *P. polystachyum*¹⁷ as well as the synthetic coumarin derivatives such as 7-hydroxycoumarin, 6-nitro-7-hydroxycoumarin, coumarin 3-(*N*-aryl) sulfonamides, and 3-bromophenyl 6-acetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate.^{18–21} The mechanism of action of these compounds remains to be identified. We present here studies which indicate that the anti-inflammatory and/or anti-cancer activity of certain coumarin derivatives could be due to the inhibition of MEK1 kinase.

The mitogen-activated protein kinase (MAPK) family consists of evolutionarily conserved signaling proteins that regulate a variety of cellular activities such as cell proliferation, mitosis, cell movement, metabolism, and apoptosis. In multi-cellular organisms, there are at least four well-conserved subfamilies of MAP kinases: the extracellular-signal regulated kinase (ERK) family, the stress-activated protein kinases/c-Jun NH2-terminal kinase (SAPK/JNK) family, the p38MAPK family,

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and the ERK5/big MAP kinase 1 (BMK1) family.^{22,23} All these MAPK cascades contain three sequentially activated protein kinases (MAP3K, MAP2K, and MAPK) in which the downstream kinases are activated by the upstream kinases through the phosphorylation in the activation loop.

The ERK/MAPK pathway plays an important role in cell proliferation and cytokine production, and therefore is the focus for the development of novel anti-cancer and anti-inflammatory agents. In this pathway, RAFs (a-Raf, b-Raf, and c-Raf), COT (also known as TPL-2), and MOS are the known MAP3Ks that directly phosphorylate and activate MEK1/2.^{24–28} MEK1/2 in turn activates ERK1/2. Several highly specific allosteric MEK1/2 inhibitors such as PD98059, U0126, and PD184352 have been identified in recent years.^{29–32} These compounds inhibit the activation of MEK1/2 by upstream MAP3Ks but do not (or weakly) inhibit the activity of the activated MEK1/2.³³ All these known allosteric MEK inhibitors inhibit the MEK activation in an ATP-non-competitive fashion. The crystal structure of the human MEK1/2 complexed with the biarylamine-based compounds (PD184352 derivatives) demonstrated that these compounds bind to an allosteric site adjacent to the ATP binding pocket, explaining the ATP-non-competitive nature of these compounds.³⁴ In addition to the MEK inhibitors, compounds targeting other members of this pathway (e.g., RAF, COT, and ERK) are also in various stages of development.^{35,36}

In order to identify novel inhibitors of the ERK/MAPK pathway, we developed a TR-FRET-based *in vitro* coupling assay for this pathway using a commercially

available Eu-labeled anti-phospho-Ser/Thr antibody (AD0176, PerkinElmer) that can specifically recognize a phosphorylated ERK substrate peptide (Fig. 1A). Compared with a published scintillation proximity assay (SPA) for RAF*–MEK–ERK assay,³⁷ the TR-FRET assays are non-radioactive and can be easily miniaturized.

Using this antibody and the ERK substrate peptide, a TR-FRET-based activity assay for ERK, and coupling assays for MEK and the MAP3Ks (RAF, COT) were then established (Fig. 1B). In the ERK assay, activated ERK2 was used directly to phosphorylate the peptide substrate. In the MEK assay (MEK1*–ERK2 coupling), activated MEK1 was used to phosphorylate and activate the unactivated ERK2. In the COT (COT*–MEK–ERK coupling) and RAF (BRAF*–MEK–ERK coupling) assays, the MAP3Ks were used to activate the unactivated MEK1. Activated MEK1 can phosphorylate and activate the unactivated ERK2 (Fig. 1B). In all these assays, the conversion of the ERK substrate peptide was detected by the TR-FRET methods. As showed in Figure 2A, ERK2, MEK1, and RAF/COT were able to activate their downstream targets in a concentration dependent manner. These assays are very sensitive and picomole-concentrations of activate ERK2, activated MEK1, and activated BRAF can be detected. We also noticed that the Baculovirus-expressed COT is at least 1000-fold less efficient than BRAF in activating MEK1 at the assay conditions used (Fig. 2A). It is possible that full activation of COT requires other factors (or steps).

The COT*–MEK–ERK coupling assay was then optimized in a 1536-well plate and used to screen a diverse

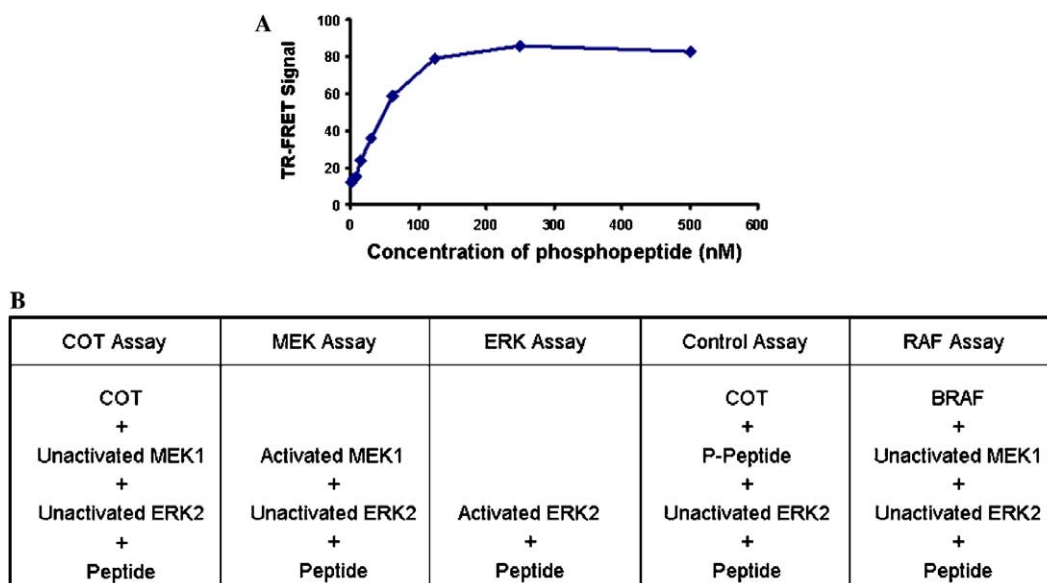


Figure 1. (A) Detection of a phosphorylated ERK substrate peptide by TR-FRET. Phosphorylated ERK substrate peptide (LCB-FFKNIVTPRT³PPP) was diluted in 1 μ M of unphosphorylated peptide and detected with TR-FRET using a Eu-labeled anti-phospho-Ser/Thr antibody and SA-APC. (B) Main components of the TR-FRET-based assays. In each assay, 1 μ M of unphosphorylated peptide was used. The COT assay (COT*–MEK–ERK coupling) mixture contains activated COT, unactivated MEK1, and unactivated ERK2. The MEK assay (MEK1*–ERK2 coupling) mixture contains activated MEK1 and unactivated ERK2. The ERK assay mixture contains activated ERK2. The control assay mixture contains active COT, unactivated ERK2, and the phosphorylated peptide. No unactivated MEK was added in the control assay. The RAF assay (RAF*–MEK–ERK coupling) mixture contains active BRAF, unactivated MEK1, and unactivated ERK2.

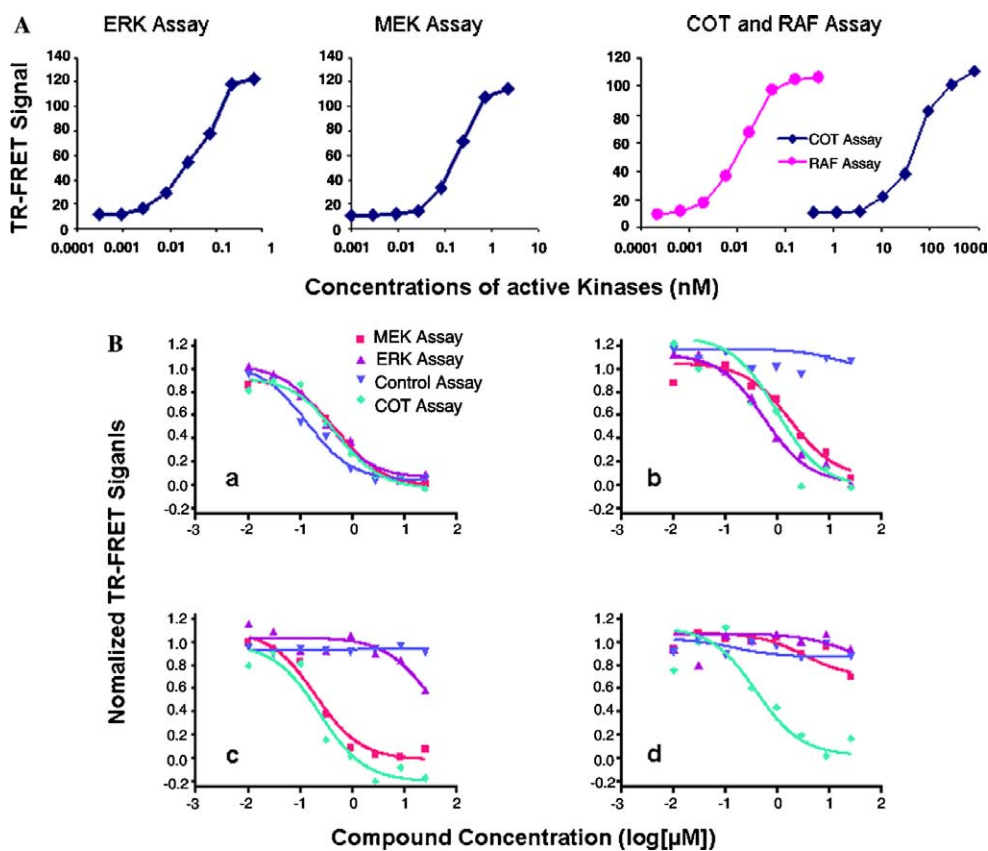


Figure 2. (A) Concentration curve of activated ERK2 in the ERK assay, activated MEK1 in the MEK assay (MEK*–ERK coupling), active COT in the COT assay (COT*–MEK–ERK coupling), and active BRAF in the RAF assay (RAF*–MEK–ERK coupling). (B) Activity profiles of the identified hits from the screen. Group a compounds showed activities in all the four assays and are non-specific inhibitors of the TR-FRET assay format. Group b compounds showed activities in the three kinase assays and are likely to be ERK inhibitors. Group c compounds showed activities in the COT coupling assay and MEK coupling assay, and are likely to be competitive MEK inhibitors. Group d compounds showed activities only in the COT coupling assay and are likely to be inhibitors of COT or allosteric inhibitors of MEK.

library of 1 million compounds. For confirmation and further characterization, identified hits were re-tested in the COT*–MEK–ERK coupling assay, the MEK*–ERK coupling assay, and the ERK assay in an eight point 3-fold dilution series in 1536-well plates. In order to eliminate compounds that interfere with the TR-FRET assay format, these compounds were also tested in a control assay in which the phosphorylated peptide was added to the assay mixture.

The activities of the compounds in all these four assays were compared. Based on the activity profiles, the identified hits can be divided into four different groups (Fig. 2B). Group a compounds showed activity in all four assays, suggesting that these compounds interfere with the TR-FRET assay format. Compounds in group b showed activity in the three kinase assays (the COT coupling assay, the MEK coupling assay, and the ERK assay) but no activity in the control assay suggesting that these compounds inhibit ERK2 (and possible COT, MEK1 as well). Several well-known kinase inhibitor scaffolds including staurosporine analogs, purines, pyrimidines, and oxindoles were identified in this group. The third group of compounds (Group c) showed good activity in the COT coupling assay and the MEK coupling assay, but much lower activity in the ERK assay,

suggesting that these compounds are MEK1 inhibitors. This group of compounds includes resorcylic acid lactones and quinolines, two well-known competitive MEK inhibitor scaffolds.^{38,39} The fourth and the most interesting group of compounds (Group d) showed activity in the COT coupling assay, but much lower activity in the MEK coupling assay and the ERK assay. This group of compounds could either be specific COT inhibitors or ATP-non-competitive MEK inhibitors. PD184352, a known ATP non-competitive MEK inhibitor, was identified in Group d.

To further characterize the group d compounds and to distinguish the non-competitive MEK inhibitors from the COT inhibitors, we then tested Group d compounds in the RAF*–MEK–ERK coupling assay. We expect that a non-competitive MEK inhibitor should inhibit both the COT*–MEK–ERK coupling assay and the RAF*–MEK–ERK coupling assay with equal potency, while a COT inhibitor is likely to inhibit the COT*–MEK–ERK coupling assay only. Indeed, both types of compounds were identified from Group d. Of the compounds showing activity in both the COT*–MEK–ERK and the RAF*–MEK–ERK coupling assays are a number of coumarin derivatives. Figure 3 shows the structures of two of the coumarin hits identified,

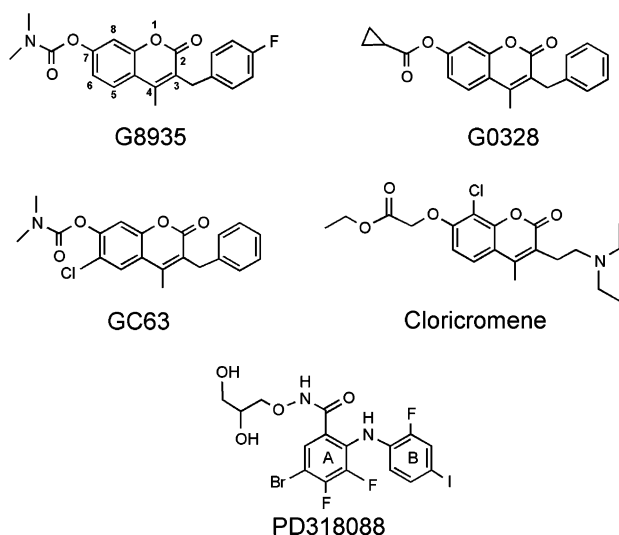


Figure 3. Structures of the compounds mentioned in the text. G8935 and G0328 are the two coumarin hits identified from the screen. GC63 is a published inhibitor of TNF α production (Ref. 11). Cloricromene is a known compound with anti-inflammatory activity. PD318088 is a biarylamine compound co-crystallized with MEK (Ref. 34).

G8935 and G0328. Their activities in the RAF*–MEK–ERK coupling, COT*–MEK–ERK coupling, and MEK*–ERK coupling assays are shown in Figure 4. Both compounds potently inhibit the RAF*–MEK–ERK and COT*–MEK–ERK coupling assays with equal potency (IC_{50} for G8935 = 0.3 μ M, IC_{50} for G0328 = 0.2 μ M), but show no significant activity (up to 10 μ M) in the MEK*–ERK coupling assay, suggesting that these compounds inhibit the activation of MEK1 by upstream MAP3Ks but not the activity of the activated MEK1. The known non-competitive inhib-

itor U0126 (Fig. 4) and PD184352 (data not shown) also showed similar activity profiling in our assays, that is, inhibiting the activation of MEK more potently than inhibiting the activity of activated MEK1.

The two coumarin derivatives inhibit the RAF and COT coupling assay in an ATP-non-competitive fashion. No significant difference in IC_{50} was noticed whether the assay was run in the presence of 1 mM ATP or 50 μ M ATP (data not shown). In addition, these coumarin derivatives are very specific for unactivated MEK1. At 10 μ M, they showed no significant activity against 55 other kinases (including BRAF, activated MEK1, and activated ERK2; data not shown).

We noticed that compound G8935 is very similar to a series of TNF α production inhibitors reported recently by Cheng et al.¹¹ Those reported compounds were initially identified from a cellular assay measuring LPS-induced TNF α production, and the exact molecular target was not known. Using the cellular assay, extensive SAR studies were performed for this series of coumarins.¹¹ We synthesized one of the published compounds (GC63 in Figure 3, Compound #63 in reference 11). This compound has a chlorine at position 6 and is one of the more potent compounds in the published series that inhibit TNF α production. C-6 chloro substitute was shown to be responsible for the high potency of this compound.¹¹ As shown in Figure 4, GC63 potently inhibits the activation of MEK1 by COT in vitro with an IC_{50} of around 70 nM. As expected, GC63 showed much lower activity at inhibiting activated MEK1 (IC_{50} around 10 μ M).

Another published compound that shows significant structural similarity to the identified coumarins is clori-

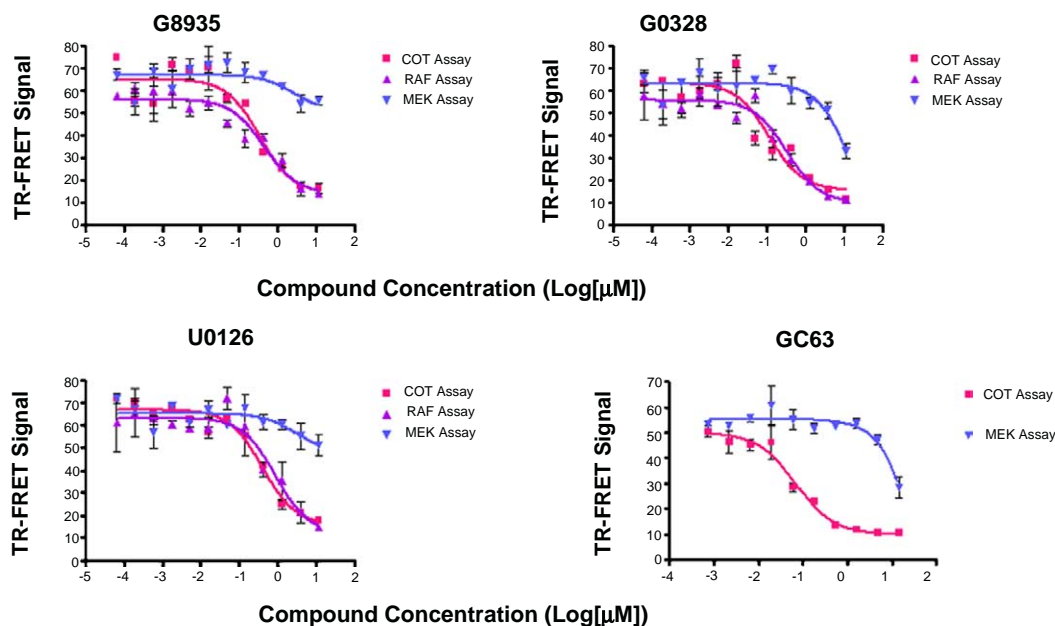


Figure 4. Activities of the compounds in the COT assay (COT*–MEK–ERK coupling), RAF assay (BRAF*–MEK–ERK coupling), and MEK assay (MEK*–ERK coupling). The coumarin-based compounds showed good activities at inhibiting the MAPK3–MEK–ERK coupling assays but are less active in the MEK*–ERK coupling assay, suggesting that they interact with the inactivate conformation of MEK1. The known allosteric inhibitor U0126 also showed a similar activity profile. Assays were done in triplicate and error bars represent standard deviations.

chromene (compd 2432 in MERCK Index, 13th edition, and see Fig. 3 for structure). Cloricromene has been shown to reduce TNF α production and has anti-inflammatory activities in several animal models.^{7–9} We therefore tested cloricromene in our in vitro assays. Cloricromene, up to 100 μ M, did not show significant activity in either the COT/RAF coupling assays or the MEK coupling assay (data not shown), suggesting that cloricromene itself is not a MEK inhibitor. However, we cannot rule out the possibility that its metabolites might inhibit MEK1.

The identified coumarin compounds were tested in an LPS-induced TNF α production assay in a human monocyte THP-1 cell line. As shown in Figure 5A, the carbamate-substituted coumarins, G8935 (IC₅₀, 200 nM) and GC63 (IC₅₀, 90 nM), block the LPS-induced TNF α production in a dose dependent manner. The cellular IC₅₀ for G8935 and GC63 are very similar to their in vitro IC₅₀, strongly suggesting that the mechanism of action for these compounds is the inhibition of MEK activation. As expected for MEK inhibitors, G8935 and GC63 also block ERK1/2 phosphorylation without affecting the level of total ERK1/2 (Fig. 5B). Unlike the carbamate-substituted compounds, the ester-substituted compound G0328 is much less potent in inhibiting TNF α production and ERK phosphorylation in cells, despite its good in vitro potency. This discrepancy between the in vitro and cellular activities for G0328 may be due to hydrolysis of the ester bond and the deactivation of this compound inside the cells.

Crystal structures of MEK complexed with the biarylamine compounds have recently been published revealing an allosteric binding site for this class of compounds.³⁴ The newly identified compound-binding site is adjacent to, but does not overlap with, the ATP binding site, explaining the non-competitive nature of these compounds with respect to ATP.³⁴ We have successfully docked the identified coumarins (both the carbamate-substituted and the ester-substituted) into the allosteric site of the MEK1 structure (PDB code: 1S9J) using the docking program GOLD.⁴⁰ Figure 6 shows the docking model for the carbamate-substituted coumarin G8935. The docked G8935 shows considerable overlap with PD318088. The coumarin ring occupies a similar position as the A ring of PD318088. Most importantly, the carbonyl oxygen from the coumarin ring replaces the 4-fluoro from the A ring of PD318088 to form the critical hydrogen bond with the backbone amide of Ser212 in the activation loop. The carbamate at C7 position partially overlaps with the PD318088 B ring, which forms numerous van der Waals interactions with the protein. The carbamate carbonyl group also makes a hydrogen bond with the backbone of Asp208 in the DFG motif. Interestingly, the benzyl group at C3 position occupies a pocket formed by Ile216, Phe209, Arg189, and Asp190, a pocket that is not used by PD318088. Occupation of this pocket seems to be important for the activity of the coumarin series. Overall, the docking model agreed very well with the SAR for this series of compounds (Ref. 11 and our unpublished data).

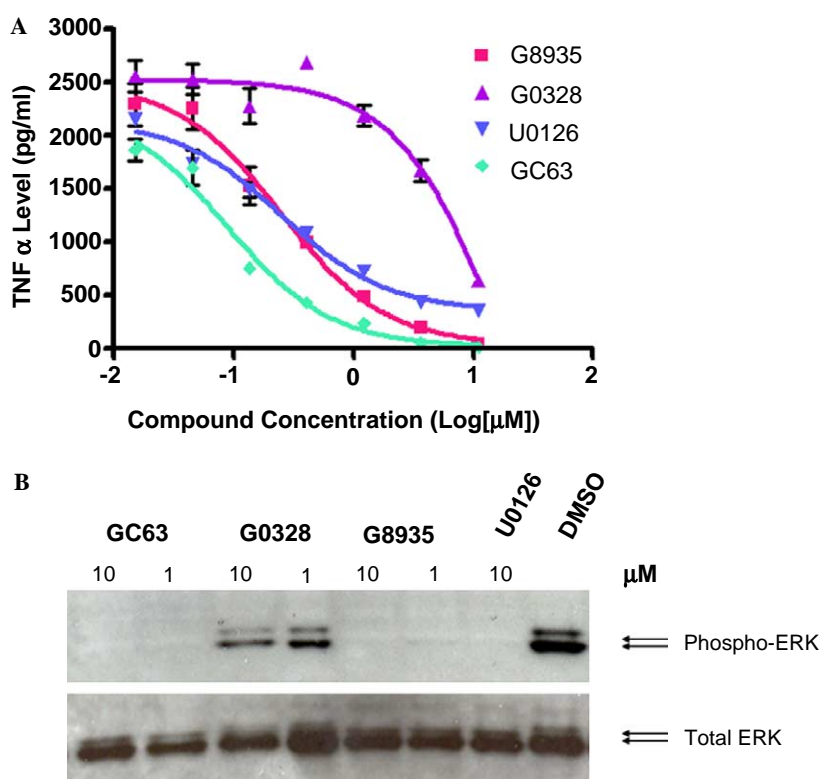


Figure 5. (A) Activities of the coumarin compounds in LPS-induced TNF α production in THP-1 cells. Cells were treated with the compounds for 30 min before the addition of LPS. TNF α levels were measured after 4 h of LPS-treatment using a TNF α kit from Meso-scale Discovery. Assays were done in triplicate and error bars represent standard deviations. The more potent coumarin compound (GC63) showed an IC₅₀ of 90 nM in this assay. (B) Activities of the coumarin derivatives in inhibiting ERK phosphorylation in THP-1 cells.

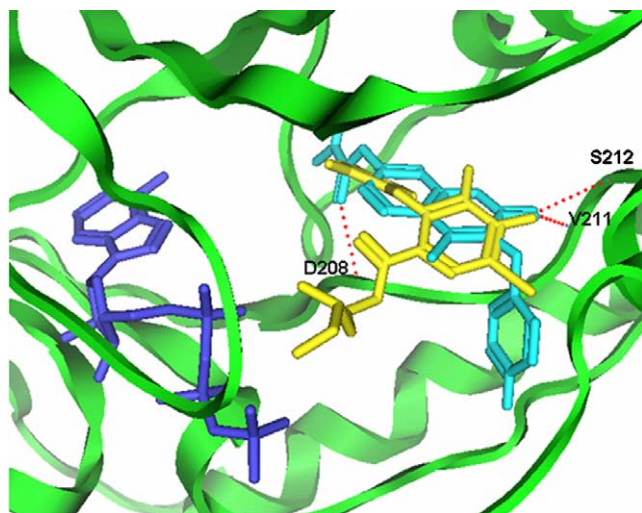


Figure 6. Docking model of G8935 binds to the PD318088-binding pocket on MEK1. MEK1 is a ribbon diagram in green. G8935 (cyan) occupies the unique pocket adjacent to ATP binding site and is superimposed with PD318088 (yellow). ATP from the crystal structure (1S9J) is shown in blue. Proposed hydrogen bonds between G8935 and MEK are shown as dotted lines.

In conclusion, we have identified coumarin-based compounds as a novel class of allosteric MEK1 inhibitors. This discovery identified the mechanism of action for a previously reported series of compounds and raised the possibility that other natural or synthetic coumarin derivatives might exert their anti-cancer and anti-inflammatory activities through the inhibition of MEK1. In addition, these coumarins provided us a lead scaffold for the development of novel MEK inhibitors. The binding mode of the coumarins may also provide new insight for the further optimization of other MEK inhibitor scaffolds.

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