Published in final edited form as:

Chem Biol Drug Des. 2013 November ; 82(5): 520–533. doi:10.1111/cbdd.12177.

Synthesis and SAR studies of dual AKT/NF- κ B inhibitors against melanoma

Elisa Barile^{#†}, Surya K. De^{#†}, Yongmei Feng[†], Vida Chen[†], Li Yang[†], Ze'ev Ronai[†], and Maurizio Pellecchia^{†,*}

[†]Sanford-Burnham Medical Research Institute, La Jolla, California, 92037, USA

[#] These authors contributed equally to this work.

Abstract

The AKT and NF- κ B pathways are central regulators of cellular signaling events at the basis of tumor development and progression. Both pathways are often up-regulated in different tumor types including melanoma. We recently reported the identification of compound **1** (BI-69A11) as inhibitor of the AKT and the NF- κ B pathways. Here we describe SAR studies that led to novel fluorinated derivatives with increased cellular potency, reflected in efficient inhibition of AKT and IKKs. Selected compounds demonstrated effective toxicity on melanoma, breast and prostate cell lines. Finally, a representative derivative showed promising efficacy in an *in vivo* melanoma xenograft model.

Introduction

The AKT and NF- κ B cascades are two pro-survival pathways known to be up-regulated in tumor growth, including melanoma.(1-6) The NF- κ B family of transcription factors regulates several cellular processes, including inflammation, cell migration, cell cycle regulation, and apoptosis.(7) Stimulation of the NF- κ B pathway leads to the activation of the IKK complex, which in turn phosphorylates I κ B, inducing its proteasomal degradation and NF- κ B translocation to the nucleus, where it 'turns on' the expression of target genes such as IAP, Bcl-xL, FLIP, and cyclin D.(8-11)

The PI3K/AKT signaling pathway is also involved in critical cellular events responsible for cell growth and proliferation, protein synthesis, cell survival, as well as glucose uptake and glycogen metabolism.(12, 13) A key regulator of this cascade is the phosphatidylinositol-3-kinase (PI3K), that initiates a series of downstream events which lead to full activation of AKT (through the phosphorylation of Thr308 by the upstream kinase PDK1 and of Ser473 by the mammalian target of rapamycin complex 2 (mTORC2)).(14, 15) Among its diverse spectrum of effects, AKT activation results in increased protein synthesis rate by phosphorylation at Thr246 of the proline-rich substrate of 40 kDa (PRAS40). Three different isoforms of AKT have been reported (AKT1, AKT2 and AKT3) with AKT1 being the most relevant in cancer.(4)

We have initiated a drug discovery program aimed at the identification of compounds with cellular and *in vivo* efficacy targeting these pathways. Recently, we have reported the

*Corresponding author. mpellecchia@sanfordburnham.org Phone: 858-646-3159; Fax: 858-713-9925.

Supporting Information. Additional Supporting Information including proton NMR spectra, HPLC traces and elemental analysis data for key compounds, supporting figures, tables may be found in the online version of this article

The authors declare no conflict of interests.

identification from a virtual docking approach of BI-69A11, here named as compound **1** (Table 1) as a micromolar inhibitor of AKT.(16) Interestingly, however, the compound showed a more profound effect when tested in cell, due to its peculiar ability of inhibiting not only phosphorylation of the AKT substrates but also the activity and stability of AKT itself. Most recently, we reported its selectivity profile and, from this panel, compound **1** also inhibited IKK, SPHK, and few other kinases out of the 315 tested.(17) Further characterizations using cellular and *in vivo* models of melanoma confirmed the efficacy of compound **1** that may explain the simultaneous targeting of both the AKT and NF- κ B signaling pathways.(17-19)

While the precise mechanism of action and cellular targets remain still not fully understood, the observed cellular activity and *in vivo* efficacy of compound **1** provided the impetus for the synthesis and cellular testing of additional derivatives aiming at further improving potency and drug-like properties. We report a comprehensive structure activity relationship study describing novel small molecules **1** derivatives, with a focus on further characterizations of cellular potency and *in vivo* oral efficacy against melanoma.

Results and discussion

Scheme 1 reports our general procedure for the synthesis of compound **1** and our initial series of derivatives. Compound **4** and its analogs (Scheme 1) were either synthesized according to the published literature (20) or commercially available. Compounds **5a-5l** were prepared through Friedlander condensation by microwave irradiation under solvent free conditions, in presence of catalytic amount of cerium chloride (Scheme 1). Final compounds (**7-55**, Table 1 and Supporting Information) were obtained by condensation of **5a-5l** with the appropriate aldehydes in the presence of sodium hydroxide in ethanol as shown in Scheme 1 for a general compound **6**. From our hit compound **1**, we first replaced the benzoimidazole with a simple phenyl group as in compound **7** or with different substituted phenyl rings as for compounds **8-18** (Supporting Information). Unfortunately all of them resulted completely inactive in the AKT1 *in vitro* inhibition assay up to 100 μ M (Supporting Information). Similarly, introducing different aryls in lieu of the benzoimidazole of **1** resulted in compounds **19-36** (Supporting Information), but these also failed to show any significant inhibition of AKT1 *in vitro* with the exception of compound **29** (imidazole substitution) and compound **36** (β -pyridyl substitution) that showed moderate inhibition (IC₅₀ values of 29.5 μ M and 9.72 μ M respectively). However, these compounds did not show any improvement in cellular activity compared to **1** (not shown), corroborating our previous observation of a parallel between cellular potency and *in vitro* AKT1 inhibition. Similar trends were observed for compounds **37-38**, *N*-methylated on the quinolinone ring, that again resulted practically inactive from both *in vitro* and cell based assays (Supporting Information and data not shown). Based on these data we decided to make only smaller changes on the core structure of **1** such as introducing fluorine, chlorine, or bromine atoms, or methyl, methoxyl, ethoxyl groups on different positions of the molecule, as shown in Table 1 (compounds **39-55**). *N*-methylation of the benzoimidazole resulted in compound **39** that also was inactive in the kinase inhibition assay at the tested concentration (Table 1). As mentioned, because we recognize that the activity of our compounds in cell and *in vivo* is likely due to inhibition of multiple kinases, most predominantly AKT and IKK isoforms, selected compounds that resulted active in the kinase activity assay against AKT1 were further profiled against AKT2, AKT3 and IKKs (Table 1). It can be observed that the SAR relative to AKT and IKK parallel in the compound tested hence did not provide a basis for discriminating the activities between these two targets. Nonetheless, introducing a methyl group at the 5-position on the benzoimidazole resulted in compound **40** that showed modest activity against AKT1 with an IC₅₀ value of 43.3 μ M, but it retains the same activity against IKKs, while substitution at the same position with a bromine (**41**) or a fluorine atom (**42**)

resulted in compounds with retained inhibition against both targets (Table 1), suggesting that possibly some small differences in mode of binding exists. Similarly, replacement of the chlorine in compound **1** with a hydrogen atom (**43**), a bromine atom (**44**) or a methyl group (**45**), retained both AKTs and IKKs inhibition (Table 1). The simple *N*-methylation of compound **42**, instead, resulted in compound **46**, less active than its close analogue against AKT1.

As mentioned, to evaluate the effect of the putative Michael acceptor group present in this series, by using different synthetic routes we obtained compounds **58** and **59** (Schemes 2 and 3, respectively) that lack of that putative reactive functionality. Both molecules resulted to be completely inactive against AKT1 and IKKs in the *in vitro* kinase assay, again suggesting that the Michael acceptor in **1** and its derivatives could potentially render these molecules covalent inhibitors. We are currently investigating further this hypothesis.

Furthermore, because the structure of **1** is reminiscent of the natural product viridicatin,⁽²¹⁾ which is also known to inhibit the TNF- α mediated pathway among others,^(22, 23) we designed and synthesized thio-, oxa- and amino- viridicatin derivatives based also on the structure of **1**. These efforts resulted in compound **60-86** (Supporting Information) synthesized according to the previously published procedures (Supporting Information).^(22, 23) Unfortunately, none of these compounds showed any significant inhibition of AKT1 at 100 μ M (Supporting Information).

Finally, we thought to fluorination as a way to improve the cellular potency of **1** as the introduction of fluorine atoms in proper positions of a given molecule is a successful strategy used in drug design to improve the physicochemical and ADME properties of a lead compound.⁽²⁴⁻²⁶⁾ Indeed, several fluorinated drugs entered clinical trials^(27, 28) and it has been reported that approximately 20% of the marketed drugs contain at least one fluorine atom, including the top-selling drug Lipitor.⁽²⁹⁾ We indeed synthesized the polyfluorinated compounds **47-55** of which compounds **47-49** showed *in vitro* AKT1 inhibition (Table 1). Among these we deduced that introducing a methyl (**50**), chlorine (**51**), methoxyl (**52**), ethoxyl (**53**) or even an additional fluorine atom (**54, 55**) on the second phenyl ring resulted in compounds that have poor activity. For these reasons we selected for further cellular evaluation only those fluorinated analogs which retained the *in vitro* AKT inhibitory activity (compounds **42, 47-49**) and excluded those with poor potency.

Taken together, our extensive SAR studies indicate that the benzoimidazole, the Michael acceptor and fluorination at proper positions constitute the structural features important for retaining *in vitro* inhibition of AKT. In particular, the SAR data on the benzimidazole substitutions seem to suggest that electron withdrawing groups, that may enhance the reactivity of the Michael acceptor, may result in more active molecules. Based on these observations, we selected compound **42** (Table 1) for further *in vitro* selectivity, by testing it against 21 protein kinases, which were inhibited by compound **1** previously profiled against 315 kinases.⁽¹⁸⁾ As expected, compound **42** inhibited with IC₅₀ values in the low micromolar range all the selected kinases including SPHK1, SPHK2, FES and AURKB (Supporting Information), while it is also similarly effective in inhibiting both isoforms of IKK (Table 1).

These data suggest that compound **42** and possibly other fluorinated analogs retaining AKT1 inhibition, such as **47-49**, could be subjected to further assays to assess their ability to inhibit the AKT and NF- κ B pathways in cell. Indeed, in agreement with our previous observations with compound **1**,⁽¹⁸⁾ cell based evaluations for inhibition of AKT and NF- κ B pathways with these derivatives showed that the compounds of this series exhibit a remarkable activity in cell, despite the apparent modest inhibition of AKT *in vitro* (Figure 1). While presumably

this is due to the dual inhibition of AKT and IKKs (Table 1), the detailed mechanism of action of these compounds is still not entirely clear, and may possibly involve covalent inhibition to these or other possible targets. Initial molecular modeling studies against both putative targets cannot account unambiguously for the observed SAR studies (not shown), unless a possible covalent interaction is evoked, which is possible especially with IKKs given the presence of a cysteine residues near the ATP binding pocket. We are currently evaluating these hypotheses experimentally. The fluorinated compounds **42**, **47-49** effectively inhibited both the AKT levels and phosphorylation of its substrate PRAS40 (Figure 1A), as well as phosphorylation of IKK Ser176/180 and total I κ -B levels (Figure 1B). This prompted us to further compare the activity of **49** with the parent compound **1**, by testing these molecules side by side against a set of cancer cell lines including melanoma, prostate and breast cancer cells (Figure 2). Western blot analyses revealed that compound **49** is consistently more active than **1** in reducing AKT protein expression and in inhibiting PRAS40 phosphorylation (Figure 2). Taken together, our data suggest that fluorinated compounds **42**, **47-49** possess improved cell potency compared to the previously reported compound **1**.

Hence, we selected compound **42** to evaluate its ability in inhibiting growth/proliferation of melanoma UACC903 spheroids, which better mimic the cell-to-cell contacts, oxygen and nutrient levels within tissues.(30) Therefore UACC903 spheroids, generated through the previously reported hanging drop method,(31) were treated daily, for a total of 7 days, with different concentrations of compound **42**. As shown in Figure 3A, treatment with 1 μ M, 3.3 μ M and 10 μ M of compound **42** every day strongly and significantly suppressed cell growth/proliferation, evaluated by measuring spheroid volumes. Compound **1**, tested as positive control, showed significant proliferation/growth inhibition only at 3.3 μ M and at 10 μ M (Supporting Information), corroborating that compound **42** is superior to compound **1**.

Therefore, we evaluated *in vitro* ADME properties of compounds **42** and few analogs (including compound **1**), confirming that these molecules present favorable plasma and microsomal stability (Supporting Information) and used the Hot Rod Chemistry (Pharmatek, San Diego, see Experimental Section) to select a possible preliminary vehicle formulation to use in *in vivo* experiments.

Hence, we assessed the anti-cancer properties of **42** in a mouse xenograft model of melanoma UACC903 cells implanted subcutaneously into the flank of nude mice. Remarkable tumor growth/suppression was observed administering **42** orally and as single agent twice a week at doses of 10 mg/kg and 25 mg/kg (Figure 3B).

From TUNEL staining experiments, tumors from treated mice exhibited higher levels of apoptosis compared to vehicle-treated animals, suggesting that the inhibition of tumor growth is caused by increased apoptosis (Figure 3C). In addition, tumors from **42**-treated mice exhibited higher I κ B expression than vehicle-treated controls, consistent with our observations with NF- κ B pathway inhibition found in cultured cells.

Conclusions and future directions

While our studies strongly point at AKT and IKK as possible main targets for the observed activity of this intriguing compound series, the precise mechanism of action of these molecules is not fully understood. Hence, we believe that novel derivatives described in this manuscript and relative data will help these further evaluations. Nonetheless, our studies and the pre-clinical evaluations of this compound series provide a possible platform for the translation of these putative AKT/ NF- κ B cellular inhibitors into promising drug candidates

for the treatment of melanoma and potentially other cancers that depend of the activation of these pathways for their onset and proliferation.

Experimental Section

Chemistry

Unless otherwise indicated, all anhydrous solvents were commercially obtained and stored in Sure-seal bottles under nitrogen. All other reagents and solvents were purchased as the highest grade available and used without further purification. Thin-layer chromatography (TLC) analysis of reaction mixtures was performed using Merck silica gel 60 F254 TLC plates, and visualized using ultraviolet light. NMR spectra were recorded on Varian 300 or Jeol 400 or Bruker 500 MHz instruments. Chemical shifts (δ) are reported in parts per million (ppm) referenced to ^1H (Me_4Si at 0.00). Coupling constants (J) are reported in Hz throughout. Mass spectral data were acquired on an Esquire LC00066 for low resolution, a Micromass 70 SEQ for high resolution, or a JEOL LC-mate tuned for either low resolution or high resolution. Purity of all compounds was obtained in a HPLC Breeze from Waters Co. using an Atlantis T3 $3\mu\text{m}$ 4.6×150 mm reverse phase column. The eluant was a linear gradient with a flow rate of 1 ml/min from 95% A and 5% B to 5% A and 95% B in 15 min followed by 5 min at 100% B (Solvent A: H_2O with 0.1% TFA; Solvent B: acetonitrile with 0.1% TFA). The compounds were detected at $\lambda=254$ nm. Purity of key compounds was established by elemental analysis as performed on a Perkin Elmer series II-2400 and HPLC analysis and determined to be > 95%. Combustion analysis was performed by NuMega Resonance Labs, San Diego, CA, USA.

Synthesis of 3-acetyl-4-phenylquinolin-2(1H)-one (5a)

A mixture of (2-aminophenyl)(phenyl)methanone (394 mg, 2 mmol), ethyl acetoacetate (260 mg, 2 mmol), and $\text{CeCl}_3\cdot 7\text{H}_2\text{O}$ (149 mg, 0.40 mmol, 20 mol%) into a test tube (10 mL) was subjected to microwave irradiation at an output of 350 W for 8 min. After it was cooled to room temperature, water (50 mL) was added followed by extraction with ethyl acetate (50 mL x 2). The combine organic layer was washed with water and brine. The organic layer was dried (MgSO_4) and concentrated *in vacuo*. The residue was chromatographed over silica gel (10 to 20 % ethyl acetate in hexane) to afford a pure product **5a** (315 mg, 60%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 2.19 (s, 3 H), 7.03 (dd, $J = 7.9$ Hz and 1.2 Hz, 1 H), 7.08-7.14 (m, 1 H), 7.28-7.31 (m, 2 H), 7.39 (d, $J = 7.9$ Hz, 1 H), 7.45-7.57 (m, 5 H), 12.21 (br s, 1 H); HRMS calcd for $\text{C}_{17}\text{H}_{14}\text{NO}_2$ (M+H) 264.1025, found 264.1028.

Compounds **5b** to **5l** were prepared following the above mentioned procedure and the use of the appropriate starting materials and reagents (Scheme 1). Yields refer to the final step.

Synthesis of 3-acetyl-6-chloro-4-phenylquinolin-2(1H)-one (5b)

Yield 61%; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 2.21 (s, 3 H), 6.93 (d, $J = 2.1$ Hz, 1 H), 7.30-7.36 (m, 2 H), 7.42 (d, $J = 8.7$ Hz, 1 H), 7.46-7.58 (m, 3 H), 7.63 (dd, $J = 8.7$ and 2.1 Hz, 1 H), 12.27 (br s, 1 H); HRMS calcd for $\text{C}_{17}\text{H}_{13}\text{ClNO}_2$ 298.0635 (M + H), found 298.0639.

Synthesis of 3-acetyl-6-bromo-4-phenylquinolin-2(1H)-one (5c)

Yield 58%; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 2.20 (s, 3 H), 7.06 (d, $J = 1.8$ Hz, 1 H), 7.30-7.33 (m, 2 H), 7.35 (d, $J = 8.5$ Hz, 1 H), 7.48-7.52 (m, 3 H), 7.72 (dd, $J = 8.5$ and 1.8 Hz, 1 H), 12.37 (br s, 1 H); HRMS calcd for $\text{C}_{17}\text{H}_{13}\text{BrNO}_2$ 342.0130 (M+H), found 342.0138.

Synthesis of 3-acetyl-6-methyl-4-phenylquinolin-2(1H)-one (5d)

Yield 61%; ^1H NMR (400 MHz, DMSO- d_6) δ 2.18 (s, 6 H), 6.80 (d, J = 1.2 Hz, 1 H), 7.27-7.29 (m, 3 H), 7.38 (dd, J = 8.5 and 1.7 Hz, 1 H), 7.46-7.50 (m, 3 H), 12.14 (br s, 1 H); HRMS calcd for $\text{C}_{18}\text{H}_{16}\text{NO}_2$ 278.1181 (M+H), found 278.1186.

Synthesis of 3-acetyl-6-fluoro-4-(4-fluorophenyl)quinolin-2(1H)-one (5e)

Yield 56%; ^1H NMR (400 MHz, DMSO- d_6) δ 2.23 (s, 3 H), 6.71 (dd, J = 27 and 9 Hz, 1 H), 7.31-7.51 (m, 6 H), 12.33 (br s, 1 H); HRMS calcd for $\text{C}_{17}\text{H}_{12}\text{F}_2\text{NO}_2$ 300.0836 (M+H), found 300.0820.

Synthesis of 3-acetyl-6-chloro-4-(4-fluorophenyl)quinolin-2(1H)-one (5f)

Yield 55%; ^1H NMR (400 MHz, DMSO- d_6) δ 2.22 (s, 3 H), 6.94 (d, J = 2.2 Hz, 1 H), 7.31-7.44 (m, 5 H), 7.62 (dd, J = 2.2 and 8.4 Hz, 1 H), 12.39 (br s, 1 H); HRMS calcd for $\text{C}_{17}\text{H}_{12}\text{ClFNO}_2$ 316.0541 (M+H), found 316.0533.

Synthesis of 3-acetyl-4-(3,4-difluorophenyl)-6-fluoroquinolin-2(1H)-one (5g)

Yield 50%; ^1H NMR (400 MHz, DMSO- d_6) δ 2.28 (s, 3 H), 6.78 (dd, J = 2.2 and 8.7 Hz, 1 H), 7.18-7.19 (m, 1 H), 7.41-7.58 (m, 4 H), 12.37 (br s, 1 H); HRMS calcd for $\text{C}_{17}\text{H}_{11}\text{F}_3\text{NO}_2$ 318.0742 (M+H), found 318.0748.

Synthesis of 3-acetyl-6-chloro-4-(*p*-tolyl)quinolin-2(1H)-one (5h)

Yield 52%; ^1H NMR (400 MHz, DMSO- d_6) δ 2.18 (s, 3 H), 2.37 (s, 3 H), 6.96 (d, J = 2.2 Hz, 1 H), 7.19 (d, J = 8.5 Hz, 2 H), 7.31 (d, J = 8.5 Hz, 2 H), 7.40 (d, J = 8.1 Hz, 1 H), 7.60 (dd, J = 2.2 and 7.8 Hz, 1 H), 12.35 (br s, 1 H); HRMS calcd for $\text{C}_{18}\text{H}_{15}\text{ClNO}_2$ 312.0791 (M+H), found 312.0782.

Synthesis of 3-acetyl-6-chloro-4-(4-chlorophenyl)quinolin-2(1H)-one (5i)

Yield 48%; ^1H NMR (400 MHz, DMSO- d_6) δ 2.25 (s, 3 H), 6.93 (d, J = 2.1 Hz, 1 H), 7.36 (d, J = 8.4 Hz, 2 H), 7.42 (d, J = 7.8 Hz, 1 H), 7.57 (d, J = 8.6 Hz, 2 H), 7.63 (dd, J = 2.2 and 7.8 Hz, 1 H), 12.41 (br s, 1 H); HRMS calcd for $\text{C}_{17}\text{H}_{12}\text{Cl}_2\text{NO}_2$ 332.0245 (M+H), found 332.0241.

Synthesis of 3-acetyl-6-chloro-4-(3,4-difluorophenyl)quinolin-2(1H)-one (5j)

Yield 44%; ^1H NMR (400 MHz, DMSO- d_6) δ 2.27 (s, 3 H), 6.99 (d, J = 2.4 Hz, 1 H), 7.18-7.20 (m, 1 H), 7.41 (d, J = 7.8 Hz, 1 H), 7.50-7.64 (m, 3 H), 12.43 (br s, 1 H); HRMS calcd for $\text{C}_{17}\text{H}_{11}\text{ClF}_2\text{NO}_2$ 334.0446 (M+H), found 334.0441.

Synthesis of 3-acetyl-6-chloro-4-(4-methoxyphenyl)quinolin-2(1H)-one (5k)

Yield 48%; ^1H NMR (400 MHz, DMSO- d_6) δ 2.17 (s, 3 H), 3.82 (s, 3 H), 7.01 (d, J = 2.1 Hz, 1 H), 7.06 (d, J = 8.2 Hz, 2 H), 7.24 (d, J = 8.2 Hz, 2 H), 7.40 (d, J = 7.8 Hz, 1 H), 7.61 (dd, J = 2.4 and 7.9 Hz, 1 H), 12.32 (br s, 1 H); HRMS calcd for $\text{C}_{18}\text{H}_{15}\text{ClNO}_3$ 328.0740 (M+H), found 328.0743.

Synthesis of 3-acetyl-6-chloro-4-(4-ethoxyphenyl)quinolin-2(1H)-one (5l)

Yield 43%; ^1H NMR (400 MHz, DMSO- d_6) δ 1.35 (t, J = 6.5 Hz, 3 H), 2.17 (s, 3 H), 4.08 (q, J = 6.6 Hz, 2 H), 7.01 (d, J = 2.2 Hz, 1 H), 7.04 (d, J = 8.2 Hz, 2 H), 7.22 (d, J = 8.2 Hz, 2 H), 7.41 (d, J = 8.6 Hz, 1 H), 7.60 (dd, J = 2.3 and 7.8 Hz, 1 H), 12.32 (br s, 1 H); HRMS calcd for $\text{C}_{19}\text{H}_{17}\text{ClNO}_3$ 342.0897 (M+H), found 342.0891.

Synthesis of (*E*)-3-(3-(1*H*-benzo[d]imidazol-2-yl)acryloyl)-6-chloro-4-phenylquinolin-2(1*H*)-one (BI-69A11, **1**)

To a solution of compound **5c** (511 mg, 1.72 mmol) in ethanol (17 mL), NaOH (688 mg, 17.2 mmol) solution in water (3 mL) was added at room temperature. After stirring for 15 min, 1*H*-benzoimidazole-2-carboxaldehyde (252 mg, 1.72 mmol) was added to the reaction mixture and resulting reaction mixture was stirred for 16 h at room temperature. After almost completion of the reaction, it was neutralized with 1N HCl, giving a precipitate, which was collected, and purified by flash chromatography (60 to 80 % ethyl acetate in hexane) to furnish the final compound **1** (438 mg, 60%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 6.99 (d, *J* = 2.1 Hz, 1 H), 7.05 (d, *J* = 16.5 Hz, 1 H), 7.18-7.24 (m, 2 H), 7.25-7.38 (m, 3 H), 7.41-7.50 (m, 3 H), 7.55-7.60 (m, 3 H), 7.67 (dd, *J* = 8.7 and 2.1 Hz, 1 H), 12.50 (br s, 1 H), 12.85 (br s, 1 H); MS *m/z* 428 (M+H)⁺, 426 (M+H)⁺, 398, 253, 169, 120, 107, 85; HRMS calcd for C₂₅H₁₇ClN₃O₂ 426.1009 (M+H), found 426.1006.

Compounds **7** to **55** (Table 1 and Supporting Information) were prepared following the above mentioned procedure and the use of the appropriate starting materials and reagents (Scheme 1). NMR and MS data for compounds reported in Table 1 and Schemes 2 and 3 are provided below. HPLC profiles and NMR spectra of selected compounds are reported in Supporting Information.

Synthesis of (*E*)-6-chloro-3-(3-(1-methyl-1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-phenylquinolin-2(1*H*)-one (**39**)

Yield 65%; ¹H NMR (300 MHz, DMSO-*d*₆) δ 3.47 (s, 3 H), 6.86-6.96 (m, 4 H), 7.12-7.20 (m, 5 H), 7.27-7.46 (m, 4 H), 7.63 (dd, *J* = 7.8, 2.4 Hz, 1 H), 12.22 (br s, 1 H); MS *m/z* 442 (M+H)⁺, 440 (M+H)⁺, 419, 253, 169, 121, 107, 85, 67; HRMS calcd for C₂₆H₁₉ClN₃O₂ (M+H), found 440.1180.

Synthesis of (*E*)-6-chloro-3-(3-(5-methyl-1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-phenylquinolin-2(1*H*)-one (**40**)

Yield 55%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 2.37 (s, 3 H), 6.86-7.02 (m, 2 H), 7.27-7.32 (m, 3 H), 7.39-7.46 (m, 6 H), 7.62-7.66 (m, 2 H); 12.40 (br s, 1 H), 12.43 (br s, 1 H); MS *m/z* 442 (M+H)⁺, 440 (M+H)⁺, 254, 126, 121, 107, 85; HRMS calcd for C₂₆H₁₉ClN₃O₂ 440.1166, (M+H) found 440.1160.

Synthesis of (*E*)-3-(3-(5-bromo-1*H*-benzo[d]imidazol-2-yl)acryloyl)-6-chloro-4-phenylquinolin-2(1*H*)-one (**41**)

Yield 60%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 6.95 (d, *J* = 2.7 Hz, 1 H), 7.04 (d, *J* = 16.5 Hz, 1 H), 7.26-7.30 (m, 3 H), 7.34 (d, *J* = 1.6 Hz, 1 H), 7.40-7.47 (m, 5 H), 7.4 (dd, *J* = 8.7 and 2.2 Hz, 2 H), 12.26 (br s, 1 H), 12.91 (br s, 1 H); HRMS calcd for C₂₅H₁₆BrClN₃O₂ 504.0114 (M+H), found 504.0108.

Synthesis of (*E*)-6-chloro-3-(3-(5-fluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-phenylquinolin-2(1*H*)-one (**42**)

Yield 62%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 6.96 (d, *J* = 2.1 Hz, 1 H), 7.21-7.31 (m, 4 H), 7.40-7.47 (m, 6 H), 7.62-7.68 (m, 2 H), 12.43 (br s, 1 H), 12.94 (br s, 1 H); MS *m/z* 446 (M+H)⁺, 444 (M+H)⁺, 247, 217, 142, 120, 101, 85, 79, 67; HRMS calcd for C₂₅H₁₆ClFN₃O₂ 444.0915 (M+H), found 444.0910.

Synthesis of (*E*)-3-(3-(1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-phenylquinolin-2(1*H*)-one (**43**)

Yield 56%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.01 (d, *J* = 15.9 Hz, 1 H), 7.07-7.19 (m, 2 H), 7.25-7.7.31 (m, 4 H), 7.36-7.45 (m, 6 H), 7.56-7.62 (m, 2 H), 12.27 (br s, 1 H), 12.89 (br

s, 1 H); MS m/z 392 (M+H)⁺, 253, 148, 126, 121, 107, 85, 67; HRMS calcd for C₂₅H₁₈N₃O₂ 392.1399 (M+H), found 392.1394.

Synthesis of (*E*)-3-(3-(1*H*-benzo[d]imidazol-2-yl)acryloyl)-6-bromo-4-phenylquinolin-2(1*H*)-one (44)

Yield 51%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.11 (d, *J* = 15.9 Hz, 1 H), 7.12 (d, *J* = 2.2 Hz, 1 H), 7.26-7.31 (m, 5 H), 7.33 (d, *J* = 15.9 Hz, 1 H), 7.41-7.46 (m, 3 H), 7.59-7.63 (m, 2 H), 7.77 (dd, *J* = 8.5 and 2.1 Hz, 1 H), 12.48 (br s, 2 H); MS m/z 472 (M+H)⁺, 470 (M+H)⁺, 253, 198, 126, 121, 107, 85, 67; HRMS calcd for C₂₅H₁₇BrN₃O₂ 470.0504, (M+H) found 470.0498.

Synthesis of (*E*)-3-(3-(1*H*-benzo[d]imidazol-2-yl)acryloyl)-6-methyl-4-phenylquinolin-2(1*H*)-one (45)

Yield 61%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 2.20 (s, 3 H), 6.88 (d, *J* = 1.8 Hz, 1 H), 7.02 (d, *J* = 16.4 Hz, 1 H), 7.17-7.43 (m, 11 H), 7.57-7.62 (m, 1 H), 12.21 (br s, 1 H), 12.81 (br s, 1 H); MS m/z 406 (M+H)⁺, 254, 192, 126, 121, 107, 85; HRMS calcd for C₂₆H₂₀N₃O₂ 406.1556 (M+H), found 406.1548.

Synthesis of (*E*)-6-chloro-3-(3-(5-fluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)-1-methyl-4-phenylquinolin-2(1*H*)-one (46)

Yield 58%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.70 (s, 3 H), 7.02 (d, *J* = 1.8 Hz, 1 H), 7.06 (d, *J* = 16.4 Hz, 1 H), 7.25-7.33 (m, 4 H), 7.40-7.45 (m, 5 H), 7.69-7.77 (m, 3 H), 12.32 (br s, 1H, NH); MS m/z 458 (M+H)⁺; HRMS calcd for C₂₆H₁₈ClFN₃O₂ 458.1072, (M+H) found 458.1076.

Synthesis of (*E*)-6-fluoro-3-(3-(5-fluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-(4-fluorophenyl)quinolin-2(1*H*)-one (47)

Yield 54%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 6.66-6.79 (m, 2 H), 6.90 (d, *J* = 8.1 Hz, 1 H), 7.05 (d, *J* = 16.2 Hz, 1 H), 7.22-7.52 (m, 8 H), 12.21 (br s, 1 H), 12.89 (br s, 1 H); HRMS calcd for C₂₅H₁₅F₃N₃O₂ 446.1116 (M+H), found 446.1108.

Synthesis of (*E*)-3-(3-(5,6-difluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)-6-fluoro-4-(4-fluorophenyl)quinolin-2(1*H*)-one (48)

Yield 45%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 6.66-6.74 (m, 2 H), 6.91 (d, *J* = 8.1 Hz, 1 H), 7.07 (d, *J* = 16.2 Hz, 1 H), 7.22-7.55 (m, 7 H), 12.38 (br s, 1 H), 12.96 (br s, 1 H); HRMS calcd for C₂₅H₁₄F₄N₃O₂ 446.1116 (M+H), found 464.1011.

Synthesis of (*E*)-6-chloro-3-(3-(5,6-difluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-(4-fluorophenyl)quinolin-2(1*H*)-one (49)

Yield 41%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 6.97 (d, *J* = 2.1 Hz, 1 H), 7.03 (d, *J* = 16.3 Hz, 1 H), 7.22-7.45 (m, 7 H), 7.62-7.67 (m, 2 H), 12.28 (br s, 1 H), 13.01 (br s, 1 H); HRMS calcd for C₂₅H₁₄ClF₃N₃O₂ 446.1116 (M+H), found 480.0727.

Synthesis of (*E*)-6-chloro-3-(3-(5,6-difluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-(*p*-tolyl)quinolin-2(1*H*)-one (50)

Yield 40%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 2.28 (s, 3 H), 6.97 (d, *J* = 1.8 Hz, 1 H), 7.02 (d, *J* = 16.4 Hz, 1 H), 7.12-7.17 (m 2 H), 7.21-7.27 (m, 2 H), 7.43-7.45 (m, 1 H), 7.60-7.66 (m, 2 H), 12.45 (br s, 1 H), 13.01 (br s, 1 H); HRMS calcd for C₂₆H₁₇ClF₂N₃O₂ 476.0977 (M+H), found 476.0979.

Synthesis of (*E*)-6-chloro-4-(4-chlorophenyl)-3-(3-(5,6-difluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)quinolin-2(1*H*)-one (51)

Yield 40%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 6.99 (d, *J* = 2.2 Hz, 1 H), 7.09 (d, *J* = 16.6 Hz, 1 H), 7.32-7.38 (m, 3 H), 7.48 (d, *J* = 7.8 Hz, 1 H), 7.53-7.56 (m, 2 H), 7.66-7.69 (m, 3 H), 12.50 (br s, 1 H), 13.20 (br s, 1 H), HRMS calcd for C₂₅H₁₄Cl₂F₂N₃O₂ 496.0431 (M+H), found 496.0428.

Synthesis of (*E*)-6-chloro-3-(3-(5,6-difluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-(4-methoxyphenyl)quinolin-2(1*H*)-one (52)

Yield 46%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.75 (s, 3 H), 6.98-7.08 (m, 4 H), 7.22-7.25 (m, 2 H), 7.30 (d, *J* = 16.1 Hz, 1 H), 7.46 (d, *J* = 8.1 Hz, 1 H), 7.65-7.71 (m, 3 H), 12.41 (br s, 1 H), 13.01 (br s, 1 H); HRMS calcd for C₂₆H₁₇ClF₂N₃O₃ (M+H) 492.0927, found 492.0916.

Synthesis of (*E*)-6-chloro-3-(3-(5,6-difluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-(4-ethoxyphenyl)quinolin-2(1*H*)-one (53)

Yield 36%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.26 (t, *J* = 6.8 Hz, 3 H), 3.98 (q, *J* = 6.7 Hz, 2 H), 6.94 (d, *J* = 7.8 Hz, 1 H), 7.01 (d, *J* = 16.5 Hz, 1 H), 7.04 (d, *J* = 2.2 Hz, 1 H), 7.16-7.19 (m, 2 H), 7.27 (d, *J* = 16.1 Hz, 1 H), 7.43 (d, *J* = 8.1 Hz, 1 H), 7.62-7.67 (m, 2 H), 12.38 (br s, 1 H), 13.02 (br s, 1 H); HRMS calcd for C₂₇H₁₉ClF₂N₃O₃ (M+H) 506.1083, found 506.1071.

Synthesis of (*E*)-3-(3-(5,6-difluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-(3,4-difluorophenyl)-6-fluoroquinolin-2(1*H*)-one (54)

Yield 45%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 6.83 (dd, *J* = 2.2 and 8.2 Hz, 1 H), 7.08 (d, *J* = 16.4 Hz, 1 H), 7.12-7.17 (m, 1 H), 7.32 (d, *J* = 15.8 Hz, 1 H), 7.44-7.54 (m, 4 H), 7.66-7.73 (m, 2 H), 12.42 (br s, 1 H), 13.08 (br s, 1 H); HRMS calcd for C₂₅H₁₃F₅N₃O₂ 482.0928 (M+H), found 482.0921.

Synthesis of (*E*)-6-chloro-3-(3-(5,6-difluoro-1*H*-benzo[d]imidazol-2-yl)acryloyl)-4-(3,4-difluorophenyl)quinolin-2(1*H*)-one (55)

Yield 40%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.03 (d, *J* = 2.2 Hz, 1 H), 7.07 (d, *J* = 16.5 Hz, 1 H), 7.12-7.27 (m, 1 H), 7.33 (d, *J* = 16.1 Hz, 1 H), 7.43-7.45 (m, 3 H), 7.63-7.67 (m, 3 H), 12.48 (br s, 1 H), 13.04 (br s, 1 H); HRMS calcd for C₂₅H₁₃ClF₄N₃O₂ 498.0632, (M+H) found 498.0639.

Synthesis of ethyl 6-chloro-2-oxo-4-phenyl-1,2-dihydroquinoline-3-carboxylate (56)

A mixture of **4b** (600 mg, 2.597 mmol), diethyl malonate (623 mg, 3.895 mmol) and DBU (79 mg, 0.519 mmol) was stirred at 160 °C under microwave condition (Power 350) for 1 hour. The reaction mixture was extracted with ethyl acetate (100 mL), washed with 1 N HCl (2 × 50 mL), saturated sodium bicarbonate solution (2 × 40 mL), and brine (2 × 50 mL), and dried over MgSO₄, and concentrated. The residue was purified over silica gel chromatography (20 to 30 % ethyl acetate in hexane) to afford compound **56** (508 mg, 60%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 0.81 (t, *J* = 7.3 Hz, 3 H), 3.90 (q, *J* = 7.3 Hz, 2 H), 6.96 (d, *J* = 2.3 Hz, 1 H), 7.28-7.32 (m, 2 H), 7.39 (d, *J* = 8.6 Hz, 1 H), 7.48-7.52 (m, 3 H), 7.60 (dd, *J* = 8.7 and 2.3 Hz, 1 H), 12.21 (br s, 1 H, NH); HRMS calcd for C₁₈H₁₅ClNO₃ (M+H) 328.0740, found 328.0743.

Synthesis of 6-chloro-2-oxo-4-phenyl-1,2-dihydroquinoline-3-carboxylic acid (**57**)

To a solution of compound **56** (450 mg, 1.377 mmol) in THF (10 mL) and methanol (3 mL) was added LiOH (564 mg, 13.77 mmol) in water (3 mL). The resulting mixture was stirred at room temperature for 16 hours. The reaction mixture was acidified with 1 N HCl, precipitated, and filtered, washed with water and benzene and dried under high vacuum. This compound was used for the next step without any further purification. ¹H NMR (400 MHz, DMSO-*d*₆) δ 6.82 (d, *J* = 2.3 Hz, 1 H), 7.23-7.46 (m, 7 H), 12.29 (br s, 1 H, NH); HRMS calcd for C₁₆H₁₁ClNO₃ 300.0427 (M+H), found 300.0432.

Synthesis of *N*-((1*H*-benzo[*d*]imidazol-2-yl)methyl)-6-chloro-2-oxo-4-phenyl-1,2-dihydroquinoline-3-carboxamide (**58**)

A mixture of compound **57** (200 mg, 0.668 mmol), (1*H*-benzo[*d*]imidazol-2-yl)methanamine (98 mg, 0.668 mmol), EDC (153 mg, 0.801 mmol), HOBt (108 mg, 0.801 mmol), DIEA (0.5 mL) in DMF (3 mL) was stirred at room temperature for 20 h. The reaction mixture was extracted with dichloromethane (100 mL), washed with saturated sodium bicarbonate (50 mL × 3), water (40 mL × 3), brine (40 mL × 3), dried over MgSO₄, and concentrated. The residue was purified over silica gel chromatography (5 to 10 % methanol in dichloromethane) to afford a pure product of **58** (114 mg, 40 %). ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.89 (d, *J* = 6 Hz, 2 H), 6.83 (d, *J* = 2.1 Hz, 1 H), 7.20-7.36 (m, 3 H), 7.43 (d, *J* = 8.4 Hz, 2 H), 7.48-7.56 (m, 6 H), 8.66 (t, *J* = 6 Hz, 1 H, NH), 11.89 (br s, 1 H, NH), 12.24 (br s, 1 H, NH); HRMS calcd for C₂₄H₁₈NCIN₄O₂ 429.1118, (M+H) found 429.1126.

Synthesis of 3-(3-(1*H*-benzo[*d*]imidazol-2-yl)propanoyl)-6-chloro-4-phenylquinolin-2(1*H*)-one (**59**)

To a solution of **1** (100 mg, 0.235 mmol) in ethyl acetate (20 mL) and methanol (20 mL) was added Pd/C (50 mg). The reaction mixture was stirred at room temperature under hydrogen atmosphere for 12 hours. After completion of the reaction, the mixture was filtered over celite to remove Pd/C, filtrate was concentrated and the residue was purified over silica gel (70-100% ethyl acetate in hexane) to give a pure product of **59** (76 mg, 75%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.05 (t, *J* = 6 Hz, 2 H), 3.33 (t, *J* = 6 Hz, 2 H), 6.81-6.85 (m, 1 H), 6.91 (d, *J* = 8.4 Hz, 1 H), 7.03 (d, *J* = 8.4 Hz, 1 H), 7.08-7.41 (m, 5 H), 7.43 (d, *J* = 8 Hz, 1 H), 7.47-7.61 (m, 3 H); 12.24 (br s, 1 H, NH), 12.34 (br s, 1 H, NH); HRMS calcd for C₂₅H₁₉ClN₃O₂ 428.1166 (M+H), found 428.1171.

Assays

AKT1, AKT2, AKT3 *in vitro* kinase assay

The AKT inhibition was tested by using the Z'-LYTE® biochemical assay technology from Life Technologies. The assay was performed in Corning 3676 black 384-well assay plate. The final 10 μL Kinase Reaction consisted of 0.34 - 7.65 ng AKT1 (PKB alpha) or 1 - 40 ng AKT2 (PKB beta) or 0.56 - 8.3 ng AKT3 (PKB gamma), 2 μM of the substrate Ser/Thr 06 in 50 mM of HEPES buffer at pH 7.5, containing 0.01% BRIJ-35, 10 mM MgCl₂, 1 mM EGTA ATP concentration at the *K_m* value was used. After the 1 hour Kinase Reaction incubation, 5 μL of a 1:2048 dilution of Development Reagent A was added. After the development reaction, where a site-specific protease recognizes and cleaves the nonphosphorylated peptide, a ratiometric read-out of the donor emission (Coumarin, 445 nm) over the acceptor (Fluorescein, 520 nm) was detected by a PerkinElmer EnVision fluorescence plate reader (Waltham, MA).

IKK α (CHUK) *in vitro* assay

The IKK α kinase was tested by using the ADAPTA® biochemical assay technology from Life Technologies. The assay was performed in Corning 3676 black 384-well assay plate. The final 10 μ L Kinase Reaction consisted of 5 - 60 ng CHUK (IKK α) in 32.5 mM HEPES pH 7.5, 0.005% BRIJ-35, 5 mM MgCl₂, 0.5 mM EGTA. After the 1 hour Kinase Reaction incubation, 5 μ L of Detection Mix consisting of a europium labeled anti-ADP antibody, an Alexa Fluor® 647 labeled ADP tracer, and EDTA (to stop the kinase reaction) was added to the assay well. ADP formation has been determined by calculating the emission ratio from the assay well.

IKK β (IKKBK) *in vitro* assay

The IKK β inhibition was tested by using the Z'-LYTE® biochemical assay technology from Life Technologies. The assay was performed in Corning 3676 black 384-well assay plate. The final 10 μ L Kinase Reaction consisted of 0.93 - 8.02 ng IKKBK (IKK β) and 2 μ M Ser/Thr 05 in 50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM MgCl₂, 1 mM EGTA. After the 1 hour Kinase Reaction incubation, 5 μ L of a 1:64 dilution of Development Reagent B was added. The development and detection steps followed the same protocol described above (see AKT *in vitro* assay).

For all the assays described above data analysis and curve-fitting were performed using XLfit4 and GraphPad Prism4 software.

Cell culture

UACC903, Lu1205, Mel501, WM1366 melanoma cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Mediatech, Manassas, VA) with 10% fetal bovine serum. PC3 prostate cancer cells were cultured in RPMI 1640 (Invitrogen, Carlsbad CA) with 10% fetal bovine serum. MDA-MB-231 breast cells were cultured in DMEM/F12 (50:50) (Mediatech, Manassas, VA) with 10% fetal bovine serum. All cells were maintained at 37°C in 5% CO₂.

3D culture assay

UACC903 cells were induced to form spheroids via a hanging drop method.⁽³¹⁾ Cells were plated at 200 cells per well (20 μ L) of a Nunc-60 well microwell MiniTray (Polystyrene). The trays were covered, inverted and placed in a humidity chamber for 5 days till one spheroid formed in each well. The spheroids were then transferred into a 48 well plate coated in 1% low melting point agarose to prevent them from adhering. The spheroids were measured and DMSO or drug was added every 24 hours for 7 days. Spheroid length and width (measured with an optical micrometer) were used to calculate spheroid volumes (μ m³).

Antibodies, reagents

Antibodies against phospho-IKK α β (S176/S180), IKK α β , phospho-I κ B (S32), I κ B, phospho-AKT (S473), phospho-AKT (T308), AKT (pan), phospho-PRAS40(T246) and PRAS40 were purchased from Cell Signaling Technology (Danvers, MA). Human TNF- α was purchased from Cell Signaling Technology (Danvers, MA).

Cell treatment and immunoblotting

To determine the effect of compounds on phospho-AKT, cells were plated at 75% confluence and grown overnight on 60 mm plates. Compounds were added to the plate, incubated for 4 hours and harvested for immunoblot analysis. For phospho-IKK/phospho-I κ B experiments, cells were plated as described above and grown overnight. Compounds were added to cells for 1 h prior to stimulation with 20 ng/ml TNF- α and harvested for

immunoblotting. For harvest, cells were rinsed with PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10 µg/ml aprotinin, 1 µg/ml pepstatin A, 10 µg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 µM microcystin-LR, 2.5 mM sodium orthovanadate). The protein concentration was determined using Bio-Rad protein assay solution. Equal amounts of cell lysate (50 µg) were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences, Waltham, MA). Membranes were blocked with 3% BSA/PBST for 1 h and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C with shaking. Following three times wash with PBST, membranes were incubated with second antibody for 1 h at room temperature. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling) for polyclonal antibodies or horseradish peroxidase-conjugated anti-mouse antibody (Cell Signaling) for monoclonal antibodies followed by enhanced chemiluminescence using Western Lightning reagent (PerkinElmer Life Sciences, Waltham, MA).

***In vivo* xenograft studies**

All animal studies were conducted in the Animal Facility at Sanford|Burnham Medical Research Institute in accordance with the Institutional Animal Care and Use Committee guidelines. Female 6- to 7-week-old nude mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) and allowed to acclimatize for 1 week. UACC 903 cells (1×10^6 , suspended in 200 µl sterile PBS) were injected into the subcutaneous tissue of the flank. When xenograft size reached a volume of between 10 and 20 mm³, the mice were sorted into eight mice per group with the average tumor volumes distributed equally between the groups. Vehicle control (Hot Rod Chemistry Formulation #1; Pharmatek, San Diego, CA, USA) or compound **42** at 10 and 25 mg / kg was administered by oral gavage twice a week. The mice were maintained in a pathogen-free environment with free access to food. Body weight and tumor volume were measured twice a week. Tumor size was measured with linear calipers and calculated using the formula: [length in millimeters × (width in millimeters)²] / 2. 3 mice in control group, 2 in the treated group with 10 mg/Kg dose and 4 mice in the 25 mg/kg treated group died by day 28, during the gavage administration. The mice were killed after 4 weeks, and the tumors were fixed in Z-Fix (Anatech, Battle Creek, MI, USA) and embedded in paraffin for immunohistochemical analysis.

Immunohistochemistry

Immunohistochemical studies were performed on paraffin-embedded tissue sections of tumor extracted at the time of killing. Serial sections (5-mm thick) of paraffin-embedded tissues were fixed on silane-coated glass slides, deparaffinized, and subjected to antigen retrieval (Target Retrieval Solution; Dako, Carpinteria, CA, USA). Following blocking, slides were incubated with the indicated antibodies overnight, washed in PBS, and incubated with the appropriate HRP-conjugated secondary antibody (anti-rabbit polymer-HRP; Dako). Bound antibodies were visualized with DAB+ chromogen solution (Dako) and counterstained with hematoxylin. For the TUNEL assay, tumor sections were stained using the ApopTag Peroxidase. In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer's protocol. Slides were scanned with the ScanScope XT system (Aperio Technologies, Vista, CA, USA), and quantitation was carried out using ImageScope software (Aperio Technologies).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge financial support from the NIH (grant CA128814 to MP and ZR, and grants CA149668 and CA081534 to MP). We also thank Dr. Gary Chiang for support with the initial cellular and in vivo experiments.

Abbreviations list

AKT1	Protein Kinase B alpha
AKT2	Protein Kinase B beta
AKT3	Protein Kinase B gamma
AURKB	Aurora Kinase B
FES	Feline Sarcoma Oncogene
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B cells
PI3K	Phosphatidylinositol 3-kinase
PRAS40	Proline-rich AKT Substrate of 40 kDa
SPHK	Sphingosine Kinase

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Figure 1.

Inhibition of the AKT and NF-κB pathways by BI-69A11 (**1**) and compounds **42**, **47-49**. (A) Melanoma UACC903 cells (0.8×10^5 in 60mm plate) were treated with vehicle (DMSO) or the indicated concentrations of test compounds for 4 hours. Whole cell lysates (50 μg) were subjected to SDS-PAGE followed by Westerns using the indicated antibodies. The same membrane was stripped and reprobed with anti-PLCγ antibody for loading control. (B) Melanoma UACC903 cells were incubated with either vehicle (DMSO) or 10 μM of the test compounds for 1 hour prior to TNF-α stimulation (20ng/ml). Cells were harvested 5 minutes following stimulation and whole cell lysates were immunoblotted with the indicated antibodies. The same membrane was stripped and reprobed with anti-PLCγ antibody for loading control.

Figure 2.

Inhibition of the AKT pathway by BI-69A11 (1) and compound 49 in melanoma, prostate and breast cancer cell lines. Melanoma UACC903, Lu1205, Mel501 and WM1366 cells, along with prostate PC3 and breast MDA-MB-231 cancer cell lines were treated with vehicle (DMSO) or 10 μM of BI-69A11 (1) and compound 49 for 4 hours. Whole cell lysates were immunoblotted with the indicated antibodies. The same membrane was stripped and reprobed with anti-tubulin antibody for loading controls.

Figure 3.

Compound **42** inhibits melanoma cell growth in 3D cultures and it is orally active in a xenograft model of melanoma. (A) Melanoma UACC903 cells were grown as spheroids (generated by the hanging drop method(31)) and treated with the indicated concentrations of **42** every day, for a total of 7 days. Spheroid volumes (μm³) were calculated by measuring spheroids length and width with an optical micrometer. (B) Human UACC903 melanoma cells (10⁶) were implanted subcutaneously into the flank of nude mice. Vehicle or the indicated doses (10 and 25 mg/Kg) of compound **42** were administered by oral gavage twice per week, starting when tumor size reached 10 mm³ (measured at each indicated time point by calipers). Error bars represent the Standard Error of the Mean. (C) TUNEL and IκB staining of tumor sections from vehicle and **42**-treated UACC903 xenograft mice after 28 days. Representative pictures for TUNEL (10x) and IκB staining (20x) are shown. TUNEL and IκB staining were quantitated by ImageScope software and represented as TUNEL-positive nuclei/area and IκB immunoscore/area. Error bars represent the Standard Error of the Mean.

(a) $R_1 = H$, $R_2 = H$, $R_3 = H$;

(b) $R_1 = Cl$, $R_2 = H$, $R_3 = H$;

(c) $R_1 = Br$, $R_2 = H$, $R_3 = H$;

(d) $R_1 = Me$, $R_2 = H$, $R_3 = H$;

(e) $R_1 = F$, $R_2 = H$, $R_3 = F$;

(f) $R_1 = Cl$, $R_2 = H$, $R_3 = F$;

(g) $R_1 = F$, $R_2 = F$, $R_3 = F$;

(h) $R_1 = Cl$, $R_2 = H$, $R_3 = Me$;

(i) $R_1 = Cl$, $R_2 = H$, $R_3 = Cl$;

(j) $R_1 = Cl$, $R_2 = F$, $R_3 = F$;

(k) $R_1 = Cl$; $R_2 = H$, $R_3 = OMe$;

(l) $R_1 = Cl$, $R_2 = H$, $R_3 = OEt$.

Structures of compounds **1-55** are reported in Table 1 and Supporting Information.

Reagents and conditions. (A) DIEA, DMF, room temperature, 16 hours; (B) Fries Rearrangement: $AlCl_3$, 150 °C, 3 hours; (C) $CeCl_3 \cdot 7H_2O$, Microwave, 350 W; (D) R_4CHO , NaOH, EtOH, room temperature.

Scheme 1.

General synthetic scheme for compounds **1-55**.

Reagents and conditions: (A) Diethyl malonate, DBU, 160 °C, Microwave, 350 W, 1 hour; (B) LiOH, THF, MeOH, room temperature, 16 hours; (C) (1*H*-benzo[*d*]imidazol-2-yl)methanamine, EDC, HOBt, DIEA, DMF, room temperature, 20 hours.

Scheme 2.
Synthetic scheme for compound **58**.

Reagents and conditions: Pd/C, H₂, room temperature, 12 hours.

Scheme 3.
Synthetic scheme for compound **59**.

Table 1Chemical structures and in vitro AKT inhibition assay results for compounds **1**, **39-55**.

<div> </div>							
compd ID	R ₁	R ₂	R ₃	R ₄	X	Kinase assay ^a IC ₅₀ (μM)	
						AKT1 AKT2 AKT3	IKKα IKKβ
BI-69A11 1	Cl	H	H		H	6.22 4.24 10.90	3.85 2.30
39	Cl	H	H		H	>100 nd nd	nd nd
40	Cl	H	H		H	43.30 5.00 8.40	8.03 [*] 9.60 [*]
41	Cl	H	H		H	14.00 nd nd	nd nd
42	Cl	H	H		H	5.01 3.43 6.49	6.03 1.90
43	H	H	H		H	5.20 nd nd	nd nd
44	Br	H	H		H	4.48 1.72 8.85	6.02 [*] 5.60 [*]

compd ID	R ₁	R ₂	R ₃	R ₄	X	Kinase assay ^a IC ₅₀ (μM)		
						AKT1 AKT2 AKT3	IKKα IKKβ	
45	CH ₃	H	H		H	6.26 1.64 5.53	29.10 [*] 7.90 [*]	
46	Cl	H	H		CH ₃	17.21 nd nd	nd nd	
47	F	H	F		H	15.58 nd nd	6.10 [*] 13.10 [*]	
48	F	H	F		H	15.90 nd nd	13.10 [*] 16.00 [*]	
49	Cl	H	F		H	30.40 nd nd	7.12 [*] 15.40 [*]	
50	Cl	H	CH ₃		H	>50 nd nd	nd nd	
51	Cl	H	Cl		H	>50 nd nd	nd nd	
52	Cl	H	OCH ₃		H	>50 nd nd	nd nd	

<div> </div>								
compd ID	R ₁	R ₂	R ₃	R ₄	X	Kinase assay ^a IC ₅₀ (μM)		
						AKT1 AKT2 AKT3	IKKα IKKβ	
53	Cl	H	OCH ₂ CH ₃	<div> </div>	H	>50 nd nd	nd nd	
54	F	F	F	<div> </div>	H	44.46 nd nd	7.20 [*] 16.40 [*]	
55	Cl	F	F	<div> </div>	H	>50 nd nd	12.10 [*] 17.80 [*]	

^a Values are means of at least three or more experiments with a typical standard deviation of less than $\pm 20\%$. nd: not determined (compound was not tested).

* represent predicted IC₅₀ values derived from experimentally determined % of inhibition at a given inhibitor concentration (10 μM for IKKβ or 1 μM for IKKα) which are means of two experiments.