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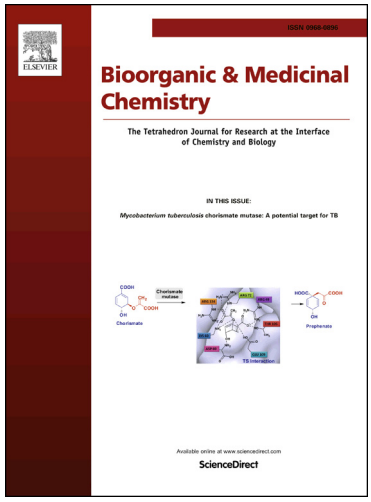

Discovery of a Potent Inhibitor of MELK that Inhibits Expression of the Anti-apoptotic Protein Mcl-1 and TNBC Cell Growth

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Graphical Abstract

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Discovery of a Potent Inhibitor of MELK that Inhibits Expression of the Anti-apoptotic Protein Mcl-1 and TNBC Cell Growth

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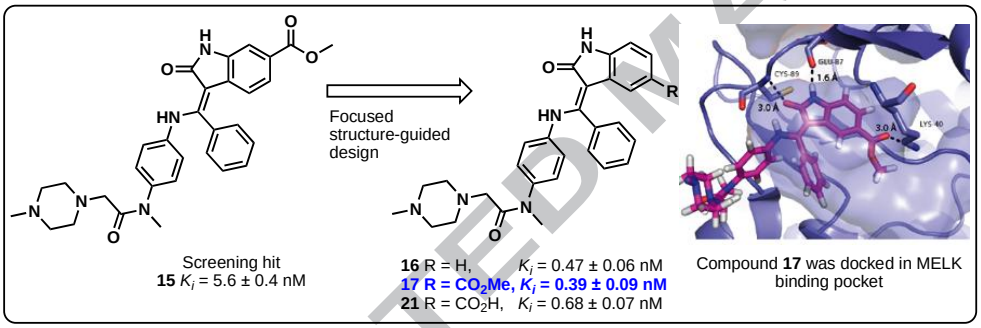

Screening hit
15 K_i = 5.6 ± 0.4 nM

16 R = H, K_i = 0.47 ± 0.06 nM
17 R = CO_2Me, K_i = 0.39 ± 0.09 nM
21 R = CO_2H, K_i = 0.68 ± 0.07 nM

Compound **17** was docked in MELK binding pocket

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ABSTRACT

Despite recent advances in molecularly directed therapy, triple negative breast cancer (TNBC) remains one of the most aggressive forms of breast cancer, still without a suitable target for specific inhibitors. Maternal embryonic leucine zipper kinase (MELK) is highly expressed in TNBC, where level of overexpression correlates with poor prognosis and an aggressive disease course. Herein, we describe the discovery through targeted kinase inhibitor library screening, and structure-guided design of a series of ATP-competitive indolinone derivatives with subnanomolar inhibition constants towards MELK. The most potent compound, **17**, inhibits the expression of the anti-apoptotic protein Mcl-1 and proliferation of TNBC cells exhibiting selectivity for cells expressing high levels of MELK. These studies suggest that further elaboration of **17** will furnish MELK-selective inhibitors with potential for development in preclinical models of TNBC and other cancers.

1. Introduction

MELK, a member of the AMPK-RK family which mediates cell survival under conditions of metabolic stress,[1-2] is a mitotically regulated kinase implicated in the cell cycle and the promotion of cell proliferation[3-4] (see recent reviews[5-7]). Its expression is elevated in the early stages of murine embryonic development[8-9] and is found to be highly expressed in immature proliferating cells, such as hematopoietic cells in bone marrow,[4] multipotent neural progenitors,[10] myoblasts,[11] and mammary progenitors.[12] In mice, MELK is detectable in the thymus, lung and testis, but in general, exhibits a limited expression profile in the adult.[1, 9] MELK knockout is reported to have no impact on the development of C57BL/6 mice,[13] suggesting that its expression is not essential.

MELK is upregulated (mRNA and protein) in a wide array of cancer cell types and clinical tumor samples,[14-19] and gene expression analysis has identified it as a possible glioma stem cell marker.[20-21] Its expression correlates with poor prognosis in several cancers, including glioblastoma multiforme (GBM),[21] breast cancer,[22] especially triple negative breast cancer (TNBC),[13, 23-24] prostate cancer,[25] lung adenocarcinoma,[17] acute myeloid leukemia,[26] and gastric cancer.[27] siRNA-mediated knockdown of MELK impedes the renewal of glioma stem cells[10] and tumor growth.[14, 21] Furthermore, MELK is also reported to induce apoptosis in glioma stem cells[21, 28] as well as in basal breast cancers,[13] but not normal neural stem cells or luminal breast cancers.[13] It is also implicated in the resistance of cancer cells to radiation.[29-30] Taken together, these data suggest that MELK may have value as a therapeutic target for treating various cancers, especially those where it might serve a potentially critical survival function (e.g. glioblastoma,[21] medulloblastoma[21] and triple negative breast cancers, TNBCs[13]).

To date, no inhibitors developed with MELK as a primary target are FDA-approved, and no selective inhibitor of MELK with good oral bioavailability has been reported. A primary goal of this study was to identify scaffolds from which to develop new inhibitors of MELK. To this end, we describe a highly potent indolinone inhibitor of MELK. Furthermore, we report on its selectivity, relative to several related kinases, and show that it preferentially inhibits the proliferation of TNBC cell lines expressing high levels of MELK. MELK contributes to the survival of TNBC cells by inducing the expression of the Bcl-2-family anti-apoptotic protein Mcl-1 (induced myeloid leukemia cell differentiation protein).[31] We demonstrate suppression of Mcl-1 expression by the inhibitor in HCC70 cells in a dose-dependent manner. The inhibitor also shows no activity against non-invasive, non-tumorigenic MCF-10A human breast epithelial cells. This inhibitor (**17**) therefore represents a useful starting point from which to develop a new class of MELK inhibitor.

2. Results and discussion

We initiated an off-target/cross-screening assay of an in-house library of approximately 800 known kinase inhibitors to identify inhibitors of MELK. Compounds were ranked according to average percent inhibition at both 10 and 1 μM, revealing 18 compounds with ≥ 50% inhibition at 1 μM. The ten most potent compounds were further characterized by determining each compound's IC_{50} (Table S1 in supporting data). We found that the indolinone scaffold or a similar bicyclic core structure populated the top hits. Indeed, indolinone motifs represent a common pharmacophore in ATP-competitive inhibitors.[32-33]

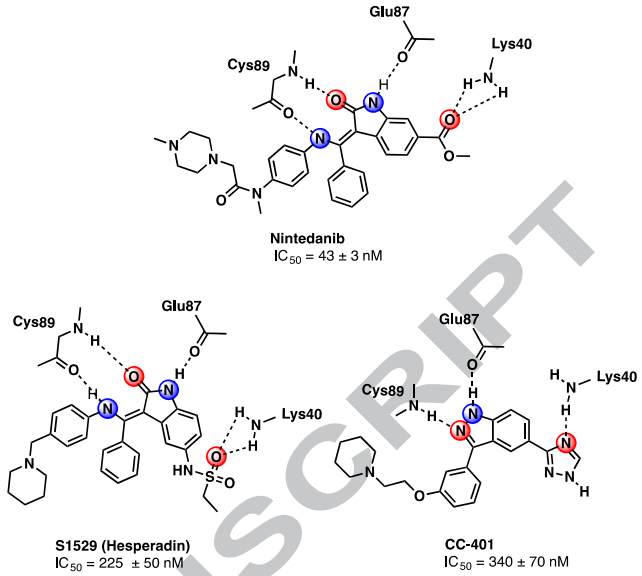


Figure 1. Top 3 screening hits with key predicted hydrogen-bonding interactions with MELK binding site and their IC_{50}'s

The most potent of the top hits was nintedanib (BIBF-1120, Vargatef®/Ofev®), which displayed an IC_{50} of 43 nM (Figure 1). Nintedanib is already FDA-approved for the treatment of idiopathic pulmonary fibrosis and is currently undergoing clinical trials for treatment of non-small cell lung cancer, metastatic colorectal cancer, and ovarian cancer. However, it is a multi-kinase inhibitor whose primary mechanism of action is inhibition of the growth factor receptors VEGFR, PDGFR, and FGFR. Given its favorable drug-like properties, we decided to improve upon nintedanib's potency and selectivity towards MELK.

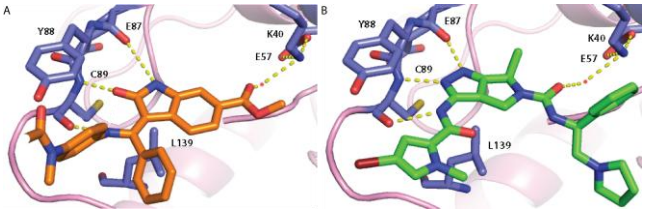


Figure 2. A) Docking of nintedanib (IC_{50} ~ 43 nM) into the ATP-binding site of the MELK conformation PDB 4BKY. B) Crystal structure of a benzodipyrazole inhibitor (**Cpd2**) in complex with the MELK catalytic domain (PDB: 4BKZ).

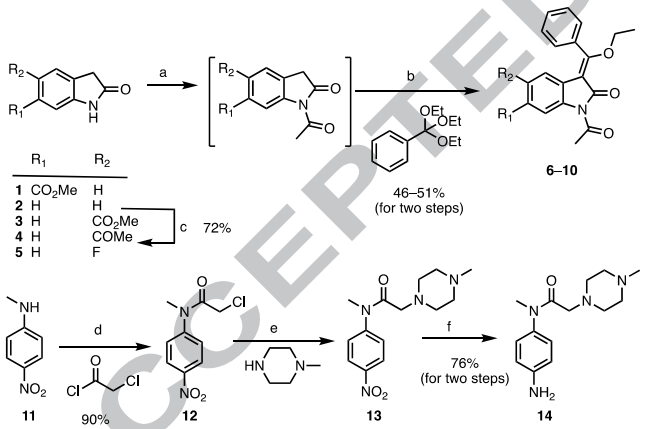
While nintedanib was clearly the most potent molecule, (IC_{50} = 43 nM), additional observations from our screen directed our medicinal chemistry design strategy. The top three most potent inhibitors (Figure 1) all exhibit a putative alternating donor/acceptor hydrogen-bonding pattern that has also been observed with previously published inhibitors. Heteroatoms and substituents of the bicyclic core are spatially oriented to interact with both the hinge region and the conserved catalytic lysine, respectively. In addition to nintedanib, an indolinone scaffold forms the core of three other candidate compounds (Table S1, entries 2, 8, and 9 in supporting data), while others contained similar heterocyclic motifs. Notably, several of the indolines contain substituents at the 5th or 6th position, which are predicted through modeling

studies to interact via hydrogen bonding with K40 of the binding pocket.

Molecular modeling studies using Gold 5.1 (Cambridge Crystallographic Data Center) reinforce the similarities in binding pose and electrostatic contacts between nintedanib and other published inhibitors (Figure 2). In the hinge region, the backbone of C89 is predicted to interact with the enamine nitrogen and the carbonyl of the indolinone ring of nintedanib (Figure 2A), mirroring the hydrogen bonding pattern seen in the crystal structure of MELK with **Cpd2** (Figure 2B, PDB 4BKY).[34] With both nintedanib and **Cpd2**, K40 is positioned to interact with bicyclic core substituents, either directly or via a water molecule. Interaction with K40 has been previously described as an "activity cliff" with other MELK inhibitors, in that loss of this interaction results in loss of compound activity towards MELK.[35-36] Taken together, the screening and modeling data suggest that MELK not only accommodates various substituents at the 5th and 6th positions of the indolinone ring, but also it may be a critical element for maintaining inhibitor potency. Thus, we elected to explore 5- and 6-substituted indolinones.

2.1. Synthesis and biological evaluation of indolinone derivatives

The general synthetic routes are outlined in Schemes 1 and 2. The key intermediates **6**–**10** were prepared by acylation of indolinones **1**–**5** and subsequent condensation with *ortho*-benzoic acid triethylester.[37] Aromatic amine intermediate **14** was prepared by literature procedures as illustrated in Scheme 1.[37] Acylation of *N*-methyl-4-nitroaniline **11** gave chloroactyl amide **12**, which was then treated with *N*-methylpiperazine to displace chloride; followed by catalytic reduction of the nitro group which gave the key aromatic amine intermediate **14**.

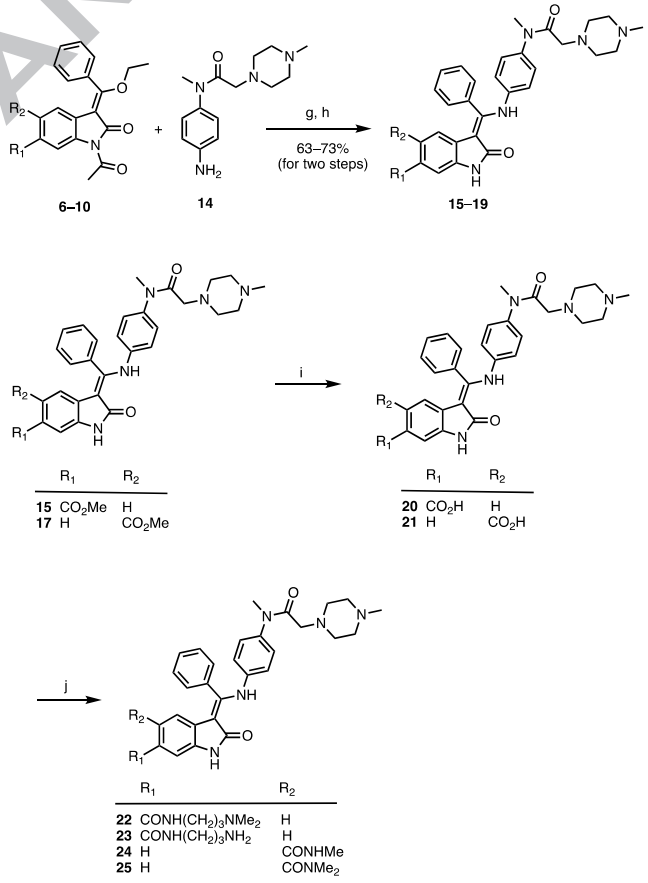


Reagents and conditions: (a) Ac_2O, 130 °C, 8 h; (b) $PhC(OEt)_3$, Ac_2O, 120 °C, 6 h, 46–51% (for two steps a and b); (c) AcCl, $AlCl_3$, 1,2-dichloroethane, below 10 °C, 1 h then RT, 12 h, 72%; (d) chloroacetyl chloride, ethyl acetate, 70 °C, 1 h, 90%; (e) *N*-methylpiperazine, toluene, 55 °C, 2 h; (f) Pd/C, H_2, ^{i}Pr-OH, RT, 12 h, 76% (for two steps e and f).

Scheme 1. Synthesis of intermediates **6**–**10** and **14**.

Final indolinone analogs **15**–**19** were prepared by addition of **14** to substituted indolinones **6**–**10** and subsequent elimination of ethanol, followed by acetyl cleavage using piperidine in one-pot (Scheme 2). Additional 5 or 6-substituted amide analogs **22**–**25** were synthesized from the corresponding 5 or 6-substituted methyl ester derivatives by hydrolysis using aqueous 1 N NaOH and subsequent standard amide coupling reactions using *N*-methyl amine and *N*, *N*-dimethylamine after TBTU or HBTU activation (Scheme 2). The 5 or 6-substituted indolinone derivatives **15**–**25** in Table 1 were evaluated for their ability to inhibit MELK.

First, we focused our efforts on 6-substituted indolinone derivatives. After synthesizing nintedanib (hereafter called compound **15**), we hydrolyzed the methyl ester to the carboxylic acid **20**. We then made longer amine containing amides **22** and **23** (similar to the amine motif in the known MELK inhibitor **Cpd1**).[34] Unfortunately, all the 6-substituted indolinone derivatives were found to be less potent compared to the lead compound **15** (Table 1). We subsequently shifted our attention to the 5th position of the indolinone ring. We made 5-substituted methyl ester, methyl ketone and fluoro indolinones **17**–**19**. Gratifyingly, both 5-CO_2Me **17** (K_i = 0.39 nM) and the 5-F derivative **19** (K_i = 3.1 nM) were found to be more potent compared to the lead compound **15** (K_i = 5.6 nM). The unsubstituted indolinone **16** was also found to be active (K_i = 0.47 nM), challenging the notion that the substituents at the 5th or 6th position are critical for binding MELK. Hydrolysis of the methyl ester produced inhibitor **21**, which retained MELK activity (K_i = 0.68 nM). 5-substituted amide analogs **24** (K_i = 2.3 nM) and **25** (K_i = 8.8 nM) showed lower activities compared to the 5-methyl ester analog **17** (K_i = 0.39 nM).



Reagents and conditions: (g) DMF, 80–100 °C, 1 h; (h) piperidine, RT, 63–73% (for two steps g and h); (i) 1N aq. NaOH, dioxane:methanol (1:1), 80 °C, 5 h; (j) an amine reactant, TBTU or HBTU, HOBT, DIPEA, DMF, RT. For yields, see experimental section.

Scheme 2. Synthesis of final compounds **15**–**25**

2.2. Computer modeling

To further understand the increase in potency by shifting the methyl ester from the 6th to the 5th position, both compound **15** (5-CO_2Me) and compound **17** (6-CO_2Me) were docked with MELK (PDB: 4BKY) and analyzed using Gold5.2 with the ChemPLP scoring function. The methyl ester substituents of both compounds are within direct or water-bridged hydrogen bonding distance of K40 (Figure 3). A methyl ester in the 5th position, however is predicted to force **17** deeper into the narrow groove leading to K40. This would possibly shorten the H-bond distance and strengthen the interaction.

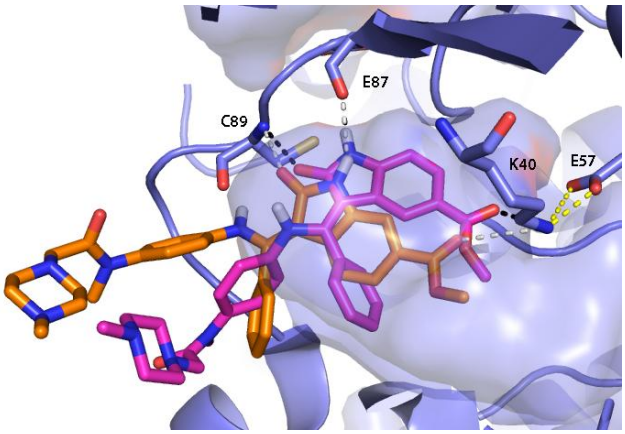


Figure 3. Top-down view of the ATP binding pocket of MELK (PDB: 4BKY) and superposition of predicted binding poses of compounds **15** (orange sticks) and **17** (magenta sticks). Compound **15** forms hydrogen bonds with C89 (2.7 Å), E87 (2.9 Å), and K40 (3.7 Å) of the ATP binding pocket. 6th position substitution sterically prevents **15** from achieving optimal complementarity with the curvature of the binding site. 5th position methyl ester substitution in **17** forms the same hydrogen bonds, but allows deeper penetration of the compound into the binding site, shortening hydrogen bond lengths between MELK and the compound by over an angstrom in some cases (C89 = 3.0 Å, E87 =1.6 Å, K40 = 3.0 Å). Measurements were generated using Pymol software. Inhibitor-protein H-bonds are shown as dashed gray lines for compound **15** and dashed black lines for compound **17.** Other H-bonds are shown as yellow dashes. ATP binding pocket is shaded in light purple. Ribbon backbones of some residues are excluded for clarity.

Moreover, this modification is predicted to enable **17** to adopt a conformation more complementary to the shape of the binding pocket, facilitating the interaction of the indolinone core with E87 through an additional hydrogen bond. The potency of **16** (unsubstituted indolinone) suggests that suboptimal substituent position significantly affects inhibitor binding, or alternatively, that an unsubstituted indolinone can adopt a greater number of binding modes.

2.3. Selectivity

As a preliminary assessment of selectivity, we chose to evaluate our inhibitors against MELK and five other kinases. These included MELK's most closely related family members AMPK and NUAK1, as well as the cell cycle regulated kinase CHK1, the AMPK family activator CAMKKII, and ERK2.

We subjected the three most potent MELK inhibitor derivatives (**16**, **17**, and **21**) to inhibition assays to determine their IC_{50}'s against the six kinases. Using apparent K_m 's of ATP, $K_{m\ (ATP)}^{app}$we calculated a selectivity $\varphi = K_i^{kinase} / K_i^{Melk}$ based on the calculated K_i for each kinase (Table 2). Compound **16**, which contains no substituent at either the 5- or 6-position of the indolinone, was the least selective inhibitor of the three. Addition of a methyl ester at the 5-position (compound **17**) increased selectivity without compromising potency towards MELK and the hydrolysis of the methyl ester to the carboxylate (compound **21**) yielded a further improvement in selectivity, displaying > 100-fold selectivity towards CHK1, CAMKK2, and ERK2. It is possible that the methyl ester of **17** is hydrolyzed *in vivo* due to the prevalence of esterases at the cellular and whole organism level, thus **17** may serve as a metabolic precursor for **21** *in vivo*. While **17** and **21** do not yet have the selectivity desired, our modeling data suggests that further elaboration of the indolinone scaffold will afford lead compounds exhibiting both higher potency and selectivity.

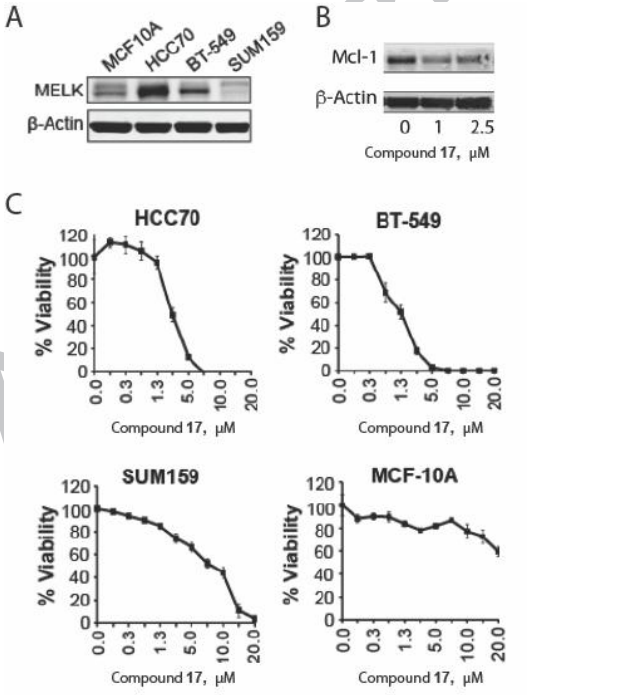


Figure 4. Compound **17** suppresses cell proliferation of TNBC cells in vitro. A. Expression levels of MELK. B. **17** suppresses expression of Mcl-1. C. **17** suppressing cell proliferation, and cells expressing high levels of MELK are more sensitive to **17** than cells expressing low levels of MELK. Cells were seeded in 96-well plates and the next day treated with inhibitors at the indicated concentrations. Seventy-two hours later, cell viability was determined using the CellTiter-Blue Cell Viability Assay.

2.4. Cellular selectivity

As **17** is a potent inhibitor of the MELK catalytic domain, we sought evidence that it engages MELK in TNBC cells. Figure 4A shows that MELK is expressed in various TNBC subtypes,[38] HCC70 (BL2 subtype), BT-549 (mesenchymal) and SUM-159 (mesenchymal stem-like), as well as the immortalized mammary epithelial cell line MCF10A (classified as Basal B by molecular profiling[39]). **17** induces a decrease in Mcl-1 expression in HCC70 cells, consistent with the notion that it inhibits the MELK-eIF4B signaling axis to abrogate Mcl-1 expression (Figure 4B)).[31] As inhibition of MELK was found to induce apoptosis in basal-like breast cancer cells,[13, 35] we used the CellTiter-Blue assay to compare the ability of **17** to inhibit the proliferation of cell lines with varied levels of MELK expression. While proliferation of the HCC70, BT-549, and SUM-159 cells were impacted by treatment with **17**, the MCF10A cells remained largely

unaffected at 10 μM (Figure 4C). Furthermore, TNBC cell lines expressing higher levels of MELK protein (HCC70 and BT549) were more sensitive to **17** than the SUM159 cells which express low levels of MELK. These data support the notion that **17** inhibits MELK in TNBC cells to suppress Mcl-1 expression and reduce TNBC cell viability.

3. Conclusions

We have identified 5-substituted indolinones as inhibitors of MELK and identified three tight binding inhibitors (**16**, **17**, and **21**) with K_i^{Melk}< 1nM. The most potent compound was shown to inhibit Mcl-1 expression in the MELK-expressing HCC70 TNBC cell line, and shown to preferentially decrease the proliferation of TNBC cells expressing high levels of MELK. It also shows little effect on the immortalized breast epithelial cell line MCF-10A, which also expresses lower levels of MELK. There are currently no FDA-approved inhibitors of MELK and no selective, orally bioavailable inhibitors have been reported. These studies suggest that further elaboration of **17** will furnish MELK-selective inhibitors with potential for development in preclinical models of TNBC and other cancers.

4. Experimental section

4.1. Chemistry

General Information. Reagents and starting materials including indolinones **1**–**3**, and **5**, and *N*-Methyl-4-nitroaniline **11** were purchased from various commercial sources including Sigma-Aldrich or Matrix Scientific and used without further purification unless otherwise stated. 5-Acetylindolinone **4** and aromatic amine intermediate **14** were prepared by literature procedures.[40, 37] All reactions were carried out in oven- or flame-dried glassware under argon. Thin-layer chromatography (TLC) was performed using pre-coated TLC plates with silica gel 60 F_{254} (EMD) or with aluminum oxide 60 F_{254} neutral. Flash column chromatography was performed using 40–63 μm (230–400 mesh ASTM) silica gel (EMD). Melting points were recorded on a Thomas Hoover capillary melting point apparatus. NMR spectra were recorded on a Varian NMR spectrometer. High-resolution mass and liquid chromatography mass spectral data were obtained at the University of Texas at Austin. Compounds were characterized by NMR and HRMS or LCMS.

5-Acetylindolin-2-one (**4**). By following acylation procedure reported in a patent,[40] 5-acetylindolin-2-one was prepared from indolin-2-one and $AlCl_3$. The crude was recrystallized from ethyl acetate to obtain 72% of the title compound. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.76 (br s, 1H, NH), 7.85 (m, 1H, Ar-H), 7.80 (m, 1H, Ar-H), 6.90 (d, J = 8.0 Hz, 1H, Ar-H), 3.55 (s, 2H), 2.50 (s, 3H, Ac).

General procedure for the synthesis of *N*-acetyl-3-(ethoxy(phenyl)methylene)-2-oxoindolines (Compounds **6**–**10**). **Methyl (*Z*)-1-acetyl-3-(ethoxy(phenyl)methylene)-2-oxoindoline-6-carboxylate** (**6**).[37] Indolinone **1** (1000 mg, 52.3 mmol) was suspended in acetic anhydride (10 mL) and refluxed at 130 ^{o}C for 8 h. The reaction mixture was allowed to cool to 50 ^{o}C and (triethoxymethyl)benzene (2930 mg, 131 mmol) was added. The resulting reaction mixture was stirred at 120 ^{o}C for 6 h. Then, volatiles were removed *in vacuo* and petroleum ether was added to the obtained residue. After triturating for 15 minutes, the separated solids were filtered and washed with petroleum ether and then dried under vacuum to afford 974 mg (51%) of title compound. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.75 (s, 1H), 8.10 (d, J = 8.0 Hz, 1H), 7.89 (d, J = 8.0 Hz, 1H), 7.49–7.58 (m, 5H), 4.01 (q, J = 7.2 Hz, 2H), 3.87 (s, 3H), 2.44 (s, 3H), 1.35 (t, J = 8.0 Hz, 3H). HRMS *m*/*z* found 365.1260, calcd for $C_{21}H_{19}NO_5$ $[M]^+$ 365.1263.

(*Z*)-1-acetyl-3-(ethoxy(phenyl)methylene)indolin-2-one (**7**).[37] The title compound was synthesized in 46% yield using similar procedure as described for the synthesis of compound **6** by substituting indolinone **2** for indolinone **1**. Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.17–8.13 (m, 1H), 8.02–7.99 (m, 1H), 7.56–7.46 (m, 5H), 7.30–7.22 (m, 2H), 3.94 (q, J = 7.2 Hz, 2H), 2.43 (s, 3H), 1.33 (t, J = 7.4 Hz, 3H). LCMS *m*/*z* found 308.1, calcd for $C_{19}H_{18}NO_3$ $[M+H]^+$ 308.1.

Methyl (*Z*)-1-acetyl-3-(ethoxy(phenyl)methylene)-2-oxoindoline-5-carboxylate (**8**).[41] The title compound was synthesized in 48% yield using similar procedure as described for the synthesis of compound **6** by substituting indolinone **3** for indolinone **1**. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.61 (d, J = 1.6 Hz, 1H), 8.23 (d, J = 8.4 Hz, 1H), 7.92 (dd, J = 8.4 Hz, J = 1.6 Hz, 1H), 7.58–7.49 (m, 5H), 3.99 (q, J = 7.0 Hz, 2H), 3.87 (s, 3H), 2.45 (s, 3H), 1.37 (t, J = 7.0 Hz, 3H). HRMS *m*/*z* found 346.1058, calcd for $C_{19}H_{17}NNaO_4$ $[M\text{-}Ac+Na]^+$ 346.1055.

(*Z*)-1,1'-(3-(Ethoxy(phenyl)methylene)-2-oxoindoline-1,5-diyl)bis(ethan-1-one) (**9**).[37] The title compound was synthesized in 47% yield using similar procedure as described for the synthesis of compound **6** by substituting indolinone **4** for indolinone **1**. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.58 (d, J = 2.0 Hz, 1H), 8.23 (d, J = 8.4 Hz, 1H), 7.94 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H), 7.58–7.49 (m, 5H), 3.99 (q, J = 7.0 Hz, 2H), 2.61 (s, 3H), 2.45 (s, 3H), 1.39 (t, J = 8.0 Hz, 3H). LCMS *m*/*z* found 350.1, calcd for $C_{21}H_{20}NO_4$ $[M+H]^+$ 350.1.

(*Z*)-1-acetyl-3-(Ethoxy(phenyl)methylene)-5-fluoroindolin-2-one (**10**). The title compound was synthesized in 48% yield using similar procedure as described for the synthesis of compound **6** by substituting indolinone **5** for indolinone **1**. Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.16 (m, 1H), 7.71(m, 1H), 7.57–7.47 (m, 5H), 7.12 (m, 1H), 3.98 (q, J = 7.0 Hz, 2H), 2.41 (s, 3H), 1.33 (t, J = 8.0 Hz, 3H). ^{19}F NMR (376 MHz, DMSO-d_6): δ - 117.6 (m). LCMS *m*/*z* found 326.1, calcd for $C_{19}H_{17}FNO_3$ $[M+H]^+$ 326.1.

***N*-(4-Aminophenyl)-*N*-methyl-2-(4-methylpiperazin-1-yl)acetamide** (**14**). The title acetamide compound was prepared from *N*-methyl-4-nitroaniline **11** using similar procedure as described in literature.[41] ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.10 (s, 3H), 2.14–2.43 (m, 8H), 2.79 (s, 2H), 3.03 (s, 3H), 5.23 (s, 2H), 6.52–6.57 (m, 2H), 6.88–6.92 (m, 2H). HRMS *m*/*z* found 263.1866, calcd for $C_{14}H_{23}N_4O$ $[M+H]^+$ 263.1872.

General procedure for the synthesis of final compounds 15–**19**. **Methyl (*Z*)-3-(((4-(*N*-methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)(phenyl)methylene)-2-oxoindoline-6-carboxylate** (**15**).[37] To a suspension of methyl (*Z*)-1-acetyl-3-(ethoxy(phenyl)methylene)-2-oxoindoline-6-carboxylate (**6**) (500 mg, 1.368 mmol) in DMF (3.5 mL) was added *N*-(4-aminophenyl)-*N*-methyl-2-(4-methylpiperazin-1-yl)acetamide (**14**) (395 mg, 1.505 mmol, 1.1 equiv.) at RT. After heating the reaction mixture at 80 ^{o}C for 1 h, it was allowed to cool to RT. Piperidine (297 μL, 3.010 mmol, 2.2 equiv.) was then added and stirred for 2 h. Volatiles were removed *in vacuo* and water was added to the obtained residue

and stirred for 15 min. The precipitate was then filtered under suction and cake was washed with water, then with minimum amount of cold methanol, and then ether. The obtained product was purified by column chromatography (neutral Al_2O_3, 0–10% methanol in CH_2Cl_2) to afford 532 mg (72%) of target molecule **15**. Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.22 (s, 1H), 10.98 (s, 1H), 7.66–7.47 (m, 5H), 7.42 (s, 1H), 7.24–7.09 (m, 3H), 6.89 (d, J = 8.0 Hz, 2H), 5.83 (d, J = 8.0 Hz, 1H), 3.77 (s, 3H), 3.06 (s, 3H), 2.69 (s, 2H), 2.34–2.06 (brs, 8H), 2.10 (s, 3H). HRMS *m/z* found 540.2606, calcd for $C_{31}H_{34}N_5O_4$ $[M+H]^+$ 540.2605.

(*Z*)-*N*-Methyl-2-(4-methylpiperazin-1-yl)-*N*-(4-(((2-oxoindolin-3-ylidene)(phenyl)methyl)amino)phenyl)acetamide (**16**). The title compound was synthesized in 64% yield using similar procedure as described for the synthesis of compound **15** by substituting (*Z*)-1-acetyl-3-(ethoxy(phenyl)methylene)indolin-2-one (**7**) for indolinone derivative **6**. A 58:42 mixture of conformers ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.19 and 12.02 (s, 1H), 10.74 and 10.59 (s, 1H), 7.61–5.75 (m, 13H), 3.17–2.64 (m, 5H), 2.36–2.07 (m, 11H). HRMS *m/z* found 482.2408, calcd for $C_{29}H_{32}N_5O_2$ $[M+H]^+$ 482.2400.

Methyl (*Z*)-3-(((4-(*N*-methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)(phenyl)methylene)-2-oxoindoline-5-carboxylate (**17**). The title compound was synthesized in 73% yield using similar procedure as described for the synthesis of compound **15** by substituting Methyl (*Z*)-1-acetyl-3-(ethoxy(phenyl)methylene)-2-oxoindoline-5-carboxylate (**8**) for indolinone derivative **6**. Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.97 (s, 1H), 11.13 (s, 1H), 7.63–7.47 (m, 6H), 7.13 (d, J = 8.4 Hz, 2H), 6.94 (d, J = 8.4 Hz, 1H), 6.88 (d, J = 8.4 Hz, 2H), 6.51 (s, 1H), 3.63 (s, 3H), 3.06 (s, 3H), 2.69 (s, 2H), 2.19 (brs, 8H), 2.10 (s, 3H). HRMS *m/z* found 540.2613, calcd for $C_{31}H_{34}N_5O_4$ $[M+H]^+$ 540.2605.

(*Z*)-*N*-(4-(((5-Acetyl-2-oxoindolin-3-ylidene)(phenyl)methyl)amino)phenyl)-*N*-methyl-2-(4-methylpiperazin-1-yl)acetamide (**18**). The title compound was synthesized in 63% yield using similar procedure as described for the synthesis of compound **15** by substituting (*Z*)-1,1'-(3-(Ethoxy(phenyl)methylene)-2-oxoindoline-1,5-diyl)bis(ethan-1-one) (**9**) for indolinone derivative **6**. Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.94 (s, 1H), 11.14 (s, 1H), 7.63–7.50 (m, 6H), 7.13 (d, J = 8.4 Hz, 2H), 6.94 (d, J = 8.4 Hz, 1H), 6.90 (d, J = 8.4 Hz, 2H), 6.39 (s, 1H), 3.06 (s, 3H), 2.71 (s, 2H), 2.32–2.16 (brs, 8H), 2.14 (s, 6H). HRMS *m/z* found 523.2585, calcd for $C_{31}H_{33}N_5O_3$ $[M]^+$ 523.2583.

(*Z*)-*N*-(4-(((5-Fluoro-2-oxoindolin-3-ylidene)(phenyl)methyl)amino)phenyl)-*N*-methyl-2-(4-methylpiperazin-1-yl)acetamide (**19**). The title compound was synthesized in 69% yield using similar procedure as described for the synthesis of compound **15** by substituting (*Z*)-1-acetyl-3-(Ethoxy(phenyl)methylene)-5-fluoroindolin-2-one (**10**) for indolinone derivative **6**. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.09 (s, 1H), 10.76 (s, 1H), 7.63–7.48 (m, 5H), 7.12 (d, J = 8.4 Hz, 2H), 6.90–6.79 (m, 3H), 6.72 (m, 1H), 5.37 (dd, J = 10.4 Hz, 2.4 Hz, 1H), 3.05 (s, 3H), 2.67 (s, 2H), 2.18 (brs, 8H), 2.10 (s, 3H). ^{19}F NMR (376 MHz, DMSO-d_6): δ - 123.3 (m). HRMS *m/z* found 500.2455, calcd for $C_{29}H_{31}FN_5O_2$ $[M+H]^+$ 500.2456.

General procedure for the synthesis of final compounds 22–25. (*Z*)-*N*-(3-(Dimethylamino)propyl)-3-(((4-(*N*-methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)(phenyl)methylene)-2-oxoindoline-6-carboxamide (**22**).

6-carboxylic acid methyl ester **15** (250 mg, 0.46 mmol) was added to 1:1 mixture of methanol and dioxane (8 mL). The resulting suspension was heated to 50 °C and then 1N aqueous NaOH solution (2.5 mL) was added. The solution was stirred for 5 h at 80 °C and then allowed to cool to RT. Volatiles were removed *in vacuo* and water was added to the obtained residue. After stirred for 10 minutes, the separated solids were filtered and washed with water and diethyl ether, and then dried under vacuum to afford crude (*Z*)-3-(((4-(*N*-Methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)(phenyl)methylene)-2-oxoindoline-6-carboxylic acid (**20**). Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.20 (s, 1H), 10.96 (s, 1H), 7.63–7.48 (m, 5H), 7.42 (s, 1H), 7.17 (dd, J = 8.0 Hz, 1.2 Hz, 1H), 7.13 (d, J = 8.4 Hz, 2H), 6.88 (d, J = 8.0 Hz, 2H), 5.81 (d, J = 8.4 Hz, 1H), 3.06 (s, 3H), 2.67 (s, 2H), 2.20 (brs, 8H), 2.12 (s, 3H). HRMS *m/z* found 525.2371, calcd for $C_{30}H_{31}N_5O_4$ $[M]^+$ 525.2376.

To a suspension of crude 6-carboxylic acid **20** (1.0 equiv.), HBTU or TBTU (1.2 equiv.), HOBt (1.2 equiv.) in dimethylformamide was added DIPEA (16 equiv.) at RT. *N*,*N*-dimethylpropane-1,3-diamine (1.5 equiv.) was then added and continued stirred for 2h. Volatiles were removed *in vacuo* and the obtained product was purified by column chromatography (neutral Al_2O_3, 0–10% methanol in CH_2Cl_2) or HPLC to afford the target molecule 2-oxoindoline-6-carboxamide **22**. Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.10 (s, 1H), 10.93 (s, 1H), 8.24 (t, J = 5.2 Hz, 1H), 7.64–7.49 (m, 5H), 7.33 (d, J = 1.6 Hz, 1H), 7.12 (d, J = 8.4 Hz, 2H), 7.05 (dd, J = 8.4 Hz, 1.6 Hz, 1H), 6.87 (d, J = 8.4 Hz, 2H), 5.74 (d, J = 8.0 Hz, 1H), 3.20 (q, J = 8.0 Hz, 2H), 3.05 (s, 3H), 2.69 (s, 2H), 2.33–2.05 (m, 19H), 1.59 (m, 2H). HRMS *m/z* found 610.3494, calcd for $C_{35}H_{44}N_7O_3$ $[M+H]^+$ 610.3500.

(*Z*)-*N*-(3-Aminopropyl)-3-(((4-(*N*-methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)(phenyl)methylene)-2-oxoindoline-6-carboxamide (**23**). The title compound was synthesized using similar procedure as described for the synthesis of compound **22** by substituting *tert*-butyl (3-aminopropyl)carbamate for dimethylpropane-1,3-diamine followed by removal of Boc protecting group with treatment of 20% TFA in dichloromethane at RT for 3 hr. Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.15 (s, 1H), 10.98 (s, 1H), 8.37 (t, J = 5.2 Hz, 1H), 7.72–7.50 (m, 7H), 7.35 (s, 1H), 7.16 (d, J = 8.4 Hz, 2H), 7.08 (dd, J = 8.0 Hz, 1.6 Hz, 1H), 6.89 (d, J = 8.4 Hz, 2H), 5.74 (d, J = 8.4 Hz, 1H), 3.48–2.69 (m, 20H), 1.74 (m, 2H). HRMS *m/z* found 582.3186, calcd for $C_{33}H_{40}N_7O_3$ $[M+H]^+$ 582.3187.

(*Z*)-*N*-Methyl-3-(((4-(*N*-methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)(phenyl)methylene)-2-oxoindoline-5-carboxamide (**24**). (*Z*)-3-(((4-(*N*-Methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)(phenyl)methylene)-2-oxoindoline-5-carboxylic acid (**21**) was synthesized using similar procedure as described for the synthesis of compound **20** by substituting indolinone **17** for indolinone derivative **15**. Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.00 (s,

1H), 11.09 (s, 1H), 7.60–7.47 (m, 6H), 7.12 (d, *J* = 8.4 Hz, 2H), 6.92 (d, *J* = 8.0 Hz, 1H), 6.85 (d, *J* = 8.8 Hz, 2H), 6.59 (s, 1H), 3.05 (s, 3H), 2.69 (s, 2H), 2.19 (brs, 8H), 2.11 (s, 3H). HRMS *m*/*z* found 526.2453, calcd for $C_{30}H_{32}N_5O_4$ $[M+H]^+$ 526.2449.

The title compound **24** was synthesized using similar procedure as described for the synthesis of compound **22** by substituting methylamine hydrochloride for dimethylpropane-1,3-diamine. Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.03 (s, 1H), 10.97 (s, 1H), 7.93 (m, 1H), 7.62–7.46 (m, 5H), 7.39 (dd, *J* = 8.4 Hz, 1.6 Hz, 1H), 7.11 (d, *J* = 8.4 Hz, 2H), 6.85 (d, *J* = 8.4 Hz, 1H), 6.83 (d, *J* = 8.8 Hz, 2H), 6.53 (s, 1H), 3.05 (s, 3H), 2.69 (s, 2H), 2.63 (d, *J* = 4.8 Hz, 3H), 2.19 (brs, 8H), 2.11 (s, 3H). HRMS *m*/*z* found 539.2782, calcd for $C_{31}H_{35}N_6O_3$ $[M+H]^+$ 539.2765.

(*Z*)-*N*,*N*-Dimethyl-3-(((4-(*N*-methyl-2-(4-methylpiperazin-1-yl)acetamido)phenyl)amino)(phenyl)methylene)-2-oxoindoline-5-carboxamide (**25**). The title compound was synthesized using similar procedure as described for the synthesis of compound **22** by substituting dimethylamine hydrochloride for dimethylpropane-1,3-diamine. Major conformer ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.03 (s, 1H), 10.94 (s, 1H), 7.62–7.48 (m, 5H), 7.11 (d, *J* = 8.4 Hz, 2H), 7.03 (dd, *J* = 8.0 Hz, 1.6 Hz, 1H), 6.88 (d, *J* = 8.0 Hz, 1H), 6.84 (d, *J* = 8.0 Hz, 2H), 5.86 (s, 1H), 3.05 (s, 3H), 2.76 (s, 6H), 2.69 (s, 2H), 2.18 (brs, 8H), 2.10 (s, 3H). HRMS *m*/*z* found 553.2930, calcd for $C_{32}H_{37}N_6O_3$ $[M+H]^+$ 553.2922.

4.2. Inhibitor library screening

MELK and its substrate, Bcl-G_L were both recombinantly expressed and purified for use in screening assays as detailed in supplementary data. 752 compounds from an in-house curated inhibitor library were subjected to a p81-based kinase assay. 10 nM MELK 340 and 10 μM Bcl-G_L in kinase assay buffer (50 mM HEPES pH 7.5, 100 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM $MgCl_2$, 10 μg/mL BSA) with 10 mM DTT were added to either 10 μM or 1 μM inhibitor aliquoted into 96 well plates (final 1% DMSO). The mixture was incubated at RT for 30 minutes prior to initiation of the assay with 40 μM γ-^{32}P-ATP (100 – 1000 CPM/pmol). 40 μL aliquots were spotted onto a p81 96 well filter plate (Unifilter, Whatman), quenched, and washed with 75 mM O-phosphoric acid 8 times, followed by a final wash with acetone for drying. Wells were then filled with scintillation fluid, sealed, and quantified using a MicroBeta TriLux liquid scintillation counter (PerkinElmer). Each inhibitor plate was assayed in duplicate, with at least 4 wells without MELK to establish background and at least 4 wells without inhibitor as a negative control. All readings were corrected for background signal based on the average counts from the wells without enzyme. Percent inhibition, defined as [(1 – (CPM + inhibitor/average CPM of negative controls))*100], was determined first at 10 μM inhibitor. The top 50 inhibitors were then re-screened in duplicate at 1 μM. Additional methods for inhibitor screening and selectivity assays are in supplementary data.

4.3. Cell culture and reagents

HCC70, BT-549, and SUM159 human TNBC cell lines and MCF10A human breast epithelial cell line were purchased from American Type Culture Collection. HCC70 and BT-549 cells were maintained in RPMI 1640 medium (Life Technologies Inc., Grand Island, NY, USA) supplemented with FBS (10%) and antibiotic/antimycotic (1%). SUM159 cells were maintained in Ham's F-12 medium (Life Technologies Inc.) supplemented with FBS (5%), antibiotic/antimycotic (1%), insulin (5 μg/mL), and hydrocortisone (1 μg/mL). MCF10A cells were maintained in Dulbecco's modified Eagle's medium/F12 medium (Life Technologies Inc.) supplemented with horse serum (10%), antibiotic/antimycotic (1%), insulin (10 μg/mL), EGF (20 ng/mL), cholera toxin (100 ng/mL), and hydrocortisone (500 μg/mL). All cell lines used in this study were validated in September 2016 by the Characterized Cell Line Core Facility at MD Anderson Cancer Center by using a short tandem repeat method based on primer extension to detect single base deviations.

4.3.1. Western blotting

For the analysis of MELK expression, cells (1 x 10^6) were seeded in 10-cm plates and incubated at 37^{o}C with 5% CO_2 for 48 h. The cell lysates were then prepared and used for Western blotting analysis as described previously.[42] For the analysis of impact of compound 17 treatment on expression of Mcl-1, HCC70 cells (1 x 10^6) were seeded in 10-cm plates overnight and the next day serum-starved for 6 h and then treated with compound 17 (0, 1, and 2.5 μM) for 18 h under serum starvation condition. Following compound 17 treatment, the cells were stimulated with TGF-β (5 ng/mL) for 1 h, and then the cell lysates were prepared and used for Western blotting analysis as described previously.[42] Proteins of interest were probed using the following primary antibodies: anti-MELK (1:750 dilution; R&D Systems, Inc., Minneapolis, MN, USA), anti-Mcl-1 (1:1000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-β-actin (1:8000 dilution; Sigma-Aldrich, St. Louis, MO, USA). Secondary antibodies were horseradish peroxidase-conjugated IgG (1:10,000 dilution; Life Technologies Inc.) for chemiluminescent signal detection and the corresponding Alexa Fluor-conjugated IgG (1:5000 dilution; Life Technologies Inc.) for fluorescence signal detection.

4.3.2. Cell proliferation assay

Cell proliferation was determined using the CellTiter-Blue viability assay as described previously.[43] Cells were seeded in 96-well plates and treated the next day with compound 17 (0-20 μM). At 72 h after treatment, optical density at 595 nm was determined.

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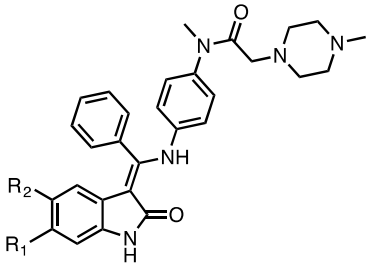
Supplementary data

Additional details of assays, experimental procedures, ^{1}H & ^{19}F NMR spectra, and either LCMS or HRMS are included in the Supplementary data.

Table 1. MELK inhibitory activity of indolinone derivatives **15**–**25**



15 – 25

Compound	R_1	R_2	IC_{50} (nM)	K_i [a] ± SE (nM)
15	CO_2Me	H	43 ± 3.4	5.6 ± 0.4
16	H	H	3.6 ± 0.4	0.47 ± 0.06
17	H	CO_2Me	3 ± 0.8	0.39 ± 0.09
18	H	COMe	354 ± 35	46 ± 4.6
19	H	F	24 ± 4.7	3.1 ± 0.6
20	CO_2H	H	1145 ± 168	149 ± 22
21	H	CO_2H	5.2 ± 0.5	0.68 ± 0.07
22	$CONH(CH_2)_3NMe_2$	H	2700 ± 540	358 ± 71
23	$CONH(CH_2)_3NH_2$	H	> 5000	> 650
24	H	CONHMe	18 ± 3.8	2.3 ± 0.5
25	H	$CONMe_2$	67 ± 11	8.8 ± 1.4

[a] K_i calculated using Equation 4, where [ATP] = 40 μM, $K_{M\,(ATP)}^{app}$ = 6 μM

Table 2. Selectivity of MELK inhibitor derivatives against selected kinases

Kinase	Compound **16**		Compound **17**		Compound **21**	
	K_i (nM)	φ[a]	K_i (nM)	φ[a]	K_i (nM)	φ[a]
MELK	0.47 ± 0.06	1.00	0.39 ± 0.09	1.00	0.68 ± 0.07	1.00
AMPK	4.2 ± 0.3	8.9 ± 0.2	9.2 ± 1.7	24 ± 0.3	4.9 ± 0.6	7.2 ± 0.2
NUAK1	1.7 ± 0.17	3.6 ± 0.2	11 ± 0.86	28 ± 0.2	8.6 ± 1.6	13 ± 0.2
CHK1	10 ± 0.5	18 ± 0.2	7 ± 0.17	18 ± 0.2	81 ± 5.6	120 ± 0.1
CAMKK2	15 ± 1.8	32 ± 0.2	31 ± 11	80 ± 0.4	85 ± 9.2	125 ± 0.2
ERK2	1160 ± 320	2500 ± 0.3	3000 ± 1300	7700 ± 0.5	19000 ± 3800	28000 ± 0.2

[a] $\varphi = K_i^{kinase} / K_i^{Melk}$

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