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DNA-dependent protein kinase (DNA-PK) inhibitors: Structure–activity relationships for *O*-alkoxyphenylchromen-4-one probes of the ATP-binding domain

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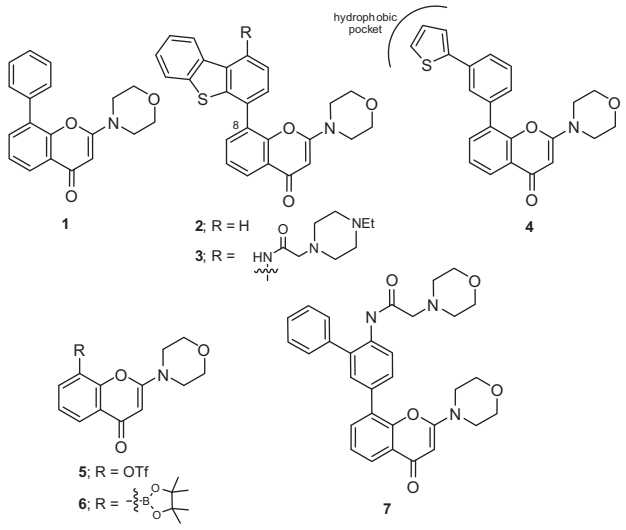
ABSTRACT

Introduction of an *O*-alkoxyphenyl substituent at the 8-position of the 2-morpholino-4*H*-chromen-4-one pharmacophore enabled regions of the ATP-binding site of DNA-dependent protein kinase (DNA-PK) to be probed further. Structure–activity relationships have been elucidated for inhibition of DNA-PK and PI3K (p110α), with *N*-(2-(cyclopropylmethoxy)-4-(2-morpholino-4-oxo-4*H*-chromen-8-yl)phenyl)-2-morpholinoacetamide **11a** being identified as a potent and selective DNA-PK inhibitor (IC_{50} = 8 nM).

DNA-dependent protein kinase (DNA-PK), a member of the phosphatidylinositol (PI) 3-kinase related kinase (PIKK) family, is a multi-component serine/threonine protein kinase that plays a key role in the repair of mammalian DNA double-strand breaks (DSBs) via the non-homologous end joining pathway of DNA repair.[1,2] Human cell lines defective in DNA-PK function are hypersensitive to agents that elicit DNA DSBs.[3,4] By impeding DNA DSB repair, selective DNA-PK inhibitors have potential application as radio- and chemo-potentiators in the treatment of cancer.[5–9] In the absence of structural biology information for the enzyme, we have conducted extensive structure–activity relationships studies (SARs) using homology modelling based on the known crystal structure of PI3Kγ[10] and employing 2-morpholino-8-phenyl-4*H*-chromen-4-one (**1**, LY294002) as a template for inhibitor design. Interestingly, a number of potent DNA-PK inhibitors have been developed from this structural class.[11–15] Previously we have described the incorporation of a dibenzothiophen-4-yl substituent at the 8-position of 2-morpholino-4*H*-chromen-4-one, which conferred excellent inhibitory activity against DNA-PK (**2**, IC_{50} = 28 nM). Crucially, chromenone **2** has been demonstrated to sensitise a human tumour cell line to both ionising radiation and the topoisomerase II inhibitor etoposide in vitro and in vivo.[16] Further refinement of this template has provided the highly potent DNA-PK inhibitor KU-0060648 (**3**; IC_{50} = 5 nM).[17]



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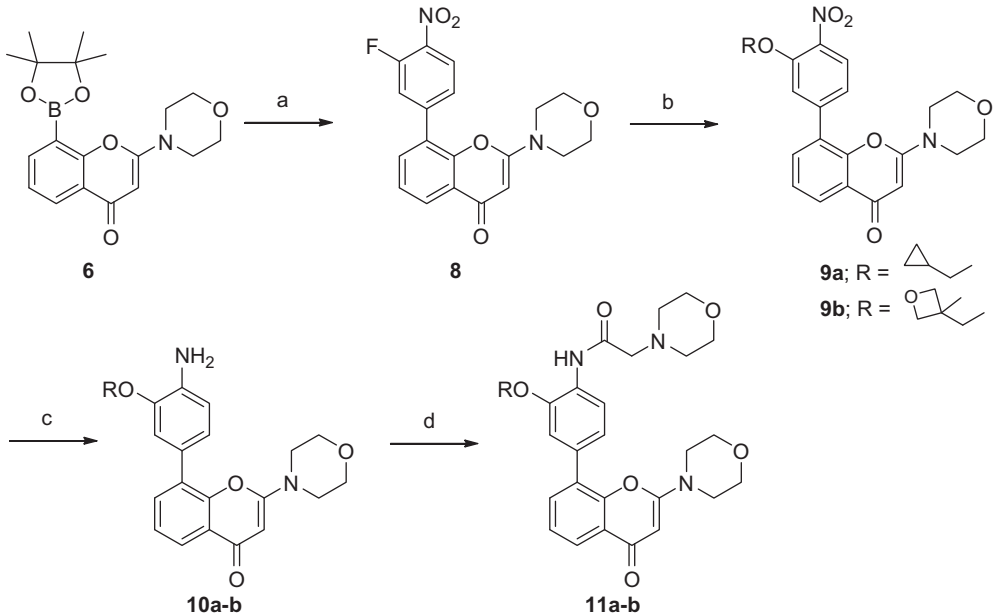
With a view to optimising the biological and pharmaceutical properties of NU7441 (**2**), and to expand SARs, both the core chromenone scaffold and the dibenzothiophen-4-yl moiety have been systematically modified. To this end, we have recently reported the identification of 8-biarylchromen-4-ones (e.g., **4**; IC_{50} = 18 nM), which offered the opportunity to further probe regions of the ATP-binding domain of the enzyme and exhibited good potency against DNA-PK.[18] Interestingly, subsequent homology modelling studies suggested that the heteroaryl substituent may occupy a putative hydrophobic pocket that could be further exploited. In this communication, we report studies designed to probe this region of the ATP-binding site of the kinase, through the synthesis and biological evaluation of a focused library of chromen-4-one DNA-PK inhibitors bearing *O*-alkoxyphenyl substituents at the 8-position.

Our previous studies have utilised the chromenone triflate **5**[11–13] or boronic ester **6**[19] as key intermediates for the preparation of 8-substituted chromenone libraries, employing Suzuki–Miyaura cross-coupling reactions. This strategy was also amenable for the synthesis of the target *O*-alkoxyphenylchromen-4-ones (**11a–b**, **15** and **21a–j**). Introduction of the 8-phenyl substituent was achieved by coupling of **6**, formed in situ by reaction of **5** with bis(pinacolato)diboron, with 4-bromo-2-fluoronitrobenzene to give the chromen-4-one **8** in 58% yield (Scheme 1). The 3-alkoxy side chain was then introduced in good overall yield by a fluoro-displacement reaction, using an alkoxide generated through treatment of the respective alcohol with sodium hydride in DMF. Reduction of the nitro intermediates **9a** and **9b** to the required arylamines (**10a**, **10b**) proceeded in excellent yields using zinc in acetic acid. Finally, in a one-pot two-step process, acylation of **10a–b** with chloroacetyl chloride was followed by reaction with morpholine to provide inhibitors **11a–b** (Table 1). In this series, the choice of a morpholine-substituted side chain was based upon previous studies which demonstrated that the parent biaryl derivative **7** exhibited good inhibitory activity against DNA-PK (IC_{50} = 14 nM) and potentiated the DNA damage elicited by ionising radiation at 2 Gy (HeLa cells, DMR (0.5 μM) = 8 and DMR (0.1 μM) = 11).[20]

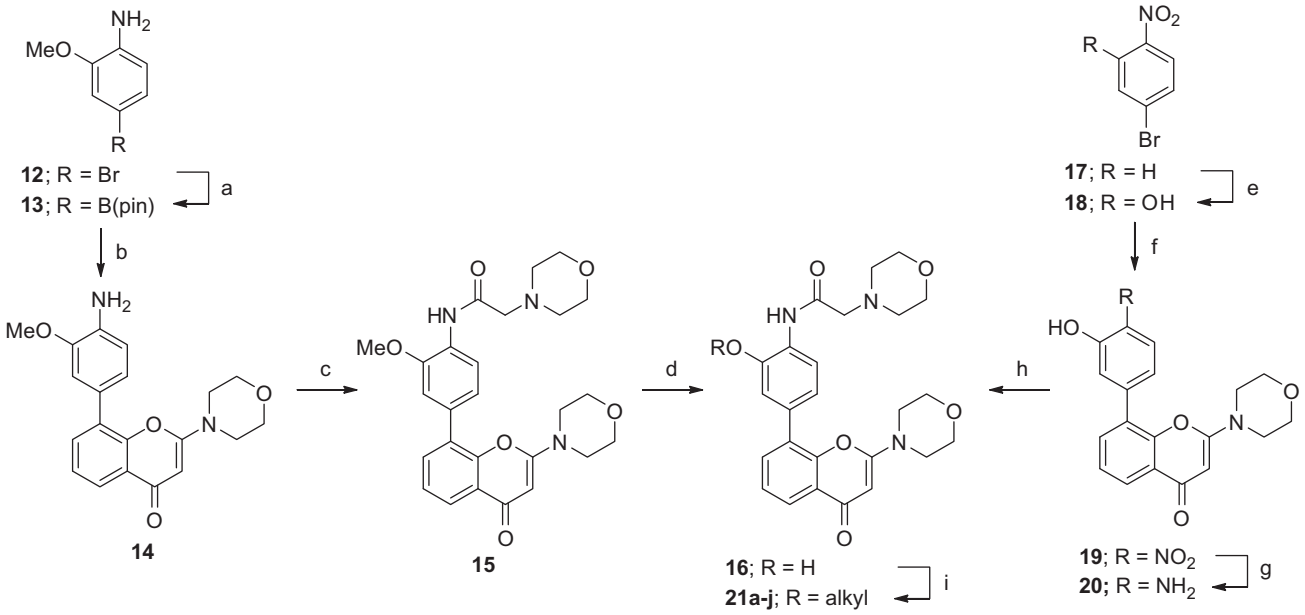
Additional derivatives were synthesised using a modified approach (Scheme 2). Commercially available 4-bromo-2-methoxyaniline (**12**) was converted into the boronic ester **13**, followed by a Suzuki–Miyaura cross-coupling with chromenone triflate **5** to afford the corresponding arylamine **14**. Acylation of **14** with chloroacetyl chloride, followed by reaction with morpholine, provided the methoxy-variant **15** and subsequent deprotection gave the phenol **16**. The key intermediate **16** was also accessed using an alternative synthetic route (Scheme 2). Commercially available 1-bromo-4-nitrobenzene (**17**) was converted into 5-bromo-2-nitrophenol (**18**) in 79% yield, using cumene hydroperoxide and *tert*-BuOK in the presence of ammonia.[21] Again, Suzuki–Miyaura cross-coupling with **5** followed by reduction of the nitrophenol intermediate gave aniline **20**. Finally, phenol **16** was obtained in 78% yield by chloroacetylation followed by reaction with morpholine. The effect upon biological activity of alkylation of hydroxyphenylchromen-4-one **16** was investigated through the preparation of a small series of *O*-alkoxyphenyl derivatives **21a–j** (Scheme 2 and Table 1).[22,23]

To enable further SAR studies, the 2-hydroxyethoxy (**23**) and aminoethoxy (**26**) derivatives were also synthesised (Scheme 3). Alkylation of phenol intermediate **16** with 2-(*tert*-butyldimethylsilyloxy)ethyl bromide[24,25] proceeded in 92% yield, followed by TBDMS removal giving the desired alcohol **23** in quantitative yield. Tosylation of **23** and treatment with NaN_3 in DMF at 60 °C gave azide **25**, which was reduced with triphenylphosphine in the presence of water to afford the desired amine **26** in 34% yield.

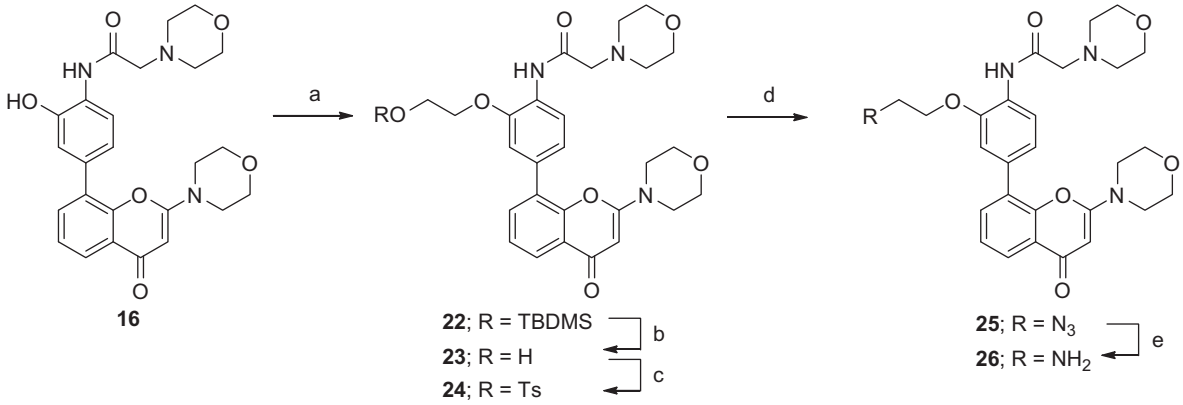
Our previous SAR studies have highlighted the importance of an appropriate 8-aryl substituent for chromenone-based inhibitors (e.g., dibenzothiophen-4-yl in **2** or a 3-arylphenyl moiety in **4**). The overall objective of this study was to investigate the effect upon DNA-PK inhibitory activity of replacing the planar dibenzothiophenyl group by a conformationally flexible and less lipophilic *O*-alkoxyphenyl system, to probe the hydrophobic pocket of the ATP-binding site. In addition, the analogues were chosen to investigate whether alkyl substituents were tolerated at this position, given that all other known derivatives are substituted with an aryl or heteroaryl group at C-8. The chemical structures and inhibitory



Scheme 1. Reagents and conditions: (a) bis(pinacolato)diboron, $PdCl_2$(dppf), dppf, KOAc, 1,4-dioxane, reflux, 18 h,[19] then 4-bromo-2-fluoronitrobenzene, $Pd(PPh_3)_4$, Na_2CO_3, H_2O, reflux, 3 h, 58%; (b) cyclopropanemethanol (for **9a**) or 3-methyl-3-oxetanemethanol (for **9b**), NaH, DMF, 25 °C, 15 min, then **8**, 100 °C, 20 min, **9a** 89%, **9b** 58%; (c) Zn powder, AcOH, 25 °C, 4 h, **10a** 82%, **10b** 71%; (d) (i) chloroacetyl chloride, Et_3N, DMA, 25 °C, 30 min; (ii) morpholine, 25 °C, 18 h, **11a** 15%, **11b** 65%.



Scheme 2. Reagents and conditions: (a) bis(pinacolato)diboron, $PdCl_2$(dppf), dppf, KOAc, DMSO, 80 °C, 2 h, 36%; (b) **5**, $Pd(PPh_3)_4$, Cs_2CO_3, 1,4-dioxane, DMA, MW, 140 °C, 15 min, 43%; (c) (i) chloroacetyl chloride, Et_3N, DCM, 25 °C, 3 h; (ii) morpholine, 25 °C, 18 h, 74%; (d) BBr_3, DCM, −78–25 °C, 1 h, 13%; (e) cumene hydroperoxide, *tert*-BuOK, NH_3, THF, −78 °C to reflux, 15 min, 79%; (f) **5**, $Pd(PPh_3)_4$, 2 M Na_2CO_3, 1,4-dioxane, MW, 150 °C, 1 h, 59%; (g) Zn powder, AcOH, 25 °C, 3.5 h, 92%; (h) (i) chloroacetyl chloride, DCM, 30 °C, 3 h; (ii) morpholine, 25 °C, 18 h, 78%; (i) R–X or R-OTs, K_2CO_3, DMF, MW, 100 °C, 15 min, 43–90% (general procedure in accordance with Ref. 22).



Scheme 3. Reagents and conditions: (a) 2-(*tert*-butyldimethylsilyloxy)ethyl bromide,[24,25] K_2CO_3, DMF, MW, 100 °C, 15 min, 92%; (b) TBAF, THF, 25 °C, 1.5 h, quantitative; (c) TsCl, Et_3N, DMAP, DCM, 25 °C, 18 h, 48%; (d) NaN_3, DMF, 60 °C, 2.5 h; (e) PPh_3, H_2O, 25 °C, 2.5 h, 34% over two steps.

activities of the library compounds are summarised in Table 1. With a view to delineating SARs and designing a selective DNA-PK inhibitor, all compounds were also tested against the related enzyme PI3-kinase α.

For the series of arylchromenone-4-ones bearing small *O*-alkoxy substituents at the phenyl 3-position (**15**, **21a**–**21d**), it is evident that substitution improved neither activity nor selectivity compared with the dibenzothiophenyl analogue **2**. Overall, a 6- to 12-fold reduction in potency was observed, and with the exception of the ethoxy derivative **21a**, which showed approximately a twofold selectivity for DNA-PK, all compounds proved to be equipotent for DNA-PK and PI3Kα.

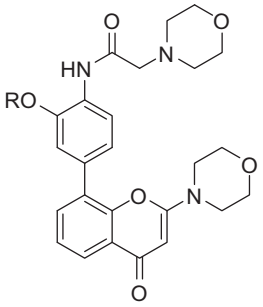
Increasing the length and steric bulk of the side chain (e.g., **21f**–**21i**) proved detrimental to inhibitory activity for both DNA-PK and PI3Kα. For example, with the cyclopropylethoxy derivative **21i**, a noticeable reduction in potency towards DNA-PK and PI3Kα was observed. This is consistent with homology modelling studies and previous SARs around this position, that indicate a limited steric tolerance in this region of the ATP-binding domain.[18]

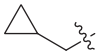
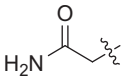
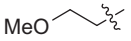
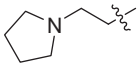
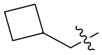
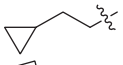
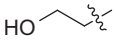
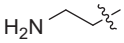
Disappointingly, the incorporation of hydrogen bond donor and acceptor groups onto the alkoxy side chain (e.g., **21e**, **23** and **26**) was not beneficial for DNA-PK inhibitory activity, and resulted in up to 117-fold reduction in potency compared with the parent 8-dibenzothiophenyl chromen-4-one **2**. These compounds also proved to be non-selective being equipotent for DNA-PK and PI3Kα. Replacement of the methoxy group of **21f** by a hydroxyl function (**23**) resulted in a modest improvement in activity, but both compounds were non-selective for DNA-PK versus PI3Kα.

Interestingly, removal of the alkoxy side chain conferred good DNA-PK inhibitory activity, with phenol **16** exhibiting a fivefold selectivity profile for DNA-PK (IC_{50} = 0.08 μM) versus PI3Kα (IC_{50} = 0.43 μM).

In light of the overall loss of activity and selectivity observed with this series of arylchromenone-4-ones bearing small *O*-alkoxy

Table 1
DNA-PK and PI3Kα inhibitory activity of *O*-alkoxyphenyl chromen-4-ones



Compound	R	DNA-PK IC_{50}[a] (μM)	PI3Kα[b] IC_{50} (μM)
1 (LY294002)	—	1.3	0.50
2	—	0.03	0.13
11a		0.008	0.07
11b		0.69	0.30
15	Me	0.29	0.35
16	H	0.08	0.43
21a	Et	0.28	0.47
21b	*i*-Pr	0.33	0.29
21c		0.18	0.15
21d		0.17	0.15
21e		3.3	1.1
21f		2.6	1.5
21g		>10	4.4
21h[23]		0.16	0.29
21i		0.5	0.4
21j		0.11	0.27
23		1.0	1.6
26		12	3

[a] IC_{50} values were determined in accordance with Ref. 13 and are the means of three separate determinations.
[b] IC_{50} values were determined in accordance with Ref. 26 and are the means of three separate determinations.

substituents, we were pleased to identify a potent and relatively selective inhibitor **11a**, exhibiting IC_{50} values of 0.008 μM and 0.07 μM for DNA-PK and PI3Kα, respectively. The fact that **11a** is approximately ninefold selective for DNA-PK versus PI3Kα and fourfold more potent than the parent 8-dibenzothiophenyl chromen-4-one **2**, suggests that the cyclopropylmethoxy group of **11a** might be making productive interactions with a hydrophobic region of the ATP-binding domain of DNA-PK. It is notable that compared to **11a**, the *sec*-butyl analogue **21c** is ca. 20-fold less potent against DNA-PK but only ca. twofold less potent against PI3Kα.

In summary, we have identified a novel series of *O*-alkoxyphenyl chromen-4-ones that exhibit a range of potencies against DNA-PK. These compounds represent the first exemplified chromenone-based DNA-PK inhibitors that lack an aryl substituent directly attached at the C-2 position of the phenyl ring. With the exception of the cyclopropylmethoxy derivative **11a**, the most potent and a modestly selective inhibitor, achieving selectivity for DNA-PK over PI3Kα has proven challenging. Overall, this study has further elucidated our understanding of SARs around a putative hydrophobic region of the ATP-binding site, indicating a limited steric tolerance and hydrophobic complementarily. Further studies are currently underway to elucidate the binding mode of this class of chromen-4-one based DNA-PK inhibitors.

Acknowledgements

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20. *DMR clonogenic assay*: HeLa cells were seeded per well into a 6-well tissue culture treated dish and incubated overnight at 37 °C/5% CO_2. Following a 1 h pre-treatment with either vehicle or compound the plates were exposed to 2 Gy ionising radiation using a Faxitron 43855D X-ray source and incubated overnight at 37 °C/5% CO_2. The media was replaced with fresh media in the absence of compound or vehicle and incubated for a further 6–8 days. The media was removed and the cell colonies were fixed and stained with Giemsa and scored with an automated colony counter (Oxford Optronics Ltd, Oxford, United Kingdom).
The dose modification ratio (DMR) is defined as the ratio of the number of cells that survive a single 2 Gy dose of IR to that of the number of cells that survive the same dose in combination with a given concentration of DNA-PK inhibitor. This value provides an indirect measure of the ability of a particular compound to potentiate the DNA damage elicited by IR, and also indicates whether or not the compound in question is cell permeable. IR alone at the dose used (2 Gy)

caused 40–60% survival. The DNA-PK inhibitor alone, at the concentrations used (0.5 and 0.1 μM), was not cytotoxic and hence the dose modification observed represents radiopotentiation and not additive toxicity. The Dose Modification Ratio (DMR) at 2 Gy irradiation was calculated as follows:

$$\mathrm{DMR} = \frac{\%\ \mathrm{survival} - \mathrm{compound}/+2\ \mathrm{Gy}}{\%\ \mathrm{survival} + \mathrm{compound}/+2\ \mathrm{Gy}}$$

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22. *General procedure for the library synthesis* (**21a–21j**): A mixture of *N*-(2-hydroxy-4-(2-morpholino-4-oxo-4*H*-chromen-8-yl)phenyl)-2-morpholinoacetamide **16** (1.0 equiv), K_2CO_3 (1.0 equiv), alkyl halide for **21a–g** (2.0 equiv), or alkyl tosylate for **21h–j** (2.0 equiv; alkyl tosylates were synthesised from the alkyl alcohol, using TsCl, Et_3N, DMAP, DCM, 25 °C, 18 h) in anhydrous DMF was heated under microwave irradiation at 100 °C for 15 min. Water was added to the resulting mixture and the product was extracted into EtOAc. The combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was removed in vacuo to give the crude product which was subsequently purified by medium pressure flash chromatography.
23. *Synthesis of N-(2-(cyclobutylmethoxy)-4-(2-morpholino-4-oxo-4H-chromen-8-yl)phenyl)-2-morpholinoacetamide* (**21h**): Following the general procedure[22] using **16** (40 mg, 0.086 mmol), cyclobutylmethanol *O*-toluene-4-sulfonate (43 mg, 0.18 mmol), K_2CO_3 (12 mg, 0.086 mmol) and DMF (3 mL). Purification by medium pressure chromatography on Si-NH using EtOAc gave the title compound as a white solid (35 mg, 76%): mp 229–231 °C; λ_{max} (EtOH)/nm 209.0, 302.5; IR (cm^{-1}), 3309, 2851, 1687, 1626, 1568, 1525, 1404; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.88–1.94 (2H, m, cyclobutyl-CH_2), 1.98–2.09 (2H, m, cyclobutyl-CH_2), 2.23–2.29 (2H, m, cyclobutyl-CH_2), 2.66 (4H, t, *J* = 4.4 Hz, NCH_2-morpholine), 2.87 (1H, septet, *J* = 7.0 Hz, cyclobutyl-C*H*), 3.21 (2H, s, N(H)C(O)CH_2), 3.38 (4H, t, *J* = 4.8 Hz, NCH_2-morpholine), 3.74 (4H, t, *J* = 4.8 Hz, OCH_2-morpholine), 3.81 (4H, t, *J* = 4.4 Hz, OCH_2-morpholine), 4.06 (2H, d, *J* = 7.0 Hz, Ar-OCH_2cyclobutane), 5.52 (1H, s, C-3 chromenone), 7.03 (1H, d, *J* = 1.6 Hz, H-Ar), 7.14 (1H, dd, *J* = 8.3 Hz, *J* = 1.6 Hz, H-Ar), 7.40 (1H, t, *J* = 7.6 Hz, H-Ar), 7.55 (1H, dd, *J* = 7.6 Hz, *J* = 1.7 Hz, H-Ar), 8.17 (1H, dd, *J* = 7.7 Hz, *J* = 1.7 Hz, H-Ar), 8.54 (1H, d, *J* = 8.3 Hz, H-Ar), 9.74 (1H, s, N(*H*)C(O)); ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.9, 25.2, 34.7, 44.9, 54.0, 63.0, 66.1, 67.3, 73.0, 87.3, 112.2, 119.3, 122.3, 123.6, 124.9, 125.1, 127.4, 130.3, 132.0, 133.7, 147.8, 150.8, 162.8, 168.3, 177.4; HRMS calcd for $C_{30}H_{36}N_3O_6$ $[M+H]^+$ 534.2599, found 534.2589.
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26. *PI3K alpha enzyme assay*: To generate IC_{50} values, compounds were solubilised in 100% DMSO at 10 mM, and then serially-diluted in 100% DMSO using a VPrep liquid handler (Agilent Technologies UK Limited, Stockport, Cheshire, UK) to give four 1:100 dilutions in a Labcyte acoustic-certified 384-well plate (Labcyte Incorporated, Sunnyvale, California, USA). A Labcyte Echo acoustic liquid dispenser was used to generate a 12-step dose range from 200 μM down to 0.2 nM (in a 6 μL total reaction volume), dispensing a total of 120 nL compound and/or DMSO into each well of a white low-volume 384-well plate (Greiner Bio-One Limited, Stonehouse, UK). The maximum signal was generated using 120 nL of a known inhibitor, and the minimum signal was generated with 120 nL of 100% DMSO. Kinase reaction buffer was prepared in deionised water to give 50 mM Tris (pH 7.4), 0.05% CHAPSO, 10 mM $MgCl_2$ and 2.1 nM DTT. Enzyme solution was made up in kinase reaction buffer to give 40 nM PI3K alpha isoform enzyme (20 nM final concentration, in-house enzyme preparation), and substrate solution was made up in kinase reaction buffer to give 16 μM ATP and 160 μM PIP2 (8 μM and 80 μM final concentration, respectively, PIP2 purchased from Cayman Chemical Company, Ann Arbor, Michigan, USA). 3 μL enzyme solution was added to each well with an Aquamax liquid dispenser (Molecular Devices, Sunnyvale, California, USA) and, after a 20 min incubation at room temperature, 3 μL per well substrate solution was added. Plates were then covered and incubated at room temperature for 80 min. At the end of the incubation period, the kinase reaction was stopped by adding 4 μL per well of Kinase-Glo® Plus Reagent (Promega Corporation, Madison, Wisconsin, USA, prepared as per manufacturers instructions and brought to room temperature prior to addition) with an Aquamax. Plates were incubated for at least 20 min at room temperature, before being read with a Pherastar microplate reader (BMG Labtech GmbH, Offenburg, Germany) with the standard luminescence filter block, and the gain set at 3400.