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# Synthesis and biological evaluation of novel phosphatidylinositol 3-kinase inhibitors: Solubilized 4-substituted benzimidazole analogs of 2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazole (ZSTK474)

Gordon W. Rewcastle [a,b,*], Swarna A. Gamage [a], Jack U. Flanagan [a,b], Jackie D. Kendall [a,b], William A. Denny [a,b], Bruce C. Baguley [a,b], Christina M. Buchanan [b,c], Mindy Chao [a], Philip Kestell [a], Sharada Kolekar [c], Woo-Jeong Lee [c], Claire L. Lill [a], Alisha Malik [c], Ripudaman Singh [a], Stephen M.F. Jamieson [a,b], Peter R. Shepherd [b,c]

[a] *Auckland Cancer Society Research Centre, School of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand*
[b] *Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand*
[c] *Department of Molecular Medicine and Pathology, School of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand*

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ABSTRACT

A range of 4-substituted derivatives of the pan class I PI 3-kinase inhibitor 2-(difluoromethyl)-1-[4,6-di-(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazole (ZSTK474) were prepared in a search for more soluble analogs. 4-Aminoalkoxy substituents provided the most potent derivatives, with the 4-$O(CH_2)_3NMe_2$ analog (compound **14**) being identified as displaying the best overall activity in combination with good aqueous solubility (25 mg/mL for the hydrochloride salt). This compound was tested in a U87MG xenograft model, but displayed less potency than ZSTK474 as a result of an unfavorable pharmacokinetic profile.



## 1. Introduction

Phosphoinositide 3-kinases (PI3Ks) are a family of three distinct classes (I, II and III) of lipid kinases that play key roles in cell and tissue physiology [1–3]. The three class-Ia PI 3-kinases (p110α/β/δ) and the sole class-Ib PI 3-kinase (p110γ) couple growth factor receptors and G-protein coupled receptors respectively to a wide range of downstream pathways [4–6]. These enzymes have different methods of activation and different kinetic properties [7], but all use phosphatidylinositol-4,5-diphosphate (PIP2) to produce phosphatidylinositol-3,4,5-triphosphate (PIP3). The cellular levels of PIP3 are tightly controlled by phosphatases including PTEN which dephosphorylates PIP3 back to PIP2 [8,9]. The importance of this pathway in cancer is highlighted by the fact that defects in both the kinase and phosphatase activities are commonly observed in tumors, and there is now increasing evidence that a high proportion of human cancers depend strongly on p110α for their survival and resistance to therapy [8–15]. Therefore the targeting of PI3K with small molecule inhibitors is one of the most promising new approaches to cancer treatment, and a number of programs to develop PI3K inhibitors are currently in progress [5,16–19], with several inhibitors in clinical trial [20–26].

2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazole (ZSTK474) (**1**) (Fig. 1) is a potent ATP-competitive pan-class I PI3K inhibitor, with high selectivity over other classes of

* Corresponding author. Auckland Cancer Society Research Centre, School of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand. Tel.: +64 9 9236147; fax: +64 9 3737502.
*E-mail addresses:* g.rewcastle@auckland.ac.nz, grewcastle@orcon.net.nz (G.W. Rewcastle).

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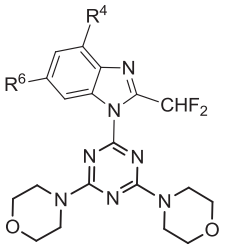


**1**; $R^4 = R^6 = H$
**2**; $R^4 = OH$, $R^6 = H$
**3**; $R^4 = OMe$, $R^6 = H$
**4**; $R^4 = NH_2$, $R^6 = H$
**5**; $R^4 = OMe$, $R^6 = NH_2$

**Fig. 1.** Structure of ZSTK474 (**1**) and substituted analogs.

PI3K and protein kinases [27–29], and demonstrated antitumor activity *in vivo* against human tumor xenografts [27,29].

We recently performed a structure activity relationship (SAR) study of this compound, and identified substitution at the 4- and 6-positions of the benzimidazole ring as having significant effects on the potency of substituted derivatives [30]. Electron donating substituents that increased the electron density on the benzimidazole 3-nitrogen were found to be particularly efficacious, and this was presumed to be due to enhanced H-bonding of the benzimidazole nitrogen to a specific lysine amino group in the ATP binding site of PI3K (Lys802 in p110α, Lys805 in p110β, Lys833 in p110γ and Lys779 in p110δ). 4-Substituents could also participate in H-bonding to the lysine amino group, and the 4-OH derivative **2** was particularly potent, although found to have poor pharmacokinetics, presumably as a result of rapid glucuronidation. The more stable 4-OMe derivative **3** was less potent than **2**, but showed greater selectivity for the p110α isoform of PI3K. Amino substituents were also efficacious, with the 4-$NH_2$ derivative **4** having similar potency to **3**, while the 4-OMe, 6-$NH_2$ derivative **5** was particularly potent. *In vivo* testing of **5** showed that it dramatically reduced cancer growth by 81% compared to untreated controls, although it suffered from solubility issues [30].

In the present paper we investigate a wider range of benzimidazole 4-substituents, focusing particularly on oxygen or nitrogen-linked solubilizing moieties that might overcome the solubility problems seen with **5**, while still retaining the ability to assist in binding to the PI3K lysine amino group.

## 2. Chemistry

The 4-oxygen linked derivatives (**6**–**17**) of Table 1 were prepared by alkylation of phenol **2** [30,31] (Scheme 1). Compounds **6**–**9**, **11**, **12** and **15** were prepared directly from **2**, but the one-step synthesis of **14** occurred in too low a yield to be viable by this route. Accordingly, **14** was prepared from alcohol **8**, via mesylation and subsequent reaction with dimethylamine. Primary amines **10** and **13** were prepared from **2** via the respective phthalimides **18** and **19**, while the amino-alcohols **16** and **17** were prepared via amine addition to epoxide **20**.

The 4-nitrogen linked derivatives (**21**–**25**) of Table 1 were prepared from amine **4** [30,32] or its Boc derivative **26** [30] (Scheme 2). Alkylation of **26** followed by Boc removal gave amine **21**, while amino-amide derivatives **22**–**25** were prepared via acylation of **4**, and subsequent reaction of intermediates **28**–**31**. Thus primary amines **22** and **24** were prepared by TFA deprotection of their Boc derivatives **28** and **29**, while tertiary amines **23** and **25** were prepared by dimethylamine addition to chloroacetamide **29** or acrylamide **31** respectively.

Scheme 3 details an alternative (higher yielding) route to alcohol **8** which was subsequently used for a larger scale preparation of amine **14**. Thus, instead of proceeding through phenol **2**, whose prior synthesis involves a moderately yielding five-step procedure [30,31], the synthesis of **8** was achieved directly in just five steps starting from commercially available 2-amino-3-nitrophenol (**32**). Alkylation of **32** gave alcohol **33** which was converted to benzimidazole **34** by reduction and subsequent ring closure with difluoroacetic acid. Protection of the alcohol group of **34** as its TBDMS derivative, followed by reaction with 2-chloro-4,6-dimorpholino-1,3,5-triazine [33] in DMSO at 130 °C gave **36** which was then deprotected with TBAF to give **8** in 56% overall yield.

## 3. Results and discussion

### 3.1. Enzyme inhibition

All new compounds were tested for their enzyme activity against the p110α, β and δ isoforms of PI3K using a lipid kinase assay (Table 1).

Compounds **6**–**17** explored a variety of solubilizing substituents attached via a 4-oxygen linker with acidic carboxylate, neutral alcohol, and basic amine substituents being investigated. The oxyacetic acid derivative **6** did not show good enzyme or cellular potency, and was not studied further. The alcohols **7**–**9** retained good enzyme and very good cellular potency, but did not markedly improve solubility. The basic amino compounds **10**–**17** also retained good cellular potencies but had varying enzyme inhibitory effects, with **14** displaying the best enzyme potency against all three isoforms. Compounds **21**–**25** explored the use of a 4-nitrogen linker, but all were less effective than the oxygen linked analogs.

Overall, the 4-O$(CH_2)_3NMe_2$ analog **14** was the most active of the basic amino compounds, and displayed good aqueous solubility properties (25 mg/mL for the hydrochloride salt), and was therefore selected for further evaluation.

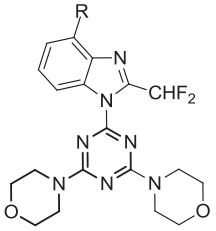
### 3.2. Cellular inhibition

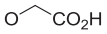
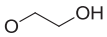
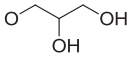
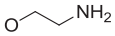
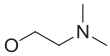
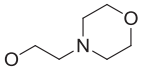
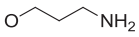
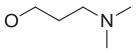
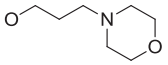
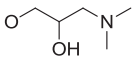
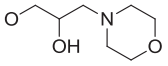
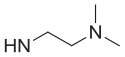
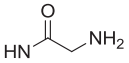
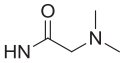
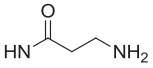
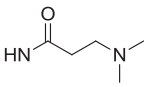
The compounds were also evaluated using two human tumor cells lines, NZOV9 (Y1021C mutation of p110α enzyme) and NZB5 (wild-type p110α enzyme) and the data are shown in Table 1. The growth inhibitory $IC_{50}$ values for some compounds were comparable with the $IC_{50}$ values for isolated enzyme studies, while others were considerably higher, suggesting variable efficiency in cellular uptake. Growth inhibitory $IC_{50}$ values for the NZOV9 line correlated significantly to enzymatic $IC_{50}$ values for p110α $IC_{50}$ values (Spearman rank correlation; $R = 0.60$; $p = 0.011$). However, NZOV9 $IC_{50}$ values did not correlate significantly with p110β or p110δ $IC_{50}$ values, and NZB5 $IC_{50}$ values did not correlate to $IC_{50}$ values of any of the enzyme assays. These results supported the hypothesis that the mutant p110α enzyme played a significant role in drug-induced inhibition of cell growth.

### 3.3. Inhibition of cell signaling

To determine whether compound **14** was capable of entering cells and attenuating signaling downstream from PI 3-kinase, the effect on phosphorylation of Akt/PKB was determined in HCT116 cells using previously described methods [34,35]. HCT116 cells were chosen for this experiment as they are commonly used in xenografts and have constitutive activation of the PI 3-kinase pathway due to activation of *PIK3CA*. We determined the $IC_{50}$ for the inhibition of phosphorylation at not only the most commonly measured Ser473 site but also for Thr308 as this site is the one most directly linked to activation of PI 3-kinase in the cell. Compound **14**

**Table 1**
Enzyme and cellular inhibition.



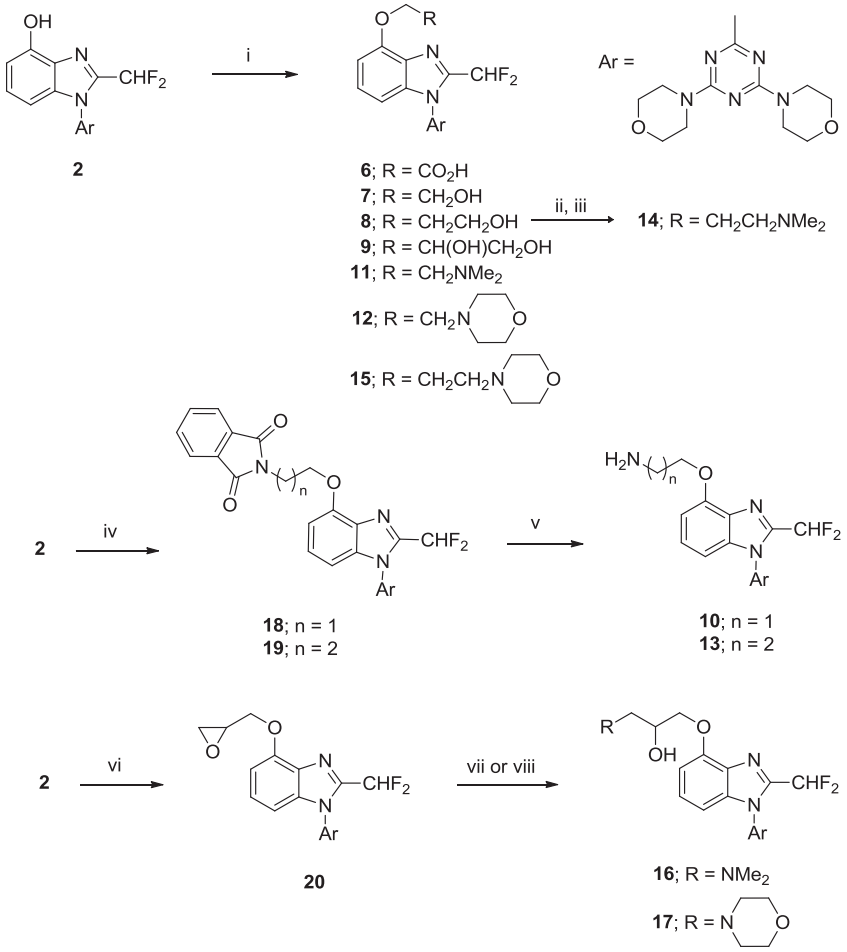
| Compound | R | p110α $IC_{50}$[a] (nM) | p110β $IC_{50}$[a] (nM) | p110δ $IC_{50}$[a] (nM) | NZB5 $IC_{50}$[b] (μM) | NZOV9 $IC_{50}$[b] (μM) |
|---|---|---|---|---|---|---|
| **6** |  | 52 | 274 | 39 | 8.5 | 3.4 |
| **7** |  | 5.3 | 16 | 2.6 | 0.06 | 0.06 |
| **8** |  | 10 | 170 | 22 | 0.071 | 0.018 |
| **9** |  | 12 | 24 | 4.7 | 0.026 | 0.025 |
| **10** |  | 17 | 130 | 20 | 0.052 | 0.022 |
| **11** |  | 35 | 105 | 19 | 0.45 | 0.15 |
| **12** |  | 130 | 150 | 53 | 0.105 | 0.071 |
| **13** |  | 28 | 75 | 28 | 0.032 | 0.009 |
| **14** |  | 11 | 7.3 | 4.5 | 0.17 | 0.04 |
| **15** |  | 29 | 76 | 18 | 0.06 | 0.03 |
| **16** |  | 33 | 30 | 13 | 0.33 | 0.11 |
| **17** |  | 41 | 53 | 5.5 | 0.054 | 0.025 |
| **21** |  | 174 | 73 | 15 | 0.20 | 0.11 |
| **22** |  | 119 | 740 | 52 | 0.14 | 0.06 |
| **23** |  | 379 | 1040 | 99 | 0.05 | 0.05 |
| **24** |  | 855 | 3015 | 210 | 0.44 | 0.12 |
| **25** |  | 486 | 1260 | 112 | 0.29 | 0.14 |

[a] *In vitro* lipid kinase assay. $IC_{50}$ values are the mean of duplicate or triplicate measurements.
[b] *In vitro* cell proliferation assay. $IC_{50}$ values are the mean of duplicate or triplicate measurements.

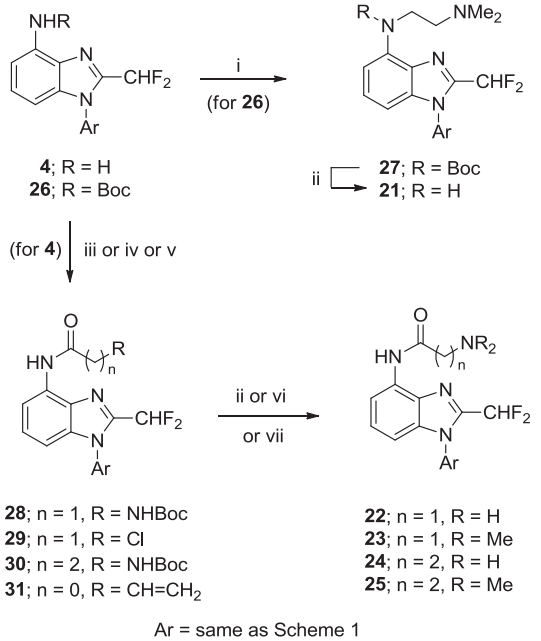
was found to inhibit phosphorylation at both sites, showing that it was capable of entering cells and attenuating downstream signaling, with a potency comparable to that of compounds **1**, and **3** [30] (Table 2), although not as potent as compound **5** with the additional 6-amino group.

### 3.4. Pharmacokinetics and antitumor efficacy

The plasma pharmacokinetic profile of **14** was determined in C57 mice following a dose of 10 mg/kg by i.p. injection (Table 3). A peak plasma concentration of 1938 nM was reached 15 min after

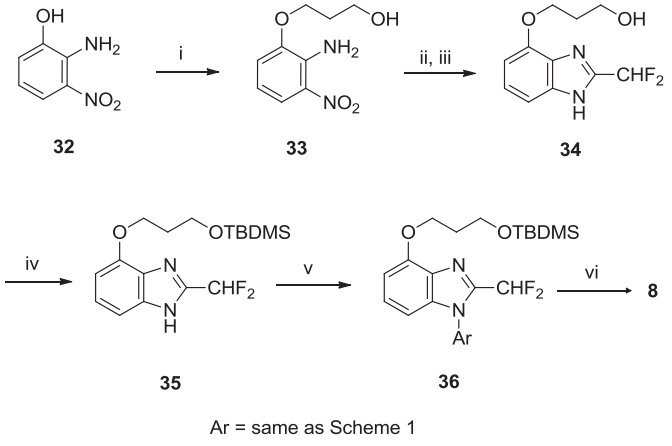



**Scheme 1.** Reagents and conditions: (i) $RCH_2X$, $K_2CO_3$, DMF, rt or 50–60 °C; (ii) MsCl, $Et_3N$, THF, 0 °C; (iii) 40% aq. $Me_2NH$, THF, rt; (iv) *N*-(bromoalkyl)phthalimide, $K_2CO_3$, DMF, 50 °C; (v) $NH_2NH_2{\cdot}H_2O$, $CH_2Cl_2$, THF, rt; (vi) epibromohydrin, $K_2CO_3$, DMF, 50 °C; (vii) (for **16**) 40% aq. $Me_2NH$, THF, rt; (viii) (for **17**) morpholine, THF, MeOH, rt.




**Scheme 2.** Reagents and conditions: (i) NaH, $Me_2N(CH_2)_2Cl$, DMF, 0 °C to rt; (ii) TFA, $CH_2Cl_2$, rt; (iii) (for **28** and **30**) $BocNH(CH_2)_nCO_2H$, EDCI, DMAP, DMF, rt; (iv) (for **29**) $ClCH_2COCl$, $K_2CO_3$, $CH_2Cl_2$, rt; (v) (for **31**) $CH_2{=}CHCOCl$, DIPEA $CH_2Cl_2$, 0 °C to rt; (vi) (for **23**) 40% aq. $Me_2NH$, EtOH, reflux; (vii) (for **25**) 40% aq. $Me_2NH$, EtOH, rt.

dosing, after which the compound was cleared with a half-life of 1.56 h, generating an $AUC_{INF}$ of 2586 nM h. The antitumor efficacy of **14** was determined in a U87MG xenograft model in Rag1$^{-/-}$ mice alongside compounds **1** and NVP-BEZ235 (**37**) (Fig. 2). Each compound was administered by i.p. injection at its maximally tolerated dose at a qdx14 dosing schedule. At a dose of 60 mg/kg, **14** delayed tumor growth relative to controls, but was less effective than both **1**




**Scheme 3.** Reagents and conditions: (i) $HO(CH_2)_3Cl$, $K_2CO_3$, acetone, reflux; (ii) $H_2$, Pd/C, MeOH; (iii) $F_2CHCO_2H$, 4 N HCl, reflux; (iv) TBDMSCl, pyridine, rt; (v) 2-chloro-4,6-dimorpholino-1,3,5-triazine, $K_2CO_3$, DMSO, 130 °C; (vi) TBAF, THF, rt.

**Table 2**
Inhibition of cell signaling in HCT116$^{+/+}$ (PIK3CA H1047R mutant) cells by **1**, **3**, **5** and **14** *in vitro*.

| Compound | pAkt/PKB Ser473 $IC_{50}$[a] (nM) | pAkt/PKB Thr308 $IC_{50}$[a] (nM) |
|---|---|---|
| **1**[b] | 97 | 78 |
| **3**[b] | 37 | 25 |
| **5**[b] | 12 | 17 |
| **14** | 36 | 74 |

[a] Compound concentration required to produce 50% inhibition of phosphorylation in a cellular assay.
[b] Data from Ref. [30]. Cells were exposed to inhibitor dissolved in DMSO for 15 min before stimulation with 500 nM insulin for 5 min. Protein isolation and immunoblotting for phospho-Akt/PKB was carried out according to the methods previously described in Refs. [34,35].

**Table 3**
Mouse plasma pharmacokinetic parameters for **14**.

| Compound | Dose (mg/kg) | Route | $C_{max}$ (nM) | $T_{max}$ (h) | $AUC_{INF}$ (nM h) | $T_{1/2}$ (h) |
|---|---|---|---|---|---|---|
| **14** | 10 | i.p. | 1938 | 0.25 | 2586 | 1.56 |

and **37** (Fig. 3A). Tumor growth inhibition relative to controls was calculated on day 7 with **14** inhibiting tumor growth by 56.3 ± 2.6% ($P < 0.001$), **1** by 67.6 ± 2.7% ($P < 0.001$) and **37** by 60.3 ± 6.0% ($P < 0.001$). Each treatment was well tolerated for the majority of animals, with only mild bodyweight loss averaging between 5 and 7% of pre-treatment size observed for the 3 treatment groups (Fig. 3B). However, significant bodyweight loss (19%) resulting in mortality was observed with 1 mouse treated with **1**, while an unexpected mortality with no preceding signs of toxicity or bodyweight loss occurred with **14**.

### 3.5. Modeling of binding

A binding mode for ZSTK474 (**1**) was predicted in the p110α kinase catalytic site where the benzimidazole moiety was bound in the affinity pocket as previously reported [30]. A possible interaction between the benzimidazole 3-nitrogen atom and Lys802 was identified, and one morpholine unit formed what we consider to be an essential interaction with the back bone amide of Val851 [30]. This predicted binding mode was in good agreement with that observed in the X-ray crystal structure of the p110δ isoform (PDB 2wxl) [36]. Further, when superimposed on ZSTK474 bound in the p110δ active site, the conformation generated upon docking into p110α had an RMSD of 0.23 Å, with most variation seen in the non-essential morpholine group. Similar binding modes were found for compounds **2**–**5** when active site flexibility was introduced at the side chains of residues Lys720, Lys776, Lys802, Asp805, Asp933 along with a soft potential interaction model at residues Leu807, Asp810, Tyr836 and Ile848. Binding models that predicted a Lys802 interaction with the benzimidazole 3-nitrogen were not necessarily the top scoring binding poses, and under the conditions used

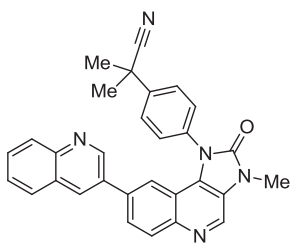



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**Fig. 2.** Structure of NVP-BEZ235 (**37**).

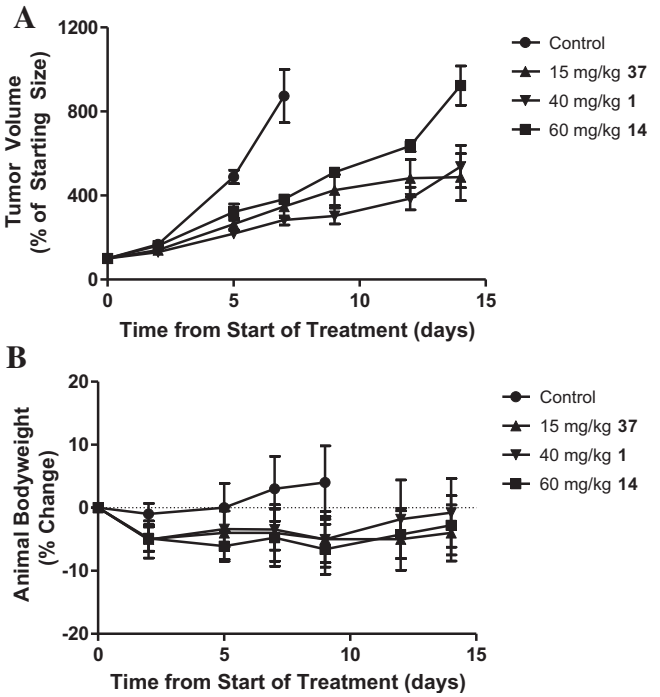


**Fig. 3.** Average tumor volume (A) and bodyweight loss (B) in Rag1$^{-/-}$ mice with U87MG tumors treated with 15 mg/kg **37**, 40 mg/kg **1** or 60 mg/kg **14**. Bars represent the mean and standard error of 5–7 mice per group.

Lys802 was also predicted to interact with oxygen atoms at the benzimidazole 4-position. The 6-amino moiety of **5** was predicted to form interactions with Asp754 and 877 in the same region. RMSD values for the ZSTK474 core of the predicted binding modes compared to that bound in p110δ was not improved from compound **1** with the 4 and 6 substitutions of compounds **2**–**5** (compound **2** RMSD 0.25, compound **3** RMSD 0.67, compound **4** RMSD 0.75 and compound **5** RMSD 0.48).

Fig. 4 shows the 4-methoxy derivative **2** (magenta stick) docked into the kinase active site of p110α, with both the oxygen atom of the methoxy group and the benzimidazole 3-nitrogen atom forming H-

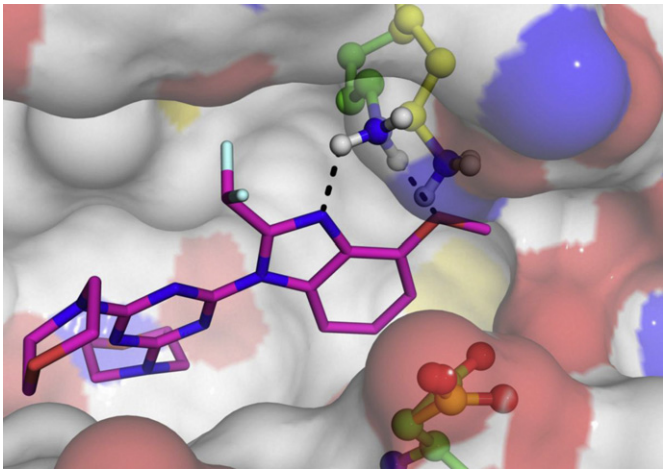

**Fig. 4.** Compound **2** (magenta stick) docked into the kinase active site of p110α. Residues Lys802 and Asp933 defining the benzimidazole binding site are shown in ball and stick. The positions of these residues in the p110α structure 2rd0 are shown in yellow, while the final positions associated with the flexible receptor docking of **2** are shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

bond interactions with Lys802. The results presented here indicate that the better inhibitory activity of O-linked solubilizing groups (compounds **6**–**17**) may result from the maintenance of an interaction with Lys802, while the less well tolerated amine and amide linked groups (compounds are **21**–**25**) are likely to disrupt this.

## 4. Conclusion

The pharmacokinetic parameters of **14**, in particular $T_{1/2}$ and to a lesser degree, $C_{max}$, are comparable with those reported for compounds **1** and **5** in CD-1 mice [30]. However, the $AUC_{INF}$ of **14** was approximately half that of **5** and a quarter that of **1**. Therefore, despite the potency of **14** against the PI3K isoforms and its improved solubility relative to **1**, it demonstrated less potency than **1** in a U87MG xenograft model as a result of its unfavorable pharmacokinetic profile. Further work is therefore ongoing to identify compounds that display both good aqueous solubility and improved PK properties that will result in a better *in vivo* profile.

## 5. Experimental

### 5.1. Chemistry

Elemental analyses were performed by the Microchemical Laboratory, University of Otago, Dunedin, New Zealand. Melting points were determined on an Electrothermal IA9100 melting point apparatus and are as read. NMR spectra were obtained on a Bruker Avance-400 spectrometer at 400 MHz for proton spectra and 100 MHz for carbon spectra, referenced to $Me_4Si$. Low-resolution atmospheric pressure chemical ionization (APCI) mass spectra were measured for organic solutions on a ThermoFinnigan Surveyor MSQ mass spectrometer, connected to a Gilson autosampler. High-resolution electron impact (HREIMS) and fast atom bombardment (HRFABMS) mass spectra were determined on a Varian VG-70SE mass spectrometer at nominal 5000 resolution. Thin-layer chromatography was carried out on aluminum-backed silica gel plates (Merck 60 F254), with visualization of components by UV light (254 nm). Column chromatography was carried out on silica gel, (Merck 230–400 mesh) unless otherwise stated. Tested compounds were ≥95% purity, as determined by combustion analysis, or by HPLC conducted on an Agilent 1100 system, using a reversed-phase C8 column with diode array detection.

#### 5.1.1. ({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)acetic acid (**6**)

A mixture of 2-(difluoromethyl)-4-hydroxy-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazole (**2**) [30,31] (275 mg, 0.63 mmol) and dry $K_2CO_3$ (800 mg) in DMF (3 mL) was stirred at 20 °C for 30 min. A premixed suspension of $K_2CO_3$ (1.0 g) and bromoacetic acid (1 mL) in DMF (3 mL) was then added. The reaction mixture was stirred at 50 °C for 24 h and then diluted with water. After filtration through a pad of celite, the filtrate was acidified with conc. HCl. The precipitate was collected by filtration, washed with water, and dried. Two recrystallizations from $CH_2Cl_2$/EtOH gave **6** (190 mg, 60% yield): mp 305–308 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 13.0 (br s, exchangeable with $D_2O$, 1H), 7.91 (d, $J$ = 8.4 Hz, 1H), 7.70 (t, $J_{HF}$ = 52.8 Hz, 1H), 7.37 (t, $J$ = 8.2 Hz, 1H), 6.86 (d, $J$ = 8.1 Hz, 1H), 4.96 (s, 2H), 3.82–3.80 (m, 8H), 3.69 (m, 8H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 170.2 (C), 164. 4 (C), 161.3 (C), 150.2 (C), 144.3 (t, $J_{CF}$ = 26.7 Hz, C-2), 134.6 (C), 131.4 (C), 126.6 (CH), 108.6 (t, $J_{CF}$ = 237.2 Hz, $CHF_2$), 108.2 (CH), 106.8 (CH), 65.9 ($CH_2$), 65.6 ($CH_2$), 43.6 ($CH_2$). Anal. Calcd for $C_{21}H_{23}F_2N_8O_5 \cdot 0.25H_2O$: C, 50.9; H, 4.8; N, 19.8. Found: C, 50.8; H, 4.5; N, 19.9%.

#### 5.1.2. 2-({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)ethanol (**7**)

A mixture of **2** (85 mg, 0.0196 mmol), 2-iodoethanol (37 mg, 0.22 mmol), and powdered $K_2CO_3$ (83 mg, 0.6 mmol) in 10 mL of DMF was stirred at room temperature overnight, and then diluted with water. The product was collected by filtration, dried, and purified by chromatography on silica eluting with EtOAc to give **7** (47 mg, 50% yield): mp (EtOAc) 243–246 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 7.89 (d, $J$ = 8.1 Hz, 1H), 7.70 (t, $J_{HF}$ = 52.8 Hz, 1H), 7.38 (t, $J$ = 8.2 Hz, 1H), 6.95 (d, $J$ = 7.9 Hz, 1H), 4.93 (t, $J$ = 5.5 Hz, exchangeable with $D_2O$, 1H), 4.24 (t, $J$ = 5.0 Hz, 2H), 3.84–3.79 (m, 10H), 3.69 (m, 8H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 164.4 (C), 161.4 (C), 151.1 (C), 144.2 (t, $J_{CF}$ = 26.7 Hz, C-2), 134.5 (C), 131.6 (C), 126.8 (CH), 108.6 (t, $J_{CF}$ = 237.4 Hz, $CHF_2$), 108.0 (C), 106.5 (C), 70.4 ($CH_2$), 68.9 ($CH_2$), 59.6 ($CH_2$), 43.6 ($CH_2$). Anal. Calcd for $C_{21}H_{25}F_2N_7O_4$: C, 52.8; H, 5.3; N, 20.5. Found: C, 52.9; H, 5.2; N, 20.5%.

#### 5.1.3. 3-({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)-1-propanol (**8**)

*5.1.3.1. Method A (Scheme 1).* A mixture of **2** (0.36 g, 0.83 mmol), 3-chloropropanol (0.40 g, 4.2 mmol), powdered $K_2CO_3$ (0.58 g, 4.2 mmol) and NaI (0.63 g, 4.2 mmol) in DMF (5 mL) was heated at 50–60 °C for 24 h, and diluted with water. The product was extracted with $CH_2Cl_2$ and chromatographed on silica, eluting with $CH_2Cl_2$–MeOH (96:4), and recrystallized from $CH_2Cl_2$/EtOH to give **8** (200 mg, 49% yield): mp 200–203 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.93 (d, $J$ = 8.0 Hz, 1 H), 7.48 (t, $J_{HF}$ = 53.5 Hz, 1H), 7.33 (t, $J$ = 8.2 Hz, 1H), 6.91 (d, $J$ = 7.8 Hz, 1 H), 4.48 (t, $J$ = 5.9 Hz, 2H), 3.97 (q, $J$ = 5.6 Hz, 2H), 3.88–3.86 (m, 8H), 3.79–3.77 (m, 8 H), 3.12 (t, $J$ = 6.0 Hz, exchangeable with $D_2O$, 1 H), 2.16–2.10 (m, 2H); $^{13}$C NMR ($CDCl_3$) $\delta$ 164.5 (C), 161.5 (C), 151.0, 144.1 (t, $J_{CF}$ = 26.8 Hz, C-2), 134. 8 (C), 132.4 (C), 126.1 (CH), 108.8 (CH), 108.6 (CH), 108.0 (t, $J_{CF}$ = 240.2 Hz, $CHF_2$), 66.5 ($CH_2$), 66.1 ($CH_2$), 58.8 ($CH_2$), 43.6 ($CH_2$), 43.4 ($CH_2$), 31.8 ($CH_2$). Anal. Calcd for $C_{22}H_{27}F_2N_7O_4$; C, 57.8; H, 5.5; N, 20.0. Found: C, 54.0; H, 5.6; N, 20.2%.

#### 5.1.4. 3-({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)-1-propanol (**8**)

*5.1.4.1. Method B (Scheme 3).* A mixture of 2-amino-3-nitrophenol (**32**) (7.2 g, 46.7 mmol), 3-chloropropanol (6.6 g, 1.5 equiv) and dry powdered $K_2CO_3$ (19.3 g, 3 equiv) in acetone (50 mL) was heated and stirred under reflux for 20 h. The solvent was evaporated to dryness and the residue was diluted with water and extracted into EtOAc and dried ($Na_2SO_4$). Evaporation of the solvent and chromatography of the residue on silica, eluting with $CH_2Cl_2$–EtOAc (4:1), gave 10.0 g (100% yield) of 2-(2-amino-3-nitrophenoxy) propanol (**33**) as a yellow solid: mp ($CH_2Cl_2$/hexanes) 72–74 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.71 (dd, $J$ = 8.9, 1.2 Hz, 1H), 6.92 (d, $J$ = 7.7 Hz, 1H), 6.60 (dd, $J$ = 8.9, 7.8 Hz, 1H), 6.42 (s, exchangeable with $D_2O$, 2H), 4.20 (t, $J$ = 6.1 Hz, 2H), 3.89 (q, $J$ = 5.7 Hz, 2H), 2.12 (pentet, $J$ = 6.1 Hz, 2H), 1.48 (t, $J$ = 5.0 Hz, exchangeable with $D_2O$, 1H). Anal. Calcd for $C_9H_{12}N_2O_4$: C, 50.9; H, 5.7; N, 13.3. Found: C, 51.0; H, 5.7; N, 13.3%.

A solution of **33** (4.3 g, 20.26 mmol) in MeOH (50 mL) was hydrogenated over 10% Pd on C and then filtered into a solution of 5 mL conc. HCl in MeOH (50 mL). After removal of the solvents, the resulting residue was dissolved in a mixture of 4 N HCl (60 mL) and difluoroacetic acid (5 mL), and the resulting mixture was heated under reflux for 6 h. After cooling to 20 °C the mixture was diluted with water (100 mL), neutralized with aq. $NH_3$, extracted into EtOAc (5 × 40 mL), and dried ($Na_2SO_4$). Evaporation of the solvent gave crude 2-{[2-(difluoromethyl)-1*H*-benzimidazol-4-yl]oxy}ethanol (**34**) which was combined with TBDMSCl (6.1 g, 2 equiv) in pyridine (15 mL) and stirred at room temperature for 3 h before being poured into water (150 mL), extracted with EtOAc, and dried

($Na_2SO_4$). Evaporation of the solvents and the chromatography of the residue on silica, eluting with $CH_2Cl_2$–EtOAc (9:1) gave 4-(2-{[*tert*-butyl(dimethyl)silyl]oxy}propoxy)-2-(difluoromethyl)-1*H*-benzimidazole (**35**) (5.79 g, 84% yield): mp ($CH_2Cl_2$/hexanes) 133–135 °C; $^1$H NMR (DMSO-$d_6$) (tautomeric mixture) $\delta$ 13.40 and 13.23 (s, exchangeable with $D_2O$, 1H), 7.36–7.05 (m, 3H), 6.86 and 6.75 (2d, $J$ = 7.8 Hz and 7.4 Hz, 1H), 4.27 and 4.22 (2t, $J$ = 6.3 Hz, 2H), 3.87 and 3.81 (2t, $J$ = 6.1 Hz, 1H), 2.05–1.95 (m, 2H), 0.85 and 0.84 (2s, 9H), 0.02 and 0.01 (2s, 6H).

A mixture of **35** (684 mg, 1.92 mmol), dry powdered $K_2CO_3$ (530 mg, 3.84 mmol) and 2-chloro-4,6-dimorpholino-1,3,5-triazine [33] (285 mg, 3.84 mmol) in DMSO (5 mL) was heated at 130 °C for 3 h. The cooled reaction mixture was diluted with water, and the resulting precipitate was filtered, washed with water, and recrystallized from $CH_2Cl_2$/EtOH to give 4-(3-{[*tert*-butyl(dimethyl)silyl]oxy}propoxy)-2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazole (**36**) (971 mg, 83% yield): $^1$H NMR (DMSO-$d_6$) $\delta$ 7.88 (d, $J$ = 8.2 Hz, 1H), 7.48 (t, $J_{HF}$ = 53.5 Hz, 1H), 7.34 (t, $J$ = 8.2 Hz, 1H), 6.87 (d, $J$ = 8.0 Hz, 1H), 4.38 (t, $J$ = 6.6 Hz, 2H), 3.88–3.84 (m, 6H), 3.81–3.78 (m, 4H) 2.16 (pentet, $J$ = 6.3 Hz, 2H), 0.91 (s, 9H), 0.62 (s, 3H), 0.02 (s, 3H).

To a solution of **36** (809 mg, 1.35 mmol) in THF (10 mL) was added TBAF (1 M, 2.5 mL) and the reaction mixture was stirred for 30 min. The mixture was then diluted with $H_2O$ (150 mL) and stirred for 1 h. The resulting precipitate was filtered, washed with water and chromatographed on silica eluting with $CH_2Cl_2$–MeOH (96:4) to give **8** (504 mg, 81% yield), identical with material prepared in Method A.

#### 5.1.5. 3-({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)-1,2-propanediol (**9**)

To a solution of **2** (208 mg, 0.48 mmol) in DMF (5 mL) was added powdered $K_2CO_3$ (331 mg, 2.4 mmol) and 3-chloro-1,2-propanediol (1 mL). The stirred mixture was heated at 50 °C for 48 h and then diluted with water. The resulting precipitate was collected by filtration, washed with water, and dried. Recrystallization from $CH_2Cl_2$/MeOH gave **9** (150 mg, 62% yield): mp 229–231 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 7.88 (d, $J$ = 8.2 Hz, 1H), 7.70 (t, $J_{HF}$ = 52.8 Hz, 1H), 7.38 (t, $J$ = 8.2 Hz, 1H), 6.94 (d, $J$ = 8.0 Hz, 1H), 5.05 (d, $J$ = 5.2 Hz, 1H), 4.71 (t, $J$ = 5.7 Hz, 1H), 4.23 (dd, $J$ = 10.0, 4.2 Hz, 1H), 4.10 (dd, $J$ = 10.0, 6.2 Hz, 1H), 3.91 (m, 1H), 3.81–3.79 (m, 8H), 3.69 (br s, 8H), 3.51 (t, $J$ = 6.0 Hz, 2H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 164.3 (C), 161.3 (C), 151.2 (C), 144.2 (t, $J_{CF}$ = 26.6 Hz, C-2), 134.5 (C), 131.6 (C), 126.8 (CH), 108.6 (t, $J_{CF}$ = 237.1 Hz, $CHF_2$), 106.5 (CH), 106.2 (CH), 70.5 ($CH_2$), 70.0 (CH), 65.8 ($CH_2$), 62.7 ($CH_2$), 43.6 ($CH_2$). Anal. Calcd for $C_{22}H_{27}F_2N_7O_5 \cdot 0.25H_2O$: C, 51.6; H, 5.4; N, 19.2. Found: C, 51.6; H, 5.4; N, 19.0%.

#### 5.1.6. 2-({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)ethanamine (**10**)

A mixture of **2** (281 mg, 0.65 mmol) and powdered $K_2CO_3$ (449 mg, 3.25 mmol) in dry DMF (5 mL) was stirred at 20 °C for 30 min, and then *N*-(2-bromoethyl)phthalimide (330 mg) was added. The resulting mixture was heated and stirred at 50 °C for 24 h, and a second batch of *N*-(2-bromoethyl) phthalimide (330 mg) was added. After an additional 24 h the reaction mixture was diluted with water, and the resulting precipitate was collected by filtration, washed with water, and dried. Recrystallization from $CH_2Cl_2$/EtOH gave 2-[2-({2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazol-4-yl}oxy)ethyl]-1*H*-isoindole-1,3(2*H*)-dione (**18**) (366 mg, 93% yield): mp 250–252 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.90 (d, $J$ = 4 Hz, 1H), 7.87–7.84 (m, 2H), 7.73–7.70 (m, 2H), 7.44 (t, $J_{HF}$ = 53.5 Hz, 1H), 7.31 (t, $J$ = 8.2 Hz, 1H), 6.90 (d, $J$ = 7.8 Hz, 1H), 4.61 (t, $J$ = 6.6 Hz, 2H), 4.22 (t, $J$ = 6.6 Hz, 2H), 3.87–3.86 (m, 8H), 3.79–3.76 (m, 8H).

To a solution of **18** (292 mg, 0.25 mmol) in $CH_2Cl_2$ (15 mL) and THF (10 mL) was added hydrazine monohydrate (2 mL), and the resulting mixture was stirred at room temperature for 24 h. After removal of the solvent under vacuum, the residue was diluted with water, and the resulting precipitate was collected by filtration, and dried. Chromatography on neutral alumina eluting with $CH_2Cl_2$/MeOH (95:5) gave **10** (133 mg, 58% yield): mp ($CH_2Cl_2$/hexanes) 201–203 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.90 (d, $J$ = 8.4 Hz, 1H), 7.49 (t, $J_{HF}$ = 53.5 Hz, 1H), 7.33 (t, $J$ = 8.2 Hz, 1H), 6.84 (d, $J$ = 7.8 Hz, 1H), 4.31 (t, $J$ = 5.2 Hz, 2H), 3.88–3.87 (m, 8H), 3.79–3.77 (m, 8H) 3.25 (t, $J$ = 5.2 Hz, 2H); $^{13}$C NMR ($CDCl_3$) $\delta$ 165.2 (C), 162.2 (C), 151.6 (C), 144.6 (t, $J_{CF}$ = 26.8 Hz, C-2), 135.4 (C), 132.60 (C), 126.7 (CH), 108.7 (t, $J_{CF}$ = 239.9 Hz, $CHF_2$), 108.5 (CH), 106.5 (CH), 71.3 ($CH_2$), 66.8 ($CH_2$), 44.1 ($CH_2$), 41.5 ($CH_2$). Anal. Calcd for $C_{21}H_{26}F_2N_8O_3 \cdot 0.5H_2O$: C, 52.0; H, 5.6; N, 23.1. Found: C, 52.2; H, 5.4; N, 22.1%.

#### 5.1.7. 2-({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)-N,N-dimethylethanamine (**11**)

A mixture of **2** (301 mg, 0.69 mmol), NaI (1 g), and $K_2CO_3$ (956 mg, 6.9 mmol) in DMF (5 mL) was stirred at 20 °C for 5 min when a yellow suspension was obtained. An excess of 2-*N*,*N*-dimethylaminoethyl chloride hydrochloride (1 g) was added, and the mixture was stirred at 50 °C for 3 days. The reaction mixture was diluted with water, extracted with $CH_2Cl_2$, and dried ($Na_2SO_4$). After solvent removal, the residue was chromatographed on silica eluting with $CH_2Cl_2$/MeOH (9:1) containing 1% aq. $NH_3$. Further chromatography on alumina eluting with $CH_2Cl_2$/MeOH (94:6) gave **11** (19 mg, 5% yield): mp ($CH_2Cl_2$/hexanes) 190–192 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.89 (d, $J$ = 8.1 Hz, 1H), 7.46 (t, $J_{HF}$ = 53.5 Hz, 1H), 7.32 (t, $J$ = 8.2 Hz, 1H), 6.84 (d, $J$ = 7.9 Hz, 1H), 4.39 (t, $J$ = 6.3 Hz, 2H), 3.88–3.87 (m, 8H), 3.79–3.77 (m, 8H), 2.91 (t, $J$ = 6.3 Hz, 2H), 2.38 (s, 6H); $^{13}$C NMR ($CDCl_3$) $\delta$ 164.5 (C), 162.1 (C), 149.4 (C), 144.4 (t, $J_{CF}$ = 26.8 Hz, C-2), 135.4 (C), 132.4 (C), 131.8 (C), 127.0 (CH), 110.0 (CH), 108.5 (t, $J_{CF}$ = 240.1 Hz, $CHF_2$), 107.9 (CH), 66.8 ($CH_2$), 65.1 ($CH_2$), 64.9 ($CH_2$), 57.1 ($CH_2$), 44.4 ($CH_3$), 44.2 ($CH_2$), 44.1 ($CH_2$); HRMS (FAB$^+$) Calcd for $C_{23}H_{30}F_2N_8O_3$ [MH$^+$]: *m*/*z* 505.2487. Found: 505.2479. Anal. Calcd for $C_{23}H_{30}F_2N_8O_3 \cdot 0.5H_2O$: C, 53.8; H, 6.1; N, 21.8. Found: C, 53.7; H, 5.8; N, 21.5%.

#### 5.1.8. 2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-4-[2-(4-morpholinyl)ethoxy]-1H-benzimidazole (**12**)

A mixture of **2** (215 mg, 0.49 mmol), $K_2CO_3$ (683 mg, 4.9 mmol), and 4-(2-chloroethyl)morpholine hydrochloride (455 mg, 2.5 mmol) in DMF (5 mL) was stirred at 50 °C for 2 h. The mixture was diluted with water and the resulting precipitate was collected by filtration, washed with water, and dried. Recrystallization from $CH_2Cl_2$/EtOH gave **12** (193 mg, 73% yield): mp 228–230 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.90 (dd, $J$ = 8.3, 0.5 Hz, 1H), 7.47 (t, $J_{HF}$ = 53.5 Hz, 1H), 7.32 (t, $J$ = 8.2 Hz, 1H), 6.83 (d, $J$ = 7.7 Hz, 1H), 4.41 (t, $J$ = 6.1 Hz, 2H), 3.88–3.86 (m, 8H), 3.96–3.74 (m, 12H), 2.95 (t, $J$ = 6.1 Hz, 2H), 2.62 (br t, $J$ = 4.6 Hz, 4H); $^{13}$C NMR ($CDCl_3$) 165.2 (C), 162.2 (C), 151.4 (C), 144.7 (t, $J_{CF}$ = 27.0 Hz, C-2), 135.4 (C), 132.6 (C), 126.7 (CH), 108.7 (t, $J_{CF}$ = 240.1 Hz, $CHF_2$), 108.6 (CH), 106.5 (CH), 67.0 ($CH_2$), 66.8 ($CH_2$), 66.3 ($CH_2$), 57.6 ($CH_2$), 54.2 ($CH_2$), 44.2 ($CH_2$), 44.1 ($CH_2$). Anal. Calcd for $C_{25}H_{32}F_2N_8O_4$: C, 54.9; H, 5.9; N, 20.5. Found: C, 55.0; H, 6.0; N, 20.4%.

#### 5.1.9. 3-({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)-1-propanamine (**13**)

Similarly to the synthesis of **10**, reaction of **2** with *N*-(3-bromopropyl)phthalimide gave 2-[3-({2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazol-4-yl}oxy)propyl]-1*H*-isoindole-1,3(2*H*)-dione (**19**) in 96% yield: mp ($CH_2Cl_2$/EtOH) 212–215 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.87 (d, $J$ = 8.1 Hz, 1H), 7.85–7.80 (m, 2H), 7.71–7.67 (m, 2H), 7.40 (t, $J_{HF}$ = 53.6 Hz, 1H), 7.30 (t,

$J$ = 8.2 Hz, 1H), 6.80 (d, $J$ = 7.8 Hz, 1H), 4.37 (t, $J$ = 6.5 Hz, 2H), 3.96 (t, $J$ = 6.7 Hz, 2H), 3.88–3.86 (m, 8H), 3.79–3.76 (m, 8H), 2.35 (pentet, $J$ = 6.6 Hz, 2H).

Deprotection of **19** with hydrazine monohydrate (as for **10**) gave **13** (28% yield): mp ($CH_2Cl_2$/hexanes) 218–221 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.91 (d, $J$ = 8.1 Hz, 1H), 7.50 (t, $J_{HF}$ = 53.6 Hz, 1H), 7.32 (t, $J$ = 8.2 Hz, 1H), 6.87 (d, $J$ = 7.9 Hz, 1H), 4.40 (t, $J$ = 6.1 Hz, 2H), 3.88–3.86 (m, 4H), 3.79–3.76 (m, 4H), 3.15 (t, $J$ = 6.5 Hz, 2H), 2.19 (pentet, $J$ = 6.2 Hz, 2H); $^{13}$C NMR ($CDCl_3$) $\delta$ 165.1 (C), 162.1 (C), 144.7 (t, $J_{CF}$ = 26.8 Hz, C-2), 151.4 (C), 135.3 (C), 132.6 (C), 126.8 (CH), 108.9 (CH), 108.7 (t, $J_{CF}$ = 240.1 Hz, $CHF_2$), 107.6 (CH), 66.8 ($CH_2$), 67.6 ($CH_2$), 44.2 ($CH_2$), 44.1 ($CH_2$), 38.7 ($CH_2$), 30.8 ($CH_2$); HRMS ($EI^+$) Calcd for $C_{22}H_{29}F_2N_8O_3$ [$MH^+$]: $m/z$ 491.22325. Found: $m/z$ 491.2316.

### 5.1.10. 3-({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)-N,N-dimethyl-1-propanamine (**14**)

To a solution of **8** (150 mg, 0.31 mmol) and $Et_3N$ (0.5 mL) in THF (15 mL) at 0 °C, was added methanesulfonyl chloride (0.37 mmol). The reaction mixture was stirred at 0 °C for 45 min, and then 3 mL of a 40% aqueous solution of dimethylamine was added. The resulting mixture was stirred at room temperature for 24 h. The solvent was removed under vacuum, the residue was diluted with water, and the resulting precipitate was collected, washed with water, and dried. Chromatography on silica eluting with $CH_2Cl_2$/MeOH (95:5) containing 1% aq. $NH_3$ gave **14** (130 mg, 81% yield): mp ($CH_2Cl_2$/hexanes) 191–193 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.88 (d, $J$ = 8.1 Hz, 1H), 7.47 (t, $J_{HF}$ = 57.8 Hz, 1H), 7.32 (t, $J$ = 8.3 Hz, 1H), 4.33 (t, $J$ = 6.7 Hz, 2H), 3.88–3.87 (m, 8H), 3.79–3.77 (m, 8H), 2.56 (br, 2H), 2.32 (br s, 6H), 1.16 (pentet, $J$ = 6.4 Hz, 2H); $^{13}$C NMR ($CDCl_3$) $\delta$ 165.2 (C), 162.2 (C), 151.7 (C), 144.5 (t, $J_{CF}$ = 27.0 Hz, C-2), 135.4 (C), 132.6 (C), 126.72 (CH), 108.8 (t, $J_{CF}$ = 240.0 Hz, $CHF_2$), 108.2 (CH), 106.3 (CH), 67.6 ($CH_2$), 66.8 ($CH_2$), 56.5 ($CH_2$), 45.7 ($CH_3$), 44.1 ($CH_2$), 27.5 ($CH_2$). HRMS ($FAB^+$) Calcd for $C_{24}H_{32}F_2N_8O_3$ [$MH^+$]: $m/z$ 519.2644. Found: 519.2644.

Hydrochloride: $^1$H NMR (DMSO-$d_6$) $\delta$ 9.97 (br s, exchangeable with $D_2O$, 1H), 7.92 (d, $J$ = 8.1 Hz, 1H), 7.70 (t, $J_{HF}$ = 52.8 Hz, 1H), 7.41 (t, $J$ = 8.2 Hz, 1H), 6.98 (d, $J$ = 7.9 Hz, 1H), 4.33 (t, $J$ = 6.1 Hz, 2H), 3.82–3.80 (m, 8H); 3.69 (m, 8H), 3.25 (m, 2H), 2.81 (s, 6H), 2.27–2.20 (m, 2H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 164.3 (C), 161.3 (C), 150.5 (C), 144.1 (t, $J_{CF}$ = 26.7 Hz, C-2), 134.5 (C), 131.6 (C), 126.8 (CH), 108.5 (t, $J_{CF}$ = 237.0 Hz, $CHF_2$), 108.4 (CH), 107.0 (CH), 66.0 ($CH_2$), 66.8 ($CH_2$), 54.2 ($CH_2$), 43.6 ($CH_2$), 42.2 ($CH_3$), 24.0 ($CH_2$). Anal. Calcd for $C_{24}H_{32}F_2N_8O_3$1.2 HCl $H_2O$: C, 49.7; H, 6.1; Cl, 7.3; N, 19.3. Found: C, 49.6; H, 6.0; Cl, 7.4; N, 19.1%.

### 5.1.11. 2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-4-[2-(4-morpholinyl)propoxy]-1H-benzimidazole (**15**)

Similarly to the synthesis of **12**, reaction of **2** with 4-(3-bromopropyl)morpholine hydrobromide gave **15** (29% yield); mp ($CH_2Cl_2$/EtOH) 188–190 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.88 (d, $J$ = 8.3 Hz, 1H), 7.47 (t, $J_{HF}$ = 53.5 Hz, 1H), 7.32 (t, $J$ = 7.9 Hz, 1H), 6.85 (d, $J$ = 8.0 Hz, 1H), 4.34 (t, $J$ = 8.0 Hz, 2H), 3.90–3.87 (m, 8H), 3.79–3.76 (m, 8H), 3.73–3.71 (m, 4H), 2.60–2.58 (m, 2H), 2.49 (m, 4H), 2.15–2.10 (m, 2H); $^{13}$C NMR ($CDCl_3$) $\delta$ 165.2 (C), 162.2 (C), 151.7 (C), 144.6 (t, $J_{CF}$ = 26.8 Hz, C-2), 135.4 (C), 132.6 (C), 126.7 (CH), 108.8 (t, $J_{CF}$ = 240.1 Hz, $CHF_2$), 108.3 (CH), 106.4 (CH), 67.5 ($CH_2$), 67.1 ($CH_2$), 66.8 ($CH_2$), 55.6 ($CH_2$), 53.8 ($CH_2$), 44.1 ($CH_2$), 26.3 ($CH_2$). Anal. Calcd for $C_{26}H_{34}F_2N_8O_4$: C, 55.7; H, 6.1: N, 20.0. Found: C, 55.7; H, 6.1; N, 20.1%.

### 5.1.12. 1-({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)-3-(dimethylamino)-2-propanol (**16**)

A mixture of **2** (288 mg, 0.66 mmol) and powdered $K_2CO_3$ (454 mg, 2.4 mmol) in DMF (5 mL) was stirred at room temperature for 30 min and epibromohydrin (0.5 mL) was added. The resulting mixture was heated at 50 °C for 30 min, cooled, and diluted with water. The resulting precipitate was filtered, washed with water, and dried to give 2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-4-(2-oxiranylmethoxy)-1*H*-benzimidazole (**20**) (341 mg, 97% yield): $^1$H NMR ($CDCl_3$) $\delta$ 7.92 (d, $J$ = 7.9 Hz, 1H), 7.48 (t, $J_{HF}$ = 53.4 Hz, 1H), 7.33 (t, $J$ = 8.1 Hz, 1H), 6.89 (d, $J$ = 7.9 Hz, 1H), 4.51 (dd, $J$ = 11.5, 3.8 Hz, 1H), 4.35 (dd, $J$ = 11.5, 5.4 Hz, 1H), 3.88–3.87 (m, 8H), 3.79–3.77 (m, 8H), 3.52–3.48 (m, 1H), 2.93 (t, $J$ = 4.6 Hz, 1H), 2.80 (dd, $J$ = 4.9, 2.6 Hz, 1H).

Reaction of the above epoxide **20** with excess 40% aqueous dimethylamine in THF (10 mL) and MeOH (10 mL) at room temperature gave **16** (96% yield): mp ($CH_2Cl_2$/hexanes) 197–200 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.91 (d, $J$ = 7.9 Hz, 1H), 7.48 (t, $J_{HF}$ = 53.5 Hz, 1H), 7.33 (t, $J$ = 8.3 Hz, 1H), 6.90 (d, $J$ = 7.6 Hz, 1H), 4.33–4.20 (m, 1H), 4.30 (t, $J$ = 4.9 Hz, 2H), 3.89–3.86 (m, 8H), 3.79–3.77 (m, 8H), 2.61–2.49 (m, 2H), 2.33 (s, 6H); $^{13}$C NMR ($CDCl_3$) $\delta$ 165.2 (C), 162.2 (C), 151.5 (C), 144.6 (t, $J_{CF}$ = 26.8 Hz, C-2), 135.3 (C), 132.5 (C), 126.8 (CH), 108.7 (CH), 108.6 (t, $J_{CF}$ = 240.0 Hz, $CHF_2$), 107.2 (CH), 66.8 (CH), 72.4 ($CH_2$), 66.8 ($CH_2$), 62.1 ($CH_2$), 44.9 ($CH_3$), 44.1 ($CH_2$). Anal. Calcd for $C_{24}H_{32}F_2N_8O_4$.0.25$H_2O$: C, 53.4; H, 5.9; N, 20.7. Found: C, 53.5; H, 6.1; N, 20.8%.

### 5.1.13. 1-({2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}oxy)-3-(4-morpholinyl)-2-propanol (**17**)

Morpholine (0.26 g, 3 mmol) was added to a solution of **20** (300 mg, 0.61 mmol) in THF (10 mL) and MeOH (10 mL), and the resulting mixture was stirred at room temperature for 16 h before being evaporated to dryness. The residue was triturated with water, and the resulting precipitate was collected, dried, and recrystallized from $CH_2Cl_2$/hexanes to give **17** (353 mg, 100% yield): mp 173–175 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.91 (dd, $J$ = 8.4, 0.6 Hz, 1H), 7.48 (t, $J_{HF}$ = 53.4 Hz, 1H), 7.33 (t, $J$ = 8.1 Hz, 1H), 6.90 (d, $J$ = 7.6 Hz, 1H), 4.35–4.26 (m, 3H), 3.89–3.86 (m, 8H), 3.79–3.77 (m, 8H), 3.74–3.71 (m, 4H), 2.69–2.60 (m, 4H), 2.55–2.49 (m, 2H); $^{13}$C NMR ($CDCl_3$) $\delta$ 165.2 (C), 162.2 (C), 151.4 (C), 144.7 (t, $J_{CF}$ = 26.8 Hz, C-2), 135.4 (C), 132.6 (C), 126.8 (CH), 108.9 (CH), 108.6 (t, $J_{CF}$ = 240.1 Hz, $CHF_2$), 107.3 (CH), 72.3 ($CH_2$), 67.2 ($CH_2$), 66.8 (CH), 66.1 ($CH_2$), 61.3 ($CH_2$), 54.1 ($CH_2$), 44.1 ($CH_2$). Anal. Calcd for $C_{26}H_{34}F_2N_8O_5$: C, 54.2; H, 5.9; N, 19.4. Found: C, 54.4; H, 5.9; N, 19.5%.

### 5.1.14. $N^1$-{2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}-$N^2$,$N^2$-dimethyl-1,2-ethanediamine (**21**)

*tert*-Butyl 2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazol-4-ylcarbamate (**26**) [30] (149 mg, 0.3 mmol) was suspended in dry DMF (2 mL) at 0 °C, and NaH (64 mg 2.7 mmol) was added. The reaction mixture was stirred for 30 min, and a solution of *N*,*N*-dimethylaminoethyl chloride (generated from 5 g amine hydrochloride and aq. $K_2CO_3$) in dry benzene was added. The mixture was stirred at room temperature for 24 h, and was then quenched with $H_2O$ and extracted with EtOAc. The organic fraction was dried ($Na_2SO_4$) and evaporated to dryness. Chromatography of the residue on silica eluting with $CH_2Cl_2$/MeOH (95:5) gave crude *tert*-butyl 2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazol-4-yl [2-(dimethylamino)ethyl] carbamate (**27**) (111 mg, 61% yield), which was treated with TFA (5 mL) in $CH_2Cl_2$ (5 mL) for 48 h. The reaction mixture was evaporated to dryness at 20 °C, and after being neutralized with aqueous $NH_3$, the product was purified by chromatography on silica eluting with $CH_2Cl_2$/MeOH (9:1) to give a white solid, which was recrystallized from $CH_2Cl_2$/hexanes to give **21** (25 mg, 18% yield): mp 237–239 °C; $^1$H NMR ($CDCl_3$) $\delta$ 7.55 (d, $J$ = 8.2 Hz, 1H), 7.52 (t, $J_{HF}$ = 53.7 Hz, 1H), 7.20–7.23 (m, 1H), 6.48 (d,

$J$ = 7.8 Hz, 1H), 5.33 (t, $J$ = 5.1 Hz, exchangeable with $D_2O$, 1H), 3.87 (m, 8H), 3.79–3.76 (m, 8H), 3.38 (q, $J$ = 5.6 Hz, 2H), 2.62 (t, $J$ = 6.2 Hz, 2H), 2.30 (s, 6H); HRMS ($EI^+$) Calcd for $C_{23}H_{31}F_2N_9O_2$: $m/z$ 503.2573. Found: $m/z$ 503.2575.

*5.1.15. 2-Amino-N-{2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}acetamide (**22**)*

A mixture of amine **4** [30,32] (202 mg, 0.48 mmol), [(*tert*-butoxycarbonyl)amino]acetic acid (164 mg, 0.96 mmol), DMAP (230 mg, 1.9 mmol), and EDCI (360 mg, 1.9 mmol) in DMF (5 mL) was stirred at room temperature for 6 h, and additional [(*tert*-butoxycarbonyl)amino]acetic acid (82 mg, 0.24 mmol) was added. After an additional 2 h the reaction mixture was diluted with water, and the resulting precipitate was collected by filtration, washed with water, and dried. Recrystallization from $CH_2Cl_2$/EtOH gave *tert*-butyl 2-({2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazol-4-yl}amino)-2-oxoethylcarbamate (**28**) (238 mg, 86% yield): mp 238 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 9.79 (s, 1H), 8.10 (d, $J$ = 7.7 Hz, 1H), 8.01 (dd, $J$ = 8.3, 0.7 Hz, 1H), 7.74 (t, $J_{HF}$ = 52.7 Hz, 1H), 7.44 (t, $J$ = 8.2 Hz, 1H), 7.33 (t, $J$ = 4.8 Hz, 1H), 3.87 (d, $J$ = 6.1 Hz, 2H), 3.82–3.80 (m, 8H), 3.69 (m, 8H), 1.42 (s, 9H). Anal. Calcd for $C_{26}H_{33}F_2N_9O_5$: C, 53.0; H, 5.6; N, 21.4. Found: C, 52.7; H, 5.8; N, 21.1%.

A mixture of the above carbamate (**28**) (205 mg, 0.35 mmol) and TFA (6 mL) in $CH_2Cl_2$ (10 mL) was stirred at room temperature for 16 h before being evaporated to dryness. The residue was triturated with MeOH/EtOAc and the resulting precipitate was collected, washed with EtOAc, and dried to give **22** as the trifluoroacetate salt (195 mg, 92% yield): mp 218–221 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 10.65 (s, exchangeable with $D_2O$, 1H), 8.01 (br s, exchangeable with $D_2O$, 2H), 8.13 (d, $J$ = 8.07 Hz, 1H), 8.07 (d, $J$ = 8.28 Hz, 1H), 8.07 (d, $J$ = 8.3 Hz, 1H), 7.76 (t, $J_{HF}$ = 52.6 Hz, 1H), 7.49 (t, $J$ = 8.2 Hz, 1H), 3.99 (s, 2H), 3.83–3.81 (m, 8H), 3.70 (m, 8H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 165.8 (C), 162.3 (C), 161.2 (C), 145.0 (t, $J_{CF}$ = 27.1 Hz, C-2), 133.4 (C), 132.6 (C), 129.8 (C), 126.4 (CH), 114.8 (CH), 11.4 (CH), 108.5 (t, $J_{CF}$ = 237.1 Hz, $CHF_2$), 65.8 ($CH_2$), 43.6 ($CH_2$), 41.2 ($CH_2$). Anal. Calcd for $C_{23}H_{26}F_5N_9O_5 \cdot H_2O$, C, 44.6, H, 4.5, N, 20.3. Found: C, 44.8; H, 4.5, N, 20.3%.

*5.1.16. N-{2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}-2-(dimethylamino)acetamide (**23**)*

To a suspension of **4** (158 mg, 0.37 mmol) and $K_2CO_3$ (200 mg, 2.9 mmol) in $CH_2Cl_2$ (10 mL) was added chloroacetyl chloride (1 mL), and the mixture was stirred for 4 h. The mixture was diluted with water, extracted with $CH_2Cl_2$, and dried ($Na_2SO_4$). The solvent was removed and the residue was recrystallized from $CH_2Cl_2$/hexanes to give 2-chloro-*N*-{2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazol-4-yl}acetamide (**29**) (188 mg, 100% yield): mp 287–289 °C, $^1$H NMR (DMSO-$d_6$) $\delta$ 10.39 (s, exchangeable with $D_2O$, 1H), 8.12 (d, $J$ = 7.9 Hz, 1H), 8.06 (dd, $J$ = 8.3, 0.7 Hz, 1H), 7.75 (t, $J_{HF}$ = 52.6 Hz, 1H), 7.46 (t, $J$ = 8.2 Hz, 1H) 4.53 (s, 2H), 3.83–3.80 (m, 8H), 3.70 (m, 8H). Anal. Calcd for $C_{21}H_{23}ClF_2N_8O_3$: C, 49.6; H, 4.6; N, 22.0. Found: C, 49.5; H, 4.6; N, 21.9%.

A mixture of chloroacetamide **29** (206 mg, 0.40 mmol) and 40% aq. dimethylamine (2 mL) in ethanol (10 mL) was heated under reflux for 2 h. The solvent was removed and the residue was recrystallized from $CH_2Cl_2$/EtOH to give **23** (171 mg, 83% yield): mp 259–261 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 9.96 (s, exchangeable with $D_2O$, 1H), 8.25 (d, $J$ = 8.0 Hz, 1H), 8.01 (dd, $J$ = 8.4, 0.7 Hz, 1H), 7.74 (t, $J_{HF}$ = 52.7 Hz, 1H), 7.45 (t, $J$ = 8.2 Hz, 1H), 3.82–3.80 (m, 8H), 3.70 (m, 8H), 3.19 (s, 2H), 2.37 (s, 6H); $^{13}$C NMR ($CDCl_3$) $\delta$ 169.1 (C), 164.5 (C), 161.5 (C), 144.0 (t, $J_{CF}$ = 26.8 Hz, C-2) 133.2 (C), 132.1 (C), 130.0 (C), 126.3 (CH), 112.9 (CH), 110.3 (CH), 106.8 (t, $J_{CF}$ = 239.0 Hz, $CHF_2$), 66.2 ($CH_2$), 63.6 ($CH_2$), 45.6 ($CH_3$), 43.6 ($CH_2$), 43.5 ($CH_2$). Anal. Calcd for $C_{23}H_{29}F_2N_9O_3$: C, 53.3; H, 5.7; N, 24.4. Found: C, 53.5; H, 5.6; N, 24.5%.

*5.1.17. 3-Amino-N-{2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}propanamide (**24**)*

Similarly to the synthesis of **22**, EDCI coupling of **4** and *N*-(*tert*-butoxycarbonyl)-β-alanine gave *tert*-butyl 3-({2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazol-4-yl}amino)-3-oxopropylcarbamate (**30**) (85% yield): mp ($CH_2Cl_2$/EtOH) 217–219 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 9.96 (s, exchangeable with $D_2O$, 1H), 8.10 (d, $J$ = 7.7 Hz, 1H), 8.01 (dd, $J$ = 8.3, 0.7 Hz, 1H), 7.74 (t, $J_{HF}$ = 52.65, 1H), 7.41 (t, $J$ = 8.2 Hz, 1H), 6.79 (br s, exchangeable with $D_2O$, 1H), 3.83–3.80 (m, 8H), 3.69 (m, 8H), 3.28–3.23 (m, 2H), 2.68 (t, $J$ = 7.0 Hz, 2H), 1.38 (s, 9H). Anal. Calcd for $C_{27}H_{35}F_2N_9O_5$: C, 53.7; H, 5.8; N, 20.9. Found: C, 53.7; H, 5.9; N, 21.1%.

Deprotection of **30** with TFA in $CH_2Cl_2$ gave **24** as the trifluoroacetate salt (86% yield): mp (MeOH/EtOAc) 237–240 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 10.31 (s, exchangeable with $D_2O$, 1H), 8.09 (d, $J$ = 7.8 Hz, 1H), 8.04 (d, $J$ = 8.2 Hz, 1H), 7.75 (t, $J$ = 52.6 Hz, 1H), 7.69 (br s, exchangeable with $D_2O$, 2H), 7.45 (t, $J$ = 8.2 Hz, 1H), 3.83–3.80 (m, 8H), 3.69 (m, 8H), 3.13 (t, $J$ = 6.6 Hz, 2H), 2.91 (t, $J$ = 6.2 Hz, 2H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 169.3 (C), 164.3 (C), 161.3 (C), 144.8 (t, $J_{CF}$ = 27.9 Hz, C-2), 133.3 (C), 132.7 (C), 130.4 (C), 126.2 (CH), 115.2 (CH), 110.9 (CH), 108.5 (t, $J_{CF}$ = 237.5 Hz, $CHF_2$) 65.8 ($CH_2$), 43.7 ($CH_2$), 35.0 ($CH_2$), 33.3 ($CH_2$). Anal. Calcd for $C_{24}H_{28}F_5N_9O_5$: C, 46.7; H, 4.6; N, 20.4. Found: C, 46.7; H, 4.6; N, 20.7%.

*5.1.18. N-{2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1H-benzimidazol-4-yl}-3-(dimethylamino) propanamide (**25**)*

To a mixture of **4** (198 mg, 0.45 mmol) and DIPEA (1 mL) in $CH_2Cl_2$ (10 mL) at 0 °C was added acryloyl chloride (1 mL). The reaction mixture was allowed to warm to 20 °C and then stirred for an additional 2 h. The mixture was diluted with water, extracted with $CH_2Cl_2$, and dried ($Na_2SO_4$). The solvents were removed and the residue was recrystallized from $CH_2Cl_2$/EtOH to give *N*-{2-(difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]-1*H*-benzimidazol-4-yl}acrylamide (**31**) (168 mg, 76% yield): mp 246–249 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 10.27 (s, exchangeable with $D_2O$, 1H), 8.19 (d, $J$ = 8.2 Hz, 1H), 8.04 (dd, $J$ = 8.3, 0.6 Hz, 1H), 7.75 (t, $J_{HF}$ = 52.6 Hz, 1H), 7.45 (t, $J$ = 8.2 Hz, 1H), 6.90 (dd, $J$ = 17.0, 10.2 Hz, 1H), 6.31 (dd, $J$ = 17.0, 2.0 Hz, 1H), 5.78 (dd, $J$ = 10.2, 2.0 Hz, 1H), 3.82–3.81 (m, 8H), 3.70 (m, 8H).

Reaction of acrylamide **31** with 40% aq. dimethylamine in EtOH (5 mL) at room temperature, followed by chromatography on neutral alumina, eluting with $CH_2Cl_2$/EtOAc (4:1), gave **25** (35% yield): mp ($CH_2Cl_2$/MeOH) 228–230 °C; $^1$H NMR ($CDCl_3$) $\delta$ 12.06 (s, exchangeable with $D_2O$, 1H), 8.34 (d, $J$ = 7.9 Hz, 1H), 7.94 (dd, $J$ = 8.4, 0.7 Hz, 1H), 7.52 (t, $J_{HF}$ = 56.8 Hz, 1H), 7.40 (t, $J$ = 8.2 Hz, 1H), 3.88–3.87 (br, 8H), 3.79 (br, 8H), 2.74 (t, $J$ = 5.9 Hz, 2H), 2.60 (t, $J$ = 5.9 Hz, 2H), 2.46 (s, 6H); $^{13}$C NMR ($CDCl_3$) $\delta$ 171.0 (C), 164.5 (C), 161.6 (C), 143.8 (t, $J_{CF}$ = 26.7 Hz, C-2), 133.1 (C), 132.3 (C), 131.3 (C), 126.3 (CH), 113.0 (CH), 109.8 (CH), 107.9 (t, $J_{CF}$ = 239.4 Hz, $CHF_2$), 66.2 ($CH_2$), 54.3 ($CH_2$), 44.0 ($CH_3$), 43.6 ($CH_2$), 43.5 ($CH_2$), 33.7 ($CH_2$). Anal. Calcd for $C_{24}H_{31}F_2N_9O_3$: C, 54.2; H, 5.9; N, 23.7. Found: C, 54.3; H, 5.8; N, 24.0%.

*5.2. Enzyme assays*

Compounds were evaluated for their ability to inhibit the Class I PI 3-kinase enzymes p110α/p85, p110β/p85 and p110δ/p85. The Class Ia PI3K lipid kinase assays were performed using recombinant PI 3-kinase and phosphatidylinositol as a substrate and [$^{33}$P]-ATP tracer with a final ATP concentration of 10 μM, as described

previously [34,37]. $IC_{50}$ data is normally the average of at least two determinations.

### 5.3. Cell proliferation assay

The compounds were evaluated in cellular assays by comparing NZB5, a cell line derived from a medulloblastoma and expressing the wild-type gene for p110α, and NZOV9 a cell line derived from a poorly differentiated endometrioid adenocarcinoma of the ovary and expressing a mutant p110α enzyme with a single amino acid substitution in the kinase domain (Y1021C). The derivation of the latter line has been described previously [38]. Cells were cultured in the presence of drugs in a 5-day assay as previously described and proliferation was measured by incorporation of [$^3$H]-thymidine at the end of the incubation period [37]. The $IC_{50}$ values (concentrations for 50% inhibition relative to controls were averages of at least two determinations).

### 5.4. Inhibition of cell signaling

HCT116 cells were grown in MEM alpha supplemented with 10% (v/v) FBS (fetal bovine serum), 100 units/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen). For inhibition studies, cells were seeded in 12-well plates and grown for 1 day before overnight starvation in serum-free media. Cells were then exposed to varying concentrations of inhibitor dissolved in DMSO or DMSO alone (final concentration of DMSO in media 0.1%) for 15 min before stimulation with 500 nM insulin for 5 min. Protein isolation and immunoblotting for phospho-PKB was carried out according to the methods previously described, with antibodies from Cell Signaling Technology (Ser473 catalogue# 9271, Thr308 catalogue# 9275) [34].

### 5.5. Pharmacokinetics

Age-matched specific pathogen-free male C57 mice were administered a single 10 mg/kg dose of **14** in 10% EtOH and were culled at multiple time-points after dosing. Blood was removed by cardiac puncture into EDTA collection tubes (Becton Dickinson, Auckland, New Zealand) and centrifuged for 10 min at 6000 rpm to separate plasma, which underwent protein precipitation in MeOH. Quantitative analysis was carried out as described previously [30]. Pharmacokinetic parameters were determined by noncompart mental analysis using Phoenix WinNonlin 6.2 (Pharsight, Sunnyvale, CA, USA).

### 5.6. Antitumor efficacy

Age-matched specific pathogen-free female Rag1$^{-/-}$ mice were subcutaneously inoculated on the right flank with $5 \times 10^6$ U87MG cells in phosphate buffered saline (PBS). Tumor diameter was measured by callipers to calculate tumor volume ($mm^3$) using the formula $(L \times w^2) \times \pi/6$ (where; $L$ = longest tumor diameter and $w$ = perpendicular diameter). Dosing began 3–4 weeks after inoculation when tumors were well established, averaging approximately 8 mm in diameter. The dosing vehicle for compound **14** was D5W (5% dextrose), while **1** was administered in 10% DMSO, 15% Cremophor EL, 15% EtOH, 60% saline and **37** was administered in 10% EtOH. All drugs were dosed by i.p. injection as the free base equivalent at a dosing volume of 10 mL/kg. Tumor volume and animal bodyweight were measured every 2–3 days. Tumor growth inhibition was calculated as the difference between the average tumor volume of treatment mice vs control mice at the final control measurement expressed as a percentage of tumor volume in control mice. Statistical significance was determined by 1-way ANOVA with Holm-Sidak multiple comparison analysis using SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, USA). Mice were culled if they developed moderate signs of toxicity or if bodyweight loss exceeded 20% of starting weight. All animal experiments followed protocols approved by the Animal Ethics Committee of The University of Auckland.

### 5.7. Molecular modeling

The protein was prepared for docking as previously described [39,40]. Ligands were prepared using the SKETCHER module of SYBYL8.0.3 with optimisation by MAXIMIN2 using the MMFF94s force field with MMFF94 charges and a distance dependent dielectric function with a dielectric constant of 80, and converged to 0.05 kcal/mol*A. Ligands were docked into an 18 Å cavity centered on Ile800 using GOLD [41] (v5.0.1) with search efficiency set at 200%. The Chemscore scoring function with kinase specific modification was used, and 10 dockings were performed with all poses kept. Residues Lys720, Lys776, Lys802, Asp805, Asp933 were treated as flexible and side chain conformations restricted to library entries, while residues Leu807, Asp810, Tyr836 and Ile848 were assigned to an alternative soft potential model using the 4-8 2-4 option available in GOLD.

### 5.8. Aqueous solubility determinations

The solid compound sample was mixed with water (enough to make a 2 mM solution) in an Eppendorf tube, and the suspension was sonicated for 15 min and then centrifuged at 13,000 rpm for 6 min. An aliquot of the clear supernatant was diluted 2-fold with water, and then centrifuged again at 13,000 rpm for 6 min. A 100 μL aliquot of the clear supernatant was injected into the HPLC and the peak area measured. The solubility was calculated by comparing the peak area obtained with that from a standard solution of the compound in DMSO (after allowing for varying dilution factors and injection volumes). HPLC was conducted on an Agilent 1100 system using an Altima C18 column with a gradient elution from an organic phase of 80% v/v acetonitrile and water (40%), and an aqueous mobile phase of 45 mM ammonium formate solution (pH 3.5) (60%), to 100% organic phase.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.03.038.

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