Dual and/or selective DNA-PK, PI3K inhibition and isoform selectivity of some new and known 2-amino-substituted-1,3-benzoxazines and substituted-1,3-naphthoxazines

ARTICLE *in* MEDICINAL CHEMISTRY RESEARCH · MAY 2014

Impact Factor: 1.4 · DOI: 10.1007/s00044-014-1037-6

Med Chem Res (2014) 23:4680–4691
DOI 10.1007/s00044-014-1037-6

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Dual and/or selective DNA-PK, PI3K inhibition and isoform selectivity of some new and known 2-amino-substituted-1,3-benzoxazines and substituted-1,3-naphthoxazines

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Received: 9 February 2014 / Accepted: 9 May 2014 / Published online: 24 May 2014

Abstract The 2-morpholino-substituted-benzoxazines **7a** and **7b** were used in the synthesis of 2-morpholino-di-*O*-benzyl, *O*-pyridin-2yl, 3-yl and 4yl-methoxy)-1,3-benzoxazines **8a–8d,** and *N*-(2-morpholino-4-oxo-4H-benz[e][1,3]oxazin-7-yl)-*N*-(pyridin-2-and-3-ylmethyl)acetamides **8e** and **8f**. The DNA-dependent protein kinase (DNA-PK) and phosphatidylinositol 3-kinase (PI3K) α, β, γ, and δ isoforms were studied for the new compounds **8a**–**8f** and PI3K for the 18 previously synthesized compounds **9**–**26**. The most active DNA-PK inhibitors were the 2-morpholino-*O*-substituted linear or angular naphthoxazine compounds **18**–**20** and **21**–**22** which showed potent and selective DNA-PK activity (IC_{50} from 0.01 to 2.43 μM) over PI3K. 8-(2-(4-Methylpiperazin-1-yl)ethoxy)-2-morpholino compound **13**, and 8-methyl-2-(pyridin-3-yl(pyridin-3-ylmethyl)amino)-7-(pyridin-3-ylmethoxy) compound **25** showed selective DNA-PK inhibition. 2-morpholino-8-substituted-benzoxazine **9** (8-ph) and **10**–**12** (8-(pyridine-2-, 3-, or 4-ylmethoxy) showed high-to-moderate inhibition of PI3K and DNA-PK. A similar pattern for DNA-PK nonselectivity over PI3K was observed for compounds with 7,8-*O*-bis-substituted **8a, 8c**, and **8d**. No DNA-PK selectivity over PI3K was observed regardless whether the substitution was phenyl, pyridin-2-ylmethoxy, pyridin-3-ylmethoxy, and pyridin-4-ylmethoxy.

Keywords Synthesis · 2-Amino-substituted-1,3-benzoxazines · 2-Amino-naphthoxazines · DNA-PK · PI3K isoform inhibition and selectivity

Introduction

Our improving understanding of DNA damage responses is providing new avenues for disease management (Jackson and Bartek, 2009).

Restoration of DNA double-strand breaks (DSB) within mammalian cells is a key component of the DNA damage-response pathway and arises principally via the processes of homologous recombination and nonhomologous end joining (Jackson and Bartek, 2009; Khanna and Jackson, 2001; Collis *et al*., 2005). DNA repair inhibitors are, therefore, of increasing interest as chemo- and radio-sensitizing agents in cancer treatment (Smith and Jackson, 2003; Helleday *et al*., 2008; Finlay and Griffin, 2012; Curtin, 2012).

A major objective of our research is the development of potent and selective DNA-dependent protein kinase (DNA-PK) and phosphatidylinositol 3-kinase (PI3K) inhibitors, suitable for clinical evaluation as chemo- and radio-sensitizers in the treatment of cancer.

Structure activity relationship (SAR) studies around the nonselective PI3K-related kinase PIKK inhibitor,

Electronic supplementary material The online version of this article (doi:10.1007/s00044-014-1037-6) contains supplementary material, which is available to authorized users.

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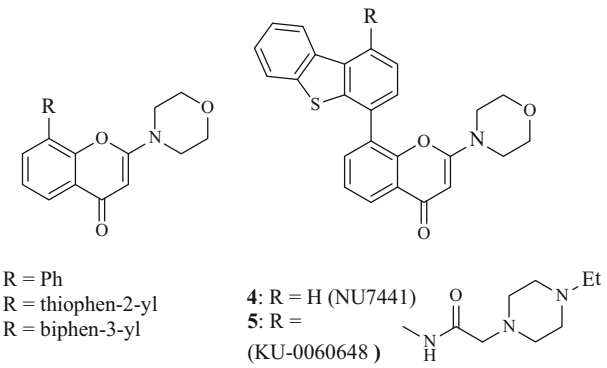


Fig. 1 Structures of some chromones as DNA-PK and PI3K inhibitors

2-morpholino-4H-chromen-4-one **1** (LY294002) (Izzard *et al*., 1999) resulted in the identification of a number of potent DNA-PK inhibitors, including the thiophen-2-yl **2** (Hardcastle *et al*., 2005) and 8-biphenyl derivatives **3** (Desage-El Murr *et al*., 2008) as well as NU7441 **4** (IC_{50} = 40 nM,) (Cano *et al*., 2013). Elaboration of this structure by Cano *et al*. (2013) has led to the highly potent water-soluble DNA-PK inhibitor KU-0060648 **5** (DNA-PK IC_{50} = 5 nM) and potentiation of the cytotoxicity to ionizing radiation (IR) in vitro 10-fold. Furthermore, compound **5** was shown to potentiate not only IR in vitro but also DNA DSB inducing cytotoxic anticancer agents, both in vitro and in vivo. Counter-screening against other members of the PIKK family unexpectedly revealed that some of the compounds were potent mixed DNA-PK and PI-3K inhibitors (Cano *et al*., 2013) (Fig. 1).

Recently, we found that 2-morpholino-substituted-1,3-benzoxazine compounds showed high-to-moderate DNA-PK inhibition activity (ca. 0.28–6.80 μM IC_{50}) in which the most active compound was pyridin-3-ylmethoxy derivative **15** with an IC_{50} = 0.28 μM. We also found that the 2(-pyridin-2-y1methyl(pyridine-2, and,-3-yI)amino)-substituted-1,3-benzoxazin-4-ones showed moderate-to-low (ca. 2.5–25.2 μM IC_{50}) inhibitory activity, with the most potent being the *N*-(pyridin-3-yl) derivative **25** with an IC_{50} = 2.5 μM (Ihmaid *et al*., 2012).

Furthermore, PI3K inhibition studies revealed that compound **4** is potent (IC_{50} for PI3K α = 0.13 μM, PI3K β = 0.016 μM, PI3K γ = 0.22 μM, PI3K δ = 0.03 μM) (Cano *et al*., 2013).

Inhibition of PI3K signaling can diminish cell proliferation, and in some circumstances, promote cell death. Consequently, components of this pathway present attractive targets for cancer therapeutics.

A number of PI3K pathway inhibitors have been developed and are being evaluated in preclinical studies and in early clinical trials (Ma and Adjei, 2009; Shuttleworth *et al*., 2011).

The PI3K inhibitors can be divided into isoform-specific inhibitors or pan-PI3K inhibitors. Pan-PI3K inhibitors target all class IA PI3K in the cancer serum half-life (Blois *et al*., 2008; Howes *et al*., 2007; Smith *et al*., 2009).

Due to the diverse essential functions of PI3K, drugs that bind to and inhibit a broad range of kinase isoforms and complexes with low specificity can lead to deleterious side effects.

For example, the α isoform has been implicated in a variety of human cancers, and angiogenesis has been shown to selectively require the α isoform in the control of endothelial cell migration (Graupera *et al*., 2008).

The δ isoform has been implicated in a number of diseases and biological processes which express primarily in hematopoietic cells including leukocytes such as T-cells, dendritic cells, neutrophils, mast cells, β-cells, and macrophages. In addition, the γ isoform plays a role in leukocyte signaling and has been implicated in inflammation, rheumatoid arthritis, and autoimmune diseases such as lupus. PI3K β has been implicated primarily in various types of cancer including PTEN-negative cancer and HER2-overexpressing cancer such us breast cancer and ovarian cancer (Edgar *et al*., 2010).

Therefore, it is important to provide an alternative approach that effectively targets disease-related pathways, while limiting undesirable side effects.

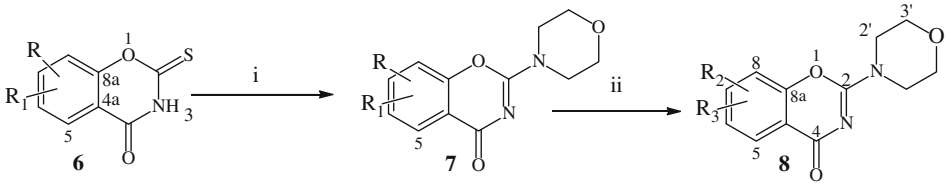
In this work, we are reporting the synthesis and structural elucidation of 6 new substituted-1,3-benzoxazines and measurement of their DNA-PK, PI3K potency. PI3K isoform selectivity of new and previously synthesized substituted-1,3-benzoxazines and substituted-1,3-naphthoxazines (18 compounds) was also described.

Results and discussion

Chemistry

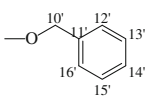
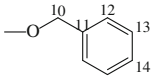
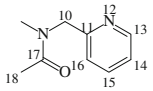
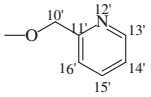
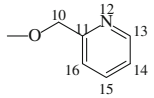
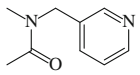
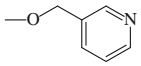
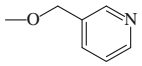
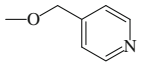
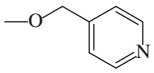
*Synthesis of substituted 2-thio-1,3-benzoxazine **6a** and **b***

Substituted 2-thio-1,3-benzoxazines **6a** and **b** were synthesized from the reaction of the appropriate substituted 2-hydroxybenzoic acid with freshly prepared $Ph_3P(SCN)_2$ according to the previously reported procedure with no further modification (Pritchard *et al*., 2005). The structures of the newly prepared oxazines **6a** and **b** were confirmed from their IR, ^{1}H NMR, ^{13}C NMR, and microanalysis. The IR spectra of compounds **6a** and **b** showed absorption at ν_{max} 1,120 and 1,188 cm^{-1}, respectively, indicating the presence of C=S, which was confirmed from the ^{13}C NMR singlet at δ 182.0 and 181.9 ppm.



Scheme 1 Synthesis of new 2-amino-benzoxazine. (*i*) Reflux morpholine in dry dioxane; (*ii*) benzylbromide, hydrohalogen salt of 2, 3 and 4-(halo-methyl)-pyridine, Cs_2CO_3, acetonitrile, reflux

Comp. **6** and **7**
a R = 7-OH, R_1 =8-OH
b R = H, R_1 = 7-NHCOCH$_3$

Comp.	R_2-7	R_3-8	Comp.	R_2-7	R_3-8
8a			**8e**		H
8b			**8f**		H
8c					
8d					

*Synthesis 2-morpholino-substituted-benzoxazines **7a** and **b***

Compounds **7a** and **b** were prepared from the reactions of the corresponding 2-thio-benzoxazine **6a** and **b** with morpholine according to the previously reported procedures (Scheme 1) (Pritchard *et al*., 2005; Ihmaid *et al*., 2010). The structures of the new products **7a** and **b** were confirmed by the analysis of their IR, ^{1}H NMR and ^{13}C NMR spectroscopy in addition to the microanalysis. The proton-NMR spectra of products **7a** and **b** showed a broad signal around δ 3.7 (8H), indicating the presence of the morpholine group, while the 13C NMR spectra showed disappearance of the signal around δ 182.0 for C=S.

*Synthesis of 2-morpholino-bis-O-bezyl, pyridin-2yl, 3-yl and 4yl-methoxy)-1,3-benzoxazine 8a-d and N-(2-morpholino-4-oxo-4H-benz[e][1,3]oxazin-7-yl)-N-(pyridin-2-and-3-ylmethyl)acetamide **8e** and **8f***

The synthesis of compounds **8a**–**8d** was carried out following the previously reported procedure from 2-morpholino-substituted-1,3-benzoxazine **7a** with benzyl bromide, hydrohalogen salt of 2, 3, and 4-(halo-methyl)-pyridine with Cs_2CO_3 in acetonitrile (Scheme 1) (Ihmaid *et al*., 2011; Pritchard *et al*., 2007).

Similarly, we synthesized the 7-acetylamino-substituted (pyridin-2yl and 3-yl)-1,3-benzoxazine **8e** and **8f** from the reaction of 7-*N*-acetylamido-2-morpholino-1,3-benzoxazine **7b** with 2- and 3-halomethylpyridinium halides, respectively, in the presence of Cs_2CO_3.

The structures of the new products **8a**–**8f** were confirmed by the analysis of their IR, ^{1}H NMR and ^{13}C NMR spectroscopy in addition to the microanalysis. The assignment of the ^{1}H NMR spectra of compounds **8a**–**8f** was achieved using hydrogen–hydrogen homo decoupling (HHHD), while the protonated signals of the ^{13}C NMR spectra were assigned using heteronuclear single quantum coherence spectroscopy (HSQC). It is worth noting that the ^{1}H NMR of compounds **8b–8d** showed 2 sets of 4 signals for the bis-*O*-7,8-substituted pyridine groups. Compound **8a** shows a doublet at δ 7.57 (H-5) and a broad multiplet from δ 7.47–7.28 (10H) for the 2 aryl groups, δ 7.90 doublet (H-6), δ 5.26 and 5.07 were assigned to H-10′ and H-10, respectively. These assignments were justified by the effect of the 2 electron donating oxygen atoms attached at ortho positions which put H-10 as higher field while the H-10′ is deshielded as a result of being para to the carbonyl group (C-4). A multiplet (8H) corresponding to the morpholine group is observed at δ 3.63. Similarly, the assignment of ^{1}H NMR for compound **8b** was achieved; however, the bis-*O*-pyridin-2yl groups showed two sets of signals each consisting of 4 proton signals. These were assigned using HHHD at the 4 deshielded signals of the 2 sets from which we determined that the lower field sets correspond to the pyridine group substituted at position 7. While the higher field signals of the second set represent the pyridine group at position 8. Therefore, δ 8.55 and δ 8.50 broad

doublets H-13′ and H-13, respectively, δ 7.78 and δ 7.75 double triplets assigned for H-15′ and H-15, respectively. The doublet at δ 7.58 was assigned H-5; δ 7.54 and δ 7.50 doublets were assigned for H-16′ and H-16. Signals at δ 7.31 and δ 7.30 were assigned to H-14′ and H-14, finally δ 5.36 and δ 5.22 were assigned for H-10′ and H-10, respectively. A similar approach using HHHD and HSQC NMR experiments assisted in assigning the ^{1}H and ^{13}C spectra for **8c**–**8f**, see experimental and supplementary information.

Biological activity

PI3K and DNA-PK inhibition activity (Tables 1, 2, 3, 4)

Structure activity relationship (SAR)

Table 1 shows that 2-morpholino-8-substituted-benzoxazine **9** (8-ph) and **10**–**12** 8-(pyridin-2-, 3-, or 4-ylmethoxy) showed high-to-moderate inhibition of PI3 Kα (IC_{50} 4–7 μM). The most active compound was **9** PI3 Kα (IC_{50} 4.07 μM), which is about 10-fold less active than the chromen-4-one analog (**1**).

The above PI3Kα activity for compounds **9–12** showed the same pattern of activity for PI3K β, γ, δ inhibition. Moving the 8-(pyridin-3-ylmethoxy)-group to position 6 **14**, or replacing it with 8-(2-(4-methylpiperazin-1-yl)ethoxy) **13** showed no PI3Kα inhibition $IC_{50} > 100$ μM. Compound **9,** however, was the most active and has activity for α, β, and δ isoforms which is comparable with inhibition of LY294002 **1** and more active than compound **1** for PI3 Kγ (IC_{50} **=** 1.44 μM).

2-Morpholino-7-*N*-(pyridin-2- and 3-ylmethyl)acetamide **8e** and **8f**, respectively, showed no DNA-PK and PI3K activity ($IC_{50} > 100$ μM).

8-Substituted 2-morpholine-compounds **9–13** showed high potency of DNA-PK with small ranges of variation in the IC_{50} from 0.3 to 1.7 μM regardless of the type of substitution. Compound **9** (8-Ph) was found to be the most potent product and had higher potency than the chromen-4-one analog **1** (LY294002). However, moving the substitution (pyridin-3-ylmethoxy) from 8- to 6-compound **14** resulted in the effective loss of DNA-PK inhibition ($IC_{50} > 100$ μM).

2-Morpholino-7,8-bis(Benzyloxy, pyridine-2-,-3- and 4-ylmethoxy)-4Hbenz[e][1,3]oxazin-4-one 8a-d PI3K and DNA-PK activity and isoform selectivity

Compound **8a**, **c**, and **d** (Table 2) showed a similar activity pattern for PI3K and DNA-PK and in some cases were less potent than compounds **9–12**.

7,8-Bis-benzyl compound **8a** showed PI3K isoform ratio selectivity of δ:γ:α isoforms as 1:2:175, respectively, with no activity observed for the β isoform ($IC_{50} > 100$ μM).

For 7,8-bis-(pyridin-2) compound **8b** showed no DNA-PK activity and moderate PI3 K activity with some β isoform selectivity. While 7,8-bis-(pyridin-3) compound **8c** also showed moderate γ:δ:α:β isoform activity ratio as 1:1.2:3.1:6.6, respectively, with moderate DNA-PK activity (Table 2).

On the other hand 7,8-bis-(pyridin-4) compound **8d** showed high ratio selectivity toward the γ isoform with a selectivity ratio of γ:δ:α:β (1:39:120:166), respectively.

8-Methyl-7-(pyridin-3) **15** showed potent DNA-PK and PI3K activity with no clear isoform selectivity (Table 2). However, 8-methyl-7-(pyridin-4) **16** showed strong DNA-PK activity, but moderate PI3K activity with isoform ratio selectivity of β:δ (1:2.8) and no α or γ activity ($IC_{50} > 100$ μM).

Finally replacing the pyridine group at position 7 by 4-methylpiperazine group to form 8-methyl-7-(2-(4-methylpiperazin-1-yl) compound **17** resulted in the loss of PI3K activity but showed high DNA-PK activity.

*2-Morpholino-O-substituted-naphthoxazines **18-22** (Table 3)*

2-Morpholino-*O*-Substituted linear or angular naphthoxazine compounds **18–20** and **21–22,** respectively showed very good selectivity of DNA-PK versus PI3K. It is worth mentioning that 2-morpholino-6-(pyridin-2-ylmethoxy)-4H-naphtho[2,1-e][1,3]oxazin-4-one **21** showed specific β isoform activity (IC_{50} 8.94 μM).

The PI3Kγ X-ray crystallography of LY294002 compound **1** (3-CH isoster of compound **9**) showed that the morpholine ring partially overlaps the volume occupied by the adenine in the ATP/enzyme complex (Walker *et al*., 2000). There is a hydrogen bond between the morpholino oxygen and the backbone amide of residue Val882, and this bond mimics the interaction that N1 of ATP makes with the enzyme.

The oxygen of the carbonyl at position 4 in compound **1** interacts with Lys-833 by putative hydrogen bonding. Removal of the 8-phenyl ring decreases inhibition approximately 3-fold. Occupying a similar space as the ribose of ATP, the 8-phenyl ring packs against Met-804 and Trp-812 on one side and Met-953 the other side (Walker *et al*., 2000).

Furthermore, PI3Kγ docking of 8-phenyl-2-morpholino-chromen-4-one **1** and its 8-benzloxy analog (TGX-102) revealed the binding free energies with PI3Kγ are –10.12 and –9.49 (kcal/mol), respectively, which indicates the

Table 1 DNA-PK and Class I PI3K inhibition IC_{50} of 6-, 7- and 8-substituted-2-morpholino-1,3-benzoxazines

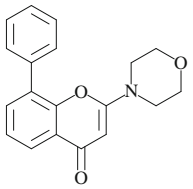
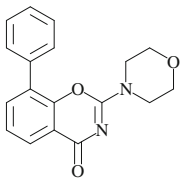
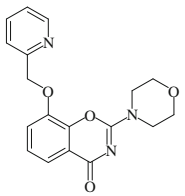
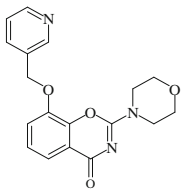
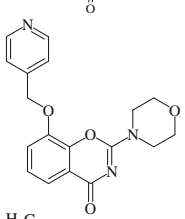
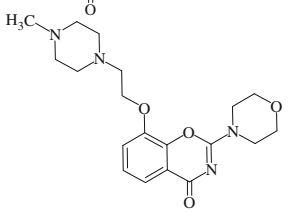
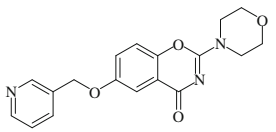
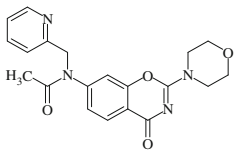
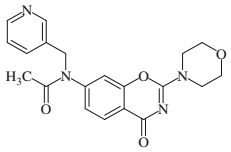
Name	Structure	PI3Kα IC_{50} (μM)	PI3Kβ IC_{50} (μM)	PI3Kγ IC_{50} (μM)	PI3Kδ IC_{50} (μM)	DNA-PK IC_{50} (μM)
1[a]		0.3	0.27	3.02	0.22	1.40
9		4.07	3.36	1.50	0.95	0.30[b]
10		5.20	14.6	18.1	1.62	0.62[b]
11		4.71	4.22	14.7	2.73	0.36[b]
12		7.24	11.6	24.4	3.78	1.60[b]
13		>100	>100	>100	>100	1.70[b]
14		>100	>100	>100	>100	>100
8e		>100	>100	>100	>100	>100

Table 1 continued

Name	Structure	PI3Kα IC_{50} (μM)	PI3Kβ IC_{50} (μM)	PI3Kγ IC_{50} (μM)	PI3Kδ IC_{50} (μM)	DNA-PK IC_{50} (μM)
8f		>100	>100	>100	>100	>100

[a] IC50 values were previously reported (Cano *et al*., 2013)

[b] IC50 values were previously reported (Ihmaid *et al*., 2012)

similarity of binding of compound **1** and **TGX-102** (Kuang *et al*., 2006).

From the discussion above and comparing the PI3K values in Tables 1 and 2 with those of compound **1** and **TGX-102**, compounds **9–12** and **8a–8d** show possible binding interaction similarities.

However, the 6,7- and 7,8-phenyl-fused linear and angular naphthoxazines **18–20** and **21–22** are rigid rings. Their binding modes differ from that of the benzoxazine analogs **8a–8d** and **9–12** which resulted in the loss of PI3K activity ($IC_{50} > 100$ μM). This could be as a result of the PI3K complex being flipped 180°, therefore, the above result could be explained by analogy to the results from the change in the PI3Kγ docking of Ly293646 and Ly293684 (3-CH isoster) naphthoxazines (Kunang *et al*., 2006).

2-(2-(Pyridin-2-and 4-yl)-1-(pyridin-3-yl)ethyl)7,8- and 8-substituted-4H-benzo[e][1,3]oxazin-4-one 23-26 (Table 4)

Replacing the 2-morpholino group by 2-(2-(pyridin-2-and 4-yl)-1-(pyridin-3-yl)ethyl) caused the loss of PI3K activity ($IC_{50} > 100$ μM) for **23–26** except specific δ isoform for compound **23** (IC_{50} 8.67 μM).

Experimental protocols

The starting reagents 3-aminopyridine, sodium hydrogen carbonate, cesium carbonate, methyl iodide, 2-(bromomethyl)-pyridine hydrobromide, 3-(chloromethyl)-pyridine hydrochloride, 4-(bromomethyl)-pyridine hydrobromide, substituted 2-hydroxybenzoic acids, and substituted hydroxynaphthoic acids were purchased from Sigma Aldrich Chemical Company and were used as received. Synthesis of 2,3,4-trihydroxybenzoic acid was achieved from the reaction of pyrogallol with CO_2 and $KHCO_3$ according to a previously reported method (Brown *et al*., 1992). 4-(acetylamino)-2-hydroxybenzoic acid was synthesized using selective *N*-acetylation of 4-amino-2-hydroxybenzioc acid according to the reported procedure (Hrdina *et al*., 2007). Infrared spectra were obtained using a Perkin Elmer FT-IR 1720× spectrometer. ^1H and ^{13}C NMR spectra were obtained using a Bruker AC 200 NMR spectrometer at 200 and 50 MHz, respectively, or a Bruker AVANCE 300 NMR spectrometer at 300 and 75 MHz, respectively. All ^1H and ^{13}C NMR spectral results are recorded as chemical shifts (δ) relative to the internal TMS for proton and 77.0 ppm in $CDCl_3$ solvent and 39.4 ppm in DMSO-d_6 solvent ^{13}C NMR. Microanalysis was performed by Chemical and Micro Analytical Services (CMAS), Australia. Melting point determinations were carried out using a Stuart Scientific (SMP3) melting point apparatus and all melting points are uncorrected.

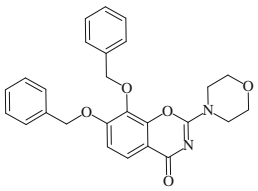
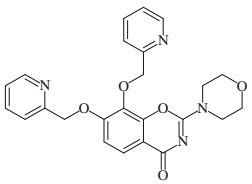
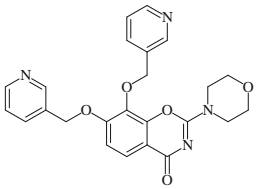
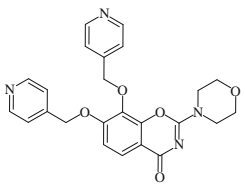
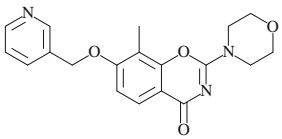
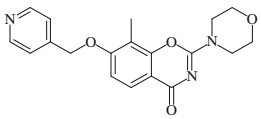
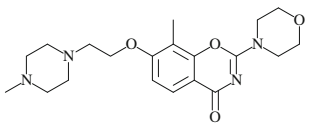
7, 8-Dihydroxy-2-thioxo-2H-benz[e][1,3]oxazin-4(3H)-one **6a**

2,3,4-trihydroxybenzoic acid (1.36 g, 8 mmol) was allowed to react with freshly prepared triphenylphosphine thiocyanate according to Pritchard *et al*. (2005). The crude solid was filtered and recrystallized from acetic acid to give **6a** as light yellow crystals (0.86 g, 51 %), mp 258–260 °C decomp. ν_{max} (KBr)/cm^{-1} 3500–3200 br (OH), 3078, 2928 (NH), 1686 (C=O), 1629 (C=C), 1120 (C=S). ^1H NMR (200 MHz, d_6-acetone) δ 10.00 (bs, 2H, 7-OH,8-OH), 7.48 (d, 1H, $J_{H5,H6} = 8.6$ Hz, H-5), 7.02 (d, 1H, $J_{H6,H5} = 8.6$ Hz, H-6). ^{13}C NMR (50 MHz, d_6-acetone) δ 182.0 (C-2), 157.3 (C-4), 152.9 (C-7), 145.6 (C-8a), 131.7 (C-8), 118.3 (C-5), 113.9 (C-6), 108.4 (C-4a); (Found C, 45.56; H, 2.49; N, 6.71; $C_8H_5NO_4S$ requires C, 45.50; H, 2.39; N, 6.63).

N-(4-Oxo-2-thioxo-3,4-dihydro-2H-benz[e][1,3]oxazin-7-yl)acetamide **6b**

4-(Acetyl amino)-2-hydroxybenzoic acid (1.56 g, 8 mmol) was allowed to react with freshly prepared triphenylphosphine thiocyanate according to Pritchard *et al*. (2005). The crude solid was filtered and recrystallized from ethanol to

Table 2 DNA-PK and Class I PI3K inhibition IC_{50} 7,8-di-substituted-2-morpholino-1,3-benzoxazines

Name	Structure	PI3Kα IC_{50} (μM)	PI3Kβ IC_{50} (μM)	PI3Kγ IC_{50} (μM)	PI3Kδ IC_{50} (μM)	DNA-PK IC_{50} (μM)
8a		29.8	>100	0.33	0.17	6.00
8b		33.9	11.5	>100	29.7	>100
8c		11.3	24.3	3.66	4.55	14.00
8d		14.4	19.9	0.12	4.68	23.10
15		0.13	0.14	0.72	2.0	0.28[a]
16		>100	11.19	>100	30.9	1.20[a]
17		>100	>100	>100	>100	0.50[a]

[a] IC_{50} values were previously reported (Ihmaid *et al*., 2012)

give **6b** as light red crystals (1.22 g, 65 %), mp 285–287 °C decomp. ν_{max} (KBr)/cm^{-1} 3290, 3183 (9-NH), 3072, 2923 (3-NH), 1704 (C=O), 1188 (C=S). ^{1}H NMR (200 MHz, 390 K, d_6-DMSO) δ 13.38 (bs, 1H, 9-NH), 10.56 (s, 1H, 3-NH), 7.86 (d, 1H, $J_{H5,H6}$ = 8.6 Hz, H-5), 7.77 (d, 1H, $J_{H8,H6}$ = 1.8 Hz, H-8), 7.44 (dd, 1H, $J_{H6,H5}$ = 8.6 Hz, $J_{H6,H8}$ = 1.8 Hz, H-6), 2.11 (s, 3H, 11-CH_3). ^{13}C NMR (50 MHz, 330 K, d_6-DMSO) δ 181.9 (C-2), 169.2 (C-10), 156.7 (C -8a), 146.0 (C-7), 127.3 (C-5), 116.3 (C-6), 109.6 (C-4a), 104.2 (C-8), 24.0 (C-11)

155.9 (C-4); (Found C, 50.69; H, 3.53; N, 11.86; $C_{10}H_8N_2O_3S$ requires C, 50.84; H, 3.41; N, 11.86).

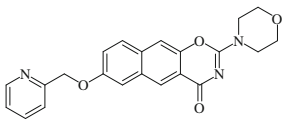
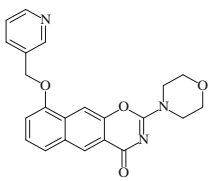
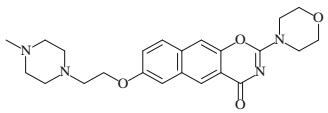
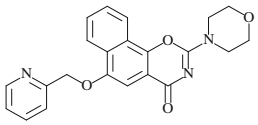
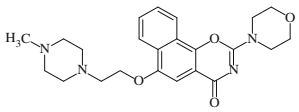
*Synthesis of 2-morpholino-substituted-1,3-benzoxazines **7a** and **b***

The reaction of substituted-2-thioxo-1,3-benzoxazine **6a** and 6**b** with morpholine was carried out as previously described in the literature (Ihmaid *et al*., 2010).

*7,8-Dihydroxy-2-morpholino-4H-benz[e][1,3]oxazin-4-one **7a*** Compound **6a** (0.53 g, 2.5 mmol) was allowed to react with morpholine (1.09 g, 12.5 mmol) according to Ihmaid *et al*., 2010. Recrystallization from methanol gave **7a** as off white crystals (0.46 g, 67 %), mp 285–287 °C. ν_{max}. (KBr)/cm^{-1} 3500–2800 br(OH), 2967, 2923, 2865 (CH), 1656 (C=O), 1625 (C=N), 1596, 1560 (C=C). ^{1}H NMR (200 MHz, 300 K, d_6-DMSO) δ 9.48 (bs, 7,8-OH, exchangeable with D_2O), 7.22 (d, 1H, $J_{H5,H6}$ = 8.4 Hz, H-5), 6.80 (d, 1H, $J = 8.4_{H6,H5}$ Hz, H-6), 3.70 (bm, 8H, 4 x CH_2 of morpholine),^{13}C NMR (50 MHz, 300 K, d_6-DMSO) δ 165.7 (C-4), 156.3 (C-2), 150.9 (C-7), 143.5(C-8a), 131.7 (C-8), 117.0 (C-5), 112.9 (C-6), 109.6 (C-4a), 65.6 (C-3′), 44.0 (C-2′); (Found C, 54.67; H, 4.68; N, 10.70; $C_{12}H_{12}N_2O_5$ requires C, 54.55; H, 4.58; N, 10.60).

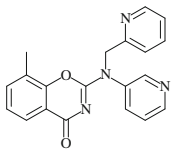
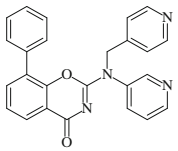
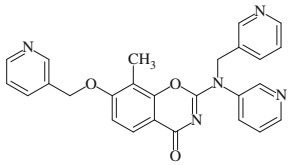
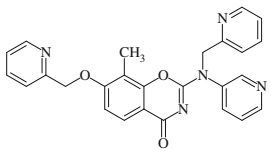
*N-(2-Morpholino-4-oxo-4H-benz[e][1,3]oxazin-7-yl)acetamide **7b*** Compound **6b** (0.59 g, 2.5 mmol) was allowed to react with morpholine (1.09 g, 12.5 mmol) according to Ihmaid *et al*. (2010). Recrystallization from ethanol gave **7b** as white crystals (0.43 g, 60 % yield), mp 284–286 °C decomp. υ_{max} (KBr)/cm^{-1} 3316,3263, 3203 (NH), 3048 (CH Ar), 2964, 2931, 2861 (CH aliphatic), 1695, 1656 (C=O), 1623 (C=N), 1569, 1542 (C=C).1H NMR (200 MHz, 330 K, d_6-DMSO) δ 10.32 (s, 1H, 9-NH), 7.90 (d, 1H, $J_{H8,H6}$ = 1.6 Hz, H-8), 7.80 (d, 1H, $J_{H5,H6}$ = 8.4 Hz, H-5),

Table 3 DNA-PK and Class I PI3K inhibition IC_{50} 2-morphlino-*O*-substituted-naphthoxazines

Name	Structure	PI3Kα IC_{50} (μM)	PI3Kβ IC_{50} (μM)	PI3Kγ IC_{50} (μM)	PI3Kδ IC_{50} (μM)	DNA-PK IC_{50} (μM)
18		>100	>100	>100	>100	0.01[a]
19		>100	>100	>100	>100	0.33[a]
20		>100	>100	>100	>100	0.19[a]
21		>100	8.94	>100	>100	2.43[a]
22		>100	>100	>100	>100	0.76[a]

[a] IC_{50} values were previously reported (Ihmaid *et al*., 2011)

Table 4 DNA-PK and Class I PI3K inhibition IC_{50} 2(-pyridin-2-y1methyl(pyridine-2, and,-3-yI)amino)-substituted-1,3-benzoxazin-4-one

Name	Structure	PI3Kα IC_{50} (μM)	PI3Kβ IC_{50} (μM)	PI3Kγ IC_{50} (μM)	PI3Kδ IC_{50} (μM)	DNA-PK IC_{50} (μM)
23		>100	>100	>100	8.67	25.20[a]
24		>100	>100	>100	>100	NA
25		>100	>100	>100	>100	2.50[a]
26		>100	>100	>100	>100	>100[a]

[a] IC_{50} values were previously reported (Ihmaid *et al*., 2012)

7.33 (dd, 1H, $J_{H6,H5} = 8.4$ Hz, $J_{H6,H8} = 1.6$ Hz, H-6), 3.71 (s, 8H, 4 x CH_2 of morpholine), 2.10 (s, 3H, 11-CH_3,). 13C NMR (50 MHz, 330 K d_6-DMSO) δ 168.9 (C-10), 164.7 (C-4), 156.3 (C-2), 153.8 (C-8a), 144.0 (C-7), 127.0 (C-5), 115.9 (C-6), 111.7 (C-4a), 104.4 (C-8), 65.3 (C-3′), 43.9 (C-2′), 23.9 (C-11); (Found C, 57.39; H, 5.30; N, 14.37; $C_{14}H_{15}N_3O_4$ requires C, 57.13; H, 5.23; N, 14.53).

Synthesis of 2-morpholino-di-O-benzyl, pyridin-2yl, 3-yl and 4yl-methoxy)-1,3-benzoxazine 8a-d and N-(2-morpholino-4-oxo-4H-benz[e][1,3]oxazin-7-yl)-N-(pyridin-2-and-3-ylmethyl)acetamide 8c and f

General Procedure A According to the previously reported procedure (Ihmaid *et al*., 2012), cesium carbonate (8.5 mmol) and the appropriate substituted-2-morpholino-4-oxo-4*H*-benz[*e*][1,3]oxazin (1 mmol) were suspended in acetonitrile (10 ml) in a two-necked round bottom flask (50 ml). The reaction mixture was heated to reflux for 2 h to form the cesium salt. After 2 h, the appropriate 2-(bromomethyl)pyridine, 3-(chloromethyl)pyridine or 4-(bromomethyl)pyridine hydrohalide (2 mmol) or benzyl bromide (4 mmol) in acetonitrile (10 mL) was added slowly via a dropping funnel, and the reaction mixture was refluxed for an additional 30 min. Upon completion, the reaction mixture was evaporated to dryness under reduced pressure. The resulting solid was extracted using chloroform (4 × 15 ml), and the combined extracts were dried over anhydrous magnesium sulfate and evaporated to dryness. Minimal diethyl ether was used to triturate the oily residue and the resulting solid collected and recrystallized from an appropriate solvent.

*7,8-bis(Benzyloxy)-2-morpholino-4H-benz[e][1,3]oxazin-4-one **8a*** Compound **7a** (0.28 g, 1 mmol) was allowed to react with benzyl bromide (0.68 g, 4 mmol) for 5 h according to general procedure A. The crude product was recrystallized from toluene to give **8a** as white crystals (0.35 g, 84 %), mp 285–288 °C. ν_{max} (KBr)/cm^{-1} 1672 (C=O), 1617 (C=N), 1597, 1562 (C=C). ^{1}H NMR (300 MHz, 340 K, d_6-DMSO) δ 7.57 (d, 1H, $J_{H5,\ H6} = 8.7$ Hz, H-5), 7.47–7.28 (m, 10H, 2 x Ar), 7.19 (d, 1H, $J_{H6,H5} = 8.7$ Hz, H-6), 5.26 (s, 2H, H-10′), 5.07 (s, 2H, 10), 3.64-3.61 (m, 8H of morpholine). ^{13}C NMR (75 MHz, 340 K, d_6-DMSO) δ 165.0 (C-4), 156.4, 155.8 (C-8a, C-2), 147.6 (C-7), 137.1, 136.4 (C-11, C-11′) 134.4 (C-8), 128.6, 128.5, 128.3, 128.2, 128.1, 127.8 (C-12, C-12′, C-13, C-13′ C-14, C-14′), 122.0 (C-5), 111.6 (C-4a), 111.5 (C-6), 75.3, 70.9 (C-10, C-10′),

65.5 (C-3′), 44.3 (C-2′); (Found C, 70.34; H, 5.54; N, 6.45; $C_{26}H_{24}N_2O_5$ requires C, 70.26; H, 5.44; N, 6.30).

2-Morpholino-7,8-bis(pyridin-2-ylmethoxy)-4H-benz[e][1,3]oxazin-4-one ***8b*** Compound **7a** (0.28 g, 1 mmol) was allowed to react with 2-(bromomethyl)pyridinium bromide (0.50 g, 2 mmol) according to general procedure A. The crude product was recrystallized from toluene (charcoal) to give **8b** as off yellow crystals (0.43 g, 98 %), mp 197–199 °C. ν_{max} (KBr)/cm^{-1} 1687 (C=O), 1630 (C=N) 1604, 1578, 1568 (C=C). ^{1}H NMR (300 MHz, 340 K, d_6-DMSO) δ 8.55 (bd, 1H, $J_{H13',H14'} = 4.2$ Hz reduced coupling as a result of nitrogen quadrupole effect, H-13′) 8.50 (bd, 1H, $J_{H13,H14} = 4.5$ Hz reduced coupling as a result of nitrogen quadrupole effect, H-13), 7.78 (dt, 1H, $J_{H15',H14'} = 7.8$ Hz, $J_{H15'\text{-}H13'} = 1.8$ Hz, H-15′) 7.75 (dt, 1H, $J_{H15,H14} = 7.8$ Hz, $J_{H15\text{-}H13} = 1.8$ Hz, H-15), 7.58 (d, 1H, $J_{H5,H6} = 9.0$ Hz, H-5), 7.54 (d, 1H, $J_{H16'\text{-}H15'} = 10.8$ Hz, H-16′), 7.50 (d, 1H, $J_{H16\text{-}H15} = 10.5$ Hz, H-16), 7.31 (bt, 1H $J_{H14',H15'} = 6.9$ Hz, H-14′), 7.30 (bt, 1H, $J_{H14,H15} = 6.6$ Hz, H-14), 5.36 (s, 2H, H-10′), 5.22 (s, 2H, H-10), 3.67-3.66 (bs, 8H of morpholine). ^{13}C NMR (75 MHz, 340 K, d_6-DMSO) δ 165.0 (C-4), 156.7, 156.4, 156.0, 155.5 (C-8a, C-2, C-11, C-11′), 149.3, 149.1 (C-13, 13′), 147.5 (C-7), 137.0, 136.8 (C-14, C-14′), 134.5 (C-8), 123.2 (C-15, C-15′), 122.6 (C-5), 122.1, 121.9 (C-16, C-16′), 111.8 (C-4a), 111.5 (C-6), 76.3 (C-10), 71.7 (C-10′), 65.5 (C-3′), 44.3 (C-2′); (Found C, 64.60; H, 5.03; N, 12.51; $C_{24}H_{22}N_4O_5$ requires C, 64.57; H, 4.97; N, 12.55).

2-Morpholino-7,8-bis(pyridin-3-ylmethoxy)-4H-benz[e][1,3]oxazin-4-one ***8c*** Compound **7a** (0.28 g, 1 mmol) was allowed to react with 3-(chloromethyl)pyridinium chloride (0.33 g, 2 mmol) according to general procedure A. The crude product was recrystallized from toluene to give **8c** as red crystals (0.23 g, 51 %), mp 129–131 °C decomp. υ_{max} (KBr)/cm^{-1} 1694 (C=O), 1632, 1602 (C=N),1577 (C=C).^{1}H NMR (300 MHz, 340 K, d_6-DMSO) δ 8.67 (d, 1H, $J_{H12',H14'} = 1.8$ Hz, H-12′), 8.56 (dd, 1H, $J_{H14',15'} = 4.8$ Hz, $J_{H14',16'} = 1.5$ Hz, H-14′) 8.54 (d, $J_{H12,H14} = 1.2$ Hz, 1H, H-12), 8.49 (dd, 1H, $J_{H14,15} = 4.8$ Hz, $J_{H14,H16} = 1.5$ Hz, H-14), 7.84 (dt, 1H, $J_{H16',H15'} = 7.8$ Hz, $J_{H16',H14'} = 1.8$ Hz, H-16′), 7.72 (dt, 1H, $J_{H16,H15} = 7.8$ Hz, $J_{H16,14} = 1.8$ Hz, H-16), 7.61 (d, 1H, $J_{H5,H6} = 8.7$ Hz, H-5), 7.40 (dd, 1H, $J_{H15',H14'} = 7.2$ Hz, $J_{H15',H16'} = 7.5$ Hz, H-15′) 7.30 (dd, 1H, $J_{H15,H14} = 7.2$ Hz, $J_{H15,H16} = 7.5$ Hz, H-15), 7.23 (d, 1H, $J_{H6,H5} = 8.7$ Hz, H-6), 5.29 (s, 2H, H-10′) 5.11 (s, 2H, H-10), 3.65-3.61 (m, 8H of morpholine). ^{13}C NMR (75 MHz, 340 K, d_6-DMSO) δ 166.9 (C-4), 156.4, 155.5 (C-8a, C-2), 149.7, 149.6, 149.5, 149.2 (C-13, C-13′, C-14, C-14′), 147.6 (C-7), 136.2, (C-14′) 135.7 (C-14), 134.1 (C-8), 132.5, 132.0 (C-11, C -11′), 123.6, 123.4 (C-15, C-15′), 122.3 (C-5) 111.8 (C-4a), 72.8 (C-10) 68.7 (C-10′), 65.5 (C-3′), 44.3 (C-2′); (Found C, 64.62; H, 5.07; N, 12.46; $C_{24}H_{22}N_4O_5$ requires C, 64.59; H, 4.97; N, 12.55).

2-Morpholino-7,8-bis(pyridin-4-ylmethoxy)-4H-benz[e][1,3]oxazin-4-one ***8d*** Compound **7a** (0.28 g, 1 mmol) was allowed to react with 4-(bromomethyl)pyridinium bromide (0.50 g, 2 mmol) according to general procedure A. The crude product was recrystallized from toluene (charcoal) to give **8d** as red crystals (0.43 g, 98 %), mp 182–185 °C. ν_{max} (KBr)/cm^{-1} 1675 (C=O), 1612 (C=N), 1599, 1562 (C=C). ^{1}H NMR (300 MHz, 340 K, d_6-DMSO) δ 8.55 (d, 2H, $J_{H13',12'} = 6.0$ Hz, H-13′, H-15′), 8.53 (d, 2H, $J_{H13,H12} = 6.0$ Hz, H-13, H-15) 7.62 (d, $J_{H5,H6} = 8.7$ Hz, H-5), 7.40 (d, 2H, $J_{H12',13'} = 5.7$ Hz, H-12′, H-16′), 7.38 (d, 2H, $J_{H12,H13} = 5.7$ Hz, H-12, H-16), 7.18 (d, 1H, $J_{H6,H5} = 8.7$ Hz, H-6), 5.31 (s, 2H, H-10′), 5.17 (s, 2H, H-10), 3.64-3.62 (bm, 8H of morpholine). ^{13}C NMR (75 MHz, 340 K, d_6-DMSO) δ 164.9 (C-4), 156.4, 155.2 (C-8a, C-2), 149.9, 149.8 (C-13, C-13′, C-15 C-15′), 147.5 (C-7), 145.9, 145.3 (C-11, C-11′), 134.4 (C-8), 122.4 (C-5), 122.4, 121.9 (C-12, C-12′, C-16, C-16′), 112.0 (C-4a), 111.4 (C-6), 73.8 (C-10) 69.2 (C-10′) 65.5 (C-3′) 44.3 (C-2′); (Found C, 64.57; H, 4.97; N, 12.55; $C_{24}H_{22}N_4O_5$ requires C, 64.57; H, 5.00; N, 12.46).

N-(2-Morpholino-4-oxo-4H-benz[e][1,3]oxazin-7-yl)-N-(pyridin-2-ylmethyl)acetamide ***8e*** Compound **7b** (0.26 g, 1 mmol) was allowed to react with 2-(bromomethyl)pyridinium bromide (0.50 g, 2 mmol) according to the general procedure A. The crude product was recrystallized from toluene to give **8e** as red crystals (0.37 g, 97 %), mp 187–190 °C decomp. ν_{max} (KBr)/cm^{-1} 1672 (C=O), 1624, (C=N), 1563 (C=C). ^{1}H NMR (300 MHz, 340 K, d_6-DMSO) δ 8.45 (dd, 1H, $J_{H13,H14} = 4.5$ Hz, H-13), 7.82 (d, $J_{H5,H6} = 8.4$ Hz, H-5), 7.70 (dt, 1H, $J_{H14,H15} = 7.5$ Hz, $J_{H14,H16} = 1.5$ Hz, H-14), 7.46 (d, 1H, $J_{H8\text{-}H6} = 1.8$ Hz, H-8), 7.32 (dd, 1H, $J_{H6,H5} = 8.4$ Hz, $J_{H6,H8} = 1.8$ Hz, H-6), 7.31 (d, 1H, $J_{H16,H15} = 8.1$ Hz, H-16) 7.20 (dd, 1H, $J_{H15,H14} = 6.9$ Hz, $J_{H15,H16} = 7.2$ Hz, H-15), 5.02 (s, 2H, H-10), 3.71 (bs, 8H of morpholine), 2.01 (s, 3H, CH_3). ^{13}C NMR (75 MHz, 340 K, d_6-DMSO) δ 169.5 (C-17), 164.7 (C-4), 156.9, 156.7 (C-2 and C-11), 153.7 (C-8a), 149.1 (C-13), 148.2 (C-7), 136.8 (C-14), 127.5 (C-5), 124.9 (C-6), 122.4 (C-15), 122.0 (C-16), 115.8 (C-4a), 115.0 (C-8), 65.6 (C-3′), 54.3 (C-10), 44.4 (C-2′), 22.6 (CH_3); (Found C, 63.10; H, 5.26; N, 14.69; $C_{20}H_{20}N_4O_4$ requires C, 63.15; H, 5.30; N, 14.73).

N-(2-Morpholino-4-oxo-4H-benz[e][1,3]oxazin-7-yl)-N-(pyridin-3-ylmethyl)acetamide ***8f*** Compound **7b** (0.26 g, 1 mmol) was allowed to react with 3-(chloromethyl)pyridinium chloride (0.33 g, 2 mmol) according to general

procedure A. The crude product was recrystallized from toluene to give **8f** as red crystals (0.21 g, 56 %), mp 189–192 °C decomp. υ_{max} (KBr)/cm^{-1} 1672 (C=O) 1621 (C=N), 1561 (C=C), 1508 (C=C). ^{1}H NMR (300 MHz, 340 K, d_6-DMSO) δ 8.40 (d, 1H, $J_{H14,H15} = 4.8$ Hz reduced coupling as a result of nitrogen quadrupole effect, H-14), 8.38 (bs, 1H, H-12), 7.83 (d, 1H, $J_{H5,\ H6} = 8.1$ Hz, H-5), 7.59 (bd, 1H, $J_{H16,H15} = 7.8$ Hz, H-16), 7.39 (d, 1H, $J_{H8,H6} = 1.8$ Hz H-8), 7.27 (dd, 1H, $J_{H15,H14} = 4.8$ Hz, $J_{H15,H16} = 6.9$ Hz, H-15), 7.22 (dd, 1H, $J_{H6,\ H5} = 8.1$ Hz, $J_{H6,H8} = 1.8$ Hz, H-6), 4.94 (s, 2H, H-10), 3.69 (bm, 8H of morpholine), 1.93 (s, 3H, CH_3). ^{13}C NMR (75 MHz, 340 K, d_6-DMSO) δ 169.4 (C-17), 164.6 (C-4), 156.6 (C-2), 153.8 (C-8a), 149.2, 148.6 (C-12, C-14), 147.3 (C-7), 135.6 (C-16), 132.9 (C-11), 127.7 (C-5), 125.2 (C-6), 123.5 (C-15), 116.2 (C-4a), 115.4 (C-8), 66.5 (C-3′), 49.7 (C-10), 44.4 (C-2′), 22.5 (CH_3); (Found C, 63.11; H, 5.37; N, 14.78; $C_{20}H_{20}N_4O_4$ requires C, 63.15; H, 5.30; N, 14.73).

DNA-PI3K inhibition assay The PI3K assays were performed by Reaction Biology Corporation, One Great Valley Parkway, Suite 2 Malvern, PA 19355 USA.

All compounds were dissolved in DMSO and tested for their ability to inhibit PI3K.

Compounds were tested in a 10-dose IC_{50} profile with 4-fold serial dilution starting at 100 μM. The control compound, PI-103, was tested in a 10-dose IC_{50} profile with 3-fold serial dilutions starting at 10 μM. Reactions were carried out at 10 μM ATP using the HTRF assay format.

DNA-PK inhibition assay The DNA-PK assays were performed by Reaction Biology Corporation, One Great Valley Parkway, Suite 2 Malvern, PA 19355 USA.

All Compounds were dissolved in DMSO and tested for their ability to inhibit human DNA-PK. Compounds were tested in a 10-dose IC_{50} profile with 4-fold serial dilution starting at 100 μM. The Control compound, LY294002, was tested in a 10-dose IC_{50} profile with 3-fold serial dilutions starting at 10 μM. Reactions were carried out using 20-μM Peptide substrate [EPPLSQEAFADLWKK], 10-μg/ml DNA, and 10-μM ATP using the HTRF assay format.

Conclusions

In this work, six new 2-morpholino-7-, and 7,8-bis-substituted-benz[e]-1,3-oxazin-4-one **8a**–**8f** were prepared and their DNA-PK activity was assessed. PI3K α, β, γ, and δ activity of the six new and 18 previously prepared and 2-amino-substituted-benz[e]-1,3-oxazin-4-one and 2-morpholino-O-substituted-naphthoxazines **9**–**26** were also reported. 2-Morpholino-*O*-substituted linear or angular naphthoxazines **18**–**22** showed potent DNA-PK activity and no PI3K activity ($IC_{50} > 100$ μM) except compound **21** which showed selective β isoform activity (IC_{50} 8.94 μM). 7,8-Bis-(pyridin-4) compound **8d** showed high ratio selectivity toward the γ isoform with a selectivity ratio of γ:δ:α:β (1:39:120:166). The aryl and *O*-CH_2-aryl groups at position 8- and 7-8-, compounds **9**–**12** and **8a**–**8d**, respectively, are important for DNA-PK and PI3K inhibition, and no selectivity of DNA-PK over PI3K activity was observed. There is a hydrogen bond between the morpholino oxygen of compound **9**–**12** and **8a**–**8d** and the backbone amide of residue Val882. The oxygen of the carbonyl at position 4 in the above compounds interacts with Lys-833 by putative hydrogen bonding. The linear and angular naphthoxazines **18**–**20** and **21**–**22** lose their PI3K activity as their binding modes differ being flipped 180° from that of the benzoxazine analogs **8a**–**8d** and **9**–**12**.

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