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Synthesis, structural elucidation, DNA-PK inhibition, homology modelling and anti-platelet activity of morpholino-substituted-1,3-naphth-oxazines

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ABSTRACT

A number of new angular 2-morpholino-(substituted)-naphth-1,3-oxazines (compound **10b**), linear 2-morpholino-(substituted)-naphth-1,3-oxazines (compounds **13b–c**), linear 6, 7 and 9-*O*-substituted-2-morpholino-(substituted)-naphth-1,3-oxazines (compounds **17–22**, **24**, and **25**) and angular compounds **14–16** and **23** were synthesised. The O-substituent was pyridin-2yl-methyl (**15**, **18**, and **21**) pyridin-3yl-methyl (**16**, **19**, and **22**) and 4-methylpipreazin-1-yl-ethoxy (**23–25**). Twelve compounds were tested for their inhibitory effect on collagen induced platelet aggregation and it was found that the most active compounds were compounds **19** and **22** with IC_{50} = 55 ± 4 and 85 ± 4 μM, respectively. Furthermore, the compounds were also assayed for their ability to inhibit DNA-dependent protein kinase (DNA-PK) activity. The most active compounds were **18** IC_{50} = 0.091 μM, **24** IC_{50} = 0.191 μM, and **22** IC_{50} = 0.331 μM.

Homology modelling was used to build a 3D model of DNA-PK based on the X-ray structure of phosphatidylinositol 3-kinases (PI3Ks). Docking of synthesised compounds within the binding pocket and structure–activity relationships (SAR) analyses of the poses were performed and results agreed well with observed activity.

1. Introduction

The DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine kinase member of the phosphatidylinositol (PI) 3-kinase-like (PIKK) family.[1]

DNA-PK becomes catalytically active on binding to DNA double-strand breaks (DSBs) and may phosphorylate a number of downstream targets including itself and the variant histone H2AX.[2–5] Cells that are defective in either DNA-PKcs or either of the regulatory Ku subunits are unable to effectively repair DNA DSBs.[6]

The search for potent and selective DNA-PK inhibitors has received particular attention, as the ability of DNA-PK to detect and signal the repair of DNA damage may also protect cancer cells from the cytotoxic effects of DNA-damaging cancer therapies.[6] A number of potent 2-morpholino-chromones have proved to be ATP-competitive DNA-PK inhibitors, including compounds **1–3** and LY294002 analogue of **4** (Fig. 1).[7] These compounds have been used for DNA-PK IC_{50} evaluation: 2-morpholino-4*H*-benzo[*g*]chromen-4-one **1** (IC_{50} = 0.4 μM), 3-morpholino-1*H*-benzo[*f*]chromen-1-one **2** (IC_{50} = 4 μM) and 2-morpholino-4*H*-benzo[*h*]chromen-4-one **3** (IC_{50} = 0.3 μM).[8–10] Recently we reported that 2-morpholino-8-phenyl-4*H*-benz[*e*]-1,3-oxazin-4-one **4**, the 3-N analogue of 2-morpholino-8-phenyl-4*H*-chromen-4-one (LY294002), shows DNA-PK inhibitor activity.[10]

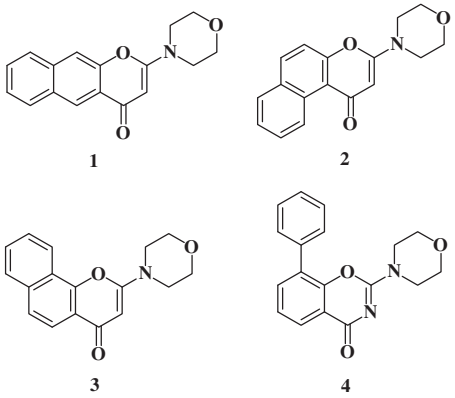

Figure 1. DNA-PK inhibitors compound **1**, **2**, **3**, and **4**.

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In order to search for more efficient inhibitors to DNA-PK, we and others have proposed that specific DNA-PK inhibitors could be used to potentiate radiotherapy and chemotherapy in cancer treatment. Lack of specificity has previously been a major hurdle in the progression of these compounds into clinical trials and diverse biological activities have been ascribed to the inhibitors thus far described.[11] As an example, the proto-type PI3K inhibitor LY294002 has been shown to inhibit both DNA-PK and the cAMP-phosphodiesterases, PDE2 and PDE3 essential for platelet function.[12] In addition PI3K p110β has been shown to play an essential role in platelet aggregation and isoform-selective inhibitors of this enzyme inhibit platelet aggregation.[13] Hence we have taken potent DNA-PK inhibition in the absence of inhibition of platelet aggregation to be a significant indicator of increased specificity for the DNA-PK enzyme. In this work we are reporting on the synthesis, inhibition of human platelet aggregation induced by collagen and IC_{50} measurements of DNA-PK inhibition of twelve newly synthesised morpholino-naphth-oxazines. Homology modelling and molecular dynamics (MD) simulation have been used to build the 3D model of DNA-PK based on the X-ray structure of phosphatidylinositol (PI) 3-kinase (PI3K). Ligands were then docked into the putative binding site of the 3D model of DNA-PK using the flexible docking method and a probable interaction model between DNA-PK and the ligands was obtained.[7] These compounds have also been docked in our newly built homology model of DNA-PK and the SAR is discussed.

2. Results and discussion

2.1. Chemistry

Thioxo-naphth-oxazines **6**, **9a–b**, and **12a–c** were prepared by allowing the relevant *ortho*-hydroxy-naphthoic acids **5**, **8a–b**, and **11a–c** to react with freshly prepared $Ph_3P(SCN)_2$ according to the previously reported procedure[14] (Schemes 1 and 2).

The morpholino-1,3-naphth-oxazines **7**, **10a–b**, and **13a–c** (Schemes 1 and 2) were prepared by refluxing the relevant thioxo-1,3-naphth-oxazines **5**, **8a–b**, and **11a–c** with morpholine in dry dioxane according to the previously reported method.[15]

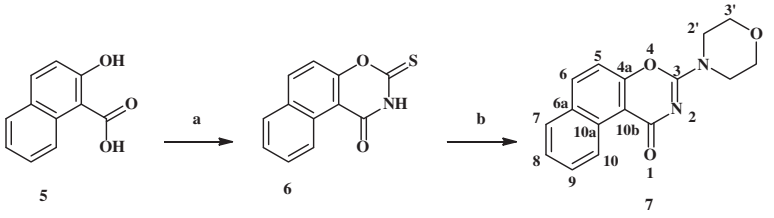
Furthermore the synthesis of 6, 7 and 9-(pyridin-2yl)-2-morpholino-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one (compounds **15**, **18**, and **21**) and pyridin-3yl analogue (compounds **16**, **19**, and **22**) (Scheme 3) were achieved by allowing the reaction of 2-(bromomethyl)-pyridinium bromide or 3-(chloromethyl) pyridinium chloride with the corresponding hydroxy substituted compounds **10b** and **13b–c** in dry acetonitrile in the presence of Cs_2CO_3 according to the earlier reported procedure.[10]

The substituted O-CH_2CH_2Br products **14**, **17**, and **20** (Scheme 4) were synthesised from the reaction of 1,2-dibromoethane with the corresponding morpholine-(hydroxy)-1,3-naphth-oxazines **10b** and **13b–c** in acetonitrile with Cs_2CO_3 according to the previously reported procedure.[15]

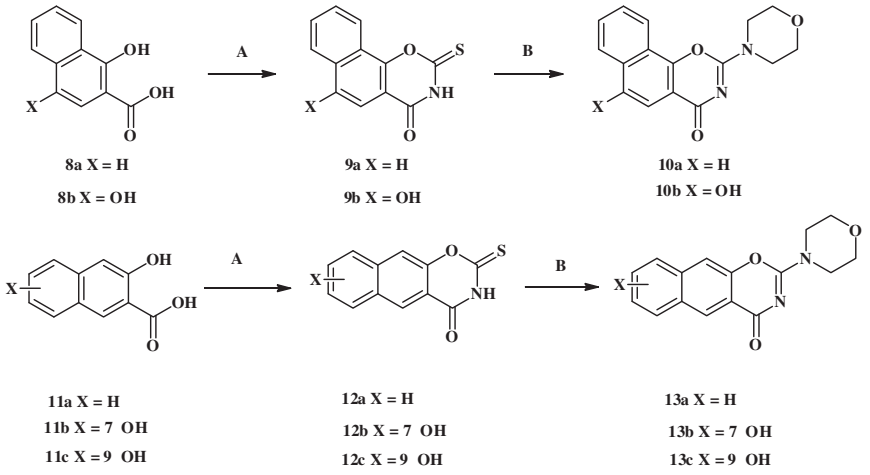
The synthesis of substituted 2-(4-methylpipreazin-1-yl-ethoxy)-morpholino-1,3-naphth-oxazines **23–25** (Scheme 4) was achieved by refluxing 1-methylpiperazine with the corresponding *O*-2-bromoethoxy-morpholino-1,3-naphth-oxazines **14**, **17**, and **20** in dry acetonitrile according to the previously reported procedure.[10]

The structures of these new products were confirmed from their IR, ^1H, ^{13}C NMR spectra and microanalysis. Assignment of the ^1H and ^{13}C NMR spectra for the new 2-thioxo-1,3-naphth-oxazines **9b** and **12b–c** and morpholine substituted 1,3-naphth-oxazines **7**, **10a–b**, and **13a–c** were confirmed by comparison with the reported assignments[14] for compounds **9a** and **12a**.

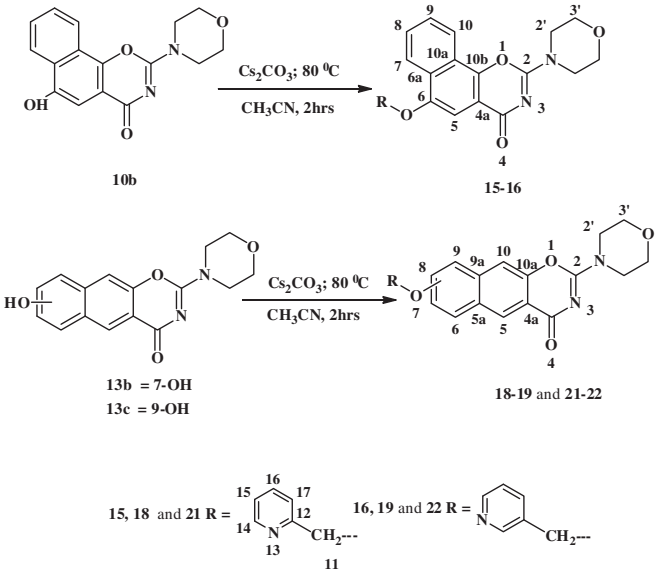
However, the analysis the ^1H and ^{13}C NMR of *O*-2-methylene-pyridine **15**, **18**, and **21** and *O*-3-methylene-pyridine



Scheme 1. Synthesis of 3-thioxo-2,3-dihydro-1*H* naphth[1,2-*e*]-1,3-oxazin-1-one **6** and the morpholino analogue **7**. Reagents and conditions: (a) freshly prepared $Ph_3P(SCN)_2$ in dry DCM; (b) reflux morpholine in dry dioxane.



Scheme 2. Synthesis of 2-thioxo-2*H*-naphth[2,1-*e*]-1,3-oxazin-4(3*H*)-one (**9a–b**), (**12a–c**) and the morpholine-analogue (**10a–b**), (**13a–b**). Reagents and conditions: (A) freshly prepared $Ph_3P(SCN)_2$ in dry DCM; (B) reflux morpholine in dry dioxane.



Scheme 3. Synthesis of 6, 7 and 9-(pyridine-2yl and pyridine-3yl-methyloxy)-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-ones **15**, **16**, **18**, **19**, **21**, and **22** using Cs_2CO_3.

morpholino-naphth-oxazine **16**, **19**, and **21** was based on the analysed ^1H and ^{13}C NMR for 2-methylpyridine and 3-methylpyridine[16] and also use the analysed spectra of the corresponding hydroxy-morpholino-naphth-oxazines **10b**, **13b**, and **13c**.

The simulated ^1H and ^{13}C NMR using Chem Draw V12 ultra were also used as references to aid the analysis of the observed ^1H and ^{13}C NMR spectra of the new products.

It's worth noting that the H-5 chemical shift in compounds **6**, **7**, **9a**, **12a**, **13a** showed a signal at δ~8.4–8.7 ppm which is a result of the long range de-shielding effect of the carbonyl group at position 4. However, the H-5 chemical shift in compounds **9b**, **10b**, **14**, **16**, **23** showed a signal at δ ~7.2–7.5 ppm which was attributed to the resonance effect of the –O– substitution at position 6.

2.2. Inhibition of DNA-PK activity

All the IC_{50} measurements for the 12 morpholino-naphth-1,3-oxazines were completed using the assay outlined in Experimental Section, Biological Activity 5.2.

It is important to note that the some of the morpholino-naphth-1,3-oxazines prepared, **7** and **18**, showed much more activity than the previously reported morpholino-4*H*-chromen-4-one analogues **1**–**3** (Fig. 1). However, some of the morpholino-1,3-naphtho-oxazines showed comparable activity (**10a**, **16**, **21**, and **22**).

Of particular interest is compound **18**, which shows no platelet inhibitory activity even at high concentrations (Table 2), yet is the most active of the 12 morpholino-1,3-naphth-oxazines in its inhibition of DNA-PK activity (Table 1). Given that non-isoform selective inhibitors of PI3K have been shown to inhibit DNA-PK activity as well as platelet aggregation and those isoform-specific inhibitors of PI3K p110 inhibit platelet aggregation these results can be taken as an indication of increased specificity for the DNA-PK enzyme, a favourable quality in any compound destined for clinical use.

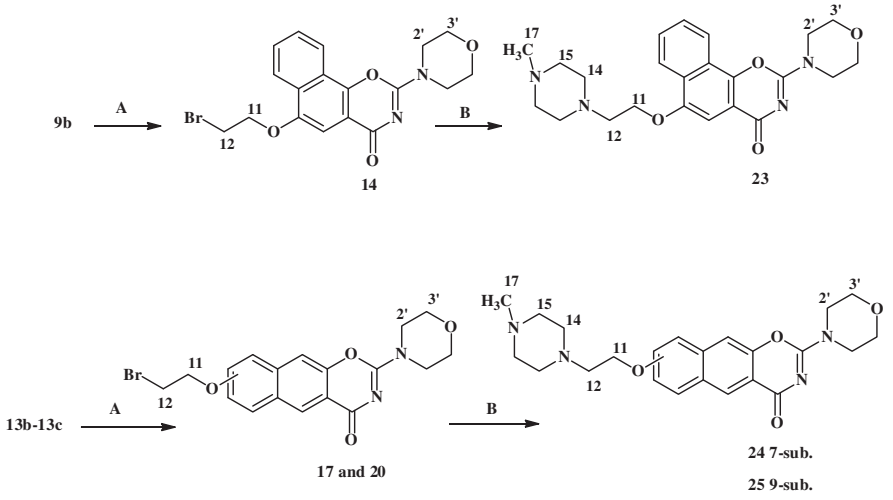
2.3. Inhibition of platelet aggregation

We previously reported the inhibition of collagen induced platelet aggregation by a series of 2-amino-1,3-benzoxazines and found the two most active products to be the 8-methyl-2-morpholin-4-yl-7-(pyridin-3-ylmethoxy)-4*H*-1,3-benzoxazin-4-one (inhibition IC_{50} 2 ± 1.5 μM) and 8-methyl-2-morpholin-4-yl-7-(pyridin-4-ylmethoxy)-4*H*-1,3-benzoxazin-4-one (inhibition IC_{50} 4 ± 1.5 μM).[10]

Here we report the in vitro testing of 12 of the amino-substituted naphth-oxazines to determine their inhibitory effect on collagen induced human platelet aggregation. Table 2 shows that the compound with the most activity was compound **19**, whilst the other compounds, except perhaps for compound **22** displayed only weak inhibitory activity at best.

2.4. Homology model of human DNA-PK

To direct future syntheses, and to better understand our structure–activity relationship, a homology model of the catalytic subunit of human DNA-PK was generated. Previously, Cao et al.[9] have reported a homology model of DNA-PK based on the template protein phosphoinositide 3-kinase (PI3K). The authors used the model as the basis of a study to understand the protein–ligand interactions of known inhibitors. Recently, researchers from Center for the Study of Systems Biology (CSSB) (http://cssb.biology.gatech.edu/kinomelhm) have published[17] homology models of proteins from the human kinome with open access for the academic community. We observed the homology between DNA-PK and



Scheme 4. Synthesis of 6, 7 and 9-(2-(4-methylpipreazin-1-yl)ethoxy)-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **23**–**25**. Reagents and conditions: (A) is Cs_2CO_3 with 1,2-dibromoethane in dry acetonitrile; (B) is 1-methylpiperazine in dry acetonitrile.

PI3K to be 26% over the primary sequence of the catalytic domain we were interested in; we sought to improve this to optimise the protein model. The primary sequences of DNA-PK and PI3K 1E7U[18] were aligned using Discovery Studio (Fig. 2; protocol detailed in Section 4). The residues of DNA-PK that corresponded to gaps in the template were the basis of a more specific blast search in which a number of proteins with secondary structures that matched the predicted structure of DNA-PK were aligned, increasing the homology to 36% (58% similarity). The resulting model was solvated and minimised prior to docking compounds listed in Table 1 using GOLD 4.1 (Fig. 2).

In Figure 2, red background indicates non-matching residues. The gaps in the PI3K sequence have been aligned to segments of crystal structures which adopt the same secondary structure as the predicted structure of DNA-PK. The secondary structure shown as orange/red bars: helix, blue arrows: β sheets, grey bars: loops Fig. 2, final 36% homology and 59% similarity.

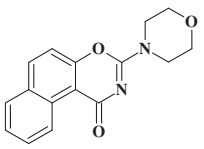
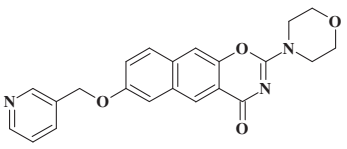
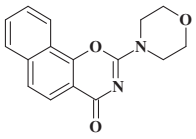
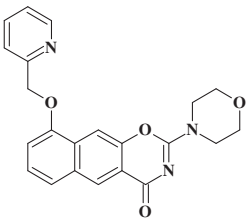
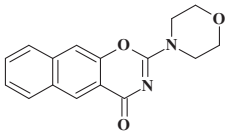
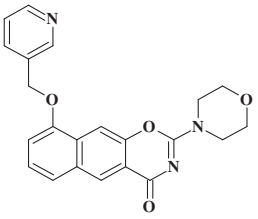
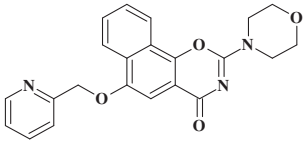
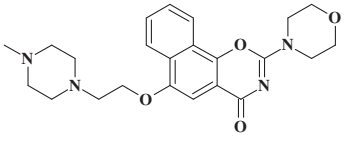
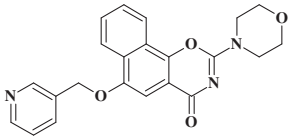
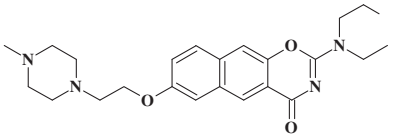
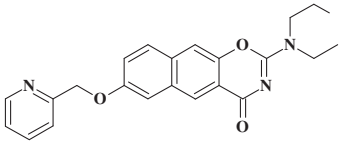
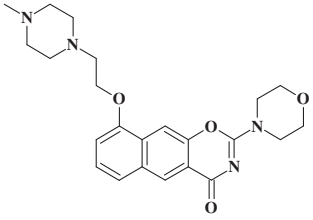
Figure 3, shows the modelled structure of DNA-PK, the green backbone indicates where the PI3K template has been used, the black areas where the additional templates have been used. The binding site is shown as a grey mesh with the residues within 5 Å in stick notation, coloured by atom.

2.4.1. Docking protocol (GOLD)

The centre of the active site was identified using find sites from receptor cavity tool and confirmed by the previously reported residue sequence.[9] Side-chain flexibility was set to free for Ser102 and Lys124, the remaining residues were kept rigid. Ligands shown in Table 1 were docked, filtered and minimised using Ligand Minimisation (default parameters). Finally corresponding protein models and ligands were rescored using GOLD.

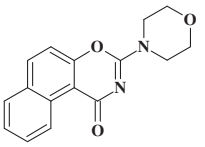
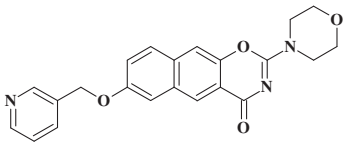
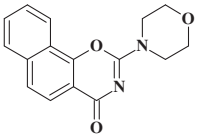
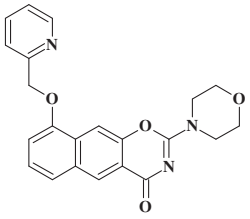
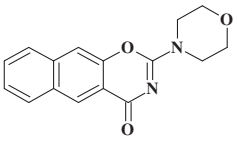
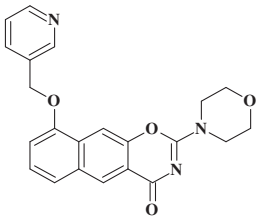
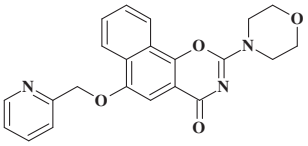
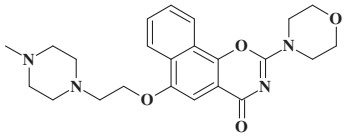
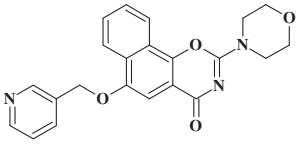
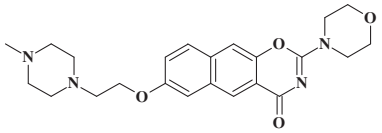
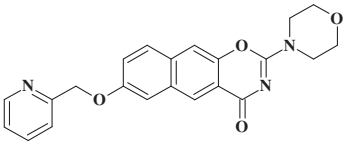
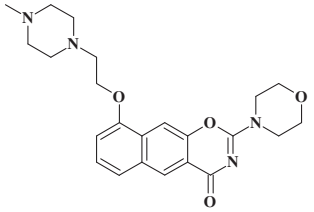
In Figure 4, A and B shows compounds **18** and **22** docked respectively in the binding site of the modelled structure of DNA-PK with surface coloured by atom type. C and D high lights the key residues interacting with **18** and **22**. Hydrogen bonds are shown as red dashed lines. Only the key hydrogens are shown to simplify the view. As expected, the docked poses show the morpholino head of the ligands buried into the active site with the oxygen hydrogen bonding to the backbone NH of Leu177. In addition to this key and common hydrogen bond, another H-bond

Table 1
Inhibition of DNA-PK IC_{50} for the morpholino-1,3-naphth-oxazines

Compd	Structure	IC_{50} (μM)	Compd	Structure	IC_{50} (μM)
7		0.72 ± 1.5*	**19**		1.70 ± 2
10a		0.42 ± 2	**21**		0.93 ± 2.5
13a		6.5 ± 2	**22**		0.33 ± 2
15		2.43 ± 2.5	**23**		0.76 ± 6
16		0.717 ± 4	**24**		0.19 ± 5
18		0.096 ± 3.5	**25**		3.09 ± 1.5

* Errors represent standard deviations of at least three measurements.

Table 2
Inhibition data for morpholino-1,3-naphth-oxazines against collagen induced human platelet aggregation

Compd	Structure	IC_{50} (μM)	Compd	Structure	IC_{50} (μM)
7		450 ± 2*	**19**		55 ± 4
10a		280 ± 1.5	**21**		⩾100
13a		155 ± 2.5	**22**		85 ± 4
15		100 ± 5	**23**		100 ± 3
16		100 ± 1.5	**24**		100 ± 3.5
18		100 ± 2.5	**25**		100 ± 1.5

* Errors represent standard deviations of at least three measurements.

is between the oxazine carbonyl and the side chain of Lys124. The additional potency achieved by the oxazine ring versus the pyranone[9] could be explained by the close proximity of the Tyr162 side-chain OH to the nitrogen. Although not shown in these examples, a potential hydrogen bond is possible here.

The SAR of closely related compounds can be justified from the poses generated using the above method. The 2-methoxy-pyridine extension at position 7 in compound **18** is located between Ser102 and Arg104. These form two hydrogen bonds with the side chain OH of Ser102 and the 2-pyridine ring may also form a π-stacking interaction with Arg104. Compound **19**, the 3-methoxy-pyridine derivative, does not allow this hydrogen bonding interaction and as shown is less active as predicted. With a similar analogy, the increase potency of compound **22** versus compound **21** can be explained by the loss of the hydrogen bond between the N of the pyridine ring and the backbone NH of Thr182. Although not shown in these models, alternative poses showed the potential of methoxy O forming a hydrogen bond with the side-chain OH of Thr182. This potential interaction could aid in the submicromolar activity of compound **21** and **22**.

3. Conclusion

In conclusion we have synthesised a number of substituted 1,3-naphth-oxazines **6**, **9a**–**b**, **12a**–**c** and their 2 or 3-mopholino-substitutes **7**, **10a**–**b**, and **13a**–**c**. Furthermore synthesis of the new *O*-substituted-morpholino-naphth-oxazines **14**–**25** was achieved and all the products were characterised.

The in vitro testing was carried out on a total of 12 new *O*-substituted-morpholino-1,3-naphtho-oxazines to determine their inhibitory effect on human platelet aggregation induced by collagen and it was found that the these structures are less potent than previously reported[10] *O*-substitued-2-morpholino-1,3-benzoxazines analogues. However, the most active was found to be the 3-methoxy-pyridine derivative **19** with IC_{50} = 55 ± 4 μM.

Inhibition of DNA-PK activity by the morpholino-1,3-naphth-oxazines (**7**, **10a**, and **13a**) and (**15**–**19**, and **21**–**25**) was evaluated and showed comparable and in some cases with higher activity than previously reported for *O*-substitued-2-morpholino-1,3-benzoxazines analogues.

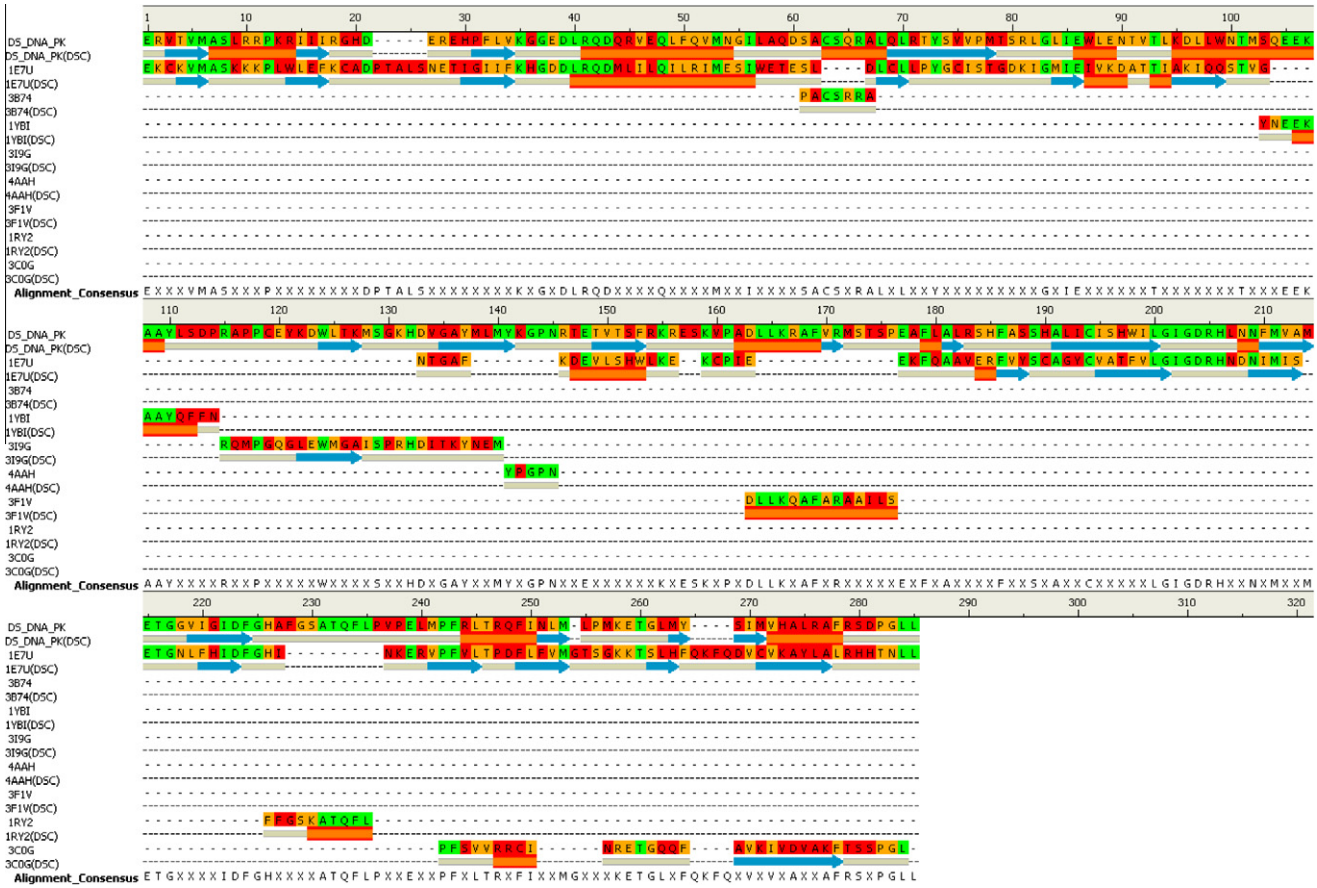


Figure 2. Sequence alignment of DNA-PK with PI3K (1E7U) which equates to 26% homology (identical residues green) 45% similarity (amber residues).

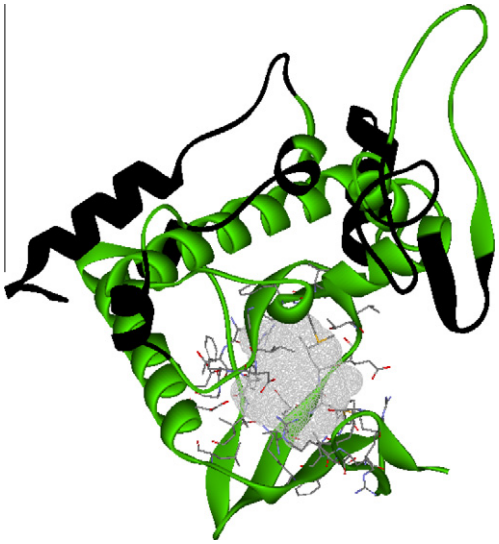


Figure 3. Modelled structure of DNA-PK, the green backbone indicates where the PI3K template has been used, the black areas where the additional templates have been used. The binding site is shown as a grey mesh with the residues within 5 Å in stick notation, coloured by atom.

The presence of a 2 or 3-O-CH_2-pyridil- group at C-7 and C-9 in compounds **18** and **22** was found to be essential to the DNA-PK inhibitory activity.

Docking of synthesised compounds within the binding pocket and SAR analyses of the poses were performed and results agreed well with observed activity.

4. Experimental

4.1. Chemistry

Infrared spectra were obtained using a Perkin Elmer FT-IR 1720x spectrometer. ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker AC 200 NMR spectrometer at 200 and 50 MHz, respectively. All ^{1}H and ^{13}C NMR spectral results are recorded as chemical shifts (δ) relative to the internal TMS. 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.

4.1.1. Starting materials

The starting reagents, morpholine, 1,2-dibromoethane, *N*-methyl piperazine, cesium carbonate, 2-(bromomethyl)-pyridine hydrobromide, 3-(chloromethyl)-pyridine hydrochloride hydrobromide and substituted hydroxynaphthoic acids were purchased from Aldrich Chemical Company and were used as received.

4.2. Synthesis of 2-thio-1,3-naphth-oxazines

General procedure A: Triphenylphosphine dibromide (5 mmol), lead thiocyanate (6 mmol) and the relevant 2-hydroxy naphthoic acid (4 mmol) in dry dichloromethane were allowed to react according to the previously reported procedure.[14] The solid was collected and recrystallised from a suitable solvent.

The known 2-thio-1,3-naphth-oxazines **9a** and **12a** were synthesised according to procedure A and the IR, ^{1}H and ^{13}C NMR data

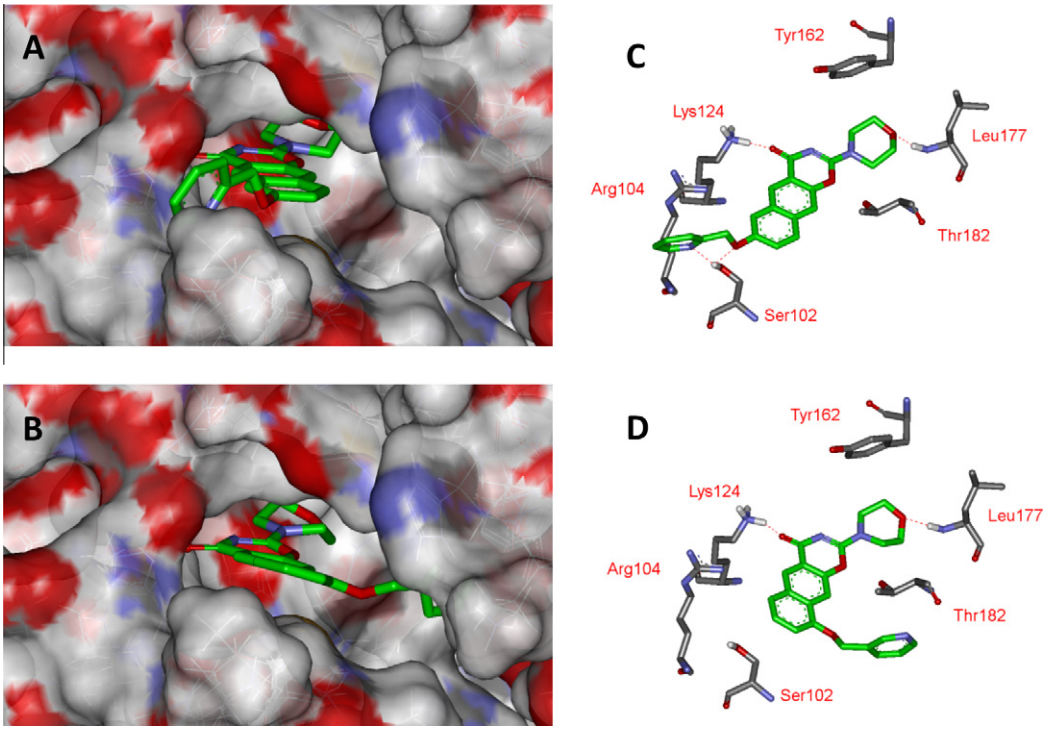


Figure 4. Compounds **18** and **22** docked respectively in the binding site of the modelled structure of DNA-PK with surface coloured by atom type.

collected for compounds **9a** and **12a** were found to agree with the previously reported data.[14]

4.2.1. 3-Thioxo-2,3-dihydro-1*H*-naphth[1,2-*e*]-1,3-oxazin-1-one 6

2-Hydroxy-1-naphthoic **5** acid was allowed to react with freshly prepared triphenylphosphine thiocyanogen according to the general procedure A. The solid was collected and recrystallised from acetic acid to give **6** (70% yield), mp 259 °C decomp. (lit.[21] 257–258 °C decomp.). ν_{max} (KBr) 3185, 2944 (N–H), 1673 (C=O), 1620 (C=C), 1139 (C=S) cm^{-1}; ^1H (DMSO-d_6) δ 13.67 (s, 1H, N–H), 9.39 (d, 1H, J = 6.3 Hz, H-5), 8.40 (d, 1H, J = 6.3 Hz, H-8), 8.15 (d, J = 6.3 Hz, 1H, H-9), 7.80 (t, J = 6.3 Hz, 1H, H-6), 7.68 (t, J = 6.3 Hz, 1H, H-7), 7.59 (d, J = 6.3 Hz, 1H, H-10); ^{13}C (DMSO-d_6) δ 181.1 (C-2), 158.5 (C-4), 156.9 (C-10a), 138.1 (C-9), 130.5 (C-4b), 129.9 (C-6), 129.3 (C-8a), 129.0 (C-8), 126.8 (C-7), 124.9 (C-5), 116.0 (C-10), 108.1 (C-4a).

4.2.2. 6-Hydroxy-2-thioxo-2,3-dihydro-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 9b

1,4-Dihydroxy-2-naphthoic acid **8b** was allowed to react with freshly prepared triphenylphosphine thiocyanogen according to the general procedure A. The solid was collected and recrystallized from DMF/acetonitrile to give **9b** (75% yield), mp 268 °C decomp. ν_{max} (KBr) 3438–2931 (O–H), 3281–2931 (N–H), 1702 (C=O), 1637 (C=C), 1148 (C=S) cm^{-1}; ^1H (DMSO-d_6) δ 13.72 (s, 1H, N–H), 10.95 (s, 1H, 6-O-H), 8.29–8.22 (m, 2H, H-8/H-9), 7.81 (d, J = 6.3 Hz, 1H, H-7/10), 7.77 (d, J = 6.3 Hz, 1H, H-10/H-7), 7.12 (s,1H, H-5); ^{13}C (DMSO-d_6) δ 181.3 (C-2), 158.0 (C-4), 151.2 (C-6), 146.5 (C-10b), 129.4 (C-8), 128.4 (C-7), 122.7 (C-10), 122.2 (C-10a), 121.8 (C-9), 111.8 (C-4a), 99.7 (C-5); (found C, 58.35; H, 3.01; N, 5.45; $C_{12}H_7NO_3S$, requires C, 58.77; H, 2.88; N, 5.71).

4.2.3. 7-Hydroxy-2-thioxo-2,3-dihydro-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 12b

3,7-Dihydroxy-2-naphthoic acid **11b** was allowed to react with freshly prepared triphenylphosphine thiocyanogen according to the general procedure A. The solid was collected and recrystallized from acetone to give **2b** (70% yield), mp 285 °C decomp. ν_{max} (KBr) 3413–2924 (O–H), 320, 2924 (N–H), 1695 (C=O), 1620 (C=C), 1155 m (C=S) cm^{-1}; ^1H (DMSO-d_6) δ 13.52 (s, 1H, N–H), 10.24 (s, 1H, 7-OH), 8.45 (s, 1H, H-5), 7.97 (d, J = 7 Hz, 1H, H-9), 7.93 (s, 1H, H-10), 7.48 (s, 1H, H-6), 7.32 (d, J = 7 Hz, 1H, H-8);^{13}C (DMSO-d_6) δ 182.2 (C-2), 157.7 (C-4), 155.8 (C-7), 149.0 (C-10a), 131.7 (C-5a), 130.9 (C-9a), 129.3 (C-5), 126.3 (C-9), 123.3 (C-8), 114.6 (C-4a), 112.3 (C-10), 109.7 (C-6); (found C, 58.80; H, 2.92; N, 5.70; $C_{12}H_7NO_3S$, requires C, 58.77; H, 2.88; N, 5.71).

4.2.4. 9-Hydroxy-2-thioxo-2*H*-naphth[2,3-*e*]-1,3-oxazin-4(3*H*)-one 12c

3,5-Dihydroxy-2-naphthoic acid **11c** was allowed to react with freshly prepared triphenylphosphine thiocyanogen according to the general procedure A. The solid was collected and recrystallized from dioxane/dichloromethane to give **12c** (65% yield), mp 315 °C decomp. ν_{max} (KBr) 3257–2867 (O–H), 3257, 2921 (N–H), 1707 (C=O), 1633 (C=C), 1165 (C=S) cm^{-1}; ^1H (DMSO-d_6) δ 13.5 (s, 1H, N–H), 10.6 (s, 1H, 9-O-H), 8.6 (s, 1H, H-5), 7.9 (s, 1H, H-10), 7.6 (d, 1H, J = 7 Hz, H-6), 7.4 (t, J = 7 Hz,1H, H-7), 7.0 (d, 1H, J = 7 Hz, H-8);^{13}C (DMSO-d_6) δ 182.3 (C-2), 157.8 (C-4), 153.0 (C-10a), 150.3 (C-9), 131.4 (C-5a), 128.6 (C-5), 128.0 (C-9a), 127.4 (C-7), 120.0 (C-6), 114.8 (C-4a), 111.3 (C-8), 107.0 (C-10); (found C, 58.80; H, 2.82; N, 5.68; $C_{12}H_7NO_3S$, requires C, 58.77; H, 2.88; N, 5.71).

4.3. Synthesis of morpholino-1,3-naphth-oxazines

General procedure B: Thio-1,3-(substituted) naphth-oxazines **6**, **9a–b**, and **8a–c** (2.5 mmol) was allowed react with morpholine (12.5 mmol) in 1,4-dioxane, according to the previously reported procedure.[15] The solid was collected and recrystallised from a suitable solvent.

4.3.1. 3-Morpholino-1*H*-naphth[1,2-*e*]-1,3-oxazin-1-one 7

3-Thioxo-2,3-dihydro-1*H*-naphth[1,2-*e*]-1,3-oxazin-1-one **6** was allowed to react with morpholine according to the general procedure B. The solid was collected and recrystallised from toluene to give **7** (65% yield), mp 248–250 °C decomp. (Lit.[20] 245 °C decomp.). ν_{max} (KBr) 3102, 2851 (C–H), 1666 (C=O), 1517 (C–N) cm^{-1}; ^1H (DMSO-d_6) δ 9.86 (d, J = 6.8 Hz, 1H, H-10), 8.05 (d, J = 6.8 Hz, 1H, H-7), 7.86 (d, J = 9.0 Hz, 1H, H-6), 7.72 (m, 1H, H-9 part of AB second order system), 7.62 (m, 1H, H-8 part of AB second order system), 7.08 (d, J = 6.8 Hz, 1H, H-5), 3.84 (br m, 8H, 4× CH_2 of morpholine); ^{13}C (DMSO-d_6) δ 168.1 (C-1), 156.0 (C-4a), 154.5 (C-2), 135.9(C-6), 131.6 (C-10a), 131.0 (C-6a), 129.6 (C-9), 128.6 (C-7), 127.5(C-8), 126.7 (C-10), 115.4 (C-5), 110.9 (C-10b), 66.7 (C-3′), 44.8 (C-2′).

4.3.2. 2-Morpholin-4-yl-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 10a

2-Thioxo-2,3-dihydro-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **9a** was allowed to react with morpholine according to the general procedure B. The solid was collected and recrystallised from toluene to give **10a** (72% yield), mp 272–274 °C decomp. (Lit.[20] 274 °C decomp.). ν_{max} (KBr) 3058, 2866 (C–H), 1681 (C = O), 1646, 1562 (C-N) cm^{-1}; ^1H (DMSO-d_6) δ 8.21-7.65 (m, 6H, ArH), 3.95 (br m, 8H, 4× CH_2 of morpholine); ^{13}C (DMSO-d_6) δ 167.1 (C-4), 156.5 (C-2), 150.5 (C-10b), 136.1 (C-6a), 129.0 (C-8), 128.3 (C-5), 127.0 (C-7), 125.2 (C-9), 122.3 (C-10), 122.0 (C-10a), 121.0 (C-6), 113.0 (C-4a), 66.2 (C-3′), 44.5 (C-2′).

4.3.3. 2-Morpholin-4-yl-4*H*-naphth[2,3-*e*]-1,3-oxazin-4-one 13a

2-Thioxo-2,3-dihydro-4*H*-naphth[2,3-*e*]-1,3-oxazin-4-one **12a** was allowed to react with morpholine according to the general procedure B. The solid was collected and recrystallised from toluene to give **13a** (72% yield), mp 248–250 °C decomp. (Lit.[20] 245 °C decomp.). ν_{max} (KBr) 3052, 2865 (C–H), 1675 (C=O), 1667, 1588 (C–N) cm^{-1}; ^1H (DMSO-d_6) δ 8.70 (s, 1H, H-10), 7.98 (d, J = 8.0 Hz, 1H, H-5/H-8), 7.81 (d, J = 8.0 Hz, 1H, H-8/H-5), 7.59-7.49 (m, 3H, ArH), 3.83 (br m, 8H, 4× CH_2 of morpholine); ^{13}C (DMSO-d_6) δ 167.1 (C-4), 157.0 (C-2), 149.8 (C-9a), 135.8 (C-8a), 130.8 (C-10a), 129.5/129.1/128.8 (C-5/C-7/C-10), 127.1/126.0 (C-6/C-8), 116.3 (C-4a), 111.4 (C-9), 66.2 (C-3′), 44.5 (C-2′).

4.3.4. 6-Hydroxy-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 10b

6-Hydroxy-2-thioxo-2,3-dihydro-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **9b** was allowed to react with morpholine according to the general procedure B. The solid was collected and recrystallized from DMF/ethanol to give **10b** (85% yield), mp 285 °C decomp. ν_{max} (KBr) 3055–2717 (O–H), 1649 (C=O), 1544 (C=N) cm^{-1}; ^1H (DMSO-d_6) δ 10.62 (br s, 1H, 6-OH), 8.29 (d, J = 6.6 Hz, 1H, H-7/H-10), 7.73 (m, 2H, H-8/H-9), 7.20 (s, 1H, H-5), 3.7 (br m, 8H, 4× CH_2 of morpholine); ^{13}C (DMSO-d_6) δ 165.7 (C-4), 156.3 (C-2), 150.2 (C-10b), 143.5 (C-6), 127.9 (C-8), 127.5 (C-10), 127.4(C-9), 122.7 (C-10a), 122.4 (C-4a), 121.1 (C-7), 117.6 (C-6a), 101.2 (C-5), 65.4 (C-3′), 44.0 (C-2′); (found C, 64.38; H, 4.68; N, 9.47; $C_{16}H_{14}N_2O_4$ requires C, 64.42; H, 4.73; N, 9.39%).

4.3.5. 7-Hydroxy-2-morpholin-4*H*-naphth[2,3-*e*]-1,3-oxazin-4-one 13b

7-Hydroxy-2-thioxo-2,3-dihydro-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **12b** was allowed to react with morpholine according to the general procedure B. The solid was collected and recrystallized from DMF to give **13b** (75% yield), mp 278 °C decomp. ν_{max} (KBr) 3043–2586 (O–H), 1665 (C=O), 1560 (C=N) cm^{-1}; ^1H (DMSO-d_6) δ 10.01 (br s, 1H, 7-OH), 8.30 (s, 1H, H-5), 7.84 (d, J = 8.3 Hz, 1H, H-9), 7.78 (s, 1H, H-10), 7.32 (s, 1H, H-6), 7.24 (d, J = 8.3 Hz, 1H, H-8), 3.74 (br m, 8H, 4× CH_2 of morpholine); ^{13}C (DMSO-d_6) δ 165.8 (C-4), 156.8 (C-2), 155.3 (C-7), 147.7 (C-10a), 131.9 (C-5a), 130.1 (C-9a), 128.8 (C-5), 125.4 (C-9), 122.2 (C-4a), 116.5 (C-8), 111.8 (C-10), 109.5 (C-6), 65.5 (C-3′), 44.3 (C-2′); (found C, 64.39; H, 4.78; N, 9.40; $C_{16}H_{14}N_2O_4$ requires C, 64.42; H, 4.73; N, 9.39).

4.3.6. 9-Hydroxy-2-morpholin-4*H*-naphth[2,3-*e*]-1,3-oxazin-4-one 13c

9-Hydroxy-2-thioxo-2,3-dihydro-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **12c** was allowed to react with morpholine according to the general procedure B. The solid was collected and recrystallized from DMF/acetonitrile to give **13c** (65% yield), mp 265–268 °C decomp. ν_{max} (KBr) 3017–2559 (O–H), 1661 (C=O), 1542 (C=N) cm^{-1}; ^1H (DMSO-d_6) δ 10.53 (br s, 1H, 9-OH), 8.45 (s, 1H, H-5), 7.97 (s, 1H, H-10), 7.57 (d, J = 8.2 Hz, 1H, H-6), 7.36 (t, J = 8.2 Hz, 1H, H-7), 6.99 (d, J = 8.2 Hz, 1H, H-8), 3.73 (br m, 8H, 4× CH_2 of morpholine); ^{13}C (DMSO-d_6) δ 165.8 (C-4), 156.8 (C-2), 152.5 (C-10a), 148.9 (C-9), 131.5 (C-5a), 127.4 (C-5), 127.4 (C-9a), 126.3 (C-7), 119.6 (C-6), 116.5 (C-4a), 110.1 (C-8), 106.6 (C-10), 65.4 (C-3′), 44.1 (C-2′); (found C, 64.37; H, 4.84; N, 9.25; $C_{16}H_{14}N_2O_4$ requires C, 64.42; H, 4.73; N, 9.39).

4.4. Reactions of 2-morpholino-*O*-substituted naphth-oxazine 10b, 13b, and 13c with 1,2-dibromoethane

The 2-morpholino-*O*-substituted naphth-oxazines **14**, **17**, and **20** were synthesised from the reactions of 2-morpholino-6, 7 and 9-hydroxy naphth-oxazines **10b**, **13b**, and **13c** (1 mmol) with 1,2-dibromoethane (10 mmol) according to the general procedure.[15] The solid was collected and recrystallised from a suitable solvent.

4.4.1. 6-(2-Bromoethoxy)-2-morpholin-4*H*-naphtho[2,1-*e*][1,3]oxazin-4-one 14

6-Hydroxy-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **10b** was allowed to react with 1,2-dibromoethane according to the general procedure.[15] The solid was collected and recrystallized from ethanol to give **14** (87% yield), mp 252 °C. ν_{max} (KBr) 2968, 2858 (C–H), 1666 (C=O), 1606 (C=C), 1561 (C=N) cm^{-1}; ^1H ($CDCl_3$) δ 8.43 (d, J = 8.4 Hz, 1H, H-7), 8.16 (d, J = 8.4 Hz, 1H, H-10), 7.73 (m, 2H, H-8/9), 7.32 (s, 1H, H-5), 4.56 (t, J = 5.8 Hz, 2H, H-11), 3.95 (br m, 8H, 4× CH_2 of morpholine), 3.83 (t, J = 5.8 Hz, 2H, H-12); ^{13}C ($CDCl_3$) δ 167.1 (C-4), 156.8 (C-2), 151.4 (C-10b), 145.6 (C-6), 128.8 (C-8), 127.8 (C-10), 127.7 (C-9), 123.3 (C-10a), 123.1 (C-7), 120.8 (C-6a), 113.3 (C-4a), 99.8 (C-5), 68.6 (C-11), 66.7 (C-3′), 44.8 (C-2′), 29.0 (C-12); (found C, 53.37; H, 4.19; N, 7.01; $C_{18}H_{17}BrN_2O_4$ requires C, 53.35; H, 4.23; N, 6.91).

4.4.2. 7-(2-Bromoethoxy)-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 17

7-Hydroxy-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **13b** was allowed to react with 1,2-dibromoethane according to the general procedure.[15] The solid was collected and recrystallized from ethanol to give **17** (75% yield), mp 225 °C decomp. ν_{max} (KBr) 2957, 2859 (C–H), 1675 (C=O), 1595 (C=C), 1564 (C=N) cm^{-1}; ^1H ($CDCl_3$) δ 8.54 (s, 1H, H-5), 7.75 (d, J = 9.5 Hz, 1H, H-9), 7.54 (s, 1H, H-10), 7.30 (d, J = 9.5 Hz, 1H, H-8), 7.20 (s, 1H, H-6), 4.42 (t, J = 5.8 Hz, 2H, H-11), 3.83 (br m, 8H, 4× CH_2 of morpholine), 3.74 (t, J = 5.8 Hz, 2H, H-12); ^{13}C ($CDCl_3$) δ 167.0 (C-4), 157.2 (C-2), 156.2 (C-7), 149.0 (C-10a), 132.0 (C-5a), 132.0 (C-9a), 128.8 (C-5), 127.5 (C-9), 122.6 (C-4a), 117.0 (C-8), 111.6 (C-10), 108.2 (C-6), 68.2 (C-11), 66.3 (C-3′), 44.8 (C-2′), 28.8 (C-12); (found C, 53.40; H, 4.15; N, 7.02; $C_{18}H_{17}BrN_2O_4$ requires C, 53.35; H, 4.23; N, 6.91).

4.4.3. 9-(2-Bromoethoxy)-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 20

9-Hydroxy-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **13c** was allowed to react with 1,2-dibromoethane according to the

general procedure.[15] The solid was collected and recrystallized from ethanol to give **20** (65% yield), mp 232 °C decomp. ν_{max} (KBr) 2925, 2863 (C–H), 1671 (C=O), 1590 (C=C), 1566 (C=N) cm^{-1}; ^1H ($CDCl_3$) δ 8.67 (s, 1H, H-5), 8.05 (s, 1H, H-10), 7.61 (d, J = 7.9 Hz, 1H, H-6), 7.41 (t, J = 7.9 Hz, 1H, H-7), 6.90 (d, J = 7.9 Hz, 1H, H-8), 4.51 (t, J = 5.7 Hz, 2H, H-11), 3.87 (br m, 8H, 4× CH_2 of morpholine), 3.80 (t, J = 5.7 Hz, 2H, H-12); ^{13}C ($CDCl_3$) δ 167.4 (C-4), 156.7 (C-2), 151.2 (C-10a), 145.5 (C-9), 128.8 (C-5a), 128.6 (C-5), 127.8 (C-9a), 127.8 (C-7), 123.1 (C-6), 120.8 (C-4a), 113.0 (C-8), 99.3 (C-10), 68.3 (C-11), 66.3 (C-3′), 44.5 (C-2′), 29.3 (C-12); (found C, 53.29; H, 4.34; N, 6.59; $C_{18}H_{17}BrN_2O_4$ requires C, 53.35; H, 4.23; N, 6.91).

4.5. Synthesis of 6, 7 and 9-(pyridin-2yl or 3yl-methyloxy)-2-morpholin-4-yl-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one compounds 15, 16, 18, 19, 21, and 22

General procedure C: A suspension of 2-morpholino-naphth[2,1-*e*]-1,3-oxazine **10b**, **13b**, and **13c** (1 mmol), cesium carbonate (8.5 mmol) and 2-(bromomethyl)-pyridine hydrobromide, 3-(chloromethyl)-pyridine hydrochloride (2 mmol) in acetonitrile, according to the previously reported procedure.[10] The solid was collected and recrystallised from a suitable solvent.

4.5.1. 2-Morpholin-6-(pyridin-2-ylmethoxy)-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 15

6-Hydroxy-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **10b** was allowed to react with 2-(bromomethyl)-pyridine hydrobromide according to the general procedure C. The solid was collected and recrystallized from toluene to give **15** (83% yield), mp 228–230 °C. ν_{max}(KBr) 3068, 2856 (C–H), 1667 (C=O), 1645 (C=C), 1561 (C=N) cm^{-1}; ^1H ($CDCl_3$) δ 8.66 (d, J = 5.0 Hz, 1H, H-14), 8.43 (d, J = 9.0 Hz, 1H, H-7), 8.14 (d, J = 9.0 Hz, 1H, H-10), 7.75 (m, 4H, H-8/H-9/H-15/H-16), 7.45 (s, 1H, H-5), 7.27 (d, J = 7.6 Hz, 1H, H-17), 5.41 (s, 2H, CH_2O) 3.91 (br m, 8H, 4× CH_2 of morpholine); ^{13}C ($CDCl_3$) δ 167.5 (C-4), 157.3 (C-2), 157.1 (C-12), 152.3 (C-10b), 150.0 (C-14), 146.1 (C-6), 137.1 (C-16), 129.4 (C-8), 129.0 (C-10), 128.1 (C-9), 123.9 (C-10a), 123.6 (C-15/C-17), 123.2 (C-7), 121.9 (C-17/C-15), 121.3 (C-6a), 114.0 (C-4a), 100.7 (C-5), 72.2 (C-11); 66.8 (C-3′), 45.2 (C-2′); (found C, 67.39; H, 4.13; N,10.66; $C_{22}H_{19}N_3O_4$ requires C, 67.86; H, 4.92; N, 10.79).

4.5.2. 2-Morpholin-6-(pyridin-3-ylmethoxy)-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 16

6-Hydroxy-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **10b** was allowed to react with 3-(chloromethyl)-pyridine hydrochloride according to the general procedure C. The solid was collected and recrystallized from toluene to give **16** (67% yield), mp 180 °C. ν_{max} (KBr) 3063, 2864 (C–H), 1664 (C=O), 1645 (C=C), 1561 (C=N) cm^{-1}; ^1H ($CDCl_3$) δ 8.85 (s, 1H, H-13), 8.67 (br dd, $J_{H15\text{-}H16}$ = reduced coupling as a result of nitrogen quadrupole effect 8.0 Hz, 1H, H-15), 8.38 (m, 1H, H-10), 8.17 (m, 1H, H-7), 7.87 (d, J = 8.2 Hz, 1H, H-17), 7.67 (m, 2H, H-8/H-9), 7.55 (s, 1H, H-5), 7.38 (dd, $J_{H16\text{-}H17}$ = 8.2 Hz, $J_{H16\text{-}H17}$ = 7.3 Hz, 1H, H-16), 5.34 (s, 2H, CH_2O) 3.94 (br m, 8H, 4× CH_2 of morpholine); ^{13}C ($CDCl_3$) δ 167.6 (C-4), 156.9 (C-2), 151.7 (C-10b), 150.0 (C-13), 149.4 (C-15), 145.6 (C-6), 135.7 (C-17), 132.3 (C-12), 129.0 (C-8), 128.7 (C-10), 128.1 (C-9), 123.9 (C-16), 123.4 (C-10a), 123.2 (C-7), 121.2 (C-6a), 113.3 (C-4a), 99.6 (C-5), 68.4 (C-11); 66.6 (C-3′), 44.8 (C-2′); (found C, 67.89; H, 4.96; N, 10.69; $C_{22}H_{19}N_3O_4$ requires C, 67.86; H, 4.92; N, 10.79).

4.5.3. 2-Morpholin-7-(pyridin-2-ylmethoxy)-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 18

7-Hydroxy-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **13b** was allowed to react with 2-(bromomethyl)-pyridine hydrobromide according to the general procedure C. The solid was collected and recrystallized from ethanol to give **18** (75% yield), mp 221 °C. ν_{max}(KBr) 3056, 2865 (C–H), 1672 (C=O), 1616 (C=C), 1559 (C=N) cm^{-1}; ^1H ($CDCl_3$) δ 8.65 (d, J = 5.0 Hz, 1H, H-14), 8.56 (s, 1H, H-5), 7.81 (d, J = 8.5 Hz, 1H, H-9), 7.75 (dt, $J_{H16\text{-}H17\text{-}H15}$ = 7.6 Hz, $J_{H16\text{-}H14}$ = 1.7 Hz, 1H, H-16), 7.57 (s, 1H, H-6), 7.48 (t, J = 8.2 Hz, 1H, H-15), 7.42 (d, J = 8.0 Hz, 1H, H-17), 7.35 (d, J = 8.0 Hz, 1H, H-8), 7.29 (s, 1H, H-10), 5.34 (s, 2H, CH_2O) 3.85 (br m, 8H, 4× CH_2 of morpholine); ^{13}C ($CDCl_3$) δ 167.7 (C-4), 157.5 (C-2), 156.9 (C-7), 156.7 (C-12), 149.8 (C-14), 149.1 (C-10a), 137.3 (C-16), 132.3 (C-5a), 132.1 (C-9a), 129.1 (C-5), 127.9 (C-9), 123.3 (C-15/C-17), 123.1 (C-4a), 121.9 (C-17/C-15), 117.0 (C-8), 111.9 (C-10), 108.7 (C-6), 71.3 (C-11); 66.7 (C-3′), 44.8 (C-2′); (found C, 68.00; H, 5.05; N, 10.61; $C_{22}H_{19}N_3O_4$ requires C, 67.86; H, 4.92; N, 10.79).

4.5.4. 2-Morpholin-7-(pyridin-3-ylmethoxy)-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 19

7-Hydroxy-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **13b** was allowed to react with 3-(chloromethyl)-pyridine hydrochloride according to the general procedure C. The solid was collected and recrystallized from ethanol to give **19** (61% yield), mp 218–220 °C. ν_{max} (KBr) 3030, 2866 (C–H), 1671 (C=O), 1614 (C=C), 1563 (C=N) cm^{-1}; ^1H ($CDCl_3$) δ 8.76 (s, 1H, H-13), 8.63 (br dd, $J_{H15\text{-}H16}$ = reduced coupling as a result of nitrogen quadrupole effect 8.0 Hz, 1H, H-15), 8.58 (s, 1H, H-5), 7.83 (d, J = 8.2 Hz, 1H, H-17), 7.58 (s, 1H, H-6), 7.83 (d, J = 8.0 Hz, 1H, H-9), 7.76 (dt, $J_{H16\text{-}H17\text{-}H15}$ = 7.3 Hz, $J_{H16\text{-}H14}$ = 1.3 Hz, 1H, H-16), 7.35 (d, J = 8.0 Hz, 1H, H-8), 7.28 (s, 1H, H-10), 5.21 (s, 2H, CH_2O), 3.84 (br m, 8H, 4× CH_2 of morpholine); ^{13}C ($CDCl_3$) δ 167.6 (C-4), 157.4 (C-2), 156.7 (C-7), 150.0 (C-13), 149.5 (C-15), 149.1 (C-10a), 137.3 (C-16), 135.8 (C-17), 132.3 (C-12), 132.2 (C-5a), 132.0 (C-9a), 129.2 (C-5), 127.7 (C-9), 123.9 (C-16), 123.0 (C-8), 121.9 (C-17/C-15), 117.1 (C-4a), 112.0 (C-8), 111.9 (C-10), 108.2 (C-6), 68.1 (C-11); 66.7 (C-3′), 44.9 (C-2′); (found C, 67.96; H, 5.00; N, 10.81; $C_{22}H_{19}N_3O_4$ requires C, 67.86; H, 4.92; N, 10.79).

4.5.5. 2-Morpholin-9-(pyridin-2-ylmethoxy)-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 21

9-Hydroxy-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **13c** was allowed to react with 2-(bromomethyl)-pyridine hydrobromide according to the general procedure C. The solid was collected and recrystallized from ethanol to give **21** (78% yield), mp 260 °C. ν_{max} (KBr) 3056, 2855 (C–H), 1677 (C=O), 1594 (C=C), 1565 (C=N) cm^{-1}; ^1H ($CDCl_3$) δ 8.72 (d, J = 5.0 Hz, 1H, H-14), 8.68 (s, 1H, H-5), 8.11 (s, 1H, H-10), 7.76 (t, J = 7.6 Hz, 1H, H-16), 7.58 (d, J = 7.6 Hz, 1H, H-6), 7.37 (t, J = 8.2 Hz, 1H, H-15), 7.28 (d, J = 8.0 Hz, 1H, H-17), 7.23 (t, J = 8.0 Hz, 1H, H-7), 6.99 (d, J = 7.6 Hz, 1H, H-8), 5.42 (s, 2H, CH_2O), 3.94 (br m, 8H, 4× CH_2 of morpholine); ^{13}C ($CDCl_3$) δ 167.6 (C-4), 157.6 (C-2), 157.0 (C-12), 153.7 (C-10a), 150.1 (C-9), 149.9 (C-14), 137.4 (C-16), 132.3 (C-5a), 129.0 (C-5), 128.8 (C-9a), 126.5 (C-7), 123.3 (C-17), 122.5 (C-15), 121.8 (C-6), 117.1 (C-4a), 108.0 (C-8), 107.3 (C-10), 71.5 (C-11); 66.7 (C-3′), 45.0 (C-2′); (found C, 68.05; H, 5.15; N, 10.85; $C_{22}H_{19}N_3O_4$ requires C, 67.86; H, 4.92; N, 10.79).

4.5.6. 2-Morpholin-9-(pyridin-3-ylmethoxy)-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 22

9-Hydroxy-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **13c** was allowed to react with 2-(chloromethyl)-pyridine hydrochloride according to the general procedure C. The solid was collected and recrystallized from ethanol to give **22** (72% yield), mp 205-208 °C. ν_{max} (KBr) 3045, 2861 (C–H), 1677 (C=O), 1594 (C=C), 1565 (C=N) cm^{-1}; ^1H ($CDCl_3$) δ 8.89 (s, 1H, H-13), 8.69 (s, 1H, H-5), 8.67 (br dd, $J_{H15\text{-}H16}$ = reduced coupling as a result of nitrogen quadrupole effect 8.0 Hz, 1H, H-15), 8.02 (s, 1H, H-10), 7.87 (d,

J = 8.2 Hz, 1H, H-17), 7.64 (d, J = 7.6 Hz, 1H, H-6), 7.46 (dt, $J_{H16\text{-}H17\text{-}H15}$ = 7.3 Hz, $J_{H16\text{-}H14}$ = 1.3 Hz, 1H, H-16), 7.36 (t, J = 8.0 Hz, 1H, H-7), 7.03 (d, J = 7.6 Hz, 1H, H-8), 5.29 (s, 2H, CH_2O), 3.84 (br m, 8H, 4× CH_2 of morpholine); ^{13}C ($CDCl_3$) δ 167.6 (C-4), 157.6 (C-2), 153.8 (C-10a), 150.3 (C-9), 150.0 (C-13), 149.5 (C-15), 136.0 (C-17), 132.6 (C-12), 132.4 (C-5a), 129.1 (C-5), 128.8 (C-9a), 126.3 (C-7), 124.1 (C-16), 122.7 (C-6), 117.3 (C-4a), 107.6 (C-8), 107.2 (C-10), 68.4 (C-11); 66.8 (C-3′), 44.7 (C-2′); (found C, 68.00; H, 5.05; N, 10.61; $C_{22}H_{19}N_3O_4$ requires C, 67.86; H, 4.92; N, 10.79).

4.6. Synthesis of 6, 7 and 9-[2-(4-methylpiperazin-1-yl)ethoxy]-2-morpholin-4-yl-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one (23–25)

General procedure D: A suspension of 6, 7 and 9-(2-bromoethoxy)- naphth[2,1-*e*]-1,3-oxazine **14**, **17**, and **20** (1 mmol), was treated with 1-methylpiperazine (4 mmol) in acetonitrile, according to the previously reported procedure.[10] The solid was collected and recrystallised from a suitable solvent.

4.6.1. 6-(2-(4-Methylpiperazin-1-yl)ethoxy)-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 23

6-(2-Bromoethoxy)-2-morpholin-4*H*-naphtho[2,1-*e*][1,3]oxazin-4-one **14** was allowed to react with 1-methylpiperazine according to the general procedure D. The solid was collected and recrystallized from ethyl acetate to give **23** (78% yield), mp 198–200 °C. ν_{max} (KBr) 2935, 2798 (C–H), 1667s (C=O), 1606s (C=C) 1562s (C=N), cm^{-1}; ^1H ($CDCl_3$) δ 8.34 (d, J = 8.4 Hz, 1H, H-7), 8.12 (d, J = 8.4 Hz, 1H, H-10), 7.69 (m, 2H, H-8/H-9), 7.34 (s, 1H, H-5), 4.34 (t, J = 5.8 Hz, 2H, H-11), 3.92 (br m, 8H, 4× CH_2 of morpholine), 3.02 (t, J = 5.8 Hz, 2H, H-12), 2.71 (br m, 4H, H-13), 2.51 (br m, 4H, H-14), 2.31 (s, 3H, H-15); ^{13}C ($CDCl_3$) δ 167.0 (C-4), 156.8 (C-2), 152.1 (C-10b), 145.4 (C-6), 128.8 (C-8), 128.6 (C-10), 127.7 (C-9), 123.2 (C-10a), 123.1 (C-7), 120.9 (C-6a), 113.3 (C-4a), 99.3 (C-5), 67.2 (C-11), 66.4 (C-3′), 57.1 (C-12), 55.2 (C-13), 53.6 (C-14), 46.1 (C-15), 44.6 (C-2′); (found C, 65.07; H, 6.70; N, 13.17; $C_{23}H_{28}N_4O_4$ requires C, 65.08; H, 6.65; N, 13.20).

4.6.2. 7-(2-(4-Methylpiperazin-1-yl)ethoxy)-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 24

7-(2-Bromoethoxy)-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **17** was allowed to react with 1-methylpiperazine according to the general procedure D. The solid was collected and recrystallized from ethyl acetate to give **24** (67% yield), mp 185 °C. ν_{max} (KBr) 2936, 2798 (C–H), 1678s (C=O), 1615s (C=C) 1588s (C=N), cm^{-1}; ^1H ($CDCl_3$) δ 8.56 (s, 1H, H-5), 7.75 (d, J = 9.5 Hz, 1H, H-9), 7.55 (s, 1H, H-10), 7.29 (d, J = 9.5 Hz, 1H, H-8), 7.23 (s, 1H, H-6), 4.23 (t, J = 5.8 Hz, 2H, H-11), 3.92 (br m, 8H, 4× CH_2 of morpholine), 2.91 (t, J = 5.8 Hz, 2H, H-12), 2.66 (br m, 4H, H-13), 2.55 (br m, 4H, H-14), 2.31 (s, 3H, H-15); ^{13}C ($CDCl_3$) δ 167.5 (C-4), 157.3 (C-2), 157.2(C-7), 149.0 (C-10a), 132.3 (C-5a), 131.6 (C-9a), 128.5 (C-5), 127.4 (C-9), 122.9 (C-4a), 117.2 (C-8), 111.5 (C-10), 108.0 (C-6), 66.6 (C-11), 66.3 (C-3′), 57.1 (C-12), 55.2 (C-13), 53.7 (C-14), 46.0 (C-15), 44.8 (C-2′); (found C, 64.77; H, 6.61; N, 12.90; $C_{23}H_{28}N_4O_4$ requires C, 65.08; H, 6.65; N, 13.20).

4.6.3. 9-(2-(4-Methylpiperazin-1-yl)ethoxy)-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one 25

7-(2-Bromoethoxy)-2-morpholin-4*H*-naphth[2,1-*e*]-1,3-oxazin-4-one **20** was allowed to react with 1-methylpiperazine according to the general procedure D. The solid was collected and recrystallized from ethyl acetate to give **25** (65% yield), mp 193 °C. ν_{max} (KBr) 2974, 2840 (C–H), 1661s (C=O), 1592s (C=C) 1553s (C=N), cm^{-1}; ^1H ($CDCl_3$) δ 8.65 (s, 1H, H-5), 7.97 (s, 1H, H-10), 7.57 (d, J = 7.9 Hz, 1H, H-6), 7.42 (t, J = 7.9 Hz, 1H, H-7), 6.93 (d, J = 7.9 Hz, 1H, H-8), 4.30 (t, J = 5.8 Hz, 2H, H-11), 3.85 (br m, 8H, 4× CH_2 of morpholine), 3.00 (t, J = 5.8 Hz, 2H, H-12), 2.71 (br m, 4H, H-13), 2.51 (br m, 4H, H-14), 2.31 (s, 3H, H-15); ^{13}C ($CDCl_3$) δ 167.0 (C-4), 157.3 (C-2), 154.0 (C-10a), 149.8 (C-9), 132.0 (C-5a), 128.6 (C-5/C-9a), 126.0 (C-7), 121.8 (C-6), 117.2 (C-4a), 107.3 (C-8), 106.9 (C-10), 67.0 (C-11), 66.4 (C-3′), 57.2 (C-12), 55.2 (C-13), 53.6 (C-14), 46.0 (C-15), 44.8 (C-2′); (found C, 65.07; H, 6.70; N, 13.17; $C_{23}H_{28}N_4O_4$ requires C, 65.08; H, 6.65; N, 13.20).

5. Biological activity

5.1. Platelet aggregometry

Venous blood was collected from ostensibly healthy, drug free volunteers into trisodium citrate 22.0 g/l. Ethics approval was obtained from La Trobe University Human Ethics Committee (HEC approval No. 07-127). Platelet aggregation was determined by the optical method in a two-channel platelet aggregometer (Chrono-Log) using the previously reported protocol.[15]

5.2. DNA-PK inhibition assay and IC_{50} μM measurements

The measurement of DNA-PK inhibition and the calculation of the IC_{50} for the 12 morpholino-naphth-1,3-oxazines was accomplished using the purified DNA-PK enzyme (Promega) and the substrate peptide EPPLSQEAFADLWKK (Shanghai Research Institute of Chemical Industry Testing co., Ltd, Shanghai). Briefly, the reaction was carried out in a final volume of 25 μl made up as follows: 2.5 μl Activation buffer (0.33 μg calf thymus DNA ml^{-1} TE buffer), 5.0 μl 5× Reaction buffer (250 mM Hepes, 500 mM KCl, 50 mM $MgCl_2$ 1 mM EGTA, 0.5 mM EDTA and 5 mM DTT), 2.5 μl peptide substrate solution (4 mM DNA-PK peptide substrate in water at neutral pH), 0.2 μl 10 mg/ml BSA, (5.0 μl, 0.5 mM ATP), 1 μl of drug to be tested (or water), 6.8 μl H_2O and 2 μl DNA-PK (15U in 1× Reaction buffer). This was incubated at 30 °C for 5 min and the ADP generated measured using the ADP-Glo Kinase Assay (Promega) as per the manufacturer's instructions. Briefly, the ADP generated in the above reaction is converted from ATP and this is used to drive a luciferase/luciferin reaction. The luminescence thus generated was read in a Flex-station 3 (Molecular Devices) and IC_{50} calculated using the Graphpad Prism curve fitting software (V5) (See Fig. 5).

5.3. Homology model of human DNA-PK

The 3D structure of 275 amino acids of the catalytic domain of DNA-PK was constructed by comparative modelling using Discovery Studio (DS) 2.5[19] on a Hewlett Packard Workstation xw4600. Suitable template proteins were obtained through a BLAST search of the Protein Databank (PDB) using the BLOSUM 62 matrix with a gap penalty of 11 and a gap extension penalty of 1. The core template protein PI3K (1E7U[18]) had 26% identity (45% similarity), however there were regions of the amino acid sequence of DNA-PK corresponding to gaps in the template of PI3K. These gaps corresponded to amino acids 589–592, 630–658, 663–670, 689–701, and 754–762 of DNA-PK. There was also poor homology in the final 32 residues so a further template was sought for those residues (768–800). Individual blast searches of the gap sequences and their flanking amino acids were performed, which identified template proteins to further refine the model (alignment shown in Fig. 2) this increased the homology to 36% and similarity to 58%.

The DNA-PK and template sequences were aligned manually and five models were built at the high optimisation level using the 'Build Homology Models' protocol. The resulting models were assessed for their total probability density function energy (PDF energy); the model with the lowest energy was further evaluated.

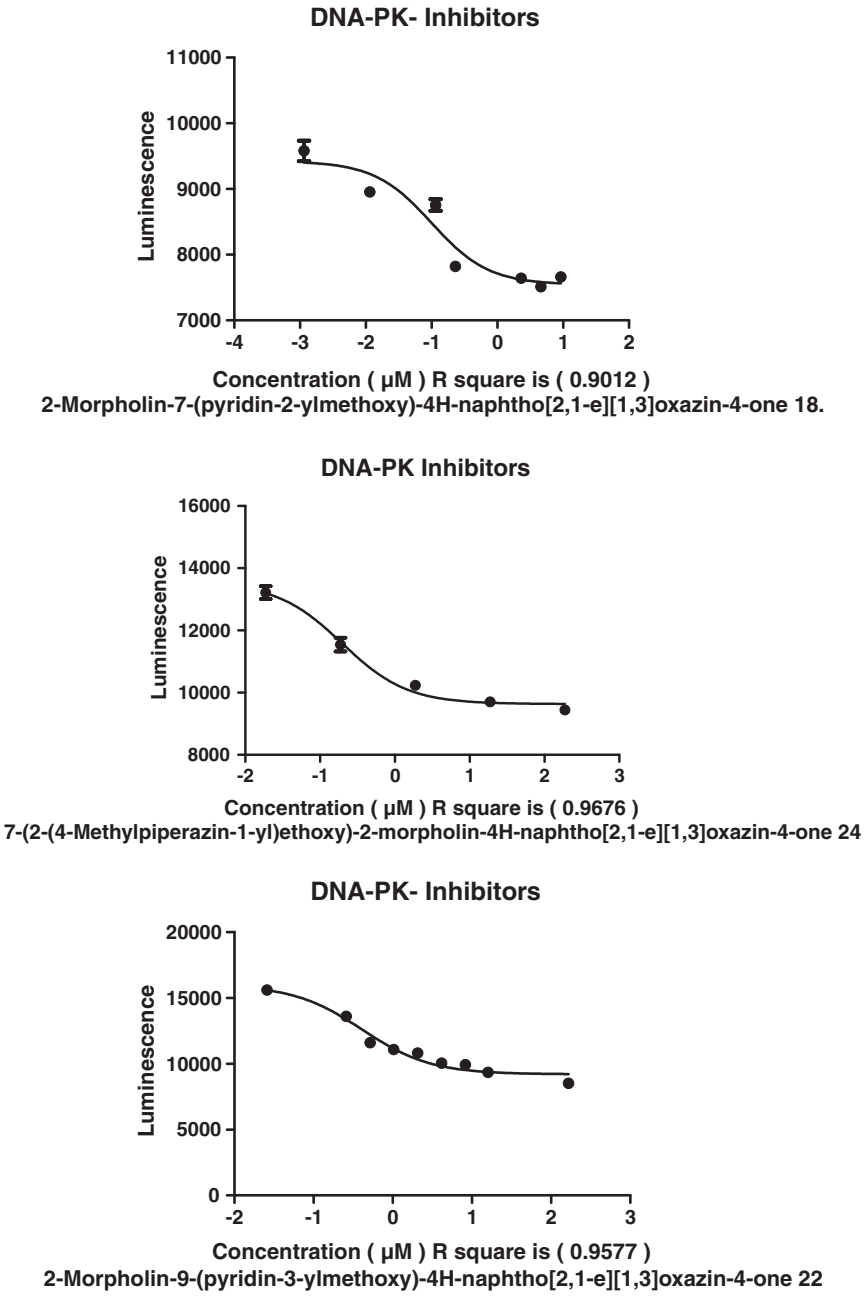


Figure 5. IC_{50} μM graphs for compounds **18**, **22** and **24** calculated using Graphpad Prism curve fitting software (V5).

Two regions of the protein were further optimised with lopper, the loop refinement protocol (amino acids 544–551 and 756–760) using the default settings. The resulting model was assessed using the 'Protein Health' protocol.

Prior to docking, the model protein was typed with the charmm 27 forcefield, solvated (default settings except the salt concentration was set to 0), subjected to a 3-stage minimisation with LY294002 in the binding site (using fixed atom constraints on the heavy atoms followed by the backbone atoms before the full structure was allowed to relax). Minimisation was effected using the Smart Minimizer algorithm until an energy convergence criterion of 0.1 kcal mol^{-1} $Å^{-1}$ was reached. The Ramachandran plot of the backbone phi/psi angles revealed 94% of the amino acids were within the allowed region. Such a high percentage indicates that the model is satisfactory.

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