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A synthesis, *in silico*, *in vitro* and *in vivo* study of thieno[2,3-*b*]pyridine anticancer analogues†

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The anticancer activity of the thieno[2,3-*b*]pyridines was explored by altering the ring size of the cycloaliphatic moiety. Five-, six-, seven- and eight-membered derivatives were tested against the NCI60 tumour cell panel. According to this assay the most active derivative **9a** has a cyclooctane moiety, which suggests that larger aliphatic ring systems are favourable. For the most sensitive tumour cell line MB-MDA-435, derivative **9a** has a GI_{50} = 70 nM and a LC_{50} = 925 nM. To explore the biological mechanism of the thieno[2,3-*b*]pyridines five derivatives were tested against tyrosyl-DNA phosphodiesterase I (TDP1), a phospholipase D enzyme, using a biochemical assay. The most potent derivative for TDP1 was **9d**, giving an excellent IC_{50} at 0.5 ± 0.1 μM. Also, derivative **12** was tested against 97 kinases and no or very limited activity was found, excluding this class of biomolecular targets. Finally, a mouse xenograft study using derivative **12** was encouraging but the tumour size/mass reduction was not quite statistically significant.

Introduction

It is now established that a class of thieno[2,3-*b*]pyridine compounds have a potent anticancer activity against a variety of tumour cell lines.[1–3] Their molecular structure is shown in Fig. 1. The efficacy of the thieno[2,3-*b*]pyridines was discovered by a virtual high-throughput screen (vHTS) against the phospholipase C-γ2 (PLC-γ2) isoform.[4] PLC-γ is an interesting anticancer biomolecular target regulating a number of cancer cellular functions including tumorigenesis,[5] metastasis development,[6] cancer cell invasion[7] and tumour angiogenesis.[8] The administration of thieno[2,3-*b*]pyridines causes the breast cancer cell line MDA-MB-231 to be severely growth restricted, rounded, with G_2/M phase population increase in the cell cycle and slowed proliferation in scratch assays, which is in line with PLC-γ inhibition, making it the putative biomolecular target.[9] Furthermore, however, it is quite possible that other biomolecular targets are affected, contributing to the overall efficacy of this class of compounds.

The substitution pattern of the phenyl moiety of the thieno[2,3-*b*]pyridines has been thoroughly explored, revealing that *ortho*/*meta* substitution on the same side of the phenyl group gives the best anticancer effect.[2,9] However, other parts of the molecular scaffold have received less attention, such as the cyclohexanone moiety, and a classical strategy in medicinal chemistry is to alter ring sizes in order to optimise the potency of ligands. Thus, systematically altering the ring size of the cyclohexane would result in a consistent structural activity relationship (SAR) series of cyclopentane, cyclohexane, cycloheptane and cyclooctane derivatives.

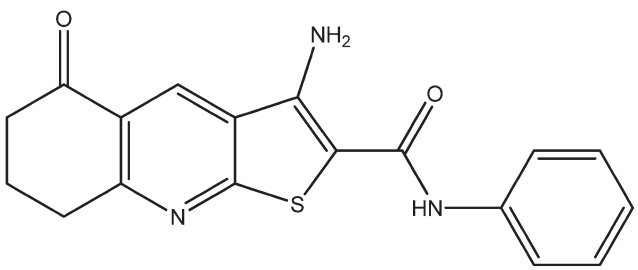

Fig. 1 The basic molecular structure of the anticancer thieno[2,3-*b*]pyridines (3-amino-5-oxo-*N*-phenyl-5,6,7,8-tetrahydrothieno[2,3-*b*]-quinoline-2-carboxamide). *Ortho*/*meta* substitution on the phenyl ring gives the most potent anticancer effect.

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The aim of this study was twofold: first, to extend the SAR of the thieno[2,3-*b*]pyridines by systematically altering the cyclohexane moiety's ring size in conjunction with testing against the National Cancer Institute's human tumour cell line panel (NCI60)[10] and second, to explore the biological mechanism of action of the thieno[2,3-*b*]pyridines led by molecular modelling investigations. Two classes of biomolecules were considered, *i.e.* the phospholipase D enzyme tyrosyl-DNA phosphodiesterase I (TDP1),[11] a promising anticancer target,[12,13] and kinases, an established class of anticancer targets (see Strebhardt, 2010, and references therein).[14] Finally, the efficacy of the thieno[2,3-*b*]pyridines was investigated in xenograph mice models.

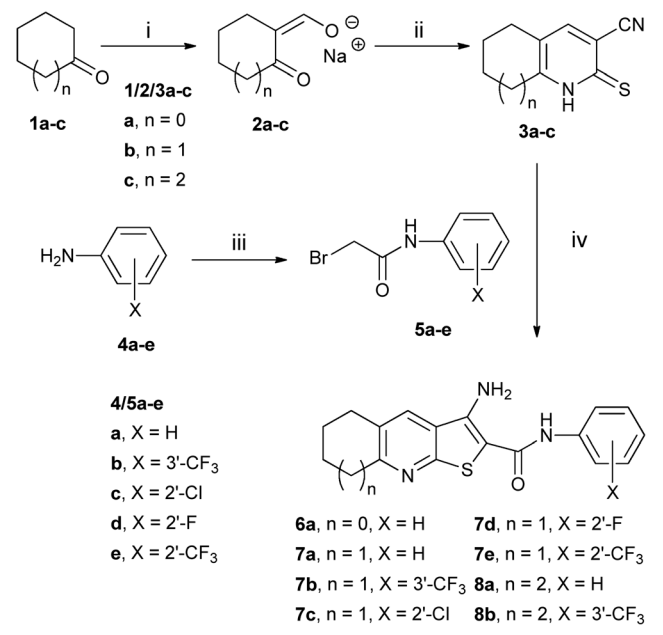
Results and discussion

Synthesis of compounds

Thieno[2,3-*b*]pyridines were prepared in three steps from the corresponding cyclic ketones **1a–c** (Scheme 1). Firstly, salts **2a–c** were formed from the reaction of ketones **1a–c** with freshly prepared sodium methoxide and methyl formate and were immediately used in the next reaction by heating with cyanothioacetamide and piperidinium acetate followed by acidification with acetic acid provided bicyclic carbonitriles **3a–c**.

Bromoanilines **5a–e** were synthesised from the reaction of anilines **4a–e** with 1 equivalent of bromoacetyl bromide in the presence of Et_3N, providing the desired products **5a–e** in very good to excellent yields. These bromoanilines **5a–e** were coupled with the required bicyclic carbonitriles **3a–c** followed by concomitant cyclisation in basic conditions to give thieno-[2,3-*b*]pyridines **6a**, **7a–e**, **8a** and **8b**.

The commercial compounds were acquired from ChemBridge (**6b**, **6c**, **7f**),[15] ChemDiv (**7g**, **7j**),[16] Specs (**7h**),[17] Key Organics (**7i**, **7k**, **8c**)[18] and InterBioScreen (**9a–d**, **10–12**).[19]



Scheme 1 Reagents and conditions: (i) Na, MeOH, methyl formate, **2a–c** 52–67%; (ii) cyanothioacetamide (1.1 equiv.), piperidinium acetate, water, reflux, 4 h, AcOH, r.t., 12 h, **3a–c** 30–59%; (iii) bromoacetyl bromide (1 equiv.), Et_3N (1.1 equiv.), 0 °C, 1 h, **5a–e** 70–100%; (iv) Na_2CO_3, EtOH, reflux, 18 h, **6a**, **7a–e**, **8a–b** 22–91%.

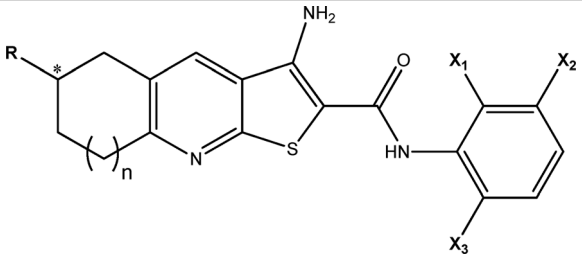
Tumour cell lines

Twenty-one derivatives of the thieno[2,3-*b*]pyridine family were tested against the NCI60 human tumour cell lines. The molecular structures are shown in Table 1 along with the average growth inhibition of the NCI60 panel compared to untreated cells, *i.e.* 100% growth is the control of untreated cells; thus, the lower the percentage number the greater the inhibition. When the ring size on the left-hand side of the derivatives is considered, a clear trend for increased inhibition is seen for the larger seven- (n = 2) and eight-membered (n = 3) rings rather than the five- (n = 0) and six-membered (n = 1) derivatives. This is reflected in derivatives **8a**, **8b**, **9a** and **9d**, being the most active with values of <15% as compared to untreated cells at 100%. Ethyl substitution (**7h–k**) on the cyclohexyl ring is not favourable as compared to the unsubstituted derivatives (**7a–g**). The substitution pattern on the phenyl ring favours the *meta* (X_2) position, with derivatives **8b** and **9d** being the most active compounds tested, *e.g.* **9d** (*m*-CF_3, 7.0%) *vs.* **9c** (*o*-CF_3, 80.2%). This is in line with a previous finding for the anticancer sensitivity of the cyclohexanone thieno[2,3-*b*]pyridine series.[1–3] Also, when the six-membered derivatives are compared to their cyclohexanone counterparts, similar NCI mean values are observed, *e.g.* for the unsubstituted phenyl ring derivatives ~30% inhibition is observed.[1] The cyclohexanone moiety has been linked to general cell cytotoxicity[20,21] and the similar growth inhibition of the two series leads to the conclusion that the biological effect of the cyclohexanone thieno[2,3-*b*]pyridine series is not caused by unspecific cytotoxic effects.

Twelve derivatives were selected for dose response testing due to their favourable inhibition at 10 μM and the results are given in Table 2. The growth inhibition at 50% (GI_{50}) and lethal concentration at 50% (LC_{50}) were derived from these measurements (for further explanation see Shoemaker, 2006).[10] GI_{50} is the concentration for 50% of maximal inhibition of cell proliferation and therefore reflects the cytostatic concentration, whereas LD_{50} is the concentration causing 50% cell death giving the cytotoxic potential of the compounds. Four tumour cell lines were particularly affected, *i.e.* MDA-MB-435 (melanoma), SF-295 (central nervous system, CNS), 786-0 (renal) and MDA-MB-468 (breast). Other cell lines also showed good response, *e.g.* SNB-75 (CNS), OVCAR-3 (ovarian) and ACHN (renal). The complete data set for the NCI data is given in the ESI.†

It can be seen from the GI_{50} values presented in Table 2 that compounds **6b** and **9a** are the most active. It has been previously observed that *ortho*/*meta* substitution on the phenyl ring with small moieties, as seen in derivative **6b**, is

Table 1 The 3-amino-*N*-phenylthieno[2,3-*b*]pyridine-2-carboxamide structural derivatives. The NCI mean percentages (%) at 10 μM as compared to untreated cells at 100% growth, *i.e.* the lower percentage numbers represent greater growth inhibition



	n	R	X_1	X_2	X_3	NCI mean
6a	0	H	H	H	H	79.5
6b	0	H	Me	Me	H	23.6
6c	0	H	F	H	H	101.7
7a	1	H	H	H	H	30.3
7b	1	H	H	CF_3	H	88.1
7c	1	H	Cl	H	H	99.4
7d	1	H	F	H	H	34.5
7e	1	H	CF_3	H	H	90.6
7f	1	H	H	Me	H	39.9
7g	1	H	H	OMe	H	28.2
7h	1	Et[a]	F	H	H	61.1
7i	1	Et[a]	Me	H	H	36.5
7j	1	Et[a]	H	F	H	60.3
7k	1	Et[a]	H	Me	H	69.7
8a	2	H	H	H	H	14.7
8b	2	H	H	CF_3	H	10.0
8c	2	H	Cl	H	Cl	52.1
9a	3	H	H	H	H	12.5
9b	3	H	Cl	H	H	29.0
9c	3	H	CF_3	H	H	80.2
9d	3	H	H	CF_3	H	7.0

[a] Tested as the racemates.

Table 2 The GI_{50} (50% growth inhibition) and LC_{50} (50% lethal concentration) in nanomolar (nM) are shown for four tumour cell lines: MDA-MB-435 (melanoma), SF-295 (CNS), 786-0 (renal) and MDA-MB-468 (breast). Derivatives **7a** and **9a** were tested twice and both values are given[a]

	MDA-MB-435		SF-539		786-0		MDA-MB-468	
	GI_{50}	LC_{50}	GI_{50}	LC_{50}	GI_{50}	LC_{50}	GI_{50}	LC_{50}
6b	52	22700	241	72400	367	14300	197	71200
7a	172/207	X/X	416/491	X/X	759/656	X/X	332/303	X/X
7f	305	X	4740	X	4130	X	1750	X
7g	150	13500	368	X	420	X	239	X
7h	133	X	10000	X	41700	X	347	X
7i	104	Y	398	X	371	X	244	X
7j	110	X	X	X	701	X	471	X
8a	205	X	307	94300	465	X	254	X
8b	2530	X	1940	11300	1580	6240	394	31000
9a	52/88	953/897	247/217	7670/3200	354/858	10200/35700	291/334	X/X
9b	275	20900	2650	46600	1750	6120	2250	X
9d	2100	59200	1710	6650	1600	6070	798	15400

[a] X: no response at 100 μM. Y: no data.

favourable in suppressing cancer cell growth for the cyclohexanone thieno[2,3-*b*]pyridines.[2] Considering the LC_{50} values, derivative **9a** is the most potent with nanomolar sensitivity for the MDA-MB-435 tumour cell line (925 nM). The MDA-MB-435 and MDA-MB-468 cell lines appear to be the most affected, which is in accordance with previous observations for the cyclohexanone thieno[2,3-*b*]pyridines.[1–3] It is interesting to note that five of the derivatives (see Table 2) are not only cytostatic but also cytotoxic (kill the cells), which makes this class of compounds even more interesting for anticancer

treatment. Close structural analogues of the compounds presented here, *i.e.* with a cyclohexanone moiety rather than a cyclohexane, are predominately cytostatic rather than cytotoxic.[1,2]

Tyrosyl-DNA phosphodiesterase I (TDP1)

TDP1 is a member of the phospholipase D (PLD) superfamily,[11] which is related to the PLC enzymes, the putative target of the thieno[2,3-*b*]pyridines.[9] Also, there is evidence of conserved regions for the sequence similarity between the PLC and PLD enzyme classes.[22,23] The idea emerged whether TDP1 could also be a biomolecular target of the thieno[2,3-*b*]-pyridines since both are phospholipases. TDP1 is a promising cancer target as it plays an important role in the removal of DNA lesions generated by DNA topoisomerase I (Top1) inhibitors, such as camptothecin.[12,13] Recently, it has been reported that TDP1 depletion can sensitize glioblastoma-resistant cancer cells to the alkylating agent temozolomide.[24] We (Arabshahi and Reynisson) were already working on the molecular modelling of TDP1 inhibitors[25] and docking of the thieno[2,3-*b*]pyridines to TDP1 (see Molecular modelling section) gave promising results, which can be attributed to serendipity. This phenomenon is known to have had a significant impact on drug discovery and development.[26] Ligand **12** was chosen for testing because it is a more potent analogue of its derivate lacking the methyl substitution on the *ortho* position (GI_{50} = 18.5 nM *vs.* 58 nM for MDA-MB-435), which has a well-defined biological profile.[2,9] Derivative **12** gave an IC_{50} of 2.2 μM using a new oligonucleotide-based fluorescence assay.[25] Four more derivatives were consequently tested and the results are given in Table 3.

From the data in Table 3 it is clear that the thieno[2,3-*b*]-pyridines do inhibit the TDP1 enzyme with derivative **9d** being the most active with nanomolar potency. Derivative **10**, with a five-membered ring (n = 0), is the least active, which fits with the trend that larger ring systems are preferable for anticancer activity.

Interestingly, few small-molecule inhibitors of TDP1 have been reported so far.[25,27–29] Thieno[2,3-*b*]pyridines are therefore a new compound class that inhibits this enzyme and further development could lead to more potent compounds. The full data set is given in the ESI.†

Molecular modelling

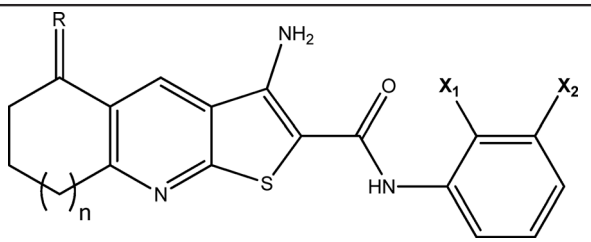
The thieno[2,3-*b*]pyridines were docked into the putative binding pocket of the TDP1 crystal structure. It is proposed that both histidine amino acid residues (His263 and His493) play a role in the biological function of TDP1 (ref. 30) and the binding pocket was defined there. This pocket has been previously used in molecular modelling work resulting in the identification of active compounds, thus lending credibility to this binding site.[27,29] Reasonable scores were observed for the four scoring functions used (see Table S1 in the ESI†). Hydrogen bonding was predicted with His263 and Asn516, as shown in Fig. 2A for derivative **9d**. The lipophilic pocket (right) and a cleft (left) shown in Fig. 2B were consistently occupied by either the phenyl moiety or the aliphatic ring system of the thieno[2,3-*b*]pyridines. The cyclopentane derivatives tended to have the aliphatic ring in the lipophilic pocket, whereas the larger eight-membered derivatives have their phenyl moiety in the lipophilic pocket as shown for derivative **9d** in Fig. 2B.

In general, a plausible binding is predicted to the TDP1 of the thieno[2,3-*b*]pyridines, supporting the results given in Table 3.

Chemical space

The calculated molecular descriptors (MW, log P, HD, HA, PSA and RB) for derivatives **6a–c**, **7a–k**, **8a–c**, **9a–d**, **10–12** are all within the boundaries of drug-like chemical space. The calculated values are given in Table S2 in the ESI.† All of the derivatives have two hydrogen bond donors and in most cases three rotatable bonds, which are within lead-like chemical space (for the definition of these regions see Zhu, 2012, references therein and Table S3 in the ESI†).[31] The polar surface area (PSA) for all derivatives is relatively low but does not quite reach lead-like chemical space.

Table 3 The inhibition of the TDP1 enzyme by the thieno[2,3-*b*]pyridine structural derivatives



	n	R	X_1	X_2	TDP1, IC_{50} (μM)
9b	3	H/H	Cl	H	1.0 ± 0.3
9d	3	H/H	H	CF_3	0.5 ± 0.1
10	0	H/H	CF_3	H	>15
11	1	O	H	CF_3	1.7 ± 0.2
12	1	O	Me	Cl	2.2 ± 0.2

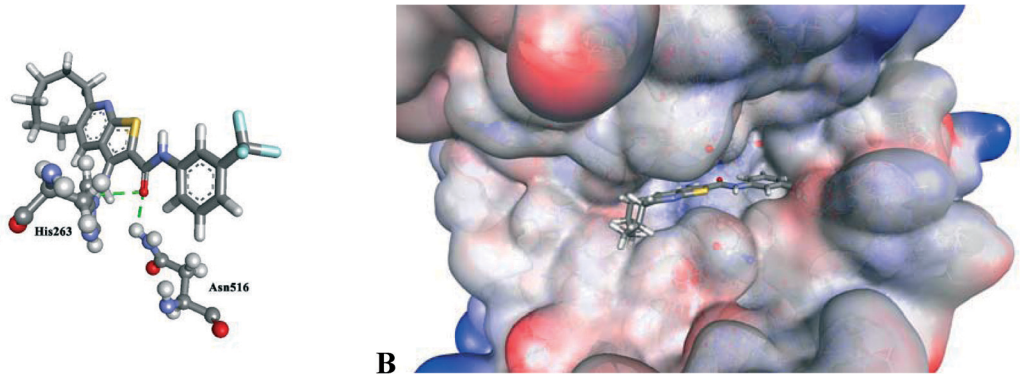


Fig. 2 The docked configuration of **9d** to the binding site of TDP1 using ASP. (A) Hydrogen bonds are depicted as green lines between ligand **9d** and the amino acid residues His263 and Asn516. (B) The phenyl group of **9d** occupies a lipophilic cavity to the right-hand side and the cyclooctane the lipophilic cleft. The protein surface is rendered. Red depicts a positive partial charge on the surface, blue depicts a negative partial charge and grey shows neutral/lipophilic areas.

Kinase screen

When the molecular structure of the thieno[2,3-*b*]pyridine molecular scaffold is considered then it contains the binding moiety mimicking the adenine moiety in ATP, *i.e.* the sulphur atom can act as a hydrogen bond acceptor and the N–H in the amine moiety as the hydrogen bond donor linked by three covalent bonds. In theory, this could make it a possible kinase inhibitor. Kinases are often targeted for anticancer therapeutics, *e.g.* checkpoint kinase 1 (Chk1).[32,33] Also, inhibition of Aurora-B kinase leads to DNA damage,[34] which might explain the potency of the thieno[2,3-*b*]pyridines. Molecular modelling investigations against the docking scaffolds of Chk1 and Aurora-B were conducted. Plausible binding configurations were predicted in the hinge regions forming hydrogen bonds to the amino acid residues Glu85/Cys87 (Chk1) and Ala213 (Aurora), *i.e.* blocking the adenine binding motif of ATP and possibly rendering the kinases inactive. This might explain the potency of the thieno[2,3-*b*]-pyridines. In order to check whether it inhibits kinases, derivative **12** was screened against 97 defined kinases using the KINOMEscan™ at 10 μM.[35,36]

In general, no hits were found against any of the 97 kinases, where a hit is defined as <35% of remaining activity compared to the untreated system at 100%. 48 of the targets have no inhibition (100%), 38 with >90% residual activity, four with >80%, three in the 70% range and two in the 60% range. The DYRK1B (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B) kinase had 57% activity. Finally, the RIO kinase 2 (RIOK2) had 43% residual activity. In view of these results it is highly unlikely that the thieno[2,3-*b*]-pyridines are exerting their anticancer effect *via* kinase inhibition and highlights the need to experimentally test modelling hypotheses. The full data set is in the ESI.†

Mouse xenograft model

In order to investigate the antitumor activity of the thieno-[2,3-*b*]pyridines derivative **12** was again selected for preliminary mouse xenograft studies. The 786-0 renal cancer cell line was chosen because **12** has a GI_{50} of ~100 nM for this cell line according to the NCI60 cell based assay.[2] A hundredfold concentration was desired *in vivo* compared to the GI_{50} *in vitro* or in this case ~10 μM. Since no pharmacokinetic information was available, a uniform and immediate distribution in the vascular system without accounting for the drug half-life or drug metabolism was assumed. Female athymic nude/beige mice were used for the subcutaneous xenograft model. An individual mouse weighs ~40 g and its volume was simply taken as 40 mL and the desired dose of **12** was derived as 0.16 mg for daily intraperitoneal injection. The poor water solubility of derivative **12** was a significant dose-limiting factor, *i.e.* the dose could only be doubled before reaching aqueous solubility limit. It was sought to determine whether **12** was able to inhibit 786-0 tumour initiation after a palpable tumour developed. As a control, the animals were injected with the vehicle (5% (vol/vol) DMSO). Despite poor solubility, intraperitoneal injection of 0.16 mg of **12** resulted in a partial inhibition of tumour growth; however, the difference did not quite reach a level of statistical

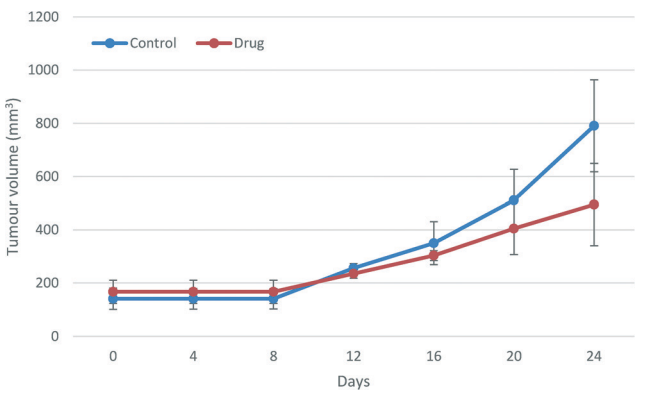


Fig. 3 The xenograft tumour volume of the renal cancer cell line 786-0 with compound **12** and a control for 24 days. The mice were administered the drug or vehicle in the morning. The error bars show the standard deviation.

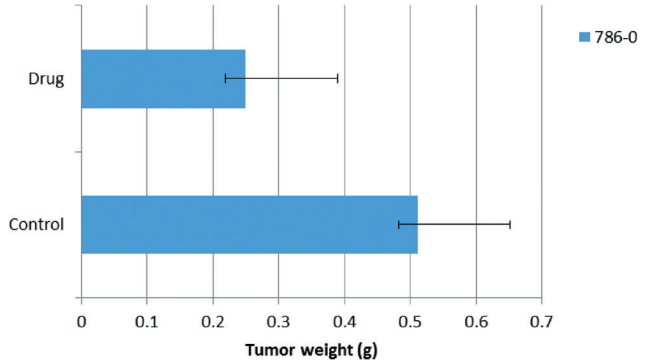


Fig. 4 The final mass of the xenograft tumours of the renal cancer cell line 786-0 with compound **12** and a control after 24 days of dosing. The error bars show the standard deviation.

significance as shown in Fig. 3 where the error bars slightly overlap.

The final mass of the tumours was determined and the results are depicted in Fig. 4. As for the tumour volumes, their mass is reduced by the dosing of compound **12** but statistical significance is not reached (p-value 0.13). The mice and the removed tumours are shown in the ESI.†

The injected mice showed evidence of mild spasm following injection, but their overall activity was not reduced. At the time of sacrifice, several mice in the treatment group showed signs of facial hirsutism (female facial hair) and dark circles around the eyes. Together, these symptoms suggest a possible effect on the adrenal glands, either by their damage by possible intraperitoneal precipitation of **12** or by direct inhibition of hormone synthesis. Due to the poor water solubility of **12**, these results can only be viewed as preliminary and interpretation is therefore limited. Nevertheless, this work has qualitatively demonstrated the anticancer potential of the thieno[2,3-*b*]pyridines and that aqueous solubility issues need to be resolved before consistent dosing of animals is achieved. Strategies to enhance water solubility are now being investigated.

Conclusions

A series of thieno[2,3-*b*]pyridines with different aliphatic ring sizes was tested against the NCI60 tumour panel. It was found that larger ring systems are favoured with derivative **9a**, containing an eight-membered ring, showing the greatest cytotoxicity. This series has a similar biological activity to their cyclohexanone counterparts,[1,2] except they have a better LC_{50} profile. Sub-micromolecular inhibition of the TDP1 DNA repair enzyme was measured for derivative **9d**, demonstrating that the thieno[2,3-*b*]pyridines inhibit this enzyme, which was supported with molecular modelling studies. In order to eliminate possible biological targets of thieno[2,3-*b*]pyridines, derivative **12** was screened against 97 kinases. No or weak affinity was observed, excluding this important enzyme class. A preliminary mouse xenograft study showed reduction of tumour size and mass upon intraperitoneal dosing with derivative **12** but the results did not reach a level of statistical significance. There are still many unanswered questions regarding this class of compounds in particular which other biomolecular targets are possibly modulated contributing to the anticancer efficacy and what is the pharmacokinetic profile? We are currently working on these topics and the results presented here have provided us with invaluable information on how to proceed.

Methodology

In silico searching. The thieno[2,3-*b*]pyridine derivatives were acquired from commercial sources using the eMolecules[37] and ZINC[38] web-based compound libraries. Substructure and Tanimoto similarity search methods were used to identify plausible inhibitors.[39] The compounds were purchased from ChemBridge,[15] ChemDiv,[16] Specs,[17] Key Organics[18] and InterBioScreen.[19] The obtained compounds were submitted to the National Cancer Institute's Developmental Therapeutic Program (DTP) where they were screened against a panel of sixty human tumour cell lines (NCI60, for further information see[10,40,41] and a complete description in the ESI†).

TDP1 assay. The recombinant TDP1 was purified to homogeneity by chromatography on Ni-chelating resin and phosphocellulose P11 as described[11,42] using plasmid pET 16B-Tdp1 kindly provided by Dr. K. W. Caldecott (University of Sussex, United Kingdom).

TDP1-biosensor 5′-(5,6 FAM-AAC GTC AGG GTC TTC C-BHQ1)-3′ was synthesized in the Laboratory of Medicinal Chemistry, Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia.

The TDP1 activity measurements were carried out as described.[25] Briefly, TDP1-biosensor with a final concentration of 50 nM was incubated in a volume of 200 μL containing TDP1 buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 7 mM β-mercaptoethanol) supplemented with purified 1.3 nM TDP1. The reactions were incubated at a constant temperature of 26 °C in a POLARstar OPTIMA fluorimeter (BMG LABTECH, GmbH) to measure fluorescence every 1 min ($Ex_{485\ nm}/Em_{520\ nm}$). The data were imported into MARS Data Analysis 2.0 program (BMG LABTECH) and the slope during the linear phase (here data from 0 to 7 min) was calculated.

Kinase assay. See Fabian *et al.*[35] and the DiscoverX website.[36] A complete description is given in the ESI.†

Mouse xenograft model. Female athymic nude/beige mice (Harlan Laboratories) were used for the subcutaneous xenograft model. All experiments were approved by the Institutional Animal Care and Use Committee at Johns Hopkins Hospital. 786-O cell lines were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinisation was stopped with medium containing 10% foetal bovine serum, and the cells were washed once in serum-free medium and re-suspended in PBS. Suspensions

comprising single cells with >90% viability were used for the injections. To establish RCC tumour xenografts, 786-O tumour cells were injected s.c. (3×10^6 cells) into the flanks of 6 week-old mice that were of 20 g average body weight. Tumours developed in all injected mice and were visible within a few days of implantation. The drug was solubilized in DMSO and further diluted in PBS/0.5% Tween-80. Drug (4.0 mg kg^{-1}) or control was administered every other day intraperitoneally beginning when the tumours had grown to a diameter of 7 mm. Tumour volumes were measured using calipers twice a week and calculated with the formula $V = 1/2(a^2b)$, where a is the short axis and b the long axis of the tumour. Treatment was continued until tumours in the control group grew to 15 mm in diameter, at which point the mice were sacrificed and the tumours were excised and weighed.

Molecular modelling. The compounds were docked to the crystal structure of TDP1 (PDB ID: 1MU7, resolution 2.0 Å),[43] which was obtained from the Protein Data Bank (PDB).[44,45] The Scigress Ultra version 7.7.0.47 program[46] was used to prepare the crystal structures for docking, *i.e.* hydrogen atoms were added, the co-crystallised tungsten(VI) ion was removed from TDP1 as well as crystallographic water molecules. The Scigress software suite was also used to build the inhibitors and the MM2 (ref. 47) force field was used to optimise the structures. The centre of the binding pocket for TDP1 was defined as the position of the hydrogen atom of His263, whose nitrogen formed a coordination bond with the tungsten ion (x = 8.312, y = 12.660, z = 35.452) with a 10 Å radius. Fifty docking runs were allowed for each ligand with default search efficiency (100%). The basic amino acid residues lysine and arginine were defined as protonated. Furthermore, aspartic and glutamic acids were assumed to be deprotonated. The GoldScore (GS),[48] ChemScore (CS),[49,50] ChemPLP[51] and ASP[52] scoring functions were implemented to validate the predicted binding modes and relative energies of the ligands using the GOLD v5.2 software suite.

The QikProp 3.2 (ref. 53) software package was used to calculate the molecular descriptors of the compounds. The reliability of the prediction power of QikProp is established for the molecular descriptors used in this study.[54]

Synthesis of compounds. All reactions were carried out under a nitrogen atmosphere in dry, freshly distilled solvents unless otherwise noted. All NMR spectra were recorded on either a Bruker Avance DRX 300 MHz or a 400 MHz spectrometer at ambient temperatures. Chemical shifts are reported relative to the solvent peak of chloroform (δ 7.26 for ^{1}H and δ 77.0 for ^{13}C), DMSO (δ 2.50 for ^{1}H and δ 39.5 for ^{13}C) or acetone (δ 2.05 for ^{1}H and δ 29.8 for ^{13}C). ^{1}H NMR data are reported as position (δ), relative integral, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of triplets; dd, doublet of doublets; tt, triplet of triplets; m, multiplet; br, broad peak; qd, quartet of doublets), coupling constant (J, Hz), and the assignment of the atom. ^{13}C NMR data are reported as position (δ) and assignment of the atom. All NMR assignments were performed using HSQC and HMBC experiments. High-resolution mass spectroscopy (HRMS) was carried out by either chemical ionization (CI) or electrospray ionization (ESI) on a MicroTOF-Q mass spectrometer. Unless noted, chemical reagents were used as purchased.

General procedure A: synthesis of salt. To a solution of sodium methoxide, which was prepared by adding sodium (1.25 equiv.) to dry methanol (7 equiv.) at 0 °C, was added dropwise a mixture of ketone **1** (1 equiv.) and methyl formate (1 equiv.) over 30 min. The reaction mixture was stirred at r.t. overnight before being diluted with ether and filtered to give the crude product **2** which was used in the next reaction without further purification.

General procedure B: synthesis of carbonitrile. A mixture of salt **2** (1 equiv.) and cyanothioacetamide (1.1 equiv.) in water with piperidinium acetate solution was heated at reflux for 4 h before being acidified with acetic acid while hot. The reaction mixture was allowed to cool to room temperature and stirred for a further 12 h before the residue was filtered off, washed with ice water and collected to give the crude carbonitrile **3** which was used in the next reaction without further purification [piperidinium acetate solution: 20% acetic acid, 45% water, 35% piperidine].

General procedure C: synthesis of bromoanilines. To a solution of aniline **4** (1 equiv.) and triethylamine (1.1 equiv.) in CH_2Cl_2 at 0 °C was added bromoacetyl bromide (1 equiv.) dropwise over 15 min and the mixture was stirred continuously for 1 h at 0 °C. The mixture was diluted with CH_2Cl_2, washed with 2M HCl, water, sat. aq. $NaHCO_3$, brine, and dried (Na_2SO_4) before the solvent was removed *in vacuo* to give the desired amides **5**.

General procedure D: synthesis of thieno[2,3-*b*]pyridine carboxamides. A mixture of 2-bromoacetamide **5** (1 equiv.), carbonitrile **3** (1 equiv.) and anhydrous sodium carbonate (1.06 equiv.) in absolute ethanol was stirred at reflux at 100 °C for 18 h. The mixture was cooled to room temperature and the solvent removed *in vacuo* to give the crude product which was washed with small amounts of ice water before being recrystallised from methanol to give the desired thieno-[2,3-*b*]pyridine **6**, **7** or **8**.

Sodium (*E*)- and (*Z*)-(2-oxocyclopentylidene)methanolate 2a. The reaction was carried out following general procedure A using cyclopentanone **1a** (1.92 ml, 21.7 mmol), methyl formate (1.34 ml, 21.7 mmol) and sodium (0.55 g, 23.9 mmol) in MeOH (4.5 ml, 0.11 mol) to give the title compound **2a** (1.5 g, 52%) as a white powder which was used in the next reaction without further purification.

Sodium (*E*)- and (*Z*)-(2-oxocyclohexylidene)methanolate 2b. The reaction was carried out following general procedure A using cyclohexanone **1b** (0.2 ml, 1.9 mmol), methyl formate (1.9 mmol) and sodium (44 mg, 1.9 mmol) in MeOH (0.43 ml, 11 mmol) to give the title compound **2b** (0.16 g, 57%) as a cream solid which was used in the next reaction without further purification.

Sodium (*E*)- and (*Z*)-(2-oxocycloheptylidene)methanolate 2c. The reaction was carried out following general procedure A using cycloheptanone **1c** (1.44 ml, 12.3 mmol), methyl

formate (0.76 ml, 12.3 mmol) and sodium (0.28 g, 12.3 mmol) in MeOH (2.8 ml, 0.069 mol) to give the title compound **2c** (1.33 g, 67%) as a white powder which was used in the next reaction without further purification.

2-Thioxo-2,5,6,7-tetrahydro-1*H*-cyclopenta[*b*]pyridine-3-carbonitrile 3a. The reaction was carried out following general procedure B using sodium salt **2a** (1.51 g, 11.2 mmol) and cyanothioacetamide (1.23 g, 12.3 mmol) in water (56 ml) with piperidinium acetate solution (8 ml) followed by acidification with acetic acid (17 ml) to give the title compound **3a** (0.76 g, 38%) as a red-brown solid which was used in the next reaction without further purification. M.p. 186–188 °C (lit. 190 °C).[46] δ_H (400 MHz, $(CD_3)_2SO$) 2.04–2.11 (2H, m, H-6), 2.72 (2H, t, J = 7.5 Hz, H-5), 2.92 (2H, t, J = 7.8 Hz, H-7), 8.01 (1H, s, H-4), 14.38 (1H, br s, NH). δ_C (100 MHz; $(CD_3)_2SO$) 22.4 (C-6), 28.9 (C-5), 31.1 (C-7), 113.1 (CN), 117.6 (C-3), 127.4 (C-4a), 141.8 (C-4), 160.3 (C-2), 176.0 (C-7a). The ^{1}H NMR data were in agreement with the literature values.[46]

2-Thioxo-1,2,5,6,7,8-hexahydroquinoline-3-carbonitrile 3b. The reaction was carried out following general procedure B using sodium salt **2b** (0.2 g, 1.4 mmol) and cyanothioacetamide (0.15 g, 1.5 mmol) in water (7.3 ml) with piperidinium acetate solution (1.4 ml) followed by acidification with acetic acid (2.2 ml) to give the title compound **3b** (77 mg, 30%) as a tan solid which was used in the next reaction without further purification. M.p. >230 °C (lit. 250–252 °C).[46] δ_H (400 MHz, $(CD_3)_2SO$) 1.62–1.73 (2 × 2H, m, H-6 and H-7), 2.47–2.51 (2H, m, H-8), 2.70 (2H, t, J = 6.3 Hz, H-5), 7.87 (1H, s, H-4), 13.88 (1H, br s, NH). δ_C (100 MHz, $(CD_3)_2SO$) 20.5 and 21.0 (C-6 and C-7), 25.5 (C-5), 26.7 (C-8), 113.4 (CN), 117.1 and 121.5 (C-3 and C-4a), 145.8 (C-4), 153.0 (C-2), 175.4 (C-8a). The ^{1}H NMR data were in agreement with the literature values.[46]

2-Thioxo-2,5,6,7,8,9-hexahydro-1*H*-cyclohepta[*b*]pyridine-3-carbonitrile 3c. The reaction was carried out following general procedure B using sodium salt **2c** (0.76 g, 4.71 mmol) and cyanothioacetamide (0.47 g, 4.71 mmol) in water (23.6 ml) with piperidinium acetate solution (4.4 ml) followed by acidification with acetic acid (7.1 ml) to give the title compound **3c** (0.57 mg, 59%) as a yellow-brown solid which was used in the next reaction without further purification. M.p. >230 °C (lit. 247 °C).[46] δ_H (400 MHz; $(CD_3)_2SO$) 1.50–1.63 (4H, m, H-8 and H-6), 1.74–1.80 (2H, m, H-7), 2.63–2.66 (2H, m, H-5), 2.94–2.96 (2H, m, H-9), 7.96 (1H, s, H-4), 13.95 (1H, s, NH). δ_C (100 MHz; $(CD_3)_2SO$) 24.9 (C-8), 26.5 (C-6), 31.1 (C-7), 31.6 (C-5), 32.3 (C-9), 112.8 (CN), 117.3 (C-3), 127.6 (C-4a), 145.8 (C-4), 159.5 (C-2), 174.9 (C-9a). The ^{1}H NMR data were in agreement with the literature values.[46]

2-Bromo-*N*-phenylacetamide 5a. The reaction was carried out following general procedure C using aniline **4a** (1.0 g, 11.0 mmol), bromoacetyl bromide (0.94 ml, 11.0 mmol) and triethylamine (1.64 ml, 12.0 mmol) in CH_2Cl_2 (25 ml) to give the title compound **5a** (2.29 g, 99%) as a cream solid. M.p. 133–135 °C (lit. 129–131 °C).[47] δ_H (300 MHz, $CDCl_3$) 4.02 (2H, s, H-2), 7.17 (1H, t, J = 7.5 Hz, H-4′), 7.36 (2H, t, J = 7.5 Hz, H-3′ and H-5′), 7.53 (2H, dd, J = 1.2, 7.5 Hz, H-2′ and H-6′), 8.19 (1H, br s, NH). δ_C (75MHz, $CDCl_3$) 29.5 (C-2), 120.1 (C-2′ and C-6′), 125.2 (C-4′), 129.1 (C-3′ and C-5′), 136.9 (C-1′), 163.4 (C═O). The ^{1}H and ^{13}C NMR data were in agreement with the literature values.[47]

2-Bromo-*N*-(3′-(trifluoromethyl)phenyl)acetamide 5b. The reaction was carried out following general procedure C using 3-(trifluoromethyl)aniline **4b** (0.40 g, 2.48 mmol), bromoacetyl bromide (0.22 mL, 2.48 mmol) and triethylamine (0.38 mL, 2.73 mmol) in CH_2Cl_2 (2 mL) to give the title product **5b** (0.70 g, 100%) as a brown solid. M.p. 78–81 °C (lit. 82 °C).[48] δ_H (400 MHz; $CDCl_3$) 4.04 (2H, s, H-2), 7.41–7.50 (2H, m, H-4′ and H-5′), 7.75 (1H, d, J = 8.0 Hz, H-6′), 7.83 (1H, s, H-2′), 8.28 (1H, s, NH). δ_C (100 MHz; $CDCl_3$) 29.2 (C-2), 116.8 (q, $^3J_{F/C}$ 3.7 Hz, C-2′), 121.8 (q, $^3J_{F/C}$ 3.8 Hz, C-4′), 123.1 (C-6′), 123.7 (q, $^1J_{F/C}$ 270.9 Hz, CF_3), 129.7 (C-5′), 131.6 (q, $^2J_{F/C}$ 32.5 Hz, C-3′) 137.4 (C-1′), 163.7 (C═O). The ^{1}H NMR data were in agreement with the literature values.[49]

2-Bromo-*N*-(2′-chlorophenyl)acetamide 5c. The reaction was carried out following general procedure C using 2-chloro aniline **4c** (2 ml, 19 mmol), bromoacetyl bromide (1.66 ml, 19 mmol) and triethylamine (2.9 ml, 21 mmol) in CH_2Cl_2 (50 ml) to give the title compound **5c** (3.79 g, 80%) as a light purple solid. M.p. 89–91 °C. δ_H (400 MHz, $CDCl_3$) 4.07 (2H, s, H-2), 7.10 (1H, dt, J = 7.6, 1.6 Hz, H-4′), 7.30 (1H, dt, J = 7.6, 1.6 Hz, H-5′), 7.40 (1H, dd, J = 8.0, 1.6 Hz, H-3′), 8.34 (1H, dd, J = 8.0, 1.2 Hz, H-6′), 8.79 (1H, br s, NH). δ_C (100 MHz, $CDCl_3$) 29.6 (C-2), 121.2 (C-6′), 123.5 (C-2′), 125.4 (C-4′), 127.7 (C-5′), 129.2 (C-3′), 133.9 (C-1′), 163.4 (C═O). ν_{max}(ATR)/cm^{-1} 3262, 3028, 2963, 1663, 1538, 1445, 1131, 761, 663. m/z (ESI$^+$) 270 (79%)/272 (100%)/274 (24%), [MNa$^+$, 100%]; 227 (7%); 159 (4%). HRMS (ESI$^+$): found 269.9289, $C_8H_7{}^{79}Br^{35}ClNNaO$ requires 269.9292.

2-Bromo-*N*-(2′-fluorophenyl)acetamide 5d. The reaction was carried out following general procedure C using 2-fluoroaniline **4d** (1 ml, 10 mmol), bromoacetyl bromide (0.9 ml, 10 mmol) and triethylamine (1.58 ml, 11 mmol) in CH_2Cl_2 (25 ml) to give the title compound **5d** (2.3 g, 96%) as a light brown solid. M.p. 73–75 °C. δ_H (300 MHz, $CDCl_3$) 4.04 (2H, s, H-2), 7.07–7.17 (3H, m, H-3′, H-4′ and H-5′), 8.21–8.26 (1H, m, H-6′), 8.42 (1H, br s, NH). δ_C (75MHz, $CDCl_3$) 29.2 (C-2), 114.8 and 115.1 (C-4′ and C-5′), 121.6 (C-6′), 124.6 (d, $^2J_{F/C}$ 3.8 Hz, C-3′), 125.3 (d, $^2J_{F/C}$ 7.7 Hz, C-1′), 152.7 (d, $^1J_{F/C}$ 243.0 Hz, C-2′), 163.5 (C═O). The ^{1}H and ^{13}C NMR data were in agreement with the literature values.[50]

2-Bromo-*N*-(2′-(trifluoromethyl)phenyl)acetamide 5e. The reaction was carried out following general procedure C using 2-aminobenzotrifluoride **4e** (1.2 g, 7.5 mmol), bromoacetyl bromide (0.65 ml, 7.5 mmol) and triethylamine (1.14 ml, 8.2 mmol) in CH_2Cl_2 (25 ml) to give the title compound **5e** (1.48 g, 70%) as a tan solid. M.p. 95–98 °C. δ_H (400 MHz, $CDCl_3$) 4.06 (2H, s, H-2), 7.28 (1H, t, J = 7.6 Hz, H-4′), 7.58 (1H, t, J = 7.6 Hz, H-5′), 7.64 (1H, d, J = 7.6 Hz, H-3′), 8.16 (1H, d, J = 8.0 Hz, H-6′), 8.60 (1H, br s, NH). δ_C (100 MHz, $CDCl_3$) 29.2 (C-2), 120.7 (q, $^2J_{F/C}$ 29.8 Hz, C-2′), 123.8 (q, $^1J_{F/C}$ 271.4 Hz, CF_3), 124.1 (C-6′), 125.2 (C-4′), 126.2 (q,$^3J_{F/C}$ 5.1 Hz, C-3′), 132.9 (C-5′), 134.4 (C-1′), 163.8 (C═O). ν_{max}(ATR)/cm^{-1} 3261, 3026,

2963, 1663, 1533, 1454, 1167, 1110, 662. *m*/*z* (ESI^+) 304(100%)/306(96%), [MNa^+, 100%]; 227(21%); 159 (10%). HRMS (ESI^+): found 303.9545, $C_9H_7{}^{79}BrF_3NNaO$ requires 303.9555. Found 305.9530, $C_9H_7{}^{81}BrF_3NNaO$ requires 305.9535.

3-Amino-*N*-phenyl-6,7-dihydro-5*H*-cyclopenta[*b*]thieno[3,2-*e*]pyridine-2-carboxamide 6a. The reaction was carried out following general procedure D using 2-bromo-*N*-phenylacetamide **5a** (60 mg, 0.28 mmol), carbonitrile **3a** (50 mg, 0.28 mmol) and anhydrous sodium carbonate (31 mg, 0.30 mmol) in absolute ethanol (2 ml) to give the title compound **6a** (40 mg, 55%) as a black solid. M.p. 199–201 °C. δ_H (400 MHz; $(CD_3)_2SO$) 2.19–2.27 (2H, m, H-6), 3.07–3.14 (4H, m , H-5 and H-7), 7.13–7.18 (1H, m, H-4′), 7.38–7.42 (4H, m, NH_2 and H-3′ and H-5′), 7.77–7.79 (2H, m, H-2′ and H-6′), 8.37 (1H, s, H-4), 9.43 (1H, br s, NH). δ_C (100 MHz; $(CD_3)_2SO$) 23.2 (C-6), 29.6 (C-5), 33.6 (C-7), 95.7 (C-2), 121.0 (C-2′ and C-6′), 123.3 (C-4′), 124.2 (C-3a), 128.4 (C-3′ and C-5′), 128.7 (C-8a), 133.3 (C-4), 139.0 (C-1′), 147.0 (C-3), 157.2 (C-4a), 164.0 (C═O), 167.8 (C-7a). ν_{max}(ATR)/cm^{-1} 3410, 3288, 2952, 1684, 1590, 1509, 1487, 1394, 1226, 742. *m*/*z* (ESI^+) 332 (100%), [MNa^+, 100%]; 310 (60%), [MH^+, 60%]; 219 (80%); 159 (15%). HRMS (ESI^+): found 332.0818, $C_{17}H_{15}N_3NaOS$ requires 332.0828.

3-Amino-*N*-phenyl-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide 7a. The reaction was carried out following general procedure D using 2-bromo-*N*-phenylacetamide **5a** (105 mg, 0.5 mmol), carbonitrile **3b** (93 mg, 0.5 mmol) and anhydrous sodium carbonate (55 mg, 0.52 mmol) in absolute ethanol (2 ml) to give the title compound **7a** (136 mg, 86%) as a green solid. M.p. >230 °C. δ_H (300 MHz, $(CD_3)_2SO$) 1.76–1.89 (2 × 2H, m, H-6 and H-7), 2.88 (2H, t, *J* = 6.3 Hz, H-5), 2.95 (2H, t, *J* = 6.2 Hz, H-8), 7.06 (1H, tt, *J* = 7.2, 1.2 Hz, H-4′), 7.28–7.34 (4H, m, NH_2, H-3′ and H-5′), 7.66–7.70 (2H, m, H-2′ and H-6′), 8.18 (1H, s, H-4), 9.33 (1H, s, NH). δ_C (75MHz, $(CD_3)_2SO$) 22.3 and 22.4 (C-6 and C-7), 28.3 (C-5), 32.5 (C-8), 95.8 (C-2), 121.1 (C-2′ and C-6′), 123.3 (C-4′), 124.4 (C-3a), 128.3 (C-8a), 128.3 (C-3′ and C-5′), 130.8 (C-4), 139.0 (C-1′), 146.8 (C-3), 155.9 (C-9a), 159.1 (C-4a), 164.1 (C═O). ν_{max}(ATR)/cm^{-1} 3448, 3333, 3180, 2943, 1607, 1590, 1522, 1495, 1318, 1166, 745. *m*/*z* (ESI^+) 346 (37%), [MNa^+, 37%]; 324 (100%), [MH^+, 100%]; HRMS (ESI^+): found 324.1161, $C_{18}H_{18}N_3OS$ requires 324.1165.

3-Amino-*N*-(3′-(trifluoromethyl)phenyl)-5,6,7,8-tetrahydrothieno-[2,3-*b*]quinoline-2-carboxamide 7b. The reaction was carried out following general procedure D using 2-bromo-*N*-(3′-(trifluoromethyl)phenyl)acetamide **5b** (138 mg, 0.5 mmol), carbonitrile **3b** (93 mg, 0.5 mmol) and anhydrous sodium carbonate (55 mg, 0.52 mmol) in absolute ethanol (2 ml) to give the title compound **7b** (43 mg, 22%) as a yellow solid. M.p. 193–194 °C. δ_H (300 MHz, $(CD_3)_2CO$) 1.79–1.95 (2 × 2H, m, H-6 and H-7), 2.87–2.98 (2 × 2H, m, H-5 and H-8), 7.09 (2H, br s, NH_2), 7.40 (1H, dt, *J* = 7.7, 0.7 Hz, H-4′), 7.54 (1H, t, *J* = 7.9 Hz, H-5′), 7.99 (1H, dt, *J* = 8.8, 0.6 Hz, H-2′), 8.04 (1H, s, H-4), 8.27 (1H, s, H-6′), 8.87 (1H, br s, NH). δ_C (75 MHz, $(CD_3)_2CO$) 23.5 and 23.6 (C-6 and C-7), 29.5 (C-5), 33.7 (C-8), 97.3 (C-2), 117.8 (q, $^3J_{F/C}$ 4.0 Hz, C-2′), 120.5 (q, $^3J_{F/C}$ 3.9 Hz, C-4′), 124.7 (q, $^5J_{F/C}$ 1.0 Hz, C-6′), 125.3 (q, $^1J_{F/C}$ 270.0 Hz, CF_3), 125.4 (C-3a), 129.6 (C-4a), 130.3 (C-5′), 131.1 (C-4), 131.2 (q, $^2J_{F/C}$ 31.6 Hz, C-3′), 141.0 (C-1′), 148.5 (C-3), 157.4 (C-9a), 160.6 (C-8a), 165.4 (C═O). ν_{max}(ATR)/cm^{-1} 3441, 3326, 2940, 1591, 1541, 1482, 1439, 1331, 1250, 1165 , 662. *m*/*z* (ESI^+) 392 (100%), [MH^+, 100%]; 381 (22%); 227 (22%); 159 (8%). HRMS (ESI^+): found 392.1033, $C_{19}H_{17}F_3N_3OS$ requires 392.1039.

3-Amino-*N*-(2′-chlorophenyl)-5,6,7,8-tetrahydrothieno[2,3-*b*]-quinoline-2-carboxamide 7c. The reaction was carried out following general procedure D using 2-bromo-*N*-(2′-chlorophenyl)-acetamide **5c** (261 mg, 1.1 mmol), carbonitrile **3b** (200 mg, 1.1 mmol) and anhydrous sodium carbonate (0.12 g, 1.1 mmol) in absolute ethanol (4 ml) to give the title compound **7c** (292 mg, 78%) as a brown solid. M.p. 228–229 °C. δ_H (400 MHz, $(CD_3)_2SO$) 1.77–1.91 (2 × 2H, m, H-6 and H-7), 2.88 (2H, t, *J* = 6.2 Hz, H-5), 2.95 (2H, t, *J* = 6.2 Hz, H-8), 7.22–7.27 (3H, m, H-4′ and NH_2), 7.36 (1H, dt, *J* = 1.2, 7.8 Hz, H-5′), 7.53 (1H, dd, *J* = 1.2, 8.0 Hz, H-3′), 7.66 (1H, dd, *J* = 1.1, 7.9 Hz, H-6′), 8.18 (1H, s, H-4), 9.05 (1H, s, NH). δ_C (100 MHz, $(CD_3)_2SO$) 22.3 and 22.4 (C-6 and C-7), 28.3 (C-5), 32.5 (C-8), 95.7 (C-2), 124.5 (C-3a), 126.7 (C-4′), 127.4 and 127.5 (C-5′ and C-6′), 128.4 (C-2′), 128.7 (C-4a), 129.4 (C-3′), 130.9 (C-4), 135.2 (C-1′), 146.8 (C-3), 155.9 (C-9a), 159.2 (C-8a), 163.8 (C═O). ν_{max}(ATR)/cm^{-1} 3404, 3283, 2928, 1645, 1585, 1504, 1432, 1303, 1187, 746, 691. *m*/*z* (ESI^+) 358 (100%)/360 (38%), [MH^+, 100%]; HRMS (ESI^+): found 358.0778, $C_{18}H_{17}{}^{35}ClN_3OS$ requires 358.0775. Found 360.0759, $C_{18}H_{17}{}^{37}ClN_3OS$ requires 360.0750.

3-Amino-*N*-(2′-fluorophenyl)-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide 7d. The reaction was carried out following general procedure D using 2-bromo-*N*-(2′-fluorophenyl)acetamide **5d** (114 mg, 0.5 mmol), carbonitrile **3b** (93 mg, 0.5 mmol) and anhydrous sodium carbonate (55 mg, 0.52 mmol) in absolute ethanol (2 ml) to give the title compound **7d** (85 mg, 51%) as a green solid. M.p. >230 °C. δ_H (400 MHz, $(CD_3)_2SO$) 1.80–1.91 (2 × 2H, m, H-6 and H-7), 2.88 (2H, t, *J* = 6.4 Hz, H-5), 2.95 (2H, t, *J* = 6.4 Hz, H-8), 7.17–7.28 (5H, m, H-3′, H-4′, H-5′ and NH_2), 7.51 (1H, t, *J* = 7.8 Hz, H-6′), 8.18 (1H, s, H-4), 9.22 (1H, s, NH). δ_C (100 MHz, $(CD_3)_2SO$) 22.3 and 22.4 (C-6 and C-7), 28.3 (C-5), 32.5 (C-8), 95.5 (C-2), 115.6 (d, $^2J_{F/C}$ 19.9 Hz, C-3′), 124.1 (d, $^4J_{F/C}$ 3.1 Hz, C-5′), 124.4 (C-3a), 125.8 (d, $^2J_{F/C}$ 12.3 Hz, C-1′), 126.7 (d, $^3J_{F/C}$ 7.7 Hz, C-4′), 127.5 (C-6′), 128.3 (C-4a), 130.9 (C-4), 146.8 (C-3), 156.2 (d, $^1J_{F/C}$ 245.4 Hz, C-2′), 156.0 (C-9a), 159.1 (C-8a), 163.9 (C═O). ν_{max}(ATR)/cm^{-1} 3411, 3275, 2938, 1645, 1606, 1507, 1447, 1319, 1244, 1186. *m*/*z* (ESI^+) 342 (100%), [MH^+, 100%]; HRMS (ESI^+): found 342.1081, $C_{18}H_{17}FN_3OS$ requires 342.1071.

3-Amino-*N*-(2′-(trifluoromethyl)phenyl)-5,6,7,8-tetrahydrothieno-[2,3-*b*]quinoline-2-carboxamide 7e. The reaction was carried out following general procedure D using 2-bromo-*N*-(2′-(trifluoromethyl)-phenyl)acetamide **5e** (138 mg, 0.5 mmol), carbonitrile **3b** (93 mg, 0.5 mmol) and anhydrous sodium carbonate (55 mg, 0.52 mmol) in absolute ethanol (2 ml) to give the title compound **7e** (124 mg, 65%) as a brown solid. M.p. 188–190 °C. δ_H (400 MHz, $(CD_3)_2SO$) 1.78–1.91 (2 × 2H, m, H-6 and H-7), 2.88 (2H, t, *J* = 6.4 Hz, H-5), 2.95 (2H, t, *J* = 6.4 Hz, H-8), 7.21 (2H, s, NH_2), 7.49 (1H, t, *J* = 7.6 Hz, H-4′), 7.58 (1H, d, *J* = 7.6 Hz, H-6′), 7.71 (1H, t, *J* = 8.0 Hz, H-5′), 7.76 (1H, d, *J* = 8.0 Hz,

H-3′), 8.18 (1H, s, H-4), 9.12 (1H, s, NH). δ_C (100 MHz, $(CD_3)_2SO$) 22.2 and 22.4 (C-6 and C-7), 28.3 (C-5), 32.5 (C-8), 95.4 (C-2), 123.7 (q, $^1J_{F/C}$ 271.9 Hz, CF_3), 124.4 (C-3a), 125.7 (q, $^2J_{F/C}$ 28.9 Hz, C-2′), 126.4 (q, $^3J_{F/C}$ 4.8 Hz, C-3′), 126.8 (C-4′), 128.3 (C-4a), 130.6 (C-6′), 130.9 (C-4), 132.9 (C-5′), 135.9 (C-1′), 146.8 (C-3), 155.9 (C-9a), 159.1 (C-8a), 164.6 (C═O). ν_{max}(ATR)/cm^{-1} 3435, 3305, 2931, 1653, 1588, 1525, 1453, 1290, 1167, 1106, 659. *m*/*z* (ESI^+) 392 (100%), [MH^+, 100%]; HRMS (ESI^+): found 392.1042, $C_{19}H_{17}F_3N_3OS$ requires 392.1039.

3-Amino-*N*-phenyl-6,7,8,9-tetrahydro-5*H*-cyclohepta[*b*]thieno[3,2-*e*]pyridine-2-carboxamide 8a. The reaction was carried out following general procedure D using 2-bromo-*N*-phenylacetamide **5a** (70 mg, 0.34 mmol), carbonitrile **3c** (70 mg, 0.34 mmol) and anhydrous sodium carbonate (38 mg, 0.36 mmol) in absolute ethanol (1.8 ml) to give the title compound **8a** (110 mg, 91%) as a brown solid. M.p. > 230 °C. δ_H (400 MHz; $(CD_3)_2SO$) 1.65–1.70 (2 × 2H, m, H-8 and H-6), 1.84–1.90 (2H, m, H-7), 2.88–2.91 (2H, m, H-5), 3.08–3.11 (2H, m, H-9), 7.08 (1H, tt, *J* = 7.3, 1.0 Hz, H-4′), 7.29 (2H, s, NH_2), 7.34 (2H, td, *J* = 8.0, 2.0 Hz, H-3′ and H-5′), 7.71 (2H, dd, *J* = 8.5, 1.0 Hz, H-2′ and H-6′), 8.21 (1H, s, H-4), 9.36 (1H, s, NH). δ_C (100 MHz; $(CD_3)_2SO$) 26.2 (C-8), 27.9 (C-6), 31.6 (C-7), 34.5 (C-5), 38.8 (C-9), 96.1 (C-2), 121.1 (C-2′ and C-6′), 123.3 (C-4′), 124.3 (C-3a), 128.4 (C-3′ and C-5′), 130.4 (C-4), 134.1 (C-4a), 139.0 (C-1′), 147.0 (C-3), 155.5 (C-10a), 164.0 (C═O), 164.8 (C-9a). ν_{max}(ATR)/cm^{-1} 3382 (NH amine), 3287 (NH amide), 1647 (C═O amide), 1596 (NH amide), 1507 (C═C aromatic), 1447 (CH alkane), 1319 (C–N aromatic). *m*/*z* (ESI^+): 360 (36%), [MNa^+, 36%]; 338 (100%), [MH^+, 100%]. HRMS (ESI^+): found MH^+ 338.1321, $C_{19}H_{20}N_3OS$ requires 338.1322.

3-Amino-*N*-(3-(trifluoromethyl)phenyl)-6,7,8,9-tetrahydro-5*H*-cyclohepta[*b*]thieno[3,2-*e*]pyridine-2-carboxamide 8b. The reaction was carried out following general procedure D using 2-bromo-*N*-(3′-(trifluoromethyl)phenyl)acetamide **5b** (60 mg, 0.20 mmol), carbonitrile **3c** (40 mg, 0.20 mmol) and anhydrous sodium carbonate (20 mg, 0.22 mmol) in absolute ethanol (1.3 ml) to give the title compound **8b** (70 mg, 90%) as a tan solid. M.p. 213–215 °C. δ_H (400 MHz; $(CD_3)_2SO$) 1.65–1.70 (4H, m, H-8 and H-6), 1.84–1.90 (2H, m, H-7), 2.88–2.90 (2H, m, H-5), 3.09–3.11 (2H, m, H-9), 7.40–7.43 (3H, m, NH_2 and H-4′), 7.57 (1H, t, *J* = 8.0 Hz, H-5′), 8.01 (1H, d, *J* = 8.0 Hz, H-6′), 8.24 (2H, br s, H-2′ and H-4), 9.68 (1H, br s, NH). δ_C (100 MHz; $(CD_3)_2SO$) 26.2 (C-8), 27.9 (C-6), 31.6 (C-7), 34.5 (C-5), 38.8 (C-9), 95.3 (C-2), 116.8 (C-2′), 119.4 (C-4′), 124.2 (C-6′), 124.2 (C-3a), 129.3 (C-5′), 129.6 (CF_3), 130.6 (C-4), 134.2 (C-4a), 140.0 (C-1′), 147.8 (C-3), 155.6 (C-10a), 164.3 (C═O), 165.1 (C-9a). ν_{max}(ATR)/cm^{-1} 3396 (NH amine), 3309 (NH amide), 1649 (C═O amide), 1597 (NH amide), 1541 (C═C aromatic), 1439 (CH alkane), 1165 (C–N aliphatic). *m*/*z* (ESI^+): 428 (20%), [MNa^+, 20%]; 406 (100%) [MH^+, 100%]. HRMS (ESI^+): found MH^+ 406.1190, $C_{20}H_{19}F_3N_3OS$ requires 406.1195.

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