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Identification of anticancer agents based on the thieno[2,3-*b*]pyridine and 1*H*-pyrazole molecular scaffolds

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Abstract

Structural similarity search of commercially available analogues of thieno[2,3-*b*]pyridine and 1*H*-pyrazole derivatives, known anticancer agents, resulted in 717 hits. These were docked into the Phosphoinositide specific-phospholipase C (PLC) binding pocket, the putative target of the compounds, to further focus the selection. Thirteen derivatives of the thieno[2,3-*b*]pyridines were identified and tested against the NCI60 panel of human tumour cell lines. The most active derivative **1** was most potent against the MDA-MB-435 melanoma cell line with GI_{50} at 30 nM. Also, it was found that a piperidene moiety is tolerated on the thieno[2,3-*b*]pyridine scaffold with GI_{50} = 296 nM (MDA-MB-435) for derivative **10** considerably expanding the structure activity relationship for the series. For the 1*H*-pyrazoles four derivatives were identified using the *in silico* approach and additionally ten were synthesised with various substituents on the phenyl moiety to extend the structural activity relationship but only modest anticancer activity was found.

Keywords: Similarity searching; phospholipase C; molecular modelling; NCI60; MTT; Structure Activity Relationship (SAR).

Introduction

The phospholipase C (PLC) family comprises a series of enzymes which regulate many cellular growth functions, making them interesting targets for cancer therapy.[1, 2] The thieno[2,3-*b*]pyridines and 1*H*-pyrazoles were initially discovered as potential inhibitors of PLC isoforms by virtual high throughput screen (vHTS) using the available crystal structure of the PLC-δ1 isoform.[1] The molecular structures of the thieno[2,3-*b*]pyridines and 1*H*-pyrazoles are shown in Figure 1. *In vitro* testing for the thieno[2,3-*b*]pyridines revealed that the most potent analogues had growth inhibitory activity at low nanomolar concentrations against the National Cancer Institute's NCI60 human tumour cell line panel.[3-7] The administration of thieno[2,3-*b*]pyridines causes the breast cancer cell line MDA-MB-231 to be severely growth restricted, rounded and blebbing of the plasma membrane, G_2/M phase population increase in the cell cycle and decrease in motility as reflected in slowed proliferation in scratch assays. [8, 9] These effects on MDA-MB-231 are in line with inhibition of the PLC-δ1 and δ2 isoforms making them the most plausible target for this class of compounds.[8, 9] The thieno[2,3-*b*]pyridine structural motive appears in many bioactive compounds with various mechanisms of action: antagonists of adenosine A_{2A} receptor antagonist (a G protein-coupled receptor),[10] as inhibitors of copper trafficking interfaces of Atox1 and CCS proteins, for inhibition of tumour growth and Wilson's disease,[11] hepatocellular carcinoma-specific anti-tumour activity[12, 13] and tubulin inhibitors.[14] The 1*H*-pyrazoles also showed, albeit more modest, activity against the NCI60 human tumour cell line panel.[4]

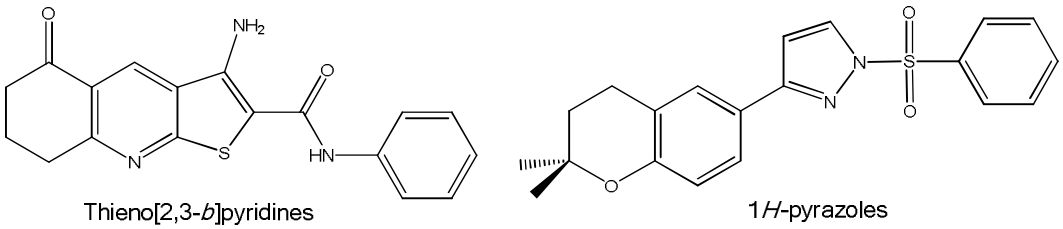


Figure 1. The basic molecular structures of the anticancer thieno[2,3-*b*]pyridines (3-amino-5-oxo-N-phenyl-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide (left) and 1*H*-pyrazoles (3-(chroman-6-yl)-1-(phenylsulfonyl)-1*H*-pyrazoles), (right).

The aim of this project is to explore the chemical space around the active thieno[2,3-*b*]pyridines and 1*H*-pyrazoles extending their structural activity relationship (SAR) profiles by identifying their commercially available analogues using similarity searching and substructure techniques. To focus the SAR study on the most promising analogues and reduce the number of compounds for testing docking against the PLC-δ1 lipase was conducted.

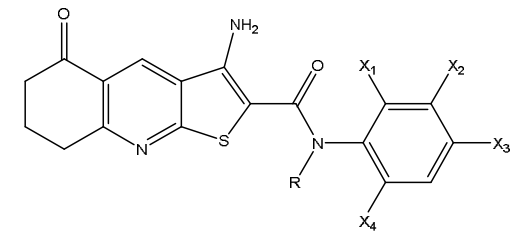
Results and Discussion

Thieno[2,3-*b*]pyridines

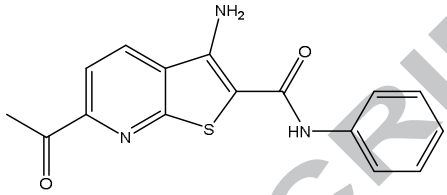
Structural similarity search was conducted on eMolecules,[15] a commercial compound database using the thieno[2,3-*b*]pyridine structure shown in Fig. 1. Six hundred and fifty-four (654) compounds were found, using similarity coefficient of 0.7, and docked against the PLC-δ1 scaffold (see Methodology). Ligands with no or weak hydrogen bonding (HB < 1) were eliminated as well as those with low predicted binding energies, (GS < 45, CS < 20, ChemPLP < 45 and ASP < 25). The remaining candidates were checked for consensus for the best predicted poses between the scoring functions used and whether a plausible binding mode was predicted, *e.g.*, unstrained poses and whether lipophilic moieties were jutting into the aqueous phase causing entropic penalty. This resulted in identification of thirteen virtual hits, which were tested using the NCI60 human tumour cell panel at 10 μM concentration. Two distinct structural classes of thieno[2,3-*b*]pyridines (**1**-**6** and **8**-**12**) and two singletons (**7**

and **13**) were found and their molecular structures are shown in Table 1 with their mean growth of the NCI60 panel.

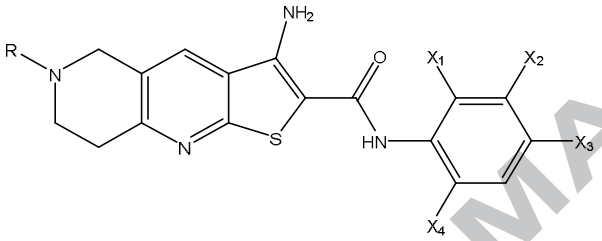
Table 1 Anti-proliferative activity of the thieno[2,3-*b*]pyridines**.**



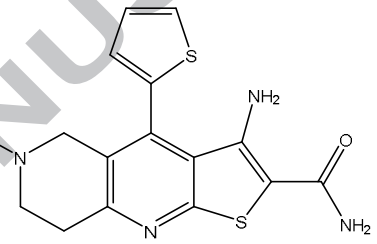
Derivatives 1-6



Derivative 7



Derivatives 8-12



Derivative 13

	R	X_1	X_2	X_3	X_4	NCI Mean[a]
1	H	H	Me	H	H	29.9
2	H	Et	H	H	H	25.4
3	H	Et	H	H	Me	25.2
4	H	H	Me	Me	H	26.3
5	H	H	Acetyl	H	H	97.9[b]
6	Phenyl	H	H	H	H	99.0
7	-	-	-	-	-	101.6
8	Benzyl	Me	H	Me	Me	27.3
9	Benzyl	H	Me	H	H	100.6
10	Benzyl	H	OMe	H	H	52.7
11	Benzyl	H	H	H	H	59.4
12	Isopropyl	H	H	H	H	98.8
13	-	-[c]	-[c]	-[c]	-[c]	24.8

[a]Values represent relative growth (%) versus control as an average for 60 human tumour cell lines (NCI60). All compounds were tested at 10 μM. [b]From ref.[5] [c]No phenyl moiety.

It is clear that derivatives **1 – 4, 8** and **13** are the most active with growth of ~25% as compared to untreated cells (100%). When the substitution patterns of the phenyl rings are

considered for derivatives **1 - 6**, it can be concluded that small alkyl substitutions at *ortho* (X_1) and *meta* (X_2) positions are the most advantageous whilst electron withdrawing groups such as acetyl (derivative **5**) at the *meta* (X_2) position is detrimental as compared to the un-substituted derivative that has a relative growth of 30.2%.[4] It has been previously reported that substitution of on the *ortho* position gives good results such as with fluoro (33.3%) and methyl (42.0%) groups but larger groups are not tolerated, *e.g.*, CF_3 (81.6%) and methoxy (61.3%).[4, 5] Furthermore, *meta* substitution is also favourable with fluoro at 31.4% and chloro at 36.3% relative growths. [4, 5] This is in line with the results presented in Table 1.[4, 5] Interestingly, methyl substitutions at both the *meta* (X_2) and *para* (X_3) positions as for derivative **4** has similar effect as derivatives **1 - 3** suggesting the importance of alkyl substitutions at the *meta* position (X_2) to anticancer activity since it has been previously seen that *para* substitution is clearly detrimental to the anticancer activity of the thieno[2,3-*b*]pyridines, *e.g.*, *para* methoxy and *para* chloro derivatives have no effect on the growth of the tumour cells .[4, 5] No anticancer activity was found for the *N,N*-diphenyl derivative (**6**).

Derivative **7** is structurally unique in that it has a bicyclic instead of tricyclic ring system. It exhibited no activity; apparently conserving the tricyclic ring system is important for anticancer activity of this class of compounds as has been previously observed.[7]

In general, the thieno[2,3-*b*][1,6]naphthyridine derivatives **9 - 13** exhibited more modest anticancer effects than the cyclohexanone-thieno[2,3-*b*]pyridines (**1**-**6**) with the exception of derivatives **8** and **13**, which had relative growth of 27% and 25%, respectively. In contrast to the cyclohexanone-thieno[2,3-*b*]pyridines (**1**-**6**), methyl *meta* (X_2) substitution is detrimental to the anticancer activity of the thieno[2,3-*b*][1,6]naphthyridines as seen for derivative **9**. A possible explanation for this effect is that the two series are inhibiting different but related enzyme classes. Changing the R group from benzyl to isopropyl (derivative **12**) had a detrimental effect indicating that the benzyl group is occupying a lipophilic pocket (see Fig.

2). Derivative **13** is different in that it does not have a phenyl group but a thiophenyl on the pyridine moiety and can therefore be considered as a novel analogue in this chemical series. Nevertheless, it is potent in reducing growth of the cancer cells.

Derivatives **1** – **4** and **8**, **10**, **11** and **13** were selected for dose response testing based on their favourable growth inhibition at 10 μM and the results are given in Table 2. The Growth Inhibition at 50% (GI_{50}) and Total Growth Inhibition (TGI) were derived from these measurements.[3] GI_{50} is the concentration for 50% of maximal inhibition of cell proliferation and therefore reflects the cytostatic concentration whereas TGI is the compound concentration resulting in total growth inhibition. Five tumour cell lines were particularly affected: MDA-MB-435 (melanoma), MDA-MB-468 (breast), A498 (renal), SF-539 (central nervous system, CNS) and K-562 (leukaemia).

Table 2 The dose response for the most active compounds shown for five tumour cell lines in nano-molar (nM) concentration.

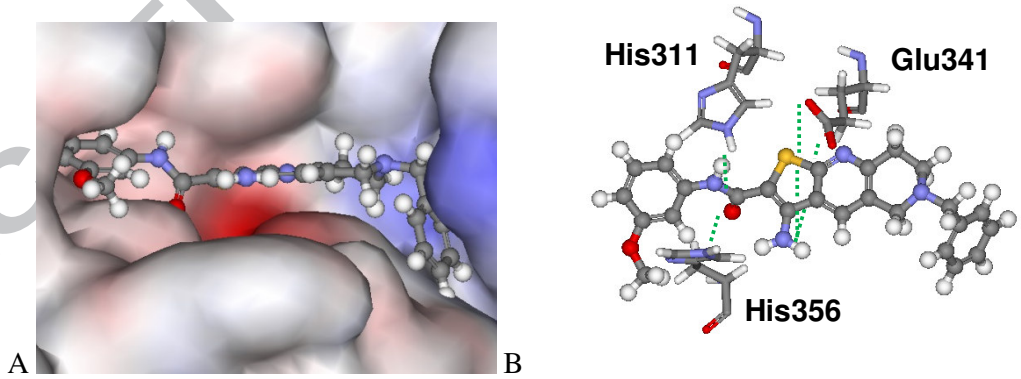
	MDA-MB-435		MDA-MB-468		A498		SF-539		K-562	
	GI_{50}[a]	TGI[b]	GI_{50}[a]	TGI[b]	GI_{50}[a]	TGI[b]	GI_{50}[a]	TGI[b]	GI_{50}[a]	TGI[b]
1[c]	31/30	115/138	143/80	Y/2660	139/31	701/915	237/167	821/462	76/144	X/X
2[c]	60/46	336/225	235/209	44700/Y	270/180	44800/682	335/416	2690/3710	372/203	X/X
3[c]	171/148	426/X	261/377	X/60600	224/178	866/665	320/334	Y/Y	341/522	31800/15800
4[c]	152/121	443/322	229/314	Y/X	274/198	Y/658	404/443	Y/Y	468/Y	35000/Y
8	399	4380	1780	32200	1310	7710	2440	7790	903	X
10	296	11000	2600	61400	3820	24400	1990	6560	530	X
11	3430	71600	17000	X	1780	Y	2780	X	9100	X
13	3130	X	1750	4860	2580	7270	2350	6790	4990	X

[a]GI_{50} (50% growth inhibition). [b]TGI (total growth inhibition). [c]Tested twice and both values are given. X: TGI not achieved at 10 μM, Y: no data.

Considering the results in Table 2, it is clear that derivative **1** is the most active compound particularly against MDA-MB-435 with an average GI_{50} of ~30 nM and inhibition in the 76 – 237 nM range for the other cell lines. The most active compound identified in this series has an *ortho* methyl and *meta* chloro substitution on the phenyl ring with GI_{50} of 18.5 nM for the MDA-MB-435 melanoma cell line and inhibition in the 23 – 38 nM range for the other cell lines.[5] Derivative **2** is second most active compound with activity in the 53 – 372 nM range

followed by derivatives **3 – 4.** Even though the thieno[2,3-*b*][1,6]naphthyridines are less active than their cyclohexanone-thieno[2,3-*b*]pyridine counterparts identifying them using the combination of similarity search and molecular modelling demonstrates the effectiveness of coupling these techniques together extending the SAR profile of the series.

Molecular modelling studies against PLC-δ1 showed that the cyclohexanone-thieno[2,3-*b*]pyridines (**1**-**6**) have hydrogen bonding to two amino acids histidine (His311) via the carboxamide and glutamic acid (Glu341) with the amine group. Also, arginine (Arg549) and lysine (Lys438) interact with the ketone group in the cyclohexanone moiety and the phenyl group is embedded into a lipophilic pocket. In general, the binding mode is the same as has been previously reported on this chemical series.[4, 5, 7, 8] The thieno[2,3-*b*][1,6]naphthyridine family had a similar predicted hydrogen bonding pattern, i.e., His311 and His356 are involved in hydrogen bonding to the carboxamide moiety, Glu341 to the amino group and the phenyl moiety occupies the lipophilic pocket to the left hand site as shown in Figure 2A for derivative **10**. The benzyl extension to the right hand side fills another lipophilic pocket, which could explain the reduction of activity for derivative **12** due to its smaller isopropyl moiety.



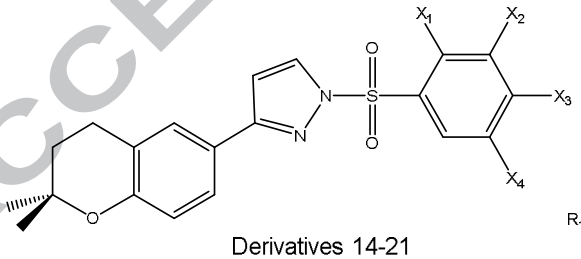
A B

Figure 2. The docked configuration of derivative **10** in the PLC-δ1 binding site. (A) The phenyl group occupies the lipophilic cavity to the left hand side and the benzyl moiety another pocket on the right hand side. Red depicts a positive partial charge on the surface, blue depicts negative partial charge and grey shows neutral/lipophilic regions. (B) Hydrogen bonds are depicted as green dotted lines between derivative **10** and amino acids His311, Glu341 and His356.

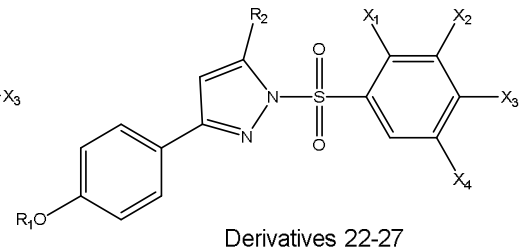
1*H*-Pyrazoles

The 1*H*-pyrazoles derivative **14** (CCT129954 – see Table 3) has a measured IC_{50} ~ 7.5 μM for PLC-γ2.[1] Structural similarity search was conducted based on derivative **14** and sixty-three compounds were found. These were docked to the PLC-δ1 binding pocket and four virtual hits were identified (**22-24** and **28,** Table 3) using the same criteria as for the thieno[2,3-*b*]pyridines. Unfortunately, no compounds with the tetrahydro-2*H*-pyran moiety were found as in derivative **14**, severely limiting the SAR study. In order to address this shortcoming seven derivatives with the tetrahydro-2*H*-pyran moiety were synthesised (**15**-**21**) with various substitutions on the phenyl ring as shown in Table 3. In particular, substitutions on the *para* (X_3) position on the phenyl ring were made since the hit (**14**) has a methoxy group there, indicating that this position was important. Also, a derivative without any substitions on the phenyl ring was made as a reference. Furthermore, for the hits **22- 24** three close structural analogues were synthesised to extend the SAR study (**25-27**) with different R_1 substituents. The molecular structures are shown in Table 3 with the total growth of the NCI60 panel at 10 μM as compared to untreated cells (100% growth).

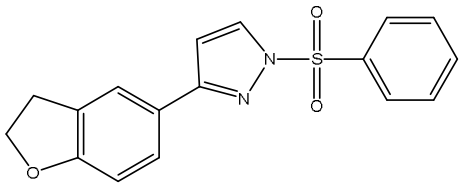
Table 3 Anti-proliferative activity of the 1*H*-pyrazole structural derivatives. Derivative **14** (CCT129954) is the lead compound for the 1*H*-pyrazole series.



Derivatives 14-21



Derivatives 22-27



Derivative 28

	R_1	R_2	X_1	X_2	X_3	X_4	NCI Mean[a]
14	-	-	H	H	OMe	H	77.3[b]
15	-	-	H	H	H	H	76.5
16	-	-	H	H	CF_3	H	89.8
17	-	-	H	H	t-Butyl	H	83.9
18	-	-	Cl	H	Cl	H	87.9
19	-	-	H	H	NHCOMe	H	64.5
20	-	-	H	OMe	OMe	H	72.4
21	-	-	H	Me	H	Me	85.5
22	$(CH_2)_3F$	H	H	H	Me	H	85.0
23	Et	NH_2	H	H	OMe	H	102.2
24	$(CH_2)_3F$	H	H	H	Br	H	87.5
25	Me	H	H	H	Me	H	84.2
26	Me	H	H	H	OMe	H	81.8
27	Isopropyl	H	H	H	OMe	H	87.0
28	2,3-dihydrofuran	-	H	H	H	H	81.9

[a]Values represent relative growth (%) versus control as an average for 60 human tumour cell lines (NCI60). All compounds were tested at 10 μM. [b]From Ref.[4]

As can been seen in Table 3 only modest reduction of growth is achieved by the 1*H*-pyrazoles with only derivatives **19** and **20** with marginally better results than **14**. Absence of the tetrahydro-2*H*-pyran ring (**22** - **27**) and ring contraction from a six membered ring to five membered tetrahydrofuran (**28**) did not result in improved anticancer activity. To further test the potency of the 1*H*-pyrazoles, derivative **14** was tested with the MTT colorimetric assay, which measures the activity of NAD(P)H-dependent redox enzymes in the cytosol reflecting the viability of the cells.[16] The breast cancer cell line MDA-MB-468 was incubated with 10 μM concentration of **14** and were compared to the activity of the anticancer drug camptothecin (topoisomerase I poison). This tumour cell line was chosen due to its low relative growth of 36.6% in the NCI60 panel.[4] Minimal effect was observed (data not shown) confirming the modest anticancer effect for this chemical class.

Chemical Space

The calculated molecular descriptors molecular weight, log P, hydrogen bond donors, hydrogen bond acceptors, polar surface area and rotatable bonds for derivatives **1** – **28** are given in Table S1 in the Supplementary Information. All are within the boundaries of '*drug* –

like' chemical space with some of the properties dipping into *lead-like* space (for definition of boundary see Table S2, Supplementary Information). It can therefore be concluded that the compounds are within favourable property space for drug development.

Conclusion

The anticancer activity of the thieno[2,3-*b*]pyridine and 1*H*-pyrazole derivatives was investigated. Tests against the NCI60 cancer cell panel revealed that the cyclohexanone-thieno[2,3-*b*]pyridines were the most active with derivative **1** giving GI_{50} = 30 nM against the MDA-MB-435 melanoma cell line emphasising the importance of *meta* substitution on the phenyl ring moiety for this chemical series, which is in line with previous findings.[4, 5, 7] By applying a combination of similarity searching and molecular modelling the thieno[2,3-*b*][1,6]naphthyridines were found to have anticancer activity extending the SAR. Finally, the 1*H*-pyrazoles only showed moderate growth inhibition for the NCI60 panel, which was verified using the MTT assay.

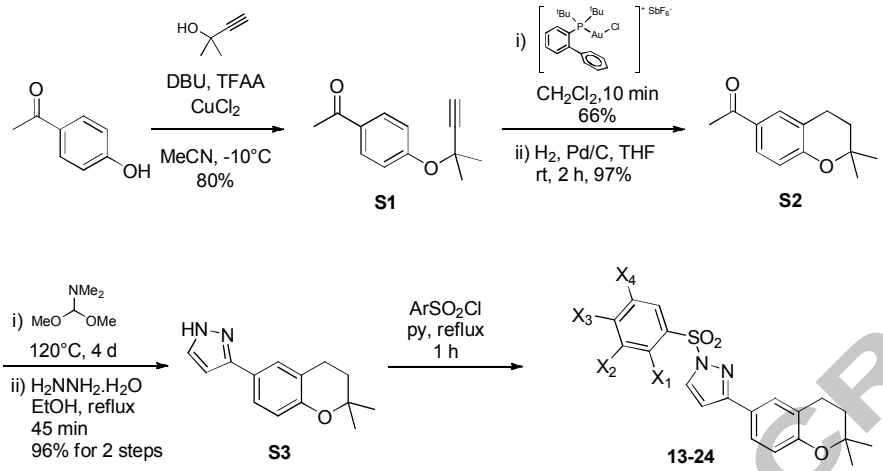
Methodology

Modelling and Screening: The thieno[2,3-*b*]pyridine and 1*H*-pyrazoles derivatives, were acquired from commercial sources using the eMolecules,[15] web based compound library. Substructure and Tanimoto similarity search methods were used to identify plausible inhibitors.[17] The structures shown in Figure 1 were used as initial search scaffolds with similarity coefficient given at 0.7 and supplemented with substructure searches.

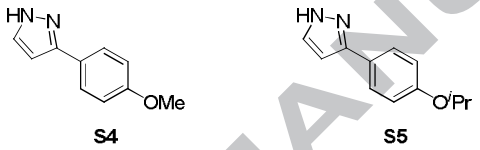
The compounds were docked to the crystal structure of PLC-δ1 (PDB ID: 1DJX, resolution 2.3 Å),[18] which was obtained from the Protein Data Bank (PDB).[19, 20] The Scigress Ultra version 7.7.0.47 program[21] was used to prepare the crystal structure for docking, i.e., hydrogen atoms were added, the co-crystallised ligand (D-Myo-Inositol-1,4,5-Triphosphate,

IP_3) was removed as well as crystallographic water molecules. The Scigress software suite was also used to build the inhibitors and the MM2[22] force field was used to optimise the structures. The centre of the binding pocket was defined as the position of the Ca^{2+} ion (x = 126.257, y = 38.394, z = 22.370) with 10 Å radius. Fifty docking runs were allowed for each ligand with default search efficiency (100%). The basic amino acids lysine and arginine were defined as protonated. Furthermore, aspartic and glutamic acids were assumed to be deprotonated. The GoldScore (GS),[23] ChemScore (CS),[24, 25] ChemPLP[26] and ASP[27] scoring functions were implemented to validate the predicted binding modes and relative energies of the ligands using the GOLD v5.2 software suite. The results were inspected visually and seventeen virtual hits selected. The QikProp 3.2[28] 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.[29]

Synthetic chemistry: Preparation of test compounds **15-21**, **25-27** was achieved via sulfonation of the corresponding pyrazole intermediates with a library of sulfonyl chlorides. Pyrazole **S3,** required for compounds **15**-**21,** was prepared using the three-step route shown in Scheme 3. The key propargylation-gold cyclisation sequence to convert 4′-hydroxyacetophenone into chroman **S2**, once optimised, gave convenient and reliable access to gram quantities of the intermediates required to carry out the study. Pyrazoles **S4** and **S5** required for synthesis of compounds **25**-**27** were prepared using analogous chemistry, beginning from 4′-methoxy or 4′-isopropoxy acetophenone, respectively.



Scheme 1. Preparation of derivatives **15**-**21** via chroman **S3**.



Scheme 2. Pyrazoles required for preparation of derivatives **25-27**.

Full experimental procedures and all characterisation data for compounds **S1-5, 15**-**21** and **25**-**27** are provided in the supporting information.

NCI60 assay: The compounds obtained 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 first at 10 μM and for active ligands dose response curves were generated (NCI60, for further information see ref.[3, 30, 31] and references therein). Furthermore, the full description of the protocol is given in the Supplementary Information.

MTT assay: The MDA-MB-468 breast cancer cell line was maintained in RPMI1640 medium and 10% heat-inactivated foetal calf serum. Twenty-five thousand cells were seeded into each well of a 96 well plate. After 24h, the cells were incubated in medium without phenol red

containing 10 µM of derivative **14** in 1% DMSO, each condition in triplicate. Camptothecin (250 nM) was used as a positive control and 1% DMSO as a negative control. Following a 24h incubation, a standard MTT assay was performed: 20 µl of 5 mg/ml MTT were added to each well and incubated for 3.5 h at 37°C, subsequent to medium removal solvent (4 mM HCl, 0.1% NP40 (Nonidet P-40) dissolved in isopropanol) was added for 15 min with agitation of the cells, followed by reading of the absorbance on a plate reader at 590 nm with a reference filter at 620 nm.

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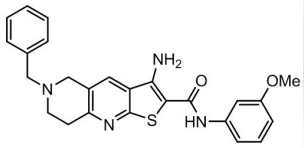
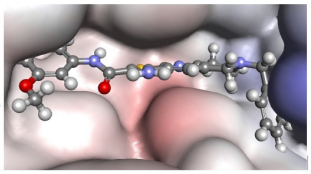


Derivative **10,** GI_{50} = 296 nM (MDA-MB-435)



Derivative **10** docked in PLC - δ1