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Original article

The effect of PLC-γ2 inhibitors on the growth of human tumour cells

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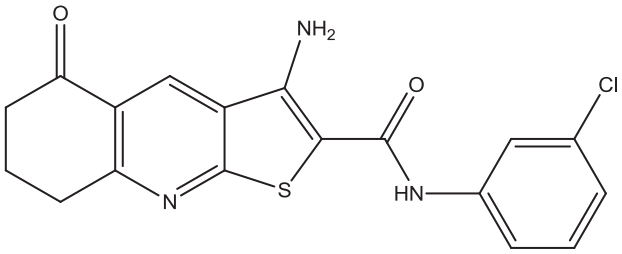
HIGHLIGHTS

- A host of known PLC-γ2 inhibitors were tested against the NCI60 tumour cell panel.
- A class of thieno[2,3-*b*]pyridines showed excellent growth arrest.
- Derivative **3** gave GI_{50} = 58 nM for the MDA-MB-435 cell line.
- Haematopoietic cells were restricted by GI_{50} = 275 nM indicating interaction to PLC-γ2.

GRAPHICAL ABSTRACT



Derivative **3**, GI_{50} = 58 nM (melanoma MDA-MB-435)

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ABSTRACT

The phosphoinositide specific-phospholipase C–γ (PLC-γ1 and 2) enzymes are plausible anticancer targets implicated in cell motility important to invasion and dissemination of tumour cells. A host of known PLC-γ2 inhibitors were tested against the NCI60 panel of human tumour cell lines as well as their commercially available structural derivatives. A class of thieno[2,3-*b*]pyridines showed excellent growth arrest with derivative **3** giving GI_{50} = 58 nM for the melanoma MDA-MB-435 cell line. The PLC-γ2 is uniquely expressed in haematopoietic cells and the leukaemia tumour cell lines were growth restricted on average GI_{50} = 275 nM by derivative **3** indicating a specific interaction with this isoform. Furthermore, a moderate growth inhibition was found for compound classes of indoles and 1*H*-pyrazoles. It is likely that the active compounds do not only inhibit the PLC-γ2 isoform but other PLCs as well due to their conserved binding site. The compounds tested were identified by applying the tools of chemoinformatics, which supports the use of *in silico* methods in drug design.

1. Introduction

Phosphoinositide specific-phospholipase C (pi-PLC) is a membrane bound protein that hydrolyses phosphatidylinositol 4,5-diphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) [1,2]. DAG activates the phospholipid-dependent serine/threonine kinase, protein kinase C (PCK), and IP_3 promotes release of Ca^{2+} from intracellular stores, which mediates cell motility and proliferation [1–3]. Motility of tumour cells is important for their invasion and dissemination leading to morbidity and death [4,5]. There are six subfamilies that belong to the mammalian pi-PLC superfamily, which are classified as PLC-β, PLC-γ, PLC-δ, PLC-ε, PLC-ζ and PLC-η [6–9]. The PLC family members contain two highly related structural domains with conserved amino acid sequences, the x- and y-boxes, that form the catalytic site [10,11]. The PLC-γ subfamily is divided into PLC-γ1, which is present in most cell types, and the minor isoform PLC-γ2, which is restricted to haematopoietic cells [8,12]. Activation of PLC-γ is an early event in pathways stimulated via

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receptor tyrosine kinases (RTKs), pathways that have been implicated in tumorigenesis [13] making PLC-γ a plausible target for anticancer therapy. E.g., inhibition of PLC-γ1 activation blocks glioma cell motility and invasion of foetal rat brain aggregates [14]. Some molecular inhibitors are known for the PLC enzymes however none of them are suitable for drug development [15–19]. Recently, Reynisson *et al.* [20] published the results from a virtual high throughput screen (vHTS) where a host of small drug-like compounds were identified as PLC-γ2 inhibitors based on a ^{3}H-PIP$_2$ flashplate biochemical assay and confirmed in a Ca^{2+} release assay in the squamous carcinoma cell line A431. The aim of this work is to establish the efficacy of the PLC-γ2 inhibitors in human tumour cells. This was achieved by testing them against the National Cancer Institute's human tumour cell lines (NCI60) [21]. Furthermore, commercially available structural derivatives of the known inhibitors were tested to elucidate structural activity relationships (SAR), which were supported with molecular modelling studies.

2. Methodology

The PLC-γ2 inhibitors [20], were acquired from commercial sources using the eMolecules [22], Hit2Lead [23] and ZINC [24] web based compound libraries. Substructure and Tanimoto similarity searching methods were used to identify the structural derivatives [25]. 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 (NCI60, for further information see refs. [21,26,27] and references therein).

For the molecular modelling the GOLD Suite 5.0.1 (Genetic Optimisation for Ligand Docking) [28] docking algorithm was implemented. Its capabilities are well documented, and of the docking algorithms available GOLD is generally considered to give the most reliable results [29–31]. The crystal structure of PLC-δ1 (PDB ID: 1DJX) [32,33] was used as a model in the absence of other crystal structures of the PLC isoforms. For further information on the modelling see Reynisson *et al.* [20].

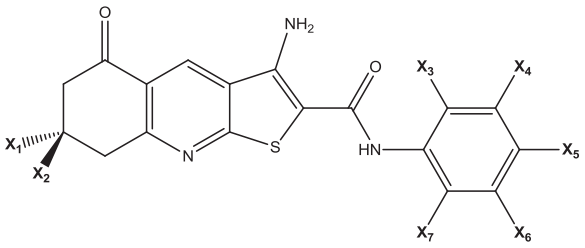
3. Results

In total, fifty nine compounds were tested in the NCI60 screen. All of the molecules are known inhibitors of PLC-γ2 or their close structural derivatives. Due to the conservation of amino acid sequences in the catalytic site of PLC [10], it was likely that the inhibitors would bind to PLC-γ1 and possibly the other PLC isoforms. Since PLC-γ2 is only present in haematopoietic cells [8,12] the growth arrest of leukaemia cells was used as an indicator of the specificity for the inhibition of this isoform. The six leukaemia cell lines within the NCI60 cell panel were used for this purpose.

4. Thieno[2,3-*b*]pyridines

Eighteen derivatives of the thieno[2,3-*b*]pyridine family were acquired from commercial sources and tested against the NCI60 human tumour cell lines. Six compounds were previously identified as PLC-γ2 inhibitors in the ^{3}H-PIP$_2$ flashplate biochemical assay where five of the compounds gave 100% inhibition at 50 μM and three of them suppressed Ca^{2+} release in A431 squamous carcinoma cells at 50 μM [20].

The most potent derivatives of this chemical family are the 3-amino-5-oxo-N-phenyl-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamides as shown in Scheme 1. It is clear that derivatives **1**–**6** are very potent and arrest the growth of all the tumour cells in



	X_1	X_2	X_3	X_4	X_5	X_6	X_7	NHI Mean	Leukaemia
1	H	H	H	H	H	H	H	30.2	24.8
2	H	H	F	H	H	H	H	33.3	41.6
3*	H	H	H	Cl	H	H	H	36.3	36.2
4	H	H	Me	H	Me	H	Me	23.1	23.6
5	H	H	Me	H	H	H	H	42.0	42.9
6	H	H	Me	H	H	Cl	H	59.2	69.0
7	H	H	H	H	OMe	H	H	103.0	108.0
8**	H	H	H	Cl	Me	H	H	105.4	103.5
9	H	H	Br	H	Me	H	H	102.4	106.5
10	H	H	H	H	CN	H	H	99.9	99.9
11	Me	Me	Me	H	Me	H	Me	101.7	97.3
12	Me	Me	H	H	F	H	H	109.8	111.1
13	Me	Me	H	H	Cl	H	H	107.6	105.2
14	Me	Me	Cl	H	H	H	H	110.2	101.6

Scheme 1. 3-amino-5-oxo-N-phenyl-5,6,7,8-tetrahydrothieno[2,3-*b*]quinoline-2-carboxamide **1**, and its derivatives. The compounds *CCT196686, **CCT196686 were identified with the vHTS method [20]. All other ligands are their derivatives. The NCI Means and Leukaemia averages are percentages (%) of growth arrest at 10 μM as compared to untreated cells (100% growth).

Table 1
The GI_{50} (50% growth inhibition) and TGI (total growth inhibition) in nanomolar (nM) are shown for four tumour cell lines and the averages for the leukaemia cell lines. Derivatives **1**–**4** were tested twice and both values are given.

	MDA-MB-435		MDA-MB-468		NCI-H522		SF-295		Leukaemia[b]
	GI_{50}	TGI	GI_{50}	TGI	GI_{50}	TGI	GI_{50}	TGI	GI_{50}
1	89/139	297/X	150/173	583/640	150/309	402/4140	372/508	X/X	520/620
2	196/237	567/771	238/256	952/959	440/516	3310/4590	570/633	85700/X	1800/2200
3[a]	45/71	270/298	45/171	622/2730	190/285	788/X	213/278	781/X	200/350
4	267/495	1160/X	596/712	3650/5260	360/1000	2010/4660	399/1430	X/X	3600/3700
5	331	1980	529	6210	895	3500	1330	X	4300
6	1100	5040	2740	8760	2170	4950	2330	7440	3500

X: no response at 100 μM or no data.
[a] CCT196686 was identified with the vHTS method [20].
[b] TGI > 100 μM for the cell lines.

the NCI60 panel by an average of 23.1–59.2% as compared to 100% growth of untreated cells. Some substitution patterns on the phenyl moiety are not tolerated; in particular the *para*-position (X_5) is often unfavourable. Methyl substitution on X_1 and X_2 in all compounds leads to no activity. Furthermore, two compounds were tested with an aliphatic link (one carbon) between the amide group and the phenyl moiety which resulted in no activity [34]. From these results a SAR is obtained without the targeted synthesis of a single compound.

Derivatives **1**–**6** were tested as a function of concentration (dose response) and their Growth Inhibition at 50% (GI_{50}) and Total Growth Inhibition (TGI) were derived (for further explanation see ref. [21] and references therein). Four tumour cell lines were particularly affected by these derivatives: MDA-MB-435 (melanoma), MDA-MB-468 (breast), NCI-H522 (non-small cell lung cancer) and SF-295 (CNS). The results for these cell lines are shown in Table 1 as well as the averages for the leukaemia cell lines.

The MDA-MB-435 and MDA-MB-468 cell lines were the most affected with GI_{50} less than 100 nM for some measurements. Furthermore, derivatives **1** and **3** are clearly the most potent.

The average GI_{50} for the haematopoietic tumour cell lines (leukaemia) for ligands **1**–**6** were in the low micromolar range and dipping into the nanomolar range for derivatives **1** and **3** as shown in Table 1. This can be interpreted that these ligands interact with the PLC-γ2 isoform. Lethal Concentration at 50% (LC_{50}) was found on three occasions: compound **1** had LC_{50} = 68 μM for NCI-H522, derivative **2** had LC_{50} = 8 μM and ligand **3** had LC_{50} = 0.9 μM for MDA-MB-435. This shows that these inhibitors not only arrest cell growth but effectively kill the cancer cells.

The molecular modelling showed that all the ligands in this family exhibited hydrogen bonding with the side chains of two amino acids: glutamate (Glu341) and lysine (Lys438). The Glu341 interacts with the amine group and the Lys438 with the carboxyl moiety in the cyclohexanone ring. Also, arginine (Arg549) and histidine (His356) appear to be of importance. Furthermore, the phenyl moiety is consistently imbedded in a lipophilic pocket as shown to the left hand side for derivative **4** in Fig. 1.

The final two compounds tested are shown in Fig. 2 [35]. Unlike the compounds depicted in Scheme 1 they have a thiophene substitution on the thieno[2,3-*b*]pyridine bicyclic ring system instead of a cyclohexanone.`Both of these compounds showed good inhibition of the MDA-MB-468 breast cell line at 10 μM concentration, i.e., 19.4% for **15** and 44.8% for derivative **16**. These ligands only had marginal activity in the leukaemia cells (~85%). These compounds follow the same binding pattern as the other inhibitors in this family.

Interestingly, derivative **2** binds to Adenosine A_{2a} receptor, a member of the G-protein coupled receptor (GPCR) family with K_i = 0.3 μM [36]. It is unlikely that the biochemical overlap between GPRC and PLCs is too extensive because some of the GPCR inhibitors reported have polar groups in the *para*-position on the phenyl ring, which is not tolerated for PLC-γ2. Nevertheless, the adenosine receptor subfamily has been implicated in cancer [37], which indicates that synergistic effects could be causing the growth arrest of the tumour cells. Further experimental work is needed to establish this.

It is well known that amino aryl compounds often display a toxicological effect through DNA adduct formation initiated by metabolic activation [38–40]. It is unlikely that the thieno[2,3-*b*] pyridines render some of their growth arrest effect via DNA adduct formation since many of the derivatives do not show any reduction of growth at all. The molecular scaffold of these ligands is quite elongated, which would make it an awkward substrate for enzymes such as cytochrome P450. Further, work is needed to verify the toxicological profile of these ligands such as the Ames test.

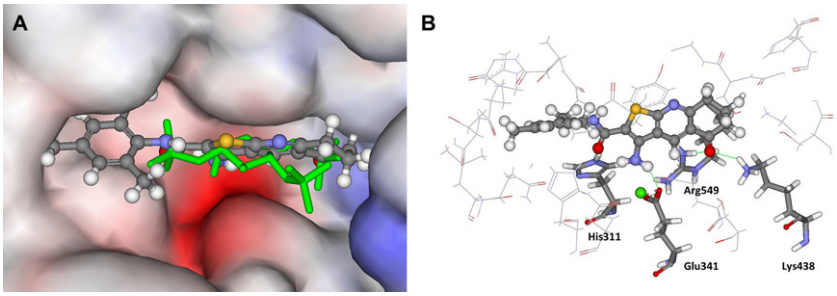


Fig. 1. The docked configuration of **4** in the binding site of PLC-δ1. (A) The protein surface is rendered and ligand **4** is overlain with the co-crystallised inositol 1,4,5-triphosphate shown in green. The phenyl group of **4** occupies a lipophilic cavity to the left hand side. Red depicts a positive partial charge on the surface, blue depicts negative partial charge and grey shows neutral/lipophilic areas. (B) Hydrogen bonds are depicted as green lines between ligand **4** and the amino acids His311, Glu341, Arg549 and Lys438. The Ca^{2+} ion is shown as a green sphere. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

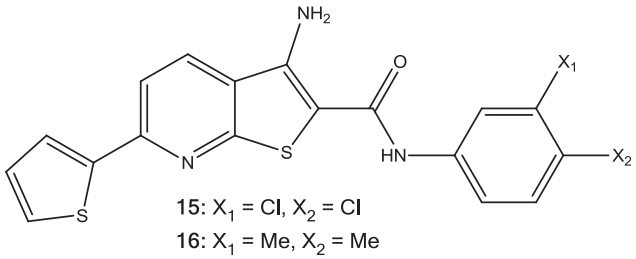


Fig. 2. The two 3-amino-N-phenyl-6-(thiophen-2-yl)thieno[2,3-*b*]pyridine-2-carboxamide derivatives.

5. Indoles

The most potent molecular family identified in the vHTS work were indole derivatives [20]. CCT129957 (**17**) was measured to have IC_{50} – ~3 μM and Ca^{2+} release inhibition in squamous carcinoma cells of ~15 μM [20]. When this compound was measured in the NCI60 screen it did not exhibit marked growth arrest with a mean of only 95.5% for all the tumour cell lines as compared to 100% growth of untreated tumour cells. Also, the leukaemia cells did not respond to these ligands as shown in Scheme 2. However, the renal UO-31 and the breast T-47D cancer cell lines were growth inhibited by ~60–70%. Five structural derivatives of **17**, derivatives **18**–**22**, were also tested and for all of them the UO-31 and T-47D cell line growth was reduced to some extent as shown in Scheme 2. Some other cell lines were inhibited such as the lung cancer cell line EKVX (see Supplementary Information).

The modelling revealed that all of the indoles form a hydrogen bond to the side chain of the Glu341 amino acid via their amine group in the indol ring system. Furthermore, the lipophilic pocket to the left hand side, as shown in Fig. 3, is filled on all occasions with the phenyl moiety. For all of the inhibitors a good binding is predicted to the PLC docking scaffold. Their lack of growth arrest can be rationalised due to the relative high polarity of the ligands resulting from their electron withdrawing (NO_2 and Cl) and donating (OMe) substituents hampering cell membrane permeability.

6. 1*H*-pyrazoles

The second most potent compound found in the virtual screen was compound CCT129954 (**23**) with IC_{50} of ~7.5 μM and an inhibition of Ca^{2+} release at 30 μM [20]. Three commercially available derivatives of **23** were tested all with different substitution pattern on the phenyl ring moiety and the results are shown in Scheme 3. Interestingly, the leukaemia cells in the NCI60 panel were affected by these ligands with an average growth inhibition between 57.9 and 68.7% as compared to 100% growth of untreated cells. This indicates that these compounds inhibit the PLC-γ2 isoform. Three tumour cell lines are particularly affected, *i.e.*, leukaemia K-562, non-small cell lung cancer HOP-92 and breast MDA-MB-468.

The four molecules in this chemical class were predicted to have hydrogen bonding with the side chain of His311 via the oxygen in the tetrahydro-2*H*-pyran moiety. Also, the amino acids Lys438 and Ser501were involved in the binding to the ligands. Little consistency was predicted for the lipophilic pockets being filled for the ligands in this family according to the modelling.

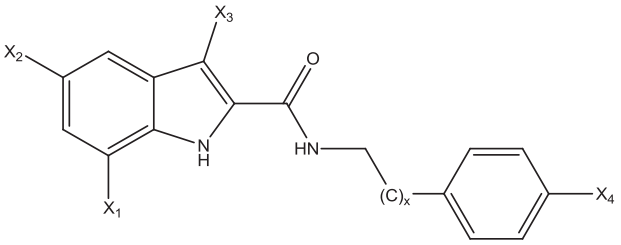
7. 1, 2, 4-oxadiazoles

Two compounds of the oxadiazoles [41] were identified in the vHTS [20]. Both display an IC_{50} of 1–2 μM but reduction in Ca^{2+} release was not seen for either in the cell based assay. Six derivatives of these compounds were tested against the NCI60 tumour cell panel and the results are shown in Scheme 4.

In general, these compounds have little growth restriction activity against the NCI60 cell panel and the leukaemia tumour cells. This indicates that these ligands are not interacting strongly with PLC-γ2. However, three strains of breast cancer cell lines were found to be affected: MCF7, T-47D and MDA-MB-468. The oxadiazole derivatives were active against these cell lines, which is of interest because the ligands are relatively small and are therefore good leads in drug discovery programmes. Derivative **27** with the doubly substituted phenyl ring is the most active whereas the unsubstituted ligand (**32**) has the least potency. According to the modelling hydrogen bonding to the amino acid His311 was significant. In half of the molecules, Asn312 bonding was also observed. There was no consistent pattern of the compounds in this family filling the lipophilic pockets again indicating that the oxadiazoles do not render their effect by PLC-γ inhibition.

8. Miscellaneous

The other inhibitors tested had inconsistent, weak or no effect on the growth of the NCI60 human tumour cells (compounds **33**–**59** in



	X_1	X_2	X_3	X_4	C_x	UO-31	T-47D	NCI Mean	Leukaemia
17*	NO_2	H	H	H	1	62.1	69.7	95.5	92.5
18	H	H	Cl	H	1	57.6	71.7	89.1	78.4
19	H	H	Cl	Cl	1	73.6	81.0	97.3	88.9
20	H	H	Cl	Cl	0	80.6	96.3	102.4	97.0
21	H	H	Cl	Me	0	110.2	84.3	100.5	101.5
22	H	OMe	H	H	0	75.5	85.9	99.7	89.9

Scheme 2. The indole derivatives tested against the NCI60 cell panel. The largest effect was observed in the renal UO-31 and breast T-47D tumour cell lines. The values are percentages (%) of growth arrest at 10 μM as compared to 100% growth of untreated tumour cells. The average of the leukaemia tumour cell lines is given. *CCT129957 was identified with the vHTS method [20].

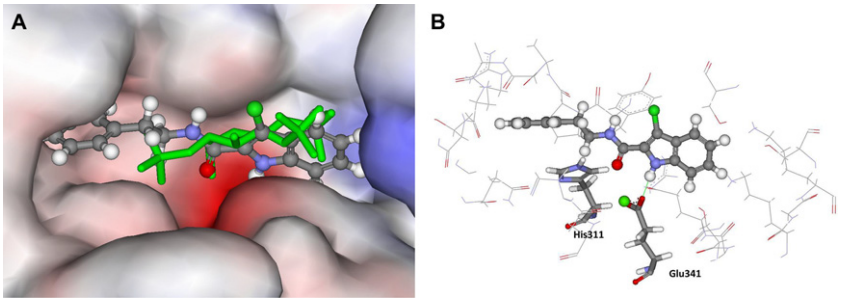
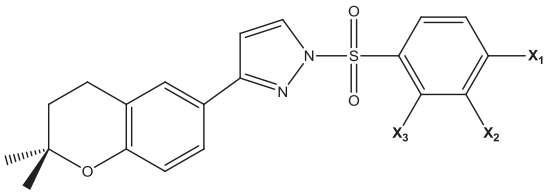


Fig. 3. The docked configuration of **18** in the binding site of PLC-δ1. (A) The protein surface is rendered and ligand **18** is overlain with the co-crystallised inositol 1,4,5-triphosphate shown in green. The phenyl group of **18** occupies a lipophilic cavity to the left hand side. Red depicts a positive partial charge on the surface, blue depicts negative partial charge and grey shows neutral/lipophilic areas. (B) Hydrogen bonds are depicted as green lines. The Ca^{2+} ion is shown as a green sphere. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



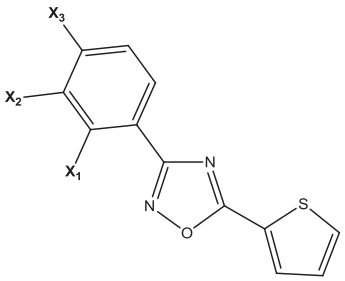
	X_1	X_2	X_3	K-562	HOP-92	MDA-MB-468	NCI Mean	Leukaemia
23*	OMe	H	H	55.6	61.4	36.6	77.3	57.9
24	Cl	H	H	52.4	8.0	48.9	75.9	58.6
25	H	CF_3	H	63.2	38.9	67.1	90.8	68.7
26	H	H	Cl	43.0	49.1	63.7	81.9	65.4

Scheme 3. The 1*H*-pyrazoles derivatives tested against the NCI60 cell panel. The largest effect was observed against the leukaemia K-562, non-small lung cancer HOP-92 and breast MDA-MB-468 tumour cell lines. The values are percentages (%) of growth inhibition at 10 μM as compared to 100% growth of untreated cells. The average of the NCI60 cells (NCI Mean) and the leukaemia tumour cell lines are given. *CCT129954 was identified with the vHTS method [20].

Supplementary Information). These included the benzo[*d*]thiazoles, 4-(quinoxalin-2-yl)phenyl benzenesulfonates, N-(2-oxo-2*H*-chromen-6-yl)-5-phenylfuran-2-carboxamides and others. Three singletons, depicted in Fig. 4, showed consistent growth arrest of the leukaemia cells when compared to other tumour types. This indicates that they interact specifically with PLC-γ2.

9. Discussion

The results show that PLC-γ2 inhibitors and their derivatives arrest growth of cancer cell lines of various/multiple origin. Interestingly, the haematopoietic leukaemia cell lines, to which PLC-γ2 expression is restricted, are not particularly affected except by



	X_1	X_2	X_3	MCF7	T-47D	MDA-MB-468	NCI Mean	Leukaemia
27	Cl	H	Cl	29.2	51.7	8.4	89.4	89.4
28	H	Me	H	39.0	53.6	40.3	90.9	94.1
29	H	Cl	H	36.8	66.7	3.4	94.0	90.7
30	H	H	Cl	33.7	56.9	44.7	94.2	98.7
31	OH	H	H	56.5	76.4	44.6	94.9	93.7
32	H	H	H	52.6	65.5	83.7	100.9	99.1

Scheme 4. The 3-phenyl-5-(thiophen-2-yl)-1,2,4-oxadiazole derivatives. All the values are given in percentages (%) as growth inhibition at 10 μM as compared to 100% growth of untreated cells. The average of the NCI60 cells (NCI Mean) and the leukaemia tumour cell lines are given.

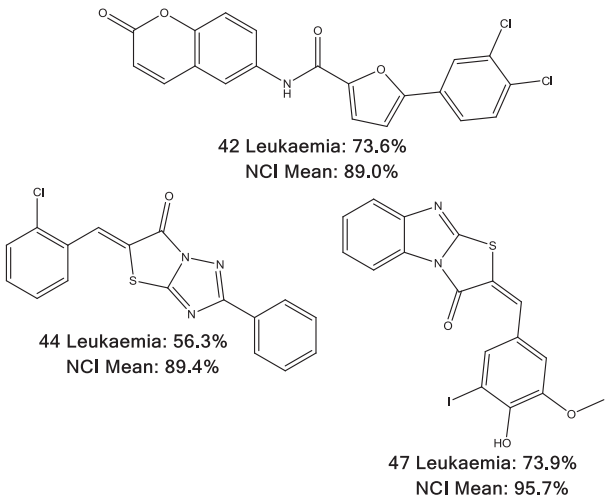


Fig. 4. The three singletons – **42**, **44** and **47** – with specific growth restriction of the leukaemia cell lines. The other tumour cell lines were marginally affected (NCI Mean). The values are given in percentages (%) as growth arrest at 10 μM as an average for leukaemia cell lines and the panel of 60 NCI cell lines (NCI mean).

ligands **1**–**6** of the thieno[2,3-*b*]pyridine family, the 1*H*-pyrazoles and some singletons indicating their specificity towards PLC-γ2. The other, non-leukaemia, cell lines showed various responses to the ligands. These cells express PLC-γ1 as well as some of the other PLC isoforms that are expected to interact with the ligands due to sequence homology with PLC-γ2 in the catalytic pocket [9,10]. Furthermore, off-target effects to related enzymes such as phosphoinositide specific-phospholipase A and B or perhaps some completely different receptors in the tumour cells are always a possibility.

It is known that the region with the highest amino acid sequence similarity between the PLC isoforms is within the binding pocket but without x-ray crystal structures of the different isoforms it is impossible to know whether they retain the same configuration or are dissimilar. Most likely they are similar but it is possible that enough differences exist to design ligands with sufficient specificity to allow discrimination between the isoforms. The results presented here could be the first sign that such a strategy is possible due to the different profiles from the NCI60 panel displayed by the chemical families. This scenario is akin to when the kinases were discovered and the challenge faced by medicinal chemists to design specific inhibitors for their many classes [42]. Obviously further experimental work is needed to verify whether such an approach is feasible for PLC.

Some of the tumour cell lines were more susceptible to the ligands tested than others. The breast cancer cell line T-47D was affected by the indoles and oxadiazoles. The breast cancer cell line MDA-MB-468 appeared to be susceptible to all the active ligands except the indoles. The thieno[2,3-*b*]pyridines not only slowed the growth of melanoma cancer cells MDA-MB-435 but actually reduced their number. The observation that ligands from different chemical families affect the same cell lines strengthens the argument that they inhibit the same enzyme.

The compounds tested in this work were identified using vHTS and other structural derivatives were found using the tools of chemoinformatics. The most potent compounds **1** and **3** have $GI_{50} < 100$ nM for the melanoma cell line MDA-MB-435. Considering that no specific synthetic work was done it is clear that the methods of computer aided drug design can produce very potent inhibitors, which serve as excellent leads in drug discovery programmes.

10. Conclusion

It is shown that known inhibitors of PLC-γ2 such as derivative **3** of the thieno[2,3-*b*]pyridines inhibit the growth of leukaemia tumour cell lines by $GI_{50} = 275$ nM, which uniquely express this isoform. Clearly, further work is needed to establish this effect however it is a very good indication that PLC-γ2 is a plausible anticancer target. Due to the similarity of the binding domains of the PLC isoforms it is likely that the inhibitors interact with more than one isoform, which is reflected in the marked growth arrest of cell lines not expressing PLC-γ2. Furthermore, only the tools of computer aided drug design were used to find these potent inhibitors. The difference between the tumour cell lines affected by the chemical families investigated could suggest that the various isoforms of the PLC enzyme are being inhibited and opens the possibility of designing specific inhibitors for the different isoforms. Finally, the oxadiazoles are small molecules and show a very good response in the breast tumour cells. However, they did not bind in a consistent way in the docking studies, which suggests that they affect other bio-molecular systems in the cancer cells.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.05.029.

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