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Article

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Authors

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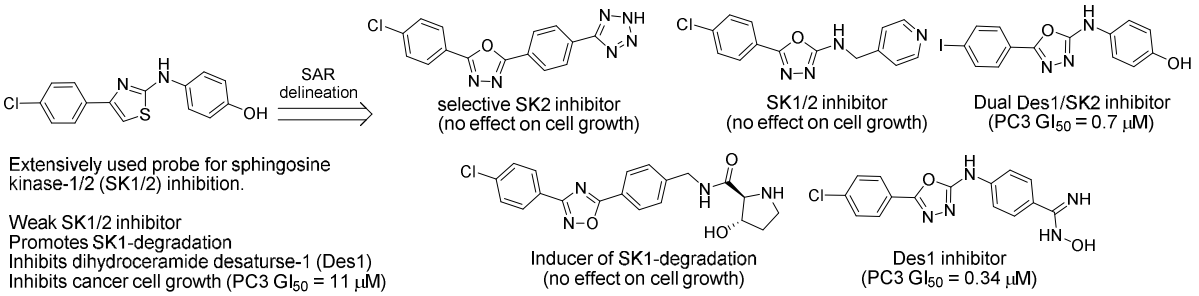
Abstract



The sphingosine kinase (SK) inhibitor, SKI-II, has been employed extensively in biological investigations of the role of SK1 and SK2 in disease and has demonstrated impressive anticancer

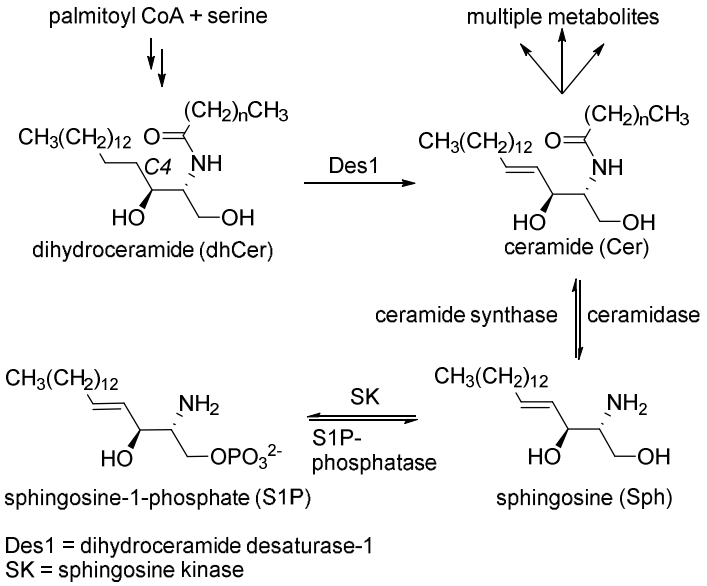
activity in vitro and in vivo. However, interpretations of results using this pharmacological agent are complicated by several factors: poor SK1/2 selectivity; additional activity as an inducer of SK1-degradation; and off-target effects, including its recently identified capacity to inhibit dihydroceramide desaturase-1 (Des1). In this study we have delineated the structure-activity relationship (SAR) for these different targets and correlated them to that required for anticancer activity and determined that Des1 inhibition is primarily responsible for the antiproliferative effects of SKI-II and its analogues. In the course of these efforts a series of novel SK1, SK2 and Des1 inhibitors have been generated, including compounds with significantly greater anticancer activity.

Introduction

Over the last fifteen years there has been considerable interest in identifying the biological functions of sphingolipid metabolites and the role they play in diseases such cancer, autoimmune disease, inflammation, pain, macular degeneration, asthma and fibrosis.[1] The *de novo* synthesis of sphingolipids commences with palmitoyl CoA, which is converted into the central lipid ceramide (Cer) in four steps, the last step being the introduction of the double bond at C4 by dihydroceramide desaturase-1 (Des1) (Scheme 1). Ceramide is regarded as the central lipid in this biosynthetic pathway from which many other bioactive metabolites are generated, usually in reversible steps from which ceramide can be regenerated by salvage pathways. Of significant interest has been the reversible conversion of Cer into sphingosine (Sph) and sphingosine-1-phosphate (S1P) (Fig 1). Cer and Sph are pro-apoptotic signaling lipids whereas S1P promotes cell growth and survival (anti-apoptotic and pro-mitogenic).[1a,g,k] Consequently, sphingosine kinases (SKs), which produce S1P, can play an important role in determining cell fate, providing a "rheostat" for cancer cell survival and death.[2] There are two isoforms of SK, SK1 and SK2. SK1 exists in the cytosol and SK2 predominantly in the nucleosome and other organelles.[1a,c,g,k] SK1 is subject to tight regulation and is upregulated in many cancers where it is thought to play a key role

in disease progression through increases in S1P and decreases in Cer and Sph levels, promoting tumour growth and survival.[1a,e,j,o,2] Thus a number of groups have pursued SK1 inhibitors in order to reverse this prosurvival effect of the Cer/Sph/S1P rheostat.[3,4] SK2 inhibitors are also being sought and may induce different mechanisms of cancer cell death to those mediated by SK1 inhibitors.[3] Notwithstanding the compelling rationale for targeting sphingosine kinases, the emergence of different SK1/2 inhibitors has met with conflicting results regarding their anticancer potential.[3] Early inhibitors included a series of Sph analogues (not shown) with mixed SK1/2 selectivity and IC_{50} values in range of 5-30 μM and the non-lipid-like inhibitors, SKI-II **1** and ABC294640 **2** (Fig 1). Compound **1** was identified by Smith and coworkers from a high throughput screen in 2003,[4a] it exhibits some selectivity towards SK2 as a kinase inhibitor (Ki: SK2 = 7.9 μM, SK1 = 16 μM) but is a more powerful promoter of SK1-degradation (100% SK1 ablation at 10 μM)[5] and has recently been identified as an inhibitor of Des1 activity (Ki = 0.3 μM).[6] The SK1-degradation induced by **1** is proposed to arise from its binding to an allosteric site, distinct from the enzymatic site, which promotes polyubquination and proteasomal degradation of SK1.[5] Compound **2**, was also discovered by Smith and co-workers and is a selective inhibitor of SK2 (Ki = 10 μM).[7] Both **1** and **2** have been used in a large number of studies directed at evaluating the therapeutic potential of targeting SKs in cancer and other indications, both have exhibited favorable efficacy in cancer models and **2** has been progressed to clinical trials.[7,8] Despite exhibiting a variety of potentially valuable anticancer properties, **1** and **2** are only moderately potent and in recent years a number of more potent (IC_{50} < 1 μM) and more selective SK1/2 inhibitors have emerged. These include the SK1 selective inhibitors **3**,[4b] **4**,[4c,d] and PF-543 **5**[4e] and a dual SK1/2 inhibitor **6**,[4h,i] (Fig 1). Like **1**, **5** is also a promoter of proteasomal degradation of SK1 and presumably also has some affinity for the allosteric site on SK1.[4g] However, in contrast to **1**, **4-6** do not to show any antiproliferative effects at concentrations that fully suppress S1P production in cells, casting doubt on the use of SK1 inhibitors as proapoptotic agents in cancer therapy.[3,4d,e,i,l]

Similar conflicts arise in the case of SK2, where the moderately potent SK2 inhibitors **6**[4h,i,] and SLR080811 **7**[4f] do not exhibit antiproliferative effects, whereas the less potent SK2 inhibitors K145 **8**[4j] and MP-A08 **9**[4k] do show increases in Cer concentrations and cancer cell apoptosis (GI_{50} = 5-30 μM). Herein, we report our SAR studies on **1** and attempts to correlate the SAR for SK1/2 inhibition to the SAR for the antiproliferative effects in androgen-independent PC3 prostate cancer cells (SK1 expression is raised in PC3 cells relative to androgen-dependent LNCap prostate cancer cells).[9] We have found that these two SARs diverge and that inhibition of these kinases cannot account the anticancer activity of **1** and our broader compound series. We have also studied the SAR of **1** in relation to SK1-degradation and inhibition of Des1 activity. The SAR of Des1 inhibition correlates to that required for inhibition of PC3 cell growth, indicating that Des1 inhibition is a key driver of the anticancer effects of **1** and it analogues, which is also supported by lipidomic studies in PC3 cells. In the course of this effort a number of substantially more potent inhibitors of some of these enzymes and of PC3 cell growth have been identified.



Scheme 1: Sphingolipid Biosynthesis

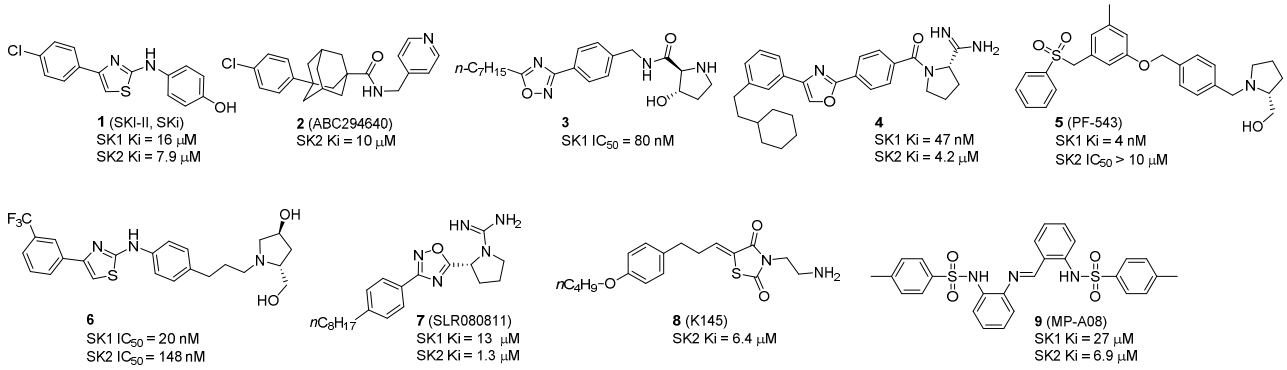
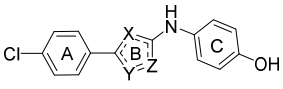


Figure 1: Structures of SK1/2 targeting agents.

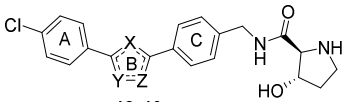
SAR Studies: Our SAR studies of **1** have been directed to modifications of the B-ring (Table 1) and of groups R^a, R^b, L and the C-ring (Table 2). As part of our SAR-guided exploration of **1**, we sought to identify useful replacements of the 2-aminothiazole and 4-aminophenol moieties as they are likely to be subject to rapid metabolism to form reactive metabolites (bioactivation), potentially compromising *in vivo* drug exposure and safety.[10] We have also avoided generating lipid-like, amphiphilic structures that are apparent in a number of the more potent SK1 inhibitors **3**-**6** and which are known to complicate cellular readouts, including through surfactant associated cytotoxicity.[4i] SK1/2 inhibition was measured at a single concentration (10 μM) and recorded as %SK1/2 activity relative to vehicle (DMSO) control.[11] PC3 cell growth was measured using an MTS colorimetric assay and is given as the concentration required to inhibit 50% of cell growth (GI_{50}). Selected compounds were also evaluated for SK1-degradation, Des1 inhibition, lipidomics analysis and clonogenic potential in PC3 cells (see below). SK1 inhibitors **1**, **3** and **5** were used as comparators in these studies. As expected, **1** gave only a modest level of inhibition of SK1 (%SK1 = 78) and SK2 (%SK2 = 90) and inhibited PC3 cell growth (GI_{50} = 11 μM) with a potency that is broadly similar to that seen with other cell lines (Table 1, entry 3).[4a,5a] Consistent with their reported high potency towards SK1, **3** (IC_{50} = 80 nM)[4b] and **5** (IC_{50} = 3 nM)[4e] gave low %SK1 values, 6% and 1%, respectively, and were much less potent towards SK2 (Table 1, entries 1 and

2). Compound **5** inhibited PC3 cell growth at a relatively high concentration (GI_{50} = 19 μM) compared to its SK1 inhibition and **3** was inactive (GI_{50} > 100 μM). The cytotoxicity observed with **5** (PC3 GI_{50} = 19 μM) may arise from its amphiphilic nature as has been reported for the similarly amphiphilic compound **6**.[4i] Interestingly, all of our novel bioisosteric B-ring analogues of **1**, **10-17** (Table 1, entry 4-11) gave higher levels of SK1/2 inhibition, with oxazole **17** giving the best SK1 potency and selectivity (Table 1, entry 11, %SK1 = 17, %SK2 = 53). The most potent PC3 cell growth inhibitor, 1,3,4-oxadiazole **10** (GI_{50} = 1.63 μM), is almost ten times more potent than **1** (Table 1, cf entries 3 and 4). Given the frequent use of the pyrrolidine ring as a polar head group in **3-7** we also prepared two hybrid structures of **1** and **3**, compounds **18** and **19** (Table 1, entries 12 and 13) but both of these were much less active than **3** towards SK1 and effectively devoid of any PC3 cell growth inhibition.

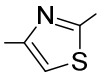
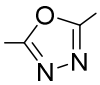
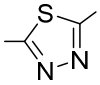
Table 1: B-ring Modifications.

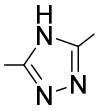


4,10-17



18, 19

Entry	B-ring	ID	%SK1 10 μM[a]	%SK2 10 μM[a]	GI_{50} PC3[b]
1.		**3**	6 ± 2	112 ± 6	>100
2.		**5**	1 ± 2	45 ± 9	19
3.		**1**	78 ± 2	90 ± 7	11 ± 2
4.		**10**	43 ± 4	44 ± 8	1.6 ± 0.4
5.		**11**	55 ± 4	70 ± 3	2.4

6.		**12**	50 ± 5	55 ± 4	40
7.		**13**	50 ± 4	77 ± 15	44
8.		**14**	34 ± 4	64 ± 8	2.6
9.		**15**	75 ± 4	78 ± 8	ND
10.		**16**	55 ± 2	68 ± 1	ND
11.		**17**	17 ± 5	56 ± 4	3.0
12.		**18**	62 ± 10	106 ± 24	77
13.		**19**	41 ± 4	118 ± 18	>100

[a] The activity of purified recombinant SK1 and SK2 were determined in the presence of 10μM compound using an *in vitro* SK enzyme assay with 10 μM Sph and 100 μM ATP (n = 3).[12]

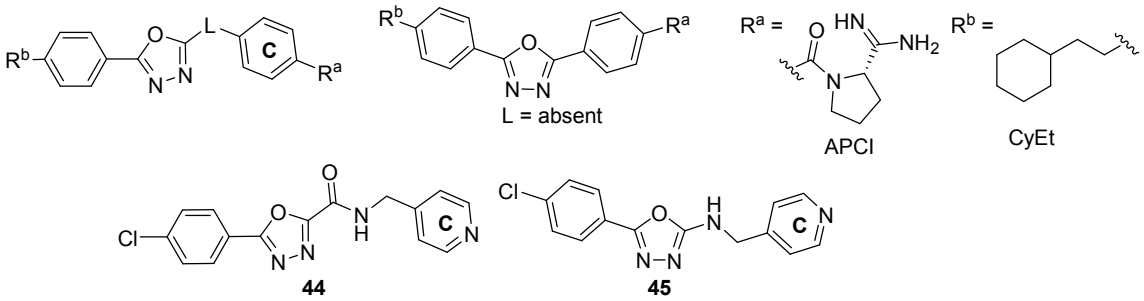
[b] PC3 cell growth was measured using an MTS colorimetric assay at eight concentrations (10^{-8} - 10^{-4} M, n = 3 per concentration) and is given as the concentration required to inhibit 50% of cell growth (GI_{50}). Where given, the standard deviations (±) is based on 3 independent GI_{50} determinations.

The 1,3,4-oxadiazole **10** was selected for further SAR studies exploring the effects of modifying the linker (L), polar head group R^a and hydrophobic tail R^b on SK1/2 activity and PC3 cell growth (Table 2). Removal of the amino (NH) L-group in **10** to give **20**, had no effect on the SK1/2 inhibition but completely removed its cell growth inhibitory activity (Table 2, cf entries 1 and 2).

Several alternative L-groups were also evaluated: L = S, C(O)NH, and CH_2NH (Table 2, entries 3-5). Of these, L = CH_2NH (**23**), gave the greatest improvement in SK1/2 inhibitory activity (Table 2, entry 5) and only **22** and **23** inhibited PC3 cell growth. A key observation in the SAR of compounds **10** and **20-23** (Table 2, entries 1-5) is the apparent requirement of a 4-aminophenol for antiproliferative activity, which is not required for SK1/2 inhibition. The 4-aminophenol is commonly linked to redox activity,[9] indicating that the antiproliferative activity of **1**, **10**, **22** and **23** may arise from a redox-active target (see below).

We also evaluated a series of polar head groups R^a to replace OH in **10** in the presence and absence of L = NH (Table 2, entries 6-18). MacDonald, Webster and Lynch have shown that the amidine ($C(NH)NH_2$) is a useful polar head group in promoting SK1/2 inhibition, in particular the acylpyrrolidine-2-carboximidamide (APCI) group, as in **4** (Fig 1).[4c-f] We evaluated the simple amidine ($C(NH)NH_2$) head-group and APCI as well as a number of other neutral, basic and acidic isosteres of the amidine and OH groups: R^a = NH_2, CH_2NH_2, CO_2H, $C(O)NH_2$, C(NH)NHOH and tetrazole (Table 2, entries 6-17). Considering first the effect these variations in R^a on SK1/2 inhibition for where L is absent (entries 6-13), the carboxamide **27** (%SK1 = 22) and tetrazole **31** (%SK2 = 14) gave the best SK1/2 inhibitory activity and were selective for SK1 and SK2, respectively (Table 2, entries 9 and 13). The amidine **28**, amidine oxime **29** and APCI **30** head groups (R^a) were least effective in SK1/2 inhibition (Table 2, entries 10 - 12). The introduction of L = NH for these different R^a groups saw little effect on carboxamide **33** SK1/2 inhibitory activity (%SK1 = 20), whereas the tetrazole **35** showed reduced activity. By contrast, the introduction of L = NH conferred a marked increase in potency of amidine **33** (%SK2 = 14) over **28** towards SK2 (Table 2, cf entries 11 and 15). With respect to PC3 cell growth of the compounds **24-36** (Table 2, entries 6-18), only four compounds showed any activity: **28** (GI_{50} = 45 μM), **34** (GI_{50} = 0.34 μM) and **35** (GI_{50} = 15 μM). Again there is no correlation between SK1/2 inhibition and PC3 cell growth inhibition in this series.

Table 2: Modifications of $R^{a/b}$, L and C-Ring.



Entry	R^a	-L-	R^b	ID	%SK1[a] 10 μM	%SK2[a] 10 μM	GI_{50} PC3[a] (μM)
1.	OH	NH	Cl	**10**	43 ± 4	44 ± 8	1.6 ± 0.4
2.	OH	absent	Cl	**20**	42 ± 8	40 ± 3	>100
3.	OH	-S-	Cl	**21**	107 ± 27	79 ± 22	>100
4.	OH	-C(O)-NH-	Cl	**22**	78 ± 8	139 ± 18	22
5.	OH	$-CH_2-NH-$	Cl	**23**	22 ± 3	39 ± 3	51
6.	NH_2	absent	Cl	**24**	53 ± 10	50 ± 11	>100
7.	CH_2NH_2	absent	Cl	**25**	51 ± 8	57 ± 4	>100
8.	CO_2H	absent	Cl	**26**	57 ± 10	40 ± 8	>100
9.	$C(O)NH_2$	absent	Cl	**27**	22 ± 5	104 ± 12	>100
10.	$C(NH)NH_2$	absent	Cl	**28**	73 ± 5	89 ± 11	45 ± 21
11.	C(NH)NHOH	absent	Cl	**29**	105 ± 13	102 ± 5	70
12.	APCI	absent	Cl	**30**	69 ± 6	135 ± 16	>100
13.	tetrazole	absent	Cl	**31**	133 ± 7	14 ± 10	>100
14.	$C(O)NH_2$	-NH-	Cl	**32**	20 ± 2	43 ± 12	>100

15.	C(NH)NH_2	-NH-	Cl	**33**	46 ± 17	14 ± 7	>100
16.	C(NH)NHOH	-NH-	Cl	**34**	93 ± 23	121 ± 8	0.34 ± 0.05
17.	tetrazole	-NH-	Cl	**35**	44 ± 8	70 ± 6	15
18.	C=N-OH	-NH-	Cl	**36**	41 ± 15	71 ± 9	>100
19.	OH	-NH-	H	**37**	53 ± 27	23 ± 2	8.1 ± 2.1
20.	OH	-NH-	CF_3	**38**	35 ± 14	17 ± 2	1.0 ± 0.8
21.	OH	-NH-	I	**39**	53 ± 1	3 ± 0.1	0.7 ± 0.14
22.	OH	-NH-	CyEt	**40**	56 ± 14	144 ± 51	50
23.	C-ring = pyr	-C(O)$NHCH_2$-	Cl	**41**	133 ± 21	73 ± 8	>100
24.	C-ring = pyr	-$NHCH_2$-	Cl	**42**	3 ± 1	39 ± 12	>100

[a] See Table 1.

We also undertook a preliminary examination of the SAR associated with hydrophobic group R^b (Table 2 entries, 19-22). Replacement of Cl with hydrophobic groups of increasing size H, CF_3, I, (**37-39**) led to successively increased inhibition of SK2, with **39** (R^b = I) proving quite potent and selective towards SK2 (%SK2 = 3) (Table 2, entries 19-21). On the other hand, the much larger (cyclohexyl)ethyl (CyEt) group, that proved effective in promoting the SK1 inhibitory activity of **4**[4c,d] and analogues of **3**[4b] (not shown) diminished its activity towards SK2 (Table 2, entry 22). Like other 4-aminophenols, **40-43** all inhibited PC3 cell growth, with **39** showing the greatest activity, GI_{50} = 0.7 μM (Table 2, entry 21). In this series (**37-40**), the SAR for SK2 inhibition does appear to coincide with inhibition of PC3 cell growth (Table 2, entries 19-22).

We next explored the use of the 4-pyridyl ring present in the SK2 inhibitor **2** (Fig 1), as an alternative C-ring. We employed two linker groups for this: L = -C(O)$NHCH_2$- and -$NHCH_2$-, **41** and **42**, respectively (Table 2, entries 23 and 24). Only **42** showed any appreciable SK1/2

inhibitory activity, but in contrast to **2**, proved to be a moderately selective SK1 inhibitor (%SK1 = 3, %SK2 = 39).

We determined the IC_{50} values of our most potent SK1/2 inhibitors **42** and **39** (Table 3) and compared these to the most potent SK1 inhibitor yet to be identified **5** (Fig 1). The previously reported IC_{50} (SK1) values for **5** of 3 and 28 nM were obtained at 3 and 5 μM of Sph, respectively,[4e,g] our value of 387 nM was obtained at 10 μM of Sph. These variations are consistent with the substrate (Sph) competitive binding kinetics of **5**.[4e] While the dual SK inhibitor **42** is not as potent as **5**, it is a more drug-like species (lower MW and cLogP) that exhibits a superior lipophilicity-corrected ligand efficiency (LELP): **5** LELP = 16 cf **42** LELP = 10 (Table 3).[13] Compounds with LELP values of > 10 are considered unlikely to achieve suitable concentrations in vivo to modulate their target.[12]

Table 3: IC_{50} values of selected compounds

Entry	Cmpnd	SK1 IC_{50}[a] (μM)	SK2 IC_{50}[a] (μM)	LELP[a]
1	**5**	0.387	17.0	16.2
3	**39**	>20	1.9	9.8
4	**42**	3.1	5.5	10.0

[a] Inhibitory constants for purified recombinant enzymes were obtained by an in vitro SK enzyme assay with 1mM ATP, 10μM Sph, and the compounds at concentrations of 0.005 - 10 μM. Data represent mean two independent experiments.

We further evaluated a selected series of our compounds for their capacity to induce SK1-degradation (Fig 2). SK1-degradation was measured using our previously described assay where expression of FLAG™ epitope-tagged SK1 is induced in HEK293 cells and the effect of the compounds on degradation of this existing protein is selectively assessed by Western blot after 16 h.[5a] The proteasome inhibitor MG132 was also included in these assays to demonstrate the

involvement of the proteasome in the SK1-degradation induced by these compounds. As previously demonstrated, **1** completely ablates SK1 levels at 10 μM in a MG132-dependent manner.[5a] In fact, SK1-degradation is likely to be the primary source of SK inhibition by **1** in cells as it is only a weak inhibitor of SK1/2 kinase activity. All other compounds used in this study that induced SK1-degradation also did so in an MG132-dependent manner (Fig 2). Comparison of **1** with the close structural analog **10**, shows **1** to be a much more effective promoter SK1-degradation than **10** despite the fact that **10** is a more potent SK1 kinase inhibitor (Table 1, entries 3 and 4). This comparison indicates that, consistent with the allosteric model,[5] SK1-degradation is independent SK1 kinase inhibition. Presumably, the exchange of the thiazole ring in **1** for the oxadiazole ring in **10** has decreased affinity for the allosteric site and increased affinity for either the ATP or Sph binding sites on SK1 and SK2. A comparison of the close structural analogues **3**, **18** and **19** further supports the finding that SK1-kinase inhibition and SK1-degradation are independent effects. **3** is a potent inhibitor of SK1 kinase activity and modest inducer of SK1-degradation, whereas **18** and **19** are much less potent SK1 kinase inhibitors but very effective in SK1-degradation. None of these compounds, **3**, **18**, **19** exhibited significant PC3 cell growth inhibition (Table 1 entries 1, 12 and 13). The Pfizer compound **5** is both a potent inducer of SK1-degradation and inhibitor of SK1 kinase activity. Also, our most potent inhibitor of PC3 cell growth, amidine oxime **34**, is a moderate promoter of SK1-degradation and has no SK1/2 kinase inhibitory activity (Table 2, entry 15). These comparisons indicate the promotion of SK1-degradation, SK1-kinase inhibition and cell growth inhibition are all independent effects though some compounds may exhibit more than one of these effects.

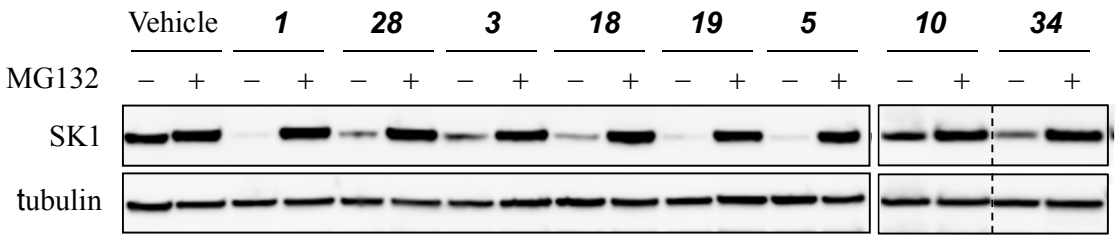


Figure 2: Effect of the compounds on SK1 protein degradation. Expression of FLAG epitope-tagged SK1 was induced HEK293 cells and the effect of applying the compounds to cells for 16 h at 10 μM on degradation of this existing protein selectively quantified by Western blot.

The close correlation of PC3 cell growth inhibition with the presence of redox active groups indicates that inhibition of a redox-active enzyme may be responsible for this effect. This overlaps with the recent finding that Des1 activity is potently inhibited by **1** (K_i = 0.3 μM).[6] To investigate this further we examined several of our antiproliferative compounds as well as several close structural analogues that do not exhibit antiproliferative effects for inhibition of Des1 activity (Fig 3). Des1 activity was obtained by measurement of the fluorescently labeled ceramide analogue, C6-NBD-dhCer, in Jurkat cells as described previously.[14] The conversion of C6-NBD-dhCer to C6-NBD-Cer is attributed to Des1 since the alternative Des-subtype, Des2, gives rise to significant C4-hydroxylation, which was not observed with this study (no detectable C4-hydroxylation of C6-NBD-dhCer) and because C4-hydroxysphingolipids are hardly present in Jurkat cells.[13] Since the assay is performed in whole cells, the direct target leading to the Des1 inhibition cannot be easily discerned and may include other elements in the electron transport pathway associated with Des1 activity. Indeed, Cingolani *et. al.*[6] propose **1** to be an indirect inhibitor of Des1, possibly targeting the upstream enzyme, NADH-cytochrome b5 reductase (CBR5), due to it being a non-competitive inhibitor of Des1 and it being structurally dissimilar to the endogenous substrate, dhCer. Our evaluation of **1** and the structurally related phenols **10**, **20** and **39** revealed all the 4-aminophenols **1**, **10** and **39** to be quite potent inhibitors of Des1, whereas the simpler phenol **20** was inactive (Fig 3). This correlates with the PC3 cell growth inhibition where **1**, **10** and **39** inhibit cell growth but **20** is inactive (Tables 1 and 2). It also overlaps with the SAR of the known inhibitor of Des1, fenretinide **43** (Fig 4),[15] which also contains a 4-aminophenol and is an inhibitor PC3 cell growth (GI_{50} ~ 2 μM).[16] **43** is believed to be a direct inhibitor of Des1 that initially shows competitive inhibition but becomes an irreversible inhibitor over longer incubation times.[14] The 4-aminophenol

in **43** group may play an important role in mediating its irreversible inhibition of Des1, where Des1 oxidation of this group generates a reactive iminoquinone intermediate **44** that reacts with nucleophilic sites on the protein, leading to time dependent irreversible inhibition of Des1 (Fig 4). A similar mechanism may be operating in the case of **1** and the related 4-aminophenols prepared in this study, in which case they may also operate as direct inhibitors of Des1. The amidine oxime **34** completely inhibited Des1 activity at 10 μM but showed little activity at 1 μM. Alkyloximes have been previously shown to undergo oxidation to nitroso groups that form high affinity complexes with the iron in the redox enzyme prostaglandin synthase.[17] Amidine oxime **34** may also undergo oxidative activation to form a nitroso intermediate **45** that forms a high affinity complex with the Fe_2O_2 in Des1. Consistent with the redox activation of **34**, is the much reduced activity of the structurally similar amidine **33** and aldimine oxime **36**, which are more resistant to oxidation. If redox activation is involved in the inhibition of Des1 activity by **34**, then it is likely to be a highly targeted effect requiring quite specific structural interactions as the structurally related amidine oxime **29** showed no Des1 inhibitory activity at 10 μM. Of these related structures, **29**, **33**, **34** and **36** only the active Des1 inhibitor **34**, showed any significant activity against PC3 cell growth, which is conststant with what was seen for the phenolic compounds (**1**, **10**, **20** and **39).** Notwithstanding the suggested possibilities for the direct inhibition of Des1 by these redox sensitive compounds, it remains a significant possibility that other enzymes in the redox cycling pathway are the target of these inhibitors.

Inhibition of Des1 activity and associated increases in dhCer are known to give rise to both apoptotic and autophagic cell death.[18] Lipidomic analysis of **1** and **34** in PC3 cells (10 μM) indicates that Des1 inhibition dominates their effects on sphingolipid synthesis, with significant increases in total dhCer levels but little effect on total Cer, Sph and S1P levels (Fig 5). Since **34** inhibited PC3 cell growth at a concentration of < 1 μM we also undertook lipidomic analysis of this compound in PC3 cells at 1.0 μM, but it showed no effect on dhCer levels (data not shown).

This indicates that while Des1 inhibition alone may explain the anticancer effects of **1** (PC3 GI_{50} = 11 μM) and many of its other 4-aminophenols analogues (Table 1 and 2), it is likely that the cell growth inhibitory effects of **34** (PC3 GI_{50} = 0.34 μM) involves another target(s) in addition to Des1. Alternatively, these different irreversible inhibitors may have different rates of oxidative activation and inhibition of Des1. The time period of drug exposure in the PC3 cell growth inhibition assay (72 h) is much longer than that in the Des1 assay (3 h) and in the lipidomics study (5 h). Further evaluation of the inhibition kinetics of **1** and **34** are required in order to verify that they are irreversible inhibitors of Des1 and if the time course of Des1 inhibition can explain current apparent differences in the level of Des1 inhibition and PC3 cell growth inhibition for **34**.

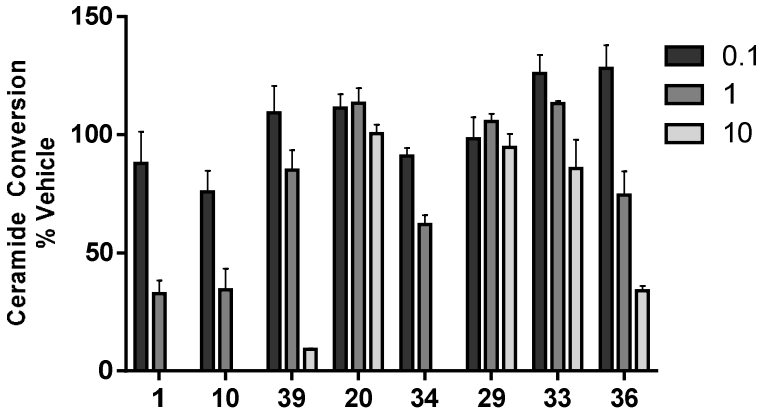


Figure 3, Inhibition of Des1: Des1 activity was obtained by measurement of C6-NBD-Cer conversion in Jurkat cells pre-labeled with C6-NBD-Cer (10 μM). Cells were treated with vehicle or inhibitor (0.1, 1, 10 μM) for 3 h. Data represent mean ± SD of three independent experiments.

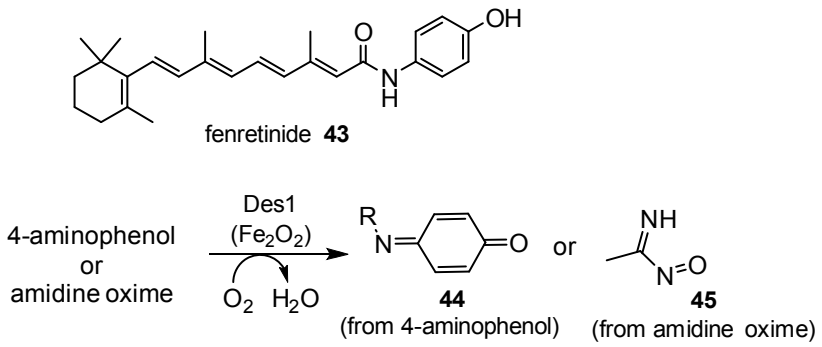


Figure 4: Fenretinide and irreversible inhibition of Des1.

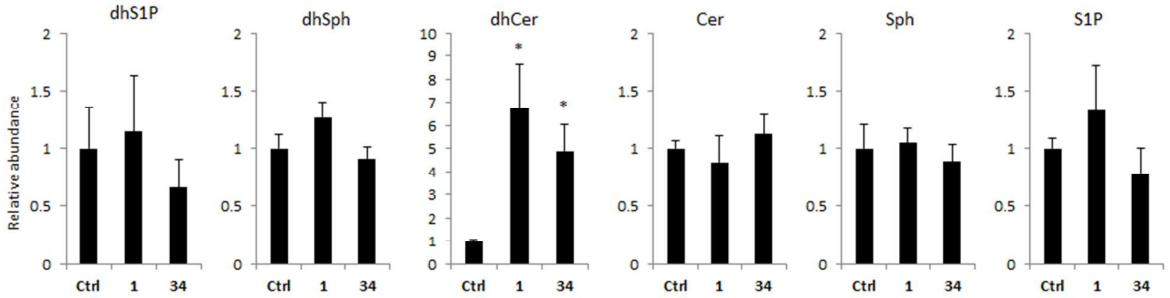


Figure 5, Lipidomic analysis: Effects on sphingolipid levels of **1** and **34** at 10 μM in PC3 prostate cancer cells.

The correlation between redox-activity and antiproliferative effects seen with **1** and its analogues does not easily extend to the SK2 inhibitor **2**, which does not contain such a moiety. Nonetheless, consistent with the Amgen group's studies,[4i] our results indicate that the antiproliferative effects seen with **2** are unlikely to arise from inhibition of SK2 kinase activity alone as the potent SK2 inhibitors **31** and **33** (%SK2 = 14 for both) had no effect on PC3 cell growth even at high concentrations (100 μM). The estrogen receptor has been identified as a potential off target for **2**, which it antagonizes in similar manner to tamoxifen.[19,] However, this may not explain all the anticancer effects seen with **2**, which may still involve contributions from SK2 inhibition. Very recently, **2** has been reported to be a weak inhibitor of Des1.[20] SK2 inhibition may act

cooperatively with Des1 inhibition in the antiproliferative effects seen with **39** (PC3 GI_{50} = 0.7 μM) and **2** (GI_{50} = 28-30 μM in several prostate cancer cells line).[20]

To test if the antiproliferative compounds **1**, **10** and **34** inhibit the clonogenic potential of PC3 cells, we performed soft agar colony formation assays (Fig 6).[21] All compounds showed qualitatively similar potency to inhibit anchorage-independent growth in soft agar compared to that obtained in the plated assays. For example, the most potent compound, **34**, has an IC_{50} of 0.5 μM and 0.34 μM in the soft agar and plated assays, respectively.

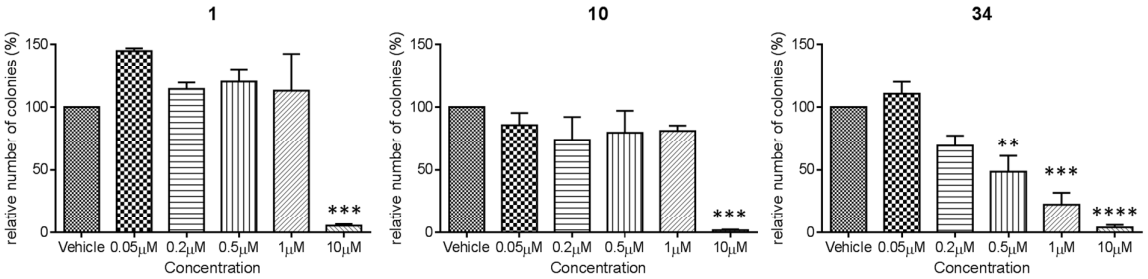
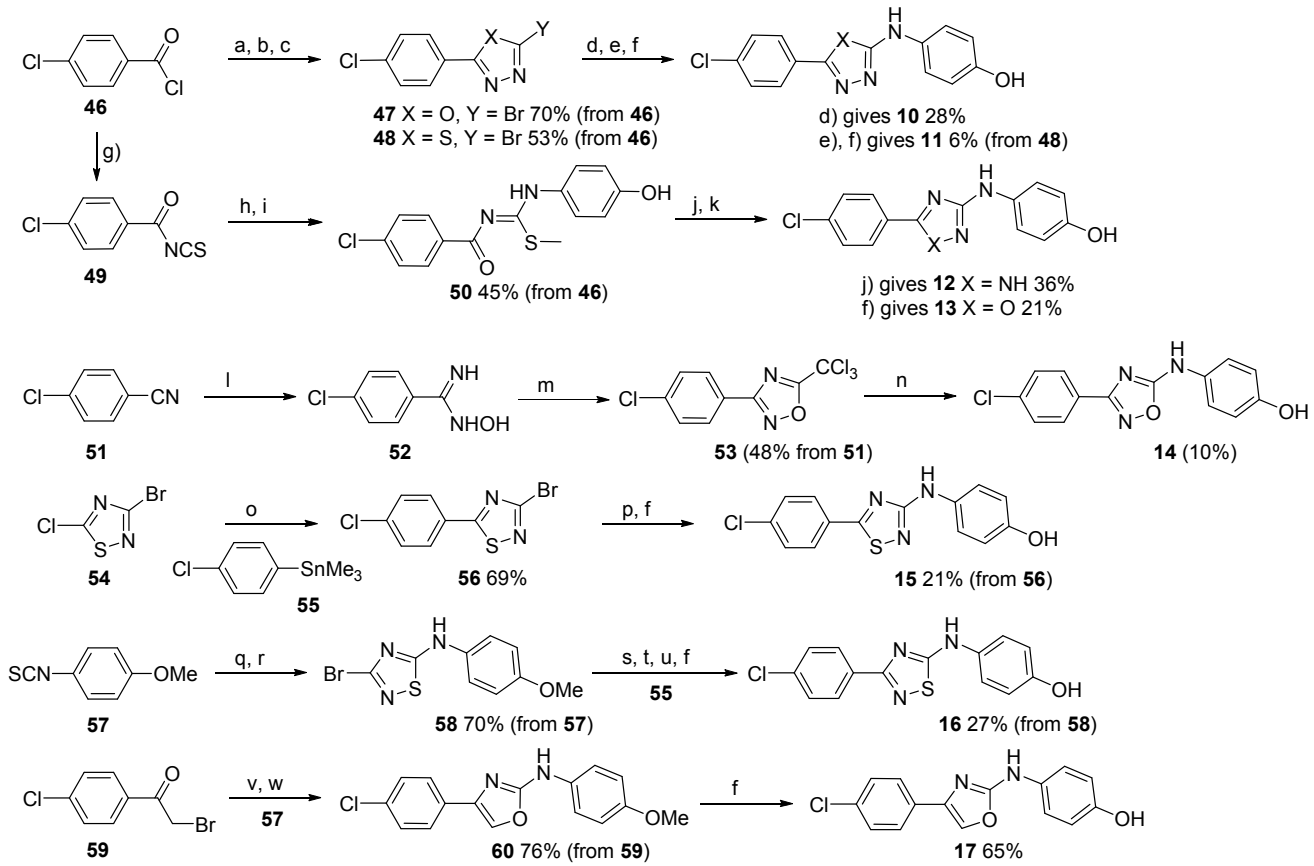


Figure 6: Inhibition of colony formation in soft agar. PC3 cells were seeded in soft agar and treated with increasing concentrations of each compound. Data represent mean ± SEM of three independent experiments performed in duplicate. ***P*<0.01. ****P*<0.001 *****P*<0.0001.

Synthetic Chemistry: The synthesis of bioisosteric replacements of the thiazole ring (Table 1) involved the preparation of a series of oxadiazoles, thiadiazoles, triazoles and oxazoles (Scheme 2). The 1,3,4-oxadiazole **10** and 1,3,4-thiadiazole **11**, were both prepared through nucleophilic substitution of the corresponding heteroaryl bromides **47** and **48**. Heteroaryl bromides **47** and **48** were prepared by known methods, involving cyclocondensation of 4-chlorobenzoyl chloride (**46**) with hydrazine carboxamide (or thiocarboxamide), followed by Sandmeyer reaction to convert the initially formed heteroaryl amines (not shown) into bromides.[22] Bromide **47** underwent direct substitution by 4-aminophenol to give **10** in a low yield (28%). This direct substitution failed altogether in the case of the corresponding thiadiazol, necessitating the use of 4-methoxyaniline in

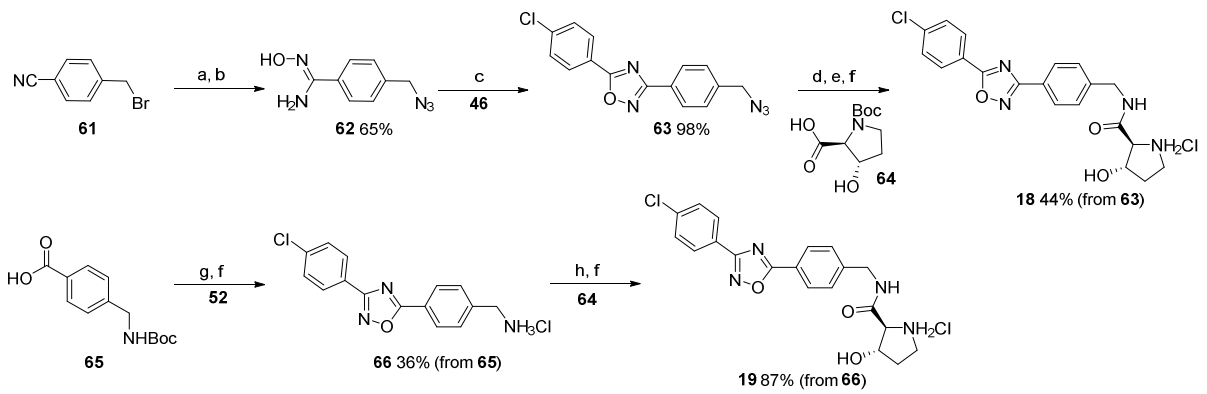
the nucleophilic aromatic substitution followed by demethylation to give **11** (6% from **48**). The triazole **12** and 1,2,4-oxadiazole **13** were also obtained from benzoyl chloride **46**, involving initial conversion to the benzoyl isothiocyanate **49** followed by reaction with 4-aminophenol and MeI to give a S-methylthiourea **50**[23] and cyclocondensation with hydrazine and hydroxylamine to give that gives **12** (36%) and **13** (21%), respectively. Preparation of the alternative 1,2,4-oxadiazole **14** involved conversion of 4-chlorobenzonitrile (**51**) into the amidine oxime **52**, followed by cyclocondensation with trichlororacetic anhydride to give **53** (71%),[24] which undergoes nucleophilic aromatic substitution with 4-aminophenol, displacing the trichloromethyl moiety to give **14** (10%). Thiadiazole **15** was obtained from selective substitution of the bromochlorothiadiazole **54** (Scheme 2). Chemoselective Pd-mediated Stille coupling of **54** with the aryl stannane **55** selectively substitutes the activated chloro group to give the bromothiadiazole **56**,[25] this is subject to nucleophilic aromatic substitution with 4-methoxyaniline and demethylation to yield **15** (21%). The isomeric thiadiazole **16** was obtained by sequential treatment of the 1-isothiocyanato-4-methoxybenzene (**57**) with sodium cyanogen and bromine, to yield the 3-bromo-1-2,4-thiadiazole **58** (70%).[26] This material was Boc-protected, coupled to stannane **55** and then deprotected and demethylated to give **16** (27%). Oxazole **17** was prepared from α-bromoketone **59**, initial displacement of the bromo group with NaN_3 and reaction with PPh_3 gives an aza-Wittig reagent (not shown) that reacts with the isothiocyanate **57** to give oxazole **60** (76%), which is demethylated to give **17** (65%).



Scheme 2: a) $H_2NC(X)NHNH_3Cl$ (X = O or S), $NaHCO_3$, AcONa, H_2O. b) H_2SO_4, rt (X = S); $POCl_3$, toluene, 140°C (X = O). c) isopentylnitrite, $CuBr_2$, MeCN, rt. d) 4-Aminophenol, DIPEA, DMF, 70 °C. e) 4-Methoxyaniline, DIPEA, DMF, 105 °C. f) BBr_3, CH_2Cl_2 . g) NH_4.SCN, acetone, rt. h) 4-Aminophenol, acetone, reflux. i) MeI, K_2CO_3, THF, 0 °C. j) $N_2H_4.H_2O$, EtOH, reflux. k) $NH_2OH.HCl$, DBU, EtOH, reflux. l) $NH_2OH.HCl$, Et_3N, EtOH, reflux. m) trichloroacetic anhydride, 110 °C. n) 4-Aminophenol, DBU, DMSO, rt. o) $Pd(PPh_3)_4$ 5 mol%, CuTC 10 mol%, **55**, 1,4-dioxane, reflux. p) 4-methoxyphenol, DIPEA, NMP, microwave: 160 °C. q) NaNHCN, acetone, rt. r) Br_2, EtAc, rt. s) Boc_2O, DMAP, 1,4-dioxane, rt. t) $Pd(PPh)_4$ 5 mol%, **55** 1,4-dioxane, reflux. u) TFA, CH_2Cl_2, rt. v) NaN_3, acetone, rt. w) **57**, PPh_3, dioxane, 100 °C.

The two hybrid structures of **1** and the Genzyme SK1 inhibitor **3**, compounds **18** and **19**, were prepared as described in Scheme 3. Preparation of **18** commenced with the 4-(bromomethyl)-

benzonitrile, the bromo group was substituted for an azide (NaN_3) and the nitrile converted into an amidine oxime **62** (65%) which was cyclocondensed with 4-chlorobenzoyl chloride (**46**) to give the 1,2,4-oxadiaxole **63** (98%).[27] Reduction of azide **63** into an amine and coupling with the Boc-protected hydroxyproline **64** and deprotection gave **18** (44%). Its oxadiazole isomer **19** was prepared by cyclocondensation of the acid **65** (activated as a mixed anhydride) with the amidine oxime **52** followed by deprotection to give **66** (36%), which is then coupled to the Boc-protected hydroxyproline **64** and deprotected to give **19** (87%).



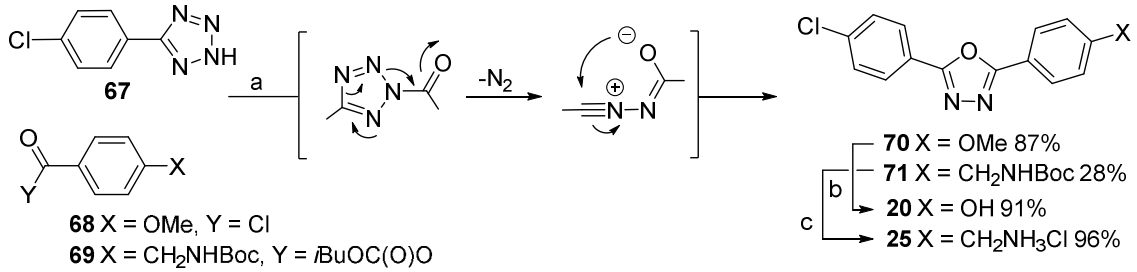
Scheme 3: a) NaN_3, DMF, rt. b) $NH_2OH.HCl$, Et_3N, EtOH, reflux. c) **46**, pyridine, xylene, reflux. d) $NH_4.HCO_2$, Zn, MeOH, rt. e) DIPEA, HATU, DMF, rt. f) EtOAc, HCl in dioxane. g) *N*-methymorpholine, isobutylchloroformate, dioxane, rt, then **52** and reflux. h) HATU, DIPEA, EtOAc.

SAR studies directed to finding alternatives to the 4-aminophenol group were all based on analogues of 1,3,4-oxadiazole ring system **10**. Direct access to systems in which the amino linker unit (L) was absent (diaryl-1,3,4-oxadiazoles) could be attained through cyclocondensation of the tetrazole **67** with benzoyl chlorides **68** and **69** to give **70** (87%) and **71** (28%) followed by demethylation and deprotection to provide test compounds **20** (91%) and **25** (96%), respectively (Scheme 4). The bromo-oxadiazole **47** also served as useful substrate for Suzuki coupling to give

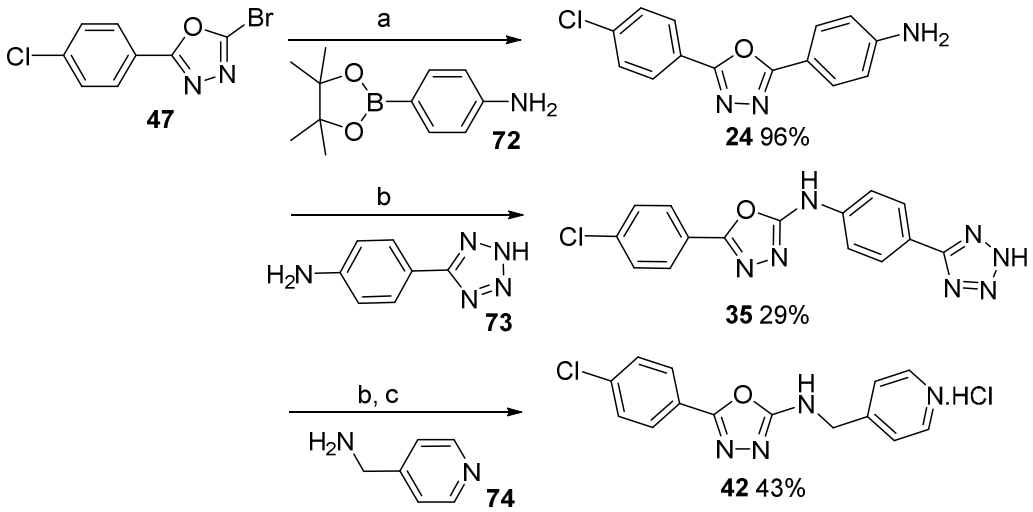
24 (96%) and through nucleophilic substitution with amines to give **35** (29%) and **42** (43%) (Scheme 5).

Many of the test compounds were achieved through cyclocondensation of the hydrazine amide **75** with different electrophiles (Scheme 6). Cyclocondensation of **75** with chloroacetic acid or ethyl oxalyl chloride in $POCl_3$ delivered the functionalised oxadiazoles **76**[28] and **77**[29], respectively, which could be converted into test compounds **22**, **23** and **41** by nucleophilic substitution (Scheme 6). Hydrolysis and decarboxylation of the ester in **77** afforded the C5-unsustituted oxadiazole **82**, which was coupled to 4-iodobenzonitrile to give **83** (75%) using a previously described Cu-mediated C-H activation process.[30] Cyclocondensation of the hydrazine amide **75** with CS_2 gave thiol **78**,[31] which was used in a Cu-mediated coupling with 4-iodoanisole to give sulfide **21** (60%). **75** was also cyclocondensed with benzoyl chloride **79** and 4-isothiocyanatobenzonitrile (**80**) to give the carboxylic acid **26** (83%) (after ester hydrolysis) and nitrile **81** (67%), respectively.

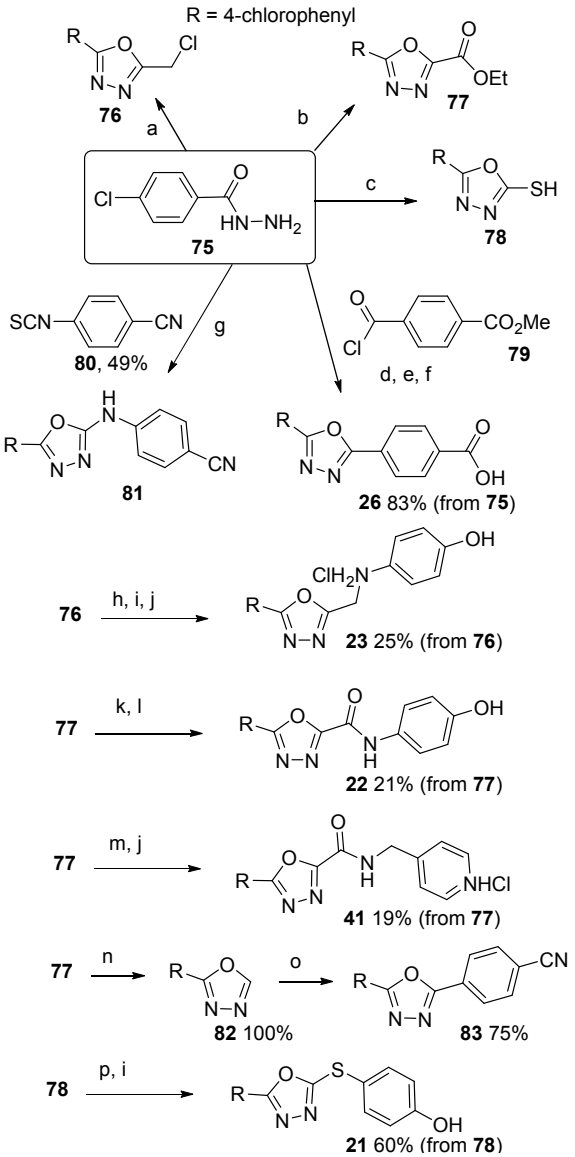
The carboxylic acid **26** and the two nitriles **81** and **83** were used in the synthesis of other test compounds (Schemes 7 and 8). The carboxylic acid **26** was converted to the simple amide **27** (43%) and the proline derivative, amidine **30** (10% over 3 steps) (Scheme 7). Nitrile **83** was converted to the tetrazole **31** (71%), by cycloaddition of NaN_3 (Scheme 8). Both nitriles **83** and **81** were converted into amidine oximes **29** (52%) and **34** (66%) and amidines **28** (66%) and **33** (27%) upon addition of LiHMDS and NH_2OH, respectively (Scheme 8). Hydration of **81** gave the simple amide **32** (94%) and DIBAL reduction afforded an aldehyde (not shown) that gave the aldimine oxime **36** (20%) upon Schiff's condensation with NH_2OH.



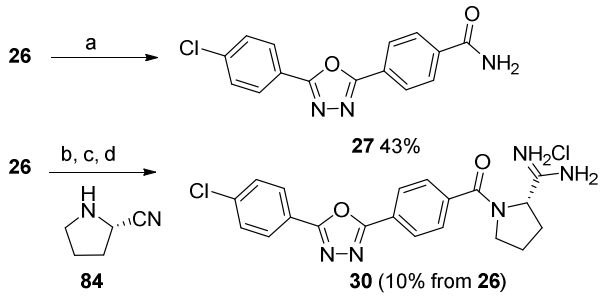
Scheme 4: a) Pyridine, toluene, reflux. b) BBr_3, CH_2Cl_2. c) HCl in dioxane, EtOAc



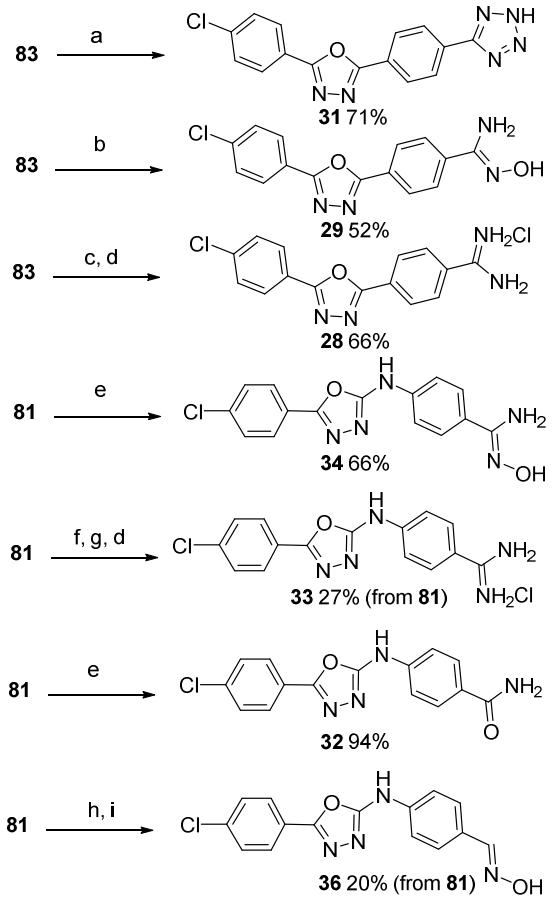
Scheme 5: a) $Pd(PPh_3)_4$ 10 mol%, Na_2CO_3, DMF, 80 °C. b) DIPEA, DMF, heat. c) HCl in dioxane, EtOAc



Scheme 6: a) Chloroacetic acid $POCl_3$, reflux. b) Ethyl oxalyl chloride, then POC_3, 100 °C. c) CS_2, KOH, MeOH, reflux. d) **79**, pyridine, rt.e) $POCl_3$, reflux. f) LiOH, THF/H_2O, then HCl(aq). g) THF, rt, then tosyl chloride, pyridine, reflux. h) 4-Methoxyaniline, DMSO, K_2CO_3, rt. i) BBr_3, CH_2Cl_2 . j) EtOAc, HCl dioxane. k) LiOH, THF/H_2O, rt. l) 4-Aminophenol, HATU, Et_3N, DMF, rt. m) **74**, EtOH, reflux. n) LiOH, THF/H_2O, rt, then HCl(aq) pH = 1-2, rt. o) 4-Iodobenzonirile, CuI 20 mol%, 1,10-phenathroline 40 mol%, Cs_2CO_3, DMSO, 100 °C. p) CuI 5 mol%, 1,10-phenathroline 10 mol%, K_2CO_3, DMF, 120 °C.

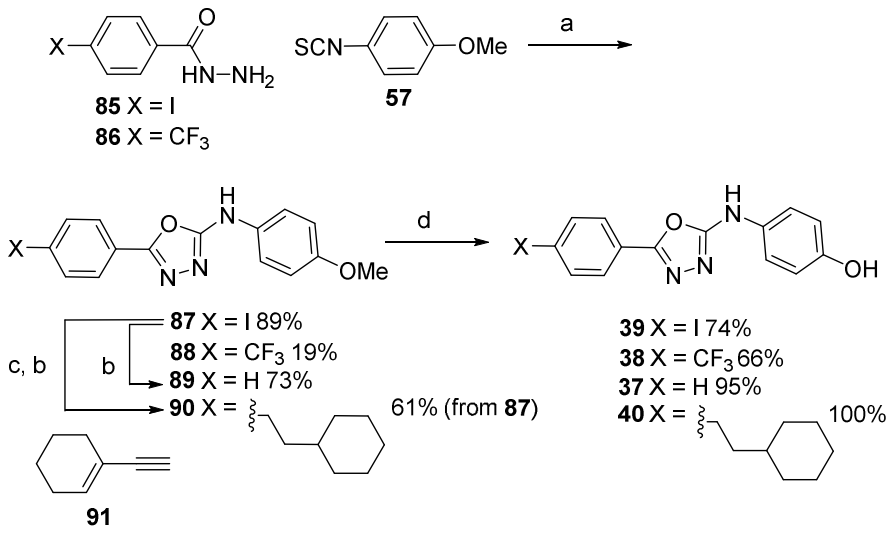


Scheme 7: a) Oxalyl chloride, CH_2Cl_2, DMF cat., NH_4OH, rt. b) **84**, HATU, Et_3N, DMF, rt. c) LiHMDS, THF, rt. d) EtOAc, HCl dioxane.



Scheme 8: a) NH_4Cl, NaN_3, DMF, 100 °C. b) $NH_2OH.HCl$, Et_3N, EtOH, reflux. c) NaOMe, NH_4Cl, MeOH, 50 °C. d) EtOAc, HCl dioxane. e) H_2SO_4, H_2O, rt. f) Boc_2O, DMAP 2 mol%, dioxane. g) LiHMDS, THF, HCl (aq). h) DIBAL, toluene, then HCl (aq). i) $NH_3OH.Cl$, Et_3N, MeOH, rt.

A small number of analogues of **1** wherein the chloro substituent was removed or replaced was also generated for this study (Scheme 9). These were prepared by reaction of the benzoyl hydrazine amides **85** and **86** with the isothiocyanate **57** to give **87** (89%) and **88** (19%), respectively. The iodo group in **87** was removed by hydrogenation to give **89** (73%) and coupled to ethynylcyclohexene **91** followed by hydrogenation to give **90** (61%). Demethylation of **87-90** yielded the test compounds **37**, **38**, **39** and **40**.



Scheme 9: a) THF, rt, then TosCl, pyridine, reflux. b) Pd/C, H_2 (g), 1 atm, MeOH / EtOAc, rt. c) **91**, $Pd(PPh_3)_2Cl_2$ 5 mol%, CuI 3 mol%, DMF, Et_3N, rt. d) BBr_3, CH_2Cl_2

Conclusion: This study delineates the SAR of the extensively utilized SK1/2 inhibitor **1** (SKI-II)[8] that is also known to induce SK1-degradation[5] and inhibit Des1.[6] SK1-kinase inhibition and induction of SK1-degradation appear to be independent effects, where different compounds give rise to either of these effects though some compounds do both. This is consistent with the previous

proposal that compounds may either bind to the substrate (Sph) domain to inhibit SK1-kinase activity or to an allosteric site to promote proteasomal degradation of SK1 and that some compounds, such as **1** and **5**, do both.[5c] However, while our data are consistent with this allosteric model, other explanations for small molecule promoted SK1-proteasomal degradation, including off-target effects, remain a possibility. Importantly, our data do show that PC3 cell growth is not inhibited by inhibition of SK1-kinase activity or by SK1-degradation and that inhibition of Des1 is the most likely source of the anticancer activity of **1** and it congeners. While SK2 inhibition in isolation does not appear to exert a cell growth inhibitory effects (e.g. **31**), the combination of Des1 and SK2 inhibition may have cooperative effects (e.g. **39**), which also may be of relevance to clinical candidate **2**,[20] but this cooperativity needs to be further verified. These findings should be borne in mind when interpreting findings made using **1** and **2** as probes for SK1/2 activity.[7,8]

In the course of this study new lead compounds have been identified exhibiting different activities: selective SK2-inhibition by **31** (Table 2, entry 13), dual SK2/Des1 inhibition by **39** (Table 2, entry 21 and Fig 3); SK1/2 kinase inhibition by **42** (Table 2, entry 24); SK1-degradation by **19** (Fig 2); and Des1 and PC3 cell growth inhibition by **34** (Table 2, entry 16 and Fig 3). These new leads provide a basis for future studies which will focus on better understanding the source of each of these activities and further optimizing them to create valuable probes and potential therapeutics.

Experimental

PC3 cell viability assays

Routine cell culture: PC3 prostate cancer cell lines were cultured in DMEM (containing 10% fetal calf serum and penicillin-streptomycin). Cells were grown at 37 °C with 5 % CO_2 and passaged when 80-90% confluent 4 times before use. Cells were harvested by trypsin treatment (5 min) then quenched with an equal volume of serum containing media and the cell suspension then centrifuged at 200 xg for 5 min and the pellet resuspended in 5 mL of media. Cells were exposed

to Trypan blue (excludes dead cells) and counted with a haemocytometer. Before treatment with drug compounds, cells were plated at 2,500 cells/well in 96 well plates and incubated at 37 °C with 5 % CO_2 in a humidified incubator for 24 h. Drug stock solutions (50 or 10 mM) were diluted x 1000 in media to a final concentration of either 50 μM or 10 μM with a DMSO vehicle concentration of 0.1%. Compounds were then serially diluted in media (containing 0.1% DMSO) to give 8 final concentrations, all at 0.1% DMSO. Cell culture supernatants were aspirated and replaced with drug containing media. Drug treatments were performed in duplicate wells, while potential plate layout-specific variation in cell growth was accounted for by addition of a vehicle control (0.1% DMSO). An untreated control (media only) and active compound control (50 μM **1**) was included in each assay. Cells were then incubated with drug compounds at 37 °C with 5 % CO_2 in a humidified incubator for 72 h prior to assay. Cell media were diluted with CellTitre AQ_{ueous} One Solution to produce a final concentration of 317 μg/mL. Cell culture supernatants were then aspirated from wells and replaced with 100 μL of CellTitre solution. Triplicate cell-free control wells containing only CellTitre solution were also included in each assay. Cells were then incubated at 37 °C with 5 % CO_2 in a humidified incubator for 1 h at which time absorbance was read at 490 nm with a microplate reader. When analysing data, background absorbance (taken from cell-free control wells) was subtracted from each reading. To determine percentage inhibition of cell viability, absorbance readings for each drug treatment were expressed as a fraction of the vehicle control (0.1% DMSO) readings. For each drug concentration the mean (± SEM) is calculated and a sigmoidal curved is fitted to the data and used to calculate the IC_{50} of each compound.

Sphingosine kinase-1/2 Activity Assay

The SphK assays employed, measures SK activity through the production of ^{32}P-labelled S1P following the addition of exogenous Sph and [γ^{32}P] ATP as described previously.[11]

Activities of SK1 and SK2 were determined using identical assay conditions with the exception that SK1 assays used 3ng/assay recombinant His-tagged human SK1, while SK2 assays used 30ng/assay recombinant human SK2.[12]

Compounds were dissolved in DMSO at 10 mM, the mixture was vortexed (and sonicated if necessary). Stock solutions were kept at 4 °C until used. Aliquots were made containing 100 µM drug compound. These aliquots were then diluted in assay buffer: 100mM Tris/HCl buffer (pH 7.4) containing, 100 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF to make up a 10 µM solution.

Measurement of dihyrdoceramide desaturase-1 (Des1) activity:

Measurement of Des1 activity was performed by HPLC using intact Jurkat cells labeled with DhCer-C6-NBD as described previously[14] with modifications to enhance sensitivity and reproducibility. These modifications included the use of parental Jurkat cells, 0.5% serum in the culture media, and cell harvesting via centrifugation at 500 xg to maximise ceramide extraction. Extracted samples (50 µl) were analysed on a Waters HPLC coupled to a fluorescence detector using a 30cm C18 reverse-phase column eluted with 1 ml/min 20% H_2O and 80% acetonitrile with 0.1% trifluoroacetic acid. NBD-labelled substrate and product were quantitated with excitation and emission wavelengths of 465 nm and 530 nm, respectively.

Lipidomics method

In vitro cultured PC3 cells were exposed to test compounds (10 µM) or vehicle control (0.1% DMSO) for five hours. Cells were lifted with trypsin, quenched and washed with cold saline (4 °C), counted and adjusted to 10^6 cells per sample. Lipids were extracted from cell pellets by addition of 200 µL chloroform/methanol/water (1:3:1) and three freeze-thaw cycles. Protein and cellular debris was removed by centrifugation and samples stored at -20 °C for <1 week until LC-MS analysis.

Lipid analysis utilized LC-MS with reversed phase chromatography (Dionex Ultimate RSLC3000; Thermo) and high resolution mass spectrometry (Q-Exactive; Thermo). 10 µL sample was injected onto an Ascentis Express C8 column (5cm x2.1mm, 2.7µm; Supelco Anaytical), with 30 minute gradient elution. Mobile phase A consisted of 40% Isopropanol, 60% H_2O, 2 mM formic acid, 8 mM ammonium formate. Mobile phase B: 2% H_2O in isopropanol, 2 mM formic acid, 8 mM ammonium formate. The gradient ran from 0% B to 20% B in 1.5min, to 28% B (7 min) to 35% B (8 min) to 65% B (24 min) at a flow rate of 200 µL/min, and increased to 100% B at 500µL/min (25-27 min), and returned to 100% A (29 min). The mass spectrometer was operated in positive and negative mode (rapid switching) electrospray ionization with a mass resolution of 140,000 and mass accuracy < 2ppm. Data-dependent high resolution (17,500) MSMS was performed on pooled extracts to confirm lipid identifications.

Lipidomics data was analysed using the IDEOM software[32] to provide relative quantification of all detected features. Lipids and other metabolites were identified according to accurate mass, with additional manual curation of significant lipids based on retention time and MSMS fragmentation spectra. Ceramides and other sphingolipids were quantified by targeted extraction of peak areas for extracted ion chromatograms using the Tracefinder software (Thermo).

Soft agar assay:

To test clonogenic potential and anchorage independent growth of PC3 cells, we performed colony formation assay as previously described.[21] Briefly, in a 6-well plate, 5,000 filtered PC3 cells were added in the presence of 0.35% low melting point agarose onto a bed of 0.7% low melting point agarose with RPMI media containing 10% Fetal Bovine Serum (FBS) final concentration and allowed to solidify as a single cell suspension at 4 °C for 4 min before incubation at 37 °C in a high CO_2 cell culture incubator. Compounds were diluted in RPMI media and added to cells 24 h after incubation. Plates were incubated at 37 °C for 21 days. Subsequently, media were removed

and colonies visualized after incubation with 0.005% crystal violet (Sigma, USA) solution for 1 hour at 37 °C. Colonies were scanned and whole wells were counted for positively stained colonies.

Synthesis of test compounds:

General: All reactions were performed under an inert atmosphere of anhydrous N_2 (g) using dry glassware. Tetrahydrofuran (THF), dimethylformamide (DMF) and dichloromethane (DCM) were dried using a commercial solvent purification system. 1,2-Dichloroethane (DCE) and diisopropylethylamine (DIPEA) were purchased in an anhydrous form and stored under nitrogen. Other solvents and reagents were used as supplied by commercial vendors without further purifications. Petroleum spirits with a boiling point range of 40-60 ºC was used in chromatography. Column (flash) chromatography was performed on either 40-60 µm silica gel or neutral alumina (activation grade III), as indicated. ^{1}H NMR spectra were recorded at 400 MHz and are reported as follows: chemical shift (δ_H) (integration, multiplicity and coupling constant (Hz)). The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublets, dt = doublet of triplets. ^{13}C NMR spectra were recorded at 100 MHz reported in terms of chemical shift (δ_C). All chemical shifts were calibrated using residual nondeuterated solvent as an internal reference and are reported in parts per million relative to trimethylsilane. LCMS was performed using a photodiode array detector (214/254 nm) coupled directly to an electrospray ionization source and a single quadrupole mass analyser (Agilent 6120 Quadrupole MS). The associated LC component employed RP-HPLC carried out at 30 °C on a 5μm C_8 column, 50 x 4.60 mm (ID). The following buffers were used; buffer A 99.9% H_2O, 0.1% formic acid and buffer B 99.9% CH_3CN, 0.1% formic acid. The following gradient was used with a flow rate of 0.5 mL/min and total run time of 12 min; 0–4 mins 95% buffer A and 5% buffer B, 4–7 mins 0% buffer A and 100% buffer B, 7–12 mins 95% buffer A and 5% buffer B. High-resolution mass spectra (HRMS) were recorded on a

time of flight mass spectrometer fitted with either an electrospray (ESI) or atmospheric pressure ionization (APCI) ion source. Compound purity was determined using HPLC and is > 95% for all test compounds, except **15** (93.2%) and **42** (93.8%).

5–(4–Chlorophenyl)–*N*–(4–hydroxyphenyl)–1,3,4–oxadiazol–2–amine 10.

2-Bromo-5-(4-chlorophenyl)-1,3,4-oxadiazole (**47**)[22] (0.1 g, 0.385 mmol), 4-aminophenol (0.105 g, 0.963 mmol) and DIPEA (164 μL, 0.963 mmol) in DMF (1.5 mL) were heated at 70 °C for 3 h. The cooled solution was partitioned between EtOAc (150 mL) and water (100 mL). The aqueous layer was removed and the organic layer was washed with water (3 × 100 mL), then brine (20 mL). The organic layer was dried over $MgSO_4$, concentrated and chromatographed on silica gel eluting with 50% EtOAc in petroleum spirit providing **10** as a beige solid (0.031 g, 28% yield). Mp 252 °C dec. ^{1}H NMR (400 MHz, DMSO) δ 10.31 (s, 1H), 9.16 (s, 1H), 7.87 (d, J = 8.6 Hz, 2H), 7.63 (d, J = 8.6 Hz, 2H), 7.39 (d, J = 8.9 Hz, 2H), 6.77 (d, J = 8.9 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 160.4, 156.7, 152.6, 135.3, 130.3, 129.5, 127.1, 122.9, 119.0, 115.5. LCMS R_f (min) = 5.44. MS m/z 288.0 (M + H). HR–ESI calcd for $C_{14}H_{11}ClN_3O_2^+$ (M + H) 288.0534, found 288.0534.

4–((5–(4–Chlorophenyl)–1,3,4–thiadiazol–2–yl)amino)phenol 11.

2-Bromo-5-(4-chlorophenyl)-1,3,4-thiadiazole (**48**)[22] (0.53 g, 1.925 mmol), 4-methoxyaniline (0.595 g, 4.815 mmol) and DIPEA (820 μL, 4.815 mmol) in DMF (1 mL) were heated at 100–105 °C for 2 days. The cooled solution was partitioned between EtOAc (150 mL) and HCl (aq) (0.5 M, 100 mL). The aqueous layer was removed and the organic layer was washed with water (3 × 100 mL), then brine (20 mL). The organic layer was dried over $MgSO_4$ then concentrated to a semi-solid that was triturated with DCM providing the intermediate methyl ether as a golden coloured solid that was filtered and washed with DCM (0.132 g, 22% yield). ^{1}H NMR (400 MHz, DMSO) δ 10.36 (s, 1H), 7.90 – 7.81 (m, 2H), 7.61 – 7.51 (m, 4H), 7.00 – 6.91 (m, 2H), 3.74 (s, 3H).

BBr_3 (19 μL, 0.2 mmol) was added at to a solution of the methyl ether (above) (0.05 g, 0.157 mmol) in dry DCM (5 mL) at 0 °C and stirred at this temperature for 3h. The reaction was quench by dropwise addition of $NaHCO_3$ (aq) (sat., 1 mL), followed by EtOAc (20 mL) and vigorously stirred for 2 min. H_2O (5 mL) was added to the stirred solution followed by 6 M HCl (aq) until pH = 2. The aqueous layer was removed and the organic layer was washed with water (3 × 30 mL), then brine (20 mL). The organic layer was dried over $MgSO_4$ and concentrated to a solid that was triturated with EtOAc that was filtered and washed with EtOAc and finally $CHCl_3$ providing **11** as a light brown powder (0.013 g, 27% yield). Mp = 252 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.22 (s, 1H), 9.23 (s, 1H), 7.84 (d, *J* = 8.7 Hz, 2H), 7.55 (d, *J* = 8.7 Hz, 2H), 7.41 (d, *J* = 8.9 Hz, 2H), 6.77 (d, *J* = 8.9 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 165.5, 155.2, 153.1, 134.4, 132.5, 129.4, 129.2, 128.2, 120.0, 115.6. LCMS R_f (min) = 5.66. MS m/z 304.0 (M + H). HR–ESI calcd for $C_{14}H_{11}ClN_3OS^+$ (M + H) 303.0233, found 303.0230.

4-((5-(4-Chlorophenyl)-4*H*-1,2,4-triazol-3-yl)amino)phenol 12.

Methyl (Z)-*N*'-(4-chlorobenzoyl)-*N*-(4-hydroxyphenyl)carbamimidothioate (**50**)[23] (0.5 g, 1.559 mmol) in EtOH (20 mL) was added $N_2H_4.H_2O$ (380 μL, 7.793 mmol) and the mixture was refluxed for 5 h. The cooled solution was concentrated to a solid that was taken up in water (30 mL) and neutralised with AcOH. The resultant solid was filtered and washed with water providing a white solid (0.290 g). The solid was chromatographed on silica gel eluting with 50% EtOAc in petroleum spirit providing **12** as a white solid (0.161 g, 36% yield). Mp = 281 °C. ^{1}H NMR (400 MHz, DMSO) δ 12.50 (bs, 1H), 9.27 – 8.56 (m, 2H), 7.96 (d, *J* = 8.5 Hz, 2H), 7.70 – 7.43 (m, 2H), 7.35 (d, *J* = 8.4 Hz, 2H), 6.69 (d, *J* = 7.9 Hz, 2H). LCMS R_f (min) = 5.23. MS m/z 287.0 (M + H). HR–ESI calcd for $C_{14}H_{12}ClN_4O^+$ (M + H) 287.0694, found 287.0693.

4–((5–(4–Chlorophenyl)–1,2,4–oxadiazol–3–yl)amino)phenol 13.

Methyl (Z)-*N'*-(4-chlorobenzoyl)-*N*-(4-hydroxyphenyl)carbamimidothioate (**50**)[23] (0.5 g, 1.559 mmol)was added to a mixture of NH_2-OH.HCl (0.542 g, 7.795 mmol) and DBU (1.63 mL, 10.913 mmol) in EtOH (20 mL) in which the mixture was refluxed for 4 h. The cooled solution was concentrated to a residue that was taken up in water (30 mL) and neutralised with 6M HCl (aq). The resultant solid was filtered and washed with water providing a white solid (0.308 g). The solid was dissolved in DCM (300 mL) and filtered through a short silica column eluting with 50% EtOAc in petroleum spirit and recrystallised from EtOH providing **15** as a white solid (0.092 g, 21% yield). Mp 119–122 °C. ^{1}H NMR (400 MHz, DMSO) δ 9.65 (s, 1H), 9.05 (s, 1H), 8.06 (d, *J* = 8.8 Hz, 2H), 7.71 (d, *J* = 8.8 Hz, 2H), 7.30 (d, *J* = 9.0 Hz, 2H), 6.74 (d, *J* = 8.9 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 171.6, 165.7, 151.9, 137.8, 131.8, 129.7, 129.4, 122.6, 118.7, 115.4. LCMS R_f (min) = 5.80. MS m/z 288.0 (M + H). HR–ESI calcd for $C_{14}H_{11}ClN_3O_2^+$ (M + H) 288.0534, found 288.0532.

4–((3–(4–Chlorophenyl)–1,2,4–oxadiazol–5–yl)amino)phenol 14.

3-(4-Chlorophenyl)-5-(trichloromethyl)-1,2,4-oxadiazole (**53**)[24] (0.613 g, 2.057 mmol) was dissolved in DMSO (9.2 mL) followed by 4-aminophenol (0.748 g, 6.859 mmol) and DBU (1.04 mL, 6.909 mmol) at rt. The mixture was stirred at for 3 h then diluted with EtOAc (50 mL) and washed with 1M HCl (aq) (20 mL), then water (4 × 20 mL) and finally brine (20 mL). The organic layer was dried over $MgSO_4$ and concentrated to a black/brown semi-solid. The crude was triturated with DCM (5 mL) providing a dark brown powder (0.164 g, 85-90% pure). The powder was chromatographed on silica gel eluting with 30% EtOAc in petroleum spirit and recrystallized from isopropanol and petroleum spirit providing **14** as an off-white solid (0.058 g, 10% yield). Mp 238–241 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.75 (s, 1H), 9.28 (s, 1H), 7.97 (d, *J* = 8.7 Hz, 2H), 7.61 (d, *J* = 8.7 Hz, 2H), 7.42 (d, *J* = 8.9 Hz, 2H), 6.79 (d, *J* = 8.9 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 168.6, 166.6, 153.5, 135.8, 129.5, 129.2, 128.6, 126.1, 120.1, 115.6. LCMS R_f (min) =

5.80. MS m/z 288.0 (M + H). HR–ESI calcd for $C_{14}H_{11}ClN_3O_2^+$ (M + H) 288.0534, found 288.0531.

4-((5-(4-chlorophenyl)-1,2,4-thiadiazol-3-yl)amino)phenol 15

$Pd(PPh_3)_4$ (0.102 g, 0.0882 mmol) and CuTC (0.034 g, 0.176 mmol) were added to a solution of (4-chlorophenyl)trimethylstannane (0.730 g, 1.76 mmol) and 3-bromo-5-chloro-1,2,4-thiadiazole (0.17 mL, 1.76 mmol) in dry 1,4-dioxane (5.5 mL) and N_2 (g) bubbled through the mixture for 10 min to remove any oxygen. The mixture was then heated under N_2 (g) to 60 °C and left to stir overnight. Upon completion of the reaction (TLC), the mixture was partitioned in EtOAc (50 mL) and washed with H_2O (2 x 30 mL). The aqueous layers were then collected and back extracted with EtOAc (2 x 30 mL). The organic layers were then combined dried over $MgSO_4$ and concentrated to a brown solid (0.994 g). The crude material was then chromatographed on silica gel eluting with 5% EtOAc in petroleum spirit to give -bromo-5-(4-chlorophenyl)-1,2,4-thiadiazole **56** as a white solid (0.336 g, 69% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (d, J = 8.8 Hz, 2H), 7.50 (d, J = 8.8 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.1, 146.2, 139.3, 129.9, 128.7, 127.9. LCMS R_f (min) = 4.14. MS m/z 275.0 (M + 2H).

A solution of **56** (0.129 g, 0.468 mmol), *p*-anisidine (0.231 g, 1.873 mmol) and DIPEA (0.38 mL, 2.107 mmol) in dry NMP (2 mL) was heated to 160 °C in a microwave reactor for 3.5 h. The resultant mixture was diluted with EtOAc (50 mL) and washed with 0.5 M HCl (aq) (20 mL), H_2O (2 x 20 mL) and brine (20 mL). The aqueous layers were then collected and back extracted with EtOAc (3 x 30 mL). The organic layers were then combined dried over $MgSO_4$ and concentrated to a dark brown solid (0.09 g). Trituration with DCM lead to a crystalline gold solid being produced (0.022 g). The filtrate was then chromatographed on silica gel eluting with 10% EtOAc in petroleum spirit. The appropriate fractions were collected and concentrated giving 5-(4-chlorophenyl)-N-(4-methoxyphenyl)-1,2,4-thiadiazol-3-amine as a gold crystalline solid (0.077 g,

51% yield). ^{1}H NMR (400 MHz, DMSO) δ 10.20 (s, 1H), 7.98 (d, *J* = 8.7 Hz, 2H), 7.67 (dd, *J* = 8.9, 2.3 Hz, 4H), 6.90 (d, *J* = 9.1 Hz, 2H), 3.72 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 183.8, 166.7, 154.0, 136.8, 133.9, 129.7, 128.7, 128.7, 118.5, 114.0, 55.2. LCMS R_f (min) = 7.03. MS m/z 318.0 (M + H).

BBr_3 (0.057 mL, 0.604 mmol) was added dropwise to a solution of 5-(4-chlorophenyl)-N-(4-methoxyphenyl)-1,2,4-thiadiazol-3-amine (0.048 g, 0.151 mmol) in dry DCM (2.5 mL) at 0 °C and the mixture left to stir at room temperature for 2 h. The mixture was then quenched with $NaHCO_3$ (aq) (sat., 5 mL), H_2O (20 mL) added and the mixture left to stir for 0.5 h. The solution was then extracted with EtOAc (3 x 20 mL). The organic layers were combined and washed with $NaHCO_3$ (2x 20 mL) and brine (10 mL), dried over $MgSO_4$ and concentrated to dark brown solid (0.037 g). The crude material was chromatographed on silica gel eluting with 15% EtOAc in petroleum spirit giving **15** as a tan crystalline solid (0.019 g, 41% yield). ^{1}H NMR (400 MHz, DMSO) δ 10.06 (s, 1H), 9.04 (s, 1H), 7.97 (d, *J* = 8.6 Hz, 2H), 7.66 (d, *J* = 8.6 Hz, 2H), 7.54 (d, *J* = 8.9 Hz, 2H), 6.71 (d, *J* = 8.9 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 183.7, 166.9, 152.0, 136.7, 132.5, 129.7, 128.8, 128.7, 118.8, 115.2. LCMS R_f (min) = 6.36. MS m/z 304.0 (M + H). HR–ESI calcd for $C_{14}H_{10}ClN_3OS^+$ (M + H) 304.0306, found 304.0316.

4-((3-(4-Chlorophenyl)-1,2,4-thiadiazol-5-yl)amino)phenol 16.

A solution of 3-bromo-N-(4-methoxyphenyl)-1,2,4-thiadiazol-5-amine **58**[26] (50 mg, 0.175 mmol), DMAP (2 mg, 0.00875 mmol) and Boc_2O (115 mg, 0.525 mmol) in dry 1,4-dioxane (0.5 mL) was heated to 60 °C for 20 min, at which point the evolution of gas ceased. The solution was cooled to rt, diluted with EtOAc (30 mL) and filtered through a short silica plug. The filtrate was concentrated under reduced pressure to produce *tert*-butyl(3-bromo-1,2,4-thiadiazol-5-yl)(4-methoxyphenyl)carbamate as a pale yellow solid (68 mg, 100% yield). ^{1}H NMR (400 MHz, DMSO) δ 7.37 (d, *J* = 9.0 Hz, 2H), 7.03 (d, *J* = 9.0 Hz, 2H), 3.82 (s, 3H), 1.40 (s, 9H). ^{13}C NMR

(101 MHz, $CDCl_3$) δ 180.3, 159.6, 153.4, 146.8, 140.8, 130.1, 128.8, 114.6, 55.5, 27.9, 27.4. LCMS R_f (min) = 4.11. EIMS m/z 387.2 (M + H).

Pd(tBu$_3$P)$_2$ (0.010 g, 0.0181 mmol) was added to a solution of *tert*-butyl(3-bromo-1,2,4-thiadiazol-5-yl)(4-methoxyphenyl)carbamate (0.140 g, 0.363 mmol) and (4-chlorophenyl)trimethyltin **55** (0.200 g, 0.725 mmol) in dry 1,4-dioxane (5 mL) and N_2 (g) was then bubbled through the mixture for 10 min then heated under N_2 (g) at reflux for 16 h. The mixture was diluted with EtOAc (50 mL), washed with H_2O (2 x 20 mL) and aqueous layers back extracted with EtOAc (2 x 20 mL). The combined organic layers were then dried over $MgSO_4$ and concentrated to a brown solid (0.254 g). This material was chromatographed on silica gel eluting with 5% EtOAc in petroleum spirit to give *tert*-butyl(3-(4-chlorophenyl)-1,2,4-thiadiazol-5-yl)(4-methoxyphenyl)carbamate as a light tan crystalline solid (0.069 g, 44% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, J = 8.7 Hz, 2H), 7.31 (d, J = 8.7 Hz, 2H), 7.20 (d, J = 9.0 Hz, 2H), 6.99 (d, J = 9.0 Hz, 2H), 3.90 (s, 3H), 1.48 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 179.6, 166.6, 159.4, 153.6, 135.9, 131.8, 131.2, 129.4, 129.2, 128.7, 114.4, 85.0, 55.6, 31.1, 28.1. LCMS R_f (min) = 4.761. EIMS m/z 418.1 (M + H).

To *tert*-butyl(3-(4-chlorophenyl)-1,2,4-thiadiazol-5-yl)(4-methoxyphenyl)carbamate (0.0224 g, 0.0536 mmol) in dry DCM (1 mL) was added TFA (0.1 mL) dropwise at 0 °C. The mixture was then left to stir at room temperature for 2.5 h. The mixture was then concentrated to produce 3-(4-chlorophenyl)-N-(4-methoxyphenyl)-1,2,4-thiadiazol-5-amine as a light brown crystalline solid (0.017 g, 100% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (d, J = 8.6 Hz, 2H), 7.52 (d, J = 8.6 Hz, 2H), 7.29 – 7.24 (m, 2H), 7.00 (d, J = 8.9 Hz, 2H), 3.85 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 179.9, 160.5, 158.9, 138.7, 130.7, 129.7, 129.2, 126.7, 122.6, 115.6, 55.8. LCMS R_f (min) = 6.97. EIMS m/z 318.0 (M + H).

BBr_3 (0.025 mL, 0.268 mmol) was added dropwise to 3-(4-chlorophenyl)-N-(4-methoxyphenyl)-1,2,4-thiadiazol-5-amine (0.0284 g, 0.0894 mmol) in dry DCM (1 mL) at 0 °C and left to stir at rt

for 2 h. The reaction was quenched with $NaHCO_3$ (aq) (sat., 3 mL) diluted with H_2O (15 mL) and extracted with EtOAc (3 x 20 mL). The organic layers were combined and washed with $NaHCO_3$ (aq) (sat., 2 x 20 mL), brine (10 mL) before, dried over $MgSO_4$ and concentrated to brown solid (0.024 g). The crude material was then chromatographed on silica gel eluting with 30% EtOAc in petroleum spirit. The appropriate fractions were collected and concentrated to give **16** light brown crystalline solid (0.017 g, 62% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (d, *J* = 8.7 Hz, 2H), 7.42 (d, *J* = 8.7 Hz, 2H), 7.21 (d, *J* = 8.8 Hz, 2H), 6.91 (d, *J* = 8.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 167.5, 153.8, 134.8, 131.7, 129.3, 128.9, 120.3, 115.9. LCMS R_f (min) = 6.31. EIMS m/z 304.0 (M + H). HR–ESI calcd for $C_{14}H_{10}ClN_3OS^+$ (M + H) 304.0306, found 304.0317.

4–((5–(4–Chlorophenyl)oxazol–2–yl)amino)phenol 17.

2-Bromo-1-(4-chlorophenyl)ethan-1-one (**59**) (1.07 g, 4.57 mmol) was dissolved in acetone (75 mL) and sodium azide (0.59 g, 9.08 mmol) was added to the stirred mixture in one portion and allowed to stir at rt overnight. The mixture was concentrated to a residue and then partitioned between DCM (50 mL) and H_2O (30 mL) and the aqueous layer extracted with DCM (2 × 30 mL). The combined organic layers were dried over $MgSO_4$ and concentrated to the intermediate azide as an orange solid (0.796 g, 89%). The azide (0.796 g, 4.069 mmol) was dissolved in anhydrous 1,4-dioxane in an N_2 atmosphere and 1-isothiocyanato-4-methoxybenzene (468 μL, 3.391 mmol) was added, followed by PPh_3 (1.07 g, 4.069 mmol). The mixture was stirred with heating on an oil bath (100 °C) for 15 min. The cooled solution was concentrated and the resulting solid recrystallized from DCM, providing 5-(4-chlorophenyl)-*N*-(4-methoxyphenyl)oxazol-2-amine (**60**) as an off-white solid (0.77 g, 76% yield). Mp 165–167 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, *J* = 8.7 Hz, 2H), 7.40 (d, *J* = 9.1 Hz, 2H), 7.34 (d, *J* = 8.8 Hz, 2H), 7.11 (s, 1H), 6.92 (d, *J* = 9.1 Hz, 2H), 6.74 (s, 1H), 3.81 (s, 3H).

BBr_3 (79 μL, 0.831 mmol) was added to a solution of **60** (0.05 g, 0.166 mmol) in DCM (5 mL) at 0 °C and the mixture then warmed to rt for 3 h. The reaction was then quench with $NaHCO_3$ (aq)

(sat.,10 mL) and diluted with EtOAc (20 mL). The organic layer was washed with $NaHCO_3$ (aq) (sat.,10 mL), H_2O (10 mL) and brine (10 mL), dried over $MgSO_4$ and concentrated to a pink solid (0.05 g). The solid was chromatographed on silica gel eluting with 5% MeOH in DCM providing **17** as a light pink solid (0.031 g, 65%). Mp 191–193 °C. ^{1}H NMR (400 MHz, DMSO) δ 9.97 (s, 1H), 9.05 (s, 1H), 7.56 (d, *J* = 8.7 Hz, 2H), 7.47 (d, *J* = 8.8 Hz, 2H), 7.45 (s, 1H), 7.41 (d, *J* = 8.9 Hz, 2H), 6.72 (d, *J* = 8.9 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 157.3, 152.1, 142.2, 131.1, 130.9, 129.0, 127.1, 123.9, 123.6, 118.5, 115.4. LCMS R_f (min) = 5.85. MS m/z 287.0 (M + H). HR–ESI calcd for $C_{15}H_{12}ClN_2O_2^+$ (M + H) 287.0582, found 287.0579.

(2S,3S)-2-((4-(5-(4-Chlorophenyl)-1,2,4-oxadiazol-3-yl)benzyl)carbamoyl)-3-hydroxypyrrolidin-1-ium chloride 18.

4-Chlorobenzoyl chloride (**46**) (332 μL, 2.615 mmol) was added to a solution of (*Z*)-4-(azidomethyl)-*N'*-hydroxybenzimidamide **63**[27] (0.5 g, 2.615 mmol) in xylene (10 mL) and pyridine (1.5 mL) and refluxed for 2 h. The cooled (rt) mixture was diluted with EtOAc (100 mL) and washed with HCl (aq) (1 M, 30 mL), water (50 mL), $NaHCO_3$ (aq) (sat., 50 mL) and finally brine (30 mL). The organic layer was dried over $MgSO_4$ and concentrated to give **63** as a white solid (0.8 g, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 – 8.12 (m, 4H), 7.55 (d, *J* = 8.8 Hz, 2H), 7.48 (d, *J* = 8.5 Hz, 2H), 4.44 (s, 2H).

Ammonium formate (0.303 g, 4.812 mmol) and freshly activated zinc dust (0.315 g, 4.812 mmol) were added to a solution oxadiazole **63** (0.2 g, 0.642 mmol) in MeOH (17 mL) and stirred at rt for 3 h. The mixture was diluted with water (50 mL) and DCM (50 mL) and vigorously stirred. Then three pellets of NaOH (~ 0.6 g) were added and vigorous stirring continued for a further 30 min. The mixture was then filtered through celite, the organic layer was separated, dried over $MgSO_4$ and concentrated to provide (4-(5-(4-chlorophenyl)-1,2,4-oxadiazol-3-yl)phenyl)methanamine as a solid (0.181 g, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (d, *J* = 8.8 Hz, 2H), 8.13 (d, *J* = 8.3 Hz, 2H), 7.54 (d, *J* = 8.8 Hz, 2H), 7.47 (d, *J* = 8.5 Hz, 2H), 3.97 (s, 2H), 1.55 (bs, 2H).

HATU (0.359 g, 0.945 mmol) and DIPEA (429 µL, 2.52 mmol) were added to a solution of (4-(5-(4-chlorophenyl)-1,2,4-oxadiazol-3-yl)phenyl)methanamine (above) (0.18 g, 0.629 mmol) and (2*S*,3*S*)-1-(*tert*-butoxycarbonyl)-3-hydroxypyrrolidine-2-carboxylic acid (**64**) (0.146 g, 0.629 mmol) in DMF (5 mL) and the mixture stirred at rt for 16 h, diluted with water (100 mL) and extracted with (2 × 50 mL). The combined organics were washed with water (20 mL), HCl (aq) (0.5 M, 50 mL), $NaHCO_3$ (aq) (sat., 30 mL) and finally brine (30 mL). The organic layer was dried over $MgSO_4$ and concentrated to provide Boc protected product as a solid (0.36 g). The solid was recrystallised from MeOH providing an off-white solid (0.16 g, 51%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, *J* = 8.8 Hz, 2H), 8.11 (d, *J* = 8.1 Hz, 2H), 7.54 (d, *J* = 8.7 Hz, 2H), 7.40 (d, *J* = 8.3 Hz, 2H), 4.83 – 4.19 (m, 4H), 3.77 – 3.43 (m, 2H), 2.23 – 1.27 (m, 13H).

HCl in 1,4-dioxane (4 M, 1.0 mL) was added to a solution of the Boc derivative (above) (0.11 g, 0.221 mmol) in EtOAc (1 mL) and the mixture stirred at rt for 18 h. The resulting precipitate was filtered and washed with EtOAc (2 mL) providing **18** as an off-white solid (0.85 g, 89% yield. Mp 278 °C dec. ^{1}H NMR (400 MHz, DMSO) δ 9.98 (bs, 1H), 9.41 (t, *J* = 5.9 Hz, 1H), 8.68 (bs, 1H), 8.19 (d, *J* = 8.8 Hz, 2H), 8.06 (d, *J* = 8.4 Hz, 2H), 7.75 (d, *J* = 8.8 Hz, 2H), 7.52 (d, *J* = 8.4 Hz, 2H), 5.92 (bs, 1H), 4.55 – 4.34 (m, 3H), 4.14 (s, 1H), 3.40 (s, 2H), 2.03 – 1.85 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 174.6, 168.1, 166.6, 142.4, 138.2, 129.8, 129.7, 128.1, 127.2, 124.7, 122.2, 73.9, 66.3, 44.1, 42.4, 32.3. LCMS R_f (min) = 4.97. LCMS R_f (min) = 4.97. MS m/z 399.1 (M + H). HR–ESI calcd for $C_{20}H_{20}ClN_4O_3^+$ (M + H) 399.1218, found 399.1217.

(2S,3S)-2-((4-(3-(4-Chlorophenyl)-1,2,4-oxadiazol-5-yl)benzyl)carbamoyl)-3-hydroxypyrrolidin-1-ium chloride 19.

Isobutylchloroformate (0.34 mL, 2.6 mmol) was added dropwise to a solution of *N*-methylmorpholine (275 µL, 2.5 mmol) and 4-(((*tert*-butoxycarbonyl) amino)methyl)benzoic acid (**65**) (0.628 g, 2.5 mmol) in 1,4-dioxane (25 mL) at 0 °C. After 5 min (*Z*)-4-chloro-*N*'-hydroxybenzimidamide (**52**) (0.427 g, 2.5 mmol) was added and stirred at rt for 2 h then reflux for

2 h. The cooled (rt) solution was concentrated to a solid and then taken up in EtOAc (150 mL) and washed consecutively with H_2O (50 mL), $NaHCO_3$ (sat., 50 mL), H_2O (50 mL), HCl (aq) (0.5 M, 50 mL) and brine (30 mL). The organic layer was dried over $MgSO_4$ and concentrated to a solid (0.94 g) and chromatographed on silica gel eluting with 20% EtOAc in petroleum spirit providing the Boc protected product as a white solid (0.48 g, 50%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (d, *J* = 8.4 Hz, 2H), 8.12 (d, *J* = 8.7 Hz, 2H), 7.51 – 7.44 (m, 4H), 5.06 – 4.88 (m, 1H), 4.42 (d, *J* = 5.7 Hz, 2H), 1.48 (s, 9H).

A solution of HCl in 1,4-dioxane (4 M, 2 mL) was added to a solution of the oxadiazole above (0.2 g, 0.518 mmol) in EtOAc (2 mL) and the mixture stirred at rt for 20 h. The resultant precipitate was filtered and washed with EtOAc (1.0 mL and diethyl ether (2.0 mL) giving **66** as a white (0.12 g, 72%). ^{1}H NMR (400 MHz, DMSO) δ 8.36 (bs, 3H), 8.26 (d, *J* = 8.4 Hz, 2H), 8.11 (d, *J* = 8.7 Hz, 2H), 7.76 (d, *J* = 8.4 Hz, 2H), 7.70 (d, *J* = 8.7 Hz, 2H), 4.18 (s, 2H).

A portion of the oxadiazole **66** (0.1 g, 0.3104 mmol) was suspended in DMF (2.5 mL) and to this mixture was added HATU (0.177 g, 0.466 mmol), (2*S*,3*S*)-1-(*tert*-butoxycarbonyl)-3-hydroxypyrrolidine-2-carboxylic acid (0.072 g, 0.3104 mmol) and finally DIPEA (264 µL, 1.552 mmol) and stirred at rt overnight. The mixture was diluted with EtOAc (50 mL), washed with H_2O (30 mL), dried over $MgSO_4$, concentrated (0.191 g) and crystallised from a small amount of EtOAc to give the Boc-**19** (0.15 g, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, *J* = 8.1 Hz, 2H), 8.11 (d, *J* = 8.8 Hz, 2H), 7.49 (d, *J* = 8.7 Hz, 2H), 7.45 (d, *J* = 8.3 Hz, 2H), 4.92 – 4.17 (m, 4H), 3.79 – 3.42 (m, 2H), 2.27 – 1.86 (m, 4H), 1.57 – 1.32 (m, 9H).

HCl in 1,4-dioxane (4 M, 3 mL) was added to a solution of the Boc protected derivative (above) (0.15 g, 0.3 mmol) in EtOAc (2 mL) and the solution at rt for 15 h. The resultant precipitate was filtered and washed sequentially with small portions of EtOAc (2.0 mL), isopropanol (1.0 mL) and diethyl ether (2.0 mL) providing **19** as an off-white solid (0.117 g, 90%). Mp 284 °C dec. ^{1}H NMR

(400 MHz, DMSO) δ 10.10 (bs, 1H), 9.51 (t, *J* = 6.0 Hz, 1H), 8.67 (bs, 1H), 8.15 (d, *J* = 8.4 Hz, 2H), 8.09 (d, *J* = 8.7 Hz, 2H), 7.68 (d, *J* = 8.7 Hz, 2H), 7.59 (d, *J* = 8.4 Hz, 2H), 5.97 (bs, 1H), 4.49 (d, *J* = 5.9 Hz, 2H), 4.43 (bs, 1H), 4.16 (d, *J* = 1.8 Hz, 1H), 3.43 – 3.24 (m, 4H), 2.03 – 1.85 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 175.5, 167.5, 166.8, 144.4, 136.4, 129.5, 128.9, 128.3, 128.1, 125.0, 121.9, 73.9, 66.3, 44.1, 42.3, 32.4. LCMS R_f (min) = 4.98. MS m/z 399.1 (M + H). HR–ESI calcd for $C_{20}H_{20}ClN_4O_3^+$ (M + H) 399.1218, found 399.1217.

4-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)phenol 20.

A solution of 5-(4-chlorophenyl)-2*H*-tetrazole (**67**) (0.5 g, 2.769 mmol), 4-methoxybenzoyl chloride **46** (750 µL, 5.537 mmol) and pyridine (269 µL, 3.322 mmol) in toluene (10 mL) was refluxed for 3 h. The cooled (rt) solution was diluted with EtOAc (50 mL) and then washed with water (30 mL), saturated $NaHCO_3$ aq (30 mL) and brine (20 mL), dried over $MgSO_4$ and concentrated to a solid (1.1 g) that was recrystallized from EtOH, providing **70** as a white solid (0.692 g, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 – 8.02 (m, 4H), 7.51 (d, *J* = 8.8 Hz, 2H), 7.04 (d, *J* = 9.0 Hz, 2H), 3.90 (s, 3H).

BBr_3 (296 µL, 3.14 mmol) was added dropwise to a solution of the oxadiazole **70** (0.3 g, 1.046 mmol) in DCM (10 mL) at 0 °C and stirred for 1 h. The mixture was quenched with $NaHCO_3$ (aq) (sat., 10 mL) then extracted with EtOAc (250 mL). The aqueous layer was separated and the organic layer was dried over $MgSO_4$ and concentrated to a solid that was triturated with DCM (7 mL) providing **20** as a white solid (0.26 g, 91% yield). Mp 262–266 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.34 (s, 1H), 8.06 (d, *J* = 8.5 Hz, 2H), 7.93 (d, *J* = 8.7 Hz, 2H), 7.64 (d, *J* = 8.5 Hz, 2H), 6.96 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 164.4, 162.5, 161.0, 136.5, 129.5, 128.7, 128.2, 122.4, 116.2, 114.0. LCMS R_f (min) = 5.84. MS m/z 273.1 (M + H). HR–ESI calcd for $C_{14}H_{10}ClN_2O_2^+$ (M + H) 273.0425, found 273.0422.

4-((5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)thio)phenol 21.

A solution of 5-(4-chlorophenyl)-1,3,4-oxadiazole-2(3*H*)-thione **78** (0.213 g, 1 mmol) in DMF (3 mL) was added to a solution of CuI (0.0096 g, 0.05 mmol), 1,10-phenanthroline (0.018 g, 0.1 mmol), K_2CO_3 (0.18 g, 1.3 mmol) and 4-iodoanisole (0.281 g, 1.2 mmol) in DMF (3 mL) and the mixture stirred at 120 °C for 10 h. The cooled (rt) solution was diluted with water (100 mL) and extracted with EtOAc (3 × 30 mL). The combined organics were washed with water (3 × 20 mL) and brine (30 mL) dried over $MgSO_4$ and concentrated to a residue that was chromatographed on silica gel eluting with 10% EtOAc in petroleum spirit providing an oil that slowly solidified to a clear solid (0.225 g, 71% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (d, *J* = 8.8 Hz, 2H), 7.63 (d, *J* = 9.0 Hz, 2H), 7.45 (d, *J* = 8.8 Hz, 2H), 6.97 (d, *J* = 8.9 Hz, 2H), 3.85 (s, 3H).

BBr_3 (200 μL, 2.12 mmol) was added dropwise to a solution of the above methyl ether (0.225 g, 0.706 mmol) in DCM (8 mL) at 0 °C then stirred for 4 h at rt. The mixture was quenched with $NaHCO_3$ (sat., 10 mL) then diluted DCM (30 mL) and the aqueous layer extracted with DCM (10 mL). The combined organic phases were dried over $MgSO_4$ and concentrated to a foam and chromatographed on silica gel eluting with 20% EtOAc in petroleum spirit providing **21** as a white solid (0.183 g, 85% yield). Mp 154–156 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36 (s, 1H), 7.92 (d, *J* = 8.7 Hz, 2H), 7.47 (d, *J* = 8.8 Hz, 2H), 7.45 (d, *J* = 8.8 Hz, 2H), 6.73 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.8, 165.3, 159.6, 138.5, 137.1, 129.7, 128.1, 121.8, 117.9, 113.4. LCMS R_f (min) = 5.89. MS m/z 305.1 (M + H). HR–ESI calcd for $C_{14}H_{10}ClN_2O_2S^+$ (M + H) 305.0146, found 305.0141.

5-(4-Chlorophenyl)-N-(4-hydroxyphenyl)-1,3,4-oxadiazole-2-carboxamide 22.

LiOH (aq) (10% w/v, 10 mL) was added to a solution of ethyl 5-(4-chlorophenyl)-1,3,4-oxadiazole-2-carboxylate **77** (1 g, 3.958 mmol) in THF (20 mL) and MeOH (39 mL) at 0 °C. The mixture was concentrated to a solid and triturated with 20% EtOAc in petroleum spirit. The solid

was collected and washed with ether providing lithium 5-(4-chlorophenyl)-1,3,4-oxadiazole-2-carboxylate as an off-white solid (0.852 g, 93% yield). ^{1}H NMR (400 MHz, D_2O) δ 7.93 (dd, *J* = 8.4, 1.2 Hz, 1H), 7.55 (dd, *J* = 8.5, 1.1 Hz, 1H).

A solution of the lithium salt (above) (0.15 g, 6.507 mmol), 4-aminophenol (0.092 g< 0.846 mmol), HATU (0.371 g, 0.976 mmol) and Et_3N (272 µL, 1.952 mmol) in DMF (5 mL) was stirred at rt for 15 h, then diluted with H_2O (10 mL) giving a precipitate. The solid was recrystallised from EtOH providing **22** as an off-white solid (0.048 g, 23% yield). Mp 259 °C dec. ^{1}H NMR (400 MHz, DMSO) δ 11.02 (s, 1H), 9.46 (s, 1H), 8.13 (d, *J* = 8.8 Hz, 2H), 7.74 (d, *J* = 8.8 Hz, 2H), 7.59 (d, *J* = 9.0 Hz, 2H), 6.78 (d, *J* = 9.0 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 164.4, 158.8, 154.7, 150.9, 137.5, 129.8, 129.0, 129.0, 122.6, 121.8, 115.2. LCMS R_f (min) = 6.03. MS m/z 316.0 (M + H). HR–ESI calcd for $C_{15}H_{11}ClN_3O_3^+$ (M + H) 316.0483, found 316.0482.

N-((5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)methyl)-4-hydroxybenzenaminium chloride 23.

2-(Chloromethyl)-5-(4-chlorophenyl)-1,3,4-oxadiazole **76** (0.1 g, 0.437 mmol) was dissolved in DMSO (440 µL) and to the mixture was added 4-methoxyaniline (0.062 g, 0.5 mmol) followed by K_2CO_3 (0.175 g, 1.264 mmol) and stirred at rt overnight. The mixture was diluted with EtOAc (20 mL) and washed with water (4 × 30 mL) and then brine (10 mL). The organic layer was dried 0ver $MgSO_4$ and concentrated to a brown/amber resin (0.139 g). The resin was chromatographed on silica gel eluting with 5% EtOAc in DCM providing the product as an off-white solid (0.057 g, 41% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, *J* = 8.8 Hz, 2H), 7.50 (d, *J* = 8.8 Hz, 2H), 6.83 (d, *J* = 9.1 Hz, 2H), 6.76 (d, *J* = 9.1 Hz, 2H), 4.63 (s, 2H), 4.09 (s, 1H), 3.77 (s, 3H).

The methyl ether above (0.057 g, 0.181 mmol) was dissolved in DCM (5 mL) and cooled in an ice-bath in an N_2 atmosphere and neat BBr_3 (85 µL, 0.903 mmol) was added dropwise and stirred ice-cold for 3h. The mixture was diluted with between EtOAc (20 mL) and $NaHCO_3$ (aq) (sat.,10 mL) and the organic layer washed water (10 mL) and brine (10 mL). The organic phase was dried over

$MgSO_4$ and concentrated to a solid residue. This material was dissolved in EtOAc (10 mL) and a solution of HCl in 1,4-dioxane (4 M, 300 μL) added to give a precipitate that was filtered and washed with EtOAc (1 mL) providing **23** as a white solid (0.037 g, 61% yield). Mp 206°C dec. ^{1}H NMR (400 MHz, DMSO) δ 7.97 (d, *J* = 8.7 Hz, 2H), 7.69 (d, *J* = 8.7 Hz, 2H), 6.77 (d, *J* = 8.7 Hz, 2H), 6.63 (d, *J* = 8.8 Hz, 2H), 4.63 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 163.8, 162.8, 153.7, 137.0, 129.76, 128.4, 124.4, 124.1, 121.9, 119.4, 115.9, 41.4. LCMS R_f (min) = 5.84. MS m/z 302.1 (M + H). HR–ESI calcd for $C_{15}H_{13}ClN_3O_2^+$ (M + H) 302.0691, found 302.0697.

4-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)aniline 24.

$Pd(PPh_3)_4$ (0.089 g, 0.0771 mmol) was added to a solution of 2-bromo-5-(4-chlorophenyl)-1,3,4-oxadiazole **47** (0.2 g, 0.771 mmol), Na_2CO_3 (aq) (1 M, 3.9 mL) and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline **72** (0.186 g, 0.848 mmol) in DMF (15 mL) and the mixture degassed by bubbling N_2 (g) then heated to 80 °C for 4 h. The resultant mixture was cooled to rt and slowly diluted with water (40 mL) providing **24** as a yellow precipitate that was filtered and washed with water and then recrystallized from DCM (0.2 g, 96% yield). Mp 197–200 °C. ^{1}H NMR (400 MHz, DMSO) δ 8.09 (d, *J* = 8.8 Hz, 2H), 7.78 (d, *J* = 8.7 Hz, 2H), 7.68 (d, *J* = 8.8 Hz, 2H), 6.70 (d, *J* = 8.8 Hz, 2H), 5.98 (bs, 2H). ^{13}C NMR (101 MHz, DMSO) δ 165.0, 161.9, 152.5, 136.2, 129.5, 128.3, 128.1, 122.6, 113.6, 109.4. LCMS R_f (min) = 6.37. MS m/z 272.1 (M + H). HR–ESI calcd for $C_{14}H_{11}ClN_3O^+$ (M + H) 272.0585, found 272.0582.

(4-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)phenyl)methanamine hydrochloride 25.

4-(Aminomethyl)benzoic acid (5 g, 33.075 mmol) and Boc_2O (7.2 g, 33 mmol) were taken up in a mixture of acetone (100 mL) and water (100 mL). Sodium hydroxide (1.32 g, 33 mmol) was added and the mixture and stirred overnight. The acetone was removed by rotary evaporation and the aqueous layer was washed with ether (2 × 50 mL) and then EtOAc (100 mL) was added to the aqueous layer and vigorously stirred while 10% citric acid was added slowly to the stirring mixture

until pH ≈ 3–4. The organic layer was removed and the aqueous layer was extracted with EtOAc (50 mL). The combined EtOAc layers were dried ($MgSO_4$), filtered and concentrated to provide 4-(((*tert*-butoxycarbonyl)-amino)methyl)benzoic acid as a white solid (7.98 g, 96% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (d, *J* = 8.3 Hz, 2H), 7.38 (d, *J* = 8.4 Hz, 2H), 5.11 – 4.76 (m, 1H), 4.40 (d, *J* = 5.7 Hz, 2H), 1.47 (s, 9H).

Isobutylchloroformate (337 µL, 2.588 mmol) was added dropwise was to a stirred solution of the above carbamate (0.65 g, 2.59 mmol) and *N*-methylmorpholine (285 µL, 2.59 mmol) in dry THF (25 mL) at 0 °C. After stirring for a further 20 min powdered 5-(4-chlorophenyl)-1*H*-tetrazole (0.467 g, 2.588 mmol) and the reactionaloowed to warm to st over 10 min and then refluxed for 2 h. The reaction mixture was then concentrated to a solid that was partitioned between EtOAc (150 mL) and $NHCO_3$ (aq) (sat., 50 mL) and vigorously stirred for 10 min. The aqueous layer was removed and the organic layer was washed with water (50 mL) and finally with brine (20 mL). The EtOAc layer was dried over $MgSO_4$ and concentrated to provide **71** as a white solid (0.997 g) that was recrystallized from EtOH (0.278 g, 28% yield). Mp 177–179 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 – 8.04 (m, 4H), 7.52 (d, *J* = 8.6 Hz, 2H), 7.46 (d, *J* = 8.3 Hz, 2H), 4.96 (s, 1H), 4.41 (d, *J* = 5.8 Hz, 2H), 1.48 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.7, 163.8, 156.1, 143.5, 138.1, 129.6, 128.3, 128.0, 127.3, 122.7, 122.5, 79.9, 44.4, 28.5. LCMS R_f (min) = 6.91. MS m/z 386.1 (M + H). HR–ESI calcd for $C_{20}H_{21}ClN_3O_3^+$ (M + H) 386.1266, found 386.1265.

A solution of HCl 1,4-dioxane (4 M, 5 mL) was added to a solution of *tert*-butyl (4-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)benzyl)carbamate **71** (0.2 g, 0.518 mmol) in 1,4-dioxane (2 mL) and the mixture stirred at rt overnight. The mixture was then diluted with EtOAc (20 mL) and stirred for 0.5 h. The resulting precipitate was filtered and washed with cold EtOAc and finally ether giving the product **25** as a white solid (0.161 g, 96% yield). Mp 295–299 °C. 1H NMR (400 MHz, DMSO) δ 8.35 (s, 3H), 8.21 (d, J = 8.4 Hz, 2H), 8.17 (d, J = 8.8 Hz, 2H), 7.77 – 7.68 (m, 4H), 4.16 (s, 2H). HR–ESI calcd for $C_{15}H_{13}ClN_3O^+$ (M + H) 286.0742, found 286.0740.

4-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)benzoic acid 26.

Oxalyl chloride (4.84 mL, 55.51 mmol) was added dropwise to the stirred solution 4-(methoxycarbonyl)benzoic acid **79** (2 g, 11.101 mmol) and DMF (5 drops) in dry DCM (20 mL) and the resultant solution stirred at rt for 3 h. The mixture was concentrated to a solid and then taken up in THF (30 mL), cooled to 0 °C and 4-chlorobenzohydrazide **75** (1.89 g, 11.101 mmol) and pyridine (987 µL, 12.211 mmol) added and after 15 min the solution warmed to rt and stirred for 16 h. The mixture was diluted with water (100 mL) and the resulting precipitate filtered, washed with water (30 mL) providing the intermediate hydrazide as a white solid (3.47 g). The solid was taken up in $POCl_3$ (20 mL) and refluxed for 3 h. The mixture was cooled to rt and concentrated to remove most of the $POCl_3$ and then crushed ice/water mixture was added to the residue providing a solid. The solid was filtered and washed with water (30 mL) to give the oxadiazole ester as an off-white solid (3.47 g). The solid was taken in THF (50 mL), MeOH (100 mL) and DMSO (55 mL). To this mixture was added dropwise LiOH (aq) (10% w/v, 20 mL and stirred at rt overnight. The mixture was then concentrated to remove volatiles and then diluted with water (150 mL). The mixture was acidified to pH = 2 with 6 M HCl (aq) and the resultant precipitate washed with water and dried giving **26** as a cream solid (2.75 g, 83% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.27 (d, J = 8.7 Hz, 2H), 8.18 (d, J = 6.0 Hz, 2H), 8.16 (d, J = 5.9 Hz, 2H), 7.73 (d, J = 8.8 Hz, 2H). The spectral data for this compound are consistent with that previously reported.[33]

4-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)benzamide 27.

Oxalyl chloride (75 µL, 0.865 mmol) was added dropwise to a solution 4-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)benzoic acid (**26**) (0.2 g, 0.665 mmol) in THF (10 mL) and DMF (one drop). The mixture was stirred at rt for 4h then cooled to 0 °C and concentrated NH_4.OH (3 mL) added dropwise with vigorous stirring, then the mixture brought to rt and stirred for a further 10 min. After this time it was diluted with water (50 mL) and the resultant precipitate filtered and washed

with $NaHCO_3$ (aq) (sat., 10 mL) and H_2O (20 mL) and dried under vacuum providing a white solid (0.163 g). The crude solid was recrystallized from EtOH and again from DMSO giving **27** as a white solid (0.085g, 43% yield). Mp 297–299 °C. ^{1}H NMR (400 MHz, DMSO) δ 8.23 (d, *J* = 8.6 Hz, 2H), 8.21 – 8.14 (m, 3H), 8.10 (d, *J* = 8.6 Hz, 2H), 7.73 (d, *J* = 8.7 Hz, 2H), 7.59 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 166.9, 163.7, 163.6, 137.2, 136.9, 129.6, 128.6, 128.46, 126.7, 125.5, 122.1. LCMS R_f (min) = 5.53. MS m/z 300.1 (M + H). HR–ESI calcd for $C_{15}H_{11}ClN_3O_2^+$ (M + H) 300.0534, found 300.0533.

Amino(4-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)phenyl)methaniminium chloride 28.

LiHMDS (1M in THF, 900 µL, 0.9 mmol) was added dropwise to a solution of 4-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)benzonitrile **83** (0.12 g, 0.426 mmol) in dry THF (5 mL) at 0 °C and left to stir at this temperature for 4 h then at rt for 12 h. The mixture was cooled to 0 °C and a solution of HCl in 1,4-dioxane (4 M, 852 µL, 3.41 mmol) added and the solution allowed to warm to rt for 20 min. The resultant precipitate was filtered and washed with EtOAc (10 mL) then diethyl ether (10 mL) providing a white solid (0.182 g). The solid was dissolved in MeOH (5 mL) and remaining precipitate removed by filtration. The filtrate was diluted with H_2O (10 mL) and 1 M NaOH (aq) added dropwise until a precipitate formed. The precipitate was collected, washed with H_2O (5 mL) and diethyl ether (5 mL). The solid was then dsuspended in EtOAc (4 mL) and a solution of HCl in 1,4-dioxane (4 M, 1 mL) added and stirred for 16 h. The precipitate was filtered and washed with EtOAc (10 mL) and diethyl ether (10 mL) providing **28** as an off-white solid (0.094 g, yield 66%). Mp > 300 °C. ^{1}H NMR (400 MHz, DMSO) δ 9.63 (s, 2H), 9.40 (s, 2H), 8.36 (d, *J* = 8.7 Hz, 2H), 8.20 (d, *J* = 8.8 Hz, 2H), 8.08 (d, *J* = 8.7 Hz, 2H), 7.73 (d, *J* = 8.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 164.9, 163.9, 163.2, 137.1, 131.0, 129.6, 129.3, 128.7, 127.6, 127.0, 122.0. LCMS R_f (min) = 4.23. MS m/z 299.1 (M + H). HR–ESI calcd for $C_{15}H_{12}ClN_4O^+$ (M + H) 299.0694, found 299.0695.

(Z)-4-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)-N'-hydroxybenzimidamide 29.

A solution of 4-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)benzonitrile **83** (0.37 g, 1.314 mmol), hydroxylamine hydrochloride (0.274 g, 3.941 mmol) and Et_3N (546 µL, 3.941 mmol) in EtOH (13 mL) was refluxed for 16 h. After cooling to rt the precipitate was filtered washed with H_2O (10 mL), EtOH (10 mL) and diethyl ether (10 mL) giving a tan sold (0.38 g). The solid was recrystallized from EtOH providing **31** as a white solid (0.215 g, 52% yield). Mp 239–241 °C. ^{1}H NMR (400 MHz, DMSO) δ 9.93 (s, 1H), 8.17 (d, *J* = 8.7 Hz, 2H), 8.14 (d, *J* = 8.7 Hz, 2H), 7.93 (d, *J* = 8.7 Hz, 2H), 7.73 (d, *J* = 8.7 Hz, 2H), 5.99 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 164.0, 163.3, 150.0, 136.8, 136.6, 129.6, 128.5, 126.6, 126.1, 123.3, 122.2. LCMS R_f (min) = 4.41. MS m/z 315.1 (M + H). HR–ESI calcd for $C_{15}H_{12}ClN_4O_2^+$ (M + H) 315.0643, found 315.0640.

(*S*)-Amino(1-(4-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)benzoyl)pyrrolidin-2-yl)methaniminium chloride 30.

4-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)benzoic acid **26** (1.92 g, 6.385 mmol) was added to a solution of DMF (30 mL) containing the trifluoroacetate salt of (*S*)-pyrrolidine-2-carbonitrile **84** [from 1.25 g of the corresponding Boc derivative that was deprotected at ice-cold mixture of DCM:TFA (10 mL, 1:1) for 0.5h] and cooled to 0 °C. To the cold mixture was added Et_3N (4.57 mL, 32.97 mmol) and HATU (3.65 g, 9.578 mmol) and the solution stirred for 10 min at 0 °C then at rt for 20 h. The mixture was diluted with EtOAc (150 mL) and washed sequentially with HCl (aq) (1 M, 100 mL), H_2O (50 mL), $NaHCO_3$ (aq) (sat., 50 mL), H_2O (3 × 50 mL) and brine (30 mL). The organic layer was dried over $MgSO_4$, concentrated to a brown solid (2.78 g) and chromatographed on a silica gel eluting with EtOAc to give a solid that was recrystallized from MeOH providing the intermediate nitrile (0.691 g, 29% yield). Mp 204–206 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (d, *J* = 7.9 Hz, 2H), 8.10 (d, *J* = 8.7 Hz, 2H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.54 (d, *J* = 8.7 Hz, 2H), 5.04 – 4.84 (m, 1H), 3.75 – 3.49 (m, 2H), 2.44 – 2.04 (m, 4H).

NaOMe (0.5 M in MeOH, 264 μL, 0.132 mmol) was added to a solution of the above nitrile (above) (0.1 g, 0.264 mmol) in anhydrous MeOH (2.64 mL) then heated to 50 °C for 24 h. Then NH_4Cl (0.028 g, 0.528 mmol) was added and left to stir at 50 °C for 72 h. The mixture was concentrated and then taken up in EtOAc (30 mL) and stirred for 0.5 h then filtered and washed with EtOAc (10 mL) to afford a cream solid (0.127 g). The solid was dissolved in 1:1 CH_3CN:H_2O (20 mL) and the mixture was slowly concentrated under vacuum to remove CH_3CN providing a solid that is filtered and washed with water. The aqueous filtrate was treated with $NaHCO_3$ (aq) (sat., 4 mL) and slowly evaporated until a solid precipitates. The solid was collected and washed with H_2O (10 mL) and diethyl ether (10 mL) to give a cream solid (0.059 g). The solid was dissolved in a minimum of MeOH and diluted with an equal volume of 1,4-dioxane then a solution of HCl in diethyl ether (2 M, 3 mL) was added dropwise with stirring. The mixture was concentrated to a residue and then dissolved in isopropanol (3 mL) (required sonication) and then diluted with EtOAc (10 mL) providing a precipitate that was filtered washed with EtOAc (5 mL) and diethyl ether (10 mL) to give **30** as a white solid (0.039 g, 34% yield). Mp 183 °C dec. ^{1}H NMR (400 MHz, DMSO) δ 9.22 – 9.07 (m, 2H), 8.87 – 8.72 (m, 2H), 8.25 (d, J = 8.2 Hz, 2H), 8.18 (d, J = 8.5 Hz, 2H), 7.95 (d, J = 8.2 Hz, 2H), 7.74 (d, J = 8.5 Hz, 2H), 4.70 – 4.61 (m, 1H), 3.87 – 3.75 (m, 1H), 3.50 – 3.41 (m, 1H), 2.44 – 2.35 (m, 1H), 1.99 – 1.84 (m, 3H). ^{13}C NMR (101 MHz, DMSO) δ 170.8, 168.6, 163.7, 163.6, 138.6, 136.9, 129.6, 128.7, 128.6, 126.6, 124.8, 122.2, 58.1, 50.1, 31.0, 25.1. LCMS R_f (min) = 4.71. MS m/z 396.2 (M + H). HR–ESI calcd for $C_{20}H_{19}ClN_5O_2^+$ (M + H) 396.1222, found 396.1220.

2-(4-(2H-tetrazol-5-yl)phenyl)-5-(4-chlorophenyl)-1,3,4-oxadiazole 31.

A solution of 4-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)benzonitrile **83** (0.3 g, 1.065 mmol) NaN_3 (0.083 g, 1.278 mmol) and NH_4Cl (0.068 g, 1.278 mmol) in DMF (1.5 mL) was heated to 100 °C for 18 h. The cooled solution was diluted with H_2O (10 mL) and acidified with HCl (aq)

(6M, 1 mL) providing a precipitate that was collected and washed with H_2O (10 mL). The solid was then suspended in NaOH (aq) (2.5 M, 10 mL) and stirred for 0.5 h at rt. The solution was filtered and the filter cake was washed with NaOH (aq) (2.5 M, 10 mL). The combined filtrate was acidified with 6 M HCl (aq) (pH ~ 1) and the precipitate was collected and washed with H_2O (10 mL). The solids were combined and triturated with boiling EtOH (10 mL) filtered and washed with EtOH and diethyl ether, providing **31** as a white solid (0.273 g, 79% yield). Mp 270 °C (decmp). ^{1}H NMR (400 MHz, DMSO) δ 8.38 (d, *J* = 8.5 Hz, 1H), 8.29 (d, *J* = 8.6 Hz, 1H), 8.19 (d, *J* = 8.6 Hz, 1H), 7.74 (d, *J* = 8.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 163.6, 163.5, 155.3, 136.9, 129.6, 128.5, 127.8, 127.6, 127.4, 125.3, 122.1. LCMS R_f (min) = 5.80. MS m/z 325.0 (M + H). HR–ESI calcd for $C_{15}H_{10}ClN_6O^+$ (M + H) 325.0599, found 325.0598.

4-((5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)amino)benzamide 32.

4-((5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)amino)benzonitrile **81** (0.05 g, 0.169 mmol) was dissolved in conc. H_2SO_4 (2 mL) at rt and stirred for 18 h. The mixture was cooled to 0 °C and crushed ice (50 g) was added to the stirred solution providing a solid. The mixture was neutralized with saturated $NaHCO_3$ (aq) and the solid filtered and washed with H_2O (10 mL) providing **32** as a white solid (0.05 g, 94% yield). Mp > 300 °C. ^{1}H NMR (400 MHz, DMSO) δ 11.03 (s, 1H), 7.94 – 7.88 (m, 4H), 7.86 (s, 1H), 7.70 – 7.62 (m, 4H), 7.23 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 167.4, 159.7, 157.3, 141.1, 135.7, 129.5, 128.8, 127.6, 127.4, 122.6, 116.3. HR–ESI calcd for $C_{15}H_{12}ClN_4O_2^+$ (M + H) 315.0643, found 315.0643.

Amino(4-((5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)amino)phenyl)methaniminium chloride 33.

4-((5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)amino)benzonitrile **81**: A solution of 4-chlorobenzohydrazide (0.532 g, 3.121 mmol) and 4-isothiocyanatobenzonitrile (0.5 g, 3.121 mmol) in THF (15 mL) was stirred at rt for 15 h. To this mixture 4-toluoylsulfonyl chloride (0.714 g, 3.748 mmol) and pyridine (530 μL, 6.554 mmol) were added and the stirred mixture refluxed for 6

h. The solution was cooled to rt and diluted with H_2O (50 mL) and the resulting solid filtered and washed sequentially with H_2O (20 mL), EtOH (5 mL) and DCM (20 mL) providing **81** as a pale yellow solid (0.45 g, 49% yield). Mp 274–276 °C. ^{1}H NMR (400 MHz, DMSO) δ 11.34 (s, 1H), 7.92 (d, *J* = 8.8 Hz, 2H), 7.84 (d, *J* = 9.0 Hz, 2H), 7.77 (d, *J* = 9.0 Hz, 2H), 7.67 (d, *J* = 8.8 Hz, 2H).

Boc_2O (1.1g, 5.055 mmol) was added was added to a solution of benzonitrile **81** (above) (0.5 g, 1.69 mmol) and DMAP (10.3 mg, 0.084 mmol) in 1,4-dioxane (0.5 mL) and the mixture stirred in at 60 °C for 20 min (the evolution of gas ceases). The cooled solution was diluted with EtOAc (30 mL) and filtered through a short silica pad and concentrated to an oily residue (1.17 g). The oil was triturated with 20% EtOAc in petroleum spirit providing a white solid that was filtered and washed with 20% EtOAc in petroleum spirit giving Boc-**81** (0.495 g, 74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, *J* = 8.8 Hz, 2H), 7.71 (d, *J* = 8.8 Hz, 2H), 7.52 (d, *J* = 6.3 Hz, 2H), 7.50 (d, *J* = 6.3 Hz, 2H), 1.51 (s, 9H).

LiHMDS (1 M in THF, 799 µL, 0.799 mmol) was added dropwise to the stirred solution of the Boc-**81** (above) (0.15 g, 0.378 mmol) in anhydrous THF (4.5 mL) at 0 °C then stirred at rt overnight. The a solution was cooled to 0 °C and HCl in 1,4-dioxane (4 M, 0.5 mL) added dropwise and stirred for 2 h at 0 °C. The mixture was partitioned between HCl (aq) (1 M, 40 mL) and EtOAc (30 mL) and the aqueous layer neutralised with saturated $NaHCO_3$ (aq) and left to stand for 50 h. The precipitate was filtered and washed with H_2O (5 mL) providing a yellow solid (0.038 g). The solid was suspended in EtOAc (10 mL) and HCl in 1,4-dioxane (4 M. 1 mL) added and the solution stirred at rt for 2 h. The hydrochloride salt was filtered and washed with EtOAc (15 mL) and diethyl ether (10 mL) providing **33** as a tan solid (0.036 g, 27% yield). Mp > 300 °C. ^{1}H NMR (400 MHz, DMSO) δ 11.46 (s, 1H), 9.25 (bs, 2H), 8.97 (bs, 2H), 7.92 (d, *J* = 8.7 Hz, 2H), 7.90 (d, *J* = 8.8 Hz, 2H), 7.82 (d, *J* = 8.9 Hz, 2H), 7.68 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 164.7, 159.4, 157.6, 143.5, 135.8, 129.5, 129.5, 127.4, 122.4, 120.3, 116.7. LCMS

R_f (min) = 4.72. MS m/z 314.1 (M + H). HR–ESI calcd for $C_{15}H_{13}ClN_5O^+$ (M + H) 314.0803, found 314.0799.

(*Z*)-4-((5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)amino)-*N'*-hydroxybenzimidamide 34.

A solution of benzonitrile **81** (above) (0.15 g, 0.506 mmol), hydroxylamine hydrochloride (0.039 g, 0.556 mmol) and $NaHCO_3$ (0.047g, 0.556 mmol) in EtOH (5 mL) and H_2O (1 mL) was refluxed for 18h, then cooled to rt giving a lemon colored precipitate that was filtered washed with EtOH (2 mL) and H_2O (4 mL) (0.132 g). The solid was recrystallized from EtOH providing **34** as a white solid (0.12 g, 66% yield). Mp > 300 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.85 (s, 1H), 9.51 (s, 1H), 7.91 (d, *J* = 8.8 Hz, 2H), 7.68 (d, *J* = 6.5 Hz, 2H), 7.66 (d, *J* = 6.3 Hz, 2H), 7.60 (d, *J* = 8.9 Hz, 2H), 5.75 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 159.9, 157.1, 150.5, 139.0, 135.6, 129.5, 127.4, 127.0, 126.3, 122.7, 116.6. LCMS R_f (min) = 4.71. MS m/z 330.1 (M + H). HR–ESI calcd for $C_{15}H_{13}ClN_5O_2^+$ (M + H) 330.0752, found 330.0754.

5-(4-Chlorophenyl)-*N*-[4-(1*H*-tetrazol-5-yl)phenyl]-1,3,4-oxadiazol-2-amine 35.

Triethylamine (0.20 mL) was added to a solution of 2-bromo-5-(4-chlorophenyl)-1,3,4-oxadiazole **47**[24] (80.5 mg, 0.310 mmol) and 4-(*2H*-tetrazol-5-yl) aniline **73** (60 mg, 0.3721 mmol) in DMF (1.6 mL) and heated the mixture heated to 90 °C for 4 h. The reaction mixture was cooled to rt and acidified (pH ~1) by addition of HCl (aq) (10% w/v). The precipitate was filtered and washed with water to give a brown solid which was retained. The filtrate was diluted with water (75 mL) extracted with EtOAc (3 x 50 mL). The organic layer was dried with $MgSO_4$ and concentrated to give leave a yellow solid. The precipitate and the solid extract of the filtrate were combined and chromatographed on silica gel eluting with EtOAc and recrystallised from EtOH to give **35** as yellow crystals (30.6 mg, 29%). Mp 210 °C (decomp.). ^{1}H NMR (400MHz, DMSO): 8.12 (d, 2H,

Ar), 7.79 (d, 2H, Ar), 7.75 (d, 2H, Ar), 6.72 (d, 2H, Ar), 5.72 (b, 2H). ^{13}C NMR (101MHz, DMSO): 164.78, 160.01, 150.93, 142.02, 138.11, 129.74, 128.82, 126.44, 122.05, 113.70, 112.16. LCMS R_f (min) = 5.97. MS m/z 312.1 (M – N_2 + H). HR-ESI could not be obtained (too low intensity).

(E)-4-((5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)amino)benzaldehyde oxime 36.

DIBAL (1.5 M in toluene, 1.67 mL, 2.5 mmol) was added dropwise to a solution of benzonitrile **83** (0.297 g, 1.0 mmol) in anhydrous toluene (5 mL) at 0 °C then stirred at rt overnight. The this HCl (aq) (10% w/v,10 mL) was added slowly and cautiously, causing a precipitate to form. The precipitate was filtered and washed with EtOAc (10 mL) and H_2O (15 mL) providing the crude aldehyde, 4-((5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)amino)benzaldehyde, as a yellow solid (0.185 g, 62% yield). ^{1}H NMR (400 MHz, DMSO) δ 11.35 (s, 1H), 9.89 (s, 1H), 7.96 – 7.89 (m, 4H), 7.81 (d, J = 8.7 Hz, 2H), 7.67 (d, J = 8.7 Hz, 2H).

A solution of the aldehyde (above) (0.1 g, 0.334 mmol), hydroxylamine hydrochloride (0.035 g, 0.51 mmol) and Et_3N (1 mL) in MeOH (5 mL) was stirred at rt overnight and the resultant precipitate filtered and washed with MeOH (10 mL) then dethyl ether (10 mL). The solid was recrystallised from MeOH providing an off-white solid (0.035 g). The solid material was dissolved in a mixture of EtOAc and MeOH and evaporated down on silica gel (10 g). The silica gel was filtered on a buchner funnel and washed with EtOAc (40 mL) and concentrated to a solid that was triturated in EtOAc giving **36** as a white solid (0.033 g, 31% yield). Mp 226–229 °C. ^{1}H NMR (400 MHz, DMSO) δ 11.04 (s, 1H), 10.92 (bs, 1H), 8.08 (s, 1H), 7.91 (d, J = 8.8 Hz, 2H), 7.69 – 7.57 (m, 6H). ^{13}C NMR (101 MHz, DMSO) δ 159.8, 157.2, 147.6, 139.4, 135.6, 129.5, 127.4, 127.3, 126.8, 122.7, 117.2. LCMS R_f (min) = 5.23. MS m/z 315.1 (M + H). HR–ESI calcd for $C_{15}H_{12}ClN_4O_2^+$ (M + H) 315.0643, found 315.0639.

4-((5-Phenyl-1,3,4-oxadiazol-2-yl)amino)phenol 37.

5-(4-Iodophenyl)-N-(4-methoxyphenyl)-1,3,4-oxadiazol-2-amine **87**: A solution of 4-iodobenzahydride (3.79 g, 14.48 mmol) and 1-isothiocyanato-4-methoxy benzene (2 mL, 14.48 mmol) in THF (100 mL) was stirred at rt for 16 h during which time a white precipitate formed. To this suspension tosyl chloride (3.312 g, 17.37 mmol) and pyridine (2.44 mL, 30.41 mmol) were added and the mixture refluxed for 20 h. H_2O (120 mL) was added to the mixture and allowed to stir for 10 min. The gold precipitate formed was then filtered and recrystallised in hot EtOH (450 mL) to produce **87** as gold crystalline solid (4.94 g, 89% yield). Mp 269 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.49 (s, 1H, NH), 7.95 (d, J = 8.6 Hz, 2H, CH), 7.64 (d, J = 8.6 Hz, 2H, CH), 7.52 (d, J = 9.1 Hz, 2H, CH), 6.95 (d, J = 9.1 Hz, 2H, CH), 3.73 (s, 3H, CH_3). ^{13}C NMR (101 MHz, DMSO) δ 160.68 (C), 157.37 (C), 154.95 (C), 138.62 (CH), 132.19 (C), 127.57 (CH), 123.71 (C), 119.09 (CH), 114.78 (CH), 98.28 (C), 55.67 (CH_3). LCMS R_f (min) = 3.510 MS m/z 394.0 (M + H).

87 (0.150 g, 0.382 mmol) was added to a suspension of Pd/C 10% (0.080 g) in EtOAc/MeOH (13.5 mL/4.5 mL) under N_2 (g). The atmosphere was exchanged for H_2 (g) through exposure to a vacuum source and backfilling with H_2 (g) (4 x). The mixture was then left to stir at rt overnight then filtered to remove Pd/C 10% catalyst before being concentrated to form a grey solid. The solid was triturated with DCM and filtered to produce a white crystalline solid (0.053 g), which was retained. The filtrate was then diluted with DCM (50 mL) and washed with $NaHCO_3$ (aq) (sat., 30 mL), dried over $MgSO_4$ and concentrated to give a white solid (0.021 g). Both solid samples of **89** were combined (74.2 mg, 73% yield). Mp 210 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.46 (s, 1H, NH), 7.89 (m, 2H, CH), 7.58 (m, J = 5.1, 1.9 Hz, 3H, CH), 7.54 (d, J = 9.1 Hz, 2H, CH), 6.97 (d, J = 9.1 Hz, 2H, CH), 3.75 (s, 3H, CH_3). ^{13}C NMR (101 MHz, DMSO) δ 160.21 (C), 157.56 (C), 154.51 (C), 131.92 (C), 130.91 (C), 129.38 (CH), 125.49 (CH), 123.92 (CH), 118.64 (CH), 114.39 (CH), 55.28 (CH_3). LCMS R_f (min) = 3.647 MS m/z 268.2 (M + H).

BBr_3 (0.0710 mL, 0.748 mmol) was added dropwise to a solution **87** (above) (0.050 g, 0.187 mmol) in DCM (3 mL) at 0 °C and stirred at rt for 2 h. The reaction was quenched with $NaHCO_3$

(aq) (sat., 1 mL) and diluted with H_2O (20 mL) then extracted with EtOAc (3 x 50 mL) and the combined extracts washed with $NaHCO_3$ (aq) (sat., 3 x 30 mL). The organic layer dried over $MgSO_4$ and concentrated giving 4-((5-phenyl-1,3,4-oxadiazol-2-yl)amino)phenol **37** as a cream solid (45.1 mg, 95% yield). Mp 231 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.29 (s, 1H, NH), 9.15 (s, 1H, OH), 7.87 (m, 2H, CH), 7.56 (m, 3H, CH), 7.40 (d, 2H, CH), 6.75 (d, 2H, CH). ^{13}C NMR (101 MHz, DMSO) δ 160.37 (C), 157.46 (C), 152.61 (C), 130.83 (C), 130.43 (C), 129.38 (CH), 125.41 (CH), 123.96 (CH), 118.95 (CH), 115.59 (CH). LCMS R_f (min) = 3.065 MS m/z 254.2 (M + H). HR-ESI calcd for $C_{14}H_{11}N_3O_2^+$ (M +H) 254.0924, found 254.0928.

4-((5-(4-(Trifluoromethyl)phenyl)-1,3,4-oxadiazol-2-yl)amino)phenol 38.

A solution of 4-(trifluoromethyl)benzohydrazine **86**[34] (0.5 g, 2.45 mmol) and 1-isothiocyanato-4-methoxybenzene (0.338 mL, 2.45 mmol) in THF (20 mL) was stirred at rt overnight. To this, tosyl chloride (0.560 g, 2.94 mmol) and pyridine (0.414 mL, 5.14 mmol) were added and the mixture refluxed for 20 h. H_2O (50 mL) was added to the mixture and the resultant cream precipitate filtered and chromatographed on silica gel eluting with 10% EtOAc in petroleum spirits yielding an off-white solid which was recrystallized from EtOH to produce **88** as a white crystalline solid (0.155 g, 19% yield). Mp 258 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.58 (s, 1H, NH), 8.09 (d, J = 8.2 Hz, 2H, CH), 7.96 (d, J = 8.2 Hz, 2H, CH), 7.54 (d, 2H, CH), 6.98 (d, 2H, CH), 3.75 (s, 3H, CH_3). ^{13}C NMR (101 MHz, DMSO) δ 160.84 (C), 156.77 (C), 154.86 (C), 131.88 (C), 130.48 (C), 127.88 (C), 126.57 (CF_3), 126.37 (CH), 126.37 (CH), 125.45 (CH), 119.02 (CH), 119.02 (CH), 114.60 (CH), 114.60 (CH), 55.47 (CH_3) (CH ortho to CF_3 group does not appear in ^{13}C NMR due to effects fluorine coupling). LCMS R_f (min) = 3.480 MS m/z 336.1 (M + H).

BBr_3 (0.0566 mL, 0.597 mmol) was added dropwise to a solution of **88** (0.050 g, 0.141 mmol) in DCM (3 mL) at 0 °C then left to stir at rt for 2 h. The reaction was quenched by dropwise addition

of $NaHCO_3$ (aq) (sat., 2 mL) at 0 °C then diluted with H_2O (20 mL) and left to stir for 15 min. The mixture was extracted with EtOAc (3 x 50 mL) and the combined extracts washed with H_2O (2 x 50 mL), $NaHCO_3$ (aq) (sat., 2 x 50 mL) and brine (1 x 30 mL), dried over $MgSO_4$ and concentrated to give a cream solid. Crude material was recrystallised using hot EtOH and petroleum spirit to afford **38** as a white solid (0.014 g, 31% yield). Mp 265 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.42 (s, 1H, NH), 9.20 (s, 1H, OH), 8.07 (d, J = 8.2 Hz, 2H, CH), 7.94 (d, J = 8.2 Hz, 2H, CH), 7.41 (d, J = 8.8 Hz, 2H, CH), 6.78 (d, J = 8.8 Hz, 2H, CH). ^{13}C NMR (101 MHz, DMSO) δ 160.81 (C), 156.50 (C), 152.81 (C), 130.58 (C), 130.19 (C), 127.74 (C), 126.39 (CF_3), 126.15 (CH), 119.17 (CH), 115.62 (CH) (CH ortho to CF_3 group does not appear in ^{13}C NMR due to effects fluorine coupling). LCMS R_f (min) = 3.267 MS m/z 322.1 (M + H). HR-ESI calcd for $C_{15}H_{10}F_3N_3O_2^+$ (M + H) 322.0798, found 322. 0802.

4-((5-(4-Iodophenyl)-1,3,4-oxadiazol-2-yl)amino)phenol 49.

BBr_3 (2.20 mL, 7.63 mmol) was added dropwise to a solution of **87** (above) (1.0 g, 2.54 mmol) in DCM (25 mL) at 0 °C then stirred at rt for 2 h. The reaction was quenched by dropwise addition of $NaHCO_3$ (aq) (sat., 4 mL) at 0 °C then diluted with H_2O (100 mL) and left to stir for 15 min. The solution was then extracted with EtOAc (3 x 70 mL), washed with $NaHCO_3$ (aq) (sat., 3 = 50 mL), dried over $MgSO_4$ and concentrated to give **39** as a yellow crystalline solid (0.713 g, 74% yield). Mp 279 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.34 (s, 1H, NH), 9.18 (s, 1H, OH), 7.95 (d, *J* = 8.6 Hz, 2H, CH), 7.63 (d, *J* = 8.6 Hz, 2H, CH), 7.39 (d, *J* = 8.9 Hz, 2H, CH), 6.76 (d, *J* = 8.9 Hz, 2H, CH). ^{13}CNMR (101 MHz, DMSO) δ 160.41 (C), 156.95 (C), 152.64 (C), 138.18 (CH), 130.26 (C), 127.11 (CH), 123.43 (C), 118.98 (CH), 115.54 (CH), 97.75 (C). LCMS R_f (min) = 3.271 MS m/z 258.3 (M + H). HR-ESI calcd for $C_{14}H_{10}IN_3O_2^+$ (M + H) 379.9890, found 379.9900.

4-((5-(4-(2-Cyclohexylethyl)phenyl)-1,3,4-oxadiazol-2-yl)amino)phenol 40.

$Pd(PPh_3)_2Cl_2$ (17.9 mg, 0.025 mmol) and CuI (2.42 mg, 0.013 mmol) were added to a deoxygenated solution (N_2 (g) was bubbled through the mixture for 10 min) of 5-(4-iodophenyl)-N-(4-methoxyphenyl)-1,3,4-oxadiazol-2-amine **87** (above) (0.2 g, 0.51 mmol) and 1-ethynylcyclohexene (0.09 mL, 0.76 mmol) in DMF (3 mL) and Et_3N (3 mL) and left to stir at rt for 18 h. The solution was diluted in EtOAc (20 mL) and washed with H_2O (2 x 30 mL) and HCl (aq) (1.0 M, 2 x 30 mL). The organic layers were then collected and washed with H_2O (1 x 30 mL) and brine (1 x 30 mL) before being dried over $MgSO_4$ and concentrated to a brown-cream solid (0.284 g). The crude material was chromatographed on silica gel eluting with 20% EtOAc in DCM affording 5-(4-(cyclohex-1-en-1-ylethynyl)phenyl)-N-(4-methoxyphenyl)-1,3,4-oxadiazol-2-amine as a gold crystalline solid (0.181 g, 96% yield). Mp 219 °C. ^{1}H NMR (400 MHz, CDCl3) δ 7.87 (d, J = 8.6 Hz, 2H, CH), 7.50 (d, J = 8.6 Hz, 2H, CH), 7.44 (d, J = 9.0 Hz, 2H, CH), 7.02 (s, 1H), 6.94 (d, J = 9.0 Hz, 2H, CH), 6.26 (m, 1H), 3.82 (s, 3H), 2.24 (m, J = 2.2 Hz, 2H), 1.66 (m, J = 23.9, 5.9, 2.1 Hz, 5H). LCMS R_f (min) = 3.867 MS m/z 372.2 (M + H).

Pd/C 10% (0.160 g) was added to a solution of the alkyne above (0.160 g, 0.4308 mmol) in EtOAc (7 mL) and MeOH (3 mL) under N_2 (g). The N_2 (g) atmosphere was replaced with H_2 (g) by evacuation and backfilling with H_2 (g) (4 cycles). The mixture was then left to stir at rt overnight then filtered to remove the catalyst and concentrated to afford 5-(4-(2-cyclohexylethyl)phenyl)-N-(4-methoxyphenyl)-1,3,4-oxadiazol-2-amine (**90**) as a white solid (0.105 g, 64%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, *J* = 8.3 Hz, 2H, CH), 7.43 (d, *J* = 9.0 Hz, 2H, CH), 7.28 (d, 2H, CH), 6.93 (d, *J* = 9.0 Hz, 2H, CH), 3.81 (s, 3H, CH_3), 2.66 (dd, *J* = 9.2, 7.0 Hz, 2H, CH_2), 1.71 (m, 9H, CH_2), 1.52 (dd, *J* = 16.3, 6.6 Hz, 4H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ 197.41 (C), 195.03 (C), 191.83 (C), 183.33 (C), 169.38 (C), 166.53 (CH), 162.88 (CH), 158.82 (C), 155.97 (CH), 151.77 (CH), 92.68 (CH_3), 75.89 (CH_2), 74.05 (CH), 70.12 (CH_2), 69.78 (CH_2), 63.59 (CH_2), 63.18 (CH_2). 63.18 (CH_2). LCMS R_f (min) = 4.127 MS m/z 394.3 (M + H).

BBr_3 (0.1 mL, 1.019 mmol) was added dropwise to a solution of **90** (0.096 g, 0.255 mmol) in DCM (4 mL) at 0 °C then left to stir at rt for 2 h. The reaction was quenched by dropwise addition $NaHCO_3$ (aq) (sat., 2 mL) at 0 °C diluted H_2O (20 mL) and left to stir for 15 min. The solution was then extracted with EtOAc (3 x 50 mL) and the combined organic layers washed with $NaHCO_3$ (aq) (sat., 3 x 50 mL), dried over $MgSO_4$ and concentrated to a brown solid (0.221 g). The crude material was chromatographed on silica gel eluting with 25% EtOAc in DCM giving **40** as a light brown solid (0.109 g, 100% yield). Mp 260 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.24 (s, 1H, NH), 9.14 (s, 1H, OH), 7.77 (d, *J* = 8.2 Hz, 2H, CH), 7.38 (dd, *J* = 8.5, 4.8 Hz, 4H, CH), 6.75 (d, *J* = 8.9 Hz, 2H, CH), 2.66 (m, 3H, CH_2), 1.69 (dd, *J* = 42.6, 11.9 Hz, 6H, CH_2), 1.49 (dd, *J* = 15.4, 7.0 Hz, 2H), 1.17 (t, *J* = 7.1 Hz, 2H, CH_2), 0.93 (t, *J* = 12.0 Hz, 2H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ 160.12 (C), 157.48 (C), 152.48 (C), 145.79 (C), 130.38 (C), 129.16 (CH), 125.45 (CH), 121.45 (C), 118.82 (CH), 115.52 (CH), 38.44 (CH_2), 36.63 (CH), 32.70 (CH_2), 32.37 (CH_2), 26.17 (CH_2), 25.79 (CH_2). LCMS R_f (min) = 3.788 MS m/z 364.2 (M + H). HR-ESI calcd for $C_{22}H_{25}N_3O_2{}^+$ (M + H) 364.202, found 364.2029.

4-((5-(4-Chlorophenyl)-1,3,4-oxadiazole-2-carboxamido)methyl)pyridin-1-ium chloride 41. Ethyl 5-(4-chlorophenyl)-1,3,4-oxadiazole-2-carboxylate (**77**) (0.2 g, 0.79 mmol) and pyridin-4-ylmethanamine (241 µL, 2.38 mmol) were refluxed in EtOH (2 mL) for 18 h. The cooled (rt) solution was filtered and the filter-cake washed with EtOH and dried to give pink solid (0.184 g). The solid was dissolved in a mixture EtOAc (1 mL) and 1,4-dioxane (3 mL) (required heating) and a solution of HCl in 1,4-dioxane (4 M, 1 mL) added. After stirring for 15 min the solution was then concentrated and resuspended in EtOAc (2.0 mL), sonicated then filtered (0.2 g). The solid was triturated in hot EtOH, filtered and then recrystallised from DMSO (54 mg, 19%). Mp 264 °C dec. ^{1}H NMR (400 MHz, DMSO) δ 10.12 (t, *J* = 6.0 Hz, 1H), 8.82 (d, *J* = 6.4 Hz, 2H), 8.11 (d, *J* = 8.8 Hz, 2H), 7.90 (d, *J* = 6.3 Hz, 2H), 7.74 (d, *J* = 8.8 Hz, 2H), 4.75 (d, *J* = 6.0 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 164.3, 158.4, 156.9, 153.6, 143.6, 137.5, 129.8, 128.86, 124.3, 121.7, 42.0.

LCMS R_f (min) = 4.69. MS m/z 315.1 (M + H). HR–ESI calcd for $C_{15}H_{12}ClN_4O_2^+$ (M + H) 315.0643, found 315.0639.

5-(4-Chlorophenyl)-N-(pyridin-4-ylmethyl)-1,3,4-oxadiazol-2-amine 42.

2-Bromo-5-(4-chlorophenyl)-1,3,4-oxadiazole (**47**)[22] (0.150 g, 0.578 mmol), 4-(aminomethyl)piperidine (0.176 mL, 1.734 mmol) and DIPEA (0.3 mL, 1.734 mmol) in DMF (3 mL) were heated to 70 °C for 3 h in which time the solution changed from yellow to orange. The mixture was diluted in EtOAc (50 mL) and washed with H_2O (3 x 30 mL) and brine (1 x 30 mL) dried over $MgSO_4$ and concentrated to a yellow semi-solid (0.177 g). The crude solid was then triturated in Et_2O and filtered to produce an orange solid (0.143 g). This solid dissolved in EtOAc (4 mL), and a solution of HCl in diethyl ether (2.0 M, 1 mL) was added at 0 °C. The mixture was left to stir at rt overnight before being filtered and washed with EtOAc (2.0 mL), forming a light brown solid (54 mg, 43% yield). Mp 191 °C. ^{1}H NMR (400 MHz, DMSO) δ 9.01 (s, 1H, NH), 8.90 (d, J = 6.7 Hz, 2H, CH), 8.08 (d, J = 6.6 Hz, 2H, CH), 7.82 (d, J = 8.7 Hz, 2H, CH), 7.62 (d, J = 8.7 Hz, 2H, CH), 4.81 (s, 2H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ 163.35 (C), 159.92 (C), 157.82 (C), 141.45 (CH), 135.75 (C), 129.78 (CH), 127.42 (CH), 125.45 (CH), 123.03 (C), 45.61 (CH_2). LCMS R_f (min) = 3.185 MS m/z 287.1 (M + H). HR-ESI calcd for $C_{14}H_{16}ClN_3O_2^+$ (M + H) 287.0694, found 287.0706.

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Supporting Information

Copies of HPLC, ^{1}H NMR and ^{13}C NMR spectra for all test compounds.

Ancillary Information

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Abbreviations Used

APCI, acylpyrrolidine-2-carboximidamide; CBR5, NADH-cytochrome b5 reductase; Cer, ceramide; CyEt, (cyclohexyl)ethyl; Des1, dihydroceramide desaturase-1; dhCer, dihydroceramide; LELP, lipophilicity-corrected ligand efficiency; GI_{50}, concentration required to inhibit 50% of cell growth; HEK293, human embryonic kidney-293; NBD, *N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)amino; PC3, prostate cancer-3; S1P, sphingosine-1-phosphate; SD, standard deviation; SEM, standard error of the mean; SK, sphingosine kinase; Sph, sphingosine.

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Table of Contents graphic