Discovery of novel inhibitors to investigate diacylglycerol lipases and α/β hydrolase domain 16A

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CHAPTER 6

Discovery of sulfonyl-1,2,4-triazole ureas as *sn*-1 diacylglycerol lipase α inhibitors by HTS-ABPP*

Introduction

The European Lead Factory (ELF), part of the Innovative Medicines Initiative (IMI), is a collaborative European drug discovery platform, aiming at facilitating European drug discovery projects.[1] The ELF provides high throughput screening facilities, expertise and access to the Joint European Compound Library (JECL), a diverse 300.000+ compound library from proprietary collections of seven pharmaceutical companies.[2] Collaborations with ten academic groups and six small and medium enterprises, all part of the ELF Chemistry Consortium, have contributed an additional 100.000 novel compounds so far. This Public Compound Collection (PCC) is developed to occupy novel explored chemical space using newly developed synthetic routes, and is expected to rise to a total of ~200.000 compounds in the future.[3] The combined JECL/ PCC can be screened in the ELF program by European academic and private parties, provided that the assay target is innovative and has relevance for disease. Furthermore, feasibility of the assay in 384-well plate format has to be demonstrated. Importantly, a milestone payment system has been created in case drug-like hits, leads or drug candidates will be identified and commercialized.[3,4]

Diacylglycerol lipases (DAGLs) are serine hydrolases responsible for the formation of the endocannabinoid 2-arachidonoylglycerol (2-AG). 2-AG is a full agonist of the cannabinoid CB1 and CB2 receptors (CB1R/CB2R) and functions as the main precursor for arachidonic acid and pro-inflammatory eicosanoids in the brain.[5] The dual role of 2-AG signifies that DAGLs could be important targets for therapeutic intervention for diseases

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where excessive 2-AG signaling (or metabolites) contributes to the specific pathophysiology (*e.g.* metabolic and neurodegenerative diseases, see Chapter 8).[6] Several DAGL inhibitors have been reported in the literature,[6] but most of these compounds do not possess the activity, selectivity or pharmacokinetic properties to act as drug candidates, or as tools to study the role of 2-AG in health and disease. Thus, there is an unmet need to identify novel chemotypes to modulate DAGLα activity.

In principle, there are two general strategies to identify new chemical matter to modulate targets: structure-based drug design (SBDD) and ligand-based drug design (LBDD). The discovery of selective DAGLα inhibitors through a SBDD-approach is, however, hampered by a lack of structural knowledge of the target, as no crystal structures are available for DAGLα. Since several biochemical assays to identify DAGL inhibitors have been reported in the literature, a LBDD-approach using high throughput screening (HTS) has been shown to be viable option. For instance, researchers from Bristol-Myers-Squibb (BMS) reported on the development of two types of surrogate substrate assays using the hydrolysis of *para*-nitrophenyl (PNP) butyrate and 6,8-difluoro-4-methylumbelliferyl (DiMFU) octanoate by membrane fractions of HEK293 cells that express recombinant human DAGLα.[7] Subsequently, BMS performed a HTS on DAGLα, using the DiMFU-octanoate as a fluorogenic surrogate substrate.[7,8] The assay was of high quality and provided signal to background (S/B) ratios of 5-8 and Z` values of ~0.7 for the most optimal conditions in 384-well format. Approximately one million compounds were screened and 314 actives were identified. During the hit triage, two deselection assays were performed to assess selectivity over monoacylglycerol lipase (MAGL) and pancreatic lipase (PL). From the DAGLα lead chemotype series that was selective over the two off-targets tested, three compounds were ultimately reported (**1**-**3**, Figure 1). Glycine sulfonamide **2** was selected for lead optimization.[8]

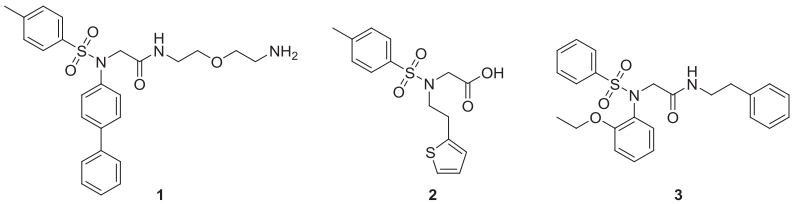


Figure 1. Three reported compounds from the lead chemotype series discovered by researchers of Bristol-Myers-Squibb (BMS).[8] Glycine sulfonamide **2** was selected for lead optimization.

DAGL, MAGL and PL all belong to the serine hydrolases, a 200+ membered family of enzymes that use an active site serine for substrate hydrolysis. Therefore, family-wide selectivity screening over many serine hydrolases is important to identify potential DAGL inhibitor off-targets, especially over targets within the endocannabinoid system. Activity-based protein profiling (ABPP) is a highly useful method to assess potency and selectivity of serine hydrolase inhibitors in complex samples, such as tissue or cell homogenates.[9] Surprisingly, very few examples in literature use ABPP in combination with HTS assays to test inhibitor

selectivity in the earliest possible stage of inhibitor discovery.[10] Baggelaar *et al*. reported on the first DAGLα targeting activity-based probe (ABP). Here, HTS-ABPP is employed to identify several novel chemotypes for DAGLα, within a lead discovery program executed in the framework of a public-private partnership with the ELF.

Results & Discussion

Optimization to 384-well-plate format and proof of principle screen

Previously, the colorimetric 96-well plate *para*-nitrophenyl (PNP) butyrate activity assay, utilizing membrane fractions of HEK293T cells overexpressing DAGLα (Figure 2A), successfully confirmed α-ketoheterocyles as DAGL inhibitors, which were identified by virtual screening of a pharmacophore model (Chapter 2).[11] To apply this assay for HTS-screening by the ELF, miniaturization to 384-wells format was required.[7,11] To this end, the assay volume (V) was reduced to highest volume possible (30 µL), to ensure optimal cross-section in 384-well plate. The enzyme concentration was varied and found to be optimal at 0.05 µg/µL.[7,11] These conditions provided optimal S/B and Z' values (Figure 2 B,C). Endpoint measurements instead of rate determination was applied to increase HTS efficiency (60 minutes as single endpoint, S/B = 2.8). Using this protocol, a proof-of-principle screen was conducted on the commercially available Library of Pharmacologically Active Compounds 1280 (LOPAC®, Sigma Aldrich). All 1280 compounds were screened at 10 µM (N = 2, n = 2) with high Z' (0.73 ± 0.08) and S/B values (3.4 ± 0.5). The screen delivered 26 actives with > 50% effect (Table S1). Eight hits were selected for full determination of dose-response curves based on their chemical structure and/or activity on their original target (Table S1, **bold**). cRaf1 kinase inhibitor **4,** containing a highly acidic phenol (carboxylic acid mimetic), was the only compound demonstrating dose-dependent reduction in DAGLα activity, but its activity could not be confirmed in detergent-containing assay buffer (Tween 0.05% m/m). This indicated that compound **4** is a false positive hit that possibly forms aggregates (Figure 2D). Nevertheless, the colorimetric 384-well assay fulfilled the assay requirements (Z' ~ 0.7 and S/B ~ 3) of the ELF for target acceptance and an application was submitted. After the target was approved by the ELF, the 384-well assay was optimized to 1536-well plate within the ELF consortium.

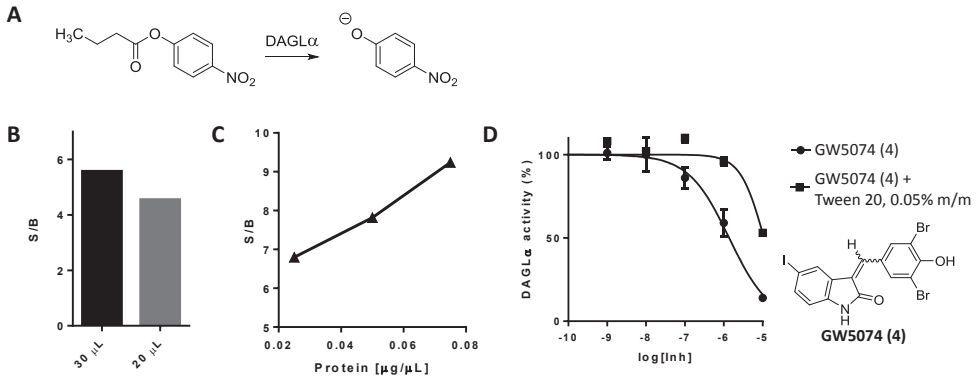


Figure 2. Development of a high throughput 384-well plate hDAGLα activity assay **A)** The assay is based on the conversion of *p*-nitrophenyl (PNP)-butyrate by HEK293T membrane fractions overexpressing hDAGLα. OD_{405} is measured over time. S/B is determined from the slope over 5-30 min. **B)** Optimization of assay volume per well; 30 µL volume provides better S/B values, N = 1 **C)** Optimization of protein concentration. Corresponding Z' values are: 0.26, 0.63 and 0.25 for a protein concentration of 0.025, 0.05 and 0.075 µg/µL respectively. Plate variance is optimal with 0.05 µg/µL DAGLα, N = 4. S/B and Z' are determined from the slope over 5-30 min. **D)** Profiling of hit compound **4** in dose response analysis with and without detergent Tween® 20 (0.05% m/m). Final screening conditions are: Clear 384-well plate, 30 µL volume, 5% DMSO, 10 µM inhibitor, 0.05 µg/µL membrane protein, 50 mM HEPES pH = 7.0, 20 minutes pre-incubation, then 300 µM PNP-butyrate. S/B is determined from the slope over 5-30 min. Endpoint at 60 minutes (OD_{405}) provides S/B values of ~2.8 (30 µL volume). Dose response analysis of hit **4** is performed in 96-well plate (as previously reported[7,11,12]). pIC_{50} for GW5074 = 5.86 ± 0.09 (normal conditions) and a ten-fold drop in potency, pIC_{50} = 4.96 ± 0.04 with Tween®20, N = 2, n = 2. This indicates **4** is a false positive. Reported concentrations are final concentrations.

Optimization to 1536-well-plate format

Substrate stability and plate edge effects: The stability of the substrate (PNP-butyrate) in assay buffer was investigated to prevent degradation, due to long storage times (up to several days) during the screening campaign. Two 0.9 mM PNP-butyrate solutions in assay buffer were stored at either room temperature or 0°C and absorption over time at 405 nm was measured over a consecutive period of 6 hours (Figure 3A). The background signal increased over time, indicating spontaneous hydrolysis of the substrate at room temperature. Substrate storage at 0°C is, therefore, essential to prevent spontaneous hydrolysis over time, especially if storage times in buffer exceed 2 h. Plate edge effect in 1536-well plates was significantly less in black clear-bottom plates compared to general clear plates as determined by a tartrazine absorption analysis (data not shown).

Substrate and protein concentration: For assay optimization to 1536-well plate, the total volume was kept at a maximum of 8.0 µL per well using the same protein and substrate concentration as in the 384-well plate assay. As expected, lower Z' and S/B values were obtained (0.64 and 2.73 respectively), presumably due to a smaller well cross section. Subsequent assay optimization focused on increasing the S/B ratios and Z' values by varying the substrate and enzyme concentrations (Figure 3B,C). First, the substrate concentration was screened in 0.3 – 1.2 mM final concentration range. Low substrate concentrations (0.3

mM) were associated with low S/B values due to lower turnover, whereas concentrations over 0.6 mM did not significantly improve S/B ratios (Figure 3B). Consequently, 0.6 mM PNP-butyrate was chosen as the optimal substrate concentration. Next, the enzyme concentration was varied and determined best at 0.025 µg/µL, which is two-fold lower than in the 384-well protocol (Figure 3C). Several validation runs using automated robotic handling and liquid dispensing demonstrated that the assay protocol was reproducible and robust. The final protocol was accepted for ultra-HTS on the Joint European Compound Library.

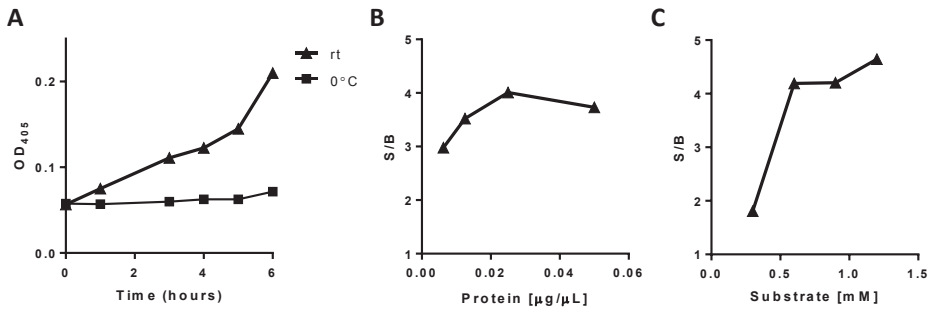


Figure 3. Development of the 1536-well plate hDAGLα activity assay. **A)** Stability test of the PNP-butyrate in assay buffer at rt and 0°C (ice). Optimal substrate storage is at 0°C. **B)** Optimization of PNP-butyrate substrate concentration. 0.6 mM was chosen as optimal since increasing the substrate concentration (1.5 or 2 fold) did not significantly improve S/B values. **C)** Optimization of protein concentration. 0.025 µg/µL was chosen as optimal. Final screening conditions are: Black Corning clear bottom 1536-well plates, 8.0 µL volume, 0.25% DMSO, 10 µM inhibitor, 0.025 µg/µL DAGLα protein, 50 mM HEPES pH = 7.0, 20 minutes pre-incubation, then 600 µM PNP-butyrate, endpoint at 90 minutes (OD_{405}). Reported concentrations are final concentrations.

High throughput screening and orthogonal ABPP assay

302.655 Compounds were screened in three days using the optimized 1536-well protocol. The primary assay resulted in 1932 hits with ≥50% effect at 10 µM inhibitor (Figure 4A, for exact values see Table S2), which corresponds to a 0.64% hit rate. The low hit rate suggested that the ELF library consists of high quality compounds with good physicochemical properties[2] and few pan-assay interference compounds (PAINS).[13] The course of the Z' and S/B values over the primary assay was monitored and is depicted in Figure 4B-C, showing the assay is robust and of good quality (S/B ~6, Z' ~0.8). Active conformation was performed at two inhibitor concentrations (10 and 1.25 µM, Table S2), resulting in a total of 263 confirmed actives (>70% eff. at 10 µM or >50% eff. at 1.25 µM)

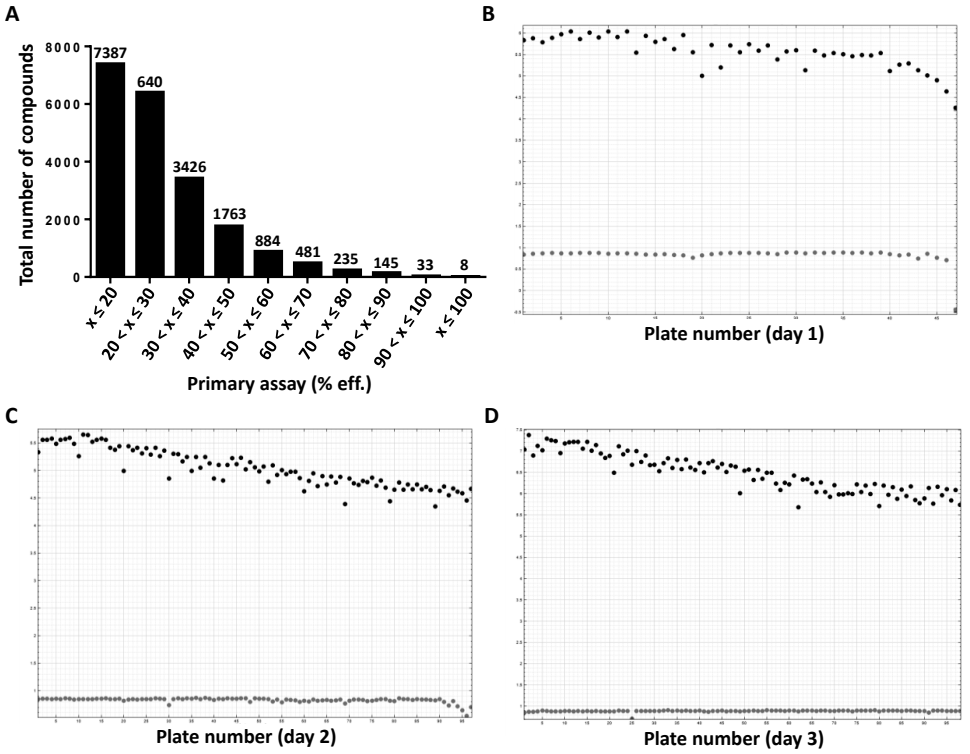


Figure 4: Overview of the HTS and assessment of screening quality over all 242 screened 1536-well plates. **A)** Total number of actives grouped per % effect. **B**-**D**) Assessment of assay quality of the primary assay over days 1-3 (B-D, respectively). A total of 47, 97 and 98 plates were measured on days 1-3. As shown, S/B (—●—) values are ~6, Z' (—●—) values are ~0.8.

To determine the activity and selectivity of the confirmed actives on endogenous DAGLα, the 263 confirmed actives were screened in an orthogonal ABPP assay on mouse brain proteome. A low throughput gel-based ABPP assay was developed based on previously reported protocols (see Experimental).[8] Using this protocol, the confirmed actives were screened at a single concentration (10 μM) for inhibition of DAGLα labeling by activity-based probe MB064 (**1**). The percentage inhibitory effect on DAGLα was calculated and normalized to control and corrected for protein loading using Coomassie staining (DMSO, N = 1).[8] Many of the compounds were less active in the ABPP assay than in the primary assay (Figure 5A, exemplary gel see 5B). However, several compounds demonstrated high activity also in the orthogonal assay, thereby confirming their cross-species DAGLα inhibitory activity. Importantly, the orthogonal ABPP assay also revealed the selectivity profile of the compounds over several other serine hydrolases, such as DDHD2, ABHD16, ABHD12, ABHD6 and LypLA2. The information of the orthogonal assay was taken into account and the hits were triaged by potency, selectivity and chemical eye (Table 1). The purity and correct m/z

of the compounds was analyzed by LC-MS. After clustering and legal clearance, the qualified hit list (QHL) contained 46 compounds (Table 2).

Table 1. Ultra-HTS assay triage overview. 46 Compounds form the Qualified Hit List (QHL).

	Compounds	Cut-off	% Total
Total screened	302.655	-	100
Primary assay hits	1932	>50% eff. (10 µM)	0.64
Active conformation hits	263	>70% eff. (10 µM) >50% eff. (1.25 µM)	0.09
Triaging and orthogonal assay[a]	46	Chemical eye, clustering, legal, purity	0.02

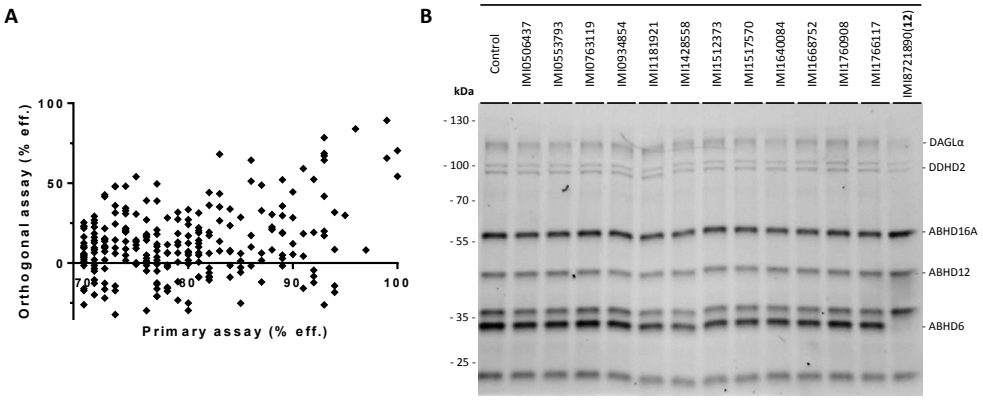


Figure 5. A) Analysis of the orthogonal assay versus primary assay (% eff. at 10 µM inhibitor). **B)** Exemplary gel of the orthogonal ABPP assay, consisting of a total of 21 analysed gels. Final ABPP screening conditions are: Incubation in 384-well plate, 20 µL Mouse brain membrane, 10 µM inhibitor (50 nL in DMSO), 30 minutes pre-incubation, then 250 nM MB064, 15 min, 2.8% DMSO, quench with 10 uL 3x Sample Buffer. All 21 gels were analysed according to the screening conditions reported. Percentage effect ABPP for DAGLα is calculated from the obtained gels, normalized for control and corrected for protein loading by Coomassie staining (N = 1). Reported concentrations are final concentrations.

Qualified hit list

The QHL contained 10 clusters of various chemotypes and 10 singletons (see Table 2) with IC_{50}-values in the range of 100-10000 nM. Actives from four distinct series were selected for resynthesis and retesting; glycine sulfonamides, α-keto amides, β-keto-α,α-difluoro amides and sulfonyl-1,2,4-triazole ureas. The two most potent compounds, IMI4906626 (**5**) and IMI4749305 (**6**) belong to the glycine sulfonamides series (Table 2, page 119), a series previously published as potent DAGLα inhibitors[8,14–16] that cross-react with ABHD6.[16] Noteworthy, compound **6** has a remarkable similarity to the previously reported LEI106 (Chapter 5) and has excellent ligand efficiency (LE = 0.36) and lipophilic efficiency (LipE = 4.3).[16] The European Screening Centre resynthesized compound **5** and confirmed its structure and activity in the PNP-assay (see experimental section). The potency of **5** is 32 nM (IC_{50}), LE is 0.29 and LipE is 3.6. Compound **5** possesses a free carboxylic acid and has

intrinsically high polar surface area (tPSA > 80 Å^2), which makes it interesting for the development of peripherally restricted inhibitors.[15] After resynthesis, compound **5** was retested on ABPP using broad-spectrum serine hydrolase probe TAMRA-FP and also MB064. This selectivity assessment showed that **5** was selective over all off-targets tested on ABPP, including ABHD6 (Figure 6B,C) which makes **5** the first known reported glycine sulfonamide inhibitor with this selectivity profile. Consequently, **5** could provide an excellent starting point for a hit optimization program.

Two additional clusters consist of α-keto amides **7**-**9** and β-keto-α,α-difluoro amides **10**, **11** (Table 2). Compounds **7-11** were resynthesized by the European Screening Centre (see experimental methods) and their structure and activity was confirmed (Table 2). Compound **7**-**9** had an IC_{50} of 1.0, 1.0 and 2.5 μM respectively, thereby making them interesting starting points for a hit optimization program with LE of 0.37 - 0.31 and LipE of 2.0 - 1.1 (Table 2). α-Keto amides are reported as peptidomimetic inhibitors of serine or cysteine proteases, such as Hepatitis C Virus serine proteases, thrombin, trypsin and cathepsin K (see [17] for a recent review). α-Keto amide motifs are incorporated in many natural products (*e.g.* Rapamycin,[18] a T-cell proliferation inhibitor) and FDA approved drugs (e.g. Boceprevir[19] and Telaprevir[20]). Moreover, this chemotype has also been used as substrate mimetic to obtain insight in anandamide hydrolysis.[21] α-Keto amides act by mechanism-based inhibition, using the activated ketone as covalent site of attachment (*i.e.* electrophilic trap) for nucleophilic residues in the catalytic site. The amide functionality can provide a template for key hydrogen bonding interactions with the target enzyme. β-Keto-α,α-difluoro amides are previously reported as porcine pancreatic elastase inhibitors[22] and are very similar to the α-keto amides in structural features. The presence of the α-keto-fluorines increases ketone reactivity. Compounds **10** and **11** have LE of 0.36 and 0.35 and LipE of 2.6 and 2.4, respectively. Both keto-amide classes show high similarity with previously published trifluoromethylketones and α-keto heterocycles, which are known chemotypes for DAGLα,[23] FAAH[24] (Chapter 2 and 3) and serine proteases.[25] Compound **7** and **10** were the most active in both the PNP- and orthogonal ABPP-assay (Table 2). After resynthesis, compound **7**-**10** were retested on ABPP using broad-spectrum serine hydrolase probe TAMRA-FP and also MB064. This ABPP analysis showed that the β-keto-α,α-difluoro amides **10** and **11** targeted ABHD6, whereas α-keto amides **7** and **8** did not (Figure 6B). Importantly, compounds **7**-**11** show excellent physicochemical properties, such as MW < 350, cLogD < 5, tPSA < 50, and HBA/HBD < 5. The α-keto amides **7**-**9** and β-keto-α,α-difluoro amides **10**, **11,** and derivatives, may provide valuable starting points for inhibitor discovery (within the serine or cysteine hydrolase/protease families).

Sulfonyl-1,2,4-triazole ureas **12**-**14** were discovered as the third novel chemotype for DAGLα. Compounds **12** and **13** were resynthesized by the European Screening Centre (see experimental) and their structure and activity was confirmed (Table 2). Compound **12** and **13** had an IC_{50} of 1.3 μM and 3.2 μM, LE of 0.35 and 0.31, respectively, and LipE of 4.2. This makes compound **12** the most optimal active among the newly discovered chemotypes (*i.e.*

best LE and LipE). The sulfonyl-1,2,4-triazole ureas are expected to be covalent irreversible inhibitors and provide a structural template bearing a reactive urea with tunable reactivity. The sulfonyl-1,2,4-triazole ureas resemble the 1,2,3-triazole ureas which were previously reported as potent DAGLα and DAGLβ inhibitors.[26,27] The sulfonyl-1,2,4-triazoles have an additional interesting feature, since their reactivity can be tuned by the sulfur oxidation state, which potentially influences the triazole pKa and, thereby, leaving group capacity. After resynthesis, compound **12** and **13** were retested on ABPP using broad-spectrum serine hydrolase probe TAMRA-FP and also MB064. Similar to the 1,2,3-triazole ureas, the sulfonyl-1,2,4-triazole ureas target ABHD6, DDHD2 and FAAH as determined by ABPP (Figure 6A,B). Compounds **12**-**14** have very good physicochemical properties (*e.g.* low MW and low cLogD), although their tPSA is relatively high (>96 $Å^2$, Table 2). Altogether, the high potency, LipE and LE of sulfonyl-1,2,4-triazole ureas **12**-**14** makes this cluster a highly interesting starting point for a hit optimization program.

Clusters 5-10 contain (fused) five-ring heterocycles such as phenyl thiazoles **15**-**19**, benzoxazoles **20**,**21** and benzimidazoles **22**,**23**. These inhibitors are expected to be non-covalent reversible inhibitors that derive their potency from specific interactions with DAGLα. Other clusters contain generally very lipophilic and linear-shaped compounds, interestingly often with multiple basic amines, including phenyl acetylene amines **24**-**26**, 4-amino piperidines **27**,**28**, pyrrolo-quinazolines **29**,**30**. Finally, a total of 10 singletons (**31**-**50**) were identified. Noteworthy, singleton **50** seems to selectively compete for labeling with a ~20 kD band on ABPP (possibly LyPLA2, Figure 6C). For DAGLα however, these compounds show a weak effect in the active conformation (Table S2), orthogonal assay (Table 2) and in retest in ABPP (Figure 6A-C). Of note, almost all of these compounds have not been resynthesized and their activity has not been confirmed.

Conclusions

The Joint European Compound Collection containing > 300.000 compounds was successfully screened using a 1536-wells high throughput assay with recombinant human DAGLα. Activity-based protein profiling (ABPP) with mouse brain proteomes was employed as an orthogonal assay to select the most interesting confirmed actives. ABPP provided highly valuable insight in activity and selectivity over many endogenously expressed brain serine hydrolases in an early hit discovery phase. This resulted in a qualified hit list of 46 compounds. Four major compound clusters were discovered, including previously published glycine sulfonamides, and three novel DAGL inhibitor chemotypes: α-keto amides, β-keto-α,α-difluoro amides and sulfonyl-1,2,4-triazole ureas. In addition, 6 minor clusters were identified together with 10 singletons. The sulfonyl-1,2,4-triazole ureas **12**-**14** were prioritized for subsequent lead optimization due to their high LE and LipE. A focused library of approximately 100 compounds of sulfonyl-1,2,4-triazoles was developed based on **12**-**14** within the European Lead Factory consortium (unpublished results). This focused library can be used for lead optimization of the sulfonyl-1,2,4-triazole as DAGL inhibitors. It is

anticipated that sulfonyl-1,2,4-triazole ureas can also serve as a novel versatile chemotype for inhibitor discovery on other unexplored serine hydrolases and related enzyme families (*e.g* cysteine proteases). In this aspect, the QHL and the focused library may serve as important screening sets. For example, compounds derived from the sulfonyl-1,2,4-triazole urea focused library were recently discovered as potent *in vivo* active compounds for α/β hydrolase domain 16A (ABHD16A, also known as BAT5), as described in Chapter 8.[28]

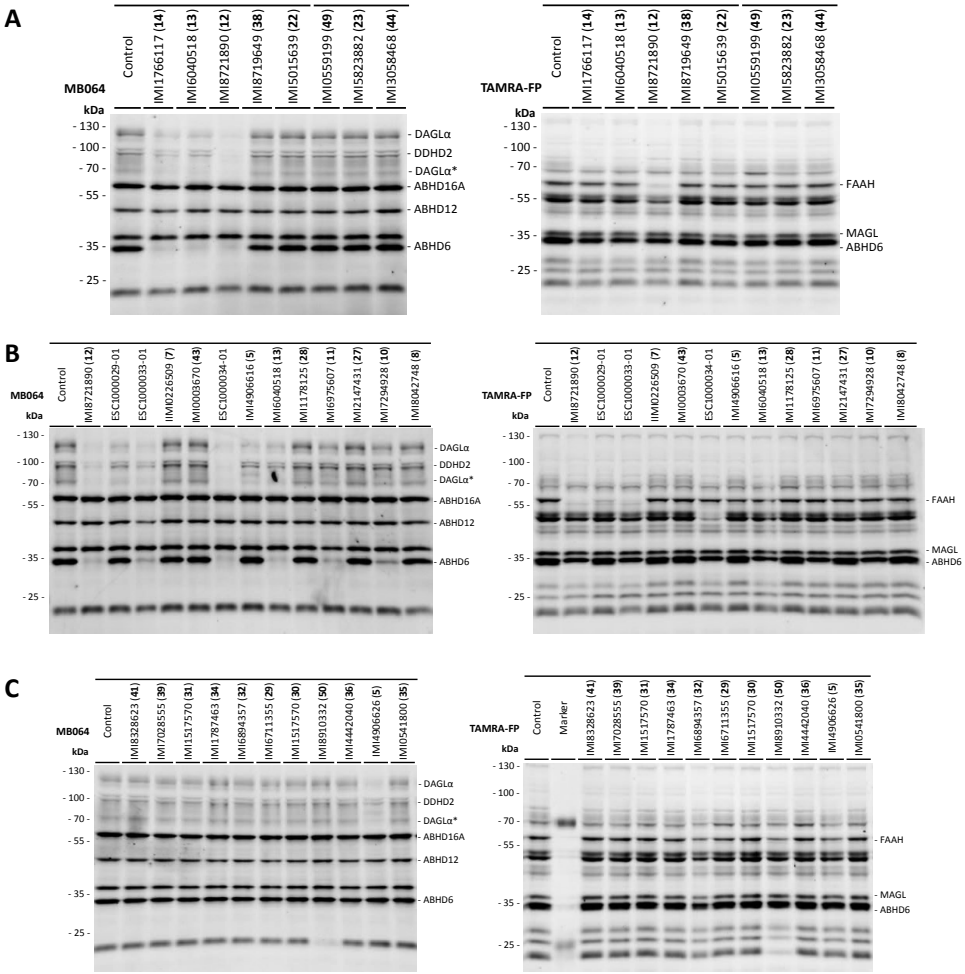
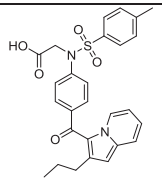
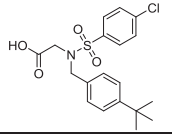
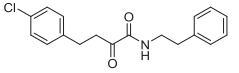
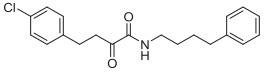
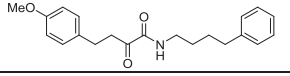
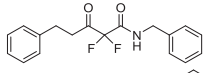
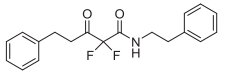
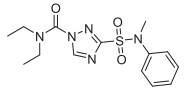
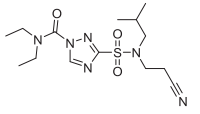
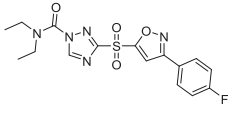
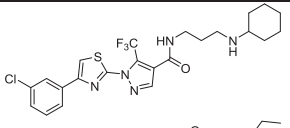
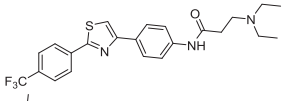
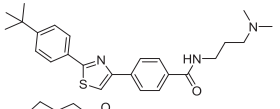
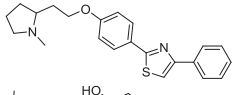
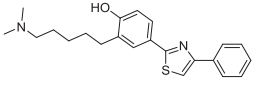
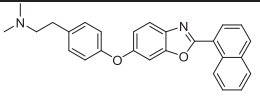
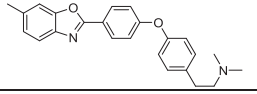
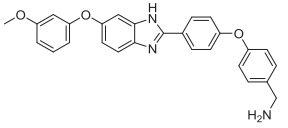
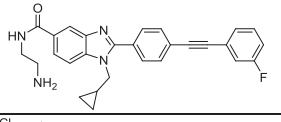
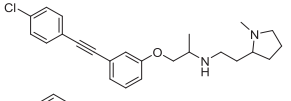
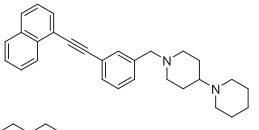
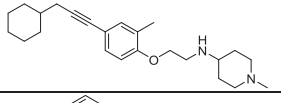
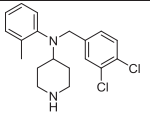


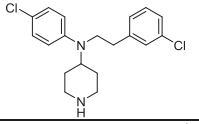
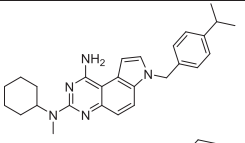
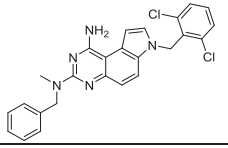
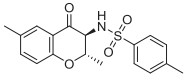
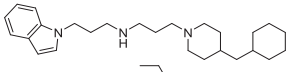
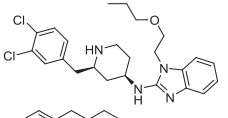
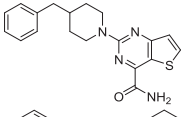
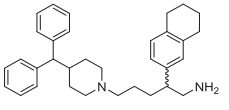
Figure 6. Overview of a select set of compounds retested on ABPP using two probes MB064 and TAMRA-FP using the standard previously reported procedures.[11] Compounds include: Glycine sulfonamide **5** (B,C), α-keto amides **7** and **8** (B), β-Keto difluoro amides **10** and **11** (B), Sulfonyl 1,2,4-triazole ureas **12-14** (A,B), Benzimidazoles **22** and **23** (A), 4-Amino piperidines **27** and **28** (B), Pyrrolo-quinazolines **29** and **30** (C) and singletons **31**, **32**, **35**, **36**, **38**, **39**, **41**, **43**, **44**, **49** and **50** (A-C). ESC1000029-01, 32-01 and 34-01 in (B) are part of the sulfonyl 1,2,4-triazole urea focused library (structures are not depicted).

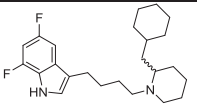
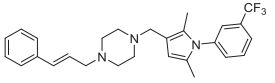
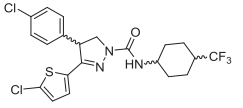
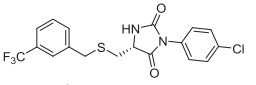
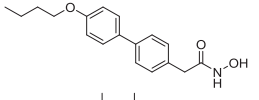
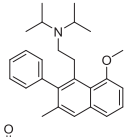
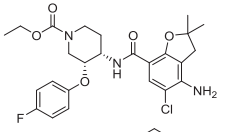
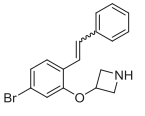
Table 2. Qualified hit list compounds **5**-**50** categorized per cluster (chemotype). pIC_{50} values originate from the colorimetric PNP assay (N = 2, n = 2). Percentage effect ABPP is calculated, normalized for control and corrected for protein loading by Coomassie staining (N = 1). IMI coded compounds are actives obtained from the HTS. IMI coded compounds that have an additional ESC code are resynthesized by the European Screening Centre (ESC) and are reconfirmed actives (*i.e.* **5**, **7**, **8**, **10**, **11**, **12**, **13**, **27**, **28**, **34** and **46**, see experimental). Lipophilic efficiency, LipE = pIC_{50} - cLogD. Ligand efficiency, LE = (1.37/HA)pIC_{50}. For % effect of the primary assay and active conformation, see Table S2 (SI).

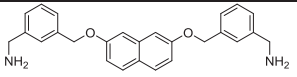
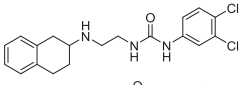
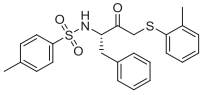
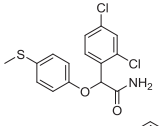
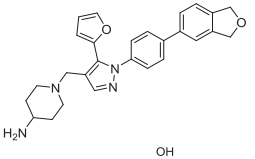
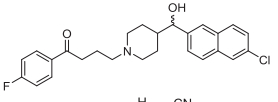
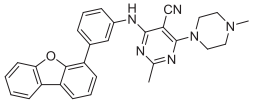
Cluster/ Chemotype	Entry	Code	Structure	pIC_{50} DAGLα (PNP)	% Eff. DAGLα (ABPP)	MW	cLogD	tPSA	HBD	HBA	LipE	LE
Glycine sulfonamide	**5**	IMI4906626 ESC1000043-01		7.5	79	491	3.91	104.5	1	7	3.6	0.29
	6	IMI4749305		6.9	69	396	2.61	83.1	1	5	4.3	0.36
α-Keto amide	**7**	IMI0226509 ESC1000025-01		6.0	42	316	3.97	46.2	1	3	2.0	0.37
	8	IMI8042748 ESC1000026-01		6.0	17	344	4.89	46.2	1	3	1.1	0.36
	9	IMI4961592		5.6	15	339	4.21	55.4	1	4	1.4	0.31
β-Keto difluoro amide	**10**	IMI7294928 ESC1000042-01		6.1	49	317	3.48	46.2	3	1	2.6	0.36
	11	IMI6975607 ESC1000044-01		6.2	21	331	3.80	46.2	3	1	2.4	0.35

Cluster/ Chemotype	Entry	Code	Structure	pIC_{50} DAGLα (PNP)	% Eff. DAGLα (ABPP)	MW	cLogD	tPSA	HBD	HBA	LipE	LE
Sulfonyl-1,2,4-triazole urea	**12**	IMI8721890 ESC1000032-01		5.9	66	337	1.69	96.8	0	8	4.2	0.35
	13	IMI6040518 ESC1000048-01		5.5	54	356	1.29	120.6	0	9	4.2	0.31
	14	IMI1766117		5.6	64	395	2.36	115.1	0	9	3.2	0.28
Phenyl thiazole	**15**	IMI1947191		5.0	35	512	4.41	100.1	2	6	0.6	0.20
	16	IMI8258632		5.1	32	448	4.07	73.5	1	4	1.0	0.23
	17	IMI4843646		5.0	28	422	3.56	73.5	1	4	1.4	0.23
	18	IMI0512143		4.9	16	365	3.33	53.6	0	3	1.3	0.26
	19	IMI2088196		5.0	14	367	4.27	64.6	1	3	0.7	0.26

Cluster/ Chemotype	Entry	Code	Structure	pIC_{50} DAGLα (PNP)	% Eff. DAGLα (ABPP)	MW	cLogD	tPSA	HBD	HBA	LipE	LE
Benzoxazole	**20**	IMI1909578		4.9	33	408	5.00	38.5	0	4	-0.1	0.22
	21	IMI7964529		4.9	-3	372	4.58	38.5	0	4	0.3	0.24
Benzimi-dazole	**22**	IMI5015639		5.5	48	437	5.44	82.4	3	6	0.1	0.23
	23	IMI3058468		< 4.7	48	453	4.05	72.9	3	5	-	-
Phenyl acetylene-amine	**24**	IMI8077796		5.2	42	397	2.54	24.5	1	3	2.7	0.25
	25	IMI4618041		5.1	33	409	4.52	6.5	0	2	0.6	0.23
	26	IMI4545816		4.9	22	369	4.07	24.5	1	3	0.8	0.25
4-Amino piperidine	**27**	IMI2147431 ESC1000046-01		5.0	31	349	4.96	15.3	1	2	0.0	0.30

Cluster/ Chemotype	Entry	Code	Structure	pIC_{50} DAGLα (PNP)	% Eff. DAGLα (ABPP)	MW	cLogD	tPSA	HBD	HBA	LipE	LE
4-Amino piperidine	**28**	IMI1178125 ESC1000054-01		4.8	23	349	4.69	15.3	1	2	0.1	0.29
Pyrrolo-quinazoline	**29**	IMI6711355		5.6	50	428	6.36	60.0	2	5	-0.8	0.24
	30	IMI2341674		5.5	33	462	6.74	60.0	2	5	0.5	0.24
Singleton	**31**	IMI1517570		5.9	54	345	3.39	80.8	1	5	2.5	0.34
	32	IMI6894357		5.4	45	396	2.98	20.2	1	3	2.4	0.26
	33	IMI1371614		N.D.	41	461	4.14	51.1	2	5	-	-
	34	IMI1787463 ESC1000055-01		5.9	36	352	4.11	100.3	2	5	1.8	0.32
	35	IMI0541800		5.7	33	467	4.92	29.3	2	2	0.8	0.22

Cluster/ Chemotype	Entry	Code	Structure	pIC_{50} DAGLα (PNP)	% Eff. DAGLα (ABPP)	MW	cLogD	tPSA	HBD	HBA	LipE	LE
	36	IMI4442040		5.4	31	389	5.81	19.0	1	2	0.4	0.26
	37	IMI2233474		5.2	30	454	4.46	11.4	0	3	0.7	0.22
	38	IMI8719649		5.6	30	490	6.17	72.9	1	4	-0.6	0.25
	39	IMI7028555		6.0	23	415	4.56	74.7	1	4	1.4	0.30
Singleton	**40**	IMI9488216		5.8	22	299	3.70	58.6	2	4	2.1	0.36
	41	IMI8328623		5.3	21	376	5.01	12.5	0	2	0.3	0.26
	42	IMI2499282		5.7	21	506	3.76	103.1	3	8	1.9	0.22
	43	IMI0003670		5.4	20	330	3.99	21.3	1	2	1.4	0.37

Cluster/ Chemotype	Entry	Code	Structure	pIC_{50} DAGLα (PNP)	% Eff. DAGLα (ABPP)	MW	cLogD	tPSA	HBD	HBA	LipE	LE
Singleton	**44**	IMI0559199		5.7	19	399	2.28	70.5	4	4	3.4	0.26
	45	IMI3277364		4.9	18	378	4.34	53.2	3	4	0.6	0.27
	46	IMI9906175 ESC100100		5.5	16	440	5.39	96.9	1	4	0.1	0.25
	47	IMI1224131		5.1	16	342	4.26	77.6	2	3	0.8	0.33
	48	IMI2003088		5.2	15	443	2.64	78.7	2	7	2.6	0.22
	49	IMI5823882		5.6	6	440	4.74	40.5	1	3	0.9	0.25
	50	IMI8910332		5.4	2	475	5.14	81.2	1	7	0.3	0.21

Experimental

General procedures biology

Cloning procedures

Cloning procedures were performed as previously reported.[11,12] In brief, full-length human DAGLα cDNA was purchased from Source Bioscience and cloned into mammalian expression vector pcDNA3.1, containing genes for ampicillin and neomycin resistance. The empty vector was used as a negative control (mock). All plasmids were grown in XL-10 Z competent cells and prepped (Maxi Prep, Qiagen). The sequences were confirmed by sequence analysis at the Leiden Genome Technology Centre.

Preperation of membrane fractions

Cell culture and membrane preparations were performed as previously reported.[11,12] In brief, HEK293T cells were grown in DMEM with stable glutamine and phenol red (PAA) with 10% new born calf serum, penicillin, and streptomycin. Cells were passaged every 2−3 days by resuspension in medium and seeding to the appropriate confluence. Membranes were prepared from transiently transfected HEK293T cells. 24 Hours prior to transfection, cells were seeded in Petri dishes (40 x 15 cm for HTS). Cells were transfected by the addition of a 3:1 mixture of polyethyleneimine (60 μg) and plasmid DNA (20 μg) in 2 mL of serum free medium. The medium was refreshed after 24 h, and after 72 h the cells were harvested by suspending them in 20 mL of medium. The supernatant was removed by centrifugation for 10 min at 1000 rpm. The cell pellet was flash frozen in liquid nitrogen and stored at −80 °C until use. Cell pellets were thawed on ice and suspended in lysis buffer A (20 mM HEPES, pH 7.2, 2 mM DTT, 0.25 M sucrose, 1 mM $MgCl_2$, 1× cocktail (Roche cOmplete EDTA free), 25 U/mL Benzonase). The suspension was homogenized by polytrone (3 × 7 s) and incubated for 30 min on ice. The membrane fraction was separated by ultracentrifuge (100.000g, 30 min, 4 °C, Beckman Coulter, type Ti70 rotor) and the pellet was resuspended in lysis buffer B (20 mM HEPES, pH 7.2, 2 mM DTT, 1× cocktail (Roche cOmplete EDTA free)). The protein concentration was determined with Qubit protein assay (Invitrogen). The total protein concentration was diluted to 1.0 mg/mL (~200 mL for HTS) and the samples were quickly frozen in liquid nitrogen and stored in 10 mL tubes at −80 °C until use.

Biochemical hDAGLα activity assay (96-well format)

The biochemical hDAGLα activity assay was performed as previously reported.[7,11] In brief, the biochemical hDAGLα activity assay is based on the hydrolysis of paranitrophenylbutyrate (PNP-butyrate) by membrane preparations from HEK293T cells transiently transfected with hDAGLα. Reactions (200 μL) were performed in flat bottom Greiner 96-wells plates. Final assay conditions; 200 μL total volume, 50 mM HEPES pH 7.2 buffer with 0.05 μg/μL hDAGLα transfected HEK293T membrane fractions, 300 μM PNP-butyrate, 5% DMSO. Controls were measured at N = 2, n = 4, inhibitors were measured at N = 2, n = 2. Slope is determined in 5-15 min.

Biochemical hDAGLα activity assay (384-well format)

The 384-well plate format assay is based on the above protocol. 30 μL reactions were performed in a 384-well plate (flat bottom, polypropylene). To each well was added 10 μL inhibitor solution (3x concentrated in 50 mM HEPES pH 7.2 assay buffer, 7.5% DMSO) and 10 μL membrane fraction solution (3x concentrated in assay buffer) after which the plates were centrifuged (5 s, 1000 rpm) and incubated at room temperature for 20 minutes. Lastly 10 μL substrate solution (3x concentrated in assay buffer, 7.5 % DMSO) was added to each well and the plate was centrifuged again (5 s, 1000 rpm). Enzymatic activity was followed by measuring the absorption at 405 nm on a PHERAstar microplate reader every 60-90 seconds, for up to 80 minutes at room temperature. Controls were measured at N = 2, n = 4. Final assay conditions; 30 μL total volume, 50 mM HEPES pH 7.2 buffer with 0.05 μg/μL hDAGLα transfected HEK293T membrane fractions, 300 μM PNP-butyrate, 5%

DMSO. Controls were measured at N = 2, n = 4, inhibitors were measured at N = 2, n = 2. Slope is determined in 5-30 min. End point determination is feasible at 60 minutes (S/B = 2.8).

Proof of principle LOPAC® 1280 library screen

Membrane fraction solution (10 µL 2 µg/µL), then 9.4 µl assay buffer and 0.6 µl inhibitor (50x concentrated DMSO stock, 500 µM) were added in a 384-well plate (flat bottom, polypropylene). The plate was centrifuged (5 s, 1000 rpm) and incubated at room temperature for 30 minutes. Subsequently 10 µL substrate mix (3x concentrated in assay buffer with DMSO) was added to each well and the plate was centrifuged again (5 s, 1000 rpm). Enzymatic activity was determined by measuring the absorption at 405 nm on a PHERAstar microplate reader after 60 minutes incubation at room temperature. Controls were measured at N = 2, n = 8, inhibitors at N = 2, n = 1.

Biochemical hDAGLα activity assay (1536-well format)

Using acoustic dispensing (Labcyte 555 Echo Liquid Handler), 20 nL of inhibitor solution (400x concentrated in DMSO) was added to a Black Corning clear bottom 1536-well plate, after which was added 2.5 uL of 50 mM HEPES pH 7.0 assay buffer (BioRAPTR FRD Microfluidic Workstation, Beckman Coulter) and 3 µL of membrane solution (2.67x concentrated in assay buffer, ThermoFisher Multidrop Combi). The plate was centrifuged (5 s, 1000 rpm) and incubated at room temperature for 20 minutes. Subsequently 2.5 µL of substrate solution (3.2x concentrated in assay buffer, 8.8% DMSO, BioRAPTR) was added to each well and the plate was centrifuged again (5 s, 1000 rpm). Enzymatic activity was determined by measuring the absorption at 405 nm on an Envision microplate reader after 90 minutes incubation at room temperature. Inhibitors were measured at N = 2, n = 1. Final assay conditions; 8.0 µL total volume, 50 mM HEPES pH 7.0 buffer with 0.025 µg/µL hDAGLα transfected membrane fractions, 600 µM PNP-butyrate, 0.25% DMSO. Primary assay; inhibitor 10 µM, Active conformation; inhibitor 10 and 1.25 µM final concentrations.

Preparation of mouse brain proteome

Mouse brains were isolated according to guidelines approved by the ethical committee of Leiden University (DEC No. 10095). Brain lysis was performed as previously reported.[9] In brief, mouse brains were thawed on ice and homogenized by Polytrone (3 × 7 s) in pH 7.2 lysis buffer A (20 mM HEPES, 2 mM DTT, 1 mM $MgCl_2$, 25 U/mL Benzonase) and incubated for 15 min on ice, followed by low speed spin (2500g, 3 min at 4 °C) to remove debris. The supernatant was subjected to ultracentrifugation (100.000g, 45 min, 4 °C, Beckman Coulter, type Ti70 rotor) to yield the cytosolic fraction in the supernatant and the membrane fraction as a pellet. The pellet was resuspended in lysis buffer B (20 mM HEPES, 2 mM DTT). The total protein concentration was determined with Quick Start Bradford assay (Biorad). Membranes were stored in small aliquots at −80 °C until use.

Orthogonal cABPP mDAGLα activity assay

The orthogonal cABPP protocol is based on the previously reported ABPP protocol.[8, 11] Using acoustic dispensing, 50 nL of inhibitor solution (400x concentrated, 4 mM) was added to a 384-well plate, after which was added 20 µL of mouse brain membrane proteome (2 mg/ mL). The mouse brain was incubated for 30 minutes with vehicle (0.5 µL of DMSO) or inhibitor and for 15 minutes with 0.5 µL of ABP MB064 (40x stock, 10 µM). Lastly was added 10 µL of standard 3 × SDS-PAGE sample buffer. The samples were loaded and resolved on commercially available SDS−PAGE gel (10% acrylamide). Final DMSO concentration: 2.8%. The gels were scanned with a ChemiDoc MP sytem (Cy3 settings, 605/50 filter) and analyzed using Image Lab 4.1. All 263 samples were measured at n = 1, N = 1.

ABPP selectivity assay (retest)

The cABPP selectivity assay was performed as previously reported.[11] In brief, mouse brain proteome (2.0 mg/mL, 20 µL) was preincubated for 30 min with vehicle (0.5 µL of DMSO) or inhibitor (0.5 µL of 400 µM inhibitor in DMSO) and subsequently treated with ABP (MB064 or TAMRA-FP, 250 nM and 500 nM final conc.). The reactions were quenched with 10 µL of standard 3x SDS−PAGE sample buffer. The samples were directly loaded and resolved on SDS−PAGE gel (10% acrylamide). Final DMSO concentration: 5.0%. The gels were scanned with a ChemiDoc MP sytem (Cy3 settings, 605/50 filter) and analyzed using Image Lab 4.1. All samples were measured at n = 1, N = 1.

General procedures chemistry

Synthesis of ESC compounds

Synthesis, characterization and retesting of the compounds in the colorimetric DAGLα activity assay is performed by the European Screening Centre.

2-Propylindolizine (51)

To a mixture of 1-bromopentan-2-one and 3-bromopentan-2-one (1.1 g, 3.34 mmol), was added 2-methylpyridine (0.66 ml, 6.67 mmol), $NaHCO_3$ (0.56 g, 6.67 mmol) and acetonitrile (6 mL) and it was heated in a microwave to 150°C for 1 hour. After full conversion, the reaction mixture was concentrated *in vacuo*, extracted with EtOAc/ H_2O, washed with H_2O and brine, dried over Na_2SO_4 and evaporated yielding 2-propylindolizine (377 mg, 71%) without additional purification. The crude product was used immediately in the next reaction.

(4-Nitrophenyl)(2-propylindolizin-3-yl)methanone (52)

2-Propylindolizine (**51**, 377 mg, 2.37 mmol) and triethylamine (0.4 ml, 2.84 mmol) were combined in DCM (15 mL). A solution of 4-nitrobenzoyl chloride (483.29 mg, 2.6 mmol) in DCM (5 mL) was added dropwise and the reaction stirred at rt for 18 hours. Sat. $NaHCO_3$ (20 mL) was added to the reaction mixture which was extracted with DCM (2 x 25 mL), washed with brine and dried over Na_2SO_4. Solvent was removed to afford crude product (743 mg) which was purified by flash chromatography to yield (4-nitrophenyl)(2-propylindolizin-3-yl)methanone (525mg, 72%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.83 (d, *J* = 7.03 Hz, 1H), 8.23 - 8.44 (d, 2H), 7.77 (d, *J* = 8.53 Hz, 2H), 7.50 (d, *J* = 8.78 Hz, 1H), 7.28 (s, 2H), 7.24 (s, 1H), 6.92 (t, *J* = 7.03 Hz, 1H), 6.43 (s, 1H), 2.14 (t, *J* = 7.65 Hz, 2H), 1.40 - 1.52 (m, 2H), 0.70 (t, *J* = 7.40 Hz, 3H).

(4-Aminophenyl)(2-propylindolizin-3-yl)methanone (53)

To a suspension of (4-nitrophenyl)-(2-propylindolizin-3-yl)methanone (**52**, 525 mg, 1.7 mmol) in ethanol (12 mL) and H_2O (16 mL) was added Zinc (400.82 mg, 6.13 mmol) and AcOH (1.46 ml, 25.54 mmol). The reaction was heated to reflux for 4 hours. The reaction mixture was allowed to cool to room temperature and then filtered. Solid residues were washed with EtOAc and the resulting filtrate extracted with EtOAc. Organics were washed with sat. NaHCO and then brine, dried over Na_2SO_4 and concentrated *in vacuo*. Purification by flash chromatography yielded (4-aminophenyl)(2-propylindolizin-3-yl)methanone (492 mg, 85%). The sample was further dried and then used in subsequent reaction without additional analysis. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.41 (d, *J* = 7.28 Hz, 1H), 7.56 - 7.68 (m, 2H), 7.42 (d, *J* = 8.78 Hz, 1H), 6.93 - 7.10 (m, 3H), 6.74 (dt, *J* = 1.25, 6.90 Hz, 1H), 6.32 - 6.44 (m, 1H), 2.37 (t, *J* = 7.65 Hz, 2H), 1.42 - 1.58 (m, 2H), 0.68 - 0.81 (m, 3H).

4-Methyl-*N*-(4-(2-propylindolizine-3-carbonyl)phenyl)benzenesulfonamide (54)

(4-Aminophenyl)-(2-propylindolizin-3-yl)methanone (**53**, 403 mg, 1.45 mmol) was dissolved in DCM (5 mL) and tosyl chloride (289.82 mg, 1.52 mmol) added followed by pyridine (0.14 mL, 1.74 mmol). The reaction was stirred at room temperature for 4 hours. LC-MS indicated a small quantity of starting material remained unreacted so additional tosyl chloride (0.1eq.) and pyridine (0.1 eq.) were added and stirring continued for 16 hours, overnight. The reaction mixture was diluted with DCM (20 mL) and washed with HCl (0.5M aq., 20 mL) and brine. Organics were dried over Na_2SO_4 and concentrated *in vacuo*. Purification by flash chromatography yielded 4-methyl-N-(4-(2-propylindolizine-3-carbonyl)phenyl)benzenesulfonamide (681 mg, quant.). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.57 (d, *J* = 7.28 Hz, 1H), 7.74 (d, *J* = 8.28 Hz, 2H), 7.55 (d, *J* = 8.53 Hz, 2H), 7.44 (d, *J* = 8.78 Hz, 1H), 7.24 - 7.32 (m, 2H), 7.18 (d, *J* = 8.53 Hz, 2H), 7.06 - 7.14 (m, 1H), 6.93 (br. s., 1H), 6.80 (dt, *J* = 1.25, 6.90 Hz, 1H), 6.38 (br. s., 1H), 2.36 - 2.45 (m, 3H), 2.12 - 2.24 (m, 2H), 1.35 - 1.50 (m, 2H), 0.65 (t, *J* = 7.28 Hz, 3H).

Tert-butyl *N*-(4-(2-propylindolizine-3-carbonyl)phenyl)-*N*-tosylglycinate (55)

4-Methyl-*N*-[4-(2-propylindolizine-3-carbonyl)phenyl]benzenesulfonamide (**54**, 92%, 681 mg, 1.45 mmol) was dissolved in THF (5 mL) and cooled to 0°C in an ice-water bath. Sodium hydride (60% dispersion in mineral oil, 69.52 mg, 1.74 mmol) was added and the reaction stirred at 0°C for 20 minutes. Tert-butyl 2-bromoacetate (0.32 ml, 2.17 mmol) was added and the reaction stirred at room temperature for 1 hour. Additional tert-butyl 2-bromoacetate (0.32 ml, 2.17 mmol), NaH (60% dispersion in mineral oil, 69.52 mg, 1.74 mmol) and THF (10 mL) were added and stirring continued for 30 minutes. The reaction was subsequently heated to reflux for 2 hours. The reaction mixture was allowed to cool to rt and split between EtOAc and H_2O. Organics were washed with H_2O and brine and dried over Na_2SO_4 Solvent was evaporated *in vacuo* and purified by flash chromatography yielding tert-butyl *N*-(4-(2-propylindolizine-3-carbonyl)phenyl)-*N*-tosylglycinate (959 mg quant.). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.67 (d, *J* = 7.28 Hz, 1H), 7.59 (dd, *J* = 8.41, 14.43 Hz, 4H), 7.46 (d, *J* = 8.78 Hz, 1H), 7.22 - 7.38 (m, 4H), 7.09 - 7.19 (m, 1H), 6.83 (dt, *J* = 1.25, 6.90 Hz, 1H), 6.39 (s, 1H), 4.37 (s, 2H), 2.43 (s, 3H), 2.15 - 2.26 (m, 2H), 1.38 - 1.52 (m, 11H), 0.72 (t, *J* = 7.28 Hz, 3H).

***N*-(4-(2-propylindolizine-3-carbonyl)phenyl)-*N*-tosylglycine (5, ESC1000043)**

Tert-butyl *N*-(4-(2-propylindolizine-3-carbonyl)phenyl)-*N*-tosylglycinate (**55**, 83%, 959 mg, 1.46 mmol) was dissolved in 4M HCl (4M in dioxane) (5 ml) and the reaction stirred at room temperature for 1 hour and heated to 40°C for 1 hour. Solvent was evaporated from the reaction mixture under reduced pressure and the resulting residue dissolved in EtOAc and washed with HCl (1M, aq.). Organics were dried over Na_2SO_4 and solvent evaporated under reduced pressure to afford crude product, 822 mg. Purification by reverse phase flash chromatography (C18 column, water 5% to 95% MeCN gradient, 0.1% TFA modifier) followed by evaporation of solvent from the appropriate fractions afforded a dark green solid. The solid was suspended in MeOH, sonicated and filtered. The resulting solid was heated in EtOH for 2 hours and then filtered, washed with diethyl ether and dried to yield *N*-(4-(2-propylindolizine-3-carbonyl)phenyl)-*N*-tosylglycine (240 mg, 34%) as a pale green solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 12.95 (br. s., 1H), 9.54 (d, *J* = 7.03 Hz, 1H), 7.64 (d, *J* = 8.78 Hz, 1H), 7.59 (d, *J* = 8.03 Hz, 2H), 7.51 (d, *J* = 8.53 Hz, 2H), 7.38 (d, *J* = 8.03 Hz, 2H), 7.31 (d, *J* = 8.53 Hz, 2H), 7.20 - 7.28 (m, 1H), 6.96 (dt, *J* = 1.25, 6.90 Hz, 1H), 6.51 (s, 1H), 4.50 (s, 2H), 2.39 (s, 3H), 1.98 - 2.16 (m, 2H), 1.23 - 1.46 (m, 2H), 0.61 (t, *J* = 7.28 Hz, 3H). LC-MS Purity = 100%, rt = 2.13 min, m/z [M+H] = 491.3.

Tert-butyl 2-[methoxy(methyl)amino]-2-oxo-acetate (56)

To a solution of oxalyl chloride (2.2 mL, 25.7 mmol) in dry THF (40 mL) at 0°C was added t-butanol (2.4 mL, 25.1 mmol). Stirring was continued for 1h, followed by addition of *N*-methoxymethanamine HCl (2.51 g, 25.7 mmol) with TEA (10.7 mL, 77.1 mmol) with stirring at 0°C. A rich precipitate formed quickly and stirring was continued at 0°C for 2h. The reaction was quenched with H_2O (20 mL) and the volatiles were removed *in vacuo*. The

product was extracted into EtOAc (2x) and the combined organic layers were washed with brine, dried with $MgSO_4$ and concentrated to dryness. The residual yellow oil was purified by flash chromatography yielding tert-butyl 2-[methoxy(methyl)amino]-2-oxo-acetate (2.2 g, 43%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18 – 7.38 (m, 2H), 3.77 (s, 3H), 3.23 (s, 3H), 1.49 – 1.70 (m, 14H). Literature protocol.[29]

Tert-butyl 4-(4-chlorophenyl)-2-oxobutanoate (57)

To a mixture of Mg turnings (0.068 g, 2.80 mmol) in dry diethylether (1 mL) was added a small piece of iodine. Then 1-(2-bromoethyl)-4-chloro-benzene was added and the mixture was subjected to ultrasound radiation for 20 minutes during which a clear yellow solution forms. The solution was stirred for an hour at rt. Then, the solution was added dropwise to a -78°C cold solution of tert-butyl 2-[methoxy(methyl)amino]-2-oxo-acetate (**56**, 0.76 g, 4.0 mmol) in 5 mL of dry diethylether. After stirring for 2h at -78°C the reaction was quenched with aq. NH_4Cl and the product was extracted into DCM (2x). The combined org extracts were dried with $MgSO_4$, concentrated *in vacuo* and purified by flash chromatography to yield tert-butyl 4-(4-chlorophenyl)-2-oxobutanoate (0.32 g, 28%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.22 – 7.35 (m, 3H), 7.16 (d, J = 8.28 Hz, 2H, 3.73 – 3.83 (m, 1H), 3.11 (s, 2H), 2.93 (s, 2H), 1.45 – 1.66 (m, 12H). Literature protocol.[29]

4-(4-Chlorophenyl)-2-oxobutanoic acid (58)

To a solution tert-butyl 4-(4-chlorophenyl)-2-oxobutanoate (**57**, 0.32 g, 1.19 mmol) in DCM (5 mL) was added TFA (0.44 mL, 5.95 mmol) with stirring at rt. Stirring was continued overnight. The reaction mixture was concentrated *in vacuo* and purified by flash chromatography to yield 4-(4-chlorophenyl)-2-oxobutanoic acid (0.22 g, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 – 7.40 (m, 2H), 7.16 (d, *J* = 8.28 Hz, 2H), 3.30 (t, *J* = 7.40 Hz, 2H), 2.99 (t, *J* = 7.28 Hz, 2H). Literature protocol.[29]

4-(4-Chlorophenyl)-2-oxo-N-phenethylbutanamide (7, ESC1000025)

To a solution of 4-(4-chlorophenyl)-2-oxobutanoic acid (**58**, 100 mg, 0.47 mmol) in DMF (5.0 mL) was added DIEA (0.09 mL, 0.52 mmol) and phenethylamine (0.065 mL, 62 mg, 0.517 mmol) followed by HATU (178 mg, 0.47 mmol) while stirring. After 1h, water was added and the product was extracted into EtOAc (2x) and the combined org extracts were dried with Na_2SO_4, concentrated *in vacuo* and purified by flash chromatography to yield 4-(4-chlorophenyl)-2-oxo-N-phenethylbutanamide (75 mg, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 – 7.47 (m, 11H), 6.75 – 7.04 (m, 1H), 3.58 (d, *J* = 6.53, 2H), 3.26 (t, *J* = 7.40 Hz, 2H), 2.89 (td, *J* =7.25, 19.89 Hz, 4H). LC-MS Purity = 94%, rt = 3.02 min, m/z [M+H] = 315. Literature protocol.[29]

4-(4-Chlorophenyl)-2-oxo-N-(4-phenylbutyl)butanamide (8, ESC1000026)

To a solution of 4-(4-chlorophenyl)-2-oxobutanoic acid (**58**, 100 mg, 0.47 mmol) in DMF (5.0 mL) was added DIEA (0.09 mL, 0.52 mmol) and phenbutylamine (77 mg, 0.517 mmol) followed by HATU (178 mg, 0.47 mmol) while stirring. After 1h, water was added and the product was extracted into EtOAc (2x) and the combined org extracts were dried with Na_2SO_4, concentrated *in vacuo* and purified by flash chromatography to yield 4-(4-chlorophenyl)-2-oxo-N-(4-phenylbutyl)butanamide (82 mg, 46%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.06 – 7.40 (m, 10H), 6.83 – 7.02 (m, 1H), 3.18 – 3.47 (m, 4H), 2.93 (t, *J* = 7.40 Hz, 2H), 2.66 (t, *J* = 7.40 Hz, 2H), 1.47 – 1.85 (m, 5H). LC-MS Purity = 100%, rt = 3.16 min, m/z [M+H] = 343. Literature protocol.[29]

Ethyl 2,2-difluoro-3-hydroxy-5-phenyl-pentanoate (59)

TMS-Cl (31 µL, 0.25 mmol) was added to a stirred suspension of Zn (165.91 mg, 2.54 mmol) in THF (5 mL), followed by 1,2-dibromoethane (4 µL, 0.05 mmol) and heated to 40°C under argon for 15 minutes. The temperature was increased to 60°C. A solution of ethyl 2-bromo-2,2-difluoro-acetate (500 mg, 2.46 mmol) and

3-phenylpropanal (330.52 mg, 2.46 mmol) in THF (1 mL) was added to the reaction and heating continued for 30 minutes. The reaction was allowed to cool to rt. Water, DCM and 1N HCl was added to the reaction mixture and stirred for 15 minutes. The layers were separated, the organic dried over Na_2SO_4, the solvent removed *in vacuo* and purified by flash chromatography yielding ethyl 2,2-difluoro-3-hydroxy-5-phenyl-pentanoate (239 mg, 38%) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16 - 7.38 (m, 5H), 4.37 (d, *J* = 7.03 Hz, 2H), 3.97 - 4.12 (m, 1H), 2.89 - 3.03 (m, 1H), 2.70 - 2.82 (m, 1H), 1.80 - 2.11 (m, 3H), 1.37 (t, *J* = 7.15 Hz, 3H).

***N*-Benzyl-2,2-difluoro-3-hydroxy-5-phenyl-pentanamide (60)**

Ethyl 2,2-difluoro-3-hydroxy-5-phenyl-pentanoate (**59**, 115 mg, 0.45 mmol) and benzylamine (145.9 µl, 1.34 mmol) were combined in THF (2 mL) and heated to 120°C in the microwave for 1.5 hours. The solvent was removed at *in vacuo* and purified by flash chromatography yielding *N*-benzyl-2,2-difluoro-3-hydroxy-5-phenyl-pentanamide (85 mg, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 (s, 10H), 6.59 - 6.83 (m, 1H), 4.45 - 4.61 (m, 2H), 4.05 - 4.25 (m, 1H), 2.89 - 3.05 (m, 1H), 2.66 - 2.81 (m, 2H), 1.84 - 2.09 (m, 2H).

***N*-Benzyl-2,2-difluoro-3-oxo-5-phenylpentanamide (10, ESC1000042)**

DMP (318 mg, 0.76 mmol) was added to a stirred solution of *N*-benzyl-2,2-difluoro-3-hydroxy-5-phenyl-pentanamide (**60**, 80 mg, 0.25 mmol) and the reaction stirred at room temperature overnight. The reaction mixture was diluted with DCM. The reaction mixture was washed with sat. $NaHCO_3$ solution and water. The organic was dried over Na_2SO_4 and the solvent removed *in vacuo* and purified by flash chromatography to yield *N*-benzyl-2,2-difluoro-3-oxo-5-phenylpentanamide (79 mg, 31%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.19 - 7.44 (m, 11H), 6.69 (br. s., 1H), 4.55 - 4.58 (m, 1H), 4.52 (d, *J* = 5.77 Hz, 2H), 3.15 - 3.23 (m, 2H), 2.95 - 3.03 (m, 2H). The compound has a weak UV chromophore and does not ionise well.

2,2-Difluoro-3-hydroxy-5-phenyl-N-(2-phenylethyl)pentanamide (61)

Ethyl 2,2-difluoro-3-hydroxy-5-phenyl-pentanoate (**59**, 151 mg, 0.58 mmol) and 2-phenylethanamine (220.95 µl, 1.75 mmol) were combined in THF (2 mL) and heated to 130°C in the microwave for 60 minutes. The solvent was removed *in vacuo* and purified by flash chromatography to yield 2,2-difluoro-3-hydroxy-5-phenyl-N-(2-phenylethyl)pentanamide (166 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.08 - 7.45 (m, 10H), 6.30 - 6.57 (m, 1H), 3.96 - 4.17 (m, 1H), 3.62 (d, *J* = 6.78 Hz, 2H), 2.88 (t, *J* = 7.03 Hz, 3H), 2.58 - 2.80 (m, 2H), 1.76 - 2.05 (m, 2H). LC-MS Purity = 100%, rt = 1.92 min, m/z [M+H] 334.20.

2,2-Difluoro-3-oxo-N-phenethyl-5-phenylpentanamide (11, ESC1000044)

DMP (572 mg, 1.34 mmol) was added to a stirred solution of 2,2-difluoro-3-hydroxy-5-phenyl-N-(2-phenylethyl)pentanamide (**61**, 150 mg, 0.45 mmol) in DCM (10 mL) at rt and stirring continued for 4 hours. The reaction mixture was diluted with DCM. The reaction mixture was washed with sat. $NaHCO_3$ solution and water. The organic was dried over Na_2SO_4 and the solvent removed *in vacuo* and purified by flash chromatography to yield 2,2-difluoro-3-oxo-N-phenethyl-5-phenylpentanamide (24.5 mg, 31%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.15 - 7.38 (m, 10H), 6.40 (br. s., 1H), 3.60 (q, *J* = 6.78 Hz, 2H), 3.08 - 3.18 (m, 2H), 2.93 - 3.01 (m, 2H), 2.87 (t, *J* = 7.03 Hz, 2H). The compound has a weak UV chromophore and does not ionise well.

3-(Benzylthio)-1H-1,2,4-triazole (62)

A solution of 1,2-dihydro-1,2,4-triazole-3-thione (1.0 g, 9.89 mmol) and benzyl bromide (1.76 mL, 9.89 mmol) in dry DMF (15 mL) was stirred at rt for 24h. Then, the mixture was diluted with EtOAc followed by saturated $NaHCO_3$. The org phase was separated, the aq. phase was extracted with EtOAc the combined org phases were

washed with water followed by brine. Concentration in vacuo yields crude 3-(benzylthio)-1H-1,2,4-triazole (1.80 g, 86%), which was used as is.

3-(Benzylthio)-*N*,*N*-diethyl-1*H*-1,2,4-triazole-1-carboxamide (**63**)

A solution of 3-(benzylthio)-1H-1,2,4-triazole (**62**, 1.72 g, 9.0 mmol) in dry DMF (20 mL) was added diethylcarbamoyl chloride (2.28 mL, 18.0 mmol) followed by K_2CO_3 (6.22 g, 45 mmol) while stirring at rt, overnight. The reaction was quenched with water and the product was extracted into EtOAc. The org phase was washed with water followed by brine, concentrated *in vacuo* and purified by flash chromatography, yielding 3-(benzylthio)-*N*,*N*-diethyl-1*H*-1,2,4-triazole-1-carboxamide (2.50 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.77 (s, 1H), 7.19 – 7.51 (m, 6H), 4.40 (s, 2H), 3.42 – 3.76 (m, 3H), 1.52 – 1.67 (m, 1H), 1.27 (t, J = 7.03 Hz, 6H). Literature protocol.[30]

1-(Diethylcarbamoyl)-1*H*-1,2,4-triazole-3-sulfonyl chloride (64)

A stirred mixture of 2M HCl (3.8 mL) and DCM (5 mL) was cooled to -5°C (internal temperature). To this mixture was added a cold 10% NaOCl solution (1.55 M, 3.25 mL, 5 mmol) at such a rate that the temp. was maintained below 0°C. Then, 3-(benzylthio)-*N*,*N*-diethyl-1*H*-1,2,4-triazole-1-carboxamide (**63**, 0.44 g, 1.50 mmol) was added in small batches while maintaining the internal temperature between -5 to -10°C. Stirring at that temp was continued for a further 15-20 minutes and the reaction mixture was used immediately in the next reaction as crude. Literature protocol.[31]

***N*,*N*-Diethyl-3-(*N*-methyl-*N*-phenylsulfamoyl)-1*H*-1,2,4-triazole-1-carboxamide (12, ESC1000032)**

To the crude reaction mixture of 1-(diethylcarbamoyl)-1*H*-1,2,4-triazole-3-sulfonyl chloride (**64**) was added N-methylaniline (401.8 mg, 3.75 mmol) while stirring at -5°C. The mixture was allowed to warm to rt and after 1h, the reaction was quenched with $NaHCO_3$. The mixture was diluted with DCM and the org phase was separated, washed with 1M HCl, then dried over $MgSO_4$ and evaporated to dryness. Normal phase flash chromatography followed by two reverse phase preparative HPLC purifications yielded *N*,*N*-diethyl-3-(*N*-methyl-*N*-phenylsulfamoyl)-1*H*-1,2,4-triazole-1-carboxamide (33 mg, 11.7%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (s, 1H), 7.15 – 7.44 (m, 7H), 4.65 (s, 2H), 3.49 (br. S., 4H), 1.01 – 1.42 (m, 6H). LC-MS Purity = 100%, rt = 1.96 min, m/z [M+H] 337.

3-(N-(2-Cyanoethyl)-N-isobutylsulfamoyl)-N,N-diethyl-1H-1,2,4-triazole-1-carboxamide (13, ESC1000048-01)

To the crude reaction mixture of 1-(diethylcarbamoyl)-1*H*-1,2,4-triazole-3-sulfonyl chloride (**64**, second batch) was added 3-aminopropanenitrile (0.56 g, 8.0 mmol) while stirring at -10°C. After stirring for 1h, the mixture was diluted with DCM and the org phase was separated, washed with 1M HCl (2x) followed by water and then dried with $MgSO_4$. The solution was concentrated *in vacuo* and purified by flash chromatography. Then, 1-bromo-2-methyl-propane (1.0 mmol) was added followed by Cs_2CO_3 (97.6 mg, 0.30 mmol) while stirring at rt over the weekend. The mixture was diluted with water and the product was extracted into EtOAc. The organic phase was washed with water twice more, followed by brine and after drying with Na_2SO_4 the volatiles are evaporated *in vacuo*. The residual material was purified on preperative HPLC yielding 3-(N-(2-cyanoethyl)-N-isobutylsulfamoyl)-N,N-diethyl-1H-1,2,4-triazole-1-carboxamide. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.89 (s, 1H), 3.48 – 3.79 (m, 3H), 3.15 (d, *J* = 7.53 Hz, 2H), 2.84 (t, *J* = 7.0 Hz, 2H), 1.87 – 2.12 (m, 1H), 1.61 (br. s., 11H), 1.34 (t, *J* = 7.03 Hz, 7H), 0.98 (d, *J* = 6.78 Jz, 7H). LC-MS Purity = 100%, rt = 1.99 min, m/z [M+H] 311.20.

Tert-butyl 4-(2-methylanilino)piperidine-1-carboxylate (65)

To a solution of tert-butyl 4-oxopiperidine-1-carboxylate (250 mg, 1.25 mmol) in DCM (2 mL) was added CH_3CO_2H (0.08 ml, 1.48 mmol) followed by 2-methylaniline (150.58 mg, 1.41 mmol). The mixture was stirred at

room temperature for 5 minutes before addition of sodium triacetoxyborohydride (276.56 mg, 1.3 mmol) as one portion. The resulting mixture was stirred at rt for 20 h then the pH was adjusted to >10 by addition of NaOH (2 M, ca. 3 mL). The mixture was extracted with DCM and the organic extracts dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash chromatography yielding tert-butyl 4-(2-methylanilino)piperidine-1-carboxylate (340 mg, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.99 - 7.18 (2 H, m), 6.61 - 6.79 (2 H, m), 3.97 - 4.16 (2 H, m), 3.46 - 3.56 (1 H, m), 3.33 - 3.46 (1 H, m), 2.91 - 3.07 (2 H, m), 2.15 (5 H, s), 1.50 (9 H, s), 1.23 - 1.46 (3 H, m).

Tert-butyl-4-[*N*-[(3,4-dichlorophenyl)methyl]-2-methyl-anilino]piperidine-1-carboxylate (66)

To a mixture of tert-butyl 4-(2-methylanilino)piperidine-1-carboxylate (**65**, 100 mg, 0.34 mmol) and K_2CO_3 (95.18 mg, 0.69 mmol) in DMF (2.6 mL) was added 4-(bromomethyl)-1,2-dichloro-benzene (0.06 mL, 0.41 mmol). The resulting mixture was heated at 100 °C for 32 h then partitioned between EtOAc and NaOH (2 M, aq.). The organic extracts were further washed with $NaHCO_3$, then brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography yielding tert-butyl-4-[*N*-[(3,4 dichlorophenyl)methyl]-2-methyl-anilino]piperidine-1-carboxylate (70 mg, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (1 H, d, *J* = 1.76 Hz), 7.26 (1 H, d, *J* = 8.28 Hz), 7.17 (3 H, d, *J* = 7.53 Hz), 7.09 - 7.14 (2 H, m), 7.06 (1 H, dd, *J* = 8.28, 1.76 Hz), 6.95 - 7.02 (1 H, m), 4.06 - 4.25 (4 H, m), 2.89 - 3.01 (1 H, m), 2.66 (5 H, t, *J* = 11.92 Hz), 2.31 (3 H, s), 1.86 (6 H, d, *J* = 12.30 Hz), 1.53 - 1.68 (4 H, m), 1.47 (9 H, s).

***N*-(3,4-Dichlorobenzyl)-*N*-(*o*-tolyl)piperidin-4-amine (27, ESC1000046)**

To a solution of tert-butyl 4-[*N*-[(3,4-dichlorophenyl)methyl]-2-methyl-anilino]piperidine-1-carboxylate (**66**, 70 mg, 0.16 mmol) in DCM (3 mL) was added TFA (1.2 mL). LC-MS analysis after 2 h indicated consumption of the starting material with formation of the desired product (Rt = 1.37 min, *m/z* 349.20). The mixture was concentrated under reduced pressure and purified by flash chromatography. The resulting oil was treated with HCl in MeOH (prepared from dropwise addition of 1 mmol acetyl chloride to MeOH) and concentrated under reduced pressure. As the salt ratio could not be determined, the mixture was concentrated under reduced pressure and purified by flash chromatography yielding *N*-(3,4-dichlorobenzyl)-*N*-(*o*-tolyl)piperidin-4-amine (40.9 mg, 75%) as an orange gum. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (1 H, d, *J* = 1.51 Hz), 7.24 (1 H, d, *J* = 8.03 Hz), 7.15 (1 H, d, *J* = 7.28 Hz), 7.08 (3 H, s), 6.91 - 6.99 (1 H, m), 4.21 (2 H, s), 3.13 (2 H, d, *J* = 12.05 Hz), 2.81 - 2.92 (1 H, m), 2.55 (2 H, td, *J* = 12.17, 2.01 Hz), 2.31 (3 H, s), 1.88 (2 H, d, *J* = 11.80 Hz), 1.61 (2 H, qd, *J* = 12.00, 3.89 Hz), LC-MS rt = 1.71 min, m/z [M+H] 349.20.

4-Chloro-*N*-[2-(3-chlorophenyl)ethyl]aniline (67)

A mixture of 4-chloroaniline (250 mg, 1.96 mmol), 1-(2-bromoethyl)-3-chloro-benzene (0.4 ml, 1.96 mmol), KI (32.53 mg, 0.2 mmol) and DIEA (0.34 ml, 1.96 mmol) in MeCN (4 mL) was subjected to microwave irradiation at 170 °C for 2.5 h. The resulting mixture was partitioned between DCM and $NaHCO_3$ (sat. aq.). The organics were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The material was purified by flash chromatography yielding 4-chloro-*N*-[2-(3-chlorophenyl)ethyl]aniline (414 mg, 79%) with an additional impurity and residual solvent. The material was carried into the next step without any further purification.

Tert-butyl 4-[4-chloro-*N*-[2-(3-chlorophenyl)ethyl]anilino]piperidine-1-carboxylate (68)

To a solution of tert-butyl 4-oxopiperidine-1-carboxylate (274.96 mg, 1.38 mmol) and CH_3CO_2H (0.09 ml, 1.63 mmol) in DCM (1.5 mL) was added a solution of 4-chloro-N-[2-(3-chlorophenyl)ethyl]aniline (**67**, 411.39 mg, 1.55 mmol) in DCM (2 mL). The resulting mixture was stirred at room temperature for 7 h then sodium triacetoxyborohydride (304.18 mg, 1.44 mmol) added in one portion. The mixture was stirred at room

temperature for 66 h then quenched with 2 M NaOH (adjusting pH to >10). The mixture was extracted with DCM and the organic extracts washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The material was subjected to flash chromatography yielding tert-butyl 4-[4-chloro-*N*-[2-(3-chlorophenyl)ethyl]anilino]piperidine-1-carboxylate (110 mg, 18%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.13 - 7.26 (5 H, m), 7.03 - 7.11 (1 H, m), 6.77 (2 H, d, J = 9.03 Hz), 4.09 - 4.35 (2 H, m), 3.53 - 3.67 (1 H, m), 3.27 - 3.41 (2 H, m), 2.65 - 2.84 (4 H, m), 1.66 - 1.77 (2 H, m), 1.48 (11 H, s). LC-MS tR = 2.64 min, m/z 449.25 [M+H]+, 393.15 [M-tBu+H]+, 349.20 [M-CO_2^{t}Bu+H]+

_N_-(3-Chlorophenethyl)-_N_-(4-chlorophenyl)piperidin-4-amine (28, ESC1000054)

To a solution of tert-butyl 4-[4-chloro-N-[2-(3-chlorophenyl)ethyl]anilino]piperidine-1-carboxylate (**68**, 110 mg, 0.24 mmol) in DCM (3 mL) was added TFA (1.5 mL). The resulting solution was stirred at room temperature for 18 h then concentrated under reduced pressure. The material was subjected to flash chromatography yielding *N*-(3-chlorophenethyl)-*N*-(4-chlorophenyl)piperidin-4-amine (82.1 mg, 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 - 7.23 (5 H, m), 7.00 (1 H, d, J = 7.03 Hz), 6.71 (2 H, d, J = 9.03 Hz), 3.49 - 3.67 (2 H, m), 3.31 (2 H, m, J = 7.50, 7.50 Hz), 3.22 (2 H, d, J = 12.30 Hz), 2.62 - 2.81 (4 H, m), 1.58 - 1.82 (4 H, m). LC-MS tR = 2.62 min, m/z 349.00.

Ethyl 2-chlorothieno[3,2-*d*]pyrimidine-4-carboxylate (69)

To a stirred solution of 2-chloro-4-(1-ethoxyvinyl)thieno[3,2-*d*]pyrimidine (318 mg, 1.32 mmol) in dioxane (21 mL) was added a solution of 0.38M $NaIO_4$ in water (6.95 ml). $KMnO_4$ (20.88 mg, 0.13 mmol) was then added, resulting in a bright pink solution. After stirring for a couple of minutes, this mixture had become a pink-brown suspension. TLC analysis after 1.5 h indicated formation of a new spot but starting material still remaining. Additional $KMnO_4$ (20.88 mg, 0.13 mmol) was then added and stirring continued for a further 2.5 h. TLC analysis indicated further conversion but starting material still remaining so additional $KMnO_4$ (20.88 mg, 0.13 mmol) was added and stirring continued for a further 1.5 h. Although starting material still remained, the mixture was quenched by addition of sat. aq. K_2CO_3 to achieve a pH of 7-8. The resulting mixture was filtered to remove a brown precipitate, washing with DCM (x4). The filtrate was diluted with water and the organic extracts washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The material was subjected to flash chromatography yielding ethyl 2-chlorothieno[3,2-*d*]pyrimidine-4-carboxylate (175 mg, 55%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (8 H, d, J = 5.52 Hz), 7.58 (8 H, d, J = 5.52 Hz), 4.61 (17 H, q, J = 7.03 Hz), 1.52 (25 H, t, J = 7.15 Hz).

Ethyl 2-(4-benzyl-1-piperidyl)thieno[3,2-*d*]pyrimidine-4-carboxylate (70)

A solution of ethyl 2-chlorothieno[3,2-d]pyrimidine-4-carboxylate (**69**, 175 mg, 0.72 mmol) and 4-benzylpiperidine (0.38 ml, 2.16 mmol) in N-methylpyrrolidinone (3.6 mL) was subjected to microwave irradiation at 130 °C for 3 h. The mixture was partitioned between EtOAc and water. The aqueous phase was further extracted with EtOAc (x2) and the combined organic extracts washed with water (x2), brine (x2), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The material was subjected to flash chromatography yielding ethyl 2-(4-benzyl-1-piperidyl)thieno[3,2-*d*]pyrimidine-4-carboxylate (171 mg, 62%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (1 H, d, J = 5.52 Hz), 7.28 - 7.35 (2 H, m), 7.13 - 7.25 (4 H, m), 4.90 (2 H, d, J = 13.05 Hz), 4.48 - 4.58 (2 H, m), 2.92 (2 H, td, J = 12.80, 2.26 Hz), 2.54 - 2.64 (2 H, m), 1.74 - 1.92 (3 H, m), 1.45 - 1.55 (3 H, m), 1.19 - 1.39 (2 H, m).

2-(4-Benzylpiperidin-1-yl)thieno[3,2-*d*]pyrimidine-4-carboxamide (34, ESC1000055)

A mixture of ethyl 2-(4-benzyl-1-piperidyl)thieno[3,2-d]pyrimidine-4-carboxylate (**70**, 171 mg, 0.45 mmol) and 7M NH_3 in methanol (0.64 mL) in MeOH (4.5 mL) was sealed in a 20 mL microwave vial and heated at 40 °C in a sand bath for 22 h. The mixture was concentrated under reduced pressure and purified by flash

chromatography yielding 2-(4-benzylpiperidin-1-yl)thieno[3,2-*d*]pyrimidine-4-carboxamide (182 mg, 115%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 - 7.99 (1 H, m), 7.73 (1 H, br. s.), 7.28 - 7.35 (2 H, m), 7.13 - 7.25 (4 H, m), 5.77 (1 H, br. s.), 4.82 (2 H, d, J = 13.30 Hz), 2.92 (2 H, td, J = 12.80, 2.51 Hz), 2.59 (2 H, d, J = 7.03 Hz), 1.73 - 1.93 (3 H, m), 1.20 - 1.37 (4 H, m). LC-MS tR = 3.04 min, m/z 353.05.

Tert-butyl *N*-[(1*S*)-1-benzyl-3-(o-tolylsulfanyl)-2-oxo-propyl]carbamate (71)

Tert-butyl *N*-[(1*S*)-1-benzyl-3-bromo-2-oxo-propyl]carbamate (310 mg, 0.91 mmol), 2-methylbenzenethiol (106.74 µL, 0.91 mmol), 2,6-lutidine (105.5 µL, 0.91 mmol) and TBAI (3.35 mg, 0.01 mmol) were combined and stirred in acetonitrile (15 mL) at rt for 1 hour. The reaction mixture was diluted with ethyl acetate and washed sequentially with 0.5M HCl, water and brine. The organic was dried over Na_2SO_4, concentrated at reduced pressure and purified by flash chromatography yielding tert-butyl *N*-[(1*S*)-1-benzyl-3-(o-tolylsulfanyl)-2-oxo-propyl]carbamate (176 mg, 50%).

(3S)-3-Amino-1-(o-tolylsulfanyl)-4-phenyl-butan-2-one hydrochloride (72)

Tert-butyl N-[(*1S*)-1-benzyl-3-(o-tolylsulfanyl)-2-oxo-propyl]carbamate (**71**, 176 mg, 0.46 mmol) was stirred in 4M HCl in dioxane (3 ml) at room temperature for 2 hours. The solvent was removed at reduced pressure to afford (3S)-3-amino-1-(o-tolylsulfanyl)-4-phenyl-butan-2-one hydrochloride (150 mg, quant.).

***N*-[(1*S*)-1-Benzyl-3-(o-tolylsulfanyl)-2-oxo-propyl]-4-methylbenzenesulfonamide (46, ESC100100)**

(*3S*)-3-Amino-1-(o-tolylsulfanyl)-4-phenyl-butan-2-one hydrochloride (**72**, 150 mg, 0.47 mmol) and tosyl chloride (88.85 mg, 0.47 mmol) were stirred at room temperature in DCM (5 mL). DIEA (162.35 µl, 0.93 mmol) was added and the reaction stirred at room temperature overnight. The reaction mixture was diluted with DCM and washed with water. The organic was dried over Na_2SO_4 and concentrated at reduced pressure. The resulting residue was purified by flash chromatography followed by acidic reverse phase preperative HPLC yielding *N*-[(1*S*)-1-benzyl-3-(o-tolylsulfanyl)-2-oxo-propyl]-4-methylbenzenesulfonamide (54 mg, 26%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 - 7.59 (m, 2H), 7.06 - 7.25 (m, 8H), 6.95 - 7.05 (m, 3H), 5.16 (d, *J* = 7.78 Hz, 1H), 4.37 - 4.47 (m, 1H), 3.58 - 3.69 (m, 2H), 2.88 - 3.02 (m, 2H), 2.26 - 2.47 (m, 6H). LC-MS: RT = 1.92, m/z 440.25 [M+H]+.

Supplementary information

Proof-of-principle LOPAC screen

Table S1. All 26 hits of the LOPAC library proof-of-principle screen, ranked on % effect (> 50% at 10 µM, 2.03% hit rate). Class and function are derived from the Sigma Aldrich library descriptions. Bold are selected for subsequent dose response analysis on DAGLα.

% Eff.	Compound	MW	Class	Function
92	Tetradecylthioacetic acid	365,9	Transcription	Peroxisome proliferator-activated receptor (PPAR)-alpha agonist
88	**GW5074 (Compound 4)**	425,1	Phosphorylation	cRaf1 kinase inhibitor
86	Cortisone 21-acetate	341,8	Hormone	Anti-inflammatory cortisol
81	Palmitoyl-DL-Carnitine chloride	606,1	Phosphorylation	Long-chain acylcarnitine; modulator of PKC activation; intermediate in mitochondrial fatty acid oxidation
77	(R,R)-cis-Diethyl tetrahydro-2,8-chrysenediol	471,7	Hormone	Potent estrogen receptor beta antagonist; potent partial agonist at estrogen receptor alpha
76	**NNC 55-0396**	464,1	Ca^{2+} Channel	Selective T-type calcium channel inhibitor
75	**SB 224289 hydrochloride**	510,1	Serotonin	Selective 5-HT1B serotonin receptor antagonist
73	Bromoacetyl alprenolol menthane	786,4	Adrenoceptor	Alkylating beta adrenoceptor antagonist
73	DL-erythro-Dihydrosphingosine	502,8	Phosphorylation	Protein kinase C, phospholipase A2, and phospholipase D inhibitor
73	Phorbol 12-myristate 13-acetate	356,3	Phosphorylation	Activates protein kinase C; strong NO promoter; lymphocyte activator
72	Tamoxifen citrate	376,6	Phosphorylation	Anti-estrogen; relatively selective protein kinase C inhibitor
68	GR 127935 hydrochloride hydrate	365,9	Serotonin	Selective 5-HT1B/1D serotonin receptor antagonist.
67	Protoporphyrin IX	425,1	Cyclic Nucleotides	Activates soluble guanylyl cyclase
66	**Amiodarone hydrochloride**	341,8	Adrenoceptor	Adrenoceptor agonist; inhibits binding of 1,4-dihydropyridine to L-type Ca^{2+} channels; coronary vasodilator
65	1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole	606,1	Hormone	Specific estrogen receptor alpha (ERalpha) agonist
64	A-77636 hydrochloride	471,7	Dopamine	Potent, orally active D1 dopamine receptor agonist
64	3',4'-Dichlorobenzamil hydrochloride	464,1	Ion Pump	Na^{+}/Ca^{2+} exchanger inhibitor
58	**PQ401**	510,1	Somatostatin	Peroxisome proliferator-activated receptor (PPAR)-alpha agonist
57	Prochlorperazine dimaleate	786,2	Dopamine	cRaf1 kinase inhibitor
57	Terfenadine	502,8	Histamine	Anti-inflammatory cortisol
55	DL-Stearoylcarnitine chloride	356,3	Phosphorylation	Long-chain acylcarnitine; modulator of PKC activation; intermediate in mitochondrial fatty acid oxidation
52	**Raloxifene hydrochloride**	376,6	Hormone	Potent estrogen receptor beta antagonist; potent partial agonist at estrogen receptor alpha
52	Ruthenium red	365,9	Ion Pump	Selective T-type calcium channel inhibitor
51	**GW7647**	425,1	Transcription	Selective 5-HT1B serotonin receptor antagonist
51	DL-Homatropine hydrobromide	341,8	Cholinergic	Alkylating beta adrenoceptor antagonist
50	**CP55940**	606,1	Cannabinoid	Protein kinase C, phospholipase A2, and phospholipase D inhibitor

Primary assay and active conformation JECL screen

Table S2. All 46 QHL hits from the ELF and their corresponding % effect in the primary screen at 10 µM inhibitor and the active conformation at 10 µM and 1.25 µM inhibitor. Concentrations are final concentrations.

Entry	Primary assay (% Eff.)	Active conformation (% Eff. 10 µM and 1.25 µM)		Entry	Primary assay (% Eff.)	Active conformation (% Eff. 10 µM and 1.25 µM)	
5	91	93	89	**28**	63	70	7
6	89	93	77	**29**	76	77	-7
7	88	72	55	**30**	67	74	9
8	78	82	36	**31**	76	58	50
9	79	80	26	**32**	86	90	29
10	86	87	54	**33**	96	93	91
11	86	77	58	**34**	72	73	17
12	99	99	81	**35**	85	80	11
13	87	86	34	**36**	64	74	19
14	76	75	22	**37**	89	89	30
15	82	83	-3	**38**	96	88	15
16	93	94	18	**39**	84	84	23
17	72	78	6	**40**	79	70	33
18	75	76	11	**41**	53	74	28
19	73	71	8	**42**	94	88	25
20	85	87	8	**43**	80	81	23
21	87	82	10	**44**	78	92	52
22	70	73	17	**45**	88	91	39
23	84	73	1	**46**	75	71	18
24	91	93	16	**47**	65	70	26
25	86	89	15	**48**	64	72	25
26	86	88	10	**49**	81	82	17
27	54	73	31	**50**	93	87	43

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