Design and Synthesis of a Screening Library Using the Natural Product Scaffold, 3-Chloro-4-hydroxyphenylacetic Acid

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ABSTRACT: The fungal metabolite, 3-chloro-4-hydroxyphenylacetic acid (**1**), was utilized in the generation of a unique drug-like screening library using parallel-solution phase synthesis. A 20-membered amide library (**3**–**22**) was generated by first converting **1** to methyl(3-chloro-4-hydroxyphenyl)acetate (**2**) then reacting this scaffold with a diverse series of primary amines via a solvent-free aminolysis procedure. The structures of the synthetic analogues (**3**–**22**) were elucidated by spectroscopic data analysis. The structures of compounds **8**, **12,** and **22** were confirmed by single X-ray crystallographic analysis. All compounds were evaluated for cytotoxicity against a human prostate cancer cell line (LNCaP) and for antiparasitic activity towards *Trypanosoma brucei brucei* and *Plasmodium falciparum* and showed no significant activity at 10 μM. The library was also tested for effects on the lipid content of LNCaP and PC-3 prostate cancer cells, and it was demonstrated that the fluorobenzyl analogues (**12**–**14**) significantly reduced cellular phospholipid and neutral lipid levels.

Natural products (NPs) have played a key role in drug discovery, which is reflected by the fact that numerous commercial drugs are either NPs, modified NPs, or compounds based on a NP pharmacophore.[1-4] The success and impact of NPs in the pharmaceutical arena is due, in part, to the fact that these natural constituents have been biologically validated, since they have been selected during evolution to bind to biosynthetic enzymes.[5-7] This inherent capacity of NPs to interact in biological space is thought to correlate with their ability to recognize therapeutic targets present in humans and/or other organisms (e.g., pathogenic microbes) that are associated with human diseases.[5,8,9]

In regard to NP drug discovery, a scaffold is a basic structural element that contains mono- or poly-functional groups that can be exploited in the synthesis of chemically diverse and unique screening libraries. The ultimate goal of such libraries is the identification of hit or lead compounds [usually following high-throughput screening (HTS)] that can be further optimized using medicinal chemistry for potential preclinical development and beyond. The use of selected NP scaffolds as the starting points for the generation of novel libraries has been touted as a means by which NPs can impact present and future drug discovery and development programs.[3,4,10-12] On the basis of this hypothesis, one of our current research objectives is the design and synthesis of "smarter" lead or drug-like screening libraries based on unique NP scaffolds isolated from Australian biota sources. To date, we have synthesized libraries based on the muurolane (source: plant),[4] tetrahydroanthraquinone (source: mushroom),[13] tambjamine (source: ascidian)[14] and 3-chloro-4-hydroxyphenylacetic acid (**1**) (source: endophytic fungus)[15] NP scaffolds. As part of our continuing research efforts in this field it was decided to evaluate

further and exploit the 3-chloro-4-hydroxyphenyl acetic acid (**1**) scaffold in medicinal chemistry studies.

In this second generation library, careful consideration was given to the design aspect of the library, paying particular attention to creating molecules with desirable drug-like physicochemical properties, based on Lipinski's "Rule of Five" criteria.[16] Furthermore, it was desired to investigate an alternative amidation reaction compared to the original library procedure,[15] in an attempt to improve yields, and potentially simplify the reaction product work-up. The fungal NP, 3-chloro-4-hydroxyphenyl acetic acid (**1**), was first isolated from the endophytic microfungus *Xylaria* sp.[17] Although this NP has shown minimal activity towards human melanoma (MM96L) and prostate (DU145) cell lines,[15] and carbonic anhydrase,[15] the 3-chloro-4-hydroxyphenyl motif present in **1** has been identified in a number of bioactive molecules such as (–)-xylariamide A,[18] coniothyriomycin,[19] and cryptophycin 53.[20,21] Owing to the limited biological profiling of the original 3-chloro-4-hydroxyphenyl acetic acid-derived library[15] it was decided to make additional synthetic analogues for a more thorough evaluation in several biological assays.

Herein the solution-phase parallel synthesis of a 20-membered amide library (**3**–**22**) using a derivative of the fungal metabolite, 3-chloro-4-hydroxyphenyl acetic acid (**1**) is reported. The biological evaluation of the library against malaria and trypanosome parasites, along with a prostate cancer cell lines is also described.

A large percentage of NPs and synthetic compounds possess the amide functional group and it is also known that ~25% of clinical drugs contain this moiety thus making the amidation reaction the most commonly used reaction in medicinal chemistry.[22] A number of reviews have been published demonstrating the use and

importance of coupling agents (e.g., HBTU, EDCI, CDI, and DCC) for amide formation.[23,24] However, for the synthesis of this second generation amide library, an amidation reaction was used whereby the products were prepared by direct aminolysis of an ester with a range of primary amines under solvent-free conditions.[25,26] The previous amidation study on the NP scaffold (**1**) reported the synthesis of a small 11-membered library using EDCI coupling.[15] Following liquid-liquid partitioning of the crude reaction product and silica flash chromatography, the yields from the EDCI first generation library ranged from 8 to 99%.

Prior to commencing the synthesis of the second generation library, a 25-membered virtual analogue (VA) library was constructed using a set of commercial amines. Using the software Instant JChem,[27] the physicochemical parameters such as cLog P, hydrogen bond donors (HBD), hydrogen bond acceptors (HBA), and molecular weight (MW) of these VAs (VA1–VA20) were determined (see Supporting Information, Table S45 and Figure S46). The molecules with favourable physicochemical properties, based on Lipinski's "Rule of Five" for drug-like molecules (MW < 500, HBD < 5, HBA < 10, cLog P < 5)[16] (see Supporting Information Table S47) were prioritized for synthesis work. On the basis of the in silico data, VA1–VA20 were selected for synthesis since all these analogues had no "Rule of Five" violations.

The synthetic route for the target amide analogues is shown in Scheme 1. The carboxylic acid moiety of the commercially available scaffold **1** was activated by esterification with anhydrous MeOH in the presence of *p*-toluenesulfonic acid (*p*TsOH) yielding methyl(3-chloro-4-hydroxyphenyl)acetate (**2**), which is more susceptible to aminolysis under mild conditions. Treatment of **2** with 20 primary amines afforded the secondary amides (**3**–**22**) as shown in Figure 1. Excess amine

was removed under N_2 and/or by high vacuum after which the crude reaction mixture was pre-adsorbed onto silica gel and chromatographed using CH_2Cl_2-MeOH or CH_2Cl_2-MeOH-1% triethylamine (TEA) (see Experimental Section) to afford compounds **3**–**22** in moderate to good yields. Compounds **3** and **9** have been synthesized previously using EDCI as the coupling agent with the yields of 98% and 69%, respectively.[15] However, using the direct aminolysis method, **3** (73%) and **9** (25%) were obtained in reduced yields when compared to the EDCI coupling procedure. Despite the lower yields, direct aminolysis under solvent-free conditions proved to be a simple and clean reaction that avoided the use of coupling reagents and complicated work-up procedures. Thus, this methodology is well suited for combinatorial chemistry studies using the scaffold, methyl(3-chloro-4-hydroxyphenyl)acetate (**2**).

The structures of all the amide analogues (**3**–**22**) were determined following 1D (^{1}H and ^{13}C) and 2D NMR (gCOSY, gHSQC, gHMBC) and HRESIMS data analysis. Slow evaporation of solutions of **8**, **12,** and **22** in CH_2Cl_2 resulted in crystals suitable for X-ray diffraction. Therefore, the structures of compounds **8**, **12,** and **22** were also confirmed by X-ray crystallography (see Figure 2 and Supporting Information S48 and Figure S49).

Owing to an ongoing interest in the discovery and development of novel small molecules that have potential anticancer[28-30] and/or anti-infective[31-33] properties the library (**3**–**22**) was profiled along with the NP scaffold (**1**), and methyl ester derivative (**2**) against a human prostate cancer cell line (LNCaP),[28] as well as *Trypanosoma brucei brucei*[34] and *Plasmodium falciparum*.[35] These in vitro assays showed that none of the compounds displayed significant activity (>50% inhibition) after 72 h at 10 μM. Additionally, all the analogues were also tested at 10 μM for effects on the lipid

content of LNCaP cells by staining with the lipophilic fluorescent dye Nile Red and fluorescence microscopy. The fluorescence intensity of lipid-bound Nile Red is linearly correlated with the concentration of cellular lipids.[36] Moreover, depending on the hydrophobicity of the bound lipids, Nile Red displays different emission maxima.[37] For example, Nile Red-stained neutral lipids like triacylglycerols and cholesterol esters, which are the main contents of lipid droplets, fluoresce with an emission maximum of ~560 nm, while polar phospholipids like phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, which are the main constituents of lipid bilayers, have an emission maximum of ~620 nm. Hence, this sensitive fluorescent stain can provide important information about alterations in the levels of these lipid classes, which is a composite representation of the highly dynamic lipid flux from various segments of cellular lipid homeostasis (uptake, synthesis, transport, storage, remodeling, and degradation). As shown in Figure 3, three of the fluorobenzyl analogues (**12**–**14**) significantly reduced the cellular levels of both phospholipids and neutral lipids when compared to the DMSO control. Similar results were observed in the metastatic prostate cancer cell line PC-3 (see Supporting Information, Figure S50). For comparison, the known acetyl-CoA carboxylase inhibitor TOFA, which antagonizes de novo fatty acid synthesis,[38] reduced the cellular neutral lipid content to similar levels as **12** and **13**, while the phospholipid levels remained unaffected (Figure 3). It is noteworthy that the *ortho*-substituted fluorobenzyl analogue **14** displayed a significantly reduced inhibitory activity towards the cellular neutral lipid levels in comparison to the *para* and *meta* fluoro-substituted isomers **12** and **13** (Figure 3A). These data indicated that the substitution pattern on the benzyl ring is important for the antagonistic activity. The present discovery that the fluorobenzyl analogues reduced cellular lipid levels in LNCaP and PC-3 cells

suggested that they affect one or multiple aspects of lipid homeostasis. The dysregulation of lipid metabolism is one of the most important metabolic hallmarks of cancer and is critical for the pathogenesis of cancer.[39] For example, most types of cancer synthesize almost all of their required saturated and mono-unsaturated fatty acids de novo through enhanced lipogenesis despite the abundance of exogenous dietary fatty acids. This lipogenic switch occurs early during the transformation into cancerous cells and becomes critical for cancer cell survival during cancer progression. Mounting evidence demonstrates that the dysregulation of lipid metabolism in cancer provides excellent chemotherapeutic targets with the potential for a large therapeutic index.[39] Hence, future experiments will investigate the molecular mechanism of the antilipogenic phenotype of the fluorobenzyl analogues.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on a Cole-Palmer melting point apparatus and are uncorrected. UV spectra were recorded on a JASCO V-650 UV/vis spectrophotometer. FTIR data were recorded using a Universal Attenuated Total Reflectance Two (UATR) attachment on a Perkin-Elmer spectrophotometer. NMR spectra were recorded at 30 °C on a Varian 600 MHz Unity INOVA spectrometer that was equipped with a triple resonance cold probe. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peak for DMSO-d_6 at δ_H 2.50 and δ_C 39.5, respectively. LRESIMS were recorded on a Waters ZQ mass spectrometer. HRESIMS data were recorded on a Bruker Daltronics Apex III 4.7e Fourier-transform mass spectrometer. Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} pre-coated aluminum plates and was observed using UV light. Alltech Davisil silica 30–40 μm 60 Å was used for pre-adsorption of the crude reaction products and was packed into an open glass column (35 × 70 mm) for silica gel flash chromatography. X-ray diffraction data were collected on an Oxford-Diffraction Gemini S Ultra CCD diffractometer utilizing CrysAlis software. All solvents used for chromatography, UV, and MS were Lab-Scan HPLC grade, and the H_2O was Millipore Milli-Q PF filtered. All the reagents were purchased from Sigma Aldrich and used without further purification.

Synthesis of Methyl(3-chloro-4-hydroxyphenyl)acetate. 3-Chloro-4-hydroxyphenylacetic acid (**1**, 1.5 g, 0.0075 mol, Sigma Aldrich, 99%) was dissolved in anhydrous MeOH (1 mL), and a crystal of *p*TsOH was added and the mixture was stirred at room temperature for 48 h.[40, 41] Excess solvent was removed under N_2 and the resulting crude mixture was partitioned between CH_2Cl_2 (2 × 100 mL) and saturated $NaHCO_3$ (100 mL). The CH_2Cl_2 layer was dried over anhydrous $MgSO_4$ then the solvent removed under reduced

pressure to yield methyl(3-chloro-4-hydroxyphenyl)acetate (**2**, 1.43 g, 95%) as a pale-brown gum. NMR and MS data were consistent with literature values.[42]

Generation of the Amide Library. Methyl(3-chloro-4-hydroxyphenyl)acetate (**2**, 50 mg, 0.25 mmol) and the relevant primary amine (0.5 mL) were added together and stirred for 16 h at room temperature. The reaction mixture was dried under N_2 and then high-vacuum before being redissolved in CH_2Cl_2-MeOH (1:1) and pre-adsorbed to silica (~ 1 g) overnight. Two different purification conditions were used during these studies. The first method was applied to the neutral amide analogues (**3**–**15**) and included the pre-adsorbed material being dry packed onto a CH_2Cl_2 equilibrated silica flash column (35 × 70 mm) and subsequently flushed using CH_2Cl_2 (100 mL), 95:5 CH_2Cl_2-MeOH (100 mL), 90:10 CH_2Cl_2-MeOH (100 mL), and 80:20 CH_2Cl_2-MeOH (100 mL). The second method was applied to the basic analogues (**16**–**22**) whereby the pre-adsorbed material was dry packed onto a CH_2Cl_2 equilibrated silica flash column and subsequently flushed using 100:1 CH_2Cl_2-TEA (101 mL), 95:5:1 CH_2Cl_2-MeOH-TEA (101 mL), 90:10:1 CH_2Cl_2-MeOH-TEA (101 mL), and 80:20:1 CH_2Cl_2-MeOH-TEA (101 mL). For both purification methods, 10 fractions were collected from each eluent flush, and then analyzed by both TLC and ^{1}H NMR spectroscopy with only high-purity fractions combined and added to the screening library. The physical and spectroscopic data of compounds **3**–**11** and **15**–**22** are summarized in the Supporting Information (S44).

*2-(3-Chloro-4-hydroxyphenyl)-N-(4-fluorobenzyl)acetamide (**12**):* light brown needles; (45.2 mg, 66%); mp 175–178 °C; UV (MeOH) (log ε) 233 (3.81), 282 (3.39) nm; IR ν_{max} 1607, 1592, 1538, 1509, 1421, 1210, 1157, 1058 cm^{-1}; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 3.38 (2H, s, H-7), 4.25 (2H, d, J = 6.0 Hz, H-9), 6.91 (1H, d, J = 8.3 Hz, H-5), 7.03 (1H, dd, J = 8.3, 2.1 Hz, H-6), 7.12 (2H, m, H-12, H-14), 7.24 (1H, d, J = 2.1 Hz, H-2), 7.26 (2H,

dd, *J* = 8.2, 5.9 Hz, H-11, H-15), 8.47 (1H, t, *J* = 6.0 Hz, NH-8), 9.96 (1H, br s, OH-4); ^{13}C NMR (150 MHz, DMSO-d_6) δ_C 41.0 (C-7), 41.6 (C-9), 114.9 (2C, d, $^2J_{CF}$ = 20.8 Hz, C-12, C-14), 116.4 (C-5), 119.3 (C-3), 128.0 (C-1), 128.6 (C-6), 129.2 (2C, d, $^3J_{CF}$ = 8.2 Hz, C-11, C-15), 130.2 (C-2), 135.6 (d, $^4J_{CF}$ = 2.3 Hz, C-10), 151.6 (C-4), 161.2 (d, $^1J_{CF}$ = 240.8 Hz, C-13), 170.2 (C-8); (–)-LRESIMS *m/z* 292 (100), 294 (30) $[M - H]^-$; (+)-LRESIMS *m/z* 294 (100), 296 (30) $[M + H]^+$; (–)-HRESIMS *m/z* 292.0550 $[M - H]^-$ (calcd for $C_{15}H_{12}ClFNO_2$, 292.0546).

*2-(3-Chloro-4-hydroxyphenyl)-N-(3-fluorobenzyl)acetamide (**13**):* brown solid; (21.4 mg, 34%); UV (MeOH) (log ε) 232 (3.79), 282 (3.33) nm; IR ν_{max} 1610, 1542, 1510, 1489, 1420, 1338, 1285, 1242, 1199, 1143, 1056 cm^{-1}; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 3.39 (2H, s, H-7), 4.28 (2H, d, *J* = 6.0 Hz, H-9), 6.90 (1H, d, *J* = 8.2 Hz, H-5), 7.03 (1H, dd, *J* = 8.2, 2.2 Hz, H-6), 7.03 (2H, overlap, H-11, H-13), 7.06 (1H, d, *J* = 7.9 Hz, H-15), 7.25 (1H, d, *J* = 2.2 Hz, H-2), 7.33 (1H, ddd, *J* = 7.9, 7.9, 6.1 Hz, H-14), 8.50 (1H, t, *J* = 6.0 Hz, NH-8), 9.95 (1H, br s, OH-4); ^{13}C NMR (150 MHz, DMSO-d_6) δ_C 41.0 (C-7), 41.7 (C-9), 113.4 (d, $^2J_{CF}$ = 20.9 Hz, C-13), 113.7 (d, $^2J_{CF}$ = 21.6 Hz, C-11), 116.4 (C-5), 119.3 (C-3), 123.1 (d, $^4J_{CF}$ = 2.9 Hz, C-15), 128.0 (C-1), 128.6 (C-6) 130.1 (d, $^3J_{CF}$ = 9.2 Hz, C-14), 130.2 (C-2), 142.5 (d, $^3J_{CF}$ = 7.1 Hz, C-10), 151.6 (C-4), 162.2 (d, $^1J_{CF}$ = 241.6 Hz, C-12), 170.3 (C-8); (–)-LRESIMS *m/z* 292 (100), 294 (30) $[M - H]^-$; (+)-LRESIMS *m/z* 294 (100), 296 (30) $[M + H]^+$; (–)-HRESIMS *m/z* 292.0548 $[M - H]^-$ (calcd for $C_{15}H_{12}ClFNO_2$, 292.0546).

*2-(3-Chloro-4-hydroxyphenyl)-N-(2-fluorobenzyl)acetamide (**14**):* off-white solid (11.4 mg, 16%); UV (MeOH) (log ε) 231 (3.86), 282 (3.40) nm; IR ν_{max} 1609, 1542, 1494, 1419, 1284, 1236, 1199, 1056 cm^{-1}; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 3.37 (2H, s, H-7), 4.30 (2H, d, *J* = 6.0 Hz, H-9), 6.88 (1H, d, *J* = 8.2 Hz, H-5), 7.02 (1H, dd, *J* = 8.2, 2.2 Hz, H-

6), 7.14 (1H, overlap, H-14), 7.15 (1H, overlap, H-12), 7.23 (1H, d, J = 2.2 Hz, H-2), 7.28 (1H, overlap, H-15), 7.30 (1H, overlap, H-13), 8.45 (1H, t, J = 6.0 Hz, NH-8), 9.96 (1H, br s, OH-4); ^{13}C NMR (150 MHz, DMSO-d_6) δ_C 36.0 (d, $^3J_{CF}$ = 4.6 Hz, C-9), 40.8 (C-7), 115.0 (d, $^2J_{CF}$ = 20.6 Hz, C-12), 116.4 (C-5), 119.2 (C-3), 124.2 (d, $^4J_{CF}$ = 3.4 Hz, C-14), 125.9 (d, $^2J_{CF}$ = 15.2 Hz, C-10), 127.9 (C-1), 128.5 (C-6), 128.7 (d, $^3J_{CF}$ = 8.1 Hz, C-13), 130.1 (C-2), 129.5 (d, $^3J_{CF}$ = 4.5 Hz, C-15), 151.6 (C-4), 160.0 (d, $^1J_{CF}$ = 244.5 Hz, C-11), 170.2 (C-8); (–)-LRESIMS *m/z* 292 (100), 294 (30) $[M - H]^-$; (+)-LRESIMS *m/z* 294 (100), 296 (30) $[M + H]^+$; (–)-HRESIMS *m/z* 292.0549 $[M - H]^-$ (calcd for $C_{15}H_{12}ClFNO_2$, 292.0546).

Cellular Lipid Content Analysis. LNCaP and PC-3 cells were obtained from the American Type Cell Culture Collection. Both cells were seeded in optical 96 well plates (ibidi) at 4,000 cells/well and 3,000 cells/well, respectively in phenol-red free RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 5% CSS (Thermo Fisher Scientific) for 72 h. The evaluation of the cellular lipid content was performed as previously described.[43] Briefly, after treatment for 48 h with the indicated compounds at 10 μM in 0.3% DMSO, the medium was removed, and cells were fixed in 4% paraformaldehyde for 20 min on ice. Cells were then stained for 20 min at room temperature protected from light in 100 μL PBS containing 1.0 μg/mL DAPI [2-(4-amidinophenyl)-1*H*-indole-6-carboxamidine, Sigma Aldrich] and 0.25 μg/mL Nile Red (Sigma Aldrich). Images in the DAPI, Cy3 and Cy5 channels were acquired on an INCell Analyzer 2200 system (GE Healthcare) at 10× magnifications. Image segmentation and quantitation of cellular mean fluorescence intensities of phospholipids (Cy5) and neutral lipids (Cy3) of ~ 1500 cells per treatment were performed with CellProfiler software (Broad Institute).[44,45] Statistical significance was analyzed with GraphPad Prism (GraphPad Software) by one-way ANOVA with Dunnett's multiple

comparison test. Control cells were treated with the equivalent dose of DMSO (negative control) or TOFA [5-(tetradecyloxy)-2-furoic acid, Sigma Aldrich] as the positive control.

ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR spectra for compounds **2**–**22**, physical and spectroscopic data for compounds **3**–**11** and **15**–**22**, structures and physicochemical properties of virtual analogues VA1–VA25, physicochemical properties of **1**–**22**, X-ray crystallography data for **8**, **12**, **22** and ORTEP drawings for **8** and **22**, cellular lipid content analysis data for PC-3 cells and protocols for bioassays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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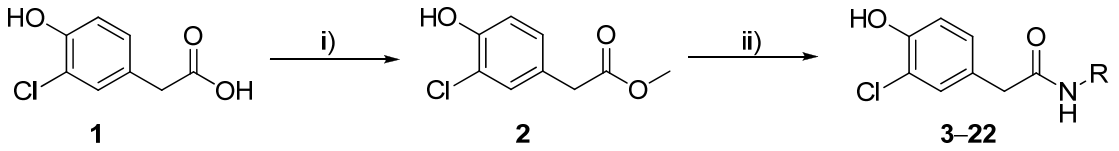
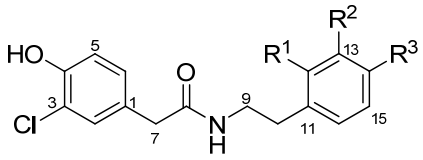
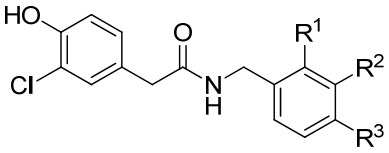


1 **2** **3–22**

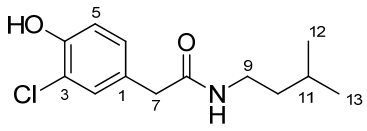
Scheme 1. Reagents and conditions. (i) anhydrous MeOH, *p*TsOH (cat.), rt, 24 h. (ii) amine, rt, 16 h.



	R^1	R^2	R^3
3	H	H	H
4	H	H	Br
5	H	H	Cl
6	H	H	F
7	H	F	H
8	F	H	H



	R^1	R^2	R^3
9	H	H	H
10	H	H	Br
11	H	H	Cl
12	H	H	F
13	H	F	H
14	F	H	H



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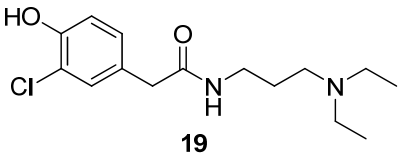

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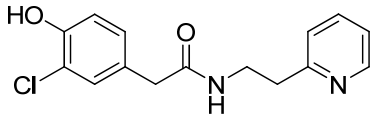

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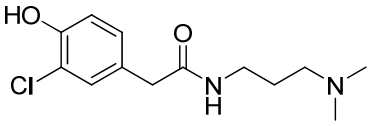

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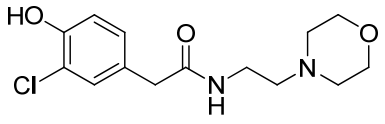

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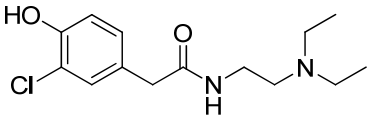

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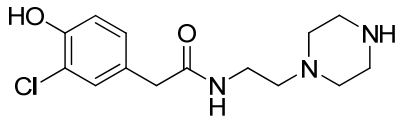

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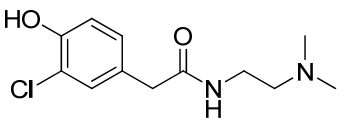

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Figure 1. Chemical structures of the amide library **3–22**.

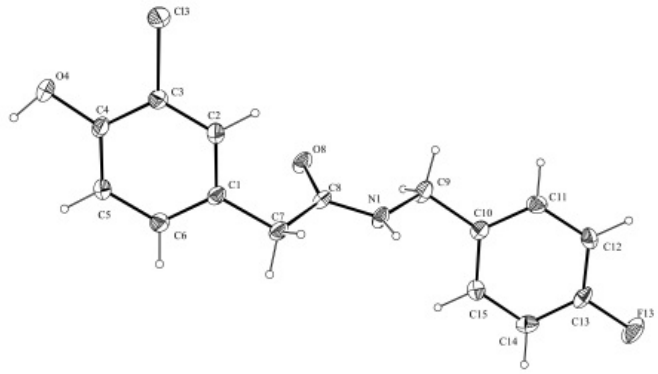


Figure 2. ORTEP drawing for compound **12**.

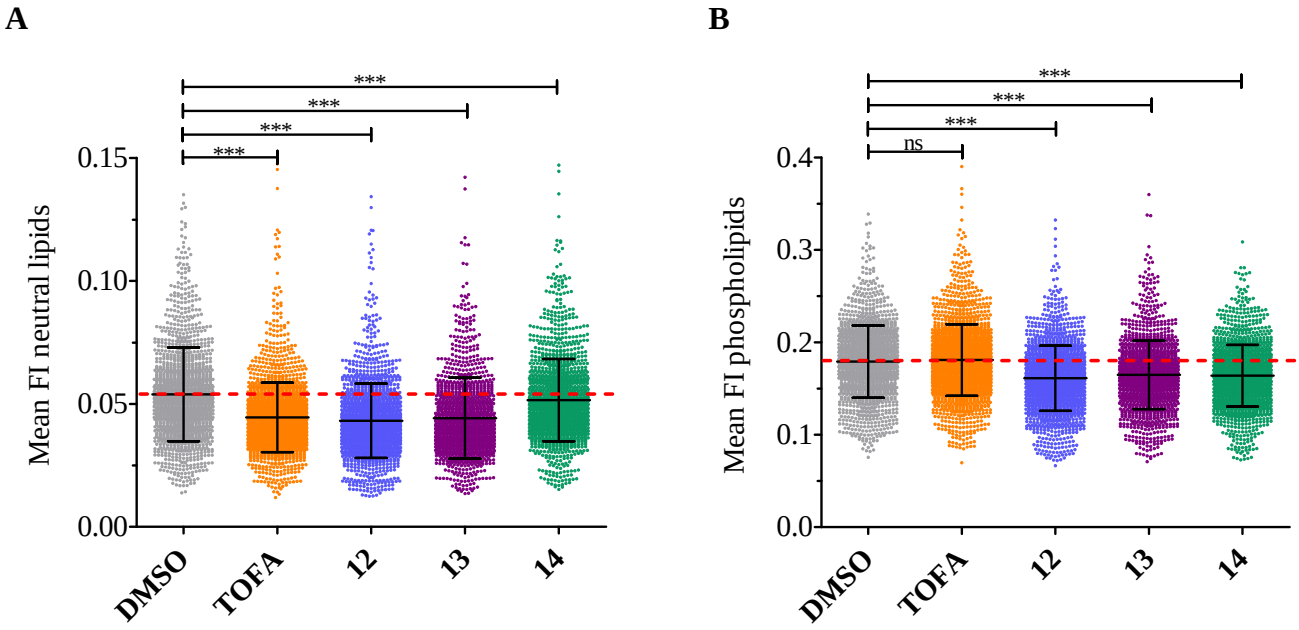
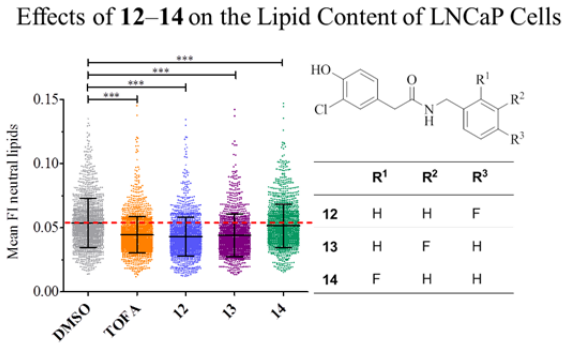


Figure 3. (**A**) Neutral lipid and (**B**) phospholipid content of LNCaP cells treated for 48 h with 10 μM of TOFA (positive control) or the library compounds **12**–**14**. The mean fluorescence intensities (FI) of cellular neutral lipids and phospholipids of Nile Red-stained LNCaP cells were quantified with CellProfiler (n~1500 cells, mean ± SD, one-way ANOVA with Dunnett's multiple comparison test, ns = not significant, *** $p < 0.001$).

TOC Graphic


Effects of 12–14 on the Lipid Content of LNCaP Cells
Mean FI neutral lipids
0.15
0.10
0.05
0.00
***
***
***
***
DMSO
TOFA
12
13
14
HO
Cl
O
N
H
R1
R2
R3
R1 R2 R3
12 H H F
13 H F H
14 F H H