Carboxylate analogues of aryl-urea-substituted fatty acids that target the mitochondrion in MDA-MB-231 breast cancer cells to promote cell death

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ABSTRACT

Selective targeting of the tumor cell mitochondrion is a viable approach for the development of anti-cancer agents because the organelle is functionally different from that in normal cells. We recently developed a novel aryl-urea fatty acid (**1**; 16({[4-chloro-3-(trifluoromethyl)phenyl]carbamoyl}amino)hexadecanoic acid) that disrupted the mitochondrion and activated apoptosis in MDA-MB-231 breast cancer cells. However, currently there is little information on the structural requirements for activity of **1** analogues. The present study evaluated the role of the carboxylic acid group on the anti-cancer activity of **1**. Bioisosteric replacement of the carboxylate in **1** maintained activity. Thus, like **1**, the sulfonic acid analogue **1-SA** and the oxo-thiadiazole analogue **1-OT** also targeted the mitochondrion and activated cell killing capacity. The hydroxamic acid analogue **1-HA** also killed MDA-MB-231 cells, but its onset of action was slower than **1-SA** and **1-OT**. In contrast, replacement of the carboxylate with non-bioisosteric amido- and methylamido-groups produced analogues that minimally altered mitochondrial function and showed little capacity to decrease tumor cell viability. These findings suggest that the carboxylate moiety in the novel mitochondrially-targeted agent **1** is an important determinant of the kinetics and efficacy of anti-cancer cell activities of **1** analogues. Further development of carboxylate-modified analogues of aryl-urea fatty acids as potential anti-cancer agents could now be warranted.

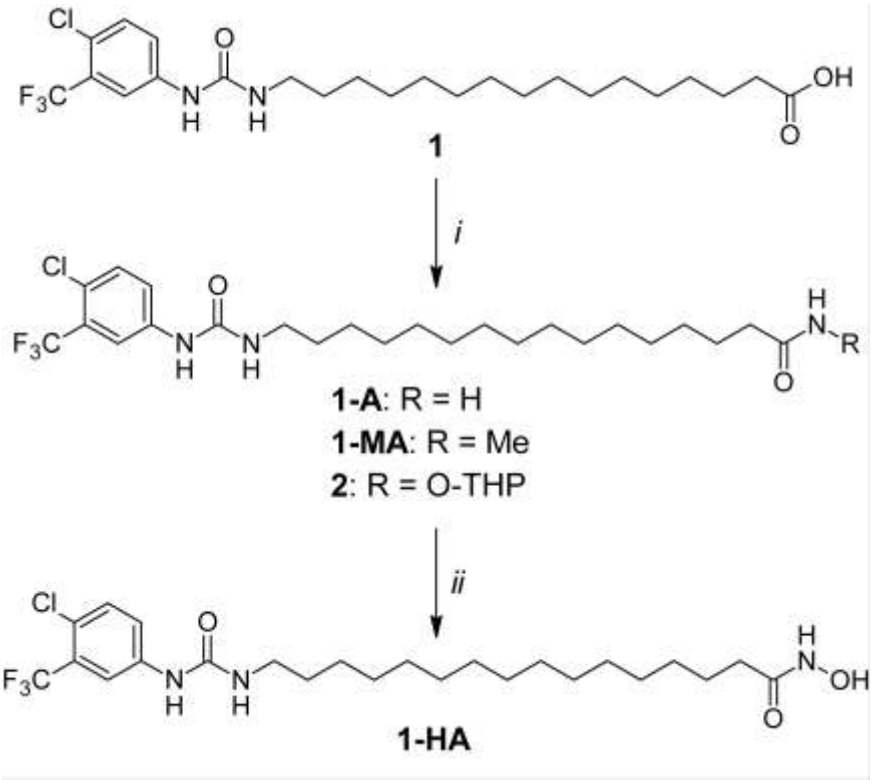
Keywords: antitumor agents, apoptosis, breast cancer, fatty acids, lipid drugs

INTRODUCTION

Agents that kill cancer cells by novel mechanisms are required to develop new anti-cancer therapeutics and to induce further remissions in cancer patients who are resistant to established agents. The tumor cell mitochondrion is functionally distinct from mitochondria in normal cells and is a promising target for anti-cancer drug development because it regulates both apoptotic cell death and the synthesis of macromolecules that are required for the rapid proliferation of tumor cells.[1]

Polyunsaturated fatty acids (PUFAs) are metabolised in cells to diverse lipid mediators, including prostaglandins, leukotrienes and epoxides, and secondary biotransformation products, that are important modulators of homeostasis.[2] While cytochrome P450 (CYP) enzymes have been most widely studied for their roles in the biotransformation of drugs, natural products and other xenobiotics,[3-6] several are also active in PUFA oxidation. CYP-derived epoxyeicosatrienoic acids (or EETs) are formed from the ω-6 PUFA arachidonic acid and promote tumorigenesis,[7] while a number of their ω-3 PUFA-derived counterparts are anti-tumorigenic.[8,9] Thus, we found that ω-3-17,18-epoxyeicosapentaenoic acid and its fully-saturated analogue impaired cell viability by decreasing proliferation and activating apoptosis.[8,10] Others have reported that docosahexaenoic acid epoxides prevent tumor progression.[9] However, unless coadministered with epoxide hydrolase inhibitors, the instability of ω-3 epoxides *in vivo*, due to rapid deactivation by soluble epoxide hydrolase, prevents their use as anti-cancer agents.[9,11] Replacement of the epoxide moiety with urea has been used to generate mimetics of ω-3[12] and ω-6[13,14] epoxides. We recently reported the development of a ω-terminal aryl-urea analogue of ω-3-17,18-epoxyeicosatetraenoic acid (termed **1;** see Scheme 1 for structure) that targeted the mitochondrion in MDA-MB-231 breast cancer cells to activate apoptotic cell death.[15]

1 is a new lead compound with promising anti-cancer activity, but we are yet to determine which pharmacophoric groups in its structure are essential for activity. The present study was designed to investigate the importance of the carboxylic acid functionality on anti-cancer activity in human MDA-MB-231 cells by preparing **1** analogues in which the carboxylate group was replaced with non-bioisosteric amide functionalities (**1-A** and **1-MA**; Scheme 1). We also prepared **1** analogues containing carboxylate bioisosteres from different structural classes. Thus, the hydroxamic acid (**1-HA**; Scheme 1), the planar heterocyclic oxo-thiadiazole (**1-OT**) and the non-planar sulfur-derived sulfonate (**1-SA**; Scheme 2) isosteres were evaluated. The principal finding to emerge was that this region was an important determinant of the potency and kinetics of the activity of **1** analogues in tumor cells; the analogues exhibited varying ability to target the mitochondrion and elicit cell death.

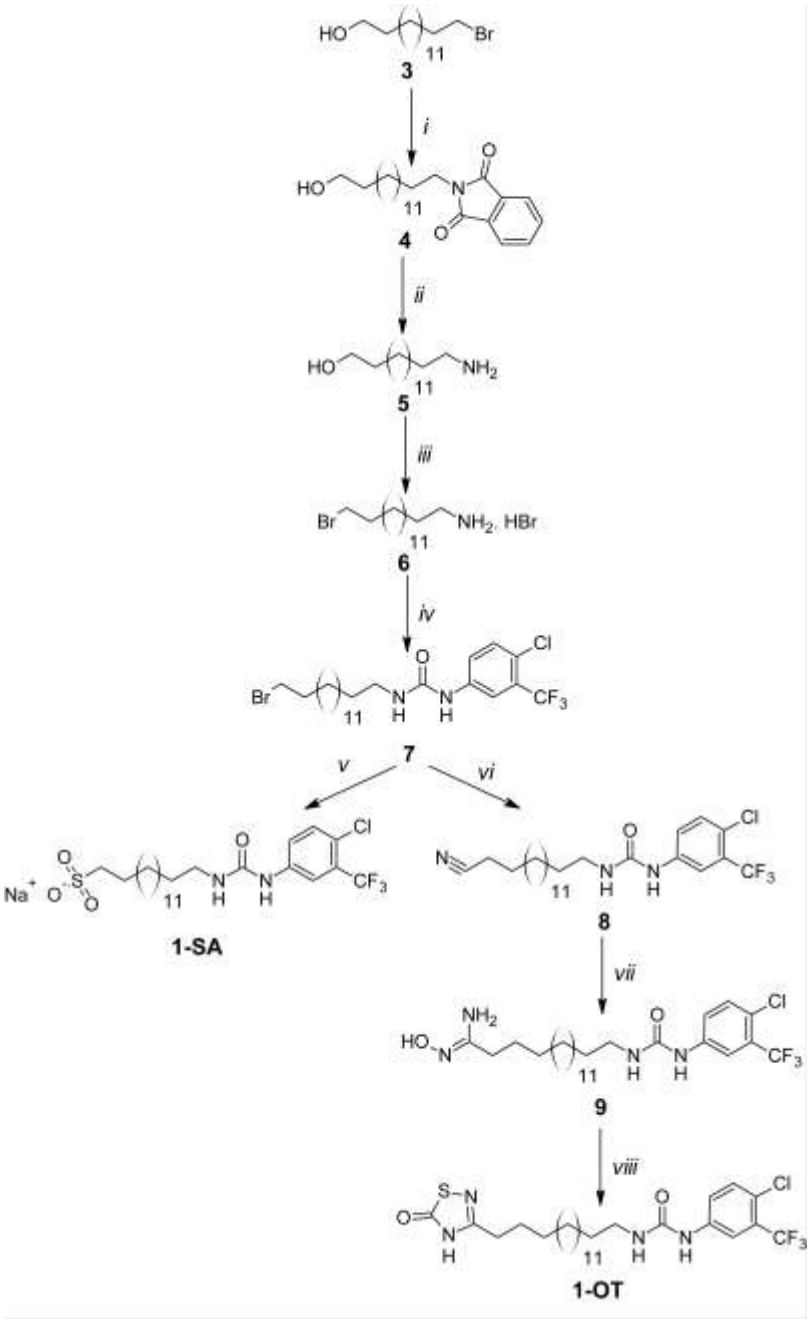


Scheme 1: Synthesis of the amide analogues **1-A** and **1-MA**, and the hydroxamic acid analogue **1-HA**. Reagents and conditions: (i) EDCI, HOBT and NMM, and then either ammonium chloride or methylammonium chloride (anhydrous DMF, rt, 18 h), (ii) 1M HCl, methanol, rt, 6 h.

RESULTS

Synthesis of aryl-urea carboxylate analogues. The amide derivatives **1-A** and **1-MA** were prepared by reactions of **1** with ammonium chloride or methyl ammonium chloride, respectively, using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI) as a coupling reagent to form the amide bonds (Scheme 1). At completion of both reactions water was added to precipitate the products, which were isolated by filtration. The hydroxamic acid **1-HA** was also synthesised from **1** using EDCI coupling (Scheme 1).[16] In the first step **1** was reacted with *O*-(tetrahydro-2H-pyran-2-yl)-hydroxylamine to yield **2**, a tetrahydropyranyl ether-protected intermediate of **1-HA**.[17] Deprotection of **2** was achieved using hydrochloric acid in methanol, yielding **1-HA** as a precipitate that was isolated by filtration and purified by trituration with methanol/water (50:50), followed by acetone.

The key intermediate in the synthesis of **1-SA** and **1-OT** was the bromourea **7**, which was prepared using an adaptation of the procedure of Miwa *et al.* (Scheme 2).[18] 15-Bromopentadecan-1-ol (**3**) was refluxed with potassium phthalimide to produce the intermediate **4** that was hydrolysed by refluxing with hydrazine to generate the primary amine **5**. The alcohol group in **5** was converted to a bromide using concentrated hydrobromic acid, yielding **6** as the hydrobromide salt that was purified by recrystallisation in acetone. The bromourea **7** was obtained by reacting **6** with 4-chloro, 3-(trifluoromethyl)phenylisocyanate in the presence of triethylamine, which served as a base to generate the free amine from the hydrobromide salt of **6**.

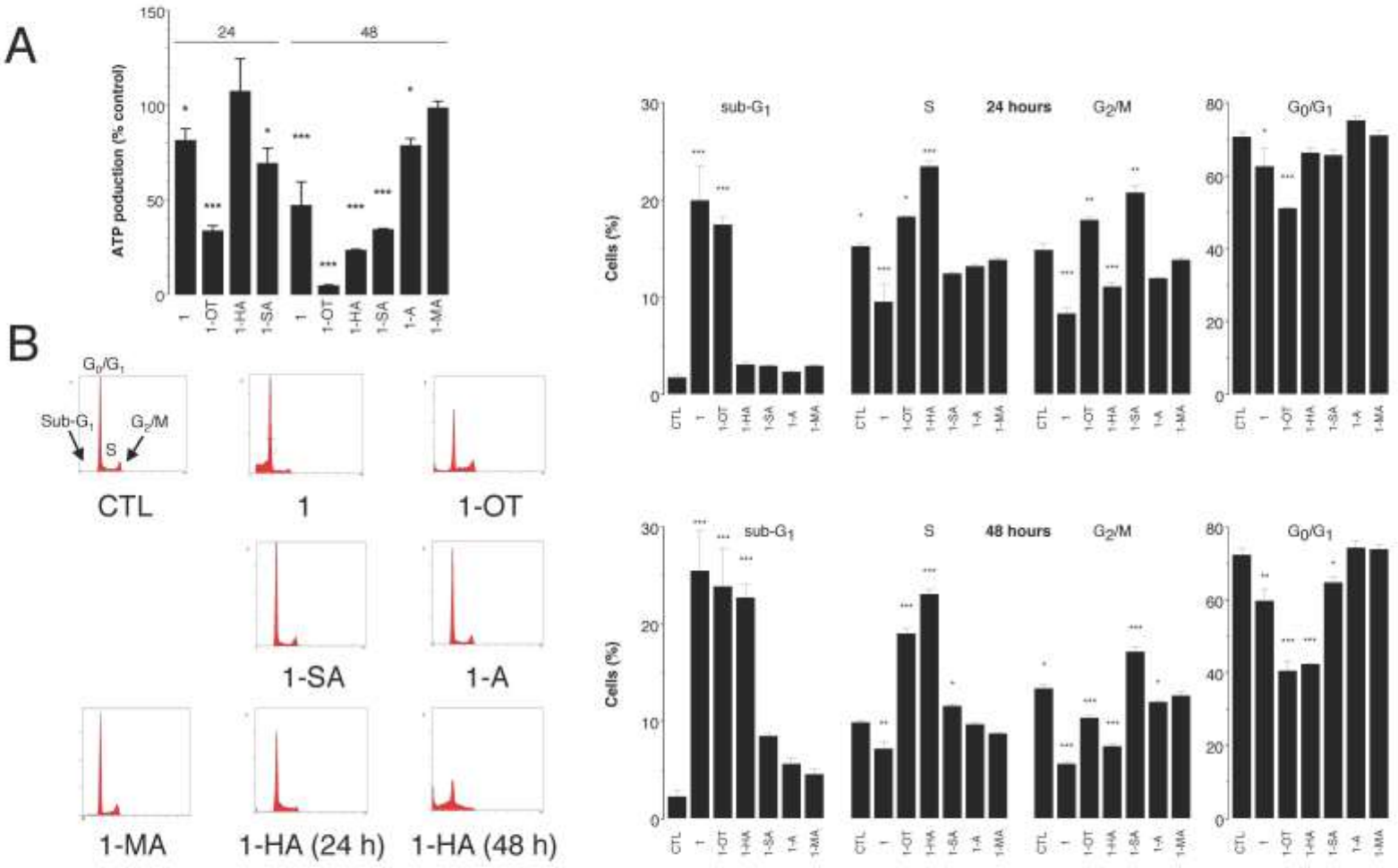


Scheme 2: Synthesis of oxo-thiadiazole (**1-OT**) and sulfonic acid (**1-SA**) isosteres. Reagents and conditions: (i) potassium phthalimide, anhydrous DMF, N_2, reflux, 18 h; (ii) hydrazine, THF, reflux, 18 h; (iii) HBr, reflux, 18 h; (iv) 4-chloro-3-(trifluoromethyl)phenyl isocyanate, triethylamine, anhydrous THF, N_2, rt, 4 h; (v) sodium sulfite, ethanol/water (3:2), reflux, 3 d; (vi) NaCN, 40°C, DMSO, 24 h; (vii) $HONH_2$, methanol, reflux, 18 h; (viii) a. 1,1′-thiocarbonyldiimidazole, anhydrous THF, rt, 2 h; b. diazabicycloundec-7-ene, ACN, 90°C to rt, 18 h.

The sulfonic acid derivative **1-SA** was prepared by refluxing bromourea **7** with sodium sulfite in aqueous ethanol for three days. The oxo-thiadiazole **1-OT** was obtained by reacting **7** with sodium cyanide in DMSO and the resultant nitrile **8** was then treated with aqueous hydroxylamine solution to generate the amidoxime **9**. Subsequently, **9** was reacted with 1,1′-thiocarbonyldiimidazole in acetonitrile to form the oxo-thiadiazole ring and yield **1-OT**.[19]

Impaired ATP production by analogues of 1 in breast cancer cells. Treatment of triple-negative MDA-MB-231 breast cancer cells with **1-OT** and **1-SA** (10 μM, 24 h) decreased ATP production to 33±4% ($P<0.001$) and 69±8% ($P<0.05$) of control, respectively. In comparison, **1** decreased ATP production to 72±14% of control ($P<0.05$; Figure 1A). Longer term treatment for 48 h produced more pronounced effects. Thus, **1-OT** strongly decreased ATP production to 5±1% of control ($P<0.001$), while the activities of **1** and **1-SA** were also greater (Figure 1A). Interestingly, the hydroxamic acid derivative **1-HA** was highly active after 48 h of treatment despite its low activity at 24 h, while **1-A** and **1-MA** exerted minimal effects on ATP production even after 48 h of treatment.

Figure 1 (below): (A) Effect of **1** analogues (10 μM) on ATP formation by MDA-MB-231 cells after 24 and 48 h of treatment; (B) Flow cytometric analysis of MDA-MB-231 cells after treatment with **1** analogues (10 μM) for 24 and 48 h. Areas in histograms corresponding to sub-G_1, G_0/G_1, S and G_2/M phases are shown in control (CTL) cells. Histograms were obtained after 24 h unless indicated otherwise. All data are mean±SEM from three separate experiments. Different from DMSO-treated control: ***$P<0.001$, **$P<0.01$, *$P<0.05$.

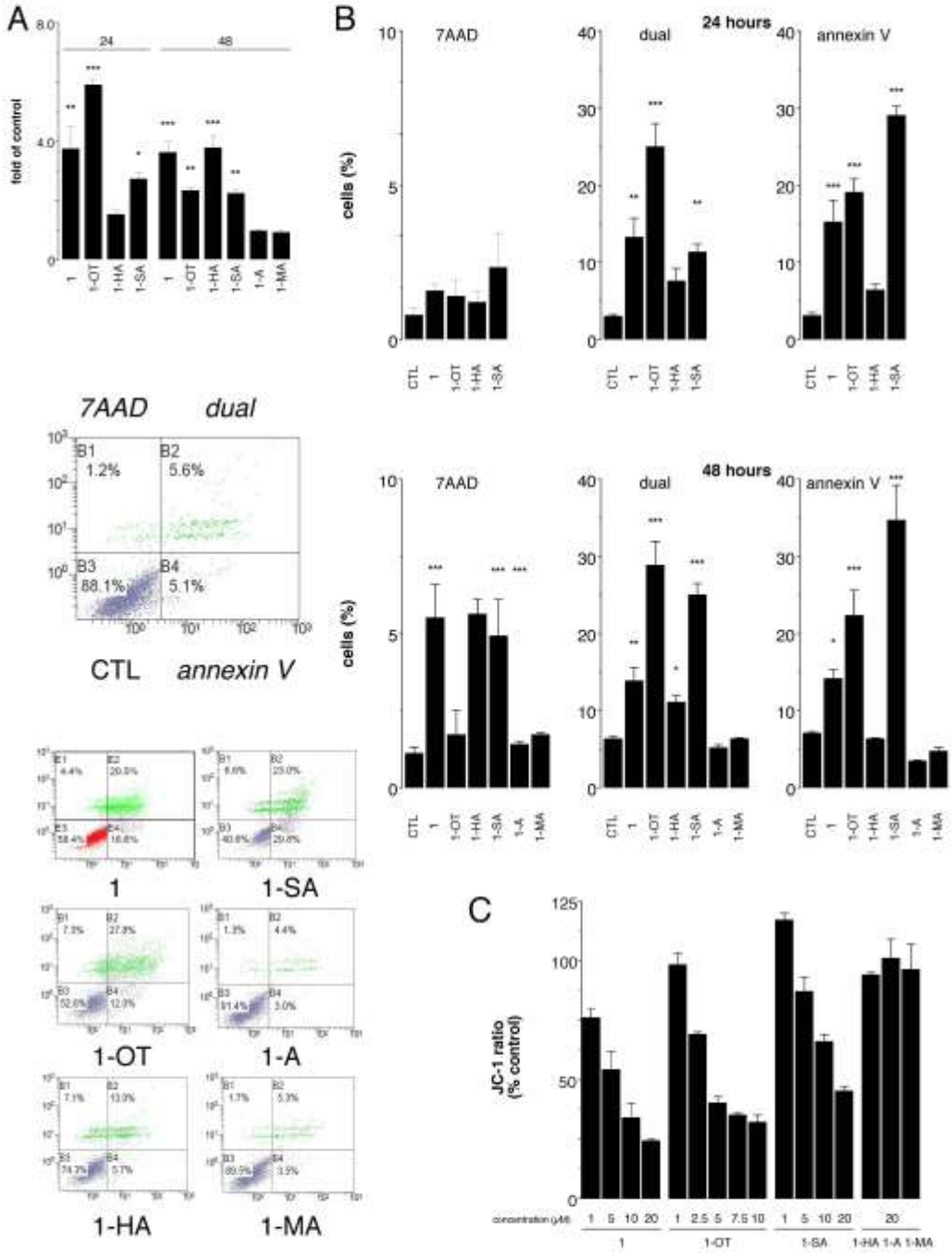

A
ATP poduction (% control)
24
48
1
1-OT
1-HA
1-SA
1-A
1-MA
B
G0/G1
Sub-G1
S
G2/M
CTL
1
1-OT
1-SA
1-A
1-MA
1-HA (24 h)
1-HA (48 h)
sub-G1
S
G2/M
G0/G1
24 hours
48 hours
Cells (%)

To corroborate the effects on ATP production, the impact of the **1** analogues on cell cycle kinetics was investigated by flow cytometry. After 24 h of treatment **1** and **1-OT** (10 μM) strongly increased the proportion of cells in sub-G_1 phase to ~10-fold of control and altered the cell populations in S and/or G_2/M phase (Figure 1B); these changes were more pronounced after 48 h. **1-SA** produced a trend toward increased sub-G_1 content to 1.8 and ~4-fold of control at 24 and 48 h, respectively, and increased the accumulation of cells in G_2/M phase. Treatment of cells with **1-HA** altered the cell cycle distribution at both 24 and 48 h, whereas treatment for 48 h was required to increase sub-G_1 phase (Figure 1B). Together these findings indicate that the carboxylate analogues of **1** - namely **1-OT**, **1-HA** and **1-SA** - impaired the viability of MDA-MB-231 cells by decreasing energy production and cell cycle progression. In contrast, **1-A** and **1-MA** (10 μM) did not markedly alter the cell cycle distribution from control after 24 and 48 h treatment (Figure 2B).

Activation of apoptosis by aryl-ureas. The increase in sub-G_1 phase elicited by several analogues implicated apoptotic cell death in the mechanism of cell impairment. Accordingly, we further assessed the capacity of the most effective analogues to activate apoptosis in MDA-MB-231 cells by measuring the activity of the executioner caspases-3/7 (Figure 2A). After 24 h, **1** (10 μM) increased caspase-3/7 activity to 3.7-fold of control ($P<0.01$). In comparison, **1-OT** (10 μM) increased caspase activity to ~5.9-fold of control at 24 h ($P<0.001$), while **1-SA** (10 μM) produced a lesser increase to 2.7-fold of control ($P<0.05$). At 48 h the activation by **1-SA** seen at 24 h was sustained, while **1-HA** also exhibited activity (~3.5-fold of control). In contrast, the marked increase produced by **1-OT** by 24 h of treatment was decreased in magnitude to ~2.3-fold of control by 48 h. The capacity of **1** and its analogues to increase caspase-3 activity was also assessed in non-cancerous MCF10A cells, which are used commonly as a control breast cell line. None of the analogues significantly increased caspase-3 activity when tested at a concentration of 10 μM.

To further substantiate the capacity of the active analogues to elicit cell death, MDA-MB-231 cells were subjected to annexin V-FITC/7-aminoactinomycin D (annexin V/7AAD) staining (Figure 2B). Strong increases in dual-stained and annexin V-stained cells after 48 h of treatment are consistent with increased late and early apoptosis produced by **1, 1-OT** and **1-SA** to ~4-5-fold of corresponding control. In contrast, **1-HA** strongly increased 7AAD-staining and, to a lesser extent, dual staining to ~2-fold of control, while annexin V staining was essentially unchanged from control. These findings suggest that necrosis may be an important death process in the case of that analogue. However, after 24 h of treatment **1-HA** did not activate cell death. In view of these time-dependent effects of **1-HA** on cell viability, we also tested the other active agents at 24 h. Similar changes to those at 48 h were also noted at 24 h with **1** and **1-SA**, but **1-OT** effected a pronounced increase in dual-stained cells to ~11-fold of control that decreased by 48 h, consistent with findings from caspase-3/7 assays (Figure 2A) and suggesting that extensive cell deletion occurred rapidly with that analogue.

Figure 2 (below): (A) Effect of **1** analogues (10 μM) on caspase-3/7 activity in MDA-MB-231 cells after 24 and 48 h of treatment; (B) annexin V-FITC/7AAD staining in MDA-MB-231 cells after treatment with **1** analogues (10 μM) for 24 or 48 h. Areas corresponding to 7AAD-stained cells (upper left quadrant), annexin V-stained cells (lower right quadrant), dual 7AAD and annexin V-stained cells (upper right quadrant) and unstained live cells (lower left quadrant) are shown in control (CTL) cells. Images showing cell distributions were obtained after 24 h of treatment; (C) JC-1 ratios in MDA-MB-231 cells treated with various concentrations of **1** analogues. All data are mean±SEM from three separate experiments. Different from DMSO-treated control: *P<0.001, **P<0.01, *P<0.05.


A
fold of control
24
48
1
1-OT
1-HA
1-SA
1-A
1-MA
B
7AAD
dual
annexin V
24 hours
48 hours
cells (%)
CTL
7AAD dual
CTL annexin V
B1 1.2%
B2 5.6%
B3 88.1%
B4 5.1%
1
1-SA
1-OT
1-A
1-HA
1-MA
C
JC-1 ratio (% control)
concentration (μM)
1 5 10 20
1 2.5 5 7.5 10
1 5 10 20
20
1
1-OT
1-SA
1-HA 1-A 1-MA

Aryl urea analogues target the mitochondrial membrane in MDA-MB-231 cells. 1 has been found previously to target the mitochondrion in MDA-MB-231 cells.[15] We assessed whether the present carboxylate-modified aryl-urea fatty acids also effected mitochondrial disruption using the membrane-permeable redox-active cationic dye JC-1. JC-1 forms aggregates that fluoresce red in the electronegative environment of the intact inner mitochondrial membrane. In damaged mitochondria that have lost their membrane potential, JC-1 remains in the cytoplasm in its monomeric form, which fluoresces green. As expected, **1** treatment (1 h) rapidly decreased the red:green fluorescence ratio in JC-1 stained cells in a concentration-dependent fashion (IC_{50} ~4.7 μM; Figure 2C). **1-OT** was similarly effective (IC_{50} ~4.6 μM) and **1-SA** also targeted the mitochondrion, but was less effective than **1** or **1-OT** (IC_{50} ~19 μM). Neither **1-HA** or the inactive amides **1-A** and **1-MA** altered the JC-1 red:green ratio over the concentration range 1-20 μM at 1 h (Figure 2C). In further studies, longer-term treatment with **1-HA, 1-A** and **1-MA** for up to 24 h also minimally altered the JC-1 ratio (decreases to 81-89% of control at a test concentration of 20 μM; data not shown).

DISCUSSION

The development of well-tolerated anti-cancer agents with novel mechanisms of action could provide valuable options in the treatment of cancer patients. The long-chain fatty acid derivative **1** was recently identified as the first in a new class of potential anti-cancer agents with activity against MDA-MB-231 breast cancer cells.[19] The present study assessed the importance of the carboxylic acid group in the activity of **1**. Replacement of this moiety with non-bioisosteric amide groups found in **1-A** and **1-MA** led to inactive compounds, indicating that the carboxylate group is required for activity. Consistent with the carboxylate being part of the pharmacophore, the carboxylate bioisosteres **1-OT**, **1-HA** and **1-SA** were active and strongly decreased the viability of MDA-MB-231 breast cancer cells.

The most active analogues (**1-OT**, **1-HA** and **1-SA**) decreased cellular energy production as ATP. Arrest of cell cycle progression by the active analogues is consistent with decreased energy metabolism, and the accumulation of cells in S and/or G_2M phases is consistent with prevention of DNA replication and cell proliferation. After 48 h of treatment **1, 1-OT, 1-HA** and **1-SA** also activated cell death, as reflected by the increases in the proportion of cells in sub-G_1 phase, caspase-3/7 activity and annexin V/7AAD staining. The increase in annexin V-FITC/7AAD staining by active carboxylate modified analogues of **1** suggests that cell killing is mediated by the dual mechanisms of apoptosis and necrosis. Apoptosis appears to be the major death mechanism that is initially activated by **1, 1-OT** and **1-SA** in treated cells. Subsequent disruption of the plasma membrane following prolonged treatment with active analogues reflects the activation of necrosis and enables DNA staining by 7AAD. Similar to aryl urea **1**, the analogues **1-OT** and **1-SA** induce cell death by collapsing the mitochondrial membrane potential, as reflected by a shift in the JC-1 red:green fluorescence ratio of cells following treatments. **1-HA** did not alter the JC-1 ratio over the 24 h timeframe of these experiments, which is consistent with a delayed onset of action that was also noted in other

assays. Taken together, the carboxylic acid functional group can be replaced with several moieties to retain the anti-proliferative and pro-apoptotic activity of **1** in MDA-MB-231 breast cancer cells.

The activity of **1-OT** suggests that planar heterocyclic moieties may also be effective carboxylate isosteres. We attempted to prepare a tetrazole-containing bioisostere of **1** but were unable to synthesise this compound from bromourea **7** using established approaches. Interestingly, the hydroxamic acid analogue **1-HA** displayed different kinetics to **1**, **1-SA** and **1-OT**, and required 48 h to produce extensive loss of cell viability. Unlike the other active agents evaluated in this study, **1-HA** did not disrupt the mitochondrial membrane potential and appeared to activate necrotic death in a delayed fashion, rather than by the rapid activation of apoptosis produced by other analogues. Our ongoing studies to identify the cellular target(s) of **1** analogues may eventually assist with a more complete interpretation of the present findings.

In cancer cells the mitochondrion could contain novel drug targets because it is structurally and functionally different from the organelle in normal cells.[1] Whereas oxidative phosphorylation is the primary pathway by which ATP is generated in normal cells, aerobic glycolysis is more important in highly aggressive tumors cells (the Warburg effect).[20] Instead, the cancer cell mitochondrion is involved in the increased production of the macromolecules that are necessary for tumor cell replication.[1] The mitochondrion is also a critical regulator of apoptotic cell death and in cancer cells these pathways are frequently less responsive to cytotoxic agents. Thus, the development of novel agents that selectively damage the mitochondrion in tumor cells could lead to the development of new therapeutic strategies.[2,23] Recently **1** emerged as the first in a new class of agents with activity against cancer cells produced by rapid targeting of the tumor cell mitochondrion. It now emerges that the analogues **1-OT** and **1-SA**, but not the amides **1-A** and **1-MA**, also disrupt the mitochondrion to promote extensive tumor cell destruction.

CONCLUSION

In summary, we have prepared analogues of the novel anticancer agent **1** in which the carboxylate group was modified with bioisosteric and non-bioisosteric groups. Substitutions with bioisosteric groups produced active analogues. Like **1** the oxo-thiadiazole and the sulfonic acid derivatives **1-OT** and **1-SA** effectively disrupted the mitochondrial potential and promoted cell death, while the hydroxamic derivative **1-HA** had a delayed onset of action relative to **1,** which appeared to be unrelated to mitochondrial targeting. The inactivity of the non-bioisosteric amide derivatives **1-A** and **1-MA** indicates that the carboxylate group of **1** is part of the pharmacophore.

EXPERIMENTAL SECTION

Chemistry.

General: All reagents and anhydrous solvents were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Dry Column Vacuum Chromatography was used to purify reaction products on silica gel with gradient elutions. TLC was performed on silica gel 60 F_{254} plates. Melting points were measured on a Stuart SMP10 melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded on an Agilent 500 MHz NMR. Spectra were referenced internally to residual solvent ($CDCl_3$; ^{1}H δ 7.26, ^{13}C δ 77.10. *d*$_6$-DMSO; ^{1}H δ 2.49, ^{13}C δ 39.52). High resolution mass spectrometry (HRMS) was performed on an Agilent Technologies 6510 Q-TOF LCMS.

General procedure for synthesis of amides 1-A and 1-MA: To a solution of **1** (0.150 g, 0.304 mmol) in anhydrous DMF (10 mL) under nitrogen were added 1-hydroxybenzotriazole hydrate (0.060 g, 0.365 mmol), NMM (0.100 mL, 0.912 mmol) and EDCI (0.082 g, 0.426 mmol). The solution was stirred at room temperature for 30 min, then NH_4Cl or methylamine HCl (0.942 mmol) was added and stirring was continued for 18 h. Distilled water (10 mL) was added, and the solid collected by filtration, washed with water and diethyl ether, affording the amide as a white solid.

16-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]hexadecanamide (1-A): White solid, yield 30%. Mp = 129-132°C. ^{1}H NMR (500 MHz, DMSO-*d*$_6$): δ 8.88 (s, 1H), 8.03 (s, 1H), 7.52-7.50 (m, 2H), 7.18 (b, 1H), 6.63 (b, 1H), 6.27 (t, J_{HH}=7.5 Hz, 1H), 3.04 (q, J_{HH}=7.0 Hz, 2H), 1.98 (t, J_{HH}=6.5 Hz, 2H), 1.45-1.39 (m, 4H), 1.24-1.20 (m, 22H). ^{13}C NMR (125 MHz, DMSO-*d*$_6$): δ 177.39, 157.95, 143.35, 134.91, 129.78 (d, J_{CF}=30.0 Hz), 127.08 (d, J_{CF}=271.2 Hz), 125.32, 124.28, 119.11 (d, J_{CF}=5.2 Hz), 38.22, 32.68, 32.16, 32.15, 32.11,

32.09, 32.06, 31.92, 31.83, 29.41, 28.21. HRMS (ESI) *(m/z):* calcd for $C_{24}H_{38}ClF_3N_3O_2$ $[M+H]^+$ 492.2599, found 492.2599.

16-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]-N-methyl-hexadecanamide (1-MA): White solid, yield 41%. Mp = 133-135°C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.88 (s, 1H), 8.04 (d, J_{HH}=2.5 Hz, 1H), 7.63 (b, 1H), 7.53-7.49 (m, 2H), 6.29 (t, J_{HH}=5.5 Hz, 1H), 3.06 (q, J_{HH}=7.0 Hz, 2H), 2.68 (s, 3H), 2.01 (t, J_{HH}=7.5 Hz, 2H), 1.45-1.38 (m, 4H), 1.24-1.20 (m, 22H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 209.60, 175.58, 157.96, 143.35, 134.91, 129.77 (d, J_{CF}=30.0 Hz, 1C), 127.06 (d, J_{CF}=271.2 Hz, 1C), 125.32, 124.27, 119.16, 38.44, 33.80, 32.68, 32.16, 32.14, 32.12, 32.00, 32.03, 31.89, 31.83, 31.81, 29.41, 28.49, 28.37. HRMS (ESI) *(m/z):* calcd for $C_{25}H_{40}ClF_3N_3O_2$ $[M+H]^+$ 506.2755, found 506.2756.

Preparation of 16-({[4-chloro-3-(trifluoromethyl)phenyl]carbamoyl}amino)-N-(tetrahydro-2H-pyran-2-yloxy)hexadecanamide (2). To a solution of **1** (0.150 g, 0.304 mmol) in anhydrous DMF (10 mL) under nitrogen were added 1-hydroxybenzotriazole hydrate (0.060 g, 0.365 mmol), and NMM (0.100 mL, 0.912 mmol). The solution was stirred at room temperature for 30 minutes, then *O*-tetrahydro-2-H-pyran-2-yl-hydroxyamine (0.110 g, 0.942 mmol) was added and stirring was continued for 18 h. The resulting mixture was diluted with water (80 mL) and extracted with chloroform (3×30 mL). The combined organic layer was washed with water (3×100 mL), saturated aqueous sodium bicarbonate ($NaHCO_3$, 30 mL), and brine (30 mL) and then concentrated in vacuo. The crude product was purified on silica gel by stepwise gradient elution with dichloromethane/isopropanol (95:5 to 80:20), affording **2** as a white solid (0.120 g, 67%). Mp = 116-118°C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.85 (s, 1H), 8.88 (s, 1H), 8.05 (d, J_{HH}=2.0 Hz, 1H), 7.54-7.50 (m, 2H), 6.29 (t, J_{HH}=5.0 Hz, 1H), 4.77 (s, 1H), 3.92-3.87 (m, 1H), 3.48-3.46 (m, 1H), 3.08 (q, J_{HH}=6.5 Hz, 2H), 1.96 (t, J_{HH}=7.0 Hz, 2H), 1.65-1.59 (m, 3H), 1.51-1.39 (m, 7H), 1.25-1.21 (m, 22H). ^{13}C NMR (125 MHz, DMSO-d_6):

δ 172.16, 157.95, 143.34, 134.93, 129.78 (d, J_{FC}=30.0 Hz), 127.07 (d, J_{CF}=271.2 Hz), 125.32, 124.27, 119.14 (d, J_{CF}=5.8 Hz), 103.25, 64.38, 35.27, 32.69, 32.16, 32.11, 32.04, 31.85, 31.56, 30.90, 29.42, 28.06, 27.80, 21.42. HRMS (ESI) *(m/z):* calcd for $C_{29}H_{46}ClF_3N_3O_4$ $[M+H]^+$ 592.3123, found 592.3123.

Preparation of 16-({[4-chloro-3-(trifluoromethyl)phenyl]carbamoyl}amino)-N-hydroxyhexadecanamide (1-HA). To a solution of **2** (0.090 g, 0.152 mmol) in methanol (10 mL) was added 1M HCl (1 mL) dropwise. The suspension was stirred for 6 h at room temperature, and then concentrated in vacuo. The off-white solid was triturated with methanol/water (50:50) followed by acetone and dried under vacuum to yield **1-HA** as an off-white solid (0.062 g, 80%). Mp = 114-117°C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.31 (s, 1H), 8.90 (s, 1H), 8.64 (d, J_{HH}=1.5 Hz, 1H), 8.05 (d, J_{HH}=2.0 Hz, 1H), 7.53 (m, 2H), 6.31 (t, J_{HH}=6.0 Hz, 1H), 3.07 (q, J_{HH}=6.5 Hz, 2H), 1.92 (t, J_{HH}=7.0 Hz, 2H), 1.45-1.39 (m, 4H), 1.24-1.21 (m, 22H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.21, 157.95, 143.34, 134.93, 130.12 (d, J_{CF}=30.0 Hz), 127.07 (d, J_{CF}=271.2 Hz), 125.32, 124.27, 119.09 (d, J_{CF}=5.8 Hz), 35.36, 32.69, 32.17, 31.85, 31.70, 29.42, 28.23. HRMS (ESI) *(m/z):* calcd for $C_{24}H_{38}ClF_3N_3O_3$ $[M+H]^+$ 508.2548, found 508.2547.

Preparation of 2-(15-hydroxypentadecyl)-1H-isoindole-1,3(2H)-dione (4). 15-bromo-1-pentadecanol (**3**, 3.000 g, 9.77 mmol) and potassium phthalimide (2.352 g, 12.70 mmol) were dissolved in anhydrous DMF (50 mL) under a nitrogen atmosphere. The solution was refluxed for 18 h and cooled to room temperature. The resulting mixture was filtered and the filter bed was washed with diethyl ether (50 mL). The filtrate was washed with distilled water (3×200 mL) and brine (2×100 mL), and then concentrated under reduced pressure. The crude product was purified on silica gel by stepwise gradient elution with dichloromethane/ethyl acetate (95:5

to 70:30), yielding **4** as a white solid (2.230 g, 61%). Mp = 105-109°C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.84 (dd, J_{HH}=5.5, 3.5 Hz, 2H), 7.71 (dd, J_{HH}=5.5, 3.5 Hz, 2H), 3.68-3.61 (m, 4H), 1.67 (p, J_{HH}=7.0 Hz, 2H), 1.58 (p, J_{HH}=8.0 Hz, 2H), 1.32-1.24 (m, 22H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 209.59, 171.14, 136.47, 134.85, 125.79, 65.76, 40.74, 35.47, 33.57, 32.26, 32.25, 32.23, 32.21, 32.19, 32.11, 32.07, 31.83, 31.25, 29.52, 28.38. HRMS (ESI) *(m/z):* calcd for $C_{23}H_{36}NO_3$ $[M+H]^+$ 374.2689, found 374.2690.

Preparation of 15-aminopentadecan-1-ol (5). To a solution of **4** (2.230 g, 5.97 mmol) in anhydrous tetrahydrofuran (THF, 70 mL) under a nitrogen atmosphere was added hydrazine monohydrate (7 mL, 95 mmol). The resulting mixture was refluxed for 18 h and cooled to room temperature. The solvent and the excess of hydrazine were removed under reduced pressure. The crude product was extracted with dichloromethane (200 mL) and aqueous sodium hydroxide (0.5M, 200 mL). The organic layer was separated and washed with aqueous sodium hydroxide (1M, 100 mL) and distilled water (2×100 mL), and then concentrated in vacuo, yielding **5** as an off-white solid (1.410 g, 96%). Mp = 58-60°C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.59 (t, J_{HH}=6.5 Hz, 2H), 2.62 (t, J_{HH}=7.0 Hz, 2H), 1.51 (p, J_{HH}=7.5 Hz, 2H), 1.38 (p, J_{HH}=7.0 Hz, 2H), 1.29-1.19 (m, 22H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 65.65, 44.93, 36.52, 35.49, 32.24, 32.22, 32.19, 32.13, 32.06, 29.53, 28.40. HRMS (ESI) *(m/z):* calcd for $C_{15}H_{34}NO$ $[M+H]^+$ 244.2634, found 244.2634.

Preparation of 15-bromopentadecan-1-aminium bromide (6). A solution of **5** (1.410 g, 5.8 mmol) and hydrobromic acid 48% (70 mL) was refluxed for 18 h. The mixture was concentrated in vacuo and then the residue was recrystallized twice with hot acetone to afford **6** as an off-white solid (1.611 g, 72%). Mp = 157-159°C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.00 (b, 2H), 3.42 (t, J_{HH}=6.5 Hz, 2H), 3.02 (t, J_{HH}=6.5 Hz, 2H), 1.87 (p, J_{HH}=7.5 Hz, 2H), 1.82 (p,

J_{HH}=7.5 Hz, 2H), 1.41 (p, J_{HH}=7.5 Hz, 2H), 1.29-1.25 (m, 20H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 62.01, 41.24, 32.83, 31.81, 28.69, 28.56, 28.54, 28.51, 28.45, 28.38, 25.86, 24.71. HRMS (ESI) *(m/z):* calcd for $C_{15}H_{34}Br_2N$ $[M+H]^+$ 306.1791, found 306.1792.

Preparation of 1-(15-bromopentadecyl)-3-[4-chloro-3-(trifluoromethyl)phenyl]urea (7). To a suspension of **6** (2.000 g, 5.16 mmol) in anhydrous THF (40 mL) under nitrogen at room temperature were added 4-chloro-3-(trifluoromethyl)phenyl isocyanate (1.372 g, 6.19 mmol) and triethylamine (0.720 mL, 5.16 mmol). The mixture was stirred for 4 h, and then concentrated under reduced pressure. The residue was purified on silica gel by stepwise gradient elution with petroleum/ethyl acetate (90:10 to 20:80) to afford **7** as a white solid (2.470 g, 91%). Mp = 88-92°C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.63 (d, J_{HH}=1.5 Hz, 1H), 7.54 (dd, J_{HH}=8.5, 2.0 Hz, 1H), 7.38 (d, J_{HH}=9.0 Hz, 1H), 6.70 (b, 1H), 4.84 (t, J_{HH}=5.5 Hz, 1H), 3.43 (t, J_{HH}=6.5 Hz, 2H), 3.26 (q, J_{HH}=7.0 Hz, 2H), 1.87 (p, J_{HH}=7.5 Hz, 2H), 1.53 (p, J_{HH}=7.0 Hz, 2H), 1.42 (p, J_{HH}=7.0 Hz, 2H), 1.28-1.24 (m, 20H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 157.48, 134.69, 133.64, 131.59 (d, J_{CF}=31.0 Hz), 128.44 (d, J_{CF}=248.0 Hz), 126.31, 124.13, 121.23, 43.33, 36.75, 35.49, 32.62, 32.56, 32.26, 32.25, 32.21, 32.19, 32.17, 32.08, 31.93, 31.41, 30.82, 29.51. HRMS (ESI) *(m/z):* calcd for $C_{23}H_{36}BrClF_3N_2O$ $[M+H]^+$ 527.1646, found 527.1647.

Preparation of sodium 15-({[4-chloro-3-(trifluoromethyl)phenyl]carbamoyl}amino)pentadecane-1-sulfonate (1-SA). A solution of **7** (0.150 g, 0.284 mmol) and sodium sulfite (0.072 g, 0.568 mmol) in 3:2 ethanol/water (10 mL) was refluxed for three days and cooled to room temperature. The volume of the reaction mixture was reduced to half under pressure. The solution was adjusted to pH = 8 with aqueous sodium hydroxide (1.5M, 2 mL), and distilled water (10 mL) was added. The water was then removed by freeze-drying. The resulting solid was triturated twice with 1:1 ethanol/water, and

then diethyl ether to yield **1-SA** as a white solid (0.070 g, 45%). Mp = 185°C (decomp.). ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.21 (s, 1H), 8.08 (d, J_{HH}=2.0 Hz, 1H), 7.59 (dd, J_{HH}=9.0, 2.5 Hz, 1H), 7.51 (d, J_{HH}=8.5 Hz, 1H), 6.68 (t, J_{HH}=5.5 Hz, 1H), 3.06 (q, J_{HH}=12.5 Hz, 2H), 2.41 (t, J_{HH}=8.0 Hz, 2H), 1.56 (p, J_{HH}=8.0 Hz, 2H), 1.40 (p, J_{HH}=6.0 Hz, 2H), 1.24-1.22 (m, 22H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 158.08, 143.62, 134.85, 129.20 (d, J_{CF}=31.0 Hz), 126.03 (d, J_{CF}=271.0 Hz), 125.24, 124.07, 119.05 (d, J_{CF}=5.2 Hz), 54.68, 32.58, 32.15, 31.78, 31.71, 31.70, 31.68, 31.67, 31.57, 31.51, 31.37, 29.36, 28.11. HRMS (ESI) *(m/z):* calcd for $C_{23}H_{36}ClF_3N_2O_4S$ $[M+H]^+$ 529.2109, found 529.2108.

Preparation of 1-[4-chloro-3-(trifluoromethyl)phenyl]-3-(15-cyanopentadecyl)urea (8). To a solution of **7** (0.300 g, 0.569 mmol) in DMSO (20 mL) was added sodium cyanide (0.084 g, 1.71 mmol) at 40°C. The resulting mixture was stirred for 18 h, and then cooled to room temperature. The resulting mixture was diluted with water (50 mL) and extracted with chloroform (3×100 mL). The organic layer was separated, washed with distilled water (2×100 mL) and brine (100 mL), and then concentrated in vacuo. The off-white solid was triturated twice with water, followed by diethyl ether and dried under vacuum to yield **8** as a white solid (0.218 g, 81%). Mp = 89-93°C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.66 (d, J_{HH}=2.5 Hz, 1H), 7.57 (dd, J_{HH}=8.5, 2.5 Hz, 1H), 7.39 (d, J_{HH}=8.5 Hz, 1H), 3.26 (t, J_{HH}=7.0 Hz, 1H), 2.37 (t, J_{HH}=7.0 Hz, 2H), 1.68 (p, J_{HH}=7.0 Hz, 2H), 1.53 (p, J_{HH}=7.5 Hz, 2H), 1.45 (p, J_{HH}=8.0 Hz, 2H), 1.30-1.26 (m, 20H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 157.64, 140.63, 134.57, 131.31 (d, J_{CF}=31.3 Hz), 127.91, 125.24 (d, J_{CF}=272.2 Hz), 125.87, 122.71, 120.82 (d, J_{CF}=5.7 Hz), 43.14, 32.61, 32.06, 32.01, 31.99, 31.90, 31.88, 31.76, 31.28, 31.24, 29.49, 27.93, 19.80. HRMS (ESI) *(m/z):* calcd for $C_{24}H_{36}ClF_3N_3O$ $[M+H]^+$ 474.2493, found 474.2495.

Preparation of (1E/Z)-16-({[4-chloro-3-(trifluoromethyl)phenyl]carbamoyl}amino)-N'-hydroxy-hexadecanimidamide (9). To a solution of **8** (0.130 g, 0.274 mmol) in methanol (20

mL) was added aqueous hydroxylamine solution (50 wt %, 1.2 mL). The mixture was refluxed for 18 h, and then cooled to room temperature. The solvent was removed under reduced pressure. The off-white solid was triturated twice with water, followed by diethyl ether and dried under vacuum to yield **9** as an off-white solid (0.140 g, 97%). Mp = 98-103°C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.88 (b, 1H), 8.63 (s, 1H), 8.04 (d, J_{HH}=2.0 Hz, 1H), 7.53-7.52 (m, 2H), 6.28 (t, J_{HH}=5.5 Hz, 1H), 5.25 (b, 2H), 3.06 (q, J_{HH}=6.0 Hz, 2H), 1.92 (t, J_{HH}=6.5 Hz, 2H), 1.44-1.40 (m, 4H), 1.25-1.20 (m, 22H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 157.96, 155.94, 143.34, 134.92, 129.67 (d, J_{CF}=30.1 Hz), 125.99 (d, J_{CF}=271.2 Hz), 125.33, 124.31, 119.15 (d, J_{CF}=5.8 Hz), 82.28, 33.89, 33.81, 32.69, 32.17, 32.16, 32.12, 32.09, 31.93, 31.84, 31.76, 29.47, 29.41. HRMS (ESI) *(m/z):* calcd for $C_{24}H_{39}ClF_3N_4O_2$ $[M+H]^+$ 507.2708, found 507.2706.

Preparation of 1-[4-chloro-3-(trifluoromethyl)phenyl]-3-[15-(5-oxo-4,5-dihydro-1,2,4-thiadiazol-3-yl)pentadecyl]urea (1-OT). To a solution of **9** (0.200 g, 0.395 mmol) in anhydrous THF (15 mL) under nitrogen was added 1,1′-thiocarbonyldiimidazole (0.141 g, 0.789 mmol). The resulting mixture was stirred for 2 h, and then concentrated under reduced pressure. The crude product was dissolved in acetonitrile (20 mL) and heated to 90°C. 1,8-Diazabicycloundec-7-ene (0.25 mL, 1.580 mmol) was added to the mixture at room temperature and stirred for 18 h. The resulting mixture was diluted with water (20 mL). The solution was adjusted to pH 1 with HCl 1M, and then extracted with chloroform (3×20 mL). The organic layer was washed with water (3×100 mL) and brine (30 mL), and then concentrated in vacuo. The crude product was purified on silica gel by stepwise gradient elution with dichloromethane/isopropyl alcohol (98:2 to 80:20) yielding **1-OT** as an off-white solid (0.030 g, 14%). Mp = 80-84°C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.87 (s, 1H), 8.03 (d, J_{HH}=2.0 Hz, 1H), 7.52-7.49 (m, 2H), 6.28 (t, J_{HH}=6.0 Hz, 1H), 3.06 (q, J_{HH}=13.0 Hz, 2H), 2.56

(t, J_{HH}=7.5 Hz, 2H), 1.59 (t, J_{HH}=6.5 Hz, 2H), 1.38 (t, J_{HH}=6.5 Hz, 2H), 1.23-1.20 (m, 22H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 187.70, 162.22, 155.29, 140.67, 132.25, 127.11 (d, J_{CF}=30.3 Hz), 124.41 (d, J_{CF}=271.7 Hz), 122.66, 121.62, 116.49 (d, J_{CF}=5.3 Hz), 30.02, 29.48, 29.46, 29.45, 29.42, 29.39, 29.20, 29.16, 28.86, 28.58, 26.74, 25.58, 23.71. HRMS (ESI) *(m/z):* calcd for $C_{25}H_{37}ClF_3N_4O_2S$ $[M+H]^+$ 549.2272, found 549.2270.

Cell-based assays

General: Human MDA-MB-231 breast cancer cells were obtained from ATCC (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Thermo Fischer Scientific, Waltham, MA) and 1% penicillin/streptomycin (Invitrogen), at 37 °C in humidified 5% CO_2 in air. Confluent cells (80−90%) were harvested with trypsin/EDTA after washing in phosphate-buffered saline (PBS; Amresco, Solon, OH). Compounds were added to cells in DMSO (Sigma-Aldrich).

ATP and caspase 3/7 assays: MDA-MB-231 cells were seeded in triplicate into black-walled 96-well plates at a cell density of $1x10^4$ cells/well and 24 h later serum was removed. Cells were treated with various concentrations of the test compounds for 24 or 48 h; control cells received serum-free DMEM alone. Following treatments, ATP formation and caspase 3/7 activity was determined using commercial kits (CellTiter-Glo® luminescent cell viability assay and Caspase-Glo, respectively; Promega; Annandale, NSW, Australia).

Cell cycle kinetics: MDA-MB-231 cells were seeded at a density of $7.5x10^4$ cells/well in 12-well plates and allowed to adhere overnight. After serum starvation for 24 h, the cells were treated with test compounds (10 µM) in DMSO for 24 or 48 h; control cells received serum-free DMEM alone. Cells were trypsinized, washed with PBS and fixed overnight with cold ethanol (80%, -20°C). The cells were washed twice with PBS and resuspended in 0.1M PBS containing 0.1 mg/mL DNase-free RNase A and 0.1% NP40. The cells were incubated on ice

for 1 h with propidium iodide and analysed as described previously in a Gallios flow cytometer (Beckman Coulter Australia, Lane Cove, NSW).[10]

Annexin V/7AAD: Annexin V/7AAD staining in treated MDA-MB 231 cells (**7**.5 x 10^{4} cells/well) was assessed in 12-well plates. Twenty four h after serum removal the cells were treated with compounds (10 μM) for 24 or 48 h. Treated cells were trypsinized and washed twice with cold PBS, stained with annexin V and 7AAD for analysis in a Gallios™ flow cytometer (Beckman Coulter Australia).[15]

JC-1 assay: MDA-MB-231 cells were seeded in triplicate in 96-well plates ($1x10^{4}$ cells/well) and 24 h later serum was removed. Cells were treated with various concentrations of the test compounds for 24 or 48 h; control cells received serum-free DMEM alone. Cells were incubated with JC-1 in serum-free medium (37°C, 20 min) and the JC-1 red:green ratio was evaluated (JC-1 Mitochondrial Membrane Potential Assay Kit; Cayman Chemical, Ann Arbor, MI).

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ABBREVIATIONS USED

7AAD, 7-aminoactinomycin; CYP, cytochrome P450; EDCI, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; EET, epoxyeicosatrienoic acid; HRMS, High resolution mass spectrometry; JC-1, 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide; NMM, N-methylmorpholine; PUFA, polyunsaturated fatty acid; THF, tetrahydrofuran.

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FIGURE LEGENDS

Scheme 1. Synthesis of the amide analogues **1-A** and **1-MA**, and the hydroxamic acid analogue **1-HA**. Reagents and conditions: (i) EDCI, HOBT and NMM, and then either ammonium chloride or methylammonium chloride (anhydrous DMF, rt, 18 h), (ii) 1M HCl, methanol, rt, 6 h.

Scheme 2. Synthesis of oxo-thiadiazole (**1-OT**) and sulfonic acid (**1-SA**) isosteres. Reagents and conditions: (i) potassium phthalimide, anhydrous DMF, N_2, reflux, 18 h; (ii) hydrazine, THF, reflux, 18 h; (iii) HBr, reflux, 18 h; (iv) 4-chloro-3-(trifluoromethyl)phenyl isocyanate, triethylamine, anhydrous THF, N_2, rt, 4 h; (v) sodium sulfite, ethanol/water (3:2), reflux, 3 d; (vi) NaCN, 40°C, DMSO, 24 h; (vii) $HONH_2$, methanol, reflux, 18 h; (viii) a. 1,1′-thiocarbonyldiimidazole, anhydrous THF, rt, 2 h; b. diazabicycloundec-7-ene, ACN, 90°C to rt, 18 h.

Figure 1. (A) Effect of **1** analogues (10 µM) on ATP formation by MDA-MB-231 cells after 24 and 48 h of treatment; (B) Flow cytometric analysis of MDA-MB-231 cells after treatment with **1** analogues (10 µM) for 24 and 48 h. Areas in histograms corresponding to sub-G_1, G_0/G_1, S and G_2/M phases are shown in control (CTL) cells. Histograms were obtained after 24 h unless indicated otherwise. All data are mean±SEM from three separate experiments. Different from DMSO-treated control: ***P<0.001, **P<0.01, *P<0.05.

Figure 2. (A) Effect of **1** analogues (10 µM) on caspase-3/7 activity in MDA-MB-231 cells after 24 and 48 h of treatment; (B) annexin V-FITC/7AAD staining in MDA-MB-231 cells after treatment with **1** analogues (10 µM) for 24 or 48 h. Areas corresponding to 7AAD-stained cells (upper left quadrant), annexin V-stained cells (lower right quadrant), dual 7AAD and annexin V-stained cells (upper right quadrant) and unstained live cells (lower left quadrant) are shown in control (CTL) cells. Images showing cell distributions were obtained after 24 h of treatment; (C) JC-1 ratios in MDA-MB-231 cells treated with various concentrations of **1** analogues. All data are mean±SEM from three separate experiments. Different from DMSO-treated control: *P<0.001, **P<0.01, *P<0.05.