# Expansion of the structure-activity relationship of branched chain fatty acids: effect of unsaturation and branching group size on anticancer activity.

Ritik Roy[1], Ariane Roseblade[1], and Tristan Rawling*.

School of Mathematical and Physical Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW, 2007, Australia.

[1]Co-first authors

*Correspondence: Dr Tristan Rawling, School of Mathematical and Physical Sciences, Faculty of Science, University of Technology Sydney, Ultimo, NSW 2007, Australia. Tel: (61-2)-9514-7956, Email: Tristan.Rawling@uts.edu.au

**Key words**: branched chain fatty acid, 13-methyltetradecanoic acid, breast cancer, MCF-7, structure activity relationship

**ABSTRACT**

Branched chain fatty acids (BCFAs) are a class of fatty acid with promising anticancer activity. The BCFA 13-methyltetradecanoic acid (13-MTD) inhibits tumour growth *in vivo* without toxicity but efficacy is limited by moderate potency, a property shared by all known BCFAs. The mechanism of action of BCFAs has not been fully elucidated, and in the absence of a clearly defined target optimisation of BCFA potency must rely on structure-activity relationships. Our current understanding of the structural features that promote BCFA anticancer activity is limited by the low structural diversity of reported BCFAs. The aim of this study was to examine the effects of two new structural modifications- unsaturation and branching group size- on BCFA activity. Thus, homologous series of saturated and *cis*-Δ9 unsaturated BCFAs were synthesised bearing methyl, ethyl, propyl and butyl branching groups, and were screened *in vitro* for activity against three human cancer cell lines. Potencies of the new BCFAs were compared to 13-MTD and an unbranched monounstaurated fatty acid (MUFA) bearing a *cis*-Δ9 double bond. The principal findings to emerge were that the anticancer activity of BCFAs was adversly affected by larger branching groups but significantly improved by incorporation of a *cis*-Δ11 double bond into the BCFA alkyl chain. This study provides new structure-activity relationship insights that may be used to develop BCFAs with improved potency and therapeutic potential.

## 1. Introduction

Breast cancer is the second leading cause of cancer-relates deaths in women, and there is a critical need to develop novel anticancer drugs to treat cancers that are refractory to established anticancer agents. Accumulating evidence suggests that lipids and fatty acids possess anticancer actions that can be harnessed to develop new drugs to inhibit tumour growth, and drug design strategies to overcome their challenging physicochemical properties have yielded clinically approved drugs (Collins et al., 1993; Murray et al., 2014; Murray et al., 2015; Van & Verheij, 2013). Thus, lipids and fatty acids are viable lead compounds for cancer drug development.

Branched chain fatty acids (BCFAs) are a naturally occurring class of fatty acids with anticancer actions. The widely studied BCFA, 13-methyltetradecanoic acid (13-MTD, Fig. 1), is an iso-$C_{15}$ branched chain fatty acid that was first isolated from a fermented soy bean product marketed as a nutritional and therapeutic supplement. In cell-based studies 13-MTD inhibited proliferation and induced apoptosis in several human cancer cell lines, including breast (MCF-7, SK-BR-3), bladder (T24, 5637), prostate (DU-145), colon (HCT-116), liver (SNU-423, LCI-D35), lymphatic (Jurkat, EL4) and pancreatic (BxPC-3) cancer with $IC_{50}$ values ranging from 26 – 159 μM (Cai et al., 2013; Lin et al., 2012; Shioiri et al., 1998; Wongtangtintharn et al., 2005; Wongtangtintharn et al., 2004; Yang et al., 2000). 13-MTD is well tolerated by mice and oral delivery of 13-MTD (35-70 mg/kg/day) inhibited tumour growth in mice carrying DU 145, LCI-D35, and Jurkat xenografts (Cai et al., 2013; Yang et al., 2000). The naturally occurring anteiso-$C_{15}$ analogue of 13-MTD, 12-methyltetradecanoic acid (12-MTA), has also been shown to inhibit proliferation of several cancer cell lines, including prostate (DU145, PC3), lung (H1299, A549) and breast cancer (MCF-7), with $IC_{50}$ values ranging from 71 – 146 μM, and like 13-MTD, reduced tumour growth *in vivo* without any apparent toxicity (Murray et al., 2014; Wongtangtintharn et al., 2004; Wright et al, 2005; Yang et al., 2003).

Although 13-MTD and 12-MTA possess promising *in vivo* activity, moderate potency limits their efficacy and therapeutic potential. Medicinal chemistry-based approaches to improve drug potency typically involve understanding the steric and electronic factors that govern the interaction of a drug with its cellular target. Several studies of 13-MTD have identified possible mechanisms of action such as disruption of mitochondrial integrity, membrane fluidity, and fatty acid biosynthesis (Cai et al., 2013; Lin et al., 2012; Wongtangtintharn et al., 2006; Wongtangtintharn et al., 2005; Yang et al., 2000), however the precise cellular target of 13-MTD remains unknown. In the absence of a defined target, a ligand-based approach to improve BCFA anticancer activity must be undertaken, which involves the development of a structure-activity relationship (SAR) that describes how drug activity is affected by structural modifications.

Some SAR insights on the BCFA scaffold have emerged. A series of $C_{12-20}$ iso-BCFAs, which are structural analogues of 13-MTD but with elongated or contracted alkyl chains, were assess for activity against MCF-7 breast cancer cells. The iso-$C_{16}$ BCFA was the most potent in the series and inhibited MCF-7 cell proliferation with an $IC_{50}$ of ~ 80 μM, and potency decreased with an increase or decrease in chain length ($IC_{50}$ concentrations from ~ 150 – 300 μM) (Wongtangtintharn et al., 2004). Iso-BCFAs exhibit greater anticancer activity relative to their anteiso-counterparts. In a direct comparison, 13-MTD exerted greater anti-proliferative and pro-apoptotic effects than 12-MTA in MCF-7 cells (Vahmani et al., 2019). Similarly, Iso-$C_{17}$ inhibited MCF-7 cell proliferation with an $IC_{50}$ of ~190 μM, whereas the anteiso-$C_{17}$ analogue only reduced cell growth to ~80% at the highest concentration tested (400 μM) (Vahmani et al., 2019; Wongtangtintharn et al., 2004). Methyl branching beyond the iso- and anteiso-positions, as well as multiple methyl branching groups, have also been explored, however cytotoxic activity against SK-BR-3 breast cancer cells remained modest ($IC_{50}$ ~ 300-350 μM) (Oku & Yanagita, 2009; Wongtangtintharn et al., 2006).

The SAR for BCFA anticancer activity is limited by the lack of structural diversity in the BCFAs tested to date. All tested BCFAs possess methyl branching groups at various positions along saturated alkyl chains, and to our knowledge the effect of extended alkyl branching and unsaturation on anticancer activity has not been investigated. In this paper we report on the synthesis of a homologous series of saturated and unsaturated BCFAs, in which terminally branched methyl groups are replaced with ethyl, propyl and butyl moieties (Fig. 1). The anticancer activity of these new BCFAs were assessed against MCF-7, MDA-MB-231 and HeLa cancer cell lines and compared to 13-MTD, and the results were used to gain SAR insights that we anticipate will assist in the design of new and more potent BCFAs.

## 2. Material and Methods

### *2.1 General Chemistry*

11-Bromoundecanoic acid was purchased from Fluorochem (Hadfield, Derbyshire, UK). All other reagents and anhydrous solvents were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Reactions were monitored by thin-layer chromatography (TLC) using silica gel 60 $F_{254}$ plates. TLC plates were visualised with UV light and potassium permanganate TLC stain. Reaction products were purified by dry column vacuum chromatography with Merck silica gel 60H as the stationary phase in a 70mm (ID) x 60mm (h) column. 12 x 50mL fractions were collected using gradient elutions of hexane:DCM. The first fraction was 100:0 and the percentage of DCM was increased by 5% increments to 50% DCM. $^{1}H$ 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. DMSO-*d*$_6$; $^{1}H$ δ 2.49, $^{13}C$ δ 39.52). High resolution mass spectra (HRMS) were recorded on an Agilent Technologies 6510 Q-TOF LCMS.

Gas chromatography-mass spectrometry (GC-MS) was performed on a Shimadzu GCMS-QP2020 gas chromatograph-mass spectrometer fitted with an SH-Rxi-5Sil MS silica fused capillary column (30.0 m x 0.25 mm x 0.25 µm). Automated injections were performed on a Shimadzu AOC-20i auto injector. The injection port temperature was 280 °C and 1.0 µL injections were made in splitless mode with a purge time of 1 min. Helium was used as a carrier gas, at a flow rate of 1.42 mL/min. The column temperature was as follows: initial temperature of 40 °C for 2 min, then ramped at 6 °C/min to 320 °C, and held for 1 min. Eluted compounds were subjected to methane chemical ionisation and detection was made in full scan mode.

The purity of all test compounds was confirmed by absolute quantitative NMR (qNMR). Briefly, DMSO-$d_6$ was spiked with the internal calibrant (IC) 1,3,5-trioxane (99.5%) to give a final IC concentration of 2.92 mg/mL. Test compounds were accurately weighed on

a 5-decimal place analytical balance, dissolved in 600 µL of IC spiked DMSO-$d_6$ and transferred to 5mm NMR tubes. $^1$H NMR spectra was recorded and the average integration of one proton from the IC and test compound was determined. The purity of the test compound (t) was then calculated using the following equation:

$$Purity_t = \frac{Integral\ area_t \times MW_t \times mass_t}{Integral\ area_{IC} \times MW_{IC} \times mass_{IC}} \times Purity_{IC}$$

*2.2 Methyl 11-bromoundecanoate (**2**)*

To a mixture of **1** (6.00 g, 22.63 mmol) in methanol (300 mL) was added AcCl (8.69 g, 113.13 mmol). The resulting solution was left to stir at room temperature for 4 h. Excess methanol was removed under pressure and the residue was dissolved in DCM (80 mL). The organic phase was washed with saturated $NaHCO_3$ in water (100 mL), water (100 mL) and brine (100 mL) and dried over $MgSO_4$. The solution was concentrated *in vacuo* affording methyl ester **2** as a yellow oil (5.88 g, 93 %) that was used without further purification. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{12}H_{23}O_2Br$ 279.0954; found 279.0946. $^1$H and $^{13}$C NMR spectra were in agreement with previously reported data (Jana et al., 2018).

*2.3 (11-methoxy-11-oxoundecyl)(triphenyl)phosphonium bromide (**3**)*

Ester **2** (5.88 g, 21.06 mmol) was left to stir with $PPh_3$ (5.52 g, 21.06 mmol) at 140ºC for 20 hours. The resulting mixture was dissolved in DCM (5 mL) and cold diethyl ether (15 mL) was added to form a precipitate. The solid was collected by vacuum filtration and washed with cold diethyl ether (2 x 15 mL) and hexane (3 x 15 mL). The product was dried *in vacuo*, affording **3** as a white solid (10.41 g, 91 %) that was used without further purification. Mp = 111-113ºC. HRMS (ESI) *m/z* $[M]^+$ calcd for $C_{30}H_{38}O_2P$ 461.2598; found 461.2599. $^1$H and $^{13}$C NMR spectra were in agreement with previously reported data (Dejarlais & Emken, 1978).

*2.4 General synthesis of the monounsaturated methyl esters **5a-d***

To a suspension of **3** (1.500 g, 2.77 mmol) in anhydrous THF (10 mL) at 0ºC under a nitrogen atmosphere was added $NaN(TMS)_2$ (1.0 M in THF, 2.77 mmol). The resulting bright orange mixture was stirred for 20 minutes at room temperature. The reaction mixture was then cooled to -78ºC, and the aldehyde (**4a-d**, 1.85 mmol) in THF (3 mL) was added dropwise. The mixture was stirred for 30 minutes at -78ºC after which the reaction was allowed to warm to room temperature for two hours. After further stirring for two hours at room temperature the reaction was quenched with saturated aqueous $NH_4Cl$ (30 mL) and extracted with EtOAc (3 x 50 mL). The combined extracts were dried over $MgSO_4$ and the EtOAc was removed *in vacuo*. The residue was purified on silica gel using a stepwise gradient elution with hexane:DCM (100:0 to 50:50) to yield the unsaturated methyl esters **5a-d**.

*2.4.1 Methyl (Z)-13-methyltetradec-11-enoate (**5a**)*

Yellow oil, yield = 42 %. $^{13}C$ NMR (125 MHz, $CDCl_3$) δ 174.31, 137.46, 127.46, 51.40, 34.09, 29.88, 29.43, 29.39, 29.24, 29.22, 29.12, 27.27, 26.41, 24.93, 23.21. *Z:E* ratio = 97.8 : 2.2. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{16}H_{31}O_2$ 255.2319; found 255.2318. The $^1H$ spectrum was in agreement with previously reported data (Fogila and Vail, 1993).

*2.4.2 Methyl (Z)-13-ethylpentadec-11-enoate (**5b**)*

Yellow oil, yield = 75 %. $^1H$ NMR (500 MHz, $CDCl_3$) δ 5.39 (dt, *J* = 10.5, 2.5 Hz , 1H), 5.00 (t, *J* = 7.5 Hz, 1H), 3.66 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 2.16-2.06 (m, 1H), 2.00 (q, *J* = 7.5 Hz, 2H), 1.61 (quint, *J* = 7.5 Hz, 2H), 1.43-1.36 (m, 2H), 1.35-1.24 (m, 12H), 1.19-1.10 (m, 2H), 0.82 (t, *J* = 7.5 Hz, 6H). $^{13}C$ NMR (125 MHz, $CDCl_3$) δ 174.32, 134.43, 130.04, 51.42, 40.62, 34.10, 29.88, 29.44, 29.40, 29.30, 29.22, 29.13, 28.30, 27.68, 24.94, 11.86. *Z:E* ratio = 100.0 : 0.0. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{18}H_{35}O_2$ 283.2632; found 283.2634.

*2.4.3 Methyl (Z)-13-propylhexadec-11-enoate (**5c**)*

Yellow oil, yield = 21 %. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 5.34 (dt, *J* = 10.5, 2.5 Hz, 1H), 5.01 (t, *J* = 7.5 Hz, 1H), 3.66 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 2.00 (q, *J* = 7.5 Hz, 2H), 1.61 (quint, *J* = 7.5 Hz, 2H), 1.34-1.25 (m, 12H), 1.24-1.17 (m, 2H), 1.16-1.08 (m, 2H), 0.86 (t, *J* = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 174.33, 135.06, 129.47, 51.42, 38.26, 36.68, 34.11, 29.91, 29.44, 29.40, 29.33, 29.23, 29.15, 27.69, 24.95, 20.45, 14.30. *Z:E* ratio = 100.0 : 0.0. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{20}H_{39}O_2$ 311.2945; found 311.2947.

*2.4.4 Methyl (Z)-13-butylheptadec-11-enoate (**5d**)*

Yellow oil, yield = 43 %. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 5.34 (dt, *J* = 10.5, 2.5 Hz, 1H), 5.01 (t, *J* = 7.5 Hz, 1H), 3.66 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 2.28-2.21 (m, 1H), 2.00 (q, *J* = 7.5 Hz, 2H), 1.61 (quint, *J* = 7.5 Hz, 2H), 1.36-1.21 (m, 20H), 1.20-1.09 (m, 4H), 0.91-84 (m, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 174.32, 135.18, 129.44, 51.42, 37.08, 35.68, 34.11, 29.90, 29.62, 29.47, 29.41, 29.34, 29.24, 29.15, 27. 70, 24.95, 22. 92, 14.12. *Z:E* ratio = 99.7 : 0.3. HRMS (ESI) *m/z* $[M+H]^+$ calcd for 339.3258; found 339.3255.

*2.4.5 Methyl (Z)-tetradec-11-enoate (**5e**)*

Yellow oil, yield: 86 %. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 5.39-5.31 (m, 2H), 3.66 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 2.06-1.99 (m, 4H), 1.62 (quint, *J* = 7 Hz, 2H), 1.37-1.25 (m, 12H), 0.95 (t, *J* = 7.5 Hz, 3H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 175.83, 131.34, 128.85, 51.38, 33.98, 29.75, 29.46, 29.35, 29.23, 29.22, 29.06, 27.02, 24.68, 20.55, 14.48. *Z:E* ratio = 100.0 : 0.0. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{15}H_{28}O_2$ 241.2162; found 241.2168.

*2.5 General synthesis of the monounsaturated branched fatty* acids ***6a-d***

To a solution of the unsaturated methyl ester (**5a-d**, 0.20 mmol) in EtOH (6 mL) was added 1.5 M NaOH (8 mL). The reaction was heated to 40ºC and stirred for four hours. The volume of the reaction mixture was reduced to 10 mL by rotary evaporation and the solution was adjusted to pH 1 with 1.0 M HCl. The solution was extracted with EtOAc (3 x 50 mL) and the combined extracts were dried over $MgSO_4$. The EtOAc was removed *in vacuo* to afford unsaturated fatty acids **6a-d** in high purity (>95%) without further purification.

*2.5.1 (Z)-13-methyltetradec-11-enoic acid (**6a**)*

Yellow oil, yield = 93%. $^1$H NMR (500 MHz, $CDCl_3$) δ 5.25-5.15 (m, 2H), 2.61-2.54 (m, 2H), 2.35 (t, *J* = 7.5 Hz, 2H), 2.01 (q, *J* = 7.5 Hz, 2H), 1.63 (quint, *J* = 7.5 Hz, 2H), 1.37-1.23 (m, 12H), 0.94 (d, *J* = 7.0 Hz, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 179.65, 137.49, 127.46, 33.99, 29.89, 29.44, 29.38, 29.24, 29.21, 29.04, 27.28, 26.42, 24.67, 23.22. FT-IR $\nu_{max}$ (neat) 2923 (O-H stretch), 2853 (C-H stretch), 1707 (C=O stretch), 1463 (C-H bend), 1411 (O-H bend), 722 (C=C bend) cm$^{-1}$. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{15}H_{29}O_2$ 241.2162; found 241.2163. qNMR purity = 95%.

*2.5.2 (Z)-13-ethylpentadec-11-enoic acid (**6b**)*

Yellow oil, yield = 97%. $^1$H NMR (500 MHz, $CDCl_3$) δ 5.40 (dt, *J* = 10.5, 2.5 Hz, 1H), 5.01 (t, *J* = 7.5 Hz, 1H), 2.35 (t, *J* = 7.5 Hz, 2H), 2.16-2.07 (m, 1H), 2.01 (q, *J* = 7.5 Hz, 2H), 1.63 (quint, *J* = 7.5 Hz, 2H), 1.44-1.37 (m, 2H), 1.37-1.21 (m, 12H), 1.20-1.09 (m, 2H), 0.83 (t, *J* = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 178.74, 134.45, 130.06, 40.63, 33.82, 29.89, 29.69, 29.45, 29.39, 29.32, 29.21, 29.05, 28.31, 27.69, 24.69. 22.69, 14.12, 11.87. FT-IR $\nu_{max}$ (neat) 2922 (O-H stretch), 2853 (C-H stretch), 1708 (C=O stretch), 1456 (C-H bend), 1412 (O-H bend), 723 (C=C bend) cm$^{-1}$. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{17}H_{33}O_2$ 269.2475; found 269.2472. qNMR purity = 97%.

*2.5.3 (Z)-13-propylhexadec-11-enoic acid (**6c**)*

Yellow oil, yield = 90%. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 5.34 (dt, *J* = 10.5, 2.5 Hz, 1H), 5.01 (t, *J* = 7.5 Hz, 1H), 2.35 (t, *J* = 7.5 Hz, 2H), 2.33-2.27 (m, 1H), 2.01 (q, *J* = 7.5 Hz, 2H), 1.63 (quint, *J* = 7.5 Hz, 2H), 1.39-1.25 (m, 16H), 1.24-1.19 (m, 2H), 1.18-1.09 (m, 2H), 0.86 (t, *J* = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 179.17, 135.08, 129.47, 38.28, 36.69, 33.89, 29.92, 29.70, 29.62, 29.51, 29.45, 29.40, 29.34, 29.22, 29.16, 29.07, 28.95, 27.71, 24.69, 20.46, 14.32. FT-IR $\nu_{max}$ (neat) 2922 (O-H stretch), 2853 (C-H stretch), 1708 (C=O stretch), 1465 (C-H bend), 1413 (O-H bend), 722 (C=C bend) cm$^{-1}$. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{19}H_{37}O_2$ 297.2788; found 297.2786. qNMR purity = 97%.

*2.5.4 (Z)-13-butylheptadec-11-enoic acid (**6d**)*

Yellow oil, yield = 81%. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 5.35 (dt, *J* = 10.5 Hz, 2.5 Hz, 1H), 5.02 (t, *J* = 7.5 Hz, 1H), 2.35 (t, *J* = 7.5 Hz, 2H), 2.30-2.21 (m, 1H), 2.01 (q, *J* = 7.5 Hz, 2H), 1.63 (quint, *J* = 7.5 Hz, 2H), 1.38-1.22 (m, 20H), 1.21-1.09 (m, 4H), 0.87 (t, *J* = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 179.34, 135.19, 129.44, 37.08, 35.68, 33.91, 29.89, 29.65, 29.62, 29.47, 29.40, 29.34, 29.23, 29.06, 27.70, 24.67, 22.92, 14.12. FT-IR $\nu_{max}$ (neat) 2922 (O-H stretch), 2853 (C-H stretch), 1708 (C=O stretch), 1465 (C-H bend), 1411 (O-H bend), 724 (C=C bend) cm$^{-1}$. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{41}O_2$ 325.3101; found 325.3103. qNMR purity = 96 %.

*2.5.5 (Z)-tetradec-11-enoic acid (**6e**)*

Yellow oil, yield: 85%. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{14}H_{26}O_2$ 227.2006; found 227.2004. qNMR purity = 99 %. $^{1}$H and $^{13}$C NMR spectra were in agreement with previously reported data (Wube et al., 2011).

*2.6 General synthesis of the saturated methyl esters 7**a-d***

To a mixture of the monounsaturated methyl ester (**5a-d**, 0.65 mmol) in EtOH (10 mL) under a hydrogen atmosphere was added Pd/C (10 wt. %, 0.20 mmol). The suspension was stirred for 20 hours at room temperature, after which the mixture was filtered over celite and washed with EtOH (2 x 25 mL). The EtOH was removed *in vacuo* and the residue was purified on silica gel with a stepwise gradient elution with hexane:DCM (100:0 to 50:50) to yield **7a-d**.

*2.6.1 Methyl 13-methyltetradecanoate (7**a**)*

Yellow oil, yield = 87 %. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 3.66 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.61 (quint, *J* = 7.5 Hz, 2H), 1.58-1.44 (m, 1H), 1.36-1.25 (m, 16H), 1.17-1.12 (m, 2H), 0.85 (d, *J* = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 174.34, 51.42, 34.12, 29.92, 29.69, 29.63, 29.58, 29.44, 29.25, 29.14, 27.96, 27.40, 24.95, 22.65. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{16}H_{33}O_2$ 252.2475; found 252.2478.

*2.6.2 Methyl 13-ethylpentadecanoate (7**b**)*

Yellow oil, yield = 68 %. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 3.67 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.61 (quint, *J* = 7.5 Hz, 2H), 1.35-1.19 (m, 21H), 1.18-1.11 (m, 2H), 0.83 (t, *J* = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 174.34, 51.41, 40.36, 34.11, 32.75, 30.13, 29.69, 29.64, 29.59, 29.44, 29.25, 29.14, 26.76, 25.43, 24.95, 10.91. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{18}H_{37}O_2$ 285.2788; found 285.2787.

*2.6.3 Methyl 13-propylhexadecanoate (7**c**)*

Yellow oil, yield = 83 %. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 3.66 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.61 (quint, *J* = 7.5 Hz, 2H), 1.28-1.15 (m, 27H), 0.87 (t, *J* = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz,

$CDCl_3$) δ 174.34, 51.42, 36.94, 36.10, 34.12, 33.67, 30.15, 29.69, 29.65, 29.59, 29.44, 29.25, 29.15, 26.67, 24.95, 19.79, 14.54. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{20}H_{41}O_2$ 313.3101; found 313.3103.

*2.6.4 Methyl 13-butylheptadecanoate (**7d**)*

Yellow oil, yield = 44 %. $^1H$ NMR (500 MHz, $CDCl_3$) δ 3.66 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.61 (quint, *J* = 7.5 Hz, 2H), 1.33-1.15 (m, 31H), 0.89 (t, *J* = 7.5 Hz, 6H). $^{13}C$ NMR (125 MHz, $CDCl_3$) δ 174.32, 51.41, 37.34, 33.70, 33.38, 30.14, 29.69, 29.65, 29.59, 29.44, 29.25, 29.15, 28.96, 26.70, 24.95, 23.16, 14.16. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{22}H_{45}O_2$ 341.3414; found 341.3415.

*2.7 General procedure for synthesis of saturated branched fatty acids **8b-d***

To a mixture of the saturated methyl ester (**7a-d**, 0.20 mmol) in EtOH (6 mL) was added 1.5 M NaOH (8 mL). The reaction was heated to 40ºC and stirred for four hours. The volume of the reaction mixture was reduced to 10 mL by rotary evaporation and the solution was adjusted to pH 1 with 1.0 M HCl. The white solid was extracted with EtOAc (3 x 50 mL) and the combined extracts were dried over $MgSO_4$. EtOAc was removed *in vacuo* to afford 13-MTD and **8b-d** in high purity (>95%) without further purification.

*2.7.1 13-methyltetradecanoic acid (**13-MTD**)*

White solid, yield = 89%. Mp = 47-49ºC (lit. Mp = 46.5-47 ºC) (Shioiri et al., 1998). FT-IR, HRMS, $^1H$ and $^{13}C$ NMR spectra were in agreement with previously reported data (Khan et al., 2018). qNMR purity = 98%.

*2.7.2 13-ethylpentadecanoic acid (**8b**)*

Yellow oil, yield = 96%. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 2.35 (t, *J* = 7.5 Hz, 2H), 1.63 (quint, *J* = 7.5 Hz, 2H), 1.37-1.20 (m, 21H), 1.19-1.11 (m, 2H), 0.83 (t, *J* = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 178.57, 40.38, 33.79, 32.76, 30.13, 29.70, 29.65, 29.59, 29.43, 29.24, 29.06, 26.77, 25.44, 24.69. FT-IR $\nu_{max}$ (neat) 2922 (O-H stretch), 2853 (C-H stretch), 1708 (C=O stretch), 1460 (C-H bend), 1412 (O-H bend) cm$^{-1}$. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{17}H_{35}O_2$ 271.2632; found 271.2635. qNMR purity = 96%.

*2.7.3 Characterization of 13-propylhexadecanoic acid (**8c**)*

Yellow oil, yield = 96%. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 2.35 (t, *J* = 7.5 Hz, 2H), 1.63 (quint, *J* = 7.5 Hz, 2H), 1.40-1.17 (m, 27H), 0.87 (t, *J* = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 1.78.73, 36.94, 36.10, 33.81, 33.67, 30.14, 29.69, 29.65, 29.61, 29.58, 29.50, 29.42, 29.35, 29.23, 29.15, 29.06, 29.67, 24.69, 19.80, 14.54. FT-IR $\nu_{max}$ (neat) 2923 (O-H stretch), 2853 (C-H stretch), 1710 (C=O stretch), 1465 (C-H bend), 1418 (O-H bend) cm$^{-1}$. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{19}H_{39}O_2$ 299.2945; found 299.2946. qNMR purity = 97%.

*2.7.4 Characterization of 13-butylheptadecanoic acid (**8d**)*

Yellow oil, yield = 89%. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 2.35 (t, *J* = 7.5 Hz, 2H), 1.63 (quint, *J* = 7.5 Hz, 2H), 1.41-1.17 (m, 31H), 0.88 (t, *J* = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 178.77, 37.35, 33.81, 33.70, 33.38, 30.14, 29.69, 29.65, 29.58, 29.43, 29.32, 29.05, 28.96, 26.70, 24.68, 23.16, 14.17. FT-IR $\nu_{max}$ (neat) 2921 (O-H stretch), 2852 (C-H stretch), 1709 (C=O stretch), 1465 (C-H bend), 1412 (O-H bend) cm$^{-1}$. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{43}O_2$ 327.3258; found 327.3256. qNMR purity = 96%.

*2.8 General cell culture*

Fetal bovine serum and trypsin/EDTA were purchased from (Invitrogen, Life Technologies, Victoria, Australia). Dulbecco's Modified Eagle Medium (DMEM), penicillin and streptomycin, phosphate-buffered saline (PBS), DMSO and general biochemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Human tumour cell lines were obtained from ATCC (Manassas, VA) and were grown at 37°C in a humidified atmosphere of 5% $CO_2$ in air in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Confluent cells (80-90%) were harvested using Trypsin/EDTA after washing in PBS.

*2.9 Cell viability assay*

The anticancer activity of the synthesized compounds was determined using the MTS assay. Cells were seeded in 96-well plates ($5\times10^3$ cells per well), 24 h later serum was removed and cells were treated with various concentrations of the test compounds, in DMSO (final concentration 0.1%), for 48 h. Control cells received serum-free DMEM and DMSO at 0.1%. Cell viability was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA) according to the manufacturer's recommendation. $IC_{50}$ values were defined as the drug concentration that prevented cell growth of more than 50% (relative to the vehicle control) and were determined using nonlinear regression analysis with Prism 7.0 (GraphPad Software, CA, USA).

*2.10 Statistical analysis*

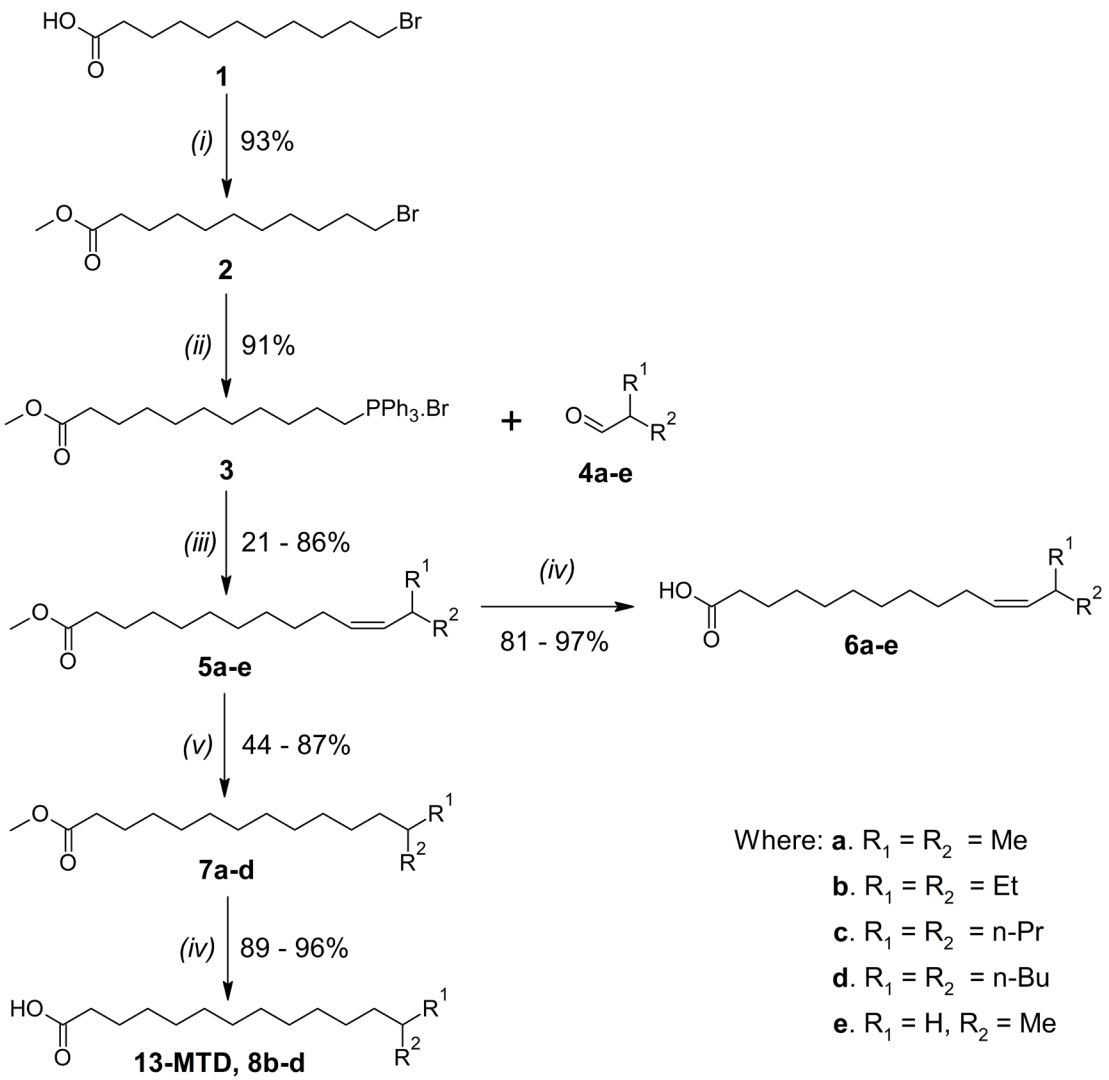
For in vitro assays, data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis. $P<0.05$ were accepted as being statistically significant. All statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, CA, USA). The results are expressed as the means±SEM unless otherwise specified.

## 3. Results

### *3.1 Synthesis*

Saturated (13-MTD and **8b-d**) and unsaturated (**6a-d**) BCFAs were synthesised as shown in Scheme 1. In the first step the carboxylic acid group in bromo fatty acid **1** was ester-protected using acetyl chloride and methanol. Under these mild reaction conditions acetyl chloride generates HCl in situ, which catalyses the esterification reaction. Methyl ester **2** was then reacted with triphenyl phosphine in a solvent-free melt to generate triphenylphosphonium bromide **3**, which was then used in Wittig reactions with commercially available aldehydes **4a-d** to yield unsaturated branched fatty acid esters **5a-d** with *cis*-double bonds in the Δ11 positions. To produce the desired *cis*-olefins the Wittig reactions were conducted in THF at -78 ºC with sodium bis(trimethylsilyl)amide as the base. These reaction conditions were chosen because they have been shown to favour formation of *cis*-olefins (*Z*:*E* ratio ≥ 97:3) (Ainai et al., 2003; Ito et al, 2006; Rawling et al., 2010). To confirm that the Wittig reactions proceeded with the desired selectivity, the *Z:E* ratio of **5a-d** was determined by GC-MS. The gas-chromatograms of these compounds contained two peaks with identical mass spectra. Based on the elution order (Aro et al., 1998; Rawling et al., 2010), the first compounds to elute were identified as the *trans*-isomers (Rt ~ 31.88 min), which were baseline separated from the *cis*-isomers (Rt ~ 31.98 min). The peak areas were then used to calculate the *Z:E* ratio, which in all cases exceed 97:3.

Olefins **5a-d** were then divided into two sets of aliquots, and alkaline hydrolysis of the ester groups in one of the sets afforded unsaturated BCFAs **6a-d** (Fig. 1). The double bonds in the remaining **5a-d** aliquots were reduced with palladium and hydrogen to give saturated BCFA esters **7a-d**. Finally, removal of the ester protecting groups yielded BCFAs **13-MTD** and **8b-d**.




**Scheme 1.** Synthesis of the fatty acids 13-MTD **8b-d** and **6a-e**. Reagents and conditions (*i*) AcCl, methanol, rt, 4 hours; (*ii*) $PPh_3$, 140 ºC, 20 hours; (*iii*) $NaN(TMS)_2$, THF, -78 ºC to rt, 2 hours; *(iv)*1.5M NaOH, ethanol, 40 ºC, 4 hours; *(v)* Pd/C, hydrogen, rt, 20 hours.

To assess the role of the methyl branching group and *cis*-Δ11 double bond in the anticancer activity of **6a**, we also prepared the monounsaturated fatty acid (MUFA) **6e**, which is the straight chain analogue of **6a**. **6e** was synthesised following the synthetic procedure outlined in Scheme 1. The Wittig reaction of phosphonium bromide **3** and straight chain aldehyde **4e** affording the ester protect MUFA **5e** with a *cis*-Δ11 double bond (*Z:E* ratio

confirmed by GC-MS analysis). Base-catalysed hydrolysis of the **5e** with sodium hydroxide provided **6e** in high purity.

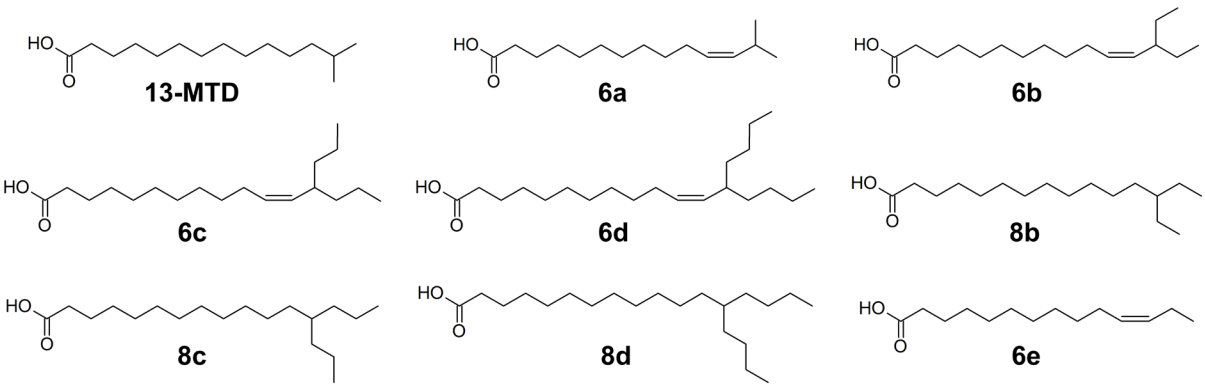



**Fig. 1.** Chemical structures of branched chain fatty acids used in this study.

### *3.2 Cell culture conditions*

Prior to testing the all synthetic BCFAs against the full panel of cancer cell lines, we first developed assay conditions using 13-MTD, which is reported to reduce MCF-7 cell viability with $IC_{50}$ concentrations of 10.03 μg/ml (~41 μM) and ~150 μM in media containing 10% fetal bovine serum (FBS) (Wongtangtintharn et al., 2004; Yang et al., 2000). In our hands, 13-MTD failed to affect MCF-7 cell viability at concentrations up to 500 μM after 72 hours under these conditions. The same result was found with 13-MTD synthesised by us or purchased commercially. One important difference between our assay conditions and those used for 13-MTD, and indeed all other previously reported anticancer BFCAs, was our choice of DMSO as the drug delivery vehicle rather than Tween 80 (Cai et al., 2013; Lin et al., 2012; Shioiri et al., 1998; Wongtangtintharn et al., 2006; Wongtangtintharn et al., 2005; Wongtangtintharn et al., 2004; Yang et al., 2000). Tween 80 has been shown to potentiate the *in vitro* effects of some anticancer agents by permeabilising the cell membrane (Riehm and Biedler, 1972), and it is possible Tween 80 also potentiates BFCA anticancer activity *in vitro*.

It is important to note that these observations do not invalidate the anticancer actions of 13-MTD and other BCFAs, as indeed 13-MTD is active *in vivo* (Yang et al., 2000), however they do indicate that Tween 80 is not an ideal vehicle for *in vitro* anticancer assays.

We next attempted cell viability assays with DMSO as vehicle but in serum free media (SFM), as SFM has been used previously in assessing the anticancer activity of the BCFA 12-methyltetradecanoic acid (Yang et al., 2003). In SFM we found that 13-MTD significantly reduced MCF-7 cell viability at concentrations ≥ 100 μM (Fig 2a). To confirm that the cells were viable and proliferative SFM we conducted cell counts from the time of serum withdrawal to the end of the 48 hour treatment period. We found that MCF-7 cell number increased 1.5 fold over the treatment period. Similarly, the other two cell lines used in this study, MDA-MB-231 and HeLa cells, grew 1.9 fold and 2.2 fold, respectively in SFM over 48 hours. Thus, all cell viability assays were performed in SFM for 48 hours with DMSO as delivery vehicle.

### *3.3 In vitro anticancer activity*

The *in vitro* anticancer activities of the test fatty acids were first assessed against MCF-7 cells. Dose-response curves were constructed (Fig 2) and $IC_{50}$ concentrations were determined (Table 1). We also calculated Hill slopes (HS) and maximum effects ($E_{max}$) for each compound as these metrics can provide mechanistic information (Fallahi-Sichani et al., 2013).

In MCF-7 cells, 13-MTD was the most potent in the series of saturated BCFAs (13-MTD, **8b-d**) and reduced MCF-7 viability with an $IC_{50}$ of 135 ± 13 μM (Table 1, Fig. 2a). $IC_{50}$ concentrations for the remaining saturated BCFAs were not able to be calculated due to limitations in the solubilities of these compounds at concentrations greater than 900 μM. From the dose-response curves obtained (Fig. 2b), the ethyl-branched analogue **8b** displayed modest activity and was able to reduce MCF-7 cellular viability to 49 ± 7% at 900 μM. Propyl-

branched BCFA **8c** only affected cellular viability at 900 μM (76 ± 4%), and the butyl-branched analogue **8d** did not affect cellular viability at any of the test concentrations.

Unlike the saturated BCFAs **8b-d**, all of the unsaturated BCFA's **6a-d** were active against MCF-7 cells. Although **6a-d** reduced MCF-7 viability with similar $IC_{50}$ values, the shapes of the dose-response curves revealed differences between these compounds (Fig. 2c). Methyl, ethyl and propyl branched compounds **6a-c** displayed relatively steep dose-response curves with Hill slopes (HS) >2.8 and maximum effect ($E_{max}$) values of 0, reflecting the ability of **6a-c** to completely abolish MCF-7 cell viability (Table 1). In contrast, the dose-response curve of the butyl-branched analogue **6d** was shallow (HS = -1.6) and the cell viability did not drop below 32% as the concentration of **6d** was increased from 150-450 μM ($E_{max}$ = 0.3).

**Table 1. $IC_{50}$ concentrations, Hill Slopes (HS) and maximum effect ($E_{max}$) of fatty acids against the MCF-7, MDA-MB-231 and HeLa cancer cell lines[a]**

| | MCF-7 | | | MDA-MB-231 | | | HeLa | | |
|---|---|---|---|---|---|---|---|---|---|
| **Fatty Acid** | **$IC_{50}$ [μM]** | **HS** | **$E_{max}$** | **$IC_{50}$ [μM]** | **HS** | **$E_{max}$** | **$IC_{50}$ [μM]** | **HS** | **$E_{max}$** |
| 13-MTD | 135 ± 13 | -3.1 | 0.0 | Inactive[c] | - | - | >900 | - | - |
| **8b** | >750 | - | - | Inactive[c] | - | - | >900 | - | - |
| **8c** | >900 | - | - | Inactive[c] | - | - | >900 | - | - |
| **8d** | >900 | - | - | Inactive[c] | - | - | >900 | - | - |
| **6a** | 49.4 ± 4.8 | -6.4 | 0.0 | 534 ± 39 | -2.6 | 0.0 | 80.7 ± 6.1 | -1.5 | 0.0 |
| **6b** | 58.9 ± 9.2 | -3.2 | 0.0 | Inactive[c] | - | - | >900 | - | - |
| **6c** | 51.8 ± 6.1 | -2.8 | 0.0 | Inactive[c] | - | - | >900 | - | - |
| **6d** | 47.6 ± 3.1 | -1.6 | 0.3 | Inactive[c] | - | - | >900 | - | - |
| **6e** | 86.4 ± 9.3 | -2.3 | 0.0 | 158 ± 42 | -2.2 | N/A[b] | 119 ± 13 | -1.2 | N/A[b] |

[a] Cell viability was measured using the MTS assay after 48 h of treatment.
[b] N/A: not applicable. $E_{max}$ values for **6e** in these cell lines could not be calculated because **6e** was not soluble in cell media at concentration above 200 μM.
[c] No effect on cell viability at concentrations up to 900 μM.

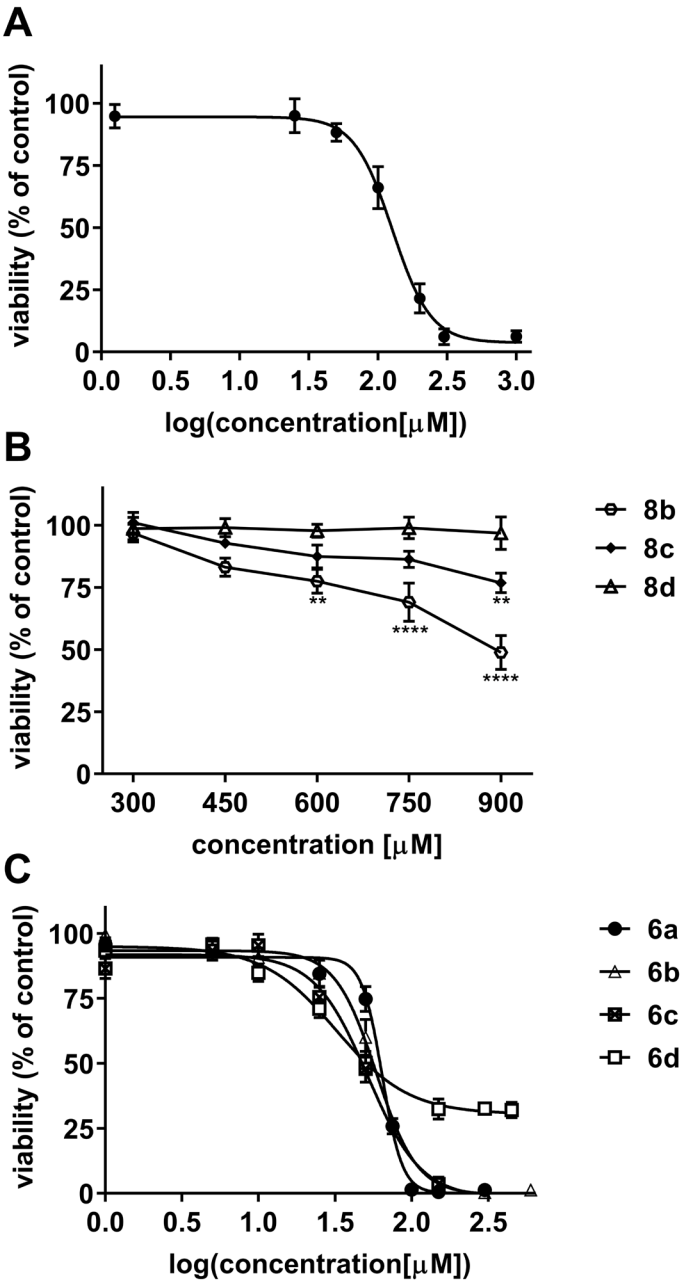



**Fig. 2.** Dose-response curves showing the effects of synthetic BCFAs on the viability of MCF-7 breast cancer cells after 48 h treatment. A) 13-MTD, B) saturated BCFAs **8b-d**, C) unsaturated BCFAs **6a-d**. Data represents the mean±SEM of at least 3 independent experiments. **P<0.01, ****P<0.0001, comparison of the indicated compound to the vehicle control.

We also assessed the anticancer activity of the BCFAs against a wider panel of cancer cell lines (Table 1 and Fig. 3). In HeLa cervical cancer cells the unsaturated and methyl branched BCFA **6a** was the most active in the series and reduced cell viability with an $IC_{50}$ of 80.7 ± 6.1 μM. All remaining BCFAs including 13-MTD were much less active that **6a** and were only able to reduce cell viability to 70 – 90% of control at 900 μM (Fig S1 and Table S1 in Supplementary Material).

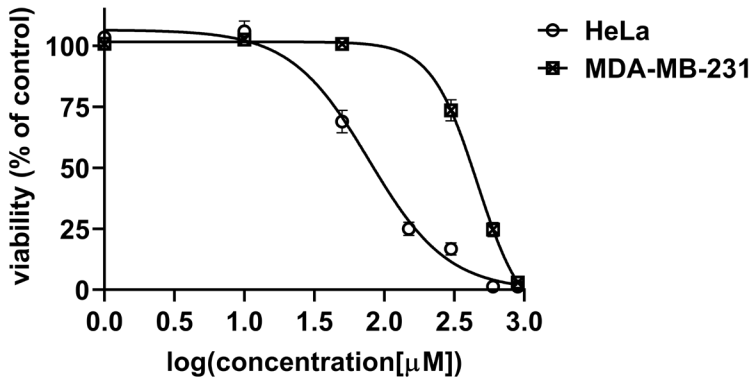



**Fig. 3.** Dose-response curves showing the effects of unsaturated BCFA **6a** on the viability of MDA-MB-231 and HeLa cancer cells after 48 h treatment. Data represents the mean±SEM of at least 3 independent experiments.

A similar pattern of activity emerged when the BFCAs were tested against MDA-MB-231 breast cancer cells. Thus **6a** fully abolished cell viability ($E_{max}$ = 0) with an $IC_{50}$ of 534 ± 39 μM (Fig. 3) while 13-MTD and the other BCFAs had no effect on cell viability at concentrations up to 900 μM (Table S2 in Supplementary Material).

MUFA **6e**, the straight-chain analogue of **6a**, was also assessed for anticancer activity in all three cell lines (Fig. 4). It was observed that **6e** reduced the viability of all cell lines with $IC_{50}$ concentrations ranging from 86 – 158 μM. Comparisons of the $IC_{50}$ concentrations of **6a** and **6e** in each cell line revealed that **6a** was approximately 1.5 - 2 fold more active that its

unbranched counterpart in MCF-7 and HeLa cells, while in MDA-MB-231 cells **6e** was 3.4 fold more active. Viability of MCF-7 cells was fully abolished by **6e** ($E_{max}$ = 0.0), however $E_{max}$ values for **6e** against MDA-MB-231 and HeLa cells could not be determined because **6e** was not soluble in cell media at concentrations above 200 μM.

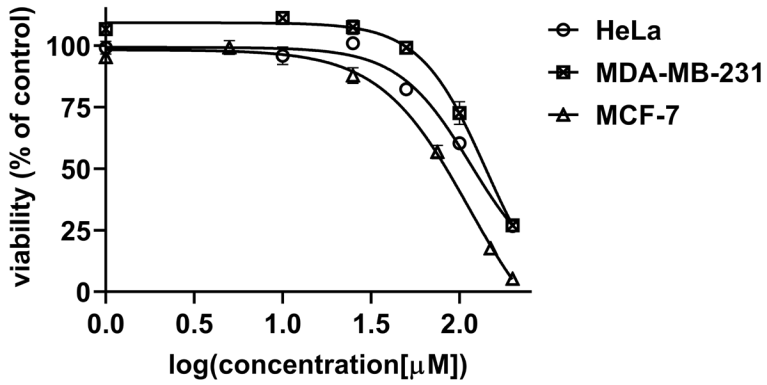



**Fig. 4.** Dose-response curves showing the effects of MUFA **6e** on the viability of MCF-7, MDA-MB-231 and HeLa cancer cells after 48 h treatment. Data represents the mean±SEM of at least 3 independent experiments.

## 4. Discussion

The development of new anticancer drugs with novel mechanisms of action and low toxicity remains an ongoing need in cancer treatment. BCFAs are structurally distinct from current oncology drugs, well-tolerated in mice and inhibit tumour growth *in vivo*, and thus present an interesting opportunity for drug development (Murray et al., 2015). The precise cellular target/s of anticancer BCFAs are not known, and medicinal chemistry approaches to improve potency must rely on the synthesis and testing of BCFA analogues, and derivation of an SAR to guide drug design. To date, antiproliferative studies have exclusively examined saturated BCFAs with methyl branching-groups and reported gains in potency have been moderate (Wongtangtintharn et al., 2004). In considering this, we developed two homologous series of BCFAs based on the structure of 13-MTD- one fully saturated series, and a corresponding unsaturated series possessing *cis*-Δ11 double bonds- and assessed their anticancer activity against three cancer cell lines.

### *4.1 Effects on MCF-7 cell viability*

13-MTD, a widely studied iso-BCFA, inhibits cell growth in various cancer cell lines both *in vitro* and *in vivo* (Cai et al., 2013; Lin et al., 2012; Wongtangtintharn et al., 2005; Yang et al., 2000). 13-MTD has previously been reported to reduce MCF-7 cell viability with an $IC_{50}$ concentrations of ~41 μM and ~150 μM (Wongtangtintharn et al, 2004), and using the assay conditions developed for this study we found similar levels of cytotoxicity (cf. $IC_{50}$ 135 μM, Table 1). In contrast, 13-MTD was essentially inactive in both MDA-MB-231 and HeLa cells. Within the saturated series, 13-MTD was the only BCFA to significantly affect cellular viability at or below 300 μM. As the size of the branching group was increased to ethyl (**8b**) and propyl (**8c**) chains, the associated activity decreased (Fig. 2b). Indeed, the butyl-branched

analogue **8d** was inactive at all test concentrations. These findings clearly illustrate a preference for smaller methyl-branching groups in saturated BCFAs.

The unsaturated BCFAs **6a-d** were all active and reduced MCF-7 viability. This activity pattern indicates that the activity of unsaturated BCFAs are not as sensitive to branching group size as their saturated counterparts. Interestingly, while **6a-d** displayed similar $IC_{50}$ concentrations, differences in the dose-response curve of butyl-branched **6d** were observed. The HS values for **6a-d** were within the range reported for a wide panel of anticancer drugs against MCF-7 cells (Fallahi-Sichani et al., 2013), however **6d** had a relatively low HS and high $E_{max}$ relative to **6a-c**. We saw no evidence that the high $E_{max}$ value of **6d** resulted from precipitation in the assays. Indeed, similar shallow dose-response curves with $E_{max}$ values > 0 have been reported for the clinical anticancer drugs docetaxel and geldanamycin in cell viability assays using the MCF-7 cell line (Fallahi-Sichani et al., 2013). Thus, the differences in HS and $E_{max}$ values calculated for **6d** suggest that **6d** reduces MCF-7 cell viability by a different mechanism of action to **6a-c** (Fallahi-Sichani et al., 2013), which may arise from the increased bulk provided by the butyl branching-group in **6d** causing it to interact with different cellular target/s to that of **6a-c**. Follow-up mechanistic studies could assess the ability of **6a-d** to inhibit COX-2 activity as MUFAs with anticancer activity have been shown to reduce COX-2 mediated prostaglandin $E_2$ ($PGE_2$) synthesis (Cui et al., 2012).

*4.2 Effects on MDA-MB-231 and HeLa cell viability*

13-MTD has been shown to reduce the viability of over 10 cancer cell lines, however the anticancer activity of 13-MTD and other BCFAs has not been studied using MDA-MB-231 and HeLa cancer cell lines. In this study we found that these two cell lines are much more resistant than MCF-7 cells to BCFA anticancer activity. 13-MTD was essentially inactive against both cell lines, and this lack of activity was also observed for all saturated BCFAs (**8b-**

**d**) and unsatutared BCFAs **6b-d**. The only exception was the unsaturated and methyl-branched BCFA **6a**, which was active across all three cell lines. Comparison of the $IC_{50}$ concentrations showed that MCF-7 cells were most sensitive to **6a**, followed by HeLa and then MDA-MB-231 cells. Consistent with this pattern of activity, HeLa cell viability was moderately reduced by the remaining BCFAs at 900 μM, while MDA-MB-231 cells were unaffected at all test concentrations.

*4.3 Effect of fatty acid branching on anticancer activity- comparison of **6a** and **6e***

Cell testing revealed that **6a** is one of the most potent BCFAs reported to date, and almost 3-fold more potent that it's saturated counterpart 13-MTD. Furthermore, within the series **6a** was the only BCFA that was active against all cell lines tested. These results indicate that inclusion of a *cis*-Δ11 double bond into the BCFA scaffold greatly increases anticancer activity. However an important SAR consideration is whether the superior activity of **6a** is dependent on the both the methyl branching group and the *cis*-Δ11 double bond, or the double bond only, because several straight chain MUFAs have been reported to reduce the viability of breast cancer cell lines (Cui et al., 2012; Miller et al, 2003). We therefore synthesised MUFA **6e**, the unbranched analogue of **6a** and compared their activities. In MDA-MB-231 cells, which were largely unaffected by the BCFAs, **6e** was more potent than **6a**. However in MCF-7 and HeLa cells, the two cell lines that were more susceptible to BCFAs, the $IC_{50}$ concentrations of **6a** was 1.5 - 2 fold lower than that of **6e**, which confirmed that both the branching group and *cis*-Δ11 double bond are critical to activity in these cell lines. Collectively, these findings indicate that unsaturation is an effective strategy to increase the potency of BCFAs, although the effects are cell line dependant.

## 5. Conclusions

By synthesising a homologous series of saturated and unsaturated BCFAs and evaluating their effects on the viability of MCF-7, MDA-MB-231 and HeLa cells, we have been able to expand the structure-activity relationship governing BCFA anticancer activity. In general MCF-7 cells were the most susceptible to the anticancer actions of the BCFAs tested, followed by HeLa and then MDA-MB-231 cells. It was shown that symmetrically extending the branching chains of saturated BCFAs is detrimental to activity in MCF-7 cells. In contrast, inclusion of a *cis*-Δ11 double bond into the BCFA chain improves activity relative to their corresponding fully saturated analogues and unsaturated BCFA **6a** was active across all the cell lines tested. These new SAR insights may assist in the development of new and more potent BCFAs as potential anticancer agents.

**Author Contributions**

TR conceived of the study. RR, AR and TR designed experiments, analysed data and wrote the manuscript. RR synthesised the branched fatty acids and AR carried out cell culture and anticancer testing.

**ACKNOWLEDGEMENTS**

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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