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The chain length of biologically produced (*R*)-3-hydroxyalkanoic acid affects biological activity and structure of anti-cancer peptides

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ABSTRACT

Conjugation of DP18L peptide with (*R*)-3-hydroxydecanoic acid, derived from the biopolymer polyhydroxyalkanoate, enhances its anti-cancer activity (O'Connor et al., 2013. Biomaterials 34, 2710–2718). However, it is unknown if other (*R*)-3-hydroxyalkanoic acids (R3HAs) can enhance peptide activity, if chain length affects enhancement, and what effect R3HAs have on peptide structure. Here we show that the degree of enhancement of peptide (DP18L) anti-cancer activity by R3HAs is carbon chain length dependent. In all but one example the R3HA conjugated peptides were more active against cancer cells than the unconjugated peptides. However, R3HAs with 9 and 10 carbons were most effective at improving DP18L activity. DP18L peptide variant DP17L, missing a hydrophobic amino acid (leucine residue 4) exhibited lower efficacy against MiaPaCa cells. Circular dichroism analysis showed DP17L had a lower alpha helix content and the conjugation of any R3HA ((*R*)-3-hydroxyhexanoic acid to (*R*)-3-hydroxydodecanoic acid) to DP17L returned the helix content back to levels of DP18L. However (*R*)-3-hydroxyhexanoic did not enhance the anti-cancer activity of DP17L and at least 7 carbons were needed in the R3HA to enhance activity of D17L. DP17L needs a longer chain R3HA to achieve the same activity as DP18L conjugated to an R3HA. As a first step to assess the synthetic potential of polyhydroxyalkanoate derived R3HAs, (*R*)-3-hydroxydecanoic acid was synthetically converted to (±)3-chlorodecanoic acid, which when conjugated to DP18L improved its antiproliferative activity against MiaPaCa cells.

1. Introduction

Polyhydroxyalkanoates (PHA) are a broad range of biological polymers with monomeric components ranging from (*R*)-3-hydroxypropionic acid to (*R*)-3-hydroxytetradecanoic acid (Chen, 2009; Grage et al., 2009; Steinbüchel and Valentin, 1995). PHA can be hydrolyzed chemically and the resultant (*R*)-3-hydroxyalkanoic acids can be purified by column chromatography (Ruth et al., 2007). Previously, we showed that the anti-proliferative activity of a cationic D-peptide DP18L was significantly enhanced by conjugation with polyhydroxyalkanoate (PHA) derived (*R*)-3-hydroxydecanoic acid (R10) and that conjugated peptides were more potent against cancer cells compared to fibroblasts and HUVECs (O'Connor et al., 2013). This enhancement is mediated through greater uptake of the acylated peptide into the cancer cell (O'Connor et al., 2013). We also demonstrated that the (*R*)-3-hydroxyalkanoic acid-conjugated peptide localised to the mitochondria and induced the intrinsic pathway of apoptosis. Finally the inhibition of cell proliferation against a broad range of cancer cells was not observed for R3HAs at concentrations up to 2 mM (O'Connor et al., 2013) and thus R3HAs alone are not able to inhibit the growth of cancer cells at concentrations 2 orders of magnitude higher than the concentrations tested with peptide-R3HA conjugates.

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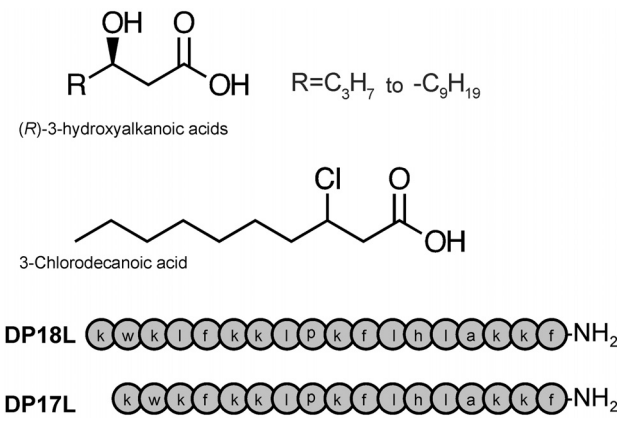

Fig. 1. (*R*)-3-hydroxyalkanoic acids ((*R*)-3-hydroxyhexanoic acid to (*R*)-3-hydroxydodecanoic acid), 3-chlorodecanoic acid, and peptides (DP18L-D-amino acid peptide, DP17L-D-amino acid peptide; L deletion at residue 4) used in the current study.

PHAs monomer ((*R*)-3-hydroxyalkanoic acid) composition is diverse with the most common easy to produce PHAs having between 4 and 12 carbons and often times the polymer is a random copolymer made up of a variety of (*R*)-3-hydroxyalkanoic acids (Steinbüchel and Valentin, 1995). In this investigation we looked at the potential of a range of (*R*)-3-hydroxyalkanoic acid for peptide biological activity enhancement (Fig. 1). We explored the effect of carbon chain length of the (*R*)-3-hydroxyalkanoic acid on peptide activity enhancement and the effect on peptide alpha helix content which is known to affect its biological activity. In doing so we are bringing together biological and synthetic technologies to synthesize a new biomolecule, 3-chlorodecanoic acid, which was subsequently conjugated to a peptide (DP18L) to assess its anti-proliferative activity against cancer cells. Pancreatic cancer cells (MiaPaCa) were selected in the current study due to limited therapies available to treat this disease, poor prognosis, and (*R*)-3-hydroxydecanoic acid acylated peptide exhibited good activity with these cells in our previous study (O'Connor et al., 2013).

2. Materials and methods

2.1. Reagents

Materials and reagents were purchased from commercial suppliers and were used without further purification unless otherwise stated. GC and HPLC grade solvents: methanol (MeOH), chloroform ($CHCl_3$), tetrahydrofuran (THF), ethyl acetate (EtOAc), methyl tert-butyl ether (MTBE), acetonitrile (ACN) for PHA monomers production were supplied by Fisher, Ireland. Silica-based reversed phase chromatography gel (75 μm COSMOSIL-C18-OPN) for separation of (*R*)-3-hydroxyalkanoic acids (R3HAs) was provided by Nacalai Tesque, Japan.

Protected amino acids and 4-methylbenzhydrylamine hydrochloride salt resin (Rink-amide MBHA resin) for peptide synthesis were obtained from Novabiochem (Merck Biosciences, United Kingdom). Peptide grade solvents: N-methyl-2-pyrrolidone (NMP), dichloromethane (DCM), dimethylformamide (DMF), reaction vessel filters and in-line filters for the peptide synthesizer (Applied Biosystems 433A) were purchased from Applied Biosystems (Warrington, United Kingdom). Cell culture based experiments were carried out with medium and components purchased from Sigma–Aldrich (Dublin, Ireland).

2.2. Production and characterization of polyhydroxyalkanoate and (R)-3-hydroxyalkanoic acids

Nitrogen limited minimal salts medium (MSM) was used for growth of bacteria to produce PHA (Schlegel et al., 1961). In order to produce the range of R3HAs required for this study, *Pseudomonas putida* KT2440 was supplied with fatty acids ranging from hexanoic acid to dodecanoic acid in a 5 L stirred tank reactor under conditions of PHA accumulation (Hume et al., 2009). The culture was harvested at 48 h using a continuous flow centrifuge (Heraeus Contifuge Stratos, ThermoFisher Scientific, Germany) at 4 °C and 25,040 × *g* in a titanium rotor (HCT 22.300, ThermoFisher Scientific, Germany). The resulting cell pellet was first frozen at −20 °C for 2–3 h, then transferred to a −80 °C freezer overnight, and lyophilised in a freeze dryer (Labconco, USA). The PHA polymer was extracted from dried cells with acetone at room temperature for 24 h (Elbahloul and Steinbüchel, 2009). The PHA polymer was subjected to acidic methanolysis (de Roo et al., 2002) and analyzed for R3HA content on an Agilent 6890N GC–MS fitted with a 5973 series inert mass spectrophotometer and a HP-1 column (12 m × 0.2 mm × 0.33 μm) (Hewlett-Packard), with an oven method of 50 °C for 3 min, increasing by 10 °C min^{-1} to 250 °C and holding for 1 min. Split ratio of 1:10 was used with helium as the carrier gas (Lageveen et al., 1988; Ruth et al., 2007).

Individual (*R*)-3-hydroxyalkanoic acids were obtained by saponification of the methyl esters mixture and subsequent preparative reversed-phase column chromatography as described by Ruth et al. (2007).

The mass of obtained individual R3HAs was confirmed by mass spectrometry on an Electron Spray Ionization MS (ESI-MS) on a Waters Micromass LCT (Massachusetts, USA). Chemical structures were identified by nuclear magnetic resonance (NMR) spectroscopy. ^{1}H and ^{13}C NMR spectra were obtained in deuterated chloroform ($CDCl_3$) with a Varian NMR system 400 MHz or 200 MHz spectrometer, respectively. For ^{1}H NMR, the following abbreviations were used: s, singlet; d, doublet; t, triplet and m, multiplet. MestRenova 5.1 (MestReLabResearc, Santiago de Compostela, Spain) programme was used to analyze ^{1}H and ^{13}C NMR spectra.

2.3. Synthesis and characterization of 3-chlorodecanoic acid

Synthesis of 3-chlorodecanoic acid is based on previously reported procedure (Amano et al., 2003) with extensive adaptations to suit biologically derived material.

Benzyl-3-hydroxydecanoate: To the solution of (*R*)-3-hydroxydecanoic acid (200 mg, 1.06 mmol) in toluene (1 ml), *p*-toluenesulfonic acid (10 mg) and benzyl alcohol (300 μl, 2.8 mmol) were added and the reaction mixture was heated at 80 °C for 18 h. Upon completion, the reaction mixture was exposed for 1.5 h to high vacuum (2 mm Hg) with heating at 60 °C to remove benzyl alcohol. Residual mixture was then dissolved in 100 ml dichloromethane (DCM) and extracted with an equal volume of H_2O. Organic phase was dried with $MgSO_4$ and the solvent evaporated under reduced pressure. Dry material (480 mg) was further purified using silica dry-flash chromatography using eluent system chloroform:methanol 98:2, yielding 220 mg (74%) of benzyl-3-hydroxydecanoate.

Benzyl-3-chlorodecanoate: To the solution of benzyl-3-hydroxydecanoate (220 mg; 0.791 mmol) in 1.7 ml diisopropyl ether (DIPE), triethylamine (17 μl; 0.12 mmol) was added and the mixture was cooled in ice bath. Thionylchloride ($SOCl_2$) (135 μl; 1.85 mmol) was then slowly added and the reaction mixture was stirred at −10 °C for 10 min. Subsequently, the mixture was gradually heated to 75 °C during 6 h and left at 75 °C for additional 10 h. Upon completion of the reaction, the mixture was dissolved

in DCM and washed with H_2O, $NaHCO_3$ (saturated solution in H_2O) and brine. Organic phase was then evaporated to dryness under reduced pressure. Dried material was further purified by dry-flash silica chromatography using petroleum ether:ethyl acetate 9:1 eluent system. Approximately 125 mg (53%) of pure benzyl-3-chlorodecanoate was obtained.

3-Chlorodecanoic acid: Hydrogenation of benzyl-3-chlorodecanoate (80 mg, 0.269 mmol) was carried out in methanol (20 ml) in Parr bottle using Pd-C (10 mg, 5%, w/v) and under pressure of 40 psi at room temperature for 24 h. Upon completion of hydrogenation, the reaction mixture was filtered and dried under reduced pressure. Dry-flash chromatography using the following eluent system: petroleum ether:acetone:formic acid 80:20:0.5 was used to further purify the acid. Approximately 56 mg (99% yield) of 3-chlorodecanoic acid was obtained.

2.4. Peptide synthesis, purification and characterization

All peptides (Table S2) were synthesized by standard solid phase peptide synthesis (SPPS) (Merrifield, 1986) on a 433A Peptide Synthesizer (Applied Biosystems, Warrington, UK). The *N*-(9-fluorenyl) methoxy carbonyl (Fmoc) chemistry was used which allows for an amino acid deprotection in very mild basic conditions created by piperidine (Merrifield, 1986). Peptides with >95% purity, as assessed by analytical reverse phase high performance liquid chromatography, were characterized by Matrix Assisted Laser Desorption Ionization–tandem Time of Flight Mass Spectrometry on an AB SCIEX 4800 Plus MALDI-TOF/TOF MS (AB Sciex, Massachusetts, USA) using α-cyano-4-hydroxy-cinnamic acid matrix.

A respective R3HA (R6–R12) was conjugated to a peptide through an amide bond between the carboxyl group of the fatty acid and the N-terminal amino of a resin-bound peptide as previously described (Falchi et al., 2000). The resin was allowed to swell for 2 h in 5 ml of dimethylformamide (DMF) with agitation at 200 rpm. The solvent was then replaced with DMF (5 ml) containing 0.4 mmol of a respective R3HA (R6–R12), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM; 0.08 g, 0.3 mmol) and N,N-diisopropylethylamine (DIEA; 75 μl, 0.6 mmol). The reaction was allowed to proceed for 30 min with agitation at 200 rpm. The resin was washed twice with 5 ml DMF (2 min) and then twice with 5 ml of dichloromethane (DCM; 2 min). Qualitative Kaiser test (Kaiser et al., 1970), used to detect free amine groups, was carried out to determine success of conjugation (negative Kaiser test). Resin-bound peptide-R3HA conjugate was further processed and characterized as described above.

2.5. Cell culture experiments

Human pancreatic carcinoma (MiaPaCa) and human lung fibroblast (MRC5) cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% penicillin/streptomycin (50 U ml^{-1}), 1% L-glutamine (2 mM), and 10% heat-inactivated fetal bovine serum (FBS). Cell lines were routinely tested for the presence of mycoplasma using MycoAlert detection kit (Lonza, UK).

Cell viability was tested by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as previously described (Hansen et al., 1989). Assays were carried out after 48 h of cell incubation in the media containing test compounds at concentrations ranging from 200 to 0.2 μM depending upon compounds being tested. In the case when R3HAs were used, concentration range was and 2.5–0.25 mM. The percentage viability values were plotted against the log of concentration and a sigmoidal dose response curve was calculated by non-linear regression analysis using Graphpad Prism software version 5.0 for Windows (Graphpad Software, CA, USA). From these curves, IC_{50} values were obtained.

2.6. Circular dichroism studies

Circular dichroism was used in order to investigate the secondary structure of peptides. Spectra were recorded in the far UV region (185–260 nm) on a JASCO J-810 spectropolarimeter (Jasco Corp., Maryland, USA), using a 0.1 cm quartz cuvette (Hellma, USA). The instrument was first flushed for 10 min with pure nitrogen gas to avoid strong absorption of light by oxygen at these wavelengths. Peptide samples were dissolved in 20 mM phosphate buffer (pH 7) and 2,2,2-trifluoroethanol (TFE) at the following concentrations of TFE: 0%, 10%, 30%, 50%, 70%. While the peptide solutions were prepared to be at 30 μM concentration, the concentration of each sample was calculated using the Beer–Lambert law ($A = \varepsilon cl$). The extinction coefficient $\varepsilon = 5500$ [cm^{-1} M^{-1}] was calculated by EXPASY online tool and the UV absorbance (A) of an analysed sample was measured at 280 nm, on a Nanodrop ND-1000 spectrometer (Thermo Scientific, Ireland). CD spectra were recorded at 20 °C, using a response time of 2 s, a scanning speed of 50 nm min^{-1}, a bandwidth of 1 nm, and data pitch of 0.2 nm. A minimum of 8 scans were accumulated per sample. Helical content was calculated using the K2D3 online programme (k2d3.ogic.ca). The input data for the K2D3 programme were calculated as described previously (Greenfield, 2006).

2.7. Statistical analysis

For the statistical analysis, we used a *t*-test to compare between two groups. Differences among the three groups were compared with one-way analysis of variance (ANOVA) and the post hoc Tukey's honest significant difference (Tukey-HSD) test for multiple comparisons. Differences were defined as significant at $p < 0.05$.

3. Results

3.1. Characterization of (R)-3-hydroxyalkanoic acids (R3HA), peptides and R3HA-peptide conjugates

Seven (*R*)-3-hydroxyalkanoic acids were produced and purified from biotechnologically obtained biopolymer (PHA) from *Pseudomonas putida* KT2440 using a previously described method for the fermentation and purification of (*R*)-3-hydroxyalkanoic acids (O'Connor et al., 2013; Ruth et al., 2007). The mass, structure and purity of all the (*R*)-3-hydroxyalkanoic acids were confirmed by MS, ^1H NMR and ^{13}C NMR (Table S1, File S1). (*R*)-3-hydroxyhexanoic acid (R6), (*R*)-3-hydroxyheptanoic acid (R7), (*R*)-3-hydroxyoctanoic acid (R8) were liquid and yellow in color (honey-like liquid), whereas (*R*)-3-hydroxynonanoic acid (R9), (*R*)-3-hydroxydecanoic acid (R10), (*R*)-3-hydroxyundecanoic acid (R11), and (*R*)-3-hydroxydodecanoic acid (R12) were in a solid state at room temperature with off-white to yellowish color. The molecular weights of individual and R3HA conjugated peptides were calculated according to their composition and confirmed through MALDI-TOF mass spectrometry (Table S2 and File S2).

3.2. In-vitro cytotoxicity of DP18L peptide and its (R)-3-hydroxyalkanoic acid conjugates

We have previously reported that conjugation of (*R*)-3-hydroxydecanoic acid to DP18L peptide improves the peptide's anti cancer activity in vitro against range of cancer cell lines (O'Connor et al., 2013). The cancer type with a poor prognosis and one of the lowest IC_{50} values for the R3HA-peptide conjugate was MiaPaCa (pancreatic) cells (O'Connor et al., 2013). Thus, we investigated the

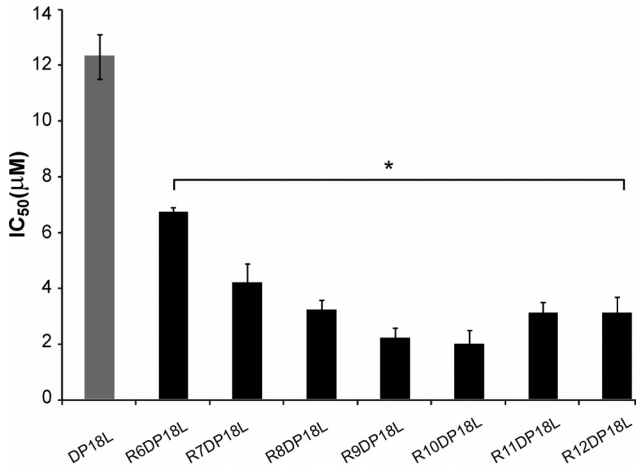

Fig. 2. IC_{50} values (μM) for synthetic peptide DP18L( ) and (R)-3-hydroxyalkanoic acid conjugates (■) incubated with MiaPaCa (pancreatic cancer). R6 = (*R*)-3-hydroxyhexanoic acid, R7 = (R)-3-hydroxyheptanoic acid etc. (* denotes $p < 0.05$).

effect of altering carbon chain length on the activity of peptide-(*R*)-3-hydroxyalkanoic acid conjugates (PHACs) with MiaPaCa cells. The IC_{50} value for unconjugated DP18L was 12.7 μM for MiaPaCa cells. Conjugation of individual (*R*)-3-hydroxyalkanoic acid, with different carbon chain length (R6–R12), to DP18L generated conjugates that reduced cell viability (Fig. 2). An incremental increase in anti-proliferation activity of the R3HA conjugated peptide occurred when the chain length of the (*R*)-3-hydroxyalkanoic acid was increased from 6 carbons up to 10 carbons (Fig. 2). This suggests that the addition of the (*R*)-3-hydroxyalkanoic acid is important for activity enhancement of the peptide against both cell lines but that enhancement is carbon chain length-dependent for MiaPaCa cells. Finally IC_{50} values for the range of R3HAs tested with MiaPaCa cells were above 1500 μM. R3HAs also inhibit the proliferation of normal (fibroblast – MRC5) cells at high concentrations (>1250 μM) (Table S3) indicating that R3HAs inhibit the growth of normal cells at concentrations 2 orders of magnitude higher than conjugated peptides.

The hydrophobicity of peptides is important for biological activity (Huang et al., 2011; Matsuzaki, 2009). By removing a hydrophobic amino acid (4th leucine) in DP18L we generated a peptide DP17L with a hydrophobic ratio of 47% compared to 50% for DP18L, while total net charge (+8) remains the same both peptides. DP17L exhibited less cytotoxicity against MiaPaCa cell lines

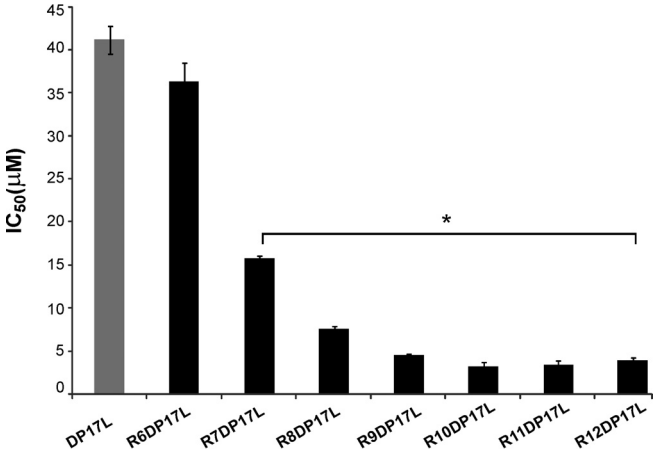

Fig. 3. IC_{50} values (μM) for synthetic peptide DP17L( ) and (*R*)-3-hydroxyalkanoic acid conjugates (■) incubated with MiaPaCa (pancreatic cancer) cells. R6 = (*R*)-3-hydroxyhexanoic acid, R7 = (*R*)-3-hydroxyheptanoic acid etc. (* denotes $p < 0.05$).

Table 1
Alpha-helical content (%) of DP18L, DP18L derivatives and (*R*)-3-hydroxyalkanoic acid-peptide conjugates.

Peptide	0% TFE	30% TFE	50% TFE	70% TFE
DP18L	3.18	48.3	42.1	38.8
DP17L	3.20	31.5	32.5	31.8
R10DP18L	2.97	46.9	44.3	46.9
R6DP18L	2.88	47.0	47.9	44.5
R10DP17L	4.60	45.1	40.9	38.8

compared to DP18L with IC_{50} values of 42 μM (Fig. 3) compared to 13 μM for DP18L (Fig. 2).

The conjugation of DP17L with (*R*)-3-hydroxyhexanoic acid did not significantly reduce viability of MiaPaCa cell lines (Fig. 3). However, increasing carbon chain length of the 3-hydroxyalkanoic acid up to 10 carbons resulted in progressively lower IC_{50} values following treatment with the conjugates, compared to DP17L alone (Fig. 3). Cells exposed to R7DP17L exhibited IC_{50} values 2.6 fold lower when compared to unconjugated DP17L (Fig. 3). Cells exposed to R8DP17L exhibited a further 2.4 fold lower IC_{50} value compared to R7DP17L with MiaPaCa cells. As observed with DP18L, the conjugation of (*R*)-3-hydroxydecanoic acid resulted in the greatest reduction in cell viability (Figs. 2 and 3; File S3). Conjugation of (*R*)-3-hydroxyalkanoic acids not only brought IC_{50} values back in line with DP18L but reduced them so they were similar to R10DP18L.

3.3. *Effect of peptide modification and (R)-3-hydroxyalkanoic acid conjugation on helical content of peptides*

The helical content of anti-microbial and anti-cancer peptides is reported to be important for biological activity (Chu-Kung et al., 2010; Huang et al., 2011; Matsuzaki, 2009; Riedl et al., 2011). Given the effect of peptide compositional change and (*R*)-3-hydroxyalkanoic acid conjugation on the activity of DP18L, the helical content of various peptides and peptide R3HA conjugates were chosen for analysis. As cationic peptides such as DP18L are able to bind membranes we dissolved peptides in this study in 2,2,2-trifluoroethanol (TFE) which is the most commonly used co-solvent for mimicing the membrane environement which promotes the formation of helices in peptides (Table 1) (Kumaran and Roy, 1999; Luo and Baldwin, 1997). Altering the concentration of TFE allows us to examine the effect of changing the hydrophobicity of the environment on peptide structure. Circular dichroism spectra were recorded and mean residue elipticity (MRE) calculated as described previously to determine helicity of the peptides and peptide R3HA conjugates (Greenfield, 2006). The online K2D3 program was used to calculate helical content of the peptides (Table 1).

All tested peptides formed a random coil in phosphate buffer (0% TFE). DP18L was composed of 48.3% helices in 30% TFE (Table 1). Interestingly, the peptide lost only 10% of its helical content at 70% TFE suggesting it is a highly stable structure in a hydrophobic environment. DP17L, which exhibited the highest IC_{50} values with MiaPaCa cells had the lowest helical content compared to other peptides (Table 1). The helical content of conjugated peptide R10DP17L was higher than the unconjugated peptides DP17L but similar to DP18L (Table 1). However, the R6DP18L had the same helical content as R10DP18L (Table 1). The structure and activity of DP18L is affected by deletion or alteration of amino acids and the reversal of the structural change occurs when (*R*)-3-hydroxyalaknaoic acids are conjugated to the peptide (Figs. 2 and 3, Table 1). However the hydrophobicity of the (*R*)-3-hydroxyalkanoic acid affects the degree to which peptide activity is altered (Figs. 2 and 3). Thus structural change is insufficient to explain the effect on biological activity and a combination

of hydrophobicity and alpha helix content is needed to observe altered peptide activity.

3.4. Chemical modification of (R)-3-hydroxyalkanoic acid to generate 3-chlorodecanoic acid and conjugation to DP18L

It is estimated that 20% of all pharmaceutical small molecule drugs are halogenated and 53% of all halogenated marketed products are chlorinated compounds (Shin et al., 2001). Among the compounds synthesized in this work, R10-hybrid peptide conjugates were amongst the most active against cancer cells. The presence of the hydroxyl moiety on the R10 offers the possibility for further synthetic manipulation. This also allowed us to test the hypothesis that bio-based (*R*)-3-hydroxyalkanoic acids could be used as synthons. Thus (*R*)-3-hydroxydecanoic acid was transformed to 3-chlorodecanoic acid and subsequently conjugated to DP18L to see if this modification could improve DP18L activity. Since the oxygen atom is slightly more electronegative than the chlorine atom (3.44 versus 3.16 according to the Pauling scale), we expected the C–Cl bond to be less polar than the C–O bond and potentially have an effect on the anti-cancer activity of R10DP18L. The 3-chlorodecanoic acid was also tested on its own to assess its anti-proliferative potential.

3-Chlorodecanoic acid (Cl10) was synthesised from (*R*)-3-hydroxydecanoic acid and its structure confirmed by ^{1}H NMR and ^{13}C NMR. The results of the ^{1}H NMR (200 MHz, $CDCl_3$) of Cl10 were as expected (File S1): 10.8 (broad s, 1H); 4.20–4.40 (m, 1H); 2.80 (d, 2H, $J = 7$); 2.75–2.90 (m, 2H); 1.70–1.85 (m, 2H); 1.20–1.38 (m, 10H); 0.89 (t, 3H, $J = 6.8$). Peaks observed in ^{13}C NMR (100 MHz, $CDCl_3$) of Cl10 were also as expected (File S1): δ 14.06 (CH3), 22.61 (CH2), 26.27 (CH2), 28.89 (CH2), 29.08 (CH2), 31.72 (CH2), 37.98 (CH2), 57.37 (CH2), 110.01 (CH), 175.53 (C). Specific rotation α_D of the compound was −4.5 indicating that obtained 3-chlorodecanoic acid was a mixture of *R* and *S* isomers. 3-Chlorodecanoic acid was conjugated to the DP18L peptide and the expected molecular mass of *m*/*z* 2489 was confirmed by MALDI-TOF MS (File S2).

3.5. Anti-proliferative activity of 3-chlorodecanoic acid and DP18L conjugated with 3-chlorodecanoic acid

3-Chlorodecanoic acid (Cl10) exhibited an IC_{50} value of 950 μM for MiaPaCa which was 2.2 fold lower compared to R10 (Table S3). Conjugation of 3-chlorodecanoic acid to DP18L resulted in the IC_{50} value of 3.5 μM for MiaPaCa cells which is 1.8 fold higher when compared to IC_{50} values of R10DP18L (Fig. 2).

4. Discussion

We have shown that the conjugation of a range of (*R*)-3-hydroxyalkanoic acids enhanced the ability of DP18L to reduce cell viability in tumour cell lines and the increase in carbon chain length coincided with increased anti-proliferative activity up to 9 and 10 carbons for MiaPaCa cells. DP18L when conjugated with (*R*)-3-hydroxyhexanoic acid exhibited improved cytotoxicity towards MiaPaCa cells. The presence of a single additional carbon in (*R*)-3-hydroxyheptanoic acid resulted in enhancement of activity of the peptide but DP17L suggesting that the threshold for enhancement of DP17L peptide activity against cancer cells by (*R*)-3-hydroxyalkanoic acids is 7 carbons (Figs. 2 and 3). Glukhov et al. have shown that a cationic anti-microbial peptide has to reach a 'hydrophobicity threshold' in order to span a membrane (Glukhov et al., 2008). DP17L required (*R*)-3-hydroxyheptanoic acid conjugation for improved cytotoxicity. Given that DP17L is less hydrophobic than DP18L, then DP17L may need a more hydrophobic (*R*)-3-hydroxyheptanoic acid to reach a hydrophobic threshold.

Helicity of the peptide is an important physicochemical parameter that controls cell selectivity and peptide activity (Huang et al., 2011; Matsuzaki, 2009). The improvement of biological activity of the peptides in the current study through conjugation of (*R*)-3-hydroxyalkanoic acids coincided with an increase in alpha helicity. However, the enhancement of biological activity of DP18L and its variants through conjugation of R3HAs is not fully explained by increased helix content e.g. despite R10DP17L, R6DP18L and DP18L having the same helix content, the IC_{50} of the R10DP17L is lower than that of DP18L while that of R6DP18L is higher. One can argue that (*R*)-3-hydroxyalkanoic acid is stabilizing the peptide as N-acylation of the Cationic anti-microbial peptide (CAP) SC4 with non-hydroxyaltaed fatty acids lauric (C12) or stearic (C18) acid caused a 30 fold increase in antibacterial activity and it was suggested that this acylation stabilized the α-helical structure of the peptide in membrane mimicking conditions (Lockwood et al., 2004). The secondary structure of AKK peptide was also stabilized upon conjugation of C12, C14, C16 or C18 fatty acids (Chu-Kung et al., 2010). There is no report of the general effect of (*R*)-3-hydroxyalkanoic acids on peptide activity.

A helical wheel representation of DP18L suggests that the relative position of amino acids within the structure of DP18L is affected by the presence of an (*R*)-3-hydroxyalkanoic acid (Fig. 4). The 4th leucine of DP18L lies on the hydrophobic face of the peptide (Fig. 4) and so disruption of that region could explain the high IC_{50} value observed for DP17L. Attaching a hydrophobic moiety to that face could again explain the reversal of the loss of helical content.

Circular dichroism analysis indicates that the helical content of the peptide is not altered significantly when DP18L is conjugated to (*R*)-3-hydroxyalkanoic acids. However, the two observations are not mutually exclusive where the secondary structure of the peptide is unaffected but the way the peptide behaves in the membrane environment could be altered. Rosenfeld et al. reported that addition of hexanoic acid (C6) or octanoic acid (C8) to K_5L_7 resulted in enhanced antibacterial activity without any major changes to the structure of the peptide (Rosenfeld et al., 2010). Peptides conjugated with fatty acids can aggregate which can possibly lead to enhancement of peptide activity (Zweytick et al., 2011), but can also lead to loss of the activity (Chu-Kung et al., 2010).

The presence of the hydroxyl moiety on 3-hydroxyalkanoic acids offers an advantage over conjugation of the non-hydroxylated alkanoic acid as it allows for further chemical manipulation and potential anti-proliferative activity enhancement. Approximately 10% of all pharmaceutical small molecule drugs are chlorinated (Meyer et al., 2011). (*R*)-3-hydroxydecanoic acid was used to synthesise 3-chlorodecanoic acid based on its superior peptide-activity enhancement characteristics. The C–Cl bond was expected to be less polar than the C–O bond and potentially have an effect on the anti-cancer activity of R10DP18L. However, the IC_{50} value of Cl10DP18L was higher than that of R10DP18L. The difference in polarity between C–O and C–Cl bonds is perhaps too small to affect the overall hydrophobicity of R10DP18L. The conjugation of other moieties through the activated beta carbon of (*R*)-3-hydroxydecanoic acid will be attempted in the future to further improve the cytotoxicity of DP18L and its derivatives.

The degree of enhancement of peptide anti-proliferative activity against pancreatic cancer cells (MiaPaCa), through conjugation of R3HAs, is dependent upon carbon chain length of the (*R*)-3-hydroxyalkanoic acid. The biological activity and helix content of DP18L was affected by altering amino acid composition but that structural change can be reversed by conjugation of R3HAs. However hydrophobicity of the peptide and the R3HA is another factor affecting peptide biological activity. We synthesised for the first time (±)3-chlorodecanoic acid from (*R*)-3-hydroxydecanoic acid which, when conjugated to DP18L enhanced biological activity but not more than when DP18L was conjugated to

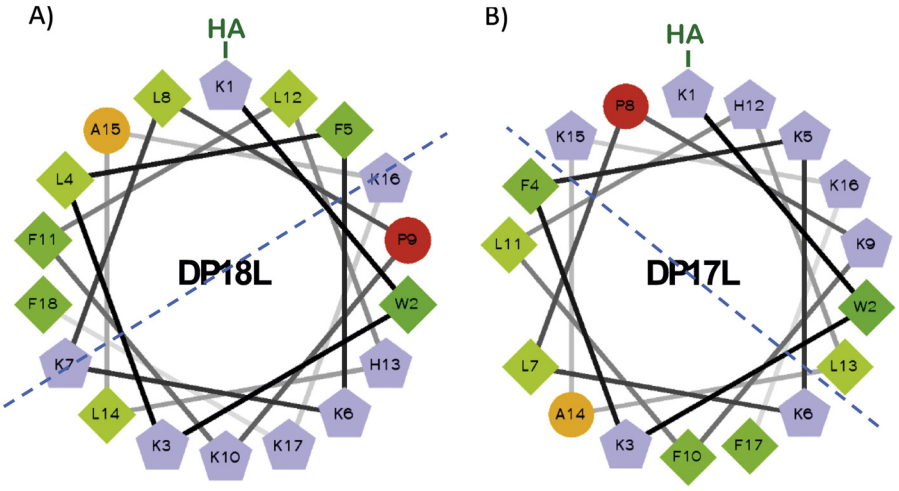

Fig. 4. Amino acid sequences of (A) DP18L and (B) DP17L plotted on a helical wheel. Hydrophobic residues are shown in green, positively charged residues are shown in grey and proline in red. 'HA' in green indicates (*R*)-3-hydroxyalkanoic acids attached to the N terminus of a peptide (K1 residue). Dashed blue line separates hydrophobic and cationic faces of the peptides (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.).

(*R*)-3-hydroxydecanoic acid. The diversity of PHA based R3HAs combined with synthetic chemistry can offer further possibilities for the generation of many new bio-synthetic compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2015.02.036.

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