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The anti-cancer activity of a cationic anti-microbial peptide derived from monomers of polyhydroxyalkanoate

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ABSTRACT

The biodegradable polymer medium chain length polyhydroxyalkanoate (mclPHA), produced by *Pseudomonas putida* CA-3, was depolymerised and the predominant monomer (*R*)-3-hydroxydecanoic acid (R10) purified. R10 was conjugated to a D-peptide DP18 and its derivatives. All peptides conjugated with R10 exhibited greater anti-cancer activity compared to the unconjugated peptides. Unconjugated and conjugated peptides were cytocidal for cancer cells. Conjugation of R10 to peptides was essential for enhanced anti-proliferation activity, as unconjugated mixes did not result in enhancement of anti-cancer activity. The conjugation of R10 resulted in more rapid uptake of peptides into HeLa and MiaPaCa cells compared to unconjugated peptide. Both unconjugated and R10 conjugated peptides localized to the mitochondria of HeLa and MiaPaCa cells and induced apoptosis. Peptide conjugated with a terminally hydroxylated decanoic acid (ω-hydroxydecanoic acid) exhibited 3.3 and 6.3 fold higher IC_{50} values compared to R10 conjugated peptide indicating a role for the position of the hydroxyl moiety in enhancement of anti-cancer activity. Conjugation of decanoic acid (C10) to peptides resulted in similar or higher IC_{50} values compared to R10 conjugates but C10 conjugates did not exhibit any cancer selectivity. Combination studies showed that R10DP18L exhibited synergy with cisplatin, gemcitabine, and taxotere with IC_{50} values in the nanomolar range.

1. Introduction

Polyhydroxyalkanoates are well-studied biodegradable polymers produced by bacteria. PHAs can be divided into two broad groups short chain length PHA (sclPHA) and medium chain length PHA (mclPHA) [1,2]. The monomers of sclPHAs are made up of between three to five carbons while the monomers of mclPHAs are between 6 and 12 carbons long [1,3]. The medical applications of PHAs have focused on sutures, drug delivery, and tissue engineering scaffolds [4,5]. However no studies have investigated the biomedical application of PHA monomers or their effect on peptide activity. We show for the first time that the application of these biopolymers extends beyond their material properties.

Antimicrobial peptides, also components of biomaterials, are found in many species (e.g. insects, fish, amphibians and mammals) and play an important role in host innate immunity to microbial pathogens [6–8]. While initially studied for their antibacterial properties, peptides have become the focus of increased research activity as anti-cancer agents [9]. The cytotoxic effect of cationic antimicrobial peptides (CAPs) on microorganisms and neoplastic cells is largely believed to be a function of their amphipathic nature

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and secondary structure [10,11]. CAPs are a class of natural macromolecules and their synthetic analogues that exhibit selective cytotoxicity against a broad spectrum of human cancer cells, including neoplastic cells that have acquired a multidrug resistant phenotype [11,12]. P18 (KWKLFKKIPKFLHLAKKF) is a hybrid L-peptide of the amino-terminal α-helical region of the 37 amino acid silk moth peptide, Cecropin A, and the amino-terminal α-helical hydrophobic region of the 23 amino acid African claw frog peptide Magainin 2 [13,14]. P18 displays cytotoxic activity against human cancer cell lines (e.g. MDA-MB-231 and Jurkat), but does not exhibit any significant haemolysis[13].

D-peptides are known to be more resistant to biological degradation *in vivo* [9,15]. With a view to developing improved and novel anti-cancer peptides, we synthesized for the first time the D-form of P18, modified its composition through conjugation of the lipophilic small molecule (*R*)-3-hydroxydecanoic acid, and investigated the anti-proliferative activity against human cancer cell lines. The conjugation of lipophilic molecules to drug compounds has been well described [16]. Conjugation of fatty acids to known anti-tumour chemotherapeutic agents resulted in improved killing potential when administered in a conjugated form [16]. However, the use of (*R*)-3-hydroxyalkanoic acids to enhance the anti-cancer activity of peptides or other potential drug candidates has not been described. The conjugation of an hydroxylated fatty acid ((*R*)-3-hydroxydecanoic acid) offers an advantage over conjugation of a fatty acid as the former contains a chiral center which can be used as a synthetic chemistry focal point for future modification of the drug molecule.

In this study, we use the monomers of the biodegradable polymer mclPHA to enhance the anti-cancer activity of a peptide and investigate the mechanism of enhanced activity. We also demonstrate synergy between the most active R10 peptide conjugate and known cancer chemotherapy drugs.

2. Materials and methods

2.1. Production of PHA, (R)-3-hydroxyalkanoic acids, peptides and peptide derivatives

PHA was synthesized by supplying glucose to *Pseudomonas putida* CA-3 in a stirred tank reactor for 48 h at 30 °C under conditions of nitrogen limitation and carbon excess (ratio C:N 18:1) [17]. Cells were lyophilized in order to remove any water and the PHA polymer was extracted in acetone at room temperature for 18 h–24 h [18]. The PHA was methanolyzed and the resulting methylated monomer samples were analysed on an Agilent 6890N gas chromatograph fitted with 5973 series inert mass spectrophotometer (Agilent Technologies, Santa Clara, USA) equipped with a BP21 capillary column (25 m by 0.25 mm, 0.32-μm film thickness; SGE Analytical Sciences) with temperature programming (120 °C for 5 min; temperature ramp of 3 °C per min; 180 °C for 10 min) to determine monomer composition [19]. The (*R*)-3-hydroxydecanoic acid monomer was purified from the biopolymer methanolysis mixture using the preparative reversed-phase column chromatography and characterized as described by Ruth and co-workers [20].

Unless otherwise specified, the D-peptide sequences were assembled by solid phase synthesis on a Rink amide-MBHA resin, yielding C-terminally amidated peptides, using Fmoc/*t*-Bu-strategy on a 433A peptide synthesizer (Applied Biosystems, Warrington, UK) as described previously [21]. Biotinylated peptides were synthesized by N-terminal modification of the sequences with Lys(ε-biotinyl) and amidation of its N^{α}-amino group with (*R*)-3-hydroxydecanoic acid. Fmoc-Lys(biotin)-OH was added to the polymer-bound, N-terminally deprotected peptides by PyBop/HOBt/DIEA coupling chemistry. The purity of the peptides was above 95%, as assessed by analytical reversed phase high performance liquid chromatography on a Varian HPLC system equipped with Phenomenex C18 Gemini column (4.6 mm D × 250 mm L; Phenomenex, Torrance, USA). Matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry on an AB Sciex 4800 Plus MALDI TOF/TOF was used to characterize all peptides with an α-cyano-4-hydroxy-cinnamic acid used as a matrix.

The conjugation of (*R*)-3-hydroxydecanoic acid (R10), decanoic acid (C10) or ω-hydroxydecanoic acid (ω10) (Fig. 1) to DP18L was through an amide bond between the carboxyl of the fatty acid and the N-terminal amino of a resin-bound peptide as previously described [22]. Briefly, 180 mg of resin primed in dimethylformamide (DMF) was placed in a solution of alkanoic acid to be conjugated (0.039 g, 0.4 mmol) in *N*-methyl-2-pyrrolidone (NMP) (5 ml). Reaction mixture also contained 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) and was stirred at ambient temperature. After 5 min of reaction the resin was isolated by filtration and washed twice with DMF (2 min, 5 ml) and twice with DCM (2 min, 5 ml). Conjugation was monitored by the qualitative Kaiser test [23].

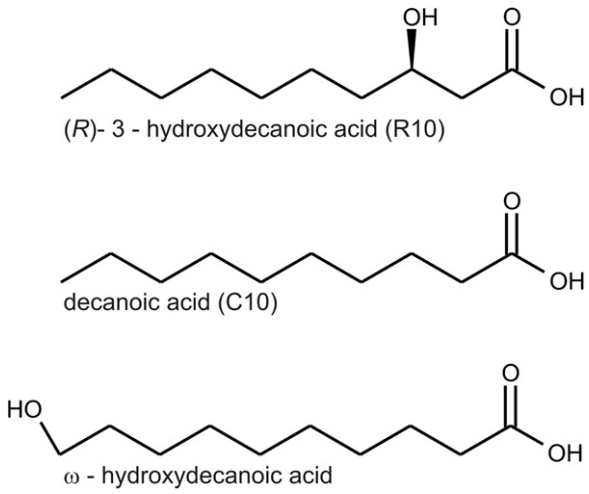

Fig. 1. Chemical structure of (*R*)-3-hydroxydecanoic acid, decanoic acid, and ω-hydroxydecanoic acid which were individually conjugated to peptides in the current study.

2.2. Cell culture

The colorectal cancer (HT-29), lung carcinoma (A549) and T-cell leukaemia (Jurkat) cell lines were maintained as monolayer cultures in RPMI-1640 supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin and 10% (v/v) foetal bovine serum (FBS) (all from Sigma, Ireland). Human glioma (SNB-19), human pancreatic carcinoma (MiaPaCa) and breast carcinoma (MDA-MB-231) cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 10% (v/v) FBS. The melanoma cell line (WM793) was originally isolated from a superficial spreading melanoma [24] and maintained in DMEM, supplemented with 10% (v/v) FBS, 50 μg/ml gentamicin and 4 μg/ml insulin (Sigma, Ireland). HeLa cells were obtained from the ATCC and maintained in DMEM supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA, Sigma), and 10% (v/v) FBS. Human umbilical vein endothelial cells (HUVEC) cells were obtained from Cambrex and maintained in an endothelial cell growth medium (Lonza, Dublin Ireland). Human Dermal fibroblast (HDF) cells were obtained from the European Collection of Cell Culture (ECACC) and were maintained in DMEM supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 10% (v/v) FBS. All cells were grown in humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.3. MTT cell viability assay

Reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by cells was assessed colorimetrically as previously described [25]. Assays were carried out after 48 h of cell incubation (72 h for combinational studies) in the media containing test compounds at concentrations ranging from 200 μM to 0.2 μM depending upon compounds being tested.

2.4. Clonogenic assay

Based on MTT assay data HeLa or MiaPaCa cells were treated at three different concentrations i.e. 10 fold lower than the IC_{50}, at the IC_{50}, and 10 fold higher than the IC_{50} value. Following treatment, cells were seeded in triplicate in 6-well plates at densities of 500 cells/well. Cells were then allowed to form colonies for approximately 2 weeks. Cells were fixed in 10% neutral-buffered formalin (Sigma, Ireland). Fixing agent was removed, and plates were allowed to air dry, before staining with a 0.25% (w/v) solution of crystal violet.

2.5. Apoptosis assay

Apoptosis was determined as per manufacturer's instructions (BD biosciences manual) using an Annexin V staining kit (BD Biosciences Ireland). Apoptosis was characterized by chromatin condensation and fragmentation when examined by