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Duramycin-porphyrin conjugates for targeting of tumour cells using photodynamic therapy

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ABSTRACT

Duramycin, through binding with phosphatidylethanolamine (PE), has been shown to be a selective molecular probe for the targeting and imaging of cancer cells. Photodynamic therapy aims to bring about specific cytotoxic damage to tumours through delivery of a photosensitising agent and light irradiation. Conjugation to biological molecules that specifically target cancer has been shown to increase photosensitiser (PS) selectivity and decrease damage to surrounding normal tissue. The aim of this study was to target tumour cells with a PE-specific PS therefore duramycin was conjugated to a porphyrin based PS which was achieved via direct reaction with the ε-amino group on the lysine residue near duramycin's N-terminal. The compound was subsequently purified using RP-HPLC and confirmed using mass spectrometry. Binding of the conjugate to ovarian and pancreatic cancer cell lines was assessed by flow cytometry. Light irradiation with a light fluence of 7.5 J/cm^2 was delivered to conjugate treated cancer cells and cell proliferation analysed by MTT assay. The conjugate detected PE on all 4 cancer cell lines in a concentration dependent manner and conjugate plus irradiation effectively reduced cell proliferation at concentrations ≥0.5 μM, dependent on cancer cell line. Reduction in cell proliferation by the irradiated conjugate was enhanced over unconjugated duramycin in A2780, AsPC-1 and SK-OV-3 ($p < 0.05$). In this study we have shown that a duramycin-porphyrin conjugate retained good binding affinity for its target and, following irradiation, reduced cell proliferation of pancreatic and ovarian cancer cell lines.

1. Introduction

Duramycin is a small tetracyclic peptide produced by the bacterium *Streptoverticillium cinnamoneus* [1]. Only 19 amino acids in length duramycin is one of very few identified, relatively, short peptides to have a defined 3-dimensional structure [2]. This structure is stable due to the presence of 3 thioether bridges formed via the unusual amino acids lanthionine and methyllanthionine. Further a covalent linkage between the cysteine at duramycin's N-terminal and an alanine residue produces lysinoalanine [3]. The strength of duramycin's structure is likely to confer its resistance to thermal and proteolytic degradation [2]. The presence of the covalent linkages also results in duramycin's stable binding site which selectively recognises the membrane phospholipid phosphatidylethanolamine (PE) through binding to its polar head group [4]. Duramycin binds to PE with high specificity with a binding molar ratio of 1:1 and a dissociation constant in the low nanomolar range [5]. The lantibiotic cinnamycin, which is structurally similar to duramycin, was shown to induce non-specific transbilayer membrane movement of phospholipids in model and cell membranes [6]. It is therefore possible that duramycin self-promotes its own binding to cells by inducing movement of PE molecules from the inner to the outer membrane. PE is an abundant membrane phospholipid which is ubiquitous in eukaryotic cells. In quiescent cells PE is predominantly located on the inner cell membrane along with phosphatidylserine [7,8]. The asymmetrical distribution of PE and phosphatidylserine is enzymatically maintained by a number of ATP-dependent aminophospholipid translocases and floppases and ATP-independent scramblases [9,10]. On activation PE can become exposed on the outer cell membrane during a number of physiologically important processes such as apoptotic and necrotic cell death, reorganisation of the membrane during cytokinesis and initiation of the coagulation cascade [11–14]. Upregulation of cell surface PE expression has been observed in cancer cells, cancer derived microparticles and on the tumour endothelium of a variety of in vitro and in vivo tumours [1,15–17]. It is well documented that upregulation of cell surface exposure of phosphatidylserine occurs after chemotherapy treatment and that this can increase the procoagulant

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activity of the tumour [18–20]. On the premise that PE and phosphatidylserine are regulated by the same mechanisms and that both are exposed during apoptosis [21] and after irradiation [1] a number of studies investigated whether PE exposure was upregulated after chemotherapy. It was demonstrated that externalisation of PE, detected by duramycin, could potentially be a marker of early apoptosis and could be utilised as a detection method for the response to chemotherapy treatment [22–24].

Photodynamic therapy (PDT) is a minimally invasive treatment that can deliver selective cytotoxic activity towards cancer [25]. PDT requires three essential components; a photosensitising agent, light and oxygen. Accumulation of the photosensitiser (PS) at the site of cancer and activation with light at a specific wavelength causes the PS to transfer energy, via its excited triplet state, to molecular oxygen which is converted from its ground triplet state to an excited singlet state. This leads to the generation of cell damaging reactive oxygen species (ROS) which ultimately causes apoptotic or necrotic cell death [25,26]. As well as its direct cytotoxic effect on cancer, PDT can have *anti*-tumour effects via induction of the inflammatory response and damage to tumour vasculature [27,28]. PDT has been approved for treatment of a variety of cancer types including premalignant and malignant skin cancers [29,30], early carcinomas of the oral cavity, pharynx, and larynx [31], cancers of the GI tract [32], prostate cancer, bladder cancer, non-small cell lung cancer and brain tumours [25]. PDT has been used as a synergistic method combined with other major therapies e.g. chemotherapy, radiotherapy and surgery [33–35] as it does not compromise the effect of the therapies and therefore can be used in conjunction without effecting future treatment options for residual or recurrent malignancy [25]. A number of PS have been approved for use in clinical oncology including m-THCP (meta-tetrahydroxyphenylchlorin, Foscan®), ALA (5-aminolevulinic acid, Levulan®) and its methyl ester (Metvix®) and porfimer sodium (Photofrin®). There are a wide range of PS still in pre-clinical and clinical trials [36,37].

Porphyrins are highly conjugated heterocyclic tetrapyrroles that were first identified as having photodynamic properties in the 1970s [38,39]. A porphyrin PS (5-[Aminobutyl-*N*-oxycarbonyl)phenyl]phenyl]-10,15,20-tris(*N*-methyl-4-pyridinium) porphyrin trichloride) plus light irradiation was able to reduce cell survival and prevent colony forming in prostate epithelial cells at concentrations of >8.75 μM [40]. The porphyrins TTP (5,10,15,20-tetra-p-tolyl porphyrin) and THNP (5,10,15,20-tetra-p-naphthyl-porphyrin) after a light irradiation dose of 4.5 J/cm^2 were able to cause cell death in a human melanoma cell line in a concentration dependent manner [37]. The main disadvantage of PDT is the low selectivity of PS to their target, which can lead to damage to surrounding normal tissue and sustained skin photosensitivity [25]. In an attempt to overcome this research in PDT in oncology has focused on developing PS's conjugated to tumour targeted biological molecules [28]. Porphyrins have been successfully conjugated to a variety of biomolecules including mAbs [26,41,42], lipoproteins [43,44] and nanoparticles [45] which allowed for more targeted delivery of PS, and therefore a more specific tissue response with limited damage to normal tissue. A number of porphyrins have been developed that conjugate to biomolecules under mild conditions through direct reaction with lysine residues [26,33,46].

The two primary amines, cysteine and lysine, at the N-terminal of duramycin offer potential sites for conjugation without interference with duramycin's binding site [3]. Using this theory a number of duramycin conjugates have been developed to take advantage of PE as a possible selective molecular probe. Fluorescent duramycin conjugates have been used to successfully target and image the lymphoma cancer cell line U937 [4], cancer derived microparticles [17], prostate tumour endothelium in rats [1] and the endothelium of aortic flow dividers [47]. Duramycin has been radiolabelled with gadolinium [3] and technetium-99 m where it was used to image cell death in a rat model of acute myocardial infarction [47–49]. The radiolabelled duramycin conjugate quickly accumulated in the PE expressing myocardium. Fast accumulation of a duramycin-PS conjugate could potentially provide an advantage over some of the current PS used in the clinical setting as a short drug-light interval could be achieved. Thus patients could receive PDT relatively quickly in the outpatient setting and would have a reduced treatment duration. In this proof of concept study we conjugated duramycin to a porphyrin based PS and assessed its ability to bind to cancer cells via cell surface PE. It has been previously shown that the level of cell surface PE expression, detected by duramycin, was increased on the pancreatic cancer cell lines AsPC-1 and CFPAC-1 when apoptotic (induced by campothecin treatment) compared to untreated viable cells and was further increased when the cells were necrotic (induced by 30% H_2O_2 treatment) [15]. As duramycin can bind to quiescent, apoptotic and necrotic cancer cells it was theorised that a duramycin conjugate could potentially provide targeted treatment of tumours at all stages of progression. We also examined its cytotoxic activity on ovarian and pancreatic cancer cell lines following irradiation with visible light.

2. Materials and Methods

2.1. Cell Culture

The cancer cell lines AsPC-1 and MIA PaCa-2 were purchased from the American Type Culture Collection (UK). The cancer cell lines A2780 and SK-OV-3 were purchased from the European Collection of Cell Cultures (UK). All cells were grown using distributors' instructions. All cells were cultured in either IMDM, McCoy's 5a Medium Modified or RPMI (Lonza, UK) substituted with 10% FBS (15% for McCoy's 5a Medium Modified) (Bio-Sera, UK) and (v/v); 100 units/mL penicillin, 100 μg/mL streptomycin (P/S) (Lonza, UK). All serum was filtered using a 0.2 μM syringe filter prior to addition to media. When not in use all media was stored between 4 and 6 °C. All cells were incubated at 37 °C in a 5% CO_2 atmosphere. Cells were cultured in tissue culture flasks (Sarstedt, UK) and removed via scraping when cells were 70–90% confluent.

2.2. Conjugation of Porphyrin to Duramycin

A solution of duramycin (Sigma-Aldrich, UK, 10 mg, 4.97×10^{-3} mmol) in DMSO (2 mL) plus trimethylamine was treated with 5-[4-(N-succinimidyloxycarbonyl)phenyl]-10,15,20-tris(4-methylpyridinium)porphyrin trichloride [50] (8 mg, 9.94×10^{-3} mmol) and the resulting solution was mixed on a rotating shaker at room temperature for 24 h. The crude material was recovered by addition of dichloromethane (5 mL) and filtration through paper. The solid was dissolved in 1 mL of 0.1% aqueous trifluoroacetic acid (TFA) and the 1:1 porphyrin/duramycin conjugate was isolated by semi-preparative HPLC and analysed by electron ionising mass spectrometry (ESI-MS) (Section 2.3). Recovery of 4.8 mg of conjugate was achieved equating to 34.4% yield. An unconjugated, "capped" porphyrin was used as a control in which the NHS ester group used for conjugation was reacted with butylamine to eliminate any non-specific conjugation [40]. All photosensitiser containing compounds were kept, protected from light, at −20 °C. Final concentrations of the re-suspended lyophilised powder of the conjugate and "capped" control were determined using UV–visible spectrophotometry.

2.3. Analyses and Purification of Duramycin-Porphyrin Conjugate

RP-HPLC-PDA analyses were performed on an Agilent 1200 HPLC system (Agilent, Germany) equipped with a G1312B BinPump SL, G13798 degasser, G1367D HiP ALS SL plus autosampler, a G1316B column compartment (set at 35 °C), and a G1315C diode array detector (acquisition rate, 80 Hz; scan rate: 380–600 nm, step: 1 nm), using a Gemini C18 column, 5 μ, 150 × 4.6 mm, 110 Å column (Phenomenex, UK), equipped with a SecurityGuard C18 (ODS) 4 × 3.0 mm ID guard