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Review article

Synthesis, photophysical and cellular characterisation of folate and methotrexate labelled luminescent lanthanide complexes

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ABSTRACT

In this work we have developed a series of highly emissive europium(III) and terbium(III) complexes tethered to either folic acid (FA) or methotrexate (MTX), with the aim of developing visual probes that enable the imaging of folate receptors in cancer cells. The synthesis, photophysical properties and cellular behaviour are reported for four new lanthanide Ln(III) complexes, where either FA or MTX are tethered to 1,4,7-tris(carbonylmethyl)-10-(4′-quinolineacetic acid, (7′-acetamido)-1′,2′-dihydro-2′-oxo)-1,4,7,10-tetraazacyclododecane Ln(III) complex, and Ln(III) = Eu(III) or Tb(III); herein referred to as **Eu-FA**, **Eu-MTX**, **Tb-FA** or **Tb-MTX**. All four complexes were found to be sensitive to the presence of the folate receptor in a range of cell lines. The MTX conjugates showed different cellular specificity in an oral adenosquamous carcinoma cell line (CAL-27) compared with the analogous FA conjugates. This suggests that it is viable to explore differences in folate receptors using folate vs. anti-folate probes, with labels that have different emissive properties (e.g. **Eu-FA** vs. **Tb-MTX**). The MTX complexes were found to be the most cytotoxic, with **Eu-MTX** showing greater cytotoxicity than free MTX or the isostructural **Tb-MTX**. This suggested that there could be a synergistic effect on toxicity for the Eu(III) chelate and the MTX components of the complex.

1. Introduction

Folates (also called vitamin B_9, folacin, pteroyl-L-glutamic acid, or pteroyl-L-glutamate) are essential for the maintenance of the human genome and cell health, due to their central role in key metabolic functions, such as RNA and DNA biosynthesis [1,2]. The critical role of folates in DNA synthesis, repair and methylation [3] has been mainly attributed to the reduced tetrahydrofolate form [4], which has an important function in rapid cell division and growth. Reduced amounts of folate have been associated with the onset of cancer, including colorectal [5], breast [6] and lung cancer, presumably due to increased DNA damage and an enhanced mutation rate [7]. Folate can be internalised into cells via at least three transport mechanisms: the reduced folate carrier (RFC) [8], which is an anion exchanger and mainly transports reduced folate; the proton-coupled folate transporter (PCFT) [9], which can transport folate in an acidic environment; and the folate receptors (FRs) [10], which have high affinity and can transport folic acid (FA) into cells via endocytosis [11,12].

In contrast to the RFC and PCFT, which are ubiquitously expressed in both tumour and normal tissues [13], the FRs usually have minimal expression in normal tissue, but increased expression in malignant cells/tissues, including, colorectal, ovarian, breast, lung, cervical, renal, kidney, brain and nasopharyngeal carcinomas; and this increased expression has been linked with tumour progression [14–16]. This, makes the FR a potential target for cancer diagnosis/therapy; [17,18] where FA (small and stable over broad range of temperatures and pH) can be

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used as a biocompatible and non-immunogenic targeting motif to covalently conjugate with an optical imaging agent or a therapeutic [19]. Anti-folates, such as methotrexate (MTX) [20], have been directly exploited as therapeutic agents [21]. MTX was first synthesised in the 1940's [22] and is still extensively used in the treatment of tumours, including acute lymphocytic leukaemia [23], breast [24], head and neck cancers [25]. It is also used in certain autoimmune diseases [26]. MTX competitively inhibits dihydrofolate reductase, a key step in the reduction of folate to its tetrahydrofolate form, inhibiting DNA and RNA synthesis to cause cell apoptosis [27]. Folates and anti-folates can also be used as targeting agents and this has been successfully employed in a wide variety of imaging applications, with clinical trials in humans underway for magnetic resonance imaging (MRI), computed tomography (CT) and positron emission tomography (PET) imaging of solid tumours [28,29]. Fluorescent agents tethered to FA have been investigated for intra-operative identification of malignant disease [30]. In contrast to the conventional modalities of MRI, CT and PET, optical imaging agents offer sub-micrometre spatial resolution, allowing for rapid results at the sub-cellular level [31]; where early stage biological changes can be more easily detected using low cost and more accessible instrumentation. This opens opportunities for the non-invasive diagnosis of cutaneous and subcutaneous cancers and for example endoscopic diagnosis of oesophageal malignancies, where the issues of tissue transparency are usually negated [28]. Furthermore, the use of imaging agents for microscopy will allow cell biologists working in the field of cancer to investigate and monitor changes at a cellular level prior to, during and post intervention, in real time. To achieve these important imaging outcomes, there is a need to be able to monitor the optical signal for long periods of time, without photo-bleaching and to be able to differentiate the signal from endogenous fluorescence. Consequently, new imaging technology is required to address this unmet need.

Luminescent lanthanide (Ln) complexes are particularly attractive as imaging agents, due to their distinctive photo-physical properties [32]. They offer extended resistance to photobleaching, have large Stokes shifts (large separation between absorption and emission wavelengths) that avoid concentration-dependant self-absorption problems, and exhibit long luminescence lifetimes (range 1 μs to 5 ms) that enable time gated measurement of luminescence to avoid either non-specific background or specific auto-fluorescence. Furthermore, compared with fluorescent bands (> 200 nm bandwidths), lanthanides have sharp emission bands (10–20 nm bandwidths) that do not overlap with one another, resulting in optimal signal to noise ratios [33]. Lanthanides have an advantage over other luminescent metal ions, as they are considered to be isostructural, which means that the emission range can be tuned by 'swapping' the metal, using the same synthetic strategy to deliver complexes with theoretically similar physiochemical and biological targeting properties, but with different wavelength emissions [34]. One key consideration with lanthanide ions is their intrinsically low extinction coefficients (in the order of 0.5–3 M^{-1} cm^{-1}), which necessitates incorporating an antenna group into the lanthanide complex, to effectively populate the lanthanide's excited state [35]. Although a wide range of lanthanide complexes have been evaluated for the optical imaging of cells, most notably by Parker and co-workers [36–39], further development is required to establish a true structure activity relationship (SAR) and to determine the critical parameters for the design and optimisation of these imaging agents. This design process may be further complicated in the case of folates as targeting groups, as there are differences in cellular uptake efficiency and receptor binding affinities for substituted analogues of folic acid [40–43].

We and others have recently explored the relationship between the folate moiety (FA consists of three moieties; pterin head group, *p*-aminobenzoate and glutamate residue), the length of the linker between the folate and lanthanide complex, and the site of conjugation of the lanthanide complex; and the effects upon cellular uptake and luminescent emission for folate targeted Eu(III) conjugates [44,45]. This has shown that each portion of FA is critical and that when a short linker is used to tether a luminescent Eu(III) complex using the γ-carboxylic acid of FA, higher cellular uptake is achieved [44]. However, to be optimal for live cell imaging, these complexes need to be highly emissive and excitable within biologically compatible wavelengths.

In this study, we report the synthesis, photophysical and cellular properties of four new imaging agents, **Eu-FA**, **Eu-MTX**, **Tb-FA** and **Tb-MTX**, which each consists of a highly luminescent antenna complex, covalently conjugated using a short linker, to one of the carboxylic acid groups present in either FA or MTX (Fig. 1). Both Eu(III) and Tb(III) complexes were explored to evaluate any differences in emission and to compare the uptake/targeting for these two isostructural ions. It is important to ascertain how changes in the core metal may influence the potential to develop near infrared emitting complexes or even to use Gd(III) as an effective MRI contrast agent. We also aimed to explore how uptake, cytotoxicity and emission varied in a range of cells when folate (in the form of FA) was substituted for the anti-folate, MTX. Differences in the uptake and cytotoxicity of FA and MTX have implications for the targeting and potential treatment of cancer, as FA is often given as an antagonist drug to lessen and limit side effect toxicity (e.g. non-tumour toxicity) during MTX treatment. Therefore, it is important and meaningful that cell biologists are equipped with tools that have the potential to clearly visualise how these two analogues behave in cells, to develop better strategies for cancer treatment.

2. Experimental

2.1. General materials and methods

1,4,7,10-tetraazacyclododecane (cyclen) was obtained commercially from Strem, USA. All other solvents and chemicals used were obtained from Sigma-Aldrich or Merck, Australia. Sephadex G10 resins were purchased from Sigma-Aldrich, Australia. Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} plates obtained from Merck, Australia. The infrared spectroscopy (IR) was recorded on a Shimadzu FTIR-8400S. Electrospray ionization mass spectrometry (ESI-MS) was recorded using a Perkin-Elmer® Scoex API 3000. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker 500 MHz NMR spectrometer. All chemical shifts are given in ppm with coupling constants in Hz. All pH measurements were conducted on an Orion Ross pH meter. Optical spectroscopy experiments were recorded in 100% water at constant ionic strength (I = 0.01 (NaCl)) using a Varian CARY 50 UV–Vis spectrophotometer or a Varian Cary Eclipse spectrophotometer at room temperature. High pressure liquid chromatography (HPLC) was performed on a Shimadzu LC-20AD with manual injection fitted with a 60 Å, 4 μM Nova-Pak phenyl analytical column (3.9 × 150 mm) with a flow rate of 0.8 mL/min and was analysed using a Shimadzu SPD-20A detector at 280 nm. Linear elution was used with mobile phases A (H_2O + 0.1% TFA) and B (CH_3CN + 0.1% TFA); 15%–60% over 10 min. Elemental analysis was conducted in Campbell Microanalytical Laboratory, University of Otago.

2.2. Synthesis

2.2.1. 2,2′,2″-(10-(2-(4-(2-(2-(tert-butoxycarbonylamino)ethylamino)-2-oxoethyl)-2-oxo-1,2-dihydroquinolin-7-ylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid.Eu (Eu.3)

Compound **Eu.1** [46] (131 mg, 0.17 mmol) was dissolved in DMSO using sonication (170 W, 30 min), after which DIPEA (45 mg, 0.35 mmol) was added. The reaction mixture was then stirred for 10 min. Then HOBt (27 mg, 0.20 mmol) and BOP (159 mg, 0.36 mmol) were added. Following this, a solution of *tert*-butyl 2-aminoethylcarbamate (60 mg, 0.37 mmol), DIPEA (23 mg, 0.18 mmol) in DMSO (2 mL) was added. The reaction mixture was then left to stir at room temperature for 24 h. The crude product was then precipitated out by the addition of diethyl ether/acetone (10 mL, 7/3 (v/v)). The