Development and Characterisation of a Tumour Specific Contrast Agent for *In Vivo* Imaging of Therapeutic Response.

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Introduction:

Tumour vasculature is distinctively different from normal vessels, normally consists of a high proportion of immature, proliferating endothelial cells and is characterized by a chaotic network of tortuous, thin-walled, leaky vessels[1,2]. In contrast, normal tissues have well-ordered vessels with quiescent endothelial cells. Therefore, therapeutic approaches that specifically target the immature tumour vasculature that rely largely on their tubulin cytoskeleton for support allows drugs to be effective against established tumours without any effect on normal tissues[3]. Colchicine is a tubulin binding agent, which when bound to tubulin inhibits tubulin polymerization resulting in microtubule destabilization and its derivatives have been utilized in a number of vascular disrupting agents (VDA).VDA's are known to produce large central necrosis within 24 hours of injection with a characteristic thin rim of viable tumour cells at the periphery which is thought to be a consequence of cells getting nutrients/oxygen from non-tumour vessels in adjacent normal tissue. Thus VDA's are not expected to produce marked tumour regression as monotherapies. In the absence of tumour shrinkage, there is a need for non-invasive methods that can provide an assessment of tumour vasculature and pathophysiology *in vivo* in response to VDA therapy. This study aimed to develop a colchicine based contrast agent to better understand VDA therapy.

Methods: Chemical synthesis:

Gd.DOTA.Colchicinic acid was synthesised using facile solution phase chemistries consisting of the conjugation of Colchicinic acid to DOTA.NHS.ester (Macrocyclics, USA). The DOTA conjugated product, referred to as DOTA.Colchcinic acid and the final Gd.DOTA.Colchicine contrast agent shown in Fig 1, obtained by the addition of 6H2O.GdCl3 to the DOTA conjugate, both compounds were synthesised in good yield and purity and characterised accordingly.

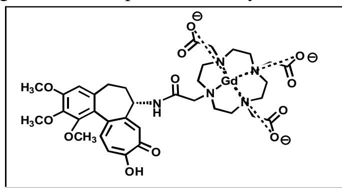

Figure 1: Chemical structure for Gd.DOTA.Colchicinic acid

In vitro experiments: *In vitro* relaxivity studies consisted of water-soluble phantoms of Gd.DOTA.Colchicinic acid compared to DOTA.Colchicinic acid and water as the control. These phantoms were placed in a quadrature ^{1}H volume coil and positioned into a 4.7T Varian Inova MRI scanner. A spin-echo sequence with the following parameters was used to assess T_1 relaxation, TR = 50, 100, 200, 300, 500, 700, 1200, 3000, 2800 ms, TE = 10 ms, FOV = 45 x 45 cm^2, averages: 1 matrix size: 256 x 128: and a 2.0 mm thickness. *In vitro* cell studies consisted of 4x10^5 OVCAR3 human ovarian carcinoma cells plated in 6 well plates and incubated for 24 hours with a range of concentrations (1nM – 1mM) of either DOTA.Colchicinic acid or Colchicine and cell death recorded by histological examination and FACS analysis to assess the efficacy of the DOTA.Colchicinc acid conjugation on tubulin binding. Gd.DOTA.Colchicinic acid uptake was then evaluated using the same plating of OVCAR3 cells at the most efficient incubation dose by taking cell pellets 2 hour intervals over the first 8 hours, 24, 48 and 72 hours after incubation and imaged as described above.

In Vivo experiments:

For *in vivo* experiments 5 x 10^6/0.1ml OVCAR3 cells were inoculated into the flanks of 6-8 weeks old Balb/c nude mice. When the tumours reached approximately 10mm^2 mice were anaesthetized with an isoflurane/O_2 mix and then placed into a quadrature ^{1}H volume coil as described above. A spin echo sequence was used to obtain T_1-weighted images using the parameters: TR = 400, 700, and 2800 ms, TE = 15 ms, FOV = 45 x 45 cm^2, averages: 1 matrix size: 256 x 128: 2.0 mm thickness, and 20 consecutive transverse slices covering the whole abdomen for evaluation of biodistribution as well intratumoural response. The mice were then removed from the magnet and a tail vein was cannulated for the administration of either 100mg/kg, 200mg/kg Gd.DOTA.Colchicinic acid, 100mg/kg, 200mg/kg DOTA.Colchicinic, or 200ul water as a control. Mice were then scanned with the same parameters 2 hours, 12 hours and 24 hours post injection. After the final scan tumours and selective organs were excised and frozen and sections where taken for Heamotoxylin and Eosin staining.

Results

In vitro results for water-soluble phantoms showed that the Gd.DOTA.Colchicinic acid contrast agent achieved a 96% T_1 reduction compared to both water and the control compound and its relaxivity was calculated to be 0.047mM^{-1}s^{-1}. The efficiency of the DOTA.Colchicinic acid compound compared to that of colchicine itself showed that colchcine was effective at significantly increasing cell death via tubulin destabilisation over a wide range of doses including that of the lowest dose whereas, DOTA.Colchicinic acid was only effective at significantly increasing cell death at the highest 1mM dose. This therefore suggests a reduction of toxicity of the tagged molecule. Cell pellets taken at specific time points after 1mM incubation with Gd.DOTA.Colchicinic also showed signal reduction at 2 hours reaching its maximum reduction at 6 hours.

In vivo MRI show that with 2 hours post injection of 100mg/kg and 200mg/kg Gd.DOTA.Colchicinic acid a slight reduction in T_1 was observed compared to that of the pre-injection, with a 40% reduction in T_1 at 12 hours post injection, which then remains unchanged after 24 hours. Whereas, both the saline control and DOTA.Colchicinic acid treated tumours presented stable T_1 signal intensities throughout. However, tumours within the 200mg/kg group showed increasing morphological changes within the tumour over the 24 hour time course, which maybe due to entrapment within the necrotic spaces after vascular occlusion (Fig 2a). Histology confirmed that all tumours treated with a colchicinic acid tagged molecule caused large central necrosis within the tumour at the 24 hour time point . This was not dependent on dose or gadolinium incorporation (Fig 2c). The liver and kidney also exhibited reductions in T_1 over the time course of the study. However, resulting histology confirmed no significant differences between treated and non-treated morphology in either organ.

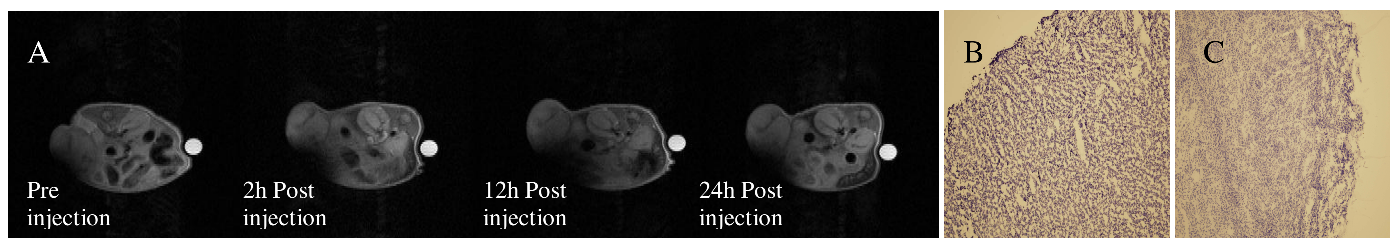

Figure 2: A) T1-weighted images prior to, 2, 12 and 24 hour post administration of 200mg/kg Gd.DOTA.Colchicinic acid (TR = 2800ms). B) H & E stained section of saline control tumour. C) H & E stained section of a tumour 24 hours after administration of 200mg/kg Gd.DOTA.Colchicinic acid.

Conclusions This project has established that Gd.DOTA.Colchicinic acid is a functional contrast agent that can bind tubulin leading to tumour vascular shutdown as well as shortening T1 both *in vitro* and *in vivo*. It has also been determined that Gd.DOTA.Colchicinic acid maintains efficiency at high concentrations enabling administration of a larger dose of Gd.DOTA.Colchicinic acid, further facilitating its function as a contrast agent and can be utilized to image vascular therapy of VDA's *in vivo*.

References 1) Fukumura D *et al.* Am. J Pathol. 1997;151:679. 2) Denekamp J. Cancer Metastases Rev.1990;9:267. 3) Siemann D.W *et al.* Cancer. 2004;100:2491