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A single-chain fragment against prostate specific membrane antigen as a tool to build theranostic reagents for prostate cancer

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Abstract Prostate carcinoma is the most common non-cutaneous cancer in developed countries and represents the second leading cause of death. Early stage androgen dependent prostate carcinoma responds well to conventional therapies, but relatively few treatment options exist for patients with hormone-refractory prostate cancer. One of the most suitable targets for antibody-mediated approaches is prostate specific membrane antigen (PSMA) which is a well known tumour associated antigen. PSMA is a type II integral cell-surface membrane protein that is not secreted, and its expression density and enzymatic activity are increased progressively in prostate cancer compared to normal prostate epithelium, thereby making PSMA an ideal target for monoclonal antibody imaging and therapy.

To obtain a small protein that can better penetrate tissue, we have engineered a single-chain variable fragment (scFv) starting from the variable heavy and light domains of the murine anti-PSMA monoclonal antibody D2B. scFvD2B was analysed *in vitro* for activity, stability, internalisation ability and *in vivo* for targeting specificity. Maintenance of function and immunoreactivity as well as extremely high radiolabelling efficiency and radiochemical purity were demonstrated by *in vitro* assays and under different experimental conditions. Despite its monovalent binding, scFvD2B retained a good strength of binding and was able to internalise around 40% of bound antigen. *In vivo* we showed its ability to specifically target only PSMA

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expressing prostate cancer xenografts. Due to these advantageous properties, scFvD2B has the potential to become a good theranostic reagent for early detection and therapy of prostate cancers.

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1. Introduction

Prostate specific membrane antigen (PSMA) has generated great interest as a tumour associated molecule suitable for diagnostic and therapeutic targeting of prostate cancer since it is highly restricted to the prostate and over-expressed in all tumour stages.[1–4] PSMA is a transmembrane protein commonly found on the surface of late-stage and metastatic prostate cancer and is a known imaging biomarker for staging and monitoring of therapy.[5]

Targeted approaches have become highly investigated fields in medicine and monoclonal antibodies (mAbs) have been widely exploited on the basis of their specific recognition of tumour associated molecules. mAbs are currently in preclinical and clinical studies for both diagnostic and therapeutic applications.[6]

However, intact antibodies, due to their large size (155 kDa) sometimes have difficulty in penetrating tumour tissues; moreover, the presence of the constant domains allows the binding to the FcR present on many circulating cells, endothelial cells and liver cells through the CH2 and to the FcRn through the CH3 giving a prolonged half life which results in a low tumour to background ratio forcing the clinicians to perform imaging several days after the injection.

Antibody fragments could be an ideal format if the recruitment of effector functions is not necessary. A variety of approaches have been undertaken to build a functional and stable complex of the variable heavy (VH) and light (VL) domains. The commonly used single chain Fv, or ‘scFv,’ is composed of the heavy and light chains of the antibody and utilises a flexible peptide linker to covalently join the VH and VL domains in a single polypeptide.[7] Unfortunately, scFvs often lack high affinity and stability, including the ability to withstand physiological concentrations of salt, pH and temperature conditions.

Herein, we characterised a scFv directed against PSMA and demonstrated that due to its intrinsic characteristics of binding strength, stability and internalisation ability, and its *in vitro* and *in vivo* targeting properties, could be successfully exploited in diagnostic and therapeutic targeted approaches.

2. Materials and methods

2.1. Cell lines and reagents

LNCaP and PC3 (human prostate cancer), MCF7 (human breast cancer) and CHO (hamster ovary) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, MD). IGROV-1 (human ovary cancer) was provided by Dr. J. Benard and PC3-PIP, stably expressing human PSMA (hPSMA),[8] was provided by Dr. W. Heston. MCF7 and CHO cell lines were used to generate stable transfectants expressing hPSMA, as described.[9]

All cell lines were subjected to short tandem repeat (STR) analysis according to ATCC guidelines and profiles were compared to publicly available databases to verify authenticity. Total cell lysates were prepared as described.[10]

Hybridomas producing the mAb 7E11, directed against the intracellular domain of hPSMA (HB-10494) and the anti-myc tag mAb 9E10 (CRL-1729) were purchased from ATCC; mAb J951, directed against the extracellular domain of PSMA,[11] was supplied in purified form by Dr. N.H. Bander (Medical College of Cornell University, New York). The mAbs were affinity purified on protein G from culture supernatants.

2.2. Anti-PSMA mAb and scFv generation, purification and in vitro testing

The IgG1 anti-PSMA mAb, hereafter IgGD2B, was obtained by conventional hybridoma technology and affinity purified on protein A/G from culture supernatant.

Total RNA was extracted from hybridoma cells using Rneasy Total RNA Purification Kit (Qiagen), cDNA was synthesised by reverse transcription and VH and VL chains were amplified by polymerase chain reaction (PCR) using the corresponding primers (Table 1).

PCR products were cloned as a scFv (VH-linker-VL orientation) into the phagemid vector pMIMO which is a derivative of pHEN1 [10717] where the most commonly used linker (Gly_4-Ser)$_3$ is added allowing the separate cloning of antibody variable domains (SFII/XHOI for VH and APALI/NOTI for VK). Electrocompetent *Escherichia coli* TG1 cells were transformed with the ligation reaction and plated on 2xTY-AGAR medium containing 100 μg/mL ampicillin. *E. coli* HB2151 cells were used for scFv soluble production as described.[12] Periplasmic preparations were purified by Ni-NTA agarose beads as described.[13]

The size, homogeneity and potential dimerisation were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), western blotting and size exclusion chromatography on a Superdex 75 HR 10/30 column (GE Healthcare). For flow cytometry refer to Fig. 2, whereas for enzyme-linked immunosorbent

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