Title

Fluorescent imaging using novel conjugated polymeric nanoparticles-affimer probes in complex *in vitro* models of colorectal cancer.

Authors

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Supporting information

Materials and Methods

Materials

Maleimide terminated CPN510B (100 μg/ml) was used as received by Stream Bio Ltd (Stream Bio, Cheshire, UK) (https://www.streambio.co.uk/products/cpn-510/). CEA Affimer was a kind gift from D Tomlinson and P Milner (University of Leeds)[1]. The Affimer was designed with a cysteine residue for maleimide conjugation, positioned away from the antigen recognition site.

Conjugation of CPN510 to CEA Affimers

500μl CEA Affimer (molecular weight ~12,600 Da) was added to HEPES/PEG solution (20mM HEPES, 0.1% (v/v) PEG) and transferred to Amicon Ultra 10KDa MWCO centrifugal filter and centrifuged at 16,000*g* for two minutes. 500μl of HEPES/PEG solution was added, and the centrifugation step repeated twice. To collect the buffer exchanged CEA Affimer, the column was inverted into a fresh collecting tube and centrifuged at 16,000*g* for five minutes. The concentration of buffer exchanged CEA Affimers was monitored using a Nanodrop 1000 with ND1000 software v3.7 and adjusted to 1g/l using HEPES/PEG buffer.

1μl of buffer-exchanged CEA Affimer was added to 9μl of HEPES/PEG solution containing TCEP (5mM) and incubated at room temperature for 30 minutes. The reduced Affimers were incubated with 50μl of Maleimide-CPN for 30 minutes at room temperature. 1μl of 1mg/ml cysteine was added to halt the reaction and further incubated at room temperature for 10 minutes. To remove unconjugated CEA Affimers, the CEA-Affimer-CPNs were purified using an AmiconUltra 100 KDa MWCO centrifugal filter. The solution was rinsed three times with HEPES/PEG and spun at 16,000*g* for 2 minutes each time.

Cell culture

Human colorectal adenocarcinoma cell lines (HT29, LoVo and LS174T) and the human vascular endothelial cell line (EA.hy926) were purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK). Cell lines were cultured in RPMI 1640 plus GlutaMAX™ (HT29), DMEM/F-12 plus GlutaMAX™ (LoVo) and DMEM plus GlutaMAX™ (LS174T and EA.hy926) media (Gibco by Life Technologies, Paisley, UK) supplemented with 10% (v/v) Foetal Bovine Serum (Sigma-Aldrich, Dorset, UK) and 1% (v/v) penicillin-streptomycin antibiotics (10,000U/mL). Cell cultures were incubated at 37°C, 5% CO_2 and 95% relative humidity. Upon 80 – 90% confluency, cell cultures were washed with phosphate buffered saline (PBS), trypsinised, centrifuged and re-suspended in fresh cell medium.

Immunofluorescence
Cells were seeded at 6 x 10^5 cells/mL onto sterile glass coverslips in 6-well plates (Corning Inc, New York, USA). After 24 hours, cells were washed and fixed with 4% paraformaldehyde (Sigma Aldrich, Dorset, UK). Fixed cells were blocked with 0.5% (w/v) skimmed milk solution (Premier Foods Group, London, UK) to reduce non-specific binding. Primary mouse anti-CEA antibody (Clare Hall Laboratories, Potters Bar, UK) was added to fixed cells and incubated for 1 hour at room temperature followed by secondary goat anti-mouse Alexa Fluor 488 antibody (Thermo Fisher) for 30 minutes at room temperature in the dark. Coverslips were washed and mounted onto glass slides using Prolong Gold plus DAPI mountant (Thermo Fisher). Slides were sealed with ethyl acetate and cured overnight at room temperature while shielded from light before being photographed using a Nikon A1R Confocal Microscope (Nikon UK Ltd, Kingston upon Thames, UK).

Mapping CPN510 excitation spectrum
CPN510 excitation spectra were mapped using the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher). CPN510 absorbance was measured between 220 nm and 600 nm.

CPN510 cytotoxicity analysis
Cells were seeded at 1 x 10^5 cells/mL into 96-well plates. After 24 hours, CPN510 was added for another 24 hours. The MTT assay was performed and absorbance of formazan was measured at 620nm using a Mithras LB940 Microplate Reader (Berthold Technologies Ltd., Harpenden, UK). One independent culture was analysed with three technical replicates for each concentration (N=3).

CEA-targeting CPN510 binding in 2D and 3D CRC models
Cells were seeded at 1.2 x 10^5 cells per well onto glass coverslips in 6-well plates. After 24 hours, cells were fixed with paraformaldehyde and washed. Cells were then incubated with CPN510-CEA-Af or unconjugated CPN510 and Hoechst 33342 nuclear stain applied. Coverslips were mounted onto glass slides and imaged using the EVOS FL fluorescence microscope (Thermo Fisher Scientific, US) and images processed using ImageJ software (National Institutes of Health, USA).

For 3D spheroid cultures, cells were seeded at of 2 x 10^4 cells per well in Ultra Low Attachment 96-well spheroid microplates (Corning Inc., New York, USA), and incubated for 72 hours. Plates were imaged using the EVOS FL fluorescence microscope every 24 hours.

Following incubation, spheroids were treated with CPN510-CEA-Af or unconjugated CPN510 and Hoechst 33342 nuclear stain applied. Spheroids were then fixed with 4% PFA for 30 minutes and embedded into Cryo-M-bed OCT (Bright Instruments, Luton, UK). OCT embedded spheroids were sectioned at 8µm thickness using the Leica CM3050 S research cryostat (Leica Microsystems, Milton Keynes, UK). Sectioned spheroids were imaged using an EVOS FL fluorescence microscope and processed using ImageJ.

Statistical analysis
Cytotoxicity data was analysed using one-way ANOVA. The fluorescence intensity signal for both 2D cells and 3D spheroids was analysed using the Mann-Whitney U

test. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, Inc., California, USA) with $p<0.05$ considered to be statistically significant. Data are presented as the mean ± standard error of mean (SEM).

Calculating fluorescence signal intensity

Fluorescence was quantified using ImageJ software. Background fluorescence was normalised to the pre-treated fluorescence signal of each cell line and the outline of CPN510 treated cells or spheroids was mapped and highlighted as regions of interest (**Fig S1**). Outlined regions of interest were superimposed onto the fluorescent images taken using the GFP filter, and fluorescence signal intensity measured. There were at least nine regions of interest for each cell lines.

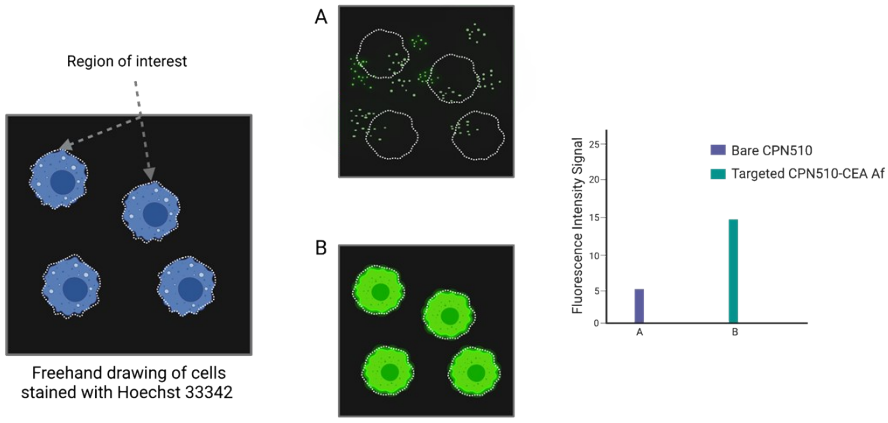

Fig S1. Diagram showing method used for fluorescence intensity calculation. Cells stained with Hoechst 33342 were visualised using a DAPI fluorescence filter. Freehand drawings were used to outlined 'regions of interest', which were superimposed on to the cells treated with (A) unconjugated CPN510, and (B) CPN510-CEA-Af. ImageJ was used to process and quantify the fluorescence intensity signal.

List of reagents

Reagent/Material	Company (Location)
CPN510B	Stream Bio Ltd (Cheshire, UK)
CEA Affimer	University of Leeds (Leeds, UK)
HEPES Buffer Solution	Thermo Fisher
Poly(ethylene glycol)	Sigma Aldrich
Amicon Ultra 10KDa MWCO centrifugal filters	Sigma Aldrich
Amicon Ultra 100 KDa MWCO centrifugal filter	Sigma Aldrich
Phosphate Buffered Saline (PBS)	Sigma Aldrich
Tris-(2-Carboxyethyl)phosphine, Hydrochloride (TCEP)	Thermo Fisher
HT29 cell line	European Collection of Authenticated Cell Cultures (Salisbury, UK)
LoVo cell line	European Collection of Authenticated Cell Cultures (Salisbury, UK)
LS174T cell line	European Collection of Authenticated Cell Cultures (Salisbury, UK)
EA.hy926 cell line	European Collection of Authenticated

	Cell Cultures (Salisbury, UK)
RPMI 1640 plus GlutaMAX™	Thermo Fisher
DMEM/F-12 plus GlutaMAX™	Thermo Fisher
DMEM plus GlutaMAX™	Thermo Fisher
Foetal Bovine Serum	Sigma Aldrich
Penicillin-Streptomycin	Thermo Fisher
Trypsin/EDTA	Thermo Fisher
Dulbecco's PBS	Thermo Fisher
Paraformaldehyde	Thermo Fisher
Powdered Milk	Premier Foods Group (London, UK)
Mouse anti-CEA antibody	Clare Hall Laboratories (Potters Bar, UK)
Goat anti-mouse Alexa Fluor 488 antibody	Thermo Fisher
ProLong Gold Antifade Mountant with DNA Stain DAPI	Thermo Fisher
Ethyl acetate	Thermo Fisher
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	Sigma Aldrich
Dimethyl Sulfoxide	Sigma Aldrich
Hoechst 33342	Thermo Fisher
Cryo-M-bed OCT embedding medium	Bright Instruments (Luton, UK)

References

1. S. H. Shamsuddin, D. G. Jayne, D. C. Tomlinson, M. J. Mcpherson and P. A. Millner, *Scientific Reports*, 2021, **11**.