Drug delivery, biodistribution and anti-EGFR activity: theragnostic nanoparticles for simultaneous in vivo delivery of tyrosine kinase inhibitors and kinase activity biosensors

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In vivo delivery of small molecule therapeutics to cancer cells, assessment of the selectivity of administration, and measuring the efficacy of the drug in question at the molecule level, are important ongoing challenges in developing new classes of cancer chemotherapeutics. One approach that has the potential to provide targeted delivery, tracking of biodistribution and readout of efficacy, is to use multimodal theragnostic nanoparticles to deliver the small molecule therapeutic. In this paper, we report the development of targeted theragnostic lipid/peptide/DNA lipopolyplexes. These simultaneously deliver an inhibitor of the EGFR tyrosine kinase, and plasmid DNA coding for a Crk-based biosensor, Picchu-X, which when expressed in the target cells can be used to quantify the inhibition of EGFR in vivo in a mouse colorectal cancer xenograft model. Reversible bioconjugation of a known analogue of the tyrosine kinase inhibitor Mo-IPQA to a cationic peptide, and co-formulation with peptides containing both EGFR-binding and cat-ionic sequences, allowed for good levels of inhibitor encapsulation with targeted delivery to LIM1215 colon cancer cells. Furthermore, high levels of expression of the Picchu-X biosensor in the LIM1215 cells in vivo allowed us to demonstrate, using fluorescence lifetime microscopy (FLIM)-based biosensing, that EGFR activity can be successfully suppressed by the tyrosine kinase inhibitor, released from the lipopolyplexes. Finally, we measured the biodistribution of lipopolyplexes containing 125I-labelled inhibitors and were able to demonstrate that the lipopolyplexes gave significantly higher drug delivery to the tumors compared with free drug.

Introduction

Current challenges in next-generation medicine have stimulated the rapid development of theragnostic agents. These are of increasing importance for diseases such as cancer, where no two patients will have exactly the same biomarkers and oncogenic mutations, and where most currently available therapeutic agents have limited target selectivity, poor localization, and undesirable side-effects. Theragnostic agents can be based on small molecules conjugated to a targeting moiety, such as an antibody; on engineered mammalian cells; and on various types of nanoparticles (e.g. iron oxide, gold, polymeric or liposomal).1 Whilst all of these have limitations,2 liposome-based nanoparticles have several key advantages. These include the ability to design multimodal nanoparticles with several functionalities contained in, or attached to, a single liposome: rapid cellular uptake; a wide range of cellular compatibilities and low toxicity; and long circulating half-life combined with eventual biodegradability.3
Many theragnostic agents have now been developed which are capable of diagnosing, through imaging, the presence of cancerous cells and then delivering a precisely targeted therapeutic intervention.\(^4\) Whilst these approaches can be used to measure biodistribution and accumulation of small molecule drugs at the tumor, none of the theragnostic agents reported to date can quantify the effectiveness of the therapeutic at the molecular level \textit{in vivo}. This is particularly important in targeted cancer therapy. Although diagnostic assays are available to determine protein expression status for some types of cancer, for many tumor types there are either no validated assays, or the expression status of the protein cannot in practice be correlated with the clinical response to the drug.\(^5\) The development of theragnostic nanoparticles which would improve drug delivery, whilst also allowing both the biodistribution and response to the drug to be measured, would be a major step towards the development of more effective cancer therapies, tailored towards the driving molecular phenotype of an individual tumor.

Epidermal growth factor receptor (EGFR) is found to be overexpressed and/or constitutively activated in a variety of tumors including breast, lung and colon\(^6\) and is a validated target for drug development. The EGFR family\(^7\) consists of four members (EGFR, HER2, HER3 and HER4) with more than 10 different ligands\(^8\) able to activate downstream signaling, leading to growth proliferation and inhibition of apoptosis, angiogenesis and metastasis. Several therapeutic strategies targeting EGFR have been devised. In particular, small molecule inhibitors of the EGFR intracellular tyrosine kinase activity (TKI), and antibodies blocking ligand interaction have been developed and tested in preclinical and clinical studies.\(^9,10\) The development of theragnostic nanoparticles which would improve drug delivery, whilst also allowing both the biodistribution and response to the drug to be measured, would be a major step towards the development of more effective cancer therapies, tailored towards the driving molecular phenotype of an individual tumor.

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A strategy for overcoming the poor pharmacokinetic profiles and low target selectivity of anticancer drugs is to use liposomes, liposome-based nanoparticles and polymersomes as delivery vehicles.\(^16\) The polyethylene glycol (PEG)-shielded liposomal doxorubicin (DOXIL\textregistered) is clinically approved for the treatment of ovarian and breast cancer, with significantly longer circulation times and lower cardiotoxicity compared to the free drug, and several other liposomal drug formulations are in Phase II trials for other cancers.\(^17\) At the preclinical level, radiolabeling\(^18\) of liposome-encapsulated dasatinib with \(^{18}\text{F}\) and liposome-encapsulated Mo-IPOA labeled\(^19\) with \(^{124}\text{I}\) have enabled quantification, demonstrating some of the highest tumor uptakes \textit{in vivo} of TKI so far observed. Non-targeted liposomal drug formulations were believed to rely on the passive accumulation of these nanoparticles (100–200 nm) in tumors through the Enhanced Permeability and Retention (EPR) effect. However, many clinical studies have failed to show either increased nanoparticle accumulation in tumors or increased efficacy, and it is now clear that this effect is, at best, heterogeneous in humans.\(^20,21\) Ligand-mediated cell-selective targeting offers additional advantages in terms of target cell specificity and cellular uptake. For example, immunoliposomes have been prepared using anti-EGFR antibodies as targeting moieties and were used to deliver drugs such as 5-fluorouracil,\(^22\) cisplatin\(^23\) and doxorubicin\(^24\) to cancer cells \textit{in vivo}, with moderate to significant improvements in cytotoxicity and cell selectivity compared to non-targeted liposomes. Likewise, polymersomes encapsulating platidipin and targeted with an anti-EGFR antibody showed greatly increased cytotoxicity and cellular uptake in colorectal cancer cell lines compared to an untargeted polymersome.\(^25\) Liposomes targeted with EGF-binding peptides have also been used to deliver doxorubicin\(^26\) and cisplatin\(^27\) effectively to cancer cells. Finally, lipoplexes targeted with anti-EGFR antibodies have been formulated to deliver siRNA to non-small cell lung cancer cell lines\(^28\) and into EGFR-overexpressing hepatocellular carcinoma.\(^29\) Surface modification was necessary for specific uptake of the siRNA into the cells and resulted in enhanced tumor accumulation when compared to untargeted lipoplexes.

We have previously reported\(^30\) the development of EGFR-targeted lipopolyplexes for the delivery of pDNA encoding an EGFR biosensor.\(^31\) This allowed us to monitor EGFR activity in a breast cancer model \textit{in vivo}, before and after separate treatment with a TKI, Mo-IPOA.\(^30\) As well as successfully demonstrating the inhibition of tyrosine phosphorylation by EGFR \textit{in vivo}, and showing a significant degree of intratumoral heterogeneity in EGFR activity, our results showed that small molecule TKIs have a very poor uptake in cancer cells, and that the uptake is unselective. In this paper, we report the development of the first targeted, lipopolyplex-based theragnostic nanoparticle that improves drug delivery to cancer cells, allows quantification of the biodistribution, and also provides a readout of TKI mediated enzyme inhibition. As the reasons for the heterogeneity of response of colorectal cancer cells to EGFR-targeted therapies, and the rapid emergence of chemotherapy-resistant strains, are still poorly understood\(^15\) these nanoparticles have been used for preliminary investigation of biodistribution and uptake in a murine xenograft colorectal cancer model.

Results and discussion

Design of lipopolyplex-based theragnostic nanoparticles

In our previous work\(^32–38\) we have developed lipopolyplexes formulated from a ternary mixture of lipids, peptides and DNA for the targeted delivery of pDNA to tumor cells. The peptide component is a trifunctional sequence comprising: a linear

\[\text{EGFR-peptide-pDNA}\]

This compound forms a lipopolyplex with DNA that is targeted with a tripeptide sequence (EGFR) and linked to DNA using a polyethylene glycol (PEG) linker. The lipopolyplex is designed to be internalized by the target cell and to deliver the DNA payload into the cell nucleus. This results in the expression of the EGFR biosensor, which can be measured \textit{in vivo} using imaging techniques. This design enables the monitoring of EGFR activity in real-time \textit{in vivo}, allowing for the assessment of the efficacy of the targeted delivery system and the potential for personalized therapy.
K16 domain to condense pDNA; a cell-targeting sequence that is displayed at the surface of the nanoparticle; and a linker sequence, RVRR, that is a substrate for the endosomal enzyme furin. Mixtures of cationic and neutral lipids are used and are co-formulated with the neutral lipid dioleoylphosphatidylethanolamine (DOPE). The cationic lipids include both DOTMA\textsuperscript{39} and the analogue DODEG\textsuperscript{42} which has a short ethylene glycol oligomer headgroup. These give shielded nanocomplexes that have better stability within the systemic circulation but do not impede cellular uptake.

The targeting peptide sequence at the surface of these lipopolyplexes binds to the cell of interest and mediates internalisation via receptor-mediated endocytosis: cleavage of the peptide at the RVRR sequence allows partial disassembly of the nanoparticle after internalisation,\textsuperscript{33} and the neutral lipid DOPE promotes fusion with the endosomal membrane and mediates escape of the pDNA/peptide complex from the endosome.\textsuperscript{40} These nanoparticles are able to transfet tumor cells in vivo with transfection efficiencies comparable to viral methods,\textsuperscript{34} and lipopolyplexes generally have lower cytotoxicity than commercially available transfection reagents such as Lipofectamine2000.\textsuperscript{37,38,41,42} We have previously carried out biophysical characterisation of these lipopolyplexes by a variety of techniques including TEM,\textsuperscript{37} freeze-fracture EM\textsuperscript{35} and cryo-EM.\textsuperscript{36} These EM studies suggest that these lipopolyplexes form spherical particles with a dense internal core and an irregular outer layer.

To report the levels of EGFR activity in cells using these ternary lipopolyplexes, we used a genetically encoded CrkII-based biosensor (Picchu-X) which undergoes conformational changes upon phosphorylation of tyrosine-221 by EGFR.\textsuperscript{31,43} These changes are quantified by fluorescence resonance energy transfer (FRET) as monitored by fluorescence lifetime imaging microscopy (FLIM). We transfected this biosensor into EGFR-positive tumor cells using targeted liposome-base nanoparticles bearing EGFR-binding peptides at the surface, and measured the response of these tumor cells to a TKI, Mo-IPQA, administered separately.\textsuperscript{30} In order to achieve our goals of selectively delivering a TKI to colorectal cancer cells, imaging the inhibition of EGFR within the tumor and evaluating the biodistribution of the TKI, we designed enhanced multifunctional lipopolyplex-based nanoparticles (Fig. 1). These lipopolyplex formulations had to include: mixtures of lipids that would enhance the particle stability and provide steric shielding; peptide targeting sequences that would promote uptake from colorectal cancer cells; and a cationic peptide–TKI bioconjugate that would enable incorporation of high levels of both the TKI and the pDNA within the lipopolyplex. Additionally, we needed to develop synthetic strategies to ensure that the cationic peptide–TKI bioconjugate could be radiolabelled with \textsuperscript{125}I,\textsuperscript{185} thus allowing the biodistribution of both the TKI and the lipopolyplex to be assessed.

With this next generation of theragnostic lipopolyplexes, in addition to DOPE, DOTMA and DODEG-4 we included cholesterol to further increase bilayer stability,\textsuperscript{44} and Cy-5-DOTMA\textsuperscript{10} to enable the uptake of the nanoparticles to the tumor cells to be easily monitored. In order to target the nanoparticles efficiently to cell lines over-expressing EGFR, we synthesized trifunctional peptides with DNA-binding linear K16, RVRR and cell-targeting sequences, as before. We have evaluated three peptide sequences for their ability to target colorectal cancer cells. Peptides P1 and P4 use the LARLT (D4) sequence,\textsuperscript{45} P2 and P5 the AELRY\textsuperscript{46} and P3 and P6 the YHWGYGTPQQV (GE11) sequence.\textsuperscript{47,48} These sequences had been previously reported to bind tightly to EGFR and, when displayed at the surface of nanoparticles, mediate uptake to EGFR+ tumor cells. Indeed, we have previously demonstrated that lipopolyplexes formulated with P1 effectively deliver pDNA encoding for a biosensor to HCC1954 and MDA-MB-231 breast cancer cell lines in vivo,\textsuperscript{30} and that lipopolyplexes formulated with P1, P2 or P3 effectively transfet HCC1954 cells in vitro.\textsuperscript{49}

The major challenge to the design of these self-assemblying nanoparticles was the co-encapsulation of the small molecule TKI with pDNA. Whilst several groups have developed multifunctional nanoparticles for combination therapy with small molecules and nucleic acids,\textsuperscript{50–54} most of these approaches have focused on the co-delivery of siRNA, and many of the reported approaches are based on dendrimers, which have associated toxicities and result in the formation of heterogeneous mixtures.\textsuperscript{55} In some cases where liposomal co-formation of both nucleic acid and small molecule was attempted, aggregation of the liposome and leakage of the drug was seen.\textsuperscript{56} In order to ensure that the TKI was successfully encapsulated in the self-assemblying nanoparticles, and that we could there-fore prepare lipopolyplexes using a mixture of 1 and peptides P1–P6. After internalization of the lipopolyplex, we predicted that the disulfide linkage would be cleaved in the reducing environment of the endosome,\textsuperscript{59,60} releasing PEG\textsubscript{6}-IPQA into the cytoplasm.

**Synthesis of K\textsubscript{16}Cys-S-S-[PEG\textsubscript{6}-IPQA] 1 and MeO-[PEG\textsubscript{6}-IPQA] 9**

In order to prepare K\textsubscript{16}Cys-S-S-[PEG\textsubscript{6}-IPQA] 1, we required the key intermediate NPys-S-PEG\textsubscript{6}-IPQA 2, with the SH group activated as the NPs derivative\textsuperscript{61} for coupling to the peptide (Scheme 1). The previously reported 17-(4-((3-iodophenyl)
Fig. 1 (a) Structures of the previously reported $^{18}$F PET radiotracer F-[PEG$_6$-IPQA]$^{52}$ and the EGFR TKI Mo-IPQA$^{51}$ (b) peptide, lipid and plasmid DNA components of the lipopolyplex nanoparticles used in this work.
This was subsequently reacted with the sodium salt of triphenylmethanol to yield the trityl protected thiol compound 4. Reduction of the nitro group was carried out using tin(II)chloride which gave the desired amino protected thiol compound 5 in 61% yield. Using a previously reported procedure58, compound 5 was reacted with acryloyl isobutyl carbonate, prepared in situ from acrylic acid and isobutyl chloroformate, to afford compound 6 in 57% yield. At this point, the trityl protecting group was replaced by NPyS in a one pot reaction under acidic conditions using 2,2′-dithiobis(5-nitropyridine)62 to afford 2 in 48% yield. In order to prevent competing Michael reaction between the thiol group and the unsaturated amide, it was necessary to maintain the thiol protection throughout the synthetic sequence, and to carry out the replacement of Trt by NPyS at acidic pH. Finally, reaction63 with the cationic peptide CysLys167 in MeOH followed by HPLC gave 1 in 59% yield.

**In vitro optimization of nanoparticle formulation**

To test the ability of lipopolyplexes to deliver pDNA and inhibitor we used LIM1215 colon cancer cells. These are known to have a high level of EGFR expression and response to the TKI inhibitor,64,65 and we had previously demonstrated that a reliable xenograft model could be established.30 As a control, to check the response of the colorectal cancer cell lines to this class of TKI, we prepared an analogue of 1, with the disulfide-linked peptide replaced with an −OMe group. TKI analogue MeO-[PEG6-IPQA] 8 was synthesized from intermediate 3 via a similar route to that of 1 (ESI†) and demonstrated to act as a potent inhibitor of EGFR activation in SW48 cells (Fig. S1†).

Initially, the sequence of the trifunctional peptides was investigated to see whether placing the targeting sequence at the N or the C terminal had any effect on lipopolyplex uptake and expression in colorectal cancer cells. Preliminary experiments (ESI, Fig. S2†) suggested that the expression levels were lower for all peptides with the AEYLR sequence (P2, P5), and higher for LARLLT (P1, P4) and GE11 (P3, P6) in DLD1 colorectal cancer cells. In this cell line, the C-terminal LARLLT (P1) and N-terminal GE11 (P3) peptides yielded the highest expression levels. The best performing formulation (CLA1, using the P1 sequence) was tested in DLD1 and LIM1215 cells (Fig. S3†). Expression levels of Picchu-X biosensor were far higher in LIM1215 cells, which were selected for further optimisation of the lipopolyplex formulations. Subsequently, the impact of plasmid DNA concentration and targeting peptide (P1-P6) on the transfection efficiency in LIM1215 cells were assessed (Fig. S4 and S5†). Although formulations with the C-terminal LARLLT peptide (P1) still showed the best transfection efficiency, formulations with N-terminal AEYLR (P5) and N-terminal GE11 (P6) also performed well in this cell line. In order to optimize the receptor binding and transfection properties of these lipopolyplexes, we then investigated formulations using different targeting peptides P1, P5, P6 and differing lipid compositions (Table 1), but initially without K16Cys-S-S-[PEG6-IPQA] 1. While we observed that all lipopolyplex formulations were equally internalized by the target cells, changes in the lipid composition and in the concentrations of
Table 1. Lipopolyplex formulations. CLA lipopolyplexes are formed from C-terminal LARLLT bifunctional peptide P1 and varying amounts of cholesterol. NAE lipopolyplexes are formed from N-terminal AEYLR bifunctional peptide P5 and varying amounts of cholesterol. NGE lipopolyplexes are formed from N-terminal [GE11] bifunctional peptide P6 and varying amounts of cholesterol (Table 2a). The F1LA and F2LA formulations are based on the CLA1 and CLA2 lipopolyplexes, but with the addition of the cationic peptide–EGFR inhibitor bioconjugate K₁₆Cys-S-S-[PEG₆/IPQA] 1 at varying concentrations (Fig. 2b).

| DODEG₄ | DOPE | DOTMA | Cholesterol | Cy₅ | Targeting peptide | K₁₆Cys-S-S-[PEG₆/IPQA] |
|--------|------|-------|-------------|-----|-------------------|-----------------------|
| CLA1   | 39   | 21    | 15          | 23  | P₁                | —                     |
| CLA2   | 39   | 44    | 15          | 0   | P₁                | —                     |
| CLA3   | 39   | 37    | 23          | 0   | P₁                | —                     |
| CLA4   | 39   | 37    | 23          | 8   | P₁                | —                     |
| NAE1   | 39   | 21    | 15          | 23  | P₁                | —                     |
| NAE2   | 39   | 44    | 15          | 0   | P₁                | —                     |
| NAE3   | 39   | 37    | 23          | 0   | P₁                | —                     |
| NAE4   | 39   | 37    | 23          | 8   | P₁                | —                     |
| NGE1   | 39   | 21    | 15          | 23  | P₁                | —                     |
| NGE2   | 39   | 44    | 15          | 0   | P₁                | —                     |
| NGE3   | 39   | 37    | 23          | 0   | P₁                | —                     |
| NGE4   | 39   | 37    | 23          | 8   | P₁                | —                     |
| F1LA1  | 39   | 21    | 15          | 23  | P₁                | —                     |
| F1LA2  | 39   | 21    | 15          | 23  | P₁                | —                     |
| F1LA3  | 39   | 21    | 15          | 23  | P₁                | —                     |
| F2LA1  | 39   | 44    | 15          | 0   | P₁                | —                     |
| F2LA2  | 39   | 44    | 15          | 0   | P₁                | —                     |
| F2LA3  | 39   | 44    | 15          | 0   | P₁                | —                     |

bifunctional peptides P₁, P₅, P₆, had an impact on the transfection of the Picchu biosensor.

In contrast to our initial screen, these studies demonstrated that the CLA1 formulation, formulated with P₁ (Table 1) gave the maximum transfection efficiency (Fig. 2A, S3 and S4†), and the optimal final concentration of lipopolyplexes in the media was found to be 10 µM (based on the concentration of the lipids) (Fig. S6†). We then prepared variant lipopolyplex formulations, based on CLA1 and including the EGFR inhibitor K₁₆Cys-S-S-[PEG₆/IPQA] 1 (Table 1). These lipopolyplex formulations (with increasing amount of inhibitor per particle) gave slightly decreased transfection efficiency (Fig. 2B) compared to particles without the drug (Fig. 2A), however the overall number of transfected tumor cells was not affected. For efficient perfusion through the leaky endothelial cells of the tumor, lipopolyplexes must be <150 nm in diameter. The lipopolyplex formulations were characterised using dynamic light scattering (DLS) and zeta potential measurements (ESI Table S1†) and were found to have particle sizes within the ideal diameter for tumor delivery, reasonable polydispersities, and positive zeta potentials which would minimize particle aggregation and afford a reasonable degree of serum stability. We also carried out in vitro studies to check that the disulfide linker between K₁₆Cys and PEG-IPQA was labile under pseudointracellular conditions (GSH/GSSG, HEPES, pH 7.2). These indicated that under reducing conditions the linkage would be cleaved within 5 min (ESI Fig. S7†). Next, we assessed the effectiveness of K₁₆Cys-S-S-[PEG₆/IPQA] 1 as an inhibitor of EGFR activity as a free therapeutic or within lipopolyplexes by utilizing FRET-FLIM and validating our results by western blot (WB) techniques. Activation of EGFR in cells treated with EGF (natural ligand for EGFR) was evident by decreasing lifetime of green fluorescent protein (GFP) in Picchu-X biosensor (Fig. 2c) and by appearance of phosphorylated EGFR band in WB image (Fig. 2d). In both cases pre-treatment with MeO-[PEG₆-IPQA] 8 for 1 h abolished these changes: lifetime of GFP remained at baseline levels (Fig. 2c) and the level of EGFR phosphorylation was significantly lower than in the sample without inhibitor (Fig. 2d, line 8 vs. line 2).

Furthermore, incorporation of K₁₆Cys-S-S-[PEG₆IPA] 1 within lipopolyplexes not only decreased EGFR activity to equally low levels as the free drug but was effective for longer duration (Fig. 2d, lines 3–7 vs. line 2). We observed EGFR inhibition when 1 was delivered within lipopolyplexes for up to 48 h, whereas free MeO-[PEG₆-IPQA] 8 was effective only for 24 h (Fig. 2d). These results clearly reveal F1LA1 to be the optimal formulation for lipopolyplexes to achieve maximum transfection efficiency and prolong inhibition of EGFR in cells stimulated with ligand.

Radiolabeling and biodistribution

In order to assess the in vivo biodistribution of these liposome-based theragnostic nanoparticles, we required a [¹²⁵I]-labelled analogue of 1. As we particularly wished to measure the biodistribution of the IPQA-peptide bioconjugate, we elected to attach the radiolabelled PEG-[¹²⁵I]IPQA moiety to K₁₆Cys via a maleimide linkage, which would not be subject to reduction in vivo. The analogue K₁₆Cys-SMAL-[PEG₃-¹²⁵I]IPQA 9 was therefore designed with a similar linker length between the K₁₆ DNA-binding peptide and IPQA analogue 1. PEG-IPQA precursor 10 was prepared using a modification of the literature procedure, reported for PEG-IPQA (ESI†). Similar methodology to that developed for the synthesis of 6 was used to access the key stannylated intermediate 16. Thus, the free
PEG terminus of 10 was protected to give TBDPS (‘butyldiphenylsilyl) ether 11, followed by reduction to give amine 12. Stannylation to give 13 and amide formation to afford 14 were followed by TBDPS deprotection to give 15. N-Maleoyl-β-alanine was then coupled to the PEG chain to give the key intermediate 16. Conjugation of 16 to K₁₆Cys (7) gave 17 as a precursor for radiolabeling. Incubation of 17 with sodium [¹²⁵I]iodide in an iodogen coated tube (30 min, ambient temperature), followed by purification with semi-preparative radio-HPLC, provided the desired K₁₆Cys-Smal-[PEG₃-[¹²⁵I]-IPQA] 9 in 28% radiochemical yield (Scheme 2).

Radiolabeled [¹²⁵I]-9 was subsequently formulated as the F1LA1 K₁₆Cys-Smal-[PEG₃-[¹²⁵I]-IPQA]-lipopolyplex, which was obtained in 64% radiochemical yield. Radiolabeled (MeO-[PEG₆-[¹²⁵I]-IPQA] 8 (Scheme 1) was prepared using the same protocol as for [¹²⁵I]-9, and was obtained in 38% radiochemical yield. The two radiolabeled tracers, [¹²⁵I]-8 and F1LA1 K₁₆Cys-Smal-[PEG₃-[¹²⁵I]-IPQA]-lipopolyplex, were synthesized in order to determine the biodistributions of the small molecule TKI inhibitor and the nanoparticle construct, respectively.

In vivo results
LIM1215 xenografts were established in standard immunocompromised CD1 nude mice (Materials and methods section). The biodistribution data (Fig. 3) showed that the free drug, radiolabeled MeO-[PEG₆-[¹²⁵I]-IPQA] 8 was rapidly cleared via the hepatobiliary route with high uptake in gallbladder and intestines at 3 and 6 h.

Whereas F1LA1 K₁₆Cys-Smal-[PEG₃-[¹²⁵I]-IPQA]-lipopolyplex showed uptake within the liver and spleen as well as high uptake in the gallbladder and intestines consistent with lipopolyplex uptake and degradation by the Kupffer cells of the reticuloendothelial system. Tumor uptake was highest for both MeO-[PEG₆-[¹²⁵I]-IPQA] 8 and F1LA1 K₁₆Cys-Smal-[PEG₃-[¹²⁵I]-IPQA]-lipopolyplex at 3 h with gradual reduction over the 24 h time course. However, the uptake of F1LA1 K₁₆Cys-Smal-[PEG₃-[¹²⁵I]}
The radioactivity of [125I]-IPQA-lipopolyplex within tumors was higher than free MeO-[PEG6-[125I]-IPQA] at all time points.

After gamma counting, the tumors were snap frozen, allowing simultaneous expression of biosensor and inhibition of EGFR activity by the liposome-encapsulated K16Cys-SMal-[PEG3-[125I]-IPQA]. Fig. 4 shows representative images of tissue from mice injected with CLA1 lipopolyplexes, which only contain the pDNA encoding for the Picchu-X biosensor.

**Scheme 2** Reagents and conditions: (i) TBDPSCl, imidazole, CH₂Cl₂, 0 °C to rt, 3 h (98%); (ii) SnCl₂·2H₂O, THF, 60 °C, 3.5 h, (65%); (iii) Pd(PPh₃)₄, Sn₂Me₆, dioxane, reflux, 2.5 h (95%); (iv) isobutyl chloroformate, acrylic acid, Et₃N, −40 °C, 30 min (53%); (v) TBAF, THF, 0 °C to rt, 1 h (95%); (vi) N-maleoyl-β-alanine, DCC, DMAP, CH₂Cl₂, rt, 2 h, (89%); (vii) CysLys₁₆, NaHCO₃, rt, 1 h (55%); (viii) sodium [125I]iodide, iodogen, 30 min rt, 28% radiochemical yield.
measurements of GFP revealed that EGFR activity can be successfully suppressed (Fig. 4) by PEG-IPQA activity can be successfully suppressed (Fig. 4) by PEG-IPQA released from the F1LA1 K16Cys-SMal-[PEG3-125I]-IPQA-lipopolyplexes. Tissues treated with inhibitor had significantly higher GFP lifetime than control tissues (Fig. 4), which can also be seen on the images (shift from red-yellow to blue colours in pseudocolor map).

**Conclusions**

Liposome-based theragnostic nanoparticles hold considerable promise for the delivery of small molecule therapeutic drugs to tumours, and for determining their biodistribution and efficacy in vivo. A major barrier to the success of such approaches is the difficulty encountered in loading sufficient quantities of the drug into the liposome-based formulation, particularly where co-delivery of DNA or RNA is required. In this work, we present a novel solution to this problem. The reversible bioconjugation of the small molecule therapeutic to a cationic peptide allows the co-formulation of both a TKI EGFR inhibitor and pDNA coding for a biosensor into a targeted lipopolyplex. The lipopolyplex is designed to disassemble on internalisation into the target cells. Our results indicate that release of the components from the endosome is accompanied by release of the TKI from the bioconjugate and inhibition of EGFR.

This approach also allowed us to further investigate the basis for the enhanced circulation and better tumour selectivity afforded by nanoparticle delivery of small molecule therapeutics. The biodistribution data in Fig. 3 suggests different kinetics for either uptake of free drug vs. liposomes, and/or elimination of the free drug vs. drug encapsulated/shielded in liposome in the cells. This phenomenon will need further investigation of potential differences in trafficking of the drug within the cell post-uptake, both in terms of the subcellular compartments involved and kinetics of intracellular trafficking. However, we believe that the in vivo trafficking/clearance differences may be more significant and therefore relevant for the therapeutic effectiveness. The clearance in vivo of radio-labelled Mo-IPQA has recently been studied, and has shown that this highly lipophilic inhibitor has limited tumour selectivity and undergoes significant hepatobiliary clearance. More hydrophilic PEGylated analogues, such as [18F]F-PEG6-IPQA, which formed the basis for the design of our own bioconjugate, show improved pharmacokinetics and quicker in vivo clearance, but still have little selectivity for tumour cells. In contrast, liposomal formulations of the structurally-related, radiolabelled quinazoline TKI inhibitor, SKI-212243 had a significantly longer circulation time, which is accompanied by release of the TKI from the bioconjugate and inhibition of EGFR.

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However, translation to clinically viable agents, particularly for the treatment of colorectal cancer, has generally been unsuccessful, and the reasons for the poor clinical efficacy of these treatments remains elusive. Knowing how these therapies actually act at a molecular level when administered to patients would be a crucial step towards improving the success rate of such inhibitors. Our results demonstrate that lipopolyplexes are a successful delivery system for both an EGFR biosensor and a TKI inhibitor, which will allow surveillance of EGFR activity while testing the potency of such drugs in vivo. This approach in turn opens a new possibility for manipulating and monitoring the activity of EGFR in situ, which in turn will allow a deeper understanding of the dynamics of EGFR activation/inhibition and sensitivity of target cells to EGFR inhibition. Whilst we have previously demonstrated that it is possible to image EGFR activity in vivo utilizing an endoscopy-based fluorescence lifetime imaging methodology, this was able to image EGFR activity due to o...

Experimental section

Chemical synthesis

General methods for chemical synthesis and solid phase peptide synthesis, sources of chemicals, purification and analytical methods are in the ESL. Peptides P1, P2, P3, P4, and P6 were synthesized according to the published literature procedures. Experimental procedures for the synthesis of 3, 8, 10, S6, P5 and 7; HPLC/HRMS for peptides 1, 17, P5, 7 and 1H/13C NMR spectra for novel compounds can be found in the ESL.

Synthesis of K18Cys-S-S-[PEG6-IPQA] (1)

N-(3-Iodophenyl)-6-nitro-7-((1,1,1-triphenyl-5,8,11,14,17-pentaaza-2-thianonadecan-19-yl)oxy)quinazolin-4-amine (4). Compound 3 (702 mg, 0.940 mmol) and triphenylmethane thiol (310 mg, 1.12 mg) were dissolved in THF (16 mL) and NaH (89 mg, 3.7 mmol) was added gradually. The dark purple reaction mixture was stirred for 2 h at rt and subsequently quenched with cold NaHCO3-solution. The aqueous phase was extracted (3 × CH2Cl2) and the combined organic phases washed with saturated NaCl solution (2×). The combined organic phases were dried (MgSO4) and filtered and concentrated in vacuo. Purification was achieved using flash column chromatography (3% MeOH/CH2Cl2) to give 4 (712 mg, 82%) as a yellowish oil. 1H NMR (600 MHz, CDCl3) δ 8.86 (s, 1H), 8.71 (s, 1H), 8.25 (s, 1H), 7.82 (d, J = 8.1 Hz, 1H), 7.50 (d, J = 7.9 Hz, 1H), 7.30–7.33 (m, 6H), 7.20–7.23 (m, 7H), 7.15–7.17 (m, 3H), 7.11 (t, J = 8.0 Hz, 1H), 4.22–4.23 (m, 2H), 3.84–3.85 (m, 2H), 3.69–3.71 (m, 2H), 3.61–3.64 (m, 2H), 3.47–3.52 (m, 6H), 3.40–3.44 (m, 6H), 3.25 (t, J = 6.8 Hz, 2H), 2.33 (t, J = 6.8 Hz, 2H) ppm; 13C NMR (150 MHz, CDCl3) δ 158.2, 157.4, 154.0, 144.8, 139.32, 139.29, 133.9, 131.4, 130.5, 129.5, 128.04, 127.99, 126.7, 122.3, 121.7, 110.0, 108.2, 94.1, 91.4, 73.1, 70.8, 70.7, 70.56, 70.52, 70.46, 70.42, 70.2, 69.9, 69.8, 69.3, 66.6, 31.6 ppm; HRMS (ESI) m/z: [M + H]+ Calcd for C45H48IN4O8S 931.2238; Found 931.2240.

N\(1\)-(3-Iodophenyl)-6-amino-7-((1,1,1-triphenyl-5,8,11,14,17-pentaaza-2-thianonadecan-19-yl)oxy)quinazolin-4-amine (5). Compound 4 (50 mg, 0.054 mmol) was dissolved in THF (10 mL) and SnCl2·2H2O (30 mg, 0.16 mmol) was added. The reaction was heated to reflux for 3 h then cooled to rt. Saturated NaHCO3 solution (20 mL) was added followed by H2O (50 mL). The aqueous phase was extracted (3 × EtOAc) and the combined organic phases dried (MgSO4) and concentrated in vacuo. Purification was achieved using flash column chromatography (3% MeOH/CH2Cl2) to give 5 (30 mg, 61%) as a yellowish oily solid. 1H NMR (600 MHz, CDCl3) δ 8.57 (s, 1H), 8.12–8.13 (m, 1H), 7.73 (d, J = 8.0 Hz, 1H), 7.43 (br s, 1H), 7.41–7.42 (m, 1H), 7.32–7.34 (m, 6H), 7.20–7.22 (m, 6H), 7.14–7.17 (m, 4H), 7.12 (s, 1H), 7.07 (t, J = 8.0 Hz, 1H), 4.62 (br s, 2H), 4.28–4.30 (m, 2H), 3.91–3.92 (m, 2H), 3.75–3.76 (m, 2H), 3.57–3.67 (m, 12H), 3.52–3.54 (m, 2H), 3.31 (t, J = 6.8 Hz, 2H), 2.39 (t, J = 6.8 Hz, 2H) ppm; 13C NMR (150 MHz, CDCl3) δ 155.3, 152.6, 151.5, 145.2, 144.8, 148.4, 140.6, 138.8, 132.4, 130.5, 129.7, 129.6, 128.0, 126.7, 120.4, 110.6, 107.6, 101.0, 94.2, 71.1, 70.8, 70.78, 70.76, 70.73, 70.63, 70.55, 70.2, 69.8, 69.1, 68.4, 66.7, 31.6 ppm; HRMS (ESI) m/z: [M + H]+ Calcd for C45H48IN4O8S 931.2246; Found 931.2248.

N\(4\)-(3-Iodophenyl)amino)-7-((1,1,1-triphenyl-5,8,11,14,17-pentaaza-2-thianonadecan-19-yl)oxy)quinazolin-6-yl)acrylamide (6). A solution of acrylic acid (50 µL, 0.74 mmol) in THF (2.5 mL) was cooled to 0 °C and treated with isobutylchloromate (81 µL, 0.62 mmol), added dropwise, followed by Et3N (175 µL, 1.26 mmol). The reaction was cooled to −40 °C using an acetoni/trry/dry ice bath and a solution of 5 (192 mg, 0.211 mmol) in THF (1 mL) was added in one portion. The resultant reaction mixture was stirred for 35 min and then quenched with saturated NaHCO3 solution. The solution was extracted (3 × CH2Cl2) and the combined organic phases dried (MgSO4) and filtered and concentrated in vacuo. Purification was achieved using automated flash column chromatography (0–2% MeOH/CH2Cl2) to give 6 (120 mg, 57%) as a yellowish oil. 1H NMR (600 MHz, CDCl3) δ 9.14 (s, 1H) 8.68 (s, 1H) 8.59 (s, 1H) 8.20 (t, J = 1.8 Hz, 1H) 7.74 (ddd, J = 8.2, 2.2, 0.9 Hz, 1H) 7.56 (s, br, 1H) 7.48 (ddd, J = 7.9, 1.6, 1.0 Hz, 1H) 7.37–7.43 (m, 6H) 7.24–7.33 (m, 6H) 7.17–7.22 (m, 3H) 7.12 (t, J = 8.0 Hz, 1H) 6.50 (dd, J = 16.8, 1.5 Hz, 1H) 6.43 (dd,
After this time the reaction mixture was diluted with CH2Cl2 and washed with saturated NaCl solution and H2O. The reaction mixture was allowed to warm to rt and stirred for 3 h.

With TBDPS-Cl (179 µL, 0.685 mmol), added slowly. The reaction mixture was stirred for 2 h at rt. The solvent was removed in vacuo and purification was achieved using flash column chromatography (5% MeOH/CH2Cl2) to give 2 (21 mg, 48%) as a yellow oil. 1H NMR (600 MHz, CDCl3) δ 9.23 (dd, J = 2.6, 0.7 Hz, 1H), 9.12 (s, 1H), 8.73 (s, 1H), 8.67 (s, 1H), 8.42 (dd, J = 8.9, 2.6, 1H), 8.20 (t, J = 1.8 Hz, 1H), 8.02 (dd, J = 8.9, 0.8 Hz, 1H), 7.79 (br s, 1H), 7.75 (dd, J = 8.1, 1.8, 0.8 Hz, 1H), 7.48 (dd, J = 7.9, 1.4, 0.9 Hz, 1H), 7.32 (s, 1H), 7.11 (t, J = 8.0 Hz, 1H), 6.43-6.49 (m, 2H), 5.83 (dd, J = 7.4, 4.1 Hz, 1H), 4.37-4.39 (m, 2H), 3.96-3.98 (m, 2H), 3.73-3.75 (m, 2H) 3.60-3.71 (m, 1H), 3.53-3.55 (m, 2H), 3.01 (t, J = 6.0 Hz, 2H) ppm; 13C NMR (150 MHz, CDCl3) δ 169.3, 164.3, 154.1, 152.5, 145.0, 142.1, 139.7, 133.3, 132.6, 132.0, 131.3, 130.0, 130.6, 128.6, 128.5, 128.1, 119.1, 119.5, 110.2, 109.6, 107.7, 94.2, 70.8, 70.74, 70.66, 70.642, 70.615, 70.56, 69.1, 68.9, 68.5, 39.0 ppm (3 OCH2 peaks overlapping – evident by HMQC); HRMS [ESI] m/z: [M + H]+ Calcd for C48H52IN4O7S 955.2601; Found 955.2528.

N-(4-(3-Iodophenyl)amino)-7-((1-(5-nitropyridin-2-yl)disulfanylidene)-2,5,8,11,14-pentaoxahexade-16-yl)oxoquinazolin-6-yl)acrylamide (2). To a solution of 6 (50 mg, 0.050 mmol) in CH2Cl2 (5 mL) was added DNP (24 mg, 0.078 mmol) and TFA (500 µL, 6.53 mmol). The reaction mixture was stirred for 2 h at rt. The solvent was removed in vacuo and purification was achieved using flash column chromatography (5% MeOH/CH2Cl2) to give 2 (276 mg, 98%) as a yellow oil. 1H NMR (600 MHz, CDCl3) δ 7.63-7.46 (m, 8H), 7.14 (s, 1H), 7.11 (s, 1H), 6.85 (s, 1H), 4.28-4.30 (m, 2H), 3.93-3.94 (m, 2H), 3.83 (t, J = 6.1 Hz, 2H), 3.70-3.75 (m, 4H), 3.67 (s, 4H), 3.61 (t, J = 6.1 Hz, 2H) 1.06 (s, 9H) ppm; 13C NMR (150 MHz, CDCl3) δ 151.5, 151.4, 151.0, 138.3, 133.7, 133.8, 130.5, 129.8, 129.8, 127.8, 127.7, 120.5, 110.2, 107.2, 94.2, 72.6, 70.93, 70.86, 70.8, 70.7, 69.4, 68.3, 63.6, 27.0, 19.3 ppm; HRMS [ESI] m/z: [M + H]+ Calcd for C38H46IN4O5Si 867.1335; Found 867.1337.

K16Cys-S-S-[PEG4-IPQA] (1). A solution of CysLys16 (7 mg) and 2 (3 mg) in MeOH were stirred in MeOH at room temperature for 1 h, monitored by analytical HPLC. The solvent was removed in vacuo and the resulting bicone was purified by HPLC to give 1 (5.9 mg, 59% yield).

Synthesis of K16-Cys-Smal-[PEG3-N-(4-(3-trimethylstannyl)amino)quinazolin-6-yl)acrylamide] (17).

K16Cys-S-S-[PEG4-IPQA] (1). A solution of CysLys16 (7 mg) and 2 (3 mg) in MeOH were stirred in MeOH at room temperature for 1 h, monitored by analytical HPLC. The solvent was removed in vacuo and the resulting bicone was purified by HPLC to give 1 (5.9 mg, 59% yield).

7-((2,2-Dimethyl-3,3-diphenyl-4,7,10,13-tetraoxa-3-silapentadec-15-yl)oxy)-N-(3-iodophenyl)quinazoline-4,6-diamine (13). A stream of argon was bubbled through a solution of 12 (430 mg, 0.542 mmol) in dioxane (20 mL) for 10 min. Pd(PPh3)4 (15 mg, 0.012 mmol) was added followed by hexamethylditin (355 mg, 224 µL, 1.08 mmol) and the resultant solution heated at reflux for 2.5 h. The reaction mixture was cooled to rt and the solvent evaporated in vacuo. CH2Cl2 and saturated NaHCO3 solution were added and the phases separated. The aqueous phase was washed (2 × CH2Cl2) and the combined organic phases washed with saturated NaCl solution then dried (MgSO4) filtered and concentrated in vacuo. Purification was achieved using automated flash column chromatography (0-10% MeOH/CH2Cl2) to give 13 (428 mg, 95%) as a yellow oil. 1H NMR (600 MHz, CDCl3) δ 8.57 (s, 1H), 8.5 (d, J = 2.3 Hz, 1H), 7.36-7.42 (m, 8H), 7.17 (s, 1H), 7.11 (t, J = 9.7 Hz, 1H), 6.95 (br s, 1H), 6.85 (s, 1H), 4.30-4.35 (m, 4H), 3.94-3.95 (m, 2H), 3.83 (t, J = 5.1 Hz, 2H), 3.70-3.75 (m, 4H), 3.66 (s, 4H), 3.62 (t, J = 5.1 Hz, 2H), 1.06 (s, 9H) ppm; 13C NMR (150 MHz, CDCl3) δ 155.6, 152.3, 152.1, 143.4, 138.4, 137.9, 135.7, 133.8, 131.6, 129.8, 128.7, 128.6, 127.8, 127.6, 122.0, 110.3, 107.5, 106.5, 76.2, 70.95, 70.89, 70.8, 70.7, 69.4, 68.2, 63.6, 27.0, 19.3 ppm; HRMS [ESI] m/z: [M + H]+ Calcd for C141H117O24N8Si 381.2973; Found 381.2973.

7-((2,2-Dimethyl-3,3-diphenyl-4,7,10,13-tetraoxa-3-silapentadec-15-yl)oxy)-N-(4-(3-trimethylstannyl)amino)quinazolin-6-yl)acrylamide (14). A solution of acrylic acid (63 mg, 60 µL,
0.88 mmol) in THF (8 mL) was cooled to 0 °C and treated with isobutyl chloroformate (104 mg, 99 µL, 0.76 mmol), added dropwise, followed by Et3N (163 µL, 1.26 mmol). The reaction was stirred at 0 °C for 10 min then the ice bath was replaced with an acetonitrile/dry ice bath and left for 2 min more. A solution of 13 (240 mg, 0.290 mmol) in THF (4 mL) was cooled in the dry ice bath and then added to the reaction in one portion, the resultant reaction mixture was stirred for 30 min and then quenched with saturated NaHCO3 solution. The solution was extracted (3 × CH2Cl2) and the combined organic phases were washed with saturated NaCl solution then dried (MgSO4) filtered and concentrated in vacuo. Purification was achieved using automated flash column chromatography (4% MeOH/CH2Cl2) to give 14 (137 mg, 53%) as a yellow oil. 1H NMR (600 MHz, CDCl3) δ 9.13 (s, 1H), 8.94 (br s, 1H), 8.64 (s, 1H), 7.86 (dd, J = 8.0, 2.0 Hz, 1H), 7.66–7.68 (m, 5H), 7.35–7.42 (m, 7H), 7.29 (d, J = 7.0 Hz, 1H), 7.24 (s, 1H), 6.47–6.49 (m, 2H), 5.76 (dd, J = 8.2, 3.1 Hz, 1H), 4.35–4.37 (m, 2H), 3.96–3.97 (m, 2H), 3.81 (t, J = 5.3 Hz, 2H), 3.64–3.76 (m, 8H), 3.61 (t, J = 5.3 Hz, 2H), 1.04 (s, 9H), 0.33 (s, 9H) ppm; 13C NMR (150 MHz, CDCl3) δ 154.9, 152.1, 148.4, 143.3, 131.9, 129.2, 128.5, 128.25, 128.20, 122.5, 103.0, 109.9, 108.3, 70.8, 70.7, 70.64, 70.62, 69.2, 69.1, 68.7, 63.9, 33.6, 32.9, −9.3 ppm; HRMS (ESI) m/z: [M + H]+ Calcd for C35H44N5O9Sn 798.2158; Found 798.2157.

**K16-Cys-SM-[PEG]N(4-(3-trimethylstannyl)amino)quinazolin-6-yl)acrylamide (17).** A solution of 16 (3.0 mg), CysLys16 (300 µg) and NaHCO3 (5.0 mg) in MeOH (1 mL) was stirred at room temperature for 1 h, then filtered and purified by analytical HPLC. The solvent was removed in vacuo and the resulting biconjugate was purified by HPLC to give 17 (6.1 mg, 55% yield).

**Liposome and lipopolyplex formulation**

**Lipopolyplex formulation for in vitro experiments.** All lipid components were dissolved in CHCl3 to a concentration of 1 mM. These stock solutions were mixed to obtain the desired lipid quantities. The solvent was then removed under reduced pressure to form a thin film, which was further dried on a high vacuum line for at least 1 h. The thin film was hydrated with sterilized water to the desired total lipid concentration, sonicated for 10 min, and used immediately. Lipopolyplexes used for in vitro experiments were formulated as described (including Cy5-DOTMA 2mol%). For every 100 µL of sonicated liposome solution, first peptide [4–8 µg] and/or peptide–drug conjugate (8–32 µM) was added followed by plasmid DNA (2–4 µg) coding for the Picchu-X sensor. The samples were mixed and used immediately.

**FIL1A K16-Cys-S-S-[PEG6-IPQA] lipopolyplex formulation for in vivo experiments (sample for 10 mice).** The following lipid solutions (1 mM in chloroform) were added to a round bottom flask:

- DODEG4: 50 mol% → 500 µL of 1 mM lipid stock for 1 mL of liposome solution;
- DOTMA: 20 mol% → 200 µL of 1 mM lipid stock for 1 mL of liposome solution;
- DOPE: 30 mol% → 300 µL of 1 mM lipid stock for 1 mL of liposome solution;
- CHOL: 30 mol% → 300 µL of 1 mM lipid stock for 1 mL of liposome solution.

This gave a total concentration of the lipids in the liposomes of 1.3 mM. The solvent in the lipid mixtures was slowly removed via evaporation in vacuo to form a lipid thin film and dried for at least 1 h on a high vacuum line. The appropriate amount of distilled water was added to reach a concentration of 1.3 mM total lipid in water. The sample was sonicated for 5 min in a bath sonicator and subsequently on a probe tip sonicator over 10 min using a cycle of 10 s sonication at level 3.
and 3 s cooling time. The lipid solution was kept on ice during the sonication process to avoid degradation of the lipid components and overheating of the solution. This procedure gives liposomes of <100 nm and surface charge around +20 mV or above.

The liposomes were transferred into a Falcon tube and to this solution was added targeting peptide **P1** (K16-RVRR-LARLLT (1 mM stock)) to a final concentration of 170 µM followed by the drug peptide bioconjugate (K16-Cys-S-S-[PEG6-IPQA] **1** (0.7 mM stock)) to a final concentration of 100 µM. The lipidosome peptide solution was then diluted to 3.5 mL. 680 µg Picchu-X plasmid DNA (1.68 µg mL⁻¹ stock, 404 µL) was added to a Falcon tube and diluted to 3.5 mL. The solution was then slowly (dropwise but steadily) added to the liposome–peptide solution while constantly swirling the solution, in order to avoid the concentration of DNA and peptide getting locally too high and precipitating. Once the whole solution has been added the now freshly formed lipopolyplexes have been transferred to a spin-column (MWCO above 5000 Da) and concentrated to the initial volume of 1 mL.

**Radiolabelling**

General methods for radioiodination work and HPLC traces can be found in the ESL.†

**Preparation of MeO-[PEG6-[¹²⁵I]-IPQA] (8).** A Sep-Pak C18 Plus Light Cartridge (145 mg, Waters Ltd, Cat. no. WAT036805) was washed with ethanol (5 mL), HPLC water (10 mL) and was air-dried (10 mL). TKI-precursor solution (86) (1.8 mg) was dissolved in PBS (0.18 mL, Sigma-Aldrich Ltd, Cat. no. P4417). This stock solution (40 µL) was added to an iodogen coated tube (Thermo-Fisher Ltd, Cat. no. 28601) followed by mixing with [¹²⁵I]NaI (5 µL, 34.2 MBq). After standing for 30 min at room temperature the reaction was quenched by adding HPLC mobile phase (100 µL of MeOH/H₂O/TFA 10/90/0.1 v/v/v). The reaction mixture was injected into a semi-preparative HPLC.

The product peak (tᵣₑ = 18.9 min) was collected and diluted with H₂O (10 mL). A conditioned ‘C18 SepPak Light cartridge was loaded with that solution, washed with H₂O (5 mL) and eluted with ethanol in six fractions (0.1 mL). The overall RCY was 38%. The radiochemical purity was >99% as determined by analytical HPLC. The UV channel (254 nm) did not show any stable impurities. The identity of the radiolabelled product was confirmed by co-injection with a non-radioactive reference solution of 9. The product was formulated by mixing aqueous cold compound (100 µM, 1.1 mL) with an ethanol fraction of [¹²⁵I]-8 as collected from the SepPak cartridge (6.8 MBq).

**Preparation of K16-Cys-Smal-[PEG3-[¹²⁵I]-IPQA] (9).** The radiolabelling procedure for preparing K16-Cys-Smal-[PEG3-[¹²⁵I]-IPQA] (9) starting from the corresponding tin precursor (17) (using a stock solution of 13 mg mL⁻¹ and [¹²⁵I]NaI (5 µL, 37.5 MBq) followed the same protocol as detailed for [¹²⁵I]-8. The overall RCY of [¹²⁵I]-9 was 28%. The radiochemical purity was >99% as determined by analytical HPLC. The UV channel (254 nm) did not show any stable impurities. The identity of the radiolabelled product was confirmed by co-injection with a non-radioactive K16-PEG-IPQA reference solution.

**Preparation of the radiolabeled F1LA1 K16-Cys-Smal-[PEG3-[¹²⁵I]-IPQA]-lipopolyplex.** The volume of K16-Cys-PEG-[¹²⁵I]-IPQA ([¹²⁵I]-9) in ethanol was reduced (40 µL, 7.8 MBq) using a stream of nitrogen. Pre-formulated liposome–peptide suspension (1.3 mM lipid mixture (50% DODEG, 20% DOTMA), 170 mM targeting peptide **P1** (K16-RVRR-LARLLT, 100 µM K16-Cys-S-S-[PEG6-IPQA] **1**) in distilled water (4 mL) was mixed with K16-Cys-Smal-[PEG3-[¹²⁵I]-IPQA] **9**. After slowly adding plasmid DNA (Picchu-X, 0.748 mg) in distilled water (4 mL) with vortexing, the resulting mixture was filtered using four spin columns (VIVASPIN 500, Sartorius Cat. no. VS0515) on a micro-centrifuge (TopSpin, 40 min, 5000 rpm). Please note that this procedure can result in formation of radioactive aerosol, and it must be carried out in a suitable ventilation cabinet with appropriate measures to control radioactive contamination. The final F1LA1 K16-Cys-Smal-[PEG3-[¹²⁵I]-IPQA]-lipopolyplex suspension was collected in 1.1 mL (64% RCY).

**In vivo and in vitro testing**

**Tissue culture, reagents and antibodies.** LIM1215, DiFi, SW48 and DLD1 colon cancer cell lines (independently validated by STR DNA fingerprinting at The Institute of Cancer Research, London, UK), were maintained in RPMI-1640 (Life Technologies Ltd). Antibodies: anti-EGFR (clone D38B1) and anti-p-Y1173-EGFR (clone 53A5) were from Cell Signalling Technology Ltd.

**In vitro liposomal transfection and imaging.** A10 µM solution of the non-radio-labelled F1LA1 K16-Cys-S-S-[PEG6-IPQA] lipopolyplex was applied to cells at 60% confluence in 96-well plates. Cells expressing Picchu-X biosensor were imaged on customised “open” microscope automated FLIM system. Time-domain fluorescence lifetime images were acquired via time correlated single photon counting (TCSPC) at a resolution of 256 by 256 pixels, with 256 time bins and 100 frames accumulated over 300 seconds, via excitation and emission filters suitable for the detection of GFP fluorescence (Excitation filter: Semrock FF01-470/22 nm; Emission filter: Semrock FF01-510/50 nm).

Conventional wide field fluorescence images were acquired with filter cubes for FITC (Excitation 480/30 nm, emission 535/45 nm) on a CCD camera (Hamamatsu 1394 ORCA-ERA), with an exposure time of typically 100–500 ms. FLIM analysis was performed with the TRI2 software (Version 2.7.3.9, Gray Institute, Oxford) as described previously.

**In vivo experiments.** All animal studies were approved by the University College London Biological Services Ethical Review Committee and licensed under the UK Home Office regulations and the Guidance for the Operation of Animals (Scientific Procedures) Act 1986. 12 female 6–8 weeks old CD1 nu/nu mice (Charles River Laboratories, UK) were injected subcutaneously in the right flank with 2 × 10⁶ LIM1215 cells in 100 µL PBS. Once palpable tumors were measured in three orthogonal directions using the following equation: length × height × width × π/6. Once tumors had reached approximately 70–100 mm³ mice were randomly divided into 2 groups; group 1 received an
intravenous injection of 0.5Mbq MeO-[PEG₆⁻¹²⁵I]-IPQA \( n = 6 \), group 2 received an intravenous injection of 0.5Mbq F1LA1 K₁₆Cys-SMal-[PEG₆⁻¹²⁵I]-IPQA-lipopolyplex \( n = 6 \). Mice were then culled at 3, 6 and 24 hours \( n = 2 \) per time point and all organs were taken, weighed and \( ¹²⁵I \) uptake quantified using a gamma counter (Wizard, PerkinElmer) to obtain % Injected dose per gram (%ID g⁻¹).

**Author contributions**

Robin Bofinger, Gregory Weitsman, Rachel Evans, Matthias Glaser, Kerstin Sander and Tammy Kalber performed the experiments (chemical and peptide synthesis, cancer cell biology, FRET/FLIM, radiochemistry and biodistribution). Helen Allan, Robin Bofinger, Gregory Weitsman and Alethea Tabor wrote the initial draft, and all authors contributed to writing and reviewing the final draft. Erik Arstad, Daniel Hochhauser, Tammy Kalber, Helen Hailes, Tony Ng and Alethea Tabor supervised the research groups, and Tammy Kalber, Erik Arstad, Tony Ng and Alethea Tabor acquired the financial support for the research.

**Conflicts of interest**

There are no conflicts to declare.

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