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Ultrasmall nanoparticles induce ferroptosis in nutrient-deprived cancer cells and suppress tumour growth

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The design of cancer-targeting particles with precisely tuned physicochemical properties may enhance the delivery of therapeutics and access to pharmacological targets. However, a molecular-level understanding of the interactions driving the fate of nanomedicine in biological systems remains elusive. Here, we show that ultrasmall (<10 nm in diameter) poly(ethylene glycol)-coated silica nanoparticles, functionalized with melanoma-targeting peptides, can induce a form of programmed cell death known as ferroptosis in starved cancer cells and cancer-bearing mice. Tumour xenografts in mice intravenously injected with nanoparticles using a high-dose multiple injection scheme exhibit reduced growth or regression, in a manner that is reversed by the pharmacological inhibitor of ferroptosis, liproxstatin-1. These data demonstrate that ferroptosis can be targeted by ultrasmall silica nanoparticles and may have therapeutic potential.

Nanomaterials designed to target cancer may hold therapeutic potential by enhancing the delivery of therapeutics1–4, but their interactions with cancer in animal models and their long-term effects on cells remain poorly understood5–9. To examine the effects of nanoparticle ingestion on cells, we used ~6 nm surface-functionalized poly(ethylene glycol)-coated (PEGylated) near-infrared (NIR) fluorescent silica nanoparticles, referred to as Cornell dots (C dots), with diameters controllable down to the sub-10 nm range, as reported in detail elsewhere10. This FDA Investigational New Drug (IND)-approved hybrid organo-silica particle was previously shown to be a promising cancer molecular imaging agent in metastatic melanoma patients after functionalizing its surface with αβ3-integrin-targeting peptides and radiolabels11,12. Preferential accumulation was observed within integrin-expressing primary and/or metastatic melanomatous lesions in human subjects and animal melanoma models11,12, while at the same time demonstrating rapid renal clearance.

Given its potential clinical utility and its early adaptation for drug delivery applications13, we initially performed detailed cell biological studies using the latest generation of C dots, synthesized in water, referred to as C′ dots14, surface-functionalized with a 14 mer peptide analogue, alpha-melanocyte stimulating hormone (αMSH)14, which targets a different surface receptor expressed on malignant melanoma cells (melanocortin-1 receptor, MC1-R). The resulting αMSH-PEG-C′ dots (Fig. 1a) were used here to determine whether alterations in cell survival occurred in cancer cell lines and tumour xenografts over a wide dose range relative to that seen under control conditions and whether cellular pathways were modulated by particle ingestion.

αMSH-PEG-C′ dots reside in lysosomes and are well tolerated

Live imaging of MC1-R-expressing human melanoma cells (M21) treated with particles for 24 h revealed co-localization of fluorescent αMSH-PEG-C′ dots with lysosomes, the latter visualized by expression of a green fluorescent protein (GFP)-tagged lysosomal-associated membrane protein 1 (LAMP1), indicating that ingested particles reside in lysosomal or late endosomal networks (Fig. 1b). M21 cells treated with increasing concentrations of αMSH-PEG-C′ dots up to 15 µM showed similar survival and proliferation rates to control cells (Fig. 2a), demonstrating that incubation at even high particle concentrations is well tolerated. We next examined if lysosomes were functioning properly within cells treated with αMSH-PEG-C′ dots. To determine this, we examined the autophagy pathway, which targets intracellular substrates for lysosomal degradation. Autophagy was examined by quantifying the basal levels and turnover rates of the autophagy protein microtubule-associated protein 1 light chain 3 (LC3), which is lipidated onto autophagosomal membranes and becomes degraded following the fusion of autophagosomes with lysosomes15. The accumulation of the autophagosome-associated, lipidated form of LC3, or LC3-II, identified by its faster electrophoretic mobility in SDS–polyacrylamide gel

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**αMSH-PEG-C′ dots induce death of starved cells**

Although αMSH-PEG-C′ dots were well tolerated by cells cultured under nutrient-replete conditions and the autophagy pathway and lysosome function appeared to be unperturbed, we further examined if particle treatment might affect cells cultured under nutrient-deprived conditions where autophagy is induced. Cells cultured in amino-acid-free media were treated with αMSH-PEG-C′ dots and examined by time-lapse imaging. Although amino-acid deprivation was well tolerated by M21 cells in the absence of particles, the treatment of amino-acid-deprived cells with 15 µM αMSH-PEG-C′ dots, which had no effect on cells in nutrient-replete media, surprisingly led to cell death at high rates, detected by the uptake of Sytox Green, a membrane-impermeable nucleic acid dye that labels cells with ruptured plasma membranes (Fig. 2c). This demonstrates that while αMSH-PEG-C′ dots are generally well tolerated, nutrient-deprived cancer cells are sensitive to treatment. To determine whether this finding could have consequences for tumour growth in vivo, we incubated M21 cells with αMSH-PEG-C′ dots in culture under nutrient-replete conditions, which had no effect on cell viability (Fig. 2a and Supplementary Fig. 1a), and then injected particle-exposed cells, as well as particle-unexposed cells, into mice as flank tumour xenografts, to promote a nutrient-deprived state that is known to result from a lack of vascularization in early xenograft tumours16. M21 cells loaded with αMSH-PEG-C′ dots demonstrated statistically significant growth inhibition (P < 0.001) relative to non-particle-exposed cells (Fig. 2d). In fact, no measurable tumour growth occurred from particle-exposed cells up to 10 days following cell injection. These findings suggested that treatment with αMSH-PEG-C′ dots at high concentrations may induce cell death under conditions of nutrient deprivation in culture and in vivo.

**αMSH-PEG-C′ dot-induced death occurs by ferroptosis**

We sought to identify the mechanism of how cells treated with αMSH-PEG-C′ dots undergo cell death under nutrient-deprived conditions. Careful inspection of the morphology of dying cells suggested a form of necrosis, involving cell swelling and plasma membrane rupture, in the absence of cell blebbing and fragmentation that is typically observed during apoptosis (Fig. 3a and Supplementary Fig. 1b). To more definitively identify the mechanism of cell death, we used two non-tumour cell lines, MCF10A human mammary epithelial cells and mouse embryo fibroblasts (MEF), which were also observed to die at high rates when cultured in amino-acid-free media in the presence of αMSH-PEG-C′ dots (Fig. 3b). Cells rendered resistant to apoptosis, by overexpression of the anti-apoptotic protein Bcl-2 (MCF10A-Bcl2) (Supplementary Fig. 1c) or by genetic deletion of Bax and Bak (Bax/Bak−/− MEFs)17,18, underwent cell death at rates similar to control cells, suggesting that αMSH-PEG-C′-dot-induced cell death does not occur by apoptosis (Fig. 3c,d). Next, we examined whether cell death was occurring by necroptosis, a programmed form of necrosis that requires the RIPK3 kinase19. Ripk3−/− knockout MEFs, which are resistant to necroptosis (Supplementary Fig. 1d), also underwent cell death at rates similar to controls, suggesting that nanoparticle treatment does not induce necroptosis (Fig. 3e). We then determined whether a recently described form of cell death involving the autophagy pathway, autosis20, could be involved, by treating autophagy-related gene 5 knockout MEFs (Atg5−/− MEFs), completely deficient for autophagy, with αMSH-PEG-C′ dots in the absence of amino acids. Atg5−/− MEFs underwent cell death at rates similar to those of controls, demonstrating that αMSH-PEG-C′-dot-induced cell death does not involve autophagy and is not autosis (Fig. 3e). Collectively, the foregoing data demonstrate that cell death, induced by a combination of particle treatment and amino-acid deprivation, occurs independently of apoptosis, necroptosis and autosis.

**Figure 1 | αMSH-PEG-C′-dot particles and their localization to lysosomal networks.**

a Silica-based and ultrasmall αMSH-PEG-C′-dot particles of 5-nm-diameter silica-based particles with a fluorescent (Cy5 encapsulated) core and polyethylene glycol (PEG) coating and alpha melanocyte-stimulating hormone (αMSH)-modified exterior. b αMSH-PEG-C′-dots localize to lysosomal networks in cells. M21 melanoma cells expressing LAMP1-GFP (green) were treated with αMSH-PEG-C′-dots (15 µM) for 24 h. Note co-localization between nanoparticles (Cy5 fluorescence, pseudo-coloured red) and LAMP1-GFP in the merged image. Scale bar, 10 µm.

Electrophoresis (PAGE) relative to the non-lipidated form, LC3-I, can be quantified as a measure of autophagy induction or flux through lysosomes15. Cells treated with increasing concentrations of αMSH-PEG-C′-dots for 24 h, from 0.15 to 15 µM, had similar relative LC3-II levels as control cells, suggesting that autophagy is not induced or perturbed by nanoparticle treatment (Fig. 2b). Importantly, the treatment of cells with a lysosomal inhibitor, concanamycin A (ConA), which inhibits lysosome function by raising pH, and blocks autophagosome degradation, resulted in a similar accumulation of LC3-II in particle-treated cells as compared to controls, demonstrating that lysosomes are functioning properly even when cells are loaded with high concentrations of αMSH-PEG-C′-dots (Fig. 2b).
We next examined whether ferroptosis, a recently described cell death mechanism that occurs via an iron and lipid reactive oxygen species (ROS)-dependent process and is induced by glutathione depletion\(^2\), could be involved in αMSH-PEG-C\(^-\) dot-induced cell death. We first tested whether ferrostatin-1 and liproxstatin-1, pharmacological inhibitors of ferroptosis that are scavengers of lipid ROS, could block cell death in this context. Treatment with either inhibitor rescued cell viability, reducing cell death to levels occurring under amino-acid-deprived conditions in the absence of nanoparticles (Fig. 4a and Supplementary Fig. 1e). Nanoparticle-induced cell death was also inhibited by treatment with other antioxidants, including butylated hydroxyanisole (BHA), ascorbic acid (Asc Acid) and trolox, or, alternatively, by glutathione repletion through the addition of glutathione or N-acetylcysteine (NAC), a precursor of glutathione (Fig. 4b). To examine whether lipid ROS accumulate during nanoparticle-induced cell death, we imaged particle-exposed cells in the presence of the lipid oxidation indicator C11-BODIPY. Increased fluorescence before cell death was seen to occur in response to treatment with the known ferroptosis-inducing agent erastin, in a liproxstatin-1-inhibitable manner (Fig. 4c and
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Fluorescence images show Sytox Green-labelling of dead cell nuclei. Scale bar, 10 μm.

The absence of amino acids with 15 μM αMSH-PEG-C′ dots and after 40 h (MCF10A) or 45 h (MEF), as determined by time-lapse microscopy. Error bars indicate mean ± standard deviation. n = 5 per group. Each replicate is from one biological experiment, quantified with five independent fields of view. Supplementary Fig. 6f,g shows individual experimental values. a-e. Cell death assays, as in b, indicate that inhibition of apoptosis by Bcl2 overexpression in MCF10A (c), quantified after a 38 h time-lapse experiment, or deletion of Bax and Bak in MEF (d), quantified after 45 h, or inhibition of necroptosis by deletion of ripk3 in in MEF (e), quantified after 45 h, or inhibition of autophagy by knockout of Atg5 in MEF after 45 h (e) does not inhibit cell death induced by amino-acid starvation and treatment with 15 μM αMSH-PEG-C′ dots. Error bars indicate mean ± standard deviation. n = 5 per group. Each replicate is from one biological experiment, quantified with five independent fields of view. Supplementary Fig. 6h-j shows individual experimental values.

Supplementary Fig. 1f). Like cell death induced by erasin treatment, lipid ROS detected by C11-BODIPY fluorescence accumulated several hours before the induction of cell death by nanoparticle treatment under amino-acid-free conditions (Fig. 4c). To further examine if nanoparticle-induced death is dependent on iron, a known requirement for ferroptosis, we found that cells treated with deferoxamine (DFO), an iron chelator used for treating iron overload and an agent reported to block ferroptosis21, almost completely inhibited cell death (Fig. 4d). Together, these data demonstrate that treatment of amino-acid-starved cells with high αMSH-PEG-C′ dot concentrations induces ferroptosis. Interestingly, ferroptosis in this context was also observed to propagate from cell to cell in a wave-like manner (Fig. 4e and Supplementary Movie 1), unlike that found for cells undergoing other types of death, such as apoptosis (Supplementary Fig. 1g and Supplementary Movie 2). These findings suggested cell–cell communication of a death-inducing signal, similar to a recent report of ferroptosis occurring in renal tubules in response to treatment with erasin22.

αMSH-PEG-C′ dots inhibit tumour growth

We next examined whether ferroptosis could be induced by nanoparticle treatment in a wider panel of cancer cells. Like M21 cells, BxPC3 pancreatic carcinoma cells, H1650 lung carcinoma cells, HT-1080 fibrosarcoma cells and 786-O renal carcinoma cells underwent high rates of necrosis when treated under amino-acid-free conditions with αMSH-PEG-C′ dots, indicating that cell death can be induced by a combination of particle treatment and nutrient deprivation in a variety of different cancer cell types (Fig. 5a–c and Supplementary Fig. 2a). Interestingly, HT-1080 cells underwent necrosis in response to nanoparticle treatment, even when cultured in full media (Fig. 5c), as well as in starvation media at tenfold lower particle concentrations (Supplementary Fig. 2b), suggesting that these cells are particularly sensitive to particle-induced ferroptosis. We further investigated whether particle-induced treatment responses could be generated in 786-O renal carcinoma and HT-1080 fibrosarcoma xenograft models. Using a multidosing delivery scheme, tumour growth was assessed over a 10-day period after three high-dose intravenous (i.v.) treatments of either saline vehicle, statistically significant inhibition of tumour growth was observed with multiple-dose particle treatments for both tumour types (Fig. 6a,b), and greater for HT-1080 xenografts. Surprisingly, this was accompanied by partial tumour regression exceeding 50% for all particle-treated HT-1080 tumours (range, 57–78%; mean, 64%) within a 4–5 day interval after initial injection (Fig. 6c). At study termination, statistically significant reductions in treated tumour volumes (HT-1080: mean ∼85%, P < 0.001; 786-O: mean ∼73%, P < 0.01) were found.

Figure 3 | αMSH-PEG-C′ dot particle-induced cell death is not apoptosis, necroptosis or autosis. a, MCF10A human mammary epithelial cells cultured in the absence of amino acids with 15 μM αMSH-PEG-C′ dots undergo cell death after 30 h with necrotic features. Insets: a dying cell, indicated by an arrow. Fluorescence images show Sytox Green-labelling of dead cell nuclei. Scale bar, 10 μm. b, Quantification of cell death (Sytox Green+) in MCF10A and mouse embryo fibroblast (MEF) cultures in full media or amino-acid-starved (AA-st) conditions in the presence or absence of 15 μM αMSH-PEG-C′ dots and after 40 h (MCF10A) or 45 h (MEF), as determined by time-lapse microscopy. Error bars indicate mean ± standard deviation. n = 5 per group. Each replicate is from one biological experiment, quantified with five independent fields of view. Supplementary Fig. 6f,g shows individual experimental values. e–j shows individual experimental values.
relative to control volumes (Fig. 6a,b). An intense fluorescence signal overlying the xenograft, obtained by whole-body optical imaging (Fig. 6d), suggested particle localization. Immunohistochemical staining for the macrophage marker Mac-2 revealed large numbers of recruited macrophages surrounding treated tumours relative to that seen around control tumours, both at low and high magnification (Fig. 6e–h).

To further examine if the inhibition of tumour growth resulting from nanoparticle treatment could be related to ferroptosis, we treated tumour-bearing mice with daily intraperitoneal (i.p.) doses of liproxstatin-1 for 10 days to determine the effects on particle-induced tumour shrinkage. Notably, in HT-1080 xenografted mice (n = 3) subsequently administered three high-dose particle treatments, liproxstatin-1 administration significantly reduced growth inhibition to levels nearly equivalent to those seen in non-particle-exposed tumours (Fig. 6f). Using a generalized estimating equations (GEE) model with a logit link, average daily growth in particle-exposed tumours treated with liproxstatin-1 was 14.6 mm³ (95% confidence interval (CI): 10.1–18.9), as compared with −0.87 mm³ (95% CI: −1.06 to −0.69) for particle treatment alone, a difference of 15.3 mm³ (CI: 13.1–17.6; P < 0.001, Wald test from GEE). Corresponding particle-exposed tumour specimens were significantly smaller on average than particle-treated tumours receiving daily liproxstatin-1 (Fig. 6i, inset).

Conclusions
Here, we have demonstrated that the combined treatment of cells with aMSH-PEG-C′ dots and amino-acid starvation can synergize to induce the cell death programme ferroptosis, and that high-dose particle delivery can inhibit tumour growth and cause tumour regression. These effects are reversible with liproxstatin-1. Although their role in wound repair and engulfment of cellular debris is well established, the significance of increased numbers of macrophages around particle-treated, as opposed to control tumours, remains unclear. It is known that a high degree of macrophage plasticity can occur in response to local cues from the tumour microenvironment and that, upon activation, macrophages can assume a spectrum of roles needed to maintain...
tissue homeostasis\textsuperscript{25}, including shifts in function associated with tumour shrinkage\textsuperscript{24}.

These data thus define a potential therapeutic application for molecularly targeted \textsuperscript{C} dots, already in clinical trials for cancer imaging and detection\textsuperscript{2}, but without the need for surface-attached cytotoxic agents. An important question raised by these findings is how these particles induce ferroptosis. Surface-modification of particles with \textalpha MSH peptides for targeting cancers enhances cellular internalization (data not shown). Interestingly, particle surface modification with \textalpha MSH is not required for ferroptosis in the cell lines tested. We have observed its induction, albeit more slowly, with unmodified PEGylated \textsuperscript{C} dots. The slower rate of ferroptosis induction may reflect a slower rate of internalization of unmodified particles relative to \textalpha MSH-modified platforms (Supplementary Fig. 3a).

We therefore considered that the native silica particle itself has ferroptosis-inducing activity, as deprotonated surface silanol groups and/or fractal internal structure may lead to iron adsorption and/or incorporation (that is, loading) within its structure. We indeed found iron loading of \textalpha MSH-PEG-\textsuperscript{C} dots incubated with culture media, as compared with doubly deionized water preparations (Supplementary Fig. 3b and Supplementary Table 1). Increasing amounts of iron were loaded into particles in a concentration-dependent manner following exposure to ferric oxide solutions. These findings were accompanied by a decrease in iron-loading capacity as they became saturated at high iron concentrations (Supplementary Table 1). Furthermore, increased intracellular iron levels were found for \textalpha MSH-PEG-\textsuperscript{C}-dot nanoparticle-treated cells compared to non-treated cells (Supplementary Fig. 3b and Supplementary Table 1). Consistent with a model in which particles deliver iron into cells, particle-treated cells upregulated expression of the heavy chain of ferritin (FTH1) that binds cytosolic iron (Supplementary Fig. 3c). We further found that iron loading into cells by treatment with ferric ammonium citrate (FAC) is sufficient to mimic particle treatment and induce ferroptosis in amino-acid-starved cells (Supplementary Fig. 3d), suggesting that nanoparticles may engage ferroptosis by loading iron into cells.

Increased iron uptake could lead to the depletion of glutathione, conceivably due to increased ROS generation. We do not find suppression of glutathione levels in particle-treated cells (Supplementary Fig. 3e). Pretreatment with erastin, which inhibits glutathione production by blocking cystine uptake, sensitizes cells to particle-induced ferroptosis, suggesting that glutathione depletion is rate-limiting for particle-induced death (Supplementary Fig. 3f). Taken together, our data support a model whereby particle-induced ferroptosis is executed following iron uptake into cells, suppression of glutathione, and accumulation of lipid ROS (Supplementary Fig. 4). Lipid ROS may accumulate in glutathione-suppressed cells due to lowered activity of the glutathione peroxidase 4 (GPX4) enzyme that protects cells from lipid peroxidation and inhibits ferroptosis\textsuperscript{26,27}. We have not found that particle treatment inhibits GPX4 activity in an enzymatic assay from treated cell lysates (Supplementary Fig. 3g), consistent with the model that particle treatment does not lead to lipid peroxidation by direct inhibition of GPX4. We additionally note that particle-induced ferroptosis does not require the presence of glutamine, unlike ferroptosis occurring in starved cells\textsuperscript{28}, as our studies also use amino-acid-free conditions in which enhanced iron uptake may bypass the requirement for glutamine.

The concentration of nanoparticles used here to either induce in vitro cell death or inhibit in vivo tumour growth is at least four orders of magnitude higher than what is used currently in human subjects for single-dose imaging-based studies\textsuperscript{2}, but local concentrations could be driven to much higher levels at tumour sites as part of a
combined inhibitor (liproxstatin-1) and particle treatment (T + L; dot treatments (with and without i.p.-injected liproxstatin-1) were given over a 10 day period. Relative to particle treatment alone, marked progression of tumour growth is seen following combined inhibitor and particle treatment (HT-1080: *P* < 0.001; 786-O: *P* < 0.01). P values are from a Wald test in a regression model estimated by GEEs to take into account the longitudinal nature of the data.

Small numbers of intratumoral Mac-2-positive cells are also noted. While reducing off-target toxicities and promoting efficient renal clearance, it is notable that the leakiness of tumour vasculature is thought to allow the accumulation of systemically injected nanoparticles in a regression model estimated by GEEs to take into account the longitudinal nature of the data. 

**Figure 6** | αMSH-PEG-C′ dots inhibit tumour growth in 786-O and HT-1080 xenograft models. 

**a, b.** Graphical summary of 786-O (a) and HT-1080 (b) average tumour volume measurements in αMSH-PEG-C′-dot-treated (‘Particles’; *n* = 5) and control (‘Saline vehicle’; *n* = 3) mice; error bars indicate standard deviation. Three high-dose (12 nmol per dose) αMSH-PEG-C′ dot or saline vehicle control treatments (30 μmol per dose) were i.v. injected (arrows) over a 10 day period, with particle-treated tumours, on average, demonstrating growth inhibition, greater for HT-1080, relative to saline-treated controls. 

**c.** Individual HT-1080 tumour volume measurements from b, shown for particle-treated (‘T’) and saline-treated (‘C’) mice. Relative to control tumour volumes, data show marked inhibition of tumour growth and partial tumour regression after particle treatments (HT-1080: *P* < 0.001; 786-OL: *P* < 0.01). P values are from a Wald test in a regression model estimated by GEEs to take into account the longitudinal nature of the data. 

**d.** Left: whole-body Cy5 fluorescent imaging of a representative HT-1080 tumour xenograft (arrows). Right: low-power view of H&E-stained tissue sections from representative control (top) and treated (bottom) tumours, revealing a densely cellular and invasive neoplasm exhibiting multifocal necrosis. Control specimens are noted to be disproportionately larger in size than the corresponding treated ones, without evident morphological differences. 

**e-h.** Immunohistochemical staining of tumour sections with macrophage marker Mac-2 shows scattered macrophages (arrow) surrounding control tumour sections (T) on low (e) and high (g) power views, while corresponding low (f) and high (h) power views of Mac-2-stained treated sections show large numbers of Mac-2-positive cells circumscribing the tumour at similar locations (boxes and arrows: d-f).

Small numbers of intratumoral Mac-2-positive cells are also noted. 

**i.** Graphical summary of individual HT-1080 tumour volume measurements in mice undergoing combined inhibitor (liproxstatin-1) and particle treatment (T + L; *n* = 3) versus particle treatment alone (T; *n* = 3). Three high-dose (12 nmol per dose) αMSH-PEG-C′ dot treatments (with and without i.p.-injected liproxstatin-1) were given over a 10 day period. Relative to particle treatment alone, marked progression of tumour growth is seen following combined inhibitor and particle treatment (*P* < 0.001). Image: representative particle-exposed tumours reveal specimens to be disproportionately larger in size when additionally treated with liproxstatin-1 (right tumour). Scale bars, 1 mm ([d-f], 50 μm ([g-h], 1 cm ([i]).
tumour tissues\textsuperscript{29}, while contributing to nutrient deprivation within tumours, suggesting that the synergism between nanoparticles and nutrient deprivation may be restricted to tumour sites \textit{in vivo}. Some cancers may also be particularly sensitive to this mechanism of cell death, which could lower the threshold of particle concentration needed to achieve an antitumour effect. Indeed, we have found that HT-1080 cancer cells undergo aMSh-PEG-C\texttextunderscore dOT\textunderscore dot\textunderscore induced ferroptosis, even under nutrient-replete conditions (Fig. 5c), and these cells are also killed in amino-acid-deprived conditions with tenfold lower nanoparticle concentration (Supplementary Fig. 2b). It is notable that HT-1080 tumours are well vascularized (Supplementary Fig. 5a), suggesting that the sensitivity of these cancer cells to particle-induced death, even under nutrient-replete conditions (Fig. 5c), may contribute to the strong antitumour effect that is observed following intravenous particle delivery.

Although, conceivably, the sensitivity of some cancers could lower the particle concentrations needed to exert an antitumour effect, we have also found that multiple high-dose aMSh-PEG-C\textunderscore dot treatments are well tolerated. Evaluation of tumour, hepatic, renal and haematological specimens from particle- and vehicle-treated HT-1080- and 786-O-bearing mice, performed at the termination of the study, showed no significant group differences in complete blood counts, serum chemistries and hepatic and renal histopathology, with the exception of indirect and total bilirubin serum concentrations, which were moderately elevated in particle-treated mice compared with controls (Supplementary Tables 2 and 3). However, no mechanism could be ascribed to this finding given the lack of haemolysis on complete blood counts and absence of liver injury on histopathology and serum chemistries. The extent of macrophage phagocytosis among particle-treated and vehicle-treated tumour specimens is summarized in Supplementary Table 4.

The discovery of nanoparticle-induced ferroptosis as a redox modulator of cell fate, as well as a mediator of tumour regression and growth inhibition, suggests that it may be possible to exploit this process therapeutically to synchronously and selectively kill those cancers most susceptible to this mechanism. Elucidating the basis for such differential tumour sensitivity is essential. It will be important, for instance, to assess the extent of tumour vascularity, as this parameter will critically influence particle delivery, nutrient and oxygenation status and treatment response. As such, its assessment across a variety of pre-clinical tumour types may enable better screening of models suitable for the induction of cell death by this mechanism, as well as for stratifying patients in the future to appropriate combinatorial treatment paradigms.

It will also be critical to determine whether controlled variations in C\textunderscore dot properties—structure, composition or surface chemistry—enhance, or even abrogate, the induction of ferroptosis. We found, for instance, that C\textunderscore dot size plays a critical role in the magnitude of the effect observed under amino-acid-deprived conditions (Supplementary Fig. 5b). Smaller-diameter (~6 nm) PEGylated C\textunderscore dots led to significantly higher percentages of Sytox Green-labelled HT-1080 cells (that is, 80%) than found for larger-diameter (~10 nm) C\textunderscore dots (~25%). Finally, we note that although the response to lipooxatin-1 provides evidence for ferroptosis as an antitumour mechanism \textit{in vivo}, other mechanisms may be contributory, including modulation of the tumour microenvironment, given the marked macrophage recruitment to particle-treated tumours (Fig. 6g,h, HT-1080; Supplementary Fig. 5c, 786-O)\textsuperscript{2}, an area under active investigation. Together, our findings lay the groundwork for exploiting these ultrasmall silica nanoparticles as effective ferroptosis-inducing agents in sensitive tumours.

Methods

Methods and any associated references are available in the online version of the paper.

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Author contributions

Additional information
Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.S.B. and M.O.

Competing financial interests
The authors have filed an international patent application PCT/US16/34351.
incubation chamber to maintain cells at 37 °C and 5% CO₂ and NIS Elements software (Nikon). Cell fates, including cell survival, death and proliferation, were manually quantified and processed using NIS Elements software and ImageJ.

Glutathione quantification. For glutathione measurements, HT-1080 cells were plated on 6 cm cell culture dishes, incubated with 15 μM aSM-HS-tagged nanoparticles for 48 h. DMEM + 10% dialysed FBS and collected ~2 h before the expected time of death. As controls, cells were treated with a mix of three parts full or amino-acid-free DMEM and 1 part H₂O₂, or 100 μM BSO in full media. Cells were washed three times with cold PBS and collected through cell scraping in 50 μl cold lysis buffer. Protein concentrations were measured using the BCA assay, and volumes were adjusted so that each sample had the same total protein concentration. Total glutathione was measured using the Glutathione Assay Kit (Cayman Chemicals, 703002) and reduced glutathione was measured using the QuantChrom Glutathione Assay Kit (BioAssay Systems, DGT-250) according to the manufacturer’s instructions.

Iron measurements. For iron loading capacity measurements of C dots, 50 μl aSM-HS-PEG-C dots (60 μM) were added to either 150 μl iron-containing media (8.6 μM) or 150 μl FeCl₃ solutions prepared over a range of iron (Fe³⁺) concentrations (2 μM to 2 mM, Supplementary Table 1). Solutions were spun at room temperature (160 r.p.m.) for 48 h, followed by separation of free iron from C dots using a PD-10 column and eluting in water. Cellular samples were incubated with and without particles in media for 48 h before centrifugation, pelleted and washed three times before re-suspending in PBS solution. Iron measurements (parts per billion, ppb) were determined using a microwave plasma – atomic emission spectrometry, along with iron concentrations. Iron loading capacity was computed as the ratio of the amount of iron purified particles to C dots (or cells), divided by the total amount of iron measured before purification and multiplied by 100. All experiments were performed in triplicate.

GFP activity assay. The GFP specific activity assay was performed according to ref. 31. In brief, frozen cell pellets were resuspended in 100 μl lysis buffer (100 mM KH₂PO₄/KH₂PO₄, pH 7.4, 1 mM EDTA, 150 mM KCl, 0.1% CHAPS, 3 mM β-mercaptoethanol and protease inhibitor cocktail) and homogenized by 50 pestle strokes. Samples were incubated for 15 min on ice, and cell debris was removed by centrifugation (20,000g; 10 min, 4 °C). A protein (50 μl) of the supernatant from homogenized cells was used to determine the enzymatic activity of the GFP assay buffer (100 mM Tris HCl pH 7.8 containing 5 mM EDTA, 0.1% Triton X, 3 mM GSH, 200 μM NAPD and 0.6 M 1-glutathione reductase) in the presence of 20 μM phosphatidylcholine hydroperoxide (PCOOH). GFP activity was determined by the glutathione reductase-dependent consumption of NAPDH detectable by a decrease in absorbance at 340 nm in a SpectraMax plate reader ( Molecular Device). Protein content in the samples was determined by the colorimetric 660 nm Pierce protein assay method.

Animal models and tumour inoculation. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center and followed NIH guidelines for animal welfare. Human melanoma (M21) xenografts were generated on the shaved flanks of immunodeficient male mice by subcutaneous injection of melanoma cells into the glutathione reductase-dependent consumption of NAPDH detectable by a decrease in absorbance at 340 nm in a SpectraMax plate reader ( Molecular Device). Protein content in the samples was determined by the colorimetric 660 nm Pierce protein assay method.

In vivo drug delivery strategy and examination. Two million M21 cells, cultured in serum-supplemented media, were subcutaneously implanted into the right flank of mice using a 23-gauge trocar needle to establish melanoma xenografts 2 days after particle exposure (n = 3) or without exposure (n = 3). In a subsequent study, mice were treated with i.v.-injected aSM-HS-PED-PEG-C dots (n = 5 mice; 200 μl) administered three times over a 10 day period (that is, days 0, 4 and 7). Control HT-1080 and 786-O mice (n = 3) were administered with 0.9% saline vehicle at the same time points. In a third treatment study, HT-1080 mice were assigned to two of the groups to evaluate the response to high-concentration doses (60 μM) of i.v.-injected aSM-HS-PED-PEG-C dots (n = 3 mice; 200 μl) alone or following ip. administration of lipoprotin-1 (n = 3 mice, 125 mg kg⁻¹) over a 10 day period. Tumor sizes were measured using calipers over the treatment interval. All mice were examined by palpation at the site of tumour cell inoculation and were observed daily until the termination of tumour growth studies for signs of morbidity or mortality. Two perpendicular diameters (d₁, d₂) of the tumour used to calculate the tumour volume (V = 0.4/3 × π × d₁ × d₂ × 8) were measured with calipers daily following the injection of cells.

In vivo fluorescence imaging. Animals were anesthetized using isoflurane, and whole-body optical fluorescence imaging was acquired to identify nanoparticles
fluorescence at the tumour site. Mice were scanned for 0.1–1 s using the IVIS Spectrum photon-counting device optical imaging system (Xenogen) with the blocks and filters for Cy5 fluorescence (excitation 650 nm, emission 680 nm) and for background fluorescence (excitation 465 nm, emission 600 nm), selected according to the manufacturer’s recommendations. The fluorescence background was subtracted according to the manufacturers’ instructions. Fluorescence signal was reported as radiant efficiency ((photons s⁻¹ cm⁻² sr⁻¹) μW⁻¹ cm⁻²).

Histopathological analysis. Immediately after terminating the in vivo imaging study, HT-1080 and 786-O male and female mice were euthanized by CO2 inhalation, and representative particle-exposed (n = 2) and control (n = 1) tumours, as well as hepatic and renal specimens, were excised at necropsy. Haematological specimens were also obtained for complete blood counts and serum chemistries. Excised tumours, livers and kidneys were fixed in 10% neutral buffered formalin for 24 h, processed in alcohol and xylene, embedded in paraffin, sectioned at 5 μm thickness, and stained with haematoxylin and eosin (H&E). Additional tumour sections were stained by immunohistochemistry for Mac-2 (primary antibody Cedarlane CL8942B applied at a concentration of 1:100 following heat-induced epitope retrieval (HIER) in a pH 6.0 buffer), myeloperoxidase (Dako A0398, 1:1,000, HIER pH 6.0), cleaved caspase-3 (Cell Signaling Technology 9661, 1:250, HIER pH 6.0) and Ki-67 (Abcam ab16667, 1:100, HIER pH 9.0). Mac-2 staining was performed manually with an avidin-biotin detection system (Vectastain ABC Elite Kit, Vector Laboratories, PK-6100). Other stains were performed on a Leica Bond RX automated stainer using the Bond Polymer Refine detection kit (Leica Biosystem DS9800). Tumour sections were also stained by the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) method as previously described. All slides were examined by a board-certified veterinary pathologist.

Tumour vascularity assessment. Tumour vascularity was assessed by staining HT-1080 tumours by immunohistochemistry for CD31 on a Leica Bond RX automated staining platform (Leica Biosystems). Following heat-induced epitope retrieval at pH 9.0, the primary antibody (rat monoclonal, catalogue no. DIA-310, Dianova) was applied at a concentration of 1:250 and was followed by application of a polymer detection system (Novocastra Bond Polymer Refine Detection, Leica Biosystems).

Statistics. Volume–time profiles were compared between the two treatment groups using robust standard errors calculated by a generalized estimating equations approach32. Particle-treated tumour growth profiles, with and without a pharmacological inhibitor to ferroptosis, liproxstatin-1, were compared using a linear model. The longitudinal aspect of the data was taken into account using GEEs. We assigned statistical significance at P < 0.05.

References