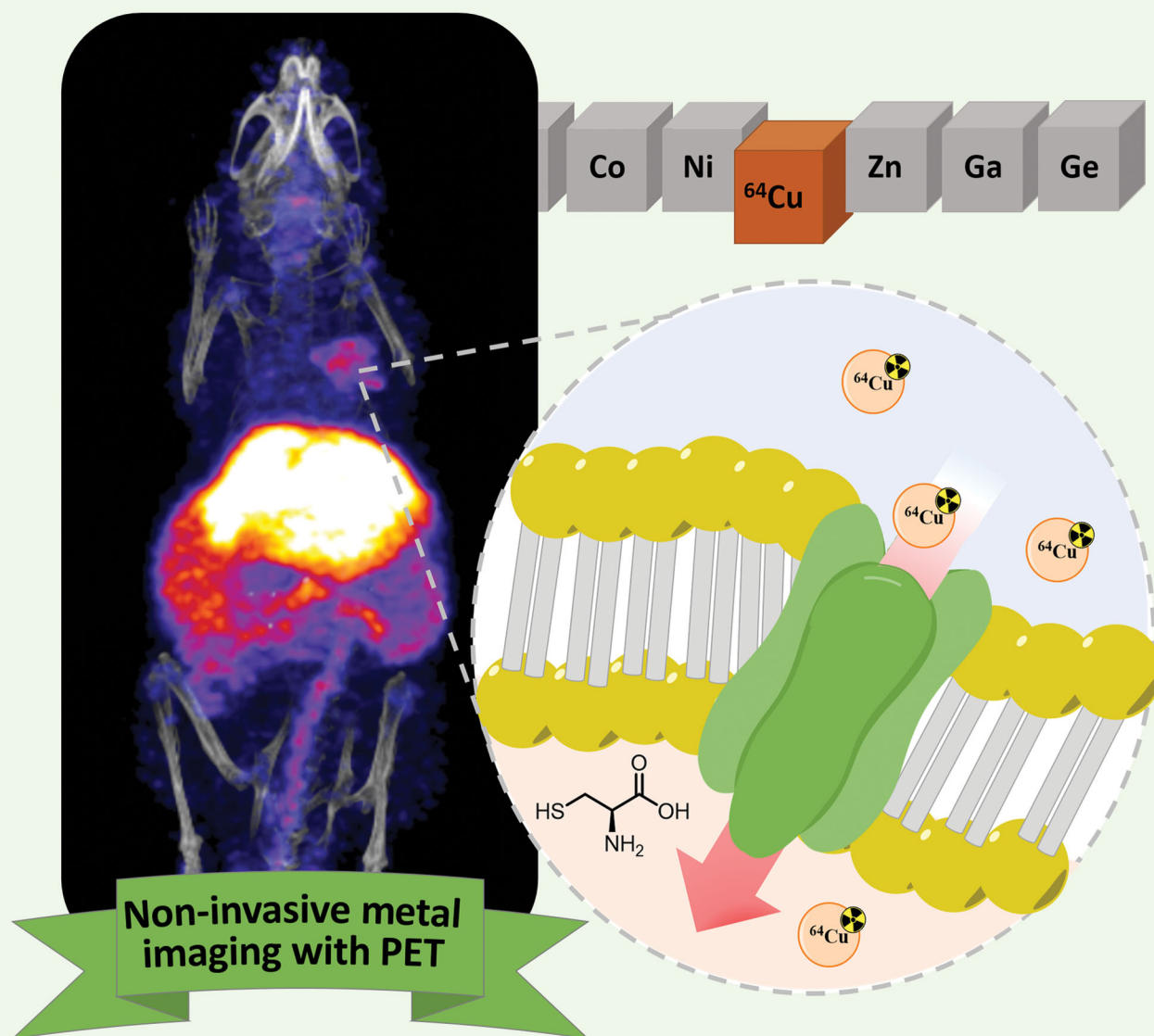


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L-Cysteine-mediated modulation of copper trafficking in prostate cancer cells: an *in vitro* and *in vivo* investigation with ^{64}Cu and ^{64}Cu -PET†

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Copper imbalance is implicated in many diseases, including cancer. Copper in blood is mainly transported by carrier proteins but a small fraction is bound to low molecular weight species, possibly amino acids. Their roles in cellular copper delivery are unknown. Our aim was to test whether accumulation of ^{64}Cu into cancer-derived cells can be influenced by copper-binding serum amino acids. *In vitro* cellular accumulation of ^{64}Cu was measured in Hank's Balanced Salt Solution in the presence of 100 μM L-histidine, L-methionine, L-cysteine and L-threonine. L-Cysteine markedly increased ^{64}Cu accumulation and retention in DU145, PC3 and SK-OV-3 cells, while some other cell lines did not show an effect. This effect was not due to ^{64}Cu delivery in the form of a ^{64}Cu -cysteine complex, nor to reduction of $^{64}\text{Cu}(\text{II})$ to $^{64}\text{Cu}(\text{I})$ by L-cysteine. Pre-incubation of cells with L-cysteine increased ^{64}Cu accumulation, even if L-cysteine was removed from HBSS before ^{64}Cu was added. The effect of L-cysteine on ^{64}Cu accumulation was not mediated by increased glutathione synthesis. Despite the demonstrable *in vitro* effect, pre-injection of L-cysteine precursor *N*-acetyl-cysteine (NAC) *in vivo* did not enhance ^{64}Cu delivery to DU145 xenografts in mice. Instead, it decreased ^{64}Cu accumulation in the DU145 tumour and in brain, as assessed by PET imaging. We conclude that ^{64}Cu is not delivered to DU145 cancer cells *in vitro* as a complex with amino acids but its cellular accumulation is enhanced by L-cysteine or NAC influx to cells. The latter effect was not demonstrable *in vivo* in the DU145 xenograft.

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Significance to metallomics

Copper is an essential element required by all cells. Its metabolism is dysregulated in many diseases. Cancer cells, especially in prostate cancer, typically accumulate more copper than normal cells. In this work we explored how copper is delivered to and retained in cancer cells. We demonstrate that L-cysteine and *N*-acetyl-cysteine supplementation increases cellular copper retention in prostate cancer cells and in some other cell lines but amino acids in serum are not required to deliver copper ions to transporters for import into the cells tested.

Introduction

Due to the indispensable roles of copper in biological processes and the toxicity of copper excess,¹ its trafficking is normally tightly controlled. Dysregulation leads to, or results from, disorders of copper deficiency (Menkes disease)² or overload (Wilson's disease).³ Copper imbalance is also implicated in other pathologies such as neurodegeneration⁴ and tumour development.⁵ Investigating copper transport is important for understanding its role in disease aetiology and for designing related diagnostic and therapeutic strategies.

In eukaryotes, the main route of cellular copper uptake across the plasma membrane is *via* copper transporter 1 (CTR1, in human cells hCTR1),⁶ though some cell types utilise alternative mechanisms.⁷ Upon entry, copper ions are transported to their subcellular destinations (*e.g.*, to copper-dependent enzymes) by specific metallochaperones,⁸ such as antioxidant 1 copper chaperone (ATOX1). These transfer copper directly from one binding site to another without release.⁹

CTR1-mediated transport of copper occurs *via* transmetallation involving coordinating methionine triads in the trans-membrane region of CTR1, which form a selectivity filter.^{10,11} While copper is mainly present in plasma as copper(II), CTR1 transports Cu(I) ions.^{6,10,12–14} Several ferric reductases were shown to also possess cupric reductase activity,^{15–17} while studies on CTR1 model peptides suggested that reduction of

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Cu(II) bound to CTR1 could also be mediated by physiological reducing agents such as ascorbate.^{18,19} Other mechanistic details, such as the identity of the direct donors of copper ions from circulation to CTR1 and whether these donors are involved in the redox of copper ions during CTR1 transport, remain speculative.²⁰ Most copper in human serum is bound to proteins.²¹ *In vitro* studies have shown that cultured cells can receive copper from all the main protein carriers: ceruloplasmin, transcuprein and albumin.^{13,22,23} Direct copper transfer from human serum albumin (HSA) to a model hCTR1 peptide has been demonstrated spectroscopically.²⁴

A small percentage of serum copper pool is bound to other biomolecules not as extensively studied as ceruloplasmin, transcuprein or albumin. This pool, isolated by gel filtration,^{25–27} ultrafiltration^{28,29} or dialysis,¹ includes species identified as small proteins (<30 kDa) and low molecular weight (LMW) components whose abundance (1–10%) and whose upper molecular weight cut-off vary depending on the analytical method used. The functions of these species in copper transport are largely unknown, except for a 2 kDa molecule playing a role in urinary copper excretion.³⁰ The LMW fraction probably includes copper-amino acid complexes, as first suggested several decades ago based on indirect methods including *in vitro* reconstitution experiments³¹ and thin layer chromatography, which detected copper-histidine and -threonine species.¹ Computer simulations based on complex formation constants demonstrated the prevalence of copper-histidine, copper-histidine-glutamine and copper-histidine-threonine complexes in plasma.³² Other work taking into account redox equilibria suggested a copper-cysteine complex as the major LMW constituent.³³ There is no consensus regarding the existence or composition of the copper-binding amino-acid pool in human serum.

An alternative strategy to illuminate the roles of amino acids in copper transport and cellular delivery is to investigate whether their presence in the extracellular medium affects copper accumulation in cells. In rat liver slices, addition of a mixture of amino acids,³⁴ or histidine alone,³⁵ markedly increased copper uptake, while in erythrocytes¹ and cultured rat hepatocytes³⁶ histidine alleviated the inhibitory effect of serum proteins on copper uptake. In lymphoid cells, by contrast, added histidine inhibited copper accumulation in the presence of albumin.¹ Copper-histidine complex was shown to donate copper to mouse embryonic fibroblasts,¹³ with uptake rates comparable to those from copper-transcuprein and -albumin complexes. Investigators of the dietary interplay of amino acids and copper found that copper-amino acid complexes had higher permeability in intestinal cells *in vitro* compared to ionic copper,³⁷ though *in vivo* studies were inconclusive.^{38,39}

There have been no systematic studies of the effects of individual amino acids other than histidine on copper uptake into tumour cells. Such studies are particularly timely due to the emerging roles of copper in tumour signalling pathways,⁵ which underpin on-going clinical trials of copper chelation therapies in cancer⁴⁰ and the use of copper-based radionuclide imaging of prostate tumours.⁴¹ Mechanisms of copper delivery from serum donors to copper membrane transporters (mainly hCTR1) are essentially unknown.

Against this background, we aimed to test the hypothesis that LMW components of the copper serum pool could affect copper uptake in cells by acting as copper donors to the cellular copper transporters. Adopting a radiotracer approach, we measured *in vitro* ⁶⁴Cu accumulation in prostate cancer cells (DU145) in the presence of individual amino acids selected based either on existing evidence¹ of their complexation with copper in serum (L-histidine, L-threonine) or presence of chemical groups capable of copper ion coordination (L-methionine, L-cysteine). We investigated *in vitro* whether amino acids can affect ⁶⁴Cu delivery to cells, and if so, by what mechanism? We then investigated whether the conclusions drawn were relevant to whole-body copper trafficking to tumours *in vivo* using Positron Emission Tomography (PET) with ⁶⁴Cu.

Methods

⁶⁴Cu production

[⁶⁴Cu]CuCl₂ was produced as previously described⁴² by proton-irradiating a solid ⁶⁴Ni target, dissolving it in concentrated hydrochloric acid and fractionating the solution by ion-exchange chromatography. Fractions with highest ⁶⁴Cu concentrations containing [⁶⁴Cu]CuCl₂ (specific activity 1313 ± 553 MBq μg⁻¹ in 0.1–1.0 M hydrochloric acid) were evaporated to dryness under a N₂ stream. For *in vitro* studies, dry ⁶⁴Cu was re-dissolved in 0.9% NaCl to give a final pH of 3–5. For *in vivo* studies, dry ⁶⁴Cu was re-dissolved in 39–51 mM sodium acetate (229873, Sigma), giving a final pH of 6–7.

Cell culture

Prostate cancer DU145 (HTB-81), PC3 (CRL-1435) and breast cancer MDA-MB-231 (HTB-26) cells were purchased from ATCC (Middlesex, UK). Melanoma A375, ovarian cancer SKOV-3 and IGROV-1 cells were generously shared by Prof Sophia Karagiannis (King's College London). Cells were cultured in RPMI 1640 medium (R0883, Sigma) with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin, except MDA-MB-231 cells which were cultured in low-glucose Dulbecco's Modified Eagle Medium (DMEM, D5546, Sigma), supplemented as above. All cells were kept in a humidified incubator at 37 °C and 5% CO₂.

In vitro cellular accumulation of ⁶⁴Cu

Cells grown as monolayer in 24-well plates were washed twice with PBS and incubated with 10 kBq [⁶⁴Cu]CuCl₂ per well at 37 °C under 5% CO₂. Three or four technical replicates were used for each condition. For the amino acid solutions prepared in Hank's Balanced Salt Solution (HBSS) no significant pH drift was observed, as monitored by the benchtop pH meter (Seven-Compact S220, Mettler Toledo, Fig. S1, ESI†). The pH of the final incubation medium after addition of amino acids and [⁶⁴Cu]CuCl₂ measured using indicator papers (Whatman, 2613-991) was close to 7 in all cases. Following incubation, cells were washed twice with phosphate-buffered saline (PBS, Sigma, 806552) and lysed using NaOH (0.1 M). Radioactivity associated



with cell lysates or extracellular medium/PBS washes was gamma counted (Wallac gamma counter). Percentage cellular accumulation of ^{64}Cu was converted to a ratio of the intracellular/extracellular concentrations of ^{64}Cu ; for some purposes these values were normalised to ratios obtained in control conditions (e.g., in HBSS or in serum without added amino acids) and expressed as fold change. Results are depicted as means \pm SD of independent biological experiments (performed on different days). Amino acids were purchased as follows: L-cysteine hydrochloride (093727) and D-cysteine hydrochloride (036386) from Fluorochem, N-acetyl-L-cysteine (A7250) and L-cystine (PHR1323) from Sigma and L-threonine (138930050) from Acros Organics. Detailed descriptions of the protocols and data analysis are in the ESI.†

In vitro cellular efflux of ^{64}Cu

DU145 cells, prepared as described above, were pre-incubated (30 minutes) with ^{64}Cu and L-Cys (100 μM) in HBSS (vs. control: ^{64}Cu in HBSS only). Radioactive HBSS was replaced with non-radioactive HBSS and cells were incubated for 1 hour (efflux step). ^{64}Cu was measured in the pellet and in the replaced HBSS.

Cell fractionation

Cell fractionation was performed after incubating DU145 cells with 10 kBq [^{64}Cu]CuCl₂ in 1% FBS in HBSS buffer for 90 minutes. Abcam's cell fractionation kit (109719) was used to prepare highly-enriched cytoplasmic, mitochondrial and nuclear fractions. Following incubation with [^{64}Cu]CuCl₂ and PBS washes as described above, cells were harvested with trypsin. Subsequent steps followed the fractionation kit protocol, except that a replacement buffer A, free of ethylenediaminetetraacetic acid (EDTA), was used. Subcellular fractions were collected and gamma counted. Purity of cytoplasmic and mitochondrial fractions was shown by western blotting (Fig. S6A; see protocols in the ESI†).

Instant thin layer chromatography (iTLC)

100 mm \times 10 mm strips of glass fibre paper impregnated with silica gel (SG, Agilent Technologies A120B12) were developed in a 50 mL plastic Falcon tube with 700 μL of iTLC mobile phase (detailed below). Radioactivity was visualised by electronic autoradiography with the Cyclone Plus Storage Phosphor Scanner (PerkinElmer, C431200) with Cyclone Plus 5.0 software. Method 1-Assessing the oxidation state of ^{64}Cu : the mobile phase was 100 μM bicinchoninic acid (BCA, Sigma D8234) in water, giving retention factor (R_f) = 0 for $^{64}\text{Cu}(\text{II})$ and R_f = 1 for $^{64}\text{Cu}(\text{I})$; for method validation see Fig. S3 (ESI†). Method 2-Distinguishing free ^{64}Cu from L-cysteine-complexed ^{64}Cu : the mobile phase was butanol:acetic acid:water, 1:2:4; R_f = 1 for free ^{64}Cu and R_f = 0.7 for ^{64}Cu -L-cysteine complex.

Size-exclusion high-performance liquid chromatography (HPLC)

L-Cysteine solutions in HBSS (150 μL) were incubated with 1–1.5 MBq [^{64}Cu]CuCl₂ (15 μL) for 20 minutes at room temperature (RT) and analysed on an Agilent Technologies 1200 Series HPLC

unit with a BioSep™ 5 μm SEC-s2000 145 Å column (300 \times 7.8 mm), coupled with a UV/vis detector at 254 nm and a LabLogic Flow-Count radioactivity detector with a sodium iodide probe (B-FC-3200). The mobile phase was 0.9% (w/v) NaCl at a flow rate of 0.8 mL min⁻¹.

Human serum preparation

Blood collection from consenting healthy volunteers was approved by a local ethics committee. Blood samples were harvested in serum-separating tubes containing clotting activator (Medisave, 367958), incubated at RT for 30 minutes and centrifuged at 22 °C, 1500 relative centrifugal force (RCF) for 10 minutes. Serum was separated, filtered through a 0.45 μm filter and stored at 4 °C for use within 72 h, or at -20 °C for later use.

Measuring thiol concentration using Ellman's reagent

HBSS buffer containing L-cysteine and [^{64}Cu]CuCl₂ was added to wells of two 24-well plates-one with and one without DU145 cells. Plates were incubated for 1 hour at 37 °C in the humidified incubator. HBSS was sampled (20 μL) before and after the incubation period and added in triplicate to a 96-well plate, followed by 200 μL of the 0.2 mM Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB, Sigma D8130) in phosphate buffer (0.1 M, pH = 8.0, 5 mM EDTA). Plates were incubated for 5 minutes at RT and monitored at 412 nm using a SpectroStar Nano plate reader. The unknown thiol concentrations were derived from a linear regression using L-cysteine standards ranging from 2.44 to 1.25 mM.

Measuring total glutathione (GSH) by an enzymatic recycling method in cells treated with buthionine sulfoximine (BSO)

DU145 cells were grown for 24 hours in 24-well plates to 80% confluency, in unmodified complete growth medium either with or without 1 mM BSO (Focus Biomolecules, 10-4572). The following day, cells were incubated in HBSS supplemented with 1% (v/v) FBS and 10 kBq [^{64}Cu]CuCl₂ (1 hour, with/without 100 μM L-cysteine), then harvested and counted with the haemocytometer, using Trypan blue to confirm viability. *In vitro* cellular accumulation of ^{64}Cu was assessed by gamma counting. Total GSH content in the cell extracts was then measured by an enzymatic-recycling method⁴³ from a linear regression using GSH standards ranging from 0.4125 to 26.4 nM. Calculated GSH content was normalised to the cell count and nominal cell volume⁴⁴ to yield intracellular GSH concentration. Glutathione reductase (G3664), β -NADPH (N7505) and DTNB (D8130) were from Sigma.

DU145 xenograft model

Animal experiments were performed in accordance with the Animals (Scientific Procedures) Act, 1986 using protocols approved by the Animal Welfare and Ethical Review Body for King's College London (St Thomas' Campus). To generate the prostate cancer xenograft model, 8 week old male SCID/beige mice (Envigo, UK) were injected subcutaneously in the left shoulder with 4 \times 10⁶ DU145 cells in PBS (100 μL). Mice were



regularly monitored for health status and imaged when tumours reached 4–8 mm diameter (3–4 weeks).

PET/CT imaging and *ex vivo* biodistribution

All administered substances were prepared <24 hours before injection and passed through a 0.22 μM filter. NAC was dissolved in saline and neutralised to pH 6–7 with 3 M NaOH, [^{64}Cu]Cu-acetate was prepared as described as above. Animals were anaesthetised with isoflurane (2.0–3.0% in O_2 , flow rate 1 L min^{-1}) and injected *via* the tail vein with either 150 mg kg^{-1} NAC ($n = 3$) or saline ($n = 3$) over the course of 2 minutes. After 5 minutes all animals were *i.v.* injected with 4 ± 2 MBq [^{64}Cu]Cu-acetate and immediately imaged by PET for 1 hour, followed by a CT scan, using a nanoScan[®] PET/CT scanner (Mediso Medical Imaging Systems, Budapest, Hungary). Images were acquired and reconstructed using Nucline software (version 1.02, Mediso Ltd, Budapest, Hungary) and analysed using VivoQuant software (Version 3.5). Mice were sacrificed 75 min post-injection (*p.i.*) using cervical dislocation and their tissues were harvested, weighed and gamma counted (Wallac gamma counter).

Statistical analysis

Statistical analysis was done with GraphPad Prism 8. Data were first assessed for normal and lognormal distribution using Shapiro–Wilk test. For *in vitro* ^{64}Cu accumulation, paired analysis was performed to match the amino acid supplemented groups with their control groups, to account for the inter-experimental variability of ^{64}Cu accumulation (Fig. S2, ESI[†]). To compare 2 groups a paired ratio *t*-test was used. In the experiments with >2 groups, data points were transformed to log values and analysed by one-way Anova with Dunnett's *post hoc* test for multiple comparisons with the HBSS control or with Sidak's correction for multiple comparisons of pre-selected columns. For longitudinal *in vitro* ^{64}Cu accumulation results, a two-way Anova was used with Dunnett's (for >2 groups) or Bonferroni's (2 groups) *post hoc* test. To compare 3 groups not following normal and lognormal distribution a rank-based paired Friedman test was used with Dunn's correction. In the *in vivo* experiment, ^{64}Cu uptake over 1 hour was calculated as an area under the curve (AUC). AUC values were then compared between the NAC pre-injection and control groups using a two-tailed unpaired *t*-test. *p* values below 0.05 were considered significant.

Results

L-Cysteine-induced increase in ^{64}Cu accumulation in cancer cell lines

The effect of individual amino acids on ^{64}Cu accumulation *in vitro* was initially measured in HBSS, since media such as DMEM already contain a mixture of amino acids. Among L-histidine, L-methionine, L-threonine and L-cysteine, only L-cysteine had a statistically significant effect on ^{64}Cu accumulation ($p = 0.001$) in DU145 prostate cancer cells (Fig. 1A). After 90 minutes of incubation, the

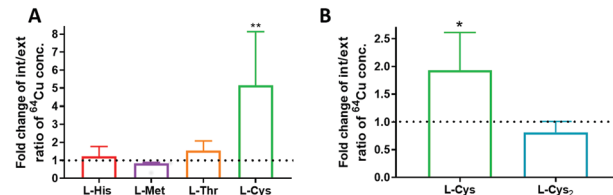


Fig. 1 The effect of added amino acids on ^{64}Cu accumulation in DU145 cells. The effect of L-cysteine on ^{64}Cu accumulation in additional cancer cell lines is shown in Fig. S1D (ESI[†]). (A) ^{64}Cu accumulation (90 min) in DU145 cells in HBSS (control) with added L-histidine (L-His), L-methionine (L-Met), L-cysteine (L-Cys) and L-threonine (L-Thr), all at 100 μM concentration, $n = 3$. (B) ^{64}Cu accumulation (60 min) in DU145 cells in HBSS (control) with added (10 μM) L-Cys or L-cystine (L-Cys₂), $n = 3$. Int./ext. ratios of ^{64}Cu conc. were analysed by one-way Anova with Dunnett's *post hoc* test for multiple comparisons with the HBSS control (A) or by a ratio paired *t*-test (B). Graphs represent mean \pm SD of control-normalised values. $p < 0.05$ is shown as *, $p < 0.01$ as **.

intracellular/extracellular concentration ratio of ^{64}Cu reached 195 ± 212 in HBSS only, while with addition of 100 μM L-Cys it reached 829 ± 634 . The high variability in accumulation of ^{64}Cu on different experimental days (Fig. S2A and B, ESI[†]) prompted us to pair results obtained in HBSS and with amino acids within each independent experiment. This analysis revealed that, on average, addition of L-cysteine to HBSS increased the ^{64}Cu intracellular/extracellular ratios by 5.15 ± 2.98 fold ($p = 0.001$).

To check for effects of oxidation of L-cysteine to its disulfide L-cystine in HBSS (considering the presence of oxidising copper ions), we compared the effects of the L-cystine and L-cysteine on ^{64}Cu accumulation. 10 μM concentration was selected due to poor L-cystine solubility. Only the reduced form increased the intracellular/extracellular ^{64}Cu ratios ($p = 0.03$, Fig. 1B). ^{64}Cu was present in HBSS at picomolar concentration and we confirmed with Ellman's reagent that under those conditions L-cysteine was not oxidised to L-cystine (Fig. S2C, ESI[†]).

Preliminary tests for this L-cysteine-specific effect were also performed in five other cancer cell lines. In prostate cancer PC3 and ovarian cancer SK-OV-3 cells, L-cysteine (100 μM) increased the intracellular/extracellular ^{64}Cu ratios but had no measurable effect in ovarian cancer IGROV-1, melanoma A375 or breast cancer MDA-MB-231 cells (Fig. S2D, ESI[†]). Since the effect was most pronounced in DU145 cells, these were selected as an experimental system to investigate its mechanisms.

^{64}Cu complexation with L-cysteine does not enhance ^{64}Cu accumulation in cells

With the aim of determining the role of the amino acid-bound copper species in cellular copper accumulation, we tested whether L-cysteine could significantly complex ^{64}Cu under the experimental conditions used for ^{64}Cu accumulation studies. Complexation of ^{64}Cu with increasing L-cysteine concentration in HBSS was monitored by HPLC (Fig. 2A) and iTLC (Fig. 2B). In the HPLC system used, unchelated ^{64}Cu does not elute and remains bound to the stationary phase. With the addition of 1 mM, but not 100 μM L-cysteine, ^{64}Cu eluted from the column, proving that ^{64}Cu was complexed more strongly by L-cysteine



than by the stationary phase only at the higher cysteine concentration. In the iTLC method, free ^{64}Cu in HBSS migrated to the solvent front ($R_f = 1$) and its behaviour was unchanged by the addition of 10 or 100 μM L-cysteine, but 1 mM L-cysteine changed the R_f to 0.7, again suggesting significant complexation of ^{64}Cu by L-cysteine only at high concentrations.

We then tested the effect of different L-cysteine concentrations on cellular ^{64}Cu accumulation. The presence of 10 or 100 μM L-cysteine (concentrations under which chromatography suggested that ^{64}Cu is only very weakly bound by L-cysteine) increased the intracellular/extracellular concentration ratio of ^{64}Cu after incubating cells with ^{64}Cu for at least 30 minutes (Fig. 2C). This suggests that ^{64}Cu -L-cysteine complexes did not contribute to the enhanced cellular ^{64}Cu accumulation. On the contrary, complexation might lead to decreased accumulation, since L-cysteine at 1 mM (a concentration at which the chromatography experiments showed strong copper complexation) had an inhibitory effect on ^{64}Cu accumulation at 7 ($p < 0.0001$) and 15 minutes ($p = 0.002$).

L-Cysteine reduces $^{64}\text{Cu(II)}$ to $^{64}\text{Cu(I)}$ in HBSS but reduction does not underlie the effect on ^{64}Cu accumulation

To test the hypothesis that the enhancement of ^{64}Cu accumulation by L-cysteine is attributable to $^{64}\text{Cu(II)}$ reduction, we repeated the above ^{64}Cu cellular accumulation experiment in the presence of other known $^{64}\text{Cu(II)}$ -reducing agents D-cysteine, glutathione (GSH) and ascorbate. The capacity of all of these agents, including

L-cysteine, to reduce $^{64}\text{Cu(II)}$ was confirmed by iTLC (Fig. S3B, ESI †), but only L-cysteine was able to increase ^{64}Cu accumulation in DU145 cells ($p = 0.03$, Fig. 3).

Cellular ^{64}Cu accumulation enhancement by L-cysteine is due to intracellular events following cellular uptake of L-cysteine

The hypotheses tested above (^{64}Cu complexation or reduction by L-cysteine) focused on plausible extracellular mechanisms by which L-cysteine might modulate ^{64}Cu accumulation in HBSS. After rejecting these hypotheses, we evaluated an alternative explanation: that L-cysteine affects ^{64}Cu retention by an intracellular rather than extracellular process. This concept was tested by pre-incubating DU145 cells with L-cysteine, followed by washing before adding ^{64}Cu (Fig. 4A). These experiments showed that pre-incubation of cells with L-cysteine enhanced subsequent ^{64}Cu accumulation (Fig. 4B). Moreover, the higher the L-cysteine concentration in HBSS during the pre-incubation step, the greater the subsequent increase in ^{64}Cu accumulation. Importantly, the effect could be partially blocked by the addition, during L-cysteine incubation, of an excess (1 mM) of L-serine and L-threonine, which are known to block L-cysteine uptake⁴⁵ (Fig. 4C). The L-cysteine effect is absent if cells are incubated in DMEM, which could be explained by the presence of competing amino acids (Fig. S5, ESI †). In the subsequent experiment (Fig. 4D), we introduced a second wash-out step following L-cysteine pre-incubation and removal. After a very short (1 minute) wash-out period, L-cysteine increased the intracellular/extracellular ^{64}Cu ratio 3.00 ± 1.24 times ($p < 0.001$). Longer wash-out periods reduced the enhancement to a non-significant level to 1.51 ± 0.14 -fold by 30 minutes ($p = 0.1$), to 1.30 ± 0.04 -fold by 90 minutes ($p = 0.47$) and by 180 minutes of wash-out there was no enhancement. These results further support the hypothesis that the L-cysteine effect depends on its cellular accumulation.

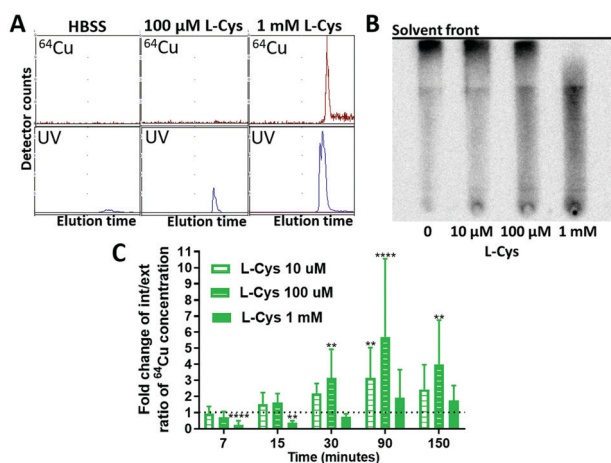


Fig. 2 The effect of increasing concentrations of L-cysteine on ^{64}Cu complexation in HBSS and on its accumulation in DU145 cells. (A) HBSS with/without L-Cys (100–1000 μM) was incubated with 1.5 MBq of [^{64}Cu]CuCl₂ (20 min) and analysed by HPLC using a size exclusion column and saline as mobile phase. Upper panels represent radiochromatograms and lower panel UV chromatograms (254 nm). (B) HBSS with/without L-Cys (10–1000 μM) was incubated with ^{64}Cu (90 min) and analysed by iTLC SG (butanol:acetic acid: water, 1:2:4). (C) ^{64}Cu accumulation in DU145 cells incubated in HBSS over time in the presence of increasing concentrations of L-Cys, $n = 3$ except for 10 μM ($n = 2$) and 100 μM at 90 min ($n = 6$). Graph represents mean \pm SD of control-normalised values. Int./ext. ratios of ^{64}Cu conc. were analysed by a two-way Anova with Dunnett's *post hoc* test for multiple comparisons with the HBSS control. $p < 0.01$ is shown as ** and $p < 0.0001$ as ****.

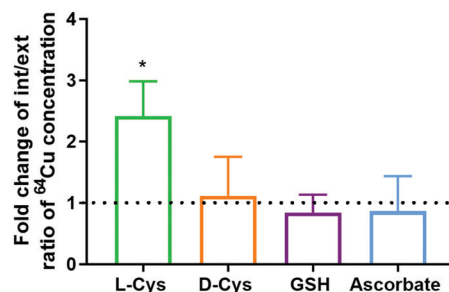


Fig. 3 The effect of reducing agents on ^{64}Cu accumulation in DU145 cells. Conditions used: L-Cys and D-cysteine (D-Cys) – 100 μM , 90 min, ascorbate – 1 mM, 90 min, glutathione (GSH) – 10 μM , 60 min, $n = 3$. $^{64}\text{Cu(II)}$ to $^{64}\text{Cu(I)}$ reduction by all reductants was demonstrated by iTLC SG method, shown in Fig. S3B (ESI †). All experiments were done in HBSS and normalised to HBSS control. A more detailed version of the figure is shown in Fig. S4 (ESI †). Graph represents mean \pm SD of control-normalised values. Int./ext. ratios of ^{64}Cu conc. were analysed by a two-way Anova with Sidak's *post hoc* test for multiple comparisons with the HBSS control. $p < 0.05$ is shown as *.



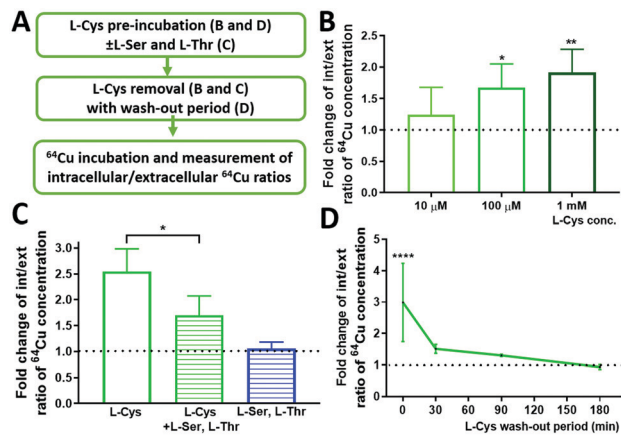


Fig. 4 The effect of pre-incubation with L-Cys on the cellular uptake of ^{64}Cu . Scheme of experimental steps is presented in panel A. (B)–(D) ^{64}Cu accumulation in DU145 cells pre-incubated for 30 min with L-Cys in HBSS, washed and incubated for 30 min with ^{64}Cu in HBSS (with 1% FBS in panel D). (B) Effect of pre-incubation with increasing concentrations of L-Cys (10–1000 μM) in HBSS, $n = 4$. (C) Effect of pre-incubation with L-Cys (100 μM) together with the competing amino acids L-serine (L-Ser, 1 mM) and L-Thr (1 mM), $n = 4$. (D) Effect of pre-incubation with L-Cys (100 μM), followed by the wash-out period, $n = 5$ for 1 min, $n = 3$ for 60 and 90 min and $n = 2$ for 180 min. Graphs represent mean \pm SD of control-normalised values. Int./ext. ratios of ^{64}Cu conc. were analysed by one-way Anova with Dunnett's *post hoc* test (panel B) or two-way Anova with Bonferroni's *post hoc* test (panel D) for multiple comparisons with the HBSS control. Two groups indicated on the graph in panel C were compared by a paired ratio *t*-test. $p < 0.05$ is shown as *, $p < 0.01$ as **, $p < 0.0001$ as ****.

L-Cysteine increases ^{64}Cu cellular retention mainly in the cytoplasm but independently from GSH synthesis

Next, we examined the possible ways in which increased intracellular L-cysteine could influence ^{64}Cu trafficking. To determine whether L-cysteine promotes ^{64}Cu delivery into cells or promotes its cellular retention, we measured ^{64}Cu efflux from DU145 cells after loading with ^{64}Cu , with and without 100 μM L-cysteine. Preloading cells with L-cysteine resulted in 4-fold decrease in % ^{64}Cu efflux compared to cells loaded with ^{64}Cu without L-cysteine ($p = 0.03$, Fig. 5A). This suggests that L-cysteine inhibits efflux of ^{64}Cu rather than promoting its uptake.

We then addressed the intracellular localisation of ^{64}Cu using a commercial cell fractionation kit employing different detergents to isolate cytoplasm, mitochondria and nuclei (Fig. S6A, ESI[†]). To circumvent potential problems caused by copper chelation, the kit was modified to exclude EDTA (which did not change the observed ^{64}Cu fractionation – Fig. S6B, ESI[†]). Cells accumulating ^{64}Cu in the presence and absence of L-cysteine had an almost identical pattern of ^{64}Cu distribution among the compartments: 85–86% in cytoplasm, 10–11% in mitochondria and 2–3% in nuclei (Fig. 5B).

A proposed cytoplasmic copper binding partner is GSH⁴⁶ and L-cysteine is a substrate for its synthesis.⁴⁷ We therefore tested the role of GSH synthesis in the L-cysteine effect on ^{64}Cu retention. We first showed that incubating DU145 cells with 100 μM L-cysteine for 1 hour (the time-scale on which L-cysteine affects ^{64}Cu accumulation) did not significantly change

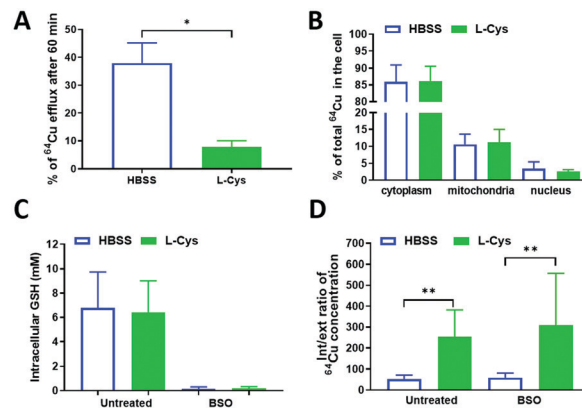


Fig. 5 Decreased efflux of ^{64}Cu with L-Cys. (A) Effect of pre-incubating DU145 with ^{64}Cu and with/without L-Cys (100 μM , 30 min) in HBSS on the subsequent efflux of ^{64}Cu over 60 min, $n = 3$. (B) Effect of incubating DU145 cells with/without L-Cys (100 μM , 90 min) in HBSS with 1% FBS on the intracellular localisation of ^{64}Cu , assessed by cell fractionation and gamma counting, $n = 3$. (C) and (D) Effect of glutathione depletion (by BSO treatment) in DU145 cells on ^{64}Cu accumulation over 1 h with/without L-Cys (100 μM) in HBSS with 1% FBS. After incubation, cells were pelleted, uptake of ^{64}Cu was measured (D) followed by GSH measurement of GSH using an enzymatic recycling method (C), $n = 3$. Graphs represent mean \pm SD. HBSS and L-Cys groups in A and C were compared by the paired ratio *t*-tests, in panel B by multiple *t*-tests with Holm–Sidak correction for multiple comparisons. Int./ext. ratios of ^{64}Cu conc. in panel D were analysed by a two-way Anova with Sidak's *post hoc* test for comparisons between the pre-selected columns. $p < 0.05$ is shown as *, $p < 0.01$ as **.

intracellular GSH levels (measured by an enzymatic recycling method as 6.79 ± 2.94 mM in control cells and 6.41 ± 2.61 mM in L-cysteine-treated cells (Fig. 5C), $p = 0.68$). Depletion of GSH in DU145 cells by BSO treatment (Fig. 5C) did not affect basal ^{64}Cu accumulation in HBSS ($p = 0.95$), nor did it affect ^{64}Cu accumulation enhancement caused by L-cysteine (Fig. 5D). Preliminary experiments exploring the molecular identity of intracellular ^{64}Cu (Fig. S7A, ESI[†]) by size-exclusion chromatography showed that the most ($87.90 \pm 1.18\%$) was tightly bound to proteins; this percentage was similar ($84.62 \pm 2.37\%$) when cells were co-incubated with L-cysteine. Thus, GSH did not directly participate in the enhancement of ^{64}Cu retention by L-cysteine.

N-Acetylcysteine (NAC) affects ^{64}Cu similarly to L-cysteine and both retain their effect in the presence of serum

So far, we used HBSS buffer to delineate the effect of incubating cells with individual amino acids. However, HBSS composition is far from physiological conditions under which copper enters human cells in serum. Therefore, some of the previous experiments were replicated in the presence of 10% FBS, which is commonly used in tissue culture. Under these conditions (Fig. 6A) our preliminary experiments showed that the effect of L-cysteine addition was even more pronounced than before (the intracellular/extracellular ^{64}Cu ratio increased 9.71 ± 0.73 fold) and D-cysteine also exhibited an effect, albeit smaller (increasing the int./ext. ^{64}Cu ratio by 4.23 ± 0.45 fold). Notably, in the presence of 10% FBS (Fig. 6B) and in 100% human (Fig. 6C) or mouse serum (Fig. 6D), co-incubation with high



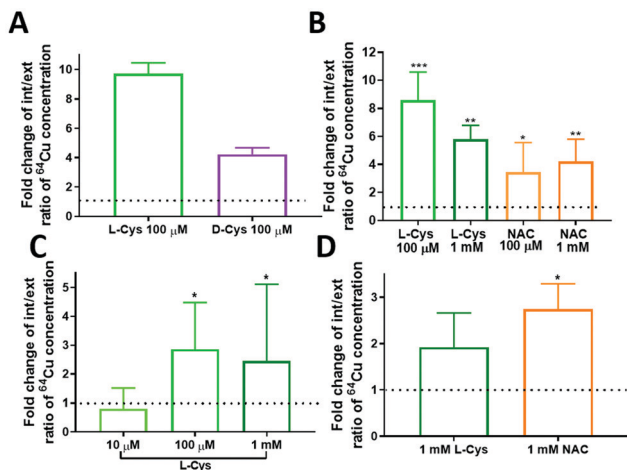


Fig. 6 The effect of added thiols on the accumulation of ^{64}Cu in DU145 cells in the presence of serum. ^{64}Cu accumulation in cells co-incubated with L-Cys, D-Cys or NAC. (A) Effect of L-Cys and D-Cys (100 μM) on ^{64}Cu uptake over 90 min in HBSS with 10% FBS, $n = 2$. (B) Effect of L-Cys or NAC (100 μM and 1 mM) on ^{64}Cu uptake over 60 minutes in HBSS with 10% FBS, $n = 3$. (C) and (D) Effect of L-Cys and NAC on ^{64}Cu uptake over 60 min in human (C) or mouse (D) sera, $n = 3$. Graphs represent mean \pm SD of control-normalised values. Int./ext. ratios of ^{64}Cu conc. in (B) and (C) data were analysed by one-way Anova with Dunnett's *post hoc* test, in (D) non-parametric paired Friedman test with Dunn's *post hoc* test was applied due to non-normality of the data. *Post hoc* comparisons were done between each treatment and control cells. $p < 0.05$ is shown as *, $p < 0.01$ as **, $p < 0.001$ as ***.

concentrations of L-cysteine (1 mM) also enhanced ^{64}Cu accumulation over a 1 hour period (contrary to what was found for serum-free experiments where such high concentrations inhibited ^{64}Cu accumulation). Addition of NAC (100 μM and 1 mM), an approved drug in humans which acts as a L-cysteine precursor in cells,⁴⁸ significantly increased ^{64}Cu accumulation in 10% FBS (4.24 ± 1.58 fold at 1 mM, $p = 0.006$, Fig. 6B), and in mouse serum (2.75 ± 0.55 fold at 1 mM, $p = 0.03$, Fig. 6D). The persistence of these effects in serum suggested that they could be physiologically relevant, which warranted progression to *in vivo* studies.

The effect of N-acetylcysteine on *in vivo* trafficking of ^{64}Cu in a prostate cancer mouse model

The enhancement of ^{64}Cu accumulation in cancer cells by added thiols encouraged us to explore the use of *in vivo* NAC supplementation as a way to improve intratumoural accumulation of ^{64}Cu (which has been used clinically, as [^{64}Cu]CuCl₂ for prostate cancer imaging).⁴¹ NAC was chosen for the *in vivo* work since it is approved for use in humans and its pharmacokinetics has been studied in mice.^{49,50} Prior work has shown that it has a more pronounced effect than L-cysteine on the replenishment of the intracellular L-cysteine levels⁵¹ and in our hands (Fig. 6D) it enhanced cellular ^{64}Cu accumulation in mouse serum more than L-cysteine did. The dose and form of NAC administration (i.v. bolus injection, 150 mg kg⁻¹) were chosen based on a previous report⁴⁹ that at 10 minutes p.i. it yielded plasma NAC concentrations approaching 1 mM. The timing of

NAC administration 5 minutes before ^{64}Cu -acetate injection was guided by the *in vitro* wash-out data (Fig. 4D). Mice bearing DU145 xenografts were injected with NAC or saline (control), followed by ^{64}Cu acetate 5 minutes later. PET images revealed ^{64}Cu -acetate biodistribution in agreement with previous reports,⁵² exhibiting pronounced liver uptake and biliary excretion (Fig. 7A). As expected, there was pronounced accumulation of ^{64}Cu in DU145 tumours, which however was reduced, not enhanced, by NAC supplementation ($p < 0.05$). At 50–60 minutes p.i. the mean uptake was $6.36 \pm 0.87\%$ ID per g in the control group and $3.58 \pm 1.38\%$ ID per g in the NAC group. NAC treatment also significantly ($p < 0.01$) decreased brain ^{64}Cu uptake, particularly before 30 minutes p.i. *Ex vivo* biodistribution analysis at 75 min p.i. confirmed that ^{64}Cu uptake values (%ID per g) in tumour and brain were both reduced in the NAC group ($p < 0.01$, Fig. 7C).

Discussion

L-Cysteine substantially increased ^{64}Cu accumulation in DU145 prostate cancer cells, whereas L-histidine, L-methionine and L-threonine did not (Fig. 1). L-Cysteine had a similar effect in PC3 (prostate cancer) and in SK-OV-3 (ovarian cancer) cells (Fig. S2D, ESI[†]). The effect is not universal as it was not seen in IGROV-1 (ovarian cancer), MDA-MB-231 (breast cancer) and A375 (melanoma) cells. The mechanism of this phenomenon was investigated in more depth using DU145 cells (the main findings are reviewed in Scheme 1).

Potentially, complexation of copper by L-cysteine could facilitate copper delivery to CTR1, or participate in an additional transport system.⁵³ However, the experiments suggested that the enhancement of ^{64}Cu accumulation in DU145 cells was not due to complexation of copper by L-cysteine in the extracellular medium, because the presence of L-cysteine at levels high enough to significantly complex copper inhibited rather than enhanced ^{64}Cu accumulation (Fig. 2).

Another possible explanation was based on the premise that CTR1 transports reduced Cu(I) ions,¹⁰ which is consistent with reports that *in vitro* uptake of ^{64}Cu is augmented in the presence of reducing agents.^{6,12,13,54} To determine whether reduction of Cu(II) by L-cysteine could be the cause of ^{64}Cu accumulation enhancement, we tested a range of alternative reducing agents reported (and proven by our experiments) to be capable of reducing Cu(II). Amongst them, only L-cysteine had an effect on ^{64}Cu accumulation (Fig. 3). These results do not refute the well-documented specificity of CTR1 for Cu(I) – it is possible that tracer amounts of ^{64}Cu used in our experimental system (0.6 nM) were already effectively reduced on the surface of DU145 cells, and did not require added reducing agents. They do, however, suggest that reduction to Cu(I) is not the key factor in enhancement of ^{64}Cu accumulation by L-cysteine.

Subsequent experiments suggested that L-cysteine, rather than inducing changes in extracellular ^{64}Cu speciation, mediates changes in the intracellular ^{64}Cu trafficking or efflux. This was first suggested by the observed delay between L-cysteine



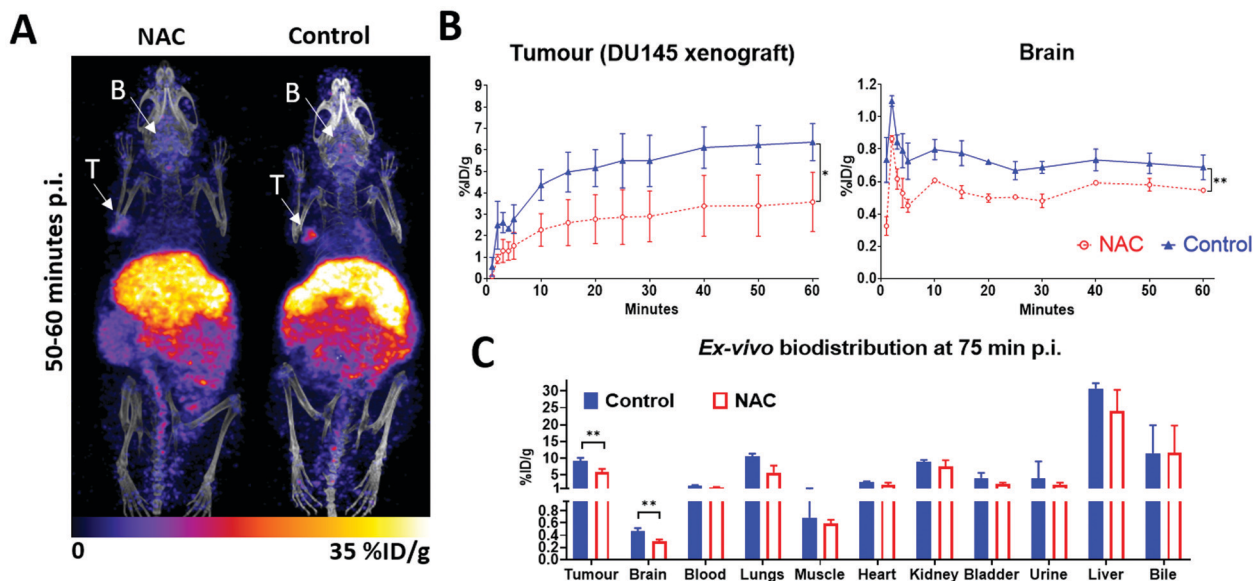


Fig. 7 The effect of NAC pre-injection on *in vivo* ^{64}Cu trafficking over 1 h in a mouse model of prostate cancer. Mice bearing DU145 xenografts were i.v. injected with 150 mg kg^{-1} *N*-acetyl-L-cysteine ($n = 3$) or saline (control, $n = 3$), followed 5 minutes later by i.v. acetate-buffered ^{64}Cu . PET images were acquired for 60 min p.i., followed by a CT scan. Uptake of ^{64}Cu in the regions of interest was expressed as % injected dose (ID) per g. (A) Images show the Maximum Intensity Projections 50–60 min p.i.; arrows point towards DU145 tumour (T) and brain (B). (B) Time-activity curves of ^{64}Cu accumulation in the brain and tumour. Area under the curve was calculated for each subject and values were compared between the NAC pre-injection and control groups using unpaired *t*-test. (C) *Ex vivo* biodistribution analysis was performed 75 minutes p.i.; radioactivity in the organs was measured by gamma counting. Groups were compared using unpaired *t*-test. Graphs represent mean \pm SD. $p < 0.05$ is shown as *, $p < 0.01$ as **.

addition and its first detectable effect on ^{64}Cu only after 30 minutes of incubation (Fig. 2), and confirmed by observing that ^{64}Cu accumulation was enhanced by pre-incubating cells with L-cysteine, followed by its removal from the HBSS before addition of ^{64}Cu (Fig. 4); hence L-cysteine did not need to be present in the HBSS to exert its effect. Addition of L-serine (1 mM) and L-threonine (1 mM) during the pre-incubation with L-cysteine partially blocked the L-cysteine (100 μM) effect. An earlier report showed that in erythrocytes, these amino acids supplied at 2.5 mM concentration blocked L-cysteine uptake by 95%.⁴⁵ The effect of L-cysteine was also diminished if the pre-incubation step was followed by a wash-out period. These results together suggest that L-cysteine is first taken up by DU145 cells and then exerts its effect on ^{64}Cu trafficking intracellularly. L-Cysteine supply to cells mainly occurs in the form of L-cystine *via* the xCT transporter.⁵⁵ However, since L-cystine supplementation did not influence ^{64}Cu accumulation in DU145 cells (Fig. 1B), we conclude that the transport of (reduced) L-cysteine itself was responsible for the downstream effect. Our experiments do not unambiguously identify which L-cysteine transport systems are operative in DU145 cells, but the blocking effect of L-serine and L-threonine suggests the involvement of the ASCT (Alanine, Serine, Cysteine Transporter) system.^{56–58} Further support for the hypothesis that L-cysteine exerts its effect from within the cell comes from the observation that efflux of previously accumulated ^{64}Cu from cells was markedly slowed by prior incubation with L-cysteine (Fig. 5).

Fractionation of DU145 cells following ^{64}Cu incubation with and without L-cysteine showed that the vast majority of ^{64}Cu

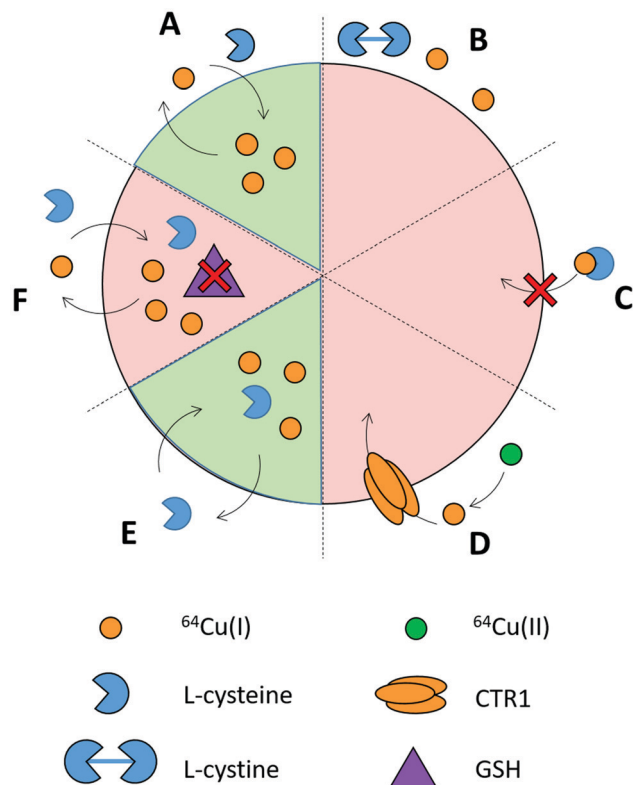
accumulating in cells was present in the cytoplasm. It should be noted that we used a fractionation kit designed for protein analysis so we cannot exclude the possibility that upon cell lysis and fractionation, some of the ^{64}Cu was exchanged between its usual binding partners or moved to different organelles – though we did remove copper chelating agents from the buffers to minimise this possibility.

We hypothesised that GSH might mediate the observed effect because L-cysteine availability is typically rate-limiting for GSH synthesis.⁵⁵ GSH is known to bind Cu(I) ⁵⁹ and may participate in intracellular copper trafficking.^{46,60–64} Our results, however, did not support this hypothesis, at least in DU145 cells: BSO treatment drastically depleted GSH levels but did not change basal ^{64}Cu accumulation or the magnitude of the L-cysteine effect on ^{64}Cu accumulation (Fig. 5). Moreover, L-cysteine incubation did not enhance intracellular GSH levels.

The intracellular events underlying the effect of L-cysteine and *N*-acetyl-cysteine on ^{64}Cu retention remain unclear. The fast onset of the effect precludes the involvement of protein synthesis in the process. Since the intracellular copper ions are bound with attomolar affinities,⁶⁵ it is unlikely that copper–L-cysteine complexes with their much lower stability constants⁶⁶ would be able to account for intracellular buffering of copper. In another line of explanation, we suggest that L-cysteine exerted an indirect effect on ^{64}Cu trafficking, which increased ^{64}Cu flux to the retention sites. Potential ways in which this could occur are outlined below.

(1) L-Cysteine could affect the kinetics of copper transfer between the intracellular chaperones. Small molecules, such as





Scheme 1 The summary of the main *in vitro* findings in DU145 cells. (A) ^{64}Cu accumulation increased with L-cysteine. (B) No change in ^{64}Cu accumulation with L-cystine. (C) L-cysteine ^{64}Cu complexes did not contribute to ^{64}Cu accumulation. (D) Reduction of Cu(II) to Cu(I) did not enhance accumulation of ^{64}Cu . (E) Increased intracellular L-cysteine augmented ^{64}Cu accumulation and retention. (F) Increased ^{64}Cu accumulation was not mediated by GSH synthesis.

glutamate,⁶⁷ glutathione or L-cysteine⁶⁸ have been shown to modulate the kinetics of copper transfer between its binding partners.

(2) An increase in the intracellular L-cysteine levels could influence the redox-sensitive residues of proteins or LMW ligands. This could change the availability of copper-binding sites or modulate protein function.⁶⁹ Intracellular redox status regulates ATOX1-mediated copper trafficking and efflux from cells.^{70,71} ATOX1 redox status is dependent on the intracellular oxidised/reduced glutathione ratio but not on the total glutathione levels.⁷⁰ This warrants further investigation to test whether ^{64}Cu accumulation enhancement is mediated by the effect of L-cysteine supplementation on oxidised/reduced glutathione ratios.

(3) Potentially, the observed effect of L-cysteine might involve a secondary effect of L-cysteine metabolism. Apart from contributing to glutathione synthesis, L-cysteine can undergo numerous intracellular catabolic pathways, such as generation of taurine, pyruvate or hydrogen sulfide.⁷² Hydrogen sulfide can trap ^{64}Cu at very low levels⁷³ or regulate copper export systems in *Mycobacterium tuberculosis*⁷⁴ and in human neuroblastoma cells.⁷⁵ S-Adenosylhomocysteine hydrolase, one of the key enzymes in the metabolism of thiol-containing amino

acids, was found to bind appreciable amounts of copper in mouse livers⁷⁶ and its expression is regulated by copper availability in mice.⁷⁷

To what extent is modulation of cellular copper accumulation by L-cysteine important *in vivo*? The finding that results obtained in HBSS were replicated in the presence of human or mouse serum (Fig. 6), suggests that L-cysteine still affects accumulation of ^{64}Cu bound to some physiological serum carriers. Moreover, the effect persists in the presence of competing non-radioactive copper and L-cysteine present in serum. Reported L-cysteine levels in human serum are in the range 10–34 μM ^{78,79} and vary in disease states such as Alzheimer's disease,⁸⁰ coronary heart disease⁸¹ and cancer.⁸² Cancer cells often show high demand for L-cysteine, or indeed L-cysteine dependency.⁸³ Both types of ASCT transporters are upregulated in various human cancers, including prostate cancer.^{84,85}

To test whether the effect of L-cysteine, or its "prodrug" form NAC could enhance ^{64}Cu accumulation in prostate cancer *in vivo*, we used PET imaging in a DU145 subcutaneous xenograft mouse model. However, i.v. injection of 150 mg kg^{-1} NAC 5 minutes before ^{64}Cu -acetate injection slightly decreased, rather than increased, ^{64}Cu accumulation in DU145 xenografts. This could be due to metabolism of NAC prior to reaching the DU145 tumour; a change in the DU145 cell phenotype in a xenograft compared to cultured DU145 cells (such as transporter expression); the effect of the other cell types and extracellular matrix at the tumour site; or differences in the ^{64}Cu speciation between serum (used in the *in vitro* experiments) and whole blood.

Unexpectedly, NAC treatment had an inhibitory effect on brain ^{64}Cu accumulation at all time-points between 0–60 minutes p.i., but most prominently between 4–20 minutes p.i. This was confirmed by both the PET image and by *ex vivo* biodistribution. Whether NAC is capable of crossing the blood–brain barrier (BBB) is unclear: studies with ^{14}C -^{50,86} or ^{13}C -labelled⁴⁹ NAC in mice were inconclusive but there is some evidence for NAC modulating brain redox status.⁴⁹ NAC could also affect ^{64}Cu speciation in serum, regional blood flow or post-transcriptional regulation of copper transporters.

Conclusions

We have shown that delivery of ionic copper to cellular transporters on prostate cancer DU145 cells can occur *in vitro* without the involvement of LMW intermediates (complexes of L-histidine, L-cysteine, L-threonine or L-methionine). This warrants further investigation of the roles of individual copper-transporting proteins as copper donors, or the ternary complexes of proteins with copper and LMW species.⁸⁷ Notwithstanding, in some cell lines we also found previously unreported effects on ^{64}Cu accumulation by thiols (L-cysteine and NAC), not due to extracellular speciation changes but *via* intracellular mechanisms that are independent of GSH synthesis. Further investigation *in vivo* of the link between thiols and copper metabolism in the context of tumour biology could help elucidate the mechanism of increased copper retention in



cancers. Additionally, gaining insight into the suppression of ^{64}Cu brain uptake by NAC supplementation may enhance understanding of dysregulated redox states and copper metabolism in the brain, and suggest strategies to modulate pathological brain copper accumulation.

Conflicts of interest

There are no conflicts to declare.

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