SUPPORTING INFORMATION

Apoferitin-encapsulated Jerantinine A for transferrin receptor-targeting and enhanced selectivity in breast cancer therapy

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SI1. Measuring concentration of protein and jerantinine A acetate (JAa)

The concentrations of all proteins used in this study were calculated using a calibration curve obtained by Bradford analysis (Figure S1a). The concentration of encapsulated JAa was assessed using UV-Vis spectroscopy of JAa solution (Figure S1b) with different concentrations. Based on these results, the calibration curve (Figure S1c) was generated and used to calculate the concentration of encapsulated JAa.

Figure S1. a) The standard curve of BSA, R² > 0.99. b) Absorbance spectra of JAa with different concentrations, with absorbance peak contred at 330 nm. c) Calibration curve showing integrated intensity of JAa absorbance peak at 330 nm, R² > 0.99.
S12. Assessment of protein recovery and encapsulation efficiency EE%

To optimize the encapsulation methods, the percentage of protein recovery was calculated using Equation (1):

\[
\text{concentrate recovery} \% = 100 \times \frac{W_c \times C_c}{W_0 \times C_0},
\]

(S1)

where \( W_c \) is the weight of the formulation after purification, \( C_c \) is the concentration of the formulation after purification, \( W_0 \) is the weight of formulation before purification and \( C_0 \) is the concentration of the formulation before purification. Encapsulation efficiency (EE%) is defined as the ratio between the encapsulated and the original amount of the drug added to the formulation:

\[
EE\% = \frac{\text{amount of encapsulated drug}}{\text{amount of added drug}} \times 100\%
\]

(S2)
SI3. Growth-inhibitory assessment of AFt-JAa

Table S1 summarizes the information about the cell lines used.

Table S1. Cell lines used and their supplemented media

| Cell line     | Medium used     | Supplements                                                                 |
|---------------|-----------------|-----------------------------------------------------------------------------|
| MCF-7         | RPMI-1640       | 10% FBS                                                                     |
| BT-474        | DMEM-high glucose | 10% FBS                                                                   |
| MDA-MB-468    | MEM             | 10% FBS                                                                     |
| SKBR-3        | McCoy's 5a      | 10% FBS                                                                     |
| MDA-MB-231    | MEM             | 10% FBS                                                                     |
| MCF-10A       | DMEM/F12        | 2.5% Horse serum, 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 10 µg/ml insulin, 1% penicillin-streptomycin |
| MRC-5         | MEM             | 10% FBS, 1% 1M HEPES, 1% 200 mM L-glutamine, 1% 0.1 mM NEA, 7.5% sodium bicarbonate, 1% penicillin-streptomycin |

The test agent concentration required to achieve 50% growth inhibition, GI$_{50}$ were calculated using Equation (S3):

$$GI_{50} = \frac{A_H - AGI_{50}}{A_H - A_L} \ast (C_H - C_L) + C_L,$$

where $AGI_{50} = \frac{A_1 - A_0}{2} + A_0$, $A_1$ is absorbance in control (untreated) wells; $A_0$ is absorbance at T0, $A_H$ and $A_L$ are absorbances higher and lower than AGI$_{50}$, at concentrations $C_H$ and $C_L$, respectively. In clonogenic assays, plating efficiency (PE%) and survival fractions (SF%) of colonies were calculated using Equations (S4) and (S5), respectively:

$$PE\% = \frac{\text{Number of colonies counted}}{\text{Number of cells plated}} \times 100\%$$

$$SF\% = \frac{PE\ of\ treated\ sample}{PE\ of\ control} \times 100\%$$
SI4. Release study of jerantinine A acetate (JAa) from apoferritin (AFt)

Release studies were performed using Slide A-Lyzer MINI Dialysis Device (Thermo Scientific, PC-8840, 0.5 mL) with 10 K MWCO); 100 mM sodium acetate buffer (NaOAc) adjusted to pH 5.3 by acetic acid; phosphate-buffered saline (PBS, pH 7.4). The formulation \((n = 4)\) was placed into the dialysis device and kept at 37 °C either in NaOAc or PBS. Direct and indirect samples were collected at different time intervals of 0 h, 1 h, 3 h, 6 h, 24 h (Figure S2). JAa concentration was estimated using the calibration curve obtained after the liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis. 

**Indirect samples:**
The JAa concentration was estimated after measuring JAa concentrations remaining in the dialysis device. First, samples were treated with 1:5 formic acid: methanol to precipitate and open the AFt cage. Samples were then centrifuged (13,000 rpm; 10 min) and the supernatant was measured using EQMS.

![Figure S2](image)

**Figure S2.** Sketch of the release study: (A) Samples dialyzed at pH 5.3 (NaOAc) or PH 7 (PBS) then (B) shaken gently \((T = 37 °C, 24 h)\) before (C) direct and indirect samples are collected at specific times (1 h, 3 h, 6 h, and 24 h) for LC-HRMS analysis.
Figure S3. The standard curve of JAa obtained from LCMS and release profile of JAa from AFt (24 h, $T = 37^\circ C$) at pH 5.3 and pH 7.4 in four independent trials, points represent means ± SD.
SI5. Stability study
Stability of the encapsulated agent was assessed using DLS measurements and by monitoring the concentration of the agent (Figure S4).

**Figure S4.** Stability study of AFt-encapsulated JAa monitored at storage conditions ($T = 4 \, ^\circ C; \, \text{pH} \, 7.4$). Hydrodynamic size (a) and zeta-potential (b) were measured using Malvern zeta sizer Nano ZS. Measurements of AFt-JAa and empty reassembled AFt (Re-AFt) were statistically comparable to AFt stock over 10 months of storage. The stability of encapsulated JAa was assessed by measuring its concentrations by UV-Vis spectroscopy at 330 nm over 5 weeks (c). Stability study of free and AFt-encapsulating JAa (0.4 µM) monitored at treatment conditions ($T = 37 \, ^\circ C; \, \text{over} \, 72 \, \text{h}$) in different media using HR-LCMS. Measurements show better stability of JAa following the encapsulation with AFt in 7 independent trials (d).
SI6. Cell culture studies

Activity and selectivity of the tested agents were assessed using MTT assays (Figures S5-S7), cell count assays (Figure S8), clonogenic (Figure S9), cell cycle analyses (Figures S12-13) and Annexin apoptosis studies (Figures 14-15). The representative MTT growth-inhibitory profiles demonstrate the ability of JAA to inhibit the growth of breast carcinoma cell lines, normal breast cells (MCF-10A) and MRC5 fibroblasts. Cancer-cell selectivity is significantly enhanced after AFt encapsulation of JAA, where significantly smaller numbers of viable cells were counted; perturbation of cell cycles was observed (with SKBR3 G2/M arrest evident; Figure S12) consistent with microtubule disruption, and large cancer cell populations undergoing apoptosis. Enhanced anticancer activity and selectivity with AFt-JAA is attributed to high expression of TfR1 (Figure S10) facilitating uptake of H-AFt (Figure S11) and encapsulated JAA in breast carcinomas.

**Figure S5:** Representative MTT graphs from a single trial (n=4 internal replicates) displaying the growth inhibitory properties of apoferitin-encapsulated JAA (AFt-JAA), JAA, and AFt in MDA-468, BT-474, MCF-7, MCF-10A and MRC-5.
Figure S6. Representative MTT growth-inhibitory profiles after treatment with JAa (a). Observed selectivity of JAa at a concentration of 5 µM to inhibit MCF-7 by ~72% and MDA-MB-231 by ~63% compared to inhibit non-cancerous (MCF-10A) µM by ~52% (b). Inhibition of the growth of MDA-MB-468 by 98% and SKBR-3 by ~86%, over non-cancerous MCF-10A (c). MDA-MB-468 by 98% and SKBR-3 show indistinguishable growth inhibition of JAa compared to MRC-5 (d). Cells were seeded in 96-well plates at a density of 3-4 x 10^3 cells/well, left to adhere for 24 h, treated and incubated for 48 h, except for BT-474 which was treated for 72 h and excluded from this comparison.

Figure S7. Representative MTT growth-inhibitory profiles in all studied cell lines after treating with Aft-JAa (a). Growth inhibition profiles of two TFR+ cells (MDA-MB-231 and SKBR-3) and non-cancerous TFR- MRC-5 cells (b). Cells were seeded in 96-well plates at a density of 3-4 x 10^3 cells/well. After allowing to adhere (24 h), all cell lines were treated and incubated for 48 h, except for BT-474 which was treated for 72 h.
**Figure S8.** *In vitro* cell viability assays in MDA-MB-231 and SKBR-3 following treatment with JAa, AFt-JAa (0.2 µM JAa), AFt (0.0017 µM) or media alone for 48 h. Cells (2 x 10^4 cells/well) were seeded in 6-well plates and incubated overnight before the treatment. Cells were harvested stained with trypan blue and counted by haemocytometer.

**Figure S9.** Representative BT-474 colonies demonstrating the effect of naked or AFt-encapsulated JAa (0.2 µM and 0.4 µM JAa), AFt (0.0033 µM) or medium alone on BT-474 clonal survival following 48 h exposure. Clonogenic survival fractions are reported as mean ± SD (n=3 internal repeats) and repeated 3 times. Significant differences from the control are expressed as **P < 0.01, ***P < 0.001 and ****P < 0.0001. Significant differences from naked JAa are expressed as #P < 0.05.
**Figure S10.** Expression of TfR1. Representative flow cytometry plots showing the shift in TfR1 after binding of PE-Anti-TfR1 in breast cancer cell lines in blue (SKBR-3, MDA-231, MCF-7, MDA-MB-468, MCF-7 and BT-474). And a non-detectable shift in MRC-5 cells.

**Figure S11.** Cellular uptake study of SKBR-3, MDA-231 and MCF-7 to 5-carboxyfluorescein-conjugated-human-apoferritin (40 nM) following 1 h, 2:30 h, 4 h and 24 h of exposure. Representative flow cytometry plots showing the shift in H-AFt fluorescence intensity indicating its cellular uptake (a). statistical analyses of H-AFt uptake with time compared to control (0 time) of exposure for each cell line (b). statistical analyses of H-AFt uptake with time of TfR1+ cells (SKBR-3 and MDA-MB-231) compared to TfR1- cells (non-cancerous MRC-5) at all exposure time (c). Data are reported as median ± SD (n...
= 3) and repeated 3 times. Significant differences from the control are expressed as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

**Figure S12.** Representative cell cycle histograms from a single trial of SKBR-3 cells following treatment with JAa, Aft-JAa (0.2 µM JAa) and (0.4 µM JAa), Aft (0.0033 µM) or media alone for 48 h. Cells (1 x 10^5 cells/well) were seeded in 6-well plates and incubated overnight before the treatment.
**Figure S13.** Representative cell cycle histograms from a single trial of MDA-MB-231 cells following treatment with JAA, AFt-JAA (0.2 μM JAA) and (0.4 μM JAA), AFt (0.0033 μM) or media alone for 48 h. Cells (1 x 10^5 cells/well) were seeded in 6-well plates and incubated overnight before the treatment.
Figure S14. Representative apoptosis quadrant plots from a single trial of SKBR-3 cells illustrating apoptotic effects following treatment with JAa, AFt-JAa (0.2 µM JAa) and (0.4 µM JAa), AFt (0.0033 µM) or media alone for 48 h. Cells (1 x 10^5 cells/well) were seeded in 6-well plates and incubated overnight before the treatment.
Figure S15. Representative apoptosis quadrant plots from a single trial of MDA-MB-231 cells illustrating apoptotic effects following treatment with JAa, AFT-JAa (0.2 µM JAa) and (0.4 µM JAa), AFT (0.0033 µM) or media alone for 48 h. Cells (1 x 10^5 cells/well) were seeded in 6-well plates and incubated overnight before the treatment.
SI7. Densitometry and semi-quantitative analyses

The apoptosis induced by free- and AF-encapsulated JAA was corroborated by Western blot studies. The densitometry semi-quantitative analyses for SKBR-3 (Figure S16) lysates revealed a dose-dependent cleavage of PARP and inhibition of anti-apoptotic MCL-1 expression in SKBR-3 cells, and a down-regulation of PLK-1 oncogenic kinase with the highest-used JAA concentration in MDA-MB-231 (Figure S17).

Figure S16. Densitometry semiquantitative analysis of SKBR-3 cells.

Figure S17. Densitometry semiquantitative analysis of MDA-MB-231 cells. Significant differences are expressed as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001.