



[HuArgI (co)-PEG5000]-induced arginine deprivation leads to autophagy dependent cell death in pancreatic cancer cells

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Summary

In this study, we examined the sensitivity of pancreatic cancer cells to [HuArgI (Co)-PEG5000]-induced arginine deprivation as well as the mechanisms underlying deprivation-induced cell death. [HuArgI (Co)-PEG5000]-induced arginine deprivation was cytotoxic to all cell lines tested with IC_{50} values in the pM range at 72 h post-treatment. Three of the five cell lines were rescued by the addition of excess L-citrulline and expressed ASS1, indicating partial arginine auxotrophy. The remaining two cell lines, on the other hand, were not rescued by the addition of L-citrulline and did not express ASS1, indicating complete auxotrophy to arginine. In addition, all cell lines exhibited G0/G1 cell cycle arrest, in the surviving cell fraction, at 72 h following arginine deprivation. Analysis of the type of cell death revealed negative staining for annexin V and a lack of caspase activation in all five cancer cell lines, following treatment, indicating that arginine deprivation leads to caspase-independent, non-apoptotic cell death. Finally, we demonstrated that arginine deprivation leads to a marked activation of autophagy and that inhibition of this autophagy greatly decreases cytotoxicity, indicating that arginine deprivation induces autophagic cell death in pancreatic cancer cells. We have shown that pancreatic cancer cells are auxotrophic for arginine and sensitive to [HuArgI (Co)-PEG5000]-induced arginine deprivation, hence demonstrating that arginine deprivation is a potentially potent and selective treatment for pancreatic cancer. We have also demonstrated that autophagy is activated following arginine-deprivation and that its prolonged activation leads to autophagic cell death.

Keywords Pancreatic cancer · [HuArgI (Co)-PEG5000] · Arginine deprivation · Autophagy · Autophagic cell death

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive types of solid malignancies with a poor prognosis and a five-year overall survival rate of 6 to 7% [1, 2]. Surgical resection presents the only viable treatment approach for PDAC given the absence of efficient therapeutics. However, in most cases PDAC displays, at diagnosis, local invasion and/or metastasis rendering it unresectable [2–4]. Therefore, there's an urgent need to develop novel, effective and selective strategies for targeting pancreatic cancer. One such strategy consists of targeting arginine auxotrophy in pancreatic cancer cells using arginine deprivation induced by a pegylated,

cobalt-substituted, human recombinant Arginase I [HuArgI (Co)-PEG5000].

In normal cells, arginine is synthesized from L-citrulline and aspartate using the urea cycle enzymes argininosuccinate synthetase-1 (ASS1) and argininosuccinate lyase (ASL), that catalyze the conversion of L-citrulline and aspartate first to argininosuccinate then to L-arginine and fumarate [5, 6]. A number of tumor types such as hepatocellular carcinoma, renal cell carcinoma, pancreatic cancer, ovarian cancer, melanoma, GBM, AML and T-ALL have been shown to downregulate their ASS1 expression, hence becoming completely auxotrophic for arginine and relying on extracellular arginine for survival and proliferation. Moreover, even cancer cells that maintain their ASS1 expression are unable to synthesize enough endogenous arginine to support their high proliferation rates, rendering them partially auxotrophic for arginine and also reliant on extracellular sources of arginine for survival and proliferation [7–14]. Therefore, depleting arginine in the tumor microenvironment can induce cytotoxicity in both ASS1 deficient (completely auxotrophic) and ASS1 expressing

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(partially auxotrophic) cancer cell lines. Consequently, arginine deprivation via the use of [HuArgI (Co)-PEG5000], an arginine-catabolizing enzyme, is a novel anti-tumor therapeutic approach that targets the discrepancy in arginine requirements between tumor cells and normal cells.

Native human L-arginase I (hArgI) is characterized by a short serum half-life, due to the loss of the two Mn^{2+} ion cofactors of its active site, which were found to rapidly dissociate in serum [14]. Replacing the two Mn^{2+} ions with two cobalt ions (Co^{2+}) largely abrogated the rapid serum dissociation of the ion cofactors, which led to a significantly enhanced stability of the modified enzyme in serum, in addition to a 10-fold increase in catalytic activity [15]. HuArgI (Co) was further conjugated to polyethylene glycol (PEG) resulting in enhanced solubility, serum stability and immunological characteristics and leading to the final pegylated, recombinant human arginase I [HuArgI (Co)-PEG5000] [15].

We and others have demonstrated the potency and selectivity of [HuArgI (Co)-PEG5000]-induced arginine deprivation in a number of tumor types, including hepatocellular, ovarian, breast, and renal cell carcinomas, melanoma, GBM, AML and T-ALL. This confirms the potential of this novel therapeutic strategy for the selective targeting of arginine-auxotrophic tumors [8–14, 16, 17]. However, targeting arginine auxotrophy in pancreatic cancer cells has only been tested in a single study and in a rather limited way. Therefore, the extent of arginine auxotrophy in pancreatic cancer cells, their potential targeting through [HuArgI (Co)-PEG500]-induced arginine deprivation and the mechanisms of arginine deprivation-induced cell death in this tumor type have not been thoroughly examined yet.

In this study, we aim to examine the mechanisms of arginine deprivation-induced cell death in pancreatic cancer cell lines in an attempt to investigate the potential usefulness of [HuArgI (Co)-PEG5000]-induced arginine deprivation as a targeted therapeutic in this tumor type.

Materials and methods

Expression and purification of HuArgI (co)-PEG5000

Pegylated human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000] (Pegzilarginase) was a gift from Aeaglea BioTherapeutics (Austin, TX, USA). Chloroquine and L-citrulline were purchased from Sigma–Aldrich.

Cells and cell lines

Human pancreatic cancer cell lines Panc-1, Capan-1, Hs 766 T, Panc 04.03 and Panc 10.05 were purchased from the American type culture collection and grown as specified.

Proliferation inhibition assay (cytotoxicity)

Cytotoxicity of [HuArgI (Co)-PEG5000] was determined using a proliferation inhibition assay as described previously [18]. Briefly, aliquots of 10^4 cells were plated per well in 100 μ l DMEM in Costar flat-bottomed 96-well plates (Corning Inc. Corning, NY). This was followed by the addition of 50 μ l [HuArgI (Co)-PEG5000] to yield 11 concentration points ranging from 10^{-7} to 10^{-13} mol/L. L-citrulline was added at a concentration of 11.4 mM and Z-VAD at a concentration of 20 μ M to the corresponding wells. The plate set-up results in having triplicate wells for every drug concentration point of each condition. Plates were then incubated at 37 °C/5% CO_2 for 24, 48, 72, 96 and 120 h, followed by the addition of XTT cell proliferation reagent (Roche, Basel, Switzerland) and a further incubation of 4 h. Absorbance at 450 nm was then determined using a plate reader (Thermo Fisher Scientific, Waltham, MA). Nominal absorbance and percent maximal absorbance were plotted against the log of concentration and a non-linear regression with a variable slope sigmoidal dose-response curve was generated along with IC_{50} values using GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

Analysis of argininosuccinate synthetase 1 (ASS1) expression

Expression of ASS1 was determined by flow cytometry as described previously [12]. Briefly, cells were fixed, permeabilized, and incubated with anti-ASS1 mouse monoclonal antibody (Sigma, Danvers, MA) for 1 h, followed by incubation with a FITC-conjugated rabbit anti-mouse polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Isotypic control consisted of a mouse IgG (Sigma, Danvers, MA) and a FITC-conjugated rabbit anti-mouse polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). ASS-1 expression was determined using the ratio of fluorescence intensity (RFI) between the mean fluorescence intensity (MFI) of the stained cells and the MFI of the isotypic control. $RFI > 2$ was considered positive, while an $RFI \leq 2$ was considered negative for ASS1 expression.

Cell cycle analysis

Cell cycle analysis was carried out using Propidium Iodide (PI)-staining as described previously [19]. In short, cells incubated with either [HuArgI (Co)-PEG5000] (10^{-7} mol/L) or with media alone (control) in flat-bottom 6-well plates (Corning Inc. Corning, NY) for 72 h at 37 °C/5% CO_2 were harvested and fixed in 70% ethanol for a minimum of 24 h, at 20 °C. Following fixation, cells were incubated in PI staining solution (50 μ g/ml) for 10 min at 37 °C. Samples were read

using a C6 flow cytometer (BD Accuri, Ann Arbor, MI) and PI staining was measured on FL2-A.

Analysis of type of cell death

Type of cell death was determined using an Annexin V-fluorescein Isothiocyanate (Annexin V-FITC)/PI apoptosis detection kit (Abcam, Cambridge, MA) and a FITC-conjugated active caspase inhibitor (ApoStat Apoptosis Detection Kit, R&D Systems, Abingdon, England) as described previously [19]. Briefly, cells were plated in flat-bottom 6-well plates and incubated either with [HuArgI (Co)-PEG5000] (10^{-7} mol/L) or media for 24 and 48 h at 37 °C/5% CO₂. Afterwards, cells were either harvested then incubated with FITC-conjugated annexin V antibody (2.5 mg/ml) and PI (5 mg/ml) in binding buffer for 5 min at 37 °C in the dark, or incubated with a FITC-conjugated active caspase inhibitor (ApoStat Apoptosis Detection Kit, R&D Systems, Abingdon, England) for 60 min and then harvested. Annexin V/PI was read on FL1-H versus FL2-H scatter plot and active caspase staining was read on FL1-H.

Western blot analysis

Panc-1 cells were plated and incubated with media alone (control), DMSO, [HuArgI (Co)-PEG5000] (10^{-7} mol/L), chloroquine (50 μM) or a combination of both for 24 and 72 h at 37 °C/5% CO₂. Cell lysates were obtained using the Qproteome Mammalian Protein Prep Kit (Qiagen Inc., US). Samples were boiled for 5 min then separated by SDS-PAGE on 6% or 12% gels and transferred to PVDF membranes for 80 min at 85 V. Membranes were then blocked with 5% BSA in PBS containing 0.05% Tween-20 for 1 h at room temperature and incubated with rabbit polyclonal anti-LC3 antibody (Cell Signaling Technology Inc., US) overnight at 4 °C. Membranes were then washed and incubated with an HRP-conjugated mouse anti-rabbit IgG (Santa Cruz Biotechnology Inc., US) for 2 h at room temperature then washed and treated with western blotting chemiluminescent reagent ECL (GE Healthcare, UK). Bands were visualized using the ChemiDoc XRS+ imaging system (BioRad Laboratories Inc., UK).

Autophagy assay

The contribution of autophagy to the arginine deprivation-induced cytotoxicity of pancreatic cancer cells was tested by incubating cells with [HuArgI (Co)-PEG5000] alone and in combination with the autophagy inhibitor chloroquine (CQ), as described previously [12]. Briefly, aliquots of 10,000 cells in 100 μl DMEM/well, were plated in a flat-bottom 96-well plate (Corning Inc. Corning, NY). In a subset of wells containing cells and culture media, CQ (50 μM) was added, followed by the addition of [HuArgI (Co)-PEG5000] at

concentrations ranging from 10^{-7} to 10^{-13} M. This was followed by incubation of the plates for 24, 48 and 72 h at 37 °C/5% CO₂ as described earlier for the proliferation inhibition assay. Nominal absorbance and percent maximal absorbance were plotted against the log of concentration and a non-linear regression with a variable slope sigmoidal dose-response curve was generated along with IC₅₀ values using GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

Statistical analysis

All experiments were carried out thrice and figures show representative graphs of each experiment. Where statistical analysis is applicable, the results represent the average values of three independent experiments. All error estimates are given as ± standard deviation (SD). The *p* values were calculated by *t*-test using GraphPad Prism. Statistical significance was set at *p* value ≤ 0.05.

Results

Cytotoxicity of [HuArgI (co)-PEG5000]

All the pancreatic cancer cell lines tested were sensitive to [HuArgI (Co)-PEG5000] with IC₅₀ values ranging from 180 to 1480 pM and percent cell death at highest concentration ranging from 50 to 76% (Percent cell death of 76%, 64%, 74%, 75%, and 50% for Panc-1, Capan-1, Hs766T, Panc 04.03 and Panc 10.05, respectively), at 72 h following treatment (Fig. 1, Table 1). Addition of exogenous L-citrulline led to the rescue of three pancreatic cancer cell lines (Hs766T, Capan-1 and Panc 04.03) from arginine deprivation-mediated cytotoxicity, indicating that these cell lines are partially auxotrophic for arginine. The remaining two cell lines (Panc-1 and Panc 10.05) were not rescued by L-citrulline, indicating complete arginine auxotrophy of these cells (Table 1, Fig. 1). Longer arginine starvation times of 96 and 120 h led to a marked increase in the sensitivity in the two cell lines that are completely auxotrophic for arginine (Panc-1, and Panc-10.05) with the partially auxotrophic cell lines (Capan-1, Hs766T and Panc 04.03) showing relatively unchanged IC₅₀ values with increasing [HuArgI (Co)-PEG5000] incubation times (Table 1, Fig. 1). Furthermore, the percent cell death at the highest concentration significantly increased for the completely auxotrophic cell lines reaching almost complete cell death (96% and 88% for Panc-1 and Panc 10.05, respectively) at 120 h, while it remained stable in the partially auxotrophic cell lines (capan-1, Hs766T and Panc 04.03) (Fig. 1). These results demonstrate that pancreatic cancer cell lines are auxotrophic for arginine and sensitive to [HuArgI (Co)-PEG5000]-induced arginine deprivation. In addition, completely auxotrophic cell lines show a time-dependent increase in cytotoxicity following arginine deprivation.

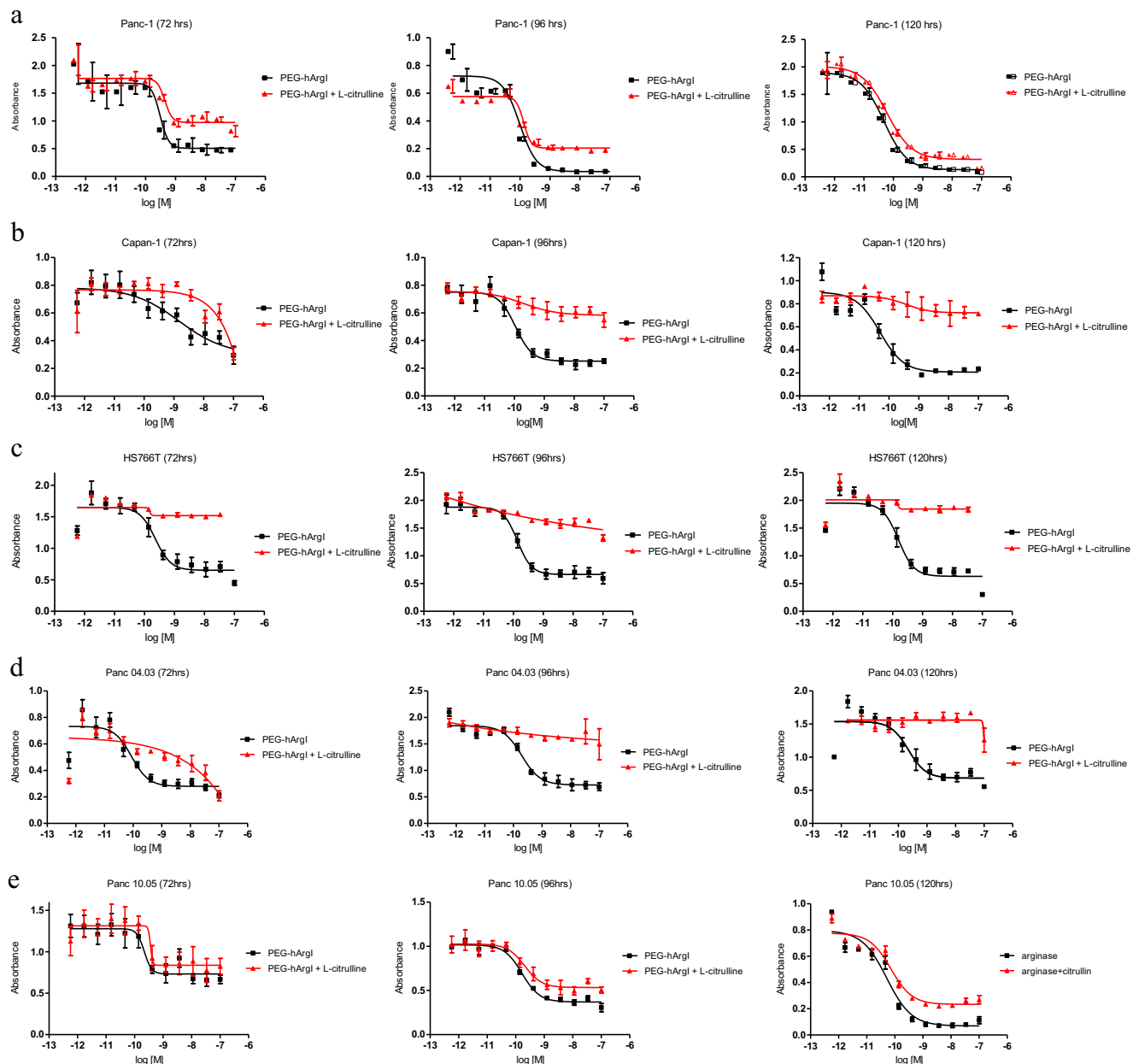


Fig. 1 Representative non-linear regression curves of the cytotoxicity of [HuArgI (Co)- PEG5000] (square) and [HuArgI (Co)-PEG5000] + L-citrulline (triangle) on human pancreatic cancer cell lines Panc-1 (a), Capan-1 (b), Hs 766T (c), Panc 04.03 (d) and Panc 10.05 (e) at 72, 96 and 120 h post-treatment

Table 1 Sensitivity of pancreatic cancer cell lines to [HuArgI (Co)-PEG5000] at 72, 96 and 120 h. Impact of the addition of 11.4 mM exogenous L-citrulline at 72 h. ASS-1 expression represented as ratio of fluorescence intensity (RFI) values

| Cells and cell lines | HuArgI (Co)-PEG5000 (72 h) | HuArgI (Co)-PEG5000 (72 h) + L-cit | HuArgI (Co)-PEG5000 (96 h) | HuArgI (Co)-PEG5000 (120 h) | ASS-1 expression (RFI) |
|----------------------|----------------------------|------------------------------------|----------------------------|-----------------------------|------------------------|
| Panc-1 | 306 pM | 436 pM | 97 pM | 48 pM | 1.79 (–) |
| Capan-1 | 1480 pM | >10,000 pM | 1331 pM | 460 pM | 4.42 (+) |
| Hs776T | 230 pM | >10,000 pM | 136 pM | 156 pM | 3.01 (+) |
| Panc 04.03 | 88 pM | >10,000 pM | 193 pM | 227 pM | 2.34 (+) |
| Panc 10.05 | 217 pM | 362 pM | 166 pM | 52 pM | 1.21 (–) |

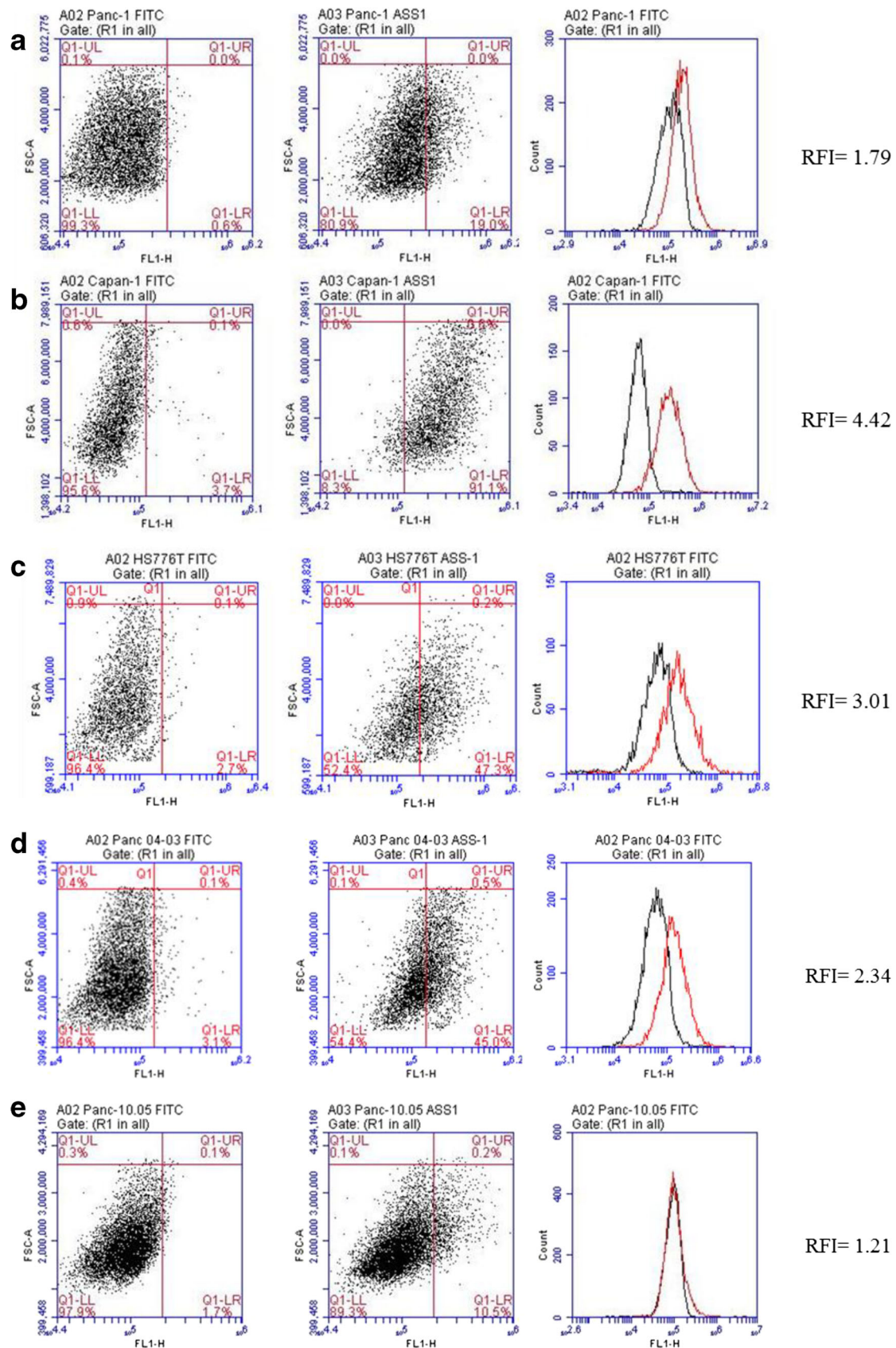


Fig. 2 Representative graphs of ASS1 expression in Panc-1 (a), Capan-1 (b), Hs 766 T (c), Panc 04.03 (d) and Panc 10.05 (e). Cells stained for ASS1 are in red and the isotopic control in black. Cells are gated on width versus forward scatter (R1)

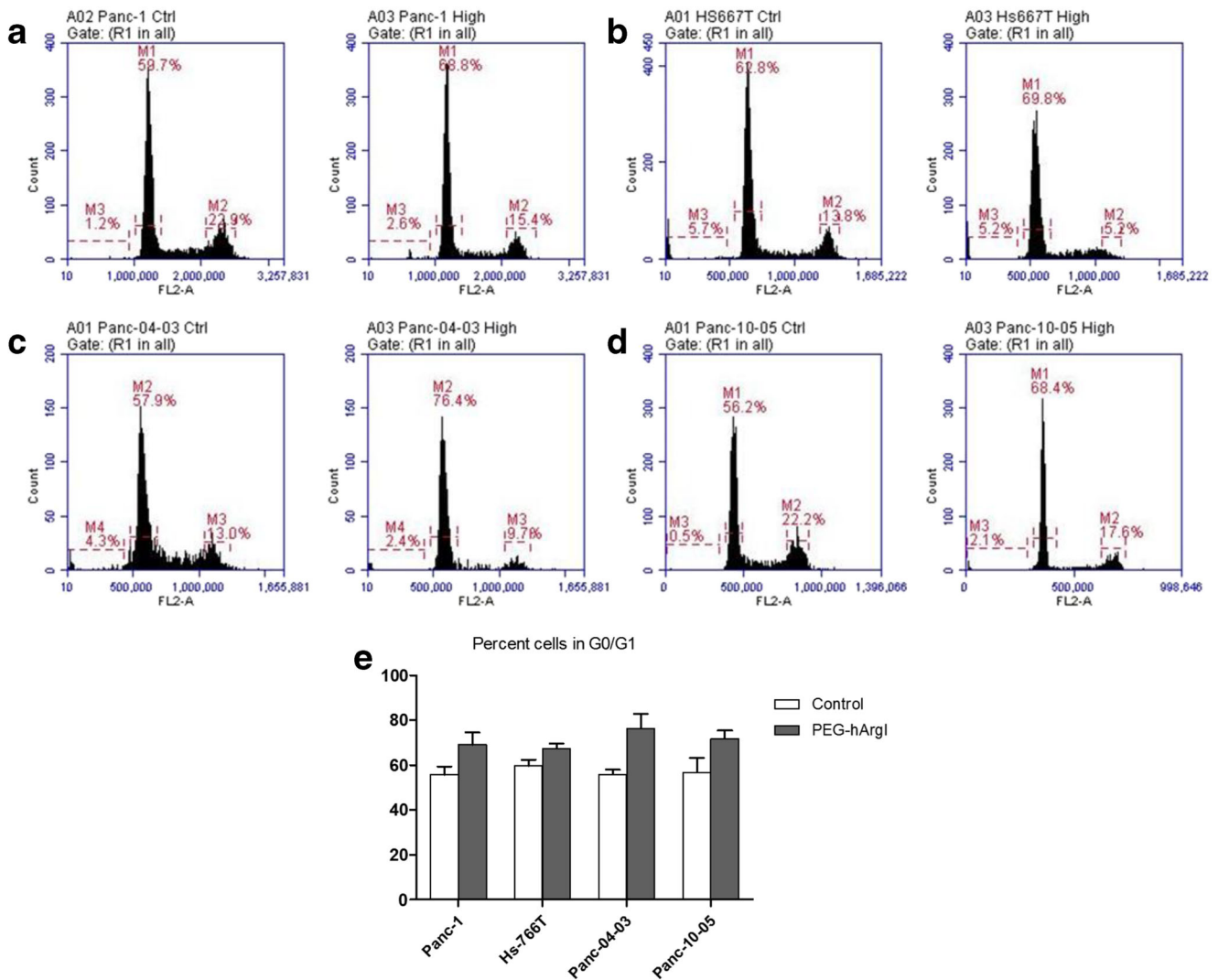


Fig. 3 Representative graphs of the cell cycle analysis (PI staining) of Panc-1 (a), Hs 766T (b), Panc 04.03 (c) and Panc 10.05 (d) cell lines treated with [HuArgI(Co)-PEG5000] for 72 h. Control samples are shown to the left and samples treated with [HuArgI (Co)-PEG5000] are shown to the right. Cells in pre-G0/G1, G0/G1 and G2/M phases are gated M1, M2

and M3, respectively. Cells are gated on width versus forward scatter (R1). (E) A bar graph representing the increase in the percentage of cells in G0/G1 after treatment for all cell lines. The increase in the G0/G1 fraction of cells following treatment is statistically significant for all cell lines

Analysis of ASS1 expression

In order to further investigate the degree of arginine auxotrophy in pancreatic cancer cells, we determined their argininosuccinate synthetase-1 (ASS1) expression levels. Three pancreatic cancer cell lines, Capan-1, Hs766T and Panc 04.03, were positive for ASS1 expression with RFI values of 4.42, 3.01 and 2.34, respectively (Fig. 2, Table 1), confirming that these cell lines are partially auxotrophic for arginine. The remaining two cell lines, Panc-1 and Panc 10.05, were negative for ASS1 expression, as demonstrated by RFI values of 1.79 and 1.21, respectively, confirming that these cell lines are completely auxotrophic for arginine (Fig. 2, Table 1). However, in these two ASS1 negative cell lines, a small percentage of cells did express ASS1 (10% to 18% of

the cells) as demonstrated by the scatter plots in Fig. 2a, e, which underlines the heterogeneity of these pancreatic cancer cell lines in terms of arginine auxotrophy and explains the slight decrease in the percentage of cell death observed when L-citrulline is added (Fig. 1a, e).

Cell cycle effect of [HuArgI (co)-PEG5000]

In order to determine whether arginine deprivation leads to cell cycle arrest in the surviving population of pancreatic cancer cells, we carried out cell cycle analysis in four pancreatic cancer cell lines. All cell lines tested showed a G0/G1 cell cycle arrest at 72 h post-treatment with [HuArgI (Co)-PEG5000]. The percent of cells in the G0/G1 phase increased significantly from $55.7 \pm 2.1\%$, $59.7 \pm 1.6\%$,

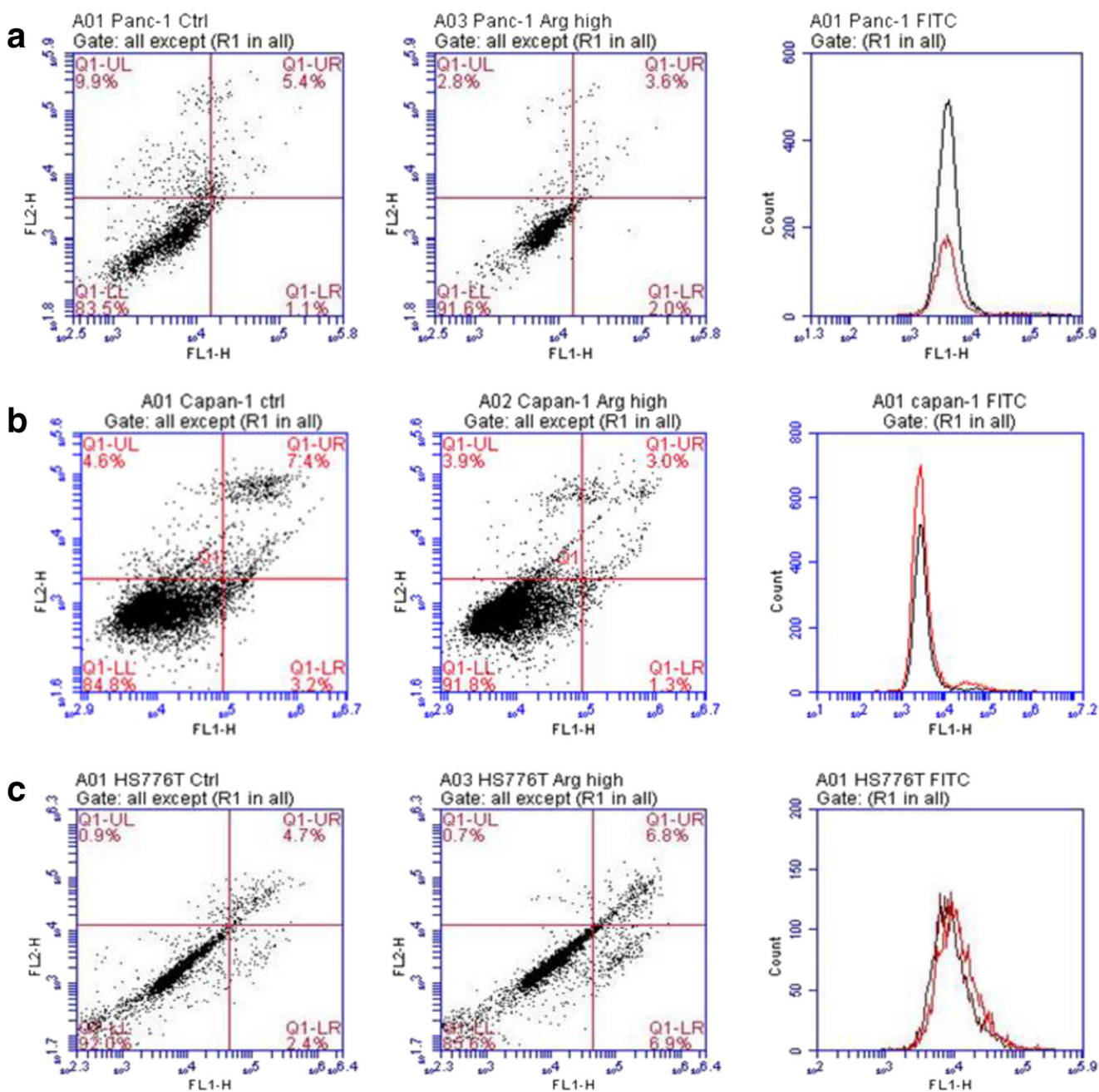


Fig. 4 Annexin V/PI (left panels) and active caspase staining (right panel) of Panc-1 (a), Capan-1 (b), Hs 766T (c), Panc 04.03 (d) and Panc 10.05 (e) cell lines treated with [HuArgI (Co)-PEG5000] for 24 h. Cells are gated on width versus forward scatter (R1). Non-Linear regression curves

of the cytotoxicity of [HuArgI (Co)-PEG5000] (square), [HuArgI (Co)-PEG5000] + Z-VAD (triangle) and Z-VAD alone (inverted triangle) on Panc-1 cells at 72 h (f)

$55.9 \pm 1.2\%$ and $56.8 \pm 3.7\%$ of the total cell population in the control to approximately $69.2 \pm 3.0\%$, $67.4 \pm 1.3\%$, $76.4 \pm 3.6\%$ and $71.8 \pm 2.1\%$ of the surviving cell fraction, for Panc-1 ($p = 0.0221$), Hs766T ($p = 0.0211$), Panc 04.03 ($p = 0.0062$) and Panc 10.05 ($p = 0.0252$) cell lines, respectively, after treatment with [HuArgI (Co)-PEG5000]. This indicates that, in addition to cytotoxicity, arginine deprivation induces cell cycle arrest in the surviving cell fraction of pancreatic cancer cells Fig. 3.

Analysis of cell death

In order to examine the type of cell death in pancreatic cancer cells following arginine deprivation, we stained for annexinV/PI and for active caspases. None of the five cell lines tested showed any signs of apoptosis following treatment with [HuArgI (Co)-PEG5000]. The percentage of annexin V-positive cells (FL1-H) did not change, at either 24 h (Fig. 4) or 48 h (data not shown) after treatment with

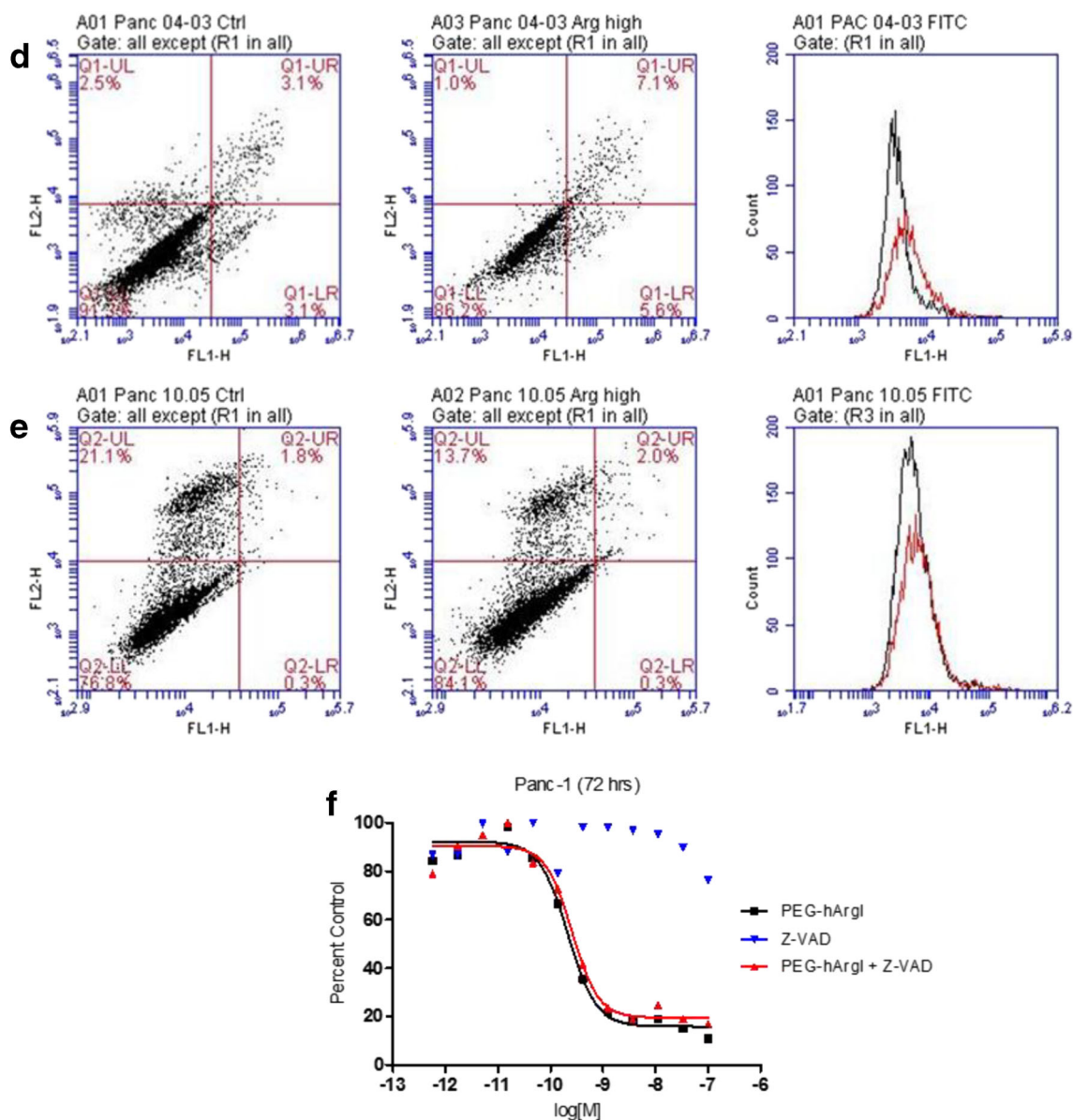


Fig. 4 (continued)

[HuArgI (Co)-PEG5000], compared to control cells. Moreover, staining for active caspases showed no sign of caspase activation following treatment, indicating that arginine deprivation-induced cytotoxicity in pancreatic cancer cells occurs through caspase-independent, non-apoptotic mechanisms (Fig. 4). In order to confirm the lack of caspase activation, we investigated the effect of caspase inhibition on the cytotoxicity of [HuArgI (Co)-PEG5000]-induced arginine deprivation. Co-incubation of Panc-1 cells with both [HuArgI (Co)-PEG5000] and the pan caspase inhibitor Z-VAD had no impact on the sensitivity of these cells to [HuArgI (Co)-PEG5000], confirming that arginine deprivation-induced cell death occurs through caspase-independent, non-apoptotic mechanisms in pancreatic cancer cells (Fig. 4).

Activation of autophagy

To further investigate the mechanisms of cell death in pancreatic cancer cells, we measured the activation level of autophagy following treatment with [HuArgI (Co)-PEG5000]. Activation of autophagy was observed in Panc-1 cells following 24 and 72 h of arginine deprivation as evidenced by the increased processing of LC3I to LC3II in treated cells (“Arg” lanes) compared to untreated, control cells (“Ctrl” lanes) (Fig. 5). This shows that [HuArgI (Co)-PEG5000]-induced arginine deprivation leads to an increase in the autophagic flux in pancreatic cancer cells. The addition of chloroquine to cells (“CQ” lanes), which blocks the downstream processing of autophagosomes, led to their accumulation, hence to a similar increase in LC3-II. As expected, accumulation of LC3-II was

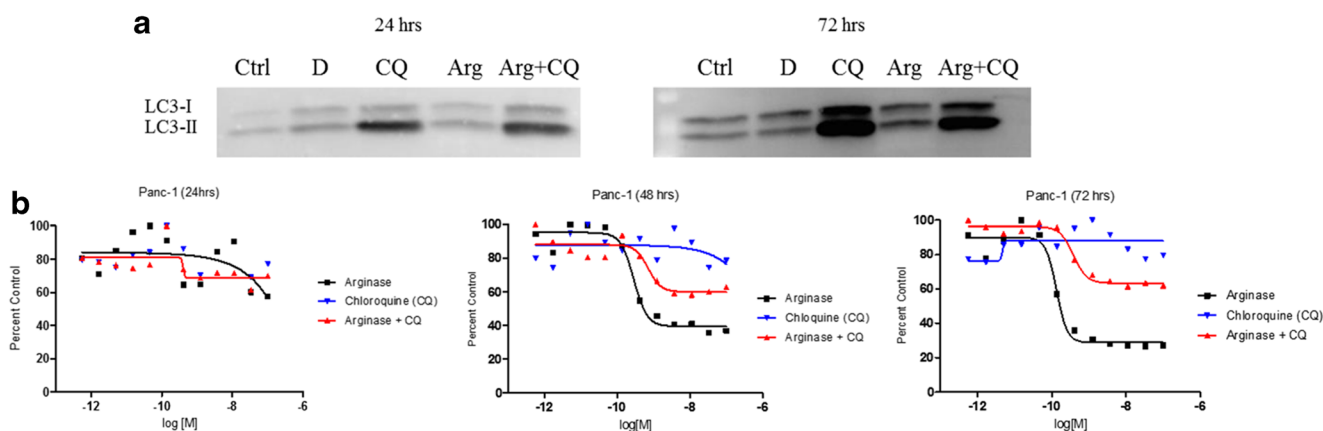


Fig. 5 Panc-1 cells, untreated and treated with [HuArgI (Co)-PEG5000], chloroquine (CQ) or a combination of both for 24 and 72 h, lysed and immunoblotted by western blot analysis for LC3 (**a**). Non-linear

regression curves of Panc-1 cells treated with [HuArgI (Co)-PEG5000] alone (square) and in combination with the autophagy inhibitor chloroquine (triangle) at 24, 48 and 72 h (**b**)

highest in cells treated with a combination of both [HuArgI (Co)-PEG5000] and CQ (“Arg + CQ” lanes) (Fig. 5). Arginine deprivation, therefore, leads to the activation of autophagy in pancreatic cancer cells.

Impact of autophagy on cell cytotoxicity

The impact of the activation of autophagy on the cytotoxicity of arginine deprivation in pancreatic cancer cells was investigated by studying the effect of the inhibition of autophagy on the sensitivity of cells to [HuArgI (Co)-PEG5000]. Panc-1 cells treated with a combination of [HuArgI (Co)-PEG5000] and CQ showed a significant decrease in cell sensitivity to arginine deprivation, compared to cells treated with [HuArgI (Co)-PEG5000] alone, at 48- and 72-h post-treatment. At the early time-point of 24 h, the presence of CQ had no impact on cell cytotoxicity (Fig. 5). This indicates that the activation of autophagy has no impact on cytotoxicity at early time points but mediates cell death (autophagic cell death) at later time points, in pancreatic cancer cells. Therefore, it appears that the prolonged activation of autophagy, following [HuArgI (Co)-PEG5000]-induced arginine deprivation, plays a deleterious role for the survival of pancreatic cancer cells by inducing autophagic cell death.

Discussion

The use of bioengineered human arginase I for the treatment of pancreatic cancer was first investigated by Glazer et al., in a pancreatic carcinoma mouse xenograft model. On the other hand, the use of bacterial arginine deiminase was investigated in an in-vitro model, in a single study by Bowles et al. [11, 20]. However, as far as we know, none of the previous studies investigated the full extent of arginine auxotrophy or the

mechanism of cell death following arginine deprivation. Hence, this is the first study to investigate the full extent of arginine auxotrophy in pancreatic cancer cells, as well as the mechanisms underlying their sensitivity to [HuArgI (Co)-PEG5000]-induced arginine deprivation.

In this study, we have demonstrated that a panel of pancreatic cancer cell lines is auxotrophic for arginine and sensitive to [HuArgI (Co)-PEG5000]-induced arginine deprivation. This is similar to findings in other tumor types such as hepatocellular carcinoma, AML, GBM, T-ALL and ovarian cancer [10–14]. Pancreatic cancer cell lines showed a combination of complete and partial auxotrophy for arginine. This was primarily determined following examination of ASS1 expression and the ability of exogenous L-citrulline to rescue cells from arginine deprivation-induced cytotoxicity. Cell lines expressing ASS1 and rescued by L-citrulline were partially auxotrophic for arginine while cell lines not expressing ASS1 and not rescued by L-citrulline were completely auxotrophic. This is in line with findings from our group and others in hepatocellular and ovarian carcinomas, GBM, AML and T-ALL cell lines [10–14]. Cytotoxicity of [HuArgI (Co)-PEG5000]-induced arginine deprivation was time dependent in completely auxotrophic cells with a decrease in IC_{50} values and an increase in percent cell death with increasing incubation times up to 120 h post-treatment. Cytotoxicity of partially auxotrophic cells varied little with increased duration of arginine deprivation. This indicates that the cytotoxic effect of arginine deprivation is cumulative, at least in completely auxotrophic cells, with prolonged arginine deprivation causing increased cell death and leading to the almost complete lack of any surviving cells after 5 days of treatment. Also, in addition to cytotoxicity, treatment with [HuArgI (Co)-PEG5000] seemed to induce cell cycle arrest in the surviving fraction of pancreatic cancer cells after 72 h of arginine deprivation.

Our findings also demonstrated that depriving cells of arginine leads to caspase-independent, non-apoptotic cell death

in pancreatic cancer cells. This was evident through the complete lack of both annexin V staining and caspase activation. In addition, cell death was associated with significant loss of membrane integrity leading to high levels of cellular fragmentation following treatment. Furthermore, inhibition of caspases, using the pan-caspase inhibitor Z-VAD, had no effect on the cytotoxic response of cells, further indicating that arginine deprivation-induced cell death was caspase-independent. These findings are similar to those we obtained in other tumor types such as AML, GBM and ovarian cancer [12–14]. This is opposite to findings from other studies that showed signs of apoptotic cell death in a number of tumor types following arginine deprivation [10, 11]. However, the difference in outcome is most likely due to the difference in the tumor types used in these studies.

Amino acid deprivation being a known activator of autophagy, we sought to investigate whether autophagy is activated following treatment of pancreatic cancer cells with [HuArgI (Co)-PEG5000]. An increase in the flux of autophagy was observed, at both early and late time points, following arginine deprivation, as demonstrated by the accumulation of LC3-II, indicating increased autophagosome formation. This is similar to findings from previous studies showing activation of autophagy in a number of tumor types following arginine deprivation [21–24]. However, the contribution of autophagy to arginine deprivation-induced cell death remains controversial with some studies showing that the activation of autophagy plays a protective role and others showing that it plays a deleterious role, leading to cell death in what can be referred to as autophagic cell death [12–14, 16, 24–27]. In the absence of evidence supporting apoptotic cell death in our study, we sought to investigate whether the observed activation of autophagy is a contributing factor to the arginine deprivation-induced cell death. We found that the activation of autophagy following arginine deprivation, while not affecting cell viability at early time points, was mediating cell death at later time points. This was demonstrated by the marked decrease in arginine deprivation-induced cytotoxicity, at late time points, following the inhibition of autophagy, hence indicating that cells subjected to arginine deprivation are dying of autophagic cell death. These findings are similar to a previous study showing that arginine deprivation leads to a non-apoptotic, cytotoxic autophagy type of cell death in prostate cancer cells [26].

In this study, we have shown that pancreatic cancer cells are auxotrophic for arginine and sensitive to [HuArgI (Co)-PEG5000]-induced arginine deprivation. In addition, we have shown that autophagy is activated following arginine deprivation and that cell death is mediated by the activation of autophagy. Hence, treatment with [HuArgI (Co)-PEG5000] leads to autophagic cell death in pancreatic cancer cells.

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Compliance with ethical standards

Conflict of interest Author Nathalie Khalil declares that she has no conflict of interest. Author Ralph J. Abi-Habib declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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