



Activation of autophagy following [HuArgI (Co)-PEG5000]-induced arginine deprivation mediates cell death in colon cancer cells

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Received: 9 May 2020 / Accepted: 17 September 2020 / Published online: 26 September 2020
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Abstract

Deregulating cellular energetics by reprogramming metabolic pathways, including arginine metabolism, is critical for cancer cell onset and survival. Drugs that target the specific metabolic requirements of cancer cells have emerged as promising targeted cancer therapeutics. In this study, we investigate the therapeutic potential of targeting colon cancer cells using arginine deprivation induced by a pegylated cobalt-substituted recombinant human Arginase I [HuArgI (Co)-PEG5000]. Four colon cancer cell lines were tested for their sensitivity to [HuArgI (Co)-PEG5000] as well as for their mechanism of cell death following arginine deprivation. All four cell lines were sensitive to arginine deprivation induced by [HuArgI (Co)-PEG5000]. All cells expressed ASS1 and were rescued from arginine deprivation-induced cytotoxicity by the addition of excess L-citrulline, indicating they are partially auxotrophic for arginine. Mechanistically, cells treated with [HuArgI (Co)-PEG5000] were negative for AnnexinV and lacked caspase activation. Further investigation revealed that arginine deprivation leads to a marked and prolonged activation of autophagy in both Caco-2 and T84 cell lines. Finally, we show that [HuArgI (Co)-PEG5000] causes cell death by sustained activation of autophagy as evidenced by the decrease in cell cytotoxicity upon treatment with chloroquine, an autophagy inhibitor. Altogether, these data demonstrate that colon cancer cells are partially auxotrophic for arginine and sensitive to [HuArgI (Co)-PEG5000]-induced arginine deprivation. They also show that the activation of autophagy does not play protective roles but rather, induces cytotoxicity and leads to cell death.

Keywords [HuArgI (co)-PEG5000] · Colorectal cancer · Arginine auxotrophy · Autophagy

Introduction

Colorectal cancer (CRC) is the third most common type of cancer and the fourth leading cause of cancer-related mortality worldwide [1]. CRC death rate has been declining due to better screening and improved treatment, however, prognosis of patients with stage IV disease remains poor with an overall 5-year survival rate ranging from 7.4 to 14.2%,

depending on the age group [1, 2]. Hence, the need for a better understanding of CRC and for more potent and selective therapeutic approaches for the treatment of late stage CRC [3–6]. One such approach consists of targeting the metabolic requirements of tumors, which are critical for their onset, survival and progression. Arginine is a semi-essential amino acid synthesized from L-citrulline and aspartate by the urea cycle enzymes, argininosuccinate synthetase-1 (ASS1) and argininosuccinate lyase (ASL) [7]. Several studies have shown that the availability of abundant arginine reserves is critical for the proliferation and survival of cancer cells [8]. Studies have also shown that many tumor types lacking the ASS1 enzyme rely on extracellular sources of arginine for survival, hence becoming completely auxotrophic for this amino acid. Moreover, even in tumors that do express ASS1, the rate of arginine synthesis is not sufficient to meet the cells growth and survival requirements of the tumor. This forces tumor cells to still depend on extracellular sources of arginine, hence rendering them partially auxotrophic for

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arginine [9–11]. Given arginine's critical role in providing the metabolic requirements to both completely and partially auxotrophic tumor cells, arginine deprivation has emerged as a promising therapeutic approach for targeting CRC tumor types that exhibit complete or partial auxotrophy for arginine.

Arginine deprivation can be achieved using the human arginine-degrading enzyme: L-arginase I (HuArgI). Native human arginase hydrolyses arginine to urea and ornithine. It is conjugated to two Mn²⁺ ion cofactors, which were found to be rapidly lost in serum, leading to a low saturation constant and a short half-life of the enzyme when administered systemically [12]. To overcome this, a recombinant human L-arginase I, in which the Mn²⁺ ions were replaced by Co²⁺ ions was designed. The cobalt-substituted, human L-arginase I [HuArgI(Co)] recombinant enzyme exhibited a significantly higher activity (approximately tenfold higher kcat/km) as well as a higher serum stability, compared to the native enzyme [12]. Serum stability was further enhanced through the addition of polyethylene glycol generating a recombinant, pegylated, cobalt-substituted, human arginase I [HuArgI (Co)-PEG5000] with improved catalytic activity, higher stability and reduced immunogenicity [13, 14].

Targeting cancer metabolic requirements, including amino-acid restriction, is currently being investigated as a potential treatment for several tumor types. Human cyst(e) inase enzyme for instance, is used to induce cysteine deprivation in a number of tumors, and L-asparaginase has been approved for the treatment of acute lymphoblastic leukemia [15, 16]. Similarly, arginine deprivation using [HuArgI (Co)-PEG5000] has shown promising results in the selective targeting of several tumor types, namely, hepatocellular carcinoma (HCC), T-cell acute lymphoblastic leukemia (T-ALL), acute myeloid leukemia (AML), glioblastoma (GBM) and ovarian cancer [10, 11, 17–21].

Despite the large amount of evidence accumulated on arginine deprivation therapy, the mechanism of cell death, as well as the contribution of autophagy to the arginine deprivation-induced cytotoxicity in cancer cells remains poorly understood. Autophagy, a self-degradative process is generally perceived as a protective survival mechanism that gets activated in response to nutrient deprivation to allow cells to manage their energy resources.

Deregulation of autophagy, however, has also been linked to non-apoptotic forms of programmed cell death [22, 23]. Autophagy activation following amino-acid deprivation has been well documented, hence the need to examine autophagy activation in this study. Furthermore, the impact of the increase in the autophagic flux on the response of cancer cells to different therapeutics remains unclear and varied from protective to death-inducing, depending on the tumor type. For example, activation of autophagy had a protective role in cisplatin-resistant ovarian cancer cells targeted with a

sphingosine analog, while it induced autophagic cell death in drug-resistant Burkitt's lymphoma cells treated with fluoxetine [24–26]. We have previously shown that autophagy plays a protective role at early time points in GBM and AML cells subjected to arginine deprivation [10, 11]. We have also recently demonstrated that the long-term activation of autophagy following arginine deprivation in ovarian cancer cells is the mechanism which leads to cell death in these cells [21].

Arginine auxotrophy in colon cancer cells has been extensively studied. However, the potential of targeting this arginine auxotrophy using arginine deprivation, in addition to the degree of activation of autophagy and its potential impact on cytotoxic cell death, have not been investigated yet. In this study, we attempt to target arginine auxotrophy in colon cancer cells using [HuArgI (Co)-PEG5000]-induced arginine depletion and determine the impact of autophagy activation on the response of these cells to arginine deprivation.

Materials and methods

Cell culture

Human colon cancer cell lines Caco-2, SK-Co-1, SW837 and T84 were purchased from the American type culture collection (Manassas, VA, USA). The cells were grown at 37 °C and 5% CO₂ in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma, Danvers, MA) and 100 U penicillin/streptomycin.

Proliferation inhibition assay (cytotoxicity)

Pegylated human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000] (Pegzilarginase) was a kind gift from Aea-glea BioTherapeutics (Austin, TX, USA). Cytotoxicity of [HuArgI (Co)-PEG5000] was determined using a proliferation inhibition assay as described previously [27, 28]. Briefly, aliquots of 10⁴ cells/well were plated in 96-well plates and treated in triplicate with or without [HuArgI (Co)-PEG5000] at concentrations ranging from 10⁻⁷ to 10⁻¹³ M. Where indicated, L-citrulline, or chloroquine (CQQ) were added to the wells at fixed final concentrations of 11.4 mM for L-citrulline and 50 or 100 μM for CQ, respectively before treatment with [HuArgI (Co)-PEG5000]. Following 24, 48, 72, 96, or 120-h treatment period, XTT cell proliferation reagent (Roche, Basel, Switzerland) was added to each well and the plates were incubated for another 4 h at 37 °C and 5% CO₂. Absorbance was read at 450 nm using a microplate reader (Varioskan). 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} using GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

Analysis of argininosuccinate synthetase 1 expression

The expression of argininosuccinate synthetase-1 (ASS1) was determined by flow cytometry and western blot, as described previously [4, 21]. Briefly, for flow cytometry, cells were fixed in ethanol then incubated with a solution of anti-ASS1 mouse monoclonal antibody (Sigma, Danvers, MA) diluted to a final concentration of 1/100 for 1 h at 37 °C. Cells were then incubated for 30 min with FITC-conjugated rabbit anti-mouse polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to the final concentration of 1/100. The isotypic control consisted of cells incubated with a mouse IgG (Sigma, Danvers, MA) and a FITC-conjugated rabbit anti-mouse polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with the secondary antibodies, cells were washed once and read using a C6 flow cytometer (BD Accuri, Ann Arbor, MI). Expression of ASS1 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. $RFI \geq 2.0$ was considered positive. For western blot, cells were lysed using the Qproteome Mammalian Protein Prep Kit (Qiagen Inc., US), boiled for 5 min, then separated by SDS-PAGE on 12% gels and transferred to PVDF membranes for 80 min at 85 V. Membranes were blocked with 5% BSA for 1 h at room temperature then incubated with an anti-ASS1 mouse monoclonal antibody (Sigma, Danvers, MA) overnight at 4 °C. Membranes were then washed and incubated with an HRP-conjugated rabbit anti-mouse 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).

Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry using Propidium Iodide (PI)-staining (Molecular Probes, OR, USA), as described previously [29]. Briefly, colon cancer cells were plated in six-well plates and treated with cell media or 10^{-7} M of [HuArgI (Co)-PEG5000] for 48 or 72 h. Following, cells were harvested and the cell pellet was washed with PBS 1X and fixed in 70% ice-cold ethanol overnight. Cells were then stained in a (50 mg/ml) PI staining solution for 40 min at 37 °C. The fluorescence intensity was then measured by flow cytometry using a C6 flow cytometer (BD Accuri, Ann Arbor, MI). Total cell DNA content was

measured on FL2-A and the percentage of cell distribution in G0/G1, S and G2/M phase was determined for control HuArgI (Co)-PEG5000 treated cells, respectively. Cells were gated on width versus forward scatter.

Analysis of the mechanism of cell death

Type of cell death was determined using Annexin V-FITC/Propidium Iodide (PI) staining (Abcam, Cambridge, MA) and staining for active caspases through a FITC-conjugated active caspase inhibitor assay (ApoStat Apoptosis Detection Kit, R&D Systems, Abingdon, England) on flow cytometry, in addition to PARP cleavage on western blot, as described previously [4, 30]. Briefly, for flow cytometry, cells were incubated with media alone or media containing the highest concentration of HuArgI (Co)-PEG5000 (10^{-7} M) for 24 h. After treatment, cells were harvested and incubated at 37 °C with annexin V-FITC antibody and PI for 45 min or with ApoStat for 30 min then harvested. Annexin V/PI was read on FL1-H versus FL2-H scatter plot and active caspase staining was read on FL1-H. For western blot, cells incubated with media alone or [HuArgI (Co)-PEG5000] (10^{-7} mol/L) for 24 and 48 h, were lysed using the Qproteome Mammalian Protein Prep Kit (Qiagen Inc., US), boiled for 5 min, then separated by SDS-PAGE on 12% gels and transferred to PVDF membranes for 80 min at 85 V. Membranes were blocked with 5% BSA for 1 h at room temperature then incubated with an anti-PARP monoclonal mouse antibody (Sigma, Danvers, MA) overnight at 4 °C. Membranes were then washed and incubated with an HRP-conjugated rabbit anti-mouse 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).

Analysis of autophagy activation

Analysis of the flux through autophagy was performed using the Cyto-ID autophagosome detection kit on flow cytometry and LC3 cleavage on western blot, as described previously [4, 21]. For flow cytometry, cells were incubated with either media alone or with 0.5 μ M rapamycin (positive control), 10^{-7} M [HuArgI (Co)-PEG5000], 50 μ M of the downstream autophagy inhibitor chloroquine (CQ), or a combination of CQ and [HuArgI (Co)-PEG5000]. At 24, 48, 72, 96 and 120 h after treatment, cells were harvested, washed and incubated with the Cyto-ID stain for 40 min before analysis using the Accuri C6 flow cytometer. Cells were gated on width versus forward scatter and the percent of cells with increased Cyto-ID staining (increased autophagosome formation) was detected on FL1-H. For Western blot, cells incubated with

media alone, [HuArgI (Co)-PEG5000] (10^{-7} mol/L), chloroquine (50 μ M) or a combination of both were lysed using the Qproteome Mammalian Protein Prep Kit (Qiagen Inc., US), boiled for 5 min, then separated by SDS-PAGE on 12% gels and transferred to PVDF membranes for 80 min at 85 V. Membranes were blocked with 5% BSA for 1 h at room temperature then incubated with a 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).

Results

[HuArgI (Co)-PEG5000] is cytotoxic to a panel of colon cancer cells

Arginine deprivation induced by treatment with [HuArgI (Co)-PEG5000] at concentrations ranging between 10^{-7} and 10^{-13} M was cytotoxic to all four colon cancer cell lines tested with IC_{50} values of 136, 246, 532 and 126 pM, for Caco-2, Sk-Co-1, SW837 and T84 cancer cells respectively at 72 h post-treatment (Table 1). Figure 1 further reveals that arginine deprivation-induced cytotoxicity is concentration dependent in all cell lines with T84 and SW837 cancer cells exhibiting most and least sensitivity to treatment with [HuArgI (Co)-PEG5000], respectively. The cytotoxic effects in response to treatment with [HuArgI (Co)-PEG5000] were also time-dependent with results showing little to no cell death at 24 h post-treatment with [HuArgI (Co)-PEG5000] as compared to later time points whereby a significant increase in cytotoxicity is detected starting 48 h and continuing through the 72, 96 and 120 h time points in all cell lines (Fig. 1, Table 1).

To determine whether colon cancer cells are completely or partially auxotrophic to arginine, we treated all colon

cancer cell lines with [HuArgI (Co)-PEG5000] alone or in combination with excess amounts of exogenous L-citrulline (11.4 mM), which has been shown to increase the intracellular levels of arginine in partially auxotrophic (expressing ASS1) but not completely auxotrophic (not expressing ASS1) cells [5–7, 17]. Figure 1 and Table 1 both demonstrate that addition of excess L-citrulline induced a significant decrease in cytotoxicity leading to the complete rescue of cells from the cytotoxic effects of arginine deprivation in all four cell lines. The ability of exogenous L-citrulline to rescue these cells indicates that colon cancer cell lines are partially auxotrophic for arginine.

ASS1 expression demonstrates the partial arginine auxotrophy of the colon cancer cells studied

To confirm the partial arginine auxotrophy of the different colon cancer cell lines used in this study, we determined the expression levels of argininosuccinate synthetase (ASS1), the rate-limiting enzyme in arginine synthesis which controls cell dependency to the semi-essential amino acid arginine, using both flow cytometry and western blot. As seen in Fig. 2a, staining for ASS1 on flow cytometry demonstrated that all four colon cancer cell lines express ASS1, with RFI values of 3.11, 2.18, 2.26 and 2.49, for Caco-2, Sk-Co-1, SW837 and T84 cell lines, respectively, as compared to the isotype control. This was confirmed by western blot, which also showed that all four cell lines express ASS1 (Fig. 2b).

Expression of ASS1 demonstrates that the tested colon cancer cells are partially auxotrophic for arginine. This finding, as well as the rescue observed upon treatment with exogenous L-citrulline, elucidate the partial auxotrophic nature of the tested cell lines as well as provide an underlying molecular basis for citrulline rescue from the arginine deprivation-induced cytotoxicity (Fig. 2, Table 1).

[HuArgI (Co)-PEG5000] treatment induces differential cell cycle arrest

To understand the mechanisms underlying arginine deprivation-induced cytotoxicity in colon cancer cells, we

Table 1 Time course of the sensitivity of colon cancer cells to [HuArgI (Co)-PEG5000] (IC_{50} values) and effect of the addition of excess exogenous L-citrulline at 72 h to all cell lines

Cell line	PEG-hArgI 24 h (IC_{50} pM)	PEG-hArgI 48 h (IC_{50} pM)	PEG-hArgI 72 h (IC_{50} pM)	PEG-hArgI + L-cit 72 h (IC_{50} pM)	PEG-hArgI 96 h (IC_{50} pM)	PEG-hArgI 120 h (IC_{50} pM)	ASS-1 (RFI)
Caco-2	1539	343	136	> 10,000	130	95	3.11 (+)
Sk-Co-1	> 10,000	689	246	> 10,000	115	67	2.18 (+)
SW837	> 10,000	779	532	> 10,000	88	45	2.26 (+)
T84	1472	856	126	> 10,000	57	71	2.49 (+)

ASS-1 expression presented as ratio of fluorescence intensity (RFI) of ASS-1 stained cells to isotype controls

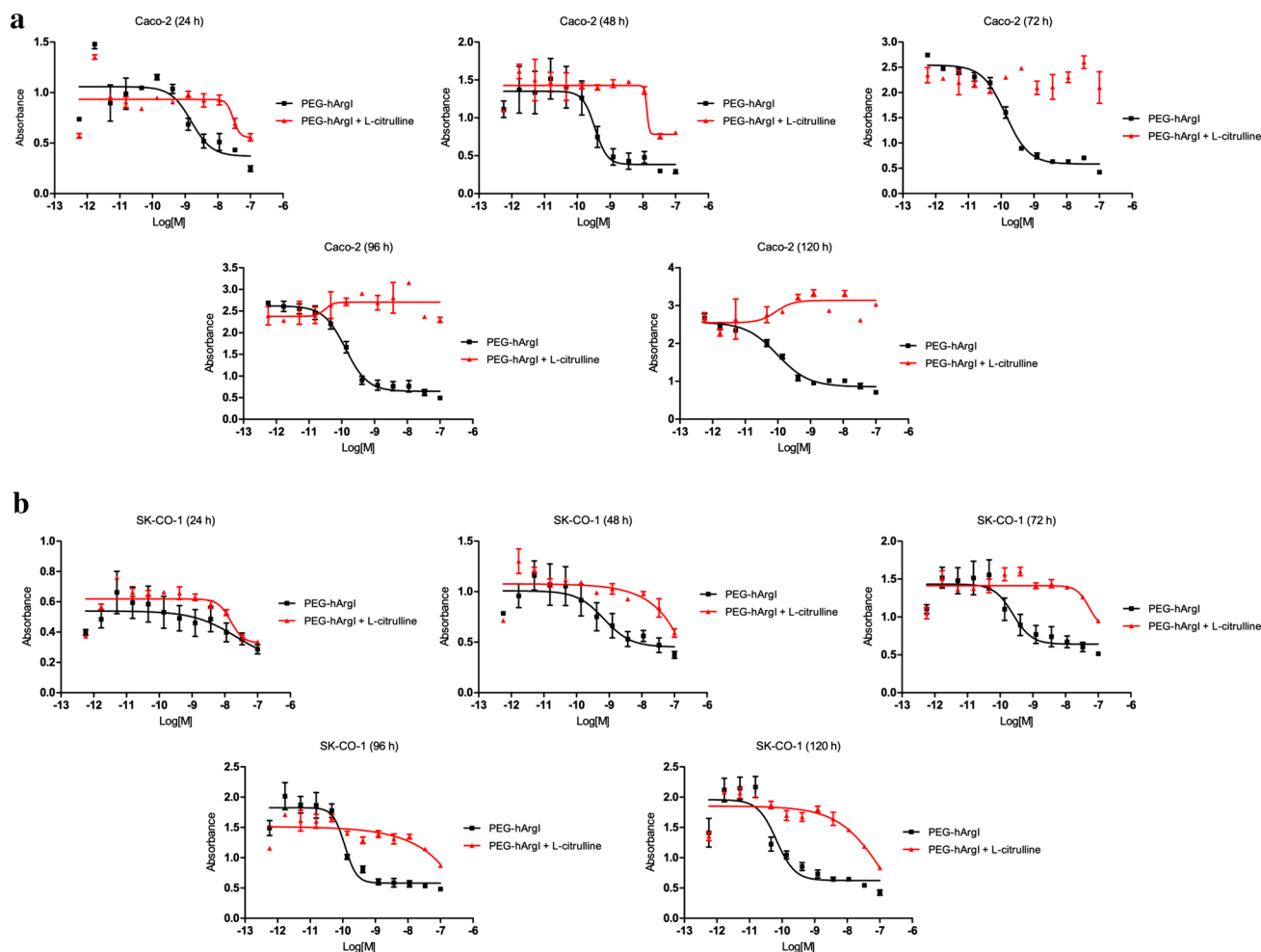


Fig. 1 Cytotoxicity of HuArgI (Co)-PEG5000 to Caco-2 (a), SK-Co-1 (b), SW837 (c) and T84 (d), alone and with L-citrulline (11.4 mM) at 24, 48, 72, 96 and 120 h

investigated cell cycle changes in response to treatment with [HuArgI (Co)-PEG5000]. Figure 3 demonstrates that only the Sk-Co-1 cell line exhibits marked G0/G1 cell cycle arrest at both 48 and 72 h following [HuArgI (Co)-PEG5000]-induced arginine deprivation. Specifically, we observe an increase in the fraction of cells in the G0/G1 phase from approximately 49.8% and 62.6% of the total cell population in control cells to approximately 79.0% and 82.5% of the surviving cell population, at 48 and 72 h post-treatment, respectively. This increase in G0/G1 phase arrest was accompanied by a corresponding decrease in the percentage of cells in the G2 phase (Fig. 3). Two of the remaining cell lines, SW837 and T84 showed cell cycle arrest at G0/G1 at 72 h but not at 48 h, while the Caco-2 cell line did not show any cell cycle arrest at any time point following treatment with [HuArgI (Co)-PEG5000] (Fig. 3). Altogether, the data demonstrates that arginine deprivation leads to cell cycle arrest in the surviving fraction of a subset of colon cancer cells.

[HuArgI (Co)-PEG5000] induces cell death in a caspase-independent, non-apoptotic manner

To determine the contribution of apoptosis to the mechanism of cell death in colon cancer cells following arginine deprivation, we stained for annexinV/PI as well as for active caspases on flow cytometry and for PARP cleavage on western blot. Figure 4a shows that none of the four cell lines exhibits any significant increase in the percentage of cells stained with annexinV, as compared to controls, at 24 h following treatment with [HuArgI (Co)-PEG5000]. Caco-2 cells, however, showed an increase in both AnnexinV and PI staining, suggesting loss of membrane integrity (Fig. 4). Similarly, staining for active caspases revealed the absence of caspase activation in all four cancer cell lines (Fig. 4b). Finally, there was no sign of PAPP cleavage in neither of the two cells lines, at both 24- and 48-h post-treatment with [HuArgI (Co)-PEG5000] (Fig. 4c). Altogether, the absence of caspase activation and PARP cleavage, in addition to the

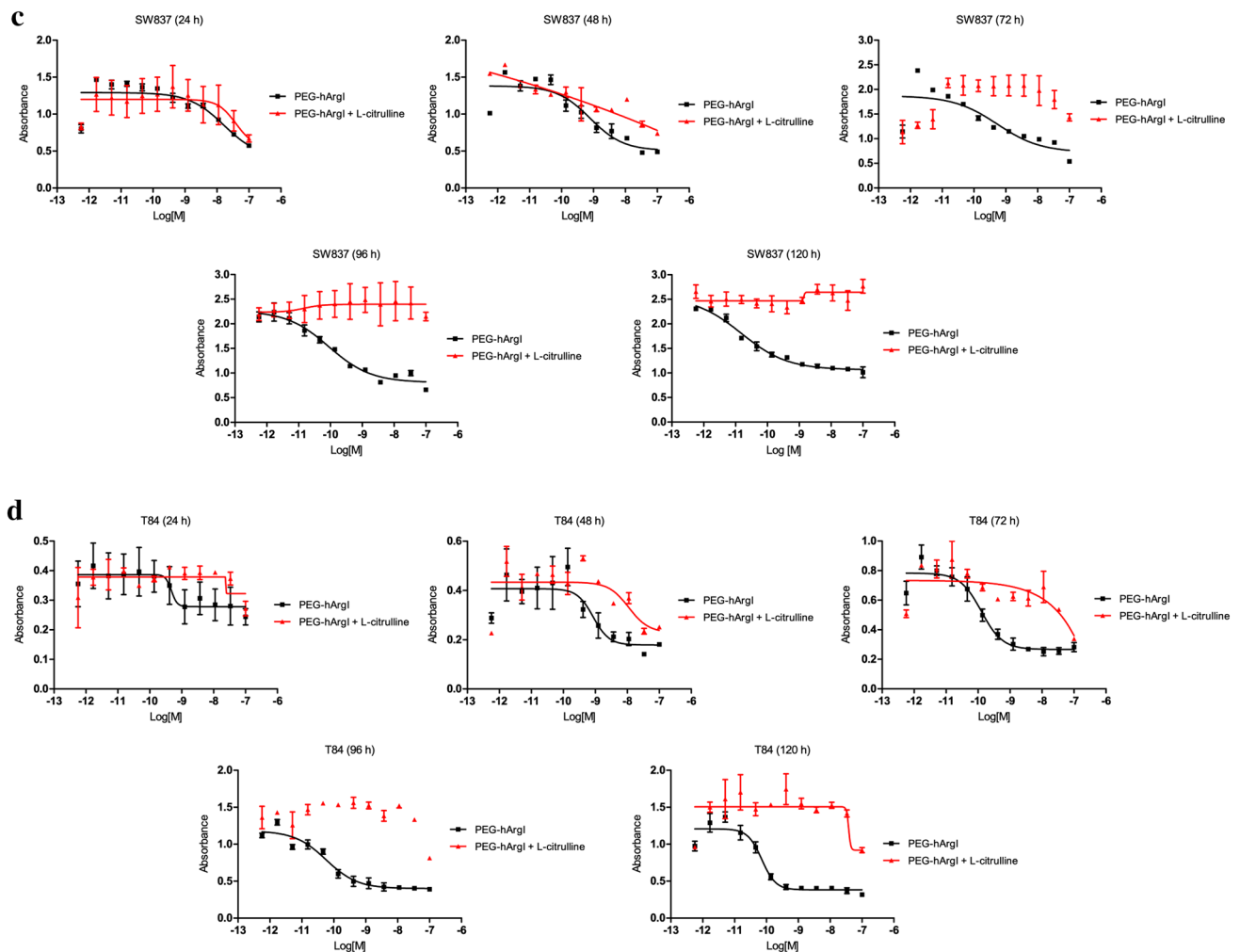


Fig. 1 (continued)

lack of staining for annexinV, indicate that arginine deprivation-induced cytotoxicity in colon cancer cell lines is mediated through a caspase-independent, non-apoptotic cell death mechanism.

[HuArgI (Co)-PEG5000]-induced arginine deprivation leads to the activation of autophagy

We next assessed the activation of autophagy in response to arginine deprivation. To this aim we examined the accumulation of autophagosomes in Caco-2 and T84 cells following treatment with [HuArgI (Co)-PEG5000] and compared it with the level of autophagosomes in untreated cells and in cells treated with rapamycin, chloroquine (CQ) or a combination of [HuArgI (Co)-PEG5000] and CQ using both an autophagosome-specific stain on flow cytometry and LC3 processing on western blot. [HuArgI (Co)-PEG5000]-induced arginine deprivation led to an increase in the formation of autophagosomes, as compared

to untreated cells, starting at 24- and 48-h post-deprivation, in Caco-2 and T84 cells, respectively, and lasting up to 120 h (Fig. 5a). The data further reveals that the formation of autophagosomes peaks at 96- and 120-h post-treatment in both cell lines (Fig. 5a). The activation of autophagy following treatment with [HuArgI (Co)-PEG5000] was similar to, and even exceeded at several time points, that triggered by the positive control rapamycin (Data not shown). Treatment with CQ, an autophagy inhibitor which prevents the fusion of autophagosomes with lysosomes, also led to the accumulation of autophagosomes and treating cells with both [HuArgI (Co)-PEG5000] and CQ in combination resulted in an accumulation of autophagosomes greater than either treatment alone (Fig. 5b). In summary, we demonstrate that [HuArgI (Co)-PEG5000]-induced arginine deprivation leads to a marked and sustained activation of autophagy in colon cancer cells starting as early as 24 and 48 h and lasting up to 120 h following arginine deprivation.

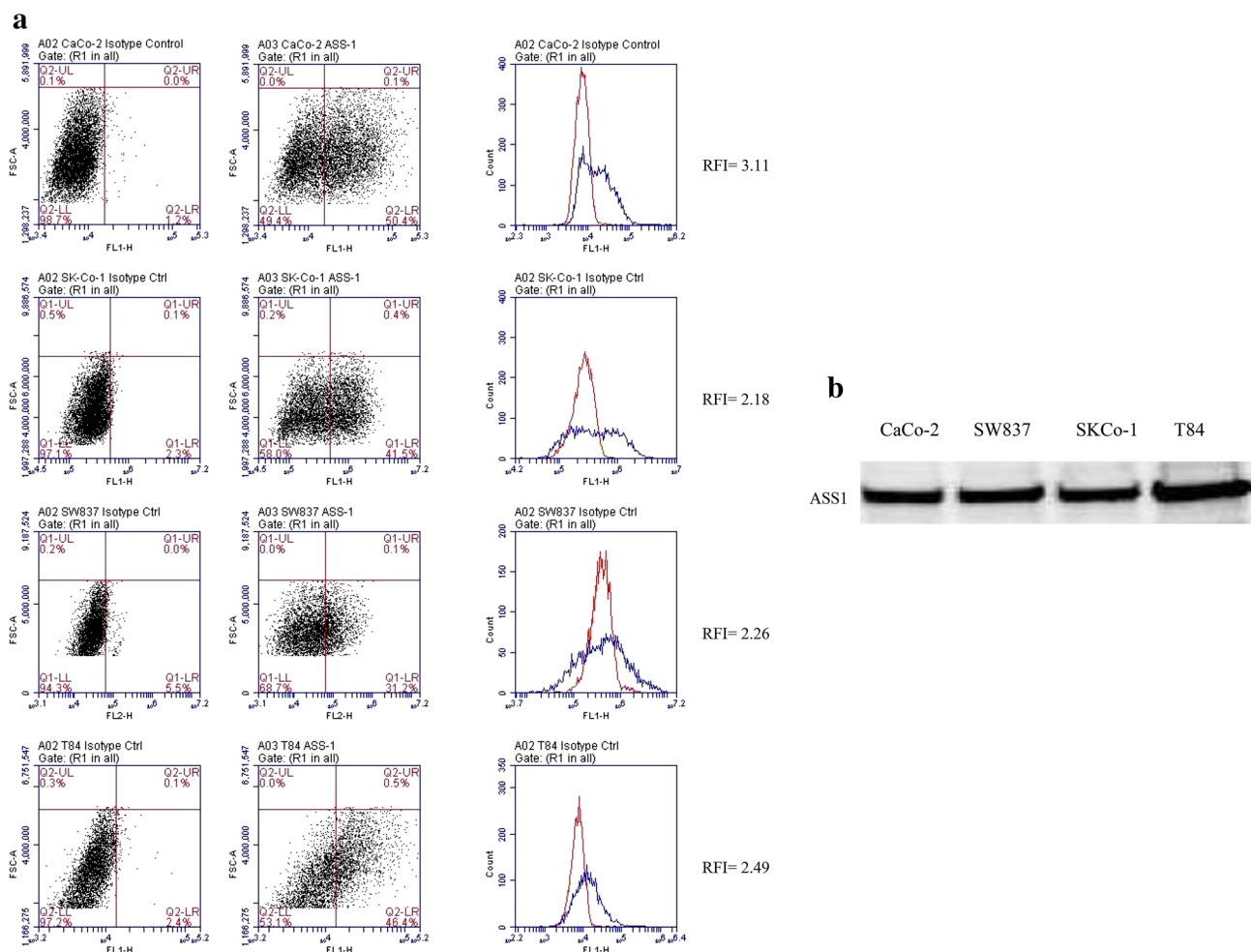


Fig. 2 ASS1 expression in CaCo-2, SK-Co-1, SW837 and T84 cells by flow cytometry (**a**). Cells stained for ASS1 are in red and the isotypic control in black. Cells are gated on width versus forward scatter.

All cell lines are positive for ASS1 expression (RFI \geq 2.0). **b** Expression of ASS1 in CaCo-2, SW837, SK-Co-1 and T84 cells by western blot

Impact of autophagy activation on cell cytotoxicity

Having ruled out apoptosis as the mechanism of cell death induced by arginine deprivation, we investigated the contribution of the prolonged activation of autophagy to the cytotoxicity induced by [HuArgI (Co)-PEG5000]. This was done by determining the impact of the inhibition of autophagy, using the downstream autophagy inhibitor chloroquine (Q), on the cytotoxicity of [HuArgI (Co)-PEG5000]-induced arginine deprivation in both CaCo-2 and T84 cells at 48- and 72-h following treatment. The findings presented in Fig. 6 show that addition of CQ significantly decreased the sensitivity of both CaCo-2 and T84 cells to [HuArgI (Co)-PEG5000] in a dose-dependent manner, at both the 48- and 72-h after arginine deprivation. Addition of CQ at a concentration of 50 μ M led to an increase in the IC₅₀ values of approximately 3.5 and sevenfold at 48 h and of 3.3 and 5.6-fold at 72 h, in CaCo-2 and T84 cells, respectively,

compared to cells treated with [HuArgI (Co)-PEG5000] alone. Addition of CQ at a concentration of 100 μ M CQ led to complete resistance of both cell lines to arginine deprivation at both time points (Fig. 6). These results demonstrate that the cytotoxicity of [HuArgI (Co)-PEG5000]-induced arginine deprivation to colon cancer cells is mediated by the prolonged activation of autophagy, a case of autophagy-mediated cell death.

Discussion

Arginine auxotrophy in colon cancer cells has been widely investigated. However, the therapeutic potential of arginine deprivation as well as the contribution of autophagy to the response of cells to arginine deprivation had not yet been determined. In this study, we demonstrate that all colon cancer cell lines assessed are partially auxotrophic for arginine

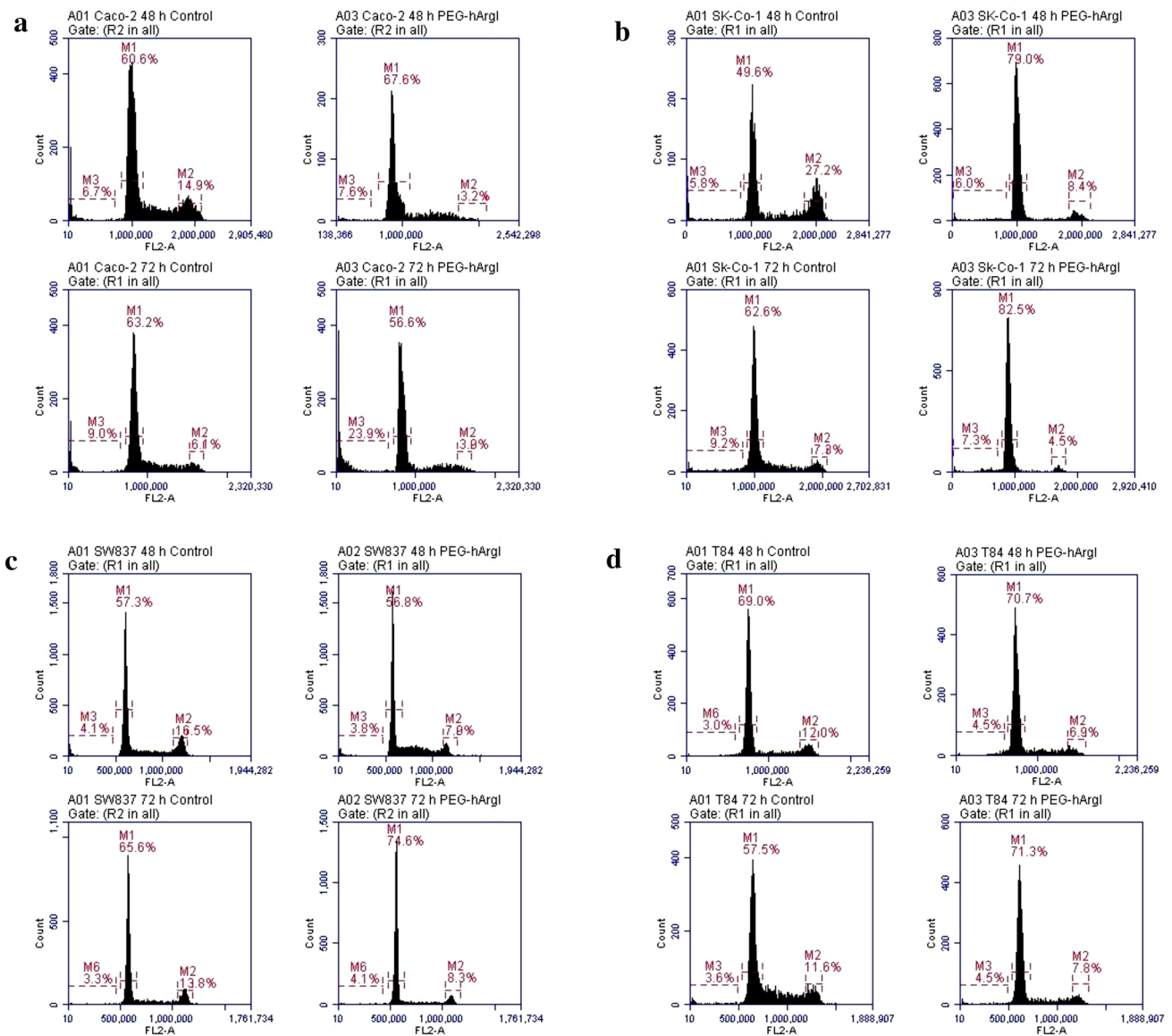


Fig. 3 Cell cycle analysis (PI staining) of Caco-2 (a), SK-Co-1 (b), SW837 (c) and T84 (d) treated with [HuArgI (Co)-PEG5000] for 48 (upper panels) and 72 h (lower panels). Cells were gated on width

versus forward scatter. Cells in G0/G1, G2/M and pre-G0/G1 phases are gated M1, M2 and M3, respectively

and that they exhibit significant sensitivity to treatment with [HuArgI (Co)-PEG5000], thus highlighting the potential of arginine deprivation for the selective targeting of colon cancer. The effectiveness of amino-acid restriction for the selective targeting of auxotrophic tumors has been amply demonstrated [10, 11, 18, 19, 21, 31, 32]. This includes asparagine depletion to target AML and acute lymphoid leukemia (ALL) [33, 34], cysteine restriction to target prostate cancer [15], as well as arginine depletion to target prostate cancer, ovarian cancer and GBM. However, unlike in other tumor types such as AML, GBM and T-ALL, all colon cancer cell lines tested in this study were partially auxotrophic for arginine [10, 11, 18, 21]. This, however, did not prevent the cells

from being sensitive to arginine deprivation. This could be, in part, due to the continuing need for exogenous arginine, in addition to the endogenous arginine, to fully support the metabolic requirements of these cancer cells. This makes them dependent on exogenous arginine resources to ensure their survival, replication and growth. Partially auxotrophic cells express the ASS1 enzyme and can thus be salvaged by the addition of excess L-citrulline, which increases the flux through the urea cycle, and by consequence, increases the levels of endogenous arginine production circumventing the need for extracellular arginine. Completely auxotrophic cells however, do not express the ASS1 enzyme and, subsequently, cannot be rescued by L-citrulline. This ability

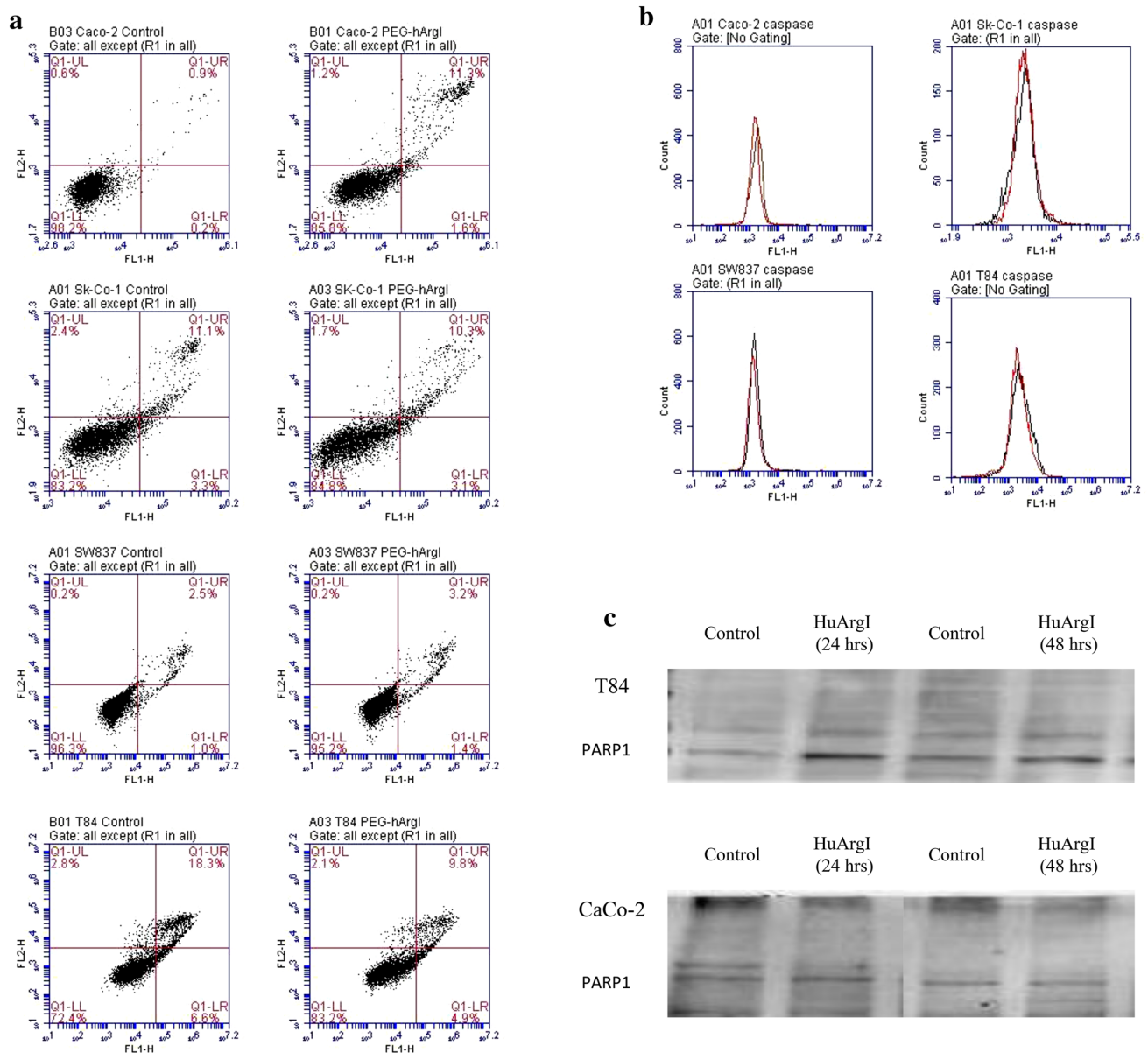


Fig. 4 Staining for Annexin V/PI (**a**) and active caspases (**b**) of CaCo-2, SK-Co-1, SW837 and T84 cells treated with HuArgI (Co)-PEG5000 for 24 h on flow cytometry. Cells are gated on width versus forward scatter. **c** PARP cleavage on western blot

of L-citrulline to rescue cells from arginine depletion has been extensively studied and demonstrated in a number of tumor types in which the rescue was directly dependent on the expression levels of the ASS1 enzyme [10, 11, 35, 36].

In addition to cytotoxicity, we show that [HuArgI (Co)-PEG5000]-induced arginine deprivation triggers a limited cell cycle arrest in the surviving fraction of some but not all colon cancer cell lines. This finding was different from our previous observations in other tumor types, namely in AML, GBM, and ovarian cancer, in which we reported a pronounced cell cycle arrest in response to arginine deprivation [10, 11, 21]. Such difference can be attributed to the

fact that all the tested colon cancer cell lines were partially auxotrophic for arginine as compared the other tumor types evaluated and which were mainly completely auxotrophic for arginine.

Investigation of the mechanism of cytotoxicity revealed that [HuArgI (Co)-PEG5000]-induced arginine deprivation leads to cell death in a non-apoptotic and caspase-independent manner, which is accompanied with evident loss of membrane integrity. This is very comparable to the findings we reported in AML, ovarian cancer and GBM where no signs of apoptotic cell death were detected in response to treatment with [HuArgI (Co)-PEG5000] [10,

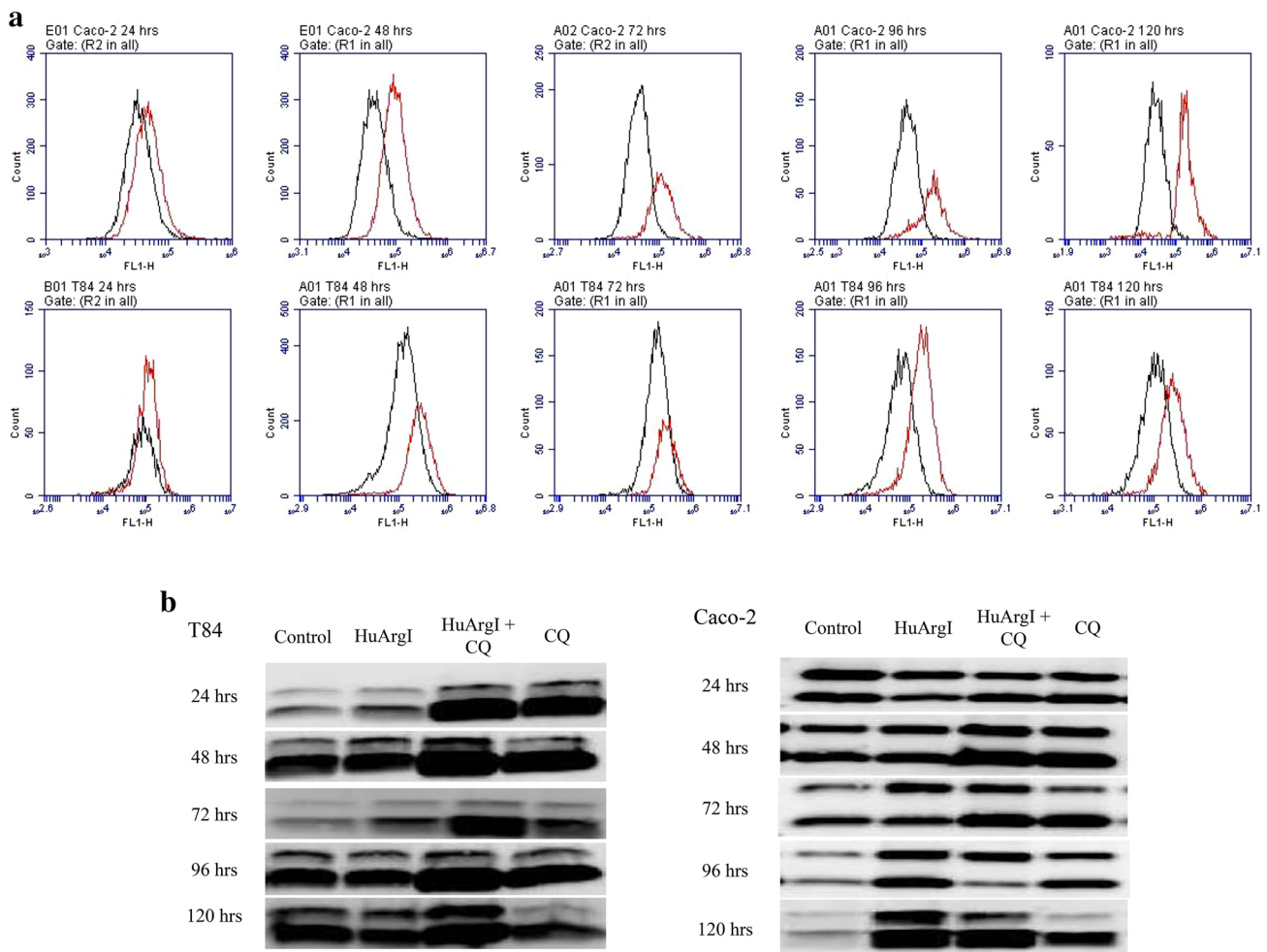


Fig. 5 CytoID (autophagosome) staining of Caco-2 and T84 cells at 24, 48, 72, 96 and 120 h following arginine deprivation on flow cytometry (a). Each panel is an overlay of control cells in black and

cells treated with [HuArgI (Co)-PEG5000] in red. Cells are gated on width versus forward scatter. **b** LC3 cleavage in T84 and Caco-2 cells at 24, 48, 72, 96 and 120 h following treatment on western blot

11, 21]. Other groups have shown apoptotic cell death following arginine deprivation, specifically in T-ALL and hepatocellular carcinoma, which might suggest that the mechanism of cell death observed might depend in part on the nature of the tumor type investigated [18, 20].

Autophagy activation in response to amino-acid deprivation is well described in literature. In addition, our previous work has provided evidence for a significant activation of autophagy in ovarian cancer cells following arginine deprivation [21]. Therefore, we sought to investigate the flux of autophagy in colon cancer cells following treatment with [HuArgI (Co)-PEG5000]. Our data demonstrate that arginine deprivation in colon cancer cells leads to a marked and sustained activation of autophagy that lasts up to 120 h following treatment with [HuArgI (Co)-PEG5000], highlighting thus the role of arginine restriction in colon cancer in the prolonged activation of autophagy.

In the absence of evidence for apoptotic cell death, and because we have shown that autophagy is significantly activated in these cells upon arginine deprivation, we sought to investigate the role played by autophagy in the cytotoxic response of colon cancer cells following treatment with [HuArgI (Co)-PEG5000]. Inhibition of autophagy resulted in a marked decrease in cell sensitivity to arginine deprivation and even led to complete resistance to [HuArgI (Co)-PEG5000]-induced arginine deprivation at the highest concentration of CQ, at both 48- and 72-h post-treatment. This demonstrates that cell death following arginine deprivation is mediated by the prolonged activation of autophagy. Recently, investigating the contribution of autophagy to the cytotoxicity of tumor cells following treatment with various anti-cancer therapeutic agents has gained importance [21, 37–40]. However, it is still unclear whether the increase in the flux of autophagy in cancer cells following treatment is protective or deleterious to the

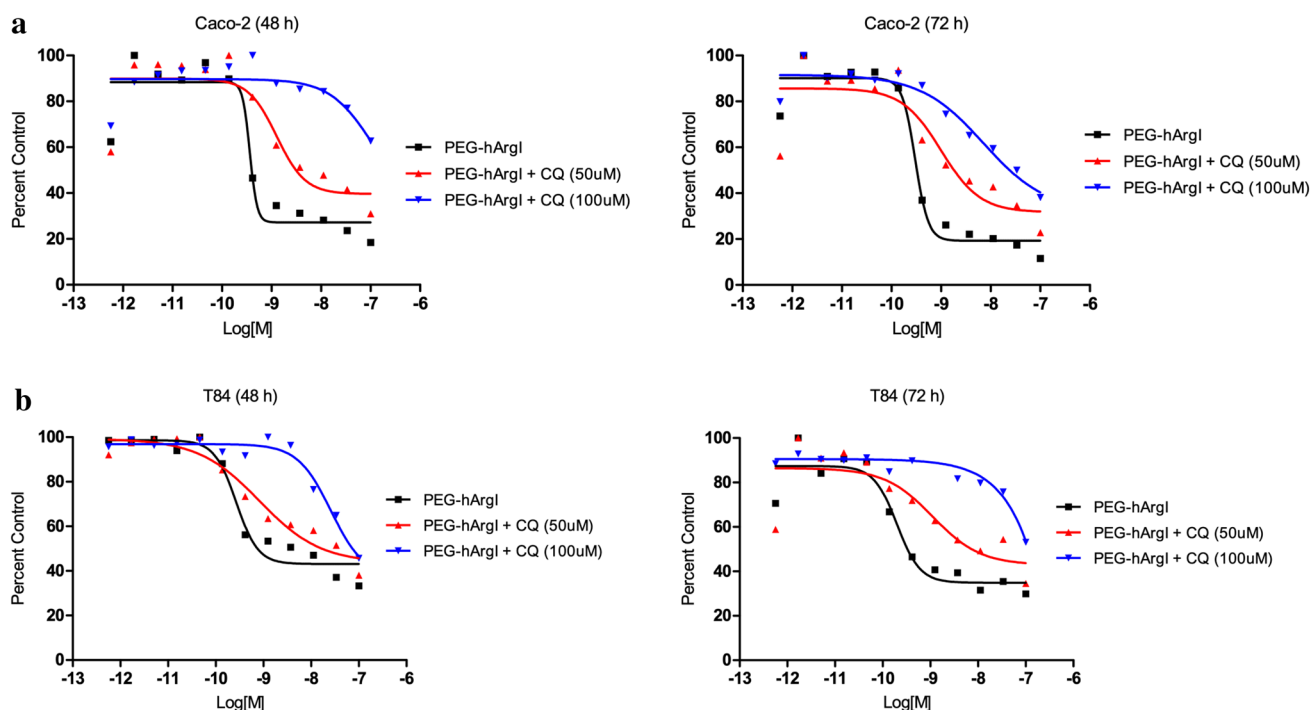


Fig. 6 Non-linear regression curves of Caco-2 (**a**) and T84 (**b**) cells treated with HuArgI (Co)-PEG5000 alone (square) and in combination with two different concentrations the autophagy inhibitor chloroquine; 50 μ M (triangle) and 100 μ M (inverted triangle) at 48 and 72 h post-treatment

cells, with both cases being documented in a number of tumors types under different treatment conditions, including arginine deprivation.

Several studies have so far shown that the activation of autophagy is protective to cells following arginine deprivation in different tumor types such as GBM, prostate cancer, sarcomas, lymphomas and laryngeal squamous cell carcinoma [37, 38, 41–43]. Though this may seem contradictory to the results obtained in this study, it is worth noting that all the previous studies had investigated the impact of the activation of autophagy at early time points while we investigate the effects of autophagy activation at later time points. On the other hand, we have previously shown that the cytotoxicity induced by arginine deprivation to ovarian cancer cells is mediated by the prolonged activation of autophagy [21]. Our findings are further supported by a study that described cytotoxic autophagy in prostate cancer cells following prolonged arginine deprivation [44]. The authors further described the autophagy-induced cytotoxicity as an atypical type of cell death characterized by mitochondrial dysfunction and chromatin autophagy, with a lack of caspase activation and other hallmarks of canonical apoptosis [44]. Therefore, it is likely that the activation of autophagy following arginine deprivation is protective in some tumor types, while it can leading to cell death in others. In addition, the impact of the activation of autophagy may be time-dependent, whereby it exerts a protective effect against cell death at early time points, but

leadings to cell death when activated in a sustained manner for prolonged times.

In this study, we have demonstrated that colon cancer cells are partially auxotrophic for arginine which makes them sensitive to [HuArgI (Co)-PEG5000]-induced arginine deprivation. All cell lines tested expressed argininosuccinate synthetase-1 (ASS1) and were sensitive to [HuArgI (Co)-PEG5000]-induced arginine deprivation. Treatment with exogenous *L*-citrulline rescued cells from the cytotoxicity of arginine deprivation suggesting partial arginine auxotrophy. Mechanistically, cells treated with [HuArgI (Co)-PEG5000] were negative for AnnexinV, and exhibited signs of loss of membrane integrity. The lack of caspase activation further supported the fact that arginine deprivation induces cell death in a caspase-independent, non-apoptotic manner.

We have also uncovered the mechanism underlying sensitivity to treatment with [HuArgI (Co)-PEG5000] and revealed that marked, long-term activation of autophagy induced in response to arginine deprivation, mediates cell death (death by autophagy) in the tested colon cancer cells. This was further evidenced by the decrease in cell cytotoxicity upon treatment with chloroquine, an autophagy inhibitor. Altogether, these data demonstrate that colon cancer cells are sensitive to [HuArgI (Co)-PEG5000]-induced arginine deprivation. They also show that the activation of autophagy does not play protective roles but rather, induces cytotoxicity and leads to cell death.

Acknowledgements The authors thank the Lebanese American University for funding and support

Author contributions MS, AB, IF, OEA and JBN performed experiments for this manuscript. ME-S collaborated on the autophagy part of the study. RJA-H is the corresponding author, he obtained funding, designed the study and wrote the manuscript.

Compliance with ethical standards

Conflict of interest All author declares that has no conflict of interest.

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