TITLE

Essential amino acid mixtures drive cancer cells to apoptosis through proteasome inhibition and autophagy activation

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Abbreviations: amino acids (AAs); essential amino acids (EAAs); non-essential amino acids (NEAAs); UPS, ubiquitin–proteasome system; ChT-L, chymotrypsin-like; T-L, trypsin-like; PGPH, peptidylglutamylpeptide hydrolyzing; AP-N, aminopeptidase N; BrAAP, branched-chain amino acids preferring; 3MA, 3-methyladenine; DMSO, dimethyl sulfoxide; AMC, amino-4-methylcoumarin; pAB, 4-aminobenzoic acid; AFC, 7-amino-4-trifluoromethylcoumarin; PCNA, proliferating cell nuclear antigen; PARP, poly (ADP-ribose) polymerase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; MDC, monodansylcadaverine.

ABSTRACT

Cancer cells require both energy and material to survive and duplicate in a competitive environment. Nutrients, such as amino acids (AAs), are not only a caloric source, as they can modulate cell metabolism and modify hormones homeostasis. Our hypothesis is that the environmental messages provided by AAs rule the dynamics of cancer cells life or death, and the alteration of the balance between essential (EAAs) and non-essential amino acids (NEAAs) (lower and higher than 50%, respectively) present in nutrients may represent a key instrument to alter environment-dependent messages thus mastering cancer cells destiny. In this study, two amino acid mixtures, one exclusively consisting of EAAs and the other consisting of 85% essential and 15% non-essential amino acids, were tested to explore their effects on the viability of both normal and cancer cell lines and to clarify the molecular

mechanisms involved. Both mixtures exerted a cell-dependent anti-proliferative, cytotoxic effect involving the inhibition of proteasome activity and the consequent activation of autophagy and apoptosis. These results, besides further validating the notion of the peculiar interdependence and extensive crosstalk between the ubiquitin-proteasome system and autophagy, indicate that variation in the ratio of EAAs and NEAAs can deeply influence cancer cell survival. Consequently, customization of dietary ratios among EAAs and NEAAs by specific amino acid mixtures may therefore represent a promising anticancer strategy able to selectively induce death of cancer cells through the induction of apoptosis via both ubiquitin-proteasome system inhibition and autophagy activation.

1. INTRODUCTION

The dissection of host-tumor relationship stimulated researchers to study the link between nutrition and altered protein/nitrogen metabolism in cancer [1, 2]. Malnutrition correlates with poor quality of life, increased mortality, morbidity, and chemotherapy-induced toxicity in cancer patients. Recent findings showed that calorie restriction counteracts the development of cancer, whereas positive results were obtained supplementing cancer cells with specific nutrients [3]. Selected nutrients, such as amino acids (AAs), besides being a caloric supply, are essential for protein synthesis, since they specifically influence gene transcription and translation, thus modulating the effects of hormones, energy availability and cell integrity [4]. AAs role in the maintenance of life is established, since they are the only source of nitrogen for synthetic and metabolic purposes. In fact, although glucose and lipids can be synthesized from AAs, the production of AAs from glucose and lipids is not possible due to the absence of nitrogen. Essential amino acids (EAAs) availability is the major limiting factor in maintaining synthesis of new proteins, since they allow endogenous formation of non-essential amino acids (NEAAs). Conversely, NEAAs are largely prevalent

and available as fuels and substrates in foods and mammal proteins. Few studies reported either on the toxicity of AAs in human health or on the safe limits for their supply and the effect of nutrients on normal and cancer tissues is not well defined [5, 6]. Among EAAs, only methionine is particularly toxic at high concentrations, and anemia [7] and a marked depression in rats growth were observed when this AA was introduced in their diet [8]. Methionine toxicity is mainly due to a primary metabolic role of the input of methionine, which should necessarily match cysteine/cystine requirements. Along this metabolic pathway, methionine is transformed in the intermediate homocysteine (Hcys) that is involved in the enhanced expression of HMG-CoA reductase in artery walls and correlates with impaired NO production and increased oxidative damage [5]. When sulfur-containing AAs needs are not matched by a well-balanced intake of cysteine/cystine [9], metabolic needs will choke S-adenosyl methionine and folate dependent methylations pathways [10, 11]. Differently, arginine and glutamine are "conditionally-essential" AAs since normal metabolism cannot maintain sufficient levels for cellular requirements in specific pathologies if EAAs would not be available in sufficient amounts [5]. Positive effects were reported upon diet supplementation with EAAs in age-associated alterations with enhanced protein synthesis, physical strength and arterial pO_2 observed in COPD-affected patients [12]. A branched-chain AAs-enriched mixture enhanced mitochondrial biogenesis and SIRT-1 expression in cardiac and skeletal muscles, increasing average life span of male mice. Additionally, upregulation of ROS defense system genes was detected, resulting in a decrease of oxidative damage [13]. EAAs oral administration to institutionalized elderly patients resulted in improved depressive symptoms, with enhanced levels of serotonin and physical performance and amelioration of health-related quality of life [14], in agreement with previous report on diabetic chronic heart failure patients [15]. Roomi *et al.* tested the effects of a nutrient mixture containing lysine, ascorbic acid, proline, green tea extract and other

micronutrients on HeLa xenograft-bearing female nude mice demonstrating the inhibition of tumor growth and enhancement of extracellular matrix proteins, suggesting its therapeutic value in the treatment of cervical cancer [16]. Tumor growth inhibition and decrease of the values of several cancer markers, including Ki67, matrix metalloproteinase-2 and -9, vascular endothelial growth factor, terminal deoxynucleotidyl transferase dUTP nick end labeling and B-cell lymphoma 2, cyclooxygenase 2, inducible nitric oxide synthase and glutathione S-transferase π were observed [17]. Furthermore, the same mixture showed therapeutic potential in the treatment of breast cancer [18]. Diet supplementation with EAAs reduced the toxic side effects of doxorubicin in *in vivo* experiments and promoted cancer cells mortality *in vitro* [19]. Since current cancer treatments based on chemotherapy and radiotherapy are toxic and associated with severe side effects, there is a need for safe, effective and natural remedies that can control cancer progression and expansion. Based on this premise, this study investigates the effects of two AAs mixtures, one entirely composed of EAAs and the other consisting of 85% essential and 15% non-essential AAs, on normal and cancer cells and dissects the molecular mechanisms involved. Both mixtures induced a cell-type dependent toxicity with proteasome inhibition and autophagy activation, ultimately driving to apoptosis. We conclude that tuning the ratio between EAA and NEAA, still being tolerable for normal mammals, may represent a promising approach in understanding cancer physiology, eventually providing additional options for the (co-)treatment of cancer through the modulation of proteolytic pathways and the activation of apoptosis.

2. RESULTS

2.1 Effects of AAs supplementation on cellular apoptosis

Cellular viability was evaluated upon treatment with both AAs formulations (1% w/v final concentration, as reported in tab. 1) in several cell lines: in detail, MCF10A (not transformed cells) and HeLa, HCT116, MCF7, HepG2, CaCo2 (cancer cell lines). Results are shown in figure 1. The concentration used allowed us to assess the occurrence of cell death and, simultaneously, the exploration of intracellular pathways. Tumor cells, in particular HCT116, showed an increased mortality compared to normal MCF10A cells, which were similarly affected by both solutions (Figure 1). Moreover, viability was assessed using the caspase inhibitor zVAD-fmk to block apoptosis to explore the link between apoptosis and the decreased viability. The addition of zVAD-fmk resulted in an increased cell viability, indicating that the two mixtures induced a caspases-mediated apoptosis. To confirm the activation of the cell death cascade additional apoptotic markers were investigated in HeLa and HCT116 cancer cells that showed the highest sensitivity to the treatment with AAs formulation. Caspase-3 is associated with the initiation of the death cascade [20]. A cell-type dependent activation of the enzyme was observed (Figure 2, panel C). Caspase-3 activity resulted significantly enhanced in tumor cell lines upon exposure to both solutions, whereas a significant increase in its activity was observed upon exposure of the control line to the 85% EAAs and 15% NEAAs solution. A remarkable increase in caspase-3 activity was observed in HCT116 cells treated with the mix, whereas in HeLa cells caspase-3 was similarly activated upon supplementation with both formulations. PARP is a nuclear protein involved in several cellular processes, including DNA repair, DNA replication, cell proliferation and differentiation. During apoptosis, PARP is cleaved by caspases and prevents DNA repair activating a calcium/magnesium-dependent endonuclease that results in internucleosomal DNA fragmentation [21]. A significant reduction of the full-length PARP was observed in the

three cell lines treated with the AAs mix, whereas, upon 100% EAAs exposure, a remarkable decrease was only observed in HCT116 cells (Figure 2, panel B). Apoptotic activation was finally confirmed by the decreased levels of the anti-apoptotic protein Mcl-1 (figure 2 panel A-B) and the analysis of DNA fragmentation and nuclear condensation [22]. Figure 2, Panel C, clearly shows DNA fragmentation in cancer cells and in particular in HeLa cells treated with the amino acids mix. The Hoechst 33342 staining confirmed that the treatment with both formulations induced morphological changes in the nuclei, peculiarly evident in HCT116 cells, and including the nuclei shrinkage and chromatin condensation (Figure 2, panel D). Effects on MCF10A, HCT116 and HeLa cells proliferation were further investigated through the detection of PCNA, an auxiliary factor for DNA polymerase σ, described as a DNA sliding clamp that acts as a polymerase processivity factor. Besides DNA replication, PCNA function is associated with chromatin remodeling, sister-chromatid cohesion, cell cycle control and DNA repair [23]. Upon treatment, a significant decrease in PCNA expression was observed in cancer cells and, in particular, in Hela cells. High levels of PCNA were detected in untreated cancer cells consistently with published data demonstrating a correlation between elevated PCNA levels and cell transformation, with cancer cells showing 5-6-fold higher levels of PCNA than immortalized non-cancer cells [24]. MCF10A cells showed no significant differences in PCNA levels after treatments (Figure 2, panel A).

2.2 Effects of AAs supplementation on proteasome functionality

The ChT-L, T-L, PGPH and BrAAP proteasomal activities were measured in cell lysates using fluorogenic substrates (figure 3). Amino acids supplementation did not affect proteasome activities in normal MCF10A cells. Conversely, a significant, cell-type and formulation-dependent inhibition was observed in cancer cells. In HeLa cells, the tested components showed the same inhibition pattern in response to the treatment with both

formulations. In HCT116 cells, the T-L and ChT-L activities were more susceptible to the 100% EAAs solution. UPS functionality was further explored by measuring the ChT-L activity of the 26S proteasome, the complex that needs ATP and ubiquitinated substrates for final degradation (figure 3, panel A). Cancer cells exhibited a remarkable decline of the enzymatic activity that in HCT116 cells was more evident upon 100% EAAs treatment. Interestingly, HCT116 and HeLa cells have higher basal levels of proteasomal activity compared to non-transformed MCF10A cells. This difference, mostly evident between MCF10A and HCT116 cells, concerns all the tested components and is in line with other reports demonstrating higher proteasomal activity in tumor cell lines [25, 26]. Treatments with both mixtures bring these higher levels down to the values evidenced in normal cells.

To gain insight into UPS modulation, the levels of a series of established ubiquitinated proteasomal substrates were determined. Total ubiquitinated proteins and p53 notably increased in HCT116 and HeLa cells treated with the EAAs/NEAAs mix. A lower, but still significant increase was observed upon supplementation of HCT116 with the 100% EAAs solution (Figure 4, Panel B). In accordance with the data on the proteasome activity, no changes were observed in the levels of ubiquitinated proteins in MCF10A cells. Proteasome inhibition resulted in the downregulation of the S-phase kinase-associated protein 2 (Skp-2), accumulation of p27, and cell cycle arrest [27]. In accordance with proteasome inhibition and with the reduced expression of PCNA, levels of p27 markedly increased in cancer cells treated with both the 100% EAAs solution and the mix (figure 4, panel B), further confirming that AAs supplementation negatively affect cell cycle progression.

2.3 Effects of AAs supplementation on autophagy

Next, we monitored the effects of both mixtures on the functionality of the autophagic pathway measuring the activity of cathepsin B and cathepsin L and the expression of the

autophagy-related proteins LC3-II, beclin-1 and p62. Globally, AAs supplementation favored a significant increase of cathepsins B and L activities in all the cell lines. In detail, the EAAs/NEAAs mix strongly activated both cathepsins in HeLa cells. In HCT116 cells, both enzymes were differently activated in response to the treatments: cathepsin L was considerably activated in the presence of 100% EAAs formulation, whereas the activity of cathepsin B was mainly increased by EAAs/NEAAs mix. A significant activation of both enzymes was measured in normal cells (Figure 5, Panel A). An intricate network of proteins orchestrates the autophagic pathway. Beclin-1 is involved in the enrolment of membranes to form the autophagosome whereas p62 binds to LC3II (the membrane-associated form of the LC3 protein) and is finally degraded in autophagolysosomes, thus its levels inversely correlate with the autophagic activity. Figure 5, panel C, shows a decrease in p62 levels in cancer cells, whereas a significant, cell-type dependent increase in beclin-1 levels was observed: in MCF10A cells, this increase was induced by the mix, but in cancer cells it was due to both formulations, mainly the one consisting of 100% EAAs. As for LC3, no changes were detected in normal cells and cancer cells showed a higher sensitivity to the 100% EAAs solution. Staining with MDC was used to additionally monitor the autophagic cascade [28]. These analyses revealed an increased uptake of MDC into vacuoles in cancer cells with the classical punctate pattern of MDC-labeled fluorescence, further corroborating the hypothesis that the AAs supplementation selectively induce autophagy (Figure 5, Panel B). In fact, by contrast, treated MCF10A cells exhibited a diffused distribution of MDC-labeled fluorescence. Interestingly, blocking autophagy using 3-MA magnified the cytotoxic effects of the AAs formulations in tumor cells (figure 1). This is likely due to the strict interdependence between UPS and autophagy, with autophagy activation occurring in response to proteasome inhibition (in cancer cells upon amino acids supplementation). The presence of the autophagy inhibitor compromises the compensatory activation of autophagy.

3. DISCUSSION

The relation between nutrition and cancer is an unresolved question with many possible answers since even its peculiar energy production from glucose is still debated [29]. Nutrients in the diet may influence cancer development [3, 6, 16, 18, 28]. Supplementation with balanced formulations of EAAs exerted positive effects in the prevention of several human pathologies, in the improvement of the quality of life, in reducing oxidative damage and in increasing average life span in mice [3, 6, 13, 14, 16, 17]. While AAs are indispensable for cells life, AAs-induced cytotoxicity is documented in cancer cells, but few data are available on the molecular mechanisms involved [6, 19, 30]. Here, we explored the effects of two AAs formulations, one composed of EAAs in ratios proved to be safe and efficient in mammals [4], and the other consisting of 85% EAAs and 15% NEAAs, on human HeLa and HCT116 cancer cells and elucidated the mechanisms responsible for their ability to trigger apoptosis. Results on cell viability showed a cell-type dependent action of both formulations, particularly evident in HCT116 cells. Additionally, the reduction of PCNA together with caspase-3 activation, decreased levels of Mcl-1 and full-length PARP, and DNA fragmentation confirm that supplementation inhibits proliferation and suggest the involvement in inducing apoptosis in cancer cells. In particular, as revealed by the MTT assay in the presence of the inhibitor zVAD-fmk, the two mixtures induced a caspases-mediated apoptosis. The anti-proliferative effects induced by some AAs mixtures on cancer cells were described in previous studies. Hagiwara *et al.* observed that essential branched chain amino acids (Leu/Ile/Val) prevented insulin-induced hepatic tumor cells proliferation by inducing apoptosis through suppression of the insulin/PI3K/Akt pathway and involving mTORC1 and mTORC2 [31]. mTORC1 drives cellular growth controlling numerous processes that regulate protein synthesis and degradation whereas mTORC2 functions downstream in the PI3K pathway to regulate cell growth, proliferation, and survival [32]. The

authors demonstrated that BCAAs suppress the PI3K/Akt pathway by inducing a negative feedback loop through mTORC1/S6K1 activation and suppressing mTORC2 kinase activity toward the kinase Akt [31]. Akt inhibition leads to the activation of FOXO3a pro-apoptotic transcription factor and, consequently, the expression of genes necessary for cell death [33, 34]. On the other hand, mTORC2 activation due to the lack of some AAs necessary for completing protein synthesis induces and controls autophagic machinery switching on [35]. Considering the close relationship between the mechanisms controlling cell cycle progression and cell death programs and the role of the proteasome as a switchboard to proceed with proliferation or to undergo apoptosis [36], we hypothesized that proteolysis modulation could be one of the pathway through which AAs exert their pro-apoptotic effect. Data here reported show that 20S and 26S proteasomes were inhibited in cancer cells and that no variations in the activities were detectable in MCF10A cells, further evidencing the selective action of the two formulations. Proteasome inhibition induces in HCT116 and HeLa cells accumulation of p27 and p53, two proteins widely associated with apoptosis [36-38]. For example, treatment with antisense p27 oligonucleotide inhibited apoptosis induced by proteasome inhibitors in cancer cells suggesting that p27 accumulation is critical in triggering apoptosis [39]. Proteasome and autophagy, another intracellular pathway for protein degradation, intensively cooperate and regulate each other's activities [40-42]. Upon treatment with both AAs formulations, cancer cells show activation of cathepsin B and L, decreased levels of p62 and Beclin-1 accumulation as well as the increase of the membrane-associated LC3-II component. Our results therefore strengthen the concept of the strict interdependence between UPS and autophagy, with autophagy activation occurring in response to proteasome inhibition in HCT116 and HeLa cells upon AAs supplementation. Interestingly, autophagy inhibition using 3-MA, compromising the compensatory activation of this pathway, magnified the cytotoxic effects of the AAs formulations in tumor cells. Autophagy is also

involved in driving cancer cells to apoptosis representing a pro-survival as well as a pro-apoptotic mechanism, depending on the cancer type, stage and microenvironment. It exerts cytoprotective effects during nutrient and growth factors deprivation due to its ability to recycle nutrients, to maintain cellular energy homeostasis degrading toxic aggregated proteins or damaged organelles. However, in other cellular settings, a continuous or excessively induced autophagy may ultimately favor cell death [43, 44]. Autophagy and apoptosis share some common pathways and sensing components, including Beclin-1 and caspases, and their activities are mutually controlled [45]. Additionally, as previously reported, autophagy activation may associate with an increased tendency of cancer cells to undergo apoptosis [46-48]. Thus, we observed that feeding with essential AAs rich mixtures selectively elicits proteasome inhibition and autophagy activation in cancer cells. Modulation of cellular autophagy inhibits cell proliferation and activates apoptotic pathway. Both formulations similarly affected the intracellular pathways investigated, with HCT116 cells more vulnerable than HeLa cells, without interfering with the same processes in normal cells. It is reasonable to think that the difference in the content of EAAs between the two mixtures (85% versus 100%) is insufficient to modify EAA effects on cell viability by autophagy activation. Indeed, serine, as main NEAA to reach a 15% ratio with 85% of EAA, was chosen with three main purposes: it is the most metabolically AA rapidly available both for energy production and transamination generating one pyruvate molecule, it has a role in maintaining folates charged of methyl groups [49], and, combined to EAA on the bases of our data, serine may have an anti-cancer effect as an allosteric activator of pyruvate kinase by tetramer formation (isoform PKM2) [50]. This may improve the drive of glucose to full oxidation into mitochondria allowing metabolic production of sufficient ROS necessary to activate the autophagic pathway [51], that we detected.

Collectively, our data show that supplementation with mixtures rich in EAAs triggers the activation of death mechanisms in cancer cells. In particular, as suggested by Corsetti G. *et al.*, being cancer cells mostly dependent on the environmental prevalent availability of NEAA for metabolic processes and cell proliferation, enriching their environment with EAAs may trigger specific pathways that are ultimately incompatible with proliferation and survival [19]. These data therefore represent an important pillar supporting the interest of studying physiological effects of nutrition in cancers cells thus characterizing peculiar fragilities of cancer. A promising source of new options for alternative treatments of this complex disease.

4. MATERIALS AND METHODS

4.1 Cell lines

MCF10A (not transformed cells) and HeLa, HCT116, MCF7, HepG2, CaCo2 (cancer cells) were grown in D-MEM supplemented with 10% FBS, antibiotic and antimycotic. To avoid results misinterpretation, no other nutrients (EAAs or NEAAs) were added to media. MCF10A cells (an *in vitro* model for studying normal epithelial cell function) were cultured in a DMEM/F12 Ham's mixture supplemented with 5% equine serum, 20 ng/mL EGF, 10 μg/mL insulin, 0.5 mg/mL hydrocortisone, antibiotics and antimycotics. Cells were incubated with growth media at 37°C equilibrated with 95% air and 5% CO_2 in flasks, 96- or 6-well plates depending on the assay.

4.2 Amino acids treatment

Cells were independently treated with a solution containing 100% EAAs or a mixture of 85% EAAs and 15% NEAAs (herein named as mix). Compositions are reported in tab 1. Suitable solubility was reached at 1% (w/v) concentration dissolving AAs in the growth medium,

followed by vortexing and sonication at 50°C for 30 minutes. Each cell line was treated with 1% (w/v) of the 100% EAAs solution and of the mixture. Control cells were treated with the growth medium. Treatments ranged from 48 to 96 h.

4.3 MTT Assay

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, an index of mitochondrial activity and cell viability. It is based on viable cell ability to convert a soluble yellow tetrazolium salt to a purple-blue formazan crystal by mitochondrial succinate dehydrogenase. After incubation with the AAs supplementations, media were replaced with serum-free media containing 0.5 mg/mL MTT. Plates were incubated at 37°C for 2h. After supernatant discard, the formazan product was solubilized in 100µL of DMSO and absorbance was measured at 550 nm [52]. MTT was also carried out in the presence and in the absence of the caspase inhibitor zVAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone, 10µM concentration in the assay, Sigma Aldrich) and of the autophagy inhibitor 3-methyladenine (5mM concentration in the assay, 3-MA, Sigma Aldrich).

4.4 Hoechst nuclear staining

Once treated, cells ($1x10^6$ cells/mL) were fixed in methanol/acetic acid (3:1), incubated with 0.05 mg/mL Hoechst 33258 nuclear stain for 15 min and observed using an inverted fluorescence microscope (Olimpus X71).

4.5 DNA fragmentation

Cell lines were seeded in six-well plates and treated with the 100% EAAs solution, the amino acid mixture and the medium as control for 72 h. The Buonanno *et al.* procedure was followed [53]. Pellets were suspended in lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 0.5% SDS, and 0.5 mg/mL proteinase K) and incubated at 50°C for 1 h. Once added 10 mg/ml of RNase, lysates were incubated for 10 min at 70°C. DNA was precipitated with NaOAc pH 5.2 and ice-cold 100% EtOH, followed by incubation on ice for 10 min, and centrifugation at 10.000×g for 10 min. Pellets were collected and dissolved in sterile water. Finally, samples were resolved on a 1.8% agarose gel stained with ethidium bromide.

4.6 Cell lysis

Upon treatment, cells were harvested in PBS, centrifuged and the pellet was re-suspended in lysis buffer (20 mM Tris, pH 7.4, 250 mM sucrose, 1 mM EDTA and 5 mM β-mercaptoethanol). Lysates were centrifuged at 12000×g for 15 min and the supernatants stocked at -80°C. Protein concentration was estimated following the Bradford method using bovine serum albumin as standard [54].

4.7 Enzymatic activities

The effects on the 20S proteasome were evaluated through fluorimetric assays using the following synthetic substrates: Leu-Leu-Al-Tyr-AMC for ChT-L, Leu-Ser-Thr-Arg-AMC for T-L, Leu-Leu-Glu-AMC for PGPH and Gly-Pro-Ala-Phe-Gly-pAB for BrAAP, whose test is performed with the addition of the aminopeptidase-N (APN) (EC 3.4.11.2) [55]. The incubation mixture contained 4 μg of cell lysates, the appropriate substrate and 50 mM Tris–HCl pH 8.0, up to a final volume of 100 μL. Incubation was performed at 37°C, and after 60 min the fluorescence of the hydrolyzed 7-amino-4-methylcoumarin (AMC) and 4-

aminobenzoic acid (pAB) was detected (AMC, λ_{exc}=365 nm, λ_{em}=449 nm; pAB, λ_{exc}=304 nm, λ_{em}=664 nm) on a SpectraMax Gemini XPS microplate reader. The 26S proteasome ChT-L activity was tested using Suc-Leu-Leu-Val-Tyr-AMC as substrate and 50 mM Tris–HCl pH 8.0 buffer containing 10 mM $MgCl_2$, 1 mM dithiothreitol, and 2 mM ATP. The effective 20S proteasome contribution to short peptide cleavage was evaluated performing control experiments using (5 μM in the reaction mixture) Z-Gly-Pro-Phe-Leu-CHO and lactacystin, specific proteasome inhibitors, and then subtracting the obtained fluorescence values from the values obtained in cell lysates.

Caspase-3 activity was measured with the fluorogenic Ac-Asp-Glu-Val-Asp-AMC synthetic substrate in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM $CaCl_2$, 1mM EDTA, 0.1% CHAPS, 5 mM β-mercaptoethanol, pH 7.5. Upon 60 min incubation at 37°C, the fluorescence of hydrolyzed AMC was measured on a SpectraMax Gemini XPS microplate reader (λ_{exc} = 365 nm, λ_{em}= 449 nm).

Cathepsin B and L proteolytic activities were measured as described by Tchoupè *et al.* [56] using the fluorogenic peptides Z-Arg-Arg-AMC and Z-Phe-Arg-AFC, respectively, at a final concentration of 5 μM. The mixture for cathepsin B, containing 7 μg of protein lysate, was pre-incubated in 100 mM PBS pH 6.0, 1 mM EDTA and 2 mM dithiothreitol for 5 min at 30°C. Upon the addition of the substrate, the mixture was incubated for 15 min at 30°C. The mixture for cathepsin L, containing 7 μg of protein lysate, was incubated in 100 mM CH_3COONa buffer pH 5.5, 1 mM EDTA and 2 mM dithiothreitol for 5 min at 30°C and, upon the addition of the substrate, the mixture was incubated for 15 min at 30°C. The fluorescence of the hydrolyzed AMC (λ_{exc}=365 nm, λ_{em}=449 nm) and 7-amino-4-trifluoromethylcoumarin (AFC, λ_{exc}=397 nm, λ_{em}=500 nm) was detected on a SpectraMax Gemini XPS microplate reader. The effective cathepsin contribution to the proteolysis was

evaluated through control experiments performed using the specific inhibitor CA074Me and subtracting these values from the fluorescence values obtained by analyzing cell lysates.

4.8 Western blotting

Cell lysates (20 µg of total proteins) were resolved by 10% or 12% SDS PAGE and electroblotted onto PVDF membranes. Membranes with transferred proteins were incubated with primary and secondary antibodies to detect p27, ubiquitin, proliferating cell nuclear antigen (PCNA), p53, Mcl-1 and poly (ADP-ribose) polymerase (PARP) (Santa Cruz Biotech, Heidelberg, Germany). The immunoblot detection was performed with an ECL western blotting analysis system. Each gel was loaded with prestained molecular mass markers in the range of 20–120 kDa (Euroclone, Milan, Italy). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as control for equal protein loading: membranes were stripped and reprobed for GAPDH. Film were analyzed using ImageJ software. Data were analyzed with Matlab (The MathWorks Inc., MA, USA).

4.9 Monodansylcadaverine (MDC)

Cells were independently treated with both AAs formulation for 72 h. The media were replaced with 50 µM MDC (Sigma) in serum-free medium. After 10 min incubation at 37°C, cells were washed three times with PBS and analyzed under a fluorescence microscope (Olympus IX71) equipped with a 380-420 nm filter.

4.10 Statistical analysis

Results are expressed as mean values and standard deviation of results obtained from five separate experiments. Statistical analysis was performed with one-way ANOVA, followed by the Bonferroni test using SigmaStat 3.1 software (SPSS, Chicago, IL, USA). p-Values <0.05 and <0.01 were considered significant.

Author contributions

E.A.M and D.S.F. planned the experiments; D.S.F. designed and provided the amino acids mixtures; B.L. performed experiments; B.L. and C.V analysed data; B.L and C.V. wrote the paper – Original Draft; E.A.M., D.S.F., M.A., C.V. and C.M. wrote the paper – Review & Editing; M.A., V.F., G.C., E.P. technical assistance; E.A.M. acquired funds.

All authors read and approved the final manuscript.

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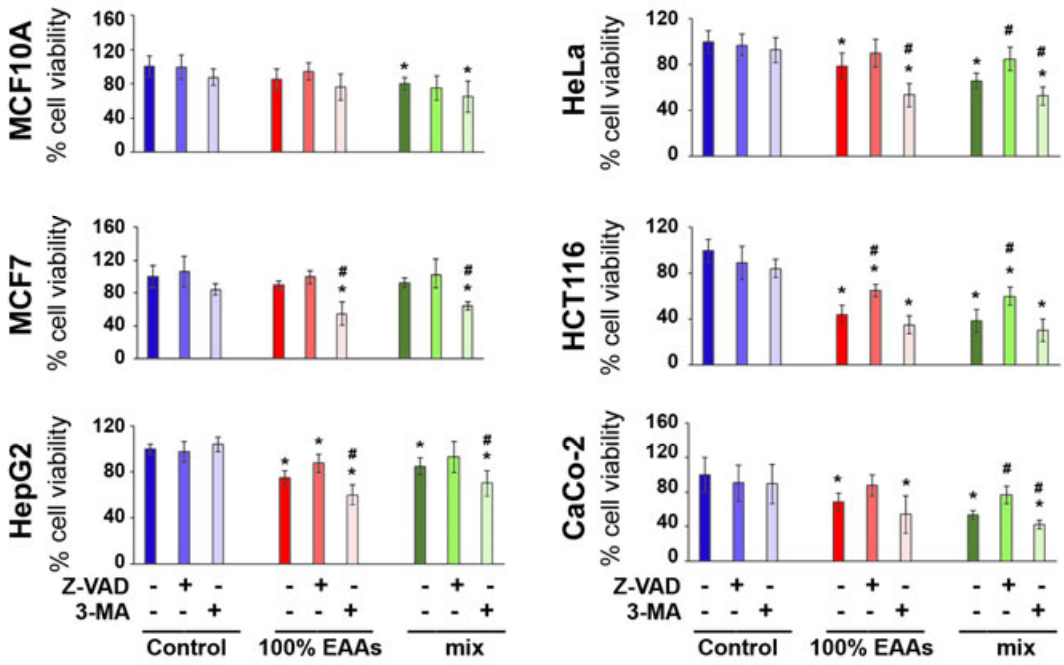
Table I: AAs ratios in the formulations tested expressed as percentage.

AAs	85% EAAs and 15% NEAAs (w/w %)	100% EAAs (w/w %)
Leucine	13.53	31.25
Isoleucine	9.65	15.625
Valine	9.65	15.625
Histidine	11.60	3.75
Lysine	11.60	16.25
Threonine	8.70	8.75
Methionine*	4.35	1.25
Phenylalanine	7.73	2.5
Triptophan	3.38	0.5
Tyrosine**	5.80	0.75
Cystine*	8.20	3.75
Serine	2.42	0
N-Acetyl Cysteine	0.97	0
Ornithine-α Ketoglutarate	2.42	0

*Both formulations contain cystine and methionine to match sulfur AAs needs without toxicity.

**Tyrosine is present in both formulations, since, when calculating phenylalanine needs, it was considered that tyrosine is a NAEE only for the liver and partially for kidneys, which can derive it by hydroxylating phenylalanine, whereas it is fully essential in any other cell of the body.



Legends

Figure 1: Effect of the two AAs mixtures on normal and cancer cells viability. The MTT assay was carried out to measure cell viability in MCF10A cells (not transformed cells) and HeLa, HCT116, MCF7, HepG2, CaCo2 cells (tumoral cell lines). Cells were treated with the two AA mixtures and with the caspase inhibitor z-VAD and the autophagy inhibitor 3-MA. Asterisks (*) represent significant data points with respect to untreated control cells (100%), hashtags (#) represent significant data points with respect to treated cells in the absence of inhibitors. Results are expressed as mean values and standard deviation obtained from five separate experiments. Statistical analysis was performed with one-way ANOVA, followed by the Bonferroni test using SigmaStat 3.1 software (SPSS, Chicago, IL, USA). p-Values <0.05 and <0.01 were considered significant.

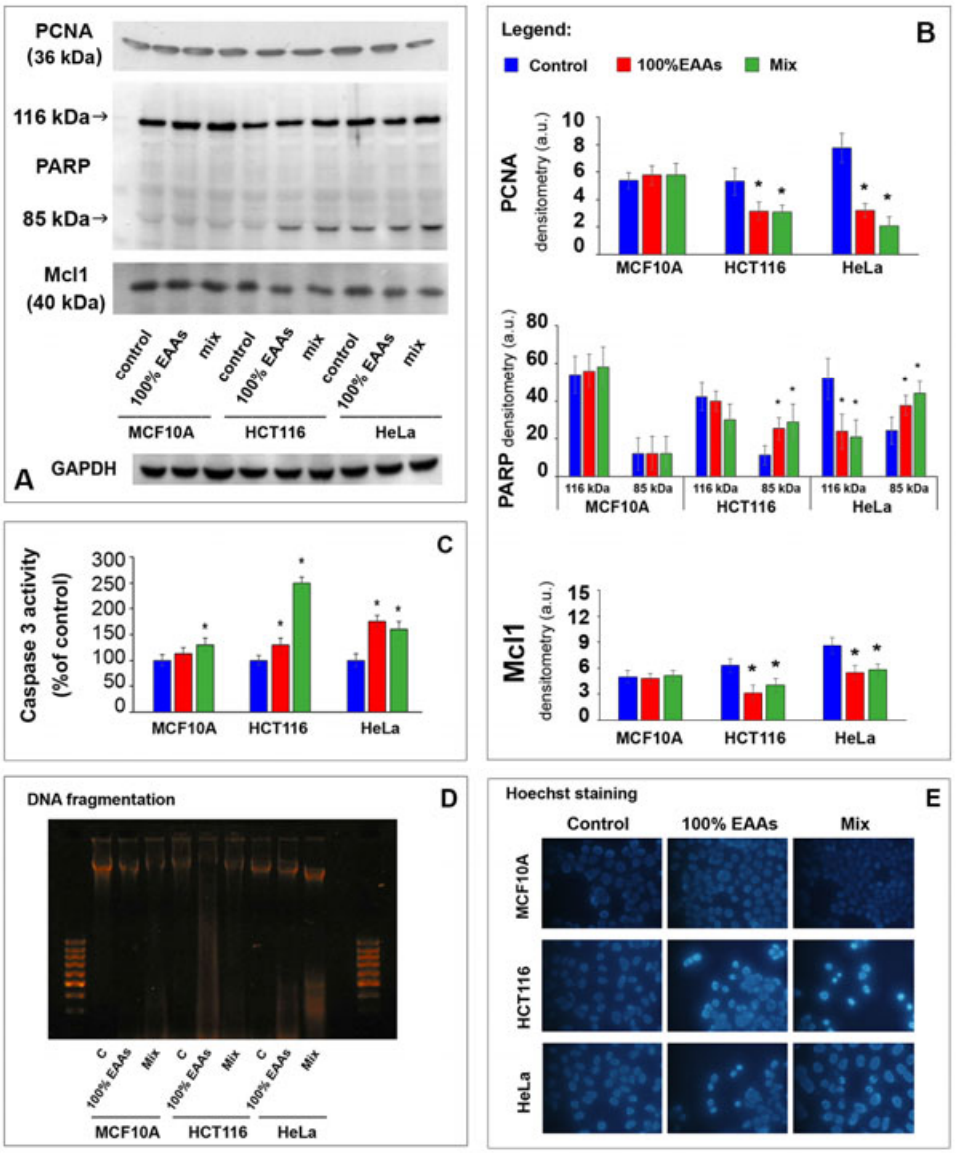


Figure 2: Effect of the two AAs mixtures on normal and cancer cells proliferation and apoptosis. Panels A and B: representative autoradiographic films obtained from the Western blotting for PCNA, PARP and Mcl-1 detection and relative densitometry. GAPDH was used as control for equal protein loading. Data points marked with an asterisk are statistically significant compared to the respective untreated control cells (*$p<0.05$). Panel C: caspase-3 activity measured using the fluorogenic substrate Ac-Asp-Glu-Val-Asp-AMC (see section 2 for details). Data are expressed as percentage of the respective untreated control cells (100%).

Panel D: gel electrophoresis of DNA samples isolated from normal and cancer cells. Each experiment was conducted in triplicate. DNA fragments were separated using 1.8% agarose gel electrophoresis and visualized under UV light following staining with ethidium bromide. Results are expressed as mean values and standard deviation of results obtained from five separate experiments. Statistical analysis was performed with one-way ANOVA, followed by the Bonferroni test using SigmaStat 3.1 software (SPSS, Chicago, IL, USA). *$p<0.05$ indicates a statistically significant difference compared with the respective untreated control cells. Panel E: upon treatment, cells were stained with Hoechst 33342 to visualize nuclei morphology. A representative microscopic field for each treatment is shown.

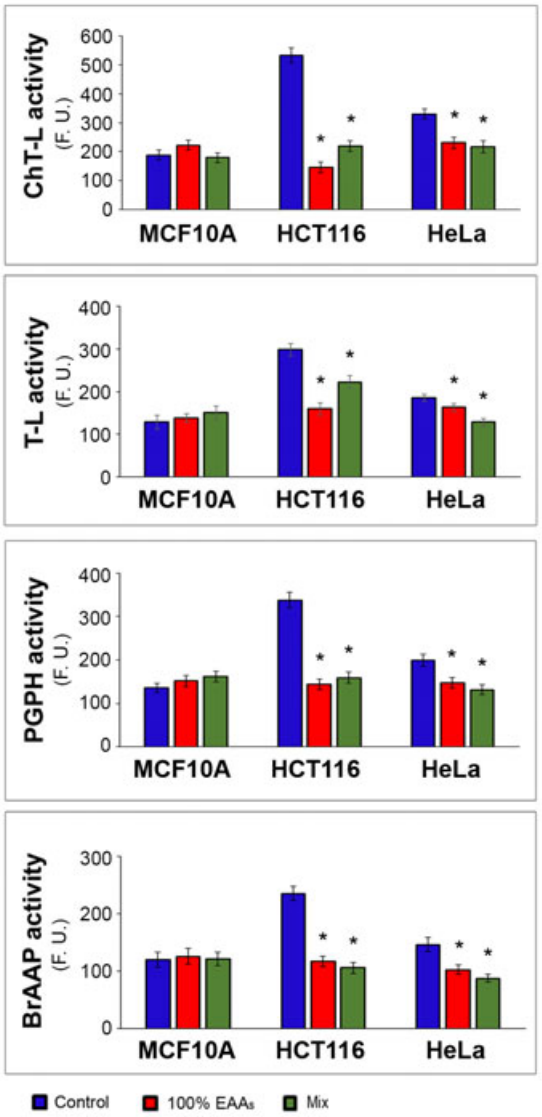


Figure 3: Effect of the two AAs mixtures on 20S proteasome. 20S proteasome activities in normal and cancer cells following treatment with 100% EAAs and the mix (1% final concentration) for 72 h (see section 2 for details). Values are expressed as fluorescence units (F.U.) and the asterisk (*, $p<0.05$) indicates significantly different values compared to respective untreated control cells. Results are expressed as mean values and standard deviation of results obtained from five separate experiments. Statistical analysis was performed with one-way ANOVA, followed by the Bonferroni test using SigmaStat 3.1 software (SPSS, Chicago, IL, USA).

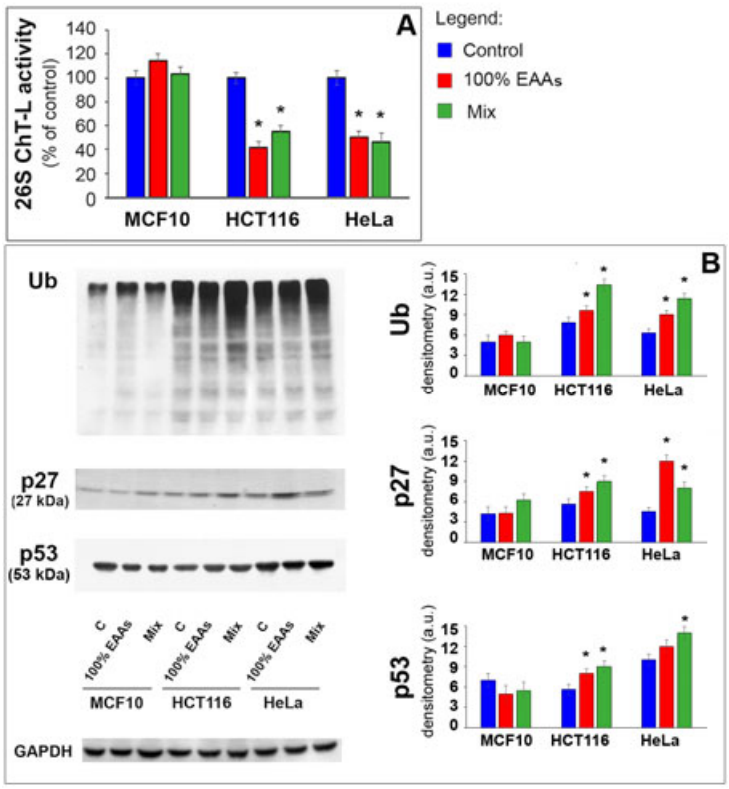


Figure 4: Effect of the two AAs mixtures on UPS. Panel A: 26S ChT-L activity measured in normal and cancer cells following treatment with 100% EAAs and the mix (1% final concentration) for 72 h (see section 2 for further details). Values are expressed as fluorescence units (F.U.) and the asterisk (*, $p<0.05$) indicates significantly different values compared to respective untreated control cells. Panel B: representative autoradiographic films obtained from Western blotting of ubiquitin, p27 and p53 and relative densitometry. GAPDH was used as equal loading control. Results are expressed as mean values and standard deviation of results obtained from five separate experiments. Statistical analysis was performed with one-way ANOVA, followed by the Bonferroni test using SigmaStat 3.1 software (SPSS, Chicago, IL, USA). *$p<0.05$ indicates significantly different values compared to respective untreated control cells.

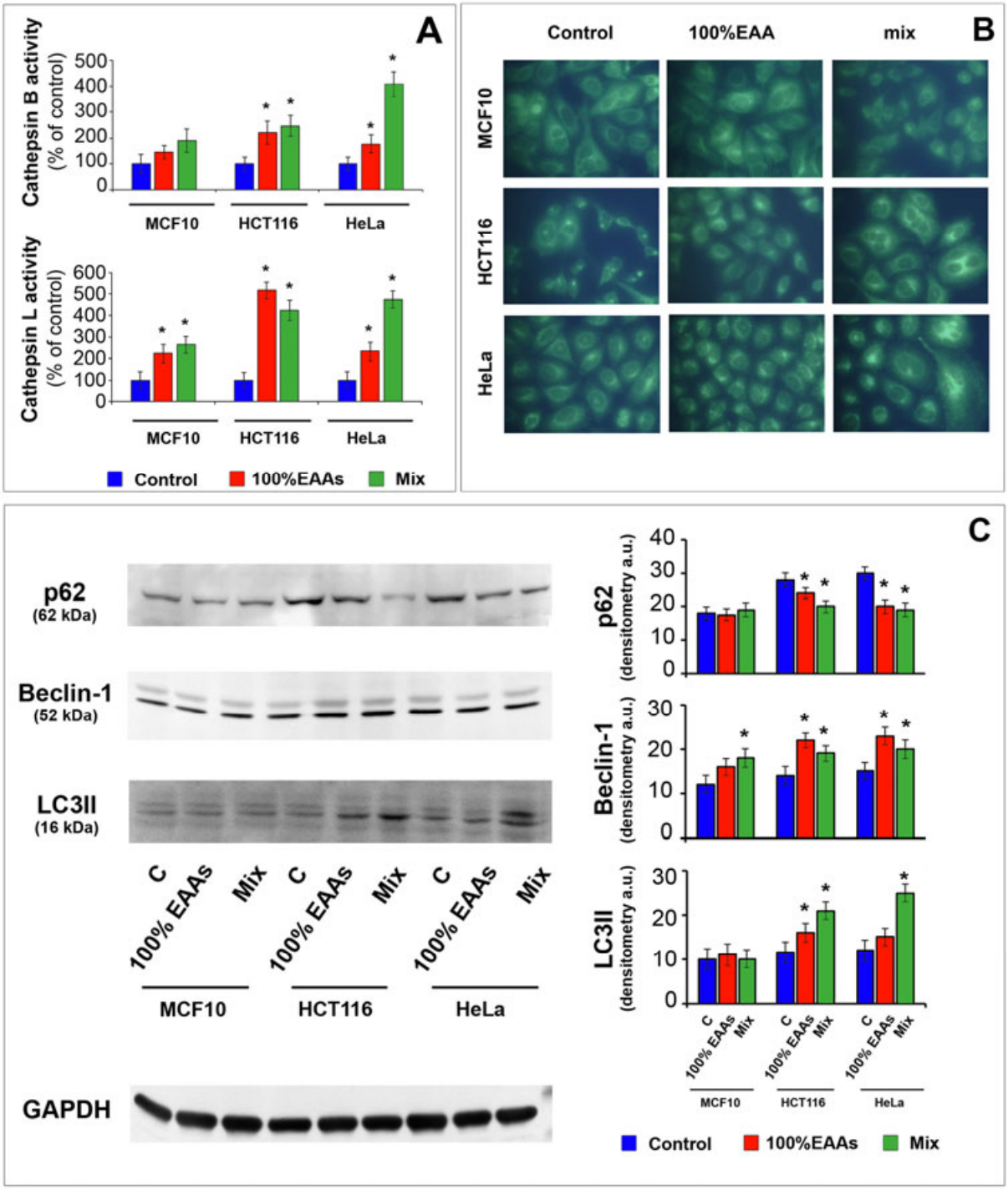


Figure 5: Effect of the two AAs mixtures on autophagy. Panel A: cathepsin L and B activity after AAs treatment were measured using fluorogenic substrates (see section 2 for details). Specific activity is expressed as the amount of released product per minute per μg of total protein in the sample. *$p<0.05$ indicates significantly different results compared to the non-treated group. Panel B: upon treatments, normal and cancer cells were incubated with MDC and analyzed by fluorescence microscopy to visualize autophagic vacuoles. Panel C: expression of autophagic markers in cell lysates. Representative autoradiographic films obtained from Western blotting for the detection of LC3-II, beclin-1, p62 and GAPDH (loading control) and densitometry. Results are expressed as mean values and standard deviation of results obtained from five separate experiments. Statistical analysis was performed with one-way ANOVA, followed by the Bonferroni test using SigmaStat 3.1 software (SPSS, Chicago, IL, USA). *$p<0.05$ indicates significantly different results compared to the non-treated group.