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

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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, but can also modulate cell metabolism and modify hormone homeostasis. Our hypothesis is that the environmental messages provided by AAs rule the dynamics of cancer cell life or death, and the alteration of the balance between essential amino acids (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 AA mixtures, one exclusively consisting of EAAs and the other consisting of 85% EAAs and 15% NEAAs, 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 (UPS) 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 AA mixtures may represent a promising anticancer strategy able to selectively induce death of cancer cells through the induction of apoptosis *via* both UPS inhibition and autophagy activation.

Abbreviations
3-MA, 3-methyladenine; AA, amino acid; AFC, 7-amino-4-trifluoromethylcoumarin; AMC, amino-4-methylcoumarin; BrAAP, branched chain amino acids-preferring; ChT-L, chymotrypsin-like; EAA, essential amino acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDC, monodansylcadaverine; mTORC, mammalian target of rapamycin complex; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEAA, non-essential amino acid; pAB, 4-aminobenzoic acid; PARP, poly(ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; PGPH, peptidylglutamylpeptide hydrolyzing; PI3K, phosphoinositide 3-kinase; T-L, trypsin-like; UPS, ubiquitin–proteasome system; zVAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

Introduction

The dissection of the 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 and 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]. The role of AAs 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. Availability of essential amino acids (EAAs) 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 mammalian proteins. Few studies have 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 into the intermediate homocysteine that is involved in enhanced expression of 3-hydroxy-3-methylglutaryl-CoA reductase in artery walls and correlates with impaired NO production and increased oxidative damage [5]. When sulfur-containing-AA needs are not matched by a well-balanced intake of cysteine/cystine [9], metabolic needs will choke *S*-adenosyl methionine- and folate-dependent methylation 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 are not 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 chronic obstructive pulmonary disease-affected patients [12]. A branched-chain AA-enriched mixture enhanced mitochondrial biogenesis and sirtuin 1 expression in cardiac and skeletal muscles, increasing average life span of male mice. Additionally, upregulation of reactive oxygen species defense system genes was detected, resulting in a decrease of oxidative damage [13]. Oral administration of EAAs 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 a previous report on diabetic chronic heart failure patients [15]. Roomi *et al.* [16] 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 the enhancement of extracellular matrix proteins, suggesting its therapeutic value in the treatment of cervical cancer. 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 cell 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 AA 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, within the range 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.

Results

Effects of AA supplementation on cellular apoptosis

Cellular viability was evaluated upon treatment with both AA formulations (1% w/v final concentration, as reported in Table 1) in several cell lines, namely MCF10A (not transformed cells) and HeLa, HCT116, MCF7, HepG2 and CaCo2 (cancer cell lines). Results are shown in Fig. 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 with normal MCF10A cells, which were similarly affected by both solutions (Fig. 1). Moreover, viability was assessed using the caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone (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 caspase-mediated apoptosis.

To confirm the activation of the cell death cascade, additional apoptotic markers were investigated in HeLa and HCT116 cancer cells, which showed the highest sensitivity to the treatment with the AA formulation. Caspase-3 is associated with the initiation of the death cascade [20]. A cell-type-dependent activation of the enzyme was observed (Fig. 2C). Caspase-3 activity was 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% EAA and 15% NEAA solution. A notable 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.

Poly(ADP-ribose) polymerase (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 AA mix, whereas upon 100% EAA exposure, a notable decrease was only observed in HCT116 cells (Fig. 2B). Finally, apoptotic activation was confirmed by the decreased levels of the anti-apoptotic protein Mcl-1 (Fig. 2A,B) and the analysis of DNA fragmentation and nuclear condensation [22]. Figure 2C, clearly shows DNA fragmentation in cancer cells and in particular in HeLa cells treated with the AAs mix. Hoechst 33342 staining confirmed that the treatment with both formulations induced morphological changes in the nuclei, particularly evident in HCT116 cells, and including shrinkage of the nuclei and chromatin condensation (Fig. 2D).

Effects on MCF10A, HCT116 and HeLa cell proliferation were further investigated through the detection of proliferating cell nuclear antigen (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's 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, consistent with published data demonstrating a correlation between elevated PCNA levels and cell transformation, with cancer cells showing 5- to 6-fold higher levels of PCNA than immortalized non-cancer cells [24]. MCF10A cells showed no significant differences in PCNA levels after treatment (Fig. 2A).

Table 1. AA ratios in the formulations tested, expressed as a percentage.

AA	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[a]	4.35	1.25
Phenylalanine	7.73	2.5
Tryptophan	3.38	0.5
Tyrosine[b]	5.80	0.75
Cystine[a]	8.20	3.75
Serine	2.42	0
N-acetylcysteine	0.97	0
Ornithine α ketoglutarate	2.42	0

[a] Both formulations contain cystine and methionine to match sulfur AA needs without toxicity. [b] Tyrosine is present in both formulations, since when calculating phenylalanine needs, it was considered that tyrosine is an NEAA 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.

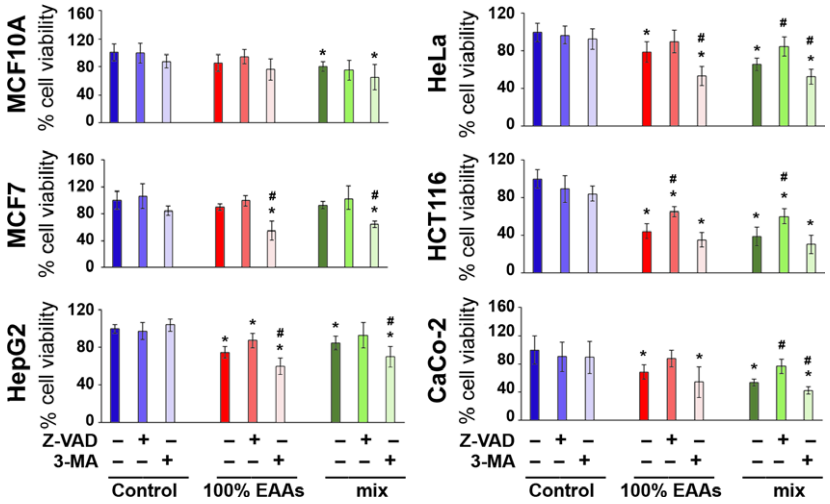

Fig. 1. Effect of the two AA mixtures on normal and cancer cell viability. The MTT assay was carried out to measure cell viability in MCF10A cells (not transformed cells) and HeLa, HCT116, MCF7, HepG2 and CaCo2 cells (tumoral cell lines). Cells were treated with the two AA mixtures and with the caspase 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. $P < 0.05$ and < 0.01 were considered significant.

Effects of AA supplementation on proteasome functionality

The chymotrypsin-like (ChT-L), trypsin-like (T-L), peptidylglutamylpeptide hydrolyzing (PGPH) and branched chain amino acid-preferring (BrAAP) proteasomal activities were measured in cell lysates using fluorogenic substrates (Fig. 3). Amino acid 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% EAA solution. Ubiquitin–proteasome system (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 (Fig. 3). Cancer cells exhibited a notable decline of the enzymatic activity that in HCT116 cells was more evident upon 100% EAA treatment. Interestingly, HCT116 and HeLa cells have higher basal levels of proteasomal activity compared with 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]. Treatment with both mixtures brought 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 EAA–NEAA mix. A lower, but still significant increase was observed upon supplementation of HCT116 with the 100% EAA solution (Fig. 4B). 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% EAA solution and the mix (Fig. 4B), further confirming that AA supplementation negatively affects cell cycle progression.

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,

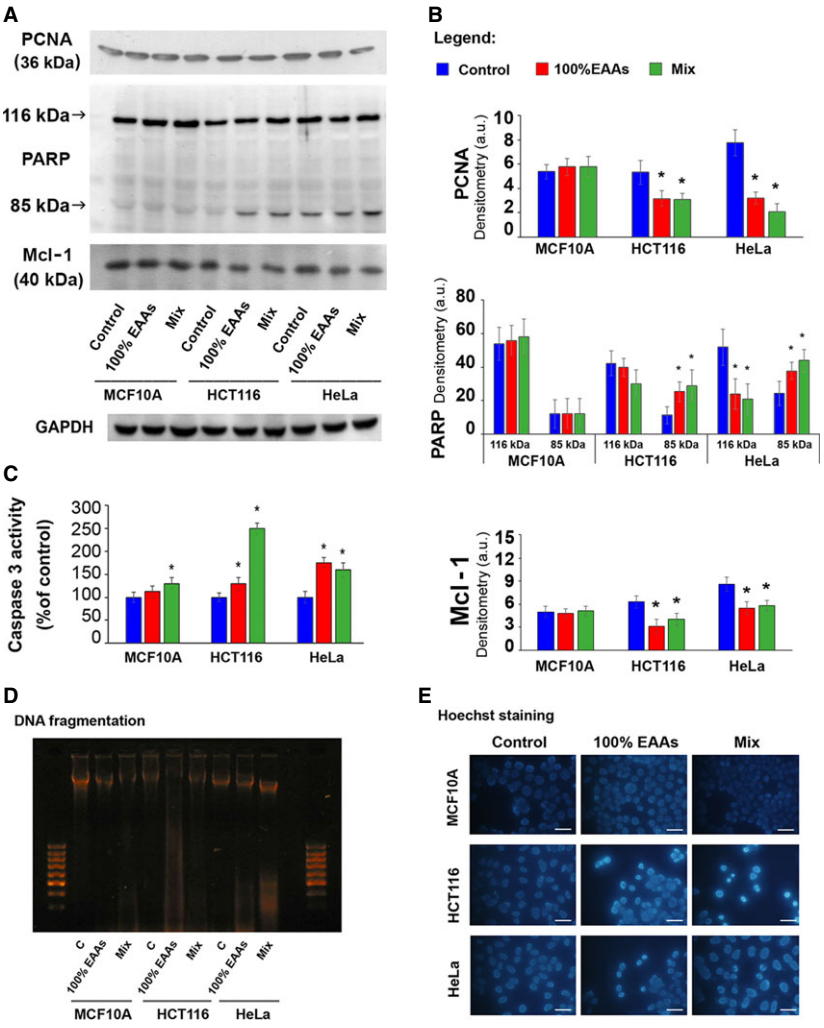

Fig. 2. Effect of the two AA mixtures on normal and cancer cell proliferation and apoptosis. (A, B) Representative western blots for PCNA, PARP and Mcl-1 detection (A) and relative densitometry (B). GAPDH was used as control for equal protein loading. Data points marked with an asterisk are statistically significant compared with the respective untreated control cells (*$P < 0.05$). (C) Caspase-3 activity measured using the fluorogenic substrate Ac-Asp-Glu-Val-Asp-AMC (see 'Results' for details). Data are expressed as percentage of the respective untreated control cells (100%). (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. *$P < 0.05$ indicates a statistically significant difference compared with the respective untreated control cells. (E) Upon treatment, cells were stained with Hoechst 33342 to visualize the morphology of nuclei. A representative microscopic field for each treatment is shown (scale bar = 10 μm).

Beclin-1 and p62. Globally, AA supplementation favored a significant increase of cathepsins B and L activities in all the cell lines. In detail, the EAA–NEAA 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 the 100% EAA formulation, whereas the activity of cathepsin B was mainly increased by the EAA–NEAA mix. A significant activation of both enzymes was measured in normal cells (Fig. 5A).

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 LC3-II (the membrane-associated form of the LC3 protein) and is finally degraded in autophagolysosomes, and thus its levels inversely correlate with the autophagic activity. Figure 5C 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% EAA solution. Staining with monodansylcadaverine (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 AA supplementation selectively induces autophagy (Fig. 5B). In fact, by contrast, treated MCF10A cells exhibited a diffused distribution of MDC-labeled fluorescence. Interestingly, blocking autophagy using 3-methyladenine (3-MA) magnified the cytotoxic effects of the AA formulations in tumor cells (Fig. 1). This is

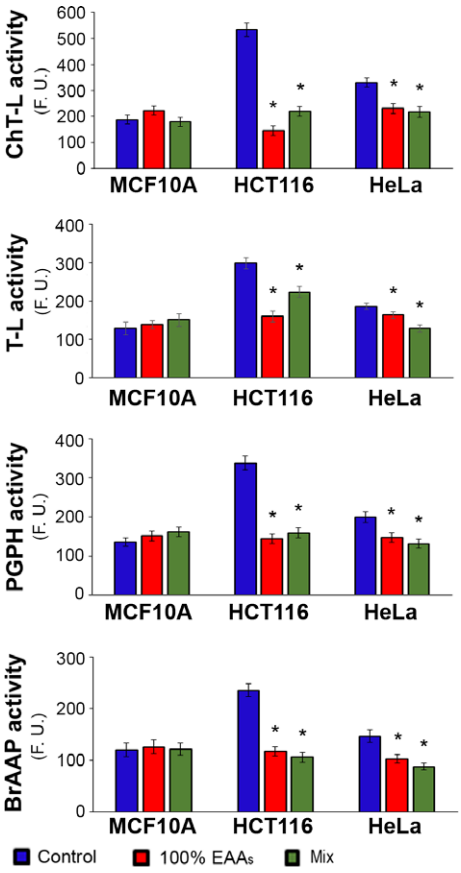

Fig. 3. Effect of the two AA mixtures on 20S proteasome. 20S proteasome activities in normal and cancer cells following treatment with 100% EAAs and the mix (1% final concentration) for 72 h (see 'Results' for details). Values are expressed as fluorescence units (F.U.) and the asterisk (*$P < 0.05$) indicates significantly different values compared with 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.

likely due to the strict interdependence between UPS and autophagy, with autophagy activation occurring in response to proteasome inhibition (in cancer cells upon AAs supplementation). The presence of the autophagy inhibitor compromises the compensatory activation of autophagy.

Discussion

The relation between nutrition and cancer is an unresolved question with many possible answers since even cancer cells' 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, AA-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 AA formulations, one composed of EAAs in ratios proven 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in the presence of the inhibitor zVAD-fmk, the two mixtures induced a caspase-mediated apoptosis.

The anti-proliferative effects induced by some AA mixtures on cancer cells were described in previous studies. Hagiwara *et al.* [31] observed that essential branched chain AAs (Leu/Ile/Val) prevented insulin-induced hepatic tumor cell proliferation by inducing apoptosis through suppression of the insulin–phosphoinositide 3-kinase (PI3K)–Akt pathway and involving mammalian target of rapamycin complex (mTORC) 1 and mTORC2. 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 branched chain amino acids 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 switching on of autophagic machinery [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

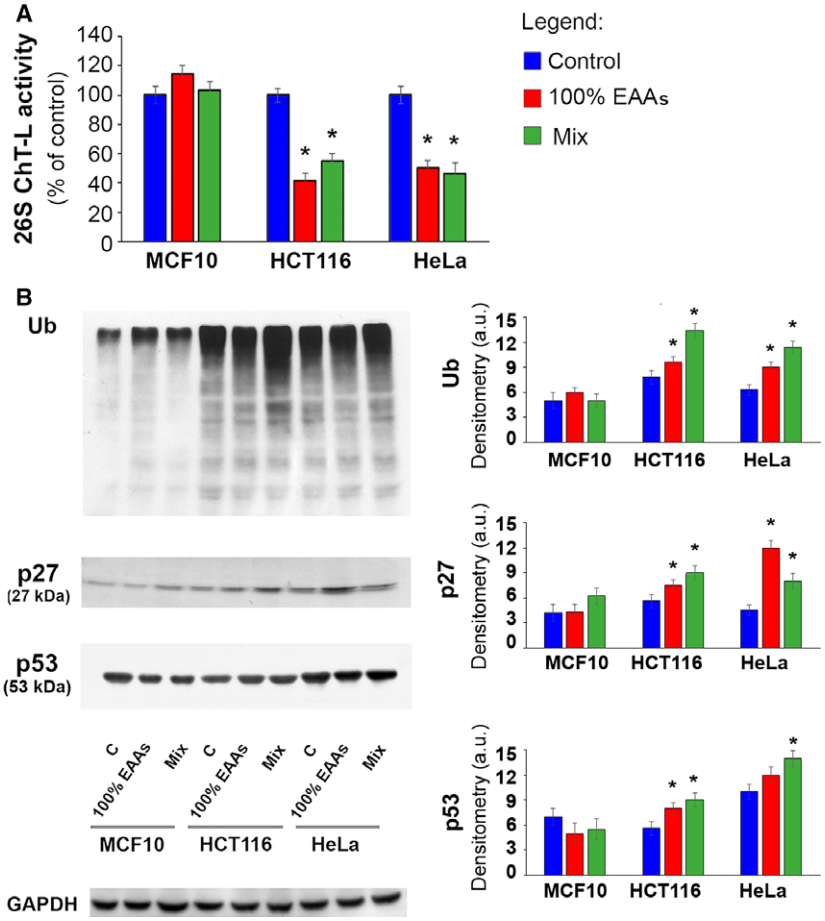

Fig. 4. Effect of the two AA mixtures on UPS. (A) 26S ChT-L activity measured in normal and cancer cells following treatment with 100% EAAs and the mix (1% final concentration) for 72 h (see 'Results' for further details). Values are expressed as fluorescence units (F.U.) and the asterisk (*$P < 0.05$) indicates significantly different values compared with respective untreated control cells. (B) Representative western blots 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. *$P < 0.05$ indicates significantly different values compared with respective untreated control cells.

hypothesized that modulation of proteolysis could be one of the pathways 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 AA formulations, cancer cells show activation of cathepsins 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 AA supplementation.

Interestingly, autophagy inhibition using 3-MA, compromising the compensatory activation of this pathway, magnified the cytotoxic effects of the AA 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 factor deprivation due to its ability to recycle nutrients, to maintain cellular energy homeostasis by 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

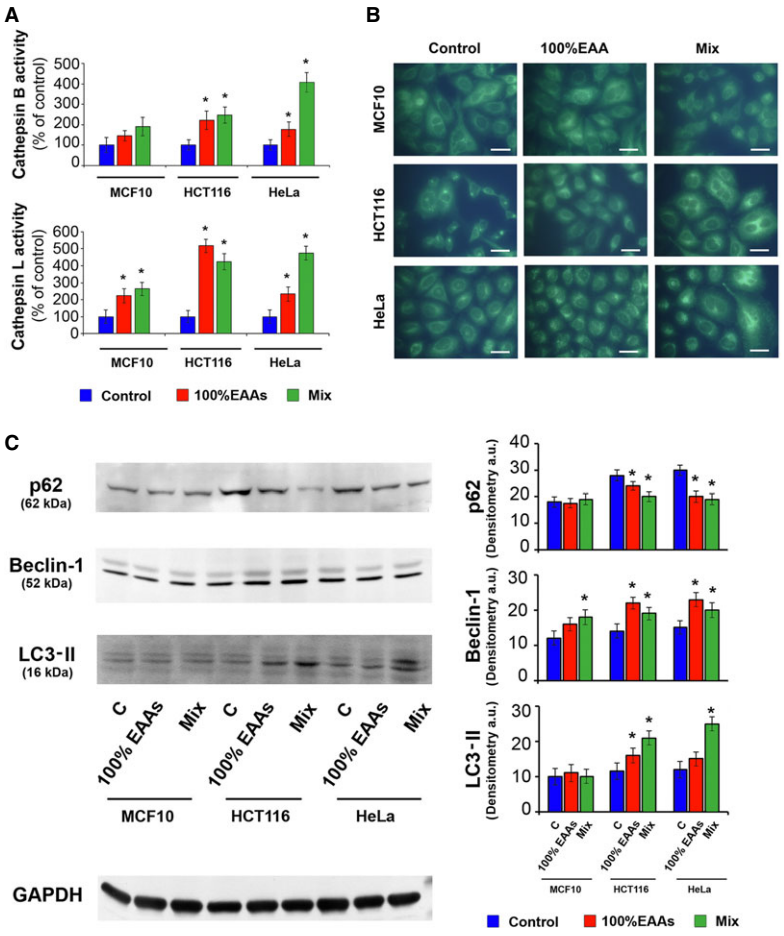

Fig. 5. Effect of the two AA mixtures on autophagy. (A) Cathepsin L and B activity after AA treatment were measured using fluorogenic substrates (see 'Results' for details). Specific activity is expressed as the amount of released product per minute per microgram of total protein in the sample. $*P < 0.05$ indicates significantly different results compared with the non-treated group. (B) Upon treatments, normal and cancer cells were incubated with MDC and analyzed by fluorescence microscopy to visualize autophagic vacuoles (scale bar= 20 μm) (C) Expression of autophagic markers in cell lysates. Representative western blots 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. $*P < 0.05$ indicates significantly different results compared with the non-treated group.

feeding with essential AA-rich mixtures selectively elicits proteasome inhibition and autophagy activation in cancer cells.

Modulation of cellular autophagy inhibits cell proliferation and activates the 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 the effects of EAAs on cell viability by autophagy activation. Indeed, serine, as the main NEAA to reach a 15% ratio, was chosen with three main purposes: it is the AA most metabolically rapidly available both for energy production and for transamination (generating one pyruvate molecule), it has a role in maintaining folates charged with methyl groups [49], and, combined with EAAs on the basis of our data, it 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 in mitochondria allowing metabolic production of sufficient reactive oxygen species necessary to activate the autophagic pathway [51], which 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 *et al.* [19], cancer cells are mostly dependent on the prevailing environmental availability of NEAAs for metabolic processes and cell proliferation, and enriching their environment with EAAs may trigger specific pathways that are ultimately incompatible with proliferation and survival. These data therefore support the study of the physiological effects of nutrition in cancers cells, thus characterizing the peculiar fragilities of cancer and promising new options for alternative treatments of this complex disease.

Materials and methods

Cell lines

MCF10A (not transformed cells) and HeLa, HCT116, MCF7, HepG2 and CaCo2 (cancer cells) were grown in Dulbecco's modified Eagles's medium (DMEM) supplemented

with 10% fetal bovine serum, antibiotics and antimycotics. To avoid misinterpretation of results, no other nutrients (EAAs or NEAAs) were added to the medium. 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 $ng \cdot mL^{-1}$ epidermal growth factor, 10 $\mu g \cdot mL^{-1}$ insulin, 0.5 $mg \cdot mL^{-1}$ hydrocortisone, antibiotics and antimycotics. Cells were incubated with growth medium at 37 °C equilibrated with 95% air and 5% CO_2 in flasks or 96- or 6-well plates depending on the assay.

Amino acids treatment

Cells were independently treated with a solution containing 100% EAAs or a mixture of 85% EAAs and 15% NEAAs (herein referred to as mix). The compositions are reported in Table 1. Suitable solubility was reached at 1% (w/v) concentration for dissolving AAs in the growth medium, followed by vortexing and sonication at 50 °C for 30 min. Each cell line was treated with 1% (w/v) of the 100% EAA solution and of the mixture. Control cells were treated with the growth medium. Treatments ranged from 48 to 96 h.

MTT assay

Cell viability was evaluated by MTT assay, an index of mitochondrial activity and cell viability. It is based on viable cells' ability to convert a soluble yellow tetrazolium salt to a purple–blue formazan crystal by mitochondrial succinate dehydrogenase. After incubation with the AA supplementations, media were replaced with serum-free media containing 0.5 $mg \cdot mL^{-1}$ MTT. Plates were incubated at 37 °C for 2 h. After discarding the supernatant, the formazan product was solubilized in 100 μL of dimethyl sulfoxide and absorbance was measured at 550 nm [52]. The MTT assay was also carried out in the presence and in the absence of the caspase inhibitor zVAD-fmk (10 μM concentration in the assay; Sigma-Aldrich, St Louis, MO, USA) and of the autophagy inhibitor 3-MA (5 mM concentration in the assay; Sigma-Aldrich).

Hoechst nuclear staining

Once treated, cells (1×10^6 $cells \cdot mL^{-1}$) were fixed in methanol/acetic acid (3 : 1), incubated with 0.05 $mg \cdot mL^{-1}$ Hoechst 33258 nuclear stain for 15 min and observed using an inverted fluorescence microscope (Olympus X71, Leica, Melville, NY, USA).

DNA fragmentation

Cell lines were seeded in six-well plates and treated with the 100% EAA solution, the AA mixture and the medium as control for 72 h. The procedure of Buonanno *et al.* [53] was followed. Pellets were suspended in lysis buffer (50 mM Tris/HCl pH 8, 10 mM EDTA, 0.5% SDS, and 0.5 $mg \cdot mL^{-1}$ proteinase K) and incubated at 50 °C for 1 h. RNase was added (10 $mg \cdot mL^{-1}$) and lysates were incubated for 10 min at 70 °C. DNA was precipitated with sodium acetate pH 5.2 and ice-cold 100% ethanol, 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.

Cell lysis

Upon treatment, cells were harvested in PBS, centrifuged and the pellet was resuspended in lysis buffer (20 mM Tris, pH 7.4, 250 mM sucrose, 1 mM EDTA and 5 mM β-mercaptoethanol). Lysates were centrifuged at 12 000 ***g*** for 15 min and the supernatants stored at −80 °C. Protein concentration was estimated following the Bradford method using bovine serum albumin as standard [54].

Enzymatic activities

The effects on the 20S proteasome were evaluated by fluorimetric assays using the following synthetic substrates: Leu-Leu-Ala-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 (where AMC is 7-amino-4-methylcoumarin and pAB is 4-aminobenzoic acid); the test for BrAAP was performed with the addition of aminopeptidase-N (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 AMC and 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 (Molecular Devices, Sunnyvale, CA, USA). 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 $CaCl_2$, 1 mM 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).

Cathepsins B and L proteolytic activities were measured as described by Tchoupe *et al.* [56] using the fluorogenic peptides Z-Arg-Arg-AMC and Z-Phe-Arg-AFC, respectively (where AFC is 7-amino-4-trifluoromethylcoumarin), 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 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.

Western blotting

Cell lysates (20 μg of total proteins) were resolved by 10% or 12% SDS/PAGE and electroblotted onto poly(vinylidene difluoride) membranes. Membranes with transferred proteins were incubated with primary and secondary antibodies to detect p27, ubiquitin, PCNA, p53, Mcl-1 and PARP (Santa Cruz Biotechnology, 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 (https://imagej.nih.gov/ij/). Data were analyzed with MATLAB (The MathWorks Inc., Natick, MA, USA).

Monodansylcadaverine

Cells were independently treated with both AA formulations for 72 h. The media were replaced with 50 μM MDC (Sigma-Aldrich) 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.

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 (Systat Software, Inc., San Jose, CA, USA). *P*-values < 0.05 and < 0.01 were considered significant.

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Conflict of interest

The authors have no conflict of interest to declare.

Author contributions

AME and FSD planned the experiments; FSD designed and provided the amino acid mixtures; LB performed experiments; LB and VC analyzed data; LB and VC wrote the paper – original draft; AME, FSD, MA, VC and MC wrote the paper – review and editing; MA, VF, GC and EP provided technical assistance; AME acquired funds. All authors read and approved the final manuscript.

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