

“Compositions comprising amino acids for prevention and/or treatment of cancer”

5 **Field of the invention**

The present description relates generally to compositions comprising amino acids for use in the prevention and treatment of cancer.

Background

10 Cancers have a different metabolic profile with respect to normal tissues and contrasting evidence are emerging on the role of mitochondrial activity in the proliferation of cancer cells. The so-called Warburg effect, the preferential use of glucose even in presence of oxygen is one of the hallmarks of tumor cells. This phenomenon, initially defined as “aerobic glycolysis” led to the earliest hypothesis of a defective mitochondrial function in cancer. However, this setting
15 has been shown to be more complex, and there is now general agreement that cancer mitochondria, during oncogenesis, engage in pleiotropic functions, such as macromolecular biosynthesis, apoptosis resistance, and activation of oncogenic signaling. Nevertheless, many mitochondrial mutations are indeed found in tumors, and a decreased oxidative phosphorylation (OXPHOS) rate has been
20 shown, owing to a reduction in reactive oxygen species (ROS), to result in a proliferative advantage and an increase in cancer cell proliferation. Furthermore, given the pivotal role of glycolysis pathway in cancer growth, and since OXPHOS and glycolysis are reciprocally regulated, it is tempting to speculate that enhancing mitochondrial function could lead to glycolysis inhibition and cancer
25 cell death. Moreover, much attention and efforts have been employed to develop glycolytic inhibitors as a pharmaceutical approach in anti-cancer therapy; some first and second generation drugs have been developed but their safety profile and development of resistance has raised some concerns.

Summary of the invention

30 The present description has the aim of providing new compositions particularly effective in the prevention and/or treatment of cancer and endowed with a safe administration profile.

 According to the present description, the above object is achieved thanks to the subject matter specifically recalled in the ensuing claims, which are
35 understood as forming an integral part of this disclosure.

An embodiment of the present description provides a composition for use in the prevention and/or in the treatment of cancer in a subject, the composition comprising an active agent, said active agent containing the amino acids leucine, isoleucine, valine, threonine, lysine and citric acid, succinic acid, malic acid.

5 In one or more embodiments, the cancer may be selected in the group consisting of melanoma, adenocarcinoma, colorectal carcinoma, breast cancer.

In one or more embodiments, the active agent of the composition may further contain one or more amino acids selected in the group consisting of histidine, phenylalanine, methionine, tryptophan, cysteine and tyrosine.

10 **Brief description of the figures**

The invention will now be described, by way of example only, with reference to the enclosed figures, wherein:

- Figure 1 shows a MTT viability assay in cancer and normal cells incubated for the times indicated, with a normal media (DMEM) or with the composition of the instant application ($\alpha 5$). * $p < 0.01$ ** $p < 0.05$ vs DMEM.

- Figure 2 shows a scratch wound assay in B16F10 melanoma cells at zero (0) hours and incubated for 24 hours (hr) in DMEM (NT) or with the $\alpha 5$ composition ($\alpha 5$).

- Figure 3 shows a colony formation assay in HeLa cells incubated for 24 hr in DMEM (NT) or in the $\alpha 5$ composition ($\alpha 5$).

- Figure 4 shows the results of western blot analysis of Cytochrome C (CytC) translocation and apoptosis markers in M14 melanoma cells incubated for 24 hr in DMEM (NT) or in the $\alpha 5$ composition ($\alpha 5$). TOP: $\alpha 5$ induces specific CytC, but not COX4, translocation in cytosol (PMS) of treated cells. BOTTOM: PARP-1 and caspase 3 cleavage in M14 cells.

- Figure 5 shows the results of western blot analysis of mTOR pathway and HIF1 α in cells incubated for 5 hr in DMEM (NT) or in the $\alpha 5$ composition ($\alpha 5$). LEFT: phospho-p70S6K (thr386) levels, as a marker of mTOR activity in M14, HeLa, B16F10 and Detroit 573 fibroblasts. RIGHT: HIF1 α expression in M14 and HeLa cells. GAPDH is shown as a loading control.

- Figure 6 shows the metabolic activity in cancer cell and in normal cells. Glycolysis (LEFT) and OCR (RIGHT) in HeLa, M14 and HL-1 cells after 1 hour of treatment with DMEM (NT) or in the $\alpha 5$ composition ($\alpha 5$). * $p < 0.01$ vs NT.

- Figure 7 is relative to MCF7 breast cancer cell proliferation. (A) Acid phosphatase assay: cells (5,000-20,000/well in 96-well plates) were treated with

1% $\alpha 5$ composition for 48 h and 1 μ M doxorubicin (DOX) for 16 h. (B) Proliferation assay: cells (50,000/well in 12-well plates) were treated as in (A) and Trypan blue exclusion assay was used. n = 3 experiments. *p < 0.05 and **p < 0.01 vs. untreated cells. All data are presented as the mean $\pm$ SD.

5 **Detailed description of preferred embodiments**

In the following description, numerous specific details are given to provide a thorough understanding of embodiments. The embodiments can be practiced without one or more of the specific details, or with other methods, components, materials, etc. In other instances, well-known structures, materials, or operations
10 are not shown or described in detail to avoid obscuring aspects of the embodiments.

Reference throughout this specification to “one embodiment” or “an embodiment” means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment. Thus,
15 the appearances of the phrases “in one embodiment” or “in an embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments. The headings provided herein are for convenience only and do not
20 interpret the scope or meaning of the embodiments.

An embodiment of the present description provides a composition for use in the prevention and/or treatment of a cancer in a subject, the composition comprising an active agent, said active agent containing the amino acids leucine, isoleucine, valine, threonine, lysine and citric acid, succinic acid, malic acid.

25 The cancer may be selected in the group consisting of adenocarcinoma, melanoma, colorectal carcinoma, breast cancer.

The disclosure further provides combined preparations comprising the composition herein disclosed and at least one chemotherapeutic agent for simultaneous, separate or sequential use in preventing and/or treating a cancer in a
30 subject.

The Inventor of the instant application has surprisingly found that the composition herein disclosed is particularly effective in reducing the proliferation rate of cancer cells, such as cervical cancer cells, melanoma cells, colon cancer cells, breast cancer cells and has no effect on the rate of proliferation of normal
35 cells. The composition has also been shown to be effective on the inhibition of

cancer cell motility and clonogenic potential. Very importantly, the composition exerts an inhibitory effect on the mTOR pathway and this evidence is noteworthy, since an increased mTOR activity is often associated with tumorigenesis and essential amino acid-based compositions are known to activate mTOR pathway.

5 Moreover, as shown in the following sections, the composition may be safely administered either alone or in combination with at least one chemotherapeutic agent. The chemotherapeutic agents may be for example at least one anthracycline, for example doxorubicin.

10 In one or more embodiments, the composition herein disclosed comprises an active agent, said active agent contains citric acid, succinic acid and malic acid in combination with leucine, isoleucine, valine, threonine, lysine, and the weight ratio between the total amount of citric acid, succinic acid and malic acid and the total amount of the amino acids leucine, isoleucine, valine, threonine, lysine is comprised between 0.05 and 0.3, preferably between 0.1 and 0.25.

15 In one or more embodiments, the composition may consist of leucine, isoleucine, valine, threonine, lysine, citric acid, succinic acid and malic acid and optionally vitamin B1 and vitamin B6.

20 In one or more embodiments, the active agent may further comprise one or more amino acids selected in the group consisting of histidine, phenylalanine, methionine, tryptophan, cysteine, tyrosine.

25 In one or more embodiments, The composition may comprise an active agent consisting of leucine, isoleucine, valine, threonine, lysine, histidine, phenylalanine, methionine, tryptophan, cysteine and optionally tyrosine, as well as citric acid, succinic acid and malic acid, said amino acids being the sole amino acids contained in the composition.

 In one or more embodiments, the composition may be free of any other active agent, such as any chemotherapeutic agent, i.e. any agent that directly or indirectly inhibit the proliferation of rapidly growing cells, exerting an antineoplastic effect.

30 In one or more embodiments, the composition may consist of leucine, isoleucine, valine, threonine, lysine, histidine, phenylalanine, methionine, tryptophan, cysteine, tyrosine, citric acid, succinic acid and malic acid and optionally vitamin B1 and/or vitamin B6.

35 The composition may comprise the amino acids isoleucine, leucine and valine in an amount between 35% and 65% by weight, preferably between 42%

and 56% by weight with respect to the active agent weight.

The weight ratio between leucine and citric acid may be comprised between 5 and 1, preferably between 2.50 and 3.50.

In a further embodiment, the weight or molar amount of citric acid is
5 higher than the weight or molar amount of each of malic acid and succinic acid. Preferably, the weight or molar amount of citric acid is higher than the weight or molar overall amount of malic acid plus succinic acid. In a further embodiment, the weight ratio between citric acid and the sum of malic acid and succinic acid is comprised between 1.0 and 4.0, preferably between 1.5 and 2.5. In a preferred
10 embodiment, the citric acid:malic acid:succinic acid weight ratio is comprised between 10:1:1 and 2:1.5:1.5, preferably between 7:1:1 and 1.5:1:1, more preferably between 5:1:1 and 3:1:1. In a preferred embodiment the citric acid:malic acid:succinic acid weight ratio is 4:1:1.

The preferred isoleucine:leucine molar ratio is comprised in the range 0.2-
15 0.7, preferably in the range 0.30-0.60 and/or the preferred valine:leucine weight ratio is comprised in the range 0.2-0.70, preferably in the range 0.30-0.65.

In a further embodiment, the threonine:leucine molar ratio is comprised in the range of 0.10-0.90, preferably in the range 0.20-0.70 and/or the lysine:leucine weight ratio is comprised in the range of 0.20-1.00, preferably in the range 0.40-
20 0.90.

In a preferred embodiment, the ratio between the overall molar amount of citric acid, malic acid, succinic acid and the overall molar amount of methionine, phenylalanine, histidine and tryptophan is higher than 1.35.

In one or more embodiments, the weight ratio between the sum of citric
25 acid, malic acid, succinic acid and the sum of the branched chain amino acids leucine, isoleucine, valine is comprised between 0.1 and 0.4, preferably between 0.15 and 0.35.

In a further embodiment, the overall weight amount of the branched chain amino acids leucine, isoleucine, valine plus threonine and lysine is higher than the
30 overall weight amount of the three acids citric acid, malic acid, succinic acid. Preferably, the weight amount of the single acids (citric acid, succinic acid or malic acid) is less than the weight amount of each of the single amino acids leucine, isoleucine, valine, threonine and lysine.

In a further embodiment, the overall molar amount of lysine and threonine
35 is higher than the overall molar amount of the three acids citric acid, succinic acid,

malic acid. Preferably, the ratio between the overall molar amount of the three acids citric acid, succinic acid, malic acid and the overall molar amount of lysine and threonine is comprised between 0.1 and 0.7, preferably between 0.15 and 0.55.

5 In one or more embodiments, the composition herein disclosed further comprises vitamins, preferably selected in the group of vitamins B, such as vitamin B₁ and/or vitamin B₆. The composition may include carbohydrates, flavouring substances, natural and artificial sweeteners, excipients. The excipients may be selected from maltodextrins, fructose, fish oil, sucralose, sucrose esters, 10 vitamin D3, group B vitamins.

 In one or more embodiments, the composition may be a pharmaceutical composition further comprising a pharmaceutically acceptable vehicle and at least one pharmaceutically acceptable excipient as disclosed above.

 The amino acids disclosed in the instant description can be replaced by 15 respective pharmaceutically acceptable derivatives, namely salts.

 Furthermore, in particular when preparing the compositions according to the instant disclosure, and specifically the active agent, the amino acid arginine is to be avoided. In addition, further amino acids specifically excluded by the composition herein disclosed are serine, proline, alanine. Such amino acids can be 20 counterproductive or even harmful in some concentrations or stoichiometric ratios within the composition.

 For oral use, the compositions according to the description may be in the form of tablets, capsules, granules, gel, jellifying powder, powder.

 The disclosure also provides a method for preventing and/or treating 25 cancer in a subject, the method comprising selecting a composition comprising an active agent, said active agent containing the amino acids leucine, isoleucine, valine, threonine, lysine, and the carboxylic acids citric acid, succinic acid, and malic acid, and administering the composition to the subject. The active agent may further comprise one or more amino acids selected in the group consisting of 30 histidine, phenylalanine, methionine, tryptophan, cysteine, tyrosine, as disclosed herein. The composition may be administered alone, thus the method consists in selecting the composition and administering the composition to the subject. In one or more embodiments, the composition may also be administered – simultaneously, separately or sequentially - with at least one chemotherapeutic 35 agent, preferably at least one anthracycline, more preferably said at least one

anthracycline being selected in the group consisting of doxorubicin, epirubicin, daunorubicin, idarubicin, pixantrone, sabarubicin, valrubicin, derivatives thereof.

Further specifications, in terms of amounts and ratios among the various amino acids provided for by the compositions are contained in the attached claims, which form an integral part of the technical teaching provided herein in relation to the invention.

EXAMPLES

Table 1 shows the composition disclosed in the instant application (named “ $\alpha 5$ ”). The composition comprises an active agent containing amino acids in combination with citric acid, succinic acid and malic acid and it is free of any chemotherapeutic agents.

Table 1

Composition (% w/w)	$\alpha 5$
L-Leucine	31.0885
L-Lysine HCl chlorhydrate	16.9030
L-Isoleucine	10.3628
L-Valine	10.3628
L-Threonine	7.2540
L-Cysteine	3.1089
L-Histidine	3.1089
L-Phenylalanine	2.0726
L-Methionine	1.0363
L-Tyrosine	0.6218
L-Tryptophan	2.0726
Citric acid	8.001
Succinic acid	2.00
Malic acid	2.00
Vitamin B1 (thiamine chlorhydrate)	0.004
Vitamin B6 (piridoxine chlorhydrate)	0.0038

The composition of Table 1 above may be prepared first by sifting all the components with a 0.8 mesh. To obtain a pre-mixture, each ingredient (in an amount <10% by weight of the total amount) is put in a polyethylene bag together with a portion of L-lysine HCl so as to obtain 10% of the weight of the total composition. The bag is then manually shaken for 5 minutes. The pre-mixture is then loaded in a mixer (Planetaria) together with the remainder of the ingredients and mixed for a period of 15 minutes at 120 rpm to obtain a homogeneous final composition.

METHODS

Cells and treatments

Cell lines used were: B16F10 (mouse melanoma, ATCC # CRL-6475), C2C12 (mouse myoblasts, ATCC # CRL-1772), HeLa (human adenocarcinoma, ATCC # CCL-2), Detroit 573 (human fibroblasts, ATCC # CCL-117), NIH3T3 (mouse embryonic fibroblasts, ATCC # CRL-1658), MCF-7 (human breast cancer cell line, ATCC # HTB-22), HCT116 (human colorectal carcinoma cells ATCC # CCL-247), M14 (human melanoma) [Chee DO et al. 1976 Cancer Res. 36(4):1503-9], HCT116 TP53 (-/-) (p53 null human colorectal carcinoma cells) [Sur S et al. 2009 Proc Natl Acad Sci U S A. 10;106(10)], HL-1 (mouse cardiomyocytes) [Sigma-Aldrich (Milan, Italy) (SCC065)].

Cells were routinely grown in standard Dulbecco's modified eagle medium (DMEM) supplemented with 4 mM glutamine, 10% fetal bovine serum (FBS) and 100units/ml penicillin/streptomycin (all reagents from Sigma-Aldrich, Milan, Italy) until 80% confluency. For $\alpha 5$ treatments, cells were incubated for the indicated periods in complete DMEM or with a 1% solution of $\alpha 5$ mixture dissolved in the same medium.

MCF-7 cells were cultured in DMEM as above and treated with 1 % $\alpha 5$ composition for 48 h and 1 μ M doxorubicin (DOX; Sigma-Aldrich, Milan, Italy) for 16 h (Figure 7).

Viability assay

The viability of cells was determined using the standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. All the treatments were done using 2×10^4 cells/well in 96 wells plate in 100 μ L of medium. The purple formazan crystals were dissolved in 5% SDS/0.1M HCl (100 μ L/well), and the absorbance was recorded on a microplate reader at a wavelength of 570 nm.

Monolayer wound healing assay

Cells were grown to full confluence in 24-well plates in their growth media and then were incubated overnight in control or in a $\alpha 5$ -containing media. Cell cultures were then scratched with a 200 μ L sterile pipette tip and extensively washed with PBS to remove detached cells and debris. To assess migration ability recovery, microscopy images were then collected after 16-24 hrs.

Clonogenic survival assay

100 to 1000 cells were seeded in triplicate in six-well plates one day before treatment with control DMEM or $\alpha 5$ composition. Cells were then allowed to

grow in DMEM or α 5 media until they formed colonies for about 10 days. Survived colonies were fixed with 100 % methanol and stained with methylene blue in methanol. At least three biological independent experiments were performed.

5 **Western blot analysis**

Whole cell extracts were isolated using Lysis Buffer (20 mM Tris-HCl pH 8.0, 400 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM Na pyrophosphate, 1% Triton X-100, 10% glycerol) supplemented with protease and phosphatase inhibitors. The concentration of the isolated proteins was determined using BCA Protein Assay. Fifteen-twenty micrograms of the protein were separated on a 4-20 % Tris-glycine gel and electrophoretically transferred to nitrocellulose or PVDF membranes (Bio-Rad, Segrate (MI) – Italy). After incubation with 5% nonfat dry milk in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 60 min, the membrane was washed once with TBS-T and incubated with the appropriate antibodies at 4 °C for 12 h. Membranes were washed three times for 10 min and incubated with a 1:3000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for 2 h. Blots were washed with TBS-T three times and developed with the ECL system. Antibodies used were: p-p70S6K (Thr389) (Cell Signaling cat # 9205), p70S6K (Cell Signaling cat # 9202), HIF1- α (BD Biosciences cat # 610958), GAPDH (Cell Signaling cat # 2118), CytC (Cell Signaling cat # 4280), COXIV (Cell Signaling cat # 4844), PARP (Trevigen # 4338), Caspase 3 (Cell Signaling cat # 9662)

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurement

25 The rate of change of dissolved O₂ (oxygen consumption rate, OCR) and rate of change of pH (extracellular acidification rate, ECAR) was measured in the XF24 Analyzer (Seahorse Biosciences) following manufacturer's instructions.

Cells ($2-4 \times 10^3$ per well) were plated in XF24 cell culture microplates (Seahorse Biosciences, MA, USA) and, after 18 hrs, were equilibrated with DMEM lacking bicarbonate (Seahorse Biosciences) supplemented with glucose at 37° C for 1 h in an incubator lacking CO₂. After basal OCR measurement, the compounds oligomycin (2 μ M), the uncoupler Carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) (1 μ M) and the electron transport inhibitors rotenone and antimycin A (R/A) (both 0.5 μ M) were then injected into the wells to monitor uncoupled, maximal and non-mitochondrial respiration,

respectively. Compound were injected into the wells at the indicated times.

Acid phosphatase assay

To quantify MCF7 cell growth, acid phosphatase assay was used as described [Yang TT et al. 1996 Anal Biochem. 241(1):103-8]. Briefly, MCF7
5 cells were placed in 96-well plates at 5,000 to 20,000 cells per well density and treated with 1% $\alpha 5$ (for 48 h) and 1 μ M DOX (for 16 h). Culture medium was removed and each well washed once with phosphate-buffered saline (PBS, pH 7.2), and added with 100 μ l buffer containing 0.1 M sodium acetate (pH 5.0), 0.1% Triton X-100, and 5 mM p-nitrophenyl phosphate (pNPP). Then, plates
10 were placed in a 37°C incubator for 2 h. The reaction was stopped with the addition of 10 μ l 1 N NaOH, and color development was assessed at 405 nm. Non-enzymatic pNPP hydrolysis was measured in wells without cells.

Statistical analysis

Pairwise comparisons between means of different groups were performed
15 using a Student *t-test* (two tailed, unpaired, unpaired). Data were considered significant with $p < 0.05$.

RESULTS

Inhibitory effect of the $\alpha 5$ composition on the proliferation, motility and clone formation ability of cancer cells

20 The effect of the $\alpha 5$ composition administration was first tested on *in vitro* cancer cell proliferation. To this end, various cancer cell lines were incubated with the $\alpha 5$ composition (1%). As shown in figure 1, $\alpha 5$ composition decreased cancer cell growth with respect to cells grown in culture with control medium only. Most importantly, $\alpha 5$ treatment did not affect growth of normal non-cancer cells. It
25 should be noted that $\alpha 5$ reduced viability of both HCT116 wild-type (WT) and p53 $-/-$, thus demonstrating its effectiveness also in absence of p53; since p53, which is one of the most important tumor suppressor genes, is mutated and inactive in many tumors, this data therefore greatly enlarges the range of action of $\alpha 5$.

30 Of note, $\alpha 5$ mixture was able to inhibit cancer cell growth as early as 24hr after starting of treatment. This rapid effect was particularly pronounced in both WT and p53 $-/-$ HCT116 cells and Hela (49%, 44% and 46% inhibition, respectively); the growth inhibitory effect of $\alpha 5$ then further increased at 48 hrs, and, by 72 hrs, reached 74%, 61% and 70% of inhibition. The mixture was,
35 however, also effective on both human and mouse melanoma cells; in particular,

at 72 hr, $\alpha 5$ inhibited mouse B16F10 mouse melanoma growth by 87%.

The administration of the $\alpha 5$ composition also reduced cancer cell motility, as assessed by scratch-wound assay in B16F10. Confluent cells were scratched (0 hr) and then incubated in control media or $\alpha 5$ composition. As shown in figure 2, 24 hrs after scratching, cells incubated in control media resumed proliferation and motility, while those incubated in $\alpha 5$ did not, thus showing reduced migration ability.

Furthermore, $\alpha 5$ composition also completely blocked clone formation ability in HeLa cells, when single-cell clonogenic potential was challenged in a colony formation assay. As shown in figure 3, while cells incubated in control medium efficiently formed single cell-derived colonies during ten days of growth, incubation in $\alpha 5$ composition greatly reduced the ability of HeLa cells to form colonies, therefore confirming that $\alpha 5$ blocks cancer cell reproductive capacity and cancer ability to undergo unlimited division.

The $\alpha 5$ composition promotes apoptotic cell death of cancer cells

To assess if the reduction in cell proliferation observed was due to a block in cell division or to induction of cell death, various apoptotic markers were analysed in M14 melanoma. It is well-known that, during apoptosis, the mitochondrial protein cytochrome C (CytC) is released into cell cytosol; to this aim, M14 cells were incubated in control or $\alpha 5$ media for 24 hr and thereafter, cell fractionation was performed by separating mitochondria (mito) from cytosolic fraction (Post-mitochondrial supernatant – PMS). CytC localization was then assessed by performing western blot analysis on both mitochondria and PMS. As a control, aimed to verify that release of CytC into the PMS was the specific result of apoptosis and not the consequence of mitochondrial damage (i.e. during fractionation process), localization of Cytochrome oxidase 4 (Cox4), which is not released during apoptosis, was also assessed.

As shown in fig. 4, immunoblot analysis showed specific CytC, but not Cox4, translocation in PMS of M14 cells incubated in $\alpha 5$ for 24 hr. Furthermore, clear Parp-1 cleavage, another well-known apoptotic marker, was evident after 24hr of $\alpha 5$ composition treatment in M14 melanoma cells. In addition, 24 hr of $\alpha 5$ composition also induced cleavage and formation of pro-apoptotic caspase 3 in M14 melanoma cells (figure 4).

These results strongly support the evidence that the $\alpha 5$ composition promotes apoptotic cell death of cancer cells.

Inhibitory effect of the $\alpha 5$ composition on the expression of phospho-p70S6k and HIF1 α

One major target of intracellular amino acids is the mechanistic target of rapamycin (mTOR) pathway, a main regulator of cell growth and division that integrates nutrient signals with induction of protein synthesis and cell growth by phosphorylating its downstream effector p70S6 kinase.

Since mTOR pathway is dysregulated in several cancers and its activation is frequently associated with the oncogenic cellular processes, thus enhancing tumorigenicity, the Inventor of the instant application also investigated the p70S6 phosphorylation status as readout of mTOR activity in cancer cells treated with the standard culture medium DMEM or with the $\alpha 5$ composition.

As shown in figure 5, p70S6 kinase phosphorylation levels were reduced in $\alpha 5$ treated M14, HeLa and B16F10 cells with respect to cells incubated in standard culture medium (column "NT" or "Non Treated" in figure 5); these results indicate the $\alpha 5$ composition surprisingly exerted an inhibitory effect on the mTOR pathway, thus ruling out its activation. Very interestingly, the p70S6k phosphorylation was not affected in normal Detroit 573 cells.

Most importantly, the $\alpha 5$ composition also downregulated, in both M14 and HeLa cells, the expression levels of the hypoxia-inducible factor 1 alpha (HIF1 α), which is one of the most important oncogenes involved in tumorigenesis (figure 5).

Since as a major oncogene HIF1 α is also a pivotal regulator of cancer glycolysis, the extracellular acidification rate (ECAR) was analyzed, as a measure of the glycolytic rate, in both cancer and normal cells, untreated (NT) or incubated with the $\alpha 5$ composition for 1 hour. As shown in figure 6, the treatment with the $\alpha 5$ composition significantly downregulated basal ECAR in cancer cells ($p < 0.01$ vs NT), while slightly decreasing glycolysis in control (NS), non tumor HL-1 cardiomyocytes. However, OCR was not upregulated in M14 and HeLa, while was greatly increased in HL-1 control cardiomyocytes ($p < 0.01$ vs NT).

These data demonstrate that acute $\alpha 5$ composition blocks glycolysis in cancer cells while simultaneously increases OCR only in normal cells.

The anti-proliferative effect of DOX is potentiated in the MCF7 breast cancer cells in the presence of the $\alpha 5$ composition

The effect of the exposure of MCF7 breast cancer cell line to the $\alpha 5$ composition alone or in combination with doxorubicin (DOX) was assessed with

two different assays (Figure 13A and 13B). The anti-proliferative effect of DOX in MCF7 cells was completely unaffected by the amino acid presence (Figure 13A and 13B): very interestingly, the anti-proliferative effect on cancer cell is potentiated when DOX is administered together with the $\alpha 5$ composition.

5 Altogether the results show that the composition herein disclosed is able to block cancer cell proliferation. Of note, the effect is specific for cancer cells, as normal cells proliferation was unaffected by the $\alpha 5$ composition, with a substantial safety profile and lack of toxic side effects.

10 Most importantly, the $\alpha 5$ composition exerts an inhibitory effect on the mTOR pathway, as assessed by reduced phosphorylation (activity) of its downstream target p70S6K. Again, this is noteworthy, since increased mTOR activity is often associated with tumorigenesis and essential amino acid-based compositions are known to activate mTOR pathway.

15 The composition herein disclosed comprises specific amino acids and tricarboxylic acids (TCA cycle intermediates); the administration of the composition may boost the mitochondrial activity and OCR and, as a consequence, shift the metabolic activity away from glycolysis towards amino acids and TCA cycle intermediates oxidation, leading to glycolysis downregulation.

20 Very surprisingly, although effectively inhibiting glycolysis in cancer cells, such a switch occurred only in non cancer cells (as shown in figure 6). This result could be ascribed to the extreme sensitivity of cancer cells to glycolysis, whose fast inhibition exerted by the $\alpha 5$ composition, most probably activates the onset of apoptosis before any switch to OCR could take place.

25 Therefore, the composition of the instant application has been shown to be effective in reducing the proliferation rate of cancer cells without presenting side effects.

30 The composition may be used alone or also in combined preparations with at least one chemotherapeutic agents; the results herein provided show that when administered together with doxorubicin, the rate of cancer cell proliferation is further reduced. Therefore, the effect of combined therapies also allows to greatly reduce dosage of chemotherapeutic drugs, thus leading to a more efficient and safe anticancer approach.