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(54) **Title:** NEW USE OF KNOWN PHARMACEUTICALLY ACTIVE CHEMICAL COMPOUNDS.

(57) **Abstract:** The invention relates to the use of at least one member selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such Na, Ca, Mg, Cu, Sr, Zn, K and others in doses 0.001-1 g/kg/day or in concentration 0.001 mM-100mM for the manufacture of a pharmaceutical preparation and/or food/ feed additive augmenting, supporting the function of the nerve cells and nervous system and simultaneously minimizing, preventing the apoptosis of nerve cells and preventing the development of diseases of nervous system in adolescent, adult and fetuses in mammals, including people, such as Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases and protecting the nervous system function against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases and protecting the growth and stimulating the reproduction and preventing the apoptosis of nerve and other cells in vitro and in biotechnological processes with the use of somatic and bacterial cells .

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NEW USE OF KNOWN PHARMACEUTICALLY ACTIVE CHEMICAL COMPOUNDS.

The present invention relates to the new use of known, pharmaceutically active chemical compounds. In particular it relates to the new use of certain acids, lipids and salts and mixtures thereof for the manufacture of a pharmaceutical and/or nutritional preparation used in the treatment and prophylaxis as a preparation augmenting, supporting the function of the nerve cells and nervous system and simultaneously minimizing, preventing the apoptosis of nerve cells and preventing the development of diseases of nervous system in adolescent, adult and fetuses in mammals, including people, and the use of the pharmaceutical or/and nutritional preparation in the process of protection of the nervous system function, e.g. against its degeneration due to apoptosis of nerve cells and the use of the preparation for the protection of the growth and stimulation of the reproduction and prevention of the apoptosis of nerve and other cells *in vitro* and in biotechnological processes with the use of somatic and bacterial cells..

Glutaminic acid is one of the main neurotransmitter in the central nervous system (CNS). Glutaminergic receptors are divided in two groups: metabotropic and ionotropic receptors. Metabotropic receptors are the membranous proteins connected, by G-proteins, with the system of secondary transmitters (e. g. IP3, cAMP). Up today, 8 subgroups of metabotropic receptors, known as mGLU1-mGLU8, were selected. On the dependence of the transmitters of the second order delivering the signal in the cell, they can be classified into specific sets. With the reference to pathology it seems that the group of ionotropic receptors, among which NMDA receptors (from agonist - N-methyl-D-asparaginic acid) and not-NMDA receptors (AMPA - alfa-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid and kainic) were selected, is more important. The main criterion of the classification, except the specificity to the antagonist,

is the sensibility to definite antagonists and the differences in the ions transmission through the channels connected with these receptors.

Glutaminergic receptors are present in all central nervous system. Their stimulation plays an important role in many physiological processes as well as in the pathology of many diseases of nervous system. An excessive activation of glutaminergic receptors leads to the toxicity by overstimulation (excytotoxicity) and than to the degeneration of the neurons. Probably, at least partly, it is responsible for neurodegenerative changes observed in Alzheimer's, Parkinson and Huntington diseases, brain ischaemia and others.

Anoxaemia, hypoglicaemia or some neurodegenerative diseases are the reason of increased concentration of glutamate in the brain - the main stimulating amino-acid in CNS. That amino-acid binds with its own receptors and activates them causing the inflow of the calcium and sodium ions to the cell, normally not present there. These proteins participate in the induction of apoptosis of neurons.

The very important part of research on receptors was the creation of specific antagonists. That made possibility to block the specific groups of receptors and the recognition of their influence on the brain regions with their highest concentration. Simultaneously, the study on the use of glutaminergic antagonists in the treatment of neurodegenerative diseases were began. Up today these researches haven't finished successfully. There is still the need to discover new substances which could prevent or inhibit neurodegenerative processes responsible for the diseases of nervous system. Such substance, according to the invention, is the preparation of alfa-keto-glutarate (AKG) and its salts.

In accordance with the present invention it was surprisigly found that alpha-keto-glutarate glutarate and its salts: ornithine-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino-acids and the salts of alpha-keto-glutarate with metal ions such Na, Ca, Mg, Cu, Sr, Zn, K and others and it augments, supports the function of nerve cells and

nervous system and simultaneously minimalizes, preventing apoptosis of nerve cells, and prevents development of nervous system diseases, e.g. Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and others, in adolescent, adult and fetuses of people and animals, and protects the function of nervous system against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases, protects the growth and stimulates reproduction and prevents apoptosis of nerve and other cells *in vitro* and in biotechnological experiences with the use of somatic and bacterial cells.

Thus according to one aspect of the present invention, there is provided the new use in doses 0.001-1g/kg/day or in concentration 0.001mM-100mM at least one member selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such as Na, Ca, Mg, Cu, Sr, Zn, K for manufacture of the pharmaceutical and/or nutritional preparation augmenting, supporting the function of nerve cells and nervous system and simultaneously minimizing, preventing the nerve cells apoptosis and preventing the development of diseases e.g. Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and others in adolescent, adult and fetuses in mammals, including people.

Thus according to two aspect of the present invention, there is provided the new use in doses 0.001-1g/kg/day or in concentration 0.001mM-100mM at least one member selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such as Na, Ca, Mg, Cu, Sr, Zn, K for manufacture of the pharmaceutical and/or nutritional preparation augmenting for protection the function of the nervous system against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases .

Thus according to three aspect of the present invention, there is provided the new use in doses 0.001-1g/kg/day or in concentration 0.001mM-100mM at least one member selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such as Na, Ca, Mg, Cu, Sr, Zn, K for manufacture of the pharmaceutical and/or nutritional preparation augmenting for protection of the growth, stimulation of reproduction and prevention of apoptosis of nerve and other cells *in vitro* and in biotechnological processes with the use of somatic and bacterial cells.

Thus according to four aspect of the present invention, there is provided the new use in doses 0.001-1 g/kg/day or in concentration of 0.001-100 mM at least one member selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such as Na, Ca, Mg, Cu, Sr, Zn, K and other for the manufacture of the pharmaceutical and/or nutritional preparation blocking the activity of glutaminergic receptor and augmenting, supporting the function of nerve cells and the nervous system and simultaneously minimizing, preventing the nerve cells apoptosis and preventing the development of diseases, e.g. Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and others, in adolescent, adult and fetuses in mammals, including people, and protecting the function of the nervous system against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases in condition of pathological, systemic overproduction of glutamate and postprandial excessive absorption of glutamate used as the gustatory addition to the food for mammals and people.

According to another aspect of the present invention there is provided a method augmenting, supporting the function of nerve cells and nervous system and simultaneously minimizing, preventing the nerve cells

apoptosis and preventing the development of diseases e.g. Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and others, in adolescent, adult and fetuses in mammals, including people, and protecting the function of the nervous system against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases and protecting the growth, stimulating the reproduction and preventing the apoptosis of nerve and other cells *in vitro* and in biotechnological processes with the use of somatic and bacterial cells and that method relates to the use of at least one member, effectively counteracting, selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such Na, Ca, Mg, Cu, Sr, Zn, K in doses 0.001-1g/kg/day or in concentration 0.001mM-100mM in a form of pharmaceutical drug and/or food/ feed additive.

According to a further aspect of the presesnt invention there is provided a method

blocking the activity of glutaminergic receptor in condition of pathological, systemic overproduction of glutamate and postprandial excessive absorption of glutamate used as the gustatory addition to the food for mammals, including people, augmenting, supporting the function of nerve cells and the nervous system and simultaneously minimizing, preventing nerve cells apoptosis and preventing the development of diseases, e.g. Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and others in adolescent, adult and fetuses in mammals, including people, and protecting the function of the nervous system against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases and that method refers to the use of at least one member, effectively counteracting, selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino

acids and salts of alpha-keto-glutarate with metal ions such Na, Ca, Mg, Cu, Sr, Zn, K and others in doses 0.001-1g/kg/day or in concentration 0.001mM-100mM in a form of pharmaceutical drug and/or food/ feed additive.

In compliance with the invention alpha-ketoglutarate and its salts: ornithyno-alpha-keto glutarate, asparagine-alpha-keto glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such Na, Ca, Mg, Cu, Sr, Zn, K and other augments, supports the function of nerve cells and nervous system and simulateneosly minimalizes, preventing apoptosis of nerve cells, and prevents the development of diseases, e.g. Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and others, in adolescent, adult and fetuses in people and animals, and protects the function of the nervous system against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other connected or not with excessive amount of glutamate in blood and cerebrospinal fluid and protects the growth, stimulates reproduction and prevents apoptosis of nerve and other cells *in vitro* and in biotechnological precesses with the use of somatic and bacterial cells.

An example of the preparation and its use is presented in the diagram, the figure 1 in the diagram presents the vitality of neurons subjected with AKG for 48 hours, and the figure 2 presents the vitality of neurons subjected with the trophic stress in the presence of AKG after 24 hours and the figure 3 - the vitality of neurons subjected with glutamate in the presence AKG after 24 hours.

Neurons culture.

Neurons culture were composed of the brains of 18-days rattish fetuses. The brain tissue was subjected with the 0.25% solution of trypsin-EDTA for dissociation to single cells. The suspension of cells with the density of 5×10^5 cells/ml was poured out on 96-pits microplates capsuled with poli-L-lysine in the concentration of 100mcl per pit, using the medium

of Neurobasal + 2% B-27 supplement (Life Technologies) with the addition of 1% antybiotic/antymycotic solution (Life Technologies). Neurons were cultured for 14 days in the temperature 37°C in the atmosphere 95% air/ 5% CO2. The medium was changed every 3 days. The identification of neurons was performed immunocytochemically by colouring cells with the characteristic marker - NSE (neuron specific enolase).

The activity of AKG in the neurons culture.

The influence of AKG on the vitality of neurons was examined in 14-days culture. For that purpose cells were subjected for 48 hours with AKG in concentrations 0.1mM-10mM.

To assess the neuroprotective effect the influence of AKG on the vitality of neurons subjected with the trophic stress (the removal from the medium the supplement B -27) and the action of the stimulating amino acid – glutamate - were examined.

The vitality of cells was assessed with MTT method.

MTT method (according to the kit "Cell proliferation kit III", Boehringer Manheim)

That method was worked out to determine the proliferation and vitality of cells in the researches on cytotoxic and antyproliferative substances. In metabolically active cells yellow tetrazolic salt MTT is reduced to blue formazane by mitochondrial dehydrases. Insoluble in water formazane crystals accumulate in cells and for their dissolution the use of organic detergent breaking the membranes and simultaneously solvent the dye is necessary. For that purpose the buffer SDS-HCl with pH 7.4 is used. The concentration of the releasing dye is assessed quantitatively in the reader for 96-pits microplates at the wavelength of 570 nm. The intensity of colouring is directly proportional to the quantity of alive cells.

15 µl of solution MTT in PBS in concentration 5 mg/ml was added to each pit on plastic plate. Plates were incubate for 3 hours in temperature 37°C, thereafter 100 µl of the buffer SDS-HCl was added to each pit and

plates were left overnight in temperature 37°C. The results were read next day with the use of reader E-max Reader (Molecular Devices Corporation, Menlo Park, CA, U.S.A.).

In compliance with the invention the research of the vitality of nerve cells showed the lack of cytotoxic activity with the use of AKG in the concentrations in the range of 0.1-10mM. Besides, AKG showed the trophic effect on the neurons culture, dependent of the dose used. At concentration of 0.25mM statistically signifficant (7.1%) increasing in the vitality of cells was obtained. This effect was greater with the increasing doses of AKG attaining the highest value of 38% at concentration of 5mM what is showed on the figure 1 of the diagram.

To demonstrate neuroprotective properties of the investigated substance the neurons cultures were subjected with neurodegenerative factors such the trophic stress and glutamate. There was shown that AKG according to the used dose reduces neurodegenerative influence of the trophic stress (serum deprivation - SD). The lowest used dose of AKG 0.1 mM caused statistically signifficant increase in neurons vitality (7.3%). Doses 0.5mM and 1mM of AKG intensified this effect to 14.3% and 19.1% respectively what was showed on figure 2 on the diagram. We should suppose that the inhibition of the neurodegenerative effect due to a trophic stress will be greater with the higher doses of AKG.

There was not show the protective action of AKG (0.1, 0.5 and 1mM) in the culture subjected with glutamate (0.5mM) what was presented on figure 3 on the diagram. We can not exclude that higher concentrations of AKG (2.5 and 5 mM) can protect neurons against neurotoxic activity of stimulating amino acids.

Performed research on the activity of alfa-ketoglutarate in neurons culture *in vitro* testifies for its potential neurotrophic and neuroprotective properties. It seems that the positive effect of AKG on nerve cells is rather due to the improvement of unefficient mitochondrial metabolism and with its antagonistic activity on glutaminergic receptors.

PATENT CLAIMS

1.The use as pharmaceutical drug or food/feed additive in doses 0.001-1g/kg/day or in concentration 0.001mM-100mM at least one member selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such as Na, Ca, Mg, Cu, Sr, Zn, K for manufacture of the pharmaceutical and/or nutritional preparation augmenting, supporting the function of nerve cells and nervous system and simultaneously minimizing, preventing the nerve cells apoptosis and preventing the development of diseases e.g. Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and others in adolescent, adult and fetuses in mammals, including people,

2. The use as pharmaceutical drug or food/feed additive in doses 0.001-1g/kg/day or in concentration 0.001mM-100mM at least one member selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such as Na, Ca, Mg, Cu, Sr, Zn, K for manufacture of the pharmaceutical and/or nutritional preparation augmenting for protection the function of the nervous system against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases .

3.The use as pharmaceutical drug or food/feed additive in doses 0.001-1g/kg/day or in concentration 0.001mM-100mM at least one member selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such as Na, Ca, Mg, Cu, Sr, Zn, K for manufacture of the pharmaceutical and/or nutritional preparation augmenting for protection of the growth, stimulation of reproduction and prevention of apoptosis of nerve and other

cells *in vitro* and in biotechnological processes with the use of somatic and bacterial cells.

4.The use as pharmaceutical drug and/or food/feed additive in doses 0.001-1 g/kg/day or in concentration of 0.001-100 mM at least one member selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such as Na, Ca, Mg, Cu, Sr, Zn, K and other for the manufacture of the pharmaceutical and/or nutritional preparation blocking the activity of glutaminergic receptor and augmenting, supporting the function of nerve cells and the nervous system and simultaneously minimizing, preventing the nerve cells apoptosis and preventing the development of diseases, e.g. Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and others, in adolescent, adult and fetuses in mammals, including people, and protecting the function of the nervous system against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases in condition of pathological, systemic overproduction of glutamate and postprandial excessive absorption of glutamate used as the gustatory addition to the food for mammals and people.

5. Method augmenting, supporting the function of nerve cells and nervous system and simultaneously minimizing, preventing the nerve cells apoptosis and preventing the development of diseases e.g. Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and others, in adolescent, adult and fetuses in mammals, including people, and protecting the function of the nervous system against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases and protecting the growth, stimulating the reproduction and preventing the apoptosis of nerve and other cells *in vitro* and in biotechnological processes with the use of somatic and bacterial cells and that method relates to the use of at least one member, effectively

counteracting, selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such Na, Ca, Mg, Cu, Sr, Zn, K in doses 0.001-1g/kg/day or in concentration 0.001mM-100mM in a form of pharmaceutical drug and/or food/ feed additive.

6.Method blocking the activity of glutaminergic receptor in condition of pathological, systemic overproduction of glutamate and postprandial excessive absorption of glutamate used as the gustatory addition to the food for mammals, including people, augmenting, supporting the function of nerve cells and the nervous system and simultaneously minimizing, preventing nerve cells apoptosis and preventing the development of diseases, e.g. Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and others in adolescent, adult and fetuses in mammals, including people, and protecting the function of the nervous system against its degeneration due to apoptosis of nerve cells in Alzheimer, Parkinson, Huntington, Creutzweld-Jakob, BSE and other diseases and that method refers to the use of at least one member, effectively counteracting, selected from the group consisting of alpha-ketoglutarate and its salts: ornithyno-alpha-keto-glutarate, asparagine-alpha-keto-glutarate and other salts with amino acids and salts of alpha-keto-glutarate with metal ions such Na, Ca, Mg, Cu, Sr, Zn, K and others in doses 0.001-1g/kg/day or in concentration 0.001mM-100mM in a form of pharmaceutical drug and/or food/ feed additive.

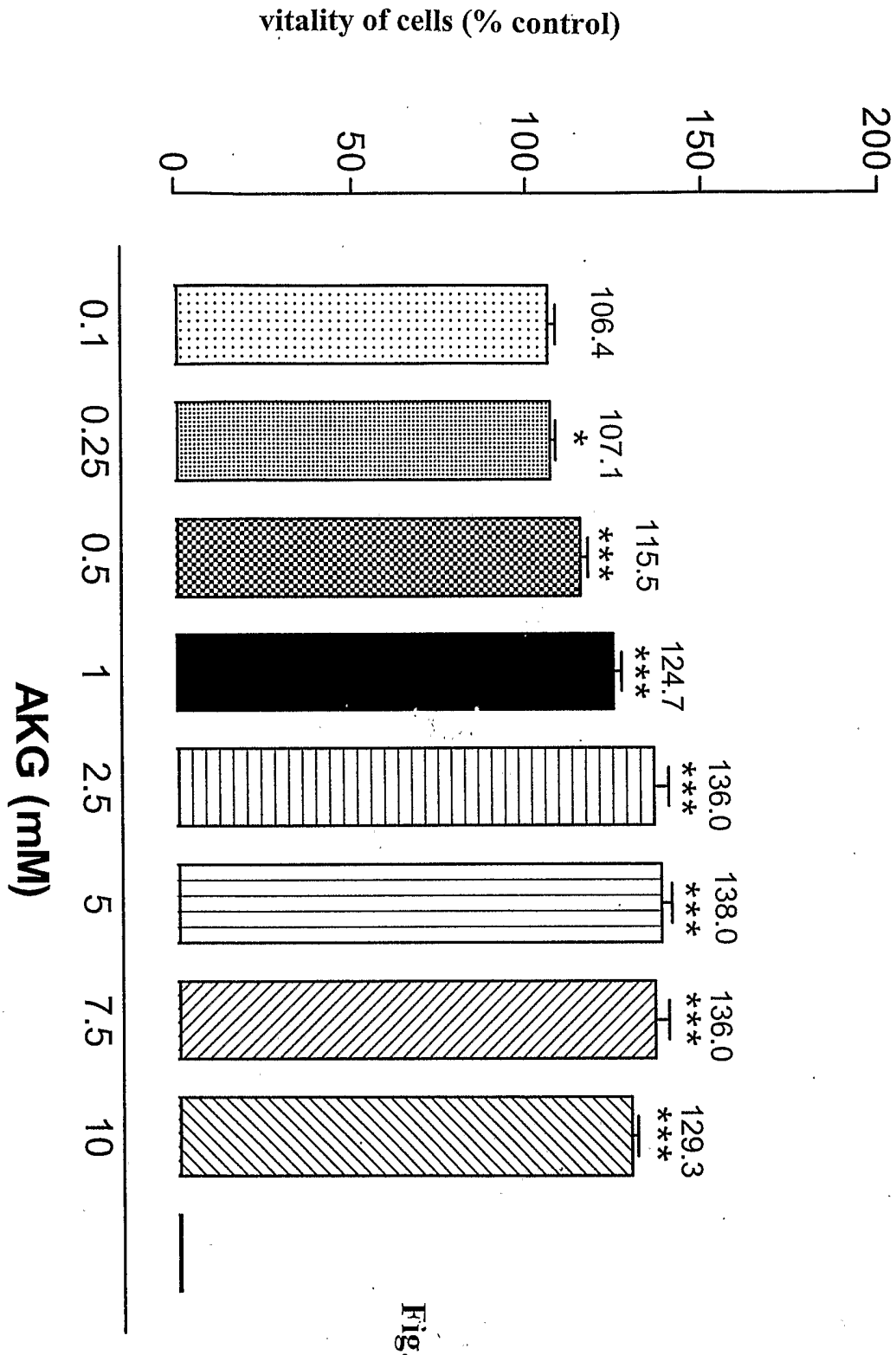
vitality of cells (% control)
0
50
100
150
200
AKG (mM)
0.1
0.25
0.5
1
2.5
5
7.5
10
106.4
107.1
*
115.5
***
124.7
***
136.0
***
138.0
***
136.0
***
129.3
***

Fig. 1

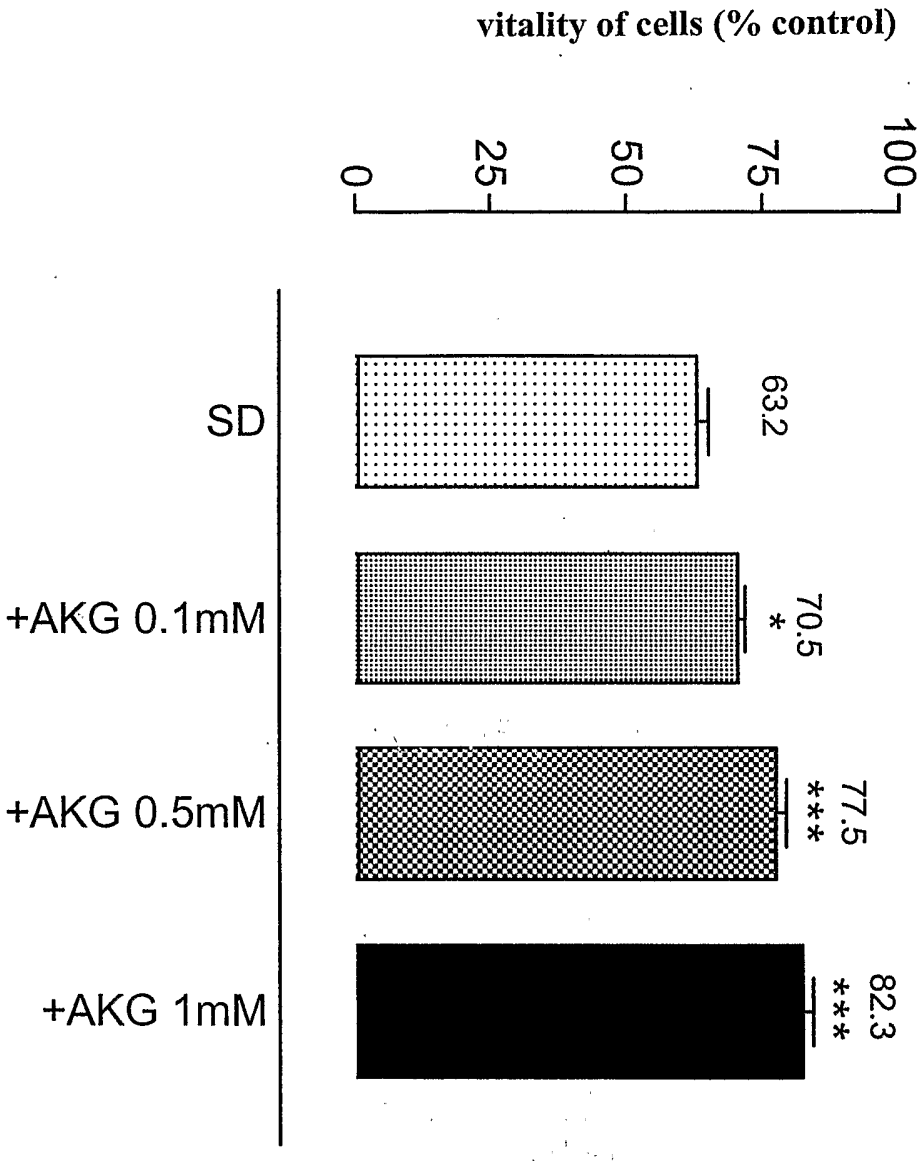
vitality of cells (% control)
0
25
50
75
100
SD
63.2
+AKG 0.1mM
70.5
*
+AKG 0.5mM
77.5
***
+AKG 1mM
82.3
***

Fig. 2

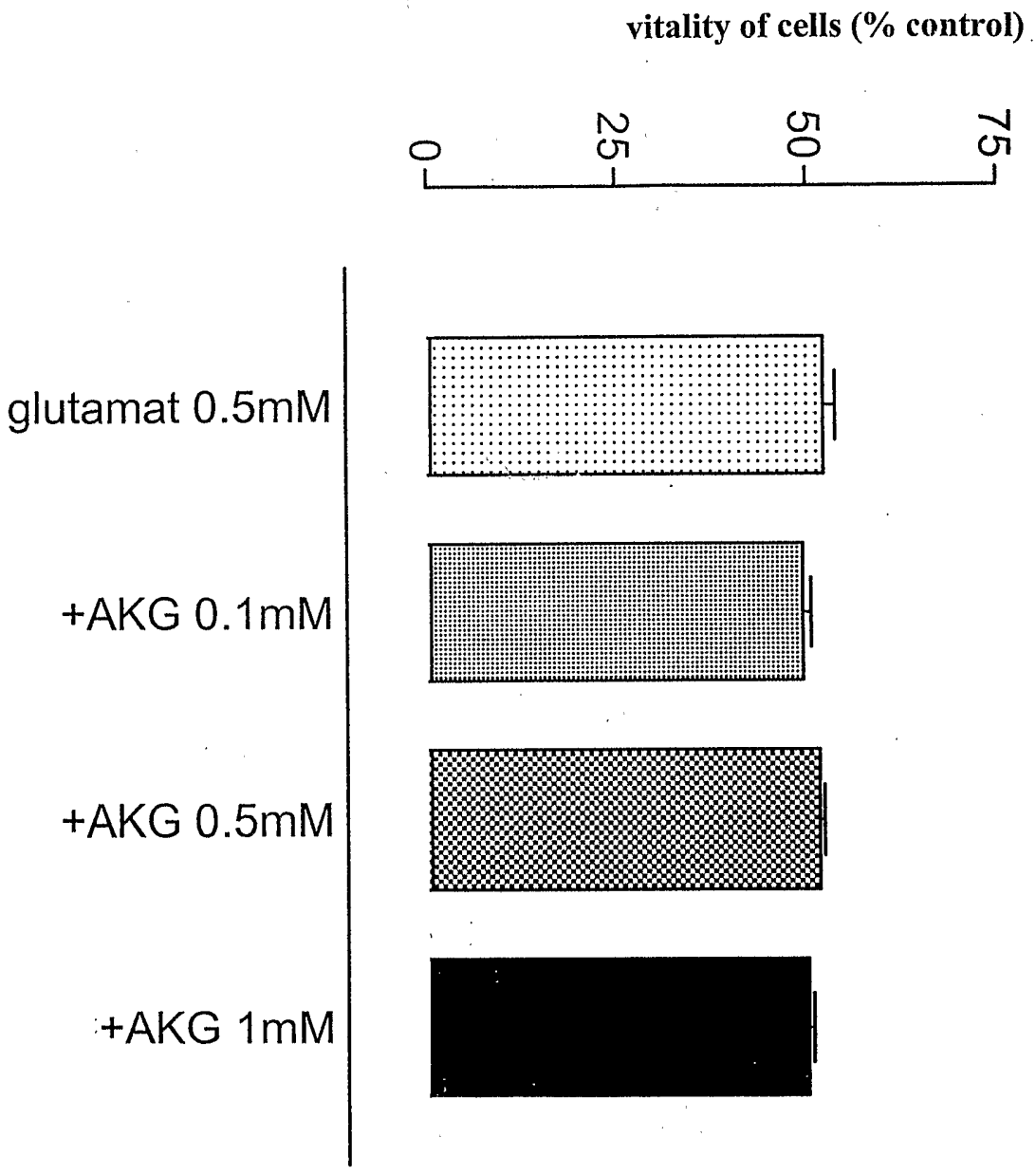
vitality of cells (% control)
0
25
50
75
glutamat 0.5mM
+AKG 0.1mM
+AKG 0.5mM
+AKG 1mM

Fig. 3