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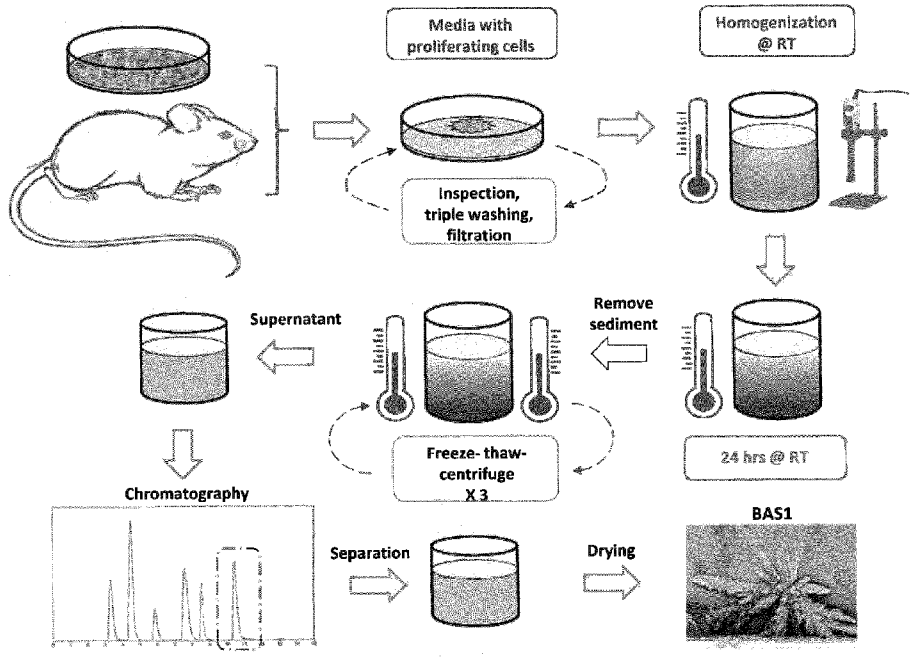
Figure 1



(57) Abstract: The present disclosure relates, inter alia, to biologically active substances extracted from animal tissue or cell culture comprising non- pathologically proliferating cells, which substances simultaneously (i) induce and/or intensify cell proliferation; (ii) induce and/or intensify apoptosis; and (iii) have antibacterial activity, and methods of extracting said substances. The present disclosure also relates to the use of such biologically active substances.

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NOVEL COMPOUNDS AND METHODS

Field of the invention

The present disclosure relates, *inter alia*, to biologically active substances extracted from animal tissue or cell culture comprising non-pathologically proliferating cells, which substances simultaneously (i) induce and/or intensify cell proliferation; (ii) induce and/or intensify apoptosis; and (iii) have antibacterial activity, and methods of extracting said biologically active substances. The present disclosure also relates to the use of such biologically active substances.

Background

Cell proliferation and factors inducing cell proliferation

Most cells that either are cultured *in vitro* or present in animal tissues can proliferate provided that suitable nutrients and other necessary conditions are supplied. However, according to accepted modern understanding, most normal cells have a limited growth potential, and after a certain number of cell divisions ("the Hayflick limit"), they can no longer proliferate (Hayflick L., *Exp. Cell. Res.* 37:614-636, 1965). This limited life span termed "replicative senescence" likely arose as a protective evolutionary mechanism in long-lived animals. Therefore, it has long been a goal to induce and support the proliferation ability of all types of cells *in vitro* and *in vivo* as a key to longevity.

"Immortalization" in the present context refers to the escape from the normal limitation on proliferation of the cell population *in vitro* or *in vivo* and is not related to the fate of individual cell and possibility of its eternal life. Once immortalized, a cell population can be continuously cultured, or can function continuously and for a long time *in vivo*. However, immortal cell lines very rarely emerge spontaneously under usual conditions. In animal tissue with correctly functioning mechanisms of the homeostasis and apoptosis in proliferation niches, activation of the nonpathogenic proliferation with simultaneous intensification of the apoptosis primarily disposing of the damaged and aging cells leads to rejuvenation of the niche. Homeostasis in the space-localized proliferation niche is understood as a continuous stabilization of the average

cell concentration. In such case, intensification of the cell division with the stabilization of the overall cell concentration means the shift of the average cell age towards niche rejuvenation: cell division increases the number of young cells, and apoptosis aims at removing old and damaged ones. In contrast, increasing the lifetime of the individual cells in the proliferation niche stabilized by homeostasis leads to the suppression of the proliferation and aging of the niche. Accordingly, in the present context, the term "immortality" does not refer to the life of an individual cell, but only to the possibility of long-term maintenance of the functioning of the cell population as a whole. Correspondingly, the rejuvenation of the homeostasis-governed proliferation niches is related to the increase in the proportion of younger cells in the cell population of the niche.

For the term "proliferation niche", we follow the definition by *The Nature* (https://www.nature.com/subjects/stem-cell-niche) "*A stem-cell niche is an area of a tissue that provides a specific microenvironment, in which stem cells are present in an undifferentiated and self-renewable state. Cells of the stem-cell niche interact with the stem cells to maintain them or promote their differentiation.*" Correspondingly, we define a "proliferation niche" as a space-defined tissue area or a restricted volume of culture with proliferating cells with specific or specialized microenvironment, in which dividing cells support the functioning of a cell population.

In order to increase the life span of cells in culture, and in attempts to achieve the rejuvenation of human and animal tissues a number of published techniques have included the use of embryonic cells. Such strategy is based upon different assumptions, including that embryonic cells are less differentiated than other cells, proliferate at a greater rate than differentiated cells, can maintain own proliferation for significant time, and can stimulate proliferation of other cells. Tumor cells are also known for their greater potential for proliferation, and some of the known methods use this property for promoting proliferation in cell cultures. However, the limited number of appropriate cell types and lines that can be used in such way, the limited number of phenotypes that they are able to generate, and their inherent

tumorigenicity, make these types of cell lines less than ideal and unsuitable for *in vivo* applications.

In culture, normal cells have been transformed by various means including the use of UV light, chemical carcinogens, and the introduction of oncogenes, which induces the cell to proliferate indefinitely. Certain viruses are also known to immortalize human cell cultures from different tissues leading to continuously growing cell lines (Sack, G. H. *In vitro.* 17:1-19, 1981). Unfortunately, it is hardly possible to immortalize the mammalian cell cultures while maintaining full range of the properties of initial cell cultures using such technologies. Although such methods can be and are used in research and testing the effects of various exogenous agents, they are so far not applicable for maintaining increased longevity or immortalization of the cell cultures while preserving their functioning in correct manner even *in vitro.*

One of the potential ways of cell population rejuvenation is related to the technology using cell reprogramming, claiming the possibility for stimulating cells for acquiring the stem cell properties (induction to pluripotent stem cell state) and increasing the cell population capacity for proliferation. Corresponding method was initially proposed by Yamanaka and Takashi, *Cell.* 126(4): 663-676, 2006, and is continuously explored and developed further (see, e.g., Guo, X-L and Chen J-S, *Int J Ophthalmol.* 8(4): 818–825, 2015). Corresponding family of the methods use so-called Yamanaka factors (four basic transcription factors in different combinations).

There are multiple applications for *in vitro* animal cell cultures depending on their longevity while preserving all properties of the initiating cell culture, including biomedicine, research and industry. One of the widely used industrial applications is in production of various proteins, peptides, hormones, growth factors, transcription factors and other biologically active substances and corresponding technologies can in some sense be referred to as ”biofactories”. Increasing longevity of such cell lines and cultures while maintaining the production of desired substances can have direct impact on increased productivity of such ”biofactories”.

In addition, many modern methods of supporting transplantation have been developed. For example, cultures of dividing cells are created and used

for the artificial cultivation of replacement tissues and even synthetic embryos (Tarazi *et al.*, *Cell*. 185: 1–17, 2022). Moreover, modern research associated with the so-called "bioprinting" when living cells are used for "additive constructing" of tissues and organs is rapidly transforming into technologies. Such technologies would benefit significantly from methods that enhance and maintain the proliferative potential of various mammalian cells in both traditional cell culture and cell culture supported by complex three-dimensional scaffolds, and their ability to multiply non-pathogenically, which allows a limited number of "seed cells" to form tissues.

Stem cells are believed to have immense potential for therapeutic purposes. Such cells can be derived from the donor sources, including, but not limited to, embryonic, blast, tissue-derived, blood, and cord-blood cells; organ-derived progenitor cells; and stromal cells among others. It is widely believed that from a therapeutic perspective alone, such cells may be useful for the treatment of a vast array of disorders. Moreover, primary stem cells that have exhibited the most plasticity are embryonic stem cells. However, obtaining large quantities of these cells is particularly problematic and raises ethical issues. In addition, introduction of foreign cells can cause various adverse effects, and in some cases, cosmetology or therapeutic use of stem cells not derived from the same individual without proper care can lead to serious health problems. In addition, technologies of deriving such cells from the same individual for further application are complex, and often do not yield adequate amounts of such cells. Stem cells supposedly also share with other cells the inability to divide indefinitely limiting intensive proliferation *in vitro*, limiting the accessibility to the needed quantities of such cells for diagnostic, investigational, or therapeutic purposes.

Stimulation of non-pathological proliferation also presumes that corresponding means of inducing proliferation *in vivo* should not disturb the mechanisms maintaining the proliferation niche homeostasis. It demands that corresponding factors should simultaneously affect the apoptosis and in correlated manner with cell proliferation, preventing uncontrollable growth of the cell numbers. As a consequence, factors that are able to induce and intensify the proliferation in the niche without disturbing the homeostasis can

lead to the ”rejuvenation” of the cell niche: intensified proliferation leads to increasing share of young cells, while the apoptosis intensify ”the removal” of older and damaged cells.

US 20110201105 discloses cDNA libraries obtained from Tammar wallaby (*Macropus eugenii*) mammary gland tissue at different times (including day 23 pregnancy, day 130 lactation, and day 260 lactation). Lactation-associated polypeptides were identified from these cDNA libraries, and bovine homologues of the wallaby proteins are also disclosed. The proteins have a range of activities including anti-apoptotic activity, pro- or anti-inflammatory activity, cathelicidin anti-microbial activity, induction of trefoil proteins (and protection of epithelial surfaces), increased cell proliferation, and induction of cell differentiation (and loss of pluripotency).

EP 0673653 A1 discloses a biologically active agent (BAA) based on components of cell membranes (CCM) of homogenized animal tissue which consists of organic substances and has immunomodulating properties. The agent contains modified components of cell membranes with antigen structure modified during the processes of embryogenesis and/or proliferation and/or differentiation of cells, and/or during a pathology preceding or accompanying the process of regeneration and/or repatriation of the tissue. A method for obtaining BAA is disclosed, as well as a pharmaceutical preparation for normalization of physiological state of human beings and animals based on the organic substances. CCM of animal tissues comprises, as active agent, BAA.

WO 9723501 A1 relates to molecules capable of modulating apoptosis of animal cells and discloses cell homologues of viral-derived, apoptotic-inhibiting molecules which are useful in modulating apoptosis of animal cells. The molecules may be used to promote or inhibit cell apoptosis depending on the exigencies of the therapeutic situation.

JP 2006246764 A discloses a method for producing an endogenous antibacterial substance; the method comprises culturing Paneths cells isolated from a crypt collected from a mammalian small intestine in the presence of an inducer by using a culture scaffold as a material to supplement a biological matrix function and secreting an endogenous antibacterial substance.

CN 112921001 A discloses an exosome containing a high-expression NAMPT protein, and a method for preparation and application thereof. The exosome containing high-expression NAMPT protein is obtained by transfecting NAMPT mRNA synthesized in vitro into iPS cells. The content of the NAMPT protein in the exosome is more than 2 times of the content of the NAMPT protein in the exosome secreted by common iPS cells, and the exosome containing high-expression NAMPT protein can improve the condition of senescent cells and has a protective effect on neurons compared with the exosome secreted by common iPS cells.

CN 111658672 discloses a human placenta stem cell extract freeze-dried powder and a preparation method thereof, in particular, a preparation method of a human placenta stem cell extract freeze-dried powder. The human placental stem cell freeze-dried powder has the effects of comprehensively regulating an aged organism; it can raise the metabolism levels of an organism, raise physiological function, raise the immunity of an organism, prolong puberty period, beautify skin, prolong life and delay senility, and at the same time, the freeze-dried powder is long in storage period, not easy to degrade and convenient for use.

CN 101152215 discloses an ovarian granulosa cell anti-aging preparation, and a method of preparation method and application of the product. The invention uses ovarian granulosa cells in preparation of anti-aging products. The ovarian granulosa cell anti-aging product is in vitro cultivated and contains amplified cultivation supernatant of female mammalian animal ovarian granulosa cells and/or the cell extract, and one or more pharmaceutically acceptable medical excipient and antioxidant. Via the ovarian granulosa cells, the invention can proliferate and secrete a plurality of bioactive substances.

RU 2728601 discloses a method of testing substances affecting aging processes by analyzing patient blood. The method involves producing patient's mononuclear cells by standard venous blood sampling with subsequent isolation of mononuclear leukocyte (MNC) population, incubation of the patient's MNC culture with the recommended doses of the test agent and determining the ratio of cells in different phases of the cell cycle by flow cytometry in the MNC culture, followed by evaluating the obtained results using

the difference in cell ratio distribution on the cell cycle. The method provides the possibility of testing the activity of substances and preparations aimed at treating various pathologies, including those associated with age.

The above description of the state-of-the-art makes it apparent that there is a need for agents that maintain the longevity of cell populations both *in vitro* and *in vivo* without damaging the homeostasis, providing larger and less costly supplies of cells maintaining their initial properties, supporting biomedical research, tissue engineering and transplantation and providing the ability to rejuvenate animal tissues. In particular, this includes cells and cell lines of mammalian origin, such as of human origin.

Summary of the invention

The present invention is based on the inventors' discovery of biologically active substances that simultaneously combine certain characteristic activities of induction and/or intensification of cell proliferation, induction and/or intensification of apoptosis and antibacterial activity, which represents a not previously described combination of properties of bioactive factors modulating cell proliferation and promoting tissue and cell culture rejuvenation. Moreover, there is a direct correlation between the biologically active substances' effects of inducing and/or intensifying proliferation, inducing and/or intensifying apoptosis and having antibacterial activity. In some embodiments, said biologically substances have anti-inflammatory activity. The biologically active substances are distinguishable from exosomes which are extracellular vesicles that are released from cells upon fusion of an intermediate endocytic compartment, the multivesicular body (MVB), with the plasma membrane.

Thus, according to a first aspect of the invention, there is provided a biologically active substance extracted from animal tissue or cell culture comprising non-pathologically proliferating cells, which substance simultaneously (i) induces and/or intensifies cell proliferation; (ii) induces and/or intensifies apoptosis; and (iii) has antibacterial activity. Optionally, said biologically active substance has anti-inflammatory activity.

Suitably, the biologically active substance has or comprises one or more characteristics selected from the group consisting of: a molecular weight of less than 1.5 kDa, long linear hydrocarbon chains, hydrocarbon fragments, steroid fragments, alkane fragments, high molecular weight unsaturated hydrocarbon fragments, phosphates, hydroxyl groups, amino groups and amide groups. Preferably, the biologically active substance has a molecular weight of less than 0.8 kDa, such as less than 0.7 kDa.

Preferably, the biologically active substance is capable of shifting the cell cycle in a cell population such that there is an increased proportion of cells in the stages just before or just after cell division.

Preferably, the biologically active substance of this aspect induces and/or intensifies cell proliferation and apoptosis such that there is a correlation between induction and/or intensification of cell proliferation and induction and/or intensification of apoptosis. Preferably such that intensification of cell proliferation is correlated with intensification of apoptosis.

The biologically active substance is suitably not carcinogenic.

In some embodiments according to this aspect of the invention, the biologically active substance is Biologically Active Substance 1 (hereinafter referred to as BAS1).

In some embodiments according to this aspect of the invention, the biologically active substance is not BAS1.

According to a second aspect of the invention, there is provided a fragment, analog or derivative of the biologically active substance according to the first aspect, which fragment, analog or derivative simultaneously possess the properties defined in the first aspect.

According to a third aspect of the invention, there is provided a composition comprising the biologically active substance according to the first aspect, or the fragment, analog or derivative according to the second aspect, and at least one pharmaceutically acceptable excipient or carrier.

According to a fourth aspect of the invention, there is provided a method of supporting, intensifying and/or activating proliferation of animal cells *in vitro* comprising the step of contacting said cells with one or more of the biologically active substances according to the first aspect, the fragment, analog or

derivative according to the second aspect, or the composition according to the third aspect. Typically, said animal cells are mammalian cells.

According to a fifth aspect of the invention, there is provided a cosmetic method of supporting, intensifying and/or activating proliferation of animal cells *in vivo* comprising the step of contacting said cells with one or more of the biologically active substances according to the first aspect, the fragment, analog or derivative according to the second aspect, or the composition according to the third aspect.

Said animal cells are typically mammalian cells, such as human cells.

According to a sixth aspect of the invention, there is provided a biologically active substance according to the first aspect, a fragment, analog or derivative according to the second aspect, or a composition according to the third aspect for use as a cell proliferation inducer and/or enhancer, an apoptosis inducer and/or enhancer, an anti-inflammatory agent or an antibacterial agent.

According to a seventh aspect of the invention, there is provided a biologically active substance according to the first aspect, a fragment, analog or derivative according to the second aspect, or a composition according to the third aspect for use as implant coating. In one particular embodiment, said implant coating is dental implant coating.

According to an eighth aspect of the invention, there is provided a method of long-term storage of the biologically active substance according to the first aspect or the fragment, analog or derivative according to the second aspect using drying and/or lyophilization and sorption on biopolymers, such that when not exposed to direct sunlight or UV-light, said substance can be stored in dry conditions at room temperature (e.g. 18-24 °C) without degrading its activity for at least 6 months, such as for at least 12 months.

According to a ninth aspect of the invention, there is provided a process of extracting a biologically active substance from non-pathologically dividing cells or cell cultures, said process comprising the steps of (i) prior to extraction, adding the biologically active substance according to the first aspect, the fragment, analog or derivative according to the second aspect, or the composition according to the third aspect to said cells or cell cultures; and (ii) extracting the biologically active substance, wherein said addition in step (i)

improves the yield of the extracted biological substance. It will be appreciated that the added biologically active substance may be the same as or different to the extracted biologically active substance. In one embodiment according to this aspect of the invention, said added biologically active substance is an autoinducer.

According to a tenth aspect of the invention, there is provided a method of identifying a substance for use as a cell proliferation inducer and/or enhancer, an apoptosis inducer and/or enhancer, an anti-inflammatory agent and/or an antibacterial agent according to the sixth aspect, said method comprising (a) providing a test substance; and (b) assessing whether the test substance shifts the cell cycle in a cell population such that there is an increased proportion of cells in the stages just before or just after cell division, wherein a test substance that shifts the cell cycle is useful as a cell proliferation inducer and/or enhancer, an apoptosis inducer and/or enhancer, an anti-inflammatory agent and/or an antibacterial agent.

In one embodiment, the method according to this aspect is an *in vitro* method.

The biologically active substance according to any one of the aforementioned aspects may be extracted from homogenized animal tissues or cell cultures containing actively and non-pathologically proliferating cells, followed by centrifugation, freezing-thawing, filtration and separation of the supernatant into fractions.

Preferably, the extraction is performed by liquid chromatography with a carrier in the form of pure water or isotonic sodium chloride solution.

Suitably, said extraction is performed by liquid phase extraction using organic solvents.

Brief description of the figures

Figure 1 is a flow chart illustrating the production of BAS1.

Figure 2 represents low (A-C) and high (D-F) resolution SEM images of typical crystallite shapes appearing after recrystallization of BAS1. Embedded bars reflect the used magnification.

Figure 3A is a characteristic IR absorption spectrum (absorbance vs wavelength number) of dry BAS1 taken in dry chamber under argon atmosphere. The bars mark corresponding peaks belonging to organic molecules. Figure 3B is an overall ^{1}H NMR spectrum of BAS1 in D_2O. Figure 3C is a ^{1}H NMR spectrum of BAS1 in D_2O between 0.9 and 5 ppm. Figure 3D is a ^{1}H NMR spectrum of BAS1 in D_2O between 4.2 and 7.8 ppm. Figure 3E is a 2D ^{1}H NMR spectrum of BAS1 in D_2O. Figure 3F is a ^{13}C NMR spectrum of BAS1 in D_2O. Figure 3G is a ^{31}P NMR spectrum of BAS1 in D_2O between -50 and 200 ppm, and its insert shows the part of the spectrum between -5 and 6 ppm.

Figure 4 is a typical SEM image of dried BAS1 with marked scan area and corresponding EDX (energy dispersive X-ray) spectrum with automated element abundance analysis.

Figure 5 is an HPLC spectrum of BAS1.

Figure 6 shows two more HPLC spectra of BAS1.

Figure 7 shows graphs that present the results of automated identification carried out for BAS1 by the gas chromatography system. Figure 7A shows alkanes (red) and phthalates (blue), without full identification. Figure 7B shows automated identification of fatty acids, cholesterol, sitosterol. Irgafos® 168 (a polymer stabilizer, most probably contamination). Figure 7C shows clear identification of squalene ($C_{30}H_{50}$) and hopane ($C_{30}H_{52}$).

Figures 8A-B illustrate the changes induced by BAS1 in the duration of the phases of the cell cycle in the culture of human dermal fibroblasts studied by flow cytometry. Figure 8A: experiment without BAS1 (Control). Figure 8B: experiment with a single addition of BAS1 at day 0. Figures 8C-D the changes induced by BAS1 in the duration of the phases of the cell cycle in the culture of human blood monocytes studied by flow cytometry, wherein Figure 8C refers to the experiment without BAS1 (Control) and Figure 8D with a single addition of BAS1 at day 0.

Figures 9A-D show the changes induced by BAS1 upon the cell distribution over the cell cycle phases as studied by flow cytometry for human blood mononuclear cells in cell culture (A- apoptosis; B-G0+G1; C-S; D-G2). Corresponding dependencies show the changes happening with addition of

different concentrations of BSA1 on day 0 as compared to the control. Horizontal axis: days of incubation. Corresponding BAS1 doses are given in mkg/ml.

Figures 10A-D show the changes induced by BAS1 upon the cell distribution over the cell cycle phases, and Figure 10E upon cell vitality as studied by flow cytometry for human fibroblasts in cell culture. Corresponding dependencies show the changes happening with addition of different concentrations of BAS1 at day 0 as compared to the control. Horizontal axis: days of incubation. Corresponding BAS1 doses are given in mkg/ml.

Figures 11A-D show typical changes induced by BAS1 in cell cycle distribution of the human mononuclear blood cells (healthy volunteers). Single dose (20 mg) of dry BAS1 was administered orally at day 0.

Figures 12A-D present the measurements of the human blood mononuclear cell cycle distribution changes performed with male and female volunteers between 16 and 86 years of age according to the method described in Russian patent no. 2728601, which suggests a quantitative criterion for the assessment of biologic age of the cell population. It is based on the distribution of the cells in the population over the cell cycle stages. It is suggested that the higher fraction of cells in the cell cycle stages immediately preceding and/or following the division, the younger is the cell population.

In one of the suggested embodiments, methodologically the measurements can be performed using flow cytometry. In practical application this can be used for the quantified assessment for action of any substance claimed to have rejuvenating or ageing activity by comparing the cell cycle distribution in the cell population before and after administering the substance. Such tests can be performed both *in vitro* and *in vivo*. In one of the embodiments, *in vivo* testing is performed using mononuclear cells extracted from venous blood.

Figure 13 illustrates the protocol used for mononuclear cell extraction from venous blood used for the tests on the presence of BAS1.

Figure 14 illustrates the differences in the intensity of cell proliferation and apoptosis (measured by flow cytometry using reference biomarkers) in the control group (CO, no treatment), and in three test groups with the

treatment using (TO) the traditional method; (TR) the BAS1 and hyaluronate gel composition; and (BAS1+GI) hyaluronate gel only (GI).

Figure 15 illustrates the differences in intensity of cell proliferation and apoptosis in the control group (with no treatment, CO, control) and the group treated with gel only (GI), used in further experiments as a carrier for BAS1.

Figure 16 illustrates the differences in intensity of cell proliferation and apoptosis (measured by flow cytometry using reference biomarkers) in the groups treated in the traditional way (TR) and with hyaluronate gel (carrier) and BAS1 as active ingredient (BAS1 + GI).

Figure 17 present the results of the WST-1 cell vitality tests in the case of undiluted and 1:10 diluted tested substances. Graphs represent the effect of undiluted (left) and tenfold diluted (right) hyaluronic acid preparations on PDLSC cell viability. The absorbance value is measured using the WST-1 cell proliferation reagent, proportional to the number of viable cells. Star indicates the results that were showing $p < 0.05$ as compared to control.

Figure 18 presents the results of the effect of tested substances on stimulating spontaneous osteogenic differentiation based on Koss densitometry. The samples were analyzed by densitometry. The darkening in the corresponding image is directly proportional to the degree of mineralization. Mineralization in the HYL and ALC cases is significantly higher compared to the control (Cont) and NTS * $p < 0.05$ vs control, # $p < 0.05$ vs NTS.

Figure 19 presents analysis of the alkaline phosphatase activity. The absorbance is proportional to ferment activity as measured using an ALP reagent.

Figure 20 presents the results of inflammation assessment after 31 days of treatment by used preparations. Control group was injected with saline solution, test groups with the solution of hyaluronate (HYL), hyaluronate (carrier) and BAS1 as an active agent (BAS1) and NST solution (NST). Induction of the periodontitis was performed at day 0 on the left side of the mouth. The difference between treatment groups is significant (p value < 0.05 versus linked side control). Within the groups, the difference between the left and right sides was compared and evaluated. A significant difference was

found between the left (induced) and right (not induced) sides only in the control group.

Figures 21A-C present the comparison of the treatment results using BAS1-hyaluronate composition and commercial composition FlexBarrier, and comparison between both results. Test results indicate that both BAS1 + hyaluronate and FlexBarrier compositions are equally suitable for the treatment of interdental papillary defects, with BAS1 + hyaluronate showing better clinical results.

Figures 22A-H illustrate correlated action of BAS1 upon the cell cycle stages and apoptosis of the cultivated human monocytes. Monocytes were extracted from the venous blood of healthy human volunteers aged between 15 and 61 years. Figures 22A-D illustrate the difference in action of the PGA and BAS1 at different concentrations (graphs represent the ratios of the corresponding values characterizing the cell stage population and apoptosis intensity); Figures 22E-F illustrate the difference in action of the PGA and BAS1 on the rest and proliferation phases. In the graphs of Figures 22A-F, the data averaged for all subjects without separation into age sub-groups). Figures 22G-H illustrate the correlation of the action of PGA and BAS1 upon the cell stages (synthesis, S and proliferation, P) for the young (age 15 to 20) and mature (aged 21 to 61) volunteer sub- groups. Graphs represent the ratios of the corresponding values characterizing the cell stage population and apoptosis intensity, which are averaged for the corresponding age sub-group.

Figure 23 shows Tables 1 and 2, wherein Table 1 provides a summary of the EDX (energy dispersive X-ray) analysis of the elemental content from different sites on the individual crystallites of the dried BAS1. Typical spectrum at the site S0 is shown in Figure 4. Table 2 provides a resulting elemental content of the BAS1 crystallites averaged from different sites analyzed by EDX.

Figure 24 shows Table 3, which presents results of the high-performance liquid chromatography (HPLC) studies summarized as a list of suggested identifiable compounds semi-automatically generated using the embedded instrument features and linked substance databases.

Figure 25 shows Table 4, which presents results of two more HPLC analyses of BAS1, summarized as the semi-automatically generated identifiable compound listings.

Figure 26 shows Tables 5A-D, which provide the list of the identifiable compounds resulting from liquid and gas chromatography studies of BAS1, separated into few categories (hormone-like substances, amides-amines, phosphates, aliphatic substances).

Figure 27 shows Tables 6-8, wherein Table 6 provides the experimental data (average values of Bactericidal Activity Index) on the antibacterial activity of BAS1 for different strains of bacteria taken from a large bacteria database. Tables 7 and 8 provide the experimental data on sensitivity of the bacteria to the action of BAS1 with the concentration 100 µg/ml for different clinical strains of bacteria.

Definitions

As used herein, the following definitions are provided to facilitate the understanding of the present invention.

The term "*biologically active substance*" can be used interchangeably with "cell extract".

"*BAS1*" or "*biologically active substance 1*" is a non-limiting example of a biologically active substance of the invention.

"Cell population" is the number of cells in a given area, both in cell culture and in live tissues and organs.

The terms "*exosomes*" can be used interchangeably with "*extracellular vesicles*".

The terms "*proliferate*" and "*multiply*" interchangeably refer to cell division. In the context of cell proliferation by contacting or otherwise acting on cells with the biologically active substances described herein, which may also be referred to herein as a proliferation factor, an inducer or an enhancer, it is contemplated that proliferation may include proliferation to the point of production of a continuous cell line (e.g., immortalization). Typically, the biologically active substances of the present invention have an effect on cells in contact with the substance as described and claimed herein. Upon contact

with the biologically active substance, cells in the population are induced to achieve a proliferation rate that is higher than the normal *in vitro* cell proliferation rate, thereby increasing the cells' potential for proliferation.

In the context of the present invention, the term "*non-pathological*" in relation to proliferation means that after division new cells do not show any abnormalities and do not have functional or structural deviations from the norm for that particular cell type.

In the context of the present invention, the term "*non-pathogenic*" in relation to proliferation means that no pathogens are generated during the division process or by the cells resulting from this process.

By the term "*autoinducer*" is meant a compound capable of inducing or stimulating its own synthesis. By the term "*animal cell proliferation inducer*" is meant a compound capable of inducing and stimulating the division of animal cells, such as mammalian cells. It will be appreciated that the animal cell proliferation inducer is able to induce and/or intensify animal cell division *in vitro* (in cell culture) and *in vivo*. In the context of the present invention, the biologically active substance may be an autoinducer and/or an animal cell proliferation inducer. It will be appreciated that while all biologically active substances of the present invention are animal cell proliferation inducers, only some of them are also autoinducers.

In the context of the present invention, the term "*apoptosis*" is primarily understood to be mitochondrial apoptosis.

"*Immortalization*" in the present context refers, as hereinbefore described, to the escape from the normal limitation on proliferation of the cell population *in vitro* or *in vivo* and is not related to the fate of individual cell and possibility of its eternal life.

"*Proliferation niche*" refers, as hereinbefore described, to a space-defined tissue area or a restricted volume of culture with proliferating cells with specific or specialized microenvironment, in which dividing cells support the proliferation functioning of a cell population.

"*Homeostasis*" is understood according the original definition given in Waddington, C. H. 1956. *Principles of Embryology*. See also Chuang, J. S. *et al. PNAS*, 2019, 116(30): 14852-14861, meaning the stabilization in time of a

chosen parameter value within a defined system. Similarly, the "*homeorhesis*" is understood as the capability of a system to maintain the value of chosen parameter following given dependency on time. Thus, *homeostasis* refers to the static and *homeorhesis*- dynamic regulation over the chosen parameter in the system.

Although the support of the cell population in the proliferation niche is essentially a dynamic process, we use the generally accepted term "*homeostasis in the proliferation niche*" instead of the more proper one referring to the homeorhesis.

"*Lysis*" refers to the disintegration of a cell by rupture of the cell wall or membrane.

In the context of the present invention, the term "*extraction*" encompasses conditioning of tissue or cells, such as cleaning, separation from damaged sections, homogenization and centrifugation, as well as more advanced separation stages (e.g. sedimentation, filtration and chromatography) and concentration (e.g. drying). Suitably, the extraction does not forcedly change the original structure of the extracted biologically active substance. Typically, the extraction does not involve any chemicals other than water, isotonic and biological buffer solutions.

"*Induction of proliferation or apoptosis*" in the context of present invention means the stimulation of these processes, including the action leading to separately or jointly starting (literally induction) or stimulation (enhancement) of the proliferation and apoptosis.

Detailed description of the invention

The present invention relates generally to biologically active substances extracted from animal tissue or cell culture comprising non-pathologically proliferating cells, which substances simultaneously (i) induce and/or intensify cell proliferation; (ii) induce and/or intensify apoptosis; and (iii) have antibacterial activity. Optionally, said substances have anti-inflammatory activity.

Thus, broadly speaking, the present disclosure is based on the inventors' discovery and development of biologically active substances that

advantageously can be used as cell proliferation inducers and/or enhancers, apoptosis inducers and/or enhancers, anti-inflammatory agents and/or antibacterial agents.

A person of skill in the art will be familiar with expressions such as "*antibacterial activity*" and "*antibacterial agent*". Nevertheless, for the sake of clarity, in the present context an antibacterial agent is to be understood to be a substance that, similar to antibiotics, reduces the growth of, or kills, undesirable bacteria (e.g. disease-causing microorganisms), which may include both gram-positive and gram-negative bacteria. See e.g., Example 5, which describes the antibacterial activity of BAS1.

An "*anti-inflammatory agent*" is to be understood as a substance that reduces inflammation, such as redness, swelling and pain, in the body, e.g. by blocking certain substances in the body that cause inflammation. See, for example, Example 4, which describes preclinical tests performed to assess the potential of BAS1 and other preparations containing BAS1 for tissue regeneration and inflammation prevention.

It has long been a goal to induce and support proliferation ability of all types of cells *in vitro* and *in vivo* as a key to longevity. However, as described in the background section, achieving this has been difficult and there is a clear need for novel agents and methods that achieve this goal.

The present inventors have advantageously managed to discover and derive, e.g. by extraction and purification, biologically active substances that possess the above-mentioned desirable characteristics and are typically suitable for both *in vitro* and *in vivo* applications.

Suitably, the biologically active substance of the invention has or comprises one or more characteristics selected from the group consisting of: a molecular weight of less than 1.5 kDa, long linear hydrocarbon chains, hydrocarbon fragments, steroid fragments, alkane fragments, high molecular weight unsaturated hydrocarbon fragments, phosphates, hydroxyl groups, amino groups and amide groups. Preferably, said biologically active substances have a molecular weight of less than 0.8 kDa, such as less than 0.7 kDa.

The biologically active substance described herein is preferably capable of shifting the cell cycle in a cell population such that there is an increased proportion of cells in the stages just before or just after cell division. In this regard, it will be appreciated that the higher the fraction of cells in the cell cycle stages immediately preceding and/or following the division, the younger is the cell population.

Suitably, the biologically active substance of this aspect induces cell proliferation and apoptosis in the proliferation niche such that there is a correlation between induction and/or intensification of cell proliferation and induction and/or intensification of apoptosis. Thus, the increase in the numbers of divided cells in a separated volume of the proliferation niche is accompanied with the correlated intensification of the apoptosis leading to the increased numbers of cells subjected to “programmed death”.

The biologically active substance is preferably not carcinogenic. Typically, the biologically active substance does not cause allergy reactions, does not show any, or only very mild, side effects and is typically compatible with most tested medicinal drugs.

In some embodiments according to this aspect of the invention, the biologically active substance is BAS1 which specific composition and properties are described in Example 1, including Figures 1-7, and Tables 1-5.

In some embodiments according to this aspect of the invention, the biologically active substance is not BAS1. Thus, while BAS1 has been exemplified herein, other biologically active substances are also encompassed by the invention as defined by the claims.

Extraction methods

A person of skill in the art will be familiar with various methods of extracting the biologically active substances of the invention from animal tissues and cell cultures. Non-limiting examples of such methods are discussed below.

Extraction from cells

The cells that the biologically active substances are extracted from may be of any type of non-pathologically proliferating cells. The proliferating cells may range in plasticity and may include, for example, blast cells, fertilized ova, non-fertilized gametes, embryonic stem cells, adult stem cells, precursor or progenitor cells, and highly specialized cells. Typically, the biologically active substances are extracted from cells that possess one or more proliferation activation receptors. See, e.g., Iatropoulos M. J. and Williams, G. M. "*Proliferation markers". Review. Exp Toxicol Pathol.* 48(2-3):175-81, 1996. A person of skill in the art will be familiar with current and more recently discovered proliferation activation receptors and cells possessing one or more of these receptors.

In some embodiments, the substances are isolated from the tissue intercellular (interstitial) fluid without cell destruction. See, for example, Wu, W., Yenkie, K.M. & Maravelias, C.T. *BMC Chem Eng* 1(21), 2019, or from the cells themselves through their destruction, followed by extraction, typically, but not exclusively, with a liquid agent. See, e.g., "Natural Bioactive Compounds. Technological Advancements". *Academic Press*, 2020. Pages 409-433, Chapter 21 – "Advances in extraction technologies: isolation and purification of bioactive compounds from biological materials".

It will be appreciated that methods with destruction of cells are more commonly used, as they are particularly suitable for industrial scaling and typically give a higher yield of the extracted substances. Suitably, the destruction of cells is achieved by osmotic destruction of membranes, also referred to as "*lysis*", under the action of enzymes, compression of tissues or homogenization of varying degrees of intensity. Freeze-thaw cycles and/or ultrasound may also be used to destruct the cells.

The substances may be extracted with a liquid agent, such as water or an aqueous solution (with varying acidity), or with organic compounds or complex mixtures at different temperatures. Suitably, the extract is subsequently filtered or separated by other methods from undissolved sediment. In particular embodiments, a centrifugation step is performed after extraction to efficiently separate the phases.

Extraction from animal tissue

Prior to extracting the biologically active substances from animal tissue, the tissue is typically preliminarily crushed or separated to remove connective tissues, fat and defective fragments. Homogenization is typically carried out with a homogenizer, e.g. a paddle homogenizer, with the addition of a cold or warm extracting agent. Suitably, homogenization is repeated if fragments of non-homogenized tissue remain. In some cases, additional agents may be added to the homogenate and mixed with it. Centrifugation of mixtures is typically undertaken at 10,000 g for 30-60 minutes. The extract is subsequently filtered through specialized filters, gauze, filter paper or glass wool.

A person of skill in the art will appreciate that the methods (with or without cell destruction), as well as the extraction agents and extraction conditions (temperature, particular type and relative amount of the extracting agent, the frequency of repetition of the process steps, etc.) are selected depending on the type of tissue used and the extracted substances. In some embodiments, liquid extraction is accompanied by sparging with gases, sonication, or microwave irradiation.

In some embodiments, crystallization and various sorption methods using molecular or ionic sorbents are used to isolate substances from the extract. As the skilled person will appreciate, the most selective sorption separation method used with liquid extracts is liquid chromatography.

As will also be appreciated, the most complex and multistage techniques are those aimed at isolating individual chemicals without destroying them. However, in many cases, such a high degree of selectivity is not required. A non-limiting example of an extraction process (from tissue or cell culture) is found in Example 6 and graphically illustrated in Figure 1.

Testing methods

Flow cytometry and cell cycle phase distribution

The skilled person will be familiar with various methods for assessing the presence and expression of the markers for both proliferation and apoptosis, such as flow cytometry. One of the particular applications of flow

cytometry relevant for the scope of present invention is assessing cell cycle phase distribution in the cell population. Corresponding technique is well established and commonly described in the manuals and reference materials provided by the manufacturers of the flow cytometry equipment and in a number of scientific publications, such as *Curr Protoc Cell Biol.* Chapter 8: Unit 8.4. Darzynkiewicz, Z. *et al.* Determining cell cycle stages by flow cytometry, 2001.

Testing for the induction of proliferation

Testing for the induction of proliferation is performed through testing the differences in the expression levels of genes that control cell proliferation without (control) and with (test) corresponding inducer compound present or added (Whitfield, M. *et al.*, *Nat Rev Cancer* 6, 99–106, 2006).

Corresponding tests are oriented towards the search and identification of characteristic proliferation markers (Iatropoulos, MJ and Williams, GM. *Exp Toxicol Pathol.* 48(2-3): 175-81, 1996). According to Iatropoulos and Williams: "*In the past, tissue/cell kinetics have been studied by thymidine histoautoradiography. Recently, monoclonal antibodies to proliferation-associated antigens have been successfully employed. These antigens are cycle-associated proteins and include (1) PCNA; (2) p53; (3) Ki67; (4) AGNOR; (5) Statin; and (6) BrdU.*" Procedures for identifying corresponding markers today are well established and are routinely performed by the laboratories. See, e.g., Scott RJ *et al.*, *J Pathol.* 165(2): 173-8, 1991 and Darzynkiewicz Z *et al. Cytometry*, 25(1):1-13, 1996.

The capacity of BAS1 to induce cell proliferation is described in Example 2, Figures 8-13 (*in vitro* results) and in Example 3, Figures 14-16 (*in vivo* results).

Testing for the autoinductive properties of proliferation inducing factors

According to the used definition, an autoinductive factor or autoinducer is capable of inducing or stimulating its own synthesis. In the context of the present invention, autoinduction of the respective biologically active factors means that the presence of these factors induces and intensifies cell division.

At the same time, in the process of cell division, these factors are synthesized, thereby supporting the process of division and "production" of these factors. As a result, the more intense cell division is, the more intensely the division-inducing factor is synthesized.

Thus, testing for the autoinductive properties of the factor can be performed by monitoring its concentration dynamics in the closed volume containing corresponding growth media and proliferating cells. If in such closed volume cell division proceeds and the concentration of the proliferation-inducing factor is stable or grows, it indicates autoinduction. If the concentration of the inducing factor in the presence of continually proliferating cells is at least stable, but in the presence of corresponding growth media and absence of proliferating cells its concentration falls with time (due to some factor degradation or destruction mechanisms, including but not limited to the oxidation or chemical reactions with the components of growth media), it also points to the presence of autoinduction.

Testing for induction of apoptosis

Testing for the induction of apoptosis is performed through testing for the presence and expression of corresponding markers without (control) and with (test) corresponding inducer compound present or added. In the context of the present invention, the inventors are following the common understanding of apoptosis, namely that apoptosis is a form of programmed cell death that occurs in multicellular organisms and cell cultures. Biochemical events lead to characteristic cell changes and death. These changes include blabbing, cell shrinkage, nuclear fragmentation, chromatin condensation, DNA fragmentation, and mRNA decay (see, e.g. Programmed Cell Death (Apoptosis). Alberts B, Johnson A, Lewis J, *et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science*, 2002). In addition to its importance as a biological phenomenon, defective apoptotic processes have been implicated in a wide variety of diseases. Certain publications expand the meaning of apoptosis to a wider interpretation as an "arrest of the proliferation" according to Iatropoulos and Williams: "Proliferation can be arrested through senescence, apoptosis, injury or even during the development of immune cells." Apoptosis

is a highly regulated process that can be initiated through one of two major pathways. In the *intrinsic pathway,* the cell kills itself because it senses cell stress, while in the *extrinsic pathway* the cell is eliminated because of external signals. Weak external signals may also activate the intrinsic pathway of apoptosis (Alberts B *et al.*, 2008). "*Chapter 18 Apoptosis: Programmed Cell Death Eliminates Unwanted Cells*". Molecular Biology of the Cell (textbook) (5th Ed.). *Garland Science*. p. 1115; Raychaudhuri S., *PLOS ONE*, 5 (8), 2010). In addition "excessive apoptosis causes atrophy, whereas an insufficient amount results in undesirable uncontrolled cell proliferation, such as cancer" (see e.g. Scott W. *et al.* Apoptosis in cancer. *Carcinogenesis*, 21(3): 485–495, 2000).The most commonly used apoptotic markers are caspase activation, cytochrome c release, and oligonucleosomal DNA fragmentation. There is a number of well-established techniques (see, e.g., Krysko D. V. *et al. Methods*. 44 (3): 205–21, 2008; Vanden Berghe T *et al.* Methods. 61 (2): 117–29, 2013; Wlodkowic D. *et al.* Recent Advances in Cytometry, Part B - Advances in Applications. *Methods in Cell Biology*. 103: 55-98, 2011). In the context of the present invention, by apoptosis is primarily meant mitochondrial apoptosis.

Testing for the induction/enhancement of proliferation or apoptosis

Induction or enhancement of the cell proliferation or apoptosis is performed through testing and quantification of the samples taken from the cell population by any accepted methods, preferably by flow cytometry.

For that, samples are taken from the cell population before and after it is subjected to the action of any substance or activation method claiming the capacity for cell proliferation or apoptosis enhancement or induction. To assess the dynamic of corresponding action, multiple samples from the cell population are taken at regular time intervals after the action of the tested substance or the activation method.

Description of the corresponding testing protocols related to proliferation and apoptosis (referred to as Cell Proliferation Assay Protocols and Apoptosis Assay Protocols) are available in the literature and from the manufacturers of the corresponding test equipment (Cell proliferation or Apoptosis protocols for imaging, Cell proliferation or Apoptosis protocols for flow cytometry).

Corresponding presented results are statistical, and in particular case relevant to the present invention provide the relative numbers (percentage) of the cells in the population at the moment of testing being in the certain stages of the cell cycle (as the percentage of all analyzed cells in the sample).

Another method for assessing the apoptosis induction/enhancement *in vitro* uses the monitoring of cell debris in the sample with cells suspended in the liquid media. Corresponding optical density of the sample according to the methods can be used as a quantitative measure or as a qualitative indicator (e.g. Image-Based Quantification of Cell Debris as a Measure of Apoptosis, M. Ölander *et al. Anal. Chem.* 91: 5548−5552, 2019).

For *in vivo* testing, one should use corresponding tissue samples extracted before and after the method claiming the actions changing the cell properties in the tissues. One of the methods is using the cells extracted from the blood. As an example, one can refer to a patented method using the flow cytometry analysis of the monocytes extracted from the venous blood samples (Russian patent no. 2728601). Corresponding method uses the samples, collected before and after the action, claiming rejuvenating activity, and analyzing the relative proportions of the cells in different cycle stages. Corresponding samples can be tested immediately after extraction, or kept for a certain time in the cell culture state. Conclusion on the presence/absence of claimed rejuvenation or anti-aging activity and intensity of such action can be derived from the changes in relative proportion (percentage) of the cells in the division cycle stages just preceding or just following the division.

Testing substances for their antibacterial properties

Methods of testing substances for the antibacterial properties are well established and may include Diffusion methods (Agar disk, Agar well and Agar plug-diffusion methods, Antimicrobial gradient method or Etest, Cross streak method, Poisoned food method); Thin-layer chromatography (TLC)–bioautography methods (Agar diffusion, Direct bioautography, Agar overlay bioassay); Dilution methods (Broth dilution method, Agar dilution method); Time-kill test or time-kill curve method; ATP bioluminescence assay and Flow cytofluorometric method. All these methods are well described in a number of

publications and manuals, and will be familiar to the skilled person. One can also refer to a review article by Balour *et al.*, Methods for in vitro evaluating antimicrobial activity: *A review. Journal of Pharmaceutical Analysis*. 6(2): 71-79, 2016. Many of these methods are well known and commonly used for the antimicrobial activity screening and evaluating of the antibacterial activity of the tested substances, for drug discovery, epidemiology and prediction of therapeutic outcomes. Most relevant methods for the scope of present invention are those used for the *in vitro* investigation of extracts and pure substances for the antimicrobial activity. The most known relevant basic methods are the disk-diffusion and broth or agar dilution methods.

According to another aspect of the invention, there is provided a fragment, analog or derivative of the biologically active substance according to the first aspect, which fragment, analog or derivative simultaneously possess the properties hereinbefore defined.

A person of skill in the art will be familiar with terms such as “fragment”, “analog” and “derivative” and the meaning of these terms in the present context. Thus, the skilled person will understand that, as referred to herein, a fragment is a shorter or truncated portion of the biologically active substance that essentially retains the activity and function of the substance. Similarly, as referred to herein, an analog or a derivative is a substance with slight variations in its composition that essentially retains the same activity and function as the biologically substance described herein.

According to another aspect of the invention, there is provided a composition comprising the biologically active substance, or the fragment, analog or derivative, and at least one pharmaceutically acceptable excipient or carrier.

The term “composition” is to be used in its widest sense, encompassing all compositions comprising at least one biologically active substance, or fragment, analog or derivative thereof, and optional excipients, carriers, adjuvants, constituents etc.

According to another aspect of the invention, there is provided a method of supporting, intensifying and/or activating proliferation of animal cells *in vitro* comprising the step of contacting said cells with one or more of the herein

defined biologically active substance, fragment, analog, derivative or composition.

The method suitably comprises the step of contacting a target cell or cells with the biologically active substance defined herein, including but not limited to BAS1, such that the proliferation factor induces or promotes the proliferation of the cells. In one embodiment, the substance is added directly to the cell culture. In another embodiment, the contact with the specified substance is indirect, for example, when the media containing the substance or the media containing the substance and the cell culture are separated by a semi-permeable membrane allowing only the passage of relatively small molecules, including the biologically active substance.

According to another aspect of the invention, there is provided a cosmetic method of supporting, intensifying and/or activating proliferation of human cells *in vivo* comprising the step of contacting said cells with one or more of the biologically active substance, fragment, analog, derivative, or composition described herein.

According to another aspect of the invention, there is provided a biologically active substance, a fragment, analog or derivative, or a composition for use as a cell proliferation inducer and/or enhancer, an apoptosis inducer and/or enhancer, an anti-inflammatory agent or an antibacterial agent.

According to another aspect of the invention, there is provided a biologically active substance, fragment, analog or derivative, or composition for use as implant coating. It will be appreciated that the biologically substance may be used for coating any type of implant that has been implanted or surgically placed in the human body. Said implant may be made of any type of material, such as metals and alloys, ceramics, polymers and composites. In one particular embodiment, said implant is a dental implant. Example 4 describe preclinical *in vitro* (Figures 17-19) and *in vivo* (Figures 20-21) tests performed to assess the potential of the preparations containing BAS1 for tissue regeneration and inflammation prevention. The aim of these studies was to assess the potential of preparations comprising BAS1 for coating of the implants and preventing and treating inflammations associated with implantation.

According to another aspect, there is provided a method of long-term storage of the biologically active substance, or the fragment, analog or derivative using drying and/or lyophilization and sorption on biopolymers, such that when not exposed to the direct sunlight or UV-light, said lyophilized substance can be stored in dry conditions at room temperature without degrading its activity for at least 6 months, such as for at least 12 months.

According to another aspect, there is provided a process of extracting a biologically active substance from non-pathologically dividing cells or cell cultures, said process comprising the steps of (i) prior to extraction, adding the biologically active substance, the fragment, analog or derivative, or the composition to said cells or cell cultures; and (ii) extracting the biologically active substance, wherein said addition in step (i) improves the yield of the extracted biological substance. It will be appreciated that the added biologically active substance may be the same as or different to the extracted biologically active substance. It will also be appreciated that said addition in step (i) improves the yield of the extracted biological substance compared to if it had been extracted by the process without step (i).

According to another aspect, there is provided a method of identifying a substance suitable for use as a cell proliferation inducer and/or enhancer, and apoptosis inducer and/or enhancer, an anti-inflammatory agent and/or an antibacterial agent, said method comprising (a) providing a test substance; and (b) assessing whether the test substance shifts the cell cycle in a cell population such that there is an increased proportion of cells in the stages just before or just after cell division, wherein a test substance that shifts the cell cycle is suitable for use as a cell proliferation inducer and/or enhancer, an apoptosis inducer and/or enhancer, an anti-inflammatory agent and/or an antibacterial agent.

It will be appreciated that the biologically active substance of the invention may be extracted from homogenized animal tissues or cell cultures containing actively and non-pathologically proliferating cells, followed by centrifugation, freezing-thawing, filtration and separation of the supernatant into fractions.

Suitably, said extraction is performed by liquid chromatography with a carrier in the form of pure water or isotonic sodium chloride solution. Said extraction may be performed in a liquid phase using organic solvents.

Applications

From the foregoing it will be appreciated that the biologically active substances of the invention may be used in various applications, and in particular for the intensification and/or continuous support of cell division in tissues, and long-term maintenance of functionally competent cell cultures; in biofactories (increasing productivity of biosynthesis); in laboratory research; for the preservation and production of cells and tissues in cell therapy and reconstructive surgery; in bioprinting (three-dimensional printing of tissues and organs); for extracorporeal division activation of cells taken from a patient with their subsequent return to the body; in medicine and in the production of drugs and other agents (in particular, anti-aging drugs, enhancers and restorers of immunity, wound healing agents, new families of antibiotics); in substances, tinctures, gels and ointments for promoting and enhancing tissue regeneration and wound healing; in activating and functionalization of coatings for implants (orthopedics, dentistry); in cosmetics and cosmetology (preparations for skin care and skin revitalization); and in veterinary applications. Continuously cultured cell lines will also be invaluable as a source of cells for cell therapy, which has been found to be effective in correcting many pathological conditions.

So that the invention may be fully understood and put into practical effect, reference is made to the following non-limiting Examples.

EXAMPLES

The following Examples disclose the properties of a biologically active substance according to the invention. The Examples also disclose the extraction of BAS1 and its role as an inducer of cell proliferation and apoptosis and as an antibacterial agent.

EXAMPLE 1
BAS1 AND ITS PROPERTIES

Introduction

A substance with the properties of proliferation inducer (including autoinducer) and simultaneous enhancer of programmed cell death (apoptosis) was extracted, and studied. Along with the two properties of supporting and most probably defining the homeostasis in the proliferation niche, BAS1 also possessed strong antibacterial properties towards both gram-positive and gram-negative bacteria, and in some applications anti-inflammatory activity. Dried BAS1 is in a form of irregular shape crystallites with light brown or light amber color. BAS1 has excellent solubility in water at 20°C (better than 200 mg/ml) and rather limited solubility in alcohols and oils. Toxicology tests of BAS1 *in vitro* and *in vivo* have shown its biocompatibility and the absence of cytotoxicity. Tests with laboratory animals have confirmed that BAS1 is not carcinogenic and that it has no tendency to promote existing lesions (e.g., it does not induce cancer and does not aggravate existing cancer). Preclinical tests have shown that BAS1 and preparations containing it do not cause allergy reactions, does not show any side effects and is compatible with most, if not all, tested medicinal drugs.

BAS1 stability and storage

To assess the stability of substances showing biological activity and determine the conditions of their storage and application a number of tests were performed including, but not limited to: storage in water solution, oils, ointments and gels, storage in solid phase, storage at freezing temperatures, with or without exposure to ambient light and UV radiation etc. For most applications, BAS1 was used in dried form, in combination with gels or solutions. BAS1 can be used both in neutral solutions (pure water, standard biological buffers, isotonic sodium chloride solution, etc.), and in different media with a pH from about 1.5 (human stomach) to about 9. Verification of the capacity for long-term storage of the substances with certain biological activity was carried out using, for example, the methods listed below.

An example of a possible verification: a liquid or gel-based sample with known biological activity was stored in one of the recognized ways (at different temperatures, in the light or in the dark, flashed with an inert gas, nitrogen or CO_2, or without it etc.) and the corresponding activity after certain periods tested. Another example of a possible verification: a liquid, gel-based or solid sample with known biological activity was frozen in test vials and stored in one of the recognized ways at various negative temperatures. After certain periods, the sample was thawed, and its activity checked.

According to the performed tests, storage time of dried or lyophilized BAS1 without exposure to ambient and UV light was more than 6 months without the loss of activity. In water solution at ambient temperature without exposure to ambient and UV light BAS1 lost its activity with characteristic lifetime (time corresponding to 50% loss of activity) of 24 to 36 hrs. BAS1 incorporated into the gel-forming biopolymers (e.g. hyaluronate, hydroxymethylcellulose etc.) kept its activity from a few days to a few months, depending on the nature and viscosity of the biopolymer. Figure 2 presents SEM images of typical crystallite shapes appearing after recrystallization of BAS1 from water solution. Dried BAS1 crystallites have characteristic distorted pyramidal shape with rough surfaces. Moreover, although XRD spectra and X-ray diffraction patterns are completely dominated by NaCl features, the crystal structure of BAS1 is far from the common prismatic one of pure NaCl or even NaCl contaminated by simple organic compounds.

Experiments show that BAS1 induces and/or intensifies non-pathological and non-pathogenic cell proliferation.

Composition analysis

A detailed structure of BAS1 was not possible to uncover despite significant efforts. The following examples summarize the results of the analysis of BAS1 by different methods.

Figure 3A shows IR absorption spectrum of the crystalline BAS1 taken in dry chamber under argon atmosphere. Corresponding conditions were chosen to exclude the potential background from NaCl.

Analysis summary

According to the simplified and quite generalized interpretation, common attribution of the most intense absorption bands to the specific bonds and chemical groups is:

3300-3500 cm^{-1} (medium)	N-H stretching (amine)
3000-2850 cm^{-1}	C–H stretching
2500-3300 cm^{-1} (broad); 3200-3600 cm^{-1} (broad)	O-H stretching (alcohol)
1700-1725 cm^{-1} (strong)	C=O stretching (carboxylic acid or ketones)
1470-450 cm^{-1}	C–H bending or scissoring
1370-350 cm^{-1}	C–H rock, (methyl)
from 725-720 cm^{-1}	C–H rock, (methyl, seen only in long chain alkanes)
<1400 cm^{-1}, so-called fingerprint area	

The simplest interpretation of the most intense bands and peaks in the IR spectrum of BAS1 suggests:

- an abundance of OH groups;
- possible presence of amino groups;
- the presence of C=O groups (carboxylic acids or ketones);
- the presence of CH_3 (methyl groups) with some indication of large alkane-like molecules;

The spectra also indicate the absence of:

- C≡C - bonds (no bands 2260-2100 cm^{-1})
- C-O bonds (no bands at 1300-000 cm^{-1}).

Analysis by experienced spectroscopists also concluded that the most intense and widest peaks belong to water molecules, while the intense band around 1000 cm^{-1} may belong to the phosphate groups. Figure 3B-G shows NMR spectra of BAS1 in solution. Both ^{1}H and ^{13}C NMR spectra were acquired with long accumulation time to provide better signal-to-noise ratio. In ^{1}H NMR spectra narrow single lines around 1.5 ppm commonly belong to CH_3 groups; the ones around 2.8-3.0 ppm commonly belong to NCH_3 groups. ^{31}P NMR

spectrum indicates the presence of phosphate groups, possible in a number of different surroundings.

Electron backscatter diffractometry (EBSD) and energy-dispersive X-ray analysis (EDX) of dried BAS1

EBSD was performed on BAS1 crystallites after it was dissolved in deionized water and recrystallized on a sample holding plate. Typical SEM image with marked scan area and corresponding EDX (energy dispersive X-ray) spectrum are shown in Figure 4. Table 1 provides a summary of the EDX data taken from different sites of individual crystallites (Figure 4 shows the analysis at the site S0) and control (pure NaCl crystals). Sites S0 and S1 do not show abundant organic content, and for the analysis of organic substances these data are inconclusive. Sites with organic (carbon) presence do not show balanced content of chlorine and potassium that is characteristic to free NaCl. Site S0 shows Na and Cl balance close to pure NaCl with some presence of oxygen, but no organic content. Table 2 provides content analysis data averaged over the sites S1 to S9.

Analysis summary

Organic part of the substance according to EBSD and EDX is dominated by carbon, oxygen and sulphur. Such atoms like hydrogen and nitrogen are not easily showing in the common EBSD and EDX spectra, so no conclusion on their presence and abundance in the samples can be drawn. Inorganic part is represented by sodium with smaller amounts of potassium and magnesium, and even smallest amounts of calcium and silicon. Presence of Ca, Si and Mg may be a reconstruction artefact or may be due to impurities coming from the sample holding glass plate. Note, that XRD and other X-ray diffractometry results are dominated by the features of pure NaCl, unlike the results of EDX analysis. It can be explained by the fact that EDX is capable of only shallow (mainly surface) analysis as the electrons have much smaller penetration depth as compared to X-rays.

Liquid chromatography

High-performance liquid chromatography (HPLC) studies of BAS1 are described and in part inconclusive. Figure 5 presents HPLC spectra of BAS1. Table 3 presents suggested automatic interpretation of the compounds (fragments) using most intense peaks as semi-automatically generated by the embedded instrument features using linked substance databases. In addition, analysis suggests presence of certain compounds that is not possible to identify with reasonable probability. Figure 6 shows two more examples of BAS1 HPLC spectra (graphs) while Table 4 below shows suggested automatic interpretation using most intense peaks done by the embedded instrument features using linked substance databases.

Analysis summary

Liquid chromatography results are rather inconclusive. It is partially because HPLC methods of detection are sensitive to both the present molecules and their fragments. Therefore, analysis reveals the presence of long hydrocarbon chain aliphatic compounds with different active groups (acids, ethers). There are indications of the presence of basic amino acids, peptides, complex amines, phosphates and pyrophosphates. There is a presence of human hormone precursors (squalene, pregnane) and their derivatives. The most useful is the conclusion about the largest possible molecules (highest molecular weight) of the substances present in the test samples.

Gas chromatography

For these studies, dried BAS1 was dissolved in different solvents (acetonitrile; deionized water etc.) for the separation of neutral, acidic and basic fractions and subjected to gas chromatography. Chromatography was performed using Agilent 6850 system with the mass-selective detector Agilent 5973N and column Phenomenex ZB-5MS. Carrier gas helium. Automated analysis was carried out using databases NIST2017, MPW5e, Designer Drugs 2019, Golm Metabolome Database. Table 5 summarizes suggested identifiable compounds (fragments) with highest intensity and largest probability from all chromatography tests. The following compounds were identified (although in

small abundance and with rather small probability): adenine, lactic acid, dehydroabietic acid, glycolic acid, oleic acid, succinic acid, nonanoic acid, decanoic acid, myristic acid, arachidic acid. Aromatic compounds were not present at all or identified in trace amounts. Analysis using linked databases revealed a significant number of unidentified compounds.

Examples of compound identification

Figure 7A-C show the elements of automatically generated report that presents the results of the automated identification carried by the system.

Residual protein analysis

Analysis using Uniport database (https://www.uniprot.org/) revealed a presence of small amounts of residual proteins listed below.

Cytoskeletal domain: Junction plakoglobin; Keratin, type I cytoskeletal; Keratin, type II cytoskeletal cochleal; Type II alpha-keratin IIA; IF rod domain-containing protein; Stathmin.

Extracellular domain: Annexin; Cytoplasmatic Actin.

Glycolysis domain: glyceraldehyde-3-phosphate dehydrogenase; pyruvate kinase; 2-phospho-D-glycerate hydro-lyase.

Programmed cell death: Cathepsin D.

Ion transport: Voltage-dependent anion-selective channel protein 1.

Adapter and regulation: 14-3-3 protein zeta.

Protection from peroxide: Catalase.

Other domains: Ubiquitin-40S ribosomal protein S27a; Polyubiquitin-B.

Additional analysis of the HPLC and gas chromatography data using semi-automatically generated brutto-formulae of the possible compounds was performed using the open data sources such as BOC Sciences “Molecular Weight Calculator” https://www.bocsci.com, NIST "Search for Species Data by Molecular Weight", (https://webbook.nist.gov/chemistry/mw-ser) and alike. The majority of the compounds specified by the semi-automated analysis by the corresponding machines and their embedded software as “unidentified” did not

generate any hits of known substances in these databases as well, indicating that such substances are “not known” and cannot be positively identified now.

Overall summary of the physicochemical analysis of BAS1

Based on the above data, BAS1 is most likely present or contained as an active part a mineral-organic complex having a complicated composition with the presumable presence of the following organic molecular fragments:
- long linear hydrocarbon chains with the possible presence of double bonds, including fragments of higher fatty acids or their esters, including those with additional simple substituents;
- long linear hydrocarbon fragments with biologically critical alternation of single and double bonds such as squalene, hopane and similar steroid precursors;
- steroids fragments (cholesterol, sitosterol) and substituted steroid fragments;
- alkane fragments;
- high molecular weight unsaturated hydrocarbon fragments;
- peptides and their fragments;
- elemental amino acid (e.g. choline, valine, taurine) fragments;

in addition, organic functional groups:
- phosphates (possibly also phosphines), pyrophosphates;
- hydroxyl (–OH) groups;
- amino and amide groups.

The following organic groups and fragments are almost certainly not present:
- aromatics - there are no indications of them in the NMR spectra, but there is a possibility of the presence of phthalates according to the results of chromatography;
- ketone fragments – could be possible, but the probability of their presence in a significant amount is very small;
- fragments with triple bonds (C≡C) are not present in detectable amounts.

Analysis of elemental atomic composition indicates the presence (in decreasing order of abundance): carbon, oxygen, sodium, chlorine, sulfur and phosphorus, with very small amounts of potassium, magnesium, calcium and silicon. Presence of sodium chloride can be partially explained by the way of substance extraction (through the isotonic solutions and NaCl solutions). The presence of small amounts and traces of potassium, magnesium, calcium and silicon can be explained by the influence of the preparation and study method (associated with laboratory glass sample holding plates). The high molecular percentage of oxygen is in good agreement with other data indicating the presence of hydroxyl (–OH) groups, acid, ether and may be some ketone fragments. The indication of presence of sulfur is in good agreement with the indication for the possible presence of taurine. A clear imbalance of the atomic percentages of sodium and chlorine (the percentage of sodium is significantly increased as compared to NaCl, with almost absence of chlorides in chromatography data), may point to a potassium salt. The presence of one of the characteristic bands in liquid chromatograms, similar to that of a sodium cation, may indicate the presence of a sodium salt of an organic acid or a metal-organic complex. Together with the possible presence of a significant amount of hydroxyl groups, this most probably determines very good solubility of BAS1 in polar solvents (water) and poor solubility in non-polar ones. The analysis indicates minimal presence of proteins and other organic molecules with a significant (more than 1000-1500 Daltons) molecular weight. Mostly BAS1 is represented by organic molecules or corresponding salts with a molecular weight of less than 700-800 Daltons.

EXAMPLE 2

BAS1 AND CELL PROLIFERATION *IN VITRO*

Cell proliferation, cell vitality and apoptosis in cell culture: human mononuclear blood cells and human fibroblasts

In cited experiments human fibroblasts and mononuclear cells were extracted from the materials donated by healthy volunteers according to the protocols described below.

Figure 8A-D shows experimental data illustrating the changes induced by BAS1 upon the duration of the phases of the cell cycle in the culture of human dermal fibroblasts studied by flow cytometry.

Figure 9A-D shows experimental data illustrating the changes induced by BAS1 upon the cell distribution over the cell cycle phases and cell vitality as studied by flow cytometry for human blood mononuclear cells in cell culture. Corresponding dependencies show the changes happening with addition of different concentrations of BAS1 as compared to control.

Figures 10A-E show the changes in the life expectancy and cell distribution over the cell cycle phases as studied by flow cytometry for human fibroblasts in cell culture. Corresponding dependencies show the changes happening with addition of different concentrations of BAS1 as compared to the control. Horizontal axis: days of incubation. Corresponding BAS1 doses are given in mkg/ml. BAS1 is added at the beginning of the 1st day of incubation (time=0). Acquired data show that it is possible to increase the cell life in culture 5 times without decreasing cell vitality applying BAS1.

Human blood mononuclear cells in vivo: age-related dynamics of the cell cycle stage distribution

Figure 11A-D show typical changes induced by BAS1 in cell cycle distribution of the human mononuclear blood cells (volunteers). Mononuclear cells were isolated from venous blood and then cultured. See below for the corresponding procedure of mononuclear cells isolation.

Figure 12A-D present the measurements of the human blood mononuclear cell cycle distribution changes performed with 18 male and 18 female volunteers between 16 and 86 years of age according to the method described in Russian patent no. 2728601 summarized above.

Although the scatter of the values is rather significant, trends in decreasing proportion of cells in G0 and increasing proportion in S with age is distinguishable and characteristic for both sexes. In addition, there is a clear tendency for the intensification of the apoptosis with the age. It should be noted that in present measurements chronologic age (as written in the documents) was used. In order to better represent the trends, one should use so-called

biologic age better representing real aging of the organism (Sukhovei, Y *et al. Biomedical Dermatology* 3:10 and 3:13, 2019).

Mononuclear cells extraction from the venous blood

Mononuclear cell extraction from the venous blood can be performed according to any protocol accepted in the microbiology. The protocol used in the present case is outlined in Figure 13. Venous blood is taken from the ulnar vein in the morning before any meals. The tests were conducted with volunteers between the ages of 16 and 86. The separation of mononuclear cells from the whole blood is performed by gradient centrifugation using DIACOLL-1077 (density gradient cell separation medium). The heparinized blood is diluted 3-fold with the Versene solution (an EDTA solution, a non-enzymatic cell dissociation reagent), then layered on top of the DIACOLL-1077 density gradient and centrifuged at 300 G for 45 minutes. The interphase containing mononuclear cells is collected and washed with RPMI-1640 medium three times.

Preparation of the human dermal fibroblast cultures

Human dermal fibroblast cultures were prepared using skin biopsies from volunteers aged 18 to 86 years according to the following protocol.

1. A skin fragment in a 0.25% trypsin-EDTA solution (PanEco) preheated to 37°C was placed in a CO_2 incubator for 30–60 minutes.
2. Using a scalpel, the epidermis and subcutaneous tissue were removed from the inside.
3. To prevent bacterial growth, skin fragments were transferred into 10% betadine solution (EGIS Pharmaceuticals Plc, Hungary) for 5 minutes, and then washed twice with medium.
4. Using scissors, the skin flap was cut to 2–3 mm and then with pointed curved scissors until a homogeneous mass was obtained.
5. A uniform consistency substance obtained from the skin was placed in a DMEM medium with type II collagenase (Gibco No. 17101-015, USA) at a

concentration of 1 mg/ml was placed in a CO_2 incubator at 37°C for 1.5 hours. Periodically, every 15-20 minutes, the tube was shaken.

6. After the end of the incubation, the solution with undispersed skin fragments was pipetted using tips with a wide and narrow lumen, in order to further mechanically disaggregate the tissue.

7. At the next stage, the DMEM medium with 10% FBS (fetal calf serum) was added to the DMEM solution with type II collagenase enzyme and a suspension of cells and undigested skin fragments, and centrifuged (325 g, 5 minutes).

8. Cells were re-suspended in 1 ml of DMEM medium containing 10% ETS, 40 µg/ml gentamicin, and 2 mM glutamine.

9. Then the culture was added to the Petri dishes (10 cm in diameter) at a density of $1x10^5$-$2x10^5$ cells/cm^2 and placed under standard incubation conditions. Standard cultivation was carried out at 37°C in an atmosphere of 5% CO_2 using a CO_2 incubator (Sanyo, Japan).

10. Next, the fibroblasts were incubated for 7 days until the formation of a complete monolayer. At this stage, microscopic examination was carried out to separate skin fragments from the attached fibroblast cells. Harvested tissue microexplants serve as additional sources of fibroblasts.

Optical inspection of fibroblasts and visualization of their preparations fixed and stained according to Romanovsky-Giemsa were performed using an inverted microscope CKX-41 (Olympus, Japan). Cell viability after each experiment was assessed by microscopy by staining them with trypan blue. Control over the processes of apoptosis and necrosis was carried out by assessing the number of apoptotic and necrotic cells by flow cytometry on a CytoFLEX flow cytometer (Beckman Coulter, USA) using the ANNEXINV-FITCKit (Immunotech, France) according to the manufacturer's instructions. Corresponding method allows the identification of living, necrotic and apoptotic cells. Immunophenotyping of cultured fibroblasts was performed with a CytoFLEX flow cytometer (Beckman Coulter, USA) using monoclonal antibodies to CD29, CD44, CD90, CD105 (markers specific for MSCs), and CD34, CD45 (markers of hematopoietic cells). The resulting suspension of cells was diluted with a complete culture medium at the rate of 1 million cells

in 1 ml of medium, and a 96-well plate was filled for studying the phases of the cell cycle.

1. In the “control”: 24 wells (200 µl of RPMI-1640 with 10% fetal calf serum is added to 200 µl containing 200,000 cells.
2. “Experiment 1”: To 200 µl, containing 200,000 cells, added 200 µl of RPMI-1640 with 10% fetal bovine serum containing the “factor” at a final concentration of 75 µg/ml.
3. “Experiment 2”: To 200 µl, containing 200,000 cells, added 200 µl of RPMI-1640 with 10% fetal bovine serum containing BAS1 at a final concentration of 150 µg/ml.
4. “Experiment 3”: 200 µl of RPMI-1640 with 10% fetal calf serum containing BAS1 at a final concentration of 300 µg/ml is added to 200 µl containing 200,000 cells.

Further:

- The entire content of one well from Control, Experiment 1, Experiment 2 and Experiment 3 is taken daily.
- After resuspension it is transferred to vials for cytometry.
- Contents of each vial is diluted 3 times with buffered saline, and centrifuged at 300 G.
- After centrifugation, the supernatant is drained
- Further, the content of the vials is transferred to cytometry.

Distribution of the cells over the cell cycle phases

For the analysis, a suspension with a concentration of 10^6 cells per 1 ml of medium was used. Analysis of the distribution of cells by phases of the cell cycle is performed using flow cytometry in accordance with any protocol adopted in the trade. In the cited studies, a Cytomics FC500 cytometer (Beckman Coulter, USA), and a test system CycleTEST™ PLUS, DNA REAGENT KIT by Becton Dickson.

EXAMPLE 3

BAS1 AND CELL PROLIFERATION *IN VIVO*

Corresponding *in vivo* (laboratory animal) model of the standard skin lesion was developed by the inventors. Laboratory animal tests were performed with mature (1 to 1.5 year old) male rabbits (weight 3-4 kg), with initial dimensions of the skin lesion diameter ca 4 cm. Corresponding lesions were monitored for up to 35-45 days and tissue samples were collected for histology and cell analysis.

The animals were divided into 4 groups of 20 rabbits each. In each group, the edges of the wounds were treated with a Lugol's iodine solution, the wound was treated with a 3% hydrogen peroxide solution, dried, and then dressing was performed after applying a substance specific for each group or no substance for control group:

Group 1, control 1, gel base only;

Group 2, control 2, traditional treatment (in the inflammation phase - traditional water-based healing preparations; in the regeneration phase - traditional fat-based healing preparations);

Group 3, control 3, no drugs;

Group 4, Experiment, Hyaluronate-based BAS1.

On days 1, 3, 5, 7,10 and 15, the size of the wound defect and the condition of the wound were assessed. At the same time, under local anesthesia, a biopsy was performed through all layers of the wound to a depth of 2 cm. Histological preparations were performed from the biopsy material to analyze the morphological picture. The preparations were also processed to detect the pro-apoptotic marker CD95+ and the proliferation marker Ki67. Figure 14 illustrates the differences in the intensity of cell proliferation and apoptosis in the control group (no treatment), and in three test groups with the treatment using: traditional method and using the BAS1–hyaluronate gel composition and hyaluronate gel only. Figure 15 illustrates the differences in the intensity of cell proliferation and apoptosis in the control group (with no treatment, CO, control) and the group treated with gel only (GI). Figure 16 illustrates the differences in the intensity of cell proliferation and apoptosis in the groups treated in traditional way (TR) and with gel, and BAS1 + hyaluronate

gel composition (BAS1+Gl). Corresponding data indicate a serious potential of preparations containing BAS1 for the intensification of tissue regeneration.

EXAMPLE 4

BAS1 AS ANTI-INFLAMMATION AGENT AND AS IMPLANT COATING

Preclinical *in vitro* and *in vivo* tests were performed to assess the potential of the BAS1-containing preparations for tissue regeneration and inflammation prevention. The aim of studies was to assess the BAS1-containing preparation's potential for coating of the implants and in preventing and treating inflammations associated with implantation.

In vitro studies

In vitro studies were carried out with the culture of Periodontal ligament stem cells (PDLSC-t) (Kadar, K. *et al. J Physiol Pharmacol*, 60(7): 167-75 (2009); Mathur, S. *et al. Int J Oral Maxillofac Implant*. 29(2): e210-9, 2014). In these tests following parameters were assessed: cell vitality and cell proliferation (WST-1 assay); cell adhesion; osteogenic cell differentiation; alkaline phosphatase activity; specific gene expression; cell apoptosis; intracellular cytokines, corresponding to specific proteins of pluripotent cells. Comparison was carried out in four groups: control (Cont), with addition of pure carrier (1% hyaluronate solution, HYL), with addition of carrier + BAS1 (BAS1) and with addition of 1% liquid gel Naturelize Tissue Support (NTS). NTS gel is a commercial hyaluronate-based composition designed and used for supporting tissue regeneration and guided bone regeneration in oral implantology. Figure 17 presents the results of the WST-1 vitality tests in the case of undiluted and 1:10 diluted tested substances. Figure 18 presents the results of the effect of tested substances on stimulating spontaneous osteogenic differentiation (measurements based on Koss densitometry). Figure 19 shows analysis of alkaline phosphatase activity for tested substances as compared to control.

Conclusions from in vitro experiments

Note that only results of a few tests are presented here. Overall results from *in vitro* experiments confirm that BAS1 in the hyaluronate (carrier) has significant potential for the coating of dental implants, and in many cases shows equally good results, and in some cases significantly better results as compared to Naturelize Tissue Support (NTS) gel regarded as one of the best available at the market.

In vivo studies

In vivo studies were carried out using laboratory rats (male Wistar rats 300±50 g weight) (Garcia V. G. *et al. Lasers Med Sci.* 25: 197-206 (2010); Oshiiwa M. and Garcia V. G. *J Periodontol.* 79: 1081-1088, 2008). Corresponding experiments were carried out by provoking the periodontal inflammation (induced periodontitis) in laboratory animals with following treatment by saline solution and the compounds containing 1% water solution of BAS1 + hyaluronate and 1% water solution of NTS. Inflammation was induced on the left side of the mouth. Each group received injection of test materials (0.02 ml / injection) locally around the left lower molar in the mesial, lingual and vestibular sites every 3 days for 31 days. Corresponding assessment of the inflammation development was carried out using Evans blue staining method. Results of the treatment were also assessed using histological studies and micro-CT. For the comparison, induction of the periodontitis was only performed at one side of the animal jaw teeth. Tissue samples for the histologic and staining analysis were removed under local anesthetic after 31 days of treatment. Figure 20 presents the results of inflammation assessment after 31 days of treatment. Control group was injected with saline solution, test groups- with the solution of hyaluronate (HYL carrier), solution of hyaluronate with BAS1 and NST solution (NST). Induction of the periodontitis was performed at the left side. Micro-CT was used to assess the long-term results of the treatment. Persisting inflammation leads to some bone loss, and the distance between the cemented enamel junction (CEJ) and the edges of the alveolar bone increases. Corresponding distances were measured after four

weeks of treatment at seven positions of the lower jawbone. Effect of the treatment by BAS1 with hyaluronate carrier was at least as good as that of the NST treatment. Tissue histology was performed after 31 days from the treatment initiation. No typical histological changes characteristic for significant inflammation were observed in the related samples. There was no difference in the degree of vascular proliferation between the groups. In addition, in the tissue samples of the control group and in the groups of animals undergoing treatment, there were no cellular elements indicative of severe inflammation.

Summary of the results and assessment of potential for implant coating

Studies have shown that all three hyaluronate-containing preparations (pure hyaluronate, hyaluronate with BAS1 and hyaluronate-based NTS) are biocompatible in the studied concentrations. In *vitro studies* have shown that BAS1-based composition is superior to the other two in its effect on PDLSC cells. BAS1-based composition also proved to be most effective in spontaneous osteogenic differentiation. Judging by cell adhesion, cytokine production and apoptosis markers, the use of BAS1-based composition has no side effects. Among other drugs used, BAS1-based composition showed the most significant changes in the expression level of the hyaluronic acid receptor CD44. All three hyaluronate-based treatments were able to prevent swelling of the gums / periodontal tissue, i.e. accumulation of excess fluid in the modeling *in vivo* tests with induced periodontitis. Based on the experimental findings BAS1-hyaluronate composition demonstrates significant potential for effective treatment and healing of severe lesions. When used together with high molecular weight hyaluronates drying to the glass consistency BAS1-based compositions have a significant potential as a bioactive coatings for implants facilitating better implant integration and preventing certain side effects such as inflammation.

Limited clinical studies

Limited clinical studies were carried out for the assessment of BAS1-hyaluronate composition potential for the non-surgical treatment of interdental papilla defects. Hyaluronate and compositions using hyaluronate are known

to be useful in treatment such defects (Jing Ni *et al. J Oral Maxillofac Surg.* 77(12): 2467-2474, 2019). In present study, the effect of BAS1-hyaluronate composition was compared with the effect of the commercial hyaluronate-based composition FlexBarrier (Naturelize GmbH).

Methods

Forty patients were selected for the study and randomly divided into test groups and administered with BAS1-hyaluronate gel (n = 20) or with FlexBarrier (n = 20). Corresponding selection criteria included absence of acute or chronic disorders who have anterior teeth with Nordland-Tarnow class 1 or 2 interdental papillae defects. The papillary defects were assessed using Nordland-Tarnow classification and measured on digital photographs using ImageJ software before treatment, one month immediately after treatment, and one month later at a follow-up visit. Treatment efficacy was quantified as the percentage of reduction in the black triangle caused by papillary defect.

Treatment was carried out using Three-Step Technique – TST:
- Injection of gel along the contour of the mucous-gingival junction at the base of the papilla at 4-5 points, creating a grid of 0.1 ml per point.
- Injection of the gel into the adjacent gingiva along the base of the papilla at 2-3 points, 0.1 ml per point.
- Injection of the gel into the papillae 2-3 mm from the tip to one point, 0.1 ml per point.

The effect of each treatment was evaluated immediately after injection, one week later and 4 weeks after administration using the following methods:
- subjective assessment of the patient, paying particular attention to side effects
- digital photos of anterior teeth
- visual classification of papillary defects.

Figures 21A-B present the comparison of the treatment results using BAS1-hyaluronate composition and commercial composition FlexBarrier, and comparison between both results.

Main conclusions

Test results indicate that both BAS1 +hyaluronate and FlexBarrier compositions are equally suitable for the treatment of interdental papillary defects, with BAS1-hyaluronate composition showing better clinical results.

EXAMPLE 5

ANTIBACTERIAL ACTIVITY OF BAS1

Antibacterial activity of BAS1 at different concentrations was tested using eight database strains of gram-negative and gram-positive bacteria (*Escherichia coli* K12, *E. coli* 25922, *Pseudomonas aeruginosa* 27853, *Staphylococcus aureus* 25923, *S. aureus* 6538P, *S. xylosus*, *S. epidermidis* 711, *Micrococcus luteus var. lysodeikticus* 15307) and 120 clinical strains of gram-negative microorganisms highly resistant to antibiotics (22 strains of *Escherichia coli*, 55 strains of *Acinetobacter baumannii* and 43 strains of *Pseudomonas aeruginosa*).

Testing method

Suspension of the bacterial cells taken from the one-day cultivated in agaric media in the isotonic solution ($5*10^8$ cells/ml) was used. 25 µl of suspension is introduced into the wells of a plastic 96-well sterile plate, and 25 µl of BAS1 water solution is added to the wells (concentrations of 50, 100, 150 and 200 µg/ml). It corresponds to the final BAS1 concentration of the wells of 25, 50, 75 and 100 µg/ml. 25 µl of isotonic NaCl solution were added to the control wells. After that, the plates were incubated for 20 minutes at 37 °C, and then 200 µl of cultivation broth is added to all wells. After that the plate is incubated for 4 hours at 37 °C, after which the optical density (OD) of bacterial cultures in the wells is measured using a Multiscan Accent microplate photometer (Thermo Electron, Finland) at a wavelength of λ = 492 nm. Each

experiment was performed in triplicate with the calculation of the average OD values.

Antibacterial activity of BAS1 are presented in Tables 6-8 using the following parameters:

Bactericidal Activity Index (BAI,%) calculated using the formula:

$BAI = (OD_c - OD_e) / OD_c * 100\%,$

where ODc and ODe are the optical densities of the control and experimental cultures, respectively. Strains with BAI> 10% were considered susceptible for the antibacterial action of BAS1.

The data were processed using variation statistics and presented as arithmetic mean and error (M ± m)

Note, that for the majority of the bacterial strains BAS1 is showing rather high antibacterial activity, with the exception of the coagulase-negative *S. xylosus* , *S. epidermidis* and *Micrococcus l*, where the activity was generally lower. Note also, that among the studied strains of the bacteria there were isolates with low sensitivity to BAS1 (BMI<10%). Additional testing for the antibacterial activity of BAS1 was performed with 84 clinical strains of *Klebsiella pneumoniae*. Testing method was similar to the one described above, but the final concentration of BAS1 in the wells was 100, 250, 500 and 1000 µg/ml. Data processing was identical. Table 8 presents the comparison of BAS1 antibacterial activity against *K. pneumoniae* and E. *coli*.

EXAMPLE 6

BAS1 EXTRACTION

Preferred extraction of BAS1 is graphically illustrated in Figure 1.

BAS1 extraction procedure

1. The primary source (raw material) for obtaining biologically active substances from animal raw materials according to present invention is any tissue or cell culture with non-pathologically proliferating cells.

2. Defective agglomerates or tissue areas are identified by visual inspection and removed.

2a. When using cell culture, extraction is commenced from the next stage.

3. Selected tissues or cell cultures are washed in clean saline solution at room temperature for 5 minutes with gentle stirring. The tissue (or cells, when extracting from the cell culture) is separated from wash solution by filtration. The operation is repeated three times.

4. The washed tissues are placed in fresh isotonic solution and homogenized at room temperature with a Medimachine automatic mechanical tissue homogenization system (Becton Dickinson). When using cell culture, the homogenization step is omitted.

5. At the next stage, a suspension with a cell concentration normalized with saline to $1x10^6$ / ml and incubated at room temperature for 24 hours.

6. In the next step, the suspension is centrifuged for 25 minutes at 1500 rpm and filtered. The filtered supernatant is frozen.

7. The completely frozen supernatant is thawed at room temperature. The supernatant is centrifuged for 25 minutes at 1500 rpm and separated from the residue by filtration for later use.

8. The procedure of centrifugation-filtration-freezing-thawing and separation of the supernatant from the residue can be repeated two to three times.

9. The collected purified supernatant is separated into fractions by liquid chromatography. Chromatographic separation is carried out using most gentle liquid carriers, such as isotonic solution or distilled water.

10. Fractions corresponding to the characteristic peaks of the chromatographic separation are collected and each fraction is tested for specific biological activity.

11. Corresponding fraction containing BAS1 is selected using the test for the characteristic set of activities, namely the influence upon cell proliferation and apoptosis and antibacterial properties.

Methods of substance testing for specific biological activity

Examples of possible tests for biological activity of biologically active compounds acquired by the described method may include (but are not limited to): tests for the influence on cell proliferation or apoptosis; antimicrobial activity test (verification is carried out using a standard method using appropriate bacterial cultures); antimycotic or antifungal activity test (verification is carried out in a standard manner using appropriate fungal cultures); antibacterial activity tests. BAS1 possesses specific biological activity, namely influence upon cell proliferation and apoptosis. Corresponding example outlined below describes one of the possible methods of testing used.

An example of a possible test for specific biological activity of BAS1: cell culture test.

Such test can be performed for any substance, having the activity similar to that of BAS1. Particular example refers to BAS1 for better clarity.

The test is performed by using the isolated fraction to the cell culture (for example, human fibroblasts, and human blood mononuclear cells). The original cell culture is divided into two identical lines maintained under identical conditions; one is used to check the activity of the corresponding fraction, the other for control. Corresponding amount of tested fraction is added to the cell culture, and amount of the sediment (dying cells and debris) is assessed. After consecutive time intervals, cells are selected from both cell lines, and compared. Comparison may include an assessment of the number of active cells and associated debris, an assessment of cell viability, an assessment of the life span of cells in culture. One of the definitive options of comparison is

carried out by determining the ratio of the number of cells in different stages of the cell cycle, for example, using flow cytometry or method similar to that described in Russian patent no. 2728601 (Method for testing substances affecting aging processes by blood analysis). Flow cytometry methods allow for both qualitative determination of the presence of substance with chosen biologic activity (activities), and for the quantification of such activity (activities).

EXAMPLE 7

BAS1 AND CORRELATED INTENSIFICATION OF THE APOPTOSIS

Influence of BAS1 upon apoptosis was studied for the human fibroblast and the human mononuclear cell cultures. The result of action from BAS1 was compared to the result of the action of Phytohaemagglutinin (PGA, e.g. D Hutchins and C M Steel, *Clin Exp Immunol*. 52(2): 355–364, 1983). Mononuclear cells and human fibroblasts were extracted according to the protocols described in EXAMPLE 2 (Mononuclear cell extraction; Preparation of the human dermal fibroblast cultures). The graphs shown in Figures 22A-H represent the data acquired for the mononuclear cells received from a group of 80 healthy volunteers aged between 15 and 61 years.

It should be noted, that the action of BAS1 is almost instantaneous (intensity of the impact peaks after few hours), while the action of PGA at chosen concentration peaks after three days of incubation (as shown in Figure 22A). It is exemplified by the action of PGA and bioactive substance upon the rest and proliferation phases (as shown in Figure 22A-F).

There was no significant difference between the action of PGA or BAS1 at studied concentration for younger age or mature age sub-groups (15-20 and 21-61 years, correspondingly) at 1 and 5 days after exposure to the active substance (‘none’- means control, nothing was added), as shown in Figures 22A-F. However, the intensification of proliferation is more pronounced for the younger sub-group of subjects at lower BAS1 concentrations, as shown in Figures 22G-H. Corresponding ratios: S/A – Synthesis/Apoptosis, P/A-Proliferation/Apoptosis.

Note that Figures 22A-H present the ratios of the cell numbers in the corresponding cell cycle stages to illustrate the correlation between the intensification of the apoptosis and proliferation and synthesis phases. Corresponding substances were added at day 0, and tests are performed after 1 to 5 days of incubation. Values at day 0 represent the initial state of the culture before adding any active substance. Corresponding values are averaged for all subjects without separating into the age sub-groups.

Summary

The substance BAS1 has five features that are simultaneously exhibited:

i. substance induces/enhances proliferation
ii. substance induces/enhances apoptosis
iii. induction/enhancement of proliferation and that of apoptosis are coupled supporting the homeostasis in the cell population (e.g. supporting cell number maintenance)
iv. substance has the property of autoinduction (self-amplification)
v. substance has antibacterial activity against gram-positive or gram-negative microorganisms or against both.

The simultaneous presence of these features can be used to identify BAS1.

Comparison between BAS1 and exosomes

BAS1 and exosomes are both derived from the supernatants extracted from the cultures of actively dividing cells and some of the properties reported for the exosomes coincide with those of the BAS1.

However, based on the common identification tests for exosomes, e.g., biochemical tests, microscopy and optical/IR spectroscopy, BAS1 is not a membrane-based vesicle. BAS1 does not lose its activity after drying and soldering. Extraction yields of BAS1 dramatically exceed those of exosomes. Although some of the properties of BAS1 are aligned with those of the extracellular vesicles, the simultaneous presence of the key features of BAS1, namely synchronized intensification of the proliferation and apoptosis,

antibacterial activity, and especially the autoinduction, has not been reported for exosomes.

CLAIMS

1. A biologically active substance extracted from animal tissue or cell culture comprising non-pathologically proliferating cells, which substance simultaneously (i) induces and/or intensifies cell proliferation; (ii) induces and/or intensifies apoptosis; and (iii) has antibacterial activity, wherein said biologically active substance optionally has anti-inflammatory activity.

2. The biologically active substance of claim 1, which has or comprises one or more characteristics selected from the group consisting of: a molecular weight of less than 1.5 kDa, preferably of less than 0.7 to 0.8 kDa, long linear hydrocarbon chains, hydrocarbon fragments, steroid fragments, alkane fragments, high molecular weight unsaturated hydrocarbon fragments, phosphates, hydroxyl groups, amino groups and amide groups.

3. The biologically active substance of claim 1 or 2, which is capable of shifting the cell cycle in a cell population such that there is an increased proportion of cells in the stages just before or just after cell division.

4. The biologically active substance of any one of claims 1-3, wherein there is a correlation between induction and/or intensification of cell proliferation and induction and/or intensification of apoptosis, preferably such that intensification of cell proliferation is correlated with intensification of apoptosis.

5. The biologically active substance of any one of claims 1-4, which is not carcinogenic.

6. The biologically active substance of any one of claims 1-5, which is BAS1.

7. The biologically active substance of any one of claims 1-5, which is not BAS1.

8. A fragment, analog or derivative of the biologically active substance of any one of claims 1-7, which simultaneously possess the properties defined in claim 1.

9. A composition comprising the biologically active substance of any one of claims 1-7, or the fragment, analog or derivative of claim 8, and at least one pharmaceutically acceptable excipient or carrier.

10. A method of supporting, intensifying and/or activating proliferation of animal cells *in vitro* comprising the step of contacting said cells with one or more of the biologically active substances of any one of claims 1-7, the fragment, analog or derivative of claim 8, or the composition of claim 9.

11. A cosmetic method of supporting, intensifying and/or activating proliferation of human cells *in vivo* comprising the step of contacting said cells with one or more of the biologically active substances of any one of claims 1-7, the fragment, analog or derivative of claim 8, or the composition of claim 9.

12. The method of claim 10, wherein said cells are mammalian cells, such as human cells.

13. A biologically active substance of any one of claims 1-7, fragment, analog or derivative of claim 8, or composition of claim 9 for use as a cell proliferation inducer and/or enhancer, an apoptosis inducer and/or enhancer, an anti-inflammatory agent and/or an antibacterial agent.

14. A biologically active substance of any one of claims 1-7, fragment, analog or derivative of claim 8, or composition of claim 9 for use as implant coating.

15. The biologically active substance of any one of claims 1-7, wherein said extraction is performed from homogenized animal tissues or cell cultures

containing actively and non-pathologically proliferating cells, followed by centrifugation, freezing-thawing, filtration and separation of the supernatant into fractions.

16. The biologically active substance of claim 15, wherein said extraction is performed by liquid chromatography with a carrier in the form of pure water or isotonic sodium chloride solution, for example wherein said extraction is performed in a liquid phase using appropriate elemental solvents or their mixtures.

17. A method of long-term storage of the biologically active substance of any one of claims 1-7, or the fragment, analog or derivative of claim 8 using drying and/or lyophilization and sorption on biopolymers, such that when not exposed to the direct sunlight or UV-light, said lyophilized substance can be stored in dry conditions at room temperature without degrading its activity for at least 6 months.

18. A process of extracting a biologically active substance from non-pathologically dividing cells or cell cultures, said process comprising the steps of (i) prior to extraction, adding the biologically active substance of any one of claims 1-7, the fragment, analog or derivative of claim 8, or the composition of claim 9 to said cells or cell cultures; and (ii) extracting the biologically active substance, wherein said addition in step (i) improves the yield of the extracted biological substance.

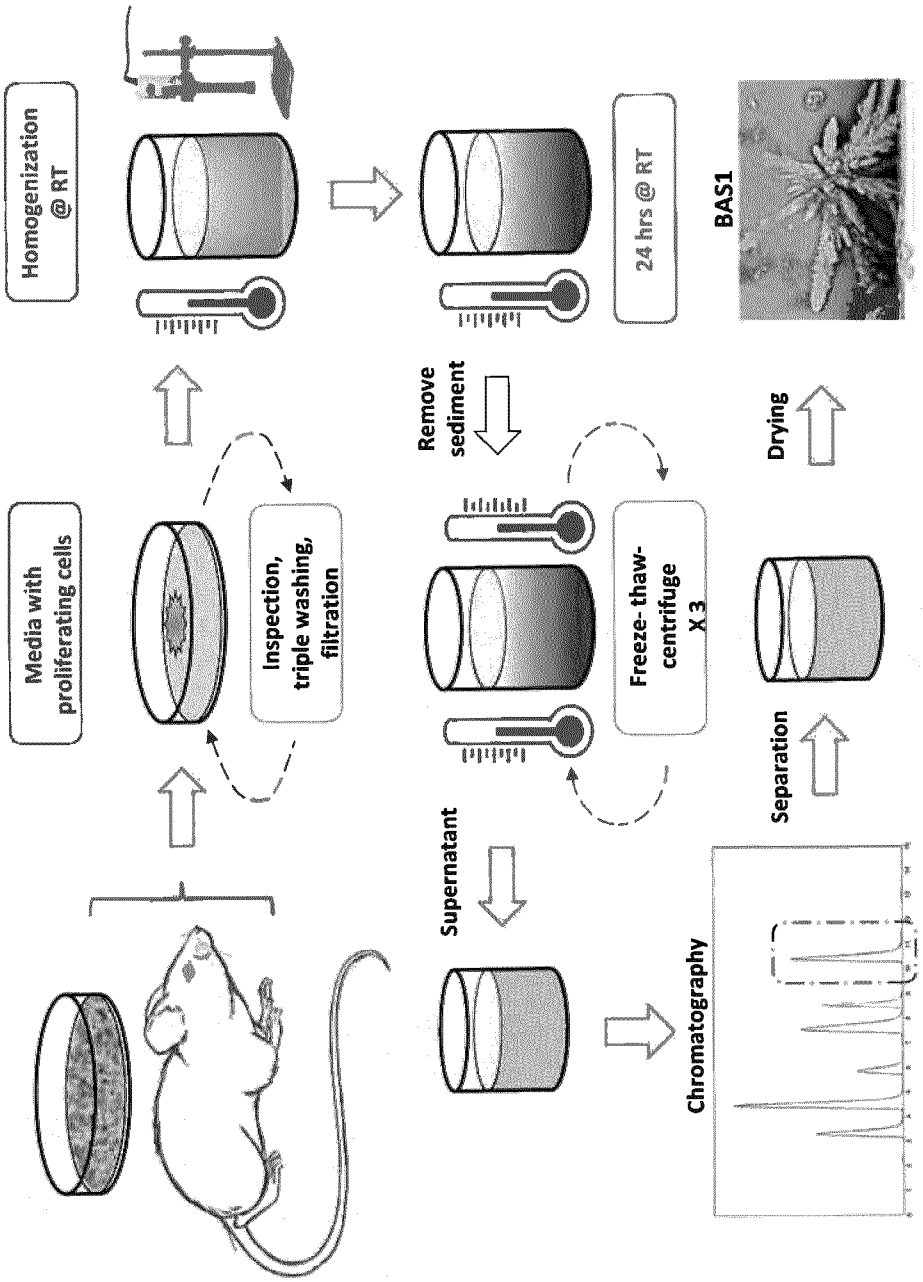
19. The process of claim 18, wherein the added biologically active substance may be the same as or different to the extracted biologically active substance.

20. A biologically active substance obtainable by the process according to claim 18 or 19.

21. A biologically active substance obtained by the process according to claim 18 or 19.

22. A method of identifying a substance for use as a cell proliferation inducer and/or enhancer, an apoptosis inducer and/or enhancer, an anti-inflammatory agent and/or an antibacterial agent according to claim 13, said method comprising (a) providing a test substance; and (b) assessing whether the test substance shifts the cell cycle as defined in claim 3, wherein a test substance that shifts the cell cycle is useful as a cell proliferation inducer and/or enhancer, an apoptosis inducer and/or enhancer, an anti-inflammatory agent and/or an antibacterial agent.

Figure 1


Homogenization @ RT
24 hrs @ RT
BAS1
Media with proliferating cells
Inspection, triple washing, filtration
Remove sediment
Freeze- thaw-centrifuge X 3
Drying
Separation
Supernatant
Chromatography

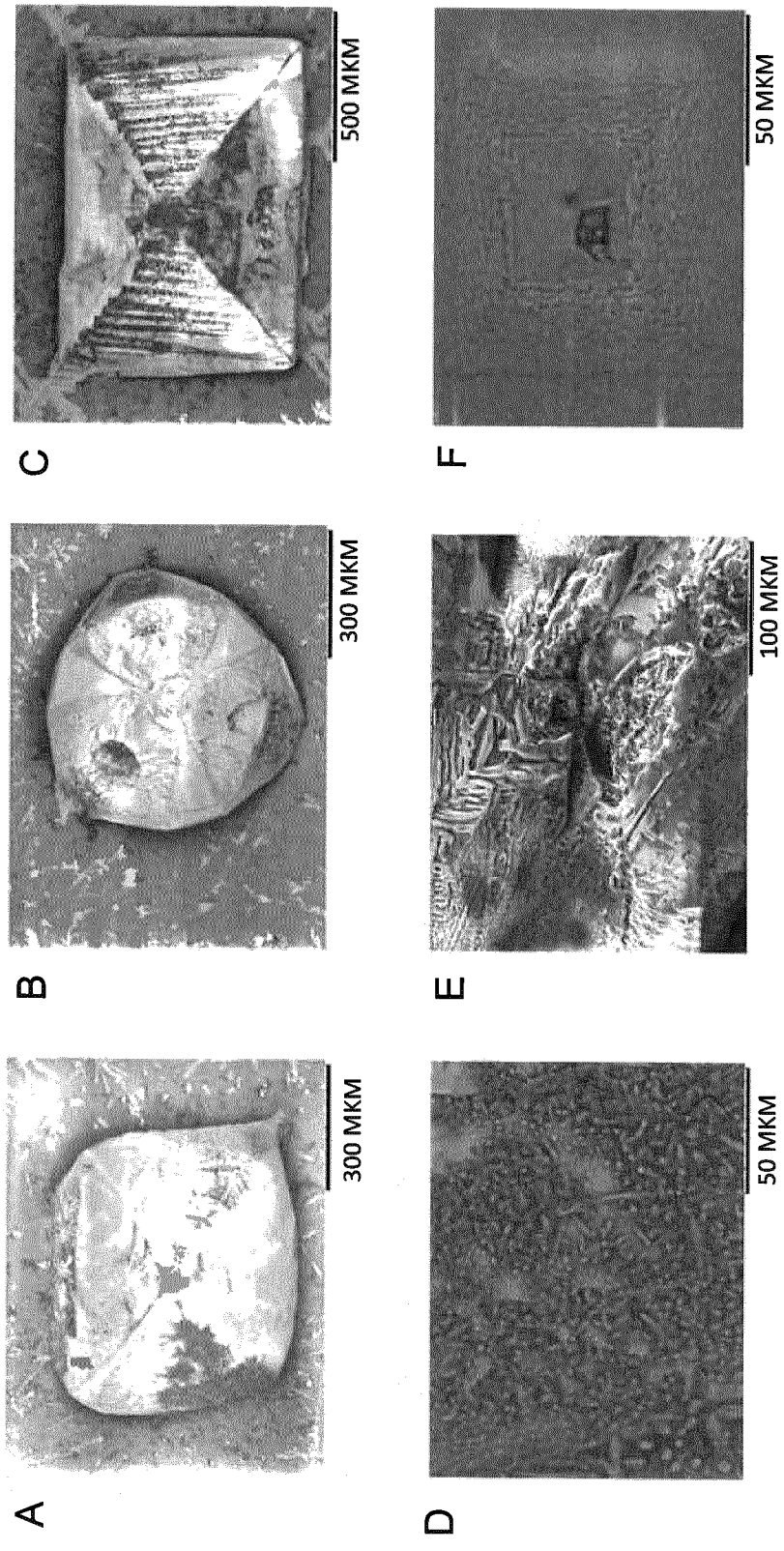
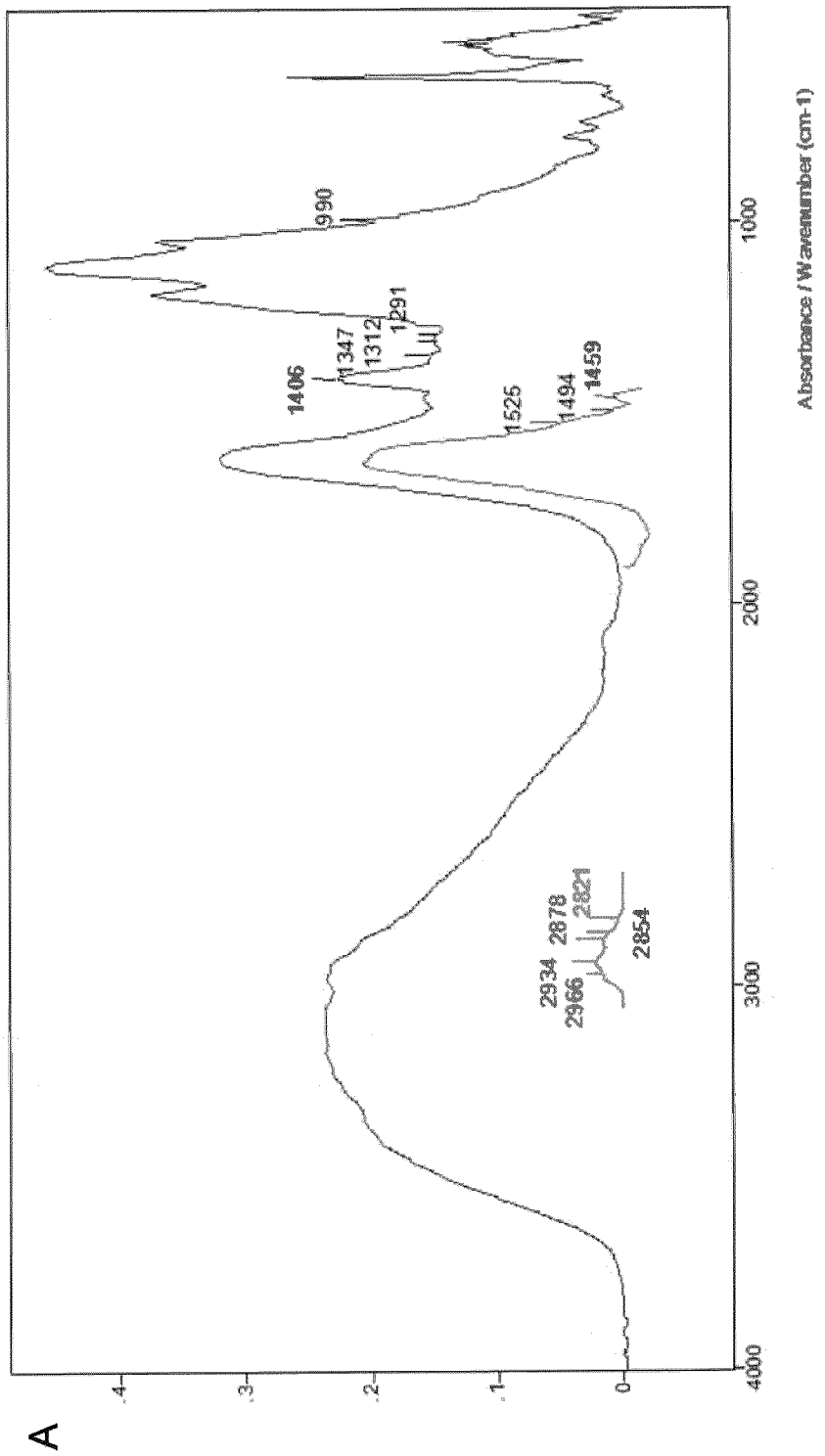


Figure 2A-F

Figure 3A


A
.4
.3
.2
.1
0
4000
3000
2000
1000
Absorbance / Wavenumber (cm-1)
2934
2878
2821
2966
2854
1525
1494
1459
1406
1347
1312
1291
990

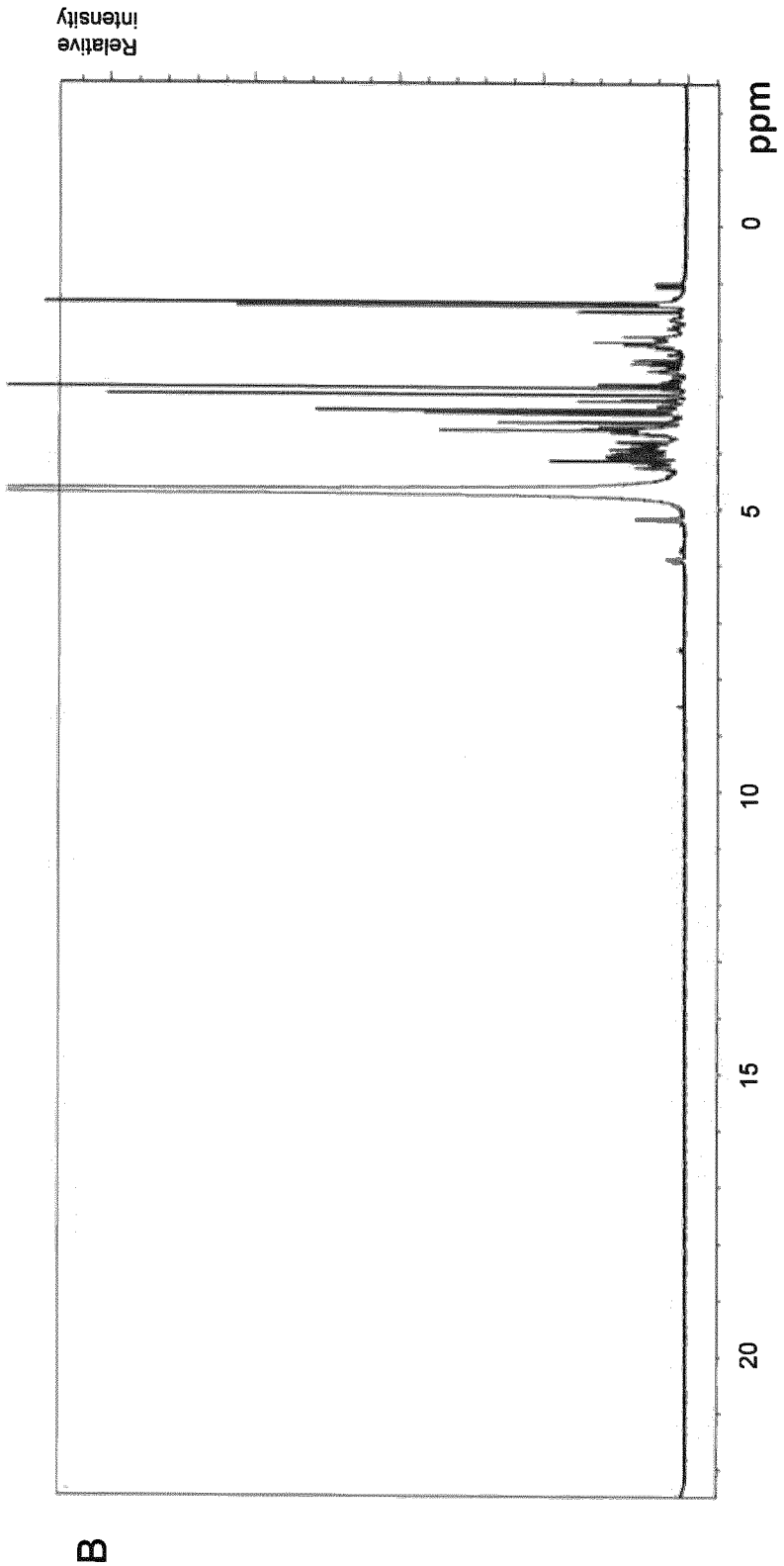
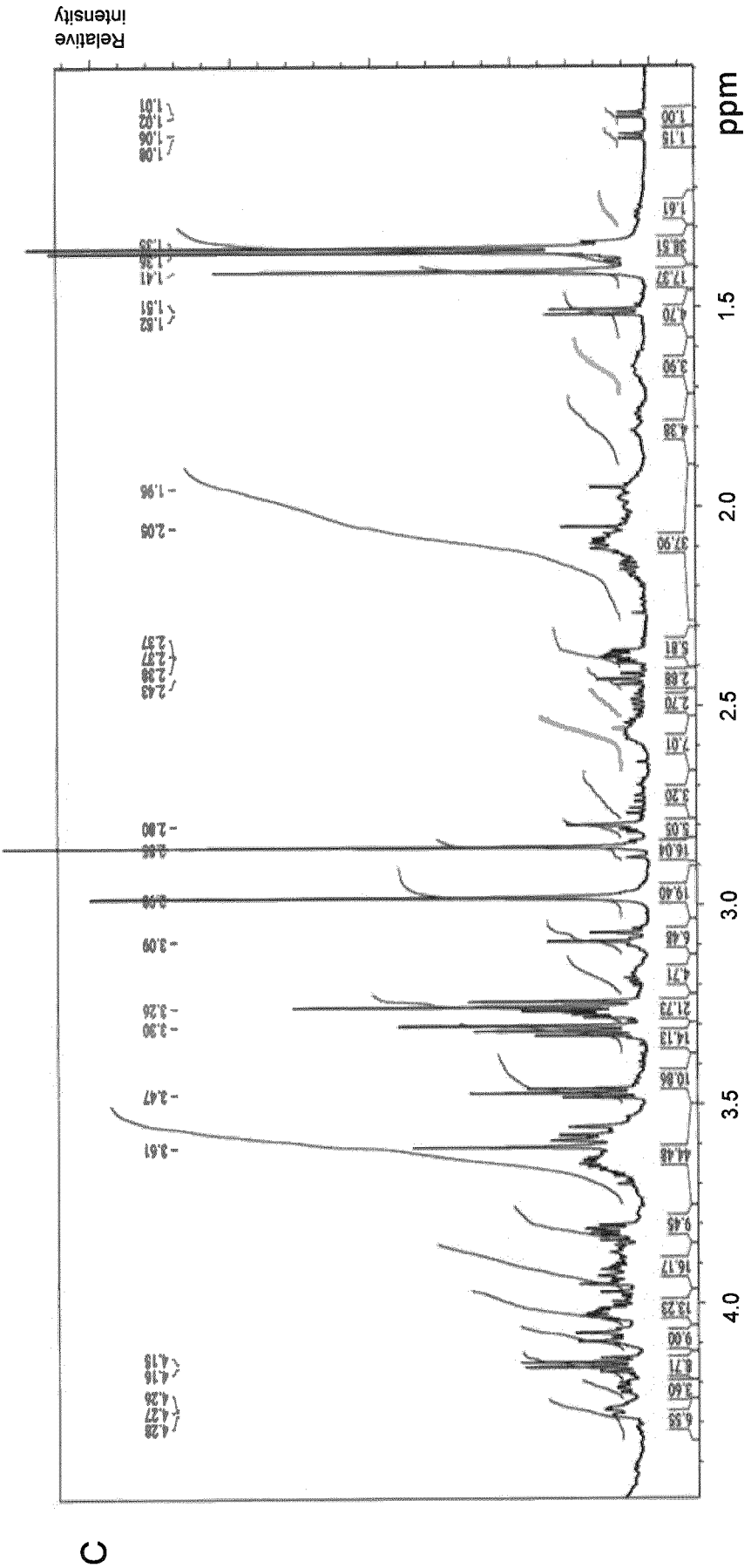


Figure 3B

Figure 3C



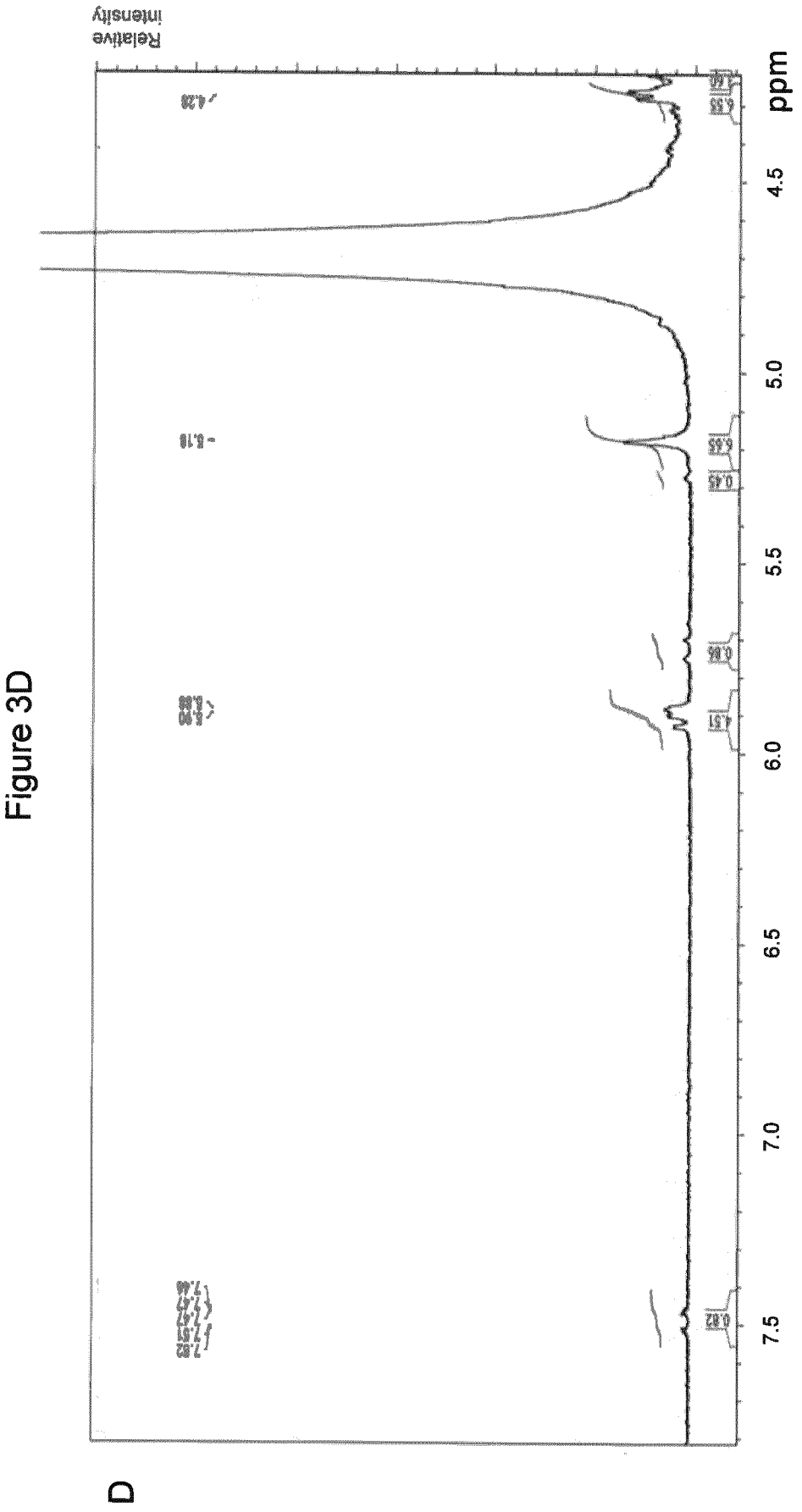


Figure 3D

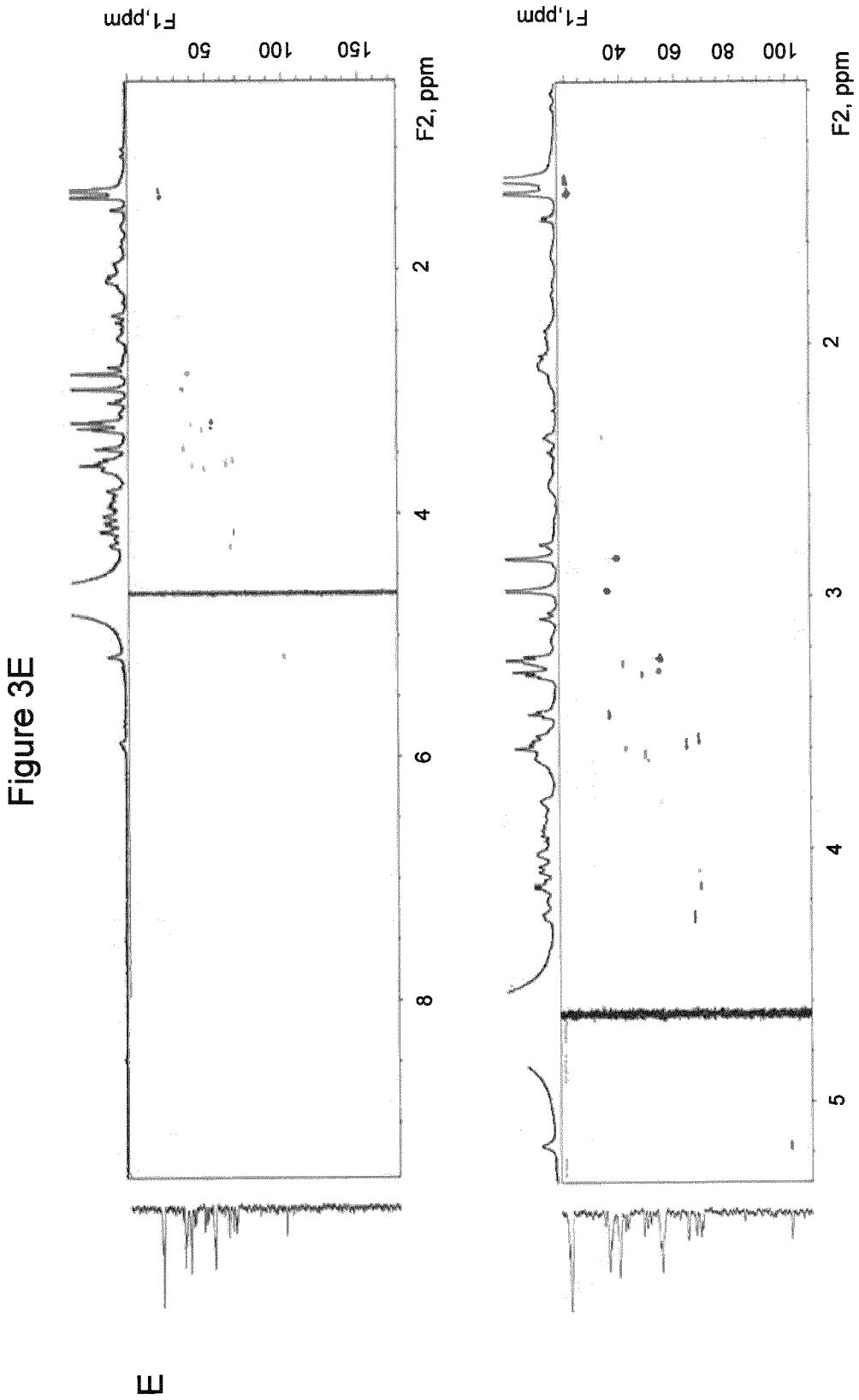


Figure 3E

E

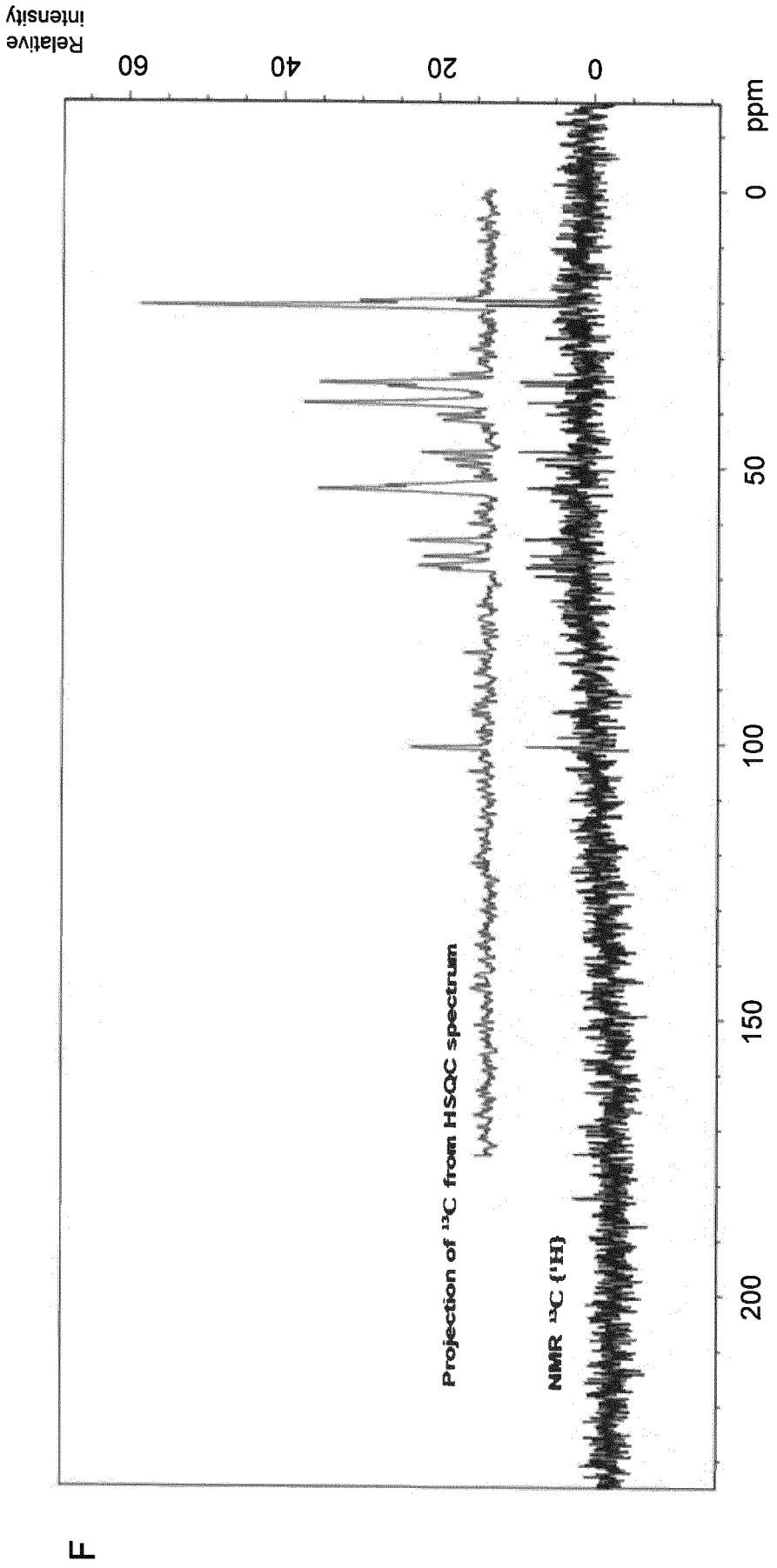

Relative intensity
60
40
20
0
ppm
0
50
100
150
200
Projection of ^{13}C from HSQC spectrum
NMR ^{13}C {^{1}H}
F

Figure 3F

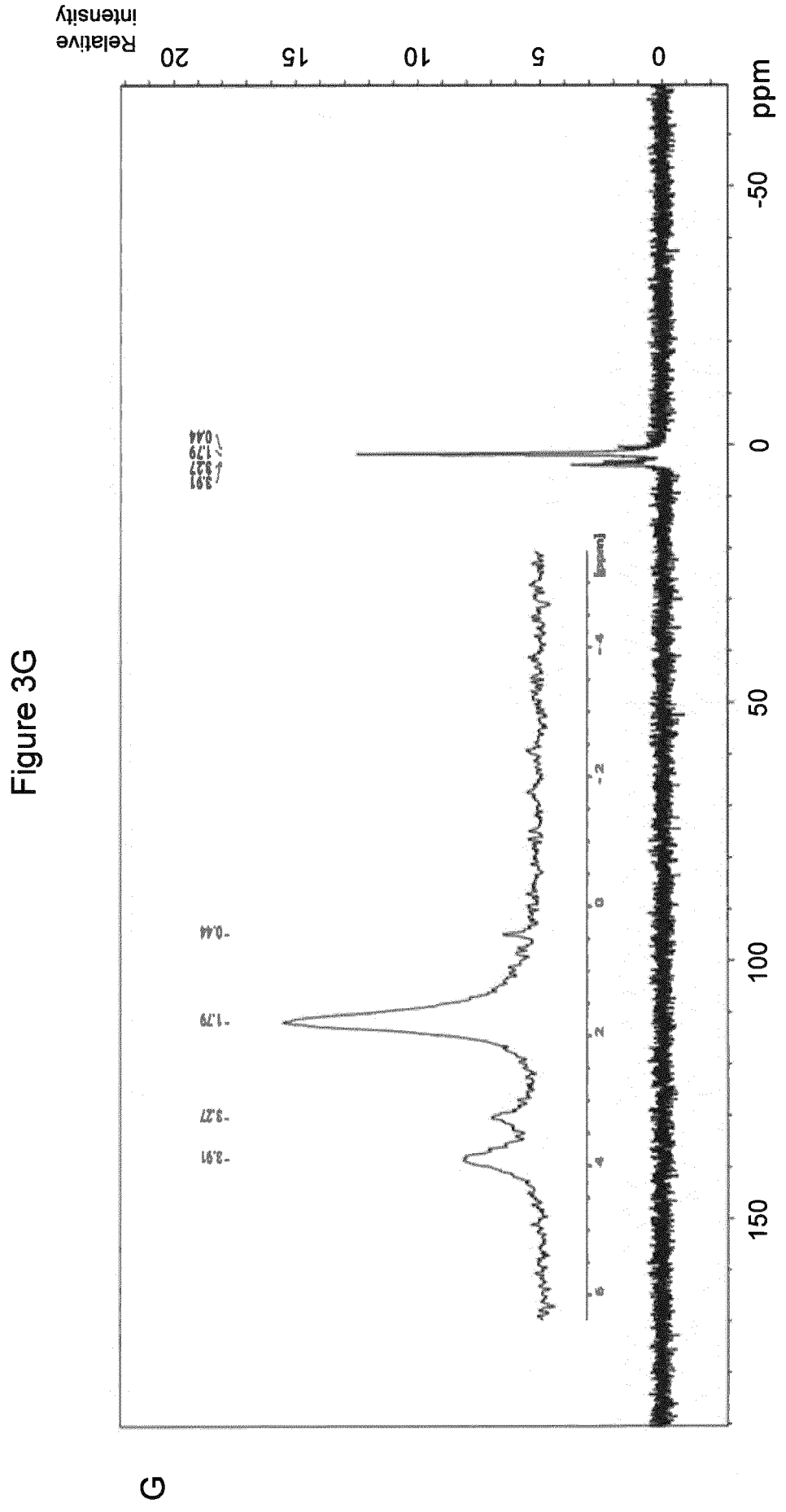
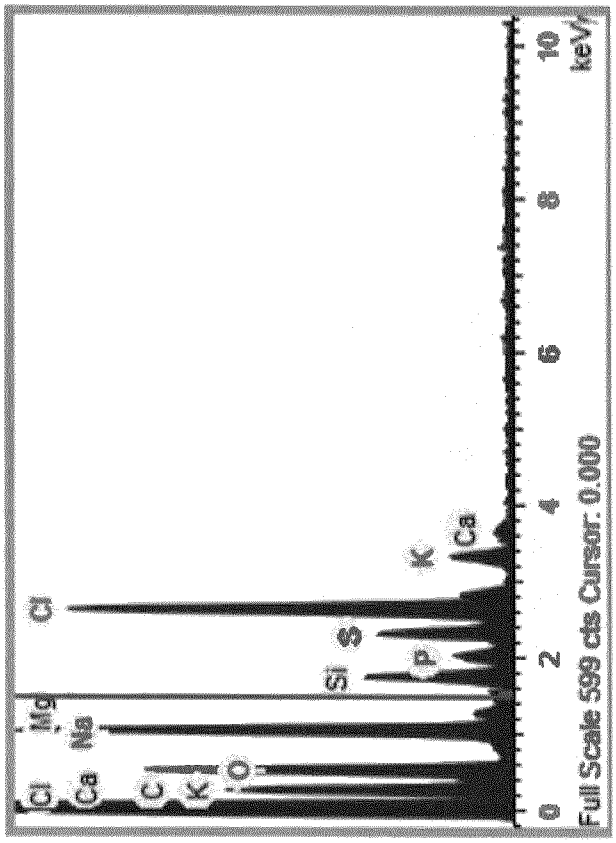

Relative intensity
20
15
10
5
0
ppm
-50
0
50
100
150
3.91
3.27
1.79
0.44

Figure 3G

Figure 4


Cl
Ca
C
K
O
Mg
Na
Si
S
P
Cl
K
Ca
0
2
4
6
8
10
keV
Full Scale 599 cts Cursor: 0.000

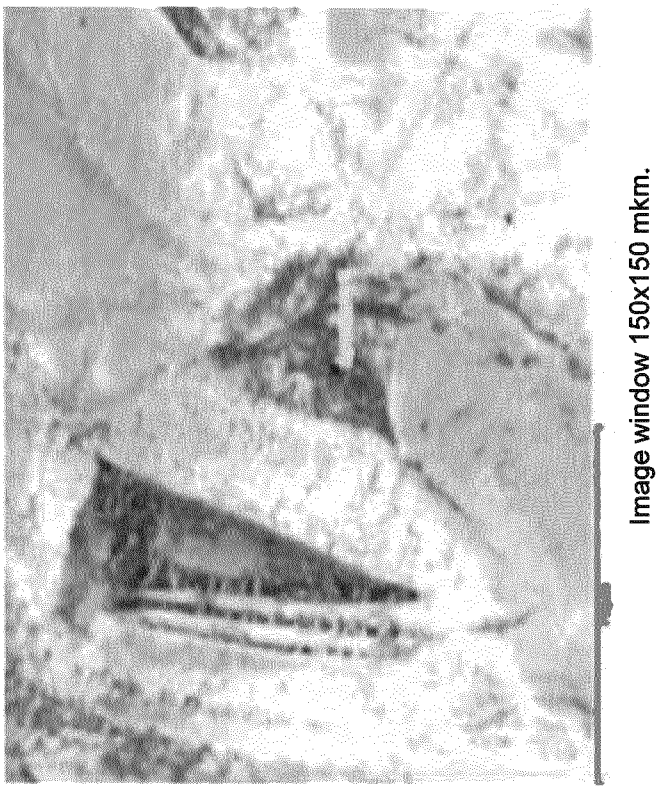


Image window 150x150 mkm.

Figure 5

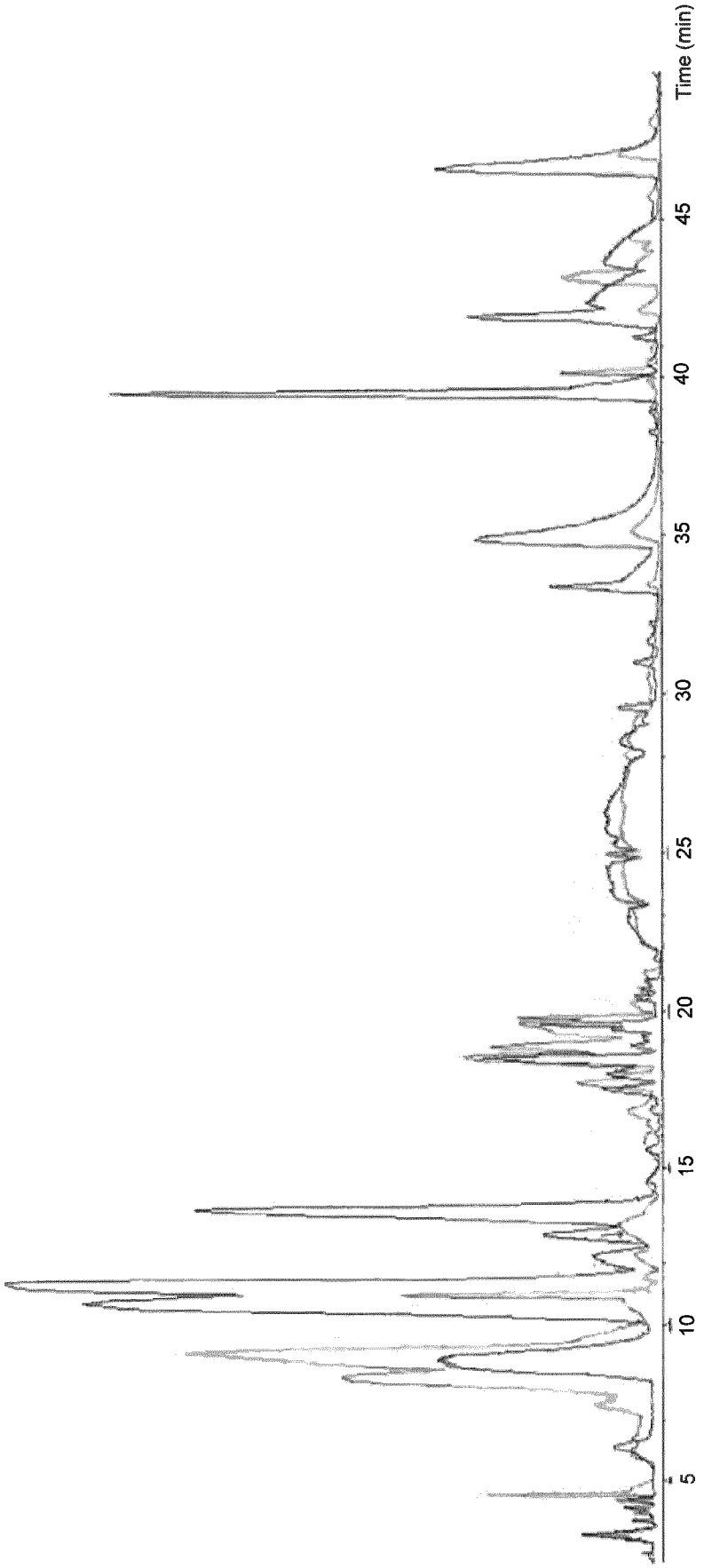


Figure 6

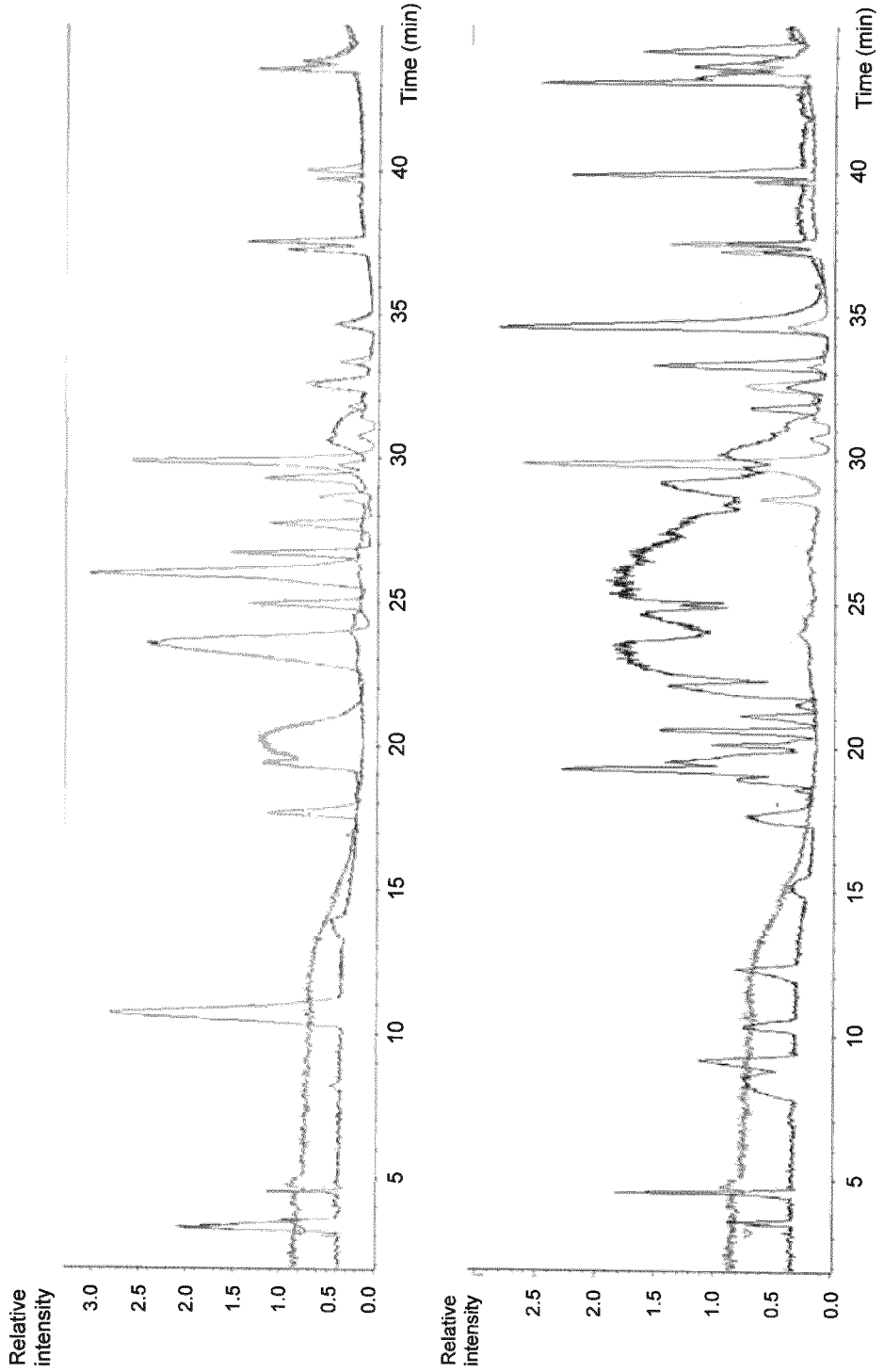

Relative intensity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5
10
15
20
25
30
35
40
Time (min)
Relative intensity
2.5
2.0
1.5
1.0
0.5
0.0
5
10
15
20
25
30
35
40
Time (min)

Figure 7A

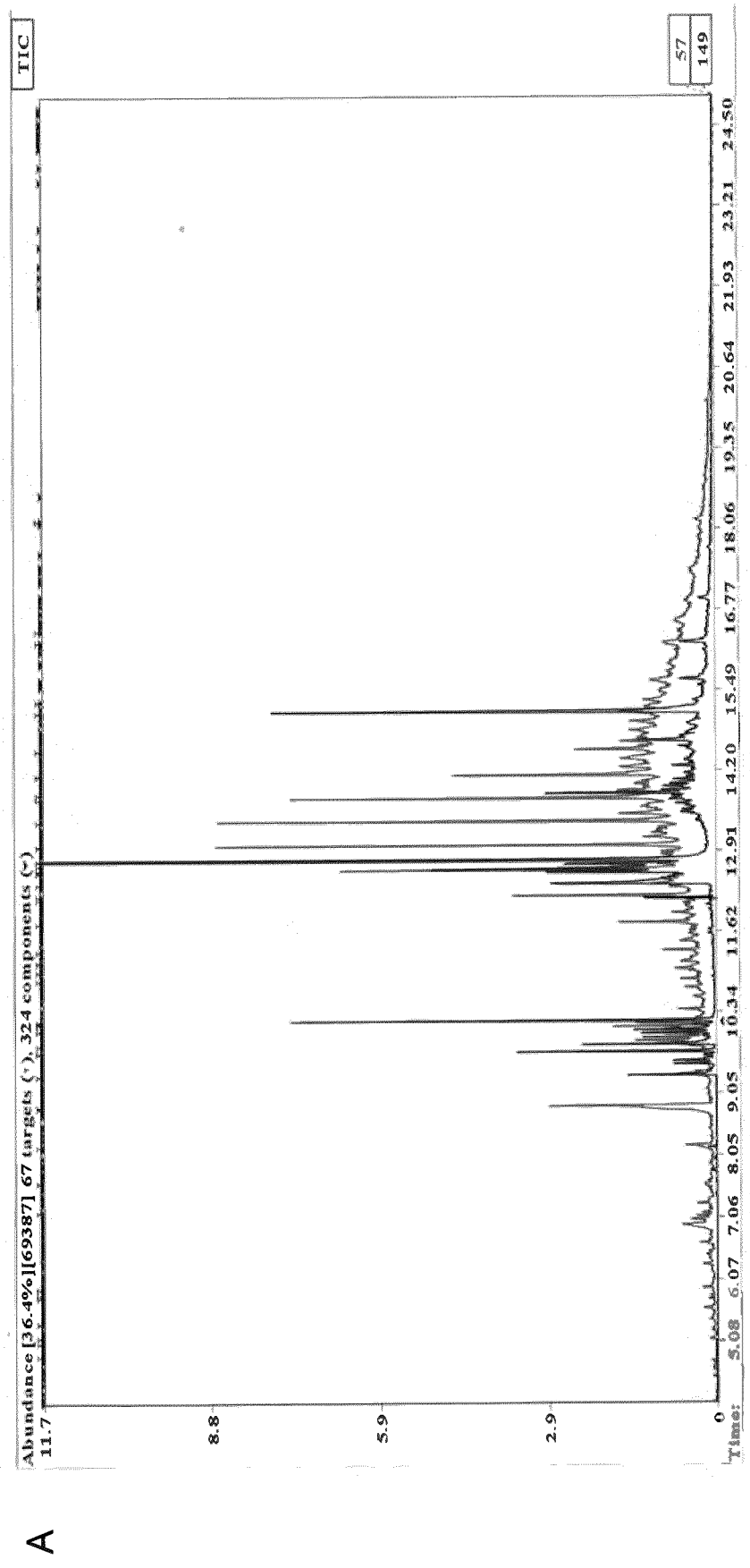

A
TIC
Abundance [36.4%][69387] 67 targets (*), 324 components (*)
11.7
8.8
5.9
2.9
0
Time:
5.08
6.07
7.06
8.05
9.05
10.34
11.62
12.91
14.20
15.49
16.77
18.06
19.35
20.64
21.93
23.21
24.50
57
149

Figure 7B

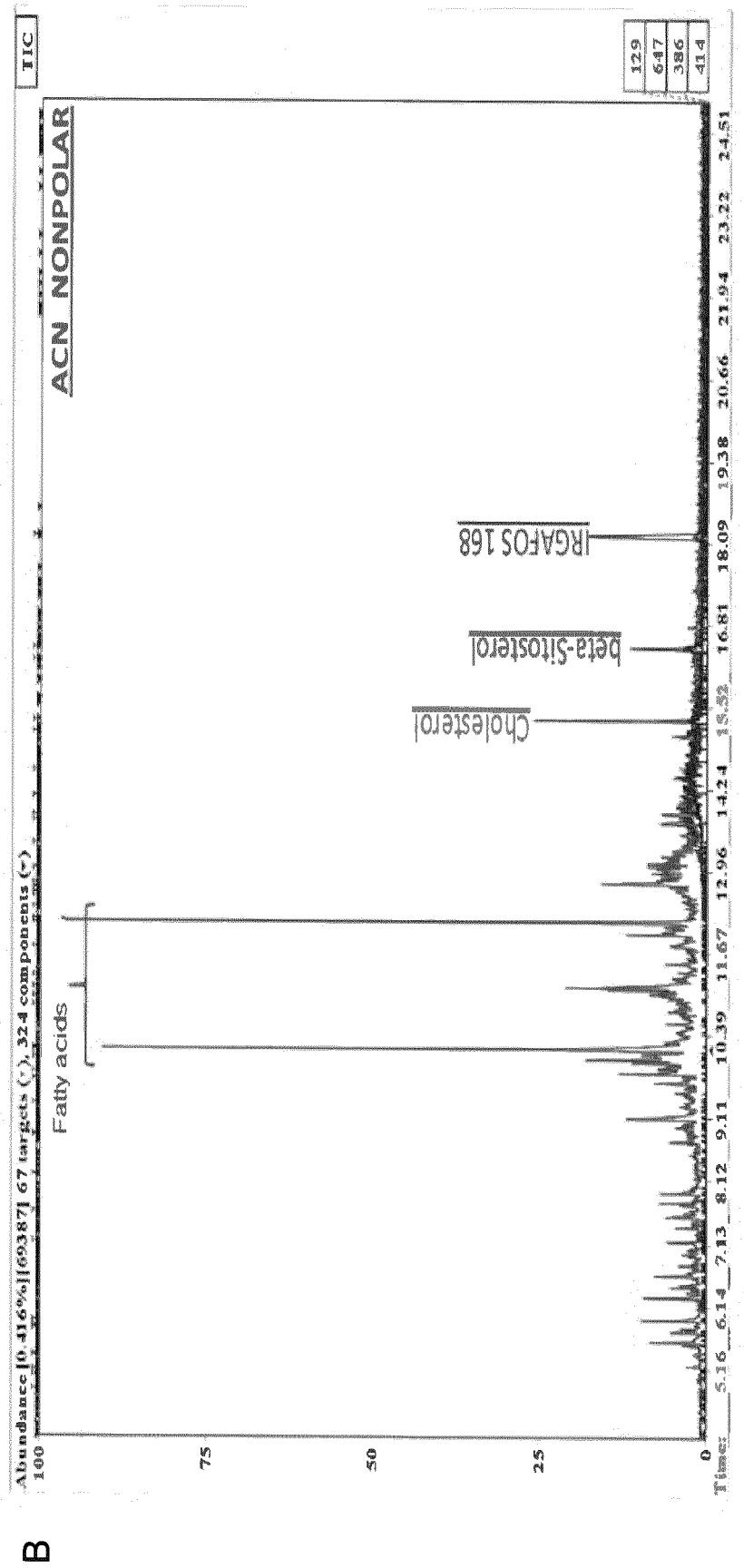

B
TIC
ACN_NONPOLAR
Abundance [0.416%][693871] 67 targets (*), 324 components (~)
Fatty acids
Cholesterol
beta-Sitosterol
IRGAFOS 168
129
647
386
414
Time
5.16
6.14
7.13
8.12
9.11
10.39
11.67
12.96
14.24
15.52
16.81
18.09
19.38
20.66
21.94
23.22
24.51
100
75
50
25
0

Figure 7C

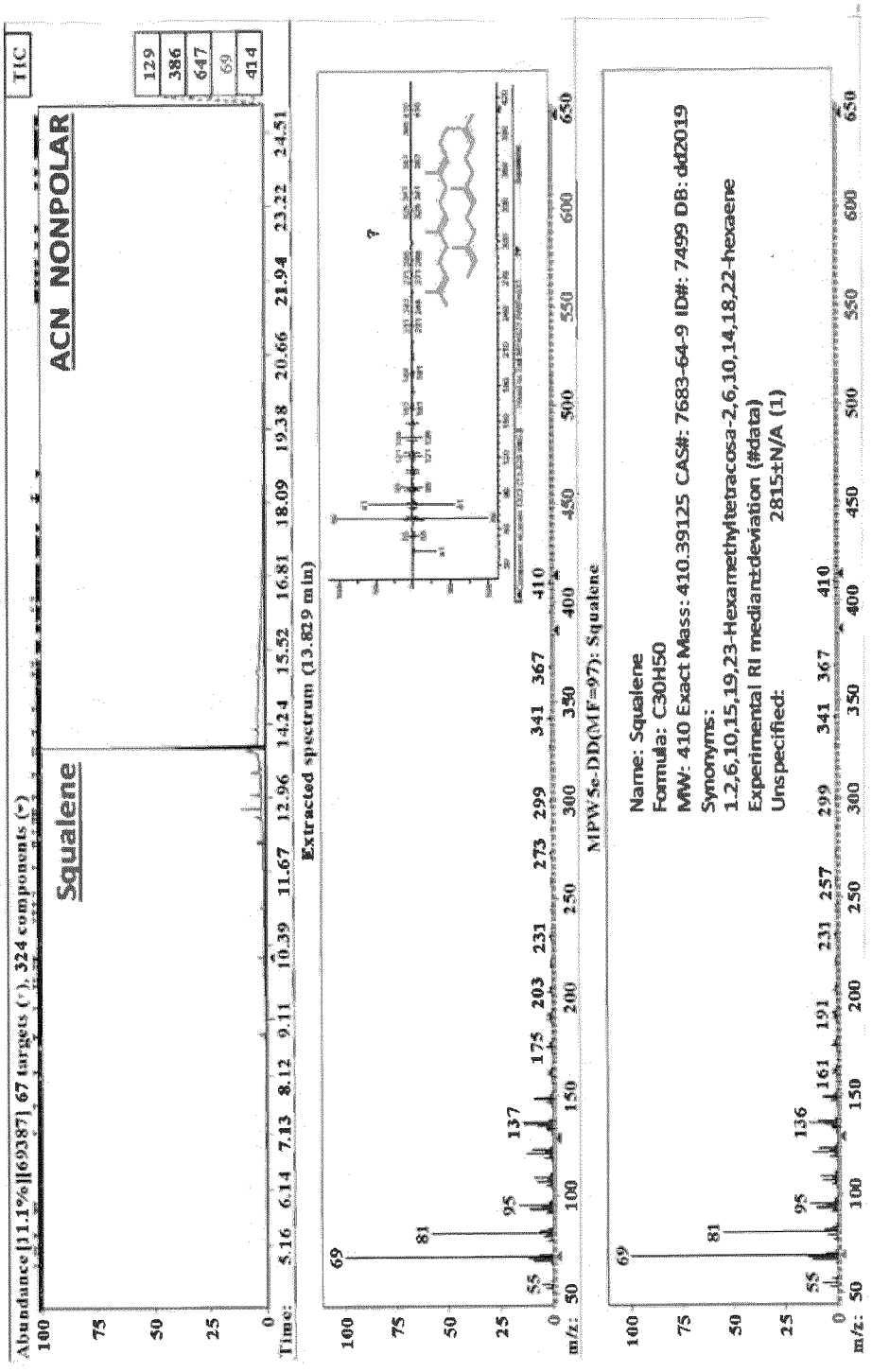
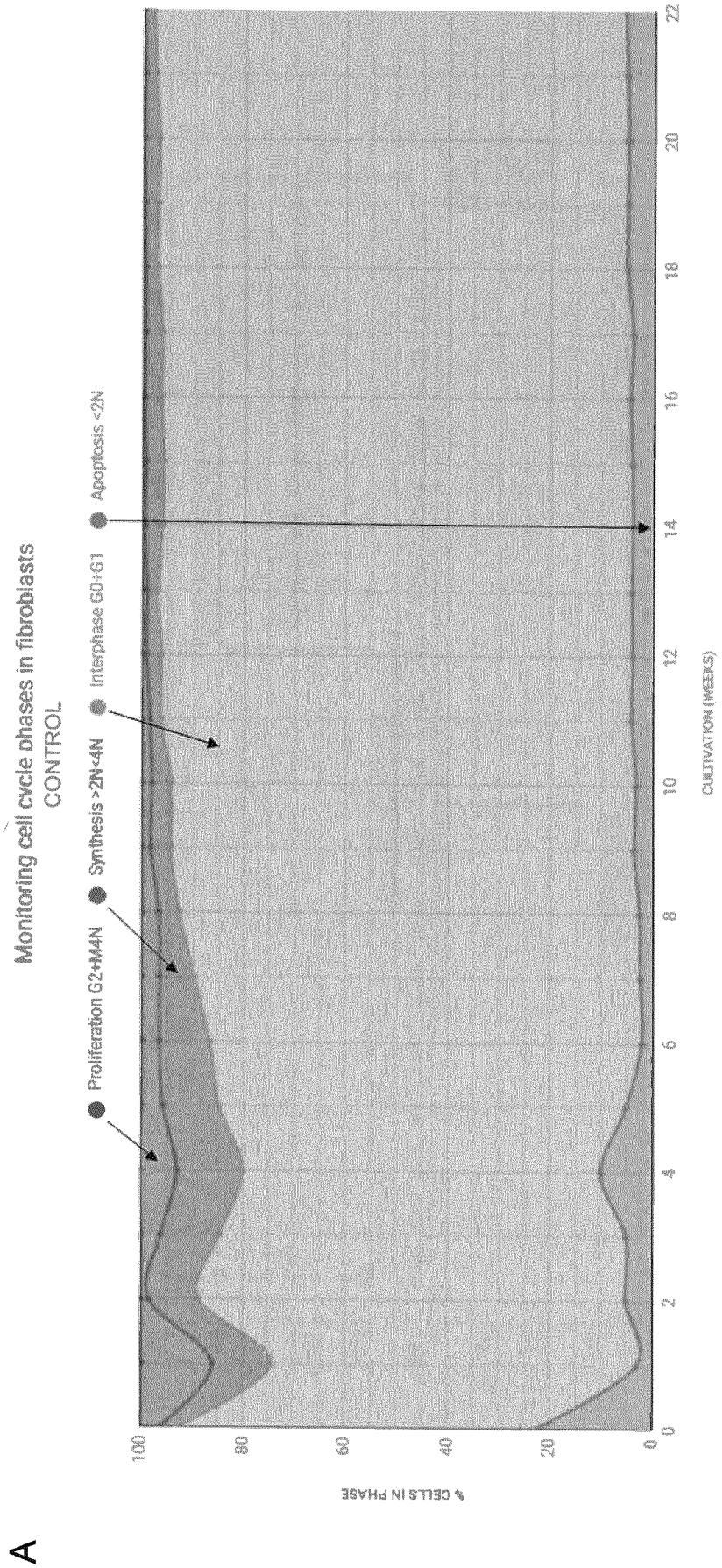

C
TIC
Abundance [11.1%][69387] 67 targets (*), 324 components (*)
Squalene
ACN_NONPOLAR
Time: 5.16 6.14 7.13 8.12 9.11 10.39 11.67 12.96 14.24 15.52 16.81 18.09 19.38 20.66 21.94 23.22 24.51
129
386
647
69
414
Extracted spectrum (13.829 min)
?
m/z: 50 100 150 200 250 300 350 400 450 500 550 600 650
MPW'Se-DD(MF=97): Squalene
Name: Squalene
Formula: C30H50
MW: 410 Exact Mass: 410.39125 CAS#: 7683-64-9 ID#: 7499 DB: dd2019
Synonyms:
1,2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22-hexaene
Experimental RI median±deviation (#data)
Unspecified: 2815±N/A (1)

Figure 8A


Monitoring cell cycle phases in fibroblasts
CONTROL
Proliferation G2+M4N
Synthesis >2N-4N
Interphase G0+G1
Apoptosis <2N
% CELLS IN PHASE
CULTIVATION (WEEKS)
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16
18
20
22

A

Figure 8B

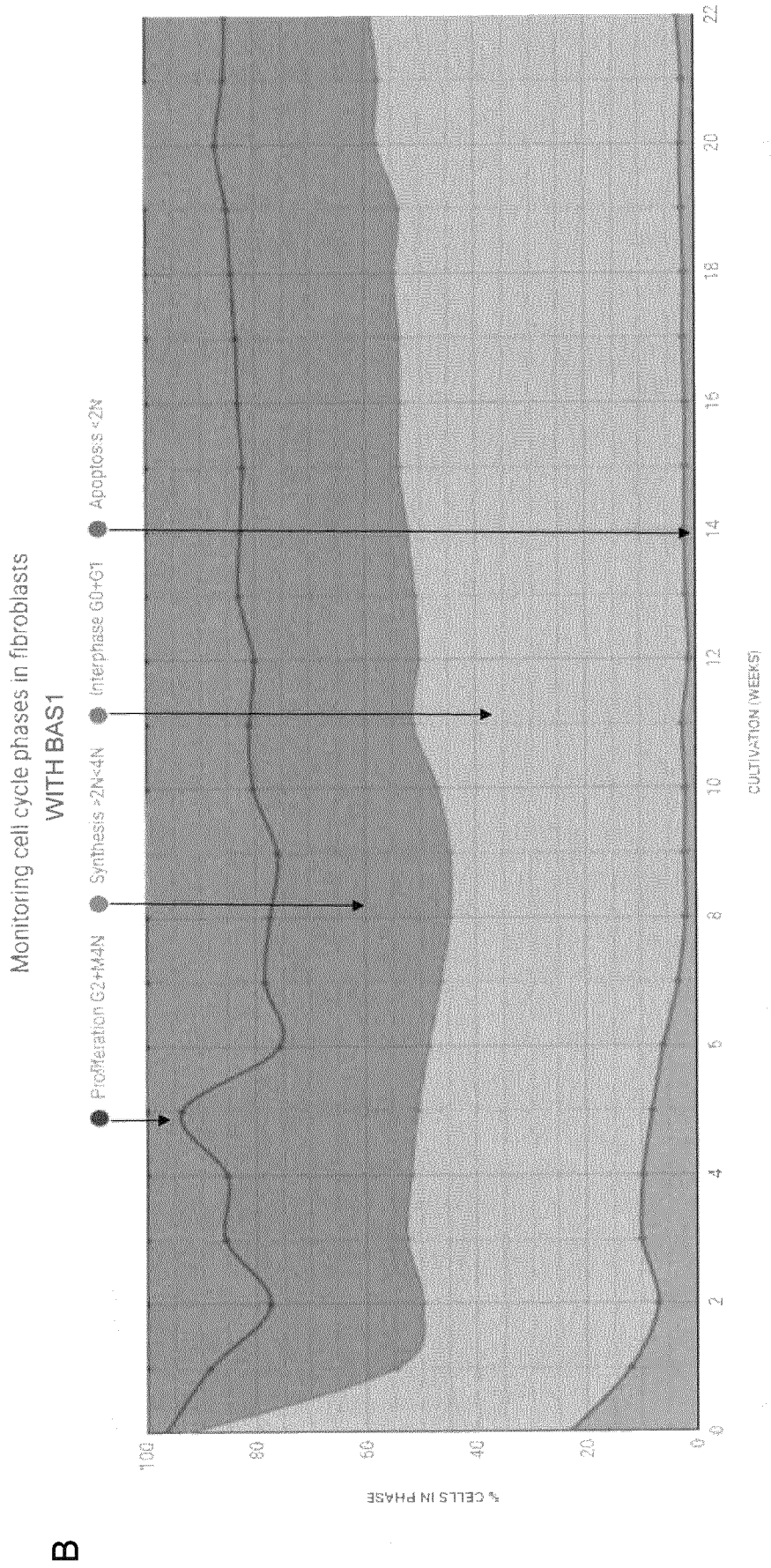
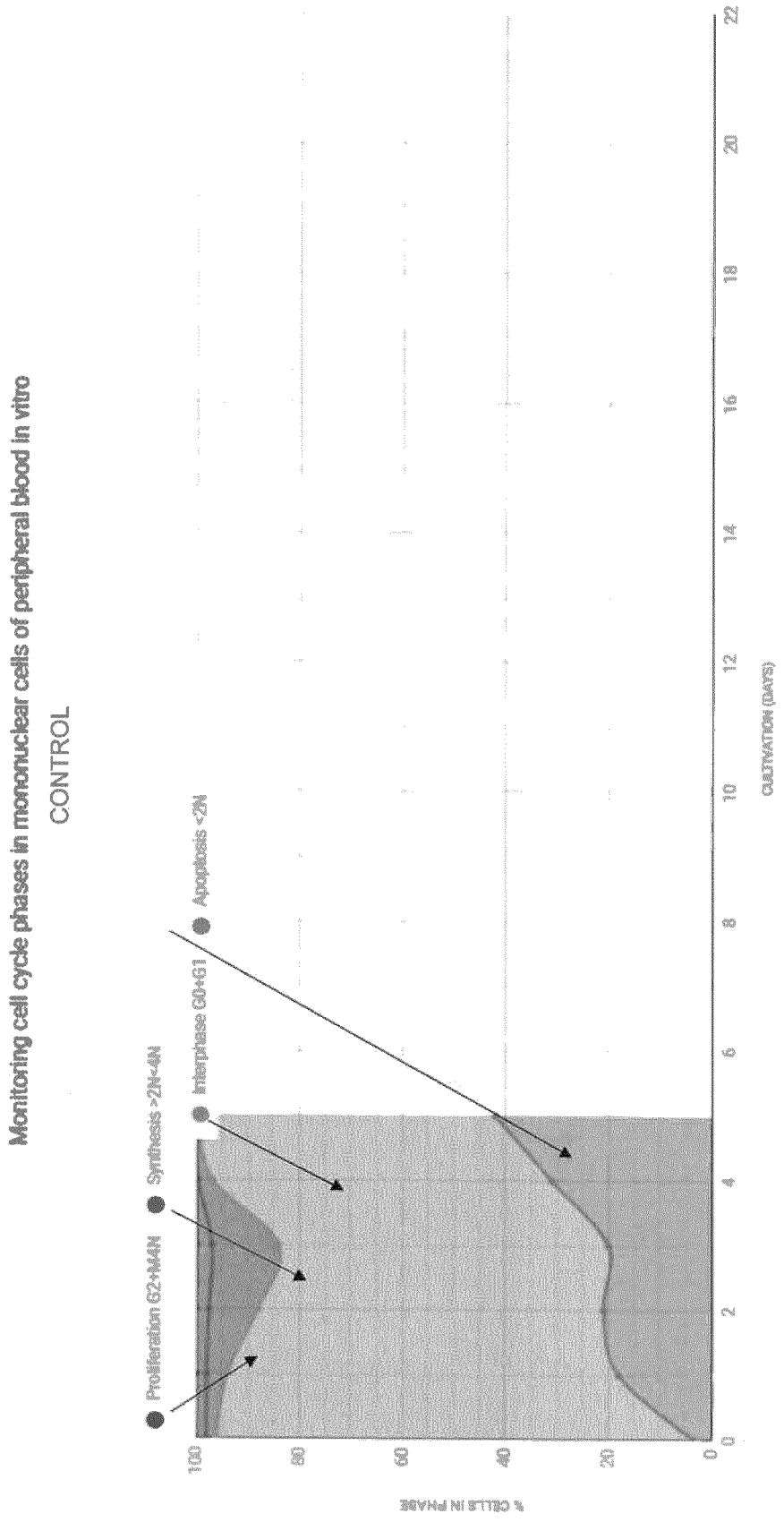

Monitoring cell cycle phases in fibroblasts
WITH BAS1
Proliferation G2+M4N
Synthesis >2N<4N
Interphase G0+G1
Apoptosis <2N
% CELLS IN PHASE
CULTIVATION (WEEKS)
0
20
40
60
80
100
2
4
6
8
10
12
14
16
18
20
22
B

Figure 8C


Monitoring cell cycle phases in mononuclear cells of peripheral blood in vitro
CONTROL
Proliferation G2+M+N
Synthesis >2N<4N
Interphase G0+G1
Apoptosis <2N
% CELLS IN PHASE
CULTIVATION (DAYS)
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16
18
20
22

C

Figure 8D

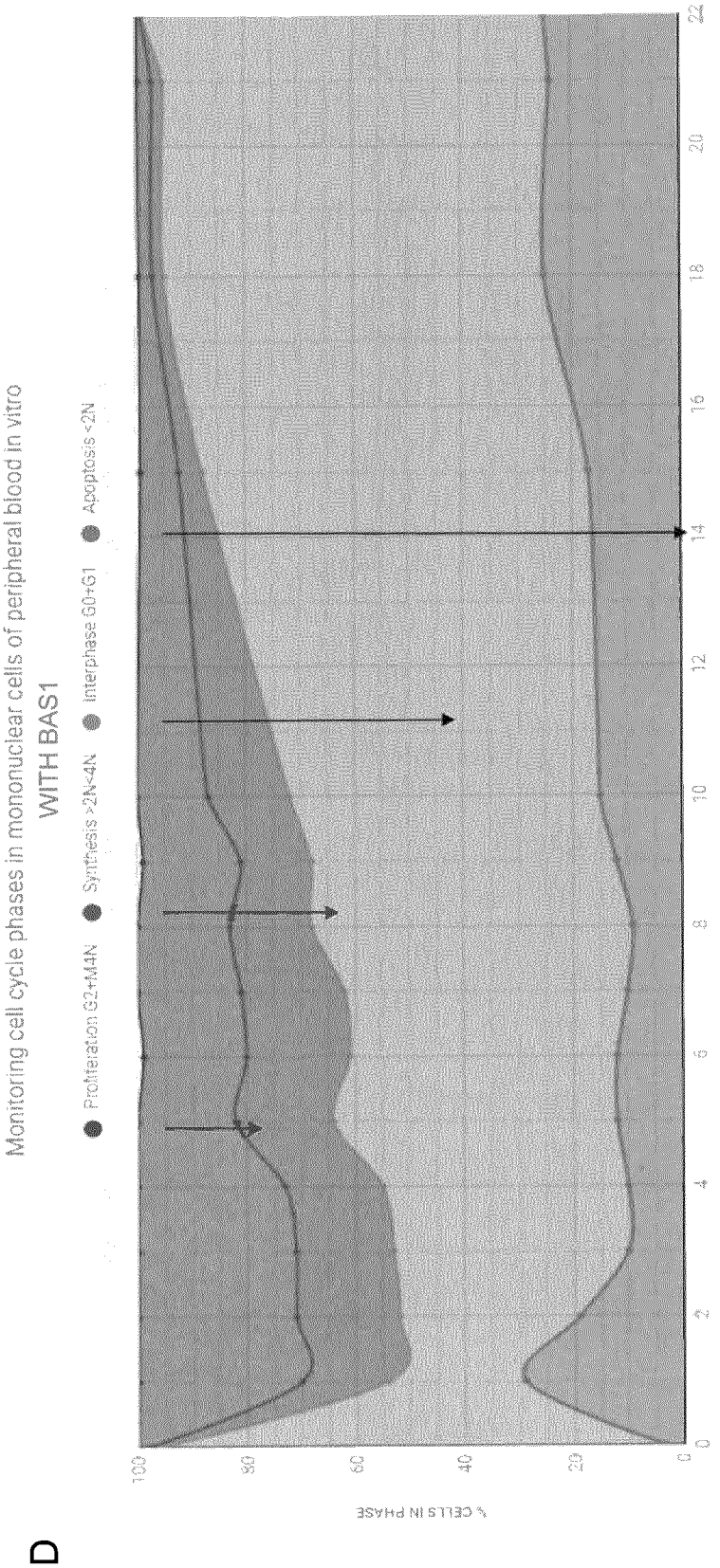

D
Monitoring cell cycle phases in mononuclear cells of peripheral blood in vitro
WITH BAS1
Proliferation G2+M4N
Synthesis >2N<4N
Interphase G0+G1
Apoptosis <2N
% CELLS IN PHASE
CULTIVATION (DAYS)
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16
18
20
22

Figure 9A-B

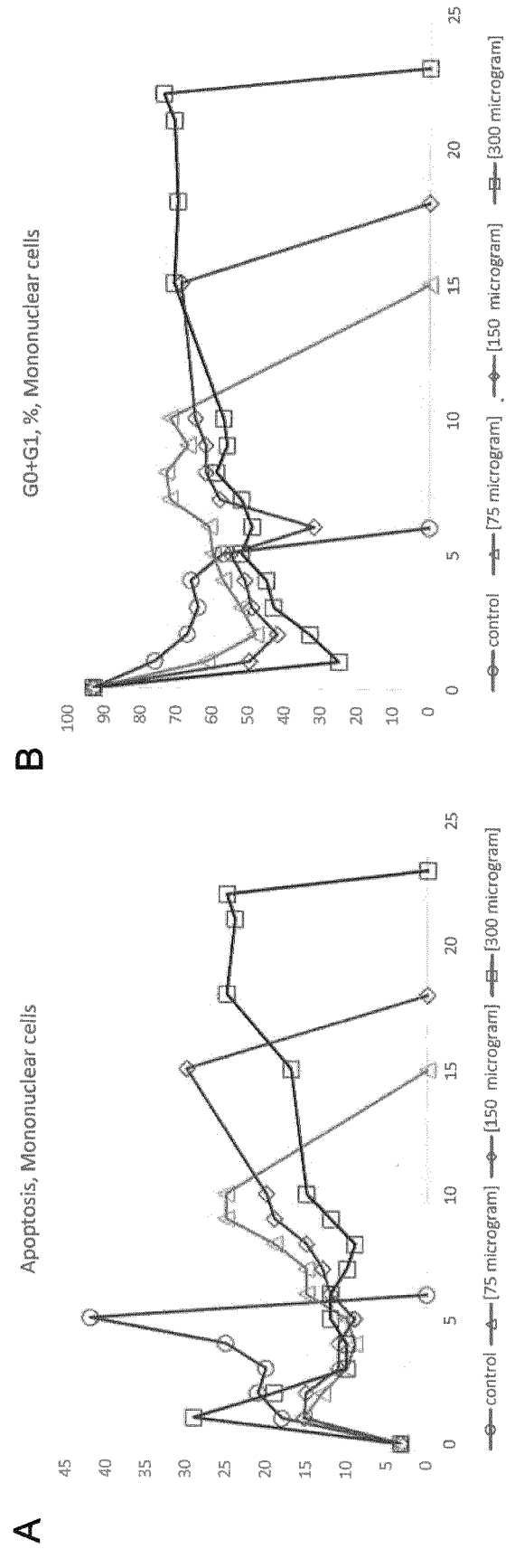

A
Apoptosis, Mononuclear cells
B
G0+G1, %, Mononuclear cells
control
[75 microgram]
[150 microgram]
[300 microgram]
BAS1 concentration in mkg/ml

Figure 9C-D

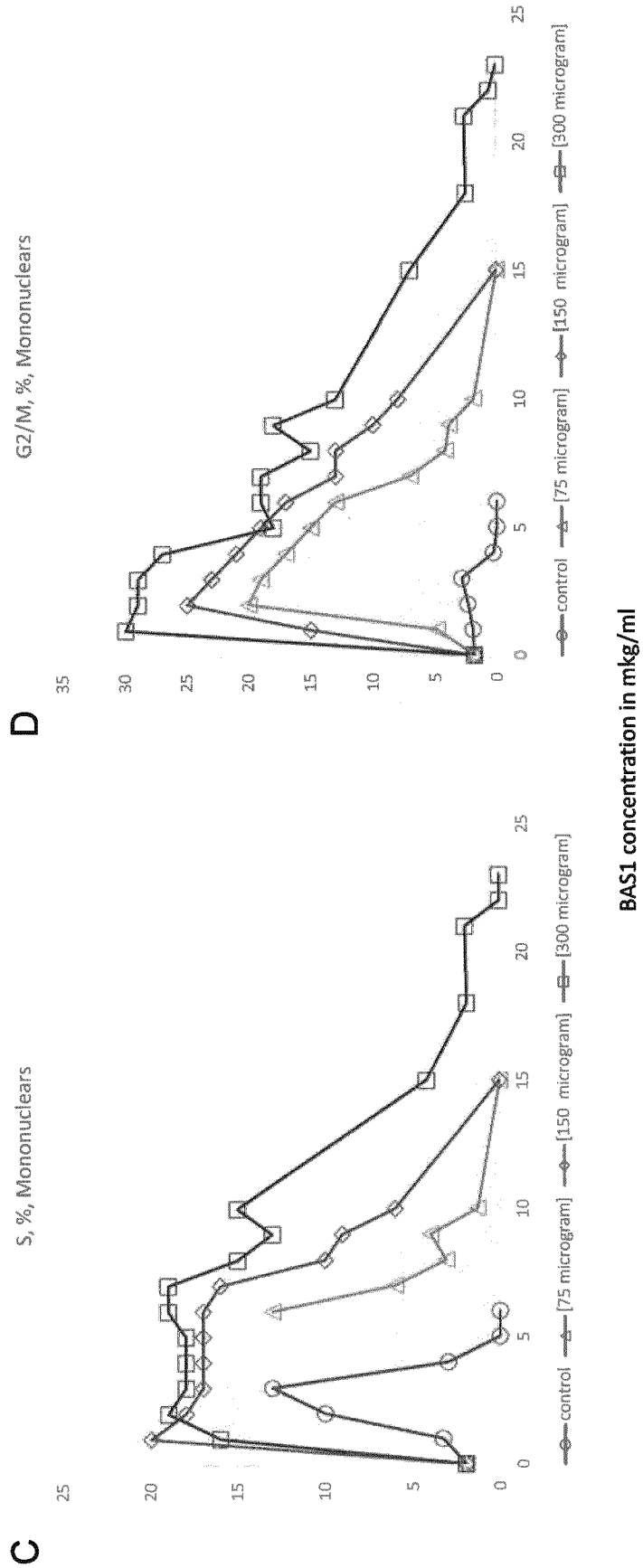

C
S, %, Mononuclears
D
G2/M, %, Mononuclears
control
[75 microgram]
[150 microgram]
[300 microgram]
BAS1 concentration in mkg/ml

Figure 10A-B

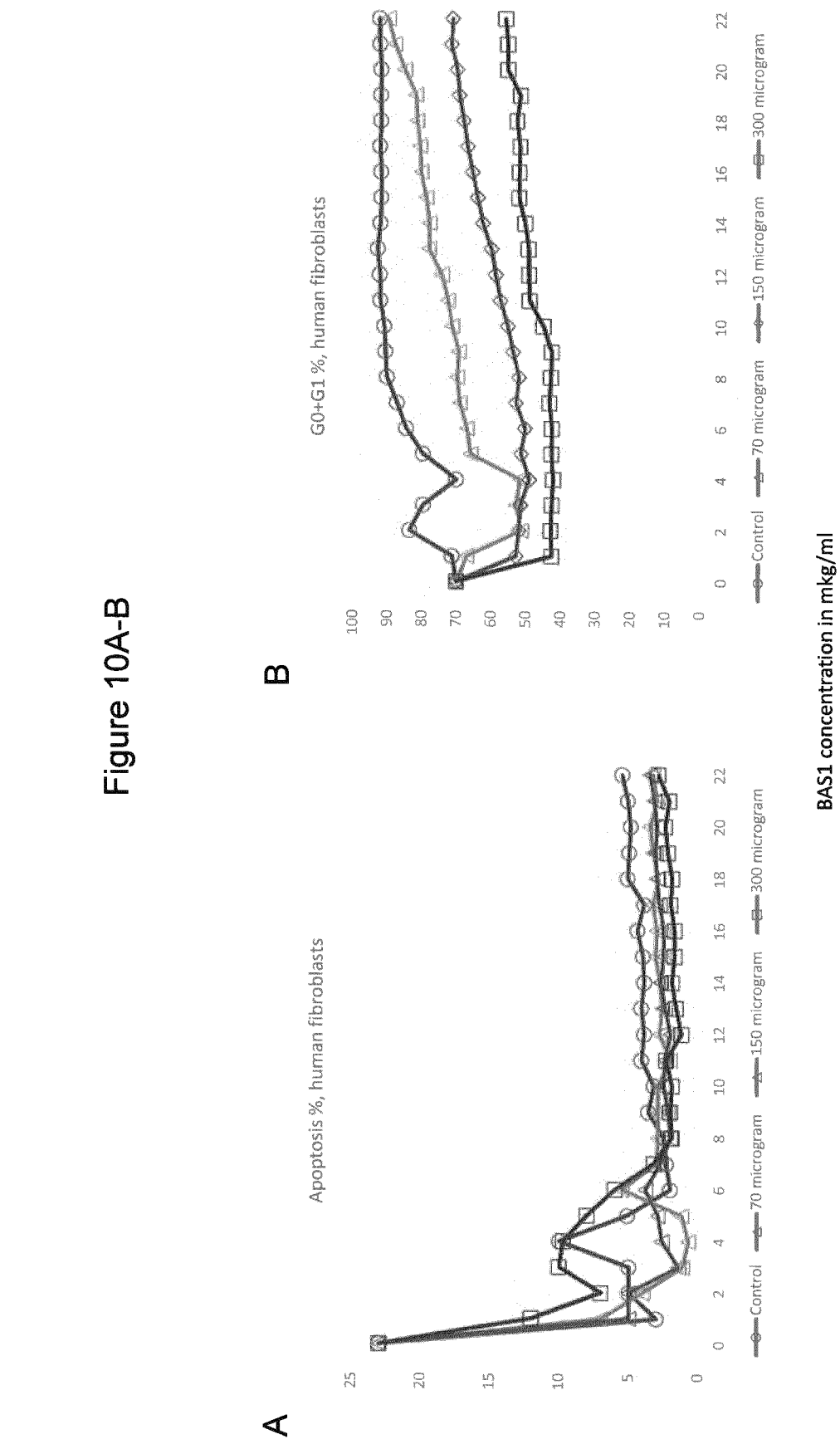

A
Apoptosis %, human fibroblasts
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20 22
Control
70 microgram
150 microgram
300 microgram
B
G0+G1 %, human fibroblasts
100 90 80 70 60 50 40 30 20 10 0
0 2 4 6 8 10 12 14 16 18 20 22
Control
70 microgram
150 microgram
300 microgram
BAS1 concentration in mkg/ml

Figure 10C-D

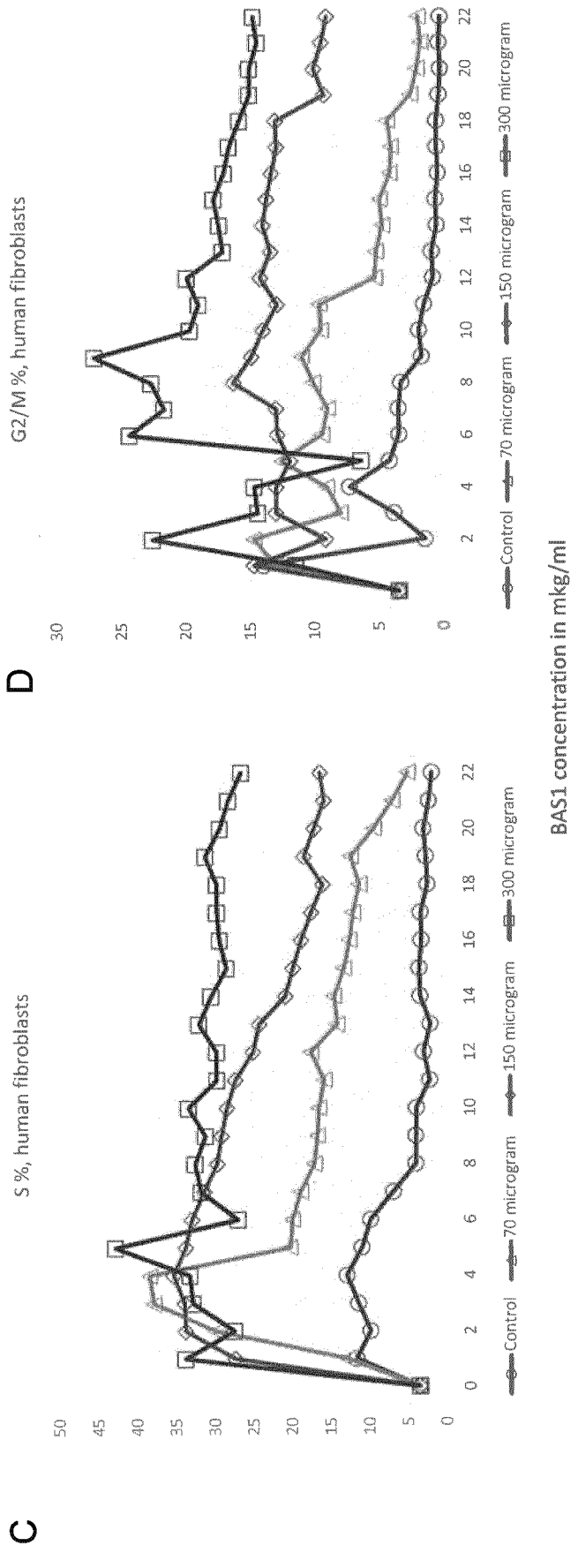
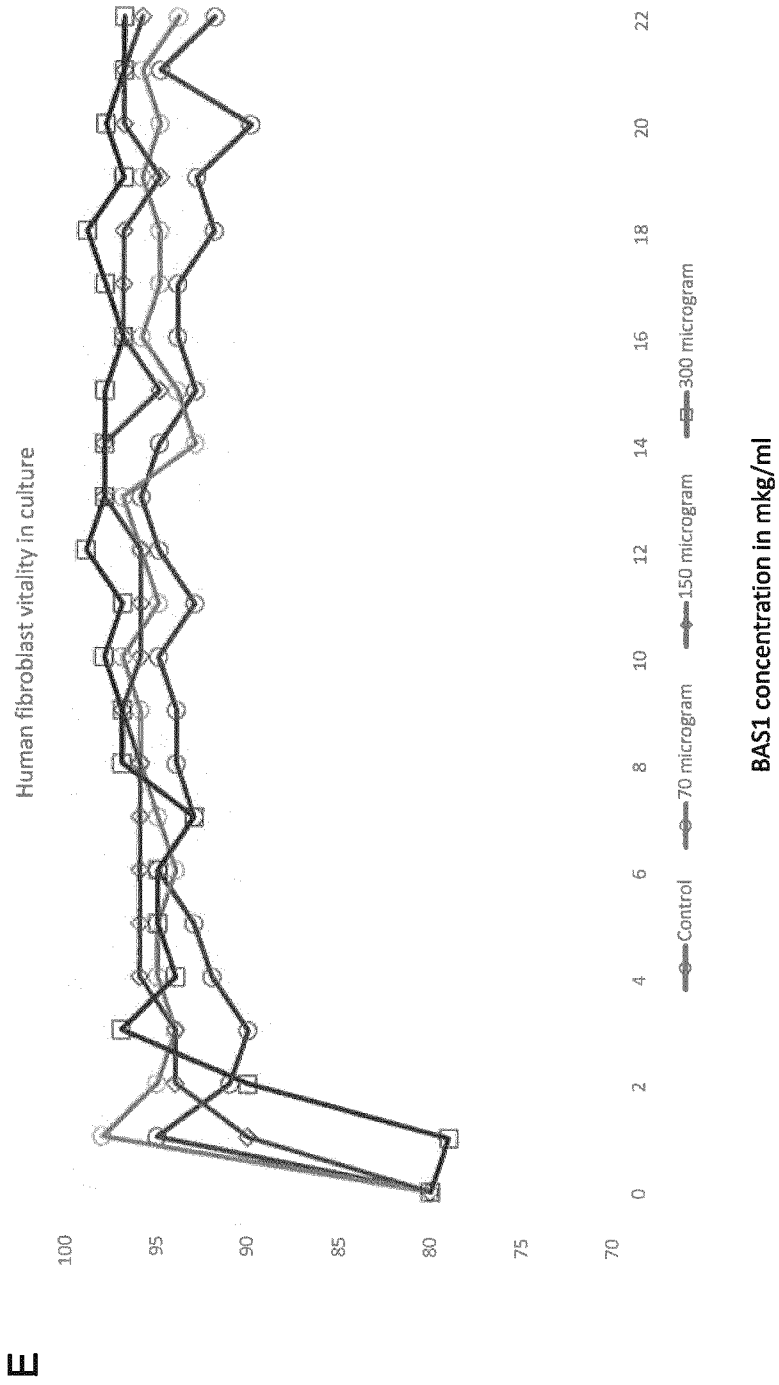

D
G2/M %, human fibroblasts
0
5
10
15
20
25
30
0
2
4
6
8
10
12
14
16
18
20
22
Control
70 microgram
150 microgram
300 microgram
C
S %, human fibroblasts
0
5
10
15
20
25
30
35
40
45
50
0
2
4
6
8
10
12
14
16
18
20
22
Control
70 microgram
150 microgram
300 microgram
BAS1 concentration in mkg/ml

Figure 10E


Human fibroblast vitality in culture
100
95
90
85
80
75
70
0
2
4
6
8
10
12
14
16
18
20
22
Control
70 microgram
150 microgram
300 microgram
BAS1 concentration in mkg/ml

E

Figure 11A-D

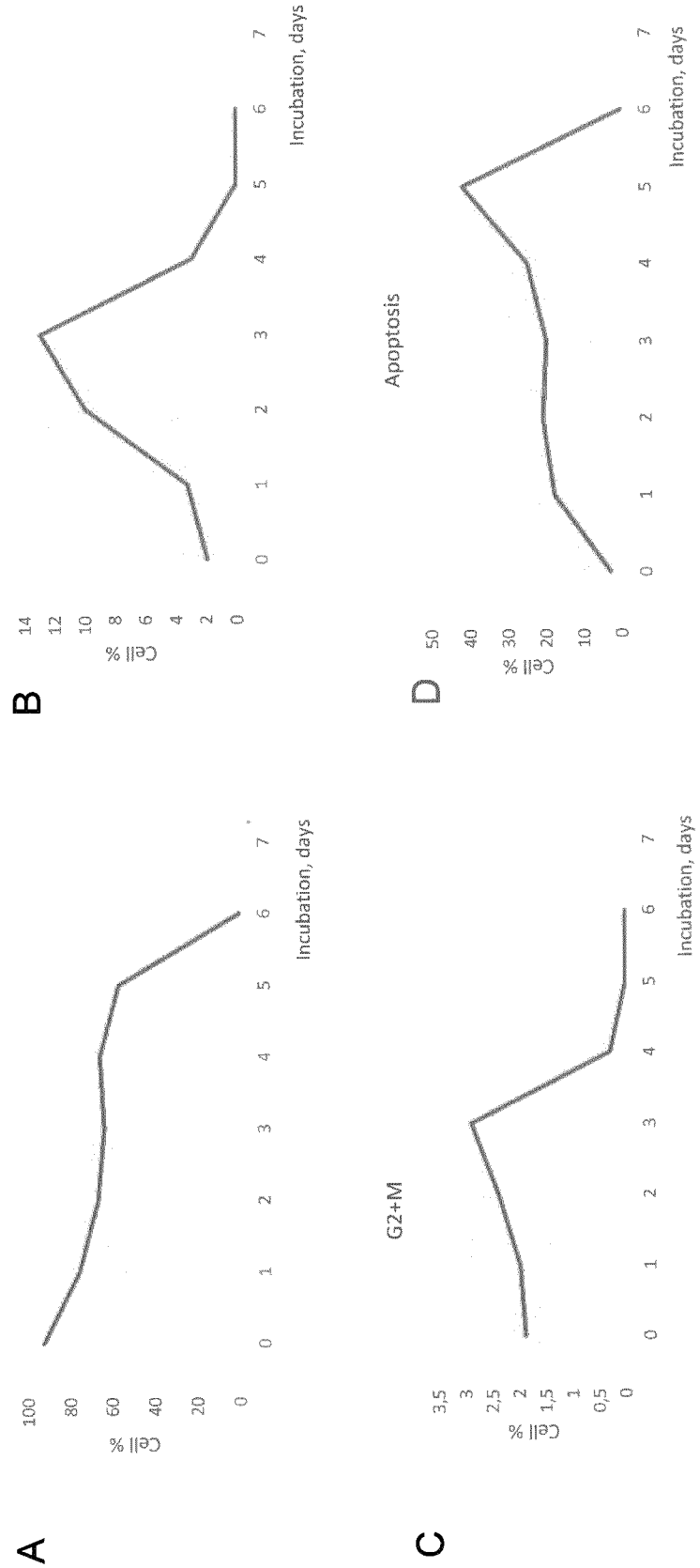

A
G0+G1
Cell %
Incubation, days
B
S
Cell %
Incubation, days
C
G2+M
Cell %
Incubation, days
D
Apoptosis
Cell %
Incubation, days

Figure 12A-B


A
G0, %, Vs Age
100
98
96
94
92
90
88
86
84
0
20
40
60
80
100
Age, years
male
female
B
S, %, Vs Age
12
10
8
6
4
2
0
0
20
40
60
80
100
Age, years
male
female

Figure 12C-D

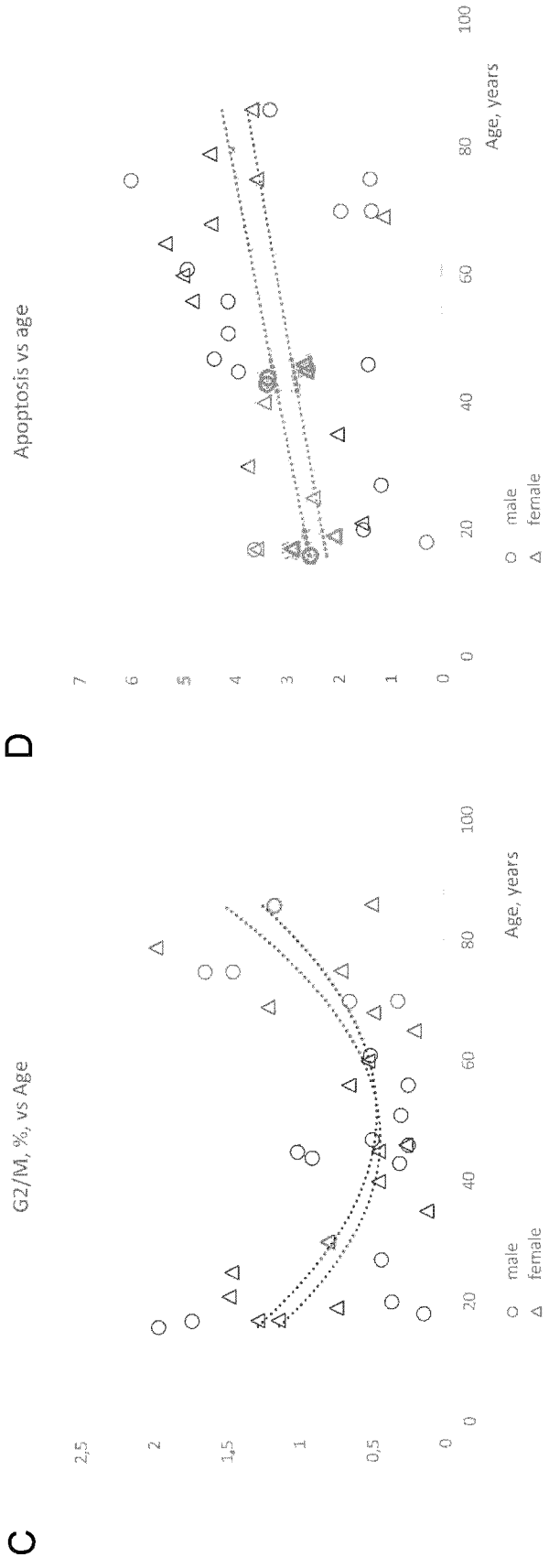

C
G2/M, %, vs Age
2,5
2
1,5
1
0,5
0
0
20
40
60
80
100
Age, years
male
female
D
Apoptosis vs age
7
6
5
4
3
2
1
0
0
20
40
60
80
100
Age, years
male
female

Figure 13

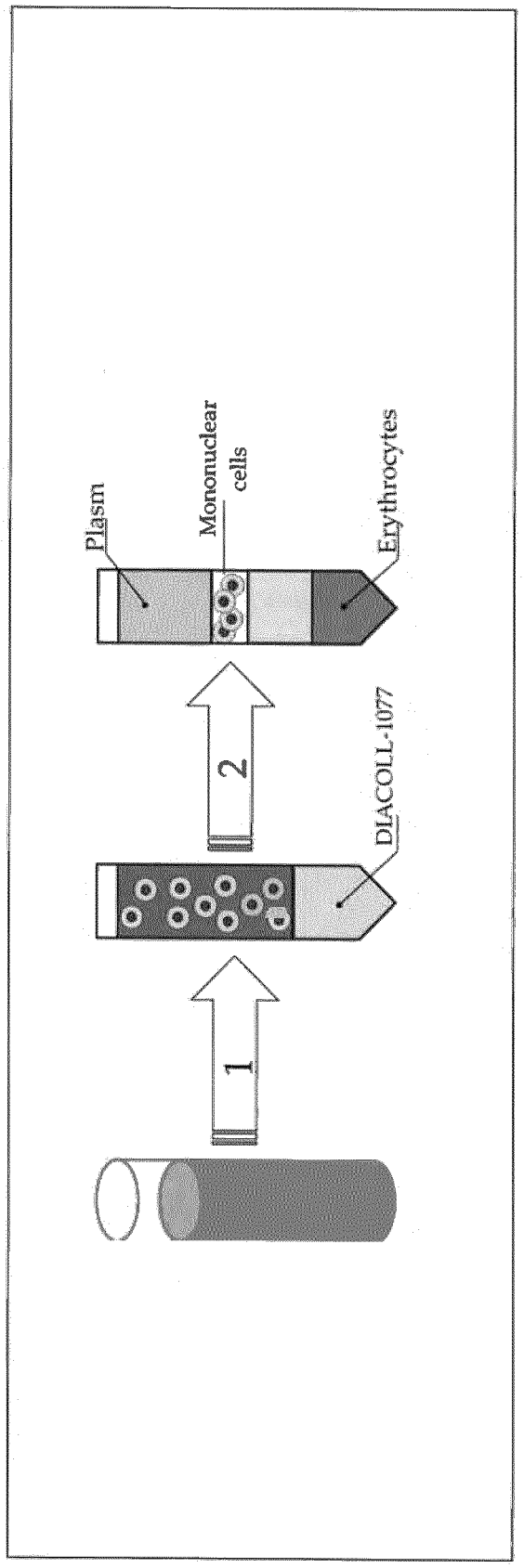


Figure 14

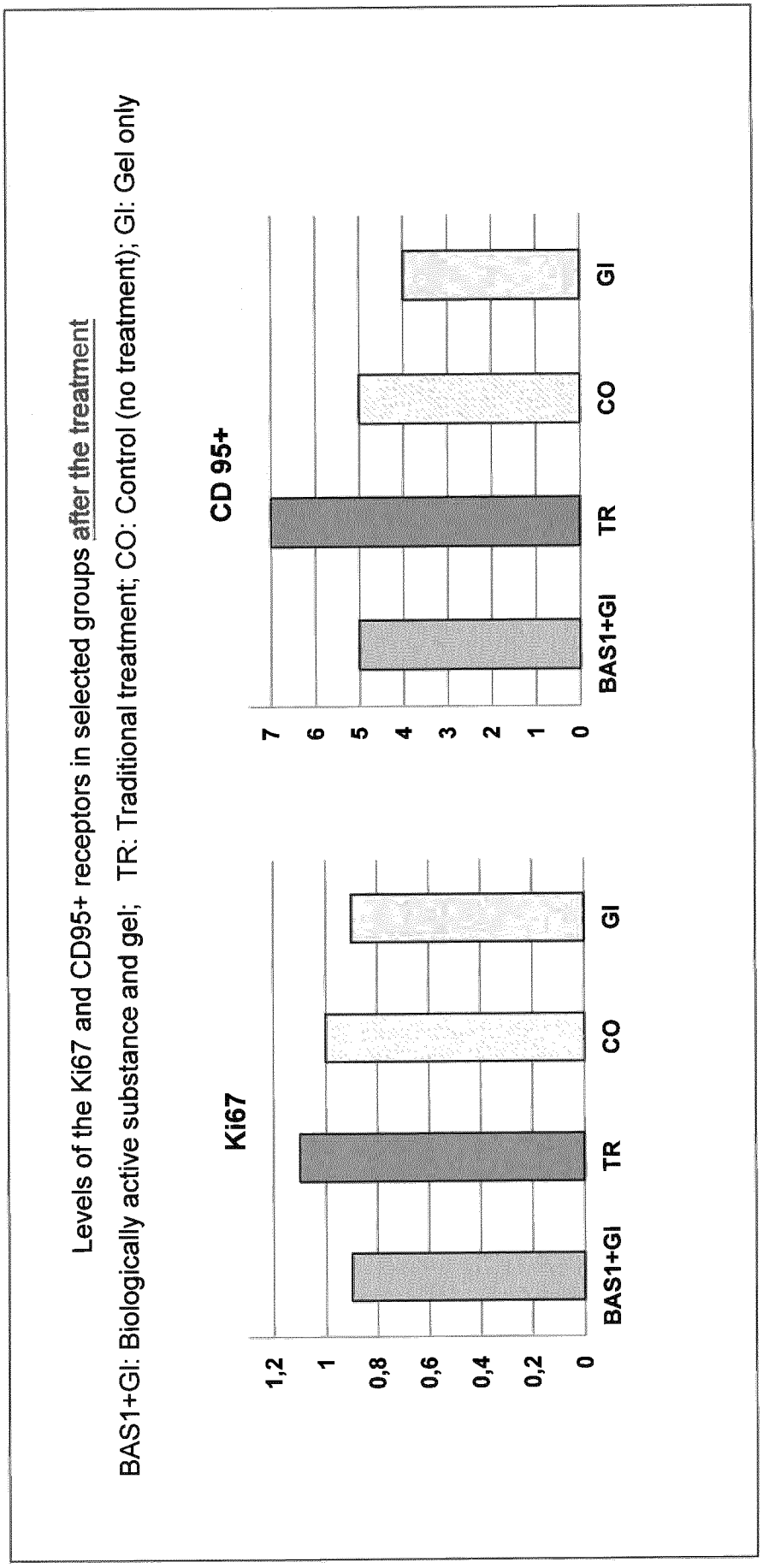
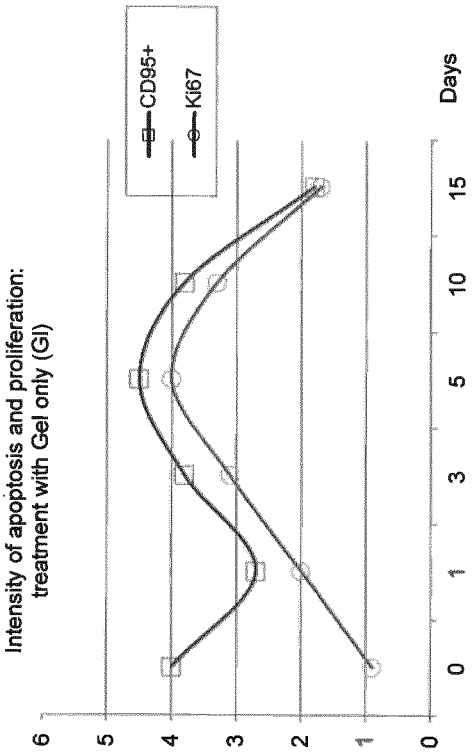

Levels of the Ki67 and CD95+ receptors in selected groups after the treatment
BAS1+GI: Biologically active substance and gel; TR: Traditional treatment; CO: Control (no treatment); GI: Gel only
Ki67
1,2
1
0,8
0,6
0,4
0,2
0
BAS1+GI
TR
CO
GI
CD 95+
7
6
5
4
3
2
1
0
BAS1+GI
TR
CO
GI

Figure 15


Intensity of apoptosis and proliferation:
treatment with Gel only (GI)
CD95+
Ki67
Days
0
1
3
5
10
15
0
1
2
3
4
5
6

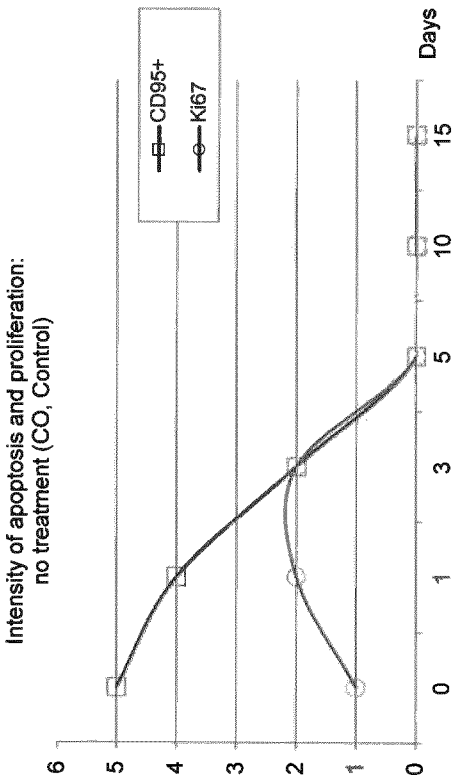
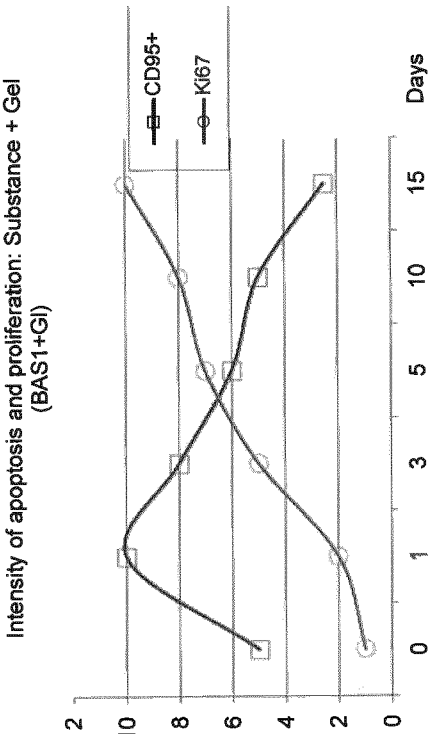

Intensity of apoptosis and proliferation:
no treatment (CO, Control)
CD95+
Ki67
Days
0
1
3
5
10
15
0
1
2
3
4
5
6

Figure 16


Intensity of apoptosis and proliferation: Substance + Gel (BAS1+Gl)
CD95+
Ki67
Days
0
1
3
5
10
15
0
2
4
6
8
10
12

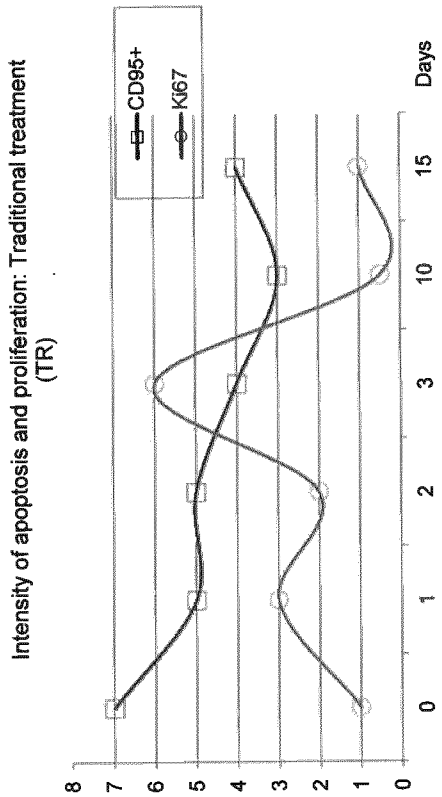
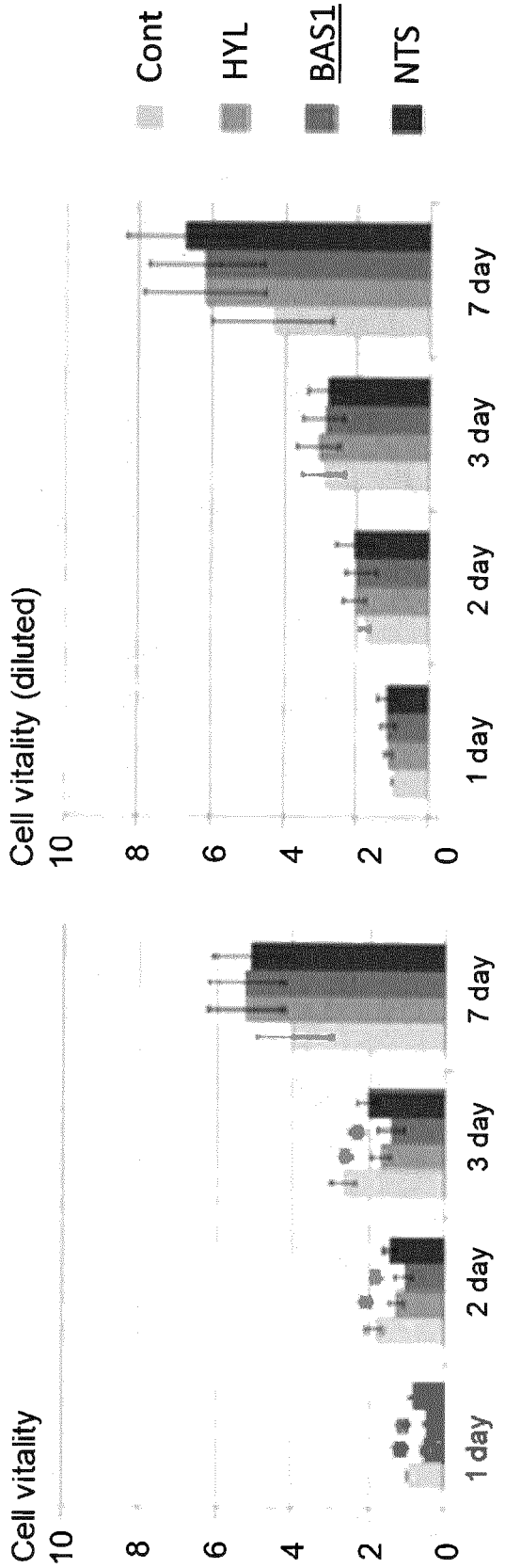
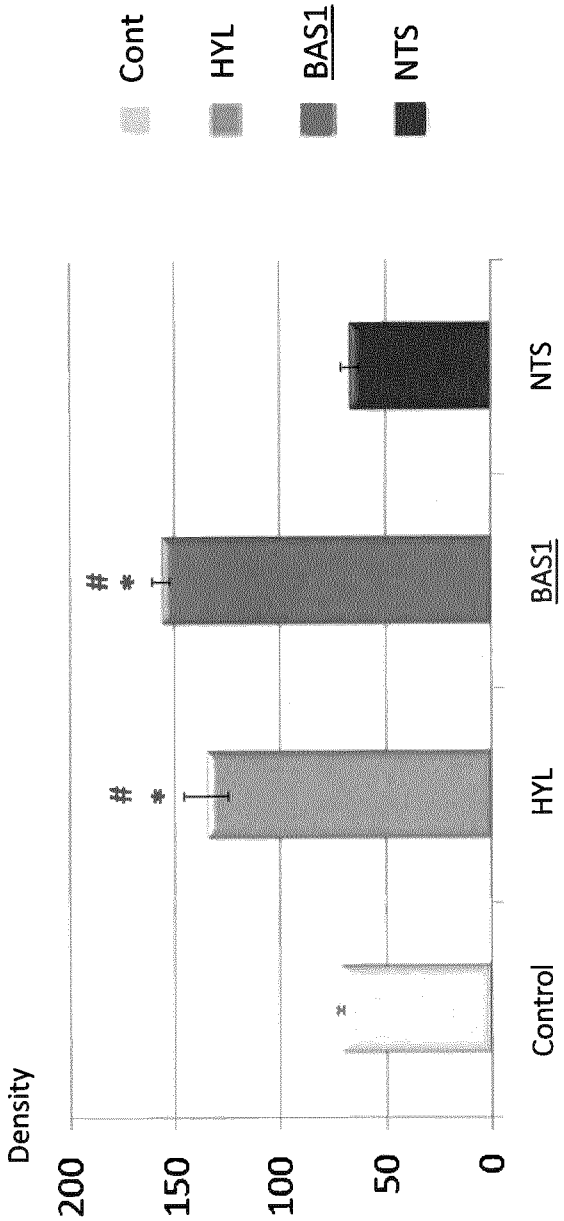

Intensity of apoptosis and proliferation: Traditional treatment (TR)
CD95+
Ki67
Days
0
1
2
3
10
15
0
1
2
3
4
5
6
7
8

Figure 17



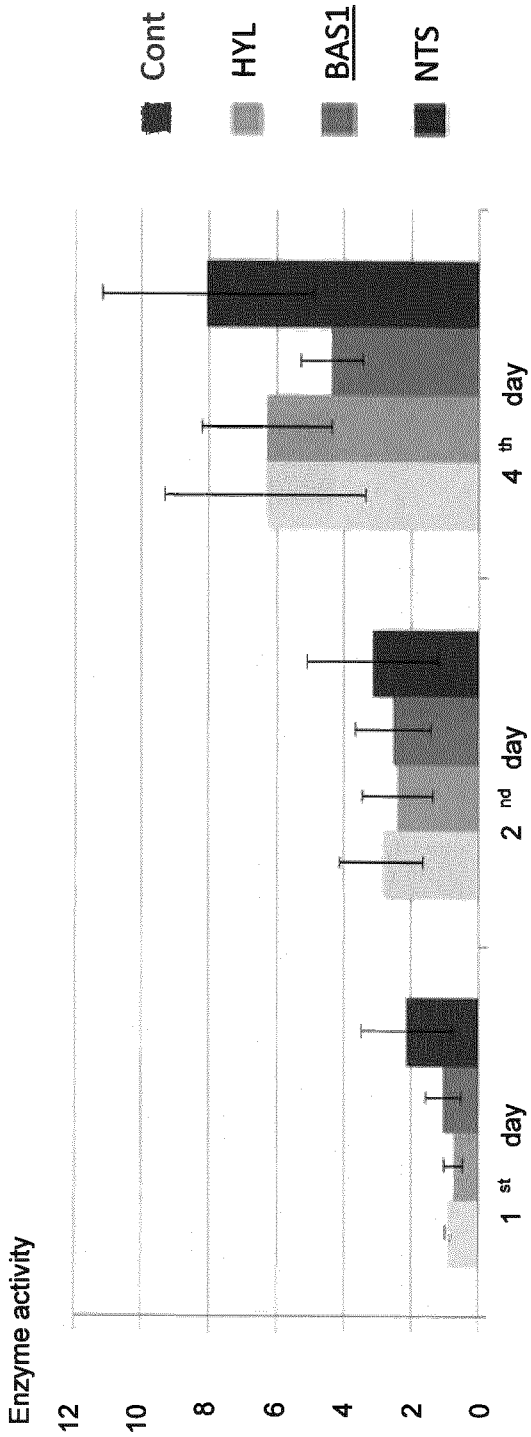
Effect of undiluted (left) and tenfold diluted (right) hyaluronic acid preparations on PDLSC cell viability. The absorbance value is measured using the WST-1 cell proliferation reagent, proportional to the number of viable cells. Star indicates the results that were showing $p < 0.05$ as compared to control.

Figure 18



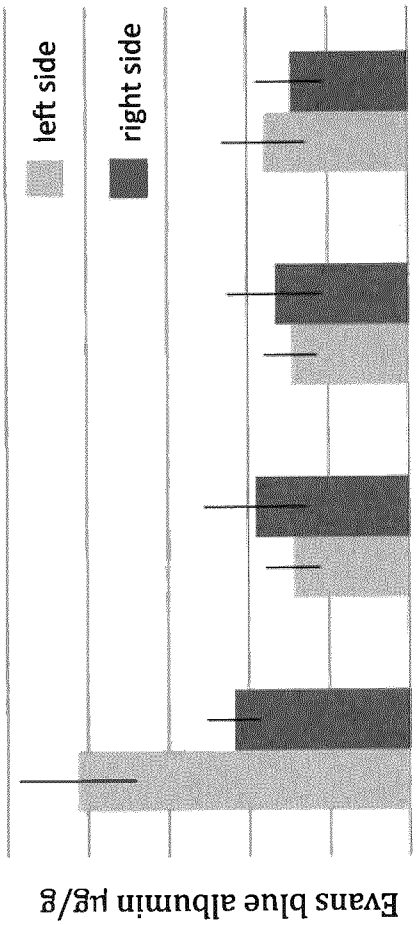
Analysis of the effect of stimulating spontaneous osteogenic differentiation based on Koss densitometry. The samples were analyzed by densitometry. The darkening in the corresponding image is directly proportional to the degree of mineralization. Mineralization in the HYL and BAS1 cases is significantly higher compared to the control (Cont) and NTS * $p < 0.05$ vs control; # $p < 0.05$ vs NTS.

Figure 19



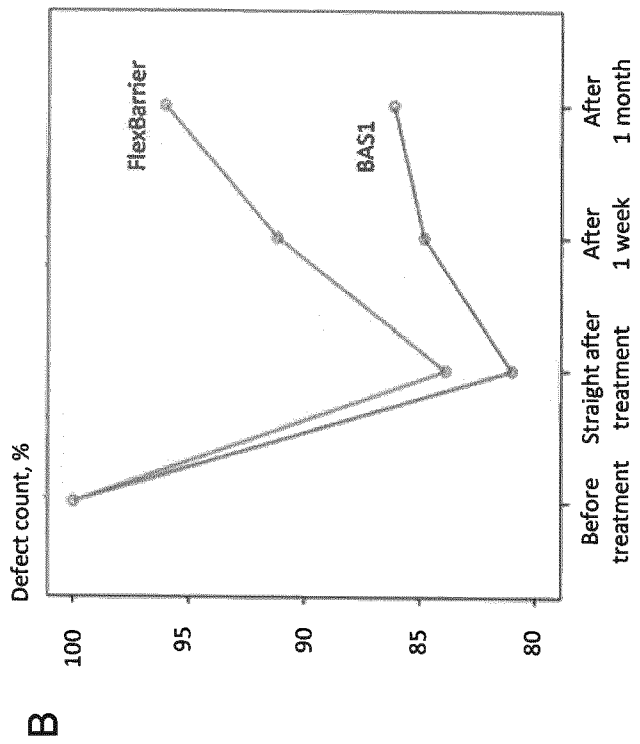
Analysis of the alkaline phosphatase activity. The absorbance is proportional to ferment activity and is measured using an ALP reagent.

Figure 20



The difference between treatment groups is significant (p value <0.05.versus linked side control). Within the groups, the difference between the left and right sides was compared and evaluated. A significant difference was found between the left (induced) and right (not induced) sides only in the control group.

Figure 21A-B


B
Defect count, %
100
95
90
85
80
FlexBarrier
BAS1
Before treatment
Straight after treatment
After 1 week
After 1 month

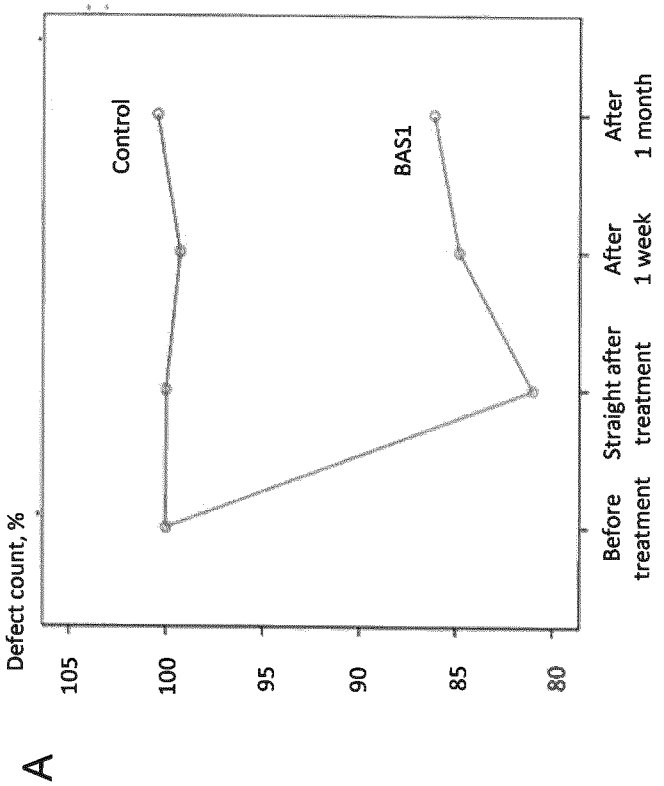

A
Defect count, %
105
100
95
90
85
80
Control
BAS1
Before treatment
Straight after treatment
After 1 week
After 1 month

Figure 22 A-B

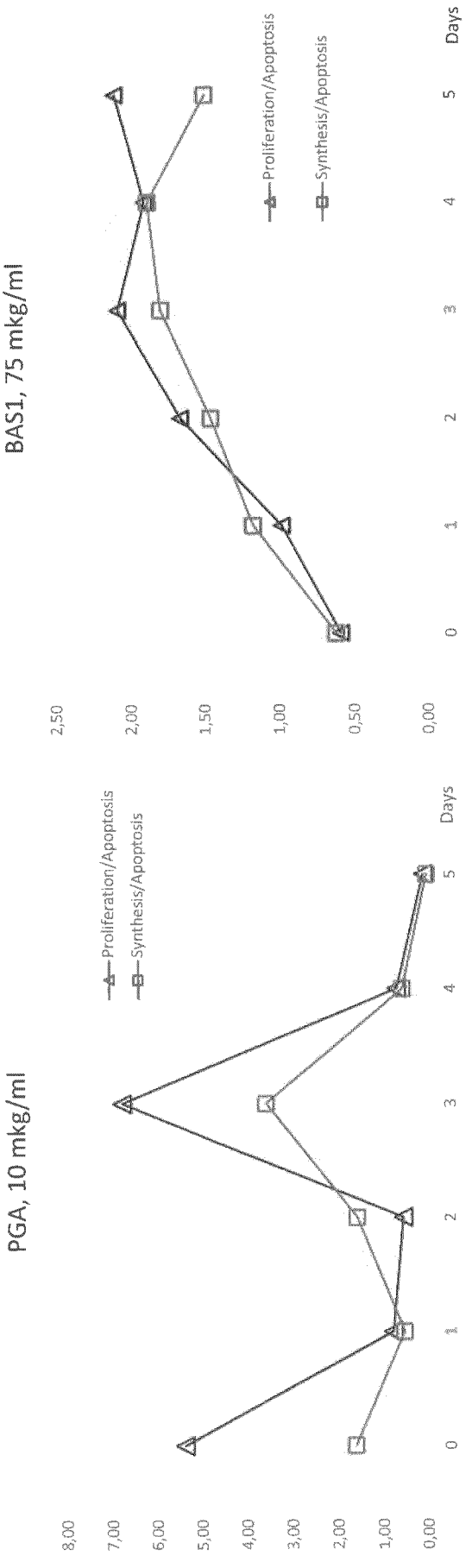

BAS1, 75 mkg/ml
Proliferation/Apoptosis
Synthesis/Apoptosis
Days
2,50
2,00
1,50
1,00
0,50
0,00
0
1
2
3
4
5
PGA, 10 mkg/ml
Proliferation/Apoptosis
Synthesis/Apoptosis
Days
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00

Figure 22 C-D

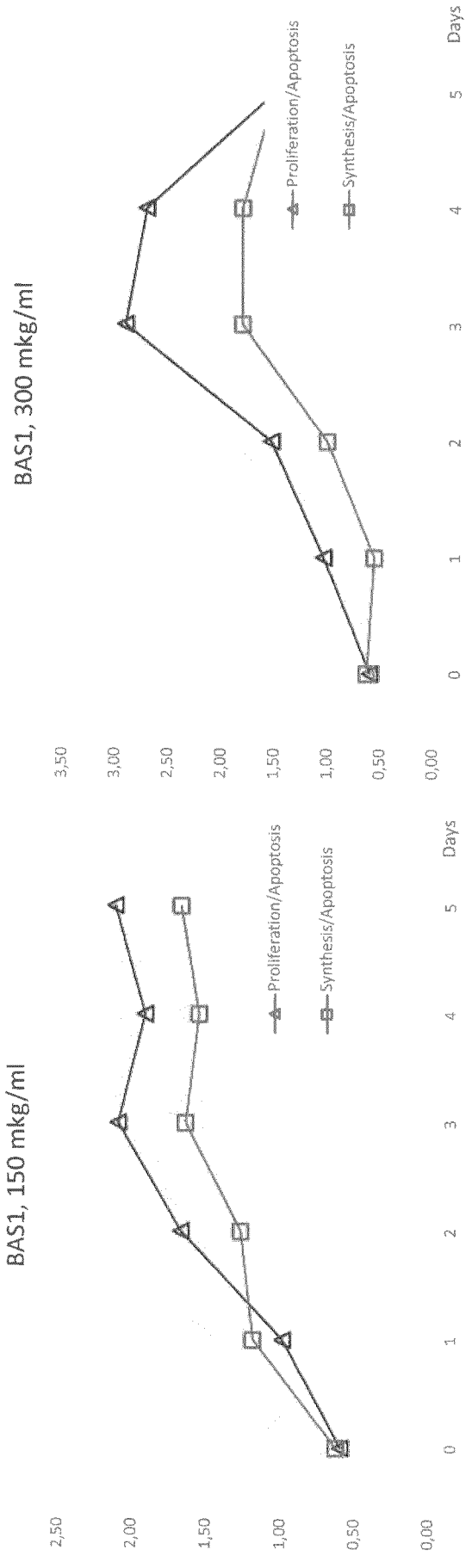
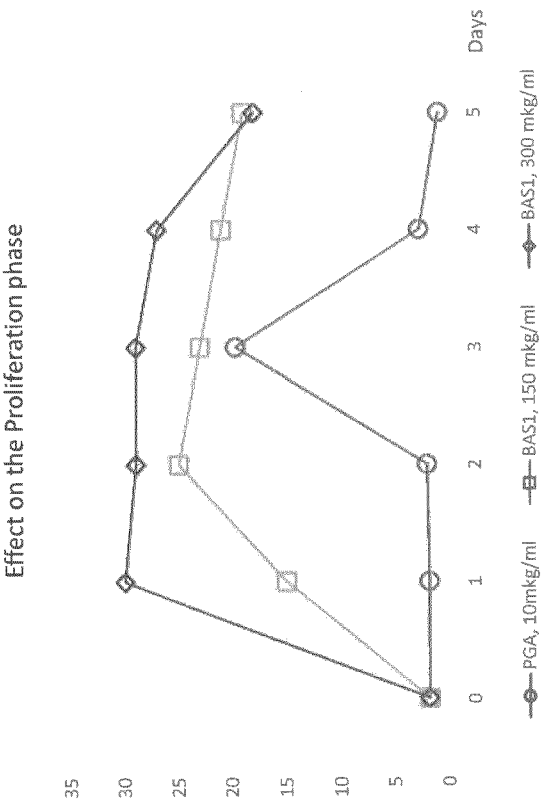

BAS1, 300 mkg/ml
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
0
1
2
3
4
5
Days
Proliferation/Apoptosis
Synthesis/Apoptosis
BAS1, 150 mkg/ml
2,50
2,00
1,50
1,00
0,50
0,00
0
1
2
3
4
5
Days
Proliferation/Apoptosis
Synthesis/Apoptosis

Figure 22 E-F


Effect on the Proliferation phase
35
30
25
20
15
10
5
0
0
1
2
3
4
5
Days
PGA, 10mkg/ml
BAS1, 150 mkg/ml
BAS1, 300 mkg/ml

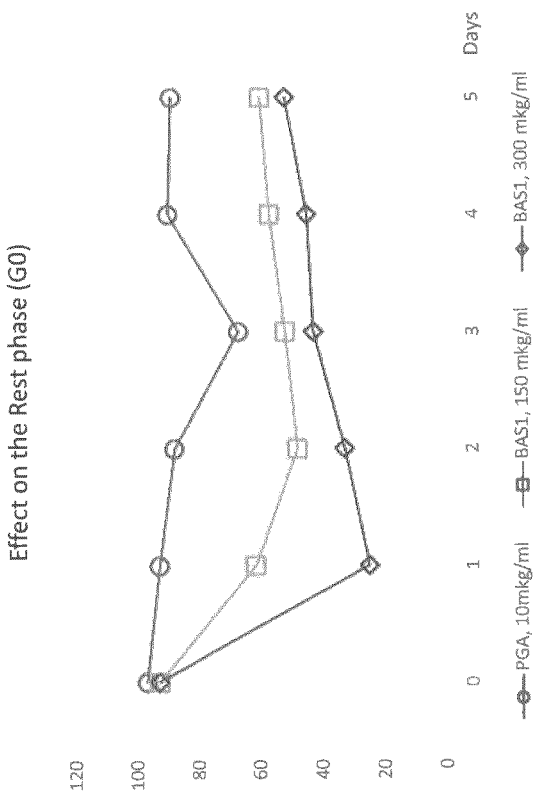

Effect on the Rest phase (G0)
120
100
80
60
40
20
0
0
1
2
3
4
5
Days
PGA, 10mkg/ml
BAS1, 150 mkg/ml
BAS1, 300 mkg/ml

Figure 22 G-H

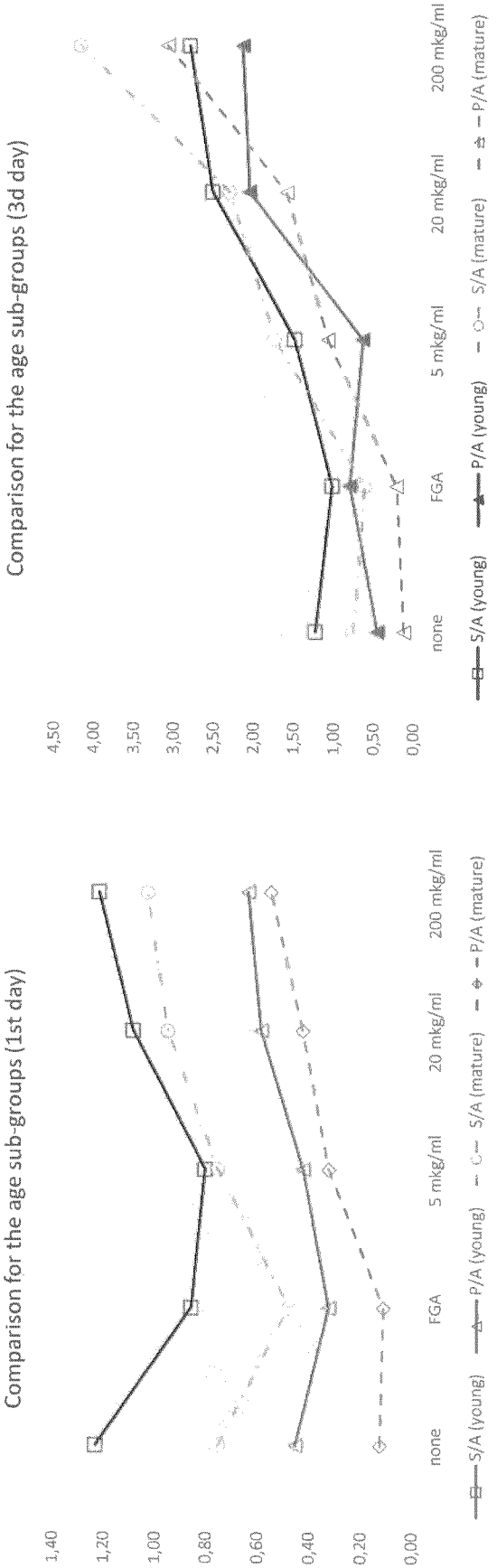

Comparison for the age sub-groups (1st day)
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
none
FGA
5 mkg/ml
20 mkg/ml
200 mkg/ml
S/A (young)
P/A (young)
S/A (mature)
P/A (mature)
Comparison for the age sub-groups (3d day)
4,50
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
none
FGA
5 mkg/ml
20 mkg/ml
200 mkg/ml
S/A (young)
P/A (young)
S/A (mature)
P/A (mature)

Figure 23

Table 1

Site	NaCl	S0	S1	S2	S3	S4	S5	S6	S7	S8	S9
Element	Atomic %	Atomic %	Atomic %	Atomic %	Atomic %	Atomic %	Atomic %	Atomic %	Atomic %	Atomic %	Atomic %
Carbon				39.835	46.742	46.583	46.316	33.191	50.064	50.502	30.609
Oxygen		6.64	31.26	35.872	39.069	36.372	42.349	49.567	34.615	28.304	38.283
Sodium	52.68	43.80	10.116	5.624	3.957	7.595	3.142	9.414	5.105	4.875	5.047
Magnesium			0.706	1.026	1.076	0.269	1.078	0.447	0.741	0.59	1.871
Silicon			1.148	0.516	0.896	1.399	0.455	0.201	0.251	0.311	1.247
Phosphorus			4.4	2.383	2.84	0.491	2.311	0.852	2.077	2.246	5.139
Sulfur			5.259	2.521	1.814	1.307	0.99	2.888	2.544	4.371	3.215
Chlorine	47.32	49.56	43.995	9.819	2.262	5.077	2.493	1.876	3.1	5.758	9.169
Potassium			3.115	2.018	1.092	0.769	0.552	1.565	1.372	2.083	4.154
Calcium				0.388	0.252	0.138	0.314		0.131	0.96	1.266

Table 2

Carbon	Oxygen	Phosphorus	Sulfur	Potassium	Chlorine	Sodium	Calcium	Magnesium	Silicon
42.98	38.05	2.29	2.46	1.7	4.94	5.59	0.43	0.89	0.66

Figure 24

Table 3

Brutto formula	Mol weight	Compound
$C_6H_{15}N$	101.19	Hexylamine
C_5H_{14}	104.17	Choline
$C_5H_7NO_3$	129.12	Pyroglutamic acid
$C_5H_{10}N_2O_3$	131.15	1-amino-D-proline
C_4H_7NO	133.103	Aspartic acid
HNa_2O_4P	141.96	disodium hydrogenorthophosphate
C_5H_7NO	145.04	L-glutamic acid dianion
$C_9H_{10}N_2$	146.189	dimethylbenzimidazole; 2-ethylbenzimidazole;
$C_6H_{14}N_4O_2$	174.2	Arginine
$C_6H_9NO_5$	175.139	Dimethyl 2-methyloxaziridine-3,3-dicarboxylate
$C_5H_{10}NO_4P$	184.15	Phosphocholine conjugate acid
$C_9H_{14}N_4O_2$	198.222	N-Butyl-1-methyl-4-nitro-1H-imidazol-5-amine;
$C_{14}H_{31}N$	213.4	2-Tetradecanamine; 12-Methyl-1-tridecanamine;
C_9H_{17}	219.235	Pantothenic acid
$C_8H_{20}NO_4P$	257.22	L-α-Glycerylphosphorylcholine
$C_{21}H_{37}N$	303.525	(3α,5α)-Pregnan-3-amine;
$C_{10}H_{17}N_2O_4P$	304.19	Phosphonic acid, (2,4-dinitrophenyl)-, diethyl ester
$C_{15}H_{23}N_5O_3$	321.38	L-Arginyl-L-phenylalanin
$C_{23}H_{41}N$	331.58	Piptamine(antibiotic); N-Benzyl-N-ethyl-1-tetradecanamine
$C_{30}H_{50}$	410.72	Squalene
$C_{37}H_{64}O_2$	540.9	Cholesteryl caprate

Figure 25

Table 4

Brutto formula	Mol weight	Compound
$C_6H_{15}N$	101.19	Hexylamine
C_5H_{14}	104.17	Choline
$C_4H_7N_3O$	113.12	Chreatinine
$C_5H_9NO_2$	115.13	Proline
$C_5H_{11}NO_2$	117.15	Valine
C2H7NO3S	125.15	Taurine
$C_5H_7NO_3$	129.12	Pyroglutamic acid
$C_5H_{10}N_2O_2$	131.15	1-amino-D-proline
C_4H_7NO	133.103	Aspartic acid
C_5H_9N	147.13	Glutamic acid
C_9H_{17}	219.235	Pantothenic acid
$C_{21}H_{37}N$	303.525	(3α,5α)-Pregnan-3-amine; N-Benzyl-3-tetradecanamine;
$C_{10}H_{13}N_2O_7P$	304.19	Phosphonic acid, (2,4-dinitrophenyl)-, diethyl ester
C18H40ClN	305.97	Octadecylamine, hydrochloride
C23H49N	339.64	Tricosylamine
??	614.77	?? Unidentified

Figure 26

Table 5A

Brutto formula	Mol weight	Compound (hormone-like)
$C_{30}H_{50}$	410.73	Squalene
$C_{30}H_{52}$	412.7	Hopan
$C_{27}H_{46}O$	386.65	Cholesterol
$C_{29}H_{50}O$	414.7	beta-Sitosterol
$C_{29}H_{50}O$	414.7	Stigmast-5-en-3-ol
$C_{20}H_{28}O_2$	300.4	Dihydroabietic acid

Table 5B

Brutto formula	Mol weight	Compound (amides, amines)
$C_{14}H_{31}N$	213.4	N,N-Dimethyldodecanamine (1-Tetradecylamine)
$C_2H_4(NH_2)_2$	60.1	Ethylenediamine
$C_{13}H_{21}N$	191.313	N-Methyl-N-pentyl-benzylamine
$C_9H_9NO_3$	179.17	2-(4-Formyl-phenoxy)acetamide
$CO(NH_2)_2$	60.06	Urea (Carbamide)

Table 5C

Brutto formula	Mol weight	Compound (phosphates)
$C_{18}H_{39}O_7P$	398.47	Tributoxyethylphosphate P1439
$C_6H_{15}O_5P$	198.15	Ethanol, 2-butoxy-, phosphate (2-butoxyethyl dihydrogen phosphate)
$C_{42}H_{63}O_4P$	662.9	Tris-(2,4-di-tert-butylphenyl)phosphate
$C_9H_{15}O_6P$	250.19	TCEP (tris(2-carboxyethyl)phosphine)

Table 5D

Brutto formula	Mol weight	Compound (cyclic alyphatic)
$C_{14}H_{28}$	196.372	Cyclopentane, nonyl-
$C_{26}H_{52}$	364.7	Cyclopentane, heneicosyl-

Figure 27

Table 6

Database strains of bacteria	Average values of Bactericidal Activity Index (M±m, %) with different BAS1 concentrations			
	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
E. coli K12	16.5±3.3	38.5±3.0	50.7±4.2	87.9±7.0
E. coli 25922	65.8±4.1	75.8±5.4	85.0±5.3	85.8±6.2
P. aeruginosa 27853	11.1±5.4	44.4±7.6	77.8±6.2	80.0±6.5
S. aureus 25923	79.3±7.2	82.6±6.1	86.0±7.6	86.5±8.1
S. aureus 6538P	54.6±6.1	84.5±7.1	87.6±8.2	88.7±6.5
S. xylosus 55/5,	61.2±7.8	61.4±6.3	68.2±6.8	68.2±5.4
S. epidermidis 711	12.8±6.6	20.5±6.3	25.6±6.4	30.8±7.2
Micrococcus luteus var. lysodeikticus 15307	22.6±5.4	24.5±5.2	30.9±6.9	39.2±6.3

Table 7

Clinical strains of bacteria (number of tested strains)	Sensitivity of the bacteria to the action of BAS1 with the concentration 100 µg/ml		
	Percentage of sensitive bacteria (%)	Spread of BAI (min... max, %)	Average of BAI (%)
E. coli (n=22)	72.7±9.7	12.1...89.1	52.1±4.1
A. baumannii (n=55)	61.8±6.6	11.0...94.7	49.7±5.1
P. aeruginosa (n=43)	48.8±7.7	12.0...78.2	33.6±4.1

Table 8

Clinical strains of bacteria (number of tested strains)	Sensitivity of the bacteria to the action of BAS1 with the concentration 100 µg/ml		
	Percentage of sensitive bacteria (%)	Spread of BAI (min... max, %)	Average of BAI (%)
K. pneumoniae (n=84)	95,2±2,3	10,5...100,0	57,3±3,2
E. coli (n=22)	72,7±9,7	12,1...89,1	52,1±4,1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/082116

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/12 A61P35/04 A61K31/00 A61K31/01 A61K31/045 A61K31/13
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROSEBOOM IGNACE C ET AL: "Skin tissue sample collection, sample homogenization, and analyte extraction strategies for liquid chromatographic mass spectrometry quantification of pharmaceutical compounds", JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS, ELSEVIER B.V, AMSTERDAM, NL, vol. 191, 28 August 2020 (2020-08-28), XP086302329, ISSN: 0731-7085, DOI: 10.1016/J.JPBA.2020.113590 [retrieved on 2020-08-28]	1-9, 13-16, 18-21
Y	page 1; tables 1-3 page 2, column 2 - page 6, column 1	1-22

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 January 2024	20/02/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Zellner, Eveline

3

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/082116

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NAPAVICHAYANUN SUPAMAS ET AL: "Effect of animal products and extracts on wound healing promotion in topical applications: a review", JOURNAL OF BIOMATERIALS SCIENCE. POLYMER EDITION., vol. 28, no. 8, 9 March 2017 (2017-03-09), pages 703-729, XP093124658, NL ISSN: 0920-5063, DOI: 10.1080/09205063.2017.1301772	1-9, 13-16, 18-21
Y	page 707, paragraph 2 page 712, paragraphs 2,4 page 713, paragraph 3 page 716, paragraph 6	1-22
X	WO 2019/040790 A1 (MERAKRIS THERAPEUTICS LLC [US]) 28 February 2019 (2019-02-28)	1,18
Y	paragraph [0035] - paragraph [0037]	1-22
Y	CN 102 316 879 B (APOSCIENCE AG) 22 January 2014 (2014-01-22) page 7 - page 8	1-22
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3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/082116

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