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(54) **Title**: POLYELECTROLYTE WITH POSITIVE NET CHARGE FOR USE AS MEDICAMENT AND DIAGNOSTIC FOR CANCER

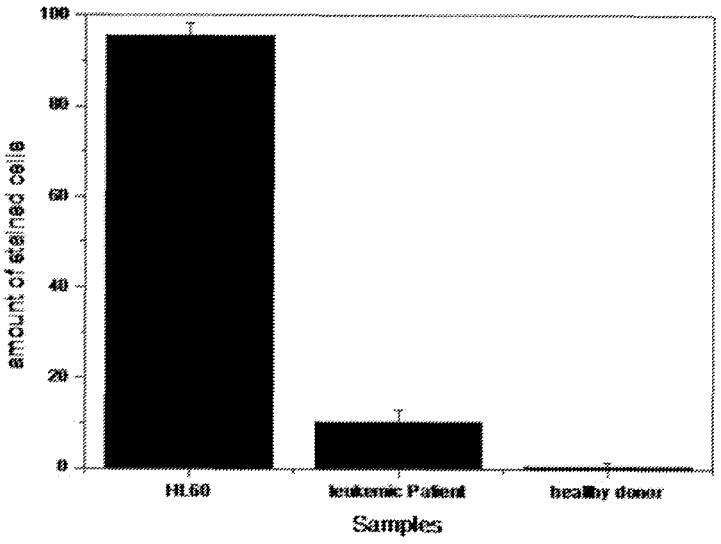

FIG 1

(57) **Abstract**: The present invention relates to the medical field, in particular to the treatment and diagnosis of cancer. More in particular, the present invention relates to the use of polyallylaminewith positive net charge as anticancer agent and as diagnostic agent for the detection and/or treatment of cancer cells in body tissues.According to the present invention, polyallylamine is useful as theranostic agent.

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POLYELECTROLYTE WITH POSITIVE NET CHARGE FOR USE AS MEDICAMENT AND DIAGNOSTIC FOR CANCER

Technical Field

[0001] The present invention relates to the medical field, in particular to the treatment and diagnosis of cancer.

[0002] More in particular, the present invention relates to the use of poly(allylamine) and its pharmaceutically acceptable salt as anticancer agents and as diagnostic agents for the detection and/or treatment of cancer cells in body tissues, as well as theranostic agent for the same purpose, a method fro treating and diagnosing cancer and an apparatus for extracorporeal treatment of blood.

Background Art

[0003] Leukemia is a broad term covering a spectrum of diseases, which are clinically and pathologically split into acute and chronic forms.

[0004] Acute leukemia is characterized by the rapid increase of immature blood cells. This crowding makes the bone marrow unable to produce healthy blood cells. Acute forms of leukemia can occur in children and young adults. Immediate treatment is required in acute leukemias due to the rapid progression and accumulation of the malignant cells, which then spill over into the bloodstream and spread to other organs of the body.

[0005] Chronic leukemia is distinguished by the excessive build up of relatively mature, but still abnormal, blood cells. Typically taking months to years to progress, the cells are produced at a much higher rate than normal cells, resulting in many abnormal white blood cells in the blood. Chronic leukemia mostly occurs in older people, but can theoretically occur in any age group. Whereas acute leukemia must be treated immediately, chronic forms are sometimes monitored for some time before treatment to ensure maximum effectiveness of therapy.

[0006] Furthermore, the diseases are classified into lymphoblastic or lymphocytic leukemias, which indicate that the cancerous change took place in a type of marrow cell that normally goes on to form lymphocytes, and myeloid or myelogenous leukemias, which indicate that the cancerous change took place in a type of marrow cell that normally goes on to form red blood cells, some types of white cells, and platelets.

[0007] Combining these two classifications provides a total of four main categories:

Table 1

Four major kinds of leukemia

Cell type	Acute	Chronic
Lymphocytic leukemia (or "lymphoblastic")	Acute lymphocytic leukemia (ALL)	Chronic lymphocytic leukemia (CLL)
Myelogenous leukemia (also "myeloid" or "nonlymphocytic")	Acute myelogenous leukemia (AML)	Chronic myelogenous leukemia (CML)

[0008] Types outside these main categories include hairy cell leukemia.

Table 2

Comparison of leukemia types

Type	Occurrence	5-year survival rate	Overall treatment
Acute lymphocytic leukemia	Most common type of leukemia in young children. This disease also affects adults, especially those age 65 and older.	85% in children and 50% in adults	Bone marrow and systemic disease control, prevention of spreading, e.g. to CNS
Chronic lymphocytic leukemia	Most often affects adults over the age of 55. It sometimes occurs in younger adults, but it almost never affects children. ⅔ of affected are men.	75%	Incurable
Acute myelogenous leukemia	Occurs more commonly in adults than in children, and more commonly in men than women.	40%	Bone marrow and systemic disease control, specific treatment of CNS, if involved
Chronic myelogenous leukemia	Occurs mainly in adults. A very small number of children also develop this disease.	90%	
Hairy cell leukemia	About 80% of affected people are adult men. No reported cases in young children.	96% to 100% at ten years	Incurable, but easily treatable

[0009] Treatment options for leukemia

[0010] Acute Lymphocytic Leukemia (ALL)

[0011] Proper management of ALL focuses on control of bone marrow and systemic (whole-body) disease as well as prevention of cancer at other sites, particularly the central nervous system (CNS).

[0012] Treatment for acute leukemia can include chemotherapy, steroids, radiation therapy, intensive combined treatments (including bone marrow or stem cell transplants), and growth factors.

[0013] Chronic Lymphocytic Leukemia (CLL)

[0014] While generally considered incurable, CLL progresses slowly in most cases.

[0015] Refractory CLL

[0016] "Refractory" CLL is a disease that no longer responds favourably to treatment. In this case more aggressive therapies are considered.

[0017] CLL is probably incurable by present treatments. But, fortunately, a large group of CLL patients do not require therapy.

[0018] Acute Myelogenous Leukemia (AML)

[0019] Treatment of AML consists primarily of chemotherapy

[0020] Chronic Myelogenous Leukemia (CML)

[0021] Chronic phase CML is treated with inhibitors of tyrosine kinase,

[0022] Blast crisis

[0023] Blast crisis carries all the symptoms and characteristics of either acute myelogenous leukemia or acute lymphoblastic leukemia, and has a very high mortality rate. This stage can most effectively be treated by a bone marrow transplant after high-dose chemotherapy.

[0024] Hairy Cell Leukemia (HCL)

[0025] Several treatments are available, and successful control of the disease is common.

[0026] Hairy cell leukemia is an incurable, indolent blood disorder in which mutated, partly matured B cells accumulate in the bone marrow.

[0027] The general medical literature, such as PORTER, et al. The Merck Manual. Merck, from which the background art is taken, provides an ample review of the treatment and diagnosis of leukemia and cancer in general.

[0028] Diagnosis of leukemia requires laboratory tests involving white blood cells count, however, bone marrow examination should often, if not always be performed. The latter requires biopsy or needle examination, which is mostly problematic to the patient, especially pediatric patients.

[0029] There is still the need of a diagnostic tool for a fast diagnosis of leukemia, possibly avoiding painful or complicated intervention on the patient.

[0030] Moreover, there is still the need for a fast, reliable diagnostic tool, which avoids sample preparation.

[0031] Cancer therapy is still a difficult task for the person expert in this field. Other than surgery, cancer therapy uses complex therapeutic protocols with anticancer drugs and combinations of said drugs and/or radiotherapy. Cancer therapy is mostly affected by severe side effects. Another strongly felt need is a medicament for treating cancer or a medicament for supplementing or

enhancing or integrating cancer therapy, which is easy to manage and has reduced side effects.

[0032] In MARIKOVSKY, Y., et al. Distribution and Modulation of Surface Charge of Cells from Human Leukemia-Lymphoma lines at Various Stages of Differentiation. Cancer . 1986, vol.58, p.2218-2223, it is reported the use of cationized ferritin (CF) to label electrical charge surface density of hematopoietic cells at various stages of differentiation, showing a correlation between the CF density/distribution and the stage of lymphoid differentiation. Treatment with retinoic acid (RA) seems to prevent CF-induced formation of CF patches. Some correlation between the distribution of surface anionic sites and the malignant potential of human leukemic cells lines could be detected. The revelation of leukemic cells requires a laborious preparation of the sample.

[0033] CHANANA, M., et al. Interaction of Polyelectrolytes and their Composites with Living Cells. Nano Letters. 2005, vol.5, no.12, p.2605-12. The Authors study interaction and toxicity between polyelectrolytes and living cells. Toxicity was found to be influenced by all of factors such as contact area, charge, transplantation site and most important the cell type in contact with the polyelectrolyte and cannot be tested easily in a model. The goal of this work is to study cytotoxicity of polyelectrolytes in order to find suitable ones for use as coating in immunoprotected transplantation, so to say, to find polyelectrolytes with reduced or minimized or null toxicity. However, the Authors conclude that a general statement about cytotoxicity of a polyelectrolyte is not possible and that the use of a model system to investigate reactions to polymers remains mainly artificial.

[0034] ARNOLD, L. J. JR, et al. ANTI NEOPLASTIC ACTIVITY OF POLY L LYSINE WITH SOME ASCITES TUMOR CELLS. Proceedings of the National Academy of Sciences of the United States Of America, vol. 76, no. 7,1979. pages 3246-3250, discloses that poly-L-lysine is effective in preventing the growth of Ehrlich ascites tumors mice and that this polycation acts by selectively binding tumor tissues in comparison with the adjacent normal tissues.

[0035] SIGURDSON, C., et al. Prion Strain Discrimination Using Luminescent Conjugated Polymers. Nature Methods. 2007, vol.42, no.12, p.1023-1030. disclose the use of luminescent conjugated polymers for characterizing prion strains.

[0036] YEUNG, T., et al. Membrane Phosphatidylserine Regulates Surface Charge and Protein Localization. Science. 2008, vol.319, p.210-213. develop a biosensor to study the subcellular distribution of phosphatidylserine.

[0037] Several methods for diagnosing leukemia exist. For a review see The Merck Manual. 18th edition. Edited by PORTER, ROBERT S., et al. Merck & Co., 2008.

[0038] Some methods are based on the determination of gene expression (WO 2008/19872 A (RICHTER, GUNTHER) 21.02.2008 , WO 2006/89233 A (WYETH) 26.08.2006 , JP 2007244377 A (OKAYAMA UNIV) 27.09.2007) or molecular marker determination (US 2007287163 A (GEUIJEN ET AL.) 13.12.2007 , US 2007264261 A (NUVELO INC.) 15.11.2007.

[0039] All these methods are complex and require sample preparation.

[0040] The need of a fast, easy and reliable method for diagnosis of cancer, in particular leukemia, still exists.

[0041] The need of an easier method for treating cancer is also felt.

[0042] Another need exists in finding an effective and selective treatment of less differentiated cancer cells, which are usually more severe in terms of long-term survival.

[0043] It is well known that cancer stem cells (CSCs) are cancer cells (found within tumors or hematological cancers) that possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer sample. These cells are therefore tumorigenic (tumor-forming), perhaps in contrast to other non-tumorigenic cancer cells. CSCs may generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types. Such cells are proposed to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors. Therefore, development of specific therapies targeted at CSCs holds hope for improvement of survival and quality of life of cancer patients, especially for sufferers of metastatic disease.

[0044] To cure cancer it is imperative to devise therapies that effectively target the cancer stem cells. Chemotherapy targets the proliferating cancer cells, and has been used as adjuvant to surgery and radiation therapies. However, chemotherapy is toxic also to normal cells. There is an obvious need for more effective cancer therapies; of which, the development is dependent on our

understanding of the disease and its progression. Cancer stem cells may be able to provide some of these answers, with the hope to develop novel therapeutics to target the tumorigenic stem cells, ultimately eradicating cancer.

Disclosure of Invention

[0045] It has been found that poly(allylamine), a polyelectrolyte bearing a positive net charge, and its pharmaceutically acceptable salts selectively enters cancer cells or cells in the process of malignant differentiation while it is excluded from healthy cells.

[0046] Advantageously, poly(allylamine) does not enter healthy cells even upon exposition of said cells for 24 h.

[0047] The selective uptake of the poly(allylamine) according to the present invention provides a fast and reliable diagnostic tool, which can advantageously be applied to body samples without complex sample preparation being necessary.

[0048] The poly(allylamine) according to the present invention is also capable of selectively killing the tumour cell.

[0049] Therefore, it is an object of the present invention a poly(allylamine) or a pharmaceutically acceptable salt thereof having a positive net charge for use as antitumor agent for cancer.

[0050] Another object of the present invention is a poly(allylamine) having a positive net charge for use as diagnostic agent for cancer.

[0051] The present invention provides the use of the above poly(allylamine) as theranostic agents. The concept of theranostic is well-known in the art and is intended as treatment strategy comprising a diagnostic test that identifies patients most likely to be helped or harmed by a new medication, and targeted drug therapy based on the test results. The test results are used to tailor treatment, usually with a drug that targets a particular gene or protein (The Scientist, 2004, 18 (16):38).

[0052] Another object of the present invention is the above poly(allylamine), which is labelled with a detectable label.

[0053] Another object of the present invention is poly(allylamine) as diagnostic, therapeutic and theranostic agent for a cancer which comprises at least one population of cancer cells selected from the group consisting of non-adhesive cancer cells, cancer stem cells, undifferentiated and less differentiated cancer

cells. Examples of said cancer are leukaemia, melanoma, liver cancer, breast cancer, ovarian cancer, glioblastoma.

[0054] Another object of the present invention is a poly(allylamine) or a pharmaceutically acceptable salt thereof for use as diagnostic agent for revealing cancer cells in an isolated tissue of a subject.

[0055] Another object of the present invention is an ex vivo method for treating a blood sample containing cancer cells from a subject affected by cancer comprising:

[0056] contacting said blood sample with a blood purification system whereby cancer cells contained in said blood sample are contacted with poly(allylamine) or a pharmaceutically salt thereof, whereby said poly(allylamine) kills said cancer cells,

[0057] removing said killed cancer cells, to provide a purified blood sample.

[0058] An apparatus for extracorporeal treatment of blood, characterized in that the part of said apparatus contacting the blood for treatment comprises poly(allylamine) or a pharmaceutically acceptable salt thereof is a further object of the present invention.

[0059] One of the main advantages of the present invention is to provide a theranostic agent which is both fast in its activity and selective towards tumor cells with respect to normal cells. From therapeutically point of view, selectivity is extremely important for safety and compliance of the drug. From diagnostic point of view, selectivity and fast activity is important for reliability of diagnosis and rapidity of response.

[0060] Another advantage of the present invention is provided by the activity on cancer stem cells and undifferentiated/low differentiated cancer cells, which provides more effective cancer treatment and also the possibility of earlier diagnosis.

[0061] Still further advantages of the present invention are in a method of treating cancer, in a method of diagnosing cancer and in a method of diagnosis and treatment of cancer, providing polyallylamine as theranostic agent.

[0062] Advantageously, in the embodiment related to leukemia, the present invention can be carried out by treating patient's blood in an extracorporeal process (ex vivo) in an equipment containing polyallylamine according to the present invention.

[0063] These and other objects of the present invention will now be illustrated in detail also by means of examples and Figures.

[0064] In the Figures:

[0065] Figure 1: PAH labelled with a fluorophore. Amount of stained cells (HL60, lymphocytes of leukemic and healthy person) after 15 min of incubation.

[0066] Figure 2: number of living HL60 cells against incubation time with or without FITC-PAH treatment.

[0067] Figure 3: Graphical representation of MTT test for cell viability vs. polyamine (polyallylamine, polyethylenimine, spermine) concentration for leukemic HL 60, Jurkat cells and normal MNC.

[0068] Modes for Carrying Out the Invention

[0069] A first object of the present invention is poly(allylamine) or a pharmaceutically acceptable salt thereof for use as antitumor agent.

[0070] In a preferred embodiment of the present invention, the poly(allylamine) is labelled with a detectable label. Preferably, the detectable label is compatible with administration in a living subject, without producing substantial damage to said subject.

[0071] Preferably, the poly(allylamine) according to the present invention is for use for treating a cancer comprising at least one population of cancer cells selected from the group consisting of non-adhesive cancer cells, cancer stem cells, solid tumors comprising undifferentiated and less differentiated cancer cells.

[0072] In a more preferred embodiment of the present invention, said cancer is selected from the group consisting of leukaemia, melanoma, liver cancer, breast cancer, ovarian cancer and glioblastoma.

[0073] In another object of the present invention, the poly(allylamine) or a pharmaceutically acceptable salt thereof is for use as diagnostic agent for revealing cancer cells in an isolated tissue of a subject. Although there is no limitation in selecting the proper tissue for diagnosis of cancer, tissues and samples easily obtainable from a subject are preferred, for example, blood, sputum, urine.

[0074] As in the case of cancer treatment, also in the diagnostic embodiment of the present invention, said cancer comprises at least one population of cancer cells selected from the group consisting of non-adhesive cancer cells, cancer stem cells, solid tumors with undifferentiated and less differentiated cancer cells.

[0075] Examples of said cancer are leukaemia, melanoma, liver, breast, ovarian, glioblastoma.

[0076] Another object of the present invention is an ex vivo method for treating a blood sample containing cancer cells from a subject affected by cancer comprising:

a. contacting said blood sample with a blood purification system whereby cancer cells contained in said blood sample are contacted with poly(allylamine) or a pharmaceutically salt thereof, whereby said poly(allylamine) kills said cancer cells,
b. removing unbound poly(allylamine) from the blood sample, to provide a purified blood sample.

[0077] The combined embodiments of the present invention, therapeutic and diagnostic, are embedded in a further object of the present invention, whereby poly(allylamine) or a pharmaceutically acceptable salt thereof is for use as theranostic agent for cancer.

[0078] The present invention comprises also the pharmaceutically acceptable salts of poly(allylamine).

[0079] Pharmaceutically acceptable salts are well known to the skilled person and don't need further explanations. See for example Wermuth, C.G. e Stahl, P. H. (eds.) Handbook of Pharmaceutical Salts, Properties; Selection and Use; Verlag Helvetica Chimica Acta, Zürich, 2002.

[0080] A preferred example is poly(allylamine hydrochloride) (PAH).

[0081] The poly(allylamine) of the present invention, when used as a medicament, must be admitted for administration to a patient, being human or animal.

[0082] The poly(allylamine) and its pharmaceutically acceptable salts according to the present invention are commercially available via common suppliers or can be prepared in the laboratory according to common general knowledge of those of ordinary skill in the art.

[0083] For the purpose of diagnostic use according to the present invention, the poly(allylamine) is labelled with a detectable label. According to a preferred embodiment of the invention, the detectable label will bind to the amine group of the polyelectrolyte. According to a more preferred embodiment, the detectable label will be a fluorophore or a chromophore.

[0084] In a general embodiment of the present invention, any sample from a subject suspected to suffer cancer disease or in need to confirm to suffer such a disease is suitable for the diagnosis according to the present invention. Blood is the preferred sample because it is the easiest to obtain and it stains cancer

cells wherever found. For example, cancer cells are detected in blood samples of leukemia patients (bone marrow affected) but also in the blood of a patient with diagnosed melanoma (skin cancer) with the risk of metastasis. The method of diagnosis according to the present invention provides contacting the body sample with the poly(allylamine) herein disclosed and revealing the irreversible adhesion of said poly(allylamine) to the cancer cell or the internalization of said poly(allylamine) into said cancer cell.

[0085] In case of diagnostic application of the present invention, a preferred embodiment provides the detection of cancer cells in blood samples. In this case, cancer cells to be detected are non-adhesive cancer cells or invading cancer cells.

[0086] In an especially preferred embodiment, cancer cells to be detected are leukemia cells or invading cancer cells (detectable in blood because they leave the primary tumour and settle somewhere, causing metastasis). In this embodiment, the method of the invention is very convenient, since only a fast centrifugation is needed to separate the lymphocytes.

[0087] Other preferred embodiments relate to the detection of cancer cells from melanoma, liver cancer, breast cancer, ovarian cancer, and glioblastoma.

[0088] A particular embodiment of the invention relates to the detection of metastatic cells. Detecting said cells in body circulation, for example blood or lymph circle is of critical importance for the management and cure of cancer disease.

[0089] In a still further embodiment, the present invention discloses a method for treating cancer comprising:

a. obtaining a blood sample from a subject affected by said cancer,
b. contacting said blood sample with a blood purification system whereby cancer cells contained in said blood sample are contacted with a solution of a poly(allylamine) according to the present invention, whereby said poly(allylamine) enters said cancer cells,
c. removing unbound poly(allylamine), to provide a purified blood sample,
d. returning said blood sample to said subject.

[0090] Another embodiment of the present invention provides an ex vivo method for treating a blood sample containing cancer cells from a subject affected by cancer comprising:

a. contacting said blood sample with a blood purification system whereby cancer cells contained in said blood sample are contacted with poly(allylamine) or a pharmaceutically salt thereof, whereby said poly(allylamine) kills said cancer cells,

b. removing unbound poly(allylamine), to provide a purified blood sample.

[0091] For the purposes of the present invention, ex vivo is intended according to the general meaning given to this term in the medical field, namely that the sample is isolated from a subject in need to be treated and processed according the above method. The method according to the present invention can be wholly performed outside the body of the subject in need of treatment. After the method is completed, the purified blood is returned to the subject.

[0092] In a preferred embodiment poly(allylamine) is labelled as disclosed before, for example with a fluorescent label. Separation of cancer cells incorporating the labelled polyallylamine is made with any method known in the art, for example with a flow cytometer.

[0093] In the above treatment providing the separation of cancer cells, it is convenient to provide the poly(allylamine) with a detectable label as above explained, in order to facilitate separation of cancer cells after incorporation of the labelled poly(allylamine).

[0094] However, a detectable label can also be used if the cancer cells, incorporating the suitable poly(allylamine), are returned to the subject, in order to perform a follow up of the treatment through imaging or any other technique. In this case, a non-toxic label is used, such as carotene or other chromophores.

[0095] Another object of the present invention is an apparatus for extracorporeal treatment of blood, characterized in that the part of said apparatus contacting the blood for treatment comprises poly(allylamine) or a pharmaceutically acceptable salt thereof.

[0096] This kind of apparatus is well-known in the art and is commercially available. Examples of these apparatuses are shown in EP1464349, EP1892001, EP1946783, EP1962833, US2007287163, WO2006123308, WO2008025467, WO2008059395, WO2008090406, WO2008125894 and the references cited therein.

[0097] Although the poly(allylamine) is not cytotoxic for the blood cells or the general population of healthy cells in the tissues next to cancer, it is highly cytotoxic for the endothelial cells (blood vessels). A systemic therapy with poly(allylamine) is excluded because of the risk to damage irreversibly the blood vessel or the myocardium but an ex vivo treatment in a blood purification system with strict control that no poly(allylamine) enters the body after treatment will have a high therapeutic potential. To this purpose, the above ex vivo method is highly preferred. For solid tumors, poly(allylamine) shall be administered directly into the tumor mass. This administration system is well-known to the person skilled in this art. Guidance can be found in the general common knowledge, for example The Merck Manual of Diagnosis and Therapy, Remington's Pharmaceutical Sciences, last edition, Mack Publishing and Co.

[0098] The following example further illustrates the invention.

[0099] Example

[00100] Material and Methods:

[00101] Materials

[00102] The polyamines, poly(allylamine hydrochloride) (PAH; MW 15kDa) and poly(ethyleneimine) (PEI; MW. 2kDa) as well as the fluorescent-labeled poly(fluorescein isothiocyanate allylamine hydrochloride) (FITC-PAH; MW. 15kDa; λ_{exc}=494 nm, λ_{em}=520 nm), Spermine (Sp; MW. 202Da), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], $MgCl_2$ and dimethylsulfoxide (DMSO) were purchased from SIGMA-Aldrich (Milan, Italy) and used as received. The plasma membrane specific dye, dialkylaminostyryl (DiA; λ_{exc}=460 nm, λ_{em}=580 nm) and nucleic acid binding dye, Propidium Iodide (PI; λ_{exc}=537 nm, λ_{em}=619 nm) were purchased from Invitrogen (Milan, Italy). All the solutions were prepared with DMEM medium (Dulbecco's Modified Eagle's Medium; Sigma-Aldrich, Milan, Italy).

[00103] Cell Culture

[00104] Leukemia: Leukemia (HL60 and Jurkat, (ATCC CCL-240™ and TIB-152™) cell lines were continuously maintained in DMEM (Gibco®,) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich,), penicillin (100 units/ml), streptomycin (100 µg/ml), gentamicin (10 µg/ml) at 5% CO_2 and 37°C. The medium was changed every second day and mycoplasma testing

was performed to exclude any possible contamination prior performing experiments.

[00105] Hepatocytes: JHH-6 cells (JCRB1030 undifferentiated hepatocellular carcinoma) were obtained from Japan Health Science Research Resources Bank (HSRRB, JCRB1030 respectively). JHH-6 were cultured in Williams E medium (Sigma-Aldrich, Missouri, USA. W4128) with 10% (v/v) fetal bovine serum (FBS) (Invitrogen), 2mM L-Glutamine (Euro-clone), and 1%(v/v) antibiotics (10,000 U/mL penicillin, and 10 mg/mL streptomycin (Euro-clone)). The cells were grown at 37°C in a humidified atmosphere 95% air and 5% CO_2 under the conditions described above.

[00106] Mononuclear Cell Extraction

[00107] Blood samples of healthy volunteers and patients were obtained from the Dermatology Department of Cattinara Hospital from healthy volunteers. Leukemic blood samples were obtained from the Hematology Department, Trieste.

[00108] Mononuclear cells (MNCs) were isolated from blood using Ficoll-Paque™ PLUS (GE Healthcare) according to manufacture's protocols and are maintained in DMEM with 10% FCS serum during experiment.

[00109] Staining of the cancer cells

[00110] To determine the binding specificity of positively charged poly-allylamine (PAH) to the cancer cells, the human leukemia (HL60, acute promyelocytic leukemia) cell line as well as the lymphocytes, granulocytes and whole blood of leukemic (n=3) and healthy volunteers (n=3) were incubated with FITC-PAH (fluorescein isothiocyanate-polyallylamine; concentration: 2 mg/ml) for 2 min, 5 min, 15 min, 30 min, 1 h and 2 h incubated at 37°C and 5% CO_2.

[00111] Fluorescence microscopy

[00112] Imaging was performed with Nikon C1 laser scanning confocal unit (Nikon D-eclipse C1Si, Japan) attached to an inversed fluorescence microscope with a 100x/1.49 oil Apo TIRF objective (Nikon, Japan). The fluorophores were excited with a multiline Argon ion laser, FITC-PAH at λ=488 nm, DiA at λ=460 nm and PI at λ=535 nm. Images were acquired and processed using the operation software EZ-C1 for Nikon C1 confocal microscope.

[00113] Analysis of the data

[00114] For the number of stained cells was determined in HL60 cells where all cells were supposed to be malign and for the lymphocytes fraction of a patient with diagnosed leukemia. The incubation was in both cases for 10 min under standard cell culture conditions (37°C, 5% CO_2). For the lymphocyte incubation a sample preparation was necessary prior to the treatment. From the whole blood sample, collected from the patient and stabilized by citrate, by Ficoll centrifugation the fraction containing the lymphocytes was separated from the monocytes and other blood components. The lymphocyte fraction was immediately after separation cultured in medium. The cell counting was always performed by two independent persons. In case of HL60 cells the experiments were repeated for 12 times and for each time at least 2 images were analyzed. For the lymphocyte analysis experiment were repeated for 4 times and for each time 4 or more images were analyzed.

[00115] Survival rate of HL60 cells

[00116] In order to determine the survival of human leukemia cells after polyelectrolyte treatment the HL60 were incubated for 15 min, 1 h or 2.5 h in presence of the polyelectrolyte under otherwise standard culture conditions (37°C, 5% CO_2). It was found that significant amount of the cells adhere to the plate surface and there was a separate counting for the dead/live assay with PI (propidium iodide, stains dead cells red) staining with adherent and suspension cells.

[00117] MTT assay

[00118] Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) dye reduction assay as previously described in Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65, 55-63 (1983).

[00119] Briefly, HL60 and Jurkat cells (20 x 10^4 cells/per well) were plated in 96 well-plates. The cells were exposed to different final concentrations of PAH (20 µg/mL, 40µg /mL, 100µg /mL, 200 µg/mL and 400 µg/mL), along with cells which received no treatment as negative control and 50µl DMSO lysed cells as positive control. Other cationic polymers like PEI (poly-(ethylenimine), an apoptosis/necrosis inducer or spermine, a natural polyamine and growth inducer were also tested in the following concentrations: 200, 400, 600, 800 µg/mL final concentration in the cell medium for spermine. Mononuclear cells

from a healthy donor were incubated with a cell density of 2 x 10^5 cells in the same concentrations of PAH solution as before. The treatment was followed by addition of 0.5 mg/mL of MTT and incubation for 1 h at 37°C. The medium was removed, the cells were lysed and the resulting blue formazan crystals were solved in DMSO. The absorbance of each well was read on a microplate reader (Beckman Coulter LD 400C Luminescence detector) at 570 nm. The absorbance of the untreated controls was taken as 100% survival. The present data represents the mean ± sd of three independent experiments.

[00120] For toxicity test with gel beads consisting of polycations and cell contents were produced by incubating 10^6 HL60 or Jurkat cells in 3.4 mL medium with 1.6 mL PAH (stock solution: 400 µg/mL). After 2hrs of incubation the gel beads were washed twice with DMEM by repeated centrifugation at 16,000 x g for 30mins at room temperature and resuspension in medium. Finally the beads were resuspended in 5 mL medium. In SEM the dried beads appear very uniform in size which is in vacuum determined to be around 150 nm. In light microscopy the swollen beads are around 300-500 nm. Different volumes (200 µL, 600 µL, 800 µL) of bead-containing solution are added to 5 mL HL60 or Jurkat cell suspension with a cell density of 20 x 10^4 cells/mL. The incubation for 2 hrs was followed by twice washing with DMEM and centrifugation at 1,000 x g for 5mins at room temperature. Then the MTT assay was performed. For the highest bead concentration (800 µl bead containing solution) the toxicity for HL60 and Jurkat cells was studied in a long-term exposure (0, 6, 24, 42, 48 h) to the beads.

[00121] FACS analysis

[00122] HL60 (260 x 10^4 cells) and Jurkat (645 x 10^4) cells were treated with different concentrations (50, 100, 200, 400 µg) of the polycation (FITC-PAH) or volumes of gel beads (VCP) for 5 min. Cells were centrifuged twice to remove the excess of the compound, the pellet was recovered and resuspended in physiologic solution. Untreated cells were used as negative control. The intracellular green fluorescence from FITC-PAH was collected by Flow Cytofluorometry using a Becton Dickinson FACSCalibur System, equipped with a single argon-ion laser, through a 530 nm band pass filter in combination with a 570 nm dichroic mirror. A minimum of 10,000 cells was analyzed for each sample.

[00123] Results:

[00124] The poly(allylamine) used according to the present invention can enter easily in non-adhesive cancer cells, like in case of leukemia or invading cancer cells, while they do not affect normal blood cells (figure 1). The increased permeability inside the cells is also observable for other cancer cells like melanoma, liver, and breast cancer.

[00125] The cell survival after 5 mins of treatment in dependence of the polycation concentration was investigated. Results of the MTT assay for HL60 and Jurkat cells in comparison to the mononuclear cells gained from healthy donors are shown in figure 3 and compared with data for PEI (poly-(ethylenimine), a different polycation and spermine, a naturally occurring polyamine. It can be clearly seen that HL60 cells are significantly more sensitive to polycation treatment in comparison to Jurkat cells. A strong concentration dependence for concentrations lower than 100 μg/mL could be observed for both cell lines. With concentrations higher than 100 μg/mL it was found that the amount of survivors remains more or less constant. This indicates that the cells contain a fraction of the cells which are resistant to treatment. The percentage is 1.5±0.7 % in case of HL60 cells and 7±1 % in case of Jurkat cells. It is noteworthy that the treated mononuclear cells isolated from healthy donors show nearly no toxic effect of PAH (survival: 80-100%). The two other tested polyamines, PEI and spermine were exemplarily included in figure 3 with one concentration. It was found that PEI is significantly less cytotoxic in short terms (2 h) to the both leukemic cell lines than PAH and spermine is a growth stimulator increasing the number of cells significantly in a concentration dependent manner. PEI is known to induce apoptosis which is a cell death visible only after several hours or days.

[00126] It was found that FITC-PAH is binding to the plasma membrane and the cytoskeletons, but in some cases also to the nucleus and the DNA. Mostly binding the strong negativity of heterochromatin nucleoli region of the nucleus within 2 min incubation. After 5 min the poly(allylamine) is seen mostly in the cytoplasm and the nucleus. After 15 min incubation almost all the nucleus are stained with FITC-PAH. Addition of FITC-PAH under microscope, showed that the poly(allylamine) enters the cells very quickly. Within 30 s poly(allylamine) are crossing the plasma membrane and staining the cytoplasm allowing the visualization of internal cell structure. An assay with Propidium Iodide (PI), a dye intercalating selectively in the DNA of dead or apoptotic cells, showed that

PI is able to enter which indicate a nanoporation of the cell membrane. No competitive effect of PI and the poly(allylamine) is expected because both are positively charged but the binding site to the DNA is different. PI is intercalating between the bases while the polymer is electrostatically bound to the negatively charged phosphate group of the DNA. After 1 h incubation, we see that the HL60 cancer cells are showing in apoptotic-like vesicles which were indentified as gel beads containing the polycation and cell contents. After 2 h of incubation with poly(allylamine) all cells are destructed and only fragments are present.

[00127] When FITC-PAH are added under microscope on whole blood, granulocytes and lymphocytes from leukemic patients and healthy volunteers, lymphocytes and monocytes (granulocytic component) of leukemic patient show similar behaviour in terms of poly(allylamine) up take as HL60 cell line. They are stained within 30 s and the polycation bind mostly to the plasma membrane and cytoskeleton. After 5 min, 1 h and 2 h incubation we see the same effect as the HL60 cell line that all the cytoplasm and nucleus is totally stained. In patients only the leukemic lymphocytes were stained, but not the healthy ones. Both, lymphocytes and granulocytes of the healthy donor are impermeable to FITC-PAH. The same is true for the red blood cells. Not even overnight incubation with FITC-PAH make the cells permeable which indicates as well that the poly(allylamine) is not cytotoxic for the blood cells. Earlier studies showed that poly(allylamine) is highly cytotoxic for the endothelial cells (blood vessels). A systemic therapy with PAH is excluded because of the risk to damage irreversible the blood vessel or the myocardium but an ex vivo treatment in a blood purification system with strict control that no poly(allylamine) enters the body after treatment will have a high therapeutic potential. For solid tumors, poly(allylamine) shall be administered directly into the tumor mass. This administration is well-known to the person skilled in this art.

[00128] The staining of the cancer cells is very fast and extremely bright as can be seen by fluorescence micrographs. The amount of cells which are stained by FITC-PAH after 10 min were compared. In the case of HL60 cells around 95% have uptake the poly(allylamine) while in case of the lymphocytes of patients with diagnosed leukemia 40-90% of the cells are affected. In a lymphocyte sample of a healthy donor the amount of stained cells is 0.5 %. Moreover it was found that the poly(allylamine) which is entering exclusively the cancer cells are leading to

their death (approx. 90 %, figure 2). So the suggested method can serve as a theranostic system, identifying the diseased cells and at the same time selectively killing them.

[00129] Interesting in this context if the cells which are affected by the FITC-PAH survive on long-terms or if the incubation with the polyelectrolyte could offer also a therapeutic application. In several incubation experiments the survival rate of the incubated cells were tested. The survival rate of cells after 2.5 h in presence of FITC-PAH is negligible (figure 2).

[00130] It has been shown that PAH had a selective cytotoxicity effect also on different cancer cell lines and cancer stem cell lines, leaving unaffected the normal cells.

[00131] In the following Table it is reported the effect of PAH on different kind of cells:

Table 3:

Cancer cells	Result with PAH
Leukemia (HL60) less differentiated	++
Leukemia (Jurkat well differentiated)	++
Hepatocarcinoma (JHH-6) less differentiated	++
Uterine cancer	+
Melanoma	+
Breast cancer	+
Ovarian cancer	+
Invading cancer in blood	++
Cancer stem cells	
Ovarian cancer stem cells	+
Glioblastoma stem (high grade)	++
Glioblastoma stem (low grade)	+
Hepatocarcinoma stem	++
Normal cells as control	
Whole blood	-
Heart stem cells	-
Adipose stem cells	-
Ovarian stem cells	-

[00132] As reported in Table 3, PAH has a cytotoxic effect on different cancer cells and cancer stem cells, while it has no effect on normal cells.

[00133] Potential application

[00134] The detection of leukemia cells can be in a fast and disposable test kit with high reliability. In a microchamber the dry fluorescent or even chromophore labelled poly(allylamine) is immobilized in a gel. In contact with a blood sample the poly(allylamine) is solving in the serum and entering in the leukemic cells. Imaging under a microscope will give after 1 min the number of cells in which the polyelectrolyte will be able to enter. The cost is very low because the polycation (PAH) used for the test kit is very cheap.

[00135] Industrial Application

[00136] The present invention relates to the medical field, in particular to the diagnosis and cure of cancer.

Claims

1. Poly(allylamine) or a pharmaceutically acceptable salt thereof for use as antitumor agent.
2. Poly(allylamine) according to claim 1, which is labelled with a detectable label.
3. Poly(allylamine) according to any one of claims 1-2, wherein said cancer comprises at least one population of cancer cells selected from the group consisting of non-adhesive cancer cells, cancer stem cells, undifferentiated and less differentiated cancer cells.
4. Poly(allylamine) according to claim 3, wherein said cancer is selected from the group consisting of leukaemia, melanoma, liver cancer, breast cancer, ovarian cancer, glioblastoma.
5. Poly(allylamine) or a pharmaceutically acceptable salt thereof for use as diagnostic agent for revealing cancer cells in an isolated tissue of a subject.
6. Poly(allylamine) according to claim 6, wherein said cancer comprises at least one population of cancer cells selected from the group consisting of non-adhesive cancer cells, cancer stem cells, undifferentiated and less differentiated cancer cells.
7. Poly(allylamine) according to claim 6, wherein said cancer is selected from the group consisting of leukaemia, melanoma, liver cancer, breast cancer, ovarian cancer, glioblastoma.
8. An ex vivo method for treating a blood sample containing cancer cells from a subject affected by cancer comprising:
    a. contacting said blood sample with a blood purification system whereby cancer cells contained in said blood sample are contacted with poly(allylamine) or a pharmaceutically salt thereof, whereby said poly(allylamine) kills said cancer cells,
    b. removing said killed cancer cells, to provide a purified blood sample.
9. Poly(allylamine) or a pharmaceutically acceptable salt thereof for use as theranostic agent for cancer.

10. An apparatus for extracorporeal treatment of blood, characterized in that the part of said apparatus contacting the blood for treatment comprises poly(allylamine) or a pharmaceutically acceptable salt thereof.

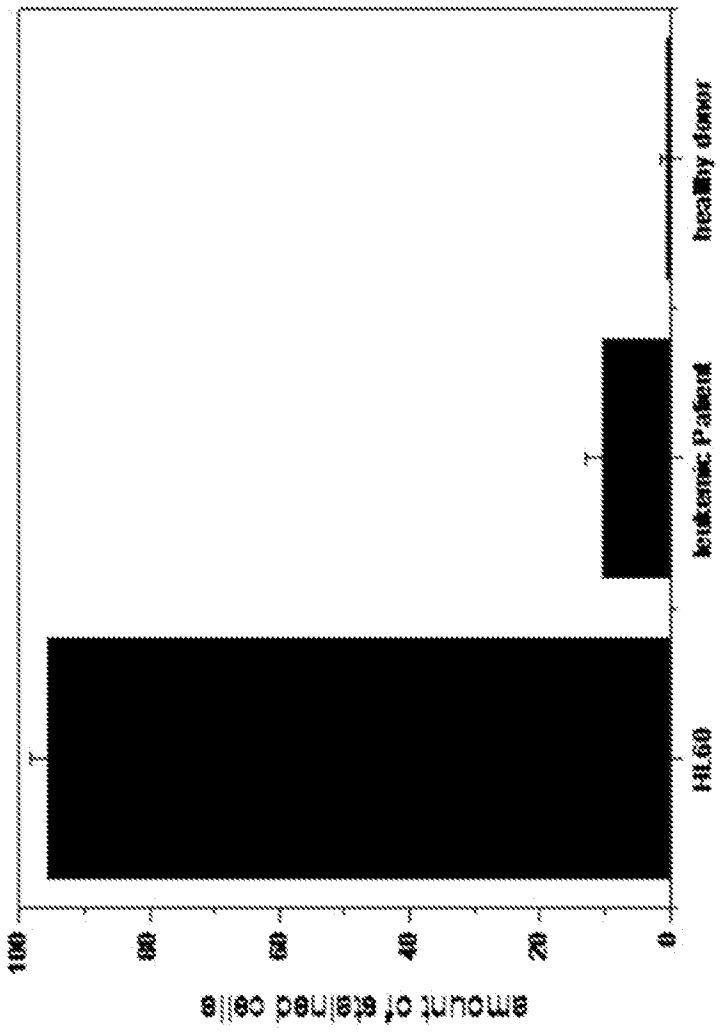
amount of eliminated cells
100
80
60
40
20
0
HL60
leukemic Patient
healthy donor
Samples

FIG 1

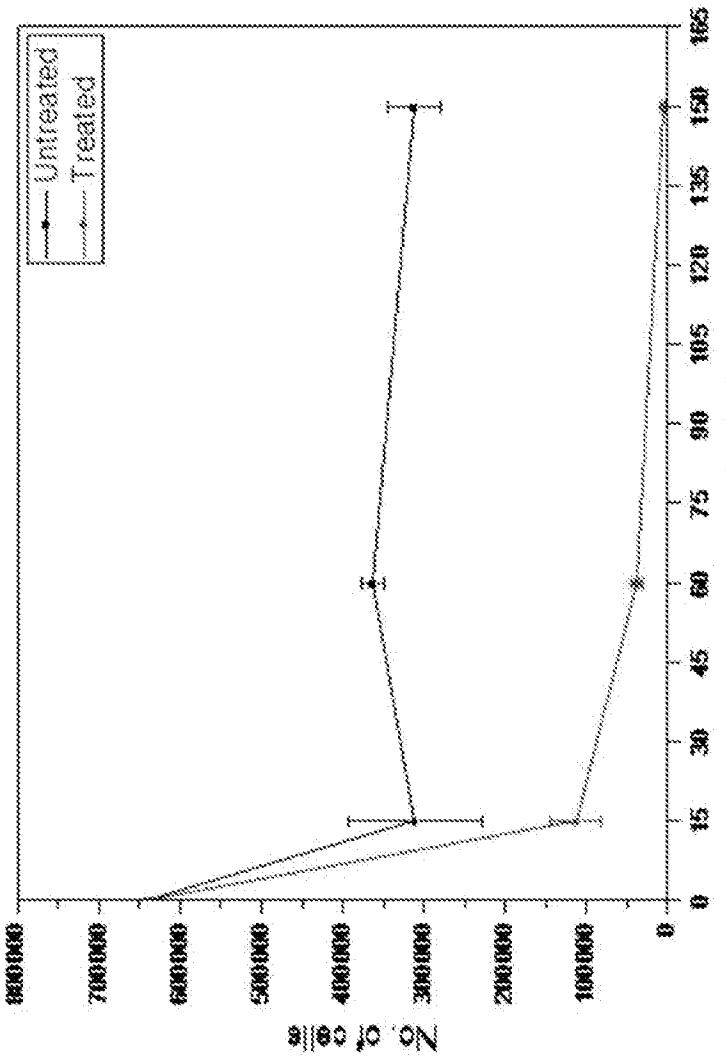
Untreated
Treated
No. of cells
800000
700000
600000
500000
400000
300000
200000
100000
0
0
15
30
45
60
75
90
105
120
135
150
165
Time (in mins)

FIG 2

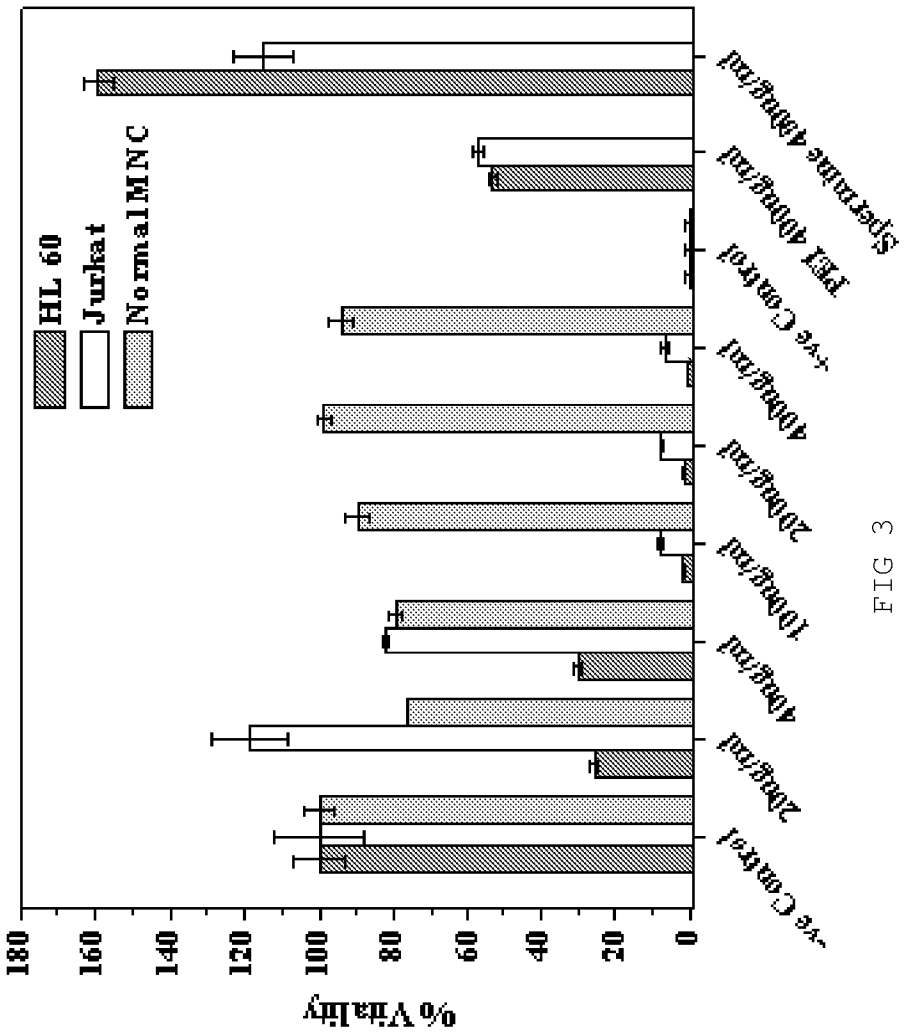
HL 60
Jurkat
NormalMNC
% Viability
180
160
140
120
100
80
60
40
20
0
-ve Control
2bbu/ml
4bbu/ml
10bbu/ml
20bbu/ml
40bbu/ml
+ve Control
PBI 40bbu/ml
Spermine 40bbu/ml

FIG 3