(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 August 2011 (18.08.2011)

PCT

(10) International Publication Number
WO 2011/100639 A2

(51) International Patent Classification:
C12Q 1/34 (2006.01) *G01N 33/573* (2006.01)
G01N 33/52 (2006.01) *G01N 33/574* (2006.01)

(21) International Application Number:
PCT/US2011/024658

(22) International Filing Date:
11 February 2011 (11.02.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/303,509 11 February 2010 (11.02.2010) US
61/412,364 10 November 2010 (10.11.2010) US

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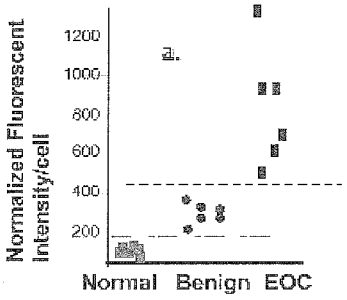
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG,

[Continued on next page]

(54) Title: PLA_2ACTIVITY AS A MARKER FOR OVARIAN AND OTHER GYNECOLOGIC CANCERS

(57) Abstract: Materials and methods are provided for diagnosis, monitoring, and personalized treatments of gynecological cancers. The methods comprise determining levels of PLA_2 activity with a patient sample and using elevated levels of this activity which correlate with diseases such as epithelial ovarian cancer (EOC) as a marker for pathology. These methods include assaying for PLA_2 activity within tissue, ascites, blood, and other tissue forms by exposing the patient sample to a fluorogenic compound such as DBPC. The methods disclosed herein further include correlating the fluorogenic detection with a disease state in the patient, including diseases such as gynecological cancers, such as EOC. The methods comprise determining levels of total PLA_2 activity, and of specific isoforms of PLA_2 such as $iPLA_2$, $iPLA_2\beta$, $cPLA_2$, among other isoforms.



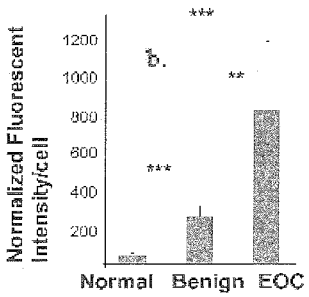


FIG. 1 E

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ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

PLA_2 ACTIVITY AS A MARKER FOR OVARIAN AND OTHER GYNECOLOGIC CANCERS

PRIORITY CLAIM

[0001] This application claims the benefit of United States Provisional Patent application Numbers 61/303,509 filed on February 11, 2010, and 61/412,364 filed on November 10, 2010, each of which is incorporated herein by reference in its entirety as if each were incorporated by reference individually.

FIELD OF THE DISCLOSURE

[0002] The present disclosure relates generally to cancer. More particularly, the present disclosure relates to Phospholiase A_2 (PLA_2) as a novel marker and as a target for diagnosing, evaluating and treating ovarian and other gynecologic cancers.

BACKGROUND

[0003] Gynecological cancers, and specifically ovarian, uterine, and cervical cancers, cause more than 26,000 deaths annually in the United States. A majority of epithelial ovarian cancer (EOC) patients present with late-stage disease. Curative treatment for late-stage EOC, and in particular for refractory and drug-resistant EOC, is often ineffective resulting in high mortality rates from EOC.

[0004] In general, there is not an acceptable method for screening patients for EOC. Current screening methods, such as ultrasound, are inadequate for early stage detection and in some cases for staging of EOC patient. Further, current markers for EOC, in general, are poor distinguishers for distinguishing between benign and malignant tumor development. Hence, development of markers for monitoring disease progress which also function as better targets for personalized treatment is urgently needed for a majority of EOC patients.

[0005] Therefore, a method and marker for better diagnosis, monitoring of gynecological cancer progression, and application in personalized gynecological cancer treatment are desirable. Some aspects of the present disclosure, disclosed herein, address these needs.

SUMMARY OF THE DISCLOSURE

[0006] Some embodiments of the disclosure include methods for evaluating a disease such a EOC including detecting and/or following the course of a disease including for example diagnosing, prognosing and evaluating the efficacy of treating a given disease, these methods may include the steps of measuring PLA_2 activity in a patient sample and providing information

associated with correlating the PLA_2 activity measured in said step of measuring, with a PLA_2 activity value indicative of a disease. Some embodiments further include the step of comparing the values measured in a given sample with valued indicative of various diseases and concluding that a given sample includes evidence of disease. Some embodiments of the methods disclosed herein include measuring the activity of PLA_2 comprising, for example, $iPLA_2$. Some further embodiments include methods of with correlating a PLA_2 activity value with a PLA_2 activity value indicative of a form of gynecological cancer. In further embodiments the form of gynecological cancer is EOC.

[0007] Some embodiments further include the step of obtaining the patient sample from a patient. In some embodiments of the disclosure, the patient sample may be selected form the group of sample consisting of tissue samples, cells, blood, bodily fluids, cellular fluids, a cellular fluids, discharges or fluids within or otherwise associated or produced by tumors, cysts or other growths and the like.

[0008] In some further embodiments of the methods disclosed herein, the step of measuring PLA_2 activity in a patient sample involves using a fluorogenic compound, including but not limited to the fluorogenic compound is DBPC. Still other embodiments include the use of still other compound that produces a detectable signal and can be used to measure or at least estimate PLA_2 activity in a given sample. In still other embodiments of the disclosed methods, the measuring step involves the use of radioactive compounds. Some embodiments may include a further separation steps and/or sample preparations to increase the sensitivity and or reproduce-ability of the activity assay. Still another embodiment includes normalizing the level of PLA_2 activity measured in a given to sample to the number of cells associated with the cells or to the level of protein in the sample.

[0009] In even further embodiments of the methods disclosed herein, the step of providing information comprises providing information correlating PLA_2 activity in range of activity that is demonstratively higher in sample for a patent with a form of GYN cancer such as EOC. In other embodiments of the methods disclosed herein, the step of providing information comprises providing information correlating PLA_2 activity in the range of about 1.5 higher than that detected in similar but health tissue of the same type. In still other embodiments the level of activity that correlates with disease may be about 2x or higher than normal activity measured in sample form non-cancerous sources include healthy samples or at least benign samples.

[0010] Still other embodiments of the methods provided herein, include the step of introducing a compound to the patient which inhibits activity of at least one isoform of PLA_2. In some embodiments the isoforms of PLA_2 in the sample includes at least one of the following $sPLA_2$, $cPLA_2$ and $iPLA_2$. Inhibitors that can be used include, but are not limited to, BEL. Some embodiments may include the steps of conducting a given PLA_2 assay with and without the presence of a given PLA_2 inhibitor and using any difference observed in the assays to assign at least a portion of the reduced activity to the forms of PLA_2 thought to be sensitive to the specific inhibitor used in the assay.

[0011] Further embodiments of the methods described herein includes a method of detecting PLA_2 activity within a sample comprising the steps of introducing a fluorogenic compound to a sample, and detecting a signal produced by the fluorogenic compound in the presence of the sample. In some embodiments of the method described herein the sample is a tissue and the method further includes the step of fixing the tissue.

[0012] In yet further embodiments of the methods disclosed herein, the tissue sample is homogenized and the fluorogenic compound is introduced to the supernant resulting from the homogenization of the tissue sample.

[0013] Still further embodiments of the methods include the step of correlating the signal value, detected in the step of detecting, with a disease. Some embodiments of these methods include the disease being a form of cancer. In some embodiments, the form of cancer is EOC.

[0014] Some embodiments include identifying a using a marker for diseases such, but not necessarily limited to EOC. These markers include phospholipase A_2 (PLA_2) activity that is demonstratively higher in samples such as fluids, tissues, blood, cells and like associated with disease such as EOC than it is in samples of healthy sources or samples from benign tumors.

[0015] In some embodiments demonstratively higher activity indicative of a disease state is at least 1.5 or in some embodiments at least 2 fold higher in a sample from a patient with a disease then it is in a patient that is without disease or that presents with a benign tumor.

[0016] In some embodiments the identification of the maker is indicative of cancer especially GYN cancers such as EOC. In some embodiments the marker can be used to diagnose a disease, prognosticate on the course of the disease, following the course of a disease or to evaluate the effectiveness of a treatment of a disease. In some embodiment the maker is activity

that due primarily to elevated iPLA$_2$β activity. In some embodiments values of PLA$_2$ activity used as markers for disease are used to personalize the treatment of a patient.

[0017] These and other features, aspects, and advantages of the present invention will become better understood with reference to the following drawings, descriptions and claims.

BRIEF DESCRIPTION OF THE FIGURES

[0018] The above-mentioned aspects of the present disclosure and the manner of obtaining them will become more apparent, and aspects thereof will be better understood by reference to the following description of the embodiments of the disclosure, taken in conjunction with the accompanying drawings, figures, schemes, graphs, charts, and the like, wherein:

[0019] **FIG. 1A.** Photomicrographs of para-nuclear PLA$_2$ activity in cells from cancerous tissue.

[0020] **FIG. 1B.** Photomicrographs of para-nuclear PLA$_2$ activity only in the surface cells in benign tumors.

[0021] **FIG. 1C.** Photomicrographs of PLA$_2$ activity in the stromal portions of from normal tissue (non-cellular).

[0022] **FIG. 1D.** Photomicrographs of cells treated with an inhibitor of cPLA$_2$ and/or iPLA$_2$ (panels b, d) and an inhibitor of sPLA$_2$ (panel c).

[0023] **FIG. 1E.** Dot plots (panel a) and a histograph (panel b) of fluorescent intensity normalized to cell count.

[0024] **FIG. 2A.** Arachidonic acid (AA) concentrations in non-malignant (N; n=9) and EOC (n=8) ascites samples.

[0025] **FIG. 2B.** Histographs of fluorescent intensity (PLA$_2$ activity) measured in the blood sample from subjects: healthy controls, patients with benign gynecologic diseases, and patients with ovarian cancer Total PLA$_2$ activities in blood samples from healthy (n=7) subjects, benign (n=8) subjects, and subjects diagnosed with EOC (n=5).

[0026] **FIG. 2C.** PLA$_2$ activities in cell-free ascites samples mentioned in **FIG. 2A**.

[0027] **FIG. 2D.** Histographs showing PLA$_2$ activities in the exosomes and the supernatant (super) portions of ascites samples. The dark bars: 1, background fluorescence; 2. sPLA$_2$ positive control; 3. TA-PC treated sPLA$_2$. **: $p<0.01$. * $p<0.05$.

[0028] **FIG. 3A.** PLA_2 activities in mouse tissues measured in mice that were injected with A2780 human EOC cells and PLA_2 activities were analyzed in tumors: a. no DBPC; b. with DBPC; c. DAPI; d. merged.

[0029] **FIG. 3B.** The PLA_2 activities from tumors obtained from control (a) and BEL-treated (b) mice. The tumors in BEL-treated mice were significantly smaller and the PLA_2 activities were reduced.

[0030] **FIG. 3C.** PLA_2 activities detected in tumors derived from human SKOV3 (C) and mouse ID-8 cells (D) EOC cells.

[0031] **FIG. 3D.** PLA_2 activities detected in tumors derived from human SKOV3 (C) and mouse ID-8 cells (D) EOC cells.

[0032] **FIG. 3E.** Normal mouse kidney tissues mainly had $sPLA_2$-like activity, which was not associated with cell nucleus and minimally affected by BEL. The numbers in all figures are the quantified PLA_2 activity/cell. Representative results are shown

[0033] **FIG. 4A.** Shows a time dependent curve demonstrating fluorescent intensity having a linear relation with time

[0034] **FIG. 4B.** Shows another time dependent curve demonstrating fluorescent intensity having a linear relation with time

[0035] **FIG. 4C.** Is a standard curve demonstrating fluorescent intensity having a linear relation with DBPC substrate concentration.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0036] For the purposes of promoting an understanding of the principles of the novel technology, reference will now be made to the preferred embodiments thereof, and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the novel technology is thereby intended, such alterations, modifications, and further applications of the principles of the novel technology being contemplated as would normally occur to one skilled in the art to which the novel technology relates are within the scope of this disclosure and what is claimed.

[0037] Unless explicitly or implicitly stated otherwise the term, 'about' as used herein means plus or minus 10 percent. For example about 1.1 encompasses the values between 0.9 and 1.1.

[0038] Unless explicitly or implicitly stated otherwise the term, 'demonstratively' as used to refer to a property that is integral to given sample and that can be reproduced with sufficient precision and accuracy to show or illustrate the similarity or difference between at least two different portions, samples and the like.

[0039] Phospholipase A_2s (PLA_2s) are enzymes commonly found in mammalian tissue. PLA_2s include several protein families, including secreted phospholipase A_2 ($sPLA_2$), cytosolic phospholipase A_2 ($cPLA_2$), and Ca^{2+} independent PLA_2 ($iPLA_2$). All PLA_2s catalyze hydrolysis of phospholipid substrates to generate a lysophospholipid (**LPL**) and a free fatty acid (**FA**), *e.g.*, arachidonic acid (**AA**). **AA** can be oxygenated to a variety of bioactive eicosanoids. Phosphatidylcholine (**PC**) is an abundant lipid substrate in cell membranes and lysophosphatidylcholin (**LPC**) is a more common product of PLA_2s. LPC is convertable to Lysophosphatidic acid (LPA) by autotaxin (ATX). Additionaly, certain PLA_2s, including $sPLA_2$ and calcium independent $iPLA_2$ isoforms can utilize phosphatidic acid (**PA**) as a substrate for directly producing LPA. The compounds, LPA and AA are shown to be important lipid mediators in chemotaxis and/or chemokinesis.

[0040] Aberrant expression of some $sPLA_2$s and $cPLA_2$ has been shown in some cancer types (not inclusive of EOC). To date, only the $sPLA_2$ isolform, $sPLA_2$-XII, has shown potential as a possible predictive indicator of patient outcomes. However, currently there exists no comparison data between malignant and benign/normal tissues.

[0041] In regard to EOC, specifically, mRNA expression levels of 19 different PLA_2 isoforms ($sPLA_2$ and $cPLA_2$s) have demonstrated decreased or unchanged transcriptional levels in EOC versus non-tumoral immortalized (EONT) cells.

[0042] $iPLA_2$s, calcium-independent phospholipase A_2 beta, the Group VIA phospholipase A_2, are intracellular enzymes that do not require Ca^{2+} for their catalytic activity. $iPLA_2$s are, in general, known to be active in phospholipid remodeling, signal transduction, and cell proliferation and apoptosis.

[0043] Various $iPLA_2$ isoforms use **PA** as a substrate and produce LPA. LPA and AA are shown to be important lipid mediators in chemotaxis and/or chemokinesis. Further, $iPLA_2$ activity is known to regulate **LPA** and other lipid concentrations in tumor microenvironment.

[0044] Lysophosphatidic acid (**LPA**) is a bioactive lipid with multiple functions. Epidemiological, animal, and cell culture studies indicate that LPA plays a role in EOC

development. Over-expression or down-regulation of LPA receptors 1, 2, and 3 in several human EOC cell lines and *in vivo* studies, indicate that LPA receptors are involved in EOC development.

[0045] LPA activity is suppressible in various manners including inhibiting its production, increasing its degradation or conversion, preventing receptor occupancy, or and interfering with distal signaling pathways.

[0046] LPA is producible by the action of lysophospholipase(s) D (lyso-PLD), *e.g.*, autotaxin (**ATX**), or via phospholipase(s) A_2 (**PLA_2**), as discussed above.

[0047] The activation of $iPLA_2\beta$ in non-apoptotic EOC cells occurs via a laminin-β_1-integrin-caspase 3 pathway resulting in LPA and AA increase from EOC cells. Also, $iPLA_2$ activity increases (in association with EOC) as disclosed herein, are not reflective of increased $iPLA_2$ expression levels in EOC and/or other GYN cancer tissues.

[0048] As detailed above, resulting LPA acts as an "oncolipid" in EOC. Unexpectedly, as disclosed herein the activity of PLA_2 in EOC tissues is increased compared to its activity in benign disease and/or normal tissues without a correlated increase in PLA_2 expression levels. The activity of the PLA_2 enzymes (there are over 20 isoforms of phospholipase A_2 enzymes) taught and described herein, are disclosed as utilizable in the assay and methods disclosed herein as useful markers for diagnosing, prognosis, and monitoring EOC progression, as well as a target for personalized therapeutics in treating EOC.

[0049] As disclosed in the Examples section provided herein, PLA_2 activities have been assessed in cancer vs. normal tissues and the differences in activity between these types of tissues are disclosed herein as usable and valuable in providing an assay for cancer diagnosis and monitoring. Similarly, PLA_2 activities have been assessed in EOC vs. tissue from benign tumors and usable differences between PLA_2 activities measured between these different tissues have been demonstrated to exit.

[0050] Further, the disclosure provided herein discloses PLA_2 activity as a robust and useful marker that can be used to distinguishing malignant EOC, and other GYN cancer tissues including, from benign and/or normal tissues. With reference to **FIG. 1E,** data is presented demonstrating PLA_2 activity in malignant and benign tissues provides distinguishability. Current markers utilized for EOC (including Ca^{125}) lack sufficient differentiation capabilities for distinguishing between benign and malignant tumor development. This common issue suggests

that the current markers' biological functions are similar, resulting in difficulty in distinguishing between benign and malignant tumor development. The methods and assays disclosed and described herein provide a further advantage over current markers by providing an assay for distinguishing malignant EOC, and other GYN cancer tissues, from benign and/or normal tissues based on PLA_2 activity.

[0051] As disclosed and described herein, PLA_2 activity provides a valuable marker with utility in personalized chemotherapy. As is noted herein, this disclosure provides a method corresponding to greater than 30 percent of all EOC patients (no other genetic changes, other than p53 mutations, is known to occur with an incidence of greater than 30% in EOC patients). Thus, personalized and targeted therapy is the promising goal for EOC treatment.

[0052] Further, PLA_2s are involved in cellular processes, sometimes associated in other forms of cancers. The major effects of $iPLA_2$, for example, in EOC cells are cell migration and invasion, two key steps of tumor metastasis, with a relatively less prominent effect on cell proliferation. Thus, the present embodiments further provides a clinical value (in utilizing of PLA_2 activity as disclosed herein) in cancer types of than EOC. For example, the instant disclosure for analysis of PLA_2 activity as a marker for metastases has a prognostic value in both EOC and other forms of GYN cancers. Hence, it is within the present disclosure to utilize the assay involving PLA_2 activity, disclosed herein, for distinguishing between metastases and primary EOC.

[0053] Metastasis is the major cause for deaths in solid cancer patients. As such, the assay disclosed herein provides a valuable marker and prognosis tool. Further, the methods associated with PLA_2 activity, disclosed and described herein, provide a valuable and necessary marker adapted for early detection in the diagnosis of GYN diseases.

[0054] One example demonstrating the value and novelty of the disclosed embodiments herein is demonstrated in early detection of Type II EOC. Recent data indicates that there exists two major types of EOC, Type I and Type II. Type I tumors are, characteristically speaking, slow growing and include low-grade micropapillary serous carcinoma, mucinous, and endometrioid carcinomas. Type I tumors are also genetically stable and are characterized by mutations in a number of different genes including KRAS, BRAF, pTEN, and beta-catenin. Type II tumors, however, are rapidly growing and highly aggressive neoplasms for which well-defined precursor lesions have not been described. Type II tumors include high-grade serous

carcinoma, malignant mixed mesodermal tumors (carcinosarcomas), and undifferentiated carcinomas and have a high level of genetic instability and are characterized by a mutation of TP53. This method and assay disclosed herein, helps to explain why current screening techniques, aimed at detecting stage I disease, have not been effective given that Type II tumors are rarely confined to the ovary and are not derived from Type I EOC. Current screening approaches for early EOC detection have utilized stage I cancers as model in early markers development. However, specifically in regard to Type II EOC, such screening approaches and models are flawed for the reasons discussed above. The diagnostic methods disclosed herein, however, provide an improved, novel, and unique method allowing for earlier diagnosis of Type II EOC.

[0055] Ascitic fluids, as disclosed herein, represents EOC tumor microenvironment and/or peritoneal washings (for those patients who do not develop ascites or are still at a relatively early stage of disease development) which can be obtained through minimally invasive ultrasound guided procedures. Accordingly, the PLA_2 activity based methods provided herein disclose a ready and less invasive means for diagnosing and monitoring the progress of these diseases.

[0056] Additionally, the single blood marker disclosed herein may be used as part of an array of diagnostic tools and has important value to existing EOC markers such as the markers marketed by Quest Diagnostics (OVA1, Ca^{125} and HE4).

[0057] Further, tissue based PLA_2 activity assays, as disclosed herein, offer information related to tumor and host cell histology, and potentially better separation for subjects in different groups. DBPC is used in assays, disclosed and described herein, for *in vitro* diagnostic applications. Additionally, development of infrared fluorescence (NIRF) PLA_2 substrates suitable for in *vivo* tumor imaging provide for *in vivo* EOC imaging. Utilization of the PLA_2 activity assays, disclosed herein, for monitoring PLA_2 activity provides not only available diagnostic tool, but also an efficient means of monitoring the efficacy of various treatments including drug treatments.

[0058] The data presented herein provides and supports a method of using PLA_2 activity as a new marker for studying a disease, such as EOC, including methods of diagnosing the diseases, predicting the course of the diseases, evaluating the effectiveness of treatments for the diseases and formulating personalized treatment regimes for this disease. As discussed herein,

PLA_2 activity provides a useful marker for diagnosis, prognosis, monitoring, and/or predicting EOC and/or other GYN cancers. These methods are especially useful when PLA_2 activity is normalized to an inherent standard such as the number of cells in a sample of tissue. Still other means of normalizing PLA_2 activity include normalizing data based on the protein content of a given sample and the like. The data indicated herein further provides that PLA_2, including $iPLA_2$ alone or in combination with other PLA_2s provides a useful target for the treatment of EOC. Further, PLA_2-related activities, disclosed herein, are highly significant in EOC management and treatment decision making, as well as in designing and testing new therapeutics. Further, it should be understood that although genetic, epigenetic, and proteomic studies regarding EOC have been conducted, those studies have focused on expression at either the RNA or protein levels, not a direct focus on PLA_2 activity. Thus, a focus directly on PLA_2 as a marker for ovarian and other gynecological forms of cancer, as disclosed herein, represents a new and highly relevant and useful method for the diagnosis, monitoring, and targeted treatment of gynecological cancers such as EOC.

EXPERIMENTAL

Example 1. Detection Assays for PLA_2 activity.

[0059] Classical methods of assaying PLA_2 activity, in general, involve the use of a radioactive-labeled phosphatidylcholine (PC) substrate. Cell or tissue lysates are incubated in a buffer with or without calcium (assays specific for a form of $iPLA_2$ are performed without calcium and include 1 mM EDTA) and the substrates [dipalmitoyl phosphatidylcholine (DPPC), and 1-palmitoyl-2-[1-^{14}C] palmitoyl-*sn*-glycero-3-phopshocholine (300,000 cpm/assay)]. Post incubation, the cleaved and ^{14}C-labled fatty acid (FA) is resolved using thin-layer-chromatography (TLC). The radioactivity associated with the cleaved FA is counted and calculated. However, this standard assay is not convenient or practical for high throughput PLA_2 activity assays.

[0060] According to the instant disclosure, a PLA_2 activity assay practical for high throughput utilizing DBPC (available from Echelon Biosciences Incorporated, Salt Lake City, UT 84108 Echelon Biosciences Incorporated, Salt Lake City, UT 84108) is disclosed. DBPC is a fluorogenic Dabcyl- and BODIPY-containing PC which functions as a PLA_2 substrate producing fluorescence. Usages of DBPC has the advantage of *not* involving radioactivity or a time-consuming and labor intensive TLC separation step. Further, the assay disclosed herein has

been adapted for use in frozen tissue sections with cell nuclei co-stained to examine the types of cells, having significant PLA_2 activity, and tissue histology, simultaneously.

Example 2: PLA_2 Activity Assay Using Tissue.

[0061] PLA_2 activity from human gynecologic (GYN) cancers (including both EOC and endometrial), benign GYN disease, and normal tissue samples were tested. Six samples from each of these groups were assayed in accordance with the instant disclosure.

[0062] Tissue samples were obtained and frozen in order to preserve enzymatic activity. The tissue samples (of average size 6-10 μm) were fixed using acetone and methanol at -20°C according to typical fixation methods known within the art. As explained above, the substrate DBPC is a fluorogenic Dabcyl- and BODIPY – containing PC which represents PLA_2 activity with fluorescence.

[0063] The PLA_2 activity in the frozen tissue sections was quantified. Quantification of the PLA_2 signals in frozen tissue sections used the software (MetaMorph). The total integrated signaling intensity, with the background subtracted (tissues processed the same way in the absence of the PLA_2 substrate), were captured and measured, and then normalized based on cell number or per cell ratio (the cell numbers were counted by DAPI-stained nuclei).

[0064] Referring now to **FIG. 1**, the activity of PLA_2 is shown illustrating PLA_2 activity being elevated in cancer (FIG. 1A) vs. benign (FIG. 1B) and normal (FIG. 1C) GYN tissue samples. Representative tissue PLA_2 activities are shown. The first two rows in FIGS. 1A to C, (each comprising four panels labeled as lower case letters a to d) present: a.) no DBPC substrate; b.) addition of DBPC; c.) DAPI staining of cell nuclei; and d.) an overlay the DAPI and PLA_2 activity staining. Referring now to **FIG. 1D**, para-nuclear PLA_2 activities are demonstrated as being sensitive to inhibitors of $iPLA_2$ and $cPLA_2$ (e.g., AACOCF3 and BEL) but not to a inhibitor of $sPLA_2$ (e.g., TA-PC) (the big bright spots were present in the absence of DBPC and thus were non-specific and were not a PLA_2 activity). Remaining with **FIG. 1D**, the numbers in each panel (a to d) are the activities expressed as fluorescent intensity/cell under each condition (as described herein).

[0065] Referring now to **FIG. 1E**. **FIG. 1E** presents PLA_2 activities measured in six samples from each of cancer (EOC and endometrial), benign GYN, and normal groups. Panel a of **FIG. 1E** presents individual values; whereas panel b of **FIG. 1E** presents mean and SD values (**, $P< 0.01$; *** $P<0.001$).

[0066] As shown herein, PLA_2 activities in EOC samples were significantly increased compared to those in either benign or normal tissues. The PLA_2's activities/cell in the three groups of samples were 881+/-395; 276 +/- 58; and 55+/-16 for human cancer, benign, and normal tissues, respectively (**FIG. 1**). The results presented herein illustrate that high levels of para-nuclear PLA_2 activities in the cancer tissues were only observed in the ovarian and endometrial cancer tissues (**FIG. 1A**). For benign tumors, these activities were limited to the superficial cell layers of the benign tumors (**FIG. 1B**). For normal tissues, very little or no para-nuclear PLA_2 activity was observed (**FIG. 1C**). The long-thin bright lines detected in some normal tissues (**FIG. 1C**) are unlikely to be cellular PLA_2 activities, since they were insensitive to any of the PLA_2 inhibitors tested (data not shown). In contrast, cancer PLA_2 activities were sensitive to inhibitors for both $cPLA_2$ and $iPLA_2$ (e.g., AACOCF3 and BEL) but not to TA-PC (an inhibitor of $sPLA_2$) (**FIG. 1D**). Thus the data presented herein illustrates that a majority (if not the total PLA_2 activity tested in tumor tissues) is derived from $cPLA_2$ and/or $iPLA_2$. It is worth noting that the individual values of PLA_2 activity from different groups were not overlapping, indicating this assays excellent separation and high specificity (**FIG. 1E**, panel a). As disclosed and described herein, a cut-off or determination value for identifying tumor tissues from both benign and normal tissues is approximately 400 (normalized fluorescent intensity values / cell). A determination or cut-off value of 400 (normalized fluorescent intensity values / cell), as disclosed herein, provides a 100% specificity in separating the various tissue states tested and described above.

Example 3: PLA_2 Activity Assay Using Tissue Homogenates.

[0067] Further, a tissue homogenization method, in which tissue lysate is utilized for measuring PLA_2 activity, is disclosed herein. The homogenization method described herein was compared against the current standard quantitative methods of tissue using a radioactive assay (as discussed above), for assessing quantification using tissue homogenates for measuring PLA_2 activity. As described herein, a tissue homogenization method suitable for PLA_2 activity analysis was developed.

[0068] According to the instant disclosure, frozen samples where pulverized and transferred to a microcentrifuge tube and mixed with 500μL lysis buffer. It is within the instant disclosure that samples may be pulverized in any known method (including using pulverizing instrumentation, bead ablation, and manual grinding). The lysis buffer comprised 10mM hepes,

pH 7.5, and 0.34 M sucrose with an additional 5μL of mammalian protease inhibitor cocktail obtained from Sigma-Aldrich, Inc. The tissue samples were next homogenized using tissue homogenization instrumentation (such as the Omni Tissue Homogenizer, Model No.: LR60902). Following lysis and homogenization, the samples were centrifuged at 16,000 x g, at a temperature of 4°C, for about 40 minutes. Following centrifugation the supernatants were transferred to a new eppendorf tube for assaying the PLA_2 activity.

[0069] PLA_2 activities measured using the standard radioactive methods (discussed above) were 1.76, 1.30, 0.52, and 0.57 [Fatty acid/(Fatty acid+PC)]%] (normalized to the *same amount of protein (1 mg)* in each sample) for the two cancer samples and two normal samples, respectively. Taken together, these data show that EOC and possibly other forms of GYN cancers have elevated PLA_2 activities when compared to those of benign GYN and normal tissues.

Example 4: PLA_2 Activity in Ascites and LPA Levels in Plasma.

[0070] The method described herein, is also adaptable for use in identifying PLA_2 activity as a marker in body fluid. For reasons discussed in detail above, a body fluid PLA_2 activity marker provides a more convenient and clinically useful marker, than a genetic number marker or a marker based on RNA.

[0071] The PLA_2 activity detection method employed herein is simple and can be easily conducted in almost any clinical laboratory. In contrast, although LPA has been shown to be a potential EOC marker, because of obstacles such as low concentration in the blood, variable samples processing procedures, as well as the rather sophisticated (less clinic-friendly) and different electrospray ionization tandem mass spectrometry (ESI-MS/MS) methods used in it, has proven slow in moving to wide spread and accepted clinical use.

[0072] Referring now to **FIG. 2**, as reported herein, PLA_2 activity is increased, with a concurrent increase in PLA_2 direct and/or indirect products (such as LPC, LPA, and arachidonic acid (AA)) in EOC ascites and blood samples. Data measured with LPC and LPA have been published, and increased LPA in EOC blood samples has been confirmed in repeated and independent studies.

[0073] According to the present disclosure, PLA_2 activity in human ascites samples were collected and assayed for detection of DBPC derived fluorescence. According to the disclosed method, ascites samples (25 μL per sample) were used in assessing PLA_2 activities. The

fluorescence detected (shown as total intensity in the accompanying figures) were approximately 2400 and 4,000 (wherein the numbers in the figures should be divided by 25, respectively, based on sample volume). The background fluorescence (intensities/μL) in the human samples assayed, according to the method disclosed herein was observed to be relatively low (~40) and did not change in a considerable amount during a 24 hour incubation time.

[0074] According to the methods disclosed and described herein, human ascite samples were centrifuged at 3,000 g for 20 minutes at 4°C. The samples were then aliquoted and stored at -80°C. Ascite samples (25 μL of each) were suspended in 50 μL buffer [comprising 80 mM hepes (pH 7.4), 150 mM NaCl, 10 mM $CaCl_2$, 4 mM Triton X-100, 60% glycerol, and 1 mg/mL BSA], respectively, and then mixed with 0.20 μg DBPC [dissolved in 50 μL DMSO/assay buffer (1:100)]. The total volume of each assay was approximately 200 μL. After incubation for 4 hrs, fluorescent intensities were measured at 485 nm/535 nm in a plate reader (Perkin Elmer Victor^{3}V 1420 Multilabel Counter).

[0075] Referring now to **FIGS. 4A** and **B**, Two time-dependent curves are presented demonstrating fluorescence observed having a linear relationship with time over a 4 hour assay time period for different samples. The data in **FIG 4 A** fit to a line with the following values: Y=230, intercept of 7468 and a R squared value of 0.9559. The data in **FIG 4 B** fit to a line with the following values: Y=352, intercept of 25,328 and a R squared value of 0.7818.

[0076] With reference now to **FIG. 4C**, the method and assay disclosed herein is demonstrated as comprising good inter-day and intra-day precision with all assays performed. FIG. 4C presents a standard curve which correlates intensity value of assayed EOC ascite samples with DBPC concentrations used.

[0077] As shown by the methods described and disclosed herein, PLA_2 is associated with human EOC ascites and exosomes (from ascites and blood). Both $cPLA_2$s and $iPLA_2$s are cytosolic enzymes, only $sPLA_2$s are secreted, data presented herein shows that elevated PLA_2 activity (which may be due to the activity of any of these three classes of PLA_2s) can be detected in the cell-free blood and/or ascites samples from patients with EOC and/or other GYN cancers.

[0078] Exosomes are 40-100-nm diameter membrane vesicles released from multivesicular bodies (MVB) by intact cells and are thought to participate in intercellular signaling. Further, cell membrane lipids, including PC (a PLA_2 substrate) and sphingolipids (in particular ceramides) are prominent lipids within exosomes.

[0079] According to methods of the instant disclosure, the ExoQuick kit from SBI System Biosciences (which has been validated to be more effective in exosome isolation than the more traditional ultra-centrifugation method, available at http://www.systembio.com/exoquick-exosomes/) was utilized in isolation of exosomes from human ascite samples. As demonstrated by the instant disclosure, significant increases in PLA_2 activities were detected in EOC blood, cell-free ascites, and exosomes vs. similar samples collected from non-malignant liver diseases or healthy controls. With reference to **FIG. 2**, approximately 80% of cell-free ascitic PLA_2 activity is shown as associated with the exosome portion in ascites. In addition, the observed exosome-associated PLA_2 activity proved sensitive to methyl arachidonyl fluorophosphonate (MAFP) (an irreversible dual $cPLA_2$ and $iPLA_2$ activity inhibitor), BEL (an irreversible and selective $iPLA_2$ activity inhibitor), and/or thioether amide-PC (TAPC) (a selective $sPLA_2$ activity inhibitor). Referring now to **FIG. 2B**, the inhibitor's effectiveness is confirmed by its ability in blocking $sPLA_2$ activity when a positive control (from bee venom; Cayman) was used (**Fig. 2D**). As such, the method and disclosure herein, provides analysis of PLA_2 activity, which is demonstrated herein to be associated with human EOC ascites and exosome (from ascites and blood), presents a novel and highly useful method for diagnostic and personalized treatment purposes in GYN cancers.

Example 5: PLA_2 Activity in Mouse Tissue.

[0080] Referring now to **FIGs. 3A**, **C** and **D**, PLA_2 activities measured from several mouse models for GYN diseases are presented. These assays demonstrate high PLA_2 activities in tumors from human A2780 and SKOV3 xenografts, as well as from tumors derived from mouse EOC ID-8 cells. Further, and as shown herein, PLA_2 activity is greatly reduced in tumors from bromoenol lactone (BEL) -treated mice compared to those from controls. Further, as shown herein, BEL targets PLA_2 enzymes (see, **FIG. 3B**, panels a - b) and reduces tumorigenesis, correlating to a reduction in PLA_2 activity as reducing tumorigensis.

[0081] Referring now to **FIG. 3E**, PLA_2 activity was measured in several other mouse tissues, including the kidney, lung, and liver. Tumors did not grow in oron these organs, and tumor cell injections did not significantly change the PLA_2 activities in these organs (data not shown). Further, it was determined that at least some (and possibly most) of the activities detected in these tissues appeared to be $sPLA_2$ activity of normal tissues (based on the activity's extracellular localization and insensitivity to BEL and AACACF3). In all experiments, controls

were run which did not include added substrate. Only the merged pictures and only kidney results among different tissues are shown in **FIGs. 3B** to **3E** (data not shown). These data demonstrate the use of PLA_2 activity for following progression of these diseases and/or to monitor the effective of various treatments for the disease.

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[0082] While the novel technology has been illustrated and described in detail in the figures and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiments have been shown and described and that all changes and modifications that come within the spirit of the novel technology are desired to be protected. As well, while the novel technology was illustrated using specific examples, theoretical arguments, accounts, and illustrations, these illustrations and the accompanying discussion should by no means be interpreted as limiting the technology. All patents, patent applications, and references to texts, scientific treatises, publications, and the like referenced in this application are incorporated herein by reference in their entirety.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for evaluating a disease, comprising the steps of:

measuring phospholipase A_2 (PLA_2) activity in a patient sample; and

providing information associated with correlating the PLA_2 activity, measured in said step of measuring, with a PLA_2 activity value indicative of a disease, wherein said PLA_2 activity is elevated in a diseased state.

2. The method according to claim 1, further including the step of:

obtaining said patient sample from a patient.

3. The method according to claim 1, wherein said patient sample is a tissue sample.

4. The method according to claim 1, wherein said patient sample includes at least one cell.

5. The method according to claim 1, wherein said sample comprises a cellular fluid.

6. The method according to claim 1, herein the patient sample is from tumorous tissue.

7. The method according to claim 1, wherein the disease is a gynecological disease.

8. The method according to claim 1, wherein the disease is EOC.

9. The method according to claim 1, wherein the measuring step includes the use of a fluorogenic compound.

10. The method according to claim 9 wherein the fluorogenic compound is DBPC.

11. The method according to claim 1, wherein the measuring step includes the use a radioactive labled substrate for PLA_2.

12. The method according to claim 1, further including the step of:

indicating the sample as being from a patient likely to have a form of cancer.

13. The method according to claim 10, wherein said information, provided in said step of providing, correlates PLA_2 activity of demonstratively higher activity in a diseased state than in a non-diseased state.

14. The method according to claim 10, wherein said information, provided in said step of providing, correlates PLA_2 activity that is about 1.5 fold higher activity in a diseased state than in a non-diseased state.

15. The method according to claim 13, wherein the form of cancer affects a mammalian reproductive tract.

16. The method according to claim 15, wherein the form of cancer is EOC.

17. The method according to claim 1 further including the step of introducing a compound to the patient which inhibits activity of an isoform of PLA_2

18. The method according to claim 17, wherein the isoform of PLA_2 is a $sPLA_2$.

19. The method according to claim 17, wherein the isoform of PLA_2 is an $iPLA_2$.

20. The method according to claim 17, wherein the isoform of PLA_2 is a $cPLA_2$.

21. The method according to claim 17, wherein the compound inhibits both a $cPLA_2$ and an $iPLA_2$s.

22. The method according to claim 1, wherein the PLA_2 includes at least one of $iPLA_2$ and $cPLA_2$.

23. The method according to claim 9, wherein the measuring step comprises a treatment step and a detection step.

24. The method according to claim 23, wherein the treatment step is performed in vivo.

25. A method of using PLA_2 activity to access the health of a patient, comprising the steps of:

obtaining a sample from an individual;

contacting the sample with a fluorogenic compound;

detecting a signal produced by the fluorogenic compound in the presence of the sample; and

correlating the size of the signal with the health of the patient.

26. The method of claim 25, wherein the sample is a tissue.

27. The method of claim 26, further comprising the step of fixing the tissue

28. The method of claim 26, further comprising the step of:

homogenizing the sample;

collecting a supernatant of the homogenized sample, wherein the fluorogenic compound is introduced, in the step of introducing, to the supernatant.

29. The method of claim 25, further comprising the step of correlating a value of the signal, detected in the step of detecting, with a disease.

30. The method of claim 29, wherein the disease is a form of cancer.

31. The method of claim 25, further including the step of introducing a compound to the sample which inhibits activity of an isoform of PLA_2.

32. The method of claim 31, wherein the isoform of PLA_2 is $iPLA_2$.

33. A marker for disease, comprising:

a maker wherein said marker is phospholipase A_2 (PLA_2) activity that is demonstratively higher in a diseased state than it is in a healthy state.

34. The marker according to claim 33, wherein the activity is at least about 1.5fold higher in a sample from a patient with a disease then it is in a patient that is without disease.

35. The marker according to claim 33, wherein the marker is elevated in at least one sample for a portion of a patient's body selected from the group consisting of: cells, tissues, and fluids.

36. The marker according, wherein said maker is indicative of cancer.

37. The marker according to claim 36, wherein the marker is EOC.

38. The marker according to claim 33, wherein said marker can be used to diagnose a disease.

39. The marker according to claim 33, wherein said maker can be used to evaluate the effectiveness of a treatment for a disease.

40. The marker according to claim 33, wherein the marker can be used to determine the prognosis of a disease.

41. The marker according to claim 33, wherein the marker can be used to determine the personalized treatment of a patient.

42. The maker according to claim 33, wherein the activity is due primarily to elevated $iPLA_2\beta$ activity.

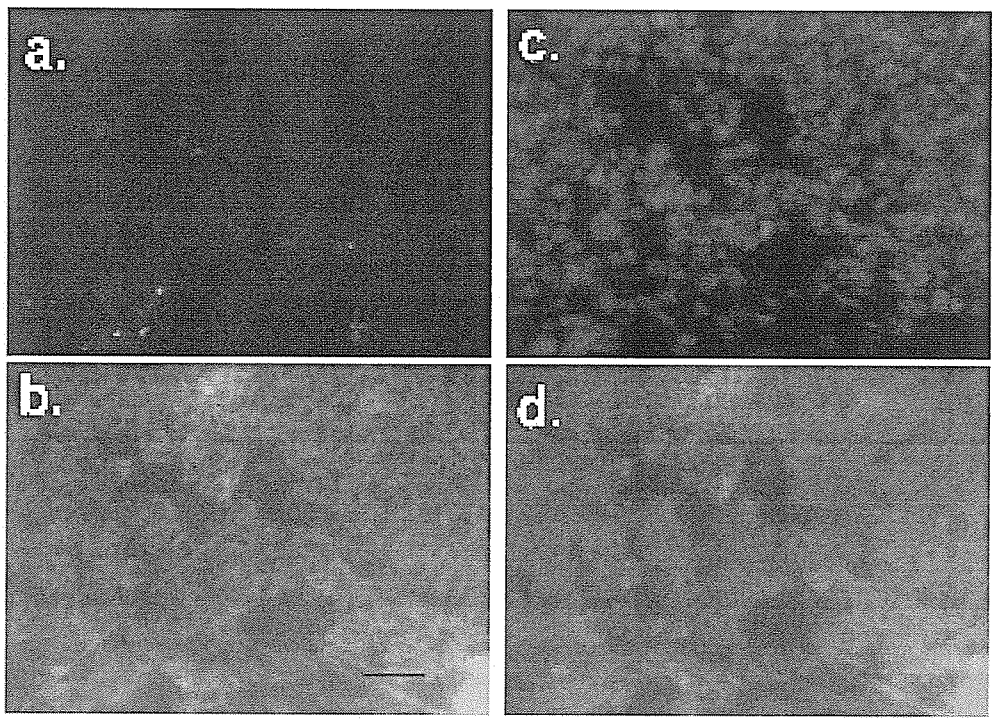


FIG. 1 A

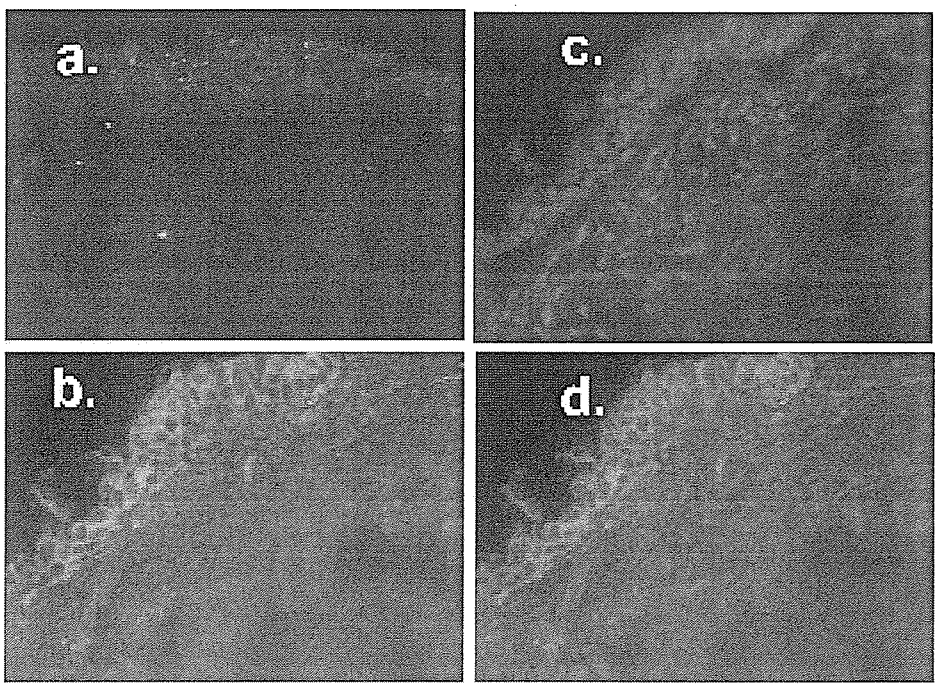


FIG. 1 B

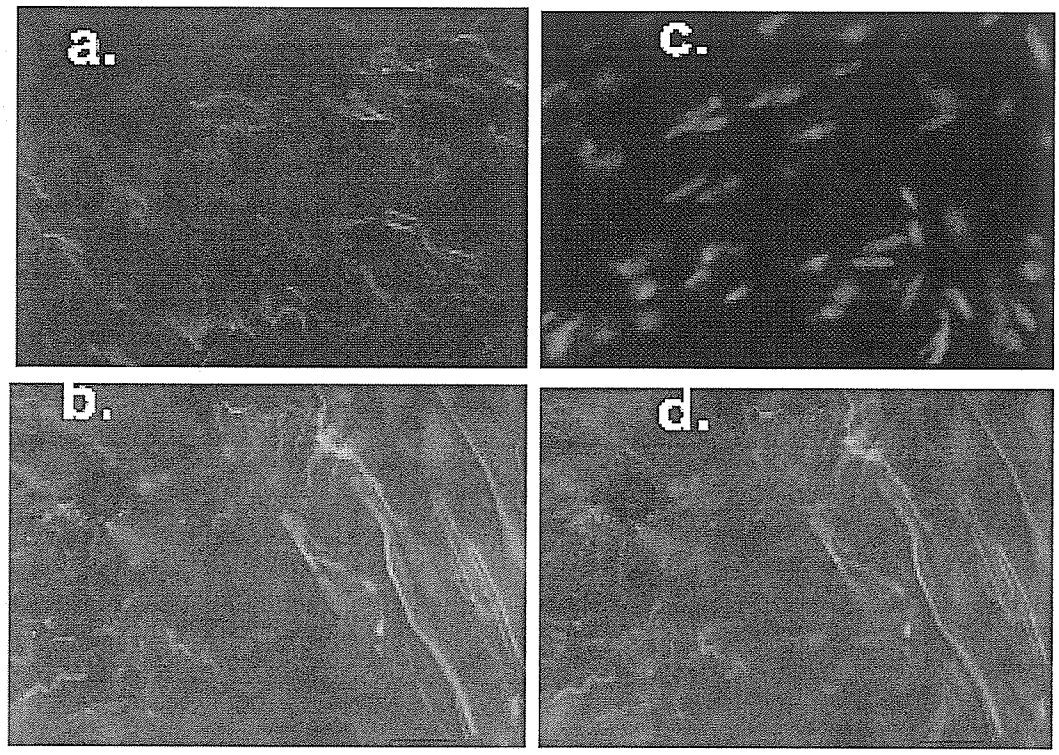


FIG. 1 C

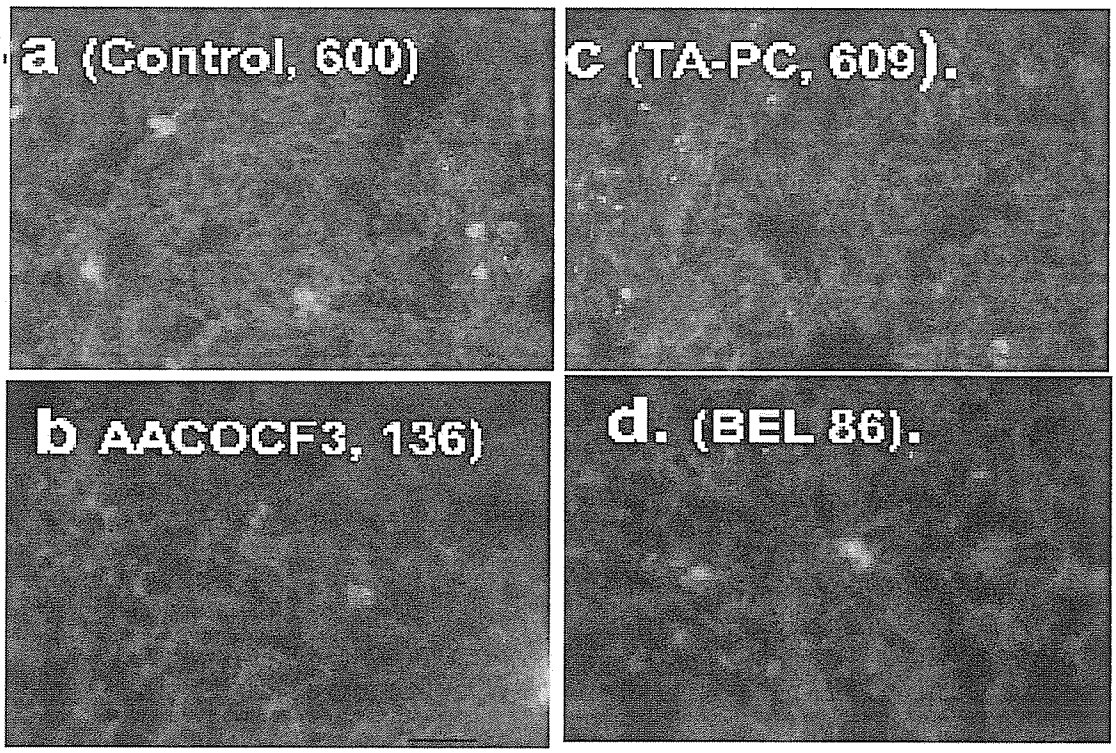
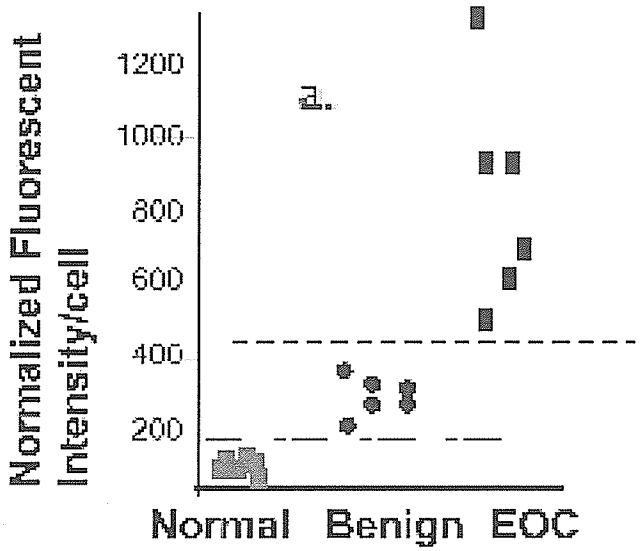


FIG. 1 D


a.
Normalized Fluorescent Intensity/cell
1200
1000
800
600
400
200
Normal
Benign
EOC

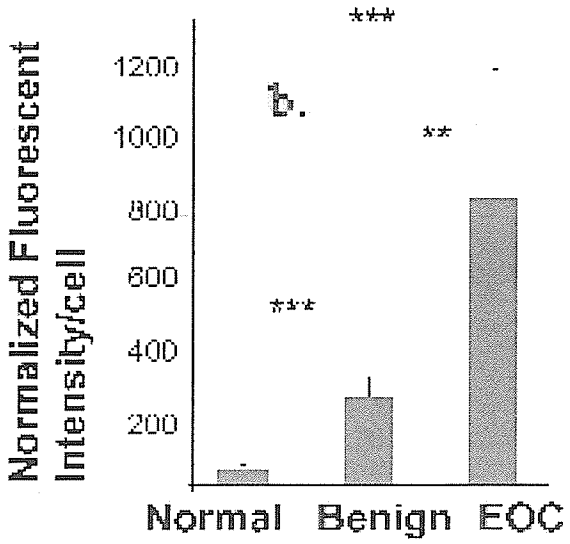

b.
***
**
***
Normalized Fluorescent Intensity/cell
1200
1000
800
600
400
200
Normal
Benign
EOC

FIG. 1 E

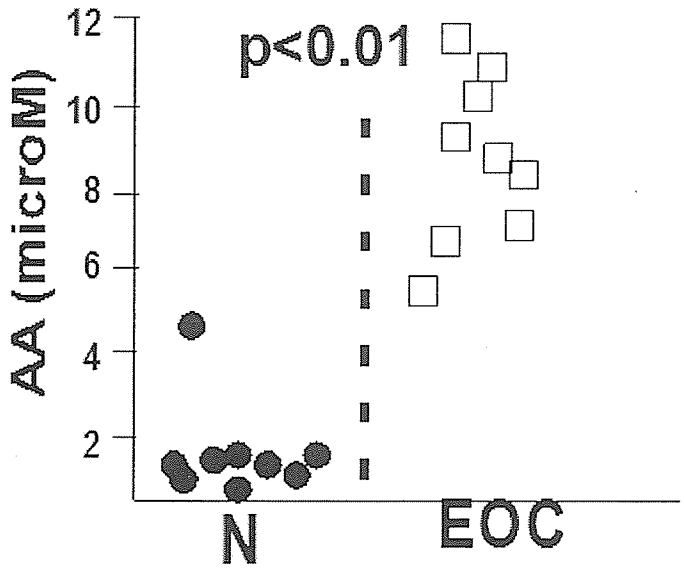


FIG. 2 A

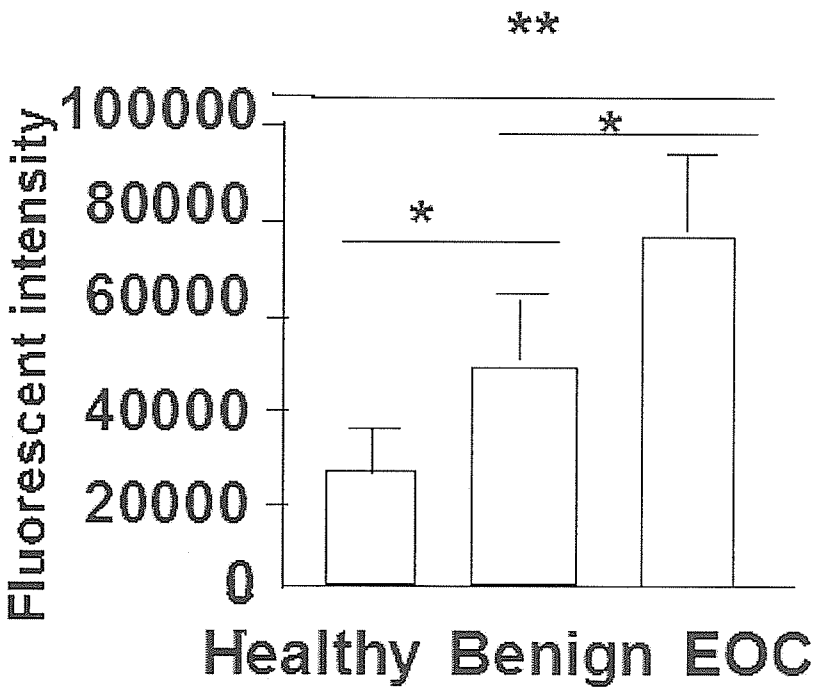

**
*
*
100000
80000
60000
40000
20000
0
Fluorescent intensity
Healthy Benign EOC

FIG. 2 B

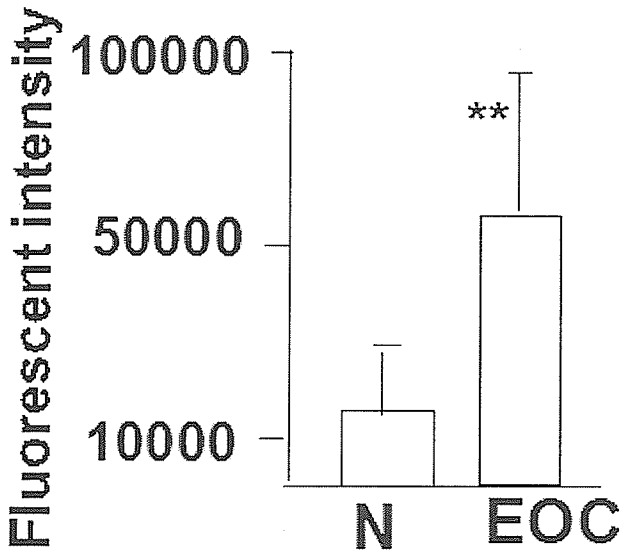


FIG. 2 C

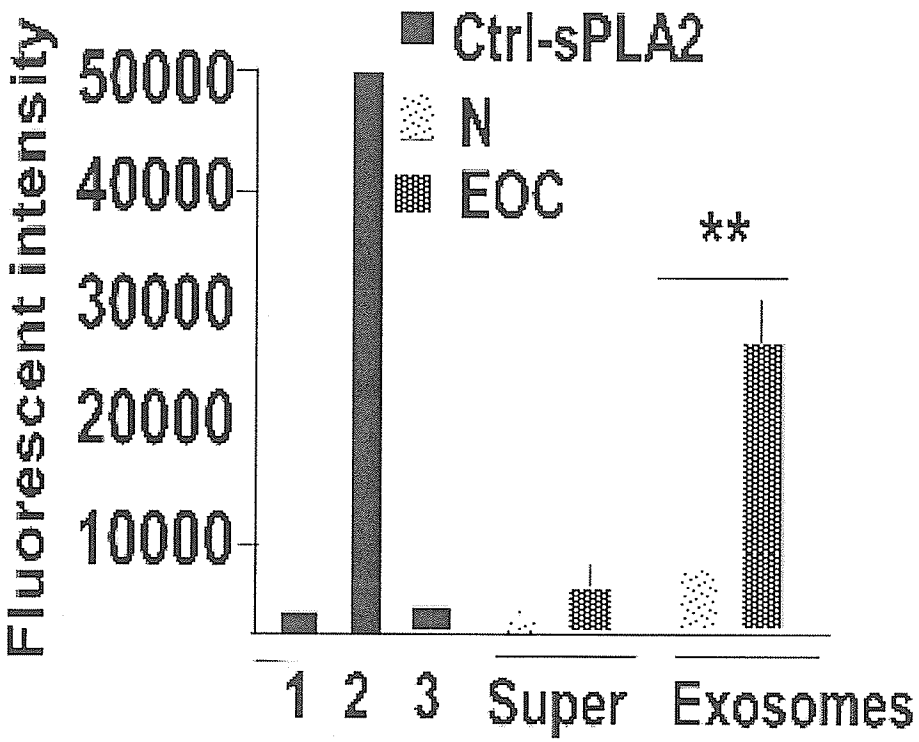

Fluorescent intensity
50000
40000
30000
20000
10000
Ctrl-sPLA2
N
EOC
**
1
2
3
Super
Exosomes

FIG. 2 D

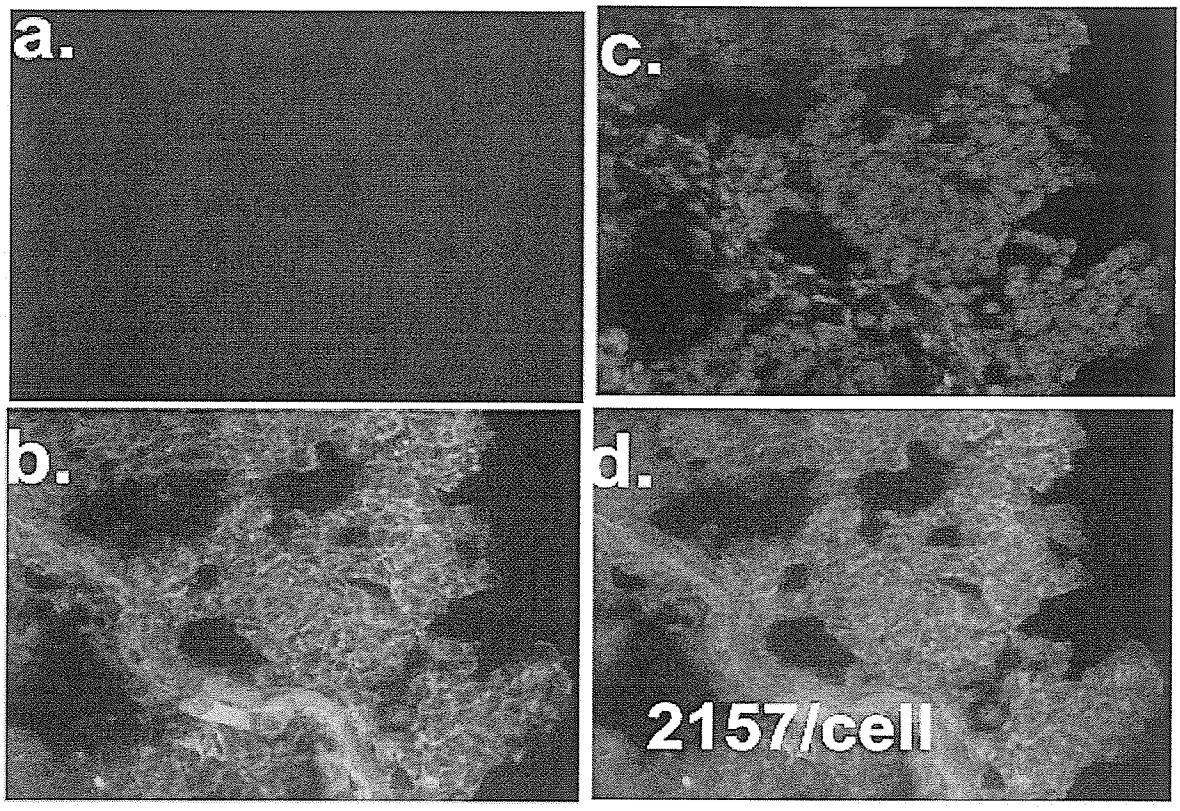


FIG. 3 A

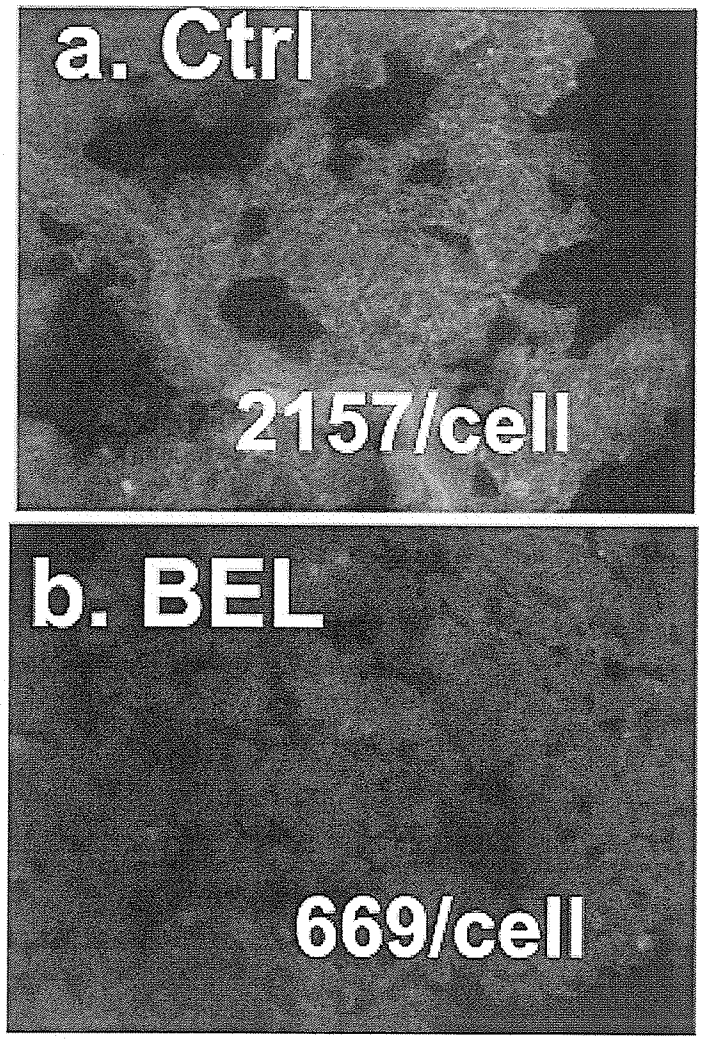


FIG. 3 B

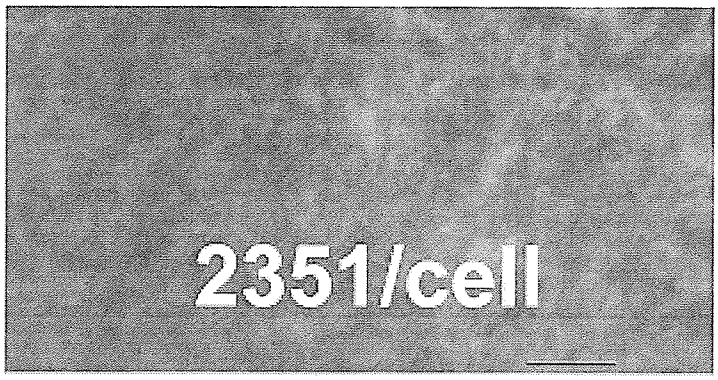


FIG. 3 C

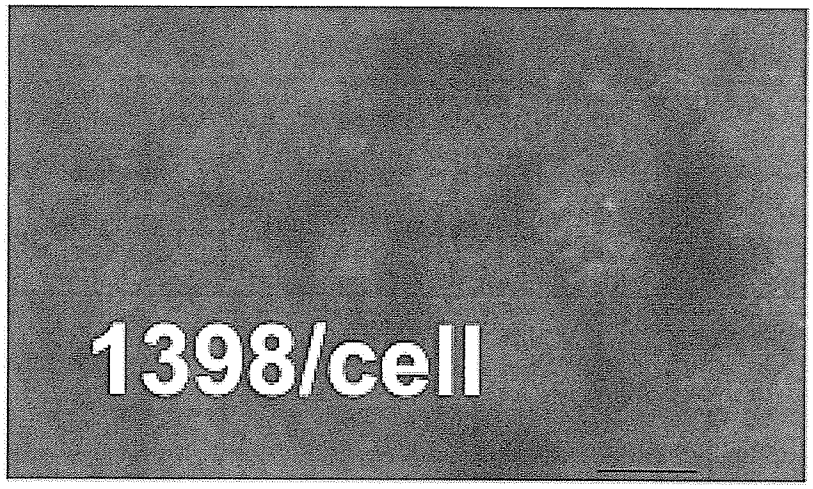


FIG. 3 D

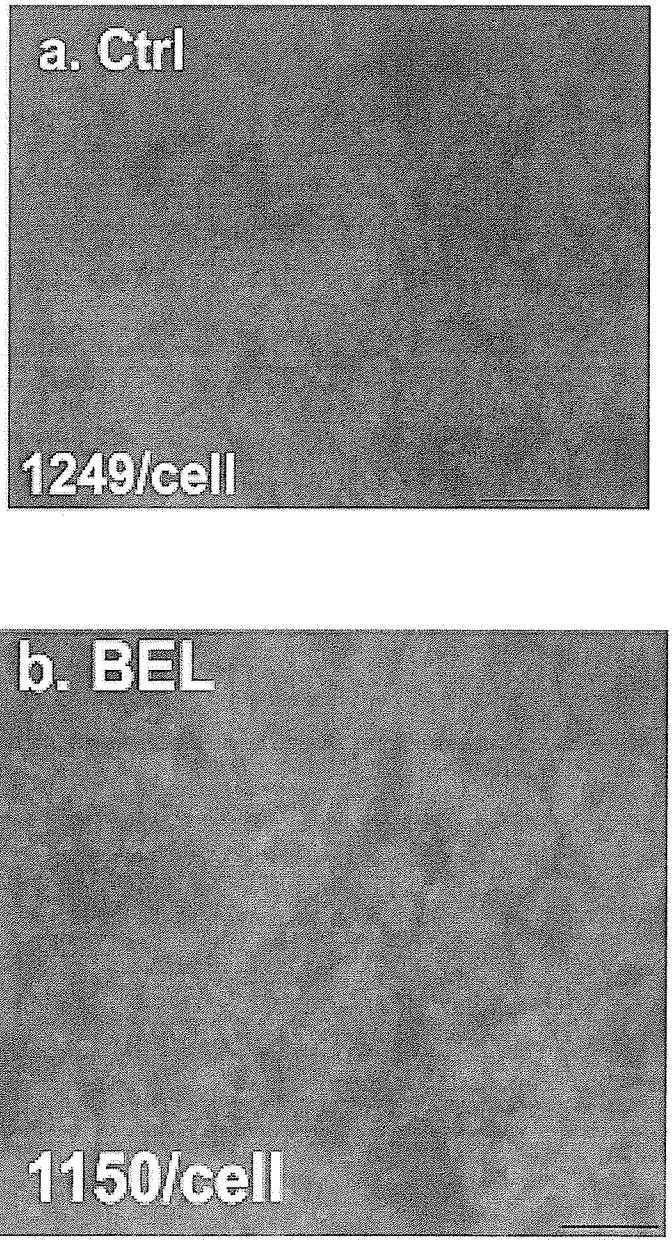


FIG. 3 E

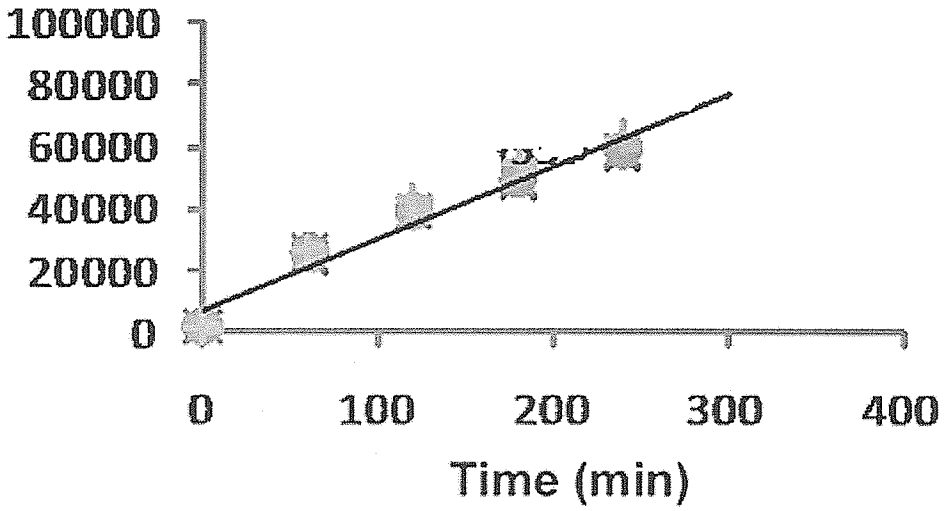


FIG. 4 A

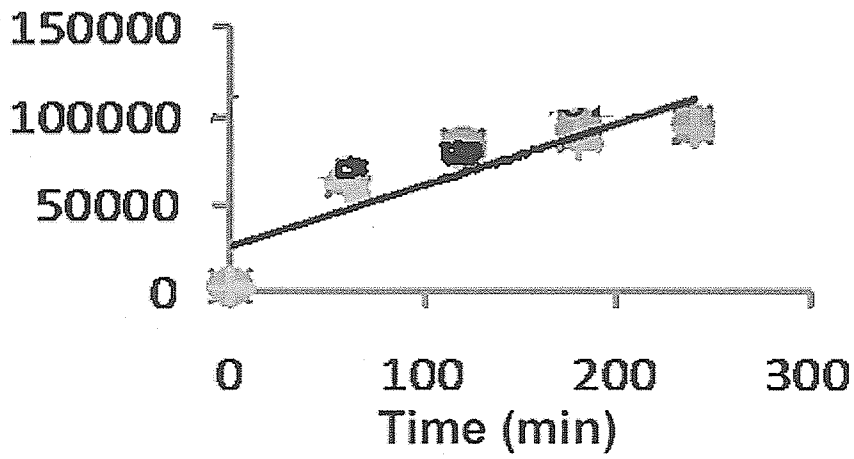


FIG. 4 B

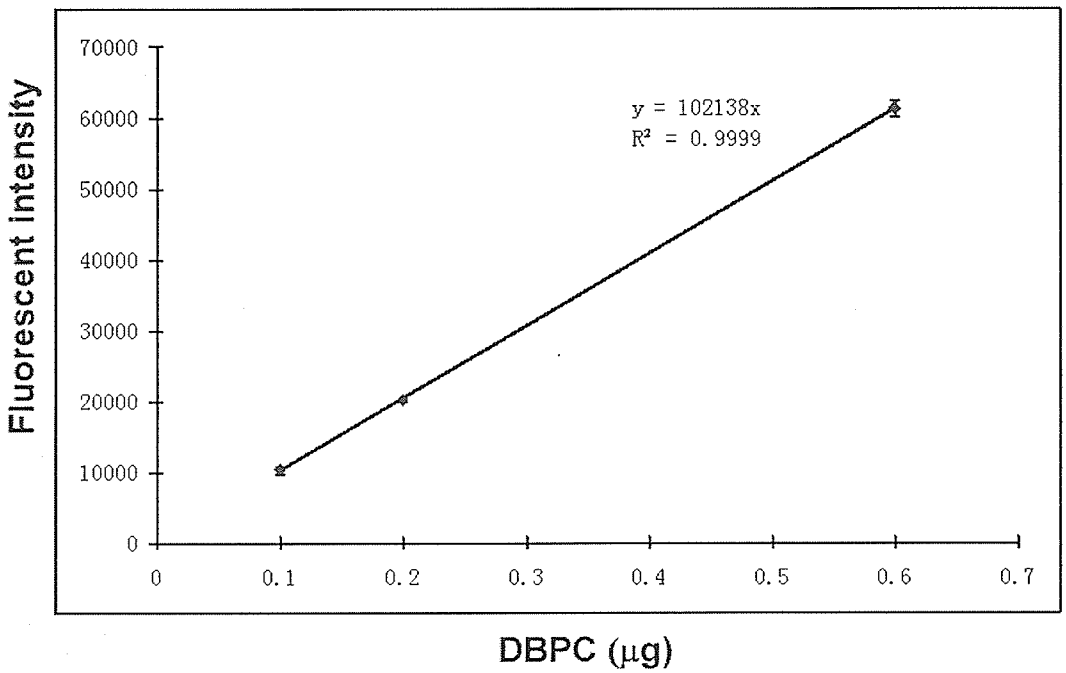

Fluorescent Intensity
70000
60000
50000
40000
30000
20000
10000
0
y = 102138x
R² = 0.9999
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
DBPC (μg)

FIG. 4 C