World Cancer Research Journal WCRJ 2015; 2 (3): e550

CANCER CELL GROWTH PROMOTION BY NDPKA/NM23 H1 PROPORTIONAL TO PHOSPHATE ION BOOK KEEPING AND ROLE OF ESTROGEN RECEPTOR

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ABSRACT – *Background:* *Previous paper results indicate that the activity of nm23H1 is independent of its NDPKA (Nucleoside Di Phosphate Kinase A) activity.*

Material and methods*: NDPKA/nm23 H1's cell viability assay, standard curve for phosphate, cell phosphate analysis, cell morphological examination, etc. were performed.*

Results: *NDPKA's effect on MDA-MB-231 cell growth and viability had significant correlation with phosphate content in cell media at the Yates's correction for continuity 0.00. NDPKA's effect on MCF-07 cell growth and viability had significant correlation with phosphate content in cell media at the Yates's correction for continuity 0.00. From both cell viability MTT assay and phosphate assay data, a triangular periodic wave function was mathematically approximated as an infinite Fourier Series as an equation. There was significant difference between cell growth increase and phosphate concentration remaining after 24 hours for both MCF-07 (p=0.00) and MDA-MB-231 cell lines (p=0.00). There was significant difference between MCF-07 cell growth increase and MDA-MB-231 cell growth increase (p=0.0472). Only in case of MCF-07, significant dose-dependent change (Yates's correction for continuity 0.00) in cell growth and cell proliferation was found (In MDA-MB-231, not found).*

Conclusions: *Since MCF-07 cell growth increase and MDA-MB-231 cell growth increase (p=0.0472) were significantly different, it proves estrogen receptor effect on cell growth. This is how estrogen receptor (ER)-positive breast cancers generally have a better prognosis and are often responsive to anti-estrogen therapy.*

Impact: *MDA-MB-231 estrogen negative cells showed significantly higher phosphate content remaining after 24 hours incubation with NDPAK/nm23 H1 than MCF-07 estrogen positive cell lines. Since ER-negative breast cancers are more aggressive and unresponsive to anti-estrogens and other targeted therapies are thus urgently needed, phosphate content might act as parameter for other therapeutic targets in estrogen negative breast cancer.*

KEY WORDS: *MCF-07, MDA-MB-231, Breast cancer, NDPKA/nm23 H1, Phosphate ion.*

INTRODUCTION

Cancer patients with high NDPKA/nm23-H1 levels (≥ 80 ng/mL) had a worse prognosis than those with low nm23-H1 levels (<80 ng/mL)[1]. This is because recombinant nm23-H1 or NDPKA (Nucleoside diphosphate Kinase A) promoted the growth and survival of cancer cells in a dose- and time dependent manner. When nm23H1 concentration in extracellular fluid of AML (Human acute

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myelogenous leukemia) patients increased from 0 to 100 ng/ml, then cell growth and survival increased. But after 100 ng/ml to higher concentration levels, the cell growth and survival falls[1]. In this study, at changing nm23H1 level in extracellular fluid of breast carcinoma cells, the change in phosphate levels measured to probe the kinetics of free phosphate ion release from simulated extracellular fluid in cell lines.

nm23-phosphohistidine is critical for phosphate transfer in the NDPK Reaction[2]-The NDPKA pathway of nm23 is illustrated as follows:

$$ATP + nm23 \leftrightarrow ADP + nm23\text{-}His\ (P)$$
$$+ NDP \leftrightarrow NTP + nm23$$

nm23 was demonstrated to exhibit nucleoside diphosphate kinase (NDPK) activity, which transfers a phosphate among nucleoside tri- and diphosphates via an nm23-phosphohistidine

intermediate. Recent data have dissociated the NDPK activity of nm23 from its phenotypic effects; therefore questions raised whether nm23 possesses additional biochemical functions.

The biological relevance of the novel phosphorylation identified herein is suggested by the direct correlation of in vivo nm23 acid-stable phosphorylation levels.

The mechanisms by which nm23-H1 protein is secreted into the extracellular environment are unclear. Hence this study focused on extracellular nm23-H1 protein derived from breast cancer cells because its clinical significance is greater than that of intracellular overexpression and elevated extracellular expression of nm23 has not been found in normal healthy plasma[3]. The study also tests cancer growth difference between estrogen positive (MCF-07) and estrogen negative (MDA-MB-231) cell lines.

MATERIALS AND METHODS

Chemicals

Recombinant Human NDPKA acquired from Novoprotein Scientific Inc., Summit, NJ, USA.

MDA-MB-231 and MCF-07 breast cancer cell lines was purchased from National Centre For Cell Science, Pune, India.

Ethical approval and consent

Novoprotein Scientific Incorporation, USA and National Centre For Cell Science, Pune, India provided signature in delivery receipt acknowledging responsibility for maintaining and delivering human material after observing full ethics and consent of appropriate authority. Drugs Control Department of India reviewed and verified safety and ethics of the reagents imported on November 2014 for this study.

Experimental Protocols

Cell viability assay

The effect of nm23 H1 protein on human breast cancer cell lines, MDA-MB-231 and MCF-07 was determined by the MTT (MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. 5 million cells was cultured in 25 cm^2 cell culture flasks at 37°C in a 5% CO_2 atmosphere. The cells are centrifuged at 1200 rpm for 10 minutes. The cells were counted using hemocytometer. The cells were seeded into 96 well plates as 1x103 cells in each well and incubated for 24 hours. The cells were incubated for 24 hours for settling and spreading on substrate unless dead. After 24 hours NDPKA/nm23 H1 proteins were applied on each well without damaging cells using pipette.

Plates were incubated for 24 hours. After incubation 10 µL MTT (5 mg/ml) was added in each well. MTT kept for 4 hours. Purple color formazone crystals formed. Crystals dissolved in 100 µL DMSO (Dimethyl sulphoxide). Then immediately read at 570 nm.

Standard curve for Phosphate

A standard curve for the phosphate assay may be generated using the phosphate standard as a source

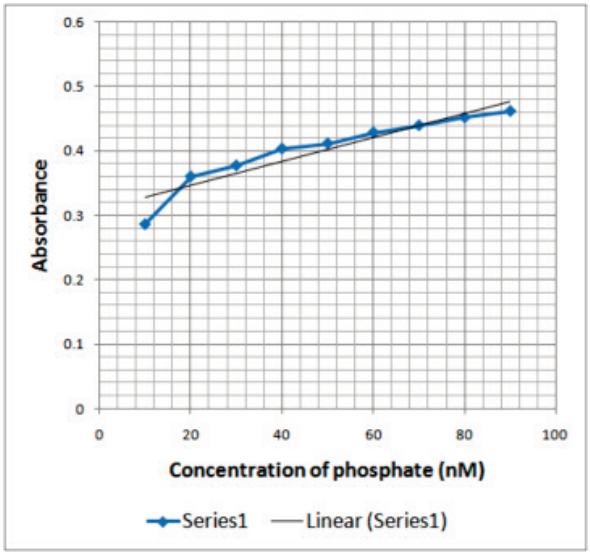

Figure 1. Phosphate standard curve. Phosphate absorbance (y axis) vs concentration in ng (x axis).

of inorganic phosphate (Figure 1). The linear range of the assay for inorganic phosphate extends from 2 µM to about 150 µM. Hence phosphate standard for 10 ng/ml to 160 ng/ml 16 concentrations taken for standard curve.

Cell Phosphate analysis

5 million cells was cultured in 25 cm^2 cell culture flasks at 37°C in a 5% CO_2 atmosphere. The cells are centrifuged at 1200 rpm for 10 minutes. The cells were counted using hemocytometer. The cells were seeded into 96 well plates as $1x10^3$ cells in each well and incubated for 24 hours. The cells were incubated for 24 hours for settling and spreading on substrate unless dead. After 24 hours proteins were applied on each well without damaging cells using pipette. Plates incubated for 24 hours. After incubation cell media were collected from each cell and phosphate content measured by phosphate assay.

Cell phosphate assay

740 µL dH_2O, 50 µL 20X reaction buffer, 200 µL MESG (2-amino-6-mercapto-7-methylpurine riboside) substrate solution, 10 µL purine nucleoside phosphorylase x µL experimental enzyme NDPKA preincubated for 10 minutes at 22°C. 200 µL of experimental substrate added and mixed well. Immediately read absorbance at 360 nm as a function.

Cell morphological examination

$1x10^6$ cells or 1 ml put into 10 mm each 6 petri dishes each. The petri dishes with cells left for 13 hours. Within 13 hours, every 1 hour the cells were examined under microscope.

RESULTS

Cell Viability and Phosphate Content Assay

The recombinant human NDPKA/nm23 H1 was evaluated for its effect on cell viability and phosphate content against human breast cancer cell lines, MCF-07 and MDA-MB-231 at different concentrations. The NDPKA/nm23 H1 showed a dose dependent increase in cell viability (Figure 2a).

Extracellular phosphate content and cell viability are moving over the same path over the course of increased dose (Figure 3a-3d). Extracellular phosphate content and cell viability not only repeats the same triangular periodic pattern, it does so in a regular fashion. The amount of dose increase it takes to complete one back and forth cycle is always the same amount i.e. when at phase starting point dose of NDPKA applied on extracellular fluid, then at first cellular viability from 40% confluence level grows and develops 24.2% more cells upto addition of 54.2 ng of NDPKA, then cells start dying out and confluence is at initial level when 108.4 ng NDPKA dose absorbed (Figure 3a). Massive cell death and nuclear breakdown generates large quantities of nucleic acids[3]. So three days before or seven days after chemotherapy phosphate > 4.5 mg/dL or 25% increase because of phosphates in nucleosides (downward curve in Figure 2) coming from cell death (Figures 2 and 4).

While the cell media Dulbecco's Modified Eagle's Medium contains no phosphate, only 4 nanomole (nM) per µl phosphate maintained in extracellular fluid by cell phosphate buffer system[4]. 888.89 and 1133.3 nM phosphate found in MCF-07 and MDA-MB-231 cell media respectively for phosphate assay at resting level.

Phosphate is the chief anion in intracellular fluid-up to 75 nanomoles of phosphate are inside

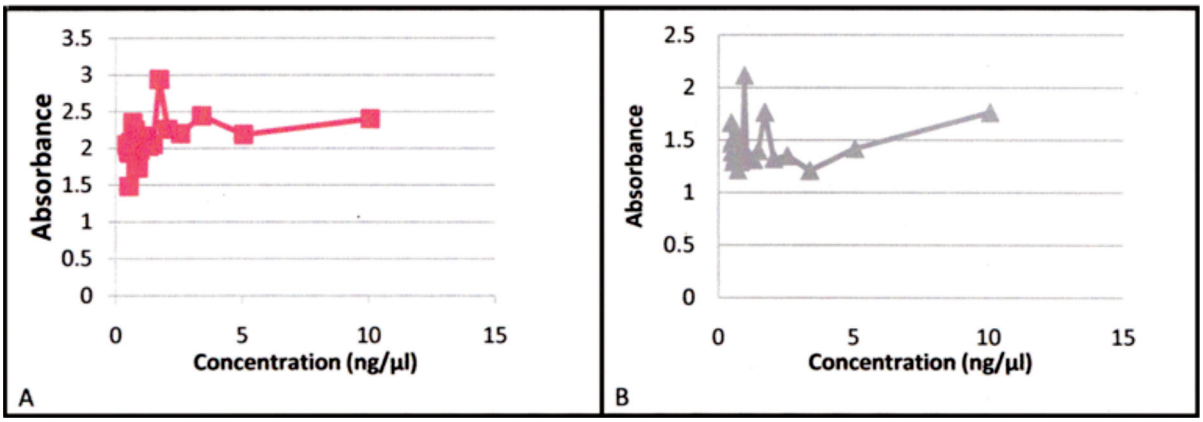

Figure 2. *A,* MCF-07 breast cancer cell line, MTT absorbance (y axis) vs NDPKA concentration. *B,* MCF-07 breast cancer cell line, phosphate absorbance (y axis) vs NDPKA concentration.

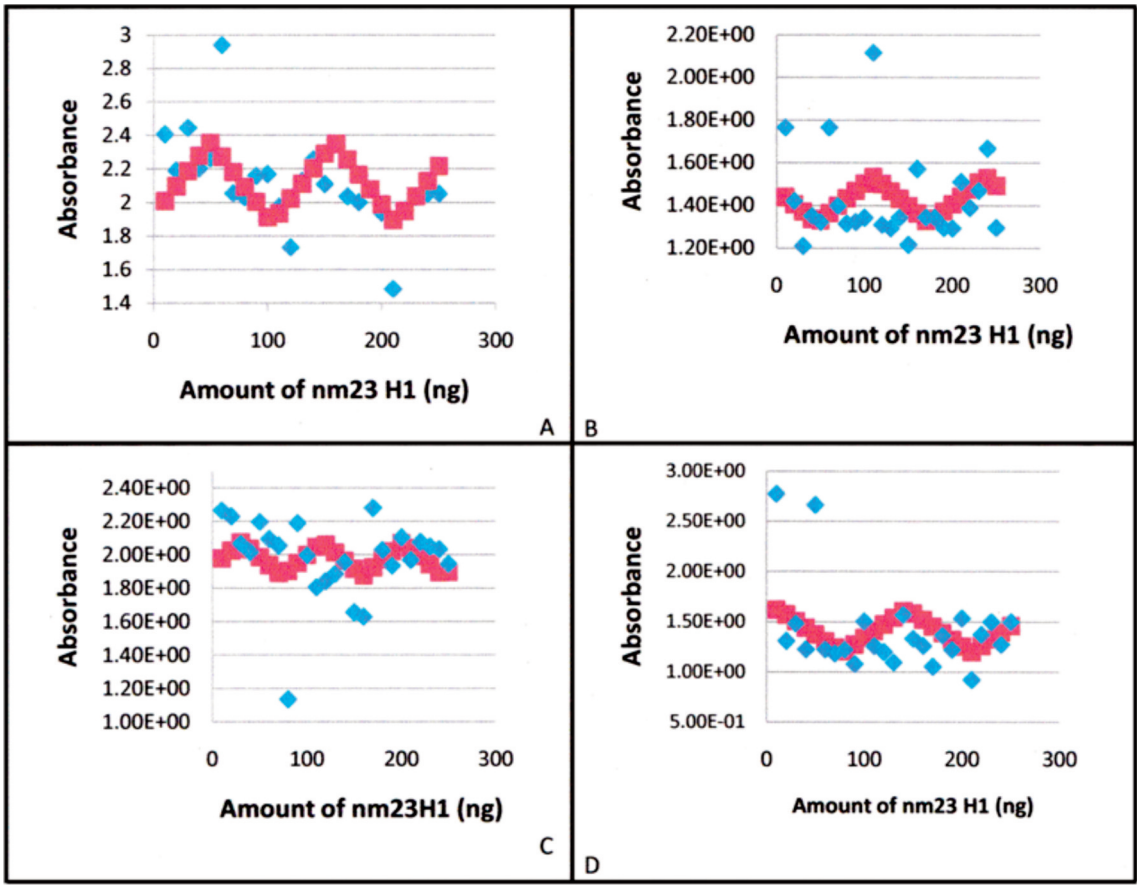

Figure 3. *A,* MCF-07 breast cancer cell line, MTT absorbance (y axis) vs NDPKA concentration in ng (x axis). Series 1: experimental data. Series 2: Triangular wave fit, the fitting yields a period T = 108.4. *B,* MCF-07 breast cancer cell line, phosphate absorbance (y axis) vs NDPKA concentration in ng (x axis). Series 1: experimental data. Series 2: Triangular wave fit, the fitting yields a period T = 127.8. *C,* MDA-MB-231 breast cancer cell line, MTT absorbance (y axis) vs NDPKA concentration in ng (x axis). Series 1: experimental data. Series 2: Triangular wave fit, the fitting yields a period T = 85.5. *D,* MDA-MB-231 breast cancer cell line, phosphate absorbance (y axis) vs NDPKA concentration in ng (x axis). Series 1: experimental data. Series 2: Triangular wave fit, the fitting yields a period T = 132.1.

cell[4]. In very high dose like 100-250 nM, no curve shift to upper level observed. At high doses the phosphate graphs (Figure 3a-3d) still stationary at periodic triangular curve.

After chemotherapy 25% of cells dies[4,5]. Thus cells grow from a certain confluence level, but returns to initial confluence level and completes a cycle—within the cycle 108.4 ng NDPKA are absorbed. On average approximately 0.0038% increase of cell viability was found in case of MCF-07 cell lines (Figure 3a). It's so predictable. Hence cell growth and cell death occurs that is regular and repeating is referred to in this paper as a periodic cycle. Most cell functions do so in a regular and repeated fashion; the cell growth and cell death are periodic.

There is a statistically significant difference between:

1. Cell growth increase and phosphate concentration remaining after 24 hours for both MCF-07 (p=0.00) and MDA-MB-231 cell lines (p=0.00)
2. MCF-07 cell growth increase and MDA-MB-231 cell growth increase (p=0.0472)

There is no statistically significant difference between:

1. MCF-07 cell media's phosphate concentration remaining and MDA-MB-231 cell media's phosphate concentration remaining (p=0.81)

3.2 Phosphate Content

From both cell viability MTT assay and phosphate assay data, a triangular periodic wave function can be mathematically approximated as an infinite Fourier Series as the following equation (1) and equation (2):

$$y_{triangular}(x) = \frac{8}{\pi^2}\sum_{i=0}^{\infty}(-1)^i \frac{sin(2\pi(2i+1)\omega x)}{(2i+1)^2} = \frac{8}{\pi^2}\left(\sin(2\pi\omega x) - \frac{1}{9}sin(6\pi\omega x) + \frac{1}{25}sin(10\pi\omega x) - \frac{1}{49}sin(14\pi\omega x) + \frac{1}{81}sin(18\pi\omega x) - \frac{1}{121}sin(22\pi\omega x) + \frac{1}{169}sin(26\pi\omega x) - \cdots\right) \quad (1)$$

where y is the cell viability, x is concentration

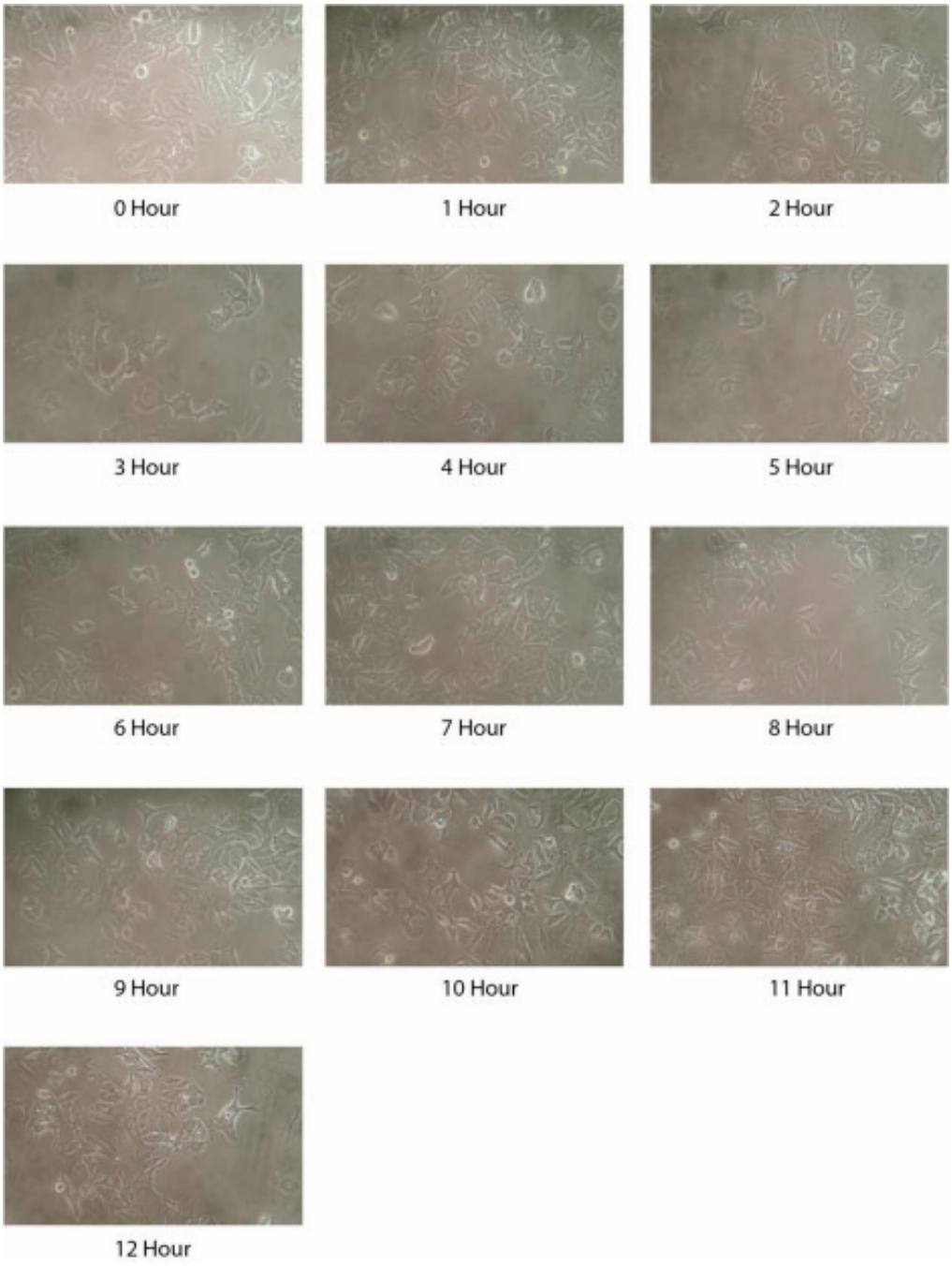

Figure 4. Phase-contrast microscopy observed the morphological changes of breast cancer cells. MCF-07 breast cancer cell lines. Magnification ×100.

$$y_{triangular}(x) = \frac{8}{\pi^2}\sum_{i=0}^{\infty}(-1)^i \frac{sin(2\pi(2i+1)\omega x)}{(2i+1)^2} = \frac{8}{\pi^2}\left(\sin(2\pi\omega x) - \frac{1}{9}sin(6\pi\omega x) + \frac{1}{25}sin(10\pi\omega x) - \frac{1}{49}sin(14\pi\omega x) + \frac{1}{81}sin(18\pi\omega x) - \frac{1}{121}sin(22\pi\omega x) + \frac{1}{169}sin(26\pi\omega x) - \cdots\right) \textbf{(2)}$$

(2) where y is the phosphate content, x is concentration

Data =
ABS(G$1)*(8/(3.1416*3.1416))*(SIN((2*3.1416*(A1+G$2))/G$3)-
(1/9) *SIN((6*3.1416*(A1+G$2))/G$3)+(1/25)*SIN((10*3.1416*(A1+G$2))/G$3)-
(1/49)*SIN((14*3.1416*(A1+G$2))/G$3)+(1/81)*SIN((18*3.1416*(A1+G$2))/G$3)-
(1/121)*SIN((22*3.1416*(A1+G$2))/G$3)+(1/169)*SIN((26*3.1416*(A1+G$2))/G$3)+G$4)

In the above formula, four variables were defined: G$1, G$2, G$3, and G$4.

— G$1: Amplitude of the Fourier series or triangular wave A.
— G$2: Phase shift of the triangular wave in the x axis φ.
— G$3: Period of the triangular wave T.
— G$4: A constant to compensate the y axis shift δ.

The following graphs (Figure 3a to Figure 3d) are fitting results from above data analysis using least square regression method, the dots are experimental data, and the lines are triangular periodic wave fitting to the data. When the G$1-G$4 (i.e., A, φ, T, δ) values are determined, the analytical function y(x) can be obtained by inserting the G$1-G$4 values back into equation (2).

For example, for MCF-07 breast cancer cell line, the MTT absorbance (y) vs NDPKA concentration (x) can be expressed as: y(x) = 0.2*[Sin(0.06x+5)-0.1Sin(0.2x+15)+0.04Sin (0.3x+25)-0.02Sin (0.4x+35)+0.012Sin(0.5x+45)-0.008Sin(0.6x+55)+0.006Sin(0.8x+65)].

Triangular Periodic Curve Analysis

This study attempts to detect the changes in cell viability over the course of increased dose. One obvious characteristic of the graph has to do with its shape. The graph has the shape of a sine wave. (Figure 3a-3d). At amplitude, the point represents the equilibrium position of the cells when rate of cell growth is equal to rate of cell death. So the cell viability is changing back and forth about the fixed resting position amplitude (Tables 1 and 2) over the course of dose increment.

A full cycle of cell growth might be thought of as the shift of the cell growth from its resting position (A) to its maximum height (B), and then back to its resting position (C). Using measurements from along the dose axis, it is possible to determine the dose for one complete cycle. The cell growth is at position A at a dose of 10 nanogram and completes its cycle when cell growth is at position C at a dose of 108.4 ng. It takes 108.4 ng to complete the first full cycle of cell

Table 1. mEq phosphate concentration after 24 hours.

	MCF-07 Cell Line		*MDA-MB-231 Cell Line*	
NDPKA/nm23H1 applied (ng/ml)	*Phosphate concentration after 24 hours (meq)*	*After 24 hours % cell growth variation between immediate higher NDPKA concentration to immediate lower NDPKA concentration*	*Phosphate concentration after 24 hours (meq)*	*After 24 hours % cell growth variation between immediate higher NDPKA concentration to immediate lower NDPKA concentration*
10.05	808.33	N/A	1368.33	N/A
5.05	616.67	4.34	554.44	2.46
3.38	499.44	-15.3	650.56	-93.64
2.55	578.33	29.16	508.33	20.31
2.05	562.22	-12.4	1307.22	12.52
1.72	808.33	-202.8	507.78	7.02
1.48	604.44	371.7	485.56	100
1.30	557.78	12.88	506.11	-2481.08
1.16	562.22	-91.44	426.67	-735.66
1.05	573.33	-8.1	661.67	-45.39
0.96	1003.33	211.2	526.11	78.28
0.88	555.00	320.76	494.44	-61.4
0.82	546.11	-619.33	435.56	-42.45
0.76	573.89	-229.32	698.33	15.01
0.72	503.33	308.7	567.22	127.12
0.68	700.00	-583.2	526.11	37.84
0.64	575.00	859.51	411.67	-316.99
0.61	573.89	107.1	585.00	-81.09
0.58	547.78	-273.6	506.67	65.96
0.55	546.11	547.2	678.89	54.52
0.53	666.11	1902.56	337.78	21.99
0.50	598.89	-2185.26	590.00	23.13
0.48	643.89	-379.52	658.89	-21.77
0.47	753.33	-110.4	533.89	6.67
0.45	547.78	0	659.44	-38.94

Table 2. Triangular periodic curve characteristics.

Cell lines used (where NDPKA applied as dose)	Amplitude	Cell growth from confluence level (%)	Phase	Period, T	Cells start dying after absorbing NDPKA ng	Cells regrow like initial growth at the initiation of cycle after consuming NDPKA ng	Constant
MCF7_MTT	0.24	24.21	85.79	108.36	54.18	108.36	10.82
MCF7_phosphate	0.11	10.98	49.95	127.77	63.88	127.77	16.03
MDA_MTT	0.10	10.32	75.98	85.51	42.76	85.51	23.61
MDA_phosphate	0.22	21.91	153.62	132.06	66.03	132.06	7.95

growth. Now if the cell growth of this cell line is periodic (i.e., regular and repeating), then it should take the same dose of 108.4 ng to complete any full cycle of cell growth. The same dose-axis measurements can be taken for the second or third full cycle of growth. In the second full cycle, the cell growth cycle moves from a resting position C up to maximum height D and then back to its resting position E consecutively as first cycle from 108.4 ng to 206.8 ng. This represents a dose of 108.4 ng to complete the second full cycle of cell growth. The two cycle doses are identical. By inspection of the table, one can safely conclude that the cell viability and phosphate in extracellular fluid is regular and repeating. Over dose increment, the cell growth is undergoing changes according to its cell function in a sinusoidal fashion. That is, the cell function of the cell growth at any given moment in dose is a function of the equation 1 of the dose. As such, the cells will both grow and die over the course of a single cycle. So to say that the MCF-07 cells are "dying" is not entirely accurate since during every cycle there are one short interval during which cell grows. A third obvious characteristic of the graph is that cell death occurs with the cell function system. Some NDPKA is being dissipated over the increment of dose. The extent to which the cell growth moves above (B,D) or below (A, C and E) varies over the increment of dose. In the second full cycle of cell growth that is shown, the cell function moves from its resting position C to 24% above. However, the amount of displacement of the cell growth at its maximum and minimum height is decreasing from one cycle to the next. This illustrates that NDPKA is being lost from the cell function system. If given enough dose, the growth of the cells will eventually cease as NDPKA is dissipated. This observation of NDPKA dissipation or NDPKA loss is the observation that could trigger the cell death discussed earlier. Authors use the phrase "NDPKA is being dissipated or lost" instead of saying the "NDPKA reaction is slowing down." So far, It has been looked at in measurements of dose and amplitude in terms of cell viability and phosphate content. The measurements were based upon readings of an Absorbance-dose graph. Previously Kado et al (2009) used Absorbance-dose graph of NDPKA of the same type as in the present study.

Periodically in triangular pattern, phosphates increase and decrease in cell media. Cell growth becomes inversely related with phosphate content. After extrapolation from standard curve when the phosphate content derived from Figure 3a-3d, from 24% cell periodically growing and dying in periodic curve in Figure 2, cell media in MCF-07 gained 15.78% increase in phosphate content, MDA-MB-231 gained 33.06% from 8.09% cell periodically growing and dying.

Many triangular periods converge (Figure 3a-3d) and yields straight line to average (Figure 2a-2d) increase or decrease.

The periods 127.77 ng for MCF-07 and 132.06 ng for MDA-MB-231 obtained after extrapolation in standard curve are almost identical. It indicates the limit of cell phosphate buffer system against dose. The two critical limits for 2 straight lines (Figure 3b, 3d) are almost identical.

The cells are having three sources of phosphates:

1. NDPKA/nm23 H1 addition
2. Dead cell destruction ingredients
3. Remaining of phosphate salt from washing with PBS (Phosphate Buffer Saline) during subculturing

Nm-23 H1 addition must be the dominant source of phosphate.

Cells might be the reservoir of escaped phosphate from cell CM/media.

4000 cells each take up phosphate inside in each of 96 wells used.

In normal human body, calcitonin and parathyroid hormone regulate phosphate concentration in blood. But in cell media, there is no hormone, so too many phosphate was found in cell media.

The MDA-MB-231 cell lines had significant correlation between dose applied and phosphate content. In case of MDA-MB-231, the predominant reason for high phosphate content in cell media was NDPKA/nm23 H1 addition.

Area under the curve (AUC) analysis

MDA-MB-231 estrogen negative cells have nearly 2.5 times higher AUC (6.55) of phosphate content than MCF-07 estrogen positive (2.52) cell lines (Table 3).

All the graphs showed similarity with Kado et al's graphs on concentration of NDPKA and cell viability. This study adds phosphate content analysis to Kado et al's findings which showed nm23 H1 is dependent on its NDPKA activity. The objective of the present study was to investigate whether similar results can be found in breast cancer cells as Kado et al's result from AML cells. Similar results came except AUC of breast cancer cells which is up to 530% higher than AML cells.

NDPKA's effect on MDA-MB-231 cell growth and viability had significant correlation with phosphate content in cell media at the Yates's correction for continuity 0.00. NDPKA's effect on MCF-07 cell growth and viability had significant correlation with phosphate content in cell media at the Yates's correction for continuity 0.00.

MDA-MB-231 cell line's cell growth and cell proliferation did not correlate (Yates's correction for continuity 0.07), with dose, but in case of MCF-07, significant dose-dependent change (Yates's correction for continuity 0.00) in cell growth and cell proliferation was found. MDA-MB-231 estrogen negative cells showed significantly higher phosphate content remaining after 24 hours incubation with NDPAK/nm23 H1 than MCF-07 estrogen positive cell lines.

Both in Okabe-Kado et al[1] and the present study, extracellular rnm23-H1 protein overall promoted the growth and survival of both AML and breast cancer cells at similar concentrations.

Data approximated from Kado et al's graph shows fraction value AUCs below 1 over 2-10 days (Table 2). The present study based on 1 day shows AUC more than 1 integer values (Table 3).

3.5 Cell morphological examination by phase-contrast microscopy

From 40-60% confluence, the cells were examined every hour for 13 hours.

Phase-contrast microscopy observed the morphological changes of breast cancer cells- fibroblastic MDA-MB-231 breast cancer cell lines (Figure 5). In 14 hours, photograph taken every 1 hour. On first 3 hours, nucleus was visible. But in final 3 hours, nucleus disappeared. Cells reduced to 40% in the 13th hour.

MCF-7 cell lines were observed. In first 3 hours, cells maintain their cell-cell contacts and adhere to the substratum. In cells of final 3 hours, cells found to lose their cell-cell contacts and detached from the substratum but nucleus was prominent. In MCF-07 cells, after 1 hour cells reduced to 70% (Figure 4). After 11 hours MCF-07 cells had huge growth.

DISCUSSION

The present study measured the free phosphate released temporarily from ATP, NDPKA reactions. nm23 protein might be released by dying tumor cells overexpressing nm23[1].

Overexpression of extracellular nm23-H1 derived from tumor cells generates a supportive microenvironment convenient for their growth and survival through cytokine production of cancer cells[1]. The supportive environment gives triangular pattern in extracellular phosphate which is periodic.

Table 3. Area under the curve (AUC).

Cases	Days	Absorbance at NDPKA/nm23 H1 Amount incorporated (ng)						Area under the curve (AUC)
		At 0 ng	At 0.45 ng	At 10 ng	At 10.05 ng	At 100 ng	At 1000 ng	
MDA-MB-231 breast cancer cell lines	1		2.052		2.407			1.512
MCF-07 breast cancer cell lines	1		1.944		2.265			2.016
AML cell line Kado et al (2009)	2	0.7		0.8		0.6	0.58	0.064
	2	0.12		0.22		0.42	0.5	0.038
	3	0.4		0.96		1	1.2	0.31
	4	0.5		1		0.6	0.58	0.432
	5	0.3		0.825		0.96	1.1	0.06
	6	0.08		0.61		0.58	0.8	0.35
	7	0.3		1.1		1	0.95	0.45
	8	0.04		0.21		0.33	0.4	0.14
	10	0.15		0.6		0.685	0.83	0.18

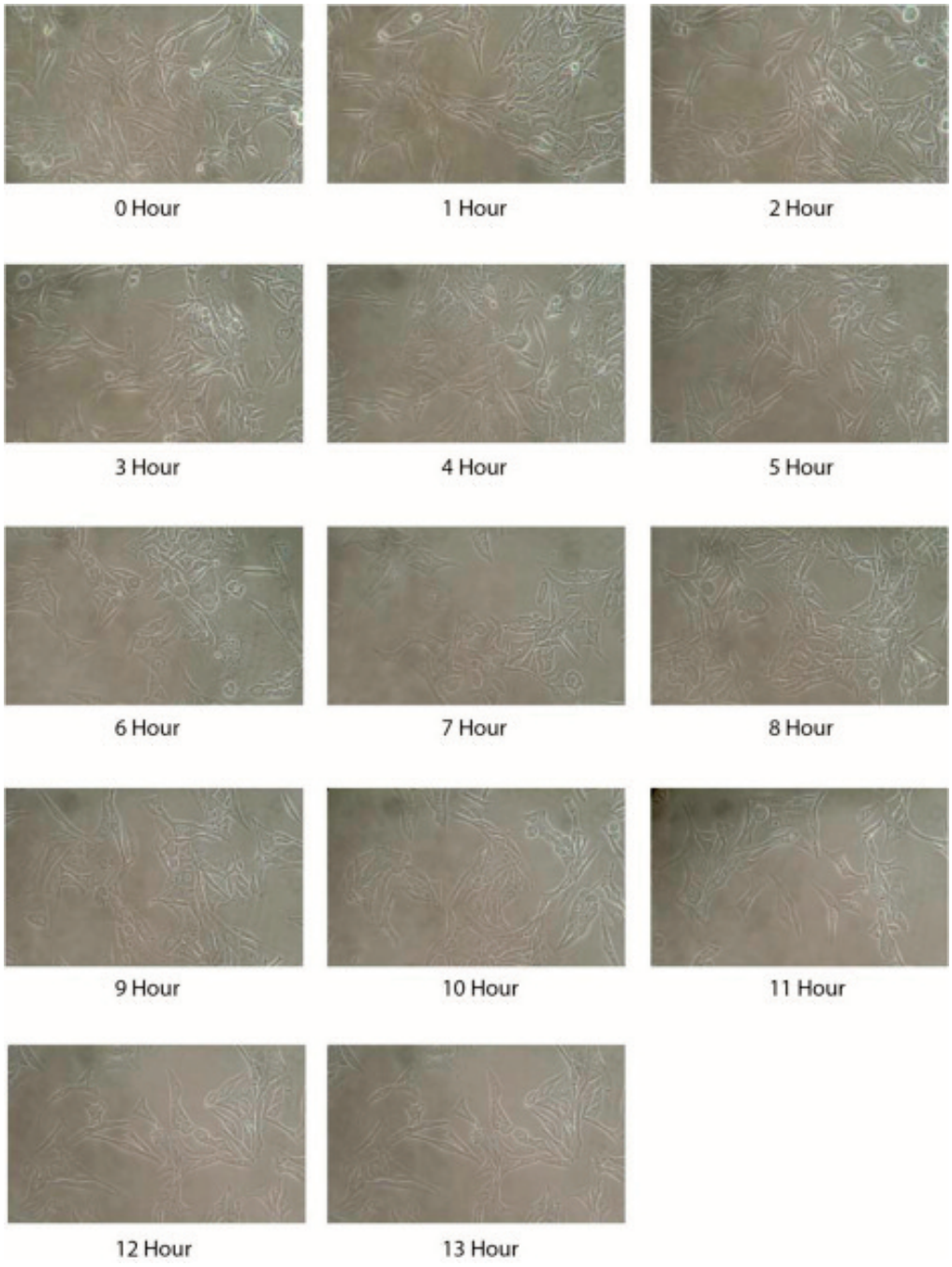

Figure 5. Phase-contrast microscopy observed the morphological changes of breast cancer cells. MDA-MB-231 breast cancer cell lines. Magnification ×100.

Although previous paper results indicate that the activity of nm23 is independent of its NDPKA activity[2], this paper finds phosphate addition and release property of NDPKA present in nm23H1 and dose-dependent phosphate addition and release reflected in triangular period curve. In positive peak maximum phosphate ions are reduced causing bond formation whereas in negative peak phosphate ions are released in extracellular fluid.

Since MCF-07 cell growth increase and MDA-MB-231 cell growth increase (p=0.0472) were significantly different, it proves estrogen receptor effect on cell growth.

MCF-07 estrogen positive yielded higher range and variation in cell viability than MDA-MB-231 estrogen negative cell lines. This is how estrogen receptor (ER)-positive breast cancers generally have a better prognosis and are often responsive to

anti-estrogen therapy[6]. MDA-MB-231 estrogen negative cells have higher phosphate content than MCF-07 estrogen positive cell lines. Since ER-negative breast cancers are more aggressive[6] and unresponsive to anti-estrogens and other targeted therapies are thus urgently needed, phosphate content might act as parameter for other targets.

Conflict of Interests:
The Authors declare that they have no conflict of interests.

Authors' Contributions
Nishat Chowdhury, Dhanasekaran Ganeshan did all experiments. Nishat Chowdhury financed the entire experiment and Chinnasamy Arulvasu provided his laboratory for use. Shingo Ueno and Yuki Mochizuki designed the study. Financial support: self funded.

REFERENCES

1. Okabe-Kado J, Kasukabe T, Honma Y, Kobayashi H, Maseki N, Kaneko Y. Extracellular NM23 protein promotes the growth and survival of primary cultured human acute myelogenous AML cells. Cancer Sci 2009; 100: 1885-1894.
2. Niitsu N, Okabe-Kado J, Kasukabe T, Yamamoto-Yamaguchi Y, Umeda M, Honma Y. Prognostic implications of the differentiation inhibitory factor nm23-H1 protein in the plasma of aggressive non-Hodgkin's lymphoma. Blood 1999; 94: 541-550.
3. Nucleoside-diphosphate kinase. https://en.wikipedia.org/wiki/Nucleoside-diphosphate_kinase. Accessed 23 July 2015.
4. Tumor lysis syndrome. https://en.wikipedia.org/wiki/Tumor_lysis_syndrome. Accessed 23 July 2015.
5. Lacombe ML, Milon L, Munier A, Mehus JG, Lambeth DO. The human Nm23/nucleoside diphosphate kinases. J Bioenerg Biomembr 2000; 32: 247-258.
6. Rochefort H, Glondu M, Sahla ME, Platet N, Garcia M. How to target estrogen receptor-negative breast cancer? Endocr Relat Cancer 2003; 10: 261-266.