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Short communication

Novel alkylphospholipid-DTC hybrids as promising agents against endocrine related cancers acting via modulation of Akt-pathway☆

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ABSTRACT

A new series of 2-(alkoxy(hydroxy)phosphoryloxy)ethyl dialkylcarbodithioate derivatives was synthesized and evaluated against endocrine related cancers, acting via modulation of Akt-pathway. Eighteen compounds were active at 7.24–100 μM against MDA-MB-231 or MCF-7 cell lines of breast cancer. Three compounds (**14**, **18** and **22**) were active against MCF-7 cells at IC_{50} significantly better than miltefosine and most of the compounds were less toxic towards non-cancer cell lines, HEK-293. On the other hand, twelve compounds exhibited cell growth inhibiting activity against prostate cancer cell lines, either PC-3 or DU-145 at 14.69–95.20 μM. While nine of these were active against both cell lines. The most promising compounds **14** and **18** were about two and five fold more active than miltefosine against DU-145 and MCF-7 cell lines respectively and significantly down regulated phospho-Akt. Possibly anti-cancer and pro-apoptotic activity was mostly due to blockade of Akt-pathway.

1. Introduction

Cancer is a group of diseases characterized by uncontrolled cell proliferation and spread of malignant cells. About 77% of all cancers are diagnosed in older persons with age 55 years or more. In US, the lifetime risk from cancer is about 1 in every 3 person [1]. The National Institute of Health (NIH) estimates that the overall costs of cancer in 2007 were US $ 226.8 billion: $103.8 for direct medical cost and $ 123.0 for indirect mortality costs [1]. The projections [2] for cancer survivors and cost of care during 2010–2020 reveal that there will be 18.1 million survivors in 2020 with the medical costs to be increased by 27% from 2010. The largest medical cost increase being in the two endocrine related cancers—prostate cancer in men (42%) and breast cancer in women (32%). These revelations have prompted the authors to design, synthesize and biologically evaluate chemical entities that may be useful in the management of both prostate and breast cancer.

Recently the concept of hybrid molecules has been attracting a lot of attention [3–6] as the presence of more than one active pharmacophore in a single molecule leads to an entirely novel class of compounds with better biological activity profile. It is supposed that a single chemical entity can modulate multiple targets simultaneously.

Alkylphosphocholines (APCs) [7] belong to a class of lipid molecules that include miltefosine (Fig. 1), perifosine (Fig. 1), erufosine (Fig. 1) and edelfosine (Fig. 1), which have known anti-tumor properties [8,9]. Unlike conventional chemotherapeutics, these compounds structurally resemble membrane lipid components, and thus, exhibit adequate drug delivery to target tumor tissues [9]. However, they can also target primarily the membrane-bound receptors associated with cancer-specific cellular phenotypes, such as, cellular proliferation and survival. More specifically, the possible mechanisms underlying the actions of APCs have been reported to be the involvement of PI3K/Akt (phosphatidylinositide 3-kinase/v-

Abbreviations: APCs, alkylphosphocholines; DDTC, diethyldithiocarbamate; HEK, human embryonic kidney; PI, propidium iodide; MMP, mitochondrial membrane potential; ATCC, American type of cell culture collection; DMEM, Dulbecco modified eagle medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI3K, phosphatidylinositide 3-kinase.

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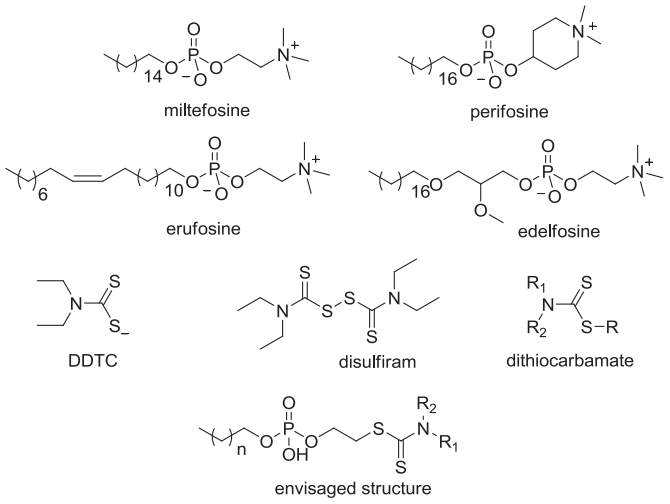


Fig. 1. Structures of known alkylphosphocholine, dithiocarbamate and envisaged structure.

Akt murine thymoma viral oncogene homolog) inhibition, their downstream signaling pathways, and the induction of apoptosis [7]. It is also known that APCs do not target the DNA, and they have cell selective effects of inhibiting the proliferation of cancer cells at low concentrations without affecting the normal cells [7]. Miltefosine (Fig. 1) shows a wide range of anti-tumor effects and as Milterx®, it has been approved for the topical treatment of skin metastases from breast cancer [7].

Likewise, dithiocarbamate is also a versatile pharmacophore exhibiting various biological activities due to its sulfur based metal chelating properties [10]. Diethyldithiocarbamate (DDTC; Fig. 1) is capable of binding accumulated copper and forming a new complex that could potently inhibit the proteasomal chemotrypsin-like activity, decrease expression of androgen receptor (AR), estrogen receptor (ER)-α and ER-β receptors proteins, and induce apoptosis in both prostate and breast cancer cells [10]. Pyrrolobenzodiazepine derivatives with DTC side chains inhibit the growth of prostate and breast cancer cell lines [11]. Tetraethylthiuram disulfide (Disulfiram, Fig. 1) and dithiocarbamate anion (Fig. 1) strongly inhibited the proliferation of cancer cells of a variety of cell types [12] by inhibiting the maturation of P-glycoprotein pump, an ATP-driven 170-kd efflux pump on the plasma membrane that pumps a variety of cytotoxic drugs out of the cell [13]. Another hybrid molecule of butenolide and dithiocarbamate have anti-tumor properties against several human cancer cell lines including the prostate and breast cancer cells [14].

Thus, it was thought worthwhile to incorporate dithiocarbamate moiety into alkylphospholipid scaffold in place of amine moiety (part of choline moeity) keeping the rest of the structure same. The hybrid structures (envisaged structure, Fig. 1) though not entirely alkylphosphocholine since it was modified in order to add the alkyldithiocarbamate moiety, were therefore addressed as alkylphospholipid. It was envisaged to arrive at a novel scaffold (Fig. 1) having anti-proliferative activity against both the breast and prostate cancer cell lines. Synthesis, biological activity and structure activity relationship (SAR) are being communicated.

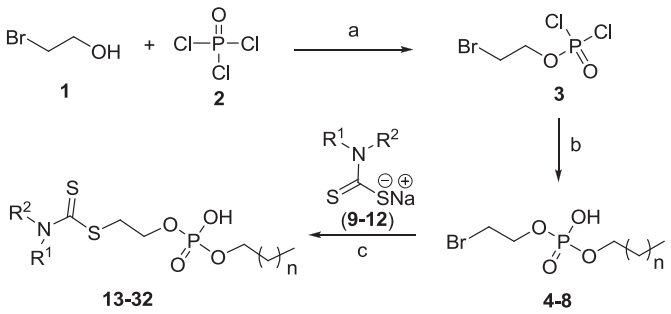
2. Chemistry

The 2-(alkoxy(hydroxy)phosphoryloxy)ethyl dialkylcarbodithioate derivatives (**13**–**32**) were synthesized according to Scheme 1. Bromoethanol (**1**) was treated with phosphorus oxychloride (**2**) to give 2-bromoethyl phosphorodichloridate (**3**). Subsequent reaction of **3** with suitable alcohol furnished 2-bromoethyl alkyl hydrogen phosphate (**4**–**8**), which after treatment with sodium salt of carbodithioic acid (**9**–**12**) provided 2-(alkoxy(hydroxy) phosphoryloxy)ethyl dialkylcarbodithioate (**13**–**32**).

3. Biological evaluation

3.1. Cell inhibitory activity in breast and prostate cancer cells

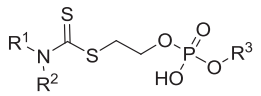
The above compounds **13**–**32** were evaluated against breast cancer cell lines, MCF-7 (estrogen responsive proliferative breast cancer model), MDA-MB-231 (estrogen independent aggressive breast cancer model); and prostate cancer cell lines, PC-3 (estrogen responsive both α and β), DU-145 (estrogen responsive β only) and non-cancer cell line, HEK-293 (human embryonic kidney) using MTT assay to assess cell inhibition [15,16]. Miltefosine, an alkylphosphocholine was used as standard drug. The results (Table 1) revealed that almost all the compounds except compounds **15** and **16** were inhibited both or either of MDA-MB-231 and MCF-7 breast cancer cell lines. Standard drug, miltefosine had IC_{50} 2.075 μM and 34.70 μM against MDA-MB-231 and MCF-7 cells respectively. Out of 20 compounds, three compounds (**14**, **18** and **22**) were active against MCF-7 cells with IC_{50} better than miltefosine. Most of the compounds were found to be less toxic towards non-cancer cell lines, HEK-293, except compound **21**, **31** and **32** which exhibited some degree of toxicity. Compound **18** exhibited potent cell growth inhibition against both MCF-7 and MD-MB-231 cells with IC_{50} of 7.24 ± 2.15 and 24.57 ± 5.69 μM respectively. It may be inferred that compound **18** is most promising compound with significant anti-proliferative activity against breast cancer cell lines. On the other hand twelve compounds (**13**–**21**, **23**, **27** and **31**) exhibited cell growth inhibiting activity against prostate cancer cell lines, either PC-3 or DU-145, at 14.69–95.20 μM while nine of these (**13**–**20** and **23**) were active against both the cell lines. The standard miltefosine had IC_{50} of 19.90 μM and 25.78 μM against PC-3 and DU-145 cell lines respectively. Compounds **14**, **15** and **19** exhibited better activity than miltefosine against DU-145 cell lines. Compound **14** and **15** were most promising as these two had lower IC_{50} values against both the prostate cancer cell lines. Sodium piperidine-1-carbodithioate was also evaluated against breast cancer cell lines, MCF-7, MDA-MB-231 and prostate cancer cell lines, PC-3, DU-145 to assess the effect of hybridization of alkylphospholipids and DTC. It has no effect against both the breast cancer cell lines and mildly inhibited the growth of prostate cancer cell lines, PC-3 and DU-145 at IC_{50} 25.5 ± 1.25 μM and 18.7 ± 0.84 μM respectively. These results



-NR^1R^2 = piperidine, pyrrolidine, azepane, N-methyl piperazine
n = 0, 4, 6, 8, 14

Scheme 1. Reagent and conditions; (a) CCl_4, reflux, 10 h; (b) (1) ROH, CCl_4, reflux, 10 h; (2) H_2O,rt, 6 h; (c) MeOH, reflux, 3 h.

Table 1
Cell inhibiting activity of the compounds (**13**–**32**).

Compd			Mean inhibition of cell proliferation (in terms of IC_{50} ± SEM in μM)				
			Anti-breast cancer activity		Cytotoxicity	Anti-prostate cancer activity	
	$-NR^1R^2$	$-R^3$	MDA-MB-231	MCF-7	HEK-293	PC-3 cells	DU-145
13		$-CH_2(CH_2)_{14}CH_3$	69 ± 2.88	72 ± 8.39	>100	65.7 ± 1.1	28.88 ± 4.8
14		$-CH_2(CH_2)_{14}CH_3$	~100 ± 5.98	29 ± 1.37	>100	34.3 ± 1.4	**14.69 ± 1.0**
15		$-CH_2(CH_2)_{14}CH_3$	>100	>100	>100	32.1 ± 0.3	17.6 ± 1.4
16		$-CH_2(CH_2)_{14}CH_3$	>100	>100	>100	95.2 ± 5.3	49.3 ± 4.8
17		$-CH_2(CH_2)_8CH_3$	67 ± 4.72	70 ± 8.34	>100 ± 16.2	56.1 ± 6.8	46.14 ± 3.0
18		$-CH_2(CH_2)_8CH_3$	24.57 ± 5.69	**7.24 ± 2.15**	70.7 ± 1.11	62.5 ± 2.5	29.27 ± 2.9
19		$-CH_2(CH_2)_8CH_3$	11.4 ± 3.72	43.3 ± 5.07	70 ± 1.71	32.9 ± 2.9	23.95 ± 1.5
20		$-CH_2(CH_2)_8CH_3$	29 ± 6.6	99.6 ± 3.65	>100 ± 1.005	80 ± 4.8	82.3 ± 8.4
21		$-CH_2(CH_2)_6CH_3$	69 ± 2.39	53 ± 4.37	28.3 ± 3.24	>100	47.1 ± 2.9
22		$-CH_2(CH_2)_6CH_3$	11.9 ± 5.87	26 ± 4.39	69 ± 8.79	>100	>100
23		$-CH_2(CH_2)_6CH_3$	27 ± 1.35	78 ± 7.56	>100 ± 5.36	56.8 ± 3.7	59.1 ± 4.8
24		$-CH_2(CH_2)_6CH_3$	58.46 ± 3.98	46 ± 5.05	86 ± 3.96	>100	>100
25		$-CH_2(CH_2)_4CH_3$	54 ± 7.13	~100 ± 6.99	>100 ± 4.75	>100	>100
26		$-CH_2(CH_2)_4CH_3$	85 ± 5.74	62 ± 6.57	>100	>100	>100
27		$-CH_2(CH_2)_4CH_3$	17.19 ± 5.00	~55	70.5 ± 6.31	>100	77.4 ± 8.4
28		$-CH_2(CH_2)_4CH_3$	73 ± 6.13	58 ± 4.96	>100	>100	>100
29		$-CH_2CH_2CH_3$	67 ± 2.38	73 ± 5.27	>100	>100	>100
30		$-CH_2CH_2CH_3$	68 ± 7.31	>100	>100	>100	>100
31		$-CH_2CH_2CH_3$	38 ± 2.98	74 ± 6.38	54 ± 6.67	>100	86.1 ± 5.6
32		$-CH_2CH_2CH_3$	62 ± 2.2	59 ± 6.09	39.6 ± 4.68	>100	>100
Miltefosine			2.075 ± 0.12	34.7 ± 11.7	–	19.9 ± 0.7	25.78 ± 1.4

Bold represents the values of most active compounds as compared to standard.

demonstrated that dithiocarbamate moiety of most active lead compounds **14** and **18** alone does not show significant anti-cancer activity.

Structure–activity relationship (SAR) for breast cancer was discussed according to the MCF-7 cell line as it is estrogen responsive. Likewise activity against DU-145 cells was chosen for SAR in prostate cancer as it is responsive to estrogen receptor β present in normal and malignant prostate. The SAR study established that both ring size of amine and length of alkyl chain significantly affected the activity. The IC_{50} values suggested that an

increase in alkyl chain from propyl (**29**–**32**) to decyl (**17**–**20**) enhanced the activity against MCF-7 cells while a further increase to hexadecyl (**13**–**16**) resulted in decrease of activity. Whereas against DU-145 cells the anti-proliferative activity increased with length of alkyl chain and most active compound **14** had sixteen carbon alkyl group. Thus, a ten carbon alkyl chain was most appropriate for anti-breast cancer activity while sixteen carbon chain was most desired against prostate cancer. The data further revealed that an increase in ring size of amine from 5-membered (**13**, **17**, **21** and **25**) to 6 (**14**, **18**, **22** and **26**) enhanced the activity against MCF-7 cells for six to sixteen carbon alkyl chain while with three carbon chain (**29**, **30**) the activity reduced. Against DU-145 cells the activity enhanced on increasing the ring size from five (**13**, **17**) to six (**14**, **18**) for long alkyl chains hexadecyl and decyl whereas lower chains were less desirable. A further increase in ring size of amine (**15**, **19**, **23**, **27** and **31**) was not beneficial irrespective of alkyl chain length and an additional nitrogen in amine residue (**16**, **20**, **24**, **28** and **32**) did not provide a better activity. The structure of most promising compounds **14** against DU-145 and **18** against MCF-7 cell lines (Fig. 2) suggested that a six membered amine at dithiocarbamate residue was most appropriate for both of these cell lines while optimum alkyl chain length was sixteen and ten respectively.

It is interesting to observe that ten compounds (**13**, **14**, **17**–**21**, **23**, **27** and **31**) were active against both the breast and prostate cancer cell lines. The most promising compounds **14** and **18** were about two and five fold more active than miltefosine against DU-145 and MCF-7 cell lines respectively and almost equally active against MCF-7 and DU-145 cells. Therefore, it was thought worthwhile to study the mechanism of anti-proliferative action of compounds **14** and **18** in respective cancer cell lines.

3.2. Effect of compound *14* and *18* on cell cycle progression

Evasion of cell cycle arrest and apoptosis or combination of both processes, is central to uncontrolled cell proliferation in cancer. Therefore, candidate molecule capable of inducing cell cycle arrest in cancer cells is considered to possibly have beneficial effect in cancer. In order to investigate effect of lead compounds **14** and **18** on cell cycle phases, we conducted propidium iodide (PI)-staining based cell cycle analysis as described in experimental section [17]. Our result showed that compound **18**, a novel miltofosine-like alkylphospholipid derivative could arrest the growth of MCF-7 cell line at G0/G1 phase (Fig. 2A). Cell cycle analysis by PI-staining showed that compound **18** at 7 μM induced cell cycle arrest at G0/G1 phase suggesting that cell cycle arrest is responsible for cell growth inhibitory activity in MCF-7 cells also there was increased sub-diploid population observed as compare to vehicle control which denotes cells undergone apoptosis. A similar arrest at G1 phase was also evident in case of DU-145 prostate cancer cells on treatment with compound **14** (Fig. 2B).

3.3. Induction of apoptosis by compound *14* and *18*

Exposure of phosphatidylserine on outer leaflet of cell membrane is considered as hallmark of apoptosis. Therefore, induction of apoptosis is commonly measured in terms of binding of Annexin-V to exposed phosphatidylserine. Annexin-V staining is further combined with PI-staining to exclude membrane intact live cells and necrotic cells [18]. Annexin-V and PI dual staining was carried out and it was observed that compound **18** induced significant apoptosis in MCF-7 cells (Fig. 3A). Annexin-V staining positive cell were significantly increased in MCF-7 cell population treated with compound **18**. Similarly, the number of apoptotic DU-145 cells increased from ~2% to ~9%, ~11% and ~13% on treatment with 5 μM, 10 μM and 15 μM of compound **14** (Fig. 3B).

3.4. Effect of compound *14* and *18* on expression of cell cycle and apoptosis-related genes

The cell cycle phase distribution analysis results showed that compound **18** induced G0/G1 arrest along with increase in sub-diploid population in MCF-7 cells (Fig. 2A). Cell cycle progression is a well-regulated process and dependent on various cyclins like cyclins A1 and A2. In order to know the effect of compound **18** in cell cycle and apoptosis-related genes which may be responsible for cell cycle arrest, gene expression analysis [19] was carried out in MCF-7 (Fig. 4A). The qPCR data showed that compound **18** treatment resulted in down-regulation of expression of cyclins A1 and A2 and anti-apoptotic gene, bcl-2. Likewise, increase expression of Bax and decreased expression of Bcl-2 was observed in DU-145 cells treated with compound **14** as evident from western blot result (Fig. 4B). A very significant increase in Bax/Bcl-2 ratio ($P < 0.001$) was evident at 15 μM concentration (Fig. 4B').

3.5. Effect of compound *14* and *18* on mitochondrial membrane potential (MMP)

The membrane-permeant JC-1 dye is widely used as an indicator of MMP. JC-1 dye exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio [19]. The mitochondrial membrane potential-sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates. When MCF-7 cells were treated with compound **18** for 24 h, a significant increase in green fluorescence was observed, suggesting loss of MMP in comparison to untreated control (Fig. 5A). Similarly, a dose dependent increase green fluorescence was also observed in DU-145 cells treated with compound **14** (Fig. 5B).

3.6. Effect of lead compound *14* and *18* on Akt phosphorylation

It is well-established that alkylphospholipid derivatives like hexadecylphosphocholines miltefosine [20], perifosine [21] and erufosine [22] downregulate Akt phosphorylation thereby blocking Akt survival pathway. Since compounds (**14** and **18**) are alkylphospholipid derivatives and share considerable homology with the other pharmacologically active compounds of this class, the effect of compound **14** and **18** was tested on phosphorylation of Akt as described in experimental section [23]. A significant reduction ($P < 0.01$) of phospho-(ser473)-Akt in MDA-MB-231 was observed upon treatment with 2 μM concentration for 24 h (Fig. 6A and A'). Significant decrease in phospho-Akt was also observed in DU-145 ($P < 0.01$) on treatment with compound **14** at 15 μM concentration (Fig. 6 B and B').

4. Conclusion

A novel series of hybrid alkylphospholipid-DTC derivatives were synthesized that exhibited considerable anti-cancer activity against breast and prostate cancer. Interestingly, these compounds did not induce any significant cytotoxicity in non-cancer cell, HEK-293. The lead compound **18** of this series showed significant inhibition of breast cancer cells, MCF-7 with IC_{50} of ~7 μM and MDA-MB-231 with IC_{50} of ~24 μM. Compound **18** induced G0/G1 cell cycle arrest, down-regulation of cyclins responsible for cell cycle progression (cyclin A1 and A2), induction of apoptosis and down-

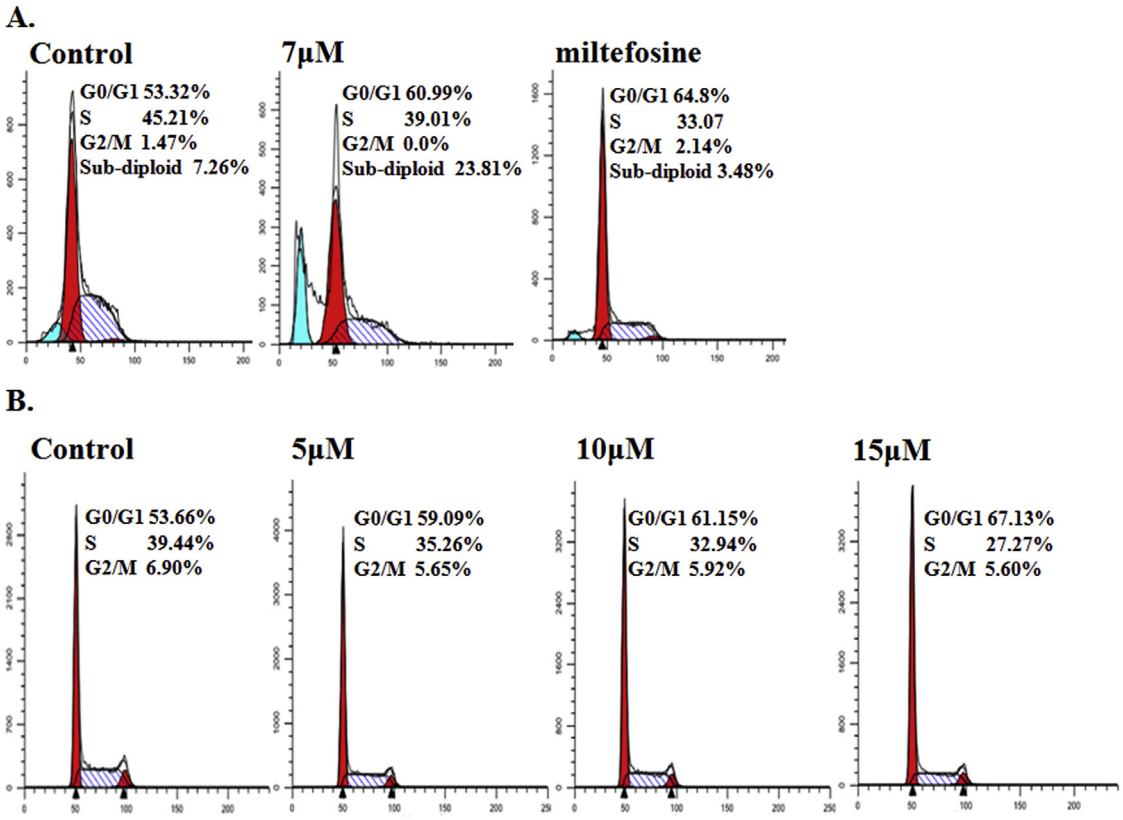


Fig. 2. Effect of compounds **14** and **18** on cell cycle progression in MCF-7 and DU-145 cells. (A). MCF-7 cells were treated with compound **18**. (B). DU-145 cells were treated with compound **14**. After 24 h, cells were harvested and stained with PI before analyzed by flow cytometry. Quantification of flow cytometry data has been shown as percent of total cells.

regulation of anti-apoptotic gene (bcl-2) and significant loss of mitochondrial membrane potential (MMP) in MCF-7 cells. Compound **18** also significantly down regulated phospho-Akt in breast cancer cells; possibly the anti-cancer and pro-apoptotic activity of compound **18** is due to blockade of Akt-pathway (Fig. 7).

Similar results were observed in prostate cancer cell, DU-145 when treated with compound **14**, indicating that its action is also probably mediated through Akt like compound **18** (Fig. 7). These new series of hybrid alkylphospholipid-DTC molecules could serve as interesting lead against endocrine-related cancers.

5. Experimental section

5.1. Chemistry

In general, all reagents and solvents were commercial quality and were used without further purification. IR spectra (ν_{max} in cm^{-1}) of the compounds were recorded on Perkin Elmer FT-IR RX1 PC spectrophotometer. ^1H NMR & ^{13}C NMR spectra were recorded on Bruker Supercon Magnet Avance DPX-200/DRX-300 spectrometers (300 MHz for ^1H; 50 and 75 MHz for ^{13}C) and Bruker Avance III HD-400 spectrometer (100 MHz for ^{13}C) in deuterated solvents with TMS as internal reference (chemical shifts δ in ppm, J in Hz). Electrospray Ionization Mass spectra (ESI-MS) were recorded on Thermo Lcq Advantage Max-IT and HR-DART MS were recorded on JEOL, JMS T100LC Accu TOF. Elemental analyses were performed on Carlo Erba EA-1108 micro analyzer/Vario EL-III C H N S analyzer. All compounds were analyzed for C, H, N and the results obtained were within ±0.4% of calculated values. The reaction progress was routinely monitored by thin layer chromatography (TLC) on precoated alumina/silica gel plates (Aldrich). Column chromatography was performed over Merck silica gel (60–120 Mesh). All chemicals and solvents were procured from Sigma–Aldrich/Merck India Ltd. 2-Bromoethyl phosphorodichloridate (**3**), [24] 2-bromoethyl alkyl hydrogen phosphate (**4**–**8**) [24] and sodium salt of carbodithioic acid (**9**–**12**) [25] were prepared by known procedure.

5.1.1. Synthesis of 2-(hexadecyloxy(hydroxy)phosphoryloxy)ethyl pyrrolidine-1-carbodithioate (**13**)

To the mixture of 2-bromoethyl hexadecyl hydrogen phosphate (**4**, 1.5 g, 3.5 mmol) and methanol (20.0 mL) was added sodium pyrrolidine-1-cabodithioate (**9**, 1.2 g, 7.0 mmol) and stirred at 65 °C for 3 h. The reaction mixture was concentrated under reduced pressure. EtOAc (20.0 mL) was added into the residue, and solid salt was filtered off. Filtrate was dried over sodium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography over SiO_2 (60–120 mesh) using chloroform/methanol (9:1) as eluent to give the title compound (1.3 g, 75%) as light brown oil; IR (neat) ν (cm^{-1}): 3405, 2925, 2854, 1641, 1218; ^1H NMR (300 MHz, $CDCl_3$) δ 4.13–4.08 (2H, m), 3.89–3.87 (4H, m), 3.67–3.60 (4H, m), 2.00–1.93 (4H, m), 1.60 (2H, bs), 1.26 (26H, bs), 0.88 (3H, *t*, $J = 6.3$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 192.2 (C=S), 66.4, 64.1, 55.1, 50.7, 32.0, 30.9, 30.0, 29.8, 29.5, 26.2, 24.4, 22.8, 14.2; ESI-MS *m/z* 518 (M+Na); Anal. Calcd for $C_{23}H_{46}NO_4PS_2$: C, 55.73; H, 9.35; N, 2.83; Found: C, 55.82; H, 9.43; N, 2.99.

The following compounds **14**–**32** were prepared using the procedure similar to that described for compound **13** from the appropriate 2-bromoethyl alkyl hydrogen phosphate and sodium salt of carbodithioic acid.

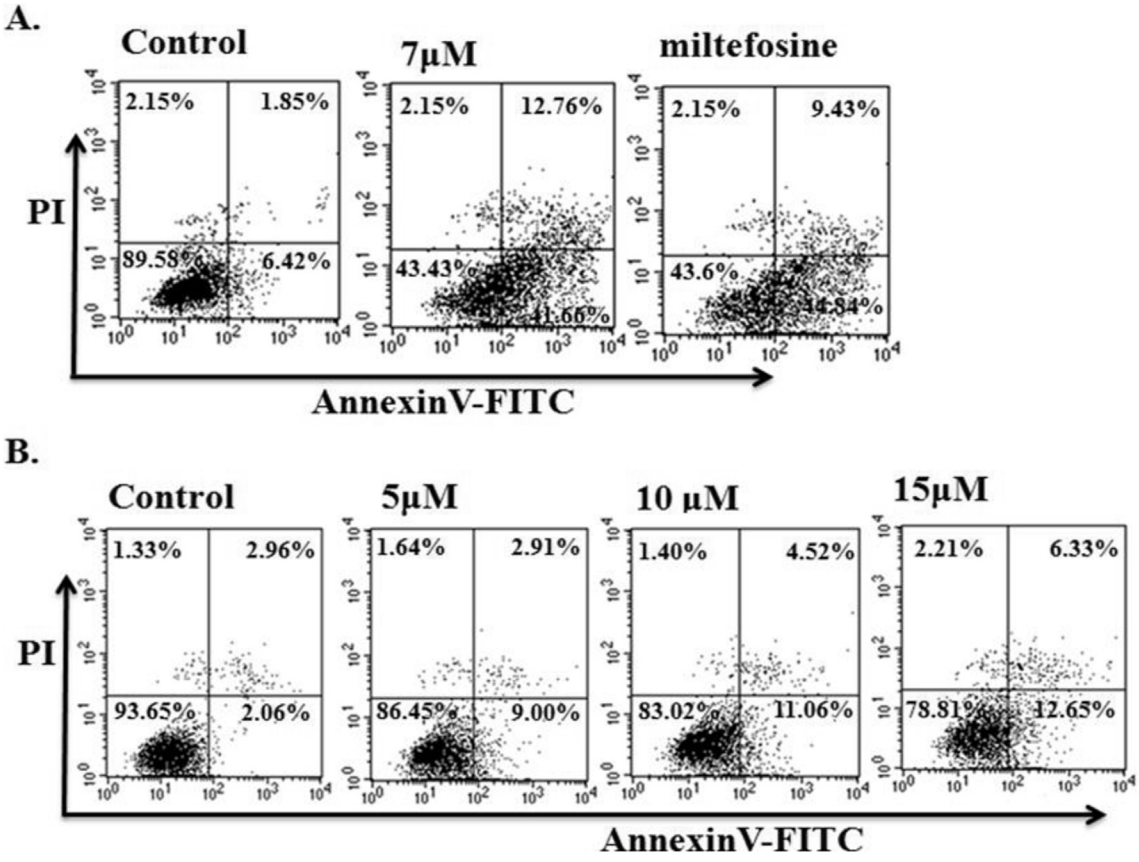


Fig. 3. Effect of compounds **14** and **18** on phosphatidylserine exposure on outer surface of plasma membrane in MCF-7 and DU-145 cells. (A). MCF-7 cells treated with compound **18** and (B). DU-145 cells treated with compound **14** at indicated concentration for 24 h. Cells were harvested and stained with Annexin-V and PI before analysis by flow cytometry. Quantification of flow cytometry data has been shown as percent of total cells.

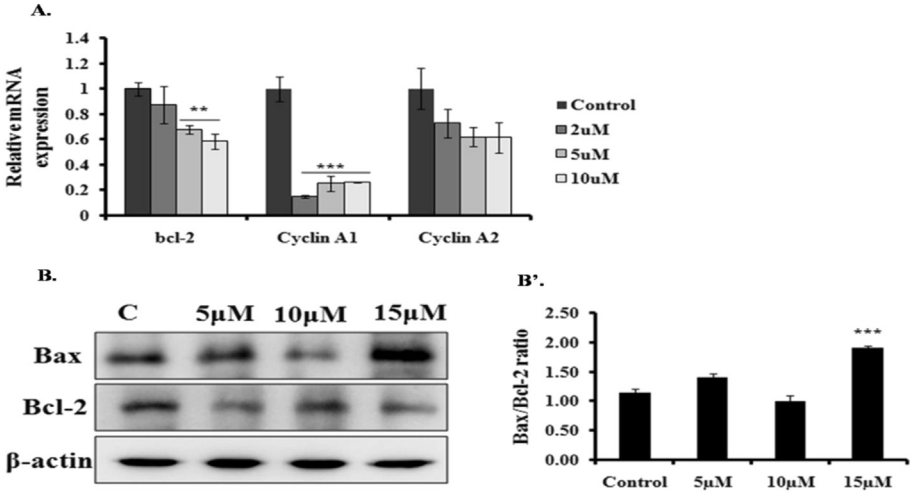


Fig. 4. Effect of compounds **14** and **18** on expression of cell cycle and apoptosis-related genes in MCF-7 and DU-145 cells. (A). MCF-7 cells treated with compound **18** at indicated concentration for 24 h. Cells were harvested and mRNA level was determined by qPCR. (B). DU-145 cells treated with compound **14** at indicated concentration for 24 h. Cells were harvested and Immunoblotting was performed to assess the protein expression in DU-145 cells. (B'). Bax/Bcl-2 ratio as mean ± SE of 3 densitometric scans, significant difference from control is indicated as $^{***}p < 0.001$.

*5.1.2. 2-(Hexadecyloxy(hydroxy)phosphoryloxy)ethyl piperidine-1-carbodithioate (**14**)*

Eluent: 12% methanol–chloroform; Yield 85%; Light yellow oil; IR (neat) ν (cm^{-1}): 3429, 2932, 2859, 1646, 1219; ^1H NMR (300 MHz, $CDCl_3$) δ 4.33–4.03 (4H, m), 3.93–3.77 (4H, m), 3.61 (2H, *t*, $J = 6.2$ Hz), 1.82–1.61 (8H, m), 1.25 (26H, bs), 0.88 (3H, *t*, $J = 6.6$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.8 (C=S), 65.7, 65.3, 52.7, 51.1, 37.2, 31.8, 29.6, 29.4, 29.2, 25.8, 22.5, 22.4, 14.0; ESI-MS *m/z* 532 (M+Na); Anal. Calcd for $C_{24}H_{48}NO_4PS_2$: C, 56.55; H, 9.49; N, 2.75; Found: C, 56.31; H, 9.58; N, 2.82.

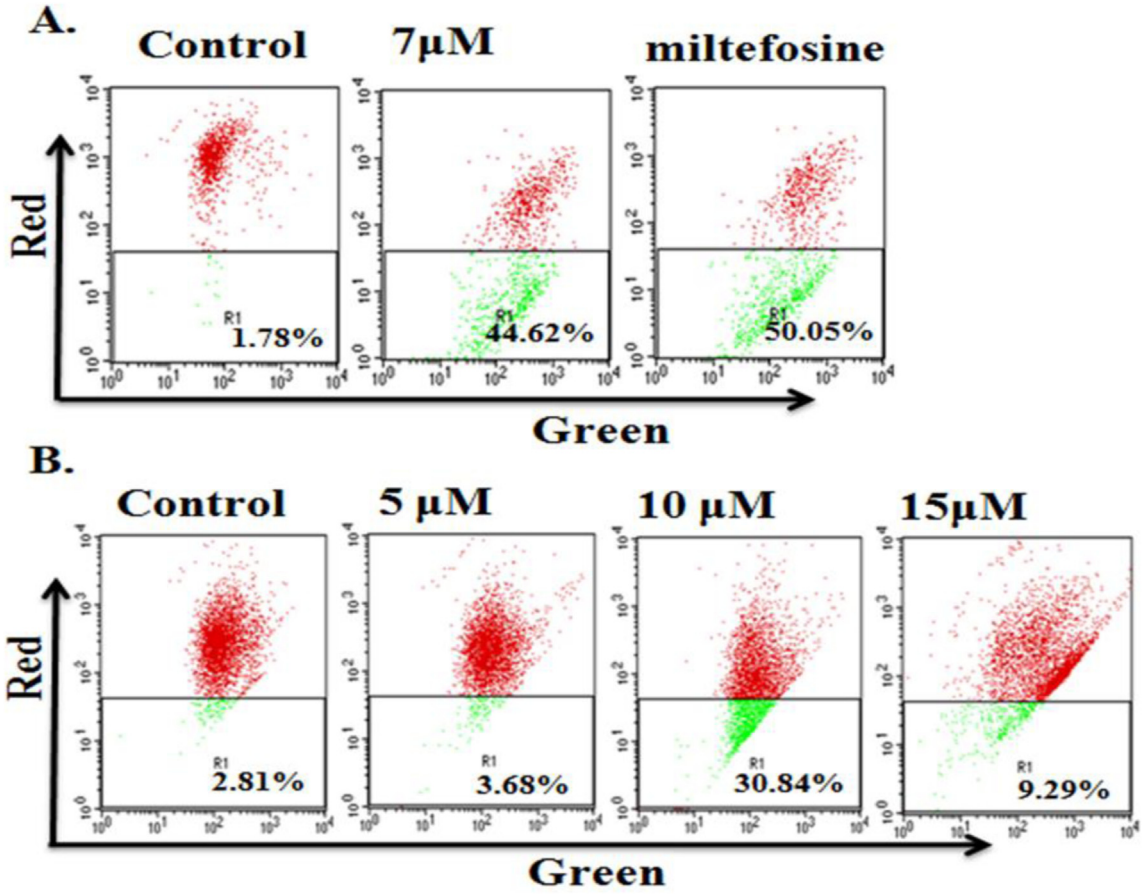


Fig. 5. Effect of compounds **14** and **18** on MMP of MCF-7 and DU-145 cells. (A). MCF-7 cells were treated with compound **18** and (B). DU-145 cells were treated with compound **14** as indicated concentration for 24 h. Cells were harvested, stained with JC-1 and analyzed by flow cytometry. Percent total JC-1 monomar (green) as measured by flow cytometry was expressed as percent MMP loss. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

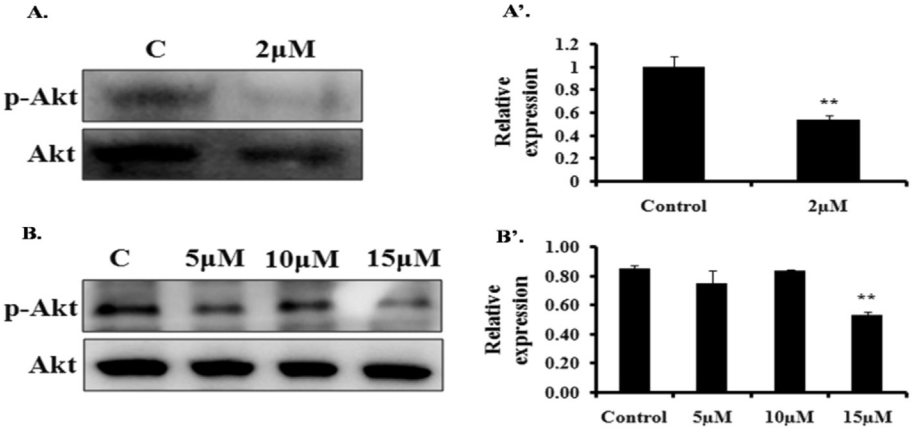


Fig. 6. Effect of compounds **14** and **18** on Akt phosphorylation in MDA-MB-231 and DU-145 cells. (A). MDA-MB-231 cells were treated with compound **18** and (B). DU-145 cells were treated with compound **14** at indicated concentration for 24 h. Cells were harvested and probed for total Akt and phospho-Akt (Ser473) by immunoblotting. (A') and (B'), histograms showing quantitative results of western blot analysis for activation of Akt protein in MCF-7 and DU-145 cells, respectively. Significant difference from control is indicated as $^{**}p < 0.01$.

*5.1.3. 2-(Hexadecyloxy(hydroxy)phosphoryloxy)ethyl azepane-1-carbodithioate (**15**)*

Eluent: 10% methanol–chloroform; Yield 78%; Brown oil; IR (neat) ν (cm^{-1}): 3427, 2926, 2855, 1630, 1215; ^1H NMR (300 MHz, $CDCl_3$) δ 4.19–4.15 (2H, m), 4.06–4.04 (2H, m), 3.91–3.82 (4H, m), 3.61–3.57 (2H, m), 1.85 (4H, bs), 1.68–1.57 (6H, m), 1.25 (26H, bs), 0.88 (3H, *t*, J = 6.5 Hz); ESI-MS *m/z* 546 (M+Na); Anal. Calcd for $C_{25}H_{50}NO_4PS_2$: C, 57.33; H, 9.62; N, 2.67; Found: C, 57.14; H, 9.48; N, 2.75.

*5.1.4. 2-(Hexadecyloxy(hydroxy)phosphoryloxy)ethyl 4-methylpiperazine-1-carbodithioate (**16**)*

Eluent: 10% methanol–chloroform; Yield 82%; Light yellow oil; IR (neat) ν (cm^{-1}): 3403, 2925, 2854, 1654, 1218; ^1H NMR (300 MHz,

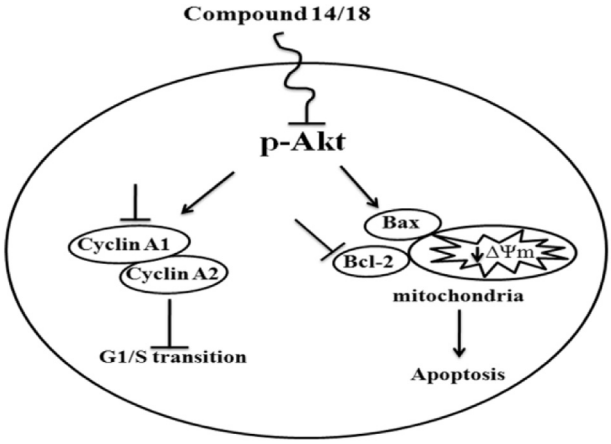


Fig. 7. Proposed mechanism of action of alkylphospholipid hybrids derivatives, compound **14** and **18** on cancer cells.

$CDCl_3$) δ 4.04–4.01 (2H, m), 3.82 (7H, bs), 3.60–3.56 (1H, m), 2.51 (4H, bs), 2.33 (3H, s), 1.59–1.57 (2H, m), 1.25 (26H, bs), 0.88 (3H, *t*, $J = 6.6$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 196.9 (C=S), 66.4, 63.9, 54.5, 50.0, 45.6, 37.3, 32.1, 31.1, 30.1, 30.0, 29.8, 29.5, 26.2, 22.8, 14.2; HRMS *m*/*z* calcd. for $C_{24}H_{49}N_2O_4PS_2$ (MH^+): 525.2950; found 525.2944; Anal. Calcd for $C_{24}H_{49}N_2O_4PS_2$: C, 54.93; H, 9.41; N, 5.34; Found: C, 54.79; H, 9.35; N, 5.27.

5.1.5. 2-(Decyloxy(hydroxy)phosphoryloxy)ethyl pyrrolidine-1-carbodithioate (**17**)

Eluent: 8% methanol–chloroform; Yield 77%; Yellow oil; IR (neat) ν (cm^{-1}): 3395, 2927, 2857, 1654, 1219; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.13–4.02 (2H, m), 3.92–3.88 (2H, m), 3.84–3.79 (2H, m), 3.68–3.57 (4H, m), 2.12–1.92 (4H, m), 1.60 (2H, bs), 1.25 (14H, bs), 0.88 (3H, *t*, $J = 6.5$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.3 (C=S), 66.1, 63.9, 55.1, 50.7, 32.0, 31.0, 30.9, 29.9, 29.8, 29.7, 29.5, 26.1, 24.5, 24.4, 22.8, 14.2; ESI-MS *m*/*z* 434 (M+Na); Anal. Calcd for $C_{17}H_{34}NO_4PS_2$: C, 49.61; H, 8.33; N, 3.40; Found: C, 49.87; H, 8.47; N, 3.54.

5.1.6. 2-(Decyloxy(hydroxy)phosphoryloxy)ethyl piperidine-1-carbodithioate (**18**)

Eluent: 12% methanol–chloroform; Yield 79%; Yellow oil; IR (neat) ν (cm^{-1}): 3425, 2929, 2858, 1641, 1219; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.34–3.97 (4H, m), 3.88–3.78 (4H, m), 3.60 (2H, *t*, $J = 6.3$ Hz), 1.81–1.59 (8H, m), 1.24 (14H, bs), 0.86 (3H, *t*, $J = 6.6$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 195.2 (C=S), 66.0, 63.8, 53.1, 51.4, 32.0, 31.0, 30.9, 29.9, 29.8, 29.7, 29.5, 26.1, 24.4, 22.8, 22.6, 14.2; ESI-MS *m*/*z* 448 (M+Na); Anal. Calcd for $C_{18}H_{36}NO_4PS_2$: C, 50.80; H, 8.53; N, 3.29; Found: C, 50.57; H, 8.61; N, 3.45.

5.1.7. 2-(Decyloxy(hydroxy)phosphoryloxy)ethyl azepane-1-carbodithioate (**19**)

Eluent: 8% methanol–chloroform; Yield 81%; Brown oil; IR (neat) ν (cm^{-1}): 3427, 2928, 2857, 1651, 1216; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.17 (2H, *t*, $J = 5.9$ Hz), 4.06–4.04 (2H, m), 3.91–3.82 (4H, m), 3.62–3.57 (2H, m), 1.85 (4H, bs), 1.68–1.57 (6H, m), 1.25 (14H, bs), 0.87 (3H, *t*, $J = 6.6$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 195.9 (C=S), 66.1, 64.0, 55.8, 52.9, 32.1, 31.0, 29.9, 29.8, 29.7, 29.5, 27.5, 27.1, 26.8, 26.7, 26.4, 26.1, 22.8, 14.2; ESI-MS *m*/*z* 462 (M+Na); Anal. Calcd for $C_{19}H_{38}NO_4PS_2$: C, 51.91; H, 8.71; N, 3.19; Found: C, 51.69; H, 8.65; N, 3.14.

5.1.8. 2-(Decyloxy(hydroxy)phosphoryloxy)ethyl 4-methylpiperazine-1-carbodithioate (**20**)

Eluent: 12% methanol–chloroform; Yield 86%; Light yellow oil; IR (neat) ν (cm^{-1}): 3383, 2926, 2854, 1655, 1227; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.08–4.04 (2H, m), 3.83–3.79 (2H, m), 3.63–3.59 (2H, m), 3.49 (4H, bs), 2.52–2.49 (4H, m), 2.34 (3H, s), 1.60 (2H, bs), 1.26 (14H, bs), 0.88 (3H, *t*, $J = 6.5$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 196.4 (C=S), 66.4, 64.0, 54.6, 45.7, 37.4, 32.2, 31.0, 30.9, 30.1, 30.0, 29.8, 29.6, 26.2, 22.9, 14.3; HRMS *m*/*z* calcd. for $C_{18}H_{37}N_2O_4PS_2$ (MH^+): 441.2011; found 441.2002; Anal. Calcd for $C_{18}H_{37}N_2O_4PS_2$: C, 49.07; H, 8.46; N, 6.36; Found: C, 49.21; H, 8.62; N, 6.51.

5.1.9. 2-(Hydroxy(octyloxy)phosphoryloxy)ethyl pyrrolidine-1-carbodithioate (**21**)

Eluent: 10% methanol–chloroform; Yield 73%; yellow oil; IR (neat) ν (cm^{-1}): 3434, 2929, 2852, 1653, 1217; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.08–4.02 (2H, m), 3.92–3.79 (4H, m), 3.69–3.57 (4H, m), 2.09–1.94 (4H, m), 1.60 (2H, bs), 1.26 (10H, bs), 0.87 (3H, *t*, $J = 6.4$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 192.3 (C=S), 66.2, 64.0, 55.2, 50.8, 36.9, 32.1, 31.1, 29.8, 26.2, 24.4, 22.9, 14.3; ESI-MS *m*/*z* 406 (M+Na); Anal. Calcd for $C_{15}H_{30}NO_4PS_2$: C, 46.98; H, 7.88; N, 3.65; Found: C, 47.15; H, 7.69; N, 3.74.

5.1.10. 2-(Hydroxy(octyloxy)phosphoryloxy)ethyl piperidine-1-carbodithioate (**22**)

Eluent: 8% methanol–chloroform; Yield 78% Yellow oil; IR (neat) ν (cm^{-1}): 3407, 2931, 2859, 1658, 1217; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.24–4.04 (3H, m), 3.84–3.80 (3H, m), 3.62–3.55 (2H, m), 3.11–3.06 (2H, m), 1.81–1.60 (8H, m), 1.25 (10H, bs), 0.87 (3H, *t*, $J = 6.4$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.0 (C=S), 65.9, 63.7, 54.9, 50.5, 36.6, 31.5, 30.6, 30.5, 25.9, 25.4, 24.3, 24.1, 22.5, 13.9; ESI-MS *m*/*z* 420 (M+Na); Anal. Calcd for $C_{16}H_{32}NO_4PS_2$: C, 48.34; H, 8.11; N, 3.52; Found: C, 48.57; H, 7.95; N, 3.68.

5.1.11. 2-(Hydroxy(octyloxy)phosphoryloxy)ethyl azepane-1-carbodithioate (**23**)

Eluent: 12% methanol–chloroform; Yield 85%; Brown oil; IR (neat) ν (cm^{-1}): 3428, 2932, 2859, 1631, 1217; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.16 (2H, *t*, $J = 5.9$ Hz), 4.06–4.02 (2H, m), 3.91–3.80 (4H, m), 3.62–3.57 (2H, m), 1.85 (4H, bs), 1.57–1.56 (6H, m), 1.26 (10H, bs), 0.89–0.85 (3H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 195.9 (C=S), 66.1, 63.9, 55.6, 52.8, 37.3, 32.1, 29.8, 29.6, 27.5, 27.2, 26.9, 26.7, 26.4, 26.1, 22.8, 14.2; ESI-MS *m*/*z* 434 (M+Na); Anal. Calcd for $C_{17}H_{34}NO_4PS_2$: C, 49.61; H, 8.33; N, 3.40; Found: C, 49.35; H, 8.19; N, 3.51.

5.1.12. 2-(Hydroxy(octyloxy)phosphoryloxy)ethyl 4-methylpiperazine-1-carbodithioate (**24**)

Eluent: 10% methanol–chloroform; Yield 88%; Light yellow oil; IR (neat) ν (cm^{-1}): 3420, 2927, 2856, 1651, 1220; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.06–4.02 (3H, m), 3.85–3.79 (2H, m), 3.63–3.59 (2H, m), 3.22 (3H, bs), 2.50–2.47 (4H, m), 2.32 (3H, s), 1.61 (2H, bs), 1.26 (10H, bs), 0.89–0.85 (3H, m); ^{13}C NMR (50 MHz, $CDCl_3$) δ 196.4 (C=S), 66.2, 63.8, 54.3, 50.5, 45.5, 37.2, 32.0, 30.9, 30.8, 29.7, 29.5, 26.0, 22.7, 14.2; HRMS *m*/*z* calcd. for $C_{16}H_{33}N_2O_4PS_2$ (MH^+): 413.1698; found 413.1692; Anal. Calcd for $C_{16}H_{33}N_2O_4PS_2$: C, 46.58; H, 8.06; N, 6.79; Found: C, 46.81; H, 8.23; N, 6.92.

5.1.13. 2-(Hexyloxy(hydroxy)phosphoryloxy)ethyl pyrrolidine-1-carbodithioate (**25**)

Eluent: 10% methanol–chloroform; Yield 76%; Yellow oil; IR (neat) ν (cm^{-1}): 3419, 2956, 2851, 1638, 1217; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.07–4.05 (2H, m), 3.93–3.80 (3H, m), 3.68–3.58 (3H, m), 3.21 (2H, bs), 2.09–1.95 (4H, m),1.60–1.58 (2H, m), 1.29 (6H, bs),

0.89–0.85 (3H, m); ^{13}C NMR (50 MHz, $CDCl_3$) δ 192.3 (C═S), 66.0, 64.0, 55.1, 50.7, 31.8, 30.8, 26.2, 25.7, 24.6, 24.4, 22.8, 14.2; ESI-MS *m/z* 378 (M+Na); Anal. Calcd for $C_{13}H_{26}NO_4PS_2$: C, 43.93; H, 7.37; N, 3.94; Found: C, 43.79; H, 7.46; N, 3.82.

*5.1.14. 2-(Hexyloxy(hydroxy)phosphoryloxy)ethyl piperidine-1-carbodithioate (**26**)*

Eluent: 12% methanol–chloroform; Yield 82%; Light yellow oil; IR (neat) ν (cm^{-1}): 3443, 2956, 2857, 1640, 1219; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.24–4.00 (3H, m), 3.90–3.80 (3H, m), 3.63–3.59 (2H, m), 3.06–3.04 (2H, m), 1.81–1.61 (8H, m), 1.28 (6H, bs), 0.87 (3H, *t*, $J = 6.4$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 195.1 (C═S), 66.0, 63.9, 53.0, 51.6, 37.4, 31.7, 30.8, 25.6, 24.4, 22.7, 14.1; ESI-MS *m/z* 392 (M+Na); Anal. Calcd for $C_{14}H_{28}NO_4PS_2$: C, 45.51; H, 7.64; N, 3.79; Found: C, 45.73; H, 7.49; N, 3.92.

*5.1.15. 2-(Hexyloxy(hydroxy)phosphoryloxy)ethyl azepane-1-carbodithioate (**27**)*

Eluent: 8% methanol–chloroform; Yield 87%; Brown oil; IR (neat) ν (cm^{-1}): 3418, 2931, 2860, 1644, 1216; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.18–4.04 (4H, m), 3.91–3.82 (4H, m), 3.62–3.58 (2H, m), 1.85 (4H, bs), 1.68–1.56 (6H, m), 1.28 (6H, bs), 0.87 (3H, *t*, $J = 6.4$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 195.8 (C═S), 66.1, 63.9, 55.7, 52.9, 37.4, 31.8, 27.5, 27.1, 26.8, 26.6, 26.3, 25.7, 22.8, 14.2; ESI-MS *m/z* 406 (M+Na); Anal. Calcd for $C_{15}H_{30}NO_4PS_2$: C, 46.98; H, 7.88; N, 3.65; Found: C, 47.16; H, 7.99; N, 3.83.

*5.1.16. 2-(Hexyloxy(hydroxy)phosphoryloxy)ethyl 4-methylpiperazine-1-carbodithioate (**28**)*

Eluent: 10% methanol–chloroform; Yield 85%; Light yellow oil; IR(neat) ν (cm^{-1}): 3394, 2955, 2861, 1646, 1217; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.33–3.98 (4H, m), 3.86–3.80 (3H, m), 3.63–3.54 (3H, m), 2.67–2.47 (4H, m), 2.32 (3H, s), 1.60 (2H, bs), 1.29 (6H, bs), 0.88 (3H, *t*, $J = 6.2$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 196.3 (C═S), 66.3, 63.8, 54.4, 51.1, 49.7, 45.6, 37.2, 31.9, 30.8, 29.7, 25.7, 22.8, 14.3; HRMS *m/z* calcd. for $C_{14}H_{29}N_2O_4PS_2$ (MH^+): 385.1385; found 385.1382; Anal. Calcd for $C_{14}H_{29}N_2O_4PS_2$: C, 43.73; H, 7.60; N, 7.29; Found: C, 43.51; H, 7.81; N, 7.15.

*5.1.17. 2-(Hydroxy(propoxy)phosphoryloxy)ethyl pyrrolidine-1-carbodithioate (**29**)*

Eluent: 8% methanol–chloroform; Yield 76%; Yellow oil; IR (neat) ν (cm^{-1}): 3409, 2928, 2858, 1652, 1217; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.08–4.02 (2H, m), 3.90 (2H, *t*, $J = 6.79$ Hz), 3.83–3.77 (2H, m), 3.68–3.57 (4H, m), 2.00–1.95 (4H, m),1.69–1.57 (2H, m), 0.91 (3H, *t*, $J = 7.4$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.0 (C═S), 67.3, 63.7, 55.0, 50.5, 36.6, 24.3, 24.1, 23.8, 10.3; ESI-MS *m/z* 336 (M+Na); Anal. Calcd for $C_{10}H_{20}NO_4PS_2$: C, 38.33; H, 6.43; N, 4.47; Found: C, 38.17; H, 6.58; N, 4.61.

*5.1.18. 2-(Hydroxy(propoxy)phosphoryloxy)ethyl piperidine-1-carbodithioate (**30**)*

Eluent: 12% methanol–chloroform; Yield 80%; Yellow oil; IR (neat) ν (cm^{-1}): 3450, 2959, 2856, 1637, 1213; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.26–4.04 (4H, m), 3.91–3.78 (3H, m), 3.63–3.59 (1H, m), 3.09–3.05 (2H, m), 1.83–1.60 (8H, m), 0.92 (3H, *t*, $J = 7.3$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9 (C═S), 67.4, 63.7, 55.0, 50.6, 36.4, 25.9, 24.3, 24.1, 23.7, 10.2; ESI-MS *m/z* 350 (M+Na); Anal. Calcd for $C_{11}H_{22}NO_4PS_2$: C, 40.35; H, 6.77; N, 4.28; Found: C, 40.59; H, 6.98; N, 4.19.

*5.1.19. 2-(Hydroxy(propoxy)phosphoryloxy)ethyl azepane-1-carbodithioate (**31**)*

Eluent: 10% methanol–chloroform; Yield 84%; Brown oil; IR (neat) ν (cm^{-1}): 3402, 2929, 2853, 1622, 1218; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.19–4.04 (6H, m), 3.90–3.86 (2H, m), 3.81–3.75 (2H, m), 1.85 (4H, bs), 1.68–1.57 (6H, m), 0.91 (3H, *t*, $J = 7.3$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 195.9 (C═S), 67.3, 63.8, 55.7, 52.8, 37.3, 27.0, 26.7, 26.6, 26.3, 25.1, 10.3; ESI-MS *m/z* 364 (M+Na). Anal. Calcd for $C_{12}H_{24}NO_4PS_2$: C, 42.21; H, 7.09; N, 4.10; Found: C, 42.37; H, 7.21; N, 4.01.

*5.1.20. 2-(Hydroxy(propoxy)phosphoryloxy)ethyl 4-methylpiperazine-1-carbodithioate (**32**)*

Eluent: 10% methanol–chloroform; Yield 87%; Light yellow oil; IR (neat) ν (cm^{-1}): 3449, 2958, 2809, 1654, 1221; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.06–4.04 (2H, m), 3.81–3.76 (6H, m), 3.61 (2H, *t*, $J = 6.1$ Hz), 2.50–2.47 (4H, m), 2.32 (3H, s), 1.69–1.57 (2H, m), 0.91 (3H, *t*, $J = 7.4$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 196.5 (C═S), 67.6, 63.8, 54.4, 50.9, 45.6, 37.4, 29.7, 23.9, 10.6; HRMS *m/z* calcd. for $C_{11}H_{23}N_2O_4PS_2$ (MH^+): 343.0915; found 343.0913; Anal. Calcd for $C_{11}H_{23}N_2O_4PS_2$: C, 38.58; H, 6.77; N, 8.18; Found: C, 38.71; H, 6.85; N, 8.32.

5.2. Biology

5.2.1. Cell culture

Human breast cancer cell lines, MCF-7 and MDA-MB-231; and prostate cancer cell lines, PC-3 and DU-145, were originally obtained from American Type of Cell Culture Collection (ATCC), USA and stocks were maintained in laboratory. HEK-293 cells were obtained from institutional cell repository, CSIR-CDRI. Cells were grown in DMEM (Dulbecco modified Eagle medium, Sigma) supplemented with 10% fetal bovine serum with 1× stabilized antibiotic-antimycotic solution (Sigma) in a CO_2 incubator (Sanyo, Japan) at 37 °C with 5% CO_2 and 90% relative humidity [15].

5.2.2. In vitro assay for anti-cancer activity

Anti-cancer activity of compounds was evaluated using MTT assay [16]. Briefly, 1×10^4 cells in 200 μL of DMEM, supplemented with 10% FBS were seeded in each well of 96-well microculture plates and incubated for 24 h at 37 °C in a CO_2 incubator. Compounds were diluted to the desired concentrations in culture medium. After 18 h of incubation, 10 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) was added to each well and the plates were further incubated for 4 h. Supernatant from each well was carefully removed, formazon crystals were dissolved in 100 μL of DMSO and absorbance at 540 nm wavelength was recorded.

5.2.3. Cell cycle analysis

Cell cycle analysis was carried out following already reported methods [17]. MCF-7 and DU-145 cells were plated and treated with test compounds for 24 h, and harvested. Cells were collected, resuspended at the rate of $1–2 \times 10^6$ cells/mL, fixed with absolute ethanol, treated with RNase A to final concentration of 10 mg/mL, and then stained with Propidium iodide to final concentration 50 μg/mL (Sigma, USA) for 30 min at room temperature. The DNA content of the cells was measured using a FACS Calibur flow cytometer (Becton–Dickinson, San Jose, CA, USA) equipped with ModFit LT 2.0 and analyzed with Cell Quest software.

5.2.4. Annexin V-PI assay for apoptosis detection

Program cell death induced by the lead compound was evaluated with Annexin-V-FITC/PI staining kit (Sigma–Aldrich) using flow cytometry [18]. Briefly, cells were trypsinized and washed in PBS, 1×10^6 cells per mL were resuspended in binding buffer (provided in the kit), and stained with FITC-conjugated annexin-V and propidium iodide. Then, cells were incubated for 10 min in the dark at room temperature, washed with binding buffer, and

analyzed by flow cytometry (FACS Calibur, Becton Dickinson) and Cell Quest software.

5.2.5. Quantitative Real Time PCR

Quantitative Real Time PCR was carried out using already published protocol [19]. Total RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer's instructions. Total RNA (5 μg) was digested using DNase I (Invitrogen). The DNase-treated RNA was then reverse transcribed using cDNA synthesis kit (Fermentas). Triplicate samples of cDNA were amplified using the Real-Time PCR System (Roche, USA). The amplification reaction mixture (10 μL total volume) contained 0.5 μL cDNA, 10 ρmol forward primer (Bcl-2: 5′-agtacctgaaccggcacct-3′, cyclin A1: 5′-ccacagtcagggagtgcttt-3′, cyclinA2: 5′-ggtactgaagtccgggaacc-3′, GAPDH: 5′-gctctctgctcctcctgttc-3′) and 10 pmol reverse primer (Bcl-2: 5′-ggccgtacagttccacaaa-3′, cyclin A1: 5′-aatgggcagtacaggagga-3′, cyclinA2: 5′-gaagatccttaaggggtgca-3′, GAPDH: 5′-acgaccaaatccgttgactc-3′), and 5 μL Taq SYBR Green Mastermix (Roche), and 2.5 μL DEPC-water. GAPDH was used as an endogenous control to normalize the quantification of the target transcripts in each sample.

5.2.6. MMP detection using Jc-1 dye

The lipophilic dye JC-1 was used to measure the mitochondrial membrane potential with flow cytometer using method described earlier [19]. MCF-7 and DU-145 cells were cultured and treated in 6-well plates, rinsed with PBS twice and stained with JC-1 dye with final concentration of 50 μg/mL in culture media for 30 min at 37 °C. Cells were rinsed with ice-cold PBS twice, resuspended in 300 μL ice-cooled PBS, and instantly assessed for fluorescence in red channel (~529 nm) and green channel (~590 nm) using flow cytometer (FACS Calibur, Becton Dickinson) and analyzed with Cell Quest software.

5.2.7. Western blotting for total Akt and phospho-Akt

Western blotting was performed following previously described protocol [23]. Briefly, compound treated cells were washed with ice-cold PBS and then lysed in RIPA buffer. Protein concentrations of the lysates were determined by Bradford method. Lysates containing equal protein amount were electrophoresed in SDS-PAGE gel and proteins were transferred onto PVDF membrane (Millipore, USA), probed against p-Akt (Ser473) and Akt (Cell Signaling Technology). Blots were then incubated for 1 h with 1:5000 HRP-conjugated anti-mouse IgG (Cell Signaling Technology), developed using HRP chemiluminescent solution (ImmobilonTM western, Millipore, USA) and scanned by chemiluminescent gel documentation system (Bio-Rad).

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Appendix A. Supplementary data

Supplementary data like scanned copies of ^{1}H NMR and ^{13}C NMR spectra can be found at http://dx.doi.org/10.1016/j.ejmech.2014.08.028.

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