Research Article

# Hydrophobic Phenoxazines shut down PI3K/Akt/mTOR/P$^{70}$S6/S6 Kinase Pathway and Induce Massive Apoptosis in Rhabdomyosarcoma Cells

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**ABSTRACT**

Akt plays an important role in many types of cancers and has been identified as a therapeutic target. Several types of cancers have posed a major threat to human health. Conventional treatments suffer from limitations of side effects, poor responses and drug-resistance. Phenoxazines have shown diverse biological activities and promising agents in anti-cancer, anti-viral and antibacterial therapy. In this study, we evaluated the effect of phenoxazine derivatives on rhabdomyosarcoma cells. Hydrophobic phenoxazines shut down Akt/mTOR/p70S6/S6 kinase pathway and induce apoptosis in rhabdomyosarcoma cells. There is activation of Akt pathway in rhabdomyosarcoma cell lines which have tumorigenic potential. These cell lines are sensitive to phenoxazines. The phenoxazine derivatives are compared for their ability to inhibit Akt phosphorylation in these cells. The lipophilicity of these compounds increased significantly by increasing the chain length to $(-CH_2)_5$ or $(-CH_2)_6$ from the corresponding $(-CH_2)_3$ or $(-CH_2)_4$ at $N^{10}$-position of the phenoxazine ring. The ability of various phenoxazine derivatives to inhibit Akt phosphorylation in rhabdomyosarcoma cells follows the order: ***N*$^{10}$-hexyl > *N*$^{10}$-pentyl > *N*$^{10}$-butyl > *N*$^{10}$-propyl**. Within the series, -Cl in C-2 position on the phenoxazine ring demonstrated a higher potency compared to phenoxazines with –H in C-2 position, suggesting that chlorine is playing a critical role on the growth inhibition.

**Keywords:** Phenoxazines, apoptosis, rhabdomyosarcoma cell lines, and Akt.

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## INTRODUCTION

Mounting evidence has confirmed the crucial role of Akt as a central player in tumorigenesis. Akt is overexpressed in a variety of human tumors and its increased expression correlates with disease progression. Therefore, intense efforts are underway to develop specific Akt inhibitors as cancer therapeutics. There is increasing evidence to support dysregulation of the PI3-kinase/mTOR pathway in the pathogenesis and progression of human cancers.[1-6] Thus, being the downstream of PI3-kinase, phosphorylation of Akt is considered as a nodal point in signal transduction pathway. Through suppression of the tuberous sclerosis complex (TSC), Akt indirectly regulates mTOR signaling, hence it regulates cap-dependent translation.[5] Akt also regulates apoptosis in response to cellular stresses including cytotoxic chemotherapy. The design and development of small molecules that specifically inhibit the Akt kinase activity and its signal transduction pathway in cancer cells are attractive approaches for the development of new cancer therapeutic agents. Akt1/2 dual inhibitors are believed to be more active because of the non-redundant role of Akt-1 and Akt-2 in cell survival and growth. [7,8]

The non-specificity and the drug related adverse effects in later stages of clinical trials hindered the development of Akt inhibitors. Therefore, there is a need for the development of Akt specific inhibitors with high degree of specificity and less adverse effects. Researchers have developed many Akt inhibitors. Currently MK-2206, GDC0068 and perifosine are in phase I and phase II trials.[9-11] Large number of compounds were developed as antitumor agents which includes PI- analogs,[12-16] H-89 analogs,[17] azapane derivatives,[18] Akt/protein kinase B signaling inhibitor-2,[19,20] and compounds containing planar aromatic heterocycles.[21] Inhibitors which selectively impede with different components of PI3K/Akt/mTOR signaling pathway were developed. Rapamycin, RAD001 and PKI-587 were developed as allosteric mTORC1 inhibitors. They show antiproliferative effects *in vitro* and *in vivo*, but are poor inducers of apoptosis.[22-33] Five main classes of PI3K/Akt/mTOR signaling pathway inhibitors namely, the pan-PI3K kinase inhibitors, the Pan-Akt (Akt-1, Akt-2, and Akt-3) inhibitors, mTORC1 inhibitors, mTORC2 inhibitors and dual PI3K and mTORC 1 or **2** inhibitors are currently being investigated in the clinic. [34,35] All five classes of PI3K/Akt/mTOR signaling pathway inhibitors are associated with hyperglycemia [36-41] and unpredicted

clinically significant side effects. A number of phenoxazines have been shown to exert anticancer effects on a variety of cancer cells both *in vitro* and *in vivo*.[42-51] Actinomycin D, an antibiotic produced by *Streptomyces antibioticus*, is one of the most well-known chemotherapy drugs that has long been used in the therapy of various malignant neoplasms.[52,53] This molecule contains a phenoxazine ring which intercalates into DNA, thus inhibiting DNA-dependent RNA polymerase.[54] But actinomycin D has marked adverse effects such as renal cytotoxicity and *myelo* suppression and unpredicted clinically significant side effects. Therefore, there is a need for the development of the anticancer agents, which suppress the proliferation and induce apoptosis of cancer cells at low doses to minimize adverse effects.

The newly-synthesized phenoxazines were found to possess antiproliferative,[55,56] immunosuppressive,[57] antibacterial,[58] anti-inflammatory,[59] and antiviral activity.[60] They have also been shown to prevent human amyloid disorders,[59] to protect neuronal cells from death by oxidative stress.[60] Azuine et al.,[60] also demonstrated a chemo preventive effect of phenoxazine in mouse carcinogenesis models. In particular, some phenoxazine derivatives exerted strong anticancer activity against various cancer cells from human melanoma,[61] pancreatic cancer,[47] neuroblastoma,[62] glioma,[48] and multiple myeloma,[63] exerting apoptotic activity against these cancer cell lines.[47,48,61-63] Moreover, one derivative of phenoxazine inhibited human leukemia cells,[55] HTLV-1 negative T cell and lymphoblastoid cells.[64] Further, Shirato et al.,[62] investigated the anticancer effects of some phenoxazine derivatives on the human glioblastoma cell lines. Subsequently, to search for more potent antitumor compounds, Suzuki et al.,[46] investigated the cytotoxicity of several phenoxazines against oral squamous cell carcinoma HSC-2, HSC-3, HSC-4 cell lines, and promyelocytic leukemia HL-60 cells, and three human normal oral cells (gingival fibroblast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF cells). There are at least three types of cell death, apoptosis, autophagy and necrosis. Tabuchi et al.,[49] found that oxidative phenoxazines induce apoptotic cell death of various cancer cells causing mitochondrial depolarization [63,65,66] and extensive reduction of intracellular pH.[66-70] This view encouraged to investigate the effects of phenoxazines on human neutrophils in terms of apoptosis induction.[71] Of note, cancer cells and neutrophils are similar in that energy requirement relies heavily on glycolysis [72-75] and in that mitochondria seem to operate more significantly in regulating apoptotic cell death than in producing ATP *via* the oxidative phosphorylation, in both these cells.[76,77] Though, it was shown that some phenoxazine derivatives cause apoptosis of some carcinoma cell lines,[47, 55,61,64,77] the detailed pathways for apoptosis caused by phenoxazines have not been clearly investigated.

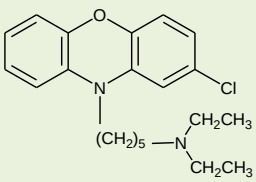
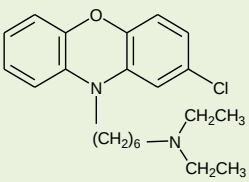
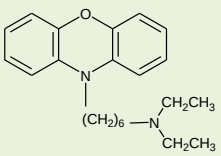
Thimmaiah et al. have reported [78-82] the chemistry and biology of $N^{10}$-substituted phenoxazines synthesized originally as modulators of P-glycoprotein-mediated multidrug resistance (MDR). Later studies revealed that at least part of the activity of some of these phenoxazine MDR modulators is mediated through an unknown, but P-glycoprotein-independent mechanism. As it was established at the time that Akt signaling protects against cellular stress, including cytotoxic agents, it was investigated whether phenoxazine derivatives inhibit Akt and induce apoptosis. The phenoxazine derivatives were screened for their effects on Akt activation in cells derived from pediatric cancers. The results demonstrated that a small group of novel phenoxazines specifically shut down the activation of Akt/mTOR/p70S6/S6 kinase pathway under normal growth conditions and induce apoptosis in rhabdomyosarcoma cells.[83] These results inspired the authors to synthesize more potent phenoxazine derivatives and evaluate them for their anticancer activity against rhabdomyosarcoma cells.

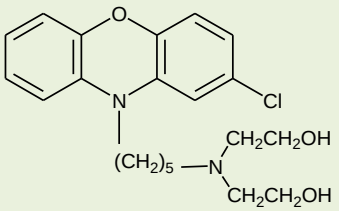
## MATERIALS AND METHODS

### Phenoxazine derivatives

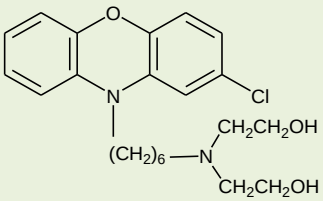
$N^{10}$-Pentyl substituted-2-chlorophenoxazines (3D-10D), $N^{10}$-Hexyl substituted-2-chlorophenoxazines (10D-16D), and $N^{10}$-Hexyl substituted phenoxazines ($10d_1$-$16d_1$) were synthesized as per the procedure reported earlier.[82] The structures of these synthesized compounds are shown in Table 1.

**Table 1:** Structures of phenoxazines

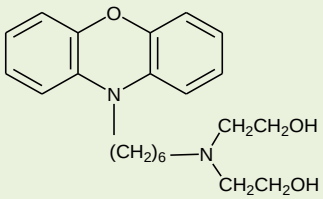
| **$N^{10}$-Pentylsubstituted-2-chlorophenoxazines (3D-9D)** | **$N^{10}$-Hexylsubstituted-2-chlorophenoxazines (10D-16D)** | **$N^{10}$-Hexylsubstituted phenoxazines ($10d_1$-$16d_1$)** |
|---|---|---|
|  10-[5'-(N-Diethylamino)pentyl]-2-chlorophenoxazine. 3D |  10-[6'-(N-Diethylamino)hexyl]-2-chlorophenoxazine. **10D** |  10-[6'-(N-Diethylamino)hexyl] phenoxazine**$10d_1$** |



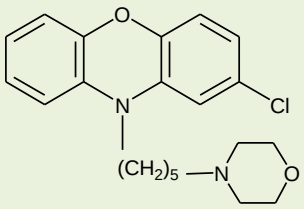
10-[5'-[N-Bis(hydroxyethyl)amino] pentyl]-2-chlorophenoxazine. 4D



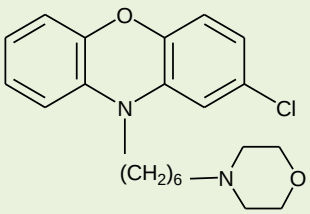
10-[6'-[NBis(hydroxyethyl)amino] hexyl] - 2-chlorophenoxazine. 11D



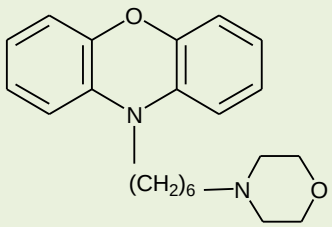
10-[6'-[N-Bis(hydroxyethyl)amino]hexyl] phenoxazine. **11d$_1$**



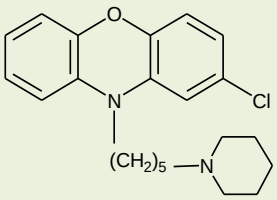
10-[5'-(*N*-Morpholino)pentyl]-2-chlorophenoxazine. **5D**



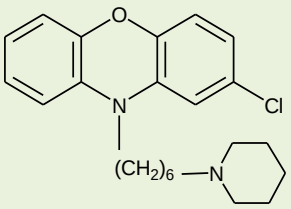
10-[6'-(N-Morpholino)hexyl]-2-chlorophenoxazine. 12D



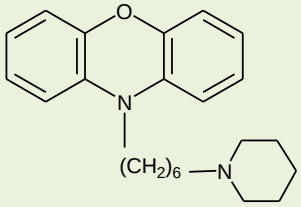
10-[6'-(N-Morpholino)hexyl] phenoxazine 12d$_1$



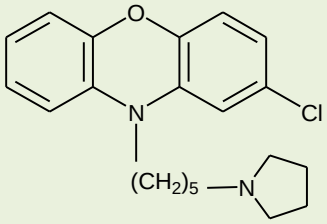
10-[5'-(*N*-Piperidino)pentyl]-2-chlorophenoxazine. 6D



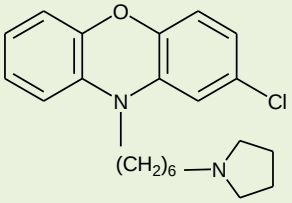
N$^{10}$-[6'-[N-Piperidino]hexyl]-2-chlorophenoxazine. 13D



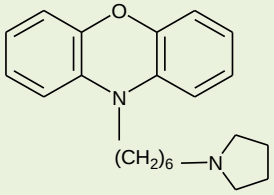
10-[6'-(N-Piperidino)hexyl]phenoxazine. **13d$_1$**



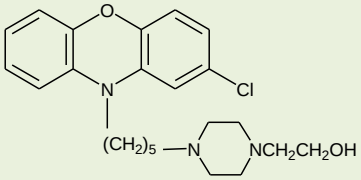
10-[5'-(N-Pyrrolidino)pentyl]-2-chlorophenoxazine. 7D



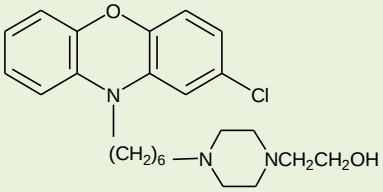
10-[6'-(N-Pyrrolidino)hexyl]-2-chlorophenoxazine. 14D



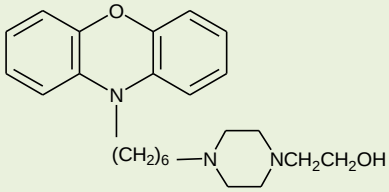
10-[6'-(N-Pyrrolidino)hexylphenoxazine **14d$_1$**



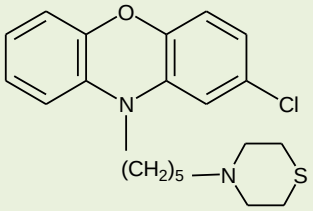
10-[5'-[N-(β-Hydroxyethyl) piperazino]pentyl]-2-chlorophenoxazine. 8D



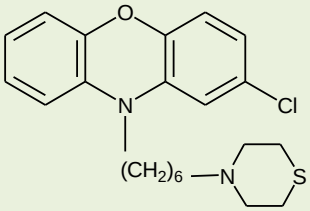
10-[6'-[N-(β-Hydroxyethyl) piperazino]hexyl]-2- chlorophenoxazine. 15D



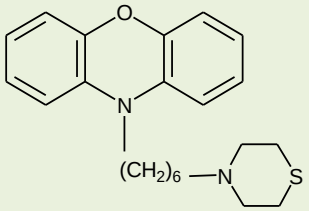
10-[6'-[N-(β-Hydroxyethyl) piperazino]hexyl]phenoxazine. **15d$_1$**



10-[5'-(N-Thiomorpholino)pentyl]-2-chlorophenoxazine. 9D



10-[6'-(N-Thiomorpholino)hexyl]-2-chlorophenoxazine **16D**



10-[6'-(N-Thiomorpholino) hexyl]phenoxazine **16d$_1$**

**Determination of pK$_a$ values**

The pK$_a$ values were determined according to the published method.[84] Briefly, 10 mL of $10^{-3}$ M solution of each compound (3D-16D or 10d$_1$-16d$_1$) was titrated against 1 M HCl. The pH was measured using pH meter with a combination glass electrode.

**Measurement of lipophilicity**

PBS (pH = 6.2) was prepared by mixing 0.1M of $Na_2HPO_4$ and 0.1M of $NaH_2PO_4$. HPLC grade 1-octanol was pre-saturated with PBS and *vice versa*. The compound whose lipophilicity has to be determined was dissolved in PBS buffer at a final concentration of $10^{-4}$M. An equal volume of 1-octanol was added to it. The tubes were then inverted continuously for 15 min. UV-spectrophotometry technique was used to measure the final concentration of the compound in octanol phase and aqueous phase. The partition coefficient, P, was determined by dividing the concentration of the phenoxazine derivative in 1-octanol by the concentration of phenoxazine derivative in the aqueous phase.

**Cell lines and growth conditions**

The human cell lines Rh1, Rh18, Rh30, Rh36, Rh41, RD and JR-1 have been described.[85] The cells were grown in an atmosphere of 5 % $CO_2$. Cells were grown in antibiotic-free RPMI 1640 medium supplemented with 10 % fetal bovine serum and 2 mM L-glutamine at 37 °C. For serum-free experiments, cells were cultured in modified N2E (MN2E) medium (DMEM/F-12; 1:1 mixture) supplemented with 1 µg/mL human holotransferrin, 30 nM sodium selenite, 20 nM progesterone, 100 µM putriscine, 30 nM vitamin E phosphate, and 50 µM ethanolamine. Cells in MN2E medium containing 5 µg/mL bovine fibronectin were plated and allowed to attach overnight at 37 °C in a humidified 5 % $CO_2$ atmosphere.

**Cellular screening**

The cells (Rh1, Rh18 or Rh30) were seeded at a density of 4 x $10^6$ /10 cm plate in serum-free medium for overnight attachment. Cells were exposed to 0.1% DMSO or each of the phenoxazine derivatives (3D-16D or $10d_1$-$16d_1$) for 1 h and then stimulated with IGF-I (10 ng/mL) for 10 min.

**Western blot analysis**

Cells were washed with ice-cold PBS, placed on ice, and lysed in mammalian protein extraction reagent containing one Complete™ mini protease inhibitor tablet, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride and 1 mM $Na_3VO_4$. Cellular debris was pelleted by centrifugation at 17,500 x g for 10 min at 4 °C. Protein concentration of the supernatants was measured by the bicinchoninic acid assay using bovine serum albumin as standard. For the analysis of Akt, Erk-1/2, mTOR, p70S6 kinase, ribosomal protein S6, and GSK-3, equivalent amounts of protein were separated on a 12 % SDS-polyacrylamide gel by electrophoresis and subsequently transferred to a nitrocellulose membrane. After 1 h incubation in 1X TBS buffer containing 0.05 % Tween-20 and 5 % blocking reagent (skim milk) at room temperature, the wet nitrocellulose membranes were then incubated with the appropriate antibodies from Cell Signaling Technologies (Waltham, MA) at the dilutions indicated: rabbit polyclonal antiserum specific for the phosphorylated Ser-473 of Akt (dilution 1:1000); rabbit polyclonal antiserum specific for the phosphorylated Thr-202/Tyr-204 of Erk-1/2 (dilution 1:1000); rabbit polyclonal antiserum specific for the phosphorylated Ser-2448 or Ser-2481 of mTOR (dilution 1:1000); rabbit polyclonal antiserum specific for the phosphorylated Thr-389 of p70 S6 kinase (dilution 1:4000); rabbit polyclonal antiserum specific for the phosphorylated Ser-235/236 of S6 (dilution 1:1000); or rabbit polyclonal antiserum specific for the phosphorylated Ser-21/9 of GSK-3α/β (dilution 1:1000). The secondary antibody was the horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (dilution 1:10,000). Immunoreactive protein was visualized by using Western Lightning chemiluminescence reagent. To ensure that equivalent amounts of protein were loaded on each gel, all immunoblots were treated with stripping buffer (62.5mM Tris-HCl, pH 6.7; 2% SDS; and 100 mM β-mercaptoethanol) for 30 min at 50 °C and then incubated with one of the appropriate antibodies as follows: rabbit polyclonal antibody to Akt (dilution 1:10,000) or mouse monoclonal antibody to β-tubulin (dilution 1:2000).

**Determination of cellular Akt kinase activity**

Rh1 cells were seeded in serum-free medium at a density of 4 x $10^6$ per 10 cm plate for 24 h. Then the cells were exposed to 0.1 % DMSO or phenoxazine derivatives (10D, 15D or $15d_1$) at 100 nM for 1 h. Cells were then stimulated with ± IGF-I (10 ng/mL) for 10 min and washed once with ice-cold PBS. Cells were lysed in 200 µL of ice-cold 1X lysis buffer and incubated for 10 min on ice. The cell lysates were centrifuged for 10 min at 20,000 x g at 4 °C. The volumes of the supernatants were adjusted so that an equal amount of protein (150 µg) was present in each sample; the supernatants were then incubated with immobilized anti-Akt antibody for 3 h at 4 °C. The immune precipitates were pelleted and washed twice in ice-cold cell lysis buffer and twice in kinase buffer. The pellets were suspended in 40 µL of kinase buffer containing 200 µM ATP and 1 µg of a GSK-3 fusion protein, which served as the substrate. After the suspensions were incubated at 30 °C for 30 min, the reaction was terminated by the addition of 3X SDS sample buffer. The samples were boiled for 5 min, and the proteins were separated on a 12 % SDS-polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. Membranes were incubated with rabbit polyclonal anti-phospho-GSK-3α/β (Ser-21/9) antibody.

**Translocation of Akt in rhabdomyosarcoma cells**

Rh1 cells (2 x $10^5$ per chamber) were grown on 2-well glass chamber slides in serum-free medium containing fibronectin (10 µg/mL). After 20 h, cells were exposed to 0.1 % DMSO or phenoxazine derivative (100 nM) for 1 h and then stimulated with IGF-I (10ng/mL) for 20 min. Cells were washed twice with PBS and fixed in 4 % formaldehyde for 30 min at room temperature. The samples were then rinsed twice with PBS and permealized with 1 % Triton X-100 for 5 min at room temperature. After rinsing twice with PBS, the cells were incubated with an anti-Akt antibody (1:50 dilution) for 45 min at 37 °C. After

rinsing three times with PBS, the slides were then incubated with an anti-IgG rabbit secondary antibody coupled to Alexa 488 at a dilution of 1:50. The slides were washed and incubated with RNase. After rinsing twice with PBS, the slides were mounted in media containing TOPRO-3 and then analyzed on a Leica confocal microscope.

### Determination of apoptosis

The detection of apoptosis and necrosis was performed by flow cytometry using the ApoAlert™ Annexin V-FITC apoptosis detection kit (Clontech) to evaluate the extent of apoptosis within cell populations. Cells (Rh1, 350,000 per 75-cm$^2$ flask; Rh 18, 800,000 per 75-cm$^2$ flask; or Rh 30, 500,000 per 75-cm$^2$ flask) were grown overnight in complete medium. On day 1, cells were treated with 0.1 % DMSO (vehicle control) or phenoxazine derivatives (10D or 15D). After 4 days, the cells were trypsinized, washed with PBS, and resuspended in 200 µL of binding buffer. Cells were incubated with 10 µL of annexin V-FITC (final concentration, 1 µg/mL) and 500 ng of propidium iodide in a final volume of 410 µL. Cells were incubated at room temperature in the dark for 10 min before flow cytometric analysis was performed as described.[85]

### Statistical analysis

Statistical analysis was done using Student's "*t*" test. The data were presented as the mean ± SD, and $P \leq 0.05$ was considered significant.

## RESULTS AND DISCUSSION

### $PK_a$ value and lipophilicity ($log_{10}$ P)

The effectiveness of any biologically active compound depends in part on its ability to accumulate in cells. Compounds 3D-16D or $10d_1$-$16d_1$ are weak bases and able to exist in both charged (protonated) and uncharged (neutral) forms. The neutral form of compounds diffuse freely and rapidly across biological membranes. On the contrary, the charged form were less membrane permeable and diffuse across membranes at a much reduced rate.[84] Further, the neutral form of the compound diffuses across the plasma membrane and enters into acidic compartment of the cell, where it gets protonated and unable to diffuse out of the cell. The extent of the biological activity of a compound depends on its $pK_a$ value besides other factors. Each of the compounds exhibited at least two $pK_a$ values in the range 4.5-9.8 as shown in the Table 2. Compounds with $pK_a$ values ranging from 7.5-9.8 lie closer to physiological pH, which may suggest that these compounds accumulate in cancer cells as free bases rather than in protonated form. The lipophilicity data varying from 2.57-4.90 expressed in $log_{10}P$ for all the compounds tested are given in Table 2. As anticipated, the lipophilicity of the compounds (3D-16D or $10d_1$-$16d_1$) increased significantly by increasing the chain length to $(-CH_2)_5$ or $(-CH_2)_6$ from the corresponding $(-CH_2)_3$ or $(-CH_2)_4$ at $N^{10}$-position of the phenoxazine ring. A close look at the data for compounds within the series tested, 10D, 15D, and $15d_1$ found to exhibit maximum hydrophobicity. In summary, within the series, at pH 7.4, the compounds are highly lipophilic and it is anticipated that these compounds will accumulate rapidly into cells.

**Table 2:** $pK_a$ value and Lipophilicity data of Phenoxazines

| 3D-9D(Chloropentyl) | | | 10D-16D(Chlorohexyl) | | | $10d_1$-$16d_1$ (Hexyl) | | |
|---|---|---|---|---|---|---|---|---|
| COMP | $PK_a$ | $log_{10}$ P | COMP | $PK_a$ | $log_{10}$ P | COMP | $PK_a$ | $log_{10}$ P |
| **3D** | 5.55<br>9.00 | 4.42 | **10D** | 5.85<br>9.15 | 4.84 | **$10d_1$** | ND | 3.61 |
| **4D** | 5.65<br>9.20 | 2.71 | **11D** | 5.70<br>9.50 | 3.13 | **$11d_1$** | 5.8<br>9.6 | 2.57 |
| **5D** | 4.20<br>7.90 | 3.35 | **12D** | 4.45<br>8.00 | 3.77 | **$12d_1$** | 4.5<br>8.5 | 3.21 |
| **6D** | 5.30<br>8.70 | 4.48 | **13D** | 5.50<br>8.90 | 4.9 | **$13d_1$** | 5.2<br>9.0 | 4.34 |
| **7D** | 5.50<br>9.30 | 4.06 | **14D** | 5.60<br>9.30 | 4.48 | **$14d_1$** | 5.9<br>9.6 | 3.92 |
| **8D** | 5.80<br>9.75 | 2.99 | **15D** | 5.85<br>9.80 | 3.4 | **$15d_1$** | 5.7<br>9.3 | 2.85 |
| **9D** | ND | ND | **16D** | ND | ND | **$16d_1$** | ND | 3.93 |
| ND: ND; not determined | | | | | | | | |

### Activation of Akt pathway in pediatric cancer cells

Rhabdomyosarcoma cells grown in immune deprived mice as xenografts are currently being used routinely as a preclinical model for evaluating new anticancer drugs. Additionally, there is activation of Akt pathway in these cell lines which are sensitive to phenoxazines. To demonstrate that Akt is activated in these cell lines, cells grown in complete medium with 10 % FBS were harvested in logarithmic phase. Samples were analyzed by western blot using the respective antibodies to detect the phospho-as

well as total protein. The data show activation of Akt, mTOR, and S6 kinase in all the cell lines (Fig 1) and level of activation follows the order: Rh36 > Rh18 > JR-1 > Rh41> Rh30 ≈ RD > Rh1 for Akt; JR-1 > RD> Rh18 > Rh30 > Rh36 > Rh41> Rh1 for S6; and RD > JR-1 > Rh36 > Rh30 > Rh18 > Rh1 > Rh41 for mTOR. The results revealed that all the cell lines tested, express the targeted enzymes to a reasonable level. Thus, to examine the efficacy of phenoxazines on cancer cells, Rh1, Rh18 and Rh30 cell lines were selected for further studies.

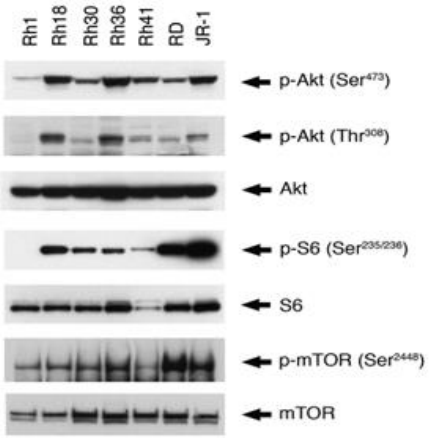



**Fig 1: Activation of Akt pathway in pediatric cancer cells.**

### $N^{10}$-pentyl or $N^{10}$-hexyl phenoxazines inhibited Akt phosphorylation in rhabdomyosarcoma cells

Phenoxazines shown in Table 3 were investigated to determine whether they would inhibit the phosphorylation of Akt at Thr 308 or Ser 473 in pediatric cancer cells. Biochemistry method is used to evaluate the effect of 3D-16D or $10d_1$-$16d_1$ compounds in this study. To determine the concentration of the various phenoxazines required for the inhibition of Akt phosphorylation, serum starved Rh1 cells were exposed to 0.1-2.0 µM phenoxazine derivatives for 1h before stimulating with IGF-I (10 ng/mL) for 10 min. Phospho-specific anti-Akt antibody or anti-Erk-1/2 antibody were respectively used to detect the phosphorylation of Akt or Erk-1/2. Increase in the expression of phosphorylated form of Akt at Ser 473 or Erk-1/2 was observed in response to IGF-I stimulation. Initially compounds 10D, 15D or $15d_1$ were screened at 0.1 µM, 0.5 µM, and 2 µM [Fig 2(A-F)] and subsequently 3D, 8D, 10D or 15D[Fig 2(E-F)] and 4D, 6D, 13D or $14d_1$[Fig3(A-D )]. As shown in Fig 2 and Table 3, compounds 10D, 15D, or $15d_1$ inhibited the phosphorylation of Akt completely at Ser 473 at 100 nM concentration (to an extent of > 95 %), without affecting the phosphorylation of Erk-1/2 (Thr-202/Tyr-204) (Fig 2B and Fig 2E), except 3D and 8D which inhibited phospho-Akt at 500 nM (Fig 2F). The results shown in [Fig 2(G-F)] revealed that at 2 µM concentration, 4D and 6D exhibited the inhibition of phospho-Akt to an extent of 50 %, and 13D or $14d_1$ exhibited the inhibition of phospho-Akt to an extent of 75 %, without affecting phospho-Erk1/2 in Rh1 cells, suggesting that these compounds are less potent than 10D, 15D, or $15d_1$. Further, additional phospho-Akt inhibition experiments were carried out for the compounds 5D, 9D, 11D, 12D, 16D, $11d_1$, or $13d_1$ at 0.1 µM, 0.5 µM or 2.0 µM concentrations. The analysis of the results revealed that these compounds at 2.0 µM either inhibited little (less than 25%) or no inhibition of phosphorylation of Akt at Ser 473 in Rh1 cells [Fig 2(K-N)]. Further, comparison of the data with previously published results revealed that the inhibition of Akt by phenoxazine derivatives follow the order: hexyl > pentyl > butyl > propyl substituted series,[83] suggesting that the lipophilicity seems to be playing an important role in increasing the potency of the compounds. On the contrary, none of the phenoxazines inhibited IGF-I stimulated phosphorylation of Erk1/2. These data imply that the phenoxazines are not inhibiting the IGF-I receptor, insulin receptor substrate proteins, or PI3-kinase, as these pathways are necessary for IGF-I-mediated activation of Erk1/2. Ring unsubstituted phenoxazines were found to be slightly less potent than 2-chlorophenoxazines. Additionally, 10D or 15D at 100 nM concentration exhibited the inhibition of phospho-Akt at Thr-308 to an extent of 75 % in Rh1 cells (data not shown). Of particular note was that morpholino and thiomorpholino derivatives of phenoxazine exhibited no inhibition on Akt activation in cells even at 5 µM concentrations, suggesting that the morpholino or thiomorpholino moiety is not the substrate for Akt enzyme. Nevertheless, the results revealed that 2.0 µM caused maximum inhibition for most of the compounds. However, compounds 10D, 15D or $15d_1$ at 100 nM concentration showed complete inhibition. Thus, further studies concentrated upon the three most active compounds, 10D, 15D and $15d_1$.

**Table 3:** Phenoxazine derivatives inhibit phosphorylation of Akt in sarcoma cells

| 3D-9D(Chloropentyl) | | | | 10D-16D(Chlorohexyl) | | | | $10d_1$-$16d_1$ (Hexyl) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| COMP | Inhibition Conc. in nM | | | COMP | Inhibition Conc. in nM | | | COMP | Inhibition Conc. in nM | | |
| | 100nm | 500nM | 2000nM | | 100nm | 500nM | 2000nM | | 100nm | 500nM | 2000nM |
| **3D** | +++ | ++++ | ++++ | **10D** | ++++ | ++++ | ++++ | **$10d_1$** | ++ | +++ | +++ |
| **4D** | N | N | ++ | **11D** | N | N | N | **$11d_1$** | N | N | + |
| **5D** | N | N | N | **12D** | N | N | N | **$12d_1$** | N | N | N |
| **6D** | N | + | ++ | **13D** | N | ++ | +++ | **$13d_1$** | N | N | + |
| **7D** | N | N | ++ | **14D** | N | ++ | ++++ | **$14d_1$** | ++ | ++ | +++ |
| **8D** | +++ | ++++ | ++++ | **15D** | ++++ | ++++ | ++++ | **$15d_1$** | ++++ | ++++ | ++++ |
| **9D** | N | N | N | **16D** | N | N | N | **$16d_1$** | ND | ND | ND |

** +, 25%; ++, 50%; +++, 75%; ++++, > 95% ; N, no inhibition, ND, not determined

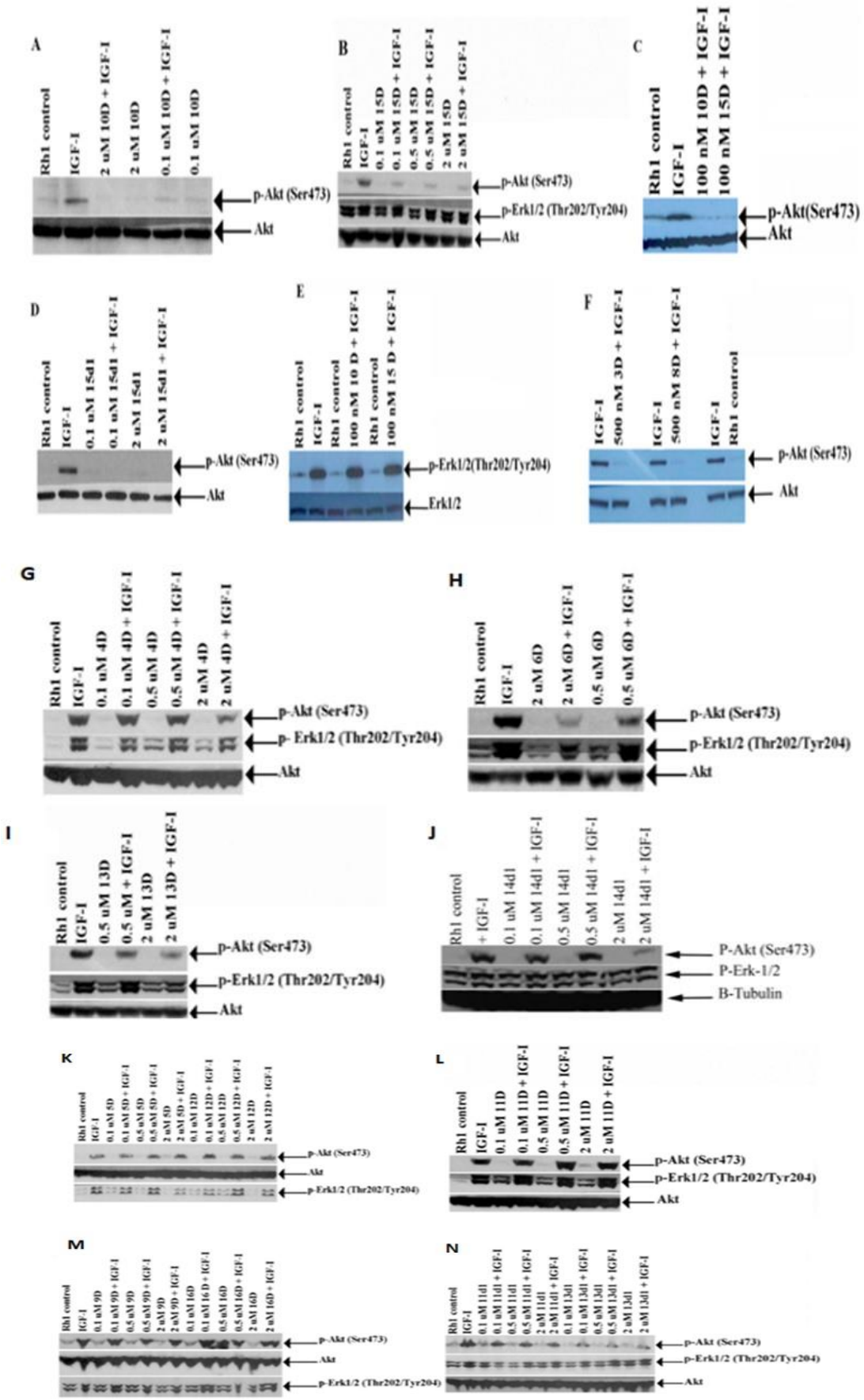



**Figure 2:** Effect of phenoxazine derivatives on Akt phosphorylation

(A-F): Phosphorylation of Akt is inhibited by pentyl or hexyl substituted phenoxazines in Rh1 cells; (G-J): Phenoxazines 4D (in G), 6D (in H), 13D (in I) and 14d1 (in J) inhibit Akt phosphorylation at 2000 nM or above in Rh1 cells; (K-N): Phenoxazines (5D,9D,11D,12D,16D,11$d_1$ and 13$d_1$ do not inhibit Akt phosphorylation at 2000 nM in Rh1 cells)

**Blockade of Akt activation abrogates the function of downstream targets such as mTOR, p-70S6 and S6 kinase**

Data demonstrate that mTOR, p-70S6 kinase and S6 kinase are the downstream targets of Akt. [86,87] To assess the role of Akt inhibition on the p-mTOR (Ser 2448 and Ser 2481 ), p70-S6 kinase (Thr389 ) or p-S6 kinase (Ser235/236), Rh1, Rh18 or Rh30 cells grown in serum-free medium were pretreated with selected phenoxazine derivatives (10D, 15D or $15d_1$) for 1 h at 100 nM and stimulated with IGF-I for 10 min. As shown in Fig 3A (Rh1), Fig 3B (Rh30) or Fig 3C (Rh18) the IGF-I induced phosphorylation of mTOR (Ser2448 and Ser2481), p-70S6 kinase (Thr389) (Rh1, Fig 3D) or S6 kinase (Ser235/236) was inhibited by phenoxazines. Phospho-Akt at Ser 473, phospho-mTOR at Ser 2448, phospho-mTOR at Ser2481, phospho-p70S6 at Thr389 or phospho-S6 kinase at Ser235/236 was checked using the respective phospho-specific antibody as shown in Fig 3(A-D). In all the three cell lines, the IGF-I induced phosphorylation of Akt, mTOR, p70S6 and S6 kinase was effectively blocked by 10D, 15D or $15d_1$. After the membrane was stripped of bound antibody, the membrane was incubated with anti-Akt antibody to determine the total amount of Akt. The data confirm that equal amounts of protein were loaded. As a whole, these results further confirm that phenoxazines derivatives blocks the Akt-mediated activation of mTOR/p70S6kinase/S6 kinase pathway in three cancer cell lines.

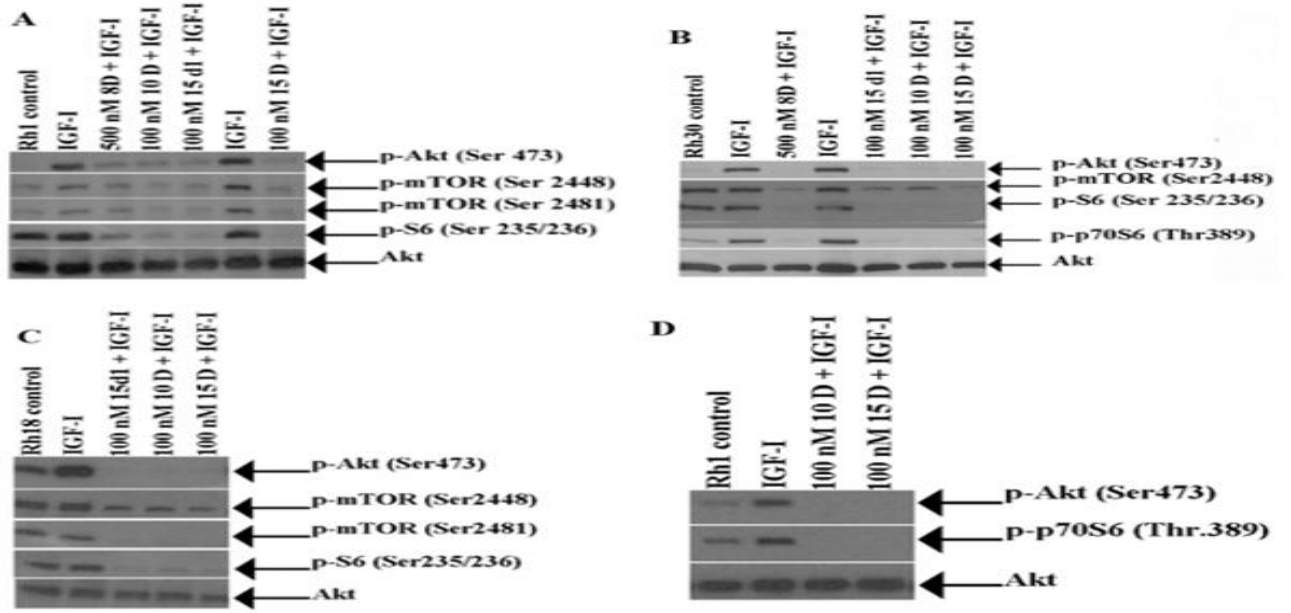



**Fig 3(A-D): Lipophilic phenoxazine derivatives inhibit IGF-I mediated activation of Akt/mTOR/p70S6/S6 kinase pathway in Rh1(A), Rh30(B) or Rh18(C) cells.**

**Phenoxazines do not inhibit the activity of PDK-1**

Although inhibition of Akt would be expected to be consistent with the cytostatic and apoptotic effects observed in cells grown in the presence of 10D or 15D, the inhibition of other AGC family members (PDK-1, SGK-1, etc.) might also contribute to these observed effects. To determine whether or not PDK1 was inhibited by 10D or 15D, an *in vitro* coupled-kinase assay was performed. Recombinant PDK1 was preincubated for 1h with phenoxazines 10D, 15D, or 0.1% DMSO. After this preincubation, the PDK1 substrate SGK1 was added along with ATP and incubated for 15 min followed by the addition of an SGK1 substrate peptide and [$\gamma$-$^{32}$P] ATP. PDK1 activity was measured by the extent to which SGK1 transferred phosphate from [$\gamma$-$^{32}$P] ATP to the substrate peptide. The amount of incorporation of phosphate into the substrate was determined by binding the substrate to phosphocellulose filters and quantitating with scintillation counting. The results indicate that PDK1 is not inhibited by the addition of either 10D or 15D (Fig 4).

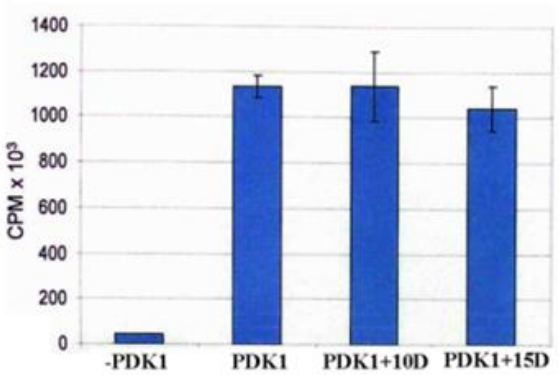



**Fig 4: Phenoxazine derivatives do not inhibit PDK1 activity**

**Phenoxazine derivatives block the translocation of Akt from cytoplasm to the nucleus in Rh1 cells**

The current model for Akt involves its translocation to the nucleus after its activation.[88-91] Therefore, it is expected that phenoxazines decrease the localization of Akt in the nucleus. Confocal microscopy experiments were performed to determine the cellular localization on treatment with phenoxazine 10D or 15D using an anti-Akt

antibody in Rh1 cells. The cells were placed in chamber well slides in serum-free medium for 20 h followed by the addition of 100 nM of 10D or 15D, or DMSO (0.1 %) for 1h, after which 10 ng/mL of IGF-1 was added for 20 min. The cells were then fixed and incubated with anti-Akt antibody as well as TOPRO-3 to identify the nucleus. As can be seen in Fig 5, when compared with IGF-I-stimulated control cells (0.1% DMSO), exposer to either 10D or 15D led to a reduction in the level of Akt present in the nucleus of Rh1 cells. These results are consistent with a block in the nuclear localization when the activation of Akt is inhibited by phenoxazines.

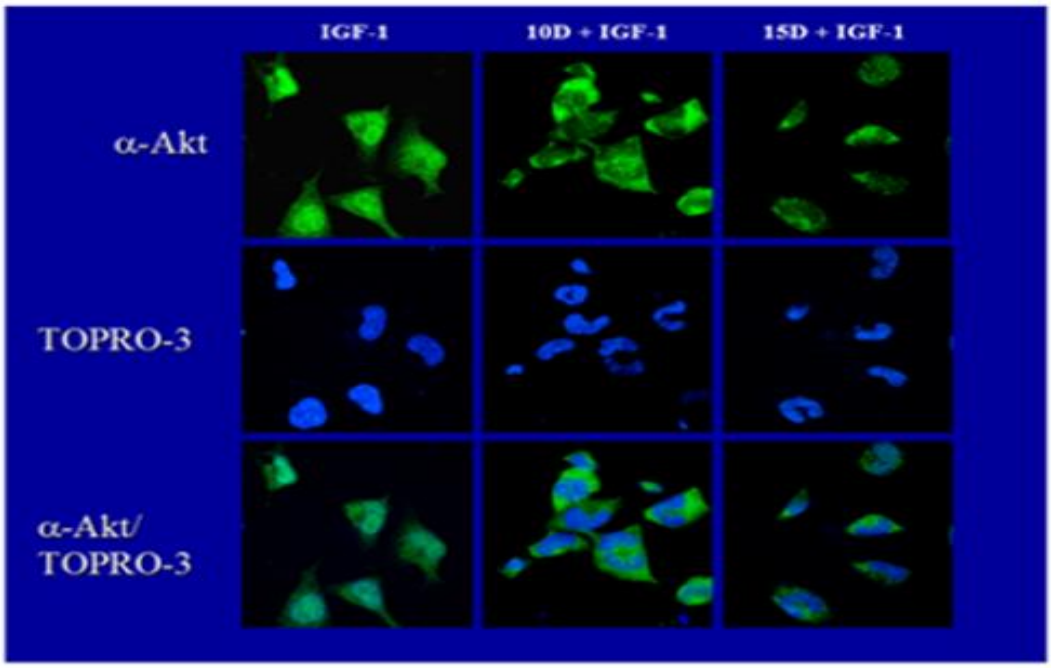



**Fig 5: Phenoxazine derivatives blocks the translocation of Akt from cytoplasm to the nucleus**

**Effects of phenoxazines on apoptosis/necrosis in human rhabdomyosarcoma cell lines**

The effects of various concentrations of phenoxazines (3D-16D and $10d_1$-$16d_1$) on the viability of human rhabdomyosarcoma cells (Rh1) after 6 days of treatment were investigated. To assess the effect on cell growth, cells grown in complete medium were exposed to graded concentrations (0.001-100 µM) of 3D-16D or $10d_1$-$16d_1$ for 6 days. Growth inhibition was assessed by lysing the cells and counting nuclei. The $IC_{50}$ values (data not shown) within the series show the order of potency: -Cl > -H (at C-2 position of the phenoxazine ring), other substituents at position $N^{10}$- being the same. The growth of pediatric cancer cells was extensively suppressed by 10 D or 15D in a dose-dependent manner. However, inhibitory effects of these phenoxazines on the viability differed according to the structure of the compounds and nature of the cell lines.

Study was made on whether the prevention of cell growth of Rh1, Rh18 or Rh30 caused by 10D and 15D was associated with induction of cell death such as apoptosis and necrosis. Since the viability of the cells was greatly reduced by phenoxazine derivatives, the presence of apoptotic cells and necrotic cells was examined in rhabdomyosarcoma cells treated with phenoxazines for 4 days, using flow cytometry (Fig 6). In this case, the staining of cells with both PI and FITC-labeled annexin-V was examined; FITC-labeled annexin-V binds specifically to the phosphatidylserine exposed to the outer membrane in apoptotic cells, and PI can penetrate necrotic cells, but not viable or early apoptotic cells. Consequently, the population of both annexin –V and PI-negative cells (viable cells: plotted in bottom left quadrant) was dominantly observed in the control cells treated with vehicle alone during 4 days (the left columns) (Fig 6). The population of annexin V-positive and PI-negative cells (early stage apoptotic cells: plotted in bottom right quadrant), that of both annexin V- and PI-positive cells (late stage apoptotic/necrotic cells: plotted in the top right quadrant), and annexin V-negative and PI-positive (equal to necrotic cells; top left) increased in rhabdomyosarcoma cells treated with

200 nM of 10D or 100 nM of 15D (details are shown in Fig 6; panel A for Rh1; panel B for Rh30; or panel C for Rh18). Since Akt is an anti-apoptotic kinase, the author investigated the effects of novel phenoxazines on apoptosis. The cells grown in complete medium were treated with 0.1 % DMSO, 10D or 15D continuously for 4 days. Cells were harvested, and the extent of apoptosis was evaluated. The apoptotic population are combined and presented in the Table 4.

**Table 4:** Lipophilic phenoxazines induce apoptosis in Rhabdomyosarcoma cells

| Cell line + treatment | %age of cells ± SD Viable | %age of cells ± SD Apoptotic |
|---|---|---|
| Rh1 control | 89.82 ± 3.49 | 10.13 ± 2.32 |
| Rh1 + 10D | 14.82 ± 6.35 | 85.12 ± 4.89 |
| Rh1 + 15D | 8.41 ± 5.14 | 91.56 ± 6.19 |
| Rh30 control | 89.74 ± 5.89 | 10.22 ± 4.49 |
| Rh30 + 10D | 21.38 ± 7.49 | 78.15 ± 6.18 |
| Rh30 +15D | 16.54 ± 5.31 | 79.10 ± 2.88 |
| Rh18 control | 92.39 ± 2.91 | 7.59 ± 7.01 |
| Rh18 + 10D | 33.13 ± 4.19 | 66.81 ± 5.47 |
| Rh18 + 15D | 18.70 ± 2.16 | 75.39 ± 6.12 |

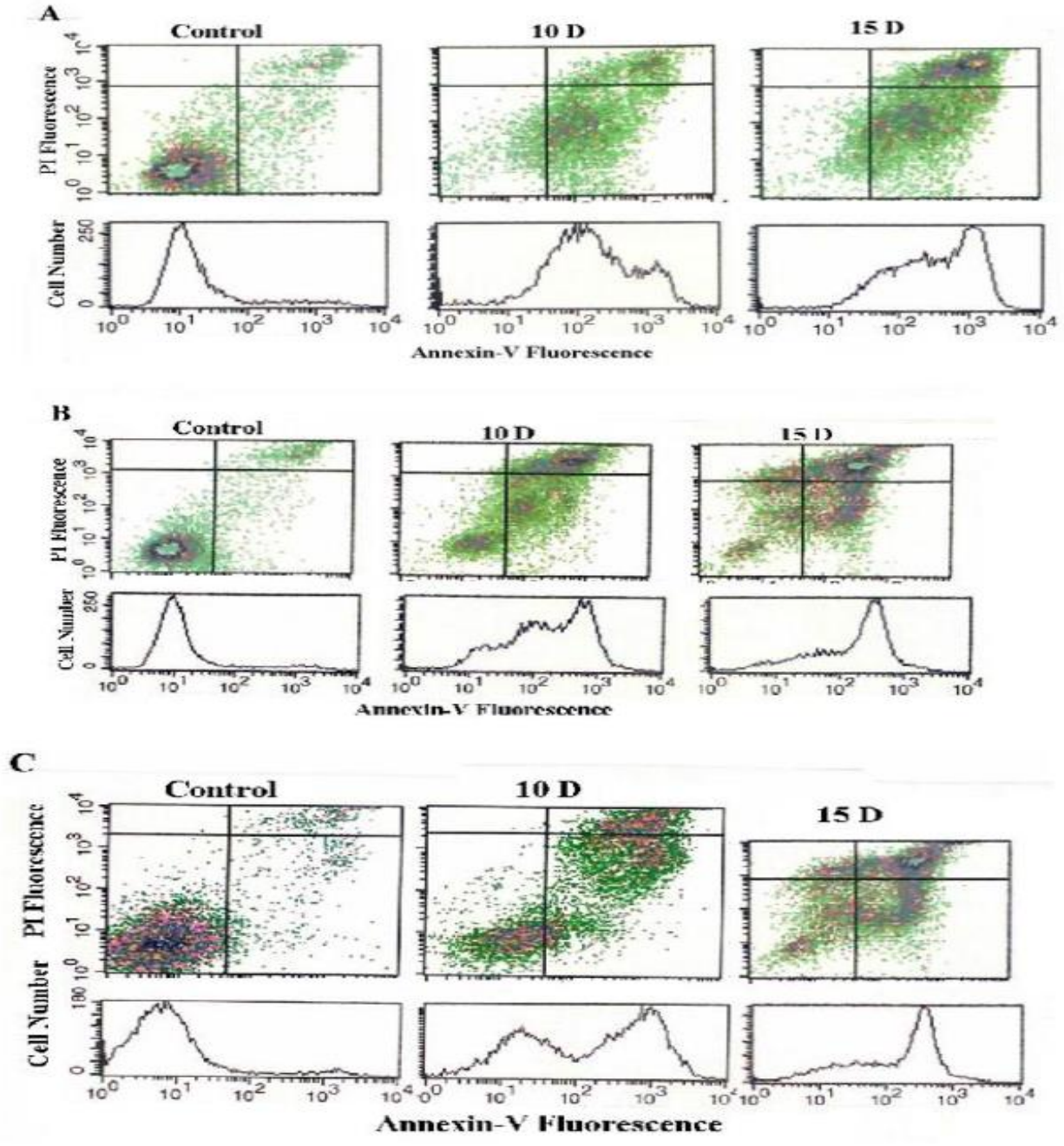



**Figure 6:** changes in the population of the viable cells and the cells in the early stage apoptosis, late stage apoptosis/necrosis and necrosis among rhabdomyosarcoma cell lines.

Approximately 8 to 10% of cells in the control population were undergoing spontaneous apoptosis at the time of evaluation (Table 4). Treatment with 10D and 15D resulted, respectively, in ≈ 85% and 92% apoptosis in Rh1 (Fig 6A), ≈ 78% and 79% apoptosis in Rh30 (Fig 6B),or ≈ 67% and 75% apoptosis in Rh18 (Fig 6C) cells. A significant increase in the proportion of apoptotic cells was evident after treatment with 6D (2.0 µM), 8D (0.5 µM), $14d_1$ (2.0 µM) or $15d_1$ (0.10 µM) (data not given). Thus, active phenoxazines caused intact rhabdomyosarcoma cells to undergo apoptosis. To assess whether the apoptosis of rhabdomyosarcoma cells in response to 10D or 15D was due to a possible general toxic effect of the compound rather than because of inhibition of Akt, the authors compared 10D or 15D with other phenoxazines that poorly inhibit Akt *in vitro* (6D, 8D, or $14d_1$). Because of their overall chemical similarity with the Akt inhibitory phenoxazines, 6D, 8D or $14d_1$ might also be expected to exhibit cellular toxicity if the mechanism was general and independent of Akt. Although, 6D, 8D, or $14d_1$ belong to the same family structurally did not inhibit the phosphorylation of Akt at 0.1 µM concentration. But in contrast to the effect observed with 10D and 15D, neither 6D nor 8D induced apoptosis (data not shown). These results indicate that 10D and 15D are capable of inducing mixed types of cell death with apoptosis and necrosis in human rhabdomyosarcoma cell lines. Thus, there is a correlation between the ability of a compound to inhibit Akt in cells and its ability to induce apoptosis upon treatment of intact cells.

In order to determine the structural requirements of phenoxazines for increased Akt inhibition, previously Thimmaiah et al.,[81] have found that parameters such as lipophilicity, a tricyclic ring system with an –NH group at position 10 and a highly electronegative atom like oxygen at position 5 seem to be essential. In the present study, a series of 21 phenoxazine derivatives are prepared. Their high lipohilicity is presumably due to increased chain length to $-(CH_2)_5$ or $-(CH_2)_6$ at $N^{10}$-position, and they are examined for their ability to inhibit phospho-Akt in rhabdomyosarcoma cells. Within the series, there are compounds with six carbon chain length attached to $N^{10}$-position were found to be more potent for inhibiting Akt activity compared to their five carbon chain length counterparts. Thus, their Akt inhibiting ability reinforces with their $IC_{50}$ values or apoptotic efficacy for Rh1, Rh18, and Rh30 cell lines. Substitution of hydrogen by

–Cl in position C-2 increased the efficacy to enhance the Akt inhibition effect and cytotoxicity in cells and this was probably due to enhanced hydrophobicity, which is expressed in $\log_{10}$ P. It is speculated that the phenoxazine nucleus with –Cl at position C-2 appears to exhibit higher affinity for membrane than those with a hydrogen atom which has been exemplified in analogous phenothiazine compounds. Of note was that compounds 10D and 15D, although exhibited the maximum effect on Akt inhibition, also exhibited the maximum antiproliferative and apoptotic effects. They inhibit Akt phosphorylation at nanomolar concentrations. In a previous publication, Thimmaiah et al.,[83] have demonstrated that propyl and butyl derivatives of 2-chlorophenoxazines effectively blocked the Akt phosphorylation at 5 µM concentration in rhabdomyosarcoma cells. The present work was undertaken with the sole purpose of enhancing their ability to block the phosphorylation of Akt at much lower than 5 µM concentration. This resulted in the synthesis of pentyl and hexyl derivatives of phenoxazines (3D-16D and $10d_1$-$16d_1$), which were found to be more hydrophobic in comparison to their respective propyl or butyl counterparts. Three compounds 10D, 15D and $15d_1$ showed the greatest potency by inhibiting the phosphorylation of Akt at Ser473 at 100 nM concentration without affecting phospho-Erk1/2 in Rh1 cells. Consistent with inhibition of Akt, the most potent inhibitors (10D, 15D or $15d_1$) blocked IGF-I-stimulated phosphorylation of mTOR, p70S6 kinase and S6 kinase, downstream of Akt in the signaling cascade. Further, neither 10D nor 15D was found to inhibit recombinant PI3-kinase, PDK1, or the closest member of the AGC kinase family SGK1. However, these compounds (10D and 15D) potently inhibited the activity of recombinant Akt and the mutant enzyme lacking the PH domain (Akt ΔPH). Taken together, these results support the view that the phenoxazines are not like the PH domain-dependent isozyme-specific Akt inhibitors as reported earlier.

The precise mechanism by which phenoxazines inhibit Akt activity is unclear. Probably, phenoxazines may lock the enzyme in an inactive conformation or acting in an allosteric manner. Studies involving co-crystallization of Akt along with 10D or 15D are planned to resolve the mode of phenoxazine binding to Akt. Consistent with inhibition of Akt activation, 10D and 15D suppressed IGF-I-stimulated nuclear translocation of Akt. The apoptogenic drugs may be beneficial for treating cancers. It is of interest that both 10D and 15D Akt inhibitors also induced very significant levels of apoptosis, even under serum-containing conditions of cell growth. This is important, as inhibition of mTOR signaling by rapamycin induces significant apoptosis only under serum-free conditions in Rh1 and Rh30 cells. It has been shown that 10D or 15D induced growth inhibition which was associated with apoptosis/necrosis for Rh1, Rh30 and Rh18 respectively, suggesting that these phenoxazines may be beneficial agents to treat cancer cells. Studies to determine the *in vivo* pharmacokinetics as well as initial studies to determine whether these compounds have anti-tumor properties are currently being undertaken.

## CONCLUSION

In summary, the drugs to induce apoptosis of cancer cells have been recently recognized as the most expectable candidates for anticancer drugs, because inflammation and release of toxic substance may be minimized in the apoptotic cell death. The authors synthesized a series of highly lipophilic phenoxazines derivatives and the compounds 10D, 15D and $15d_1$ were identified as potent

inhibitors of Akt activity *in vitro* and inhibit Akt signaling in cells. The data presented in this article provide evidence of a sustained antitumor effect and promising development for phenoxazines. These compounds induce significant apoptosis in cell lines under serum-containing conditions of culture and hence have differential cellular activities from mTOR inhibitors that induce low level apoptosis under similar conditions.

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