Identification of N^{10}-Substituted Phenoxazines as Potent and Specific Inhibitors of Akt Signaling*

A series of 30 N^{10}-substituted phenoxazines were synthesized and screened as potential inhibitors of Akt. In cellular assays at 5 μM, 17 compounds inhibited insulin-like growth factor factor 1 (IGF-I)-stimulated phosphorylation of Akt (Ser-473) by at least 50% but did not inhibit IGF-I-stimulated phosphorylation of Erk-1/2 (Thr-202/Tyr-204). Substitutions at the 2-position (Cl or CF_{3}) did not alter inhibitory activity, whereas N^{10}-substitutions with derivatives having acetyl (20B) or morpholino (12B) side chain lost activity compared with propyl or butyl substituents (7B and 14B). Inhibition of Akt phosphorylation was associated with the inhibition of IGF-I stimulation of the mammalian target of rapamycin phosphorylation (Ser-2448 and Ser-2481), phosphorylation of p70 S6 kinase (Thr-389), and ribosomal protein S6 (Ser-235/236) in Rh1, Rh18, and Rh30 cell lines. The two most potent compounds 10-[(4’-(N-diethylamino)butyl]-2-chlorophenoxazine (10B) and 10-[(4’-[β-hydroxyethyl]piperazinobutyl]-2-chlorophenoxazine (15B) 

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protein kinase B signaling inhibitor-2 (33), and compounds containing planar aromatic heterocycles (34). This latter group includes phenothiazine derivatives. Isozyme-selective inhibitors have been described and tested for their ability to inhibit both PI 3-kinase and Akt activities (35).

Previously, we have reported (36–40) the chemistry and biology of N10-substituted phenoxazinones synthesized originally as modulators of P-glycoprotein-mediated multidrug resistance (MDR). Some of these N10-substituted phenoxazinones demonstrated significant cytotoxicity per se (hence were not studied further), whereas several derivatives enhanced vincristine toxicity in cells with undetectable levels of P-glycoprotein. From these results, we conclude 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 is now established that Akt signaling protects against cellular stress, including cytotoxic agents, we have investigated whether phenoxazinone derivatives inhibit Akt and induce apoptosis. We screened a number of phenoxazinone derivatives for their effects on Akt activation in cells derived from pediatric cancers. From among these compounds, we report the identification of a small group of novel lead compounds, which at low micromolar concentrations specifically block Akt activation and downstream signaling to substrates such as mTOR, ribosomal protein S6 kinase, and ribosomal protein S6 (S6). These agents do not affect activation of the upstream kinases, PDK1, PI 3-kinase, or other kinases downstream of ras such as Erk-1/2. Furthermore, at low micromolar concentrations, under normal growth conditions, these small molecule inhibitors induce apoptosis in rhabdomyosarcoma cells.

MATERIALS AND METHODS

All the chemicals and supplies were obtained from standard commercial sources unless otherwise indicated. Wortmannin was purchased from Calbiochem. Phenoxazinone derivatives were prepared in pure form according to our procedures published previously (37, 39, 40). Each inhibitor was dissolved in MeSO before being added to culture medium (final concentration 0.1%).

Cell Lines and Growth Conditions—The human cell lines Rh1, Rh18, and Rh30 have been described (41). Briefly, Rh1, Rh18, or Rh30 cells were grown in antibiotic-free RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (HyClone), 2 mM l-glutamine (BioWhittaker) at 37 °C in an atmosphere of 5% CO2. For serum-free experiments, cells were cultured in modified N2E (MN2E) medium (DMEM/F-12, 1:1 mixture) supplemented with 1 µg/ml human transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 µM putrescine, 30 nM vitamin E phosphate, and 50 µM ethanolamine. Cells in MN2E medium containing 5 µg/ml bovine fibrinogen (Sigma) were plated and allowed to attach overnight at 37 °C in a humidified 5% CO2 atmosphere.

Cellular Screening for Inhibitors—Rh1, Rh18, or Rh30 cells were seeded at a density of 4 × 105/10-cm plate in serum-free medium for overnight attachment. Cells were exposed to 0.1% MeSO or each of the phenoxazinone derivatives for 1 h and then stimulated with IGF-1 (10 ng/ml) for 10 min.

Western Blot Analysis—Cells were rapidly washed with ice-cold phosphate-buffered saline (PBS), placed on ice, and lysed in mammalian protein extraction reagent (Pierce) containing one Complete™ mini protease inhibitor tablet (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and 1 mM NaF. Cellular debris was pelleted by centrifugation at 17,500 × g for 10 min at 4 °C. Protein concentration of the supernatants was measured by the bicinchoninic acid assay using bovine serum albumin as the standard (Pierce).

For the analysis of Akt, Erk-1/2, MTOR, p70 S6 kinase, ribosomal protein S6, and GSK-3, equivalent amounts of protein were separated on a 12% SDS-polyacrylamide gel (Bio-Rad) by electrophoresis and subsequently transferred to a nitrocellulose membrane (Bio-Rad). After a 1-h incubation in 1× TBS containing 0.05% Tween 20 and 5% blocking reagent (skim milk) (Upstate Biotechnology Inc., Lake Placid, NY) 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 or Thr-308 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 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-3 (dilution 1:1000). Immunoreactive protein was visualized by using Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences).

To ensure that equivalent amounts of protein were loaded on each gel, all immunoblots were treated with stripping buffer (62.5 mM 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 1:1000) (Cell Signaling Technology) or mouse monoclonal antibody to β-tubulin (dilution 1:2000; Sigma). The secondary antibodies and detection of bound antibody were as described above.

Determination of Cellular Akt Kinase Activity—Rh1 cells were seeded in serum-free medium at a density of 4 × 105 per 10-cm plate. After 24 h, cells were exposed to MeSO (0.1%) or phenoxazines at 5 µM for 1 h. The cells were then stimulated with 10 ng/ml IGF-1 (10 µL) and washed once with ice-cold PBS. Cells were lysed in 200 µl of ice-cold 1× lysis buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride; and 1 mM leupeptin) and incubated for 10 min on ice. The cell lysates were centrifuged for 10 min at 17,500 × g at 4 °C. The volumes of the supernatants were adjusted so that each sample contained an equal amount of protein (150 µg); the supernatants were then incubated with immobilized (cross-linked) anti-Akt antibody for 3 h at 4 °C. The immunoprecipitates were pelleted and washed twice in ice-cold cell lysis buffer and twice in kinase buffer (25 mM Tris, pH 7.5; 5 mM β-glycerophosphate; 2 mM dithiothreitol; 0.1 mM Na3VO4; and 10 mM MgCl2).

The pellets were suspended in 40 µl of kinase buffer containing 200 µg of recombinant Akt kinase from cell lysate. After the suspensions were incubated at 30 °C for 30 min, the reaction was terminated by the addition of 3× SDS sample buffer (187.5 mM Tris-HCl, pH 6.8; 6% SDS; 30% glycerol; 150 mM dithiothreitol; and 0.03% bromphenol blue). 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.

In Vitro Inhibition of Recombinant Akt, AktΔPH, and SGK1—In vitro kinase assays were performed using active recombinant full-length Akt/PKBα (Upstate Biotechnology, Inc.), active recombinant Akt lacking the pleckstrin homology domain, Akt1ΔPH (Upstate Biotechnology, Saratoga, NY), or active recombinant SGK1 (Upstate Biotechnology, Inc.). Briefly, 10 ng of recombinant enzyme in 25 µl of 1× kinase buffer (25 mM Tris, pH 7.5; 5 mM β-glycerophosphate; 2 mM dithiothreitol; 0.1 mM Na3VO4; and 10 mM MgCl2), was mixed with 2.5 µl of MeSO or phenoxazine derivative (5 µM). Samples were incubated on ice for 1 h at which time 1 µg of GSK-3 fusion protein, which served as the substrate, was added followed by ATP (200 µM) to each reaction mixture. After the suspensions were incubated at 30 °C for 30 min, the reaction was terminated by the addition of 3× SDS sample buffer (187.5 mM Tris-HCl, pH 6.8; 6% SDS; 30% glycerol; 150 mM dithiothreitol; and 0.03% bromphenol blue). 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.

Competition Experiments with ATP and Phenoxazines—Concentrations of compound 15B were prepared as 10× stocks in MeSO ranging from 25 µM to 50 µM to give a final reaction concentration range of 2.5 µM to 5 mM. An ATP master mix was prepared containing 0.75 µl of [γ-32P]ATP (PerkinElmer Life Sciences NEG302H), 0.5 µl of 10 mM ATP/γS, 0.5 µl of 1× kinase buffer for each sample. An enzyme/substrate master mix was prepared containing 10 µl of 1× kinase buffer, 5 µl of Akt peptide substrate (670 ng/µl) (Upstate Biotechnology, Inc.), and 5 µl of active Akt (10 ng/µl) (Upstate Biotechnology, Inc.) diluted from stock using 1× kinase buffer. The reactions were set up by adding 2.5 µl of the phenoxazine to the bottom of the tube followed by the addition of 2.5 µl of ATP mix near the bottom of the tube. The reaction was initiated by the addition of 20 µl of the enzyme/substrate
master mix. After the master mix was added to all of the tubes, the samples were placed at 30 °C for 30 min. After incubation the samples were centrifuged briefly and spotted onto phosphocellulose in the same order as the addition of the master mix. After 2 min these samples were added to a beaker with 0.75% phosphoric acid in the same order as above. The samples were washed for 5 min three times in 0.75% phosphoric acid followed by 5 min in acetone. The squares were then placed on Whatman paper and allowed to dry. The radioactivity was quantitated by scintillation counting.

**Molecular Modeling**—Structures of inhibitors were built, and energy was minimized by using SYBYL 6.9.1 two-dimensional modeling software (version 6.9.1, Tripos Associates, St. Louis, MO). The GOLD program (version 2.1.2) (42) from Cambridge Crystallographic Data Center, UK, was used to dock the inhibitors into the ATP site of Akt, the three-dimensional coordinates of which were imported from the Protein Data Bank (code 1O6K). GOLD uses a genetic algorithm to explore ligand conformational flexibility. The program also Optimized the torsion angles of serine and threonine hydroxyl groups as well as lysine NH$_2$ groups to achieve limited receptor flexibility. The ATP site was defined as residues lying within 15 A of Thr-292. Up to 10 different docking solutions were obtained for each molecule, the docking being terminated when the top three solutions were within a root mean square deviation of ±1.5.

**Photoaffinity Labeling of Recombinant Akt**—The photoaffinity labeling experiments were performed on ice in MES buffer (20 mM MES, pH 6.1) using 200 ng of recombinant active Akt (Upstate Biotechnology, Inc.) and 20 μM 5-azido-8-azidoadenosine 5′-[α-32P] triphosphate (SN$_5$[α-32P]ATP) (Altrcorp) in a total reaction volume of 25 μl. All of the samples, including samples containing phenoxazine 15B (250 μM), were incubated on ice for 30 min. After 30 min of incubation, SN$_5$[α-32P]ATP was added and incubated for 1 min. In the same container (200 μM or 1 μM), the ATP was premixed with SN$_5$[α-32P]ATP prior to its addition to the reaction. Immediately after binding, the samples were photo-crosslinked using a handheld model UVLS-28 (UVF) UV lamp set at 254 nm at a distance of 4 cm from the sample surface for 1 min. After cross-linking, 15 μl of 3X SDS sample loading buffer was added to each sample. The entire sample was electrophoresed on a 4–20% Tris glycine gel followed by one rinse with H$_2$O and overnight fixation (50% MeOH, 40% H$_2$O, 10% acetic acid). After fixation the gel was washed two times with 30 min with H$_2$O. The bands were detected using a STORM 860 PhosphorImager (Amersham Biosciences). To verify even loading of Akt, the gels were stained with Coomassie Brilliant Blue (Sigma).

**PI 3-Kinase Assay**—20 ng of recombinant p110α enzyme (AG Scientific, San Diego, CA), Me$_2$SO (5 μM), phenoxazine derivative (5 μM), or wortmannin (5 μM) were placed on ice for 1 h in 100 μl of ice cold buffer (10 mM Tris, pH 7.4; 100 mM NaCl; and 5 mM MgCl$_2$). 10 μg of phosphatidylinositol (Sigma) was then added to each sample, and the incubation was continued on ice for an additional 15 min. ATP (final concentration of 25 μM containing 30 μCi of [γ-32P]ATP) was added to each sample, and the reaction mixtures were incubated at 37 °C for 30 min. The samples were terminated by a 20 μl of 6 X water bath and incubated for an additional 15 min. Then SGK1 substrate peptide (245 μM) followed by ATP (40 μM containing 10 μCi of [γ-32P]ATP) were added, and the reaction mixture was gently vortexed. Samples were incubated at 30 °C for 15 min with a gentle vortexing every 2 min. Samples were centrifuged, and 40 μl of the reaction mixture was spotted onto PE 81 phosphocellulose paper square. After 30 s, the filter was washed four times with 0.75% phosphoric acid and twice with acetone. The filter was drained and transferred into a scintillation vial to which 5 ml of scintillation mixture was added. The amount of incorporated radioactivity into the substrate was determined by scintillation counting. The assay for SGK1 kinase activity was performed as described above for the PDKI assay.

**Translocation of Akt in Rh1 Cells**—Rh1 cells (2 × 10$^6$ per chamber) were grown on 2-well glass chamber slides (Falcon, Franklin Lakes, NJ) in serum-free medium containing fibronectin (10 μg/ml). After 20 h, cells were exposed to Me$_2$SO (0.1%, vehicle control) or phenoxazine derivative (5 μM) for 1 h and then stimulated with IGF-I (10 ng/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 permeabilized 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 (Rockland, West Chester, PA; 1:50 dilution) for 45 min at 37 °C. After rinsing three times with PBS, the slides were then incubated with an anti-lgG rabbit secondary antibody coupled to Alexa 488 (Molecular Probes, Eugene, OR) 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 (Molecular Probes, Eugene, OR) and then analyzed on a Leica confocal microscope.

**Cell Growth Inhibition**—Rh1, Rh18, and Rh30 cells at a density of 6000, 50,000, and 10,000 cells, respectively, were plated per well in 6-well flat-bottom tissue culture plates (Falcon) in complete medium. After 24 h at 37 °C, medium was replaced with fresh medium containing Me$_2$SO (0.1%) or phenoxazines at concentrations ranging from 100 nM to 25 μM. The cells were further incubated for 6 days. Growth was assessed after lysing cells and counting nuclei. All measurements were made in triplicate.

**Determination of Apoptosis**—We used the ApoAlertTM annexin V-FITC apoptosis kit (Clontech) to evaluate the extent of apoptosis within cell populations. Cells (Rh1, 350,000 per 75-cm$^2$ flask; Rh18, 800,000 per 75-cm$^2$ flask; or Rh30, 500,000 per 75-cm$^2$ flask) were grown overnight in complete medium. On day 1, cells were treated with Me$_2$SO (0.1%; vehicle control) or phenoxazine derivative. 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 (FACSCalibur, BD Biosciences) was performed as described (41).

**RESULTS**

**Phenoxazine Derivatives Inhibit Akt Phosphorylation in Cells**—In order to identify novel inhibitors of Akt, phenoxazines shown in Table I, were investigated to determine whether they would inhibit the phosphorylation of Akt (Ser-473 or Thr-308) in cancer cells. For these assays, serum-starved Rh1 cells were exposed to 1–5 μM phenoxazine derivatives for 1 h before stimulating with IGF-I (10 ng/ml) for 10 min. Akt or Erk-1/2 phosphorylation was detected using the phospo-specific anti-Akt antibody or anti-Erk-1/2 antibody. As shown in Fig. 1A–C, to varying degrees, all of the compounds except 5C, 2B, 5B, 9B, 12B, and 16B-21B inhibited the phosphorylation of Akt at Ser-473 or Thr-308 in cancer cells. For these assays, we screened compounds 4B, 5B, 10B, and 15B at 5 μM (Fig. 1A); subsequently, an additional 18 2-chlorophenoxazines were screened (Fig. 1B). Ring unsubstituted phenoxazines and 2-trifluorophenoxazine derivatives were examined at both 1 and 5 μM (Fig. 1C). As shown in Fig. 1A–C, to varying degrees, all of the compounds except 5C, 2B, 5B, 9B, 12B, and 16B-21B inhibited the phosphorylation of Akt at Ser-473 or Thr-308 at a concentration of 5 μM (summarized in Table I). In contrast, none of the phenoxazines inhibited IGF-1-stimulated phosphorylation of Erk-1/2. These data imply that the phenoxazines are not inhibiting the IGF-I receptor, insulin receptor substrate proteins, or PI 3-kinase, as these pathways are necessary for IGF-1-mediated activation of Erk-1/2. Further examination of the data reveals that the inhibition of Akt follows the order butyl > propyl series. Of note was that the morpholinocarbonyl and acetyl derivatives of phenoxazine exhibited little or no inhibition of cellular Akt activation at the concentrations examined. To determine the concentration of the various phenoxazines where Akt phosphorylation is inhibited, cells grown under serum-free conditions were exposed to phenoxazines at 1, 2.5, or 3.5 μM, and phospho-Akt was detected after stimulating with IGF-I. The results revealed that 1 μM caused about 60% and 3.5 μM caused maximum inhibition
for most of the compounds. However, compounds 10B and 15B showed complete inhibition at 2.5 μM (data not shown). Thus, further studies concentrated upon the two most active compounds, 10B and 15B.

Inhibition of Akt Activation Prevents Activation of Its Downstream Targets—There is substantial data indicating that activation of Akt leads to phosphorylation and activation of mTOR, p70 S6 kinase, and S6 ribosomal protein (43, 44). To assess the effect of phenoxazine treatment on phospho-mTOR (the Akt-dependent phosphorylation site Ser-2448 and the autophosphorylation site Ser-2481), phospho-p70 S6 kinase (Thr-389) and phospho-S6 (Ser-235/236), Rh1 cells grown in serum-free medium were pretreated with phenoxazine (3B, 8B, 10B, 12B, or 15B) derivatives for 1 h at 3.5–5.0 μM and stimulated with IGF-I for 10 min. As shown in Fig. 2A, the IGF-I-induced phosphorylation of mTOR (Ser-2448 and Ser-2481), S6 (Ser-235/236), and p70 S6 kinase (Thr-389) was markedly inhibited by phenoxazines 8B, 10B, and 15B, whereas inhibition by 12B was considerably less. These results indicate that phenoxazine derivatives have the ability to shut down the Akt/mTOR survival pathway in Rh1 cells.

To determine whether these effects were general, the studies were extended to other cell lines. Rh1 or Rh30 rhabdomyosarcoma cells grown in serum-free medium were exposed to compounds 10B, 14B, 15B, or 20B at 5 μM for 1 h and then stimulated with IGF-I for 10 min. Phospho-Akt (Ser-473), phospho-mTOR (Ser-2448 or Ser-2481), or phospho-S6 (Ser-235/236) was checked using the respective phospho-specific antibody as shown in Fig. 2C for Rh18 or Fig. 2D for Rh30 cells. In both cell lines, the IGF-I-induced phosphorylation of Akt, mTOR, and S6 was effectively blocked by all of the compounds except 20B. After the membrane was stripped of bound antibody, it was incubated with the anti-Akt antibody to determine the total amount of Akt. The data confirm that equal amounts of protein were loaded. Taken together, these results further confirm that Akt-mediated activation of mTOR/p70 S6 kinase/S6 pathway in three cancer cell lines is blocked by phenoxazine derivatives.

Phenoxazine Derivatives Inhibit Akt Kinase Activity in Cells—These studies strongly suggest that phenoxazines inhibit Akt activation. To determine this directly, we studied the effects of compounds 10B and 15B on the Akt kinase activity in cells. Activation of Akt by IGF-I was evaluated by assessing the phosphorylation of Akt (Ser-473) or the in vitro kinase activity of protein immunoprecipitated by the anti-Akt antibody. To determine whether changes in Akt phosphorylation were correlated with alterations in kinase activity, we examined the phosphorylation status of a target downstream from Akt, e.g. GSK-3β. Rh1 cells grown in serum-free medium were exposed to 0.1% Me2SO or 5 μM of 10B or 15B for 1 h and then stimulated with IGF-I for 10 min. Cell lysates were then immunoprecipitated with immobilized Akt antibody. Immunoprecipi-
| Compt. ID | X       | R               | Name                                      | Inhibition** |
|----------|---------|-----------------|-------------------------------------------|--------------|
| 1B       | Cl      | N               | 2-chlorophenoxazine                        | +            |
| 2B       | Cl      | -CH₂-Cl         | 10-(3'-chloropropyl)-2-chlorophenoxazine   | -            |
| 3B       | Cl      | -CH₂-NCH₂CH₃    | 10-(3'-(N-diethylamino)propyl]-2-chlorophenoxazine | ++          |
| 4B       | Cl      | -CH₂-NCH₂CH₂OH  | 10-[3'-N-bis(hydroxyethyl)amino] propyl]-2-chlorophenoxazine | ++          |
| 5B       | Cl      | -CH₂-NCH₂CH₂OH  | 10-(3'-N-morpholinopropyl)-2-chlorophenoxazine | -            |
| 6B       | Cl      | -CH₂-NCH₂CH₂OH  | 10-(3'-N-piperidinopropyl)-2-chlorophenoxazine | ++          |
| 7B       | Cl      | -CH₂-NCH₂CH₂OH  | 10-(3'-N-pyrrolidinopropyl)-2-chlorophenoxazine | +++         |
| 8B       | Cl      | -CH₂-NCH₂CH₂OH  | 10-[3'-(4-hydroxyethyl)piperazino] propyl]-2-chlorophenoxazine | +++         |
| 9B       | Cl      | -CH₂-Cl         | 10-(4'-chlorobutyl)-2-chlorophenoxazine    | -            |
| 10B      | Cl      | -CH₂-NCH₂CH₂OH  | 10-[4'-N-diethylamino]butyl]-2-chlorophenoxazine | +++         |
| 11B      | Cl      | -CH₂-NCH₂CH₂OH  | 10-[4'-N-bis(hydroxyethyl)amino]butyl]-2-chlorophenoxazine | +++         |
| 12B      | Cl      | -CH₂-NCH₂CH₂OH  | 10-(4'-N-morpholinobutyl)-2-chlorophenoxazine | -            |
| 13B      | Cl      | -CH₂-NCH₂CH₂OH  | 10-(4'-N-piperidinobutyl)-2-chlorophenoxazine | +++         |
| 14B      | Cl      | -CH₂-NCH₂CH₂OH  | 10-(4'-N-pyrrolidinobutyl)-2-chlorophenoxazine | +++         |
| 15B      | Cl      | -CH₂-NCH₂CH₂OH  | 10-[4'-(4-hydroxyethyl)piperazino]butyl]-2-chlorophenoxazine | +++         |
| 16B      | Cl      | -COCH₂Cl        | 10-(chloroacetyl)-2-chlorophenoxazine      | -            |
| 17B      | Cl      | -COCH₂Cl        | 10-[N-diethylamino]acetyl]-2-chlorophenoxazine | -            |
| 18B      | Cl      | -COCH₂Cl        | 10-(N-morpholinocetyl)-2-chlorophenoxazine | -            |
| 19B      | Cl      | -COCH₂Cl        | 10-(N-piperidinocetyl)-2-chlorophenoxazine | -            |
| 20B      | Cl      | -COCH₂Cl        | 10-(N-pyrrolidinocetyl)-2-chlorophenoxazine | -            |
| 21B      | Cl      | -COCH₂Cl        | 10-[4'-(4-hydroxyethyl)piperazino]acetyl]-2-chlorophenoxazine | -            |
| 5C       | CF₃     | -CH₂-NCH₂CH₂OH  | 10-(3'-N-morpholinopropyl)-2-trifluoromethylphenoxazine | -            |
| 11C      | CF₃     | -CH₂-NCH₂CH₂OH  | 10-[4'-(N-bis(hydroxyethyl)amino)]propyl]-2-trifluoromethylphenoxazine | +++         |
| 13C      | CF₃     | -CH₂-NCH₂CH₂OH  | 10-[4'-(N-piperidinobutyl)]-2-trifluoromethylphenoxazine | +++         |
| 4A       | H       | -CH₂-NCH₂CH₂OH  | 10-[3'-(N-bis(hydroxyethyl)amino)]propyl]-2-trifluoromethylphenoxazine | ++          |
| 8A       | H       | -CH₂-NCH₂CH₂OH  | 10-(3'-N-pyrrolidinopropyl)-2-trifluoromethylphenoxazine | ++          |
tates were used in vitro to phosphorylate a GSK-3 fusion protein. Phosphorylation was completely inhibited in cells treated with 10B or 15B (Fig. 3). These results indicate that phenoxazines effectively block the activity of endogenous Akt in Rhl cells.

**Phenoxazines Do Not Inhibit PI 3-Kinase Activity in Vitro**—As discussed above, the finding that the phenoxazine derivatives do not inhibit IGF-1-induced phosphorylation of Erk1/2 implies that they do not inhibit PI 3-kinase. However, cells treated with phenoxazines 10B or 15B exhibit many of the effects observed in cells treated with PI 3-kinase inhibitors such as wortmannin (data not shown). This is not surprising because PI 3-kinase is required for both the association of Akt with the cell membrane via the PH domain of Akt and activation of the Akt kinase function through the phosphorylation of Ser-308 by the 3-phosphoinositide-dependent protein kinase PDK1. To verify that the phenoxazines were not targeting PI 3-kinase, we performed in vitro kinase assays using recombinant enzyme. By using phosphatidylinositol (PI) as the substrate and [γ-32P]ATP as the phosphate donor, the kinase activity of samples treated with wortmannin, 10B, or 15B at 5 μM was compared with an untreated sample. The lipids were resolved by thin layer chromatography and quantitated using a PhosphorImager. As expected, the untreated sample showed robust phosphorylation of PI as can be seen by the levels of phosphatidylinositol 3-phosphate detected (Fig. 4, 1st lane). The PI 3-kinase activity in samples incubated with 10B or 15B was comparable with the untreated sample (Fig. 4, 2nd and 3rd lanes), whereas the wortmannin-treated sample had barely detectable levels of activity as shown by the lack of visible phosphatidylinositol phosphate (Fig. 4, 4th lane) and the low level of detectable counts. These results indicate that the phenoxazines do not inhibit the activity of PI 3-kinase.

**Phenoxazines Do Not Inhibit the Kinase Activity of PDK-1**—Although direct inhibition of Akt by the phenoxazines would be consistent with the observed effects on the Akt signaling pathway, inhibition of PDK1 and the consequent failure of PDK1 to phosphorylate and activate Akt would also be consistent with the observed effects on the Akt signaling pathway. Therefore, we performed an in vitro coupled-kinase assay to study the Akt inhibitory activity of phenoxazines. This assay uses recombinant enzyme to phosphorylate a GSK-3 protein, a substrate of Akt, as a read out. Akt1/PKBα active enzyme (recombinant protein expressed in Sf21 cells, 10 ng/reaction) was preincubated with compound 10B or 15B at 5 μM for 2 h on ice prior to initiating the kinase assay. The results show that compound 15B completely blocked the phosphorylation of GSK-3 (Fig. 6, 2nd and 3rd lanes), whereas inhibition by 10B was nearly complete. These results indicate that phenoxazine derivatives are targeting Akt directly and inhibit its kinase function.

**Phenoxazines Do Not Block Activation of Akt via Its PH Domain**—All three Akt isoforms consist of a conserved domain structure as follows: an amino-terminal pleckstrin homology (PH) domain, a central kinase domain, and a carboxyl-terminal regulatory domain that contains the hydrophobic motif, a characteristic of AGC kinases. The PH domain is a phosphoinositiode-binding motif found in a number of signal-transducung proteins, including Akt that gives membrane-binding properties to the host proteins. The PH domain interacts with membrane lipid products such as PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45). Biochemical analysis revealed that the PH domain of Akt binds preferentially PtdIns(3,4,5)P3 produced by PI 3-kinase (45).

**Phenoxazines Inhibit Recombinant Akt Kinase Activity in Vitro**—To study the Akt inhibitory activity of phenoxazines, we have used the phosphorylation status of GSK-3 protein, a substrate of Akt, as a read out. Akt1/PKBα active enzyme (recombinant protein expressed in Sf21 cells, 10 ng/reaction) was preincubated with compound 10B or 15B at 5 μM for 2 h on ice prior to initiating the kinase assay. The results show that compound 15B completely blocked the phosphorylation of GSK-3 (Fig. 6, 2nd and 3rd lanes), whereas inhibition by 10B was nearly complete. These results indicate that phenoxazine derivatives are targeting Akt directly and inhibit its kinase function.

**Phenoxazines Do Not Inhibit Recombinant SGK1 Kinase Activity in Vitro**—Among the AGC family of kinases to which Akt...
Me2SO or 8B, 10B, 12B, or 15B at 5 μM and allowed to attach overnight. Monolayers were treated with 0.1% Me2SO, or phenoxazines at 5 μM 10B or 15B for 1 h and then stimulated with IGF-I for 10 min. Cell lysates were then immunoprecipitated with Akt antibody. Immunoprecipitates were used in vitro to phosphorylate a GSK-3 fusion protein. Phosphorylation was completely reduced in cells treated with 10B or 15B. These results indicate that phenoxazines inhibited the activity of Akt in Rh1 cells. The results were similar in three independent experiments.

FIG. 3. Phenoxazines inhibit Akt kinase activity in Rh1 cells. Rh1 cells grown in serum-free medium were exposed to 0.1% Me2SO, or phenoxazines at 5 μM 10B or 15B for 1 h and then stimulated with IGF-I for 10 min. Cell lysates were then immunoprecipitated with Akt antibody. Immunoprecipitates were used in vitro to phosphorylate a GSK-3 fusion protein. Phosphorylation was completely reduced in cells treated with 10B or 15B. These results indicate that phenoxazines inhibited the activity of Akt in Rh1 cells. The results were similar in three independent experiments.

FIG. 4. Phenoxazines do not inhibit PI 3-kinase activity. After incubating 20 ng of recombinant p110γ with Me2SO (C), 10B (5 μM), 15B (5 μM), or wortmannin (Wort) (5 μM) in 1× kinase buffer for 1 h on ice, 10 μg of PI was added and incubated for an additional 15 min at the same temperature. 30 μCi of γ-32P]ATP (final concentration 25 μM) was added, and the kinase reaction was allowed to continue for 10 min at 37 °C. The lipids were resolved on a TLC plate and analyzed using a PhosphorImager. The results indicate that the PI 3-kinase activity in samples treated with 10B or 15B was comparable with the control sample, whereas the sample treated with wortmannin had barely detectable levels of activity. Identical results were obtained in two independent experiments.

ATP Competition with Phenoxazines—One possible mechanism of inhibition of Akt by phenoxazines is competition with ATP for binding to the kinase. To address this question, a number of activity assays were performed at various ATP and phenoxazine concentrations. These data were then modeled using equations for various types of inhibition and an experimentally determined ATP K_M of 195 μM. The modeling was unable to distinguish between competitive and noncompetitive inhibition with 50% probability for each type. However, other types of inhibition such as mixed inhibition and uncompetitive cells. Rh30 cells were treated and processed as in C. The results show that 10B or 15B effectively blocked the Akt pathway in Rh30 cells. In contrast, phenoxazine 20B was inactive. Similar results were obtained in two independent experiments.

FIG. 2. A, phenoxazines inhibit IGF-I-mediated activation of Akt kinase pathway in Rh1 cells. Cells were plated in serum-free medium and allowed to attach overnight. Monolayers were treated with 0.1% Me2SO or 3B, 8B, 10B, 12B, or 15B at 5 μM for 1 h, stimulated with IGF-I for 10 min, and processed as in A. The data show that Akt kinase pathway is completely inhibited by 10B, 14B, or 15B in Rh1 cells. Results are representative of two independent experiments. D, phenoxazines inhibit phosphorylation of Akt in Rh30
Enzyme was incubated with Me$_2$SO, 10B (5 µg), or 15B (5 µg) for 1 h on ice. 100 ng of SGK1 was added and incubated for 10 min on ice and then at 30 °C for an additional 15 min. SGK1 substrate peptide (245 µM) and ATP (40 µM) containing 10 µCi of [γ-32P]ATP was added. The kinase reaction was performed at 30 °C for 15 min. The results indicate that the activity of PDK1 was not inhibited by phenoxazines. The results were similar in at least two independent experiments.

Docking of Phenoxazines into the ATP Site of Akt——Based on the possibility that the phenoxazines might be inhibiting Akt by binding to the kinase domain, we carried out docking simulations to determine their ability to bind at the ATP site of the enzyme. The docking mode of the two most active compounds 10B and 15B is shown in Fig. 7, which was the binding mode enzyme. The docking mode of the two most active compounds 10B and 15B is shown in Fig. 7, which was the binding mode site of Akt relative to complex 10B. The addition of a 10-fold excess of ATP (200 µM) relative to 8N$_3$[α-32P]ATP does not further reduce the extent of cross-linking (Fig. 8A, 2nd lane). Most surprisingly, the addition of a 10-fold excess of 15B (200 µM) dramatically increases the extent of the 8N$_3$[α-32P]ATP-Akt complexes formed by photoactivation (Fig. 8, A, 3rd lane, and B, 2nd lane). Furthermore, if a 5-fold excess of ATP (1 mM) relative to 15B is included in the reaction, the extent of cross-linking is reduced to almost the same level as is observed in the sample without 15B (Fig. 8B, 1st lane)
Phenoxazine Derivatives Block the Translocation of Akt from the Cytoplasm to the Nucleus—Upon activation Akt translocates to the nucleus (51–54). Therefore, one of the predicted effects of inhibiting Akt phosphorylation by phenoxazines would be a decrease in localization to the nucleus in response to growth factor stimulation. To address this question, confocal microscopy experiments were performed using an anti-Akt antibody to examine cellular localization in response to treatment with phenoxazines. Rh1 cells were placed in chamber well slides in MN2E medium for 20 h followed by the addition of 5 μM phenoxazine (10B or 15B) or Me2SO (0.1%) vehicle control for 1 h, after which time 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 anti-TOPRO-3 to identify the nucleus. As can be seen in Fig. 9, when compared with IGF-1-stimulated control (0.1% Me2SO) cells, exposure to either 10B or 15B 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.

Phenoxazine Derivatives Inhibit Cell Growth and Induce Apoptosis—To assess the effect of phenoxazines on cell growth, Rh1, Rh18, and Rh30 cells grown in complete medium were exposed to graded concentrations (0.1–25 μM) of 10B or 15B for 6 days. All three cell lines were sensitive to both the compounds with an IC50 value of 2, 5, and 6 μM for Rh1, Rh18, and Rh30 cells, respectively (Fig. 10A). Thus growth inhibition appears to correlate well with the concentration of phenoxazines that inhibit Akt in cells. In contrast, compounds 12B and 20B were at least 10-fold less inhibitory, consistent with their lack of Akt inhibition. Because Akt is an “anti-apoptotic kinase,” we investigated the effects of several phenoxazine derivatives on apoptosis. We exposed Rh1, Rh18, or Rh30 cells grown in complete medium with 0.1% Me2SO, 10B, 11B, 13B, 14B, or 15B at 6.5 μM (Rh1) or 7.5 μM (Rh18 and Rh30) for 4 days. Cells were harvested, and the extent of apoptosis was evaluated by the ApoAlert flow cytometric assay. Within the apoptotic population, cells in the early stages of apoptosis were annexin V-positive and propidium iodide-negative, whereas those in the late stages were annexin V-positive and propidium iodide-positive (Fig. 10B). These populations are combined and presented in Table II. Approximately 10–19% of cells in the control population were undergoing spontaneous apoptosis (Table II). Treatment with 10B and 15B resulted, respectively, in ~52 and

![Image](https://example.com/image.png)

**Fig. 7.** The binding mode of phenoxazine analogs at the ATP site of Akt. Compounds 10B and 15B are colored orange and green, respectively. The protein is shown as a solid ribbon, and only residues interacting with the inhibitors are shown and are colored gray. Hydrogens are not shown.

**Fig. 8.** Phenoxazines facilitate photocross-linking of 8N3[α-32P]ATP to Akt and the competition of ATP with 8N3[α-32P]ATP for Akt binding. Photofinity cross-linking experiments were performed in 20 mM MES, pH 6.1, with 200 ng of recombinant Akt, and 20 μM 8N3[α-32P]ATP (1 min of incubation). The cross-linking was performed by exposing the samples to 254 nm UV light for 1 min. Phenoxazine 15B (A, 3rd lane; B, 2nd and 3rd lanes) was added prior to the 30-min ice incubation performed on all samples. ATP was pre-mixed with the 8N3[α-32P]ATP immediately prior to its addition to the samples without 15B (A, 2nd lane) and containing 15B (B, 3rd lane). The samples were electrophoresed through 4–20% gradient gels, and the amount of 8N3[α-32P]ATP cross-linked to Akt was measured by 32P PhosphorImaging (top, A and B). Coomassie staining of the gels (bottom, A and B) was carried out to show even loading of Akt.

3rd and 1st lanes, respectively). These results clearly indicate that 15B facilitates the interaction of 8N3[α-32P]ATP with Akt and that ATP is able to compete with 8N3[α-32P]ATP or possibly 15B to reduce the interaction of 8N3[α-32P]ATP with Akt.
75% apoptosis in Rh1, 34 and 33% apoptosis in Rh18, and 43 and 89% apoptosis in Rh30 cells. Cells exposed to phenoxazines appeared to lose membrane integrity late in apoptosis yet remained attached to the culture dish. Furthermore, a significant increase in the proportion of apoptotic cells was evident after treatment with 11B, 13B, and 14B (data not shown). To assess whether the apoptosis of these cells in response to 10B or 15B was because of a general toxic effect of the compound rather than because of inhibition of Akt, we compared 10B or 15B with other phenoxazines that poorly inhibit Akt in vitro (12B or 20B). Because of their overall chemical similarity with the Akt inhibitory phenoxazines, 12B and 20B might also be expected to exhibit cellular toxicity if the mechanism was general and independent of Akt. But in contrast to the effect observed with 10B and 15B, neither 12B nor 20B induced apoptosis (data not shown). 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.

**DISCUSSION**

There is increasing evidence to support dysregulation of the PI 3-kinase-mTOR pathway in the pathogenesis and progression of human cancers (55–57). Thus, activation of Akt, downstream of PI 3-kinase, becomes a nodal point in the signaling pathway. Akt indirectly regulates mTOR signaling through suppression of the tuberous sclerosis complex, hence it regulates cap-dependent translation (56). Akt also regulates apoptosis in response to cellular stresses, including cytotoxic chemotherapy. Thus, Akt has become a focus for developing small molecule inhibitors. Here we report identification of N10-substituted phenoxazines, some of which appear to inhibit Akt at low concentrations.

These compounds were originally synthesized as modulators of MDR, hence the required characteristics were for nontoxic analogs that modulated P-glycoprotein-mediated drug resistance. Consequently, analogs that exerted intrinsic cytotoxicity were excluded from further study. The observation that certain phenothiazines (structurally related to phenoxazines), such as trifluoperazine, were identified as putative weak Akt inhibitors (34) prompted us to re-evaluate some of these phenoxazines as

**FIG. 9.** Phenoxazines block the translocation of Akt from cytoplasm to the nucleus. Rh1 cells were plated in serum-free medium and exposed to 0.1% Me2SO, 10B (5 μM), or 15B (5 μM) for 1 h and then stimulated with 10% IGF-I for 10 min. The samples were processed accordingly and examined by confocal microscopy. The results show that the addition of 10B or 15B led to a reduction in the level of Akt present in the nucleus of Rh1 cells. The results were similar in at least two independent experiments.

**FIG. 10.** a, phenoxazines inhibit Akt functions and suppress cell growth. Rh1 cells were seeded in complete medium, allowed to attach overnight, and exposed continuously for 6 days to graded concentrations of 10B (●), 15B (□), 12B (△), or 20B (○). Growth was determined by lysing the cells and counting the nuclei. Data represent the mean ± S.D. values of three separate experiments. b, phenoxazines induce apoptosis. Rh1 cells (panel A), Rh18 cells (panel B), and Rh30 cells (panel C) were grown in complete medium and treated with Me2SO (0.1%; vehicle control) or 6.5 μM 10B or 15B for Rh1 or 7.5 μM 10B or 15B for Rh18 and Rh30 cell lines for 4 days. Apoptosis was evaluated by using the ApoAlert assay. Similar results were obtained in three independent experiments. Ordinate, uptake of propidium iodide (PI); abscissa, annexin V-FITC fluorescence (top panels). Viable cells are represented in the bottom left quadrant. The bottom panels show the corresponding distribution of annexin V-FITC staining of cell population.

Phenoxazine Inhibitors of Akt
Akt inhibitors. In an initial cell-based screen at 5 μM, 18 of 30 phenoxazines induced IGF-I-stimulated phosphorylation of Akt by ~50% but did not inhibit IGF-I stimulation of Erk1/2 phosphorylation. The two compounds 10-[4-(N-diethylamino)butyl]-2-chlorophenoxazine (10B) and 10-[4-(3-hydroxyethyl)piperazino]butyl]-2-chlorophenoxazine (15B) showed the greatest potency. In contrast, compounds such as 10-[4-(N-morpholino)butyl]-2-chlorophenoxazine (12B) and 10-[N-pyrrolidinoacetyl]-2-chlorophenoxazine (20B) showed no inhibitory activity in this assay. Consistent with inhibition of Akt, the most potent inhibitors blocked IGF-I-stimulated phosphorylation of mTOR, p70 S6 kinase, and S6, downstream of Akt in the signaling cascade. More importantly, neither 12B nor 20B exerted any detectable effect on phosphorylation of Akt or downstream effectors. In serum-starved cells, IGF-I rapidly activated Akt, as demonstrated by in vitro kinase assay of immunoprecipitated enzyme using a GSK-3 peptide as substrate. In contrast, Akt immunoprecipitated from cells exposed to 10B or 15B the enzyme was inactive. Although we have not undertaken an exhaustive study of other kinases, neither 10B nor 15B was found to inhibit recombinant PI 3-kinase, PDK1, or the closest member of the AGC kinase family SGK1. However, these phenoxazines potently inhibited the activity of recombinant Akt and the mutant enzyme lacking the PH domain (AktΔPH). Thus, we conclude that the phenoxazines are not like the PH domain-dependent isozyme-specific Akt inhibitors reported recently (35).

The precise mechanism by which phenoxazines inhibit Akt activity is unclear. Initial studies do not rule out the possibility that phenoxazines may be competing with ATP for binding to the enzyme. However, phenoxazines may also be locking the enzyme in an inactive conformation or acting in an allosteric manner. Modeling studies suggest that the inhibitory phenoxazines may bind in the ATP site, whereas those with shorter, more rigid amidomethyl side chains (i.e., 16B-20B) may be sterically hindered from binding. For compounds with electronegative moieties (i.e., 2D, 5B, etc.), there may be repulsion from the site.

The results from the photoaffinity cross-linking experiments, however, complicate the interpretation of the modeling data. The binding of 15B greatly facilitates the interaction of SN[α-32P]ATP with Akt. The simplest interpretation of the data is that 15B binding to Akt results in the enzyme assuming a conformation that is more favorable for the binding of SN[α-32P]ATP. The observation that ATP competes effectively with the SN[α-32P]ATP would argue against the phenoxazines acting as competitive inhibitors for ATP as the modeling data suggest but rather as noncompetitive inhibitors acting allosterically.

Clearly, further studies will be required to determine the specific interaction of the phenoxazines with Akt. Additional studies involving co-crystallization of Akt along with 10B or 15B are planned to resolve the mode of phenoxazine binding to Akt.

Consistent with inhibition of Akt activation, 10B and 15B suppressed IGF-I-stimulated nuclear translocation of Akt. At low micromolar concentrations, these compounds were effective inhibitors of cell growth in three lines examined (Rh1, Rh18, and Rh30). In contrast, compounds 12B and 20B were at 10-fold less potent. Of interest is that both Akt inhibitors 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. Thus, these results demonstrate qualitative differences in cellular responses when different kinases are targeted within the same pathway. mTOR in the rictor-Lst8 (rapamycin-insensitive) complex has been proposed as the putative PDK2 (49). Our data do not formally address whether Akt-inhibitory phenoxazines inhibit this complex. However, preliminary studies show that in vitro neither 10B nor 15B inhibits mTOR kinase activity. Also of interest is that although treatment of cells with rapamycin increases the proportion of cells in the G1 phase of the cell cycle, no such accumulation was observed in cells exposed to either 10B or 15B (data not shown), further demonstrating the differences between Akt and mTOR inhibitors.

In summary, we have identified a series of N10-substituted phenoxazines that are potent inhibitors of Akt activity in vitro and inhibit Akt signaling in cells. These compounds are growth inhibitory and induce significant apoptosis in cell lines under serum-containing conditions of culture and hence have differential cellular activities from mTOR inhibitors that induce only low level apoptosis under similar culture conditions.

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