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***In vitro* antitumor activity (NCI, USA) [SRB procedure]**

The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96 well microtiter plates in 100 μL at plating densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37° C, 5% CO_2, 95% air and 100% relative humidity for 24 hours prior to addition of experimental drugs.

After 24 hours, two plates of each cell line were fixed with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs were solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/ml gentamicin. Additional four, 10-fold or ½ log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 μl of these different drug dilutions were added to the appropriate microtiter wells already containing 100 μl of medium, resulting in the required final drug concentrations.

Following drug addition, the plates were incubated for an additional 48 hours at 37 °C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed by the gentle addition of 50 μl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4 °C. The supernatant is discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μl) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 minutes at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 μl of

80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as:

- [(Ti - Tz) / (C - Tz)] x 100 for concentrations for which Ti ≥ Tz
- [(Ti - Tz) / Tz] x 100 for concentrations for which Ti < Tz.

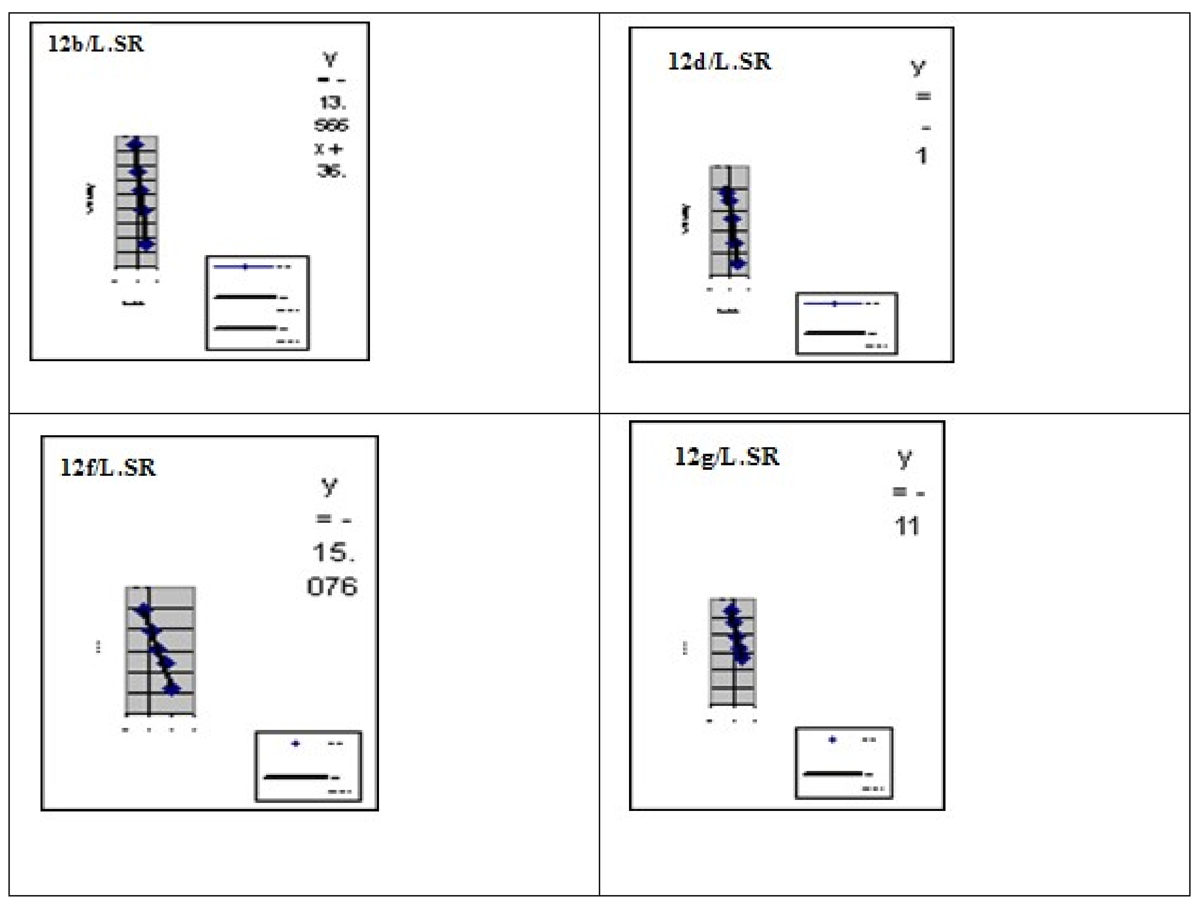
Three dose response parameters were calculated for each experimental agent. Growth inhibition of 50% (GI_{50}) was calculated from [(Ti-Tz)/(C-Tz)] x 100 = 50, which was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti = Tz. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment was calculated from [(Ti - Tz) / Tz] x 100 = -50. Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the value for that parameter was expressed as greater or less than the maximum or minimum concentration tested.

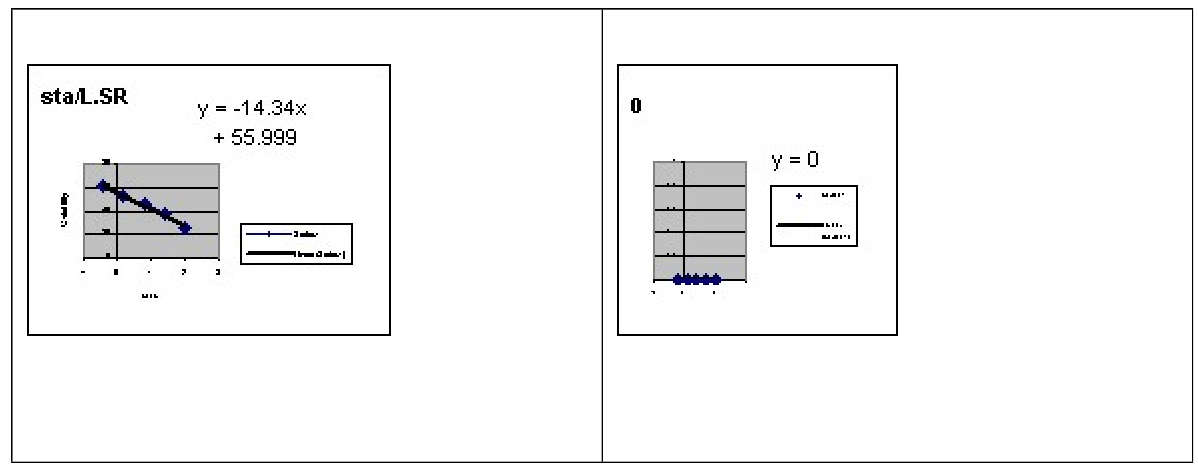
***In vitro* antiproliferative activity against leukemia cell line SR of compounds 12b,d,f,g**

MTT colorimetric assay was performed at the Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. leukemia SR cell line was acquired from American Type Culture Collection. The cells were cultured utilizing Dulbecco's Modified Eagle Medium (DMEM) obtained from Invitrogen/Life Technologies. The medium was supplemented with 10% fetal bovine serum (FBS) from Hyclone, 10 ug/ml of insulin obtained from Sigma, and 1% penicillin-streptomycin. All the remaining chemicals and reagents utilized in the study were sourced from Sigma or Invitrogen. Cell cultures were removed from the incubator into laminar flow hood and each vial of MTT M-5655 was reconstituted to be used with 3 ml of balanced salt solution without phenol red and serum. Reconstituted MTT was added in an amount equal to 10% of the medium culture volume. Cultures were returned to the incubator for 2-4 hours. After the incubation period, the

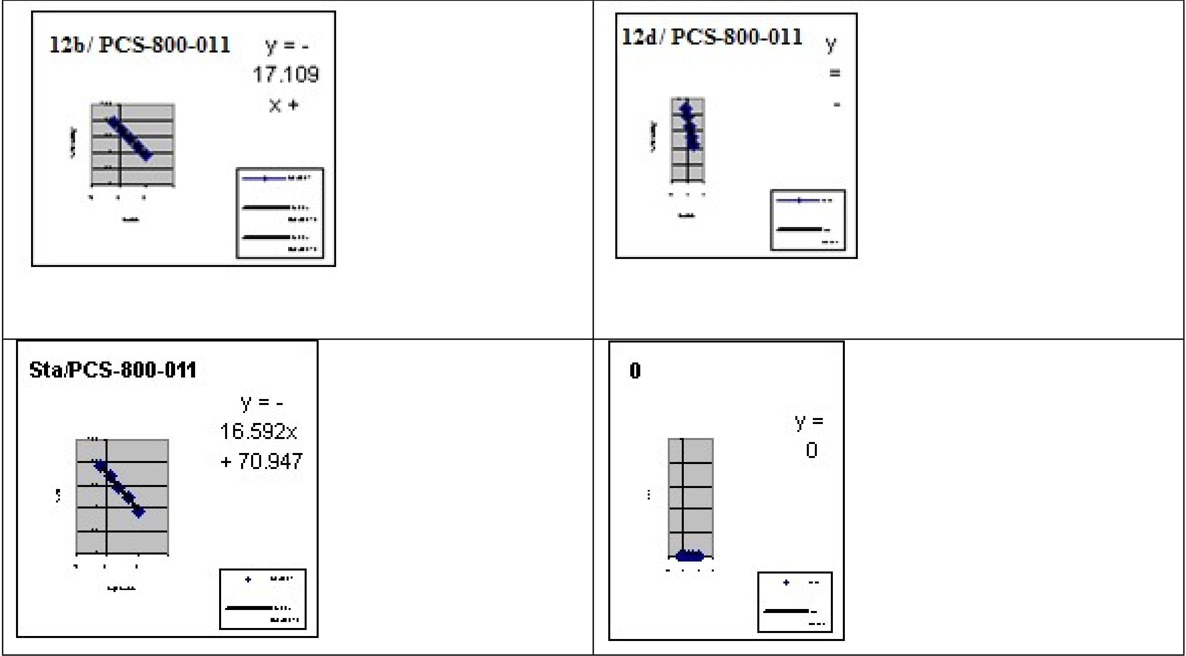
resulting formazan crystals were dissolved by adding an amount of MTT solubilization solution M-8910 equal to the original culture medium volume. The colorimetric assay is measured and recorded at the absorbance of 570 nm using a plate reader (EXL 800, USA). The percentage of the relative cell viability was calculated as (A570 of treated samples/A570 of the untreated sample) X 100.

Dose response curve of *In vitro* antiproliferative activity against leukemia cell line SR of compounds 12b,d,f,g





Dose response curve *In vitro* cytotoxicity of compound 12b and 12d against normal human primary peripheral blood mononuclear cells PCS-800-011



***In vitro* PI3K inhibition assay**

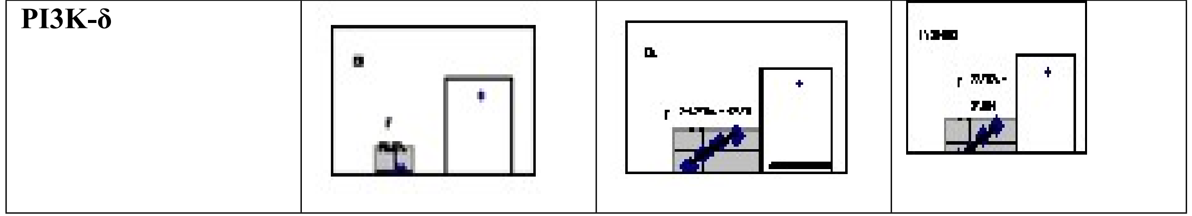
The PI3 Kinase Activity/Inhibitor Assay (ELISA Kit Merck Millipore, USA) was used to evaluate the inhibitory activity of the most active compounds **12b** and **12d** according to the manufacturer's regulations using LY294002 as appositive control.[48] The tested compounds **12b**, **12d** and LY294002 were incubated for 10 min before the addition of PIP2 (phosphatidylinositol (4,5)-bisphosphate) substrate. 5 µl/well of 5x kinase

reaction buffer and 5 µl/well of PIP2 substrate were added, respectively. Also, distilled water was added to each well to obtain 25 µl/final well then incubated for 1 hour. Excluding the control buffer, 25 µl of biotinylated PIP3/EDTA working solution was added to all wells, but 25 µL of tris-buffered saline (TBS) is added to the control wells of the buffer only, followed by the addition of 50 µL/well of GRP1 working solution to all wells then incubated at room temperature for 1 hour. Washed the wells 4 times with 200 µL/well 1X TBST, then added 50 µl (horseradish peroxide as conjugated streptavidin) SA-HRP working solution SA-HRP and incubated for 1 h at room temperature. Wells were washed 3 times with 200 µl of 1X TBST per well, then twice with 200 µl of 1X TBS per well, then 100 µl of substrate 3,3\,5,5\-tetramethylbenzidine were added to the wells and kept in dark for 5–20 min. In the final step, the blue color was assayed at 450 nm after adding 10 µl stop solution. The lower the signal, the higher the PI3 Kinase activity.

$$\text{The relative \% to B-PIP3} = \frac{\text{A450}^{*}\text{ of samples include buffer, kinase inhibitors}}{\text{A450}^{*}\text{ of Biotinylated PIP3- average}} \times 100$$

Dose response curve *In vitro* PI3K inhibition assay

PI3K	12b	12d	LY294002
PI3K-α			
PI3K-β			

PI3K-δ			

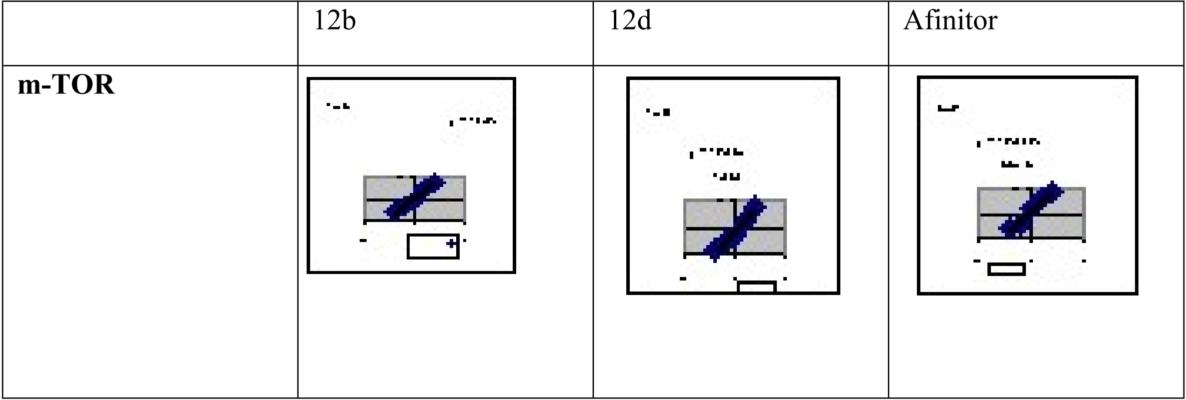
***In vitro*-mTOR inhibitory assay**

Following the arrangement of the necessary quantity of strips from the Glutathione-Coated 96-Well Plate, a volume of 100 µl of mTOR substrate WS was introduced into each well and subjected to incubation for a duration of 1 hour at ambient temperature. The contents of the wells were aspirated, followed by washing each well of the Glutathione-Coated 96-Well Plate with 200 µl of TBS. Subsequently, the contents of the wells were discarded into the sink, and the wells were dried by gently tapping the inverted plate on paper towels. This washing and drying process was repeated three times in total. The following components should be added to each well in the specified order: The mTOR standard or mTOR sample should be diluted to the assay range using a phosphate-free buffer or water. 50 µl. The 2X kinase assay buffer was added to the reaction mixture in a volume of 50 µl. Place the plate sealer over the plate, ensuring complete coverage. Utilize a plate shaker or a similar apparatus to mix the contents for 30 seconds. Subsequently, allow the mixture to incubate for 30 minutes at a temperature of 30 °C.

The kinase reaction can be halted by introducing 10 µl of Kinase Stop Solution into each well. The contents of each well were aspirated and subsequently washed with 200 µl of Plate Wash (1X). Carefully manipulate the plate by employing a plate shaker or a comparable apparatus for 5 minutes. Transfer the contents of the wells into the sink and proceed to dry the wells by gently tapping the inverted plate onto paper towels. Perform two additional wash cycles on the plate without subjecting it to any agitation. In each well, introduce 100 µl of Anti-p70S6K-T389 WS. Proceed to cover the plate with a plate sealer and incubate for 1 hour at ambient temperature. The plate underwent four washing cycles without agitation. To initiate the experiment, introduce 100 µl of HRP-Antibody Conjugate WS into each well. Proceed by securely sealing the plate with the Plate Sealer and allowing it to incubate for 1 hour at ambient temperature.

To initiate the reaction, 100 µl of TMB Substrate was added to each well. The plate was then covered with a plate sealer and incubated at room temperature for 5-20 minutes. The ELISA Stop Solution should be added to each well in a volume of 100 µl. The absorbance of the samples should be measured at 450 nm, ideally with a reference wavelength set at either 540 nm or 595 nm, as the reference recommends.

Dose response curve *In vitro* mTOR inhibitory assay

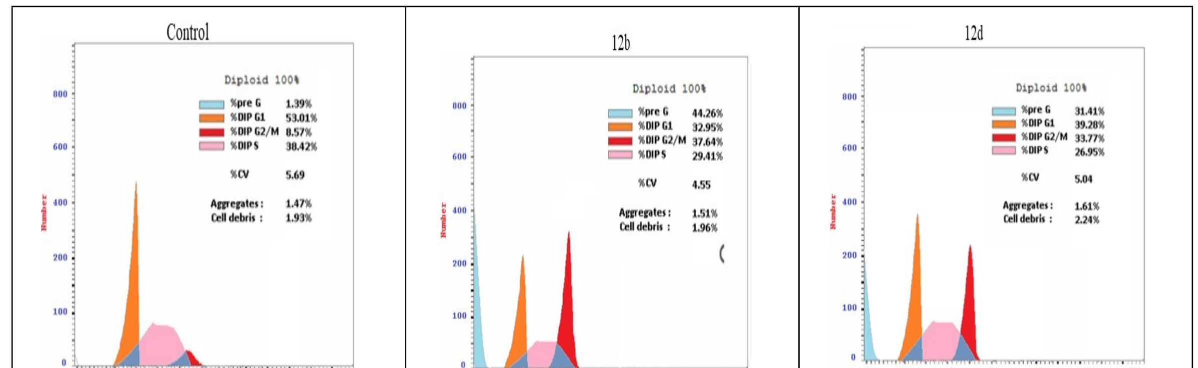


	12b	12d	Afinitor
m-TOR			

<u>Cell cycle analysis</u>

The most active compounds 12b and 12d were further assessed through cell cycle analysis in the leukemia SR cell line using Propidium Iodide Flow Cytometry Kit. The leukemia SR cells were exposed to compounds 12b and 12d at their IC_{50} concentrations for 24 hours. Following treatment, cells were washed twice with ice-cold phosphate-buffered saline (PBS), centrifuged to collect, and finally fixed at -20 °C in 70% (v/v) ethanol. Posteriorly, the cells were re-suspended with 0.1 mg/mLRNase, stained with 40 mg/mL propidium iodide (PI),and then incubated for 1 hour after washing with PBS. They were analyzed by flow cytometry using FACS Calibur (Becton Dickinson), and the distributions of the cell cycle were calculated using Cell-Quest software (Becton Dickinson).

Dose response curve of cell cycle analysis



Detection of apoptosis by Annexin V-FITC assay

To measure the apoptosis induced by compounds **12b** and **12d**, an apoptosis detection kit (BD Biosciences, San Jose, CA) was used according to the manufacturer's protocol, as reported earlier 52, 53. The leukemia SR cells were treated with IC_{50} concentrations of compound **12b** and **12d** for 24 hour, then collected by trypsinization and washed twice with PBS followed by staining with 5 µl Annexin-V-FITC and 5 µl PI in 1× binding buffer, then incubated for 15 min at room temperature in the dark. Analysis of Annexin-V-FITC binding was carried out using FACS caliber flow cyto.meter.

Dose response curve of apoptosis detection by Annexin V-FITC assay