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The potential anticancer agent PK11195 induces apoptosis irrespective of p53 and ATM status in chronic lymphocytic leukemia cells

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Supplementary data

DESIGN AND METHODS

Reagents

1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)3-isoquinolinecarboxamide (PK11195), 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (RO 5-4864), DMSO, propidium iodide (PI), and dithiocyana-tostilbene-2,2-disulfonic acid (DIDS) were from Sigma Chemicals Co. (St Louis, MO). N,N-dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN-1-27) was from Tocris Bioscience (Bristol, UK). H89, and SP600125 were from Calbiochem (San Diego, CA). Anti-Fas, clone CH11 (FasL) was from Upstate (Lake Placid, NY). Annexin V-FITC was from Bender MedSystems (Vienna, Austria). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk) was from Bachem AG (Bubendorf, Switzerland). JC-1 was from Molecular Probes, Inc. (Eugene, OR).

Cell culture

Lymphocytes were cultured immediately after thawing or isolation at a concentration of 2 to $5x10^6$ cells/mL in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Fluorescence in situ hybridization (FISH).

In order to detect prognostically relevant anomalies of chromosomal regions 11q, 13q and 17p, the following fluorescent-labelled DNA probes were used in interphase cytogenetic analyses: LSI ATM (11q23), LSI D13S319 (13q14) and LSI p53 (17p13.1); all probes purchased from Abbot Vysis (Stuttgart, Germany). Sample preparation and FISH were performed according to manufacturer's protocol.

Mitochondrial membrane potential measurements

The potential-sensitive fluorochrome JC-1 (Molecular Probes, Eugene, OR) was used to measure mitochondrial membrane potential ($\Delta\Psi_m$). Cells were incubated with 10 µM JC-1 for 30 minutes at 37°C in the dark. Cells were washed in PBS and analyzed by FACSCalibur. Data analysis was performed with CellQuest software by measuring both the green (530 ± 15 nm, FL-1) and red (585 ± 21 nm, FL-2) JC-1 fluorescence. The loss in $\Delta\Psi_m$ is seen as a shift to lower JC-1 red fluorescence accompanied by an increase in JC-1 green fluorescence. At least 10,000 events were acquired per sample.

Western blot analysis

Cells were lysed with Laemmli sample buffer and Western blot analysis was carried out using antibodies against: BIM and BAX, (BD Biosciences Pharmingen, San Diego, CA), MCL-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), PUMA (Abcam, Cambridge, UK), p53 (Ab-5, Neomarkers, Fremont, CA), BCL-2 (Dako, A/S,Glostrup, Denmark), BCL-X_L (BD Biosciences Transduction Laboratories, San Diego, CA), BMF, Cleaved Caspase-9 and mTOR (Cell Signaling technology, Danvers, MA), and ERK1/2 (Sigma Chemicals Co, St Louis, MO). For western blot analysis of ATM protein, cells were lysed by sonication in buffer containing 50 mM Tris-HCl (pH 7.5), 9 M urea, and 150 mM β-mercaptoethanol, and Western blot analysis was performed as previously described[7] using antibody against ATM (Genetex, San Antonio, TX). Antibody-binding was detected using a secondary antibody conjugated to horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK).