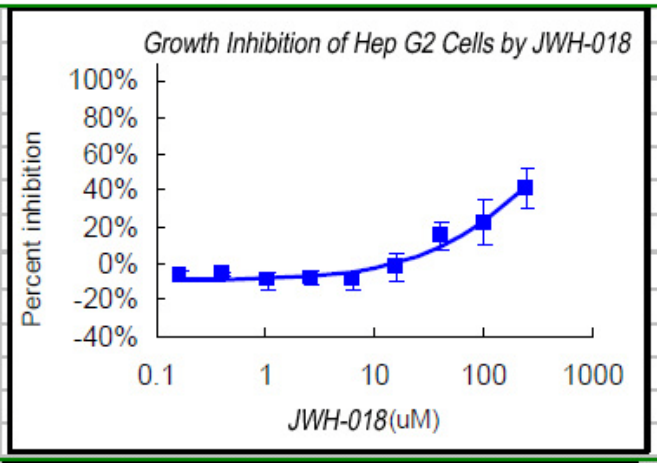
JWH-018 Cytotoxicity

Cytotoxicity Individual Data:

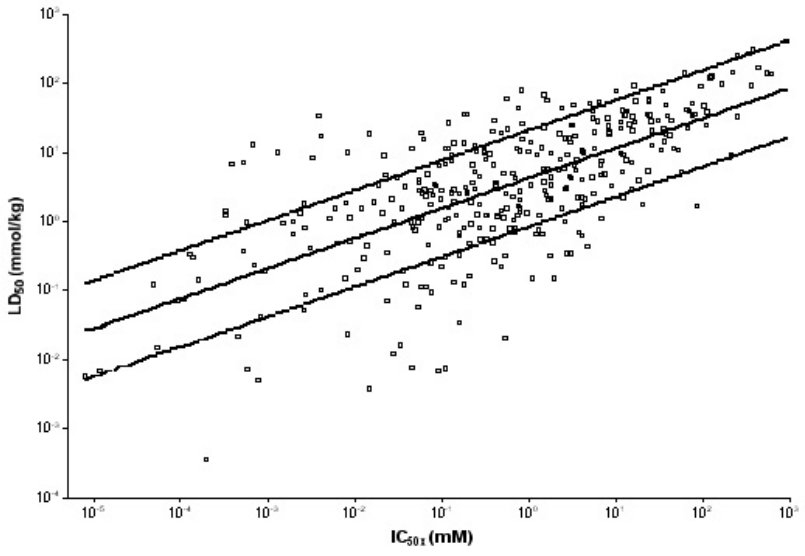


JWH-018 Concentration(uM)	Inhibition (%)
250	41%
100	22%
40	15%
16	-2%
6.4	-9%
2.56	-8%
1.02	-9%
0.41	-6%
0.16	-7%
0	0%
IC50	>100 uM

Cytotoxicity Summary:

Test Compound	Cell line	IC50(µM)	Comment
Chlorpromazine	HepG2	20	Control
Propranolol	HepG2	98	Control
JWH-018	HepG2	>250	Visible precipitant at 100 and 250 µM concentrations

Cytotoxicity IC50 Correlates with *In Vivo* Mouse Toxicity LD50:



The heavy line shows the fit of the data to a linear regression model, log (LD50) = 0.435 x log (IC50x) + 0.625; r=0.67. IC50x values are the geometric means of multiple endpoints and cell types. The thinner lines show the empirical prediction interval (± log 5, or ± 0.699) that is based on the anticipated precision for the prediction of LD50 values from cytotoxicity data (Halle 1998).

Guidance Document on Using *In Vitro* Data to Estimate *In Vivo* Starting Doses for Acute Toxicity
Based on Recommendations from an International Workshop Organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) National Institute of Environmental Health Sciences National Institutes of Health U.S. Public Health Service Department of Health and Human Services.

Standard Methods:

HepG2 human hepatocellular carcinoma cells (originally obtained from ATCC, Manassas, VA) are seeded in 96-well plates at 10,000 cells per well, and grown for 24 hr in Eagle's Modified Essential Medium supplemented with 2 mM glutamine, nonessential amino acids, 2 mM pyruvate, 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin at 37ºC in 5% CO2. NIH/3T3 mouse fibroblasts (originally obtained from ATCC) are seeded in 96 well plates at 10,000 cells per well, and grown for 24 hr in Dulbecco's Modified Essential Medium supplemented with 10% bovine calf serum, 100 U/mL penicillin, and 100 mg/L streptomycin at 37ºC in 5% CO2. . HaCaT human keratinocytes are seeded in 96-well plates at 10,000 cells per well, and grown for 24 hr in Dulbecco's Modified Essential Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/L streptomycin at 37ºC in 5% CO2.

Test article is aseptically diluted in DMSO to 200x the highest concentration, then 100-fold in the growth medium, and serial dilutions are made in 1% DMSO in growth medium. At the start of the assay, the growth medium is removed from the plates and replaced with fresh medium, and an equal volume from each test agent dilution is added. Cells are incubated with test article for 48 h, and the wells are examined microscopically to look for abnormalities.

For Neutral red staining, medium is removed, the cells are washed with PBS, and fresh medium containing 25 µg/mL neutral red (Sigma) is added. After four hours incubation, the cells are washed with PBS, and the cellular dye is solubilized with 1% acetic acid in 50% ethanol. Cellular neutral red is measured by its absorbance at 540 nm. Cytotoxicity is assessed by determining the IC50 (the concentration that causes 50% reduction in uptake of neutral red after 48 hrs exposure to compound.

For MTT staining, 20 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT, 5 mg/mL in PBS, Sigma), is added to each well. After two hours incubation, the medium is removed, and the cellular dye is solubilized with DMSO. Cellular-converted MTT is measured by its absorbance at 540 nm. Cytotoxicity is assessed by determining the IC50 (the concentration that causes 50% reduction in uptake of MTT after 48 hrs exposure to compound).