Supporting Information

Article

Design, Synthesis, Docking Study, and Antiproliferative Evaluation of Novel Schiff Base-Benzimidazole Hybrids with VEGFR-2 Inhibitory Activity

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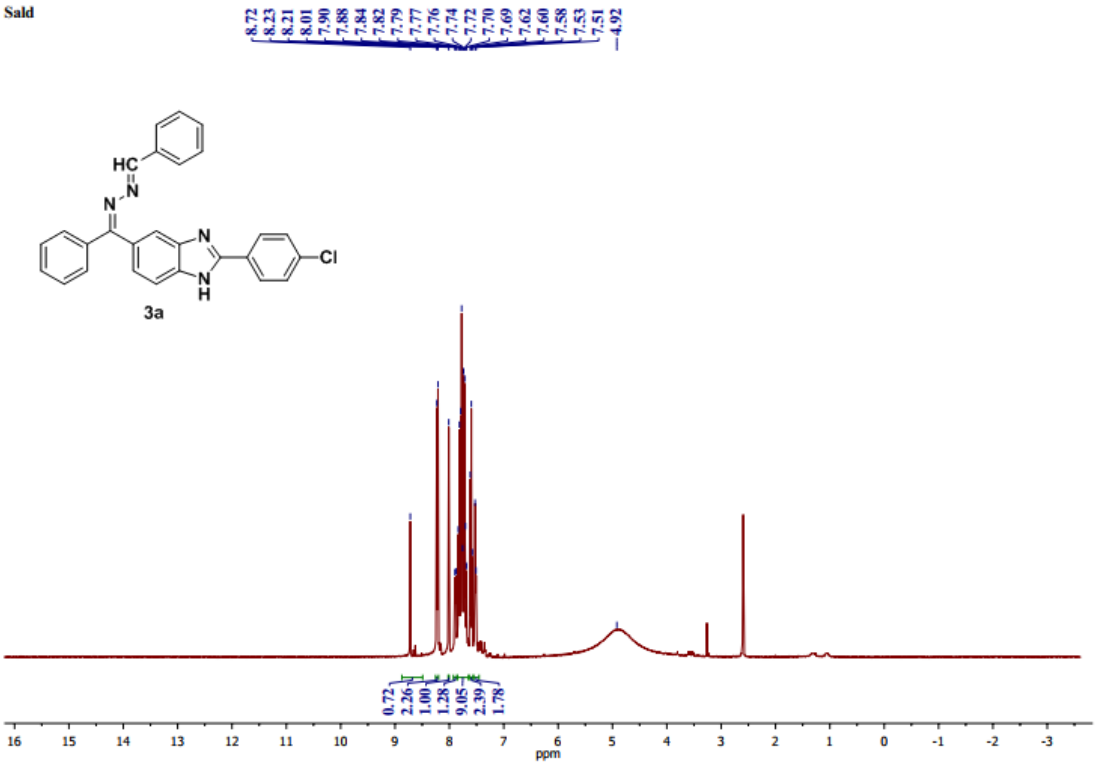

Figure S1: ^{1}H-NMR spectrum of compound **3a**

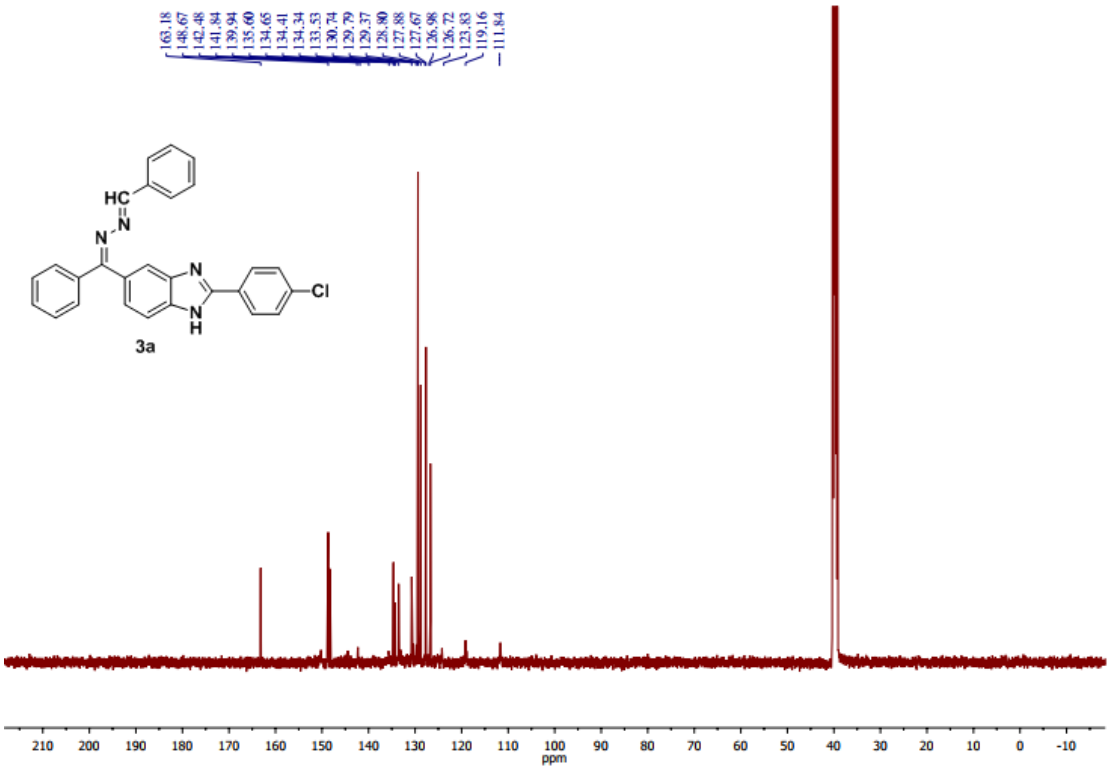

Figure S2: ^{13}C-NMR spectrum of compound **3a**

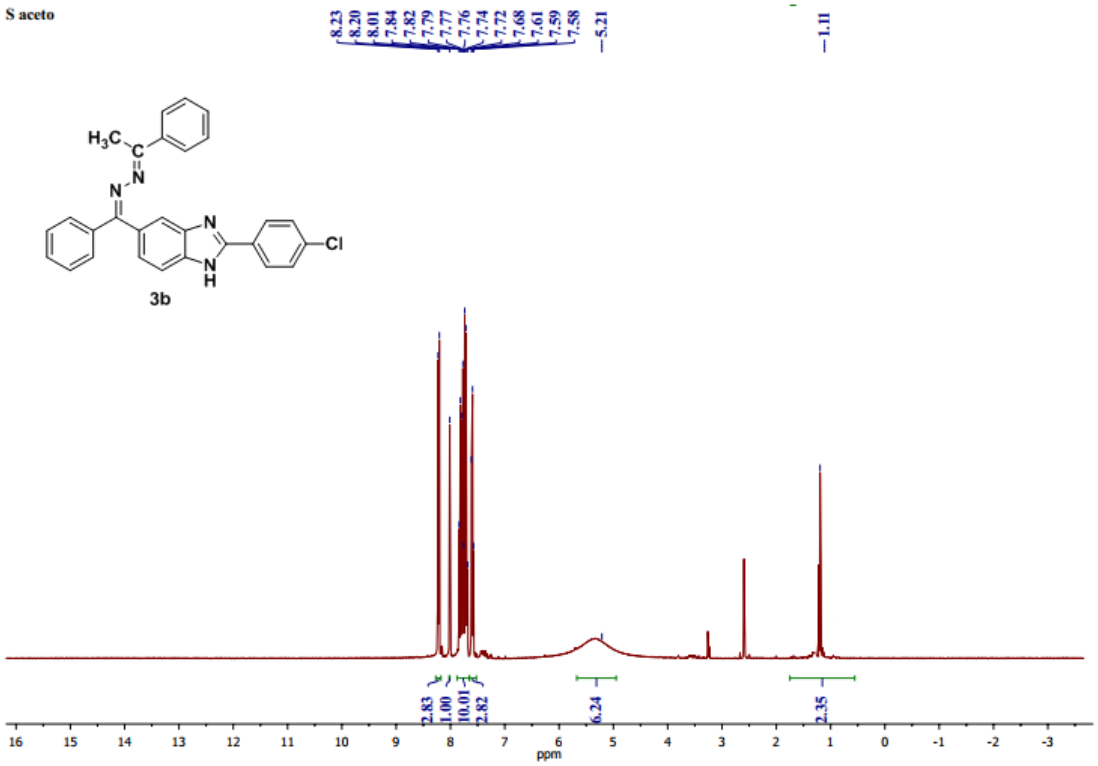

Figure S3: ^{1}H-NMR spectrum of compound **3b**

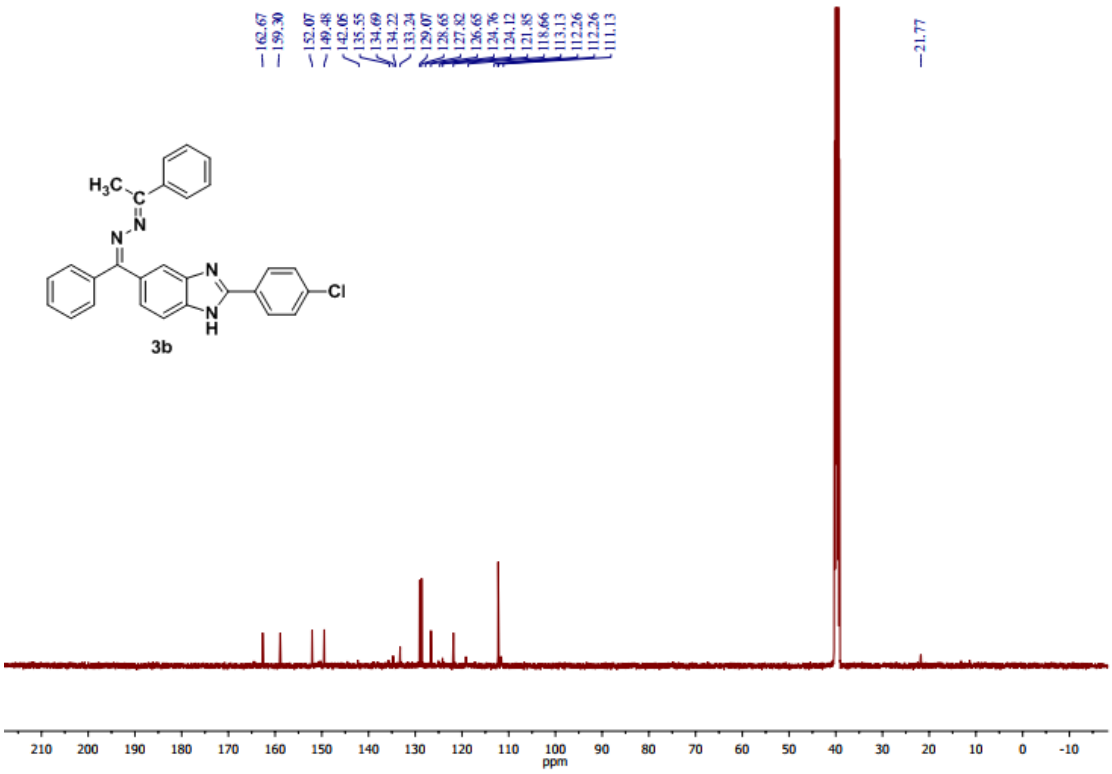

Figure S4: ^{13}C-NMR spectrum of compound **3b**

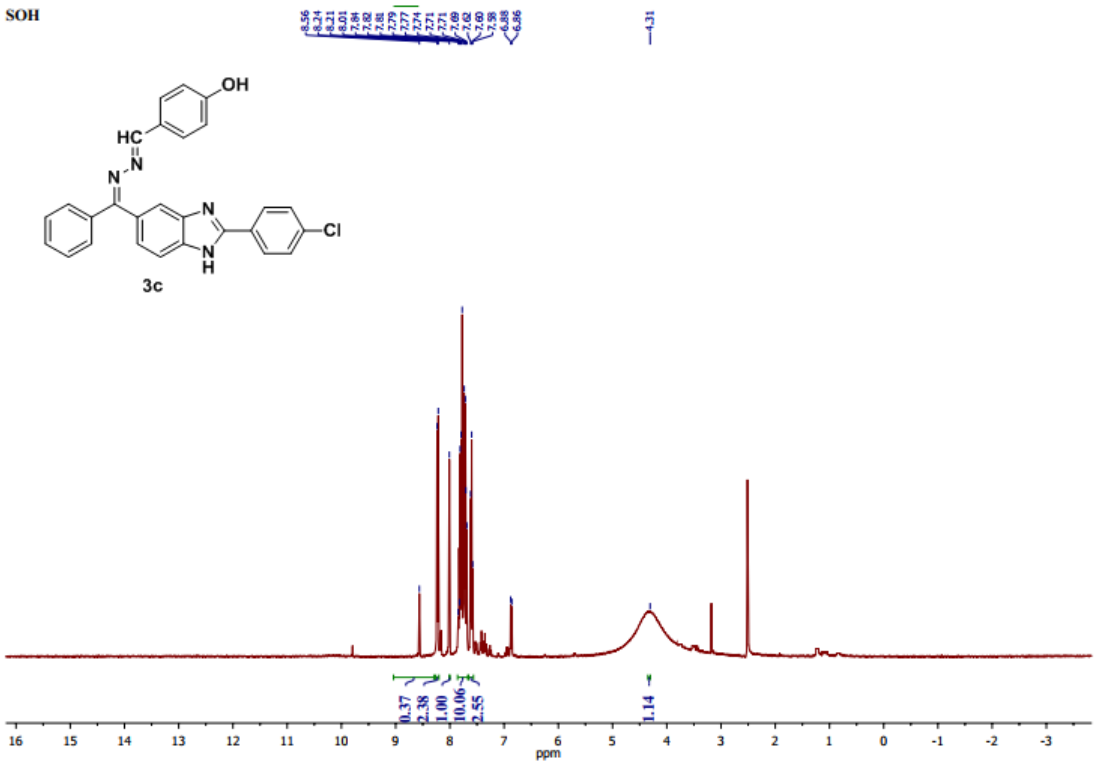

Figure S5: ^{1}H-NMR spectrum of compound **3c**

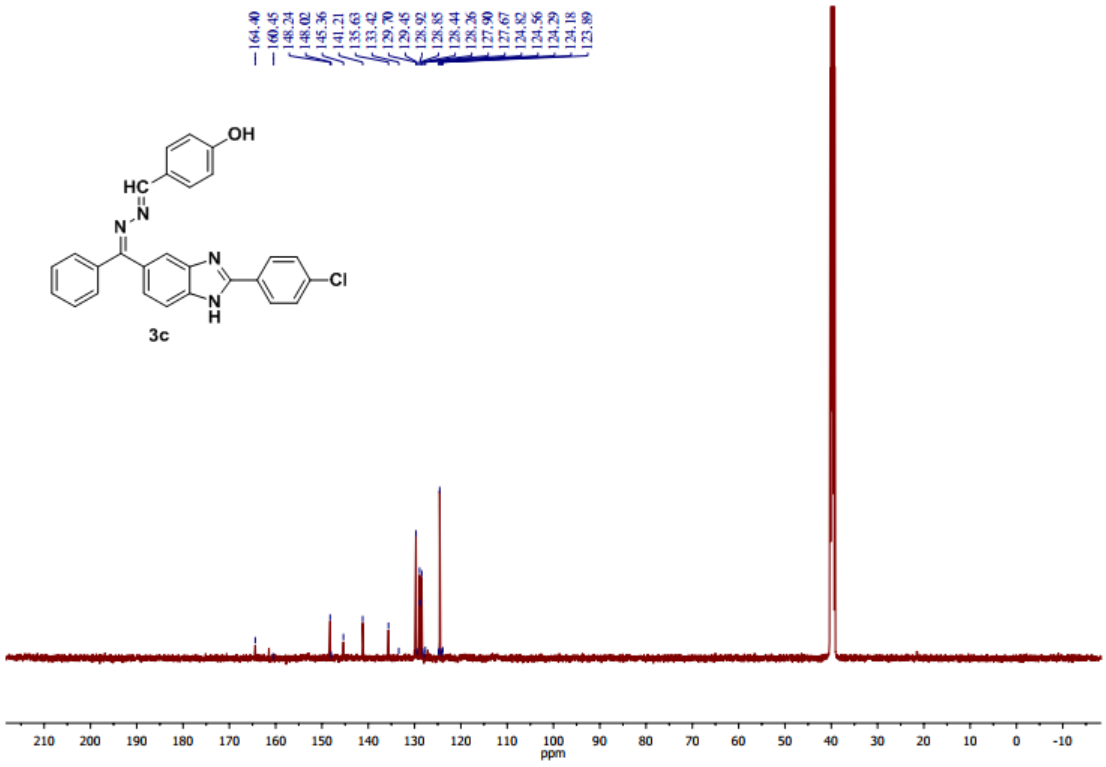

Figure S6: ^{13}C-NMR spectrum of compound **3c**

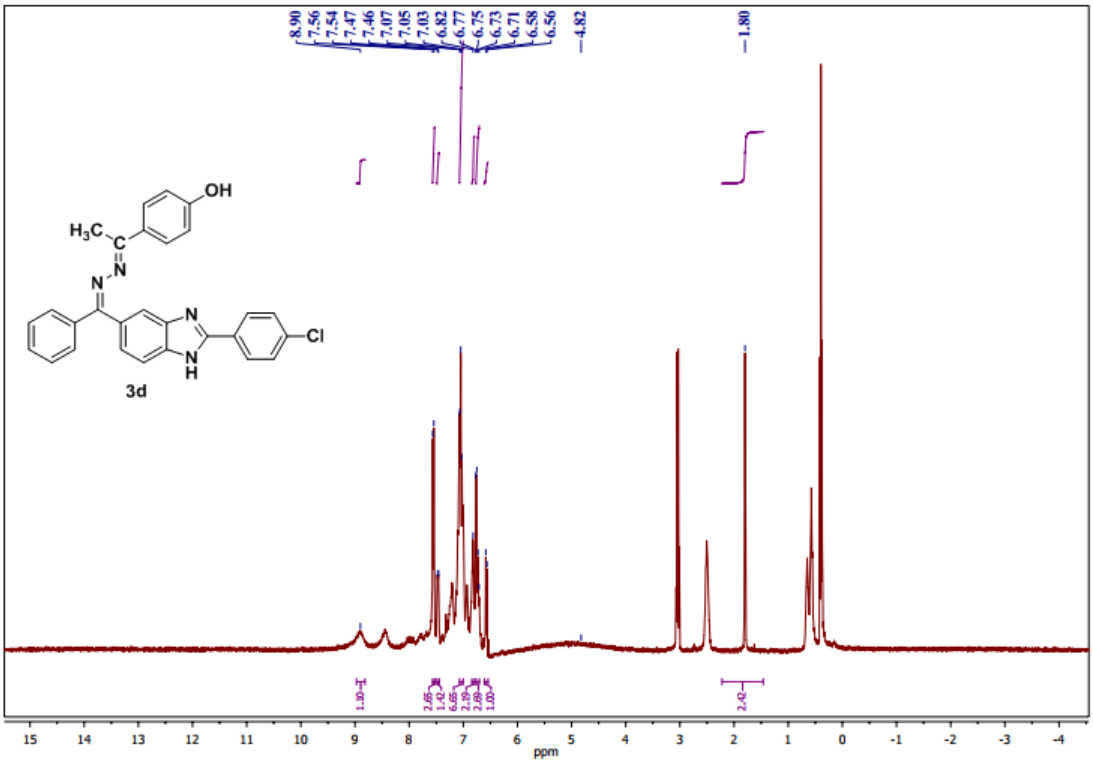

Figure S7: ^{1}H-NMR spectrum of compound **3d**

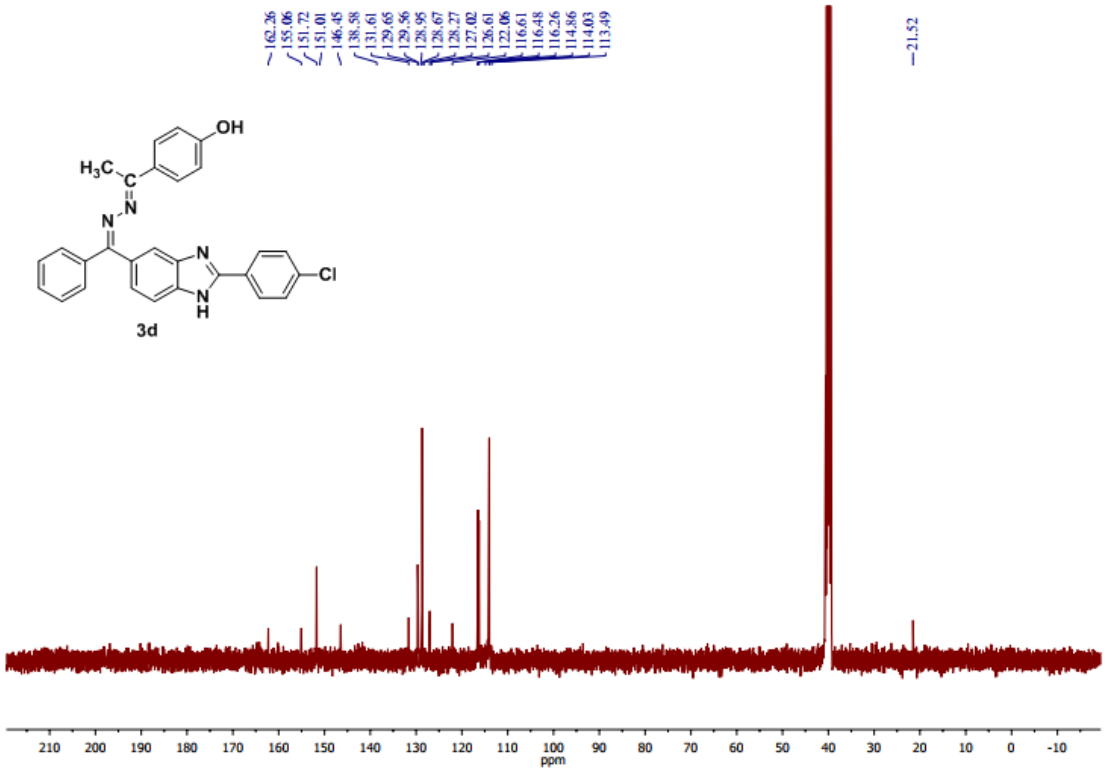

Figure S8: ^{13}C-NMR spectrum of compound **3d**

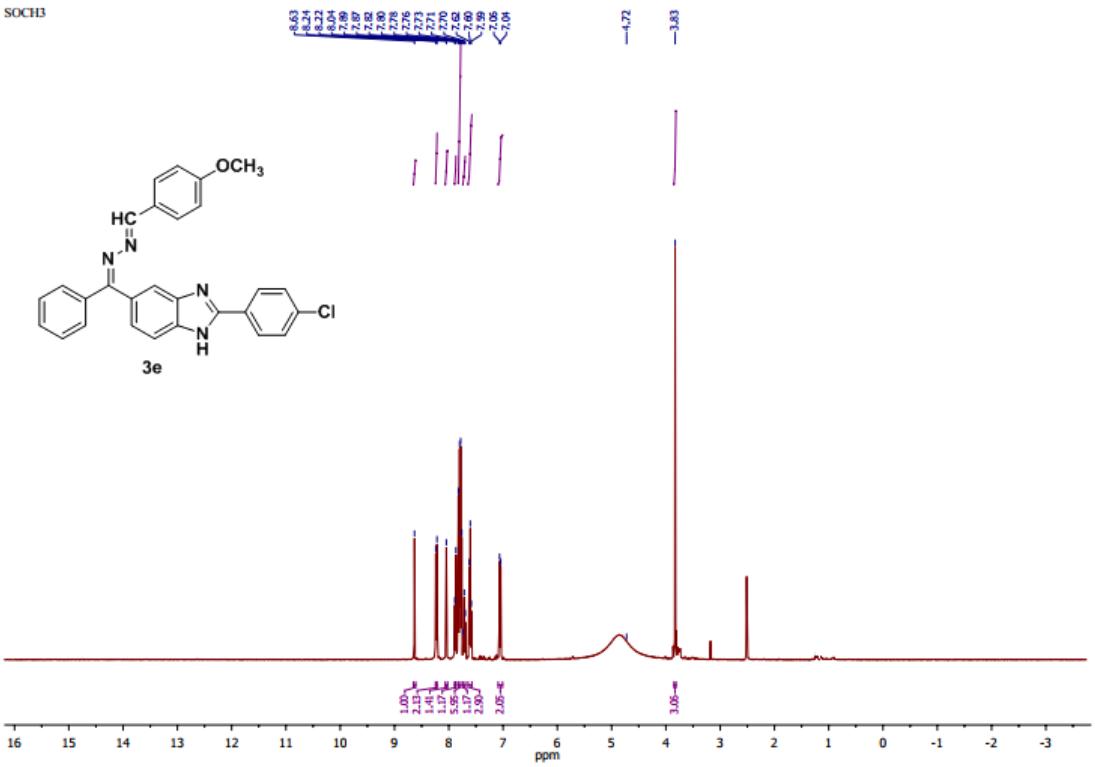

Figure S9: ^{1}H-NMR spectrum of compound **3e**

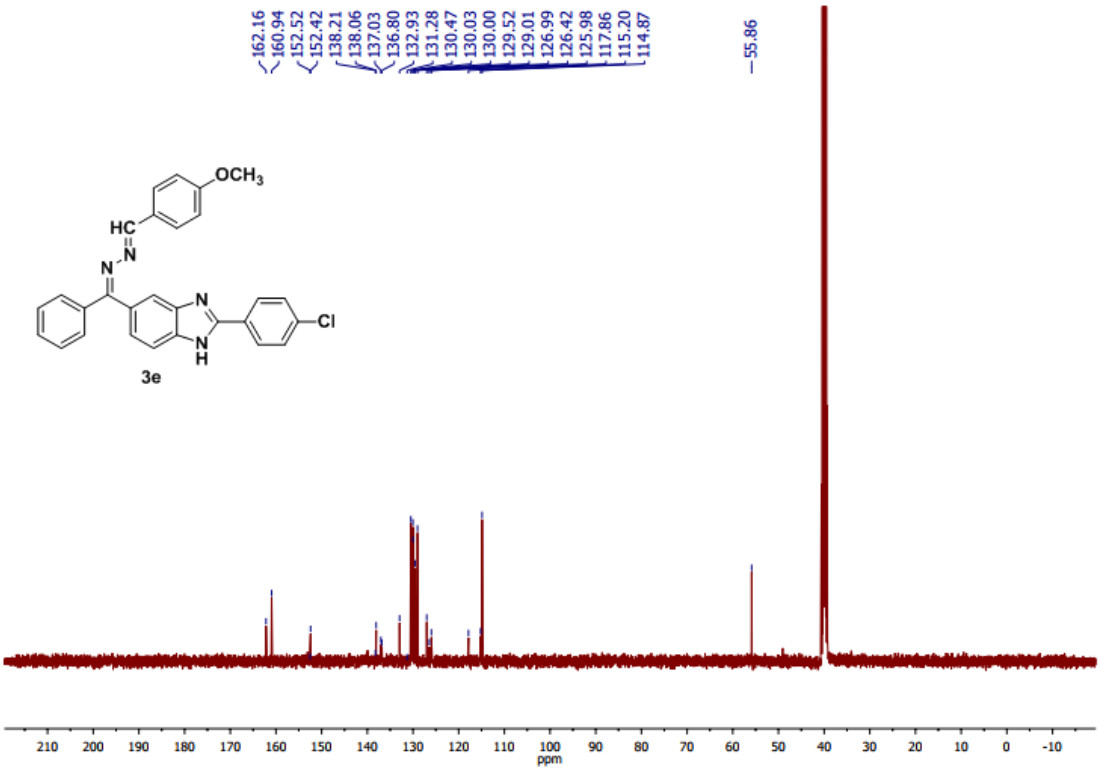

Figure S10: ^{13}C-NMR spectrum of compound **3e**

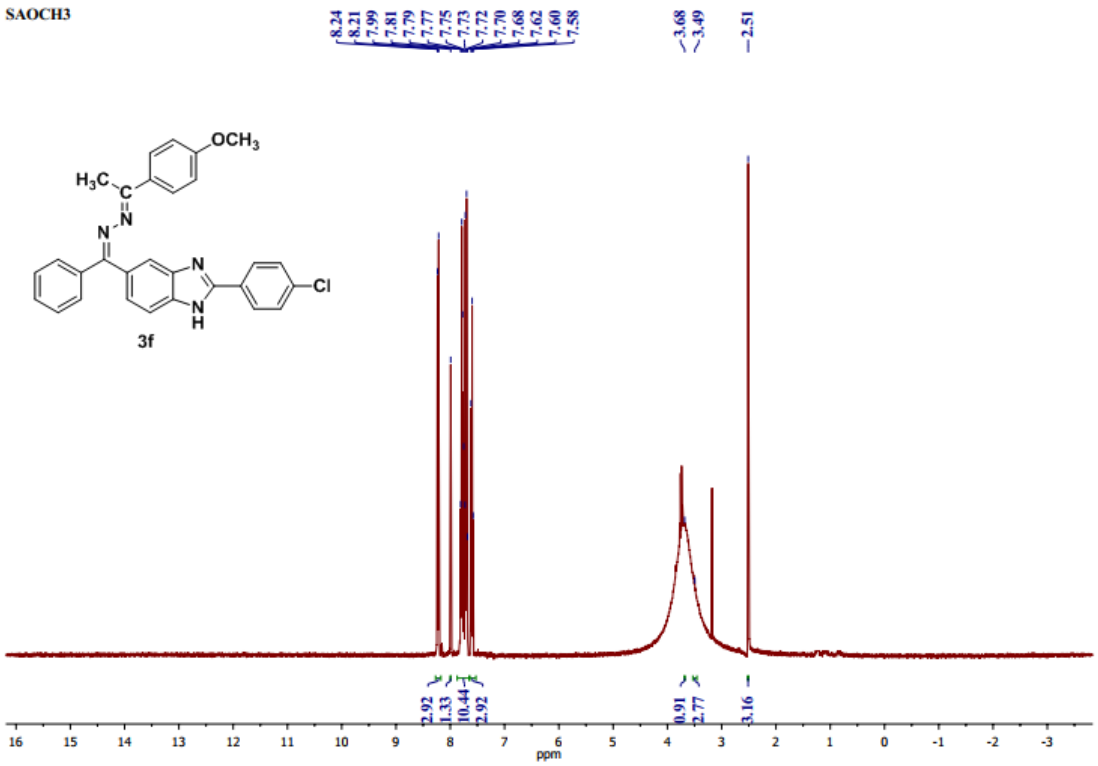

Figure S11: ^{1}H-NMR spectrum of compound **3f**

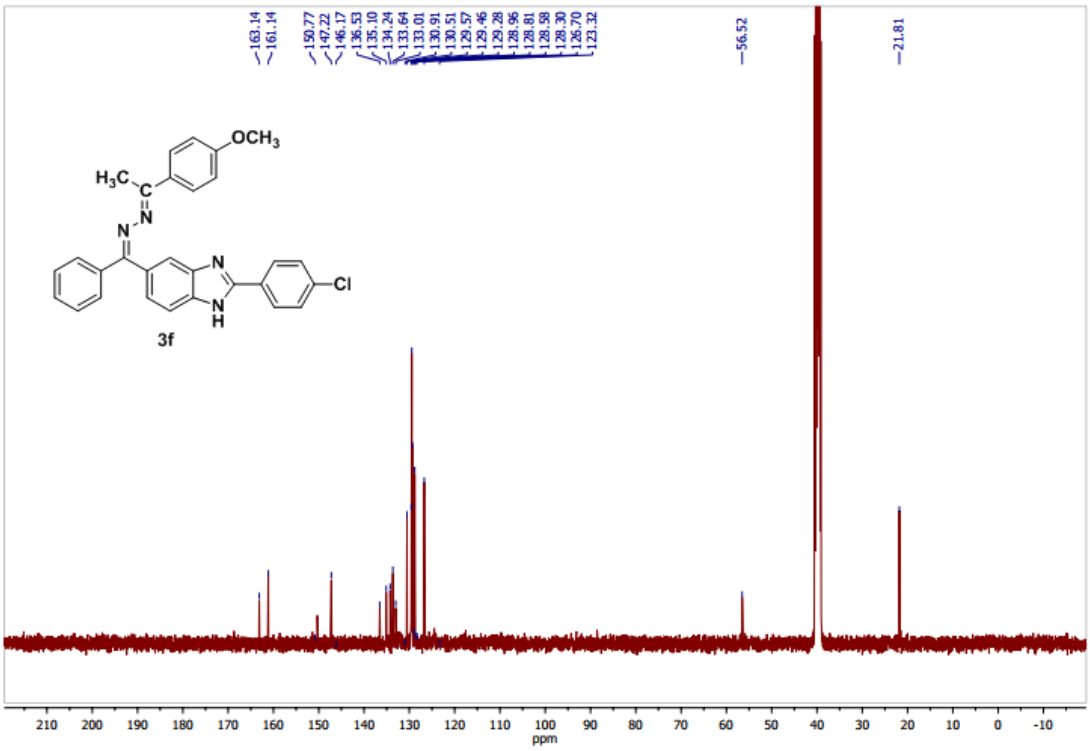

Figure S12: ^{13}C-NMR spectrum of compound **3f**

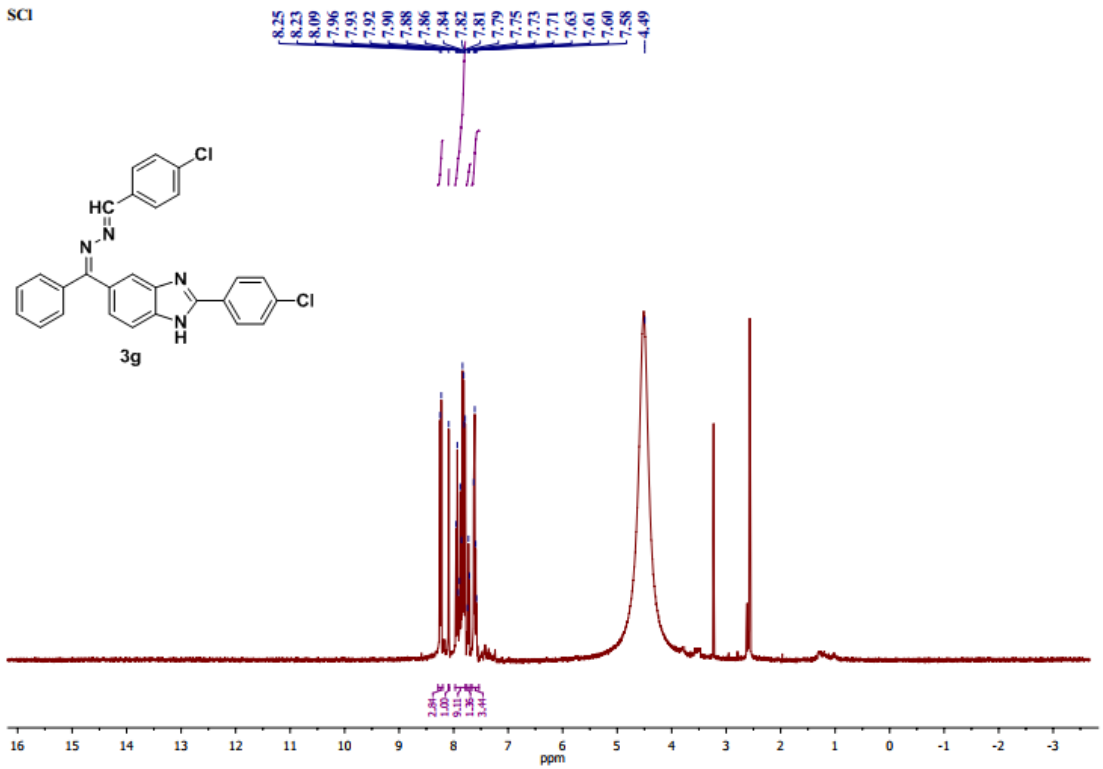

Figure S13: ^{1}H-NMR spectrum of compound **3g**

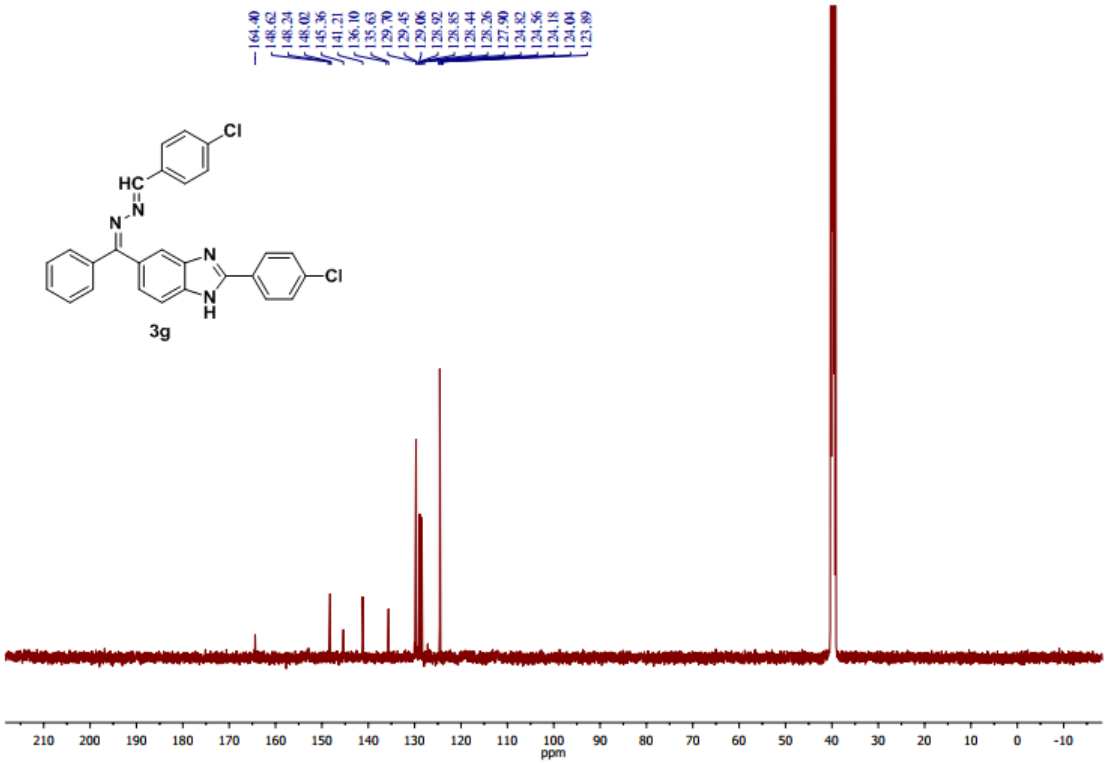

Figure S14: ^{13}C-NMR spectrum of compound **3g**

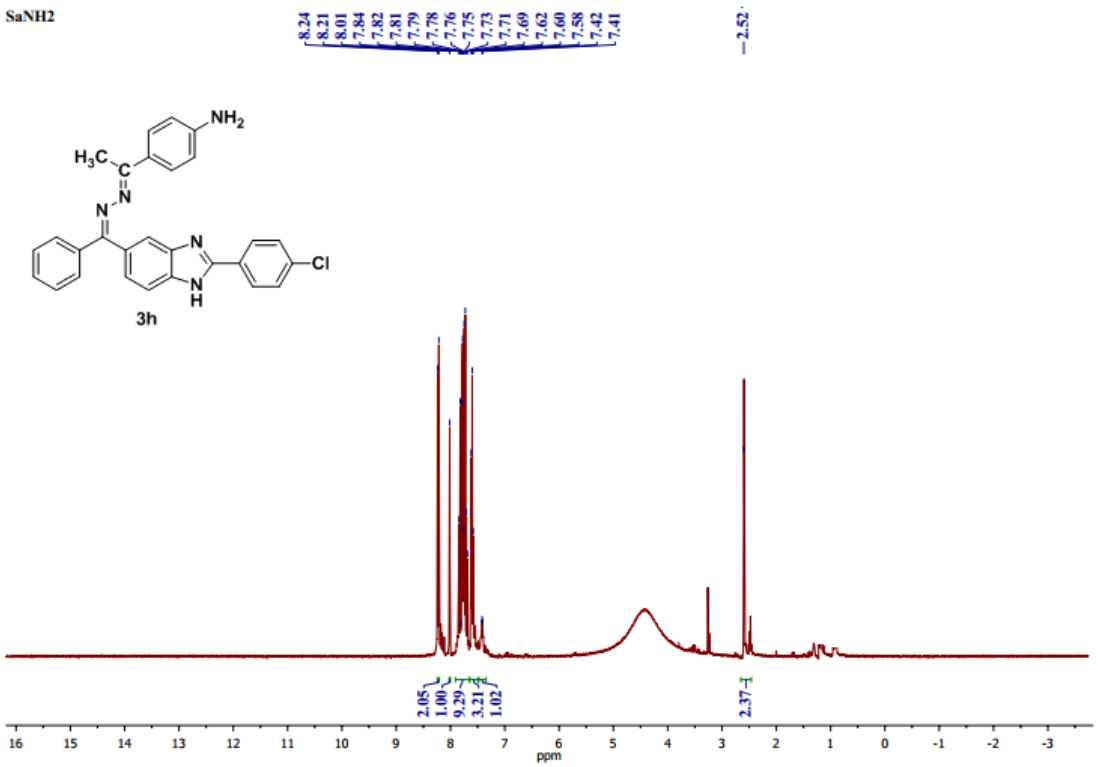

Figure S15: ^{1}H-NMR spectrum of compound **3h**

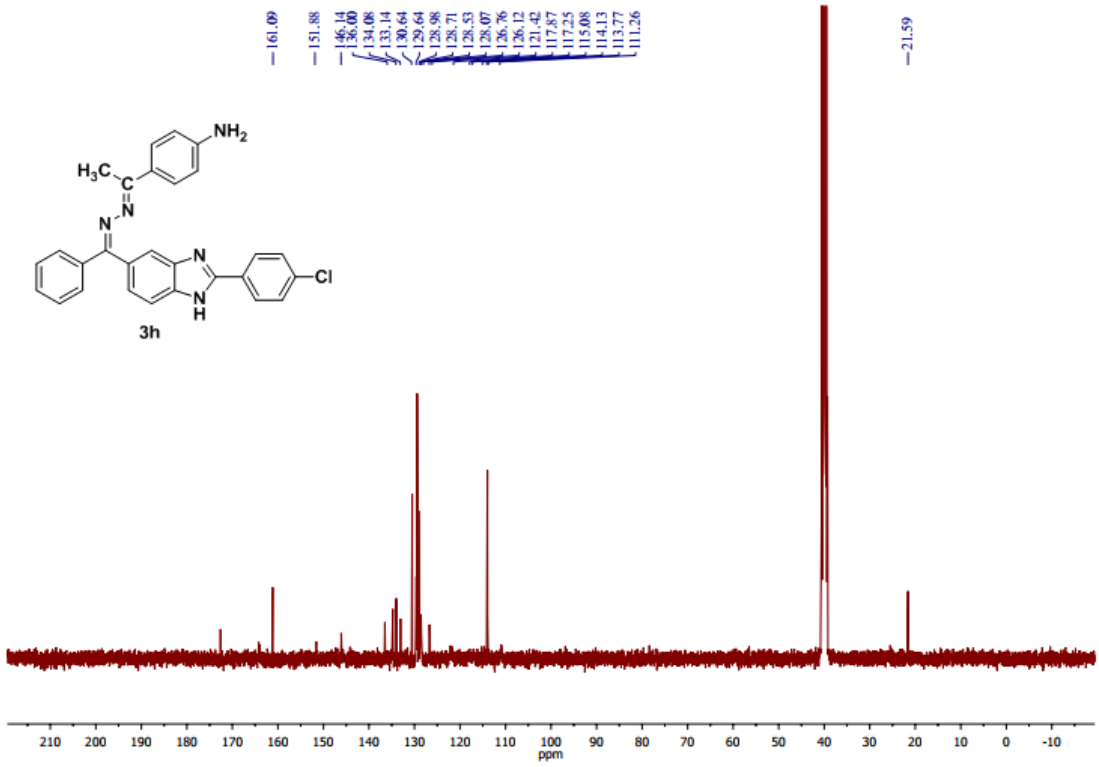

Figure S16: ^{13}C-NMR spectrum of compound **3h**

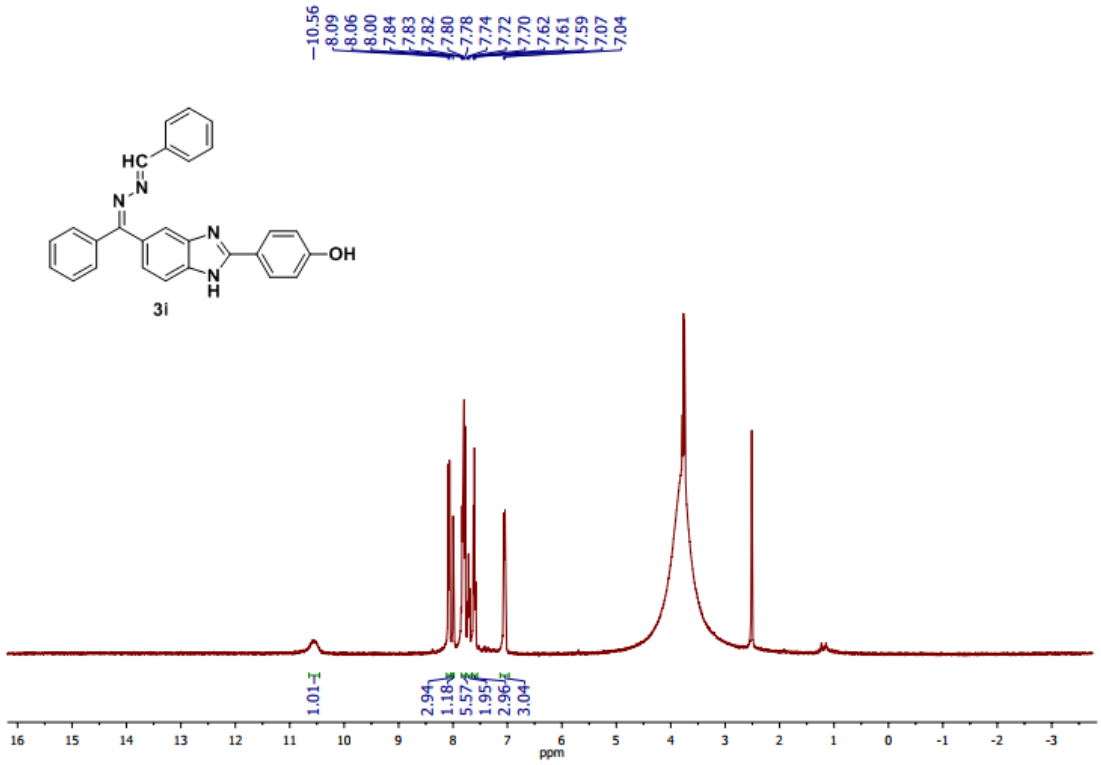

Figure S17: ^{1}H-NMR spectrum of compound **3i**

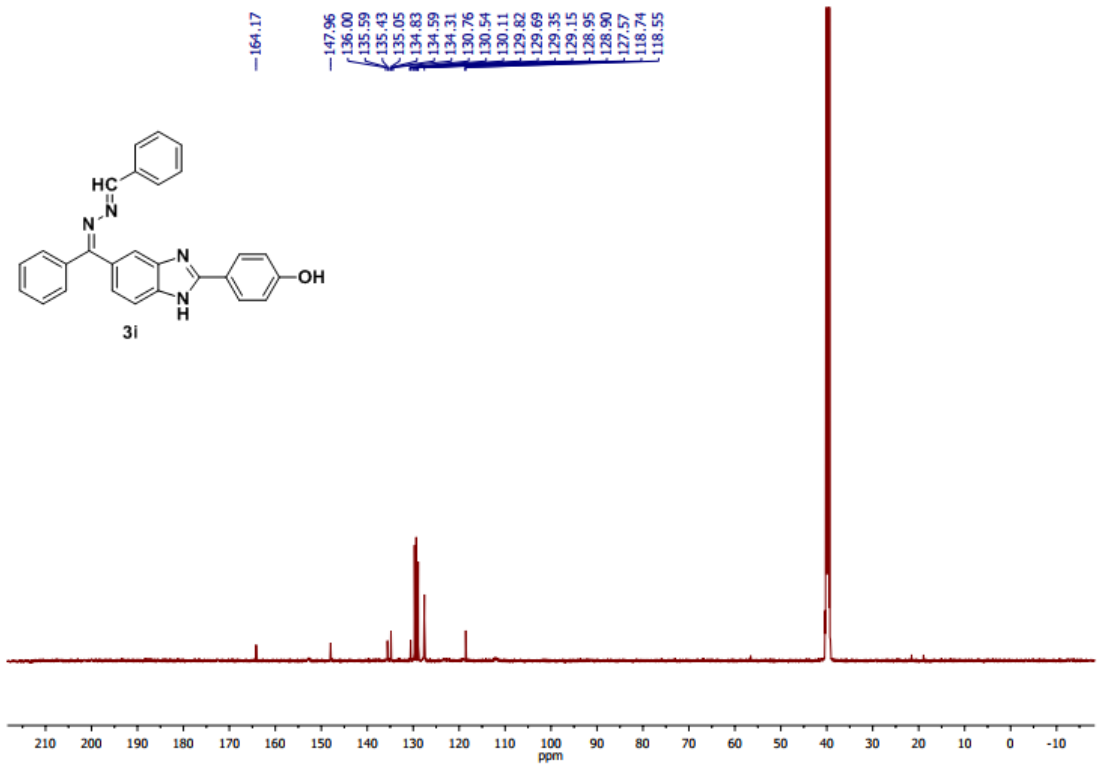

Figure S18: ^{13}C-NMR spectrum of compound **3i**

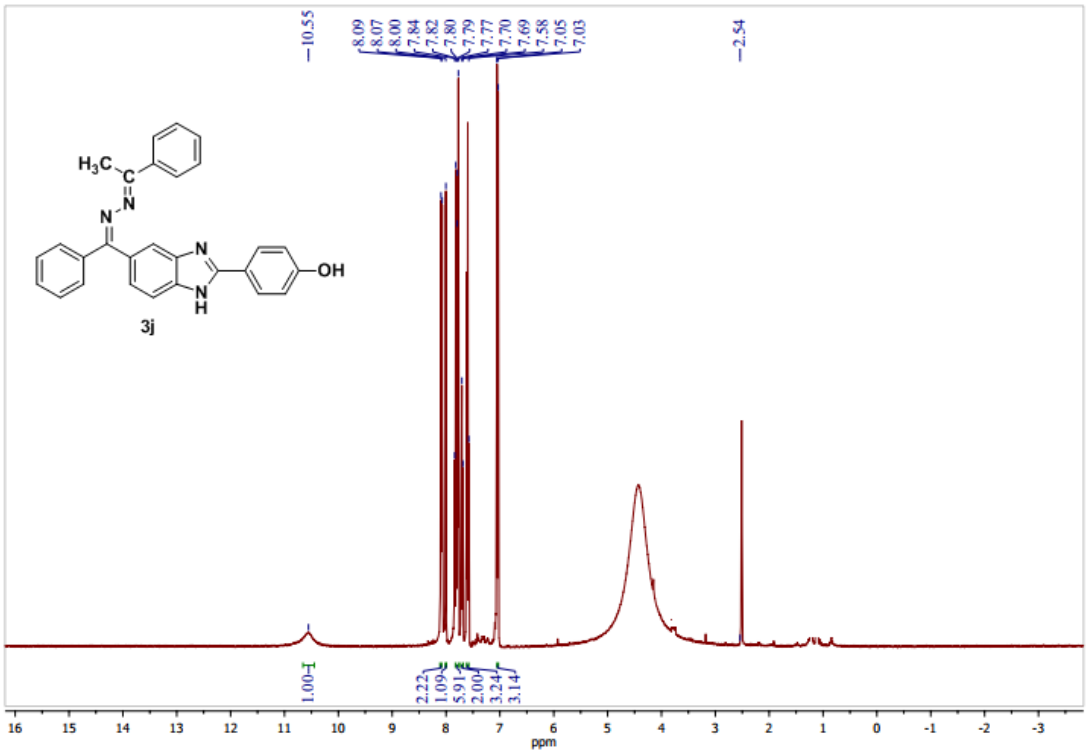

Figure S19: ^{1}H-NMR spectrum of compound **3j**

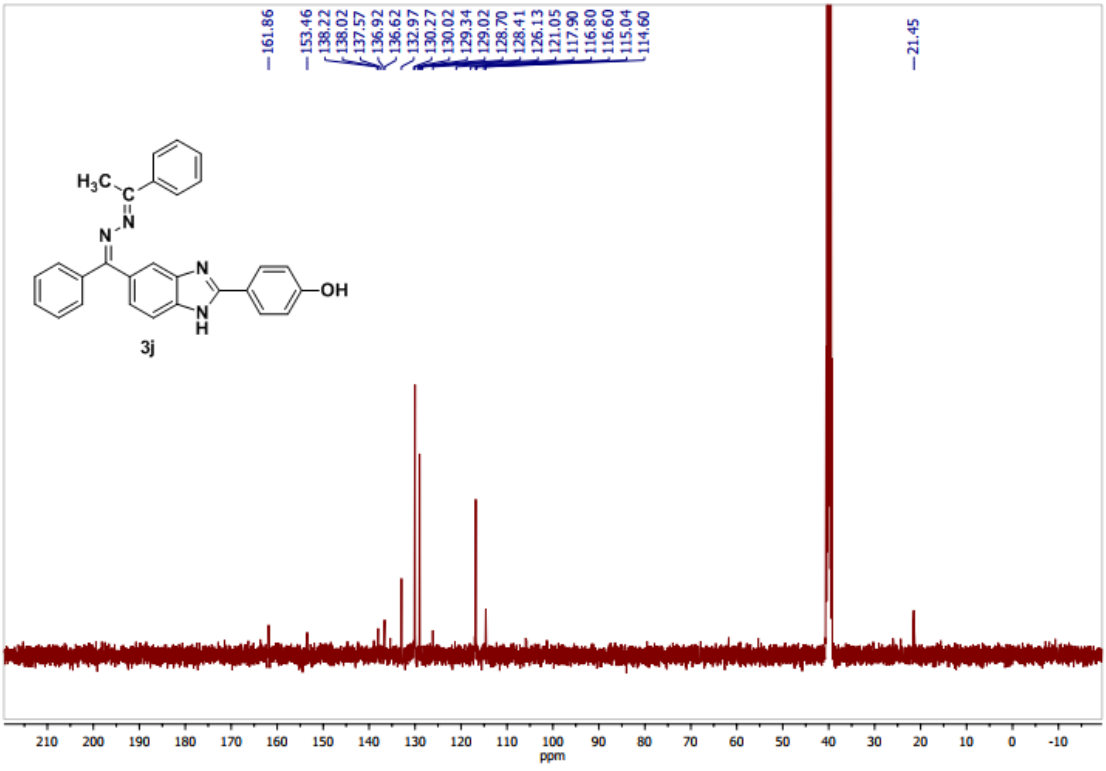

Figure S20: ^{13}C-NMR spectrum of compound **3j**

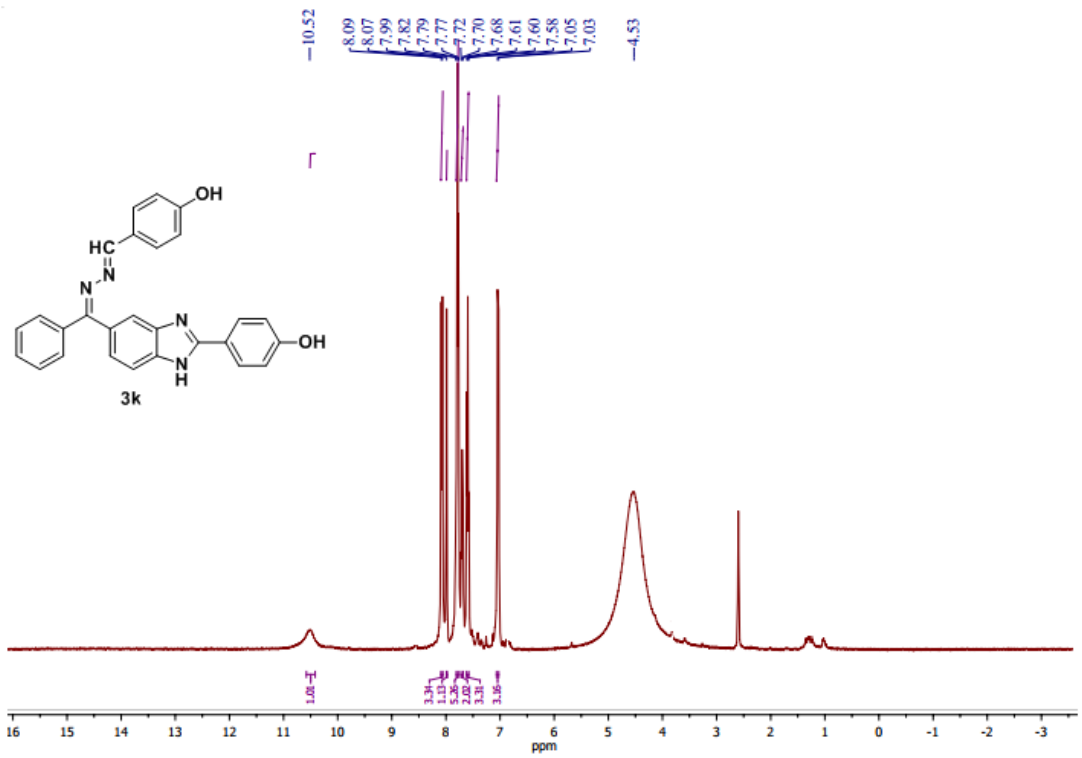

Figure S21: ^{1}H-NMR spectrum of compound **3k**

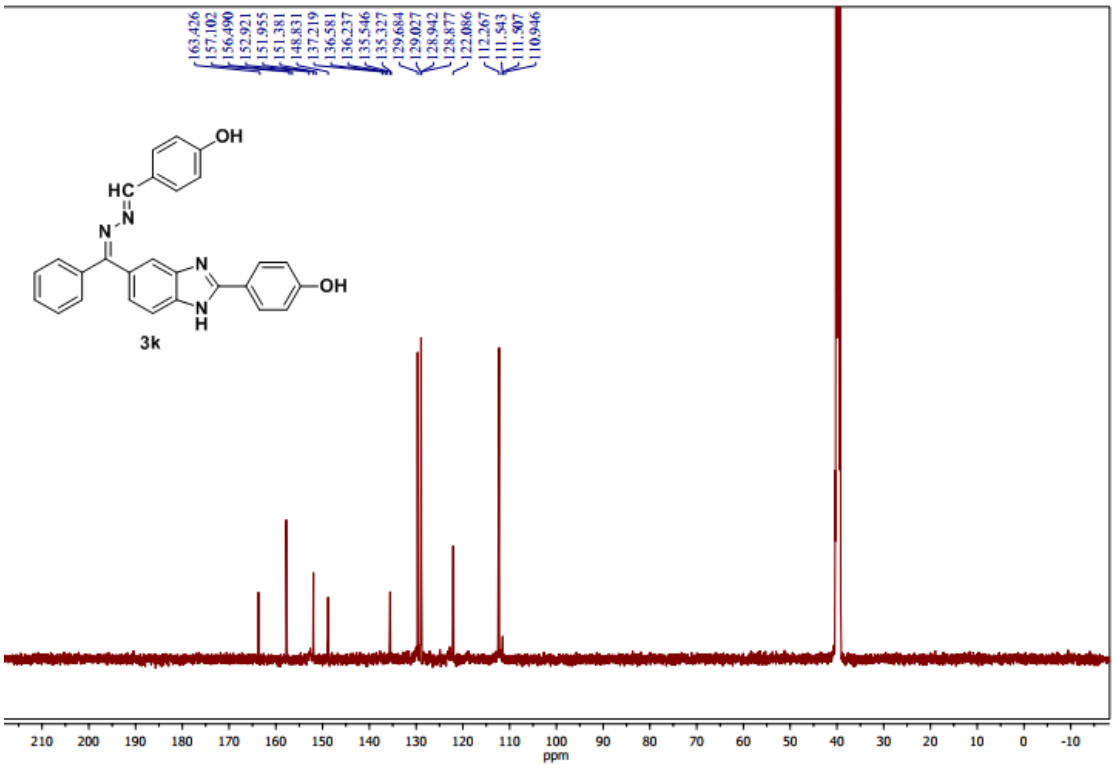

Figure S22: ^{13}C-NMR spectrum of compound **3k**

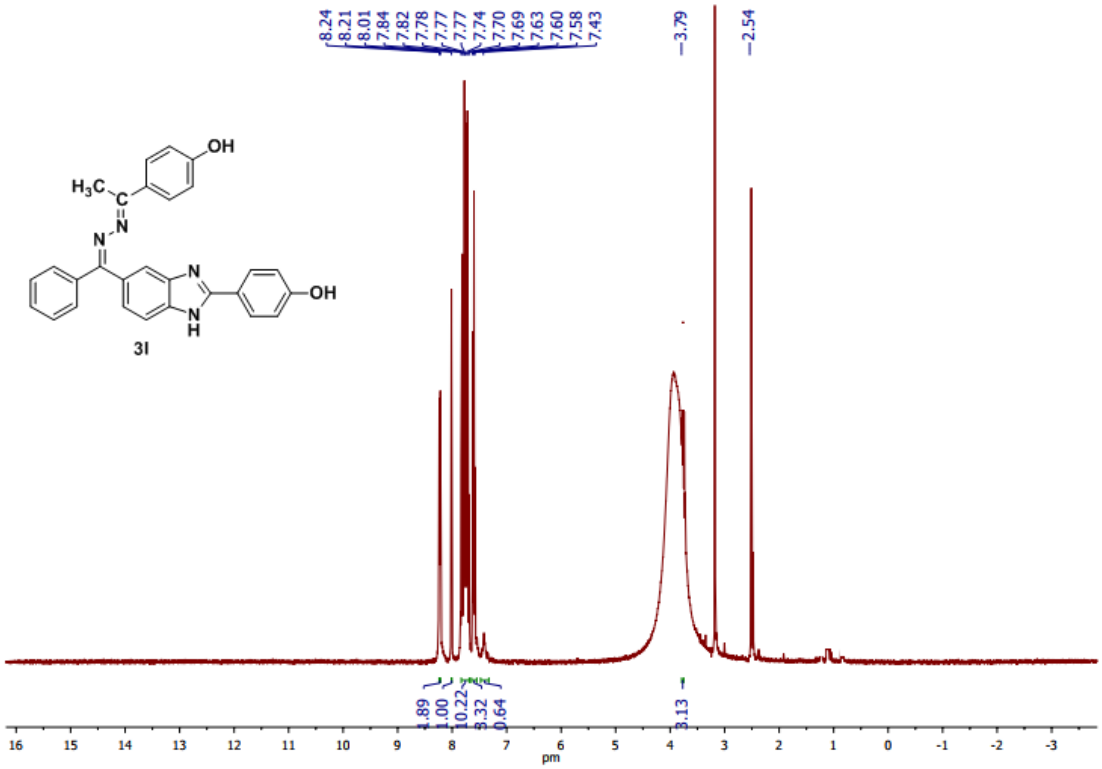

Figure S23: ^{1}H-NMR spectrum of compound **3l**

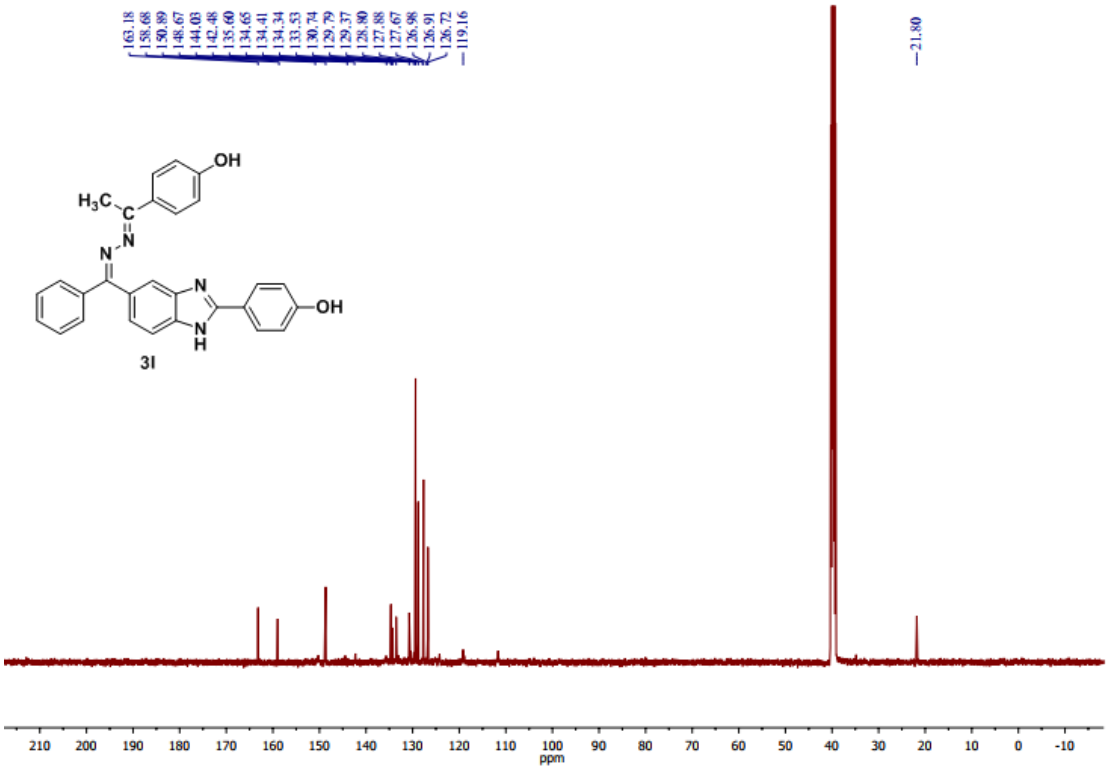

Figure S24: ^{13}C-NMR spectrum of compound **3l**

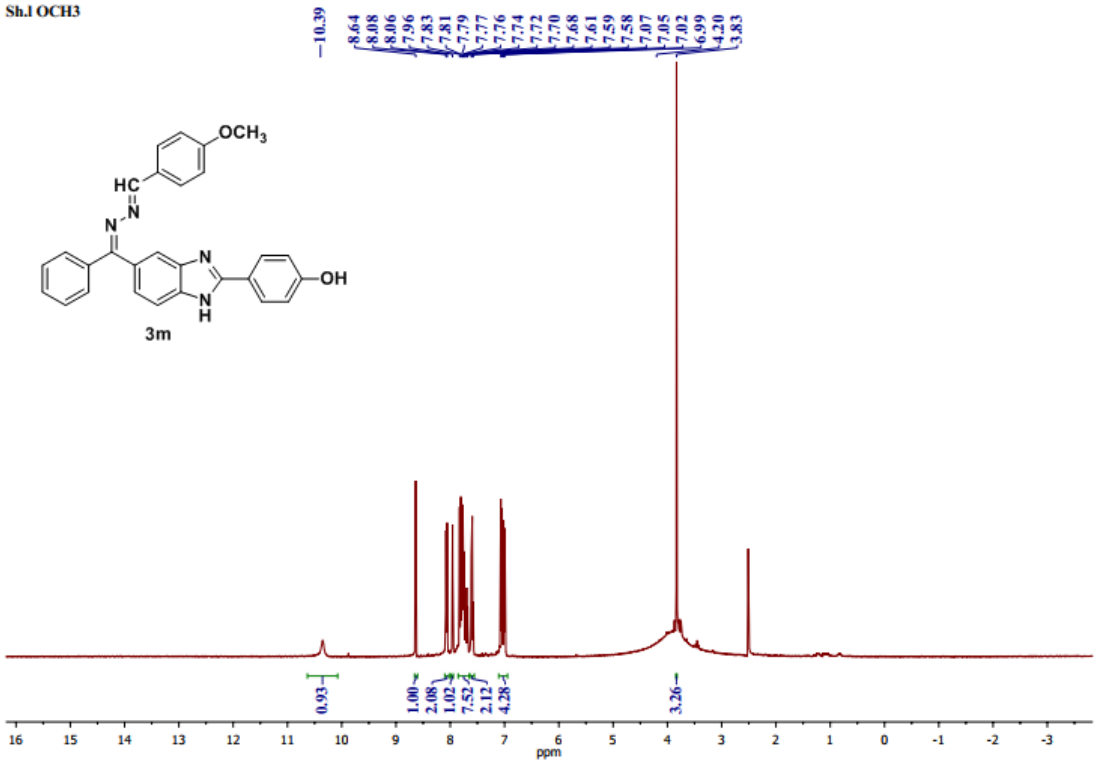

Figure S25: ^{1}H-NMR spectrum of compound **3m**

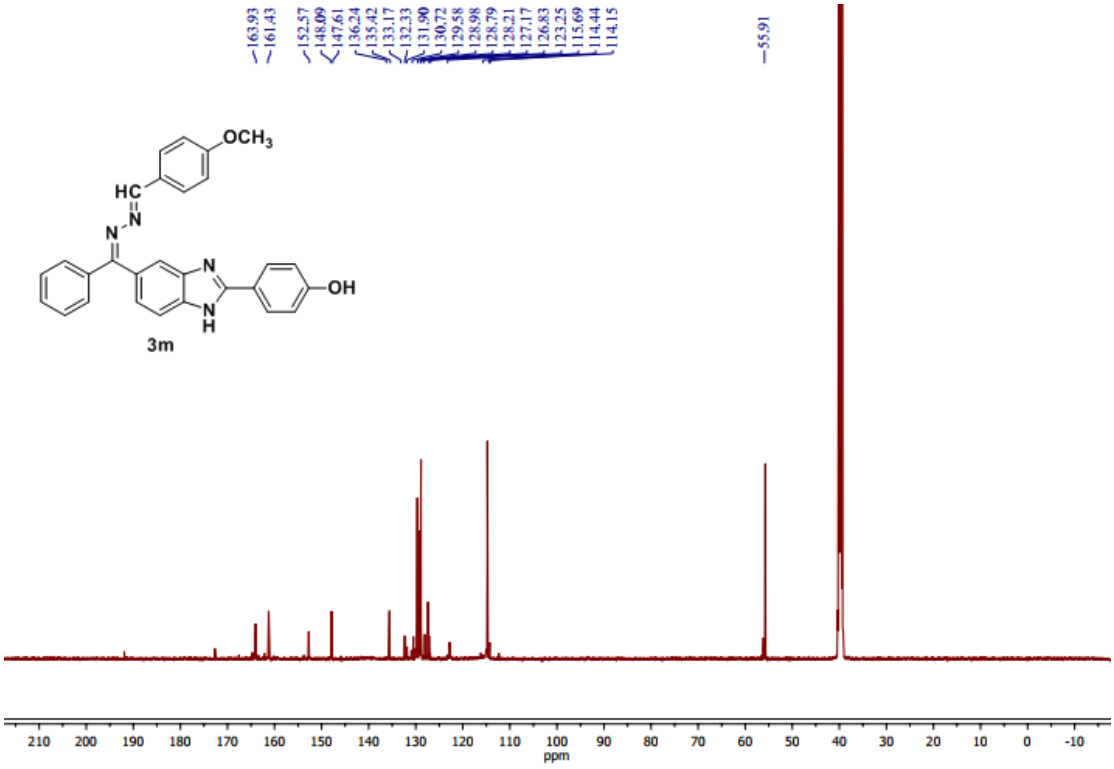

Figure S26: ^{13}C-NMR spectrum of compound **3m**

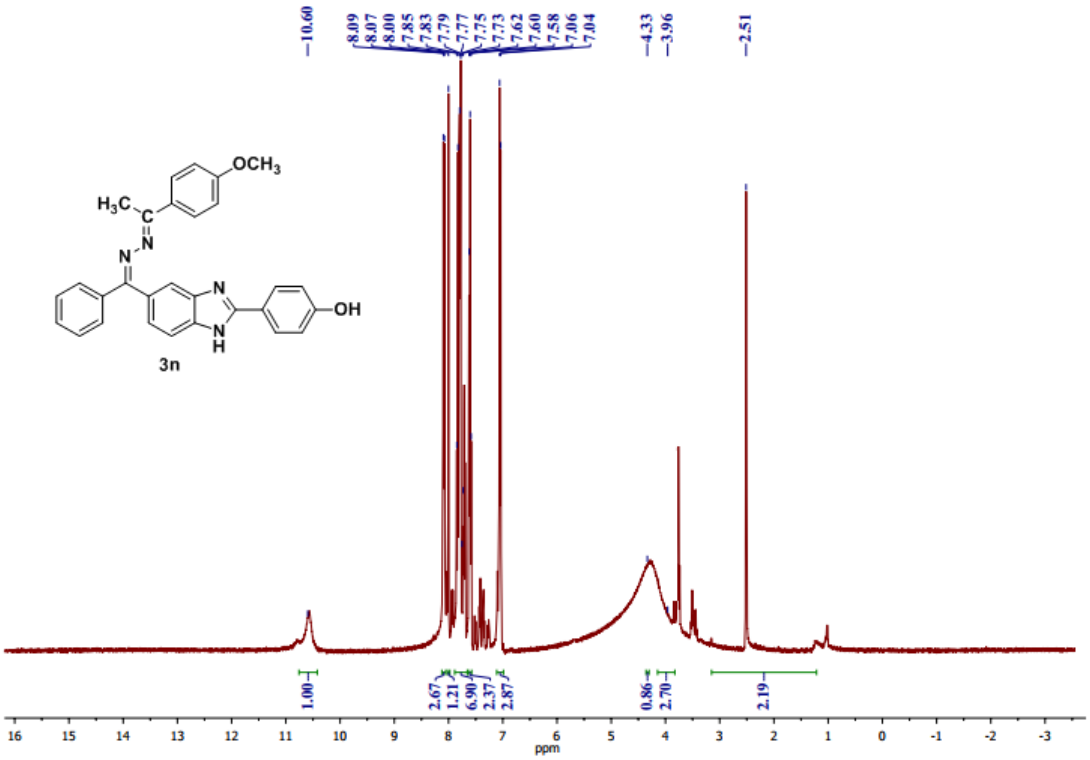

Figure S27: ^{1}H-NMR spectrum of compound **3n**

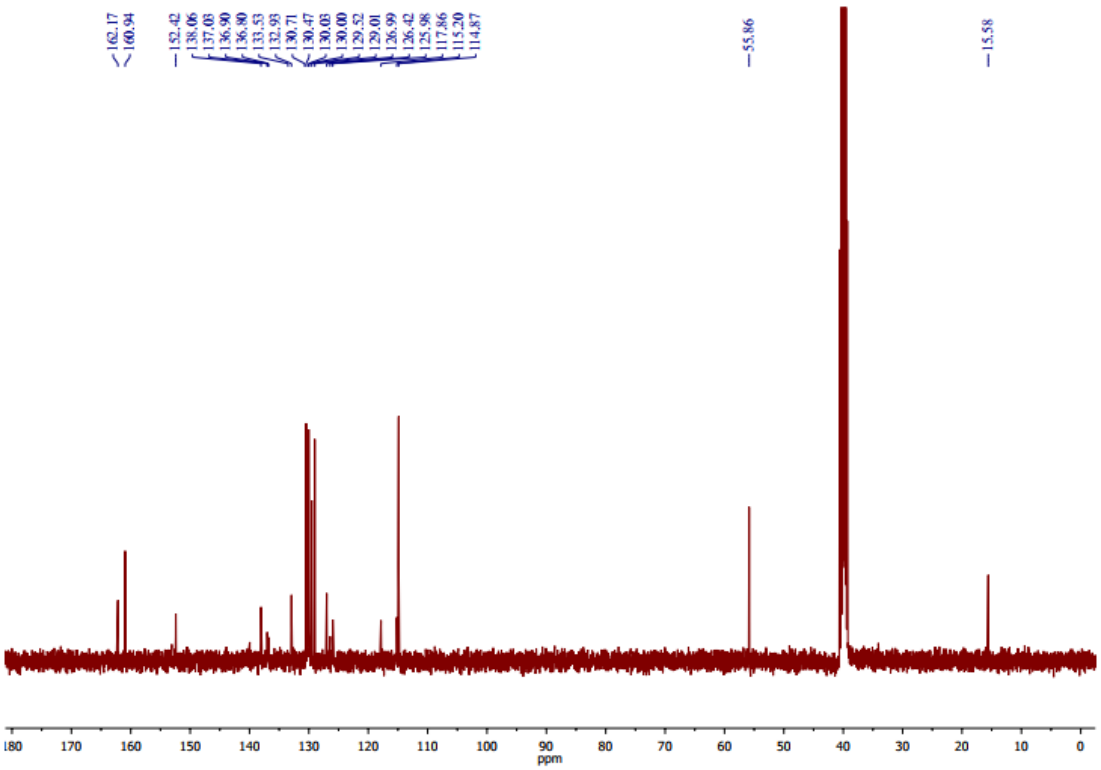

Figure S28: ^{13}C-NMR spectrum of compound **3n**

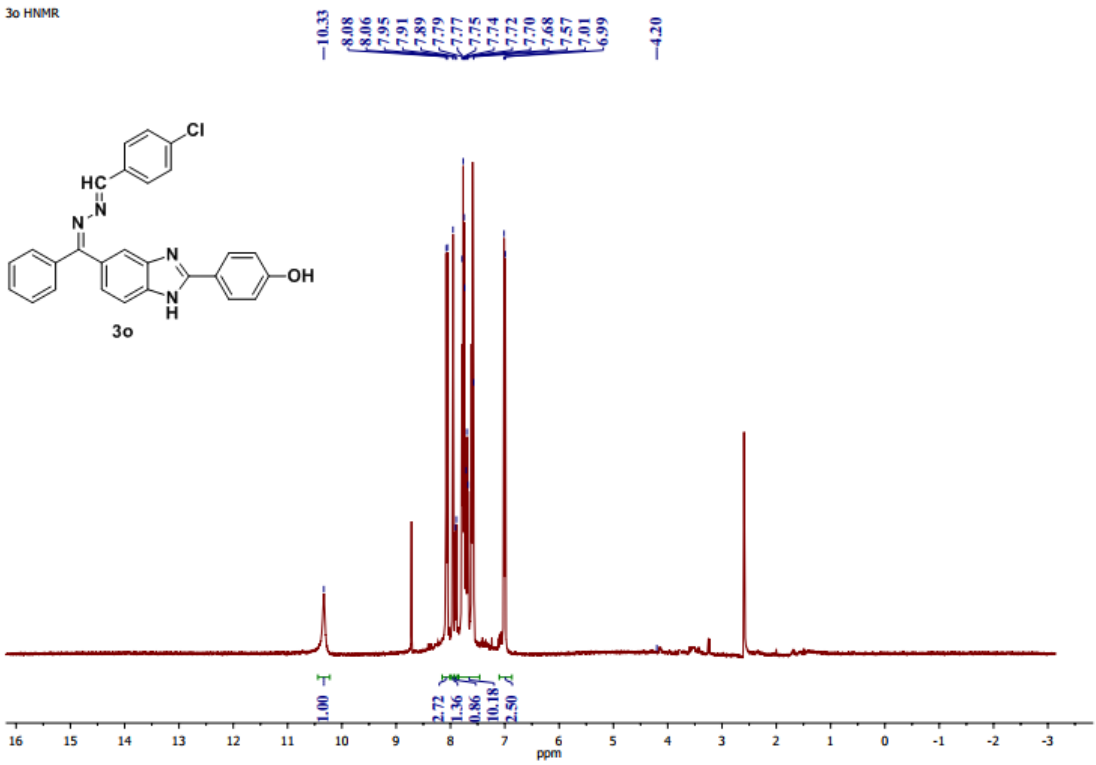

Figure S29: ^{1}H-NMR spectrum of compound **3o**

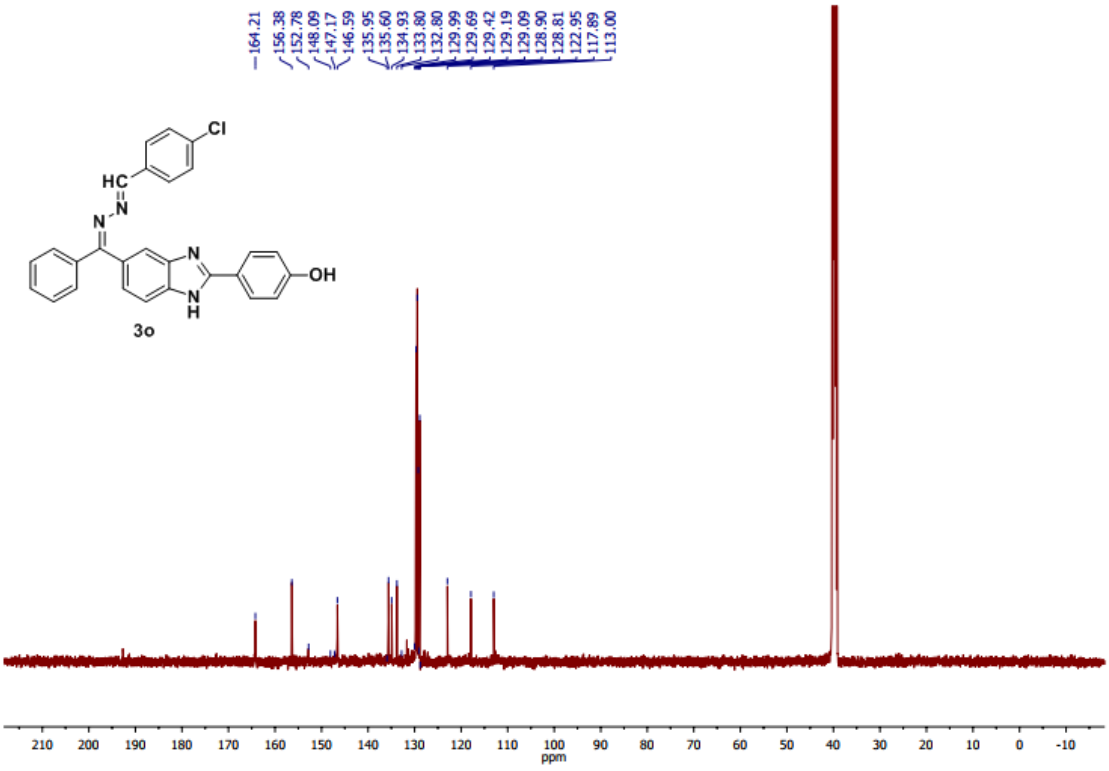

Figure S30: ^{13}C-NMR spectrum of compound **3o**

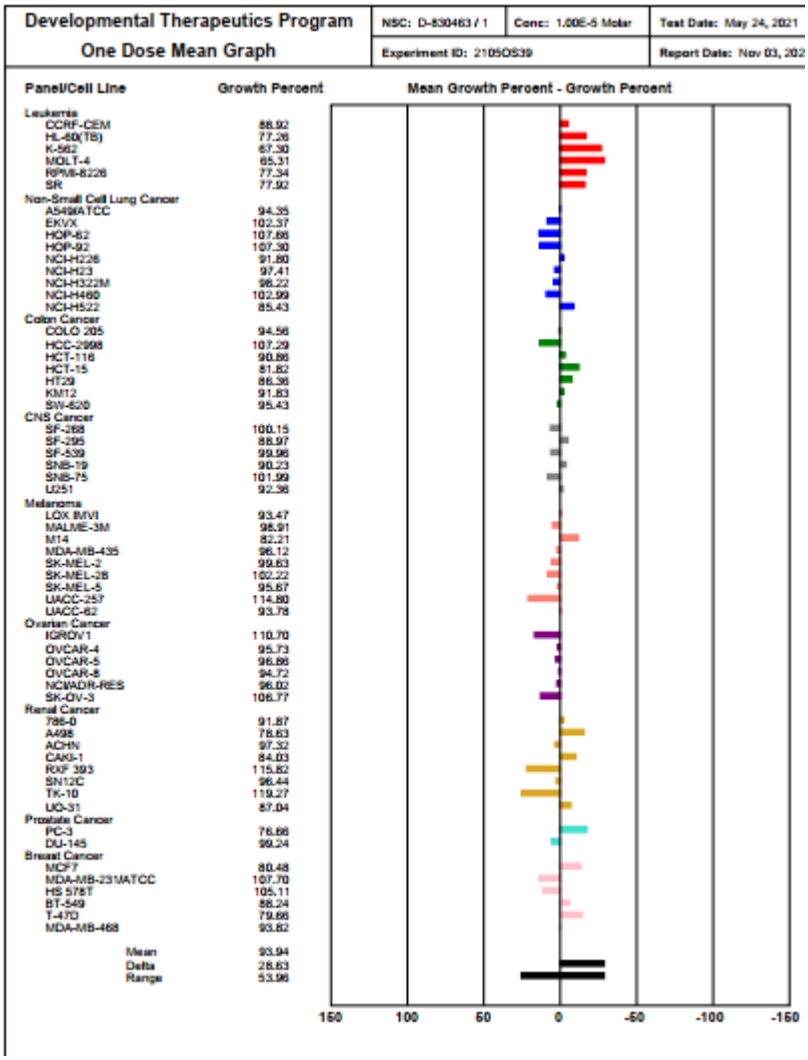

Figure S31: Results of primary *in vitro* one-dose anticancer assay of compound **3a.**

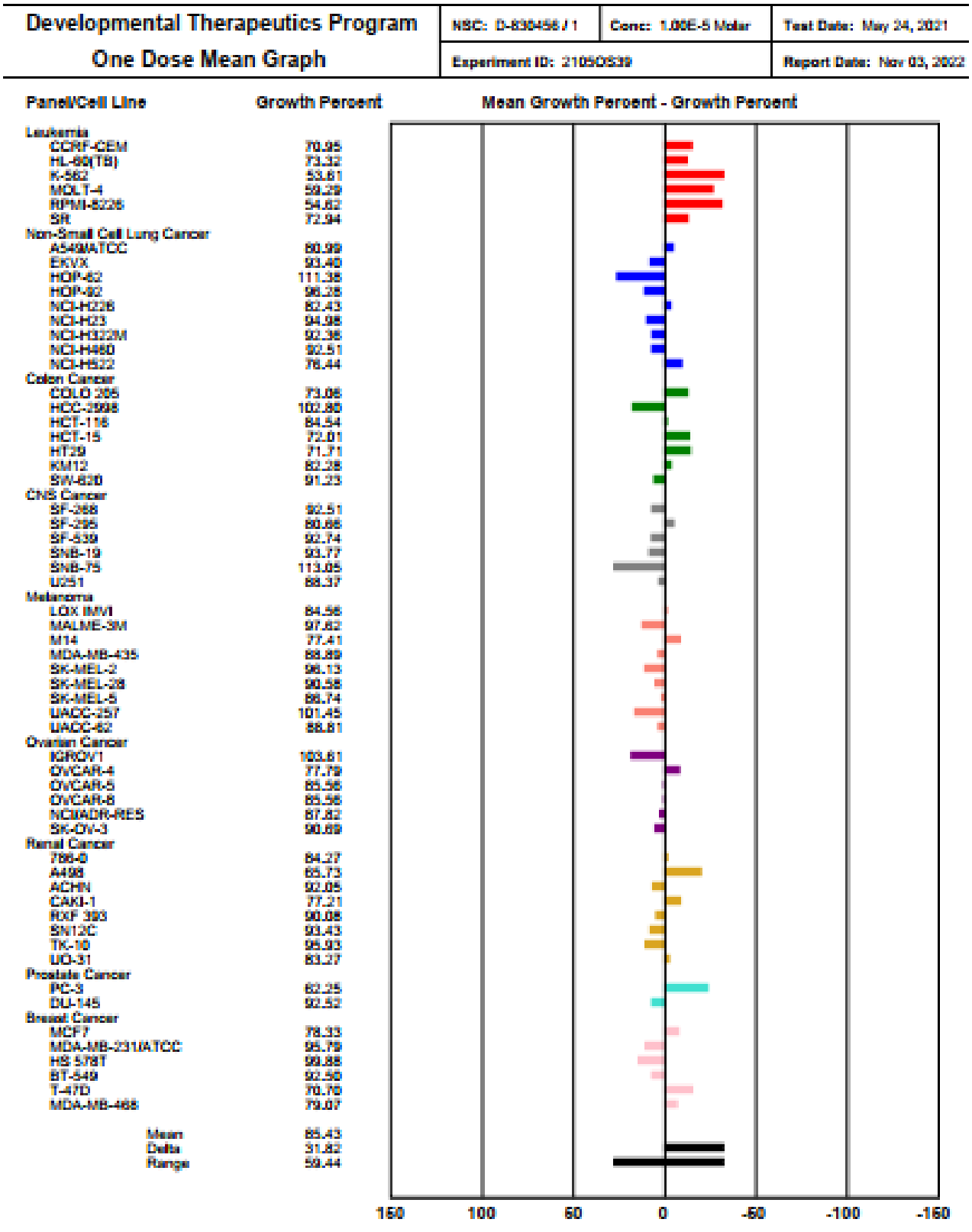

Figure S32: Results of primary *in vitro* one-dose anticancer assay of compound **3b.**

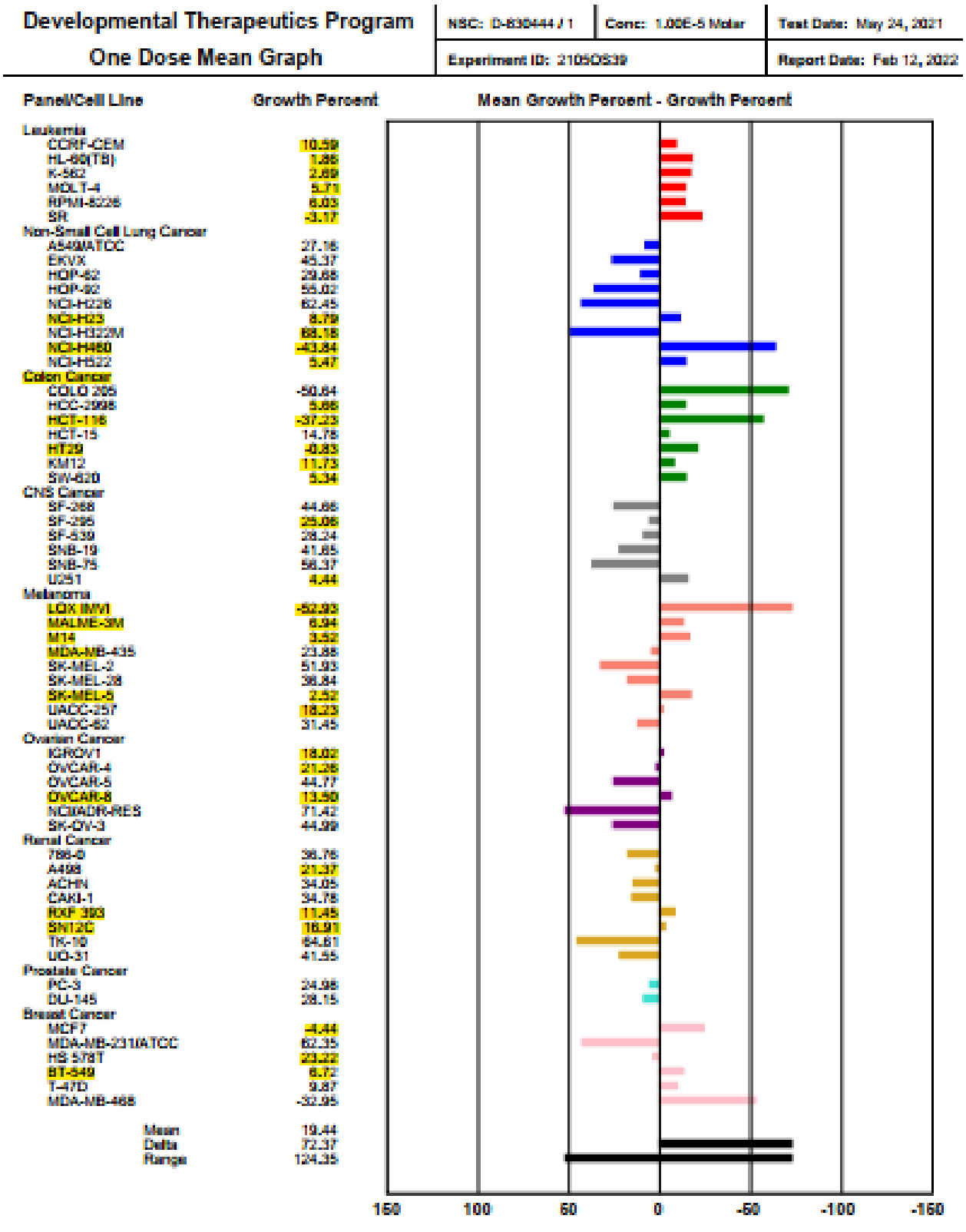

Figure S33: Results of primary *in vitro* one-dose anticancer assay of compound **3c.**

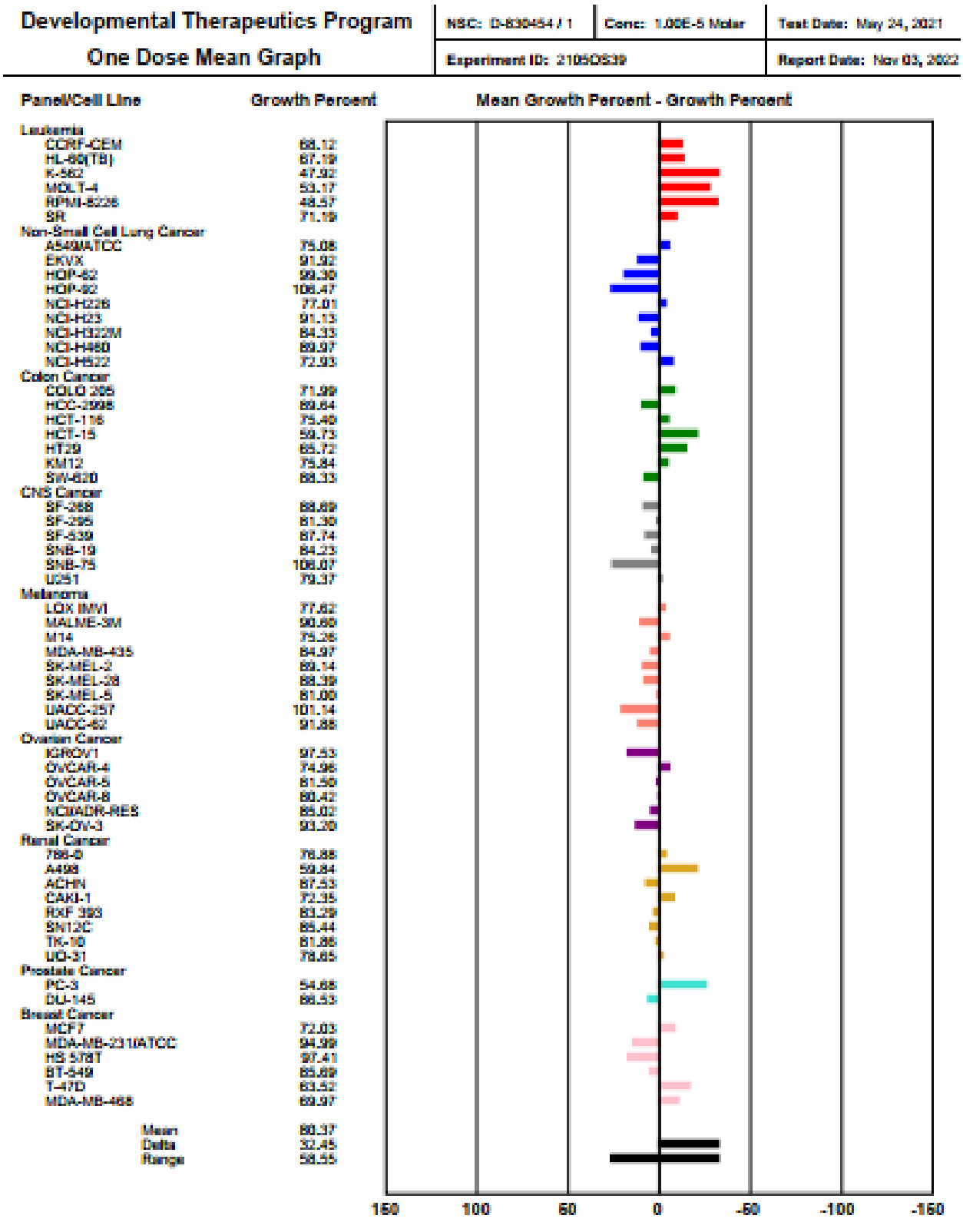

Figure S34: Results of primary *in vitro* one-dose anticancer assay of compound **3d.**

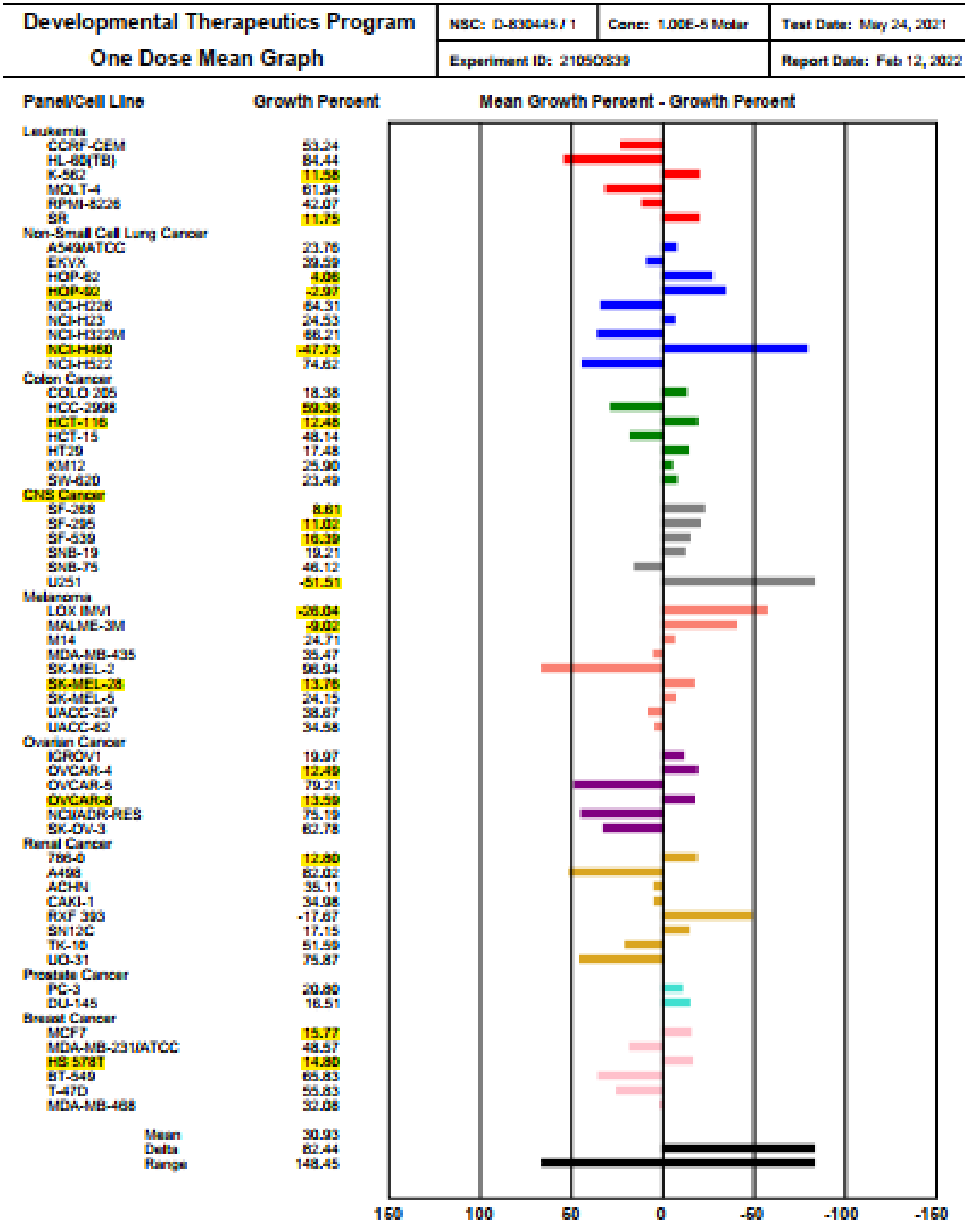

Figure S35: Results of primary *in vitro* one-dose anticancer assay of compound **3e.**

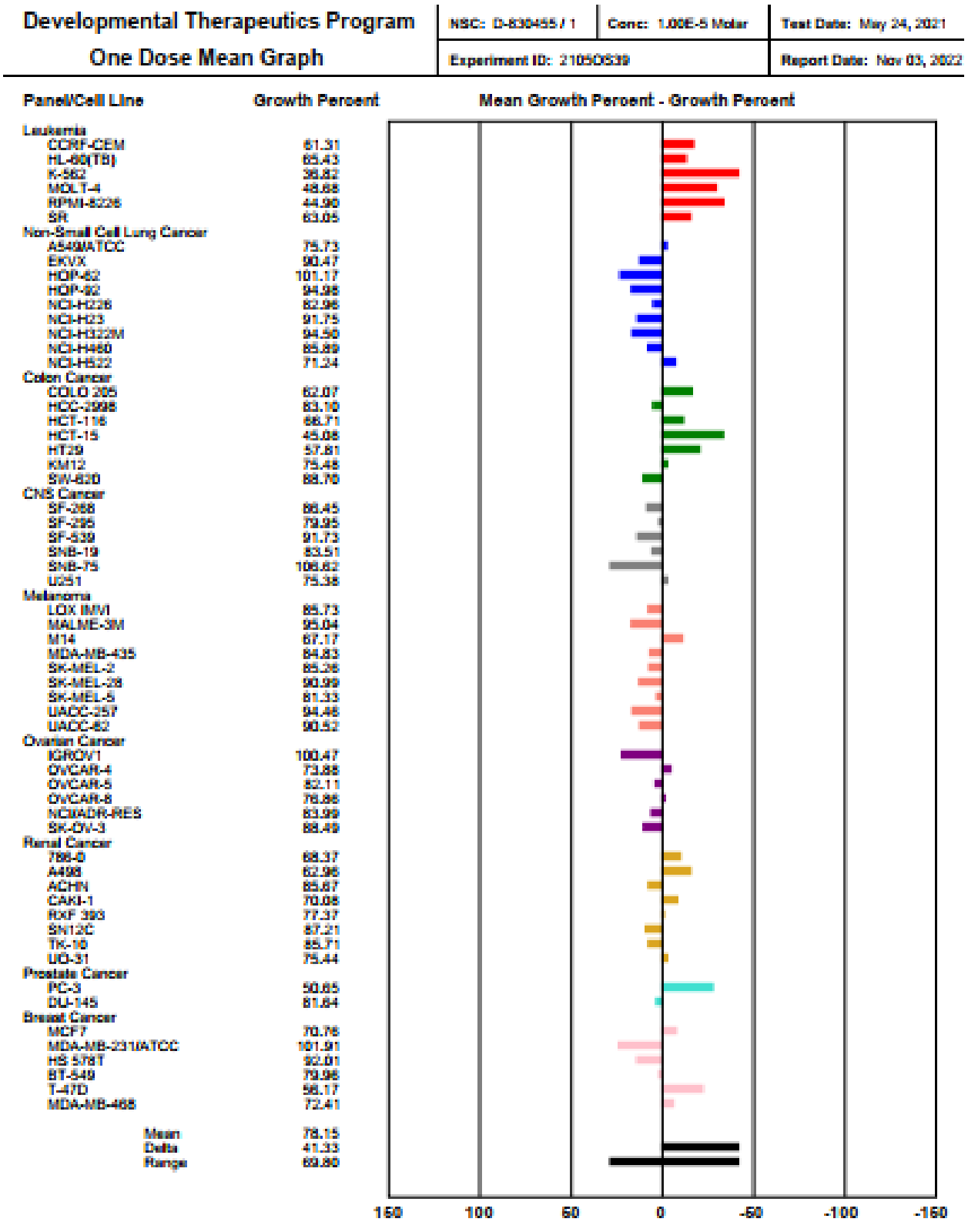

Figure S36: Results of primary *in vitro* one-dose anticancer assay of compound **3f.**

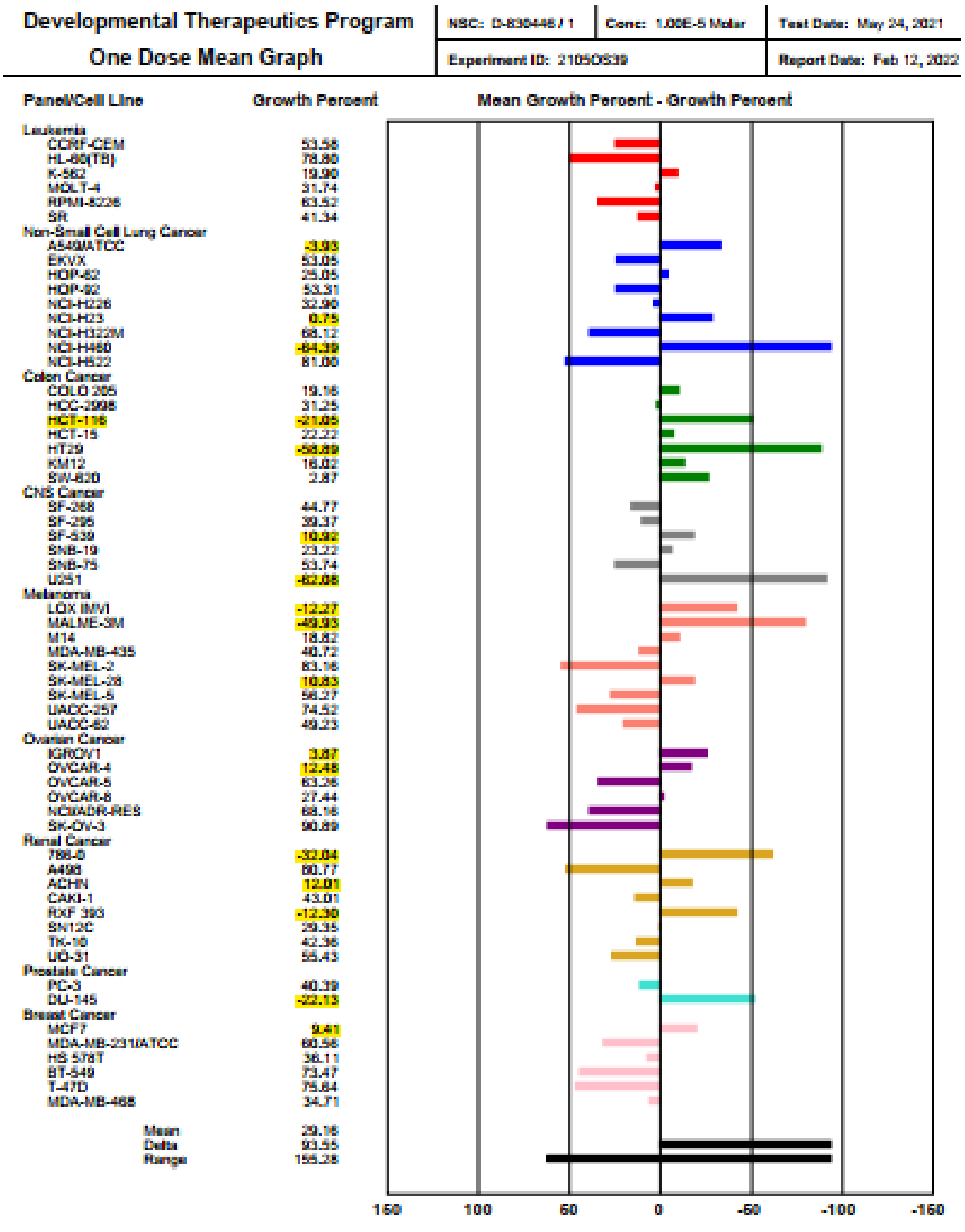

Figure S37: Results of primary *in vitro* one-dose anticancer assay of compound **3g.**

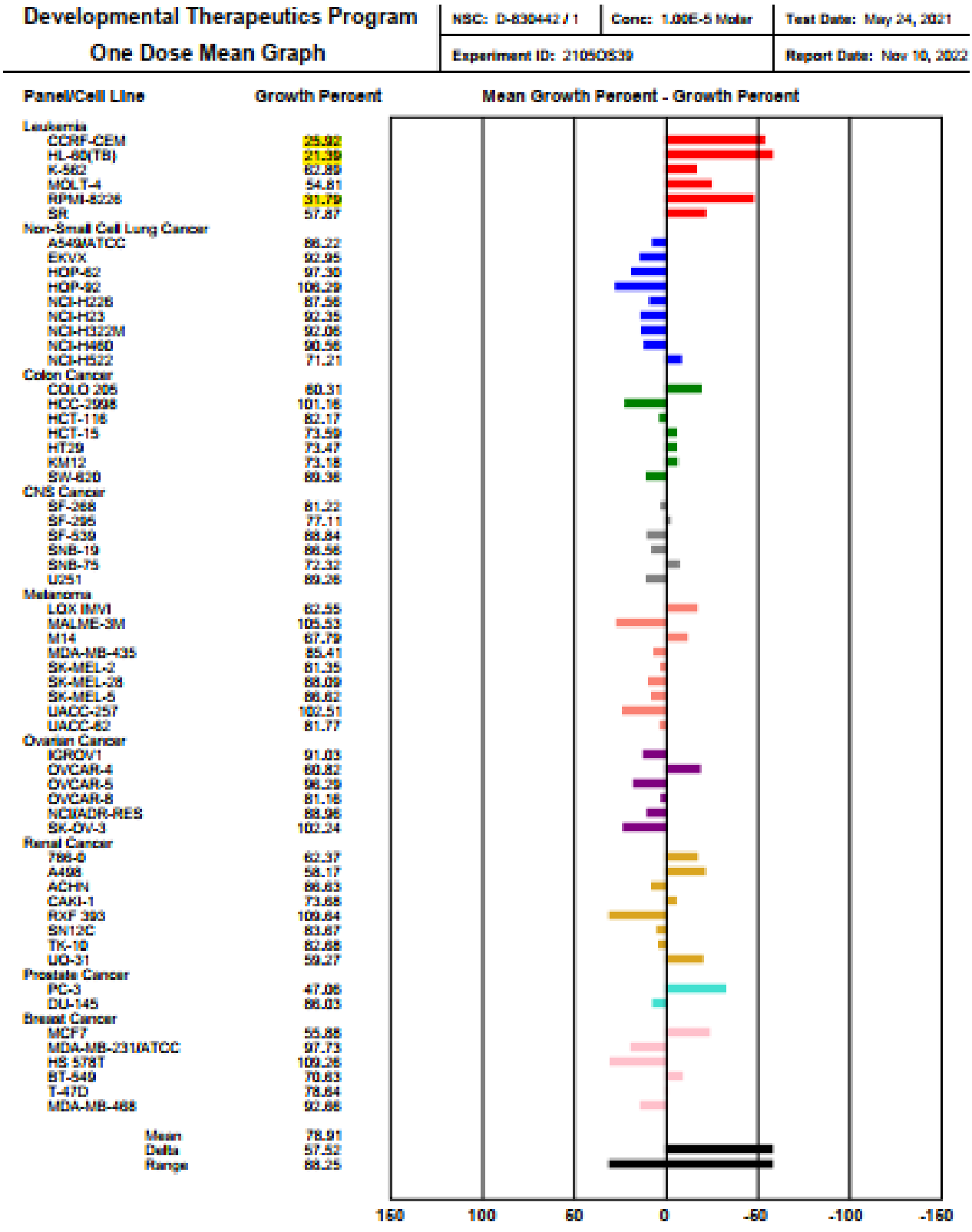

Figure S38: Results of primary *in vitro* one-dose anticancer assay of compound **3h.**

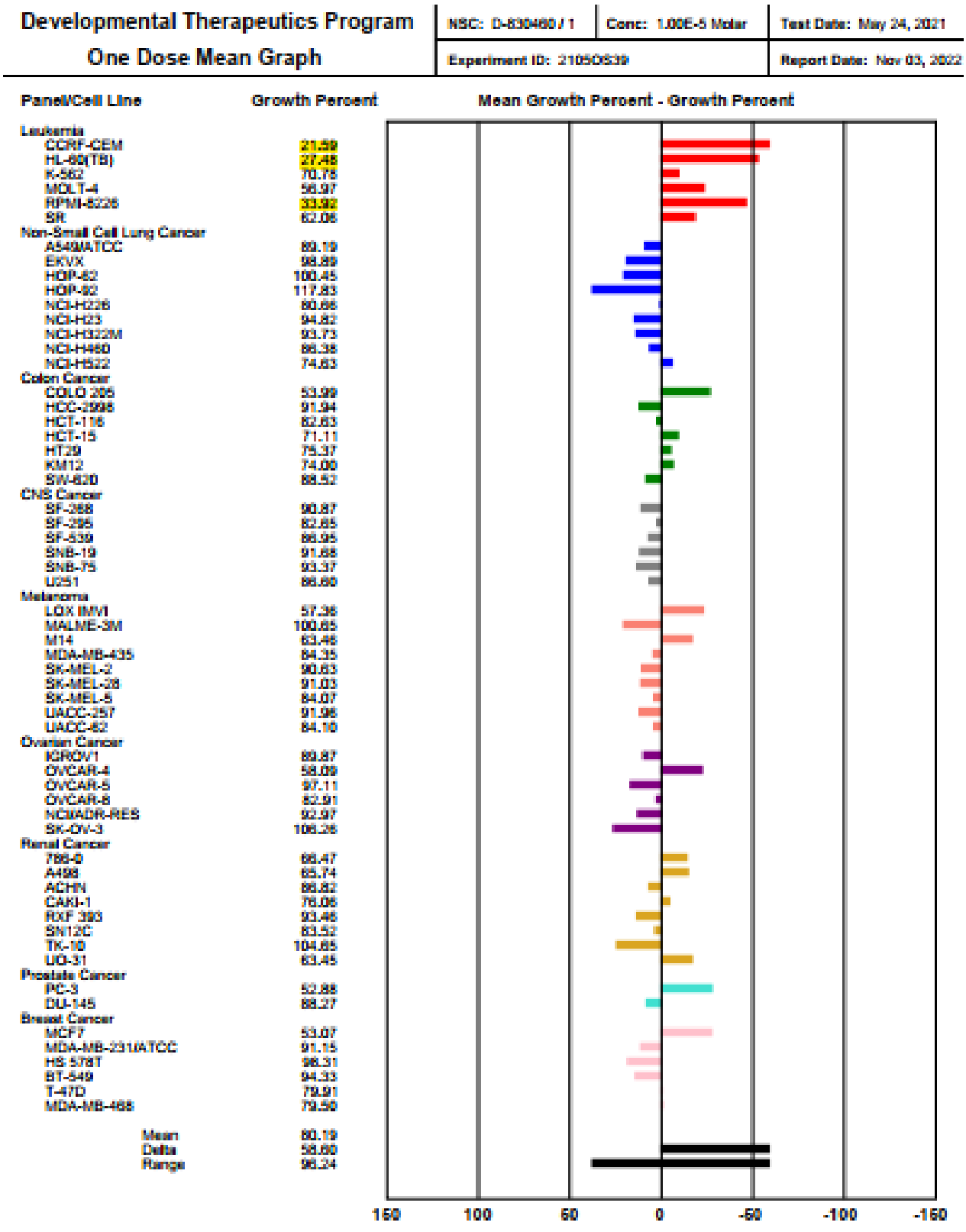

Figure S39: Results of primary *in vitro* one-dose anticancer assay of compound **3i.**

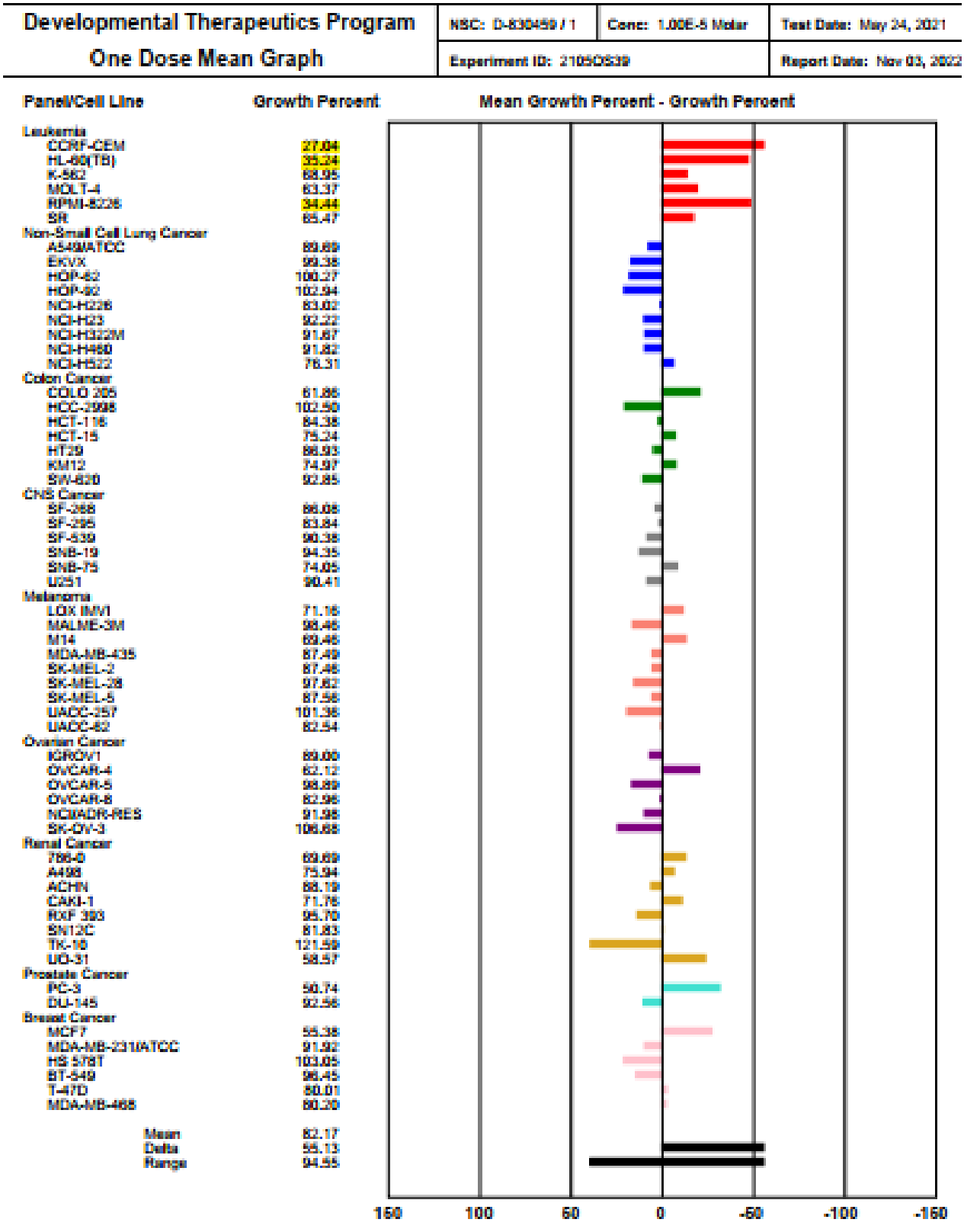

Figure S40: Results of primary *in vitro* one-dose anticancer assay of compound **3j.**

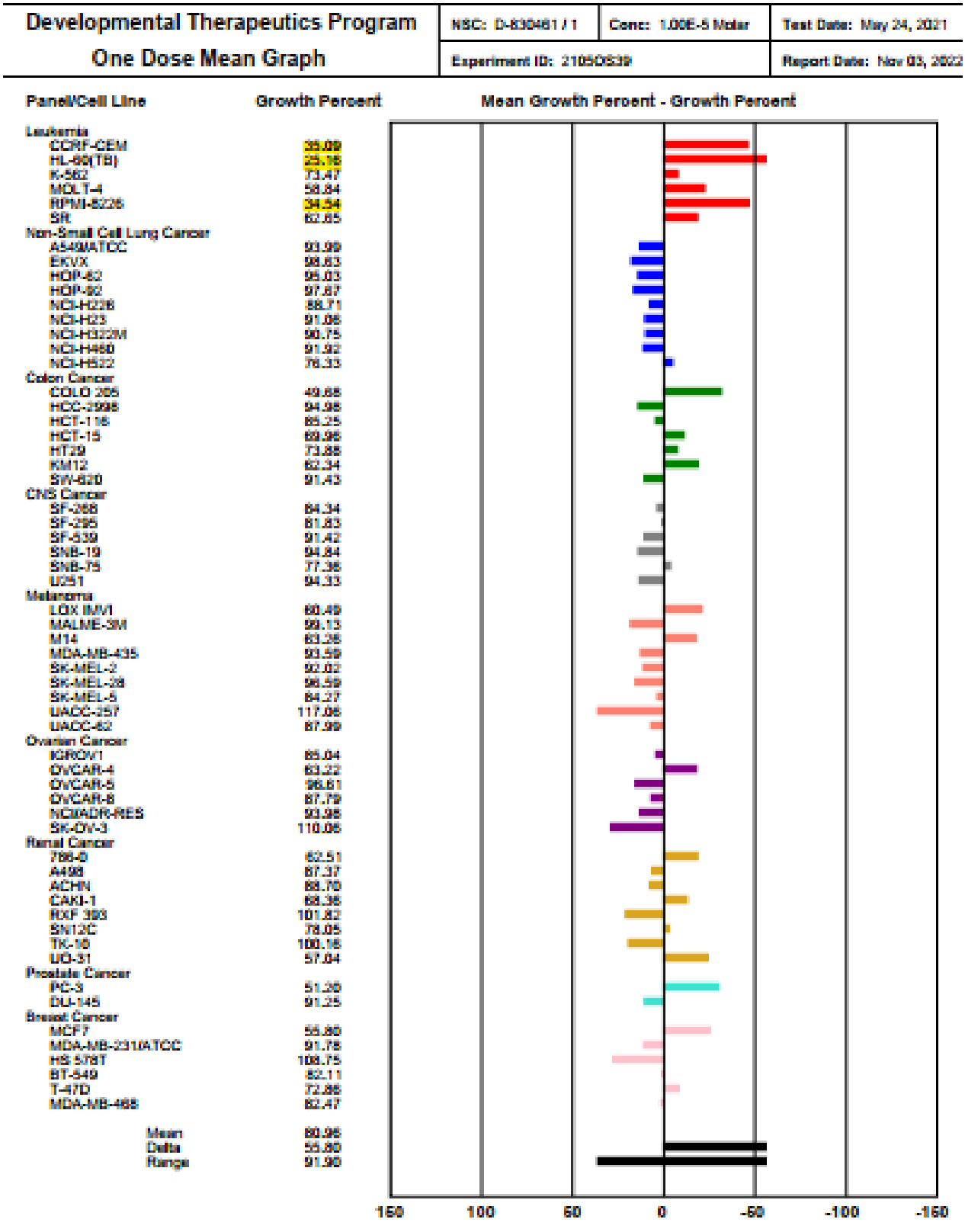

Figure S41: Results of primary *in vitro* one-dose anticancer assay of compound **3k.**

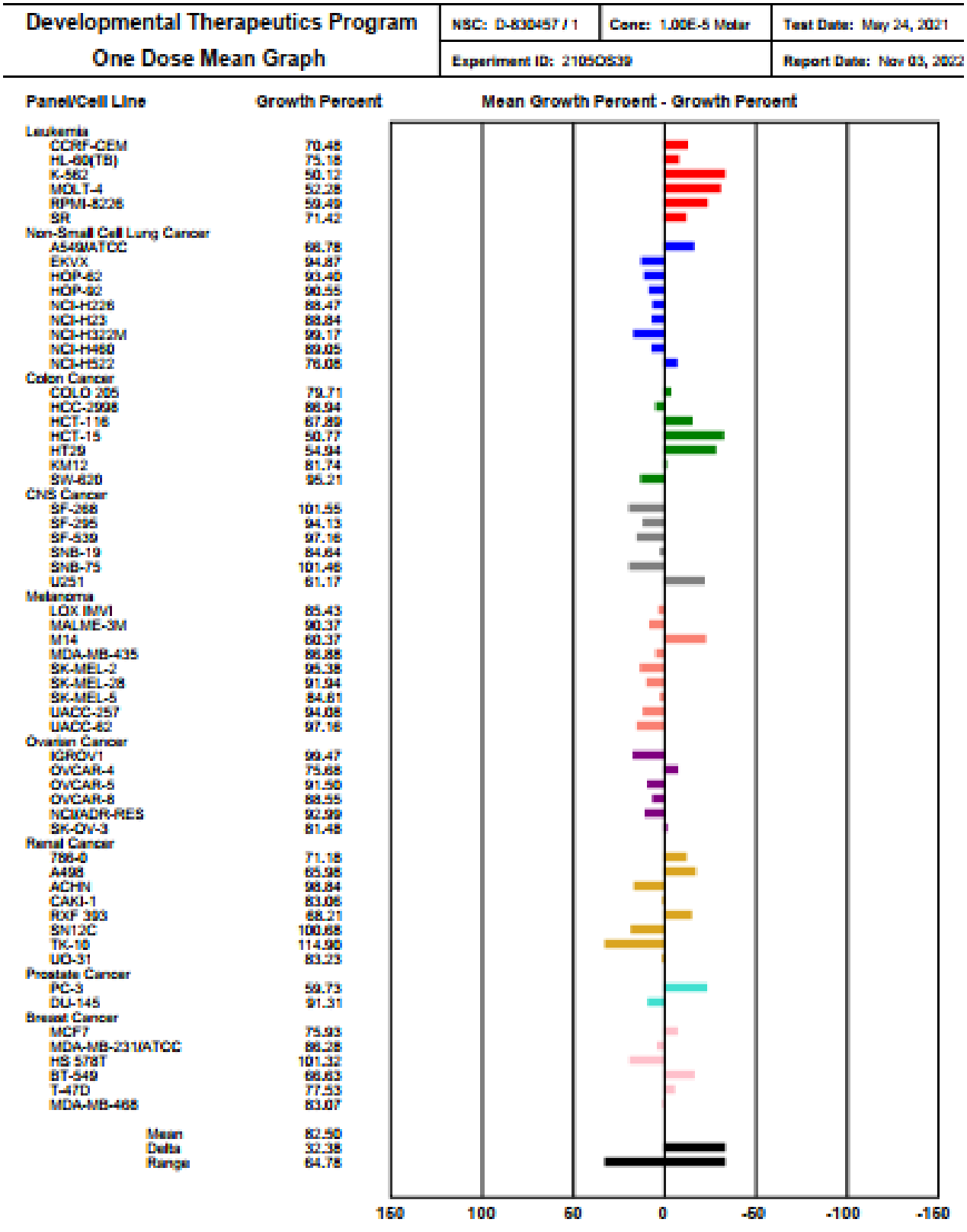

Figure S42: Results of primary *in vitro* one-dose anticancer assay of compound **3l.**

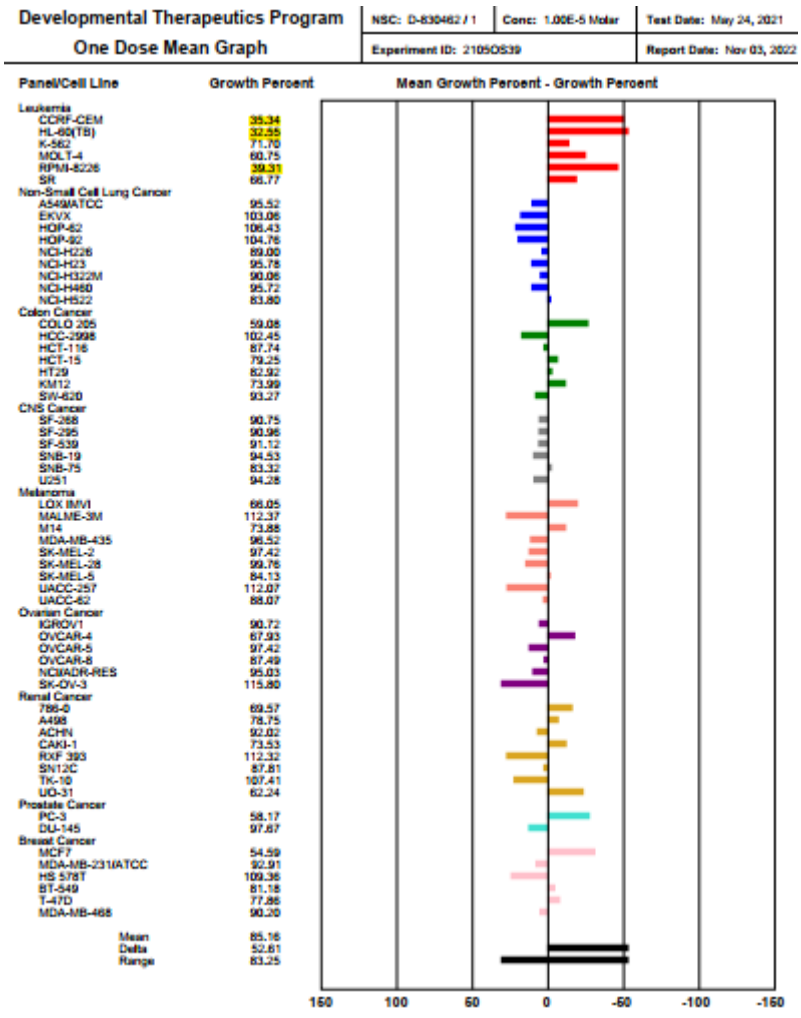

Figure S43: Results of primary *in vitro* one-dose anticancer assay of compound **3m.**

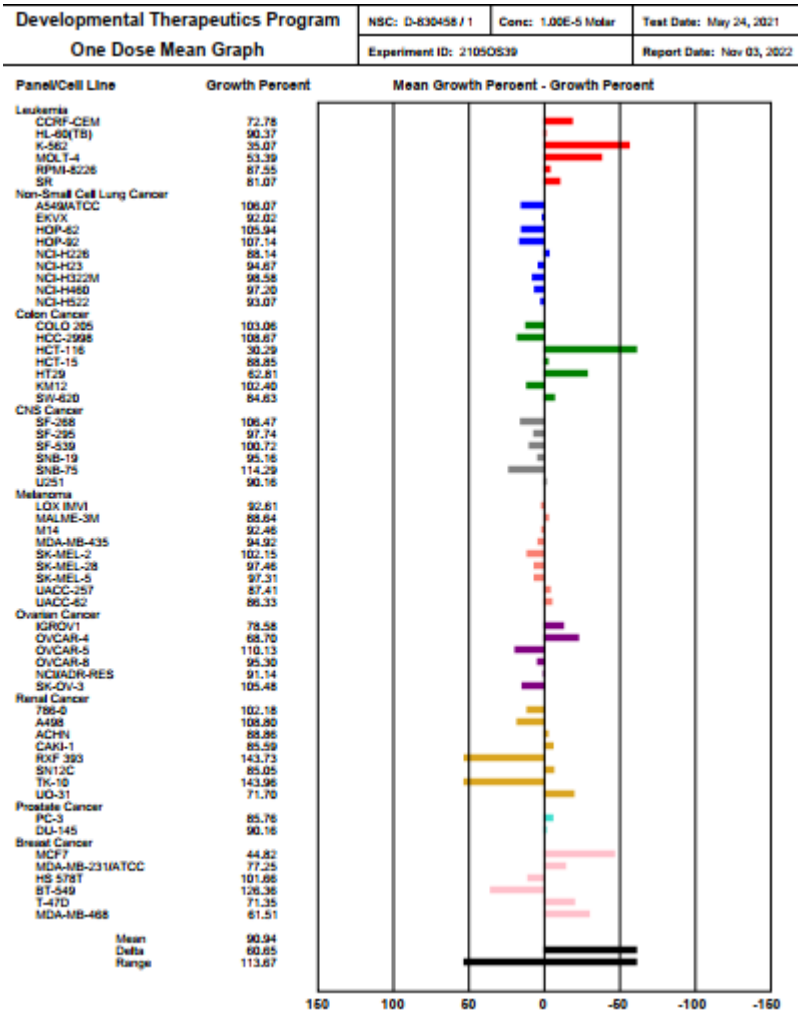

Figure S44: Results of primary *in vitro* one-dose anticancer assay of compound **3n.**

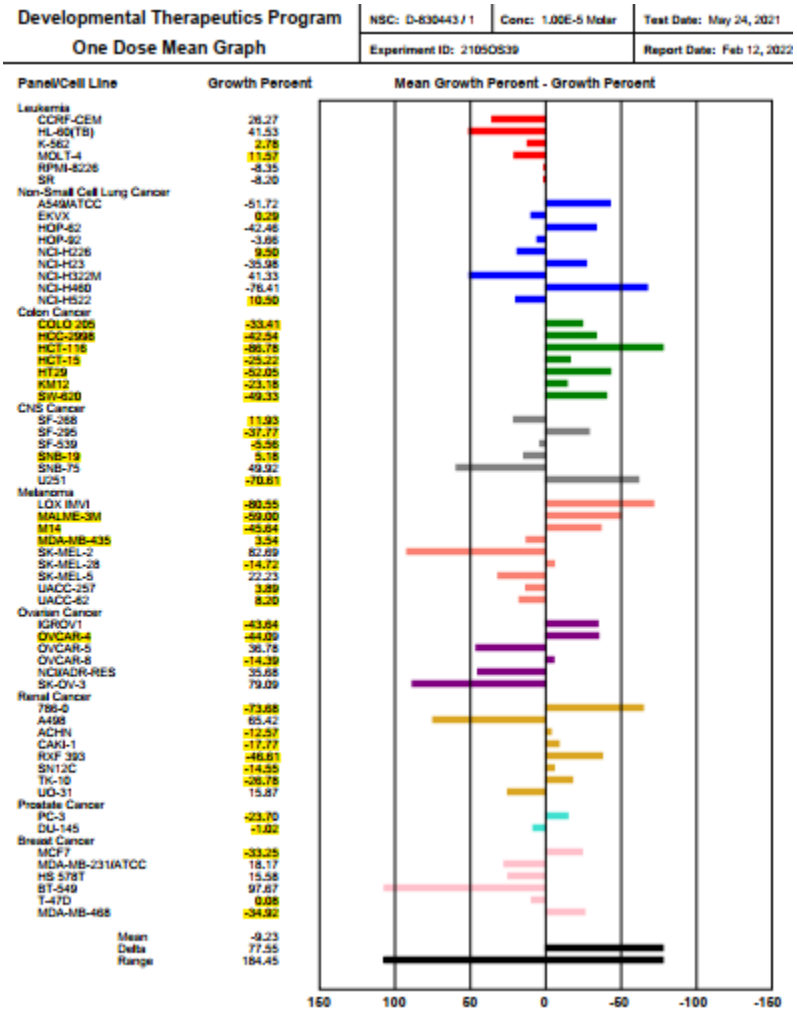

Figure S45: Results of primary *in vitro* one-dose anticancer assay of compound **3o.**

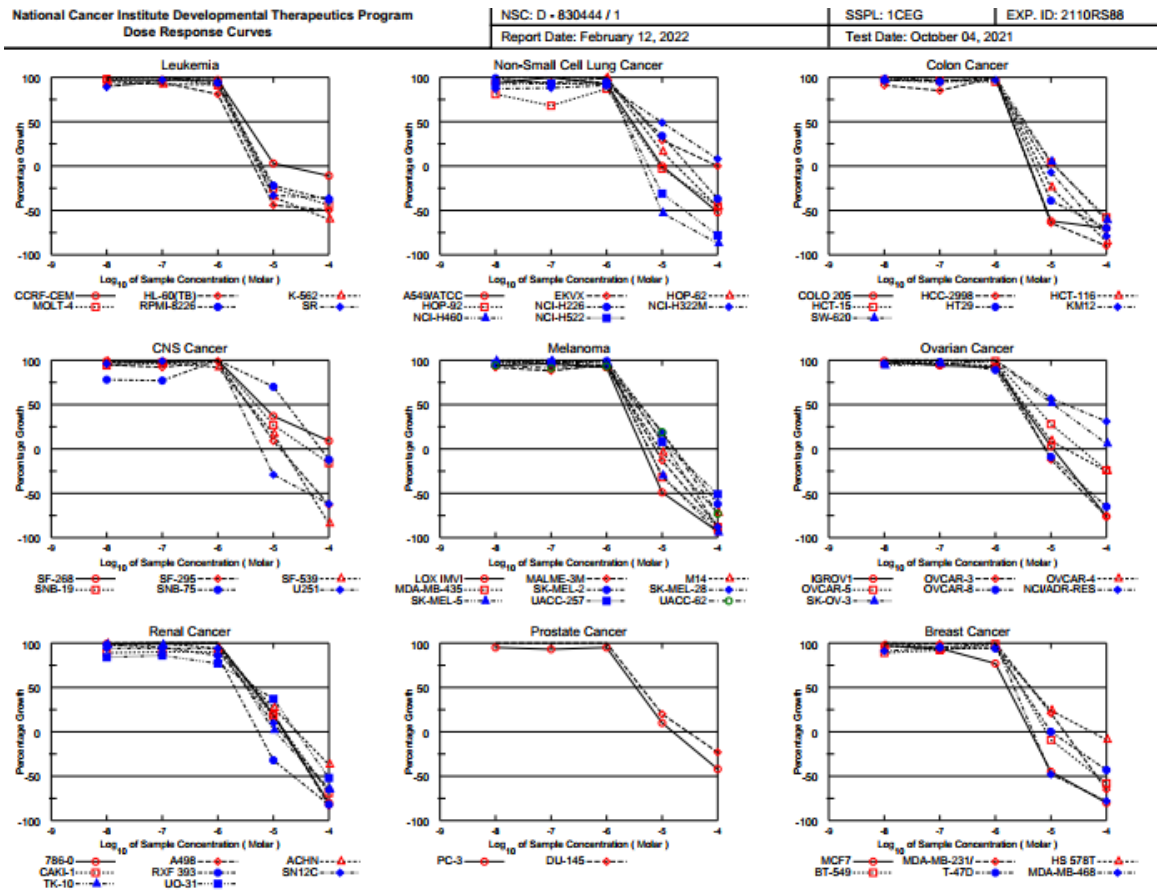

Figure S46: Dose response curve of the *in vitro* NCI five dose anticancer assay of compound **3c**.

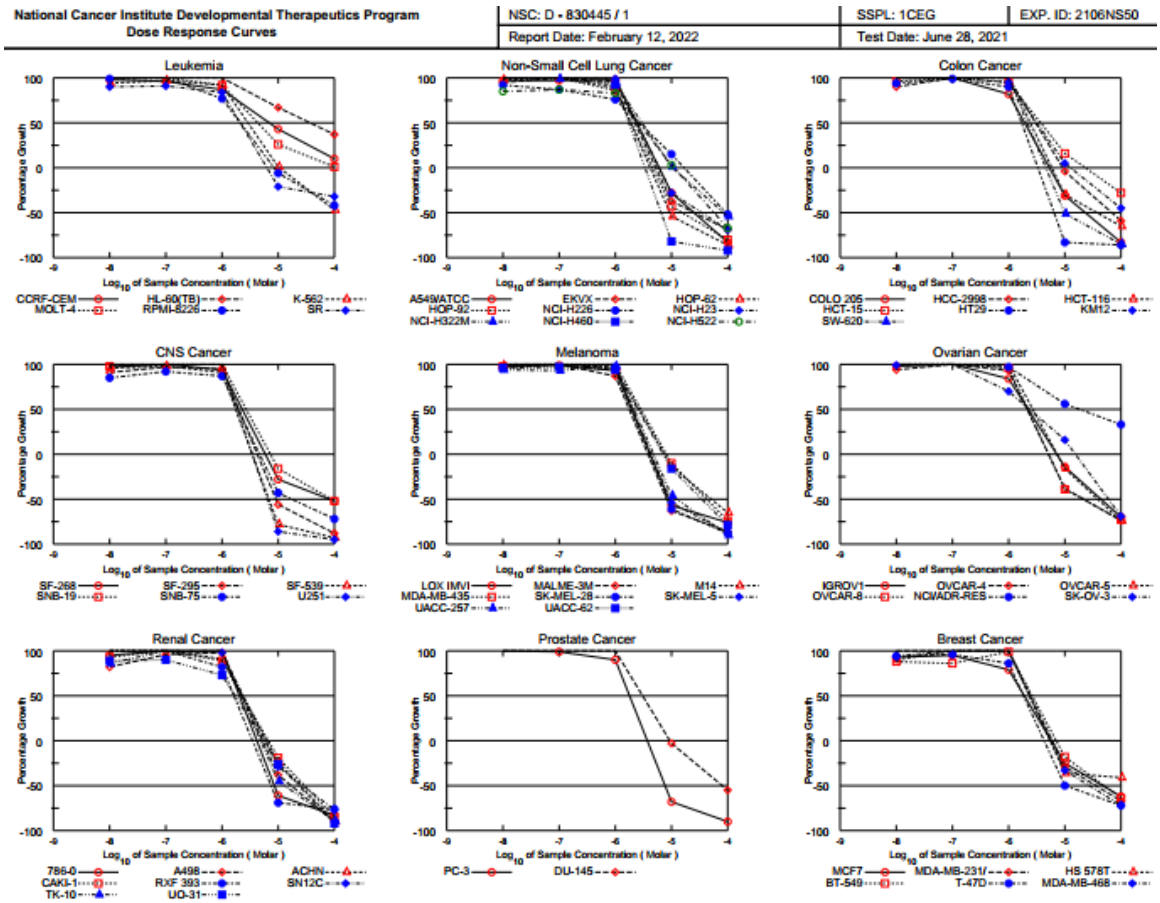

Figure S47: Dose response curve of the *in vitro* NCI five dose anticancer assay of compound **3e**.

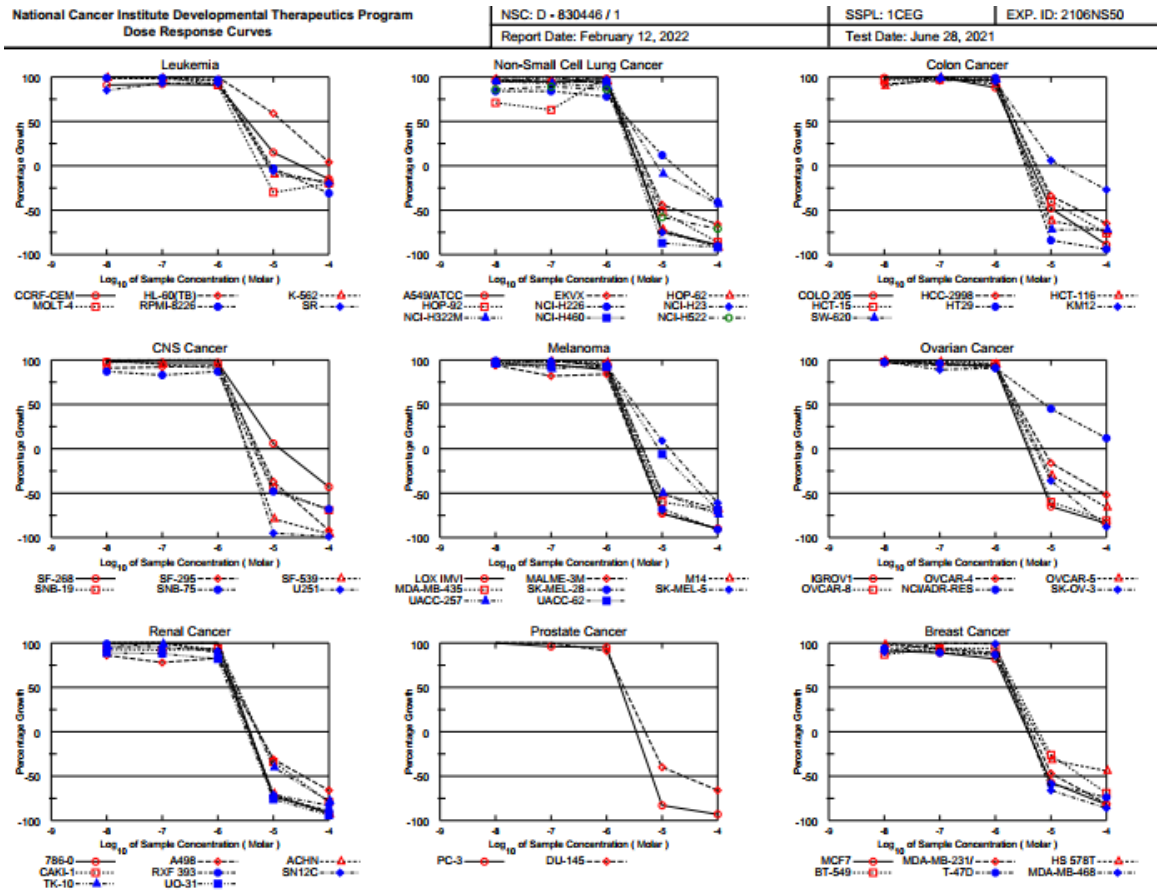

Figure S48: Dose response curve of the *in vitro* NCI five dose anticancer assay of compound **3g**.

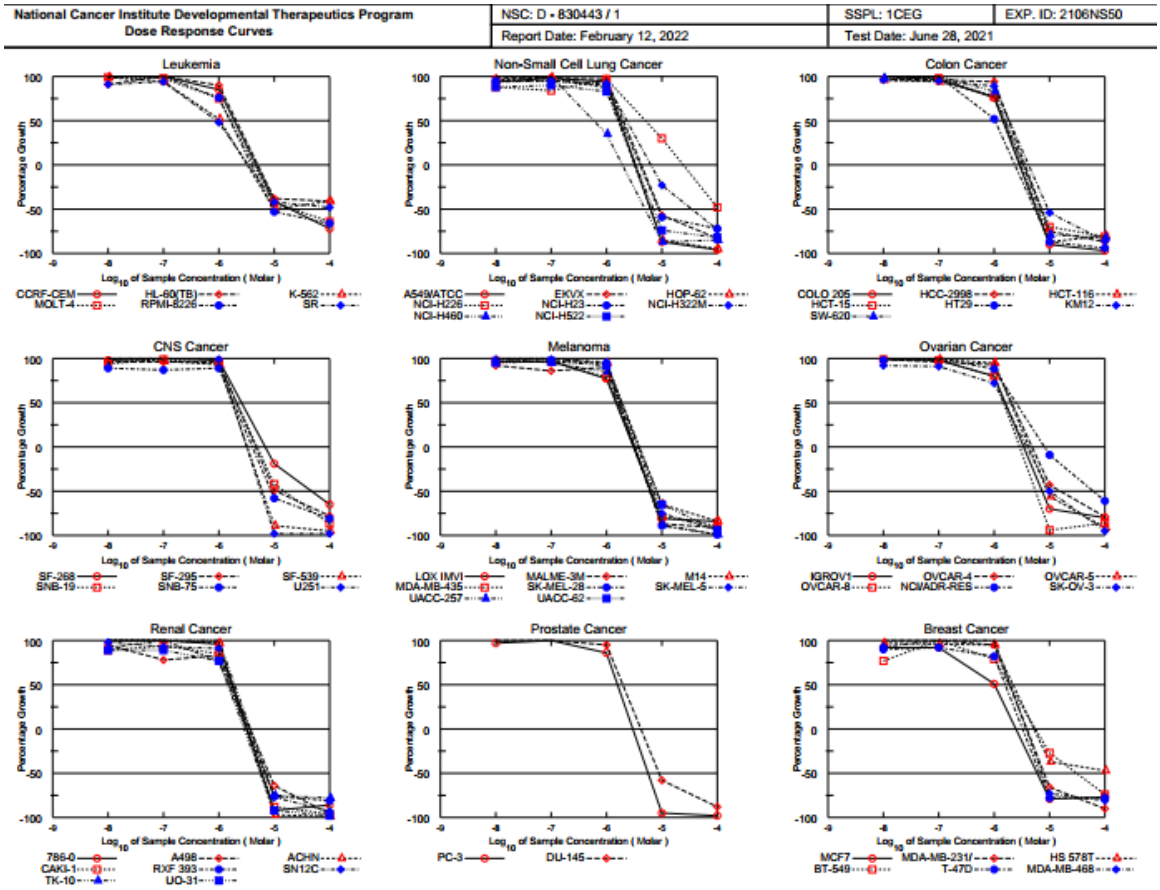

Figure S49: Dose response curve of the *in vitro* NCI five dose anticancer assay of compound **3o**.

Appendix A

4.2. Biological studies

4.1.1. Cytotoxic activity evaluation against lung cancer cell lines

To determine IC_{50}, benzimidazole molecules **3c, 3e, 3g** and **3o** were further analyzed using the standard MTT colorimetric assay against lung cancer A549 and NCI-H460 cell lines. Cells at density of 1 x 10^4 were seeded in a 96-well plate at 37 ^oC for 24 h under 5% CO_2. After incubation, the cells were treated with different concentrations of the test compounds **3c, 3e, 3g** and **3o** and incubated for 24 h, then 20 µl of MTT solution at 5 mg/mL was applied and incubated for 4 h at 37 °C. Dimethyl sulphoxide (DMSO) in volume of 100 µl was added to each well to dissolve the purple formazan that had formed. The color intensity of the formazan product, which represents the growth condition of the cells, is quantified by using an ELISA plate reader (EXL 800, USA) at 570 nm absorbance. The experimental conditions were carried out with at least three replicates, and the experiments were repeated at least three times.

4.2.2. VEGFR-2 inhibition assay

Benzimidazole compounds **3e, 3g** and Sorafenib were evaluated for their VEGFR-2 inhibitory activity according to manufacturer's instructions using # VEGFR-2 (KDR) Kinase Assay Kit Catalog # 40325 (BPS Bioscience).

4.2.3. Cell cycle analysis of compounds 3e and 3g

Cell cycle analysis in NCI-H460 cells was investigated using fluorescent Annexin V-FITC/ PI detection kit (*BioVision* EZCell^{TM} Cell Cycle Analysis Kit Catalog #K920) by flow cytometry assay. NCI-H460 cells at a density of 2×10^5 per well were harvested and washed twice in PBS. After that, the cells were incubated at 37 °C and 5% CO_2. The medium was incubated with the tested compounds **3e** and **3g** at their IC_{50} (µM) for 24 h, washed twice in PBS, fixed with 70% ethanol, rinsed again with PBS. Afterward,

medium was stained with DNA fluorochrome PI for 15 min at 37 °C. The samples were immediately analyzed using Facs Calibur flow cytometer (Becton and Dickinson, Heidelberg, Germany).

4.2.4. Apoptosis assay for compounds 3e and 3g

Apoptosis in NCI-H460 cells was investigated using fluorescent Annexin V-FITC/ PI detection kit (*BioVision* Annexin V-FITC Apoptosis Detection Kit, Catalog #: K101) by flow cytometry assay. NCI-H460 cells at a density of 2×10^5 per well were treated with compounds **3e** and **3g** at their IC_{50} (μM) for 24 h, then the cells were harvested and stained with Annexin V-FITC/ PI dye for 15 min in the dark at 37 °C. The samples were immediately analyzed using *FACS Calibur* flow cytometer (Becton and Dickinson, Heidelberg, Germany).

4.2.5. *C*aspase 9 assay for compounds 3e and 3g

To determine the effect of the synthesized benzimidazole hybrids **3e** and **3g** on apoptosis, the active *c*aspase 9 level was measured using ELISA analysis according to manufacturer's instructions. Briefly, NCI-H460 cells at a density of 2×10^4 per well were treated with compounds **3e** and **3g** at their IC_{50} (μM) for 24 h, then the cells were lysed with cell extraction buffer. This lysate was diluted in standard diluent buffer over the range of the assay. The optical density of each well was determined within 30 min using a microplate plate reader set at 450 nm to determine the human active *c*aspase 9 level.

4.2.6. Molecular modeling study

Docking studies, calculations, and investigations was conducting using Molecular Operating Environment MOE version MOE (2021.01.01 Chemical Computing Group, Canada Software) to perform molecular modeling study. Structure of **3g** was built in database of MOE. The X-ray crystal structure of Sorafenib bound to the VEGFR-2

enzyme binding site (PDB ID: 4ASD) active site was acquired from protein data bank at research collaboration for Structural Bioinformatics (RSCB) protein database [PDB].