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Supplementary Material

Thioamide Derivative of the Potent Antitubercular Decanesulfonylacetamide is Less Active Against *Mycobacterium tuberculosis* but a More Potent Antistaphylococcal Agent

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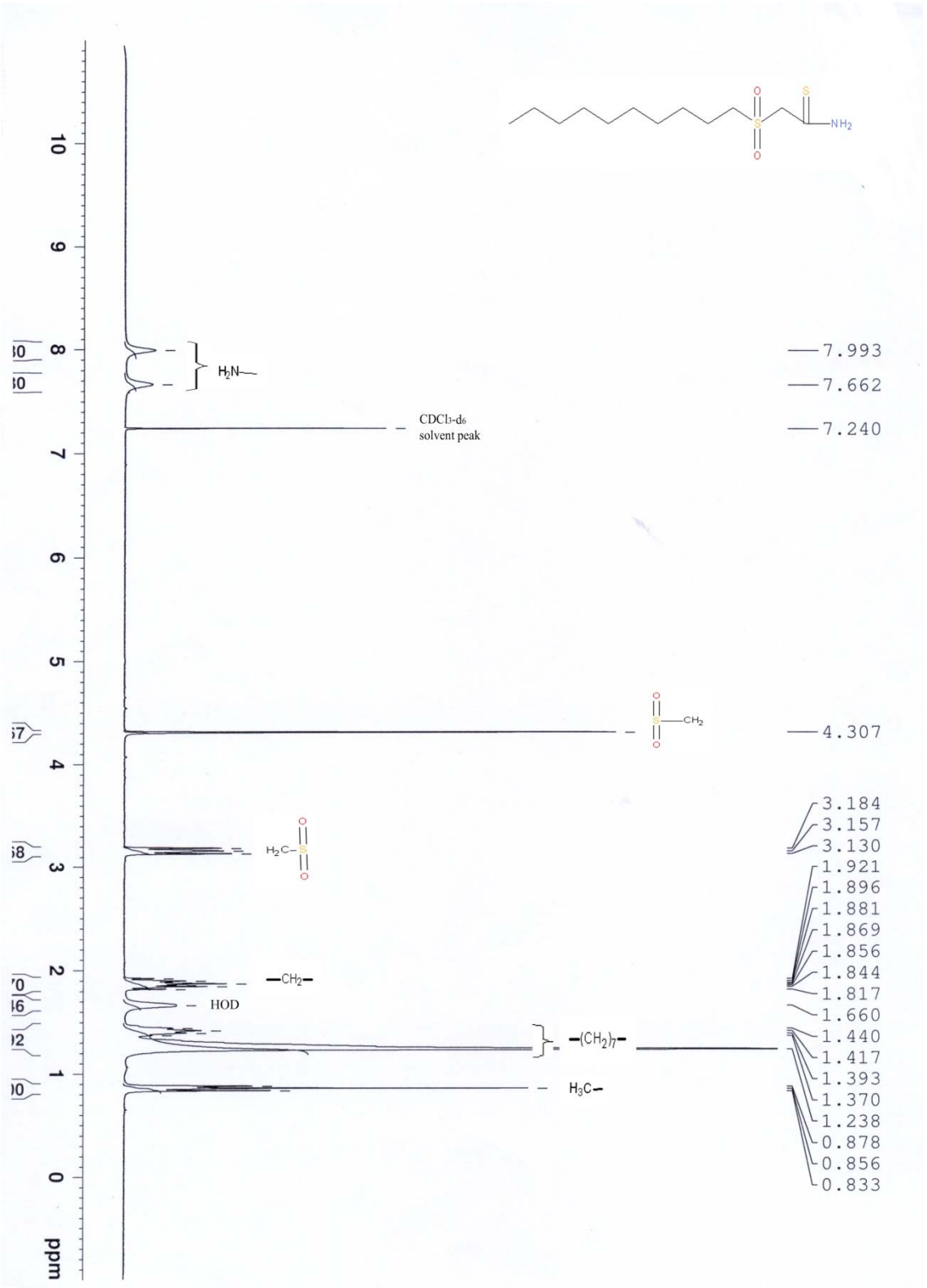
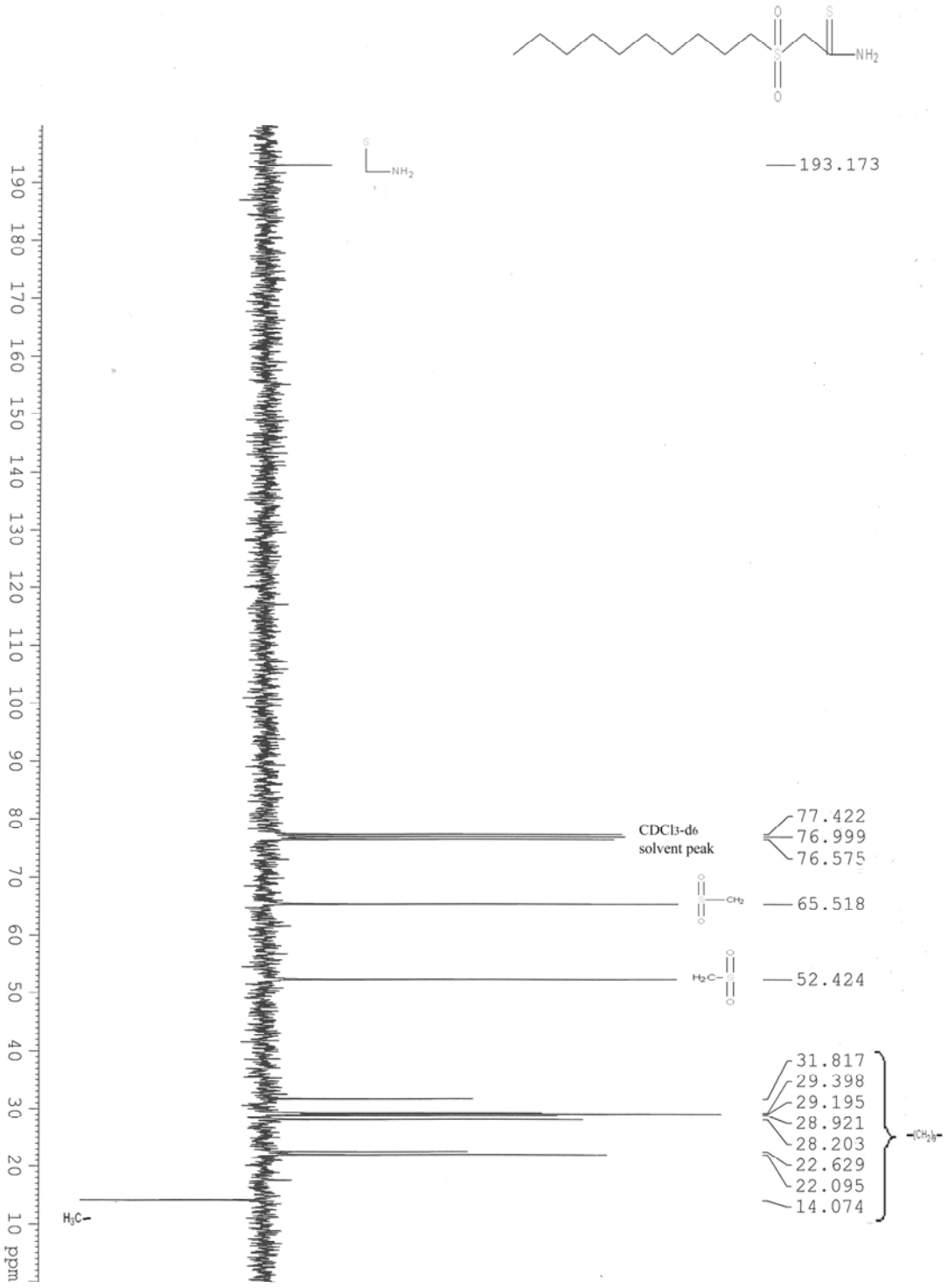
1. Proton NMR spectrum of **8**
2. DEPT NMR spectrum of **8**
3. Details of antibacterial and antifungal studies
4. Details of antitubercular studies
5. Details of cellular toxicity studies

1. Proton-NMR spectrum of **8**



2. DEPT NMR Spectrum of **8**



3. **Details of antimicrobial studies**

3.1 Reagents

S. aureus (ATCC 25923), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 27853) and *C. albicans* (ATCC 10231) were donated by Mr Zoran Klipic (Institute for Glycomics, Griffith University). Luria-Bertani (LB) broth was purchased from Oxoid (Hampshire, England) and the antibiotics (dicloxacillin sodium, vancomycin hydrochloride, gentamicin sulfate, ciprofloxacin hydrochloride monohydrate, ketoconazole) used for validating the assay were sourced from Sigma Aldrich (St Louis, MO, USA). All other chemicals and reagents were of analytical grade and sourced from Sigma Aldrich (St Louis, MO, USA). MilliQ water was sterilized by autoclaving at 121°C for 20 min. 96 well plates were purchased from Nunc (New York, USA).

3.2 Preparation of antibiotic, compound and resazurin stock solutions

Antibiotic stock solutions were prepared at 1000 mg/L in solvents recommended by Andrews. Dicloxacillin, vancomycin, gentamicin and ciprofloxacin were dissolved in water and ketoconazole was dissolved in methanol. The synthesized compounds **5-8** were dissolved in DMSO at a concentration of 1000 mg/L. Stock solutions were aliquoted and stored at 4°C and used within 1 month of preparation. Reference: J.M. Andrews, *J. Antimicrob. Chemother*. **2001**, *48 Suppl 1*, 5-16.

16.13 mg of resazurin powder was dissolved in 100 mL of sterile water to prepare a 704 μM resazurin stock solution. This resazurin stock solution was stored at 2-4°C and was used within 1 month of preparation. Reference: S.D. Sarker, L. Nahar, Y. Kumarasamy, *Methods* **2007**, *42(4)*, 321-4.

3.3 Preparation of bacterial and yeast culture

A single colony of streaked bacteria or fungi was transferred to 1 mL of sterile single strength broth and incubated overnight for 18 h. After which, the culture was adjusted to 0.5 McFarland with sterile single strength LB broth (~0.1Abs @ 600 nm). The culture was subsequently diluted 1:100 with sterile single strength LB broth for seeding of assay wells.

3.4 Antibacterial and antifungal assay

Antibacterial and antifungal assays were performed in 96 well microtitre plates. On the day of the experiment, antibiotic and test compounds stock solutions were diluted to 64 mg/L with sterile water to prepare working stock solutions. 37.5 μL of these antibiotic and test compound working solutions were transferred to the wells and sequentially diluted with water to prepare the following final concentrations: 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 mg/L. Appropriate vehicle/solvent controls were included in each assay. 37.5 μL of sterile double strength broth was subsequently transferred to all wells. 75 μL of prepared bacterial or fungal

culture was then transferred to the test wells. 75 μL of sterile single strength LB broth was transferred to the negative control wells. Final volume for each well was 150 μL.

The plates containing bacteria were covered and incubated at 37°C shaking at 100 rpm for 18 h. The plates containing yeast were covered and incubated at 30°C shaking at 100 rpm for 24 h. After incubation, 10 μL of resazurin stock solution was added to all wells. The plates were then incubated for a further 1 h at 37°C shaking at 100 rpm. Fluorescence was measured (excitation 485 nm, emission 535 nm) using a Fluoroskan Ascent microplate fluorometer (Thermo Scientific, Victoria, Australia). Each concentration of antibiotics used was repeated in triplicate and assays were performed on three separate occasions.

3.5 Data analysis

Percentage viability (relative to vehicle controls) was calculated from corrected fluorescent values. The MIC value of antibiotic controls and test compounds were determined as the first dilution at which at least 90% of growth was inhibited (MIC_{90}). Results are represented as the median value of replicate experiments.

Statistical analysis was performed using GraphPad Instat 3 (GraphPad Software, San Diego, CA, USA) on median value of the replicated experiments (triplicates). The Kruskal-Wallis non-parametric analysis of variance on ranks was used to determine whether there were significant differences among susceptibilities. Mann-Whitney tests were performed to determine specific differences between antibiotics and test compounds (Mann-Whitney test). Differences were considered significant when $P < 0.05$.

4. Antitubercular Studies

4.1 Materials and Methods

The antitubercular activity testing was conducted at the Centenary Institute of Cancer Medicine and Cell Biology (NSW, Australia) using methods adapted from Collins *et al*. The H37Ra (ATCC 25177) clinical strain of *M. tuberculosis* was used to screen for antitubercular activity. Reference: L. Collins, S.G. Franzblau, *Antimicrob. Agents Chemother.*, **1997**, *41*(5), 1004-9.

Stock solutions of the test compounds were prepared in DMSO and diluted twofold with 7H9GC broth (no Tween 80) to prepare final concentrations ranging from 2 μg/mL to 1000 μg/mL. Suitable vehicle controls were included. Antitubercular testing was performed in 96 well black plates (back view plates; Packard Instruments Company, Meriden, Conn) in order to minimize background fluorescence. The outer well of the 96 well black plates were filled with sterile water to prevent dehydration of the experimental wells.

Frozen H37Ra was initially diluted 1:20 in BACTEC 12B medium, followed by a 1:50 dilution in 7H9GC. This culture was added to the wells at a final bacteria titre of 50,000 CFU/mL. Wells containing drugs alone were included to detect and account for

autofluorescence of test compounds. Additional controls included bacteria and medium only containing wells. Plates were incubated at 37°C and at day 14, 20 μL of 10 × AlamarBlue® solution (Alamar biosciences/Accumed, Westlake, Ohio) and 12.5μL of 20% Tween 80 were added to all wells. The plates were then incubated for a further 24 h at 37°C. Following which fluorescence was measured at excitation of 530 nm and emission at 590 nm on a fluorescence microplate reader. Experiments were conducted in triplicate.

4.2 Data analysis

Results shown in Figure 1 were calculated as the percentage growth relative to vehicle controls and represented as the mean ± SD. Statistical analysis was performed with one-way ANOVA followed by Dunnett's post-test. Statistical significance was accepted at a probability level of $P < 0.05$. An MIC_{50} of 25 μM was set as the threshold for activity of interest. Only compound **5** (entry **8a** below) displayed significant activity.

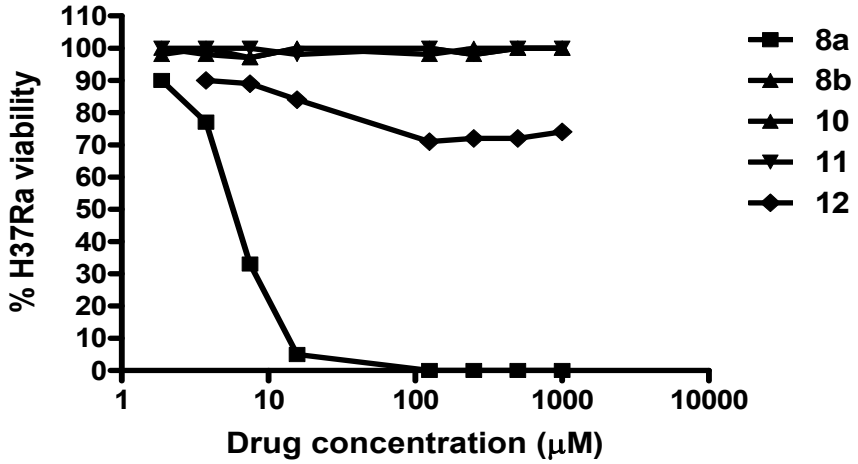


Figure S1. Percentage growth inhibition (relative to vehicle control) of the H37Ra strain of *M. tuberculosis*. Results represent mean of replicate experiments (n=3) and SD were <5%. Note: Entry **8a** is compound **5** in text, entry **8b** is compound **6** in text, entry **10** is compound **7** in text, entry **12** is compound **8** in text.

5. Cellular Toxicity Studies

5.1 Materials

The synthesised compounds **5**-**8** were dissolved in DMSO at a concentration of 1 mM. Stock solutions were aliquoted and stored at 2-4°C and used within 1 month of preparation. These were screened for cytotoxic effects against adherent A549, MCF-7, and HeLa cell lines using sulforhodamine B (SRB) colorimetric assay.

A549, MCF-7, and HeLa cell lines were obtained from ATCC (Manassas VA, USA). Complete Dulbecco's Modified Eagle Medium (DMEM)/F12, DMEM, high glucose RPMI 1640 medium, L-glutamine, sodium pyruvate, HEPES, and Penicillin-Streptomycin solution were purchased from Invitrogen (Victoria, Australia). All other chemicals and reagents were of analytical grade and sourced from Sigma Aldrich (St Louis, MO, USA). MilliQ water was sterilized by autoclaving at 121°C for 20 min. 96 well plates used in this study were purchased from Nunc (New York, USA).

5.2 Cell cultures

Adherent A549 cells were grown and maintained at 37°C with 5% CO_2 in complete DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 500 U/mL Penicillin-Streptomycin. Adherent HeLa and MCF-7 cells were grown and maintained at 37°C with 5% CO_2 in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine and 500U/mL Penicillin-Streptomycin.

5.3 Cell viability assay

For screening of the test compounds, cells were seeded at 1×10^4 trypan blue-excluding cells/mL in 96 well microtiter plates. 180 μL of cells, at appropriate cell density, were transferred to all experimental wells. 20 μL of the test compounds (at final concentrations of 5, 50 and 100 μM) or vehicle controls were added to each well, making up a final volume of 200 μL. Concentrated stock solutions of the test compounds were prepared in DMSO ensuring that the final solvent concentration remained below 1%. DMSO concentrations below 1% were shown to not affect cell viability of any of the tested cell lines during assay validation. Percentage cell viability was then assessed by either the colorimetric sulforhodamine B (SRB) or fluorescent-based resazurin assays.

The SRB colorimetric assay was used as the primary measure of cell viability for adherent A549, HeLa, and MCF-7 cells. SRB is a protein staining assay, which measures the cellular protein content of adherent or suspension cultures. SRB is a bright pink aminoxanthene dye with two sulfonic groups. Under acidic conditions, the two sulfonic groups bind to basic amino acids of cellular proteins, providing a colorimetric evaluation of the estimated total protein mass in relation to the cell number. The SRB assay has been shown to detect cell densities as low as 1,000 to 2,000 cells per well, with sensitivity comparable with several routinely used fluorescent assays. In addition, the SRB assay exhibits linearity over a dynamic range of cell densities.

The SRB assay kits were purchased from Sigma Aldrich (St Louis, MO, USA). The cells were treated with test compounds for 24 h for screening. After which, 50 μL of the 50% trichloroacetic acid (TCA) solution was added to each well to growth medium and incubated for 1 h at 4°C to fix the cells. The supernatant was removed and cells washed several times with water to remove TCA and serum proteins. Plates were subsequently air dried overnight. 50 μL of SRB staining solution was added to cells for 20 min and then removed by washing with 1% acetic acid solution until all unincorporated dye was removed. Plates were again air dried overnight. 200 μL of Tris base solution was added to each well and incubated for 5 min

at room temperature on a IKA gyrator shaker (Crown Scientific, Minto, NSW) to enhance dye mixing. Absorbance was measured using a Titertek Multiskan MKII absorbance plate reader (Huntsville, USA) at an absorbance of 492 nm.

5.4 Data analysis

The test compounds were screened at concentrations of 5, 50 and 100 μM. Cell viability was determined by comparing fluorescence of treatment wells to their respective vehicle controls. Results were calculated and represented as the percentage viable cells, relative to vehicle controls. Reported values represent the mean ± S.E.M of replicate experiments performed on different days. Statistical analysis was performed using GraphPad Instat 3 (GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by Dunnett's test were used to assess statistical differences between treatments. Statistical significance was accepted at a probability level of $P < 0.05$.

5.5 Results

The effect of compounds **5-8** on percentage viability of HeLa human cervical carcinoma cells is presented in Figure S2. These results are discussed in the paper.

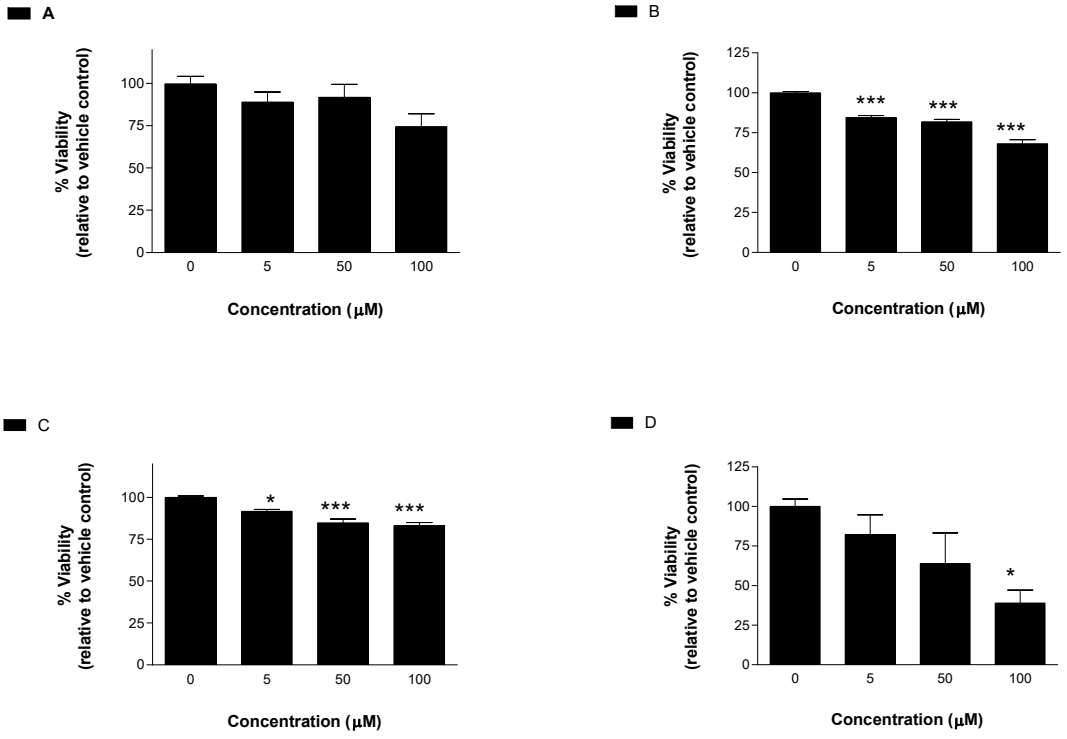


Figure S2 (Figure 2 in text). Percentage cell viability of HeLa treated with test compounds 5 (A), 6 (B), 7 (C) and 8 (D). Each bar represents mean ± S.E.M. (n = 3). Symbols (*) and (***) indicate statistical significance of $P < 0.05$ and $P < 0.001$, respectively, as compared to vehicle control.

The effect of compounds **5-8** on percentage viability of A549 human alveolar adenocarcinoma cells is presented in Figure S3. All compounds appeared to affect A549 cell viability in a concentration-dependent manner; however, observations were only statistically significant for compounds **5** and **8**. Statistically significant reductions in cell viability were observed at both 50 (28.8 ± 7.3%) and 100 μM (31.6 ± 7.4%) concentrations for compound **5**. Compound **8** produced maximal observed inhibitory effect, reducing A549 cell viability by 50 ± 12.5% at the concentration of 100 μM ($P < 0.05$). Although 50 μM of compound **8** reduced viability by 23.3 ± 13.31%, this observation was not statistically significant. Compounds **6** and **7** tended to reduce viability at 50 and 100 μM concentrations, however, these observed effects were not statistically significant when compared to vehicle controls ($P > 0.05$). Reduction in A549 cell viability at the 100 μM concentration were 30.7 ± 11.2% and 24.1 ± 12.5% for compounds **6** and **7**, respectively.

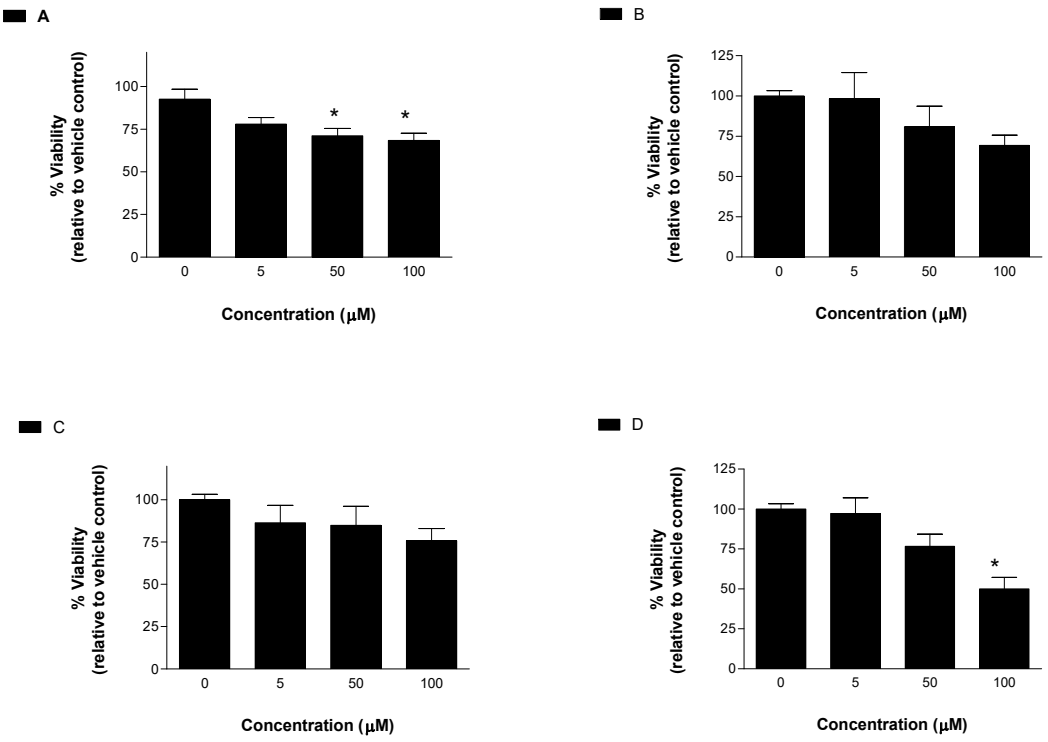


Figure S3. Percentage viability of A549 cells treated with test compounds 5 (A), 6 (B), 7 (C) and 8 (D). Each bar represents mean ± S.E.M. (n=3). Symbols (*) indicate statistical significance of $P < 0.05$ as compared to vehicle control.

The effect of compounds **5-8** on percentage viability of MCF-7 human breast adenocarcinoma cells is presented in Figure S4. Compounds **5** and **7** tended to produce a subtle, but not statistically significant, reduction in viability of MCF-7 cells. At concentrations of 100 μM, compounds **6** and **8** reduced viability by 27.9 ± 10.2% ($P < 0.05$) and 41.0 ± 9.5% ($P < 0.01$), respectively. No significant alterations in cell viability were observed for lower concentrations of compounds **6** and **8**.

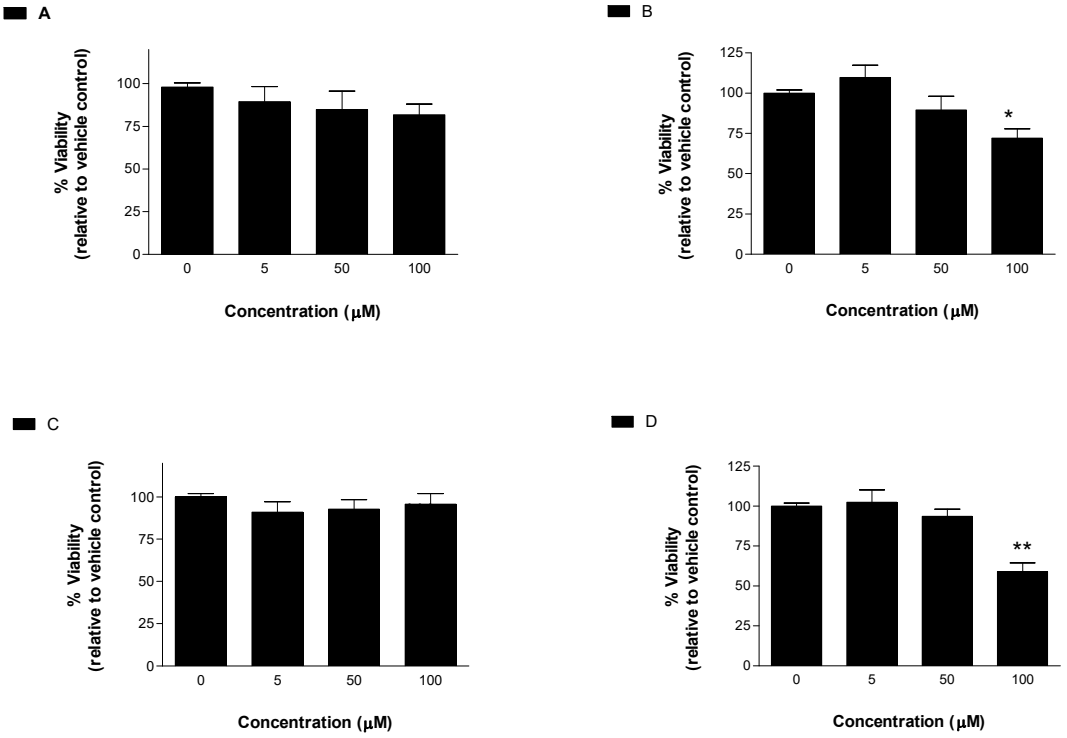


Figure S4. Percentage viability of MCF-7 cells treated with test compounds 5 (A), 6 (B), 7 (C) and 8 (D). Each bar represents mean ± S.E.M. (n = 3). Symbols (*) and (**) indicate statistical significance of $P < 0.05$ and $P < 0.01$, respectively, as compared to vehicle controls.