Design, Synthesis and *in vitro* Characterization of Novel Hybrid Peptidomimetic Inhibitors of STAT3 Protein

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S.1. Fluorescent Polarization Assay

The fluorescent polarization experiments were performed on an Infinite M1000 (Tecan, Crailsheim, Germany). All assays were performed in the same buffer conditions of 50mM NaCl, 10mM Hepes, pH 7.5, 1mM EDTA, and 2mM dithiothreitol. Fluorescent-labelled peptides were kept at a final concentration of 10nM in buffered solution. 7.5mL of labelled peptides were added to 15mL of a 300nM STAT3 protein solution (provided by SignalChem in 50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.25mM DTT, 0.1mM PMSF, 25% glycerol and stored at -80^{o}C). Inhibitory molecules were dissolved in pure DMSO and diluted using buffer solution to produce four final concentrations (250 μL, 31.25 μL, 3.90 μL, 0.49 μL). 7.5 μL of the inhibitory molecule solution was mixed with the STAT3 protein and labelled peptide solution in black 384-flat well micoplates (Corning), and incubated for 15-30 minutes. The M1000 performed a shaking cycle and then read the plates. IC_{50}'s were derived from the output using the curve-fitting software OriginPro 8 (Northampton, MA). The FP data was modelled by the following equation:

$$p(X) = \frac{p_1 \times IC_{50} + p_2 \times X}{IC_{50} + X}$$

where X was the concentration of inhibitor and p was the corresponding fluorescence at that concentration. The free parameter was half the maximal inhibitory concentration (IC_{50}) and the limiting values was the maximal measured fluorescence polarization (p_1) and the minimal fluorescence polarization (p_2). Origin curve fitting software utilizes the Levenberg-Marquardt algorithm and reduced chi-square criterion for convergence. The inhibitor dissociation constant, K_i, was calculated from the derived IC_{50} values, as per the following formula:

$$K_i = \frac{IC_{50}}{1 + \frac{[STAT3]}{K_d}}$$

where [STAT3] = 150nM and K_d= 100 nM.

S.2. Fluorescence binding curves for hybrid peptidomimetic inhibitors

Data are representative of 3 independent assays.

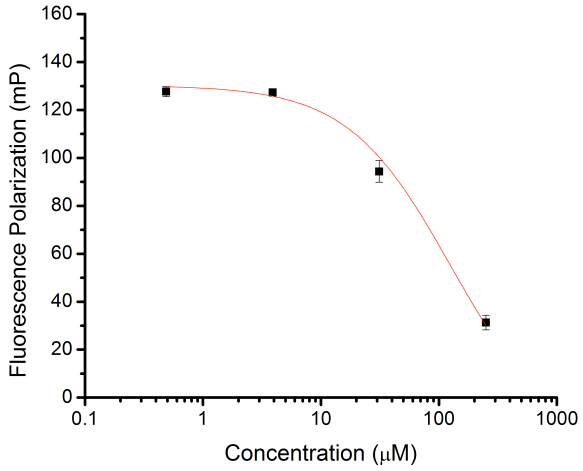

Figure 1. Competitive binding of **14bc** measured by fluorescence polarization assay, with a calculated K_i = 25 ± 6 μM. Curve fitted using ORIGIN software.

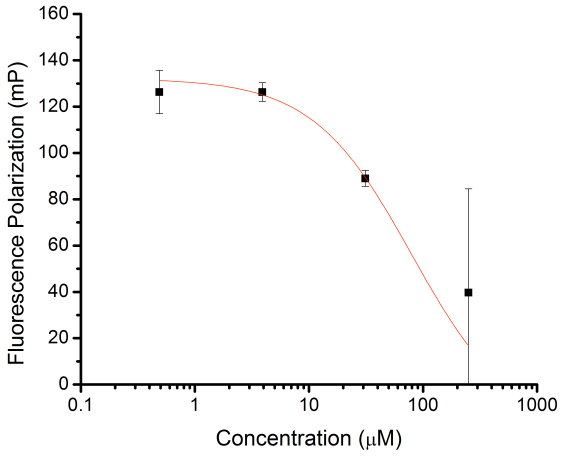

Figure 2. Competitive binding of **14bf** measured by fluorescence polarization assay, with a calculated K_i = 23 ± 2 μM. Curve fitted using ORIGIN software.

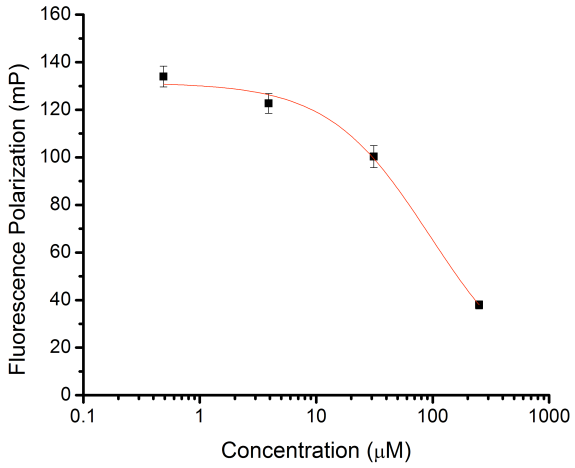

Figure 3. Competitive binding of **14bd** measured by fluorescence polarization assay, with a calculated K_i = 38 ± 16 μM. Curve fitted using ORIGIN software.

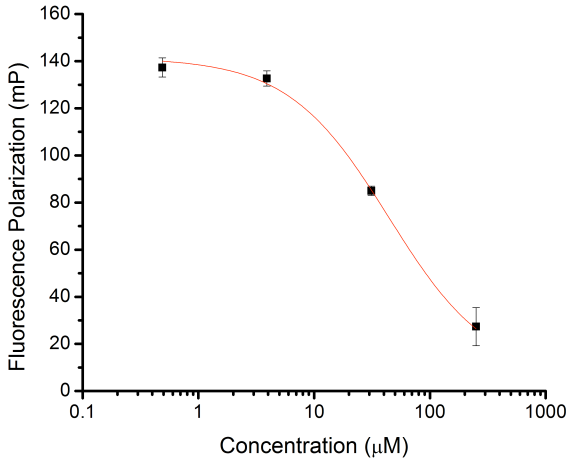

Figure 4. Competitive binding of **14be** measured by fluorescence polarization assay, with a calculated K_i = 18 ± 4 μM. Curve fitted using ORIGIN software.

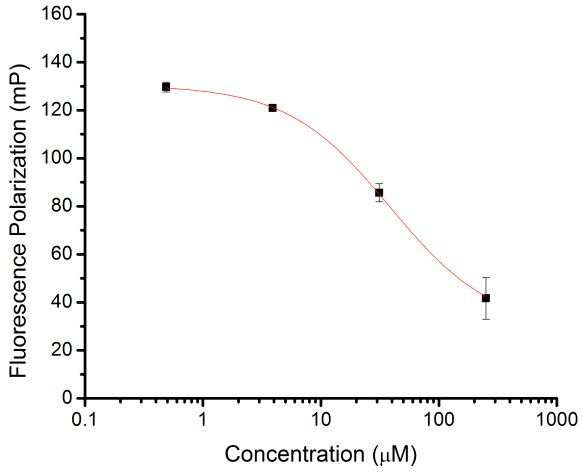

Figure 5. Competitive binding of **14ba** measured by fluorescence polarization assay, with a calculated $K_i = 9 \pm 2$ μM. Curve fitted using ORIGIN software.

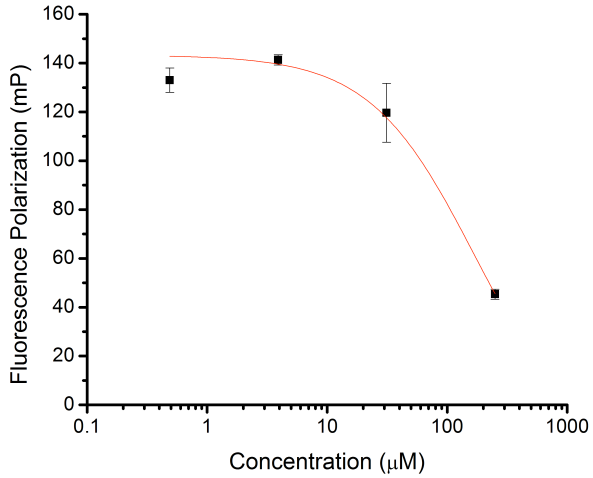

Figure 6. Competitive binding of **14bb** measured by fluorescence polarization assay, with a calculated $K_i = 36 \pm 8$ μM. Curve fitted using ORIGIN software.

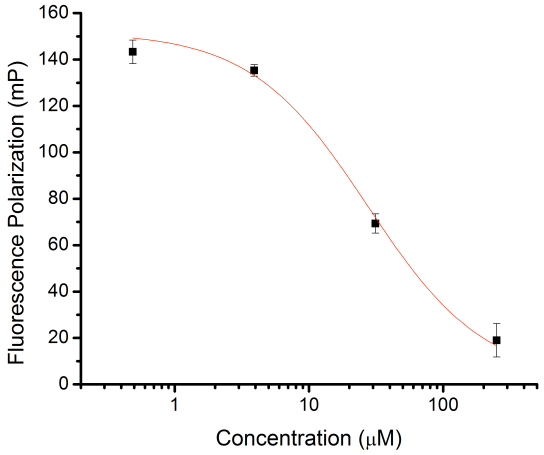

Figure 7. Competitive binding of **14ab** measured by fluorescence polarization assay, with a calculated K_i = 11 ± 4 μM. Curve fitted using ORIGIN software.

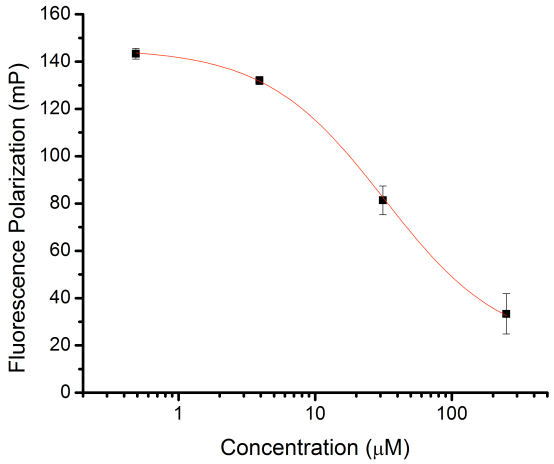

Figure 8. Competitive binding of **14ae** measured by fluorescence polarization assay, with a calculated K_i = 13 ± 1 μM. Curve fitted using ORIGIN software.

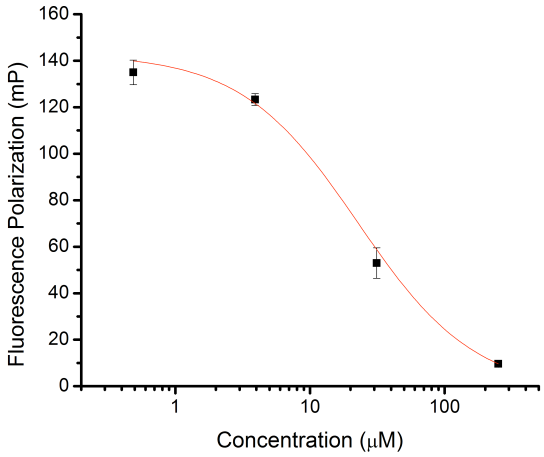

Figure 9. Competitive binding of **14aa** measured by fluorescence polarization assay, with a calculated $K_i = 5 \pm 1$ μM. Curve fitted using ORIGIN software.

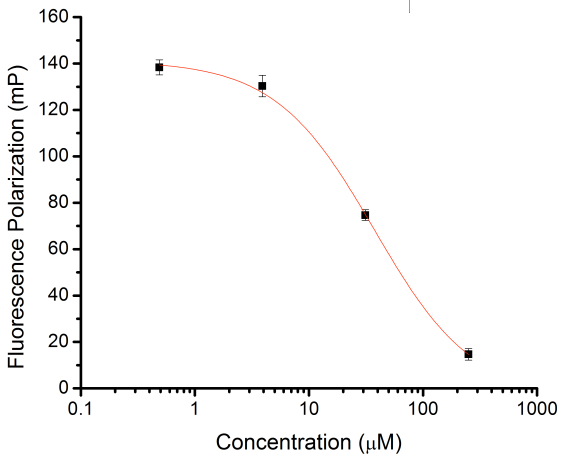

Figure 10. Competitive binding of **14ad** measured by fluorescence polarization assay, with a calculated $K_i = 15 \pm 2$ μM. Curve fitted using ORIGIN software.

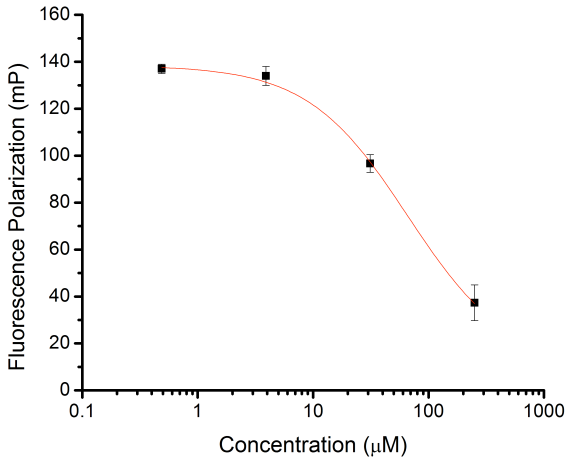

Figure 11. Competitive binding of **14ac** measured by fluorescence polarization assay, with a calculated K_i = 26 ± 5 μM. Curve fitted using ORIGIN software.

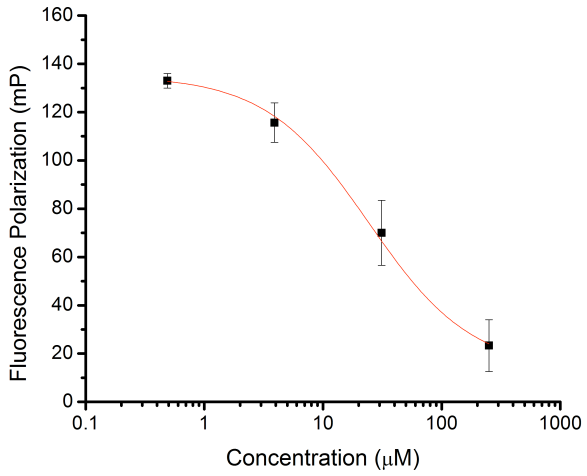

Figure 12. Competitive binding of **14af** measured by fluorescence polarization assay, with a calculated K_i = 10 ± 2 μM. Curve fitted using ORIGIN software.

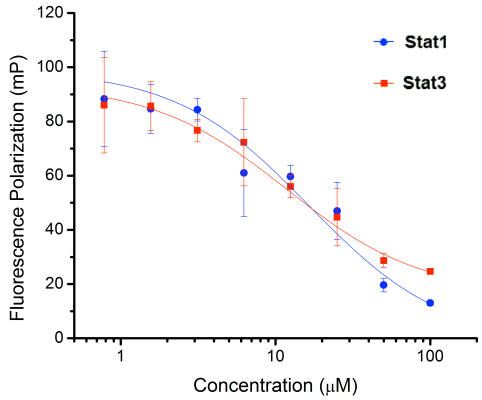

Figure 13. Competitive binding of **14ba** to STAT3 vs STAT1 measured by fluorescence polarization assay. Curve fitted using ORIGIN software.

S.3. EMSA Analysis of STAT3:STAT3-DNA Disruption

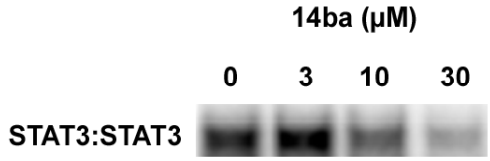

Figure 1. EMSA of STAT3 dimerization inhibition (as judged by the disruption of the STAT3–STAT3:DNA complex) via **14ba**.

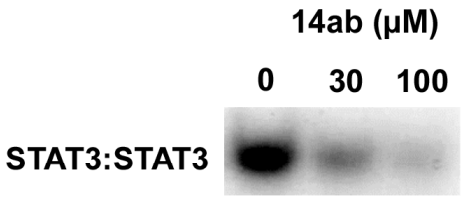

Figure 2. EMSA of STAT3 dimerization inhibition (as judged by the disruption of the STAT3–STAT3:DNA complex) via **14ab**.

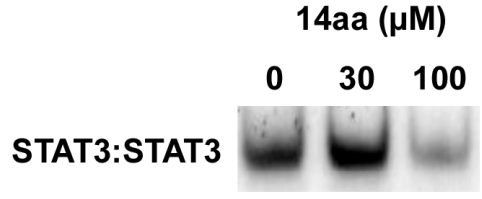

Figure 3. EMSA of STAT3 dimerization inhibition (as judged by the disruption of the STAT3–STAT3:DNA complex) via **14aa**.

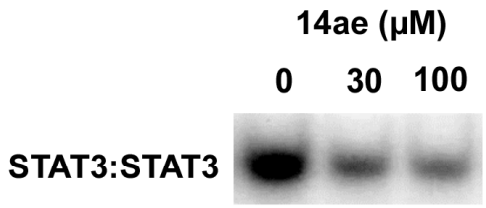

Figure 4. EMSA of STAT3 dimerization inhibition (as judged by the disruption of the STAT3–STAT3:DNA complex) via **14ae**.

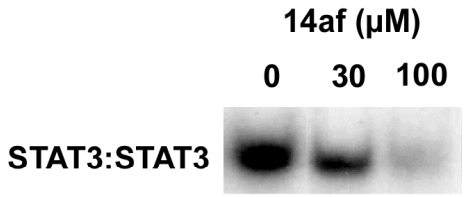

Figure 5. EMSA of STAT3 dimerization inhibition (as judged by the disruption of the STAT3–STAT3:DNA complex) via **14af**.

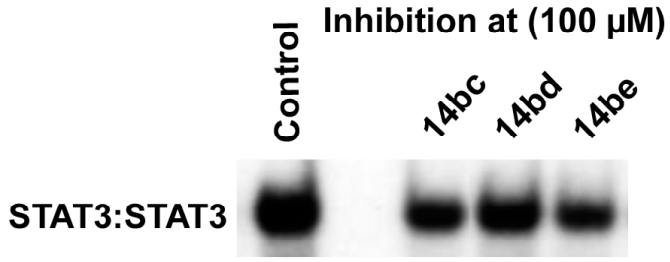

Figure 6. Inhibitors showing > 90 μM inhibition of STAT3 dimerization. EMSA of STAT3 dimerization inhibition (as judged by the disruption of the STAT3–STAT3:DNA complex) via **14bc**, **14bd**, **14be**.

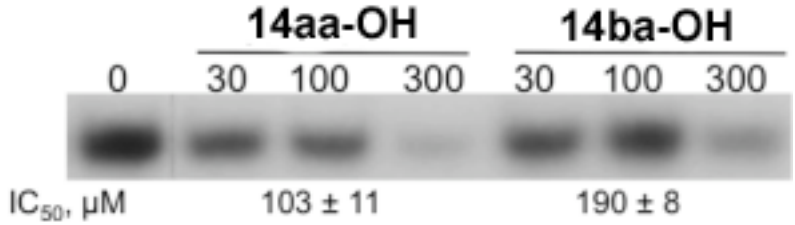

Figure 7. EMSA of STAT3 dimerization inhibition (as judged by the disruption of the STAT3–STAT3:DNA complex) via **14aa-OH** and **14ba-OH**.

S.4. Whole Cell Viability Studies

To further test activity of these molecules, a whole-cell study involving cancer lines with known abherrant STAT3 expression was conducted. These human cell lines, including prostate cancer (DU145), breast cancer (MDA468), and promyelocytic leukemia (HL-60) were treated with the peptidomimetics and incubated for 72 hours. Disruption of cell viability was measured by MTS assay and EC_{50} values for the potential inhibitors was determined. OriginPro 8 (Northampton, MA) was used to evaluate EC_{50} using the dose response curve defined as follows:

$$y = A1 + \frac{(A2 - A1)}{(1 + 10^{((log x_0 - x) \times p)})}$$

where y is the fraction of death, x is the log of drug concentration. A2 is the top asymptote, A1 is the bottom asymptote, $\log x_0$ is the center of the curve, and p is the hill slope. EC_{50} is determined by using this relationship: $EC_{50} = 10^{log x_0}$

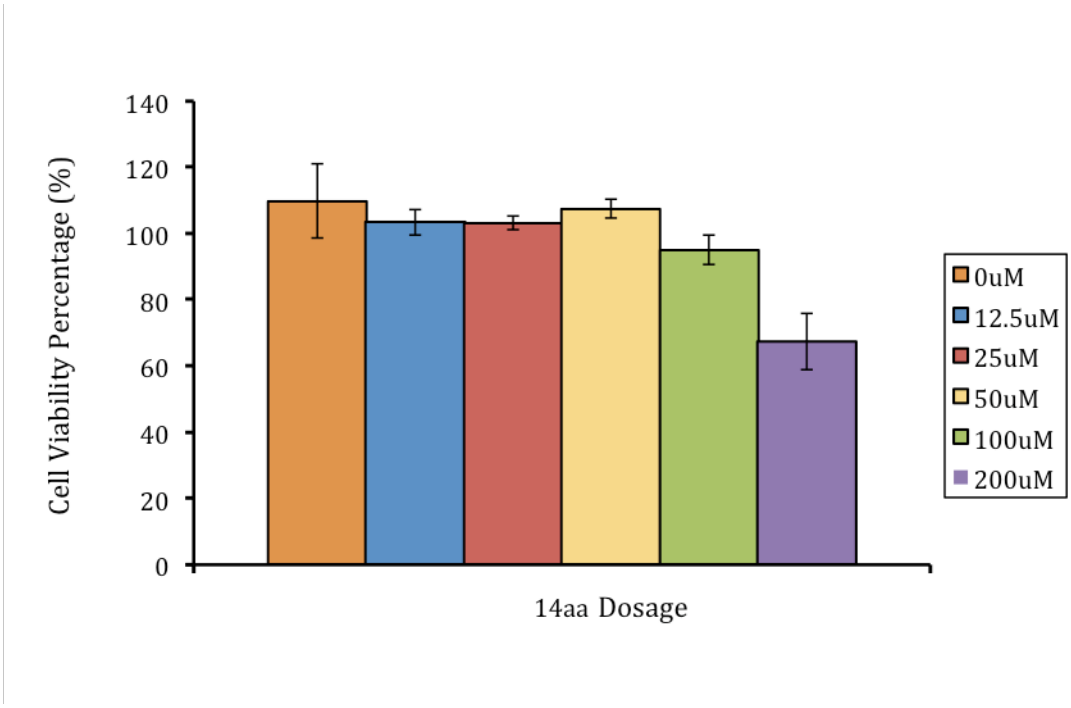

Figure 1. HL60 human promyelocytic leukemia cells treated with **14aa** (left).

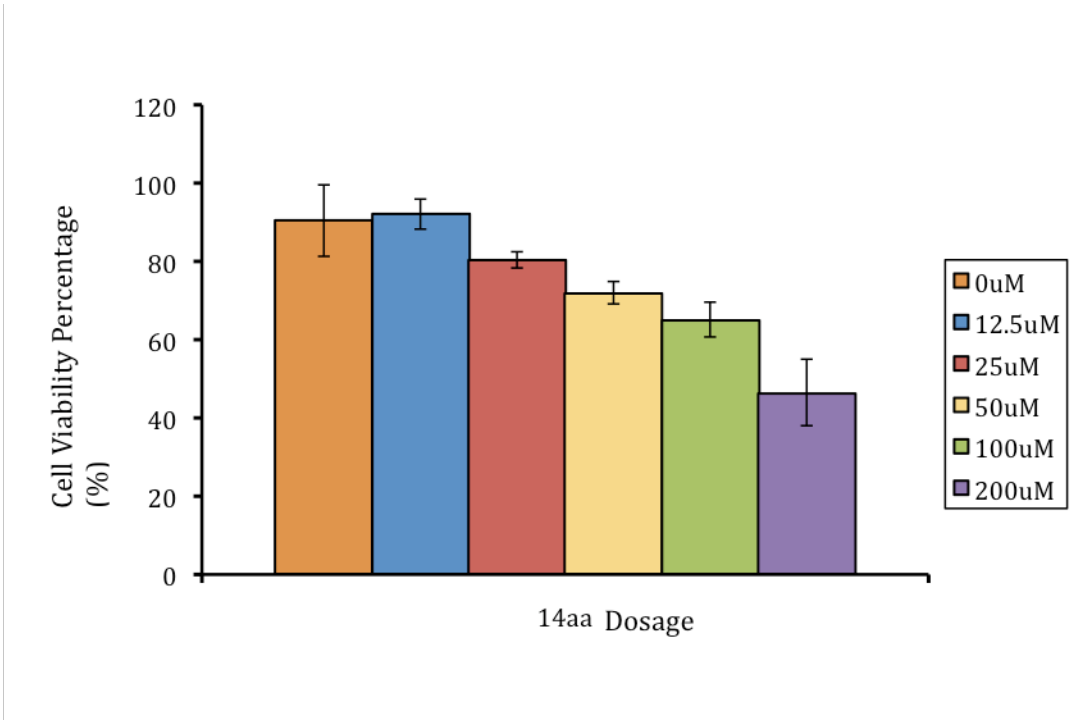

Figure 2. MDA468 human breast cancer cells treated with **14aa** (left).