IN VITRO TUMOR CELL MODEL SYSTEMS

Anticancer Drug Screening and Verification

NOBLE LIFE SCIENCES

Introduction

With the increasing incidence of patients worldwide diagnosed with cancer, the development of effective, safe, and economically viable anticancer therapeutics remains a high priority. Early anticancer drug discovery and development is very challenging resulting in a low success rate for drugs reaching clinical approval. Therefore, developing innovative, efficient, cost-effective preclinical platforms for screening and validating anticancer compounds and biologics is needed to increase the success rate of potential anticancer therapeutics to advance to clinical use.

The necessary step in drug development requires any innovative drugs to undergo extensive preclinical testing before being advanced to human clinical trials. The *in vivo* pharmacological testing of anticancer drugs is implemented with preclinical animal models to assess bioavailability, drug toxicity, and drug therapeutic efficacy. However, there are disadvantages in the use of animal models in anticancer drug screening and the initial efficacy validation, including high costs, variations in pharmacology, and restrictions on availability and feasibility of relevant animal models. Therefore, before proceeding to preclinical animal studies, it is valuable to test new molecules in the *in vitro* cancer cell model systems. The *in vitro* tumor test systems can triage molecules with insufficient anticancer activity from entering preclinical animal testing, reducing both the time and money invested in early drug development. The advantages presented by *in vitro* tumor model systems offers an opportunity to increase the efficiency of early drug development, and at the same time allows the use of effective, new *in vitro* models to assess the therapeutic efficacy of anticancer drugs.

Noble Life Sciences is a CRO that supports early drug development through IND submission to FDA. We offer a full package of services from *in vitro* tumor model systems to preclinical *in vivo* animal test systems. Noble is currently able to screen drug candidates using our OncoDiversity Cell Line Panel including NCI-60 cancer cell lines listed in the Division of Cancer Treatment and Diagnosis (DCTD) Tumor Repository Catalog, which includes a diverse collection of cell lines derived from multiple human cancer types. According to clients' needs, Noble can screen and validate drug candidates using *in vitro* two-dimensional models, three-dimensional models, and Boyden's chamber culture models for either a single cell type or cocultures of two or more cell types. This technical paper focuses on the demonstration of our capabilities in screening and validating drug candidates using the *in vitro* tumor test systems.

Assess Anticancer Efficacy Of Dasatinib Using The High-Throughput Celltiter-Glo Method

Noble is currently using the CellTiter-Glo Luminescence Cell Viability Assay to assess the anticancer efficacy of drug candidates in a high-throughput manner. The CellTiter-Glo method is a fast one-step assay to quantify the viable cells after drug treatment. The CellTiter-Glo One reagent (Promega) contains the recombinant luciferase (enzyme), luciferin (substrate), and buffer components (e.g., Mg^{2+}) for the enzymatic reaction, which is evoked by the energy molecule ATP supplied from lysed cell samples. The CellTiter-Glo Cell Viability Assay workflow is shown in Figure 1. The enzymatic reactions can be set up on 96-well cell cultures for the high-throughput assessment of drug candidates.

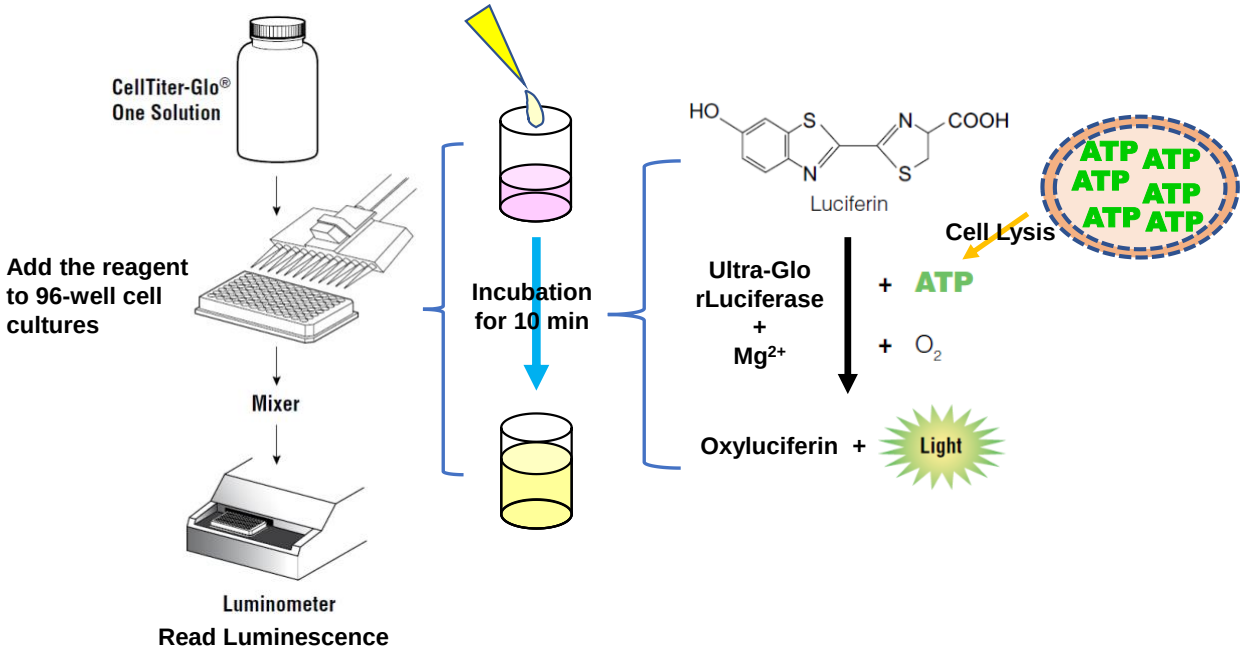

Figure 1. A diagrammatic representation of the CellTiter-Glo Luminescence Cell Viability Assay.

For assay demonstration, three cancer cell lines (K562, MEG01, and MDA-MB-231) were treated with dasatinib, a FDA-approved ATP-competitive protein tyrosine kinase inhibitor used to treat certain cases of chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL). K562 was derived from human CML and MEG01 was derived from human megakaryoblastic leukemia. K562 and MEG01 both are the suspension cell culture model. MDA-MB-231, an adhesion cell culture model, was derived from human metastatic breast cancer. To determine the IC50 of dasatinib for K562 and MEG01, a series of doses prepared by 3-fold serial dilution starting from 20 nM were used for cell treatment (Table 1). For the treatment of MDA-MB-231, 20 µM (20,000 nM) is the starting drug concentration for serial dilution (Table 1). Cell lines were treated with dasatinib for three days (72 hours) prior to being subjected to CellTiter-Glo assays. As dasatinib was prepared in DMSO, DMSO-treated cell samples were used as un-treated negative control.

Table 1. The used doses of dasatinib to determine the IC50.

Cell Line	Dasatinib Doses (from highest to lowest)
K562, MEG01	20 nM → 6.667 nM → 2.222 nM → 0.741 nM → 0.247 nM → 0.082 nM → 0.027 nM → 0.009 nM
MDA-MB-231	20000 nM → 6667 nM → 2222 nM → 741 nM → 247 nM → 82 nM → 27 nM → 9 nM

Luminescence generated from cell samples after being mixed with the CellTiter-Glo One solution was read using a luminescence reader (SpectraMAX M5, Molecular Devices). The luminescence measurements obtained from the reader were converted into cell viability percentages (the DMSO-treated cell viability data was set as default 100%) to make graphic plots of dasatinib doses against the cell viability data (Figure 2).

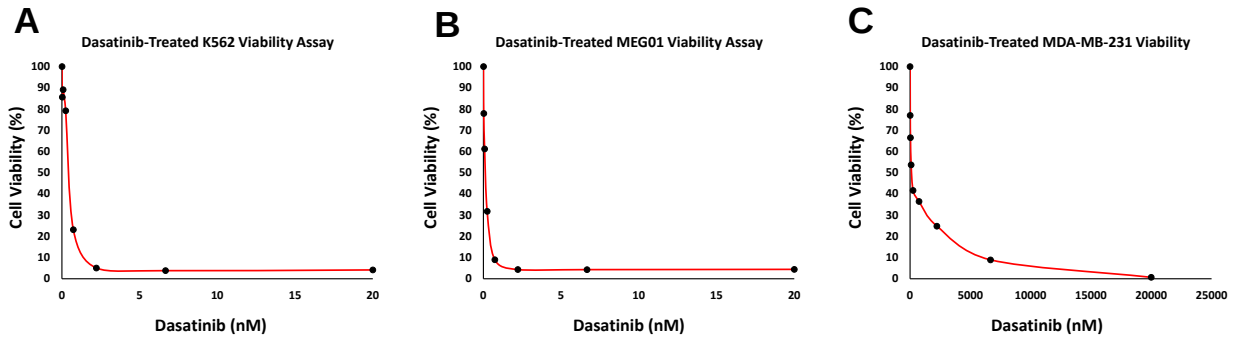

Figure 2. Cell viability analysis of cancer cell lines treated with dasatinib. The used doses of dasatinib are listed in Table 1. After dasatinib treatment for 72 hours, treated cells were subjected to CellTiter-Glo luminescence cell viability assays. Three cell lines were included in the study, including K562 (A), MEG01 (B), and MDA-MB-231 (C). Triplicate experiments were conducted, and the average cell viability data were plotted in this figure.

To determine the IC50 doses of dasatinib for these three cell lines, the dasatinib dose concentrations were log10-tranformed (x values) and plotted against the cell viability percentage data (y values) (Figure 3).

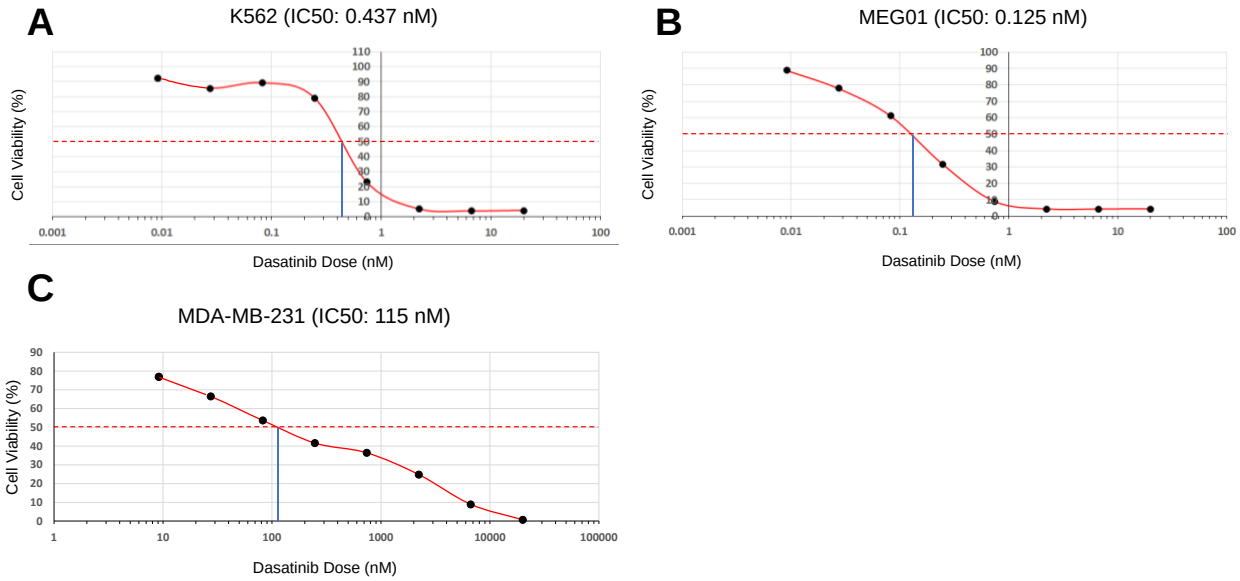

Figure 3. Cell viability analysis of cancer cell lines after log10 transformation of dasatinib dose concentrations. The used dose concentrations of dasatinib listed in Table 1 were log10-transformed and plotted against the cell viability data. Three cell lines included in analysis are K562 (A), MEG01 (B), and MDA-MB-231 (C), respectively.

The horizontal dash line in each plot indicates 50% of viability. The cross point of the 50% viability line and drug-inhibition curve can be used to determine the IC50 dose of dasatinib. which can be calculated through the two-points estimation method.

After data transformation, the two-points estimation method [1] can be used to calculate the IC50 doses of dasatinib for these three cancer cell lines (Table 2) via the following mathematical equation:

$$Drug\ Inhibition\ Percentage\ (DIP)\% = 100\% - Cell\ Viability\%$$

$$slope\ = \frac{DIP_{higher} - DIP_{lower}}{logDC_{higher} - logDC_{lower}}$$

(*DIP*$_{higher}$: The higher measured drug inhibition percentage next to 50% inhibition;

logDC$_{higher}$: The log10-transformed drug concentration causing *DIP*$_{higher}$;

DIP$_{lower}$: The lower measured drug inhibition percentage next to 50% inhibition;

logDC$_{lower}$: The log10-transformed drug concentration causing *DIP*$_{lower}$)

$$IC_{50} = \ 10^{(\frac{50\% - DIP_{lower}}{slope} + \ logDC_{lower})}$$

Table 2. The IC50 doses of dasatinib for three cancer cell lines estimated from the two-points method.

	K562	MEG01	MDA-MB-231
Dasatinib IC50 dose	0.437 nM	0.125 nM	115 nM

In addition to the two-points estimation method, non-linear regression model methods have been considered as ‘‘state-of-the-art’’ for more accurate IC50 estimation [1]. We used two non-linear regression model methods, the Spline Regression Model (SRM) and Generalized Additive Model (GAM) [2], to predict the IC50 dose of dasatinib after these two mathematical Statistics Models were trained by the actual data. The fitted SRM and GAM models are shown in Figure 4 and Figure 5, respectively. The regression coefficients (R2) and predicted IC50 doses of dasatinib from SRM and GAM models are summarized in Table 3 and Table 4, respectively. These non-linear regression modeling analyses were performed using Statistical R programming.

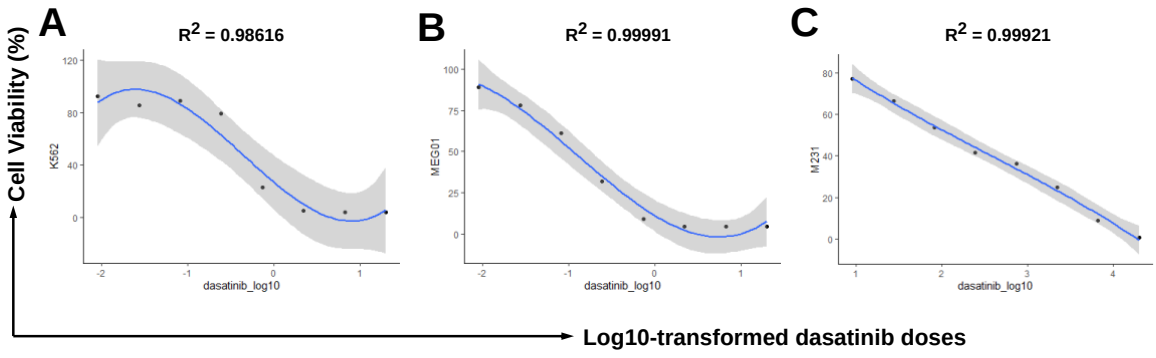

Figure 4. Non-linear Regression analysis of cell viability data vs. log10-tranformed dasatinib doses using the Spline Regression Model method. Spline Regression analysis plots for K562 (A), MEG01 (B), and MDA-MB-231 (C) are shown in the figure. The R square coefficients are indicated at the top of each regression plot. The grey shaded areas around each regression line in the plots are the confidence intervals (also called “confidence bands”) for the regression lines.

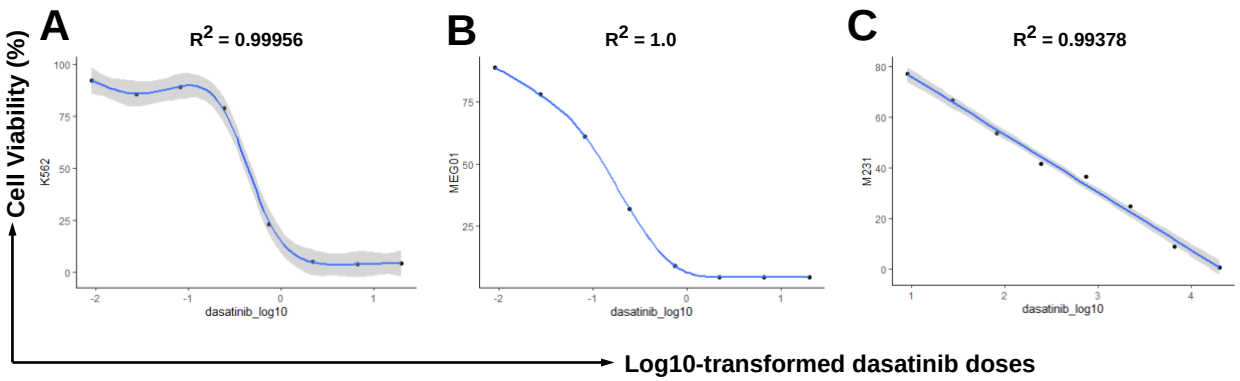

Figure 5. Non-linear Regression analysis of cell viability data vs. log10-tranformed dasatinib doses using the Generalized Additive Model method. Generalized Additive Modeling analysis plots for K562 (A), MEG01 (B), and MDA-MB-231 (C) are shown in the figure. The R square coefficients are indicated at the top of each regression plot. The grey shaded areas around each regression line in the plots are the confidence intervals for the regression lines.

Table 3. The estimated IC50 doses of dasatinib for three cancer cell lines using the Spline Regression Models.

	K562	MEG01	MDA-MB-231
Dasatinib IC50 dose	0.450 nM	0.128 nM	122 nM
R Square	0.98616	0.99991	0.99921

Table 4. The estimated IC50 doses of dasatinib for three cancer cell lines using the Generalized Additive Models.

	K562	MEG01	MDA-MB-231
Dasatinib IC50 dose	0.442 nM	0.129 nM	137 nM
R Square	0.99956	1.0	0.99378

According to R square coefficients of these non-linear regression models, Generalized Additive Models provided the better predictions of IC50 doses of dasatinib for K562 and MEG01, whereas Spline Regression Models performed better for MDA-MB-231.

The demonstration study showed that MEG01 was most sensitive to dasatinib, and K562 was three-fold more resistant to the drug when compared to MEG01. MDA-MB-231 was approximately 1000-fold resistant to

dasatinib when compared to MEG01, which was the most resistant cell line when compared to the other two leukemia cell lines.

Apoptotic Analysis of the Anticancer Effect of Dasatinib Using the Flow Cytometric Assay

To demonstrate our capabilities in delineating the mechanism underlying the anticancer action of dasatinib, we conducted Annexin V apoptotic assays to determine whether dasatinib triggers apoptosis in leukemia cell lines. The Annexin V method is based on disrupting the asymmetric organization of the cellular plasma membrane during apoptosis. The two leaflets of healthy cell membranes are composed of the asymmetric composition of phospholipids where phosphatidylserine (PS) components face the cytoplasmic side (Figure 6). During early apoptosis, the asymmetric structure of cell membranes is disrupted, and a portion of PS components flip out to the outer membranous layer, which can be bound by Annexin-FITC for apoptotic detection (Figure 6). After apoptosis proceeds to the later stage, the integrity of the cellular membranes is disrupted, and apoptotic cells become permeabilized, whose DNA can be stained with DNA-binding dyes (e.g., propidium iodide, 7-AAD) (Figure 6). By staining cells with Annexin-FITC and the DNA-binding dye (7-AAD or PI)[3], early and late apoptotic cell fractions can be detected and quantified by flow cytometry assays (Figure 6).

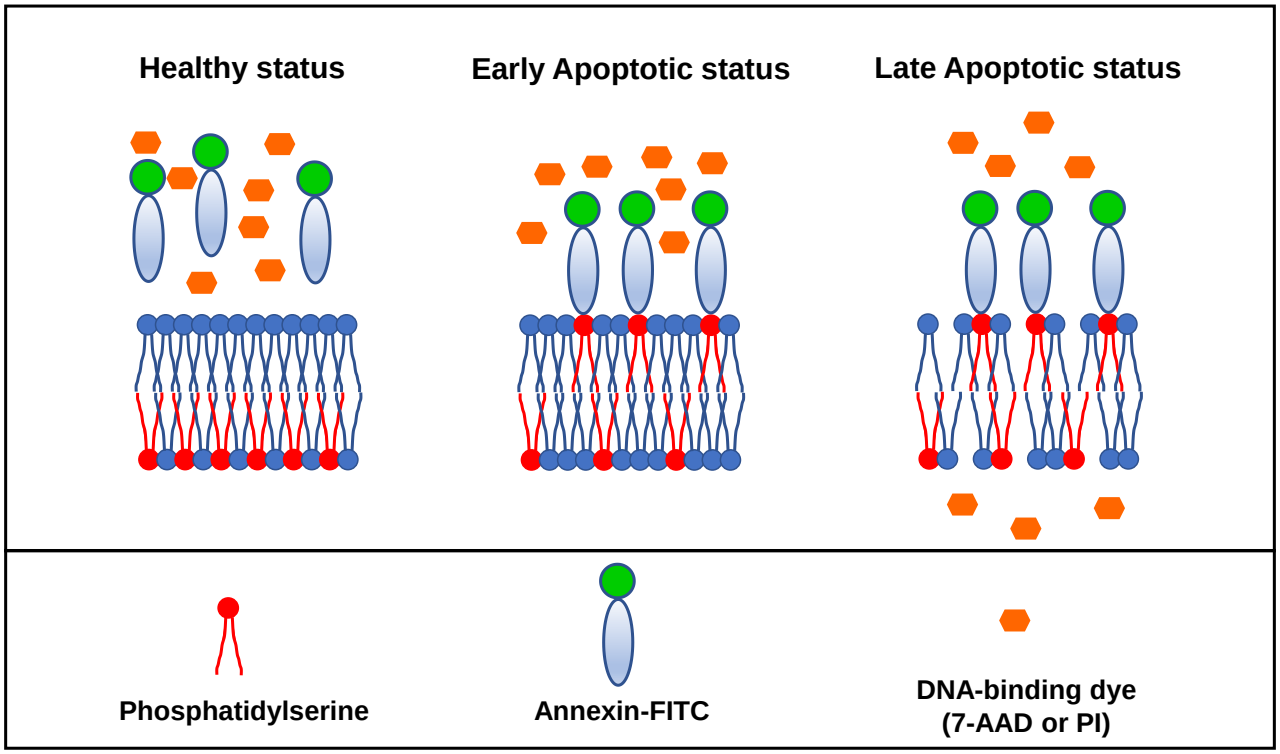

Figure 6. The diagrammatic representation of the Annexin V Apoptotic Assay.

For apoptotic analysis, K562 and MEG01 cell lines were treated with dasatinib at the doses of 0.75 nM and 0.5 nM, respectively, for 72 hours. After 3-day treatment, both cell lines were stained with Annexin-FITC and 7-AAD, and stained cell samples were analyzed by MACSQuant 10 Analyzer (Miltenyi Biotec), a flow cytometer compatible with the high-throughput 96-well platform. The FACS raw data were analyzed by using FlowJo software. Triplicate experiments were conducted. The representative flow cytometry data are shown in Figure 7.

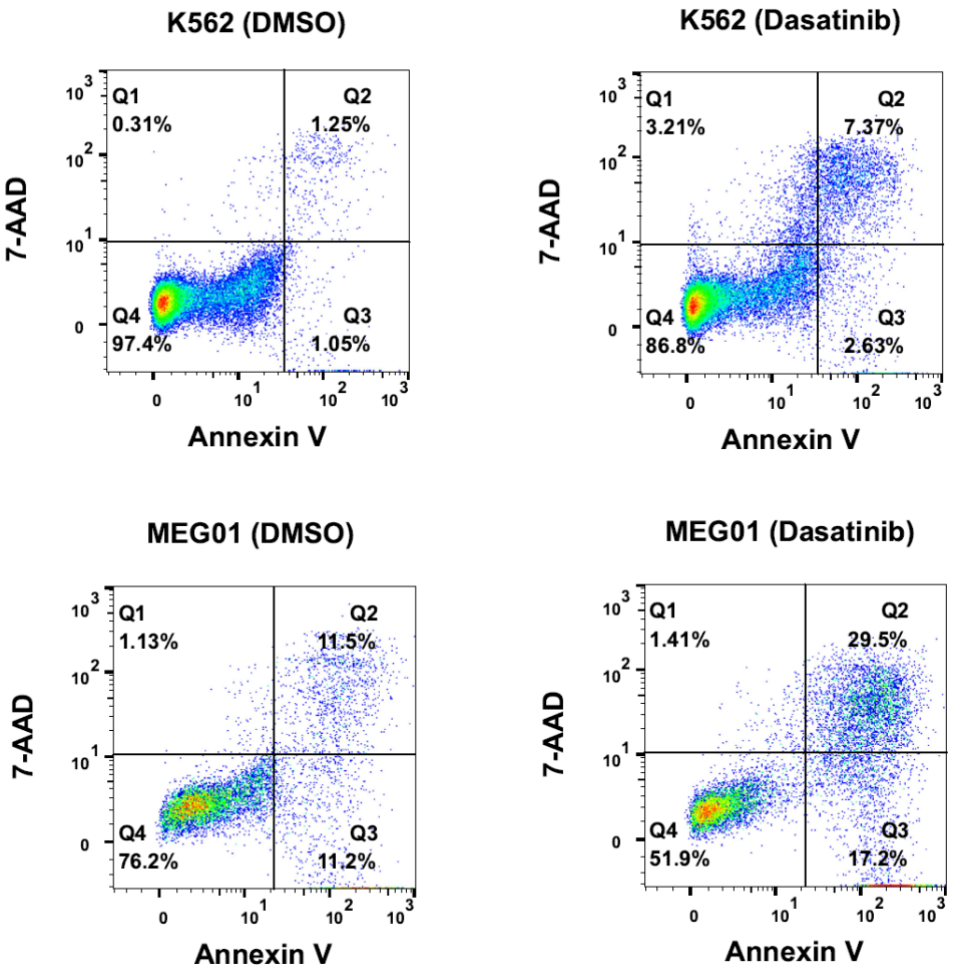

Figure 7. Flow cytometry analysis of dasatinib-treated K562 and MEG01 cells after being subjected to Annexin V Apoptotic Assays. **Q1** indicates gated necrotic cells; **Q2** indicates gated late apoptotic cells; **Q3** indicates gated early apoptotic cells; **Q4** indicates non-apoptotic live cells.

The statistical analysis of early apoptotic, late apoptotic, and necrotic cell percentages in dasatinib-treated K562 and MEG01 cells in comparison with their DMSO-treated controls was performed and the analyzed results are shown in Figure 8. The results have demonstrated that dasatinib induced apoptosis in both K562 and MEG01 cell lines.

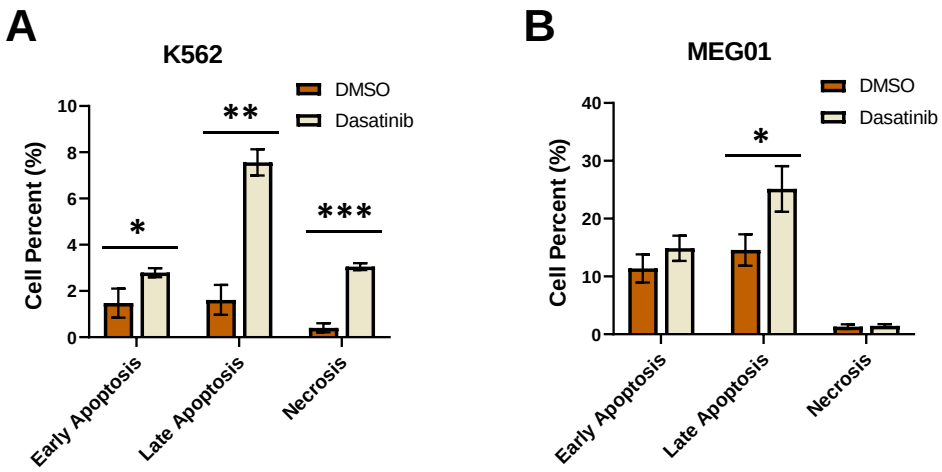

Figure 8. Statistical analysis of the Annexin V assay data from DMSO-treated and dasatinib-treated leukemia cancer cell lines. The gated early apoptotic, late apoptotic, and necrotic cell percentage data from DMSO-treated and dasatinib-treated K562 (A) and MEG01 (B) cells were plotted as bar graphs. The error bars indicate standard deviations. The Student T-test was used to evaluate the statistical significance of the cell-killing effect from dasatinib treatment. The symbol "*" indicates $p < 0.05$, "**" for $p < 0.001$, and "***" for $p < 0.0001$.

The Capabilities of Noble in Various *In Vitro* Cell Assays Using Diverse Tumor Cell Models

In addition to the two assays presented above, , Noble can conduct a variety of additional *in vitro* cell assays for drug screening and efficacy validation, including but not limited to:

- Cell Proliferation Assay
- Cell Migration/Invasion Assays
- Soft Agar Colony Formation Assay
- 3D Spheroid Formation Assay
- Cell Cycle Analysis Assay
- Co-Culturing Assay
- 3D Sphere Formation Assay for Cancer Stem Cell Studies
- Flow Cytometric Analysis of Cancer Stem Cells

Noble also conducts custom-designed *in vitro* cell assays in accordance with clients' needs.

The wide variety of cancer cell models available offers a valuable resource for efficiently and cost-effectively characterizing new molecules within your drug development pipeline. These models enable comprehensive evaluations of the efficacy and potential of novel compounds, facilitating expedited progress in your drug discovery efforts. Please feel free to contact us regarding any of your special needs or questions about our services. Our experienced experts will reach out to you to answer your questions and help with your study design and implementation. We look forward to working with you.

References

1. Nevozhay D. Cheburator software for automatically calculating drug inhibitory concentrations from in vitro screening assays. *PLoS One*. 2014 Sep 3;9(9):e106186. doi: 10.1371/journal.pone.0106186. PMID: 25184280; PMCID: PMC4153570.
2. Bruce, Peter, and Andrew Bruce. 2017. Practical Statistics for Data Scientists. O'Reilly Media.
3. Zimmermann M, Meyer N. Annexin V/7-AAD staining in keratinocytes. *Methods Mol Biol.* 2011;740:57-63. doi: 10.1007/978-1-61779-108-6_8. PMID: 21468968.