AlgiMatrix® 3D Cell Culture System As An In Vitro Tumor Model For H460 Non-Small Cell Lung Cancer Cell Line.

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OVERVIEW

INTRODUCTION

Traditionally, most cell cultures are developed in two dimensional (2-D) environments. In mammalian tissue, cells connect not only to each other, but also support structures called the extracellular matrix (ECM).

The cells grow within an organized three dimensional (3D) matrix and their behavior is dependent upon interactions with immediate neighbors and the ECM. The integrins surface receptors anchor their bearers to the ECM, and determine biochemical signals interpretation by cells to undergo differentiation, apoptosis, proliferation, or invasion .

The biological subtleties are inadvertently omitted in 2-D cell culture systems. The 3-D cultures may play a potential role in cancer drug discovery due to the lack of relevant preclinical models and advantages over 2-D cultures. Although, animal models are accurate representative of tumor environment, they are considerably less amenable to large-scale screening. In cancer research, 3D cultures are rapidly gaining importance in screening of drugs as they create a pragmatic microenvironment and mimic in vivo systems.

The development of 3D tumor model will reduce animal testing, yield more predictive data, improve cell culture efficiency, reduce cost and time to identify new drug candidates and reduce development time to market.

In cancer research, three dimensional (3D) cell culture models are rapidly gaining importance as they create a pragmatic microenvironment and mimic an in vivo system, which helps to understand cell-cell interactions.

Among 3D cultures**, AlgiMatrix®** has advantages being animal-free product and stable at room temperature (2). AlgiMatrix® is a non toxic and biodegradable ready-to-use sponge made from lyophilized alginate gel which helps to construct a cell culture model resembling normal cell characteristics and morphology.

HYPOTHESIS

The use of an AlgiMatrix® lung tumor model (6-well plate format) which will simulate in vivo conditions to screen the efficacy of drug candidates will be more effective than the use of traditional monolayer 2D cultures.

OBJECTIVE

I. To optimize , develop and evaluate the in vitro Algimatrix® 3D tumor model using H460 non small cell lung cancer cells.

II. To screen the anticancer activity of Docetaxel using the AlgiMatrix® 3D in vitro lung tumor model and 2D cell culture system.

III. To compare the anticancer efficacy of Docetaxel in AlgiMatrix® 3D lung tumor model and 2D cell culture system.

MATERIALS

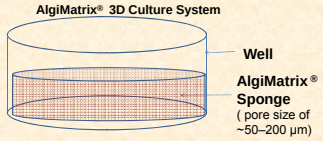
AlgiMatrix® 3D cultures, Alamar blue dye was received from Life Technologies Corporation, Carlsbad, California 92008 USA (Invitrogen Corporation, Carlsbad, California 92008). The human Non Small Cell Lung Cancer (NSCLC) cell lines H460 were obtained from American Type Culture Collection (Rockville, MD, USA). All other chemicals were either reagent or tissue culture grade.

EXPERIMENTAL METHODS

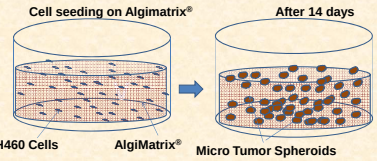
Development and Optimization of In Vitro AlgiMatrix 3D Lung Tumor Model

We did cell titration of H460 Human non small lung cancer cell line by using different cell concentrations. (0.05, 0.10, 0.15, 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, 3 & 6 million H460 cells/well using 6-well plate to determine the optimal incubation density and time for the formation of spheroids.

The 2ml of H460 cell suspension containing firming buffer was added to the AlgiMatrix® 3D cultures system . After 5 minutes the culture medium of 5 ml was added to well. A fresh medium was added every day. The growth of tumor spheroids was assessed by observing formation of the spheroids in Algimatrix® well. On 4th , 9th and 13th day post cell seeding the pictures were taken using an inverted microscope (Motic AE 31, BC, Canada) and the size of spheroids and number of spheroids were determined.



DEVELOPMENT OF AlgiMatrix® 3D LUNG TUMOR MODEL



We used 0.75 million, 1.5 million, 3 million & 6 million cells per well of 6-well plate of AlgiMatrix® to further optimize the cell seeding density. Based on results the optimized cell concentration of 0.15 million & 0.25 million cells per well of 6-well plate of AlgiMatrix® was used to establish AlgiMatrix® 3D tumor model.

AlamarBlue Assays

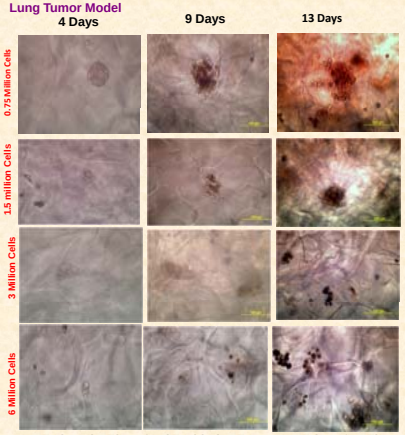
The alamarBlue® assay was performed according to the manufacturer's protocol on the 14th day. The AlamarBlue® assay was used to determine the cell viability and metabolic activity which converts non-fluorescent dye to the red fluorescent dye resorufin in response to chemical reduction of growth medium resulting from cell growth. Briefly, 10 % Alamar blue dye was used with respect to the volume of the medium in each well. After one hour of incubation, plate was read for fluorescence intensity at 530 nm & 590 nm wavelength for excitation and emission respectively.

Efficacy of Docetaxel using AlgiMatrix® 3D Lung Tumor Model

The 0.15 million cells per well of 6-well plate of AlgiMatrix ® was seeded. Docetaxel (175 µM, 250 µM, 325 µM & 400 µM) was used to treat AlgiMatrix ® seeded with 0.15 million and 0.25 million cell density on day 7, 9, 11 days post tumor cell seeding. The size of spheroids and number of spheroids was done for day 4, day 9 and day 13 using inverted microscope. Alamar Blue assay was performed to determine number of cells at the end point. The anticancer efficacy was determined in terms of the IC_{50} and IC_{90} value based on AlamarBlue® assay results. The 2D experimental protocol was similar to that of 3-D experiment.

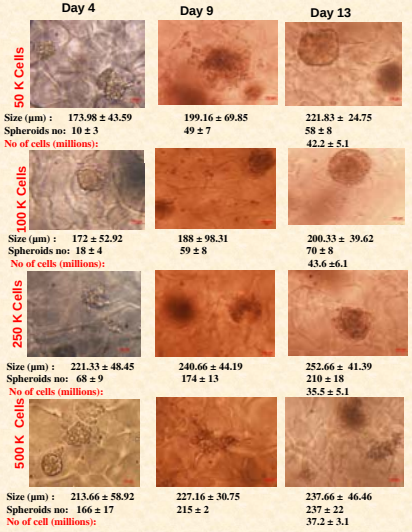
RESULTS

Development and Optimization of In Vitro AlgiMatrix® 3D H460 Lung Tumor Model

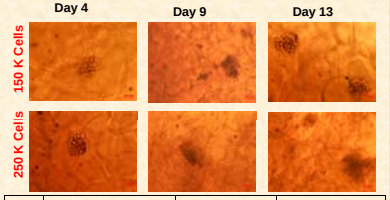


❖ **Based on the size of spheroids (Approx. 300 µM) we found out that three weeks time is the optimum time to culture cells on AlgiMatrix.**

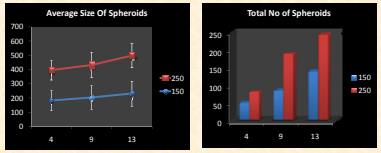
❖ **0.75 M & 1.5 M cells per well gave better response than 3 M & 6 M cells per well.**



RESULTS



Time Points Days	Average size of spheroids (µm)		Total Number of spheroids		Total Number of Cells (Million/Well)	
	0.15 million	0.25 million	0.15 million	0.25 million	0.15 million	0.25 million
4th	177.8 ± 61.7	220.7± 61.2	48 ± 4	77 ± 8	---	---
9th	205.3 ± 78.1	232.9 ± 82.8	90 ± 8	189 ± 15	---	---
13th	232.2 ± 89.1	263.9 ± 86.4	153 ± 18	228 ± 17	38.4 ± 4.9	42.9 ± 3.3



Efficacy of Docetaxel using AlgiMatrix® 3D Lung Tumor Model Established with H460 0.15 Million cells/well.

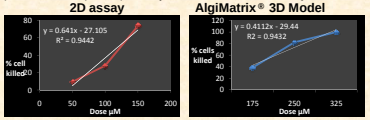
No of cells (million)	Percentage of cell killed Dose : 175 µM	Percentage of cell killed Dose : 250 µM	Percentage of cell killed Dose : 325 µM	Percentage of cell killed Dose : 400 µM
0.1 5	40.7 ± 3.4	83.3 ± 4.2	99.9 ± 2.1	99.9 ± 2.5

14th day, After Docetaxel treatment on day 7, 9 and 11

Note: One of the drug treated sample well was chosen and images were taken to observe the effect of drug on the structure of the spheroids.

❖ Using linear regression, IC_{50} & IC_{90} Values for Docetaxel was found to be 193.19 µM and 290.47 µM respectively for 0.15 million cell density in AlgiMatrix®3D lung tumor model

❖ In 2D assay IC_{50} & IC_{90} Values for Docetaxel was found to be 120.29 µM and 182.69 µM respectively for 0.15 million cell density .

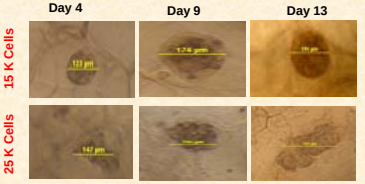


❖The higher IC_{50} of Docetaxel in 2D cultures suggests that the formed microtumors in 3D system were more resistant due to the lower penetration of drug within microtumors.

RESULTS

AlgiMatrix® 3D H460 Lung Tumor Model using 96 Well Plates for High Throughput Screening :

We evaluated H460 seeding density ranging from 1K to 35K cells/well in 96 plate format (3) to select the optimum seeding density based upon our 6 well plate results



Time Points Days	Average size of spheroids (µm)		Total Number of spheroids		Total Number of Cells (thousand/Well)	
	15 K	25K	15 K	25K	15 K	25K
4th	107.7 ± 21.4	129.9 ± 25.3	22 ± 4	31 ± 3	---	---
9th	185.3 ± 48.2	209.9 ± 42.9	32 ± 6	49 ± 5	---	---
13th	224.7 ± 59.7	251.4 ± 61.5	42 ± 7	62 ± 5	70.8 ± 9.2	117.8 ± 14.3

The AlgiMatrix® 3D H460 lung tumor model using 96 well plates with seeding density of 15K and 25K H460 cells was established and can be used for high throughput screening.

The feasibility of development of AlgiMatrix® 3D H460 lung tumor model using 96 well plates will have an advantage over 6 well plates in terms of faster throughput screening of anticancer drugs.

CONCLUSION

- **In vitro AlgiMatrix ® 3D lung tumor model was successfully developed using seeding density of 250K and 500K H460 NSCLC cells in 6 well plate and 96 well plate.**
- **These results strongly support that the AlgiMatrix 3D Cell Culture System may be used as an in-vitro tumor model and for high throughput screening of anticancer agents.**

FUTURE DIRECTIONS

- ❑ To screen the efficacy of anticancer drugs.
- ❑ To perform immunohistochemistry for hypoxia region in the spheroids.
- ❑ Perform Micro-Array to ascertain genes expression
- ❑ In vivo – In vitro correlation.
- ❑ Fabrication of co-culture model to investigate angiogenesis.

Acknowledgements

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