A Primer of an Animal Cell Culture Laboratory for Efficacy, Toxicity and Transport Screening

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Abstract

In the advent of the discovery of new molecules with potential therapeutic effect and the engineering of new drug delivery systems, the availability of *in vitro* test systems for efficacy, transport and toxicity screening are becoming of an increasing importance. In this report, we present the initiation of a cell culture system for the above mentioned purposes. The report describes some of the validated assays implemented and preliminary studies on toxicity and efficacy.

Keywords: Animal tissue culture, Screening, Toxicity, Anticancer activity.

(J Med J 2006; Vol. 40 (3): 172- 178)

Received July 13, 2005 Accepted February 14, 2006

Introduction

The progress of modern bio- and information-technology has made a vast impact on the development of new drugs; computer-aided drug design has enormously facilitated the chemical synthesis of new drug candidates. At the same time, the number of macromolecular biopharmaceuticals, such as peptides, proteins, antisense agents or gene vectors is continuously increasing. [1] Whether or not such new entities can be developed into safe and efficient medicines, is largely determined by the questions: are these molecules effective and safe? And are they able to reach their actual target (receptor) within the biological system?

For these questions to be answered, animal models have been and are still used, however, with the increasing number of new compounds, they are getting replaced by new and improved cell culture systems. Using these systems, different aspects could be approached, such as a new compound or excipient toxicity, its absorption across barriers and whether it is taken up by macrophages or not.

Cell culture has been used for preliminary screening for efficacy, where, it can give the researcher an early indication whether a new compound is actually effective in a living system or not.

Toxicity: A number of methods have been developed to study cell viability and proliferation in cell culture. [2] The most convenient assays have been optimized for the use of microtiterplates (96-well format). This allows many samples to be analyzed rapidly and simultaneously. [3]

Cytotoxicity assays have been developed which use different parameters associated with cell death and proliferation. In our laboratory, we chose two of these common assays to have a closer look on their comparability; lactate Dehydrogenase (LDH) release assay and tetrazolium salts reduction by the mitochondria of viable cells (MTT assay). Our model used was t-octylphenoxypolyethoxyethanol (Triton X-100) a non-ionic surfactant, which is widely common for the solubilization of membranes under non-denaturing conditions.

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The permeabilization of the membrane leads to a fast cell death by necrosis. [4]

The cell culture model used in these experiments is A549 cells, a human Caucasian lung carcinoma cell line that represents Type II cells in the lung.

Activity: The discovery that tumor-derived cell lines proliferate almost indefinitely promoted the present understanding of cancers, and formed the basis of pioneering *in vitro* anticancer drug discovery and testing initiatives. [5] By the early 1990s, a large number of human tumor cell lines had been established and extensive anticancer drug discovery in the newly available 96-well high-throughput (HTS) screening format was done. [6]

As a model drug, vincristine sulphate was used as an anticancerous drug with known activity and several plant extracts were screened for their cytotoxic effect (to be published). As a cell culture model, we are presenting here the MCF-7 cell line (human breast adenocarcinoma) and A549 cell line.

In this report, we present the transfer of knowledge and technology to start an animal cell culture laboratory in the Faculty of Pharmacy for the purpose of testing the toxicity of new excipients and delivery systems to be used as drug delivery systems in the future and to elaborate on the efficacy of new chemicals from in silico screening and of extracts from natural origin.

Experimental

1. Cell Culture

1.1 For cytotoxicity assays, the cell lines used were large cell lung carcinoma A549, (ECACC No.: 86012804), and human breast adenocarcinoma MCF-7 (ECACC No.: 86012803). These were obtained from the European Collection of Animal Cell Culture. Cells were cultured in RPMI 1640 medium supplement with 10% heated foetal bovine serum, 1% of 2 mM l-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin and maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity. According to their growth profiles, the optimal plating densities of each cell line were determined as 5000 cell/well for A549 and MCF-7 to ensure exponential growth throughout the experimental period (see results) and to ensure a linear relationship between absorbance at 570 nm and cell number when analyzed by SRB assay (see SRB assay).

1.2 For cell viability assays, A549 cells were used and were grown as mentioned in section 1.1. They were seeded in 96 well plate in a density of 5000 cell/well till 80-90% confluency. The medium was changed every other day.

2. Cytotoxicity Assay

For the assay, cells were washed with Phosphate Buffer Saline (PBS). The PBS was decanted and cells detached with (0.025%) trypsin-EDTA (Sigma). The cell pellet, obtained by centrifugation (1000 X*g*, 5 minutes) was resuspended in 10 ml of medium to make a single cell suspension, viable cells density being counted by trypan blue exclusion in a haemocytometer and then diluted with medium to give the previously-determined optimal plating densities for A549 and MCF-7. Of these cell suspensions, 100 µl/well were seeded in 96-well microtiter plates and incubated at 37°C to allow for cell attachment. After 24 hours, the cells were treated with the extracts or pure compounds.

Vincristine sulphate (Sigma, Lot No. 34H0447) was used as a positive control. It was used at the following concentrations, 0.1, 0.5, 1.5, 10, 25, 50, 100, 150, 200 and 400 nM.

The supplied ethanolic extracts (given the designation plant codes from 1 to 4) were diluted in medium to produce nine concentrations of 0.1,

Of each concentration, 100 µl/well was added to the plates in six replicates. The final dilution used for treating the cells contained not more than 1% of the initial solvent, this concentration is being used in the solvent control wells.

The plates were incubated for 72 hours. At the end of the exposure, time growth was then analyzed using the SRB assay. Three replicate plates were used to determine the cytotoxicity of each extract.

3. Sulphorhodamine B (SRB) Assay

The antiproliferative SRB assay was performed to assess growth inhibition by a colorimetric method which estimates cell number indirectly by staining total cellular protein with the dye SRB. [7] In brief, cells were fixed by layering ice-cold 40% Trichloroacetic Acid (TCA, Aldrich Chemical) on top of the growth medium. Cells were incubated at 4°C for 1 hour, after which plates were washed with cold water, the excess water drained off and the plates were left to dry in air. SRB stain (50 µl; 0.4 in 1% acetic acid) (Sigma) was added to each well and left in contact with the cells for 30 minutes, after which they were washed with 1% acetic acid, rinsed four times until only dye adhering to the cells was left. The plates were dried and 100 µl of 10 mM Tris base pH 10.5 (Sigma) were added to each well to solubilize the dye. The plates were shaken gently for 20 minutes on a plate shaker and the absorbance (OD) of each well was read on a ELISA reader at 570 nm. Cell survival was measured as the percentage absorbance was compared to the control (non-treated cells). The IC50 values were calculated from the Anilisa program obtained by plotting the percentage of survival versus the concentrations, interpolated by cubic spine. According to the National Cancer Institute guidelines, extracts with IC50 values < 30 µg/ml were considered active. [8]

0.5, 1, 5, 10, 25, 50, 100 and 200 µg/ml.

4. Cell Viability Assays

4.1. LDH Release

The LDH release from necrotic cells into the extracellular fluid was determined after 4 hours of treatment with the test agents by using the commercially available Cytotoxicity Detection Kit (LDH) (Cyto Tox 96, non radioactive cytotoxicity assay, Promega). After the treatment, an aliquot of 50 µl was taken to quantify the LDH. The test was performed according to the manufacturer's instructions including a positive control with LDH-standard solution. For each concentration, six replicate wells were used.

4.2. MTT Metabolism

As with LDH release, the cells were treated for 4 hours with the test agent. The dye solution was dispensed after the incubation period and the test was run using the commercially available cell proliferation assay kit from Promega (Cell Titer AQ, Non radioactive proliferation assay). Mean values from six wells were determined.

Results and Discussion

1. Cell Viability

In this report, we tried to detect cytotoxicity using two different endpoint parameters; LDH release and mitochondrial respiration in A549 cell line using Triton X-100, a non-ionic surfactant. Results are shown in Figure 1, where the viability curves adjoin in a close manner and show a parallel progression. We found a good correlation of Triton X-100 IC50-values in both models applied. This good comparability of the assays could be explained by the mechanism of action, which leads to a rapid cell death by necrosis caused by the permeabilization of the cell membranes. These assays are useful and have been adapted for testing the toxic effects of novel drug delivery systems or newly synthesized polymers for drug delivery. [9, 10]

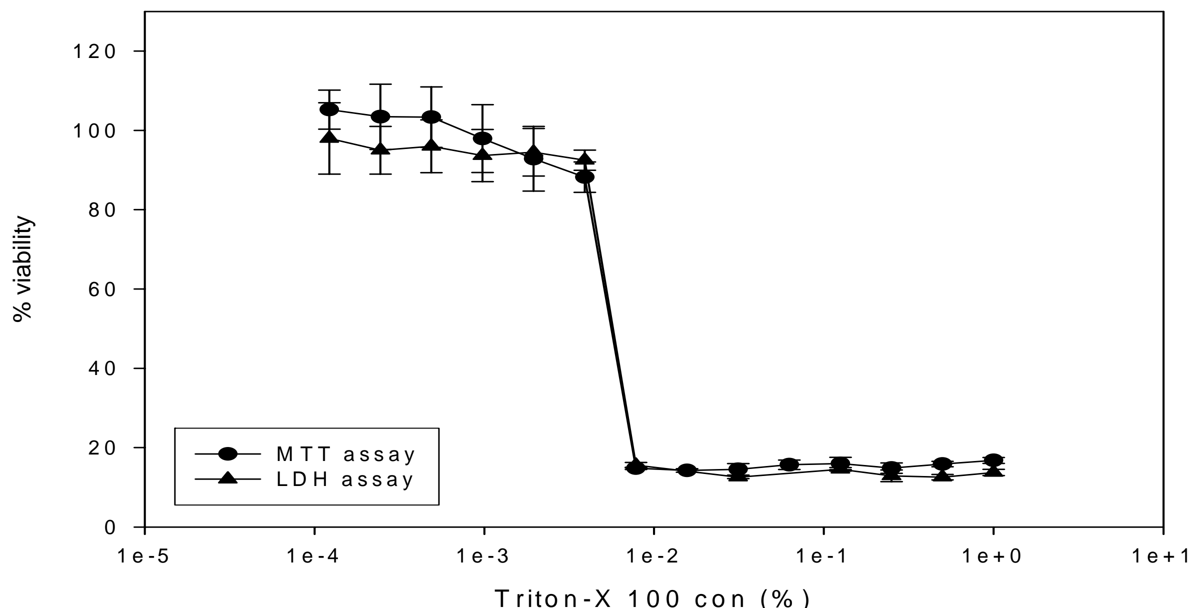

Figure 1: Concentration-viability curve of Triton X-100 on A549 cells. (Results present the average and standard deviation of six wells).

It is important to know that careful selection between these two assays is needed. It has been reported that MTT assay could detect toxicity earlier than LDH release, because a reduction in mitochondrial respiration is an expression of toxicity in cellular function that takes place before the observed release in LDH, which can be detected only after the failure of the cell membrane. [11]

For other endpoints, e.g., apoptosis, other cell markers have to be used and for those specialized kits are commercially available. [12]

2. Sulphorodamine Assay

The first step in screening compounds for cytotoxic activity is to establish the doubling time for the cells used and the right cell density that would give an acceptable level to measure total cell proteins using SRB assay.

First, MCF-7 cells were seeded with different densities and were incubated for 48 hours, after that SRB assay was performed as described in the experimental part. Results are shown in Figure 2A. Second, cells were seeded in different densities and SRB assay was done after 24 and 48 hours incubation. Results are shown in Figure 2B. Both figures demonstrate that sometime between 24 and 48 hours, the cells doubled their number and the absorbance at 570 nm increased significantly, and the seeding density of 5000 cell/well shall give a reliable absorbance value to estimate cell number after proliferation and upon incubation with substances that may influence the proliferation profile of the cells.

3. Testing the cytotoxic effects of vincristine sulphate and medicinal plants with expected activity against cancer cells.

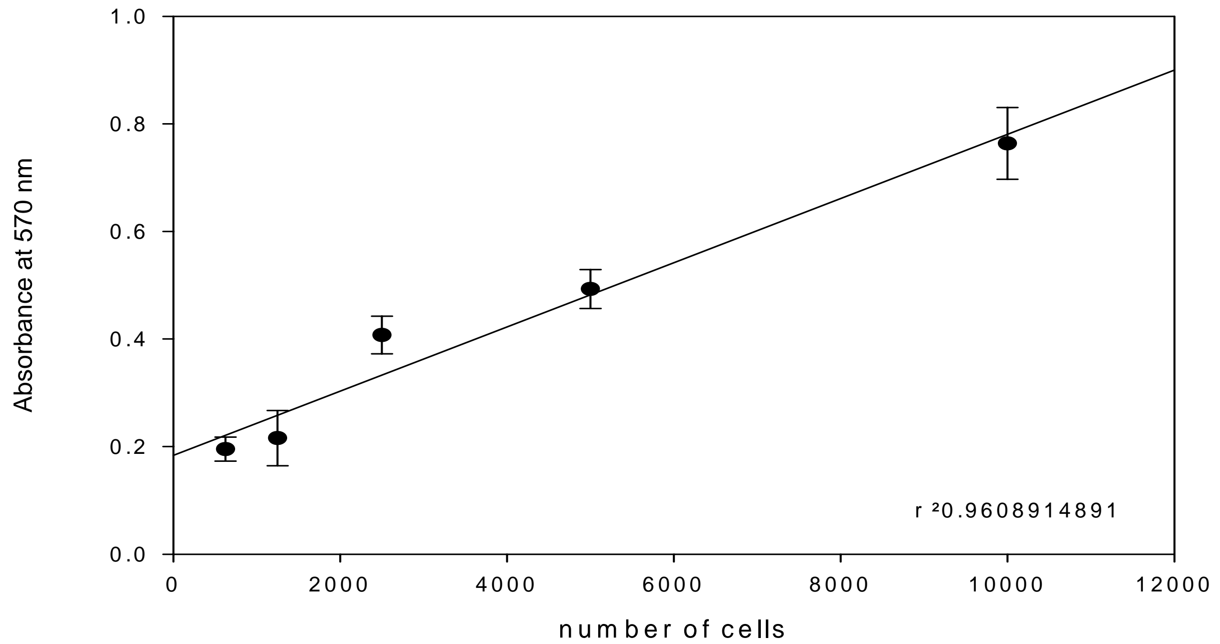

Figure 2A: Effect of cell number on the absorbance of SRB assay using MCF 7 cells. (Results present the average and standard deviation of six wells).

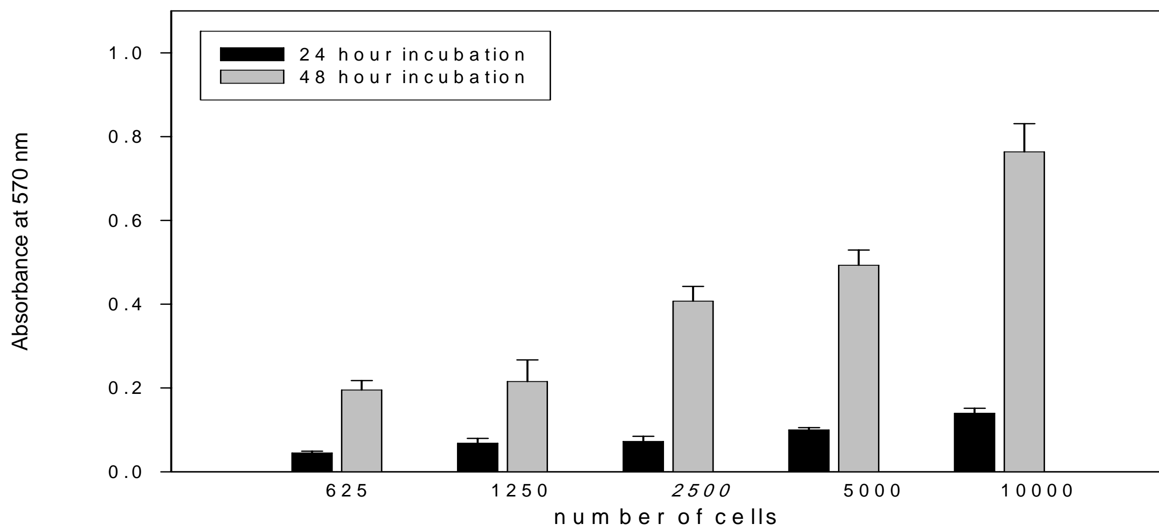

Figure 2B: Effect of incubation time and cell density on cell proliferation. (Results present the

average and standard deviation of six wells).

The percentage growth of cells (A549 and MCF-7) exposed to different concentrations of vincristine sulphate is shown in Figure 3. The toxicity profile shows different sensitivity of the cell lines to a cytotoxic agent and demonstrates the ability of the assay to detect such differences. For screening purposes, four ethanolic plant extracts were supplied by Prof. F. Afifi, Faculty of Pharmacy, University of Jordan, and the toxicity of a serial dilution was investigated on the different cells. Figure 4 represents the toxicity profile of the extracts on MCF-7 cell line and it shows that two extracts with IC50 below 30ug/ml are of value for further evaluation due to their anti proliferation capacity. The same extracts are to be run on normal epithelial cells to ensure their safety.

Conclusion

Within the context of this research project, a nucleus of a cell culture laboratory for screening purposes has been laid down. Different protocols and procedures together with the standard operating procedures have been established and are continuously evaluated and reviewed.

Assays for cytotoxicity, permeability, transport and activity are being implemented at the present time to evaluate different inputs that could be of value in the pharmaceutical industry.

Acknowledgment

The Deanship of Scientific Research at the University of Jordan is thanked for funding this project.

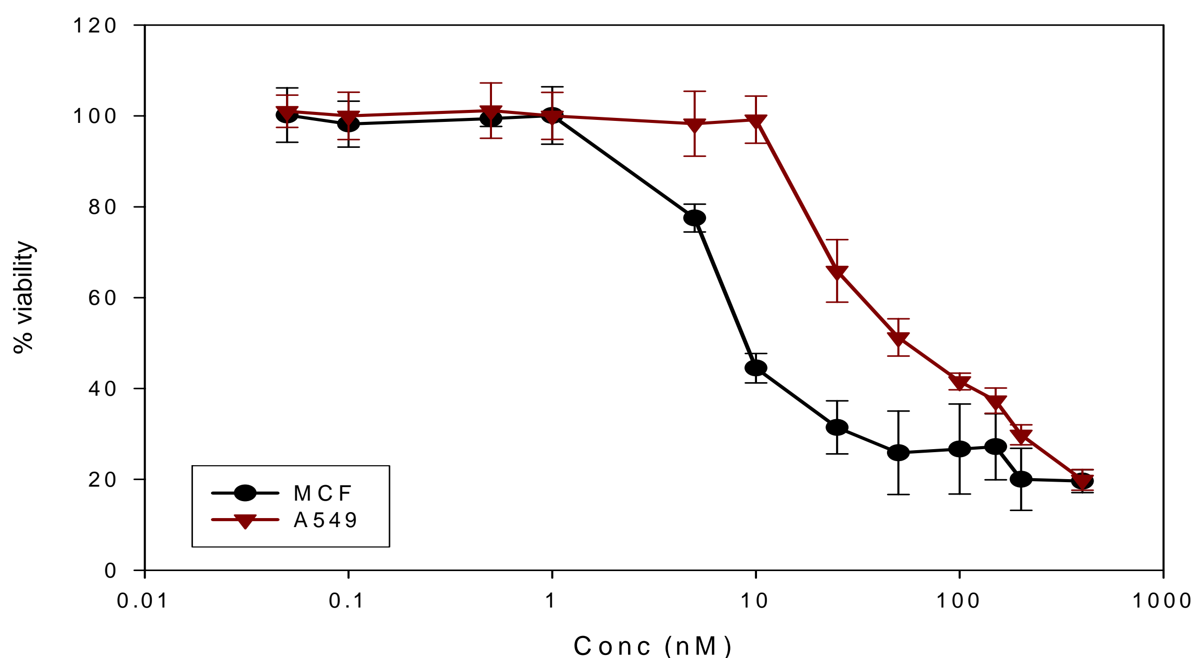

Figure 3: Toxicity of Vincristine Sulphate on A549 cell and MCF 7 cells after 72 hours incubation. (Results present the average and standard deviation of six wells).

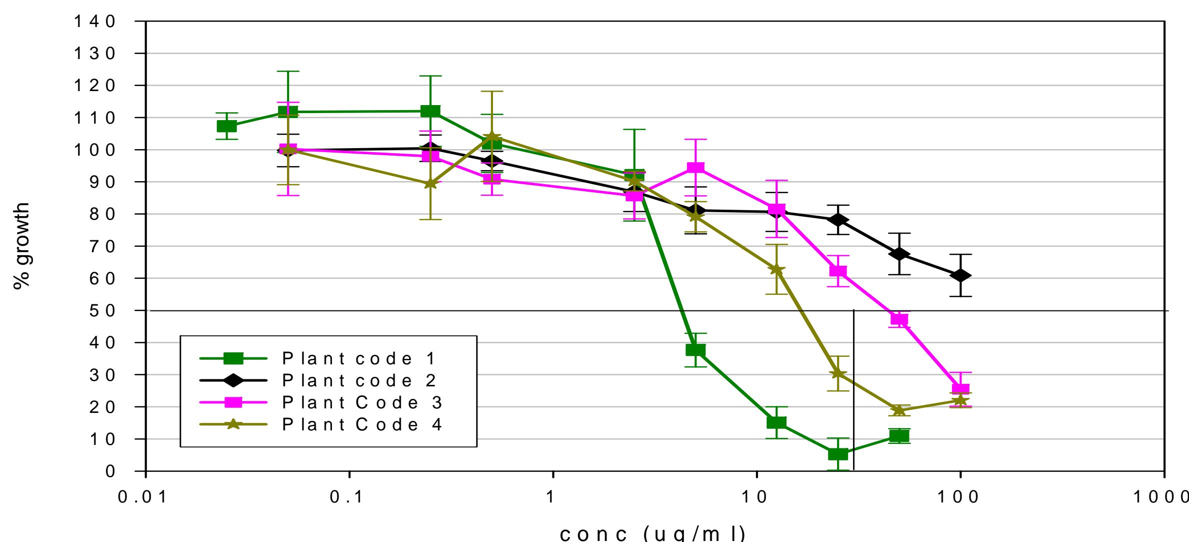

Figure 4: Toxicity of different plant extracts using MCF 7 cell line after 72 hours incubation. (Results present the average and standard deviation of six wells).

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