CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.2443347

Available online at: http://www.iajps.com ***Research Article***

SULFORHODAMINE B COLORIMETRIC ASSAY FOR CYTOTOXICITY SCREENING FOR SOME PYRAZOLE-QUINAZOLINE DERIVATIVES.

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Abstract

The sulforhodamine B (SRB) assay is a most advanced cell density determination, with the measurement of cellular protein content. The method explained here has been optimized for the toxicity screening of compounds to adherent cells in a 96-well format. After an incubation period, cell monolayers are fixed with 10% (wt/vol) trichloroacetic acid and stained for 30 min, after which the excess dye is removed by washing repeatedly with 1% (vol/vol) acetic acid. The protein-bound dye is dissolved in 10 mM Tris base solution for OD determination at 510 nm using a microplate reader. The results are linear over a 20-fold range of cell numbers and the sensitivity is comparable to those of fluorometric methods. The method not only allows a large number of samples to be tested within a few days, but also requires only simple equipment and inexpensive reagents. The SRB assay is therefore an efficient and highly cost-effective method for screening.

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Please cite this article in press Srivalli Tripuramallu and Suthakaran R et al., ***Sulforhodamine B Colorimetric Assay For Cytotoxicity Screening For Some Pyrazole-Quinazoline Derivatives.,*** *Indo Am. J. P. Sci, 2018; 05(12).*

INTRODUCTION:

A significant part of drug discovery in the last forty years has been focussed on agents to prevent or treat cancer. This is not surprising because, in most developed countries and, to an increasing extent, in developing countries, cancer is amongst the three most common causes of death and morbidity. Treatments for cancer may involve surgery, radiotherapy and chemotherapy and often a combination of two or all three is employed. In spite of these successes, there is still much activity directed to finding novel anticancer agents. The traditional cytotoxic approach is associated with severe and unpleas-ant side effects in clinical usage so a 'cocktail' of lower doses of such compounds is now often given, rather than a large, and therefore more toxic, dose of a single compound. It is relatively easy to screen compounds for cytotoxic effects and large throughput automated screening procedures are used in industry and by research organisations such as the National Cancer Institute in USA, which employs 60 different cancer cell lines. [3] Common methods for estimating cytotoxic activity are discussed below, but it should be noted that other types of experiments have to be conducted to deduce the mechanisms responsible and whether cell death is due to necrosis or apoptosis. These are beyond the scope of this paper. In the last twenty years, interest has grown in the links between dietary and environmental factors and the incidence of various cancers. [4-6] There is increasing evidence that some constituents of plants found in the diet prevent, at least to some extent, the damage to the cell or other factors in its metabolism and function, which pre-dispose people to cancer. Testing for such preventive activities is not so common as cytotoxicity testing but some approaches that have been tried are discussed below. [7-10]

MATERIALS, BUFFERS AND REAGENTS:

Stock solutions of 40 mg/ml of compounds in an appropriate solvent Dimethyl sulphoxide (DMSO) which will not harm the cells was used. Dimethyl sulphoxide (DMSO) is often used since it is cytotoxic at high concentrations, dilutions with medium were made so that the final concentration used for treating the cells was below 1% w/v. Tests had been carried out with solvent used to check its cytotoxicity, using the SRB assay. All stock solutions were sterlised by filtration (0.22 lm pore size) and store at 20 ^{o}C.

CELL LINES:

Cell lines were defined and obtained from Tata Memorial Centre [Advanced Centre for Treatment Research and Education in Cancer (ACTREC)], Mumbai. A mixture of cancer cell lines (Human Skin Cancer cell Line G361) were used. All cells should be cultured in appropriate medium and supplemented with 10% v/v foetal bovine serum (FBS), 1% w/v penicillin (104 U/ml)/streptomycin (10 mg/ml) and 1% w/v L-glutamine (200 mM). All these reagents were obtained from Sigma–Aldrich, UK.

HARVESTING CELLS:

Cells were incubated at appropriate culture medium at 37 ^{o}C in 5% v/v CO2 until 75% confluent. Cells were rinsed with 5 ml Ca2+-, Mg2+-free phosphate-buffered saline (PBS) and aspirate. Cells were incubated with 2 ml 0.05% w/v trypsin/0.5 mM EDTA (Sigma–Aldrich, UK) for 3 min at 37 ^{O}C. Cell culture flask was tapped on the edge with horizontal force until the cells detach. Incubate the flask for an additional 1 min to allow the cells to detach completely. The detached cells were triturated with a 1 ml pipette for several times to disrupt cell clumps and produce a single cell suspension. 5 ml medium containing 10% v/v foetal bovine serum was added to inactivate the trypsin. Transfer the cell suspension to a 15 ml conical tube and centrifuge at 100 g for 5 min. The supernatant layer was discarded and resuspend the cells in culture medium or PBS as appropriate.

PREPARATION OF CELLS FOR THE ASSAY:

These include the seeding density, i.e. the correct concentration of cells to be used, and the time over which the assay should be carried out. The seeding density is dependent on the cell cycle time of each cell line. There are two criteria to be considered for the selection of preferred cell density. All cell lines in control were kept in the exponential growth phase over the incubation time of bioassays. The doubling time (total cell cycle time)should be shorter or equal to the incubation time for some bioassays, otherwise a cell cycle-specific effect may not be observed.

DETERMINATION OF TOTAL CELL CYCLE TIME:

Haemocytometer is used to measure the cell density of the suspension, Suspensions of cells in the appropriate growth medium at different densities to give volumes of about 100 lL to contain from $1x10^3$ to $7x10^3$ cells/well. Seeded 100 lL a liquots of cell suspensions into 96-well plates and incubated for 5 days(120 h) and, using a haemocytometer measure the cell density of each suspension every 24 h. Plot between log of the density of cells against time was construct a growth curve. A straight line graph had showen the exponential proliferation of the cells. The cell cycle time was calculated from the graph by the slope of the line. A higher seeding density results in a

shorter doubling time. A seeding density was beeb chosen which gives a cell cycle time of about 48 h. At higher densities, contact inhibition of cells occurs and nutrients are exhausted more quickly from the medium.

THE SRB ASSAY:

The term cytotoxicity covers both cytostatic or cytocidal effects. The SRB assay can be used to determine which of these takes place for a particular test substance. Exposure to the test substance may result in a reduction of proliferation of cells at the end of the specified time compared to the control wells where no cytotoxic substance has been added. The IC50 value determined under these conditions is known as the 'Exposure' dose. At high concentrations no cells remain at this time i.e. all cells have been killed, but if some viable cells remain, it is not possible to know if they are capable of revival and proliferation once the toxic substance is removed. In order for this to be investigated, the cell medium, which contains the cytotoxic agent, is removed and replaced with fresh medium containing no cytotoxic substance. The cells
are then re-incubated for at least the same time as that used for 'Exposure' and the SRB assay carried out. IC50 determinations from this second assay are known as 'Recovery' values. If exposure to the extract does not kill the cells, they will revive when the fresh medium is added and any IC50 value will be much higher than for the exposure value. If the extract permanently affects the cells then the IC50 value will be similar to that shown by the exposure assay.

MATERIALS:

Cell lines are cultured from Tata Memorial Centre [Advanced Centre for Treatment Research and Education in Cancer (ACTREC)], Mumbai. A mixture of cancer cell lines (Human Skin Cancer cell Line G361) were used. All cells should be cultured in appropriate medium and supplemented with 10% v/v foetal bovine serum (FBS), 1% w/v penicillin (104 U/ml)/streptomycin (10 mg/ml) and 1% w/v L-glutamine (200 mM). All these reagents were obtained from Sigma–Aldrich, UK. Cultures are passaged weekly and the culture medium changed once every 5 days. Reagents. 40 w/v w/w ice-cold trichloroacetic acid (TCA) (Sigma–Aldrich, UK); 0.4% w/v sulphorhodamine B (SRB) (Sigma–Aldrich, UK) in 1% v/v acetic acid; 1% v/v acetic acid for washing cells; 10 mM Tris[hydroxymethyl] aminomethane buffer (TRIS base.

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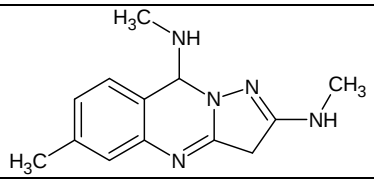
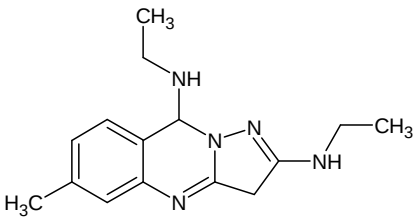
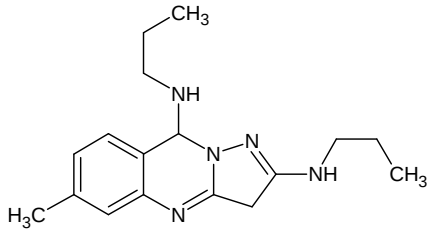
1	1	
2	2	
3	3	

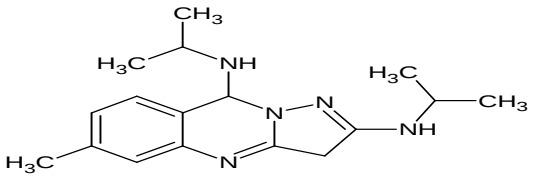
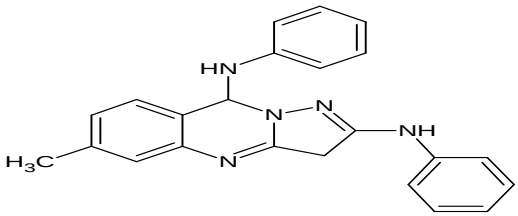
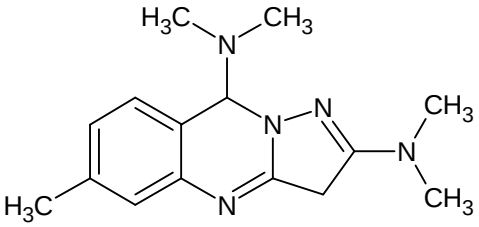
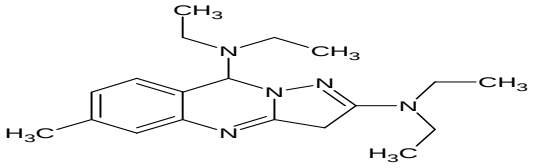
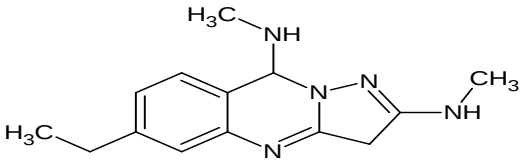
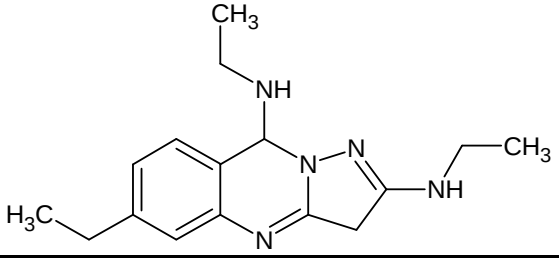
4	4	
5	5	
6	6	
7	7	
8	8	
9	9	

Table1: Molecular Structures of computational active compounds

Compound No.	Human Skin Cancer Cell Line G361			
	% Growth*			
	Molar Drug Concentration			
	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M
1	100.0	100.0	**90.9**	**-59.3**
2	100.0	100.0	99.7	48.5
3	100.0	100.0	100.0	87.7
4	100.0	99.6	100.0	75.1
5	100.0	100.0	99.7	56.5
6	99.7	97.8	94.3	47.6
7	100.0	100.0	96.1	98.1
8	100.0	98.6	100.0	75.1
9	100.0	85.7	98.6	76.5
ADR	46.9	-76.1	-73.6	-76.4

EXPERIMENTAL RESULTS:

Table2: Experimental results of SRB assay

G 361	Molar drug concentration		
	LC 50*	TGI*	G150*
1	$>10^{-4}$	$3.32*10^{-5}$	$2.08*10^{-6}$
2	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
3	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
4	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
5	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
6	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
7	$>10^{-4}$	$2.32*10^{-5}$	$1.78*10^{-6}$
8	$>10^{-4}$	$2.22*10^{-5}$	$1.08*10^{-6}$
9	$>10^{-4}$	$3.88*10^{-5}$	$2.08*10^{-6}$
ADR	$2.03*10^{-6}$	$1.27*10^{-7}$	$<10^{-7}$

*Average values of 3 experiments

Parameters calculated from Graph

Table3: Experimental results of SRB assay

DISCUSSION:

The newly synthesized compounds were screened for their anticancer activity against Human Skin Cancer Cell Line G361 by Sulforhodamine B assay. Doxorubicin was used as a standard reference drug and the results obtained were shown in (Table:1,2). All compounds (**1-9)** showed low antiproliferative activity. The % Growth inhibition of the compound **(1,7,8,9)** was found to be considerable at a concentration of 10^{-4} M. TGI_{50} (Growth inhibition of 50 % cells, calculated from drug concentration resulting in a 50 % reduction in the net protein increase) value of **(1,7,8,9)**. As heterocyclic derivative is the most active compound, it serves as a lead to further optimization in drug discovery process

CONCLUSION:

The computationally screened molecules are performed for SRB Assay and their results are found to be active and potent results. Which further leaded

in to lead optimization.

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