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Biological cytotoxicity evaluation of spiro[azetidine-2, 3'-indole]-2', 4(1'H)-dione derivatives for anti-lung and anti-breast cancer activity

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ABSTRACT

Biological in-vitro cell-based cytotoxicity assay is an easy and cost effective tool for hit ranking and lead optimization at the early stage of drug discovery, drug design and drug optimization. In the present research investigation, spiro[azetidine-2, 3'-indole]-2', 4(1'H)-dione derivatives were evaluated for cytotoxicity against HEK-293T (Human erythrocyte kidney cell line), MDA-MB453 (Human Breast carcinoma cell line), MDA-MB468 (Human Breast carcinoma cell line), NCI-H522 (Human Lung cancer cell line) and NCI-H23 (Human Lung cancer cell line) with use of short term cytotoxicity MTT and XTT assay protocol. The IC_{50} was determined by dose response curve analysis and statistical analysis using GraphPad Prism application, by plotting the graph of log concentration vs % growth Inhibition. ***Compound 7g*** *displayed significant cytotoxicity (IC_{50}) in breast cancer cell lines after 48 hrs comparable with the standard control drug doxorubicin and are good candidate for development of novel drugs based on these derivatives.*

Keywords: Spiro[azetidine-2, 3'-indole]-2', 4(1'H)-dione derivatives, cytotoxicity, lung and breast cancer cell line, MTT, XTT.

INTRODUCTION

Cancer is a class of diseases in which a group of cells display *uncontrolled growth* (division beyond the normal limits), *invasion* (intrusion on and destruction of adjacent tissues), and sometimes *metastasis* (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumours, which are self-limited, and do not invade or metastasize. [1] 2−Azetidinones, commonly known as β − lactams, are well-known heterocyclic compounds among the organic and medicinal chemists. The activity of the

famous antibiotics such as penicillins, cephalosporins and carbapenems are attributed to the presence of 2−azetidinone ring in them. Recently, some other types of biological activity besides the antibacterial activity have been reported in compounds containing 2−azetidinone ring. Such biological activities include anti-fungal, anti-tubercular, anti-tumour, cholesterol absorption inhibition and enzyme inhibition activity. The β − lactams also serve as synthons for many biologically important classes of organic compounds. [2,3,4]

Common basic steps of *in-vitro* cytotoxic screening include: (a) isolation of cells, (b) incubation of cells with drugs, (c) assessment of cell survival and (d) interpretation of the result. The trypan blue dye exclusion assay is the most commonly accepted method for the measurement of cell viability. It relies on the alteration in membrane integrity as determined by the uptake of dye by dead cells, thereby giving a direct measure of cell viability. It is now well-documented that apoptosis or programmed cell death is the key mechanism by which Chemotherapeutic agents exert their cytotoxicity. These colorimetric assays (MTT) are mainly useful in determination of cellular proliferation, viability and activation. The need for sensitive, quantitative, reliable and automated methods led to the development of standard assays. Cell proliferation and viability assays are of particular importance for routine applications. Tetrazolium salts MTT are especially useful for assaying the quantification of viable cells. Both, MTT and XTT dye work by being converted to a formazan dye only by metabolic active cells. These formazan dyes were solubilized and is directly quantified using an ELISA reader with respective reference wavelengths. [1]

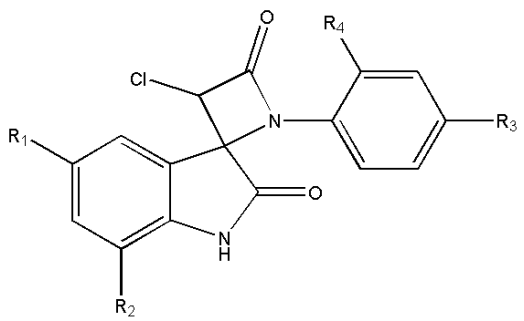
The development of *in-vitro* cytotoxicity assays has been driven by the need to rapidly evaluate the potential toxicity of large numbers of compounds, to limit animal experimentation whenever possible, and to carry out tests with small quantities of compound. Evidence for the utility of *in-vitro* cytotoxicity tests has led many pharmaceutical companies to screen compound libraries to remove potentially toxic compounds early in the drug discovery process. XTT assay and the MTT assay are the most common employed for the detection of cytotoxicity or cell viability following exposure to toxic substances. [1]

MATERIALS AND METHODS

Compounds:

A series of ten compounds **(7a-7j)** were procured from Pharmaceutical Chemistry Department, S. K. Patel College of Pharmaceutical Education & Research, Ganpat University, N. Gujarat, India as shown in Table 1. These synthesized compounds were screened for anti-lung and anti-breast cancer activity.

Table 1: Spiro[azetidine-2, 3'-indole]-2', 4(1'H)-dione derivatives used in the present investigation



Entry	Compound Code	R_1	R_2	R_3	R_4	Mol. Formula / Mol. Wt.
1	**7a**	Br	Br	CH_3	H	$C_{17}H_{11}Br_2ClN_2O_2$470
2	**7b**	Br	Br	H	CH_3	$C_{17}H_{11}Br_2ClN_2O_2$ 470
3	**7c**	Br	Br	NO_2	H	$C_{16}H_8Br_2ClN_3O_4$ 502
4	**7d**	Br	Br	H	NO_2	$C_{16}H_8Br_2ClN_3O_4$ 502
5	**7e**	Br	Br	H	H	$C_{16}H_9Br_2ClN_2O_2$ 456
6	**7f**	H	H	CH_3	H	$C_{17}H_{13}ClN_2O_2$ 312
7	**7g**	H	H	H	CH_3	$C_{17}H_{13}ClN_2O_2$ 312
8	**7h**	H	H	NO_2	H	$C_{16}H_{10}ClN_3O_4$ 344
9	**7i**	H	H	H	NO_2	$C_{16}H_{10}ClN_3O_4$ 344
10	**7j**	H	H	H	H	$C_{16}H_{11}ClN_2O_2$ 299

Media [1,5,6]
Leibovitz L-15 Medium with L-Glutamine (Biological Industries, Lot No: 928726), FBS (Fetal Bovine Serum, South American origin) (Quaditive, Lot No: 103128), SFM HEK-293 (Serum Free Media, Hyclone, Lot no: ARF26635), Thioglycollate medium (TGM) (Himedia, Lot No: YHI25), Tryptone soya broth (TSB) (Himedia, Lot No: YH031), Cell proliferation kit (MTT) 1000 tests (Biotium, Inc., Cat. No: 30006), Cell proliferation kit (XTT) 1000 tests (Biological Industries, Lot No: 910395).

Cell lines [1,5,6]
HEK-293T (Human embryonic kidney normal cell line), NCI-H23 (Human Non-Small Cell Lung cancer cell line), NCI-H522 (Human Non-Small Cell Lung cancer cell line), MDA-MB453 (Human breast adenocarcinoma cell line), and MDA-MB468 (Human breast adenocarcinoma cell line) were procured from NCCS, Pune.

Microbial and fungal culture
Candida albicans, Bacillus subtilis, Candida sporogenes, Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh.

Subculture of adherent cell lines (HEK-293T, MDA-MB453 and NCI-H23) [1,5,6]
Cultures were observed using an inverted microscope to assess the degree of confluency and the absence of bacterial and fungal contaminants was confirmed. Cell monolayer was washed with PBS without Ca^{2+}/Mg^{2+} using a volume equivalent to half the volume of culture medium. Trypsin/EDTA was added on to the washed cell monolayer using 1 ml per 25 cm^2 of surface area.

Flask was rotated to cover monolayer with trypsin. Flask was returned to the incubator and left for 2-10 mins. The cells were examined using an inverted microscope to ensure that all the cells were detached and floated. The cells were resuspended in a small volume of fresh serum containing HEK-293 medium. 100-200 µl was removed to perform a cell count. The required number of cells were transferred to a new labeled flask containing pre-warmed HEK-293 medium and incubated as appropriate for the cell line.

Determination of bacteria and fungi in normal and carcinoma cell lines [1,5,6]
Cell line was cultured in the absence of antibiotics at NCCS, Pune. Cell suspension was prepared by scrapping attached cells with the use of a cell scraper and maintained the pH 7.5-8.0. In 1.5 mL cell suspension, 2 mL thioglycollate medium (TGM) and 2 mL tryptone soya broth (TSB) were added and inoculated with two different strains; *Candida albicans* (0.1 mL) *Bacillus subtilis* (0.1 mL). Then in 1.5 mL cell suspension, 1 mL TGM was added and inoculated with 0.1 mL *Candida sporogenes* and 2 mL (TGM), 2 mL (TSB) were left uninoculated as negative controls. Broths were incubated at 32 ºC. Test and Control broths were examined for turbidity after 14 days.

Anti-cancer Activity [1,5,6]
XTT assay:
XTT assay was employed to assess cell proliferation. Viable cells were seeded into 96-well microtitre plates at 5 × 10^4 cells/well in L-15 media supplemented with FBS (fetal bovine serum), 100 units/ml penicillin, 100 µg/ml streptomycin and cultured in a humidified atmosphere of 5 % CO_2 at 37 ^{0}C. 180 µl of cell suspension was cultured with 20 µl of various concentrations of synthesized compounds (0.005-100 µg/ml) dissolved in 2 % DMSO solution and Doxorubicin as standard. Control cells were incubated in culture medium only. Wells containing only media were considered as a blank. All aliquots dilution doses were tested in duplicates.

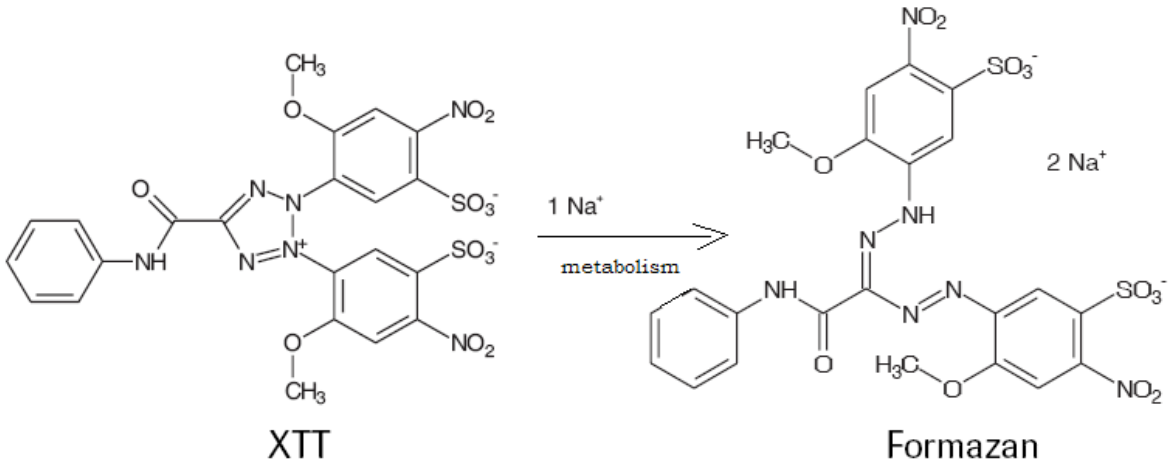


Figure 1: Principle of XTT assay

The cell proliferation is based on the ability of the mitochondrial succinate-terazolium reductase system to convert yellow tetrazolium salt XTT (sodium 3´-[1- (phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4 methoxy- 6-nitro) benzene sulfonic acid hydrate) to orange formazan dye. The test denotes the survival cells after toxic exposure. 50 µl of XTT mixture was added to each well. After 48 hrs incubation at 37 ^{0}C temperature and 5 % CO_2, the absorbance of soluble formazan product produced by viable cells was measured at 450 nm using ELISA plate reader (Thermo, USA). Reference wavelength used was 650 nm.

MTT Assay:
The cells were preincubated at a concentration of 1 × 10^6 cells/ml in culture medium for 3 hrs at 37 °C and 6.5 % CO_2. Then, the cells were seeded at a concentration of 5 × 10^4 cells/well in 100 µl culture medium and at various concentrations (0.005-100 µg/ml) of standard doxorubicin and synthesized compounds (dissolved in 2 % DMSO (dimethylsulphoxide) solution) into microplates (tissue culture grade, 96 wells, flat bottom) and incubated for 24 hrs at 37 °C and 6.5 % CO_2. The cell proliferation is based on the ability of the mitochondrial succinate-terazolium reductase system to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) to a blue colored formazan. The test denotes the survival cells after toxic exposure. Then, 10 µl MTT labelling mixture was added and incubated for 4 hrs at 37 °C and 6.5 % CO_2. Each experiment was done in triplicates. Then 100 µl of solubilization solution was added into each well and incubated for overnight. The spectrophotometric absorbance of the samples was measured using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product in between 550 and 600 nm according to the filters available for the ELISA reader (Thermo, USA) was used. The reference wavelength should be more than 650 nm.

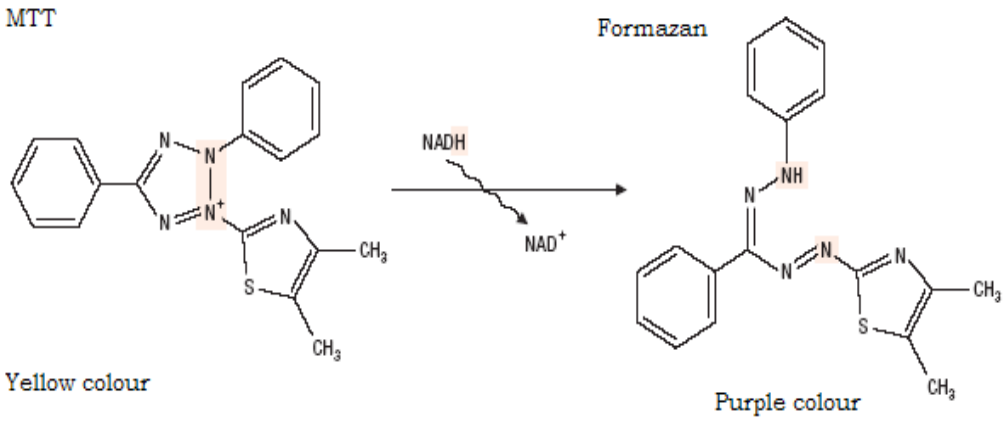


Figure 2: Principle of MTT assay

IC_{50}, the concentration of compound required to inhibit 50 % cell growth, was determined by plotting a graph of Log (concentration of compound) vs **%** cell inhibition. A line drawn from 50 % value on the *Y* axis meets the curve and interpolate to the X axis. The X axis value gives the Log (concentration of compound). The antilog of that value gives the IC_{50} value. Percentage inhibition of novel compounds against all cell lines was calculated using the following formula:

$$\% \text{ cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

Whereas,
At = Absorbance of Test,
Ab= Absorbance of Blank (Media),
Ac= Absorbance of control (cells)

$$\% \text{ cell inhibition} = 100 - \% \text{ cell survival}$$

RESULTS AND DISCUSSION

Total bacterial and fungal count

The examination of the test and control broths after 14 days incubation confirmed the absence of turbidity. Absence of turbidity in the test broth means that there was no evidence of bacterial, fungal and cross contamination.

Cytotoxicity Assay

The effect of novel compound aliquots (test) and doxorubicin (control) on the growth of MDA-MB468, MDA-MB453, NCI-H522 and NCI-H23 cell lines were examined by the MTT assay.

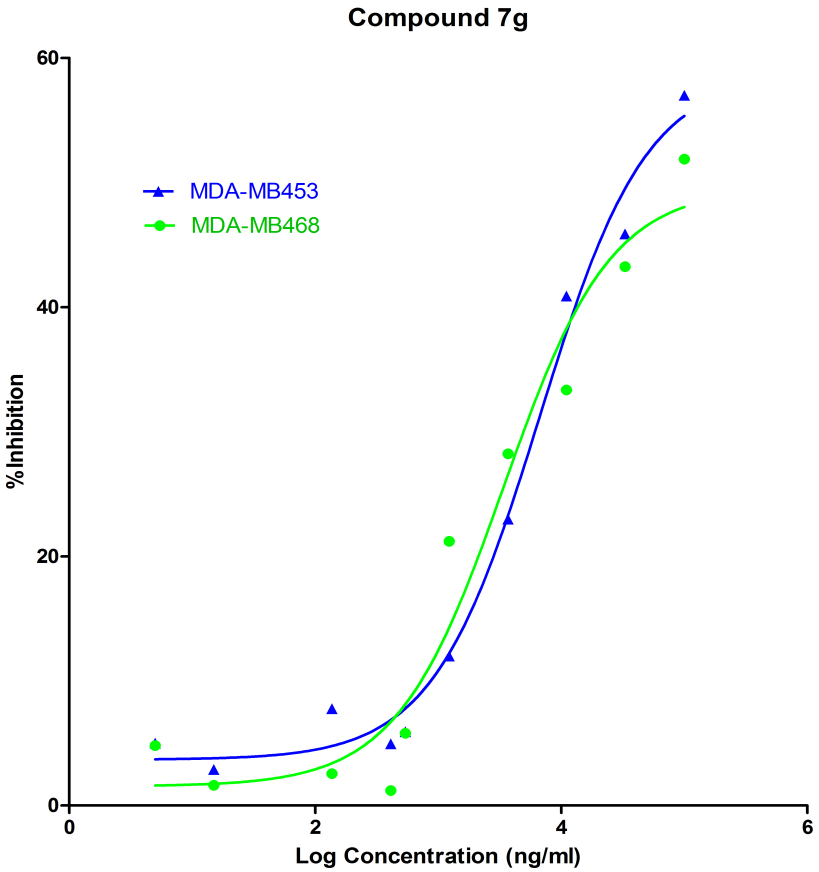


Figure 3: Activity of *Compound 7g* against various cell lines in MTT assay (48 hrs)

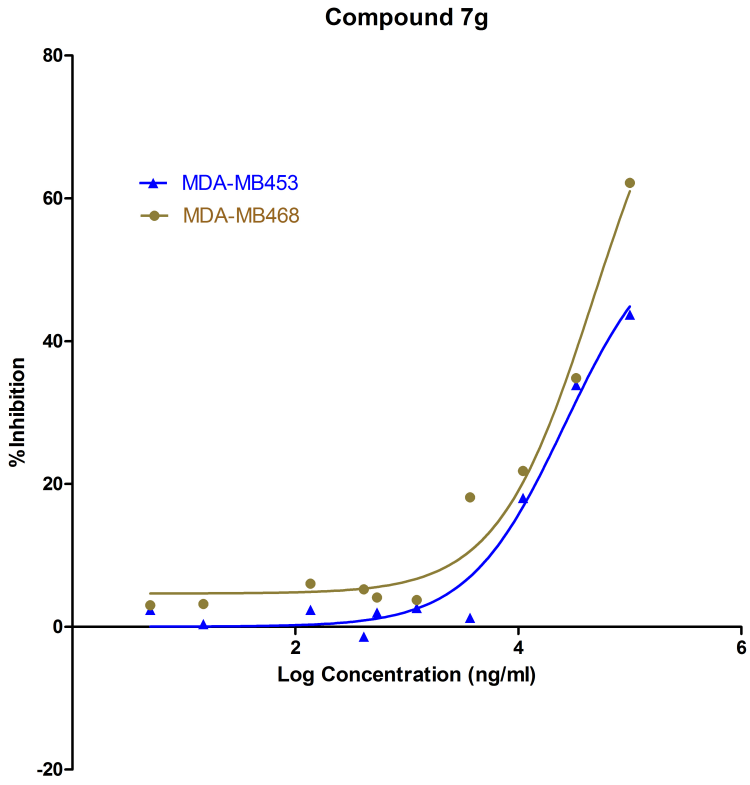


Figure 4: Activity of *Compound 7g* against various cell lines in XTT assay (48 hrs)

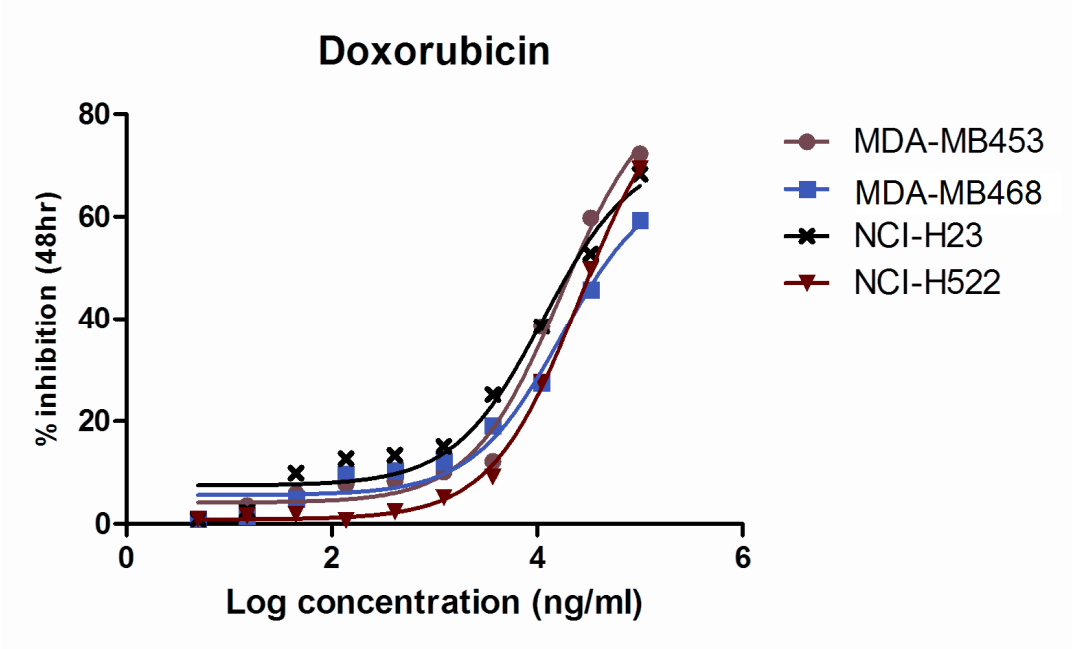


Figure 5: Activity of *Doxorubicin (control)* on various cell lines in MTT assay (48 hrs)

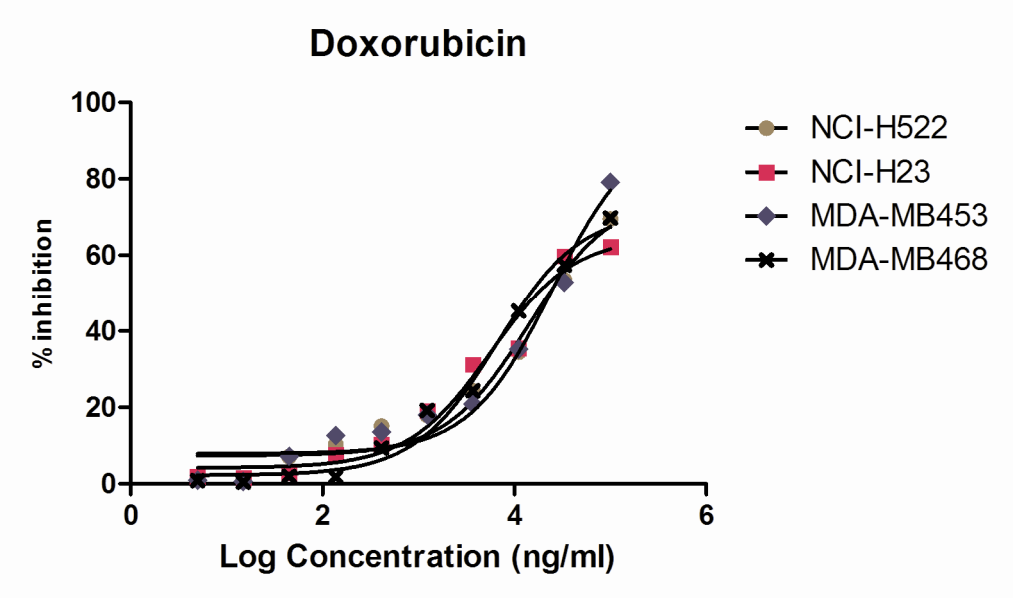


Figure 6: Activity of *Doxorubicin (control)* on various cell lines in XTT assay (48 hrs)

Table 2: IC_{50} values of Compound 7g and Doxorubicin on various cell lines by MTT and XTT Assay

Comp. No.	R_1	R_2	R_3	R_4	Assay	Breast cancer cell line		Lung cancer cell line		Normal cell line
						MDA-MB453	MDA-MB468	NCI-H522	NCI-H23	HEK-293T
7g	H	H	H	CH_3	MTT	**71.23**	**76.72**	>100	>100	**90.21**
					XTT	**71.99**	**78.82**	>100	>100	**87.98**
Doxorubicin (Standard)	*	*	*	*	MTT	**23.98**	**20.58**	**25.92**	**26.76**	**70.23**
					XTT	**22.17**	**20.18**	**25.43**	**26.61**	**74.09**

* NA = Not applicable

Dose response curves constructed between the range 0.005 – 100 µg/ml and 0.005 – 100 µM for compound aliquots and doxorubicin (control) respectively, express decreasing number of viable

cells with increasing concentration of compounds aliquots as well as doxorubicin. Calculation of IC_{50} value was done using *GraphPad Prism Software (Ver. 5.01)*. The susceptibility of cells to the compound aliquots and doxorubicin was characterized by IC_{50} values **(Table 2)**. Results indicate that the cytotoxic effect linearly and steadily strengthens with increase in the concentration.

In XTT method, the cells were treated with various compound dilutions followed by staining with XTT dye and % cell growth inhibition was calculated. Results of XTT assay have been tabulated in Table 2 and graphically presented in Figure 4. The data from graph revealed that as the concentration of test compounds increases as the % growth inhibition of cell per well increasing. IC_{50} of **Compound 7g** was 71.99 µg/ml on MDA-MB453 cell line, 78.82 µg/ml on MDA-MB468 cell line respectively; but no activity found in lung carcinoma cell lines.

In MTT assay, comparable cytotoxicity of **Compound 7g** was found against breast cancer cell line with the IC_{50} of 71.23 µg/ml on MDA-MB453 cell line and 76.72 µg/ml on MDA-MB468 cell line but no activity found in lung carcinoma cell lines. Graphical representation of the MTT results is shown in Figure 3. However, both of the compounds were found to be devoid of any activity against HEK 293T (normal) cell line but doxorubicin was found active against same lung cancer and breast cancer cell lines.

CONCLUSION

A series of new Spiro[azetidine-2, 3'-indole]-2', 4(1'H)-dione derivatives were screened for anti-cancer activity at various concentrations (0.005-100 µM/ml) using Doxorubicin as standard by MTT and XTT assay. Data indicates that among the synthesized compounds, **7g** Compound displayed greater cytotoxicity with comparable IC_{50}. The results described indicate that these compounds could serve as the basis for the development of a new group of cancer chemotherapeutics and certainly holds great promise towards good active leads.

Acknowledgement
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