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IN VITRO SULFORHODAMINE B ASSAY EVALUATION OF NOVEL 2-PHENYL BENZOFURANONE DERIVATIVES ON HUMAN SKIN CANCER CELL LINE G361

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ABSTRACT

The newly synthesized compounds are being tested for in vitro anti-cancer activity. Method used for In Vitro testing is Sulforhodamine B assay also known SRB assay. Cell lines were prepared and homoginised and disassociated with the help of trypsin. Then trypsin was inactivated with fetal bovine serum. Then cell concentration was determined. Synthesised molecules where prepared in to four different dillutions and exposed to cell lines. The procedure was also compared with standard drug doxorubicin. All the cell medium was incubated 37 degrees centigrade in a humidified incubator with 5 percentage CO. The plates were stainned and fixed with trichloroacetic acid. Finally the plates were incubated in orbital shaker incubater and absorbance was measured in micro plate reader at 510nm. All compounds (**1-30)** showed similar anticancer activity of compounds (**IA, IB, ID,IE,IF, IIB,IIC, IIIA, IVB, IVF, VA, VC, VD, VE.**) were more potent when compared to the rest of the compounds synthesized.

Keywords: Sulforhodamine B assay, trypsin, fetal bovine serum, doxorubicin, micro plate reader.

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INTRODUCTION

Cancer is also called as malignancy disease where group of abnormal cells undergo cell division uncontrollable beyond regular methods. In broad term malignancy is an illness of cellular changes that results in uncontrolled cell division, sometimes it may be rapid cell division or cell grow or divide in a slow rate. Normal cell shall be divided by controlled signals which instruct cells to divide, differentiation or to die. Whereas malignant cells builds autonomy signals causes uncontrolled growth with proliferation.[1-3] Sulfordamine B assay is an In Vivo model where an extrinsic method to estimate anti-tumour activity.[4-5] it is the method which was developed in the year 1990.[3] This Method majorly depends on the stoichiometric binding of SRB dye with proteins at mild acidic conditions which can be extracted by reverse basic canditions.[6-7] The process is majorly categorised into four stages preparation of treatment, incubation of cells with treatment of choice, cell fixation and SRB staining, and absorbance measurement.[8]

MATERIALS AND METHODS:

96, 384 well clear flat-bottom polystyrene tissue-culture plates, 96 well PCR plates, 100mm tissue culture plates,15ml falcon tubes, 1.5ml eppendorf tubes, pipette tips, 125microliter and 1250 microliter matrix pipette, human skin cancer cell line G361, culture medium, sterile and nonsterile reservoir, opti-MEM or serum free medium, phosphate buffer saline (PBS),2.5%w/v trypsin solution, fatal bovine serum, tryptan blue, trichloroacetic acid, sulforhodamine B sodium salt in 1%v/v acetic acid, acetic acid, 10mM unbuffered tris base solution, Lipofectamine RNA imax, mirvana miRNA mimics, mi RNA precursor molecules – negative control.[9]

Testing method: Sulforhodamine B assay.

Sample solution preparation:

Synthesized compounds were prepared by in the concentration of 50 microliter and other dilution and added 96-well plates at a triplicate final volume was made up to 100 microliter with DMSO.

Table 01 List of synthesized molecules and their concentration

S.NO	SAMPLE ID	DILUTION-1	DILUTION-2	DILUTION-3	DILUTION-4
1	PG-1A	10^{-7}	10^{-6}	10^{-5}	10^{-4}
2	PG-1B	10^{-7}	10^{-6}	10^{-5}	10^{-4}
3	PG-1C	10^{-7}	10^{-6}	10^{-5}	10^{-4}
4	PG-1D	10^{-7}	10^{-6}	10^{-5}	10^{-4}
5	PG-1E	10^{-7}	10^{-6}	10^{-5}	10^{-4}
6	PG-1F	10^{-7}	10^{-6}	10^{-5}	10^{-4}
7	RS-1A	10^{-7}	10^{-6}	10^{-5}	10^{-4}
8	RS-1B	10^{-7}	10^{-6}	10^{-5}	10^{-4}
9	RS-1C	10^{-7}	10^{-6}	10^{-5}	10^{-4}
10	RS -1D	10^{-7}	10^{-6}	10^{-5}	10^{-4}
11	RS -1E	10^{-7}	10^{-6}	10^{-5}	10^{-4}
12	RS -1F	10^{-7}	10^{-6}	10^{-5}	10^{-4}
13	OR-1A	10^{-7}	10^{-6}	10^{-5}	10^{-4}
14	OR-1B	10^{-7}	10^{-6}	10^{-5}	10^{-4}
15	OR-1C	10^{-7}	10^{-6}	10^{-5}	10^{-4}
16	OR-1D	10^{-7}	10^{-6}	10^{-5}	10^{-4}
17	OR -1E	10^{-7}	10^{-6}	10^{-5}	10^{-4}
18	OR -1F	10^{-7}	10^{-6}	10^{-5}	10^{-4}
19	HQ-1A	10^{-7}	10^{-6}	10^{-5}	10^{-4}
20	HQ-1B	10^{-7}	10^{-6}	10^{-5}	10^{-4}
21	HQ-1C	10^{-7}	10^{-6}	10^{-5}	10^{-4}
22	HQ-1D	10^{-7}	10^{-6}	10^{-5}	10^{-4}
23	HQ -1E	10^{-7}	10^{-6}	10^{-5}	10^{-4}
24	HQ -1F	10^{-7}	10^{-6}	10^{-5}	10^{-4}
25	HL-1A	10^{-7}	10^{-6}	10^{-5}	10^{-4}
26	HL-1B	10^{-7}	10^{-6}	10^{-5}	10^{-4}
27	HL-1C	10^{-7}	10^{-6}	10^{-5}	10^{-4}
28	HL-1D	10^{-7}	10^{-6}	10^{-5}	10^{-4}
29	HL -1E	10^{-7}	10^{-6}	10^{-5}	10^{-4}
30	HL -1F	10^{-7}	10^{-6}	10^{-5}	10^{-4}
31	ADR	10^{-7}	10^{-6}	10^{-5}	10^{-4}

Cell line preparation:

Trypsinization: Cell line monolayer's are washed with sterilized phosphate buffer saline (PBS). PBS was removed from the medium and then added 1ml of 0.25 %(w/v) trypsin to evenly cover the cell line surface. Then incubate the cell medium at 37°C for 5 min until cells start to dissociate from its surface. Trypsin was inactivated with 10 volumes of culture medium containing (fetal bovine serum) FBS by mixing up and down until single homogenous single cell suspension. Single cell suspension was transferred to a sterile falcon tube.[10]

Determination of cell concentration.

Single cell suspension was filled in a falcon tube and 0.4% (w/v) tryptan blue solution in a ratio 1:1 and then cell suspension was measured for cell concentration in hematocytometer chamber under a microscope to determine cell viability prior cell seeding and verified for cell health.

Cell concentration was adjusted with 10% FBS growth medium in 96 well with cell density of 50 microliter. The cell suspension was stored in sterile reagent container for multi-channel pipette.

Treatment exposure:

50 microliter of treatment sample was transferred in to each well of 96 well plate. Then cell suspension of 50 microliter was added to each well. Even cell distribution was observed at the bottom of the plate. A short spin was performed to the plate for 20 seconds. Three wells are incubated with above procedure with standard drug doxorubicin.

Blank preparation:

Three wells are filled with vehicle DMSO and cell suspension for untreated vehicle control, three wells are filled with DMSO only for background subtraction.

Incubation:

Plates are incubated at 37°C in a humidified incubator with 5% CO until plates are recorded.

Cell fixation and staining:

To the treated cell lines 25 microliter of cold trichloroacetic acid 50%(w/v) is added to each well directly to medium supernatant, and plates are incubated at 4°C for 1 hour. The plates are washed four times with submerging the plate in a tub with slow running water. Gently tapping was done to remove the excess water in to a paper towel and air dried at room temperature.

A 50 microliter 0.04% SRB solution was added to each well and incubated and kept at room temperature for 1 hour and quickly rinse the plates washed four times with 1%(v/v) acetic acid (200microliter) to remove unbound dye. Then plates air dried at room temperature and verified for the presence of air bubbles.

Absorbance measurement:

50-100 microliter of tris-base solution of PH 10.5 was added to each well and shakes the plate in an orbital shaker incubator for 10 min to solubilise the protein bound dye. Then absorbance was measured at 510 nm in a micro plate reader.

Calculations:

Background absorbance was removed from all vessels

Cell percentage growth = Absorbance sample/ Absorbance negative control X 100

% Growth inhibition = 100 - % Cell growth.

Growth inhibition was calculated of 50% was calculated as GI_{50}.

TGI: Drug concentration resulting in total growth inhibition (TGI).

LC_{50}: Concentration of drug resulting in a 50 % reduction in the measured protein at the end of the drug treatment (concentration of drug causing lethality to 50 % of the cells as compared to that at the beginning) indicating a net loss of cells following treatment.

Table 02 Experimental results of induvidual experiments

	Experiment - 1				Experiment - 2			
SAMPLE ID	**10^{-7}M**	**10^{-6}M**	**10^{-5}M**	**10^{-4}M**	**10^{-7}M**	**10^{-6}M**	**10^{-5}M**	**10^{-4}M**
PG-1A	100.0	100.0	100.0	-61.5	100.0	100.0	79.8	-57.0
PG-1B	95.3	94.3	63.2	29.7	100.0	92.1	65.4	34.4
PG-1C	100.0	100.0	97.3	46.6	100.0	100.0	77.2	39.5
PG-1D	100.0	100.0	100.0	-36.5	100.0	100.0	74.9	-30.0
PG-1E	100.0	100.0	100.0	-61.5	100.0	100.0	79.8	-57.0
PG-1F	100.0	100.0	100.0	-61.2	100.0	100.0	74.1	-62.9
RS-1A	100.0	100.0	100.0	21.9	100.0	100.0	89.5	19.9
RS-1B	100.0	100.0	71.2	2.2	100.0	90.8	88.0	10.2
RS-1C	100.0	100.0	100.0	-20.8	100.0	100.0	100.0	-40.9
RS -1D	100.0	100.0	100.0	22.4	100.0	100.0	100.0	9.1
RS -1E	100.0	100.0	100.0	52.5	100.0	100.0	100.0	41.6
RS -1F	100.0	100.0	100.0	25.6	100.0	100.0	86.5	15.8
OR-1A	100.0	100.0	100.0	3.5	100.0	72.4	12.7	-11.2
OR-1B	100.0	100.0	100.0	61.2	100.0	92.4	77.8	54.6
OR-1C	100.0	100.0	100.0	80.9	100.0	100.0	99.2	63.7
OR-1D	100.0	100.0	100.0	25.6	100.0	100.0	86.5	15.8
OR -1E	100.0	100.0	100.0	70.9	100.0	100.0	100.0	61.8
OR -1F	100.0	100.0	100.0	39.8	100.0	100.0	100.0	14.5
HQ-1A	100.0	100.0	100.0	30.1	100.0	100.0	100.0	25.1
HQ-1B	100.0	100.0	100.0	-12.1	100.0	100.0	100.0	-37.6
HQ-1C	100.0	100.0	100.0	15.3	100.0	100.0	100.0	12.2
HQ-1D	100.0	100.0	100.0	46.9	100.0	100.0	99.5	43.8
HQ -1E	100.0	100.0	100.0	70.9	100.0	100.0	100.0	61.8
HQ -1F	100.0	100.0	100.0	-12.1	100.0	100.0	100.0	-37.6
HL-1A	100.0	100.0	71.2	2.2	100.0	90.8	88.0	10.2
HL-1B	100.0	100.0	100.0	21.9	100.0	100.0	89.5	19.9
HL-1C	100.0	100.0	100.0	-61.2	100.0	100.0	74.1	-62.9
HL-1D	100.0	100.0	100.0	-61.5	100.0	100.0	79.8	-57.0
HL -1E	100.0	100.0	100.0	-36.5	100.0	100.0	74.9	-30.0
HL -1F	100.0	100.0	97.3	46.6	100.0	100.0	77.2	39.5
ADR	48.3	-44.4	-41.2	-55.0	-53.9	-65.2	-69.4	-73.5

	Experiment - 3				Average Values			
SAMPLE ID	**10^{-7}M**	**10^{-6}M**	**10^{-5}M**	**10^{-4}M**	**10^{-7}M**	**10^{-6}M**	**10^{-5}M**	**10^{-4}M**
PG-1A	100.0	89.6	79.1	-64.4	**100.0**	**96.5**	**86.3**	**-61.0**
PG-1B	107.6	70.1	69.0	28.6	**101.0**	**85.5**	**65.9**	**30.9**
PG-1C	100.0	89.3	75.3	34.6	**100.0**	**96.4**	**83.3**	**40.0**
PG-1D	100.0	84.0	79.2	-37.7	**100.0**	**94.7**	**84.7**	**-34.7**
PG-1E	100.0	89.6	79.1	-64.4	**100.0**	**96.5**	**86.3**	**-61.0**
PG-1F	100.0	89.4	80.2	-63.3	**100.0**	**96.5**	**84.8**	**-62.5**
RS-1A	100.0	100.0	86.1	15.8	**100.0**	**100.0**	**91.9**	**19.2**
RS-1B	100.0	86.7	73.6	8.5	**100.0**	**92.5**	**77.6**	**6.9**
RS-1C	100.0	100.0	100.0	-45.2	**100.0**	**100.0**	**100.0**	**-35.6**
RS -1D	100.0	100.0	100.0	13.4	**100.0**	**100.0**	**100.0**	**15.0**
RS -1E	100.0	100.0	100.0	42.2	**100.0**	**100.0**	**100.0**	**45.4**
RS -1F	100.0	100.0	100.0	12.0	**100.0**	**100.0**	**95.5**	**17.8**
OR-1A	100.0	100.0	17.5	-5.8	**100.0**	**90.8**	**43.4**	**-4.5**
OR-1B	100.0	100.0	99.1	39.3	**100.0**	**97.5**	**92.3**	**51.7**
OR-1C	100.0	100.0	100.0	49.8	**100.0**	**100.0**	**99.7**	**64.8**

OR-1D	100.0	100.0	100.0	12.0	**100.0**	**100.0**	**95.5**	**17.8**
OR -1E	100.0	100.0	100.0	51.8	**100.0**	**100.0**	**100.0**	**61.5**
OR -1F	100.0	100.0	100.0	21.5	**100.0**	**100.0**	**100.0**	**25.3**
HQ-1A	100.0	100.0	100.0	35.1	**100.0**	**100.0**	**100.0**	**30.1**
HQ-1B	100.0	100.0	100.0	-40.3	**100.0**	**100.0**	**100.0**	**-30.0**
HQ-1C	100.0	100.0	100.0	13.1	**100.0**	**100.0**	**100.0**	**13.5**
HQ-1D	100.0	100.0	100.0	41.0	**100.0**	**100.0**	**99.8**	**43.9**
HQ -1E	100.0	100.0	100.0	51.8	**100.0**	**100.0**	**100.0**	**61.5**
HQ -1F	100.0	100.0	100.0	-40.3	**100.0**	**100.0**	**100.0**	**-30.0**
HL-1A	100.0	86.7	73.6	8.5	**100.0**	**92.5**	**77.6**	**6.9**
HL-1B	100.0	100.0	86.1	15.8	**100.0**	**100.0**	**91.9**	**19.2**
HL-1C	100.0	89.4	80.2	-63.3	**100.0**	**96.5**	**84.8**	**-62.5**
HL-1D	100.0	89.6	79.1	-64.4	**100.0**	**96.5**	**86.3**	**-61.0**
HL -1E	100.0	84.0	79.2	-37.7	**100.0**	**94.7**	**84.7**	**-34.7**
HL -1F	100.0	89.3	75.3	34.0	**100.0**	**96.4**	**83.3**	**40.0**
ADR	-66.5	-78.0	-82.1	-83.6	**-24.0**	**-62.5**	**-64.2**	**-70.7**

Table 3 Anti-cancer activity with molar drug concentration

G361	**Molar drug concentration**		
	LC 50	**TGI**	**GI50**
PG-1A	$>10^{-4}$	$3.2*10^{-5}$	$2.02*10^{-6}$
PG-1B	$>10^{-4}$	$>10^{-4}$	$2.8*10^{-6}$
PG-1C	$>10^{-4}$	$>10^{-4}$	$3.8*10^{-5}$
PG-1D	$>10^{-4}$	$3.7*10^{-5}$	$2.2*10^{-6}$
PG-1E	$>10^{-4}$	$3.2*10^{-5}$	$2.04*10^{-6}$
PG-1F	$>10^{-4}$	$3.2*10^{-5}$	$2.02*10^{-6}$
RS-1A	$>10^{-4}$	$>10^{-4}$	$3.2*10^{-5}$
RS-1B	$>10^{-4}$	$>10^{-4}$	$2.6*10^{-6}$
RS-1C	$>10^{-4}$	$>10^{-4}$	$2.3*10^{-6}$
RS -1D	$>10^{-4}$	$>10^{-4}$	$3.2*10^{-5}$
RS -1E	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
RS -1F	$>10^{-4}$	$>10^{-4}$	$3.2*10^{-5}$
OR-1A	$>10^{-4}$	$3.7*10^{-5}$	$2.1*10^{-6}$
OR-1B	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
OR-1C	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
OR-1D	$>10^{-4}$	$>10^{-4}$	$3.2*10^{-5}$
OR -1E	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
OR -1F	$>10^{-4}$	$>10^{-4}$	$3.6*10^{-5}$
HQ-1A	$>10^{-4}$	$>10^{-4}$	$3.9*10^{-5}$
HQ-1B	$>10^{-4}$	$>10^{-4}$	$2.4*10^{-6}$
HQ-1C	$>10^{-4}$	$>10^{-4}$	$3.2*10^{-5}$
HQ-1D	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
HQ -1E	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
HQ -1F	$>10^{-4}$	$>10^{-4}$	$2.4*10^{-6}$
HL-1A	$>10^{-4}$	$>10^{-4}$	$2.6*10^{-6}$
HL-1B	$>10^{-4}$	$>10^{-4}$	$3.2*10^{-5}$
HL-1C	$>10^{-4}$	$3.2*10^{-5}$	$2.02*10^{-6}$
HL-1D	$>10^{-4}$	$3.2*10^{-5}$	$2.04*10^{-6}$
HL -1E	$>10^{-4}$	$3.7*10^{-5}$	$2.2*10^{-6}$
HL -1F	$>10^{-4}$	$>10^{-4}$	$3.8*10^{-5}$
ADR	$1.7*10^{-7}$	$<10^{-7}$	$<10^{-7}$

Fig 1 Anti-cancer results group-I

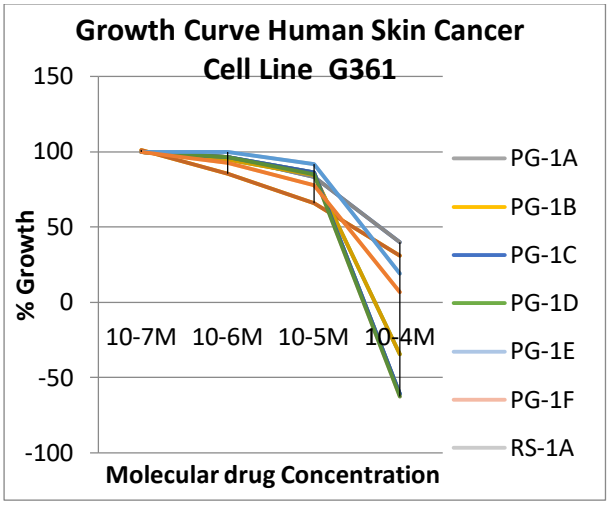


Fig 2 Anti-cancer results group-II

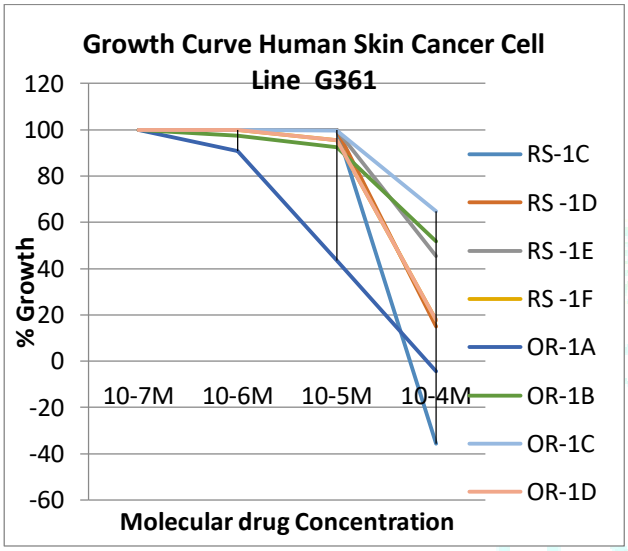


Fig 3 Anti-cancer results group-III

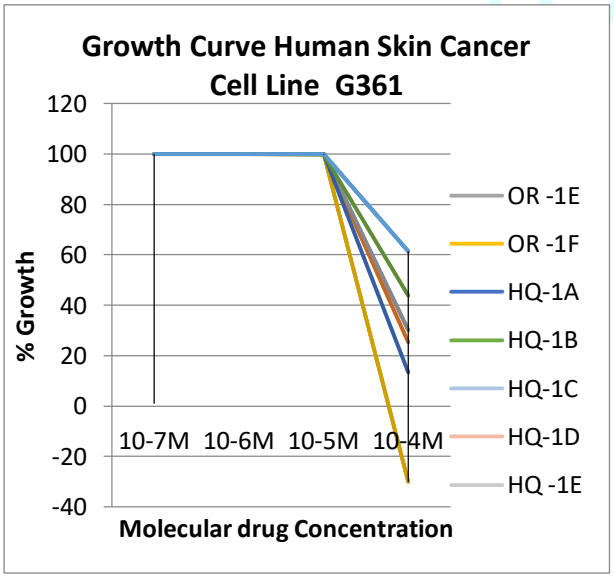
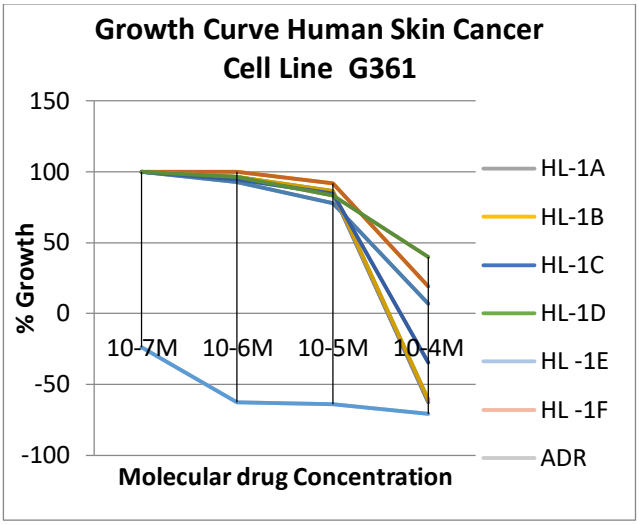


Fig 4 Anti cancer results group-IV



CONCLUSION

The synthesized molecules had been diluted at four different concentrations and Sulfordamine B assay method was opted for screening of anti-cancer activity. The results were compared with doxorubicin as standard drug at similar concentrations. The compounds exhibited good results.

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