CYTOTOXICITY STUDIES

5.1 Cell growth inhibition/MTT assay

All the compounds were screened in vitro for their anti-proliferative activity against three cell lines.

5.2 Principle

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to the given drugs. The reduction of tetrazolium salts has now been widely accepted procedure to examine cell proliferation and growth inhibition. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. The resulting purple formazan can be solubilized and quantified by specified spectrophotometric procedure. The MTT cell proliferation assay measures the cell proliferation rate and conversely the reduction in cell viability. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation [78] [79].

5.3 Protocol

Results were expressed in terms of IC_{50} (the concentration which resulted in 50% inhibition), where the gefitinib was used as positive control. IC_{50} values are the mean value of three independent experiments.

For *in vitro* evaluation, MTT assays for all compounds against three cell lines were performed. The A-549, human lung carcinoma, HCT-116, human colon cancer and MIAPaCa-2, human pancreatic carcinoma were used as cell lines for studying growth inhibition. The DMEM, RPMI-1640, DMEM-F12, were used as culture media for A-549 human lung carcinoma, HCT-116 colon cancer and pancreatic MIAPaCa-2 cell lines, respectively. 10^4 Cells per well were grown in 96-well plates and exposed to different concentrations of various test compounds for 48 h. After 44 h of treatment, 20µl of MTT solution (2.5 mg/ml) was added to each well and incubated at 37°C for 4 h in a humidified atmosphere containing 5% CO_2. In case of suspension cell lines, the

plates were centrifuged at 1500 r.p.m. for 15 min, and the supernatant was discarded while in adherent cell lines, the media was removed without centrifugation. The MTT-formazon crystals were dissolved in 150 µl DMSO. The absorbance was recorded at a wavelength of 570 nm in the microplate reader and cytotoxicity was calculated as % cell growth inhibition.

$$\% \text{ cell survival} = \{(At-Ab)/ (Ac-Ab)\} x100)$$

Where At, Ab and Ac are absorbance of test, blank and control, respectively. Concentrations 1µM, 10µM, 20µM, 30µM, and 50µM were used for assay.

5.4 Results and discussion

5.4.1 Benzyloxy)phenyl](4-benzylpiperazin-1-yl)methanone derivatives (scaffold-I)

IC_{50} values were calculated for all compounds and the results are reported in table 5.1

Table 5.1: IC_{50} values of 4-(benzyloxy)phenyl](4-phenylpiperazin-1-yl)methanone derivatives

Compound code	R	Lung A-549 IC_{50} (µM)	Colon HCT-116 IC_{50} (µM)	Pancreatic MIAPaCa-2 IC_{50} (µM)
A-1	2-Cl	>100	>100	>100
A-2	4-Cl	>100	>100	>100
A-3	2-F	>100	>100	>100
A-4	4-F	>100	>100	>100
A-5	2-NO_2	>100	>100	>100
A-6	2-OCH_3	>100	>100	>100
A-7	3-OCH_3	43.65	50	57.14
A-8	4-OCH_3	98.99	32.74	9.90
A-9	2,3-diCl	>100	>100	>100
A10	2-CH_3	29.16	18.68	44.42
A-11	3-CH_3	45.56	66.85	71.76
A-12	4-CH_3	95.75	85.63	65.66
	Gefitinib	16.56	10.51	49.50

* IC_{50} values between 100-1000 µM has been shows as >100.

In adenocarcinomic human alveolar basal epithelial cell line (Lung cancer, A-549) gefitinib showed IC_{50} value of 16.56 µM, in human colon carcinoma cell line (HCT-116 cell line) it showed IC_{50} value of 10.51 µM, whereas in human pancreatic carcinoma MIAPaCa-2 cell line, gefitinib showed IC_{50} value of 49.50 µM.

In benzyloxy)phenyl](4-benzylpiperazin-1-yl)methanone derivatives, it was observed that overall, electron withdrawing substituted compounds were devoid of cytotoxicity. Therefore, compounds A-1 to A-5 having 2-Cl, 4-Cl, 2-F, 4-F and 2-NO_2 substitution did not show cell growth inhibition up to 100 µM. However, electron donating group substitution supports growth inhibition but A-6 (2-OCH_3) was also devoid of inhibition. In A-549 and HCT-116 cell lines, compound A-10 (2-CH_3) showed the highest inhibition with IC_{50} value of 29.16 µM and 18.68 µM respectively. In MIAPaCa-2 cell line compound A-10 and A-8 (4-OCH_3) showed better inhibition than gefitinib wherein it had IC_{50} value of 9.90 µM.

5.4.2 4-(3-(4-methylpiperazin-1-yl)propoxy)-*N*-phenylbenzamide (scaffold-II) and (4-(3-methoxyphenyl)piperazin-1-yl)(4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl)methanone derivatives (scaffold-III)

IC_{50} values were calculated for all compounds and the results are reported in table 5.2

Table 5.2: IC_{50} value of compounds of scaffold II and III

Compound code	R	Lung A-549 IC_{50} (µM)	Colon HCT-116 IC_{50} (µM)	Pancreatic MIAPaCa-2 IC_{50} (µM)
Aniline substituent's (scaffold-II)				
B-1	H	41.07	6.54	25.01
B-2	4-CH_3	56.26	16.15	27.77
B-3	3-CH_3	>100	>100	>100
B-4	2,4 di-CH_3	44.35	10.11	11.54
B-5	3,4 di-CH_3	35.89	57.65	6.26
B-6	2,5 di-CH_3	7.74	18.80	14.98
B-7	4-OCH_3	43.92	35.39	41.77

B-8	4-Cl	>100	>100	>100
B-9	4-Br	>100	>100	48.35
B-10	4-F	43.92	35.39	41.77
Piperazine substituent's (scaffold-III)				
B-11	3-OCH_3	5.71	4.26	31.36
B-12	4-OCH_3	29.16	18.68	44.42
B-13	2-Cl	>100	>100	>100
B-14	4-Cl	>100	>100	>100
B-15	2,3-diCl	>100	>100	>100
B-16	4-CH3	14.28	14.42	19.88
	Gefitinib	16.56	10.51	49.23

* IC_{50} values between 100-1000 µM has been shows as >100.

In A-549 lung cancer cell line, electron donating substitution showed better inhibition. Among electron donating groups, compound B-6 (2,5 di-CH_3) showed better inhibition as compared to compound B-4 (2,4 di-CH_3) and B-5 (3,4 di-CH_3). Similarly, among scaffold III compounds, compounds B-11 (3-OCH_3) and B-16 (4-CH_3) showed the best IC_{50} value of 5.71 µM and 14.28 µM. Overall, in this cell line, meta substitution resulted in favorable activity. Compounds with EWGs such as B-8 (4-Cl), B-9 (4-Br), B-10 (4-F) resulted in very less inhibition.

In HCT-116 colon cancer line also, electron donating substituted compounds showed inhibition. Among aniline derivatives, B-1 (H), B-2 (4-CH_3) and B-4 (2,4 di-CH_3) showed better activity as compared to meta substituted compounds. Best IC_{50} value was for compound B-1 (6.54 µM). From the piperazine derivatives, compound B-11 having meta methoxy showed the best activity with IC_{50} of 4.26 µM. Compound B-16 (4-CH_3) also showed good IC_{50} value as compared to gefitinib.

In MIAPaCa-2 cell line dimethyl substituted aniline derivatives showed better results when compared to mono substituted compounds. Compounds B-4 (2,4 di-CH3), B-5 (3,4 di-CH3) and B-6 (2,5 di-CH3) showed IC_{50} values of 11.54, 6.26 and 14.98 µM respectively. From piperazine

derivatives compound B-11 (3-OCH_3) and compound B-16 (4-CH_3) showed better inhibition than gefitinib.

Overall by observing the IC_{50} values of all compounds in all the cell lines, it can be said that, compounds with electron donating substituent's showed better growth inhibition when compared to compounds with electron withdrawing groups.

5.4.3 4-(3-(4-ethylpiperazin-1-yl)propoxy)-*N*-phenylbenzamide (scaffold-IV) and (4-(3-(4-ethylpiperazin-1-yl)propoxy)phenyl)(4-(2-methoxyphenyl)piperazin-1-yl)methanone derivatives (scaffold-V):

IC_{50} values were calculated for all compounds and the results are reported in table 5.3.

Table 5.3: IC_{50} values of compounds of scaffold IV and V.

Compound code	R	Lung A-549 IC_{50} (µM)	Colon HCT-116 IC_{50} (µM)	Pancreatic MIAPaCa-2 IC_{50} (µM)
Aniline substituent's (scaffold-IV)				
C-1	H	41.80	74.76	41.18
C-2	4-CH_3	24.08	33.36	11.89
C-3	3-CH_3	>100	>100	>100
C-4	2,4 di-CH_3	33.20	11.33	>100
C-5	3,4 di-CH_3	21.22	45.89	55.08
C-6	2,5 di-CH_3	25.25	41.29	68.12
C-7	4-OCH_3	48.91	32.56	39.55
C-8	4-Cl	>100	>100	>100
C-9	4-Br	112.95	38.30	71.71
C-10	4-F	>100	59.58	84.18
Piperazine Substituent's (scaffold-V)				
C-11	3-OCH_3	>100	>100	>100
C-12	4-OCH_3	>100	>100	>100
C-13	2-Cl	NA	NA	NA

C-14	4-Cl	69.52	17.36	<1.0
C-15	2,3-diCl	>100	>100	>100
C-16	4-CH_3	>100	68.96	42.36
	Gefitinib	16.56	10.51	49.50

* IC_{50} values between 100-1000 µM has been shows as >100.

In this series also, in A-549 lung cancer cell line and HCT-116 colon cancer line, electron donating substitution showed better inhibition when compared to electron withdrawing substitution. In lung cancer cell line, most active compound was C-5 (3,4 di-CH_3) with IC_{50} of 21.22 µM. Among dimethyl substituted compounds, C-4 (2,4 di-CH_3) showed IC_{50} of 33.20 µM and C-6 (2,5 di-CH_3) showed inhibition with IC_{50} value of 25.25 µM. Among monomethylated compounds, Compound C-2 (4-CH_3) resulted in better inhibition compared to C-3 (4-CH_3).

In HCT-116 colon cancer line, compound C-2 (4-CH_3) resulted in better inhibition than C-3 (3-CH_3) and in disubstituted compounds C-4 (2,4 di-CH_3) showed comparable inhibition to that of gefitinib. Compounds C-5 (3,4 di-CH_3) and C-6 (2,5 di-CH_3) showed similar inhibition with IC_{50} values of 45.89 µM and 41.19 µM, respectively. Among piperazine derivatives, only active compound was C-14 (4-Cl), which has IC_{50} value of 17.36 µM.

In MIAPaCa-2 cell line, from aniline substituent's, compounds C-1 and C-2 showed better inhibition values than that of gefitinib. In disubstituted compounds, C-5 (3,4 di-CH_3) and C-6 (2,5 di-CH_3) resulted in comparable IC_{50} values to gefitinib. Compound C-2 (4-CH_3) also showed better IC_{50} value as compared to gefitinib. From piperazine series, compound C-14 (4-Cl) was the most active compound with IC_{50} value of less than 1µM. Compound C-16 (4-CH_3) also had inhibition values better than gefitinib.

5.4.4 4-[3-(morpholin-4-yl)propoxy]-*N*-phenylbenzamide derivative(scaffold-VI) and (4-(2-methoxyphenyl)piperazin-1-yl)(4-(3-morpholinopropoxy)phenyl)methanone derivatives (scaffold-VII)

IC_{50} values were calculated for all compounds and the results are reported in table -5.4

Table 5.4: IC_{50} values of compounds from scaffold- VI and VII.

Compound code	R	Lung A-549 IC_{50} (µM)	Colon HCT-116 IC_{50} (µM)	Pancreatic MIAPaCa-2 IC_{50} (µM)
Aniline substituent's (scaffold-VI)				
D-1	H	39.25	45.56	56.25
D-2	4-CH_3	34.81	63.12	63.12
D-3	3-CH_3	>100	>100	>100
D-4	2,4 di-CH_3	25.32	34.78	11.25
D-5	3,4 di-CH_3	61.09	16.64	>100
D-6	2,5 di-CH_3	45.20	35.41	36.51
D-7	4-OCH_3	36.23	45.58	69.25
D-8	4-Cl	50.57	59.56	65.45
D-9	4-Br	>100	>100	>100
D-10	4-F	99.86	30.48	65.34
D-11	2-Cl	NA	NA	NA
Piperazine substituent's (scaffold-VII)				
D-12	2-OCH_3	62.02	74.19	>100
D-13	3-OCH_3	78.45	68.12	>100
D-14	4-OCH_3	>100	41.37	22.72
D-15	2-Cl	NA	NA	NA
D-16	4-Cl	48.38	13.04	7.14
D-17	2,4-diCl	38.78	46.89	88.02

D-18	2-F	>100	>100	>100
D-19	4-CH_3	44.84	26.07	48.06
D-20	4-NO_2	>100	>100	>100
	Gefitinib	16.56	10.51	49.50

* IC_{50} values between 100-1000 µM has been shows as >100.

In A549 lung cancer cell line, among aniline derivatives, electron donating group substituted compounds showed better inhibition than compounds with electron withdrawing substituents. 2,4 di-CH_3 substituted compound D-4 resulted in better inhibition as compared to D-5 (3,4 di-CH_3) and D-6 (2,5 di-CH_3). D-7 (4-OCH_3) and D-2 (4-methyl) resulted in almost similar inhibition with IC_{50} values of 36.23 and 34.81 µM respectively. Among piperazine derivatives, electron withdrawing group substituted compounds D-16 (4-Cl) and D-17 (2,4-diCl) showed good inhibition but not comparable to gefitinib.

In HCT-116 colon cancer line, among aniline derivatives, anilines substituted with electron donating groups showed better inhibition. Among D-4 (2,4 di-CH_3), D-5 (3,4 di-CH_3) and D-6 (2,5 di-CH_3), D-5 showed better inhibition (16.64 µM) which is comparable to gefitinib. Compound with electron withdrawing group, D-10 (4-F) showed the IC_{50} values of 30.48 µM. In piparazine substituent's, among D-12 (2-OCH_3), D-13 (3-OCH_3) and D-14 (4-OCH_3), compound D-14 was the most active with IC_{50} value of 41.37 µM. Electron withdrawing group substituted compounds D-16 (4-Cl) and D-17 (2,4-diCl) resulted in IC_{50} value of 13.04 µM and 46.89 µM respectively.

In MIAPaCa-2 cell line, compound D-4 (2,4 di-CH_3) was most active with IC_{50} value of 11.25 µM. Compound D-6 (2,5 di-CH_3) also showed IC_{50} value of 36.51 µM, which is better than gefitinib. Electron withdrawing group substituted compounds D-8 (4-Cl) and D-10 (4-F) also resulted in similar inhibition. From among piperazine substituted compounds, compound D-14 (4-OCH_3) and D-16 (4-Cl) resulted in IC_{50} values of 22.72 µM and 7.14 µM respectively which were better than gefitinib.

Overall to conclude, electron withdrawing groups have resulted in the better activity in all cell lines and D-16 (4-Cl), a phenylpiperazine derivative, has also shown good activity in all three cell lines.