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Research Article

A novel combination therapy for treating breast cancer: Preclinical proof-of-concept

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ABSTRACT

Combining the anticancer drugs at their sub optimal doses with other non-cytotoxic agents possessing potential anticancer property is a promising strategy for effective anticancer treatment with reduced toxicity. In the present investigation we have evaluated combination of anticancer drugs SAHA (Vorinostat), and MS-275, Entinostat (histone deacetylase inhibitors HDACi), with non-cytotoxic drug sildenafil (SDFL), a Phosphodiesterases 5(PDE) inhibitor in xenograft models of breast cancer. The potential efficacies of PDEi in combination with HDACi in panel of cancer cell lines treated with SAHA or MS-275 in combination with SDFL to determine their impact on cell proliferation and in *in vivo* activities were assessed in human breast cancer xenograft model. Here in this study we have performed *in vitro* assay using cancer cell lines (HCT116 - Colon, PC3- Prostrate and MDAMB231- breast) followed by the study in human breast cancer xenograft animal models. A Panel of cancer cells treated with MS-275 and SDFL have shown enhanced anti-proliferative activity of the combination resulted in additive to synergism effect than compared to that of SAHA in combination with SDFL. Further, narrowed down the study with this combination to *in vivo* which revealed a significant inhibition ($p<0.001$) of tumor growth in severe combined immuno deficient mice bearing MDAMB-231, human breast cancer xenograft treated with the combination of low doses of SDFL and MS-275. The enhanced efficacy of combination therapy clearly demonstrates synergistic pharmacodynamics drug-drug interaction between PDE and HDAC inhibitors. Taken together, current study provided preclinical proof-of-concept for the novel, distinctive and enhanced anticancer activity of PDEi

Keywords: Breast Xenograft model; HDAC; MS-275; PDE; SAHA; SDFL

INTRODUCTION

HDACi which act by inhibiting a group of enzymes called histone deacetylase (HDACs) remove acetyl groups from histones and regulate expression of tumor suppressor genes, making them a promising therapeutic target for treatment of cancer (Bhatt S, *et al.,* 2013). Vorinostat (SAHA) a pan HDACi was approved for the treatment of cutaneous T-Cell Lymphoma (CTCL) and MS-275 a class I selective HDAC inhibitor, is currently in clinical trials for treating with solid tumors or lymphomas (Kato Y, *et al*. 2007). Though, HDAC inhibitors appear to be more effective, but they do carry some side effects like diarrhoea, thrombocytopenia, nausea and fatigue (Srividya S., *et al.,* 2010).

In order to overcome anticancer drug associated toxicity, these drugs can be combined with non-cytotoxic drug(s) which by virtue of their complementary and unique mechanism of action can enhance activity of anticancer drug by different key signal transduction pathways at their reduced doses more efficiently (Ilaria P., *et al.*, 2011)**.** One such promising approach for cancer therapy is modulation of the intracellular cyclic guanosine monophosphate (cGMP) where the impairment of which is been observed in various cancer pathologies (McEwan DG. *et al*., 2007). SDFL, a PDE5i in combination with other anticancer drugs was synergistic in different cancer suggesting that SDFL may play a broad role in promoting the activity of anticancer drugs. (Hirsh L *et al*., 2004). However, the studies have shown limited efficacy of PDE inhibitors in cancer therapy when used alone. Therefore combination of PDE inhibitors with chemotherapeutic agents may be a rational approach for the treatment of cancer (Zhi Shi, *et al*., 2011).

The variant expression of PDE and HDAC enzymes in different tumors offered to promising insight for the selective inhibition of these enzymes for combination therapy in cancer, which may allow lowering the dosage of HDACi, resulting into reduced side effects and increased anticancer activity. Hence in the current investigation, attempt has been made to evaluate the anti-tumor activity of PDEi in combination with HDACi both in *in vitro* and *in vivo* system. To the best of the

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Figure a: Combination study of SDFL with HDACi in HCT 116 cell line for 48h

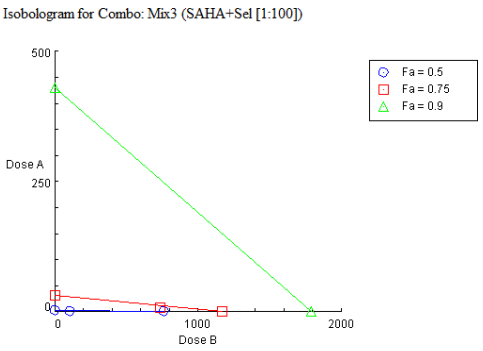


Figure A: SDFL + SAHA

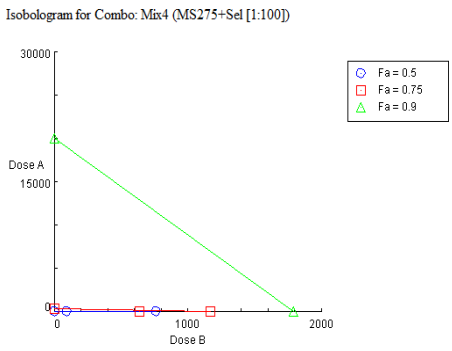


Figure B: SDFL + MS275

Figure b: Combination study of SDFL with HDACi in MDAMB231 cell line for 48h

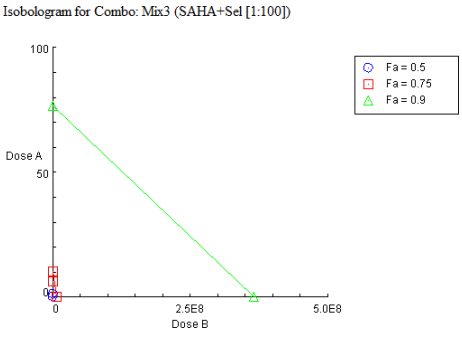


Figure A: SDFL + SAHA

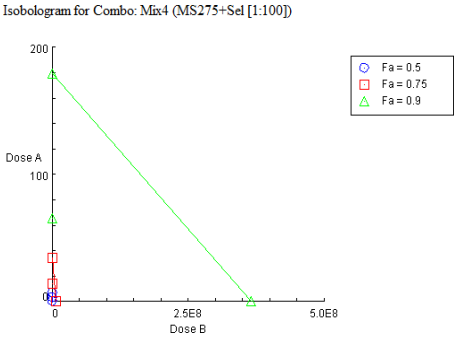


Figure B: SDFL + MS275

Figure C: Combination study of SDFL with HDACi in PC3 cell line for 48h

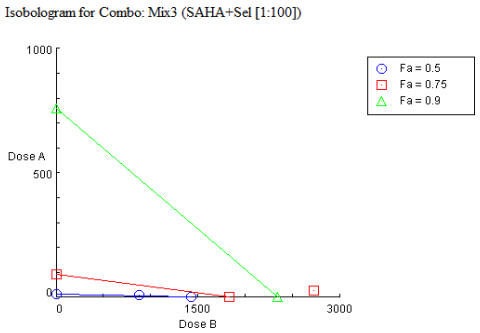


Figure A: SDFL + SAHA

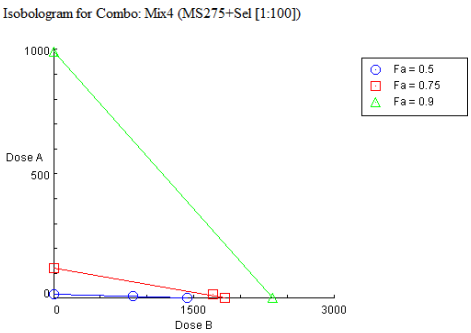


Figure B: SDFL + MS275

Figure 1 a-c: The combination of low concentrations of PPDEi with HDACi was much more potent in synergistically inhibiting the growth of MDAMB231, HCT116 and PC3 cells compared with treatment using each single agent.

Table 1: Effect of SDFL alone or in combination with HDAC inhibitors in a panel of cancer cell line – cell viability by SRB method

Drug A	Drug B	Cell lines	CI Values				
			Combo Ratio	ED50	ED75	ED90	ED95
Sildenafil	SAHA	HCT116	1:100	0.61	0.87	3.00	8.16
		MIDAMB231	1:100	0.30	0.58	10.02	68.92
		PC3	1:100	1.42	1.79	3.70	6.59
	MS275	HCT116	1:100	0.26	0.56	2.36	6.38
		MIDAMB231	1:100	0.45	0.40	0.36	0.34
		PC3	1:100	1.18	1.07	1.48	1.98

Table 2: Effect of SDFL and MS-275 on biochemical and haematological parameters of SCID mice bearing MDAMB-231 xenografts

	Vehicle Control	MS-275 - 35 mg/kg p.o.	MS-275- 5 mg/kg p.o.	SDFL - 50 mg/kg i.p.	MS 275 +SDFL 5 mg/kg p.o.+ 50 mg/kg i.p.
Urea (mg/dl)	61.6 ±7.37	57.00±6.66	54.00 ±5.55	60.17 ±7.33	59.29±10.26
Creatinine (mg/dl)	0.54 ±0.05	0.38 ±0.18	0.44 ±0.07	0.48 ±0.12	0.51 ±0.12
Protein (g/dl)	6.16 ±0.31	5.65 ±0.32	5.89 ±0.46	5.22 ±0.12	5.74±0.30
Calcium (mg/dl)	9.80 ±1.06	9.62 ±0.33	9.87 ±1.22	10.67 ±1.50	9.48±0.41
Bilirubin (mg/dl)	0.57 ±0.05	0.50 ±0.30	0.59 ±0.14	0.49 ±0.07	0.49±0.08
GOT (IU/L)	69.10±14.08	71.45 ±14.97	76.72 ±10	73.83±39.96	83.14 ±14
GPT (IU/L)	59.88±6.39	57.10±12.91	58.53±6.08	58.82±19.19	56.04±8.43
ALP (IU/L)	51.00±8.63	49.33±5.05	48.83 ±10.55	49.67±8.33	49.70±6.75
WBC (103/µl)	2.80±0.33	3.02±0.49	2.66±0.76	2.7±0.68	3.16±1.02
RBC (106/µl)	8.36 ±1.00	9.52±0.64	9.08±0.98	9.3±0.65	8.27 ±0.70
HGB (g/dl)	12.57±1.39	14.35±0.84	13.66±1.28	12.99±0.66	12.54±0.66
HCT (%)	40.95±5.50	46.61±3.24	44.57±4.68	46.7±2.93	42.24±2.98
MCV (fl)	48.93±1.11	48.98±1.21	49.11±0.59	50.17±1.24	50.66±1.52
MCH (pg)	15.05±0.23	15.11±0.46	15.07±0.42	14.98±0.53	15.34±0.70
MCHC (g/dl)	30.75±0.91	30.08±0.74	30.70±0.60	29.71±0.46	29.76±0.61
PLT (103/ml)	1812.50±322.82	**$$**1024.67±124.67	1503±190.99	1684.71±247.27	1585.86±322.42
LYM%	46.37±11.29	52.7±4.74	55.71±6.14	55.31±5.56	54.86±4.86
MXD%	12.50±3.48	12.45±0.56	11.81±1.79	12.81±1.13	12.74±1.89
NEUT%	41.13±9.94	34.85±4.34	38.47±5.09	32.17±5.20	36.03±4.77

Values are expressed as mean±SD (n=5-7). $$P<0.01 as compared to normal + vehicle, one-way ANOVA followed by Dunnets test

knowledge none have evaluated the effects of combination therapy of PDEi with HDACi in cancer models.

MATERIALS AND METHODS

Cell culture and toxicity: All the cell lines were procured from ATCC and were cultured as mentioned by ATCC. Sulphorhodamine-B assay was performed to determine the cellular growth and viability in combination (Vanich Vichai, 2006). Synergistic action of the HDACi and PDEi was studied by calculating Combination index (CI) using compusyn software.

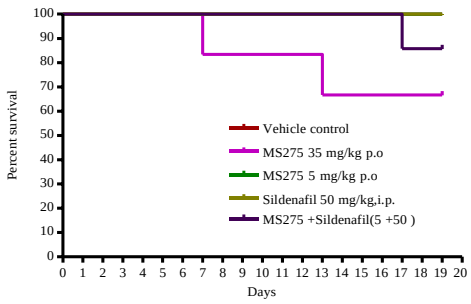
***In vivo* Anti-Tumor Efficacy Study:** Female ICR SCID mice from Taconic aged 5-6 weeks and body weight ranges 22-24 g was used to develop MDAMB231 xenograft model. The animals were grouped based on tumor volume and divided into different groups and treated with SDFL (50 mg/kg i.p) and MS-275(35 mg/kg p.o.;5 mg/kg p.o. bid) (Aninditha Dasa *et al.,* 2010) alone and in combination for 19 days. Tumor volumes were measured twice weekly and body weight recorded daily. The animals were sacrificed on day of termination, and analyzed for blood parameters followed by histopathological changes.

Statistical Analysis: The values are expressed as Mean ± SE. The statistical analysis was under taken using One-way or Two-way ANOVA with Bonferroni or Dunnett's posttest. The results were considered significant when $p<0.05$

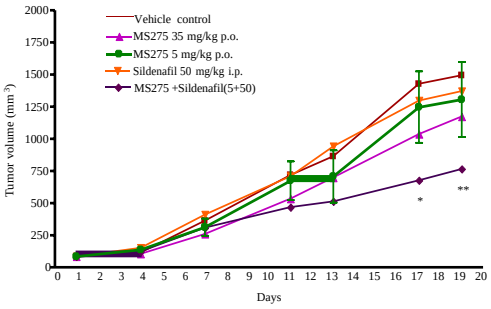
EXPERIMENTAL RESULTS

Effect of PDEi alone or in combination with HDACi, on a panel of cancer cell line

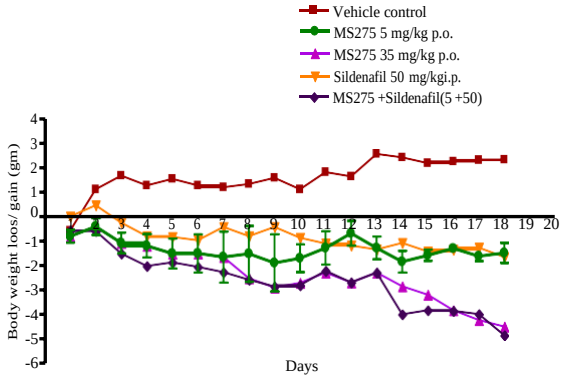
The combined effect of SDFL and HDACi on the cell viability of panel of cell lines was studied using SRB assay which showed that SDFL, combined with SAHA and MS275 in MDAMB231 cells, the CI was 0.6 to 8.1 and 0.2 to 6.3 exerting a strong to moderate synergism at ED50 and ED75 but mild to strong antagonism at ED90 and ED95. When we analyzed the synergistic, additive or antagonistic results of combination of SDFL with HDAC inhibitors, (Figure 1a-c) we found that the combination of low concentrations of SDFL with HDAC inhibitors was much more potent in synergistically inhibiting the growth of MDAMB231 cells compared with treatment using each single agent. However strong antagonistic effect was observed in HCT116 and PC3 cells, Although the cell lines exhibited varied sensitivity



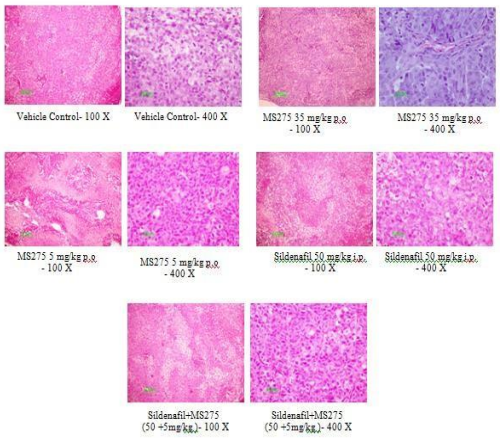
a) Survival Data



b) Tumor growth inhibition



c) Body weight Changes



d) Histopathological findings

Figure 2: Effect of SDFL in combination with MS275 in MDAMB-231 Xenograft model

to the drug combination, consistent additive to synergistic interactions was observed in SDFL when treated with SAHA and MS275 at over all drug concentrations tested in MDAMB231. With minor exception aside, the calculated CI values indicate synergy between the 2 drugs *in vitro* with a common trend towards synergy at lower combination concentrations for the cell lines HCT116, MDAMB 231.

Effect of SDFL in combination with MS275 in MDAMB231 (Breast) xenograft model

We further investigated the therapeutic potential of both SDFL and MS275, in MDAMB231 xenografts in SCID mice. On the day of termination, the mean tumour sizes were 1494.5 ± 98.3 SEM mm3, 1173.7 ± 292 SEM mm3, and 1304.7 ± 292 SEM mm3 1371.1 ±298.3 SEM in mice receiving control, MS275 at 35 and 5 mg /kg p.o, Sildenafil at 50mg/kg i.p. alone treatments, respectively, whereas the mean tumour size in mice receiving the combination of SDFL and MS275 at its low dose was 764.3 ± 80.1 SEM mm^3 (Figure 2b). Statistical analysis (TWO WAY ANNOVA) revealed significantly smaller tumours in the group of mice receiving the combination treatment than in the other treatment modality alone ($P<0.001$ for comparison to Vehicle control). Though the combination of SDFL and MS275 was well tolerated in SCID mice bearing MDAMB231 tumours, body weight loss was observed across the treatment group compared to that of the vehicle treated group however not significant. It was observed that MS275 have induced high rate of mortality at its 35 mg/kg alone and though not significant but mortality was also observed to an extent in combination treatment groups of animals bearing MDAMB231 tumors compared to that of vehicle control.

Tumours excised from euthanized animals from each treatment group were assessed for morphology by hematoxylin and eosin staining, for the treatment effects. The MDAMB231 cell morphological xenograft features were confirmed by its tubular pattern, secretory vacuolations and high mitotic index. Histopathologic examination of the xenografts demonstrated abundant mitotic figures, pleomorphic tumour cell populations. The difference in necrosis vs tumour vol/wt could be due to cell shrinkage in combination group and tumour growth kinetics in individual treatment groups. These histopathological analyses shows that the combined treatment effects of SDFL and MS275 are due in large part in the inhibition of tumour cell proliferation suggesting that tumour reduction was evident to the combination group.

DISCUSSION

Despite many advances in the field of cancer therapeutics, cancer continues to be a leading cause of death, which is in part due to the failure of chemotherapy. Clinically as most of the chemotherapeutics drugs possess, HDACi also have shown side effects. One of the approaches to minimize/mitigate treatment related side effects is to combine the above mentioned cyto-

toxic HDACi with a non-cytotoxic drug to decrease the toxicities associated with treatment and may be active against a broader range of tumour types. In the present study, we evaluated the growth-inhibitory effects of PDEi SDFL in combination with HDACi in MDAMB-231, PC3 and HCT116 carcinoma cell lines upon a trend of higher HDAC and PDE expression was observed in the advanced stage of disease

Our data on combination of HDACi and PDEi has shown the spectrum of growth inhibitory activity in human cancer cell lines *in vitro.* SDFL when combined with

MS275, the Combination index exerted a strong synergism to additive effect across all effective dose levels in MDAMB231 cell line. We found that PDE and HDAC interacted cooperatively to induce cell death in MDAMB-231.

Among the three cell lines tested, we selected MDAMB-231 for further investigation *in vivo* for MS275 and SDFL combinations (transgenic - SCID mice for Xenograft models).

In vivo studies confirmed the *in vitro* results and showed that SDFL (50 mg/kg i.p.) in combination with MS-275 (5 mg/kg p.o.bid) is very effective in suppressing MDAMB-231 tumour growth respectively. It is important to point out that although the combination is additive *in vivo* at the doses used, higher doses would produce a synergistic effect but with expected toxicities (Sabnis GJ*, et al.,* 2013). Co treatment of MS-275 and SDFL in human breast cancer xenograft was effective in inhibiting tumour growth. Though, body weight loss and other clinical signs observed, was not significant in the combination group. However, the mortality observed in combination treatment group needs to be addressed. This study was well supported by histopathological findings treatment effect on reduced mitotic figures (Suzuki T, *et al.,* 2007).

SUMMARY, CONCLUSION AND RECOMMENDATIONS

Taken together, current investigation provides preclinical proof-of-concept for the novel combination therapy of HDACi and PDEi in breast cancer model for the ongoing challenge to identify combinations of compounds that will broaden the efficacy of existing anticancer drugs like HDACi. Based on the earlier reports our work confirms that the combination indeed has greater effects on cell viability than does either compound alone at their lower doses confronting to better efficacy. Drug treatment combinations in xenograft models were very well tolerated, as no significant animal weight loss was observed with significant inhibition in tumor volume between the treatment groups when compared to vehicle control. Our observation suggests that the combination of PDEi with HDACi would be a novel potent antitumor treatment**.**

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