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Comparative Cytotoxicity of Standard of Care Treatment Drugs on Human Breast, Prostate, Colon, Cervical and Hepatic Cancer cell lines

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Abstract - Currently there are various standard of care drugs used for the treatment of various cancers. Understanding effectiveness of standard treatment over a broad range of cancer cell lines helps in selecting the most appropriate drug for a particular cancer type.

We have performed a comprehensive study for seven standard of care drugs namely Cisplatin, Sunitinib, Carboplatin, Doxorubicin, 5-Fluorouracil, Tamoxifen and Paclitaxel over a panel of Breast (MDAMB231, MDAMB468, T47D), Prostate (DU145, PC3, LNCaP), Colon (COLO205, COLO320DM, HCT-15), Cervical (SiHa, HeLa) and Hepatic (HEP3B) Cancer cell lines for anticancer (tested by MTT Assay) and anticancer stem cell activity (tested by Sphere Assay and Wound Healing Assay, WHA).Our MTT results have indicated that Doxorubicin and Sunitinib are highly potent drugs exhibiting highest activity on SiHa {IC50- 0.24uM (±0.28)} and COLO205 {IC50 -2.64uM (±0.09)}. Paclitaxel also demonstrated very high activity in MTT assay for all cancer types' especially high potency on colon: COLO205 {IC50- 0.045uM (±0.03)}, COLO320DM {IC50- 0.058uM (±0.01)}, HCT15 {IC50- 0.007uM (±0.4)}.

Interestingly, these three drugs have also demonstrated very good anti-CSC activity as indicated by ≥ 50% sphere reduction at 250nM for all cancer types and have exhibited remarkable reduction in growth (<30%) at scratch in WHA at the end of 48hrs.Overall, our findings suggest that Doxorubicin, Sunitinib and Paclitaxel have very high anticancer stem cell potential thereby qualifying to be used in combination therapy regimes.

Keywords **-** MTT, Sphere Assay, Wound Healing Assay, Anticancer activity, Anticancer stem cell activity, Standard of care drugs.

I. INTRODUCTION

Cancer involves rapid cell division without control and differentiation [1]. When there is a triggering of cell to divide and grow out of control it results in a mass or tumour called cancer [2]. The medicines used to treat cancer are called chemotherapeutic drugs [3] and treatment is called chemotherapy [4].Chemotherapy attacks dividing cells and thus it is more likely to kill cancer cells than normal cells. Some normal cells in our body such as hair and skin divide frequently [5]. These cells are also killed by chemotherapy and thus hair loss is the major side-effect of most of the chemotherapeutic regimes [6]. Chemotherapy may result in other side effects like digestive issues, vomiting, severe weakness etc. [7].

In order to minimize the side-effects of chemotherapy, chemotherapeutic agents effective at lower dose can be selected. Most of the chemotherapeutic drugs are used for a wide range of cancers [8] and understanding the effectiveness of these drugs over a range of cancer types gives better treatment options to an oncologist.

In our study, we report comparative cytotoxicity of seven standard of care treatment drugs namely, Cisplatin, Sunitinib, Carboplatin, Doxorubicin, 5-Fluorouracil, Tamoxifen and Paclitaxel over a panel of Breast (MDAMB231, MDAMB468, T47D), Prostate (DU145, PC3, LNCaP), Colon (COLO205, COLO320DM, HCT-15), Cervical (SiHa, HeLa) and Hepatic (HEP3B) Cancer cell lines. Though there are studies reported on the activity of these drugs for a few cell lines [9, 10, 11], such an extensive comparative study is not reported till date. In order to have a comparative anticancer efficacy we have performed MTT assay. Further to understand the anti-cancer stem cell effect of these standards of care treatment drugs we have performed Sphere assay and Wound Healing Assay (WHA).

In the current study, section I comprises the detailed methodology used for MTT assay. In this assay, the efficiency of the "study drug" was determined by calculating IC50 values (i.e. the concentration at which a drug kills 50% of cancer cells). A drug with higher potency will have a lower IC50 value. Using MTT assay, we have done an extensive comparative study of IC50 values for all the study drugs.

In section II, we have done an in depth explanation of sphere assay. Sphere assay measures the potential of cancer stem cells to establish in specially designed media. We have used this assay to understand the comparative anti CSC effect of the "study drugs" across the cancer cell lines.

In section III, we have described the wound healing assay. The wound healing assay determines the potential of CSC to close the wound formed in the confluent monolayer. We have used this assay to measure the ability of the "study drugs" to inhibit cancer stem cells.

While designing a treatment regimen an oncologist tries to select an effective drug required in low doses thereby minimizing the side-effects of the drug. Thus our extensive study can help an oncologist to select the most efficacious drug for the particular type of cancer.

II. RELATED WORK AND RESEARCH CONTRIBUTION

Authors have been extensively working on screening and development of small molecules exhibiting anticancer and anticancer stem cell activity. They have filed patents for some novel molecules and their research work is published in peer-reviewed international journals. One of the novel lead molecules is in the stage of IND filing.

III. METHODOLOGY

All the cancer cell lines used for the study were procured from NCCS Pune. The study was performed from June 2018 to June 2019.

MTT Assay

The standard of care drugs used in our study were initially screened for their anticancer activity by MTT assay. This assay is a simple and sensitive assay wherein the metabolic activity of a cell is measured using MTT dye. The increase in metabolic activity is taken as a parameter of cell growth [12]. The protocol for this assay was followed as described by us earlier [13]. Briefly, the plating efficiencies for each cancer cell line were initially determined as highlighted in Table 1. The cells 200ul with the desired plating efficiency were plated in 96 well adherent plates with respective media as shown in Table 2. The cell culture media was made complete by adding 10% FBS (Hi Media Catalog no.RM1112) with appropriate antibiotic. The cell culture plate seeded with respective cell lines was then incubated at 37^0C for one day in 5% CO_2. Different dilutions of standard of care drugs ranging from 100uM to 0.1uM prepared in DMSO. Appropriate controls like Growth, Solvent and Media Control were also run with each experiment. The experiment was performed in six data points. Post drug addition the plates were re-incubated for 48 hours under similar conditions and then the assay was terminated by centrifuging the plates at 3000rpm for 3 minutes and supernatant was discarded .100ul of MTT (5mg/ml) solution prepared in respective cell culture media was then added to the plates which were then further incubated. In the incubation period there was a metabolic reduction of MTT to a blue insoluble product by viable cells. This blue color was measured spectrophotometrically at 570nm. Percent viable cells were calculated (minus the background error) and plotted on to the graph. For determining IC50 value (concentration at which drug kills 50% of cells). An X-Y graph of log% dead v/s log concentration was plotted and calculation of IC50 value was done by using regression analysis.

Sphere Assay

Spheres or spheroids, enriched from cancer cell lines in specially defined serum-free media represent CSC [14].
Preliminary sphere assay was performed for the study drugs in the concentration range of 25uM to 2.5nM. Since most of the drugs exhibited activity at 250nm, this concentration was selected for comparing activity of drugs and the experiment was repeated (n=6) for confirmation.
Briefly the sphere assay was performed as follows, the trypsinized cells at concentration of 2000 cells/100ul were passed through cell strainers of 100& 40micron to obtained a single cell suspension and were suspended in appropriate media like for breast cancer cell lines-Mammosphere, for prostate cancer cell lines- Prostosphere or for colon, hepatic and cervical cancer cell lines- DMEM: F12 was used. The cell suspension was then added to the respective wells of suspension plate & incubated at 37^0C for 24 hours. The first feeding involved an addition of 2ul drug into respective wells with 100ul of the culture medium. Plates were incubated at 37^0C, 5% CO_2 for 72 hours. A second drug addition involved 2.5ul of the respective drug with 50ul of culture medium and plates were re-incubated for 72 hours under similar conditions. The third drug addition was addition of 3ul of the respective drug with 50ul of culture medium and plates were incubated for 24 hours. At the end of the experiment, the spheres formed for each concentration were counted. % viability was then estimated.

% viability = Number of spheres formed at particular concentration / Number of spheres obtained in control * 100,
where Control refers to Growth control with DMSO. Other appropriate controls like GC (Growth Control - only cells and media) and MC (Media Control) were also kept during the experiment.

Wound Healing Assay (WHA)

WHA or Scratch assays are used to check the anticancer stem cell effect of a drug [15]. To understand the effect of our study drugs on the CSC population we performed WHA as described earlier [16].

Briefly, $6*10^5$ cells of MDAMB231, PC3, Hep3B and HeLa were plated per well of 6 well adherent tissue culture plates. These cell lines were selected because they formed a confluent monolayer without a gap in between which is a prerequisite of WHA. A scratch at 0 hours was made on the confluent monolayer using a sterile 200ul tip. Cells were washed twice with DPBS (Hi Media, Cat No. PL1006). The width of the wound created was recorded immediately with IS Capture Software. The wells were then supplemented with assay medium. IC10 concentrations of each drug (calculations from MTT assay) were added for each cell line in duplicates. Dilutions of drugs were prepared in DMSO and suitable experimental controls were set up during the experiment for each cell line. The experimental plates were incubated at 37^0C, 5% CO_2 and width of scratch was measured at 24 and 48 hours respectively.

IV. RESULTS AND DISCUSSION

Chemotherapeutic drugs are used for different types of cancers [17]. Understanding the effectiveness of a standard of care drug in *in-vitro* assays helps in designing the drug therapy regime for a particular type of cancer.

We have performed an extensive study of the standard of care drugs Cisplatin, Sunitinib, Carboplatin, Doxorubicin, 5-Fluorouracil, Tamoxifen, Paclitaxel on various types f cell lines viz, Breast, Prostate, Colon, Hepatic and Cervical.

It can be seen in Table III that different standards of care drugs are exhibiting varying potencies in various cell lines tested. The potency of a compound is calculated by its IC50 value i.e. the concentration at which the compound is able to inhibit 50% of cancer cells. Thus, a compound with a lower IC50 value indicates higher potency as it would be needed in a very small amount to inhibit 50% of the cancer cell population. Comparing IC50 values thus help us to select a most potent drug for a particular type of cancer.

A close observation of Table 3 indicates that Doxorubicin is exhibiting very high potency on breast cancer cell lines MDAMB231 and MDAMB468. Both MDAMB231 and MDAMB468 are very aggressive breast cancer cell lines. Currently Doxorubicin has been frequently used treatment for triple negative breast cancer TNBC [18]. Also it does not work very effectively on ER+ve cancers as is reflected by its IC50 value on T47D which is ER+ve cell line. Similarly, Sunitinib and Paclitaxel are also exhibiting good activity on breast cancer cell lines and thus are a part of the therapy regime [19]. 5-Fluorouracil has exhibited least activity among all the study drugs for breast cancer. 5-Fluorouracil is an antimetabolite and generally not a choice for breast cancer [20]. Paclitaxel and Sunitinib are very effective on prostate cancer cell lines; our finding is in agreement with current therapy regime for prostate cancer treatment [21]. Again our results indicate that 5-Fluorouracil is not very effective on prostate cancer, which is in agreement with the published results [22]. In the case of colon cancer also these drugs have proved to be highly effective. This is reflected in their use as combination treatment in multidrug resistant colon cancer [23]. Similarly Paclitaxel and Doxorubicin have also proved to be very effective in cervical cancer [24]. From our results, Doxorubicin is indicated as the most effective drug for hepatic cancer, most of the time it is used in combination regime [25].

Sphere assays are used to understand the effect of an agent to inhibit sphere formation [26]. CSC's have the ability to form spheres when cultured in a serum free condition [27]. We have used this assay to understand the comparative activity of standard of care drugs on prostospheres, mammospheres, colonospheres, cervical spheres and hepatospheres (Figure 6). Figure 1 indicates the comparative activity of standard of care drugs on prostospheres at 250nm. It can be clearly observed that Sunitinib, Doxorubicin, Tamoxifen and Paclitaxel are able to inhibit 50% of prostospheres at 250nM. In fact in our MTT assay Tamoxifen has not indicated very high potency, this suggests that it specifically acts on CSC's and can be used in the combination regime. Similarly the activity of standard of care drugs on mammospheres (Figure 2), Sunitinib and Doxorubicin are exhibiting very high activity on mammospheres indicating their activity on breast CSC's. In case of colonospheres and cervical spheres also (Figure 3 & 4) it can be seen that Sunitinib and Tamoxifen are more active compared to other standard of care drugs. In case of hepatospheres also (Figure 5) it can be seen that Sunitinib is the most active standard of care drug inhibiting almost 50% spheres at 250nM.

Within the tumour CSC are present a very small population which is eventually responsible for tumour growth & metastasis. The CSC's have unique physiological characteristics which make them treatment resistant and metastatic. Wound Healing Assay reflects the stemness of cancer cells [28] and therefore to further understand the effect of these "study drugs" on the CSC population we further performed WHA.

Figure 7 indicates the comparative activity of standard of care drugs in WHA assay. As it can be clearly seen that Sunitinib and Doxorubicin are most effective in inhibiting % growth at the scratch compared to other "study drugs" again indicating their higher anti-CSC effect. The behavior of Paclitaxel was remarkable in WHA. At the end of 48 hours, the cells were dead and dispersed for all the four cell lines i.e. MDAMB231, PC3, Hep3B and HeLa indicating its striking high anti CSC effect (Figure 8).

Table 1: Plating efficiency of cell lines for MTT assay

Sr. No.	Cell line	Origin	Cell count (Per 200 ul)
1	MDAMB231	Breast	10000
2	MDAMB468		15000
3	T47D		20000
4	DU145	Prostate	5000
5	PC3		10000
6	LNCaP		10000
7	COLO205	Colon	20000
8	COLO320DM		15000
9	HCT-15		15000
10	SiHa	Cervical	10000
11	HeLa		5000
12	Hep3B	Hepatic	10000

Table 1 indicates the standardized plating efficiencies of the various cancer cell lines used in the MTT assay.

Table 2: Cell culture media used for each cell line along with catalog number

Sr. No.	Cell line	Origin	Medium (1X complete)	Catalogue Number
1	MDAMB231	Breast	DMEM	Himedia, AL006
2	MDAMB468		DMEM	Himedia, AL006
3	T47D		RPMI 1640 with 25mM Hepes	Himedia, AL028
4	DU145	Prostate	DMEM	Himedia, AL006
5	PC3		RPMI 1640	Himedia, AL028
6	LNCaP		RPMI 1640	Himedia, AL028
7	COLO205	Colon	RPMI 1640 with 10mM Hepes	Himedia, AL028
8	COLO320DM		RPMI 1640	Himedia, AL028
9	HCT-15		RPMI 1640	Himedia, AL028
10	SiHa	Cervical	MEM	Himedia, AL047
11	HeLa		RPMI 1640	Himedia, AL028
12	Hep3B	Hepatic	MEM	Himedia, AL047

Table 2 indicates the different media used for the cancer cell lines

Table 3: IC50 values of standard of care drugs (in uM) on different cell lines in MTT assay

Cell lines		Cisplatin	Sunitinib	Carboplatin	Doxorubicin	5Fluoro uracil	Tamoxifen	Paclitaxel
Breast	MDAMB 231	28.77(± 1.87)	2.99(± 0.03)	277.33(±3.61)	0.3(± 0.07)	1000*	37.1(± 0.02)	4.54(± 0.72)
	MDAMB 468	24.15(±1.64)	3.05(± 0.24)	61.38(± 4.5)	0.807(± 1.07)	1000*	14.48(± 1.98)	4.6(± 3.25)
	T47D	39.63(±0.52)	2.9(± 0.81)	266.69 (±1.73)	13.93(± 3.7)	1000*	40.49(± 1.44)	3.6(± 0.63)
Prostate	DU145	31.41 (±1.02)	3.17(± 0.09)	6.85(± 0.78)	1.35(± 0.08)	268.9(±5.19)	6.02(± 0.11)	0.22(± 0.04)
	PC3	30.55(± 5.3)	3.04(± 0.3)	41.21(± 4.59)	8.3(± 4.08)	319.5(±3.21)	35.97(± 4.84)	4.53(± 1.9)
	LNCaP	20.41(± 0.3)	3.38(± 0.1)	148.94(±3.52)	2.27(± 0.08)	340.27(±4.12)	66.44(± 2.79)	3.35(± 0.5)
Colon	COLO 205	26.73(±0.04)	2.64(± 0.09)	61.09(± 0.83)	0.29(± 0.11)	57.89(±5.27)	92.29(± 3.73)	0.045(±0.03)
	COLO 320DM	59.3(± 0.09)	3.03(± 0.09)	109.55(±2.14)	3.94(± 0.04)	326.92(±0.53)	31.2(± 5.1)	0.058(±0.01)
	HCT-15	18.24(±0.09)	2.81(± 0.09)	68.23(± 2.75)	0.63(± 0.05)	141.41(±0.79)	33.24(± 1.16)	0.007(± 0.4)
Cervical	SiHa	17.95(±1.58)	2.97(± 0.28)	54.3(± 5.15)	0.24(±0.28)	1000*	42.34(± 4.15)	0.119(±1.52)
	HeLa	32.14(±5.46)	2.79(± 0.2)	16.22(±1.15)	1(±0.8)	337.6(±3.11)	36.97(± 1.54)	0.003(±0.04)
Hepatic	Hep3B	25.64(±0.75)	2.99(± 0.14)	8.58(±1.18)	0.25(±0.15)	385.48(±1.29)	6.304(± 0.08)	0.65(±0.86)

Table 3 indicates the IC50 values in uM for Breast (MDAMB231, MDAMB468, T47D), Prostate (DU145, PC3, LNCaP), Colon (COLO205, COLO320DM, HCT-15), Cervical (SiHa, HeLa) and Hepatic (Hep3B).
1000* indicates the maximum concentration tested in assay and still the activity of the drug was not found.

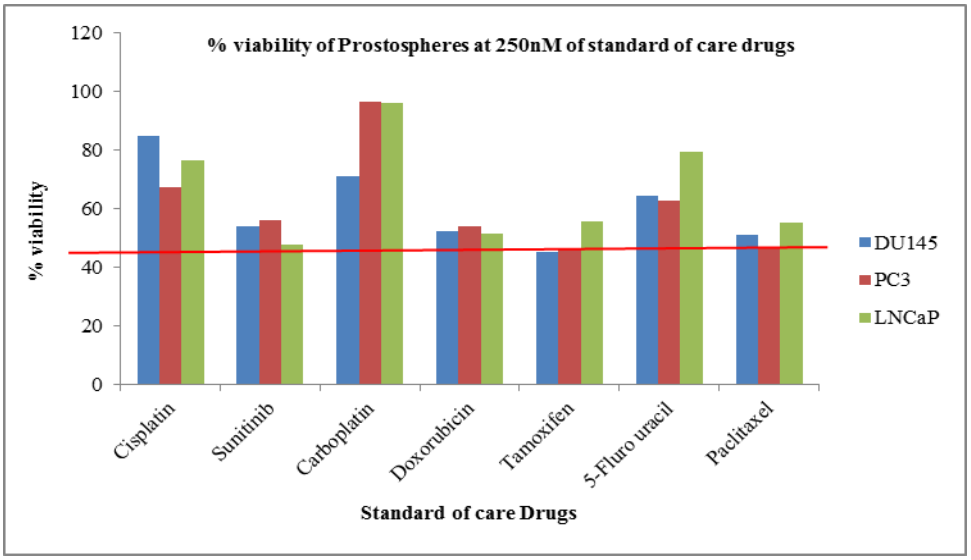


Figure 1: Comparative activity of standard of care drugs on prostospheres at 250nM

Figure 1 indicates that Sunitinib, Doxorubicin, Tamoxifen and Paclitaxel have better potential to indicate prostospheres compared to other standard of care drugs.

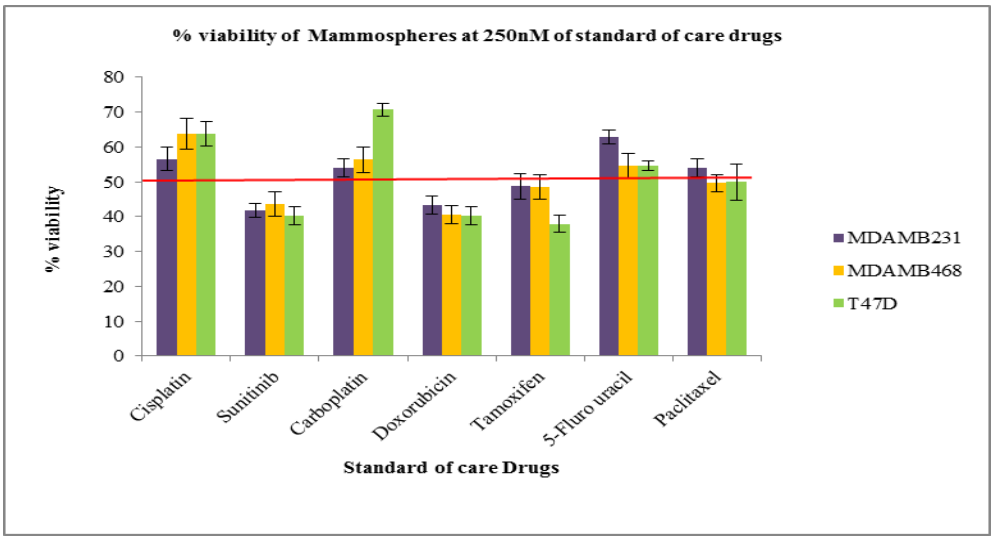


Figure 2: Comparative activity of standard of care drugs on mammospheres at 250nM

Figure 2 indicates that Sunitinib and Doxorubicin are highly active on mammospheres at 250nM compared to other standard of care drugs.

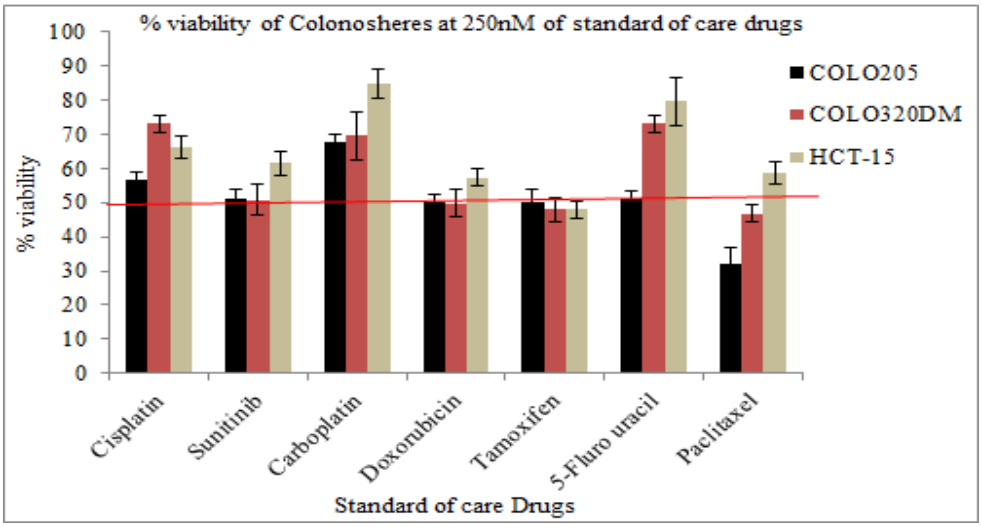


Figure 3: Comparative activity of standard of care drugs on colonospheres at 250nM

Figure 3 indicates that Sunitinib, Doxorubicin and Tamoxifen are able to inhibit colonospheres effectively at 250nM. Paclitaxel is able to inhibit colonospheres of only COLO205 and COLO32DM.

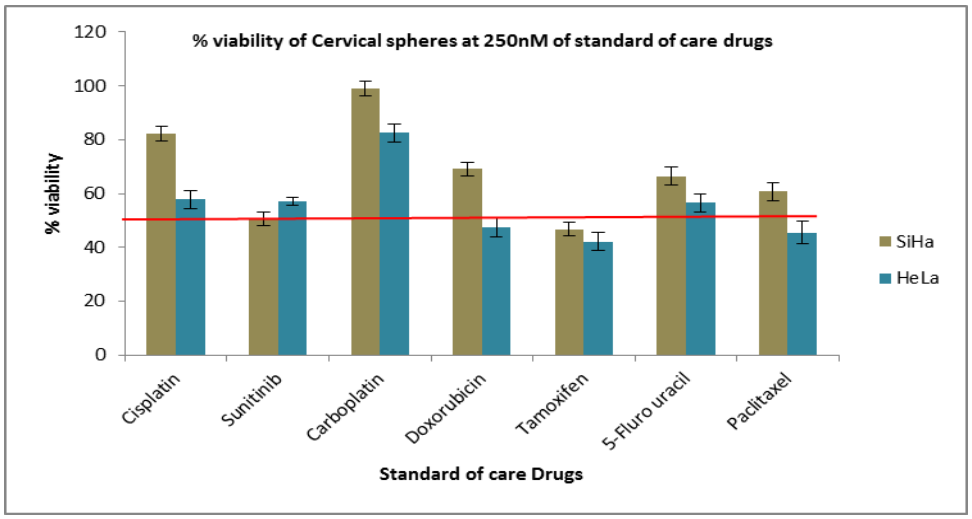


Figure 4: Comparative activity of standard of care drugs on cervical spheres at 250nM

Figure 4 indicates that Sunitinib and Tamoxifen are able to inhibit cervical spheres at 250nM compared to other standard of care drugs.

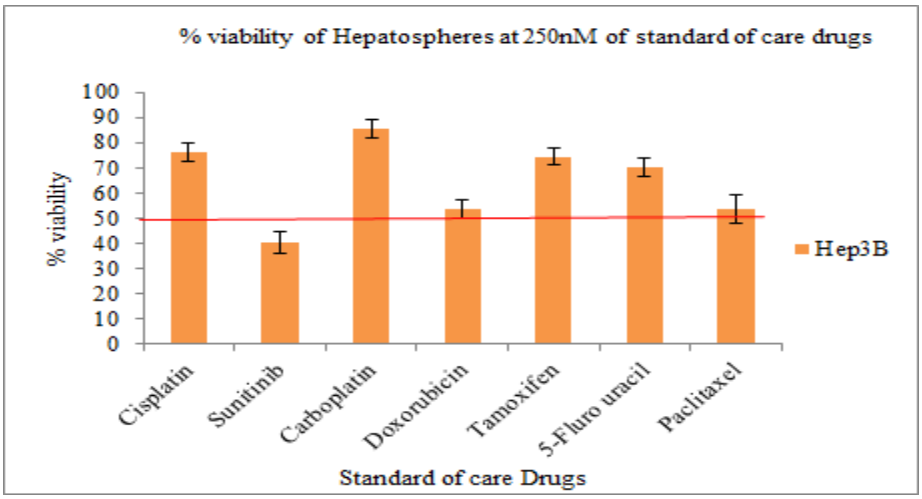
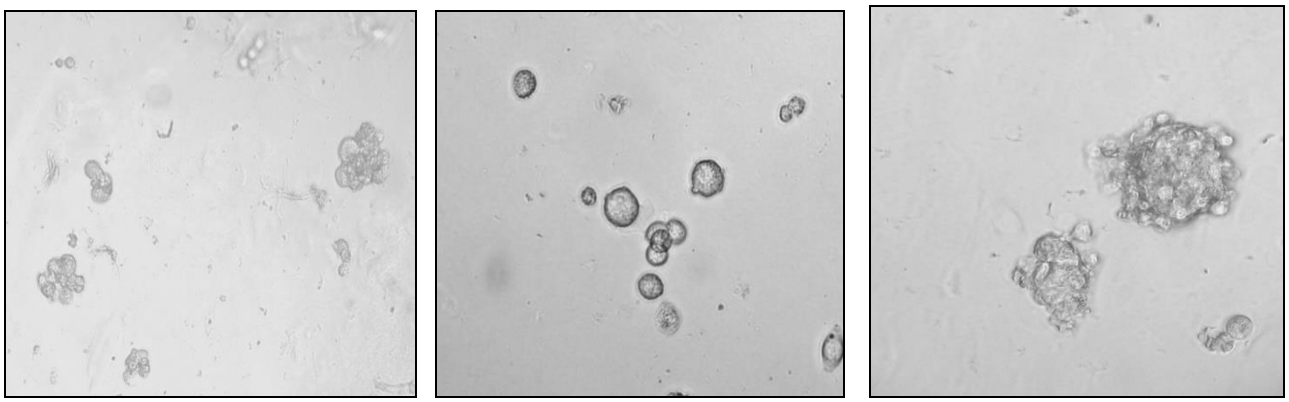


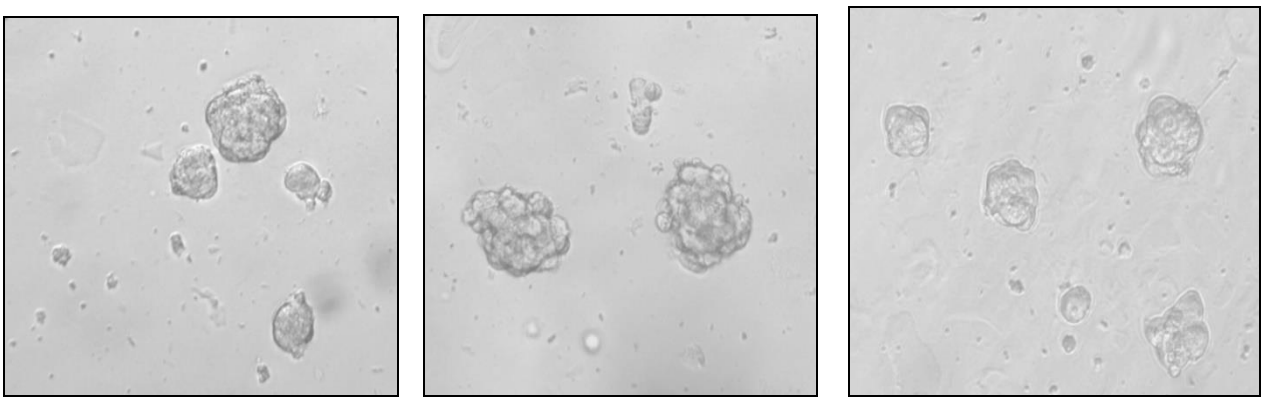
Figure 5: Comparative activity of standard of care drugs on hepatospheres at 250nM

Figure 5 indicates that Sunitinib is highly active on hepatospheres compared to other standard of care drugs at 250nM.



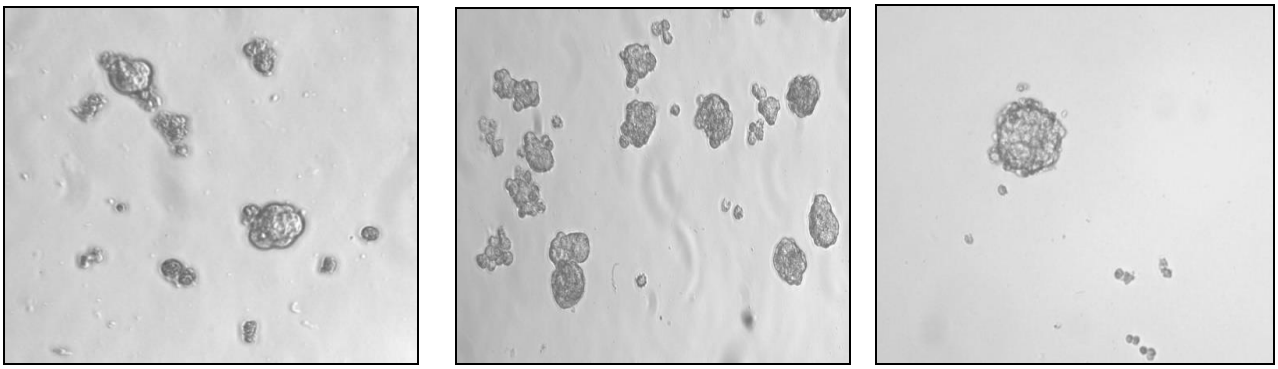
MDAMB231 MDAMB468 T47D

Figure 6 (a) indicate mammospheres of MDAMB231, MDAMB468 and T47D formed on day 9 in sphere assay (taken at 20X magnification by inverted microscope)



DU145 PC3 LNCaP

Figure 6 (b) indicate prostospheres of DU145, PC3 and LNCaP formed on day 9 in sphere assay (taken at 20X magnification by inverted microscope)



COLO205 COLO320DM HCT-15

Figure 6 (c) indicate colonospheres of COLO205, COLO320DM and HCT-15 formed on day 9 in sphere assay (taken at 20X magnification by inverted microscope)

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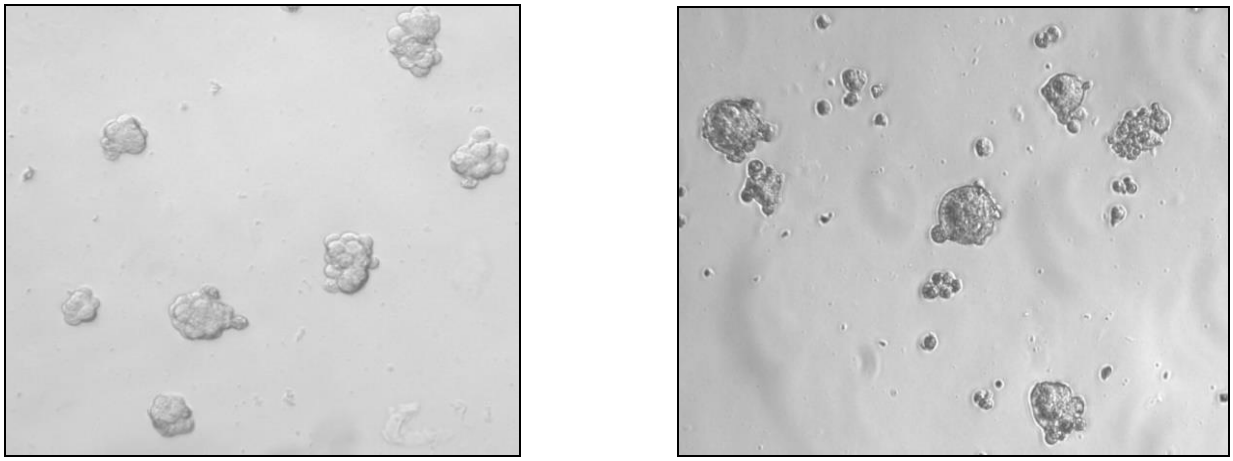
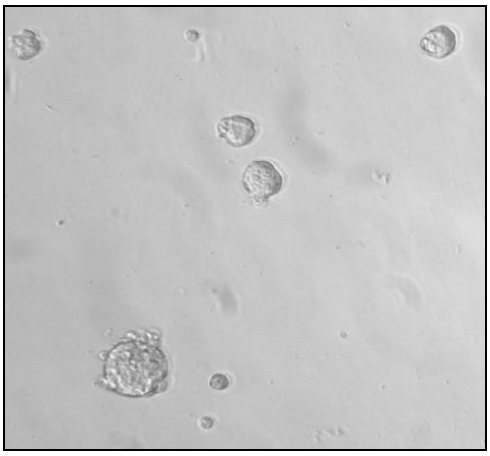


SiHa HeLa

Figure 6 (d) indicate cervical spheres of SiHa and HeLa formed on day 9 in sphere assay (taken at 20X magnification by inverted microscope)



Hep3B

Figure 6 (e) indicate hepatospheres of Hep3B formed on day 9 in sphere assay (taken at 20X magnification by inverted microscope)

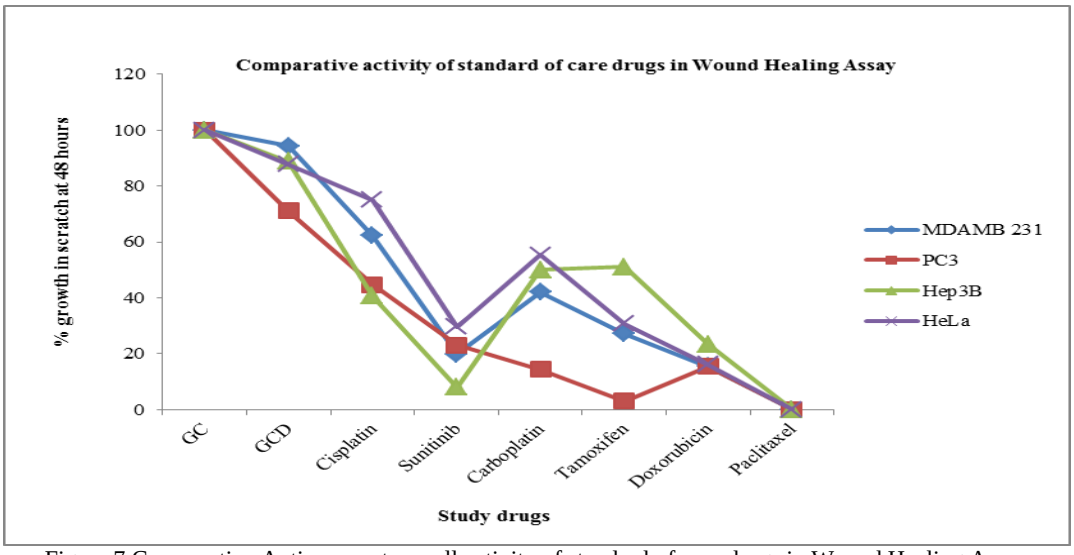


Figure 7 Comparative Anticancer stem cell activity of standard of care drugs in Wound Healing Assay

Figure 7 indicates the comparative wound healing activity of standard of care drugs on confluent monolayers of MDAMB231, PC3, HeLa and Hep3B.

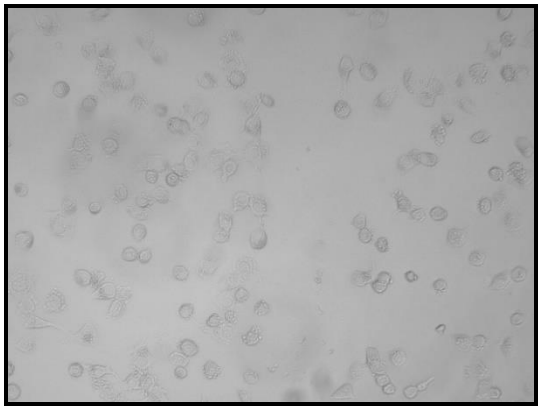


Figure 8 a) Indicates Effect of Paclitaxel in MDAMB231 cells

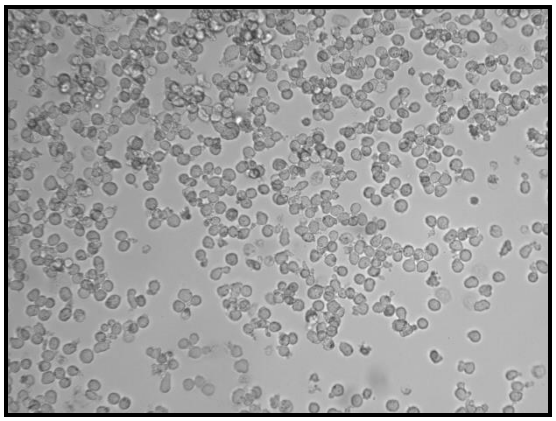


Figure 8 b) Indicates Effect of Paclitaxel in PC3 cells

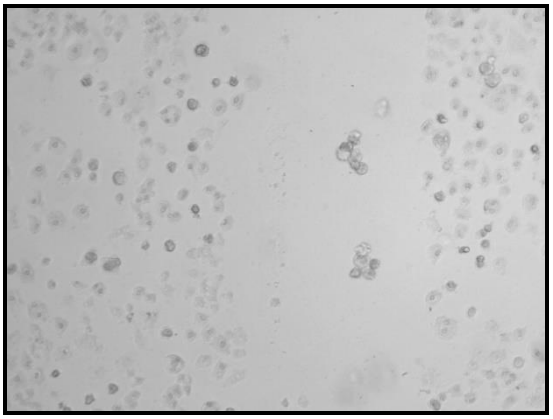


Figure 8 c) Indicates Effect of Paclitaxel in HeLa cells

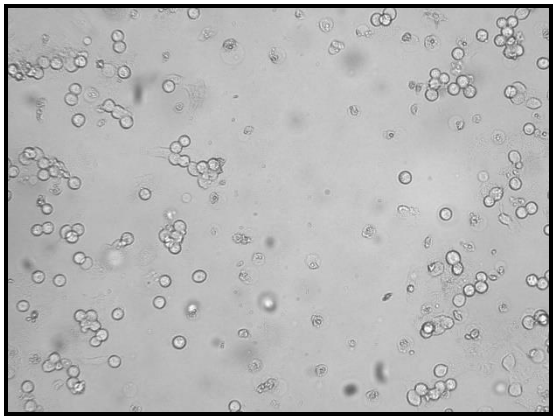


Figure 8 d) Indicates Effect of Paclitaxel in Hep3B cells

Figure 8 Effect of Paclitaxel in Wound Healing Assay at the end of 48 hours

Discussion

Presently, there are various standard of care drugs available in the market. These drugs have different modes of action [29]. In the case of current study also the standard of care drugs selected by us have exhibited different modes of action to kill cancer cells. For example, Sunitinib is a target therapy inhibiting cellular signaling .It also targets receptors for platelet-derived growth factor (PGF-Rs) and vascular endothelial growth factor receptors (VEGFR's), which are responsible for tumour growth. [30]. Carboplatin is known to modify the DNA structure by forming reactive platinum complexes and thus

inhibiting DNA synthesis [31] and thus affecting the cell in all the phases of its cycle. Cisplatin also acts in a similar way by forming DNA-DNA adducts resulting in cell death [32]. After the DNA chain is broken by topoisomerase II, Doxorubicin acts by stabilizing it, thus preventing the DNA double helix from being resealed and thereby stopping the process of replication [33]. The chemotherapeutic drug Tamoxifen acts in a different manner. It binds the mammary epithelial cell through ER receptor and blocks the proliferation actions of estrogen.5FU inhibits thymidylate synthase which is the nucleotide required for DNA replication [35] and lastly Paclitaxel acts on cell division by destabilizing mitotic spindle assembly. It is thus very important to understand the efficacy of these drugs when analyzed in-vitro on various types of cancer cell lines under similar experimental conditions.

Though there are study reports of these drugs on various cell lines there is no consolidated and comparative study done till date across various types of cell lines. Further in our study we focused not only on anticancer activity (determined by MTT assay) but we were also curious about anticancer stem cell activity (determined by sphere assay and WHA) of these drugs.

Summarizing our MTT results, Doxorubicin and Sunitinib are highly potent drugs which act on all the cancer cell types studied. Paclitaxel is exhibiting very high potency on colon, cervical and hepatic cell lines, thus these drugs can ideally be used in combination for various types of cancer. Further in sphere assay and WHA also these drugs have exhibited very high potency compared to others indicating their activity on CSC.

CSC's are responsible for chemo-resistance [37] and hence an ideal chemotherapeutic agent should kill CSC's along with differentiated cancer cells. Our study has shown that Sunitinib, Doxorubicin and Paclitaxel have shown very good anti CSC activity as indicated in sphere assay and WHA experiments. Due to this they can be used in combination therapies for a patient undergoing chemotherapy. However further research work on these three drugs can throw light on these facts.

V. CONCLUSION AND FUTURE SCOPE

Our present "in-vitro" study revealed that Sunitinib, Doxorubicin and Paclitaxel act on CSC's of Breast, Prostate, Colon, Hepatic and Cervical cell lines. These drugs have also exhibited very high anticancer activity across the cell lines tested. CSC's are tumor initiating cells. They exhibit several characteristics such as tumorigenicity, pluripotency and self renewal. They are a main cause of cancer relapse hence there is an urgent need to use these agents for a successful chemotherapy.
As our results have indicated, Sunitinib Doxorubicin and Paclitaxel have potent anti-CSC ability along with anticancer activity. Further, these drugs should be studied in combination with other chemotherapeutic drugs in in-vitro assays to understand their synergistic effect and hence to provide scientific bases for their "in-vivo" use.

CONFLICT OF INTEREST

There is no conflict of interest in the reported research work.

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- Smera Satish – Planned & performed all the experimental work (MTT, WHA and sphere assay), data analysis and data presentation included in the manuscript, also manuscript writing.
- Gayatri Gore – Performed MTT, Sphere Assay and Wound Healing Assay along with first author.
- Akshay Ganpule – Assisted first and second author in preparing reagents and performed MTT and Sphere assay with the first author.
- Sangeeta Srivastava – Assisted in prior search and graphical representation of the data in the manuscript.
- Maithili Athavale – Conceptualized research idea, planned experiments and manuscript writing.

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