

Comparative Effect of Tyrosine Kinase Inhibitors in Human Cancer Cell Lines

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Abstract

Purpose: This study compared the in vitro activity of small molecule tyrosine kinase inhibitors (TKI's) erlotinib, dasatinib and sorafenib in human cancer cell lines of different tumor origins HCT 116 (colon carcinoma), MCF7 (breast carcinoma) and H460 (non-small cell lung carcinoma).

Methods: Cytotoxicity assay was performed to determine the IC₅₀ concentrations of TKI's-erlotinib, dasatinib, sorafenib against HCT 116, MCF7 and H460 cell lines. Cell cycle analysis was done using Flow cytometry (FACS). Anti-proliferative effect was analyzed by colony forming assay. Western blot technique was used to investigate the effect of TKI's on the expression of cyclin D1 protein in cancer cell lines.

Results: Results indicated that erlotinib was less potent having IC₅₀>30μM compared to dasatinib and sorafenib in all three cancer cell lines. Dasatinib was more potent than sorafenib. The data indicated dasatinib's IC₅₀ as 0.14 μM, 0.67 μM, 9.0 μM and sorafenib's IC₅₀ as 18.6 μM, 16.0 μM, 18.0 μM in HCT 116, MCF7 and H460 respectively. This study showed that sorafenib was very potent in inducing cytotoxicity in HCT 116, MCF7, H460 cells (P < 0.05, < 0.01, < 0.001), however dasatinib was found to be highly effective in MCF7 cells only (P < 0.001). Our results in clonogenic assay indicated that dasatinib and sorafenib showed decrease in colony growth in all three cell lines. Moreover, western blot analysis showed that dasatinib and sorafenib significantly decreased cyclin D1 expression in MCF7 H460cells (P < 0.001) (P < 0.05) respectively.

Conclusion: We can conclude that our results are in accordance with reported findings of sorafenib and dasatinib. We established the hypothesis that sorafenib and dasatinib can be used in colon, breast and lung cancer. However sorafenib and dasatinib have the potential in the clinic, but in order to overcome the limitations of multi targeted tyrosine kinase inhibitors detailed studies can be performed using more cell lines as well as cell signalling mechanism needs to be elucidated.

Keywords: Tyrosine kinase inhibitors; Erlotinib; Dasatinib; Sorafenib; Cytotoxicity; Sub G₀/G₁; Cyclin D1; Cell lines

Abbreviations: TKI's: Tyrosine Kinase Inhibitors; RTK's: Receptor Tyrosine Kinases; EGFR: Epidermal Growth Factor Receptor; PDGF: Platelet-Derived Growth Factor; VEGF: Vascular Endothelial Growth Factor; ATP: Adenosine Triphosphate; CML: Chronic Myelogenous Leukaemia; pRB: Retino blastoma Protein; PI: Propidium Iodide; DMSO: Dimethyl Sulfoxide

Introduction

Cancer cells are characterized with the traits of uncontrolled growth, invasion (intrusion and destruction of adjacent tissues) and metastasis [1]. The cell evades apoptosis, override DNA damage checkpoints, continue proliferation to propagate existing mutations and acquire new mutations. It maintains high growth with rapid cell division. The function of signaling molecules is important for cell growth and cell division is often deregulated in cancer cells, which leads to their aberrant

proliferation. Thus, components of the cell signaling machinery that regulate cell growth and cell division are potential therapeutic targets for drug design [2]. Receptor tyrosine kinases (RTK's) are the high-affinity cell surface receptors for many polypeptide growth factors, cytokines, and hormones. Of the 90 unique tyrosine kinase genes identified in the human genome, almost 58 encode receptor tyrosine kinase proteins [3]. Receptor tyrosine kinases have been shown not only regulates the normal cellular processes but also to have a critical role in the development and progression of many types of cancer [4].

EGFR (Epidermal growth factor receptor) is highly expressed and prominently mutated in various forms of cancer. For the signal to be transmitted, two members of the EGFR family need to come together to form a homodimer. These then use the molecule of ATP to auto phosphorylate each other, which causes a conformational change in their intracellular structure, exposing a further binding site for binding proteins that cause a signal cascade to the nucleus [5]. By inhibiting the ATP, auto phosphorylation is not possible and the signal is stopped. ErbB-1 and ErbB-2 are found in many human cancers and their excessive signaling may be critical factors in the development and malignancy of these tumors [6]. Vascular endothelial growth factor (VEGF) is one of the main inducers of endothelial cell proliferation and permeability of blood vessels. VEGF ligand binds to receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/ Flk-1) causing phosphorylation of RTK's [7]. Protein kinases are overactive in many of the molecular pathways that cause cells to become cancerous. These pathways include Raf kinase, PDGF (platelet-derived growth factor), VEGF receptor 2 and 3 kinases and c Kit the receptor for stem cell factor. A growing number of drugs target most of these pathways [8]. Cyclin D activity is crucial for the progression into S phase and is one of the central components of the mammalian restriction point leading to S phase initiation. The major downstream target of Cyclin D is the retinoblastoma protein (pRB). The Cyclin D/Cdk 4/6 complex directly phosphorylates pRB [9]. This relieves the inhibitory effects of pRB on the transcription factor, E2F resulting in the expression of a large number of cell cycle regulated genes and eventual progression into S phase [10]. Amplification or over expression of Cyclin D1 is important in the development of many cancers including parathyroid adenoma, breast, prostate, colon cancer, lymphoma and melanoma [11]. Erlotinib hydrochloride is a drug used to treat non-small cell lung cancer, pancreatic cancer and several other types of cancer. Erlotinib acts as tyrosine kinase inhibitor by targeting EGFR similar to gefitinib. It binds in a reversible fashion to the adenosine triphosphate (ATP) binding site of the receptor [12]. Dasatinib, also known as BMS-354825. Dasa is an oral dual BCR/ABL and SRC family tyrosine kinases inhibitor approved for use in patients with chronic myelogenous leukemia (CML) after imatinib treatment and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL) [13]. It is also being assessed for use in metastatic melanoma. The main targets of Dasatinib are BCR-ABL, SRC, Ephrins, GFR [14]. Sorafenib, is a drug approved for the treatment of primary kidney cancer (advanced renal cell carcinoma) and advanced primary liver cancer (hepatocellular carcinoma) [15]. Sorafenib is unique in targeting the Raf/ Mek/ Erk pathway (MAP Kinase pathway). Sorafenib is a small molecular inhibitor of several tyrosine protein kinases [16-18]. In this study, we compared the activities of the tyrosine kinase inhibitors erlotinib, sorafenib, and dasatinib in a panel of adult human colon, lung and breast cancer cell lines using cytotoxicity assay, cell cycle analysis

with apoptosis, cyclin D1 down regulation and colony forming potential. The cell lines used in this study were HCT116, H460 and MCF 7 which represented different genetic variants such as mutation in codon13 of ras proto-oncogene, chromosomal abnormalities and estrogen sensitivity respectively, to determine which inhibitor is best used for the treatment of heterogeneity associated with clinical samples. Additionally there are several challenges in the clinic as far as selection of multi-targeted TKIs and our comparative study may support future selection of a specific or selective candidate for cancer treatment.

Materials and Methods

Cell lines

The human cancer cell lines, HCT 116, MCF7 and H460 were purchased from American type culture collection (ATCC, Rockville, MD, USA). The cell lines MCF7, H460 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and HCT 116 was cultured in McCoy's medium supplemented with 10% fetal bovine serum (Hyclone, UT, USA), 2 mmol/L L-glutamine (Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Chemical agents

Sterile medium RPMI-1640 and McCoy's, fetal bovine serum, Trypsin-EDTA, antibiotic solutions (penicillin and streptomycin), Phosphate buffer solution and Propidium Iodide (PI), RNase-A solution (1mg/ml) were purchased from reagent Sigma-Aldrich (Saint Louis, USA). TKI's-Erlo, Dasa and Sora (LC Laboratories, Woburn, MA, USA) were procured from Piramal Life Sciences Ltd. (PLSL) Mumbai. Aliquots of 10 mM stock solution of all three compounds were prepared using dimethyl sulfoxide (DMSO: Sigma-Aldrich) solvent. Appropriate dilutions were prepared using RPMI-1640 medium for H460 and MCF7 cell lines and McCoy's medium for HCT 116 cell lines as recommended by ATCC to expose cells to the drugs.

In vitro cytotoxicity

Cancer cell lines were seeded in 96 well cell culture plates (3×10³ cells/well) and were exposed to Erlotinib, Dasatinib and Sorafenib at 30 μM, 10 μM, 3 μM, 1 μM, 0.3 μM, 0.1 μM concentrations for 48 h. Cells were maintained at 37°C in 5% CO₂. A modified propidium iodide (PI) assay was used to assess the effect of compounds on the growth of the human tumor cell lines. Following continuous drug exposure, cell culture medium with or without drug was replaced by 200 μl of an aqueous PI solution (7 μg/mL). After thawing the plates, fluorescence was measured using the POLAR star OPTIMA from BMG Technologies (excitation, 448 nm; emission, 620 nm), giving a direct relationship to the total cell number. IC₅₀ values were determined by plotting compound concentration versus

cell viability. The IC_{50} value of the concentration resulting in 50 percent cell growth inhibition by 48 h exposure to drug compared with untreated control cells was calculated [19,20].

Cell cycle analysis

Cell cycle distribution and apoptosis was analyzed using PI stained cells. Human cancer cells (0.2×10^6 cells/ T 25 culture flasks) were cultured and allowed to grow to 90 % confluency followed by compound treatment, cells harvesting. The cells were then exposed to dasatinib and sorafenib at their respective IC_{50} and three times IC_{50} concentrations for 24 h and 48 h time period and compared with control samples not exposed to drug. The cell cycle analysis was done using FlowJo software (Tree Star Inc, USA). Cells with DNA content between 2N and 4N were designated as being in G0/G1, S, and G2/M phases of the cell cycle, as defined by the level of PI fluorescence. Cells exhibiting $<2N$ DNA content were designated as sub G0/G1 cells (apoptotic cells) [21]. The number of cells in each cell cycle compartment was expressed as a percentage of the total number of single cell [22].

Clonogenic assay

Human cancer cells were seeded in ATCC recommended media and 10 percent FBS (MCF7, H460 in RPMI-1640 and HCT 116 in McCoy's) in 6 well plates (1000 cells per well). Cells were seeded in two separate plates each for dasatinib and sorafenib. After overnight incubation, cells were exposed to dasatinib and sorafenib at their respective IC_{50} and three times IC_{50} concentrations for 24 h and 48 h. Media was replaced at time points of drug treatment with fresh media. Treated cells were incubated at 37°C in 5% CO_2 for 10 days. Standard protocol of colony forming assay was followed [23]. Colonies were observed under Zeiss microscope and calculated as percent inhibition as compared to untreated control groups. As erlotinib showed precipitation in culture medium above 30 μM concentration, it was not studied further.

Western blot analysis

Cancer cell lines were seeded at 0.8×10^6 cells/mL in T-75 flasks and exposed or not exposed to dasatinib and S at IC_{50} and three times IC_{50} concentrations in the above cell lines. Cells were removed at 48h, washed twice with PBS and thereafter cell pellets were stored at -70°C [24]. Antibodies used in this study were: cyclin D1 (Santa Cruz Biotechnology, CA, USA) actin (Sigma, MO, USA), anti-mouse HRP secondary antibodies (Santa Cruz Biotechnology, CA, USA). The blots were quantified using densitometric analysis using ImageJ and normalized with actin.

Statistical Analysis

All statistical analysis was performed using Graph pad prism software. The results expressed as means \pm SD or SEM were compared using Student's t test to determine statistical

significance between control and test groups as applicable. $P < 0.05$, < 0.01 , < 0.001 was considered as statistically significant.

Results

Cytotoxic effect of erlotinib, dasatinib and sorafenib on human cancer cell lines

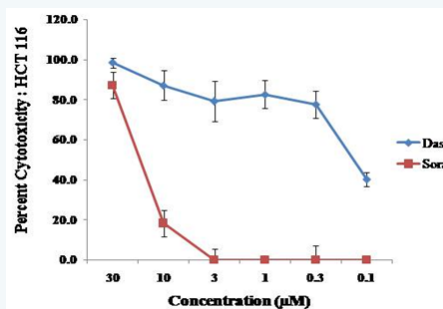


Figure 1A: Comparative analysis of cytotoxicity of dasatinib and sorafenib in colon cancer cells. Values are expressed as mean \pm SD from four independent experiments.

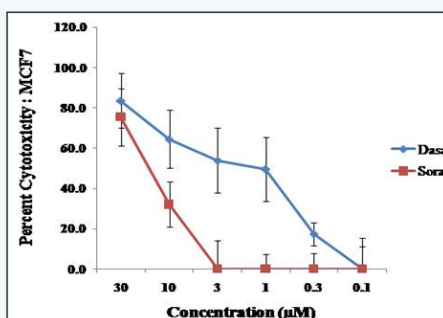


Figure 1B: Comparative analysis of cytotoxicity of dasatinib and sorafenib in breast cancer cell line. Values are expressed as mean \pm SD from four independent experiments.

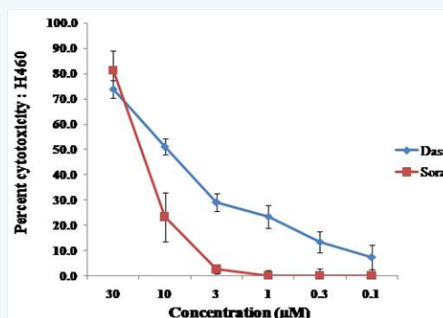


Figure 1C: Comparative analysis of cytotoxicity of dasatinib and sorafenib in non-small cell lung carcinoma cells respectively. Values are expressed as mean \pm SD from four independent experiments.

Our results indicated that erlotinib did not show any cytotoxic activity in HCT116, MCF7 and H460 cells until 30 μM (Table 1).

Moreover, erlotinib solution above 30 μM was precipitated and further omitted from the study. Dasatinib was found to be more potent than sorafenib. Sorafenib showed concentration which is 18 fold lesser (0.14 μM) in HCT 116, 16 fold lesser (0.67 μM) in MCF7 and 9 fold lesser IC_{50} (9.0 μM) in H460as compared to sorafenib (Figure 1A-1C) (Table 1). Hence, it is concluded that dasatinib which target BCR-ABL, SRC pathway inhibits cell proliferation at lower IC_{50} concentration as compared to sorafenib which targets Raf/ Mek/ Erk pathways (Figure 1A-1C).

Table 1: An IC_{50} value (μM) of TKI's in human cancer cell lines.

Samples	H460	MCF7	HCT 116
Erlotinib	> 30 μM	> 30 μM	> 30 μM
Dasatinib	9.0 \pm 2.9 μM	0.67 \pm 0.2 μM	0.14 \pm 0.04 μM
Sorafenib	18.0 \pm 4.1 μM	16.0 \pm 3.6 μM	18.6 \pm 1.9 μM

IC_{50} values (μM) of drugs are expressed as mean \pm SD, n = 4. IC_{50} value is the amount of a compound required to inhibit 50 percent of cells.

Effect of dasatinib and sorafenib on cell cycle progression of human cancer cell lines

We investigated the effect of dasatinib (entire range of concentration 0.14-27 μM) on HCT 116, H460 and MCF 7 cells at 24 and 48 h respectively. Interestingly, dasatinib inhibited followed by H460 model.

cell cycle progression thereby leading to a decreased cell proliferation. Cell cycle analysis exhibited an increase of HCT116 cells in G0/G1phase which was accompanied by a reduction of cells in S- and M phase from 65.83%, 49.48% (control) to 14.33 %, 23.64 % (0.14 μM) and 58.76%, 17.1% (0.14 μM) at 24 and 48 h. Similarly, dasatinib at higher concentration (0.42 μM) reduced cells in G0/G1 phase by 52.06 % and 56.92 respectively at 24 h and 48h (Figure 2) (Table 2). Dasatinib could not induce apoptosis until 48 h in HCT 116 model. However, on comparison with MCF 7 cells we found that dasatinib at 0.67 μM arrested 61.47% of the cells in G0/G1 phase at 24 h. Additionally we also found that in MCF 7 cells dasatinib is able to induce apoptosis in the range of 31.47-76.45 % at 24 and 48 h respectively. In addition to this Dasatinib could not induce apoptosis in H460 cells, but was able to arrest the cells in G0/G1phase in the range of 44.72-60.83% at 24h and 48h respectively (Figure 3,4) (Table 3,4). Furthermore we evaluated the effect of sorafenib (16-55.8 μM) on HCT 116 H460 and MCF 7 cells at 24 and 48h respectively (Figure 1). Incubating the HCT116 cells with sorafenib at range of 18.6-55.8 μM induced apoptosis to an extent of 40.31-98.36% respectively. Although in MCF 7 cells sorafenib at 16 and 48 μM induced apoptosis to an extent of 23.88-73.01% at 24 and 48 h respectively. Similarly in H460 cells sorafenib at 18 and 54 μM induced apoptosis to an extent of 17-90.15% at 24 and 48 h respectively. On comparison we observed that sorafenib is more effective in inducing apoptosis in following sequence HCT116

Table 2: Effect of dasatinib and sorafenib on cell cycle progression of HCT 116 cells as compared to control.

Condition	G0/G1 (%) (M1)	G2/M (%) (M2)	S (%) (M3)	Sub G0/G1 (%) (M4)
Control 24 h	49.48 \pm 1	23.64 \pm 0.26	24.89 \pm 0.95	1.58 \pm 0.22
Dasa 0.14 μM	65.83 \pm 6.68	14.33 \pm 3.12*	13.28 \pm 3.56*	5.79 \pm 0.11***
Dasa 0.42 μM	52.06 \pm 0.94	16.76 \pm 1.52*	24.03 \pm 0.79	7.58 \pm 0.95**
Sora 18.6 μM	29.53 \pm 8.22	10.92 \pm 0.48**	19.25 \pm 5.66	40.31 \pm 3.36**
Sora 55.8 μM	14.96 \pm 5.97**	5.58 \pm 2.62**	13.31 \pm 5.82	66.06 \pm 14.45*
Control 48 h	51.79 \pm 1.91	21.68 \pm 2.22	25.05 \pm 0.34	1.39 \pm 0.27
Dasa 0.14 μM	58.76 \pm 3.68	18.16 \pm 2.47	18.14 \pm 0.65***	4.23 \pm 0.78*
Dasa 0.42 μM	56.92 \pm 2.42	13.57 \pm 2.52	22.54 \pm 0.77*	7.29 \pm 0.73**
Sora 18.6 μM	16.58 \pm 0.60***	6.8 \pm 1.12**	13.19 \pm 3.23*	62.85 \pm 4.63***
Sora 55.8 μM	0.69 \pm 0.69***	0.15 \pm 0.14***	0.76 \pm 0.76***	98.36 \pm 1.63***

Values are expressed as mean \pm SEM relative to Control for 24 h and 48 h respectively, n=3.
*P=0.01 to 0.05, **P= 0.001 to 0.01, ***P=0.0001 to 0.001.

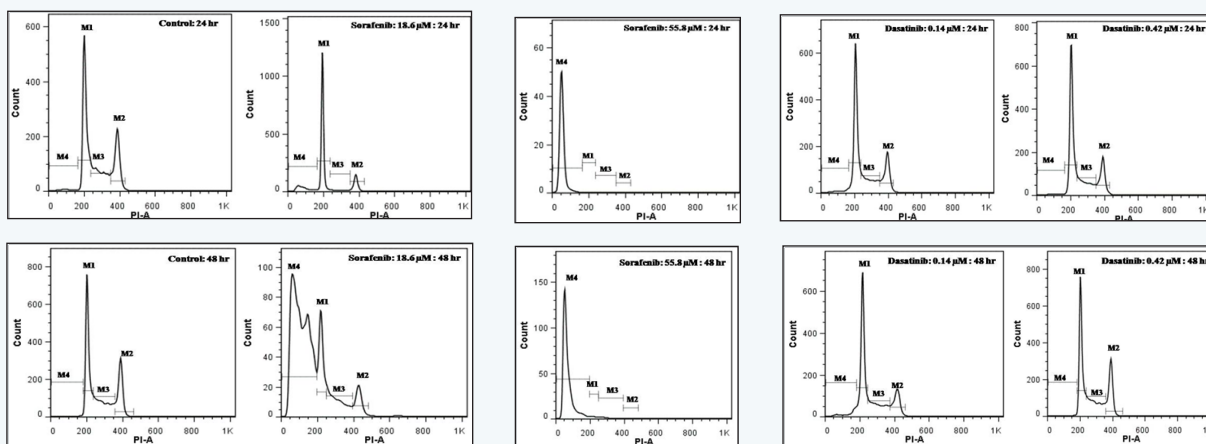


Figure 2: Cell cycle analysis of HCT 116 cells exposed to dasatinib and sorafenib at IC_{50} and three times IC_{50} concentrations for 24 h and 48 h by flow cytometry. M1: G0/G1, M2: G2/M, M3: S, M4: sub G0/G1 phases. Histogram is representative of one of three independent experiment

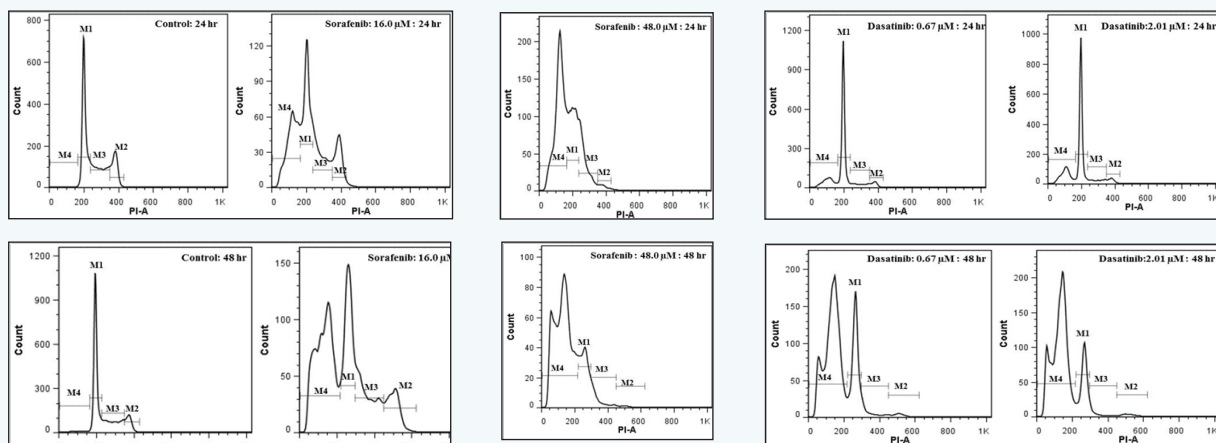


Figure 3: Cell cycle analysis of MCF7 cells in the presence of dasatinib and sorafenib at IC_{50} and three times IC_{50} concentrations for 24 h and 48 h by flow cytometry. M1: G0/G1, M2: G2/M, M3: S, M4: sub G0/G1 phases. Histogram is representative of three independent experiments.

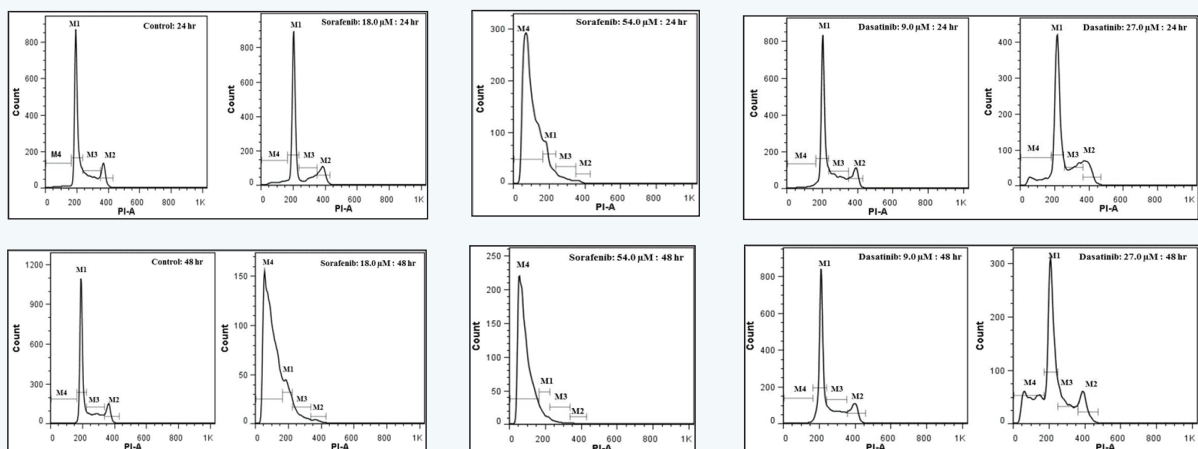


Figure 4: Cell cycle analysis of H460 cells in the presence of dasatinib and sorafenib at IC_{50} and three times IC_{50} concentrations for 24 h and 48 h by flow cytometry. M1: G0/G1, M2: G2/M, M3: S, M4: sub G0/G1 phases. Histogram is representative of three independent experiments.

Table 3: Effect of dasatinib and sorafenib on cell cycle progression of MCF 7 cells as compared to control.

Condition	G0/G1 (%) (M1)	G2/M (%) (M2)	S (%) (M3)	Sub G0/G1 (%) (M4)
Control 24 h	53.07 ± 0.84	16.17 ± 0.72	29.17 ± 0.30	1.26 ± 0.35
Dasa 0.67 µM	61.47 ± 1.75*	8.26 ± 1.15**	6.39 ± 0.65***	23.88 ± 1.61***
Dasa 2.01 µM	35.63 ± 4.09*	4.72 ± 0.92***	8.22 ± 1.96***	48.93 ± 4.66***
Sora 16.0 µM	46.18 ± 7.24	13.29 ± 4.12	8.89 ± 1.64***	31.47 ± 2.24***
Sora 48.0 µM	29.17 ± 5.45*	5.78 ± 3.07*	13.55 ± 4.32*	50.97 ± 11.85*
Control 48 h	59.69 ± 0.96	14.57 ± 1.16	23.55 ± 0.64	1.81 ± 0.13
Dasa 0.67 µM	17.69 ± 3.86***	1.1 ± 0.16***	14.12 ± 5.75	67.18 ± 1.79***
Dasa 2.01 µM	16.00 ± 3.37***	0.73 ± 0.39***	6.38 ± 4.89*	76.45 ± 1.11***
Sora 16.0 µM	23.47 ± 5.20**	11.75 ± 2.61	24.34 ± 4.78	39.24 ± 4.68**
Sora 48.0 µM	18.20 ± 5.79**	1.06 ± 0.23***	7.75 ± 4.01*	73.01 ± 9.41**

Values are expressed as mean ± SEM relative to Control for 24 h and 48 h respectively, n=3.
*P=0.01 to 0.05, **P= 0.001 to 0.01, ***P=0.0001 to 0.001.

Table 4: Effect of dasatinib and sorafenib on cell cycle progression of H460 cells as compared to control.

Condition	G0/G1 (%) (M1)	G2/M (%) (M2)	S (%) (M3)	Sub G0/G1 (%) (M4)
Control 24 h	72.2 ± 1.68	10.57 ± 0.95	14.93 ± 0.66	1.93 ± 0.14
Dasa 9.0 µM	58.04 ± 0.88**	14.82 ± 1.66	24.34 ± 2.07*	2.57 ± 0.54
Dasa 27.0 µM	56.93 ± 2.91*	14.27 ± 2.09	17.87 ± 1.82	10.18 ± 0.72***
Sora 18.0 µM	42.13 ± 3.17**	16.13 ± 1.88	24.17 ± 1.84**	17 ± 0.87***
Sora 54.0 µM	10.00 ± 2.51***	0.88 ± 0.21***	4.19 ± 0.26***	85.07 ± 2.56***
Control 48 h	65.37 ± 1.10	13.1 ± 0.61	20.3 ± 0.55	0.86 ± 0.04
Dasa 9.0 µM	60.83 ± 1.30	15.34 ± 0.55	21.99 ± 0.75	1.43 ± 0.45
Dasa 27.0 µM	44.72 ± 2.18**	10.86 ± 0.35*	17.70 ± 1.38	26.71 ± 1.39***
Sora 18.0 µM	19.35 ± 3.66***	1.92 ± 0.90***	11.02 ± 5.22	67.94 ± 9.36**
Sora 54.0 µM	7.03 ± 2.05***	0.41 ± 0.18***	2.52 ± 0.89***	90.15 ± 3.05***

Values are expressed as mean ± SEM relative to Control for 24 h and 48 h respectively, n=3.
*P=0.01 to 0.05, **P= 0.001 to 0.01, ***P=0.0001 to 0.001.

Effect of dasatinib and sorafenib on colony forming potential of human cancer cell lines

The percentage viability of HCT 116, MCF 7 and H460 control cells at 24 and 48 h is close to 80-90 %. Results indicated that dasatinib and sorafenib showed dose, concentration and time dependent decrease in colony formation of all three cell lines. HCT 116, MCF 7 and H460 cells were treated with dasatinib, sorafenib at IC₅₀ and 3X IC₅₀ values for 24 and 48 h. Figure 5A-5C highlights the effect of dasatinib and sorafenib on cell proliferation and viability of HCT 116, MCF 7 and H460 cells. Results indicated that in HCT 116 cell model, dasatinib at 24h did not inhibit the cell proliferation. In contrast at 48 h Dasatinib (0.42µM) increased the loss of viability to 40% dose dependently. Further Dasatinib at 24 h in MCF7 model depicted 50 & 70 % loss of viability at 0.67 µM & 2.01 µM respectively. Similarly at 48 h Dasatinib (at 0.67 µM & 2.01 µM) reduced

the cell proliferation by 70 and 85 % (Figure 5B) respectively. Dasatinib when exposed to H460 cells at 9 and 27µM the cell proliferation was decrease to 50-70 %. Overall from our findings we can reveal that on Dasatinib was more sensitive to ER positive MCF 7 cells than colon (HCT116, kras mutated) and lung cancer H460 cell line. However treatment of HCT 116, MCF7 and H460 cells with sorafenib at 24 and 48 h is potent inhibitor of cellular proliferation. Our findings are in accordance with Shuiying Hu et al. It was observed that, in HCT116 kras mutated cancer cell model, Sorafenib reduced the ability to form the colonies by 50 and nearly close to 100% at 18.6 & 55.8 µM respectively at 24 and 48h (Figure 5A). This trend was more prominent with MCF7 cells at 16.0 µM and 48.0 µM (Figure 5B), indicating more sensitivity of sorafenib to ER positive cells. Interestingly the effect of sorafenib at 18 and 54 µM on H460 cells is also very significant and cell proliferation capacity was reduced by 80 and 100% (Figure 5C).

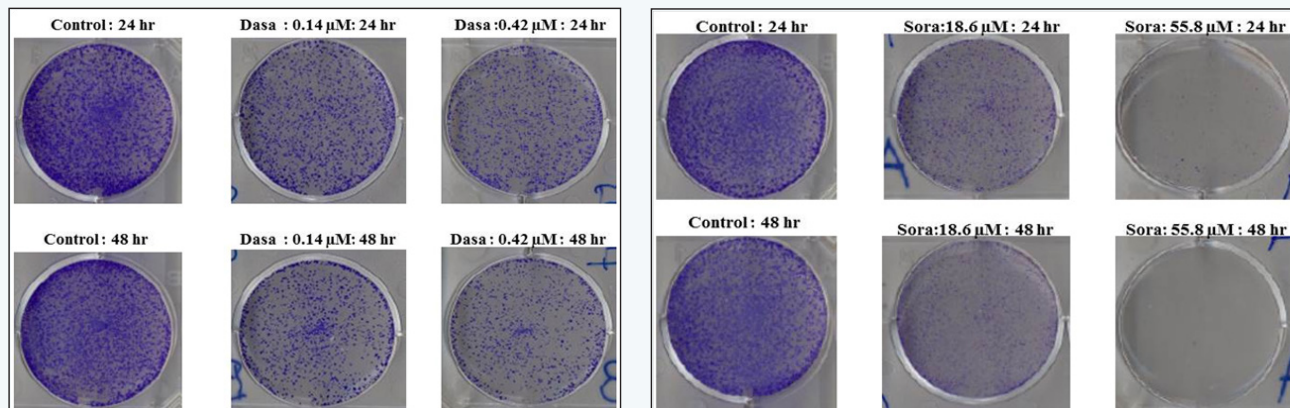


Figure 5A: Colony forming potential of HCT 116 cells upon exposure to dasatinib and sorafenib at IC_{50} and three times IC_{50} concentrations for 24 h and 48 h. Figure is representative of one of three independent experiments.

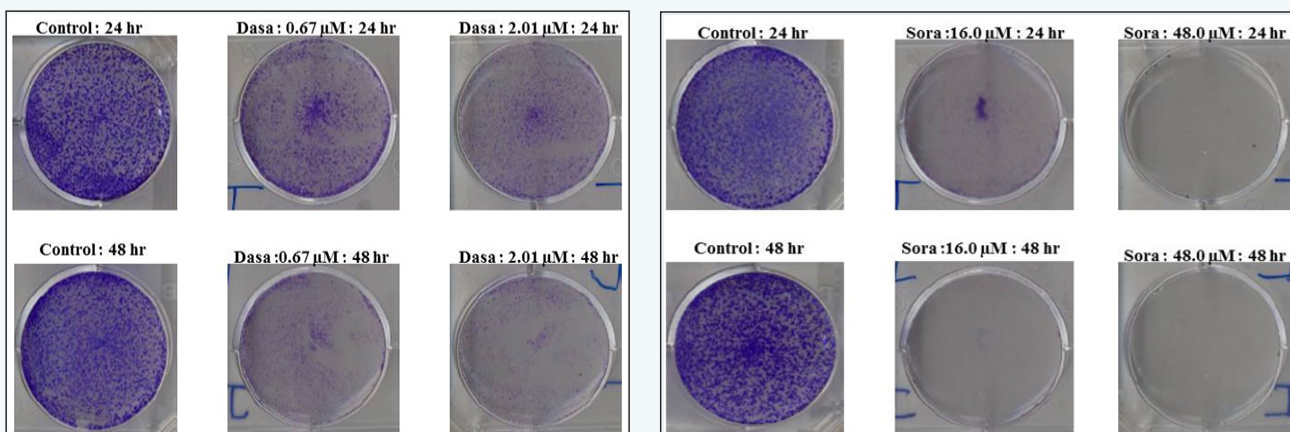


Figure 5B: Colony forming potential of MCF7 cells upon exposure to dasatinib and sorafenib at IC_{50} and three times IC_{50} concentrations for 24 h and 48 h. Figure is representative of one of three independent experiments.

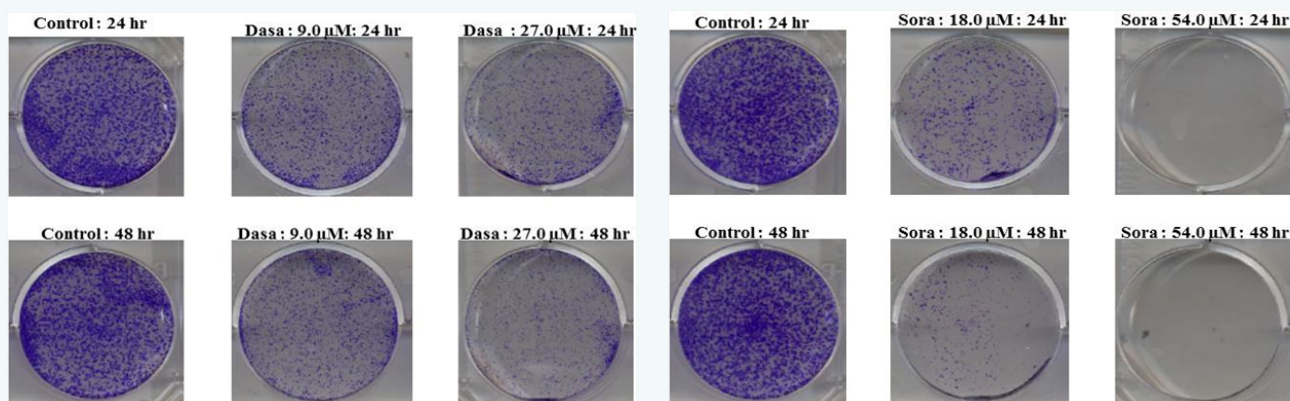


Figure 5C: Colony forming potential of H460 cells upon exposure to dasatinib and sorafenib at IC_{50} and three times IC_{50} concentrations for 24 h and 48 h. Figure is representative of one of three independent experiments.

Effect of dasatinib and sorafenib on cyclin D1 expression in human cancer cell lines

Multi kinase inhibitors are known to inhibit cell proliferation [14]. MCF7 cells were found to be significantly ($P < 0.001$) sensitive to dasatinib and down regulated cyclin D1 levels by 0.5fold compared to control. However down regulation of cyclin

D1 expression by dasatinib in H460 is significant as compared to HCT116. In regard to effect of sorafenib in western blot analysis, cyclin d1 expression levels did not alter, suggesting some other plausible mechanism of sorafenib in colon cancer. Surprisingly we found that sorafenib down regulated cyclin d1 expression levels at IC_{50} concentration in MCF 7, ER positive breast and H460 lung cancer model (Figure 6A,6B).

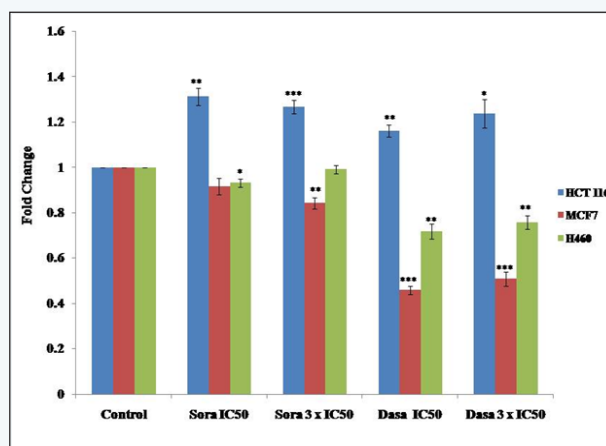
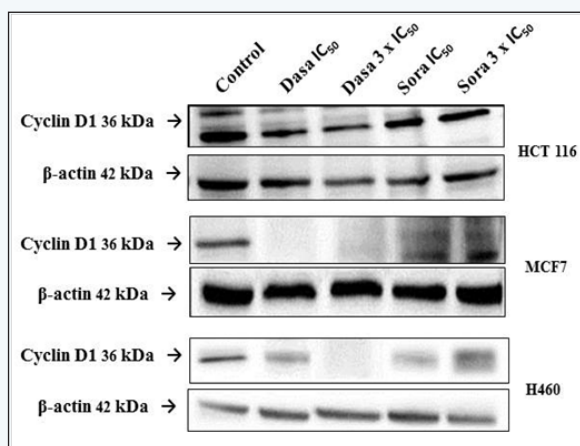


Figure 6A & 6B: Western blotting was performed to study the effect of TKI's on cyclin D1 expression in human cancer cell lines. MCF7 cells were exposed to dasatinib (0.67 μ M, 2.01 μ M), Sorafenib (16.0 μ M, 48.0 μ M) for 48h. HCT116 cells were exposed to dasatinib (0.14 μ M, 0.42 μ M), Sorafenib (18.6 μ M, 55.8 μ M) for 48h. H460 cells were exposed to dasatinib (9.0 μ M, 27.0 μ M), Sorafenib (18.0 μ M, 54.0 μ M) for 48h. The western blot is representative of three independent experiments. Cyclin D1 expression was normalized against β -actin. The data was expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control.

Discussion

In this study, we evaluated and compared the effect of three kinase inhibitors erlotinib, sorafenib and dasatinib in 3 different cancer cell lines such as HCT116, MCF7 and H460 representing colon (kras mutated), breast (ER positive) and lung cancer. Tyrosine kinases are especially important targets because they play an important role in the modulation of growth factor signaling and thus allow target specific therapy for selected malignancies. Conventional chemotherapy, although directed toward certain macromolecules or enzymes, typically does not discriminate effectively between rapidly dividing normal cells (e.g., bone marrow and gastrointestinal tract) and tumor cells, thus leading to several toxic side effects. Thus targeted therapies have high specificity toward tumor cells, providing a broader therapeutic window with less toxicity [25]. Several studies have been conducted for erlotinib, sorafenib and dasatinib. In our study, we attempted to compare multi targeted tyrosine kinase inhibitors erlotinib, sorafenib and dasatinib using specific population of cancer cell type, to determine which inhibitor is best suited for the specific cancer types. Various methods viz. cytotoxicity, cell cycle analysis and apoptosis, clonogenic assay and western blotting were used to assess the effect of TKI's on human cancer cell lines. In cytotoxicity studies erlotinib is not effective against HCT116, MCF7 and H460 cell lines, thus erlotinib

being an EGFR inhibitor will be effective against the EGFR over expressing cells. HCT 116 has a moderate levels and other two cell lines (MCF7 and H460) lack the EGFR. Interestingly, in our study we observed that erlotinib exhibited 49 % cytotoxicity at 30 μ M for HCT 116 and at the same concentration it is ineffective in H460 and MCF7 cancer cell lines. We concluded that erlotinib is effective only if EGFR is over expressed in the cells. Dasatinib is a multiply-targeted tyrosine kinase inhibitor. The main targets of dasatinib are BCR-ABL, SRC, Ephrins and GFR [13,14]. Tyrosine kinases affect systems of cellular division and survival, and are frequently over expressed or abnormally active in cancer cells. By targeting these systems, dasatinib is designed to reduce the growth and viability of various types of cancer. Cytotoxicity studies indicated that it is highly effective against HCT 116 and MCF7 cell lines. However, IC_{50} in H460 cell line was significantly high. Cell cycle analysis and apoptosis studies indicated that dasatinib induced apoptosis >70 % in MCF7 cell line, H460 (26% at three times IC_{50} for 48 h) and did not induce apoptosis in HCT 116 cell line. These observations clearly confirmed in clonogenic assay. The results indicated that, it is indeed potent towards MCF7 cells. Dose and time dependent inhibition of colonies were observed. However, it also showed moderate inhibition of colonies for HCT 116 and H460. HCT 116 is a k-ras mutated and H460 expresses very high levels of

angiogenic signaling molecules. We may suggest that dasatinib being BCR-ABL/SRC inhibitor, not effective in K-ras mutated cell line HCT 116. Because of lack of antiangiogenic potential, it is also ineffective against H460 cell line. Interestingly, cyclin D1 was down regulated in MCF7 and H460 cell lines at higher concentrations. Sorafenib, a multi kinase inhibitor targeting several serine/threonine and receptor tyrosine kinases [26]. It targets growth signaling and angiogenesis. Sorafenib blocks the enzyme RAF kinase, a critical component of the RAF/ MEK/ ERK signaling pathway that controls cell division and proliferation; in addition, sorafenib inhibits the VEGFR-2/PDGFR-beta signaling cascade, thereby blocking tumor angiogenesis [16-18]. In our study, sorafenib was found to be equipotent in cytotoxicity assay against all three cancer cell lines. However, concentrations needed to inhibit proliferation are very high as compared to dasatinib. Sorafenib being an antiangiogenic agent is very effective in the cell lines, which expresses higher levels of angiogenic signaling molecules. Our study is in accordance with above findings. In all three cell lines sorafenib revealed potent activation of apoptosis and inhibition of colony growth. In addition, cyclin D1, a key regulator of the cell cycle was also down regulated by sorafenib in MCF7 and H460 cell lines.

Conclusion

It is concluded that, multi targeted tyrosine kinase inhibitor dasatinib is more cytotoxic to HCT116. Cytotoxicity of the sorafenib is same across the panel of cell line. Overall, we can conclude that dasatinib can down regulate cyclin D1 expression significantly, this probably is linked with inhibiting cell proliferation, which further arrested the cells in Go/G1 phase. Sorafenib in HCT116 model may activate death mechanism and induces apoptosis as well as G0/G1 arrest. Although sorafenib and dasatinib have the potential in the clinic, but in order to overcome the limitations of multi targeted tyrosine kinase inhibitors detailed studies can be performed using more cell lines as well as cell signaling mechanism needs to be elucidated. For the tyrosine kinase inhibitors to have a primary role in therapy there has to be a clear hypothesis for their use, relevant preclinical data, and demonstrated use in well characterized groups of patients. So far, these criteria have not been met for most of the presently available tyrosine kinase inhibitors

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Author's Contribution

Amol Shindikar: Performed cytotoxicity assay, colony forming assay and cell cycle analysis. Analyzed the data. Wrote the first draft of the manuscript.

Umesh Chaudhari: Finalized experimental protocols, taught to perform experiments to AS and discussed the study design with KJ.

Kalpna Joshi: Conceived and designed the study. Critically reviewed the manuscript, suggested changes, contributed for writing discussion part.

Dimple Bhatia: Acquired samples of flow cytometry.

Gandhali Deshpande: Performed western blotting and analyzed the data and wrote the method. Suggested modifications, data were interpreted, critically reviewed and finalized entire manuscript.

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