Published in final edited form as:

Pharm Res. 2016 January ; 33(1): 137–154. doi:10.1007/s11095-015-1771-6.

Novel Gefitinib Formulation with Improved Oral Bioavailability in Treatment of A431 Skin Carcinoma

Chandraiah Godugu^{1,2}, Ravi Doddapaneni¹, Apurva R Patel¹, Rakesh Singh³, Roger Mercer³, and Mandip Singh¹

Mandip Singh: mandip.sachdeva@fam.u.edu

¹College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, Florida, USA

²Department of Regulatory Toxicology, National Institute of Pharmaceutical Education and Research, (NIPER), Hyderabad, Telangana, India

³Translational Science Laboratory, Florida State University, College of Medicine, Tallahassee, Florida 32306, USA

Abstract

Purpose—Oral administration of anticancer agents presents a series of advantages for patients. However, most of the anti-cancer drugs have poor water solubility leading to low bioavailability.

Methods—Controlled released spray dried matrix system of Gefitinib with hydroxypropyl β -cyclodextrin, chitosan, hydroxy propyl methyl cellulose, vitamin E TPGS, succinic acid were used for the design of formulations to improve the oral absorption of Gefitinib. Spray drying with a customized spray gun which allows simultaneous/pulsatile flow of two different liquid systems through single nozzle was used to prepare Gefitinib spray dried formulations (Gef-SD). Formulation was characterized by *in vitro* drug release and Caco-2 permeability studies. Pharmacokinetic studies were performed in Sprague Dawley rats. Efficacy of Gef-SD was carried out in A431 xenografts models in nude mice.

Results—In Gef-SD group 9.14-fold increase in the AUC was observed compared to free Gef. Improved pharmacokinetic profile of Gef-SD translated into increase (1.75 fold compared to Gef free drug) in anticancer effects. Animal survival was significantly increased in Gef formulation treated groups, with superior reduction in the tumor size (1.48-fold) and volumes (1.75-fold) and also increase in the anticancer effects (TUNEL positive apoptotic cells) was observed in Gef-SD treated groups. Further, western blot, immunohistochemical and proteomics analysis demonstrated the increased pharmacodynamic effects of Gef-SD formulations in A431 xenograft tumor models.

Conclusion—Our studies suggested that Gefitinib can be successfully incorporated into control release microparticles based oral formulation with enhanced pharmacokinetic and pharmacodynamic activity. This study demonstrates the novel application of Gef in A431 tumor models.

DISCLOSURES

Chandraiah Godugu, Ravi Doddapaneni and Mandip Singh contributed equally to this work.

Keywords

A431; control release formulations; epidermal growth factor receptor; gefitinib; spray drying

INTRODUCTION

The transmembrane glycoprotein epidermal growth factor receptors (EGFR) are specific tyrosine kinase receptors involved in the regulation of cellular differentiation and proliferation with high expression in a lot of cancers (1). Several studies demonstrated the correlation of EGFR overexpression to increased malignancy and poor prognosis in many types of human cancer (2). In human solid tumors, over activation and/or dysregulation of EGFR promote tumor progression including invasion, angiogenesis, metastasis, drug resistance with blocking of apoptosis (3,4). The EGFR expression is increased in several solid tumors such as lung, colorectal and brain tumor types (2,5). Due to the EGFR's complex role in tumor cell proliferation, invasion, angiogenesis and metastasis, EGFR inhibitors may become as promising anticancer drugs (6). Gefitinib (Gef), a low-molecular-weight EGFR inhibitor was found to be effective in many tumor types (7). Diverse studies have reported significant inhibition of tumor cell proliferation and angiogenesis as well as the induction of apoptosis by small molecule inhibitors (Gef, erlotinib) by virtue of their ability to inhibit EGFR (5). Gef was developed as an orally active EGFR inhibitor intended to use in patients with locally advanced non- metastasized or metastatic non-small cell lung cancer (5). Gef when combined with chemotherapeutic drugs and radiation therapy resulted in potentiation of anticancer effects. Since EGFRs are frequently over-expressed in several tumors types cells, the use of EGFR receptor inhibitors (Gef) in different tumor types may be a novel treatment strategy (8).

Gef is a water insoluble dibasic compound with pKa values of 5.28 and 7.17, which shows a pH-dependent solubility in gastrointestinal fluids. Gef has an extremely low aqueous solubility and its oral absorption is limited by its dissolution. The bioavailability could be greatly increased by improving the solubility and dissolution of the drug (9–16). This would help reduce the dose administered as well as the dose-related adverse effects including vomiting and diarrhea (17). The solubility and dissolution of Gef can be improved by complexation with cyclodextrins (CDs) (18). There are several approaches to improve the solubility and oral bioavailability of poorly water soluble drugs among which the spray drying (SD) solid dispersions possess several advantages (19–23). SD has gained lot of importance owing to its relative simplicity, low cost and large scalability. The drug of interest is dispersed in solution using various polymers, solvents and co-solvents. The liquid composition is then atomized and sprayed into a drying chamber where droplets are dried by heated air. This process can be optimized and used in the production of numerous stable formulations (24). In the past, SD was not preferred for drugs which are thermo labile, since elevated temperatures may potentially damage the active substance. However, several recent studies have demonstrated that SD can also be used to prepare microparticle formulations with labile therapeutics (25,26). Because of ease of administration, which increases the patient and physician compliance, the SD formulations have been used by pulmonary, oral, nasal mucosal routes. Further, SD technology is comprehensively used to prepare control

release polymeric microparticles for systemic drug delivery to increase the pharmacokinetic properties and reduce toxicity (27).

Our laboratory has developed various nanoparticle approaches (28–32) and dual channel SD technology to prepare solid dispersions for lipophilic drugs to increase oral bioavailability of various drugs. The dual channel SD system termed as dual channel *single spray gun* is modified from conventional single channel, where only one liquid may be spray dried. This modification allows us to spray two separate liquid systems containing one or more active pharmaceutical agent(s). The unique nature of our dual channel spray drying technology enables the spray drying of two different liquid compositions simultaneously as a single formulation.

Due to limited oral bioavailability of Gef because of its poor aqueous solubility, the SD solid dispersion formulations were prepared. In the current study, we used hydroxy propyl methyl cellulose (HPMC), chitosan, hydroxypropyl β -cyclodextrin (HP β -CD), succinic acid and vitamin E TPGS as formulation ingredients to prepare molecularly dispersed Gef-SD formulations. Inclusion of different polymers in SD formulations exhibit several benefits such as sustained control release and mucoadhesive properties etc.

The anticancer effects of these SD formulations were studied in A431 (human epithelial carcinoma cells, overexpressing the EGFR) induced skin cancer xenograft models. Further, the mechanisms of anticancer effects of Gef-SD formulations were studied by western blotting, immunohistochemical (IHC) and proteomic analysis. The objectives of the present work was to improve the oral bioavailability of Gef through spray dried control release microparticles drug delivery system and evaluate its therapeutic potential in A431 induced skin carcinoma model. We hypothesize that Gef-SD formulations containing HPMC, chitosan and HP β -CD solid dispersions may improve the oral bioavailability, which may lead to enhanced anticancer activity in A431 xenograft models.

Materials

Gefitinib was purchased from AK Scientific, Inc. USA. Hydroxy Propyl Methylcellulose (HPMC, E3 grade) was procured from Dow Chemicals, USA. Chitosan was purchased from Sigma-Aldrich, Inc, USA. Hydroxypropyl β -cyclodextrin (HP β -CD) was procured from Cargill Inc, USA. Vitamin E-TPGS was a kind gift from Antares health products, Inc, USA. The antibodies Ki67, Cyclin D1, p53, survivin, cleaved caspase- 3, (vascular endothelial growth factor) VEGF and vimentin were purchased from Santa Cruz Biotechnology Inc, USA. VEGF ELISA kit was procured from Thermo Fisher scientific Inc, USA. Caco-2 cells and human epidermoid A431 cells were purchased from ATCC, USA. Fetal bovine serum (FBS), Trypsin–EDTA, antibiotic-antimycotic solutions, HBSS and HEPES buffer were purchased from Invitrogen, USA.

Gef *In-Vitro* Activity on A431 Cells

To demonstrate the anticancer activity of Gef on A431 cells, *in vitro* cytotoxicity and clonogenic studies were performed. A431 cells were grown in DMEM media containing 10% fetal bovine serum (FBS). For cytotoxicity assay, 5% FBS was used, A431 cells were

plated (1000/well) in 96 well plates, after 24 h cells were treated with various concentrations of Gef and 24, 48 and 72 h after the treatment cytotoxicity was measured by staining with 0.05% crystal violet. To demonstrate the cell reproductive inhibition of Gef on A431 cells, clonogenic assay was performed. For this assay, A431 cells were placed at a density of 100 cells/well in 24 well plate, after 24 h cells were treated with Gef free drug and Gef-SD. Cells were washed with media 48 h after the treatment and cells were continued to grow for 2 weeks. The individual cells were grown into colonies, which were fixed using glutaraldehyde and stained with 0.05% crystal violet. The groups of cells more than 50 in number were considered as colonies. Colonies were counted under microscope and their sizes were recorded.

Gef-SD Formulation Preparation

By using optimized composition of following ingredients Gef-SD formulations were prepared. Chitosan, HPMC, HP β -CD, succinic acid, vitamin E TPGS and tween 80 were used to prepare the spray dryable liquid dispersion slurry. The composition of the final formulation was as follows: Gef 500 mg, HP β -CD 500 mg, HPMC 500 mg, chitosan 500 mg, succinic acid 180 mg, vitamin E TPGS 200 mg and tween 80 130 mg. In 50 ml of Millipore water required amounts of HP β -CD and Gef were dissolved. Initially, Gef was dissolved in HP β -CD in one container. In a separate beaker, chitosan (2%) solution was mixed with HPMC suspension. Drug (Gef) containing solution and polymer suspensions were mixed together. Vitamin E TPGS was dissolved in few drops of ethyl alcohol and added to the above formulation and finally tween 80 was added through proper mixing to make it a homogenous suspension. The final optimized formulations were spray dried by using custom designed dual channel spray drying system. In dual channel spray gun, one of the liquid channels was fed with the SD formulation homogenous liquid slurry (prepared as described above) and in another liquid channel of spray gun was fed with 2% chitosan solution; this will coat a mucoadhesive chitosan layer on the microparticles during the dual channel spray drying process. The liquid feed stocks are atomized into droplets *via* dual channel nozzle (single spray gun). Nozzles with the help of compressed air pressure atomize the feed. Both drug containing liquid and 2% chitosan liquids were feed simultaneously *via* separate pumps through pulsatile action. Rest of the dual channel spray drying process was carried as per our previous studies (33). Where outer channel of the gun consist of chitosan polymer solution and inner channel will consist of SD formulation liquid slurry, which will be atomized at steady flow rate and pressure. Heated process gas (nitrogen) is brought into contact with the atomized feed using a gas disperser leading to evaporation of liquid portion of the formulation (fixed temperature 140°C).

Gef-SD Drug Release Study

In vitro release was performed in PBS containing 1% Volpo at pH 6.8. In brief, equal quantity of Gef as free drug, Gef-SD and Gef together with formulation components physical mixture were suspended in 500 ml of dissolution medium. Dissolution study was conducted at 37 $\pm$ 0.5°C to see the *in vitro* drug release profile according to the USP type II paddle method at paddle rotation of 50 rpm. Withdrawn samples at different time intervals were analyzed by HPLC method (34). The equal volume of buffer was replaced after every withdrawal of samples for analysis.

Characterization of Gef-SD Formulations

By using differential scanning calorimetry (DSC) instrument (TA instrument, DE) thermograms were recorded. DSC analysis was performed in the temperature range of 30 to 300°C, at the temperature rate of 10°C/min and constant nitrogen purge was maintained at 50 ml/min flow rate. Samples were analyzed as per our reported method (33). With the help of scanning electron microscopy (SEM, Zeiss 1540 XB field emission), the surface characteristics were analyzed. SEM was performed on the carbon support film. The entrapment efficiency of Gef in SD microparticles was studied after extracting the drug in to mobile phase and drug content was analyzed by HPLC method.

Maintenance of Cell Cultures

Caco-2 cells maintained and permeability was performed as per our previous reported method (9). Permeability study was performed at 37°C for 120 min with 100 µM of Gef free drug solution and SD formulations containing equivalent amount of Gef in the donor compartment at pH 5.8 (9).

Animals

Sprague Dawley (SD) rats (300–330 g body weight) and Nu/nu male mice (5–6 weeks old) were procured from Harlan, Inc, USA. Rats were used in pharmacokinetic studies and nude mice were used for anticancer studies. Animal studies were performed as per the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Florida A&M University, protocol number 005–12. Animals were maintained and studies performed at standard experimental condition conditions of 37°C temperature and relative humidity of 60%.

Oral Pharmacokinetic study of Gef-SD

Overnight fasted animals were used for pharmacokinetics (PK) studies. Oral PK of Gef-SD was studied at the dose of 50 mg/kg. At predetermined time points (0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h), blood samples (0.25 ml) were withdrawn from tail vein. Blood samples were collected into heparinized microvet tubes and centrifuged at 10,000 rpm for 10 min separate plasma. Plasma was stored at –80°C until drug analysis performed. Protein precipitation by methanol method was used to extract Gef from plasma and drug content was analyzed by HPLC method. The gradient phase mobile composition was followed to analyze Gef as per reported method elsewhere. The composition of the mobile used was ammonium acetate (20 mM, pH 4.5) acetonitrile. Mobile phase was kept at 45% ammonium acetate and 55% acetonitrile at the time of injection. Five min after the run, the acetonitrile composition was gradually increased to 75% over 6 min. Then, the composition was changed back to ammonium acetate–acetonitrile (45:55) within 6 s. Finally, the chromatographic system was equilibrated (17). The pharmacokinetic parameters such as area under the curve (AUC), C_{max} , $t_{1/2}$, t_{max} , and mean residence time (MRT), etc. were analyzed by using non-compartmental techniques with WinNonlin® 5.0 software, USA.

Anticancer Studies in A431 Cells Induced Epidermoid Carcinoma Models

Gef sensitive A431 epidermoid carcinoma subcutaneous xenograft model was used for anticancer evaluations of Gef-SD. A431 cells (1 million in 100 µl of phosphate buffer) were

subcutaneously injected into the right flank of the mice. Two weeks after the cancer cell injection, drug treatment was started. Gef, 50 mg/kg free drug and Gef-SD formulations were given daily orally for 4 weeks and tumor sizes were monitored every week. Mice were sacrificed 4 weeks after the treatment, tumor volumes and weights were recorded.

Tumor Size and Volume Analysis

Two weeks after the tumor cells implantation, tumor sizes were measured using vernier caliper. At the time of first dose of drug administration, the tumor volumes were approximately 1000 mm³. The greatest longitudinal diameter (length) and the greatest transverse diameter (width) were measured. Tumor volumes were monitored weekly till the termination of the study. At the end of the treatment animals were sacrificed and tumor volumes and weights were measured for assessment of therapeutic efficacy of the formulation. Tumor volumes were calculated by the modified ellipsoidal formula.

$$\text{Tumor volume} = 1/2 (\text{length} \times \text{width}^2)$$

Evaluation of Anticancer Activity

During the study, nude mice body weight changes and survival rates were recorded. Tumor weights and volumes were determined, at the end of study. Representative tumor samples were stored at -80°C for western blotting and proteomics analysis. Tumors and small intestine segments were fixed in formaldehyde and used for histopathology and immunohistochemical (IHC) analysis.

Western Blot Analysis

Protein samples for western blot analysis were extracted from tumor tissues by using RIPA lysis buffer containing protease inhibitors and 500 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration was estimated by using BCA protein assay reagent kit. Equal amounts of 50 µg protein from different groups were denatured by heating for 5 min in SDS sample buffer and protein samples were separated by 10% SDS-PAGE. The protein samples separated on SDS-PAGE were transferred to nitrocellulose membranes for immunoblotting. After blocking the membranes with skim milk (5% skim milk in 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.5% Tween 20) probed with primary antibodies. The primary antibodies such as Bcl-2, Cyclin D1, VEGF, Vimentin and β-actin were used in 1:1000 dilutions. Survivin was used in 1:500 dilution. HRP-conjugated secondary antibodies were used. The detail procedure for western blotting was followed according our published methods (35)

IHC for Cleaved Caspase 3, Ki67 and Vimentin

Slides for IHC studies were processed according to the standardized protocols published in our labs (35). The expression of cleaved caspase-3, Ki67 and vimentin was studied by this method (18).

Estimation of VEGF

In both plasma and tumor lysates VEGF levels were estimated by ELISA method. By using RIPA lysis buffer containing protease inhibitors, tumors were homogenized and tissue debris were pelleted and the supernatant was used for VEGF analysis. By BCA protein assay (Pierce, Rockford, IL), concentration of total protein was determined. ELISA assay was performed according to the suppliers recommendations and VEGF levels were expressed as pg/ml plasma or pg/mg tumor lysates protein. In each group minimum of 4 samples were analyzed.

Histopathology

A431 tumors and segment of ileum specimens were fixed in formalin saline then processed for routine histopathological procedures such as dehydration and rehydration steps. Specimens were immersed in liquid paraffin blocks and 5–10 μ m sections were cut using microtome. Sections after deparaffinization and further rehydration steps, stained with Hematoxylin & Eosin (H&E) stain. Stained Sections were observed under bright field microscope to study the histological changes.

PROTEOMIC ANALYSIS OF TUMOR SAMPLES

Preparation of Samples for Proteomic Analysis

For proteomics analysis samples were prepared according to the FASP protocols are described in Jacek R Wi niewski et al. Nature methods, 2009 (36). Small portion of tumor pieces were collected from tumors of all the groups, 6–8 pieces from each tumor were pooled in each group. Tissues were lysed in SDT-lysis buffer using 1:10 sample to buffer ratio at 95°C for 3–5 min. Then the lysates were centrifuged at 16,000 $\times$ g for 5 min and used for sample processing. Then in a filter unit, the protein extract (30 μ l) was mixed with urea lysis buffer A (UA, 200 μ l) and centrifuged at 14,000 $\times$ g for 40 min. The follow through from the filter unit was discarded. Iodoacetamide solution in UA (100 μ l) was added and mixed at 600 rpm for 1 min and incubated without mixing for 5 min. After centrifuging the filter units at 14,000 $\times$ g for 30 min, 100 μ l of UB (8 M urea in 0.1 M Tris/HCl pH 8.0) was added and centrifuged twice at 14,000 $\times$ g for 40 min. Then Lys-C in UB (40 μ l) was added and mixed at 600 rpm for 1 min. These filter units were incubated in humidified chambers. After overnight incubation, these units were transferred to new collection tubes, then 120 μ l ABC (0.05 M NH₄HCO₃) with trypsin (enzyme to protein ration 1:100) was added and mix at 600 rpm in thermo-mixer for 1 min. The units were incubated at RT for 4 h. then the filter units were centrifuged at 14, 000 $\times$ g for 40 min. 50 μ l 0.5 M NaCl was added and centrifuged the filter units at 14,000 $\times$ g for 20 min. Acidified with CF₃COOH and desalted the filtrate. Solutions like urea A (UA), urea B (UB), and indole acetic acid (IAA) were prepared freshly and used within a day.

Analysis of Samples by Mass Spectrometry

An externally calibrated (445.120025 ambient lock mass) Thermo LTQ Orbitrap Velos nLC-ESI-LIT-Orbitrap (high-resolution electrospray tandem mass spectrometer) was used with the following parameters: nLC-MS/MS was run in technical triplicate to enable normalization and analysis. A 2 cm, 100 μ m i.d. trap column (SC001 Easy Column from

Thermo Scientific) was followed by a 10 cm analytical column of 75 μm i.d. (SC200 Easy Column from Thermo Scientific). Both trap column and analytical column had C18-AQ packaging. Separation was carried out using a Proxeon Easy nLC-II (Thermo Scientific) with a continuous, vented column configuration. The LC eluent was directly nanosprayed into an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). The LTQ Orbitrap Velos was operated in a data-dependent mode and under direct control of the Xcalibur software (Thermo Scientific) during the separation. Full MS scans were acquired in the Orbitrap mass analyzer over the range m/z 400–2000 with a mass resolution of 60 000 (at m/z 400). The 10 most intense peaks with charge state ≥ 2 were selected for sequencing. Further, fragmented in the ion trap with a collision energy of 35%, activation $q=0.25$, activation time of 10 ms, and one microscan. The signal threshold for triggering an MS/MS event was set to 500 counts. Charge state screening was enabled and precursors with unknown charge state or a charge state of 1 were excluded from this study. All measurements were performed at room temperature and three technical replicates of each sample were run to allow for statistical comparisons between samples, which are necessary for label-free quantification.

The raw data (.RAW files) were analyzed by Proteome Discoverer (version 1.4, Thermo Scientific). The search workflow used dual search engines (Mascot and Sequest HT). The resultant msf (mass spectral format) files were used as input to further validate the results using Scaffold (version 4.0, Proteome Software, Inc.).

Statistics

All data are presented as the mean $\pm$ standard error of mean (sem). The significance of difference in treatment groups was determined using one-way ANOVA and Tukey's Multiple Comparison Test using GraphPad prism version 5.0 (San Diego, CA), where value of $p < 0.05$ between the groups was considered as statistically significant difference between these groups. Proteomics statistical analysis was performed in Excel (Microsoft, Redmond, WA) to determine significantly altered proteins. Two parameters determined significance, 1) p -value of < 0.05 and 2) the ratio value. For all the ratios in the control sample the standard deviation (SD) was determined and then significance was defined as ($\pm 2 \times \text{SD}$).

RESULTS

Characterization of Spray Dried Formulation

Thermal properties of Gef-SD formulations were studied by differential scanning calorimetry (DSC) analysis. The characteristic melting endotherm peak of Gef was recorded at $\sim 194^\circ\text{C}$ for free drug. In the physical mixture also Gef exhibited corresponding melting peak at the same temperature point, this indicates that there was not complete interaction of Gef with formulation components upon simple co-mixing. For Gef-SD formulations, the corresponding melting peak of Gef was disappeared, which indicates the dispersion of Gef with SD formulation excipients (Fig. 1a). The DSC analysis indicated that the characteristic melting peak of Gef was not present in SD formulation due to complete molecular level dispersion of Gef in formulation excipients such as HP β -CD, HPMC etc. Where as in physical mixture this type of molecular dispersion was not possible, that is why Gef

characteristic peak is still visible on thermogram but the magnitude is lower than free Gef. The SEM analysis suggested that liquid dispersion of formulation components after spray drying process obtained spherical shaped microparticles with size range of 1–10 μm (Fig. 1b). SEM analysis also suggested the presence of heterogeneous sizes distribution of microparticles. Further, the zeta size analysis indicated that mean size of the particles were 4.76 μm and the entrapment efficiency of Gef into Gef-SD formulation was found to be 98.37%.

***In Vitro* Drug Release Study**

Drug release study indicated that Gef in SD microparticle form produced significant increase in the Gef release. There was initial 20% burst release followed by sustained release over the period of up to 48 h. The Gef-SD behaved like a zero order release profile. The time limit of 48 h was chosen based on the >95% drug release profile in Gef-SD formulations. After 48 h, approximately 95% of drug was released ($p<0.001$) in Gef-SD formulation compared to Gef free drug (only 16% of drug was released even after 48 h). The drug release from the physical co-mixing of all the formulation ingredients used in SD formulation also showed similar release profile like free drug with maximum release of 24% achieved at 48 h (Fig. 2a and b). The lower release profile observed in Gef free drug and physical mixture was due to poor solubility of Gef in these forms compared to Gef-SD, in which drug is released at higher amounts by virtue of solid dispersion pattern. In absence of succinic acid, only 74.81% drug release was observed and in absence of HP β -CD, Gef release was found to be <50%. Based on the individual contributions of excipients to increase drug release, authors selected combination of excipients to prepare final Gef-SD formulation.

Caco-2 Permeability Studies

The trans-epithelial electrical resistance (TEER) values across the tightly packed Caco-2 monolayers were found to be >350 $\Omega\cdot\text{cm}^2$. Further, the average permeability of lucifer yellow (paracellular control) was found to be <0.15 $\times 10^{-6}$ cm/s, both these control experiments conformed the paracellular integrity of Caco-2 cell monolayers. The mean P_{eff} , A–B of Gef free drug across caco-2 cell monolayers was 0.34 $\pm$ 0.09 $\times 10^{-6}$ cm/s, where as in Gef-SD it was found to be 0.69 $\pm$ 0.06 $\times 10^{-6}$ cm/s. The TEER values which are indicators of Caco-2 cell monolayer tight junction integrity was not change significantly upon treatment with Gef free drug and Gef-SD. Before application of Gef free drug and Gef-SD, the TEER values were found to be 362.35 $\pm$ 42.97 $\Omega\cdot\text{cm}^2$, after Gef treatment, TEER values were found to be 365.44 $\pm$ 36.79 $\Omega\cdot\text{cm}^2$, the unaltered TEER values confirms the integrity of monolayers during the Gef-SD permeability studies. The *in vitro* permeability studies indicated the 2-fold increase in the permeability in Gef-SD formulations (Fig. 2c). Since, Gef is BCS class II drug, the observed increased permeability is mainly due to increased solubility of drug in SD form, whereas in freed drug whose aqueous solubility is less than 1 mg/ml, enough drug was not available in apical compartment to permeate in to basolateral side. Further, use of chitosan and vitamin E TPGS in the formulation may also work as permeation enhancers.

Cytotoxicity and Clonogenic Studies

Gef exhibited concentration and time dependent cytotoxicity on A431 cancer cells. The IC_{50} of Gef on A431 cells after 72 h exposure was found to be $1.86 \pm 0.45 \mu M$ suggesting that A431 cells are susceptible to Gef treatment (Fig. 3a and b). The colony formation (clonogenic) study also demonstrated the significant reduction in the number of colonies and colony sizes in Gef (0.5, 2 μM treated groups). Gef-SD formulation also produced significant inhibition in the colonies (Fig. 3c and d). Figure 1e shows the representative A431 colonies after treatment with Gef. Based on these *in vitro* anticancer studies with Gef in free drug and SD formulation, A431 cells were selected for the pharmacodynamic studies of the Gef-SD formulations *in vivo*.

Oral Pharmacokinetics of Gef-SD

In the oral bioavailability studies, Gef-SD at the dose of 50 mg/kg exhibited significant increase in the oral absorption profile compared to Gef free drug (Fig. 4). The plasma C_{max} levels in Gef-SD formulation was 955.28 ± 82.86 ng/ml, whereas in free drug it was 248.43 ± 89.47 ng/ml. The area under the curve (AUC) $_{0-\infty}$ was significantly increased in Gef-SD formulation compared to Gef free drug. The (AUC) $_{0-\infty}$ in Gef formulation treated group was $12,285.24 \pm 1767.38$ ng/ml.h and in the Gef free drug group it was 1342.85 ± 244.45 ng/ml.h (Fig. 4). Similarly, the plasma half-life in Gef-SD was significantly increased. Gef $t_{1/2}$ was found to be 1.25 ± 0.25 h, whereas in Gef-SD, $t_{1/2}$ was increased to 4.25 ± 0.81 h. Mean residence time (MRT) was also increased from 8.85 ± 1.83 h (Gef) to 16.96 ± 1.12 h in Gef-SD formulations (Table I). These improved pharmacokinetic parameters suggested the superior oral bioavailability profile of SD Gef formulation.

Anticancer Study in A431 Xenograft Models

In subcutaneous xenograft mice models, Gef-SD formulation at the dose of 50 mg/kg significantly reduced tumor volumes. The tumor volumes started to decrease 1 week after the treatment initiation and by the end of the treatment period (4 weeks) the final tumor volumes were found to be 6663.01 ± 1113.23 mm³ in vehicle treated controls, where as in Gef free drug groups, these values were 3534.49 ± 896.16 mm³. The significant decrease ($p < 0.001$) in tumor volume was evidenced in Gef SD formulation treated group when compared to Gef free drug and control groups (Fig. 5a and b). Correspondingly, the weights of tumors in Gef-SD group were also significantly reduced (Fig. 5b). Figure 5c shows the representative tumors isolated from control, Gef and Gef-SD treated animals. That means these increased oral absorption of drug in Gef-SD formulation ultimately translated into increased anticancer effects.

Western Blot Analysis

Western blot analysis also further confirmed the superior anticancer effects of Gef-SD. Immuno blot analysis for Bcl-2 expression suggested that the antiapoptotic marker Bcl-2 was decreased in Gef-SD groups compared to other groups (Fig. 6a and b). Quantitative analysis of western blot images suggested that in Gef free drug and Gef-SD groups 2.44 and 7.45-fold, respectively resulted in significant reduction of Bcl-2 expression compared to untreated control tumors. Compared to Gef free drug, in Gef-SD treated tumors, 3.13 fold

significant reduction ($p<0.05$) in the Bcl-2 expression was observed. Significant ($p<0.001$) down regulation of survivin expression was observed in Gef treated tumors. Compared to untreated control tumors, the relative survivin expression were found to be 4.46 and 7.15 fold decreased in Gef free and Gef-SD formulation treated tumors, respectively. Cyclin D1 expression, which plays role in tumor cell proliferation is over-expressed in A431 tumors cells and its expression was significantly down regulated ($p<0.05$) in Gef-SD groups. In comparison with untreated tumors, the relative expressions of cyclin D1 was found to be 1.04 and 5.89 fold decreased in Gef free drug and Gef-SD groups, respectively. Upon comparison with Gef free drug treated tumors, in Gef-SD formulation treated groups, 1.60 (survivin) and 5.66 (cyclin D1) fold significant down regulation was observed. The p53 (tumor suppressor) expressions analysis also further confirmed the superior anticancer effects of Gef-SD. The p53 expression was found to be 2.33 and 6.51 fold significantly upregulated ($p <0.001$) in Gef free drug and Gef-SD treated tumors, respectively (Fig. 6a and d). A 2.78 fold increased in p53 expression was noticed in Gef-SD treated tumors compared to Gef free drug treated groups. Compared to untreated groups, the relative VEGF expression was found to be 1.29 and 5.09 fold significantly decreased ($p <0.01$) in Gef free drug and Gef-SD groups, respectively (Fig. 6a and e). The relative expression of vimentin was found to be 1.18 and 2.20 fold down regulated ($p <0.05$) in Gef free drug and Gef-SD groups, respectively (Fig. 6a and f). Upon comparison with Gef free drug groups, in Gef-SD groups, a 3.94 and 1.86 fold significant down regulation of VEGF and vimentin expression was observed, respectively. All the markers (Bcl-2, survivin, cyclin D1, p53, VEGF and vimentin) studied by western blot analysis demonstrated the enhanced anticancer effects of Gef-SD compared to Gef free drug treated groups in A431 xenograft models.

Immunohistochemical (IHC) Analysis

The IHC analysis of tumors for cleaved caspase 3, Ki-67 and vimentin expression also further confirmed the significant increase of anticancer effects of Gef-SD formulation in A431 xenograft model. The cleaved caspase 3 positive apoptotic cells/field was demonstrated to be 241.33 ± 32.41 in Gef-D formulation whereas in free drug group it was 145.33 ± 28.44 and in untreated control groups it was 34.00 ± 6.26 . Gef-SD formulation treatment resulted in 7.09-fold and 1.66-fold significant increase in the cleaved caspase 3 expression compared to untreated control and Gef free drug groups, respectively (Figs. 7 and 8a). Similarly, the cell proliferation marker Ki67 expression also evidenced the increased anticancer effects of Gef-SD. The significant reduction in Ki67 expression ($p<0.001$) was found in Gef-SD groups compared to other groups. Compared to untreated control tumors, in Gef-SD formulations 4.01-fold decreased expression of Ki67 was observed. Further, the expression of Ki67 was found to be 2.51-fold decreased in Gef-SD treated tumors compared to Gef free drug groups (Figs. 7 and 8). Further, to validate our proteomics data, from the group of 30 significantly altered protein expression patterns, the authors have chosen vimentin to study their expression by IHC analysis. The significant decrease in the EMT mediated metastatic marker vimentin expression ($p<0.001$) was observed in Gef-SD treated tumors compared to free drug and untreated tumors (Fig. 7). Gef-SD formulation produced 3.09 and 1.85-fold reduced expression of vimentin, respectively compared to control and Gef free drug groups (Figs. 7 and 8c). Therefore, it was evidenced that Gef treatment also

works as antimetastatic therapy. This IHC vimentin data further validate/support our reported proteomics data (Table II).

VEGF Analysis

The VEGF concentrations measured by ELISA kits indicated that in control animal serum and in tumor homogenates VEGF levels were higher. In serum and tumor lysates these levels were found to be 572.90 ± 38.51 pg/ml and 355.35 ± 49.93 pg/mg of protein respectively. In serum samples the VEGF levels were significantly decreased after treatment with free drug and SD formulation, these levels were found to be 336.40 ± 55.84 and 170.55 ± 40.40 pg/ml serum, respectively. In tumor lysates also VEGF levels were significantly decreased in Gef free drug and formulation treated tumors. These levels were reported as 175.65 ± 25.76 and 69.53 ± 18.55 pg/mg protein ($p < 0.001$), respectively in Gef free drug and Gef-SD treated groups (Fig. 9a).

Proteomic Analysis

The mass spectroscopic proteomics analysis of our samples suggested that many number of proteins involved in skin cancer pathology were significantly altered in Gef-SD formulation treated tumors compared to untreated and free Gef treated animal tumors. This study identified numerous protein groups, from 151 differentially expressed proteins observed in proteomics study, the authors have chosen 30 most relevant proteins involved in A431 skin cancer, which includes heat shock proteins, tumor suppressors, protein specific binding proteins, angiogenic proteins, metastatic proteins, tumor extracellular matrix related proteins, EMT proteins, metabolic proteins, cancer biomarkers etc. (Fig. 10). When compared to control, the authors have chosen average of 1.5 fold change as the selection criteria to consider that particular protein is significantly altered ($p < 0.05$).

Upon treatment with Gef free drug and Gef-SD, out of selected 30 proteins, 24 were significantly down regulated and 6 proteins were upregulated, and have been listed in Table II. Comparative proteomic analysis indicated the down regulation of several proteins which were found to have crucial role in the tumor initiation and progression. The down regulation of nucleolin ($P < 0.05$) by Gef-SD indicates the proapoptotic potential of Gef to produce anticancer effects. Many of the proteins may have a role in tumor initiation, development, progression, adhesion, invasion and metastases like Annexin A1 ($P < 0.05$), Poly [ADP-ribose] polymerase 1 ($P < 0.05$), Transforming growth factor-beta-induced protein ig-h3 (BIGH3) ($P < 0.05$), were found to be significantly down regulated in Gef-SD treated animals.

Gef in free and Gef-SD formulation forms exhibited significant down regulation of oncoprotein, Filamin-A ($P < 0.05$), Protein disulfide-isomerase A3 ($P < 0.05$), 60 kDa heat shock protein ($P < 0.05$), Prelamin-A/C ($P < 0.05$), and Thioredoxin ($P < 0.001$) suggesting the promising anticancer effect of our formulation. The proteomic analysis further confirmed the significant down regulation of EMT mediated metastatic marker vimentin ($P < 0.05$) expression in Gef and Gef-SD. The proliferating cell nuclear antigen (PCNA) ($P < 0.05$) over expression is a hall mark of many cancers; its expression was significantly down regulated in Gef treated groups. Our studies also demonstrated the significant down regulation of

collagen alpha-1 ($P<0.01$), Cluster of growth arrest-specific protein 6 (Gas6) ($P<0.001$) and Plectin ($P<0.01$) in Gef treated groups.

Plexin-B1 ($P<0.001$) expression was significantly down regulated in Gef-SD treated tumors. Periostin plays role in tumor angiogenesis, partly through activation of VEGF receptors and this protein is significantly ($P<0.05$) decreased in Gef free drug and Gef-SD formulation treated groups. In our tumor model, marked increase in the VEGF ($P<0.001$) levels were found in both serum and tumor lysates, suggesting the role of angiogenesis in the aggressive growth behavior of A431 induced tumor model. In Gef-SD formulation treated groups, in plasma and tumor lysates, significant decrease in the VEGF levels were observed ($P<0.001$).

PPAR- γ coactivator 1- α is reported to be down regulated in several cancer types and this protein levels were significantly increased in Gef treated groups. Peroxisome proliferator-activated receptor gamma (PPAR γ) coactivator-1 alpha (PGC-1 α) significantly ($P<0.05$) increased in Gef-SD treatment. Desmoplakin ($P<0.05$), involucrin ($P<0.05$), Serine protease inhibitor A3K ($P<0.05$) expression were found to be significantly up regulated in Gef-SD treated animals. The cell adhesion proteins like periplakin ($P<0.05$) and envoplakin ($P<0.05$) expressions were also significantly increased in Gef-SD treated animals (Table II).

Chronic Toxicity of Gef-SD

The long-term administration of Gef as free drug or Gef-SD formulation did not produce any sign of toxicity, no change in the body weights were observed among all the groups (Fig. 9b). The Histopathological analysis of (H&E staining) of GIT suggested no abnormalities in histology. Chronic 4 weeks of daily administration of these Gef-SD formulations did not exhibit any sign of toxicity on intestinal tract. These safety studies affirm the safety of our SD formulation (Fig. 9c).

DISCUSSION

Oral administration of anticancer drugs presents a series of advantages for patients. However, most of the drugs are hydrophobic (poor water solubility) and are associated with low bioavailability. The bioavailability of Gef could be greatly increased by improving its solubility and dissolution. This is the first attempt to prepare a Gef-SD formulation in an effort to enhance its pharmacokinetic characteristics in rats and pharmacodynamic properties in A431 xenograft model. This approach would allow a reduction in dose and oral dose-related side effects, such as diarrhea and vomiting (18). Controlled released matrix system with polymers (HPMC, chitosan), surfactants (vitamin E PTGS) and solubility enhancers (succinic acid, HP β -CD) has been used to improve the oral absorption of Gef. Spray drying was used to prepare these formulations and the authors used a customized spray gun which allows simultaneous/pulsatile flow of two different liquid systems through single nozzle. This modification allowed us to formulate controlled released matrix by using drug solution with excipients as one liquid system and chitosan solution as second liquid system to form a mucoadhesive coat on spray dried microparticles.. We observed that succinic acid incorporation in the formulation imparted better solubility for Gef. As Gef exhibits pH dependant solubility, whose solubility is increased in acidic conditions, succinic acid due to its acidic nature increased the solubility of Gef. Another explanation for increased solubility

of Gef in presence of succinic acid is possible co-crystallization process. Further, inclusion of HP β -CD resulted in further improvement in the solubility of Gef. This is expected because HP β -CD has been routinely used for the solubilization of various water insoluble drugs (18,36–38). Other studies also demonstrated the increased dissolution of Gef upon inclusion complex made with cyclodextrins (18). Further, incorporation of HPMC in the SD formulation imparts the control releasing behavior and has also role in increasing the dissolution of Gef (18). The SEM analysis suggested that upon spray drying, microparticles attained spherical shapes of varying sizes. Due to spray drying process at 140°C, fraction of these microparticles loss smooth surface and attained varying shapes..

In vitro drug release studies showed that Gef in SD micro-particles form produced significant enhancement in the dissolution profile. Vitamin E TPGS, Tween 80 and succinic acid improve the stability and solubility of Gef and use of beta cyclodextrin partially increased the solubility of Gef. The sustained release in SD formulation might be due to slow release of drug from microparticle composed of HPMC and chitosan. Though we had seen initial 20% burst release of Gef in SD form, it is not because of un-entrapped drug on the microparticle surface. Because the entrapment efficiency was found to be approximately 98%, and total drug is in solid dispersion form, the dispersed drug loses its characteristic melting peak. Further, varied chitosan coating may also contribute to this type of behavior (chitosan coating delays the drug release) and even varied particle sizes may also result in altered release profile. Since poor solubility is the primary cause for the low absorption of Gef, the dramatic improvement in drug release and dissolution profile demonstrated the increased in apical to basolateral transport of Gef in Gef-SD.

The PK analysis of Gef SD formulation demonstrated superior oral absorption profile than free Gef treated groups. The sustained effect of Gef SD formulations can be attributed to HPMC and Chitosan. HPMC imparts this effect by controlling the drug release from the SD microparticles, whereas chitosan by virtue of its mucoadhesive property increases the residence time of the microparticles (39). These improved pharmacokinetic parameters indicate the increased oral bio-availability of SD Gef formulation. As universally believed, spray dried solid dispersions increase the aqueous solubility of poorly soluble drugs, The increased solubility resulted in significant increase in the oral pharmacokinetic (PK) properties. It is obvious that once the oral bioavailability is increased that will translate into superior therapeutic properties. It is also possible of change of form of drug in spray dried form to an amorphous state which leads to increase in solubility thus resulting in increased PK/PD. There may be possibility of change in the microparticle size distribution contributing to the PK/PD profile, however in solid dispersion, maximum PK/PD is achieved due to solid dispersion mediated increased solubility not because of the change in particle distribution.

In a variety of tumors, EGFR is highly expressed (1) and is therefore a promising target for cancer therapy (6,40,41). The A431 cell line is an epidermoid carcinoma that is known to have increased expression of the EGFR and this cell line is commonly used for assessing the anti-EGFR activities (42). Our *in vitro* cytotoxicity and clonogenic data suggest that A431 cells are sensitive to Gef. This is in agreement with previous studies, which have illustrated the anticancer activities of Gef in A431 cells (43). Our pharmacokinetic studies suggested

that the maximum concentration of Gef achieved with Gef free drug (50 mg/kg) was 248.43 ± 89.47 ng/ml (approximately 0.55 μ M) which is also less than the *in vitro* effective anticancer concentrations, suggesting the need of higher *in vivo* concentrations in order to produce the anticancer effects in A431 tumor models. Further, in Gef-SD formulations, the maximum plasma concentration was found to be 955.28 ± 82.86 ng/ml (approximately 2.13 μ M). This improved plasma concentrations of Gef however does not indicate that optimum anticancer effects would be attained because the biodistribution of the drug into the tumor also plays crucial role in exerting the anticancer actions. Therefore, it is necessary to study the pharmacodynamic effects of Gef-SD formulations.

Significant anticancer effects were noticed in Gef-SD formulation groups as early as 1 week and western blot analysis of Bcl-2, survivin, p53, VEGF and vimentin corroborated the better anticancer effects of Gef-SD. Abundant expression of cell survival marker survivin was seen in untreated control tumors, suggesting the inherent aggressive growth behavior of the A431 tumor types, further, Gef free drug treatment decreased the survivin levels. The Gef-SD formulation brought about the superior anticancer effects by decreasing the survivin expression and increase in cleaved caspase 3 expressions.

The mass spectroscopic proteomics analysis of our samples suggested that number of proteins which are involved in the A431 cancer pathology were significantly altered in Gef-SD groups. The down regulation of nucleolin by Gef-SD indicates the proapoptotic potential of Gef to produce anticancer effects (44). Annexin A1 may play multifaceted role in cancer development, progression, and metastases and its expression was found to be down regulated in Gef-SD treated animals, indicating the superior anticancer potential of Gef-SD (45). Over expression of Poly [ADP-ribose] polymerase 1 was found to be significantly down regulated in Gef-SD groups (46). BIGH3 promotes adhesion and invasion potential of cancer cells (47) and its expression was also significantly down regulated in Gef-SD treated animals.

Peptidylprolyl cis-trans isomerase (Pin1) is over expressed in many cancer types. Both Gef free drug and Gef-SD formulations resulted in significant down regulation of this oncoprotein (48). The metastatic protein filamin-A was significantly down regulated in Gef treated groups suggesting the antimetastatic effects of this drug (49).

The protein disulfide-isomerase A3 which play role in tumor cell invasive is over expressed in A431 tumors was significantly down regulated in Gef-SD treated groups (50). Down regulation of antimetastatic and anti-invasive protein cofilin-1 was observed in Gef treated groups (51). The 60 kDa heat shock protein which was over expressed in various cancer types was significantly down regulated in Gef treated groups (52). Prelamin-A/C has role in invasiveness and potentially a more stem cell-like phenotype of cancer cells and its levels were decreased in Gef groups (53).

Thioredoxin is a small redox-regulating protein, is highly expressed in many cancers, the significant down regulation of this protein in Gef-SD formulation treated groups (54). The proteomic analysis further conformed the significant down regulation of EMT mediated metastatic marker vimentin expression in Gef and Gef-SD (55).

The proliferating cell nuclear antigen (PCNA) over expression is a hall mark of many cancers; its expression was significantly reduced in Gef treated groups (56). Increased expression of collagen alpha-1 may play role in increased extracellular matrix mediated tumor fibrosis and metastasis (57). Gas6 acts as an ligand to the Axl/Tyro3 family of tyrosine kinase receptors and exerts mitogenic activity and are overexpressed in cancer (58). The overexpressed Gas6 protein was significantly decreased in Gef-SD groups suggesting the superior anticancer effects of formulation. Plectin has role in tumor invasion and migration of cancer cells and its expression was demonstrated to be decreased in Gef treated tumors (59).

Plexin-B1 ($P<0.001$) expression pattern was significantly down regulated in Gef-SD treated groups. Periostin plays role in tumor angiogenesis, is significantly ($P<0.05$) down regulated in Gef-SD formulation treated tumors (60). In the Gef-SD treated groups, both the plasma and tumor lysates, showed a significant decrease in the VEGF levels suggesting the anticancer effects observed with Gef-SD formulations might partly be mediated through VEGF inhibition and anti-angiogenesis. Our observations are in agreement with previous reported observations (61) who also reported the anticancer effect of Gef by inhibiting VEGF levels and its receptors. The authors observed that VEGF levels estimated by ELISA in serum and tumor lysates to be reproducible in proteomics analysis of tumor lysates, confirming the role of VEGF inhibition in bringing about the increased anticancer effect of Gef-SD. Further the authors hypothesize that the increased expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2, Pyruvate kinase M1/M2 and L-lactate dehydrogenase A-like 6A play an important role in altered metabolic profile of cancer cells (62–64), as we observed these levels to be significantly down regulated in Gef-SD treated animals (Table II).

PGC-1 α induces apoptosis in several cancer cells, and its expression was found to be decreased in several cancers (65,66), In our study also significant increase in PGC-1 α levels were observed with Gef-SD treatment. We also observed increased desmoplakin expression in Gef-SD, which acts as a tumor suppressor by inhibition of the Wnt/ β -catenin signaling pathway (67).

Epidermal differentiation marker involucrin is reported to be down regulated in squamous cell carcinoma cells (68), further treatment with Gef demonstrated significant increase in the expression of involucrin. We also observed that the serine protease inhibitor A3K which has antiangiogenic effect through inhibition of VEGF production, to be increased in our Gef-SD treatment group (69).

We observed a high expression of vimentin indicating the aggressive behavior of A431 epidermoid tumors (70). Therefore, due to high metastatic ability of subcutaneously injected A431 tumor xenografts to other vital organs, the survival might have decreased in untreated control tumors. Where as in Gef-SD treated animals, significant increase in the animal survival was observed, which can be correlated to the decreased metastasis by reducing the vimentin expression as revealed by western blot, IHC and proteomic analysis. All the three techniques augmented that the vimentin expression was significantly decreased in Gef-SD treated tumors compared to other groups.

The safety of the Gef-SD formulation upon chronic administration was studied by body weight changes and H&E staining on small intestine and both these parameters indicated no signs of toxicity. Further, chronic oral administration of SD formulations did not induce any observable gastric damage indicating their safety. Therefore, Gef-SD formulations developed in this study could be promising suitable oral delivery systems for poorly water soluble anticancer drugs like Gefitinib.

CONCLUSION

Our studies clearly demonstrated that Gefitinib can be successfully incorporated into control release microparticles based oral formulations with significant 9.14- fold increase in the bioavailability. In A431 xenograft tumor model, Gef-SD treatment produced significant increase in the anticancer activity. The molecular studies by western blotting, IHC and proteomics analysis also further confirmed the superior anti-cancer effects of Gefitinib formulation. Based on superior pharmacokinetic and pharmacodynamic profiles of Gefitinib spray dried formulations, we propose that this spray drying technology can become as a potential drug delivery system for poorly water soluble anticancer drugs to treat different cancer types by oral route. The results emanating from these studies demonstrate the potential application of our dual channel spray drying system to improve the therapeutic application of Gefitinib.

Acknowledgments

The authors acknowledge the financial assistance of this research from the National Institute on Minority Health and Health Disparities (NIMHD) P20 program [Grant # 1P20MD006738-03; to M.S.]; and the Department of Defense (DOD) Breast Cancer Program [Grant # W81XWH-11-1-0211 to M.S] and the National Institutes of Health (NIH) SC-1 grant [Grant # 5SC1CA161676-03 to M.S]. The authors thanks for the support of Dr. Ramesh Katam, Assistant professor, FAMU for proteomic analysis and Dr. Jaganmohan Somagoni, Research associate, FAMU for pharmacokinetic experiments during this study.

ABBREVIATIONS

EGFR	Epidermal Growth Factor Receptor
Gef	Gefitinib
Gef-SD	Gefitinib Spray Dried Formulation
HPβ-CD	Hydroxy Propyl Beta-Cyclodextrin

References

1. Huang SM, Harari PM. Epidermal growth factor receptor inhibition in cancer therapy: biology, rationale and preliminary clinical results. *Invest New Drug*. 1999; 17(3):259–69.
2. Johnston JB, Navaratnam S, Pitz MW, Maniate JM, Wiechec E, Baust H, et al. Targeting the EGFR pathway for cancer therapy. *Curr Med Chem*. 2006; 13(29):3483–92. [PubMed: 17168718]
3. Wakeling AE, Guy SP, Woodburn JR, Ashton SE, Curry BJ, Barker AJ, et al. ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res*. 2002; 62(20):5749–54. [PubMed: 12384534]
4. Hara F, Aoe M, Doihara H, Taira N, Shien T, Takahashi H, et al. Antitumor effect of gefitinib ('Iressa') on esophageal squamous cell carcinoma cell lines in vitro and in vivo. *Cancer Lett*. 2005; 226(1):37–47. [PubMed: 16004931]

5. Penne K, Bohlin C, Schneider S, Allen D. Gefitinib (Iressa (TM), ZD1839) and tyrosine kinase inhibitors - The wave of the future in cancer therapy. *Cancer Nurs.* 2005; 28(6):481–6. [PubMed: 16330971]
6. Woodburn JR. The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol Ther.* 1999; 82(2–3):241–50. [PubMed: 10454201]
7. Herbst RS, Fukuoka M, Baselga J. Timeline - Gefitinib - a novel targeted approach to treating cancer. *Nat Rev Cancer.* 2004; 4(12):956–65. [PubMed: 15573117]
8. Djerf EA, Trinks C, Abdiu A, Thunell LK, Hallbeck AL, Walz TM. ErbB receptor tyrosine kinases contribute to proliferation of malignant melanoma cells: inhibition by gefitinib (ZD1839). *Melanoma Res.* 2009; 19(3):156–66. [PubMed: 19434003]
9. Chougule MB, Patel AR, Patlolla R, Jackson T, Singh M. Epithelial transport of Noscapine across cell monolayer and influence of absorption enhancers on in vitro permeation and bioavailability: implications for intestinal absorption. *J Drug Target.* 2014; 22(6):498–508. [PubMed: 24731057]
10. Patel AR, Chougule MB, Townley I, Patlolla R, Wang G, Singh M. Efficacy of aerosolized celecoxib encapsulated nanostructured lipid carrier in non-small cell lung cancer in combination with docetaxel. *Pharm Res.* 2013; 30(5):1435–46. [PubMed: 23361589]
11. Chougule MB, Patel A, Sachdeva P, Jackson T, Singh M. Enhanced anticancer activity of gemcitabine in combination with noscapine via antiangiogenic and apoptotic pathway against non-small cell lung cancer. *PLoS One.* 2011; 6(11):e27394. [PubMed: 22102891]
12. Patel AR, Chougule MB, Lim E, Francis KP, Safe S, Singh M. Theranostic tumor homing nanocarriers for the treatment of lung cancer. *Nanomed Nanotechnol Biol Med.* 2014; 10(5):1053–63.
13. Patel AR, Chougule M, Singh M. EphA2 targeting pegylated nanocarrier drug delivery system for treatment of lung cancer. *Pharm Res.* 2014; 31(10):2796–809. [PubMed: 24867421]
14. Patel AR, Doddapaneni R, Andey T, Wilson H, Safe S, Singh M. Evaluation of Self-emulsified DIM-14 in dogs for oral bioavailability and in Nu/nu mice bearing stem cell lung tumor models for anticancer activity. *J Control Release.* 2015; 213:18–26. [PubMed: 26079185]
15. Andey T, Sudhakar G, Marepally S, Patel A, Banerjee R, Singh M. Lipid nanocarriers of a lipid-conjugated estrogenic derivative inhibit tumor growth and enhance cisplatin activity against triple-negative breast cancer: pharmacokinetic and efficacy evaluation. *Mol Pharm.* 2015; 12(4):1105–20. [PubMed: 25661724]
16. Patel AR, Godugu C, Wilson H, Safe S, Singh M. Evaluation of spray BIO-Max DIM-P in dogs for oral bioavailability and in nu/nu mice bearing orthotopic/metastatic lung tumor models for anticancer activity. *Pharm Res.* :1–9.
17. Trummer BJ, Iyer V, Balu-Iyer SV, O'Connor R, Straubinger RM. Physicochemical properties of epidermal growth factor receptor inhibitors and development of a nanoliposomal formulation of gefitinib. *J Pharm Sci-U.S.* 2012; 101(8):2763–76.
18. Lee YHP, Sathigari S, Lin YJJ, Ravis WR, Chadha G, Parsons DL, et al. Gefitinib-cyclodextrin inclusion complexes: physico-chemical characterization and dissolution studies. *Drug Dev Ind Pharm.* 2009; 35(9):1113–20. [PubMed: 19640249]
19. Friesen DT, Shanker R, Crew M, Smithey DT, Curatolo WJ, Nightingale JAS. Hydroxypropyl methylcellulose acetate succinate-based spray-dried dispersions: an overview. *Mol Pharm.* 2008; 5(6):1003–19. [PubMed: 19040386]
20. Shah PP, Desai PR, Channer D, Singh M. Enhanced skin permeation using polyarginine modified nanostructured lipid carriers. *J Control Release.* 2012; 161(3):735–45. [PubMed: 22617521]
21. Shah PP, Desai PR, Patel AR, Singh MS. Skin permeating nanogel for the cutaneous co-delivery of two anti-inflammatory drugs. *Biomaterials.* 2012; 33(5):1607–17. [PubMed: 22118820]
22. Shah PP, Desai PR, Singh M. Effect of oleic acid modified polymeric bilayered nanoparticles on percutaneous delivery of spantide II and ketoprofen. *J Control Release.* 2012; 158(2):336–45. [PubMed: 22134117]
23. Desai PR, Shah PP, Patlolla RR, Singh M. Dermal microdialysis technique to evaluate the trafficking of surface-modified lipid nano-particles upon topical application. *Pharm Res-Dordr.* 2012; 29(9):2587–600.

24. Vehring R. Pharmaceutical particle engineering via spray drying. *Pharm Res-Dordr*. 2008; 25(5): 999–1022.
25. Bowey K, Neufeld RJ. Systemic and mucosal delivery of drugs within polymeric microparticles produced by spray drying. *BioDrugs*. 2010; 24(6):359–77. [PubMed: 21043544]
26. Sollohub K, Cal K. Spray drying technique: II. Current applications in pharmaceutical technology. *J Pharm Sci*. 2010; 99(2):587–97. [PubMed: 19862804]
27. Nekkanti V, Karatgi P, Prabhu R, Pillai R. Solid self-microemulsifying formulation for candesartan cilexetil. *Aaps Pharmscitech*. 2010; 11(1):9–17. [PubMed: 20013081]
28. Desai PR, Shah PP, Hayden P, Singh M. Investigation of follicular and non-follicular pathways for polyarginine and oleic acid-modified nanoparticles. *Pharm Res-Dordr*. 2013; 30(4):1037–49.
29. Boakye CHA, Doddapaneni R, Shah PP, Patel AR, Godugu C, Safe S, et al. Chemoprevention of skin cancer with 1,1-Bis (3'-Indolyl)-1-(Aromatic) methane analog through induction of the orphan nuclear receptor, NR4A2 (Nurr1). *Plos One*. 2013; 8(8)
30. Andey TA, Marepally S, Singh P, Singh M. Knockdown of annexin A2 by liposomal formulation of annexin A2 small hairpin RNA induces apoptosis and inhibits angiogenesis in lung cancer stem cells. *Cancer Res*. 2013; 73(8):3738.
31. Andey T, Patel A, Jackson T, Safe S, Singh M. 1, 1-Bis (3'-indolyl)-1-(p-substitutedphenyl) methane compounds inhibit lung cancer cell and tumor growth in a metastasis model. *Eur J Pharm Sci*. 2013; 50(2):227–41. [PubMed: 23892137]
32. Andey T, Marepally S, Patel A, Jackson T, Sarkar S, O'Connell M, et al. Cationic lipid guided short-hairpin RNA interference of annexin A2 attenuates tumor growth and metastasis in a mouse lung cancer stem cell model. *J Control Release*. 2014; 184(2):67–78. [PubMed: 24727000]
33. Godugu C, Patel AR, Doddapaneni R, Somagoni J, Singh M. Approaches to improve the oral bioavailability and effects of novel anticancer drugs berberine and betulinic acid. *PLoS One*. 2014; 9(3):e89919. [PubMed: 24614362]
34. Faivre L, Gomo C, Mir O, Taieb F, Schoemann-Thomas A, Ropert S, et al. A simple HPLC-UV method for the simultaneous quantification of gefitinib and erlotinib in human plasma. *J Chromatogr B Anal Technol Biomed Life Sci*. 2011; 879:2345–50.
35. Godugu C, Patel AR, Doddapaneni R, Marepally S, Jackson T, Singh M. Inhalation delivery of Telmisartan enhances intratumoral distribution of nanoparticles in lung cancer models. *J Control Release*. 2013; 172(1):86–95. [PubMed: 23838154]
36. Wi niewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods*. 2009; 6(5):359–62. [PubMed: 19377485]
37. Jablan J, Szalontai G, Jug M. Comparative analysis of zaleplon complexation with cyclodextrins and hydrophilic polymers in solution and in solid state. *J Pharm Biomed*. 2012; 71:35–44.
38. Yuvaraja K, Khanam J. Enhancement of carvedilol solubility by solid dispersion technique using cyclodextrins, water soluble polymers and hydroxyl acid. *J Pharm Biomed*. 2014; 96:10–20.
39. Shelma R, Sharma CP. In vitro cell culture evaluation and in vivo efficacy of amphiphilic chitosan for oral insulin delivery. *J Biomed Nanotechnol*. 2013; 9(2):167–76. [PubMed: 23627043]
40. Knight LA, Di Nicolantonio F, Whitehouse P, Mercer S, Sharma S, Glaysher S, et al. The in vitro effect of gefitinib ('Iressa') alone and in combination with cytotoxic chemotherapy on human solid tumours. *BMC Cancer*. 2004; 4:83. [PubMed: 15560844]
41. Ranson M, Hammond LA, Ferry D, Kris M, Tullo A, Murray PI, et al. ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, is well tolerated and active in patients with solid, malignant tumors: results of a phase I trial. *J Clin Oncol*. 2002; 20:2240–50. [PubMed: 11980995]
42. Engelman JA, Janne PA, Mermel C, Pearlberg J, Mukohara T, Fleet C, et al. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. *Proc Natl Acad Sci U S A*. 2005; 102:3788–93. [PubMed: 15731348]
43. Kaur J, Tikoo K. p300/CBP dependent hyperacetylation of his-tone potentiates anticancer activity of gefitinib nanoparticles. *Bioch et Biophy Acta*. 2013; 1833:1028–40.
44. Ishimaru D, Zuraw L, Ramalingam S, Sengupta TK, Bandyopadhyay S, Reuben A, et al. Mechanism of regulation of bcl-2 mRNA by nucleolin and A+U-rich element-binding factor 1 (AUF1). *J Biol Chem*. 2010; 285:27182–91. [PubMed: 20571027]

45. Wang LP, Bi J, Yao C, Xu XD, Li XX, Wang SM, et al. Annexin A1 expression and its prognostic significance in human breast cancer. *Neoplasma*. 2010; 57:253–9. [PubMed: 20353277]
46. Goncalves A, Finetti P, Sabatier R, Gilabert M, Adelaide J, Borg JP, et al. Poly(ADP-ribose) polymerase-1 mRNA expression in human breast cancer: a meta-analysis. *Breast Cancer Res Treat*. 2011; 127:273–81. [PubMed: 21069454]
47. Yamanaka M, Kimura F, Kagata Y, Kondoh N, Asano T, Yamamoto M, et al. BIGH3 is overexpressed in clear cell renal cell carcinoma. *Oncol Rep*. 2008; 19:865–74. [PubMed: 18357369]
48. Lee TH, Pastorino L, Lu KP. Peptidylprolyl cis-trans isomerase Pin1 in ageing, cancer and Alzheimer disease. *Exp Rev Mol Med*. 2011; 13:e21.
49. Jiang X, Yue J, Lu H, Campbell N, Yang Q, Lan S, et al. Inhibition of filamin-A reduces cancer metastatic potential. *Int J Biol Sci*. 2013; 9:67–77. [PubMed: 23289018]
50. Goplen D, Wang J, Enger PO, Tysnes BB, Terzis AJ, Laerum OD, et al. Protein disulfide isomerase expression is related to the invasive properties of malignant glioma. *Cancer Res*. 2006; 66:9895–902. [PubMed: 17047051]
51. Wang W, Eddy R, Condeelis J. The cofilin pathway in breast cancer invasion and metastasis. *Nat Rev Cancer*. 2007; 7:429–40. [PubMed: 17522712]
52. Hwang YJ, Lee SP, Kim SY, Choi YH, Kim MJ, Lee CH, et al. Expression of heat shock protein 60 kDa is upregulated in cervical cancer. *Yonsei Med J*. 2009; 50:399–406. [PubMed: 19568603]
53. Willis ND, Cox TR, Rahman-Casans SF, Smits K, Przyborski SA, van den Brandt P, et al. Lamin A/C is a risk biomarker in colorectal cancer. *PLoS One*. 2008; 3:e2988. [PubMed: 18714339]
54. Karlenius TC, Tonissen KF. Thioredoxin and cancer: a role for thioredoxin in all states of tumor oxygenation. *Cancers*. 2010; 2:209–32. [PubMed: 24281068]
55. Misra A, Pandey C, Sze SK, Thanabalu T. Hypoxia activated EGFR signaling induces epithelial to mesenchymal transition (EMT). *PLoS One*. 2012; 7:e49766. [PubMed: 23185433]
56. Zhao H, Lo YH, Ma L, Waltz SE, Gray JK, Hung MC, et al. Targeting tyrosine phosphorylation of PCNA inhibits prostate cancer growth. *Mol Cancer Ther*. 2011; 10:29–36. [PubMed: 21220489]
57. Cox TR, Bird D, Baker AM, Barker HE, Ho MW, Lang G, et al. LOX-mediated collagen crosslinking is responsible for fibrosis-enhanced metastasis. *Cancer Res*. 2013; 73:1721–32. [PubMed: 23345161]
58. Sawabu T, Seno H, Kawashima T, Fukuda A, Uenoyama Y, Kawada M, et al. Growth arrest-specific gene 6 and Axl signaling enhances gastric cancer cell survival via Akt pathway. *Mol Carcinog*. 2007; 46:155–64. [PubMed: 17186543]
59. Katada K, Tomonaga T, Satoh M, Matsushita K, Tonoike Y, Kodera Y, et al. Plectin promotes migration and invasion of cancer cells and is a novel prognostic marker for head and neck squamous cell carcinoma. *J Proteomics*. 2012; 75:1803–15. [PubMed: 22245045]
60. Shao R, Bao S, Bai X, Blanchette C, Anderson RM, Dang T, et al. Acquired expression of periostin by human breast cancers promotes tumor angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression. *Mol Cell Biol*. 2004; 24:3992–4003.
61. Pore N, Jiang Z, Gupta A, Cerniglia G, Kao GD, Maity A. EGFR tyrosine kinase inhibitors decrease VEGF expression by both hypoxia-inducible factor (HIF)-1-independent and HIF-1-dependent mechanisms. *Cancer Res*. 2006; 66:3197–204. [PubMed: 16540671]
62. Ros S, Schulze A. Balancing glycolytic flux: the role of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatases in cancer metabolism. *Cancer Metab*. 2013; 1:8. [PubMed: 24280138]
63. Goldberg MS, Sharp PA. Pyruvate kinase M2-specific siRNA induces apoptosis and tumor regression. *J Exp Med*. 2012; 209:217–24. [PubMed: 22271574]
64. Xie H, Hanai J, Ren JG, Kats L, Burgess K, Bhargava P, et al. Targeting lactate dehydrogenase—a inhibits tumorigenesis and tumor progression in mouse models of lung cancer and impacts tumor-initiating cells. *Cell Metab*. 2014; 19:795–809. [PubMed: 24726384]
65. Yu H, Xin Y. Down-regulated expressions of PPARgamma and its coactivator PGC-1 are related to gastric carcinogenesis and Lauren's classification in gastric carcinoma. *Chin J Cancer Res*. 2013; 25:704–14. [PubMed: 24385698]

66. Zhang Y, Ba Y, Liu C, Sun G, Ding L, Gao S, et al. PGC-1alpha induces apoptosis in human epithelial ovarian cancer cells through a PPARgamma-dependent pathway. *Cell Res.* 2007; 17:363–73. [PubMed: 17372612]
67. Yang L, Chen Y, Cui T, Knosel T, Zhang Q, Albring KF, et al. Desmoplakin acts as a tumor suppressor by inhibition of the Wnt/beta-catenin signaling pathway in human lung cancer. *Carcinogenesis.* 2012; 33:1863–70. [PubMed: 22791817]
68. Anand S, Honari G, Hasan T, Elson P, Maytin EV. Low-dose methotrexate enhances aminolevulinate-based photodynamic therapy in skin carcinoma cells in vitro and in vivo. *Clin Cancer Res.* 2009; 15:3333–43. [PubMed: 19447864]
69. Zhu B, Lu L, Cai W, Yang X, Li C, Yang Z, et al. Kallikrein-binding protein inhibits growth of gastric carcinoma by reducing vascular endothelial growth factor production and angiogenesis. *Mol Cancer Ther.* 2007; 6:3297–306. [PubMed: 18089723]
70. Kao WT, Lin CY, Lee LT, Lee PP, Hung CC, Lin YS, et al. Investigation of MMP-2 and -9 in a highly invasive A431 tumor cell sub-line selected from a Boyden chamber assay. *Anticancer Res.* 2008; 28:2109–20. [PubMed: 18751383]

Fig. 1. Characterization of spray dried formulation. **(a)** Differential scanning calorimetric analysis of Gef in free drug, Physical mixture and Gef-SD forms. **(b)** Scanning electron microscopic analysis of Gef-SD microparticles by Zeiss FESEM.

Fig. 2.

In vitro characterization of Gef-SD. **(a)** Drug release profile of Gef in free drug, physical mixture and Gef-SD forms. **(b)** Drug release profile of Gef after 48 h. **(c)** Permeability of Gef through Caco-2 monolayers, percentage of Gef permeated into basolateral compartment. Data was represented as mean $\pm$ sem ($n=3-4$). *** $p<0.001$ a Vs control and b Vs Gefitinib free drug groups.

Fig. 3.

Gef *in vitro* cytotoxicity and effect on colony formation. **(a)** The cytotoxicity of Gef on A431 cells 24, 48 and 72h after treatment. **(b)** IC₅₀ values of Gef 24, 48 and 72 h after the treatment. **(c)** Effect of Gef on spheroid number and **(d)** on spheroid sizes. **(e)** Microscopic images of A431 colonies from *a*) control, *b*) Gef 0.5 μM, *c*) Gef free drug 2 μM and *d*) Gef-SD 2 μM treated groups. Data was represented as mean±sem ($n=6-10$). *** $p<0.001$ Vs respective untreated controls.

Fig. 4. Pharmacokinetic profile of spray dried formulation. (a) Plasma concentration profiles of Gef-SD. Sprague Dawley Rats were administered orally at a dose equivalent to Gef 50 mg/kg. Each data point was shown as mean $\pm$ sem ($n=4-5$).

Fig. 5.

Anticancer effects of Gef-SD formulation in A431 xenograft model. (a) A431 tumor volume changes upon Gef free drug and Gef-SD treatments for 4 weeks, (50 mg/kg dose, orally), (b) Final tumor volumes 4 weeks after the Gef free drug and Gef-SD treatment, (c) Final tumor weights 4 weeks after the Gef free drug and Gef-SD treatment. (d) Representative A431 tumor images collected from different groups. Data was presented as mean $\pm$ sem ($n=6-8$). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ a Vs respective untreated groups and b Vs Gefitinib free drug treated groups.

Fig. 6.

Western Blot analysis of A431 tumor lysates. (a) Western blot images of different protein expressions in tumor lysates. Quantitative analysis of expressions of (b) Bcl-2 (c) Survivin (d) Cyclin D1 (e) p53 (f) VEGF and (g) vimentin. Data was presented as mean±sem ($n=3-4$). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ a Vs respective untreated groups and b Vs Gefitinib free drug treated groups.

Fig. 7. IHC analysis of A431 tumors. IHC analysis of tumor sections for cleaved caspase 3, Ki-67 and vimentin expression. In the first row, images indicate cleaved caspase-3 expression in different groups. In second row, images show the Ki67 expression. Third row images represent vimentin expression. In all three markers studied, brown color stained cells designate the positive expression.

Fig. 8.

IHC analysis and H&E staining. (a) Quantification of cleaved caspase 3, Ki67 and vimentin expression by IHC analysis. 10 fields were randomly counted in each slide and 4 slides per group were used. Data was shown as mean $\pm$ sem ($n=3-4$). * $p<0.05$ and *** $p<0.001$ a Vs respective untreated groups and b Vs Gefitinib free drug treated groups. (b) Representative H&E stained histopathological images of A431 xenograft tumor sections.

Fig. 9. VEGF levels and toxicity study. **(a)** VEGF levels in serum and tumor lysates ($n=3-4$). **(b)** Body weights changes throughout Gef free drug and Gef-SD treatment. Data was represented as mean $\pm$ sem ($n=6-8$). ** $p<0.01$ and *** $p<0.001$ a Vs respective untreated group and b Vs Gefitinib free drug treated group. **(c)** Representative microscopic images of H&E stained ileum sections after treatment with Gef-SD.

Fig. 10. Proteomic analysis of tumor samples. Total 151 proteins were identified and classified according to their function. The number of proteins per category is indicated in brackets.

Table I**Pharmacokinetic Profile of Gef Free Drug and Gef-SD**

Parameter	Gef free drug	Gef-SD
C _{max} (ng/ml)	248.43±89.47	955.28±82.86
t _{max} (h)	1.25±0.25	4.25±0.81
AUC (0-∞) ng/ml*h	1342.85±244.45	12,285.24±1767.34
AUMC (0-∞) ug/ml*h ²	11,570.60±2688.19	208,100.72±30,906.52
t _{1/2} (h)	7.46±1.75	13.79±2.77
V _{ss} (ml/kg)	0.45±0.22	0.08±0.009
CL (ml/kg/h)	0.08±0.0017	0.004±0.0005
MRT (h)	8.85±1.83	16.96±1.12

The pharmacokinetic parameters of Gef in Gef free drug and Gef-SD formulations groups upon administration of 50 mg/kg orally

Table II

Proteomics Analysis of Tumor Samples

S. No	Protein name	Fold change Gef/Ctrl	t-test P value (Gef/Ctrl)	Fold change Gef-SD/Ctrl	t-test P value Gef-SD/Ctrl	Fold change Gef-SD/Gef	t-test P value Gef-SD/Gef
Proteins which are down regulated after treatment with Gefitinib							
1	Cluster of Isoform L-VEGF165 of Vascular endothelial growth factor A	1.0055	P>0.05	4.11	P<0.001	4.11	P<0.001
2	Vascular endothelial growth factor C	0.96	P>0.05	2.62	P<0.001	2.72	P<0.001
3	Nucleolin	1.76	P<0.001	1.96	P<0.05	1.11	P>0.05
4	Annexin A1	1.31	P<0.05	2.06	P<0.01	1.57	P<0.05
5	Peptidylprolyl cis-trans isomerase A	2.15	P<0.001	1.87	P<0.001	0.86	P>0.05
6	Filamin-A	3.88	P<0.001	4.91	P<0.001	1.26	P<0.05
7	Protein disulfide-isomerase A3	1.07	P>0.05	1.63	P<0.01	1.51	P<0.01
8	Histone H2B type 1	1.19	P>0.05	3.64	P>0.001	3.03	P<0.001
9	Cofilin-1	1.79	P>0.01	1.56	P>0.05	0.86	P>0.05
10	60 kDa heat shock protein, mitochondrial	2.42	P>0.001	1.96	P>0.001	0.81	P>0.05
11	Prelamin-A/C	2.42	P<0.001	2.45	P<0.001	1.01	P>0.05
12	Thioredoxin	1.61	P<0.01	4.91	P<0.001	3.03	P<0.001
13	Vimentin	3.68	P<0.001	4.49	P<0.001	1.21	P<0.05
14	Proliferating cell nuclear antigen	1.60	P<0.01	1.93	P<0.001	1.20	P<0.05
15	Isoform 2 of Collagen alpha-1	2.38	P<0.001	3.48	P<0.001	1.46	P<0.01
16	Cluster of Growth arrest-specific protein 6	1.61	P<0.01	10.44	P<0.001	6.48	P<0.001
17	Plexin-B1	1.79	P<0.001	3.86	P<0.001	2.15	P<0.001
18	Cluster of Periostin	2.64	P<0.001	3.09	P<0.001	1.17	P>0.05
19	Cluster of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2	1.26	P<0.05	3.39	P<0.001	2.68	P<0.001
20	L-lactate dehydrogenase A-like 6A	1.02	P>0.05	2.05	P<0.001	1.01	P>0.05
21	Pyruvate kinase isozymes M1/M2	1.42	P<0.05	1.50	P<0.05	1.05	P>0.05
22	Cluster of Poly [ADP-ribose] polymerase 1	2.01	P<0.001	2.34	P<0.001	1.16	P>0.05
23	Cluster of Isoform 4 of Plectin	1.48	P<0.01	2.09	P<0.001	1.41	P<0.01
24	Cluster of Transforming growth factor-beta-induced protein ig-h3	2.64	P<0.001	3.09	P<0.001	1.17	P>0.05

Proteins which are up regulated after treatment with Gefitinib

S. No	Protein name	Fold change Gef/Ctrl	t-test P value (Gef/Ctrl)	Fold change Gef-SD/Ctrl	t-test P value Gef-SD/Ctrl	Fold change Gef-SD/Gef	t-test P value Gef-SD/Gef
25	Desmoplakin	3.70	$P<0.001$	2.37	$P<0.001$	0.64	$P<0.05$
26	Serine protease inhibitor A3K	4.32	$P<0.001$	5.49	$P<0.001$	1.26	$P<0.05$
27	Involucrin	25.11	$P<0.001$	29.77	$P<0.001$	1.18	$P>0.05$
28	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	6.98	$P<0.001$	7.68	$P<0.001$	1.10	$P>0.05$
29	Periplakin	7.07	$P<0.001$	8.69	$P<0.001$	1.23	$P<0.05$
30	Cluster of Envoplakin	3.08	$P<0.001$	3.70	$P<0.001$	1.20	$P<0.05$

Details of the proteins up-regulated or down regulated following treatment with Gefitinib free drug or Gef-SD. The change in the expression was represented as fold change compared to control groups.

* $p<0.05$,

** $p<0.01$ and

*** $p<0.001$ Vs respective control groups or respective Gefitinib free drug treated groups