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Antitumor activity of NPB001-05, an orally active inhibitor of Bcr-Abl tyrosine kinase

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1. ABSTRACT

Scientists are constantly searching for phytochemical compounds with anti-cancer activity. In this study, activity of plant extract NPB001-05 from *Piper betle* was tested on human chronic myelogenous leukemia (CML) xenograft models. NPB001-05 was active when dosed orally (500 mg/kg) once or twice a day in xenograft tumor models. NPB001-05 showed activity to T315I tumor xenograft, where imatinib failed to show antitumor activity. NPB001-05 showed no relevant toxicity in animal models during 2 weeks exposure to drug. Responsive tumor showed inhibition of tyrosine kinase activity with lowered Bcr-Abl protein levels and increased apoptosis. Microarray based transcription profiling studies demonstrated that both imatinib and NPB001-05 dysregulated imatinib- responsive genes. NPB001-05 showed additional genes selectively dysregulated from ER stress, PI3K/AKT, MAPK pathways. Additionally, we tested gene expression of *PI3K*, *AKT1*, *JUN*, *CASP3* and *DDIT3* in K562, BaF3P210$^{BCR-ABL}$ and BaF3 P210$^{BCR-ABLT315I}$ cell line treated for 6- and 12- hours with NPB001-05 and imatinib. The data indicates that NPB001-05 mediated cell death in K562 affects the function of ER stress. NPB001-05 shows antitumor activity with favorable toxicity profile.

2. INTRODUCTION

Due to the raising concern of the general public and efforts in finding new biologically active elements, scientist around the world started screening plants for discovering new phytochemicals. Phytochemicals derived form plants are known to have various bioactivities, including anticancer properties (1). Several studies claim that extracts from natural products have ameliorating effect on cancer as compared to chemotherapy and characterized by their multiple mechanism of action and also minimal side effects (2-5). *Piper betle* is a evergreen and perennial herb native to South East Asia (India, China, Bangladesh and Sri Lanka). Due to the variety of chemical and aroma composition, betel leaves are widely used as masticatory in Asia. They are valued as mild stimulant, digestive and for numerous medicinal properties such as catarrhal and pulmonary affection (6). The phenolic constituents (allylpyrocatecol) of the plant showed anti-microbial activity against obligate oral anaerobes that cause halitosis (7) and crude extract of leaf has stimulatory influence on pancreatic lipase activity (8). It was observed that the leaf extract of *Piper betle* has antioxidant activity and increased the activity of superoxide dismutase, in a dose-dependent

manner in Swiss albino mice (9). Piper leafs are also shown with significant hepatoprotective effect, that improved the tissue antioxidant status by increasing the levels of non-enzymatic antioxidants (Vitamin C and E, glutathione) and activities of free radical-detoxifying enzymes in liver and kidney in rats (10). Extracts are also reported to feature antimicrobial, anti-larvicidal, antioxidant, tyrosinase inhibition activity (11-13), in addition to healing property against stomach ulceration (14) and anti-diabetic activity (15).

Piper betle belongs to the family *Piperaceae*, and chemical studies of the plant extract have shown molecular components such as flavonoids, alkaloids, liganans, aristolactams, unstaturated amides, trepenes, steroids and prophenylphenols (16). Current study was done to explore the varied pharmacological components of *Piper betle* for use as chemotherapeutic or chemopreventive agent particularly for chronic myelogenous leukemia (CML). Thus, making a research effort focusing on novel drugs from plant kingdom in search of tyrosine kinase inhibitors and cancer cures (1, 17).

The leukemogenic events in chronic myelogenous leukemia takes place in hematopoietic stem cells by a reciprocal chromosomal translocation t(9;22)(q34;11.2) leading to chromosome 22 denoted as the Philadelphia chromosome (Ph). The translocation events contributes to the formation of Bcr-Abl fusion protein that confers a constitutive tyrosine kinase activity, essential for the transforming capacity and eventually CML pathophysiology (18, 19). The discovery of selective tyrosine kinase inhibitors such as imatinib has revolutionized the treatment of CML and has initiated a new paradigm for the molecularly-defined cancer therapy (20) as compared to conventional drugs such as cytarabine and alpha-interferon (21). Clinical trail reports of imatinib treatment resulted in event-free survival of 83% and the overall survival of close to 90% in CML patients (22). However, a substantial number of patients receiving imatinib develop resistance to the drug (23, 24), due to mutations in the Bcr-Abl kinase domain which can be circumvented in part by second-generation tyrosine-kinase inhibitors (25, 26). It has been realized that imatinib and second-generation inhibitors are not believed to cure CML, suggesting some limitation of this therapeutic approach (26-28).

The cancer drug discovery program at Piramal Life Sciences Limited (PLSL) has resulted in a flavone (P276-00), Cdk4 kinase inhibitor currently in clinical evaluation against myeloma tumors (29, 30). The goal of the current study was to evaluate natural products in tyrosine kinase inhibition and test antitumor activity, especially by blocking the mutant Bcr-Abl kinase activity which would offer an alternative approach to the treatment of imatinib-refractory CML. NPB001-05 is a natural product from *Piper betle* identified as inhibitor of Bcr-Abl tyrosine kinase. NPB001-05 shows antitumor activity in T315I mutant and wild type Bcr-Abl xenograft tumor models. The present study evaluates the potential anti-CML effects of NPB001-05 on Bcr-Abl expressing tumor xenograft, and the possible mechanisms are also investigated. The conclusion from this study suggests that NPB001-05 exhibits potential antitumor effect and merits further clinical investigation as a potential therapeutic agent against CML.

3. MATERIALS AND METHODS

3.1. Cell culture and drug treatments

Bcr-Abl positive K562 cell line (31) were purchased from American Type Culture Collection (LGC Promochem, Bangalore, India) where as $BaF3^{P210Bcr\text{-}Abl}$ and $BaF3P210^{Bcr\text{-}AblT315I}$ cell lines (32) were obtained from Dr. Brian Druker (Oregon Health and Science University, Oregon, USA). All cell lines were maintained as suspension cultures in RPMI1640 medium supplemented with 10 % fetal bovine serum (FBS) as per standard cell culture procedures (33).

3.2. Animals

Severe Combined Immune-Deficient (SCID strain-CBySmn.CB17-Prkdcscid/J) male mice, 5-8 weeks old, weighing 16 – 24 g was obtained from Jackson Laboratory, USA (Stock number 001803). Six to eight weeks old male Swiss mice were procured from the animal house facility at PLSL were used in preliminary toxicity study for NPB001-05. All animal experiments were conducted as per institutional animal ethics committee protocols, accepted by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines (34) and in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1985). Animals were maintained under clean-rooms conditions in sterile filter-top cages (Tecniplast SpA, Italy).

3.3. Drug preparation and administration

For *in-vivo*, doses of NPB001-05 (PLSL, India) and imatinib (Natco Pharma Limited, Hyderabad, India) were prepared in appropriate amount of sterile water to adjust injectable volume of less than or equal to 10 ml/kg body weight. NPB001-05 and imatinib were dissolved in sterile water, if required NPB001-05 aggregate suspension was homogenized to obtain uniform slurry. All mice were administered via per oral (p.o) route using a gavage needle (Harvard-Apparatus, South Natick, MA, USA) with an injection volume not exceeding 10 ml/kg of body weight. Control animals (untreated) groups were dosed with water. For *in-vitro* assays, NPB001-05 and imatinib were prepared as stock solution of 100 mg/ml and 100 mmol/L in DMSO, respectively and diluted in the culture media. Vehicle control were kept with 0.1% DMSO treatment.

3.4. *In-vivo* experiments and mouse xenograft models

A preliminary dose finding experiments were performed in Swiss mice where a single (acute) and multiple (chronic) maximum tolerated dose study for NB001-05 was conducted in 6-8 weeks old Swiss mice, weighing 20 - 26 g. Animals were observed for relevant clinical signs for every day till 25 days. For efficacy study 6-8 x 10^6 (K562, BaF3 $P210^{BCR\text{-}ABL}$ and BaF3 $P210^{BCR\text{-}ABLT315I)}$ cells were injected subcutaneously (s.c.) on the

right hind flank of SCID mice. When the tumors attained an average diameter of 5-6 mm, the mice were randomized into experimental group of 10 mice each and the dosing was initiated by oral route. NPB001-05 was administered 500 twice daily (b.i.d) or 500 and 250 once daily (o.i.d) mg/kg/day, whereas imatinib was given at 150 mg/kg/day for a period of 14 days. Control animal groups received water as vehicle control. Studies were terminated when tumor in vehicle treated animals reached an average size of approx. 2000 mg or when the tumor were judged to adversely effect the well being of the animals. Tumor growth was evaluated by 2 - 4 times weekly measurement of tumor volumes using a vernier-caliper (Mitotoya, Japan) and continued until 25 day. Tumor volume was calculated according to the formula of prolate ellipsoid: Tumor volume (mm^3) = [length (mm) X $width^2$ (mm)]/2 (35). Relative tumor volumes (RTV) representing tumor growth curves, measured from mean tumor volume from day 0 (treatment start), were normalized to tumor volumes for following days (+/- SD) *vs.* days after drug injection, were plotted. *In-vivo* antitumor efficiency was determined as percent treated to control ratio (% T/C) at different time points according to the formula: % T/C (day X) = [(T_tX-T_t0)/ (T_cX-T_c0) X 100] where T_tX is mean tumor volume of test drug on day X, T_t0 is mean tumor volume of test drug on day 0, T_cX is mean tumor volume of control on day X, T_c0 is mean tumor volume of control on day 0. Since, tumor was established and often large at the time of treatment beginning, relative percentage of tumor growth inhibition (%TGI) due to drug treatment was calculated as: %TGI = [100-T/C %]. The minimum antitumor activity was defined as a continuous %TGI more than or equal to 50 % for any test set. Treated animals were checked daily for the treatment related toxicity or mortality.

3.5. RNA extraction and microarray analysis

Total RNA was isolated from four tumor of control, NPB001-05 and imatinib treated animals as well as drug treated cells (NPB001-05: 10 µg/ml and imatinib: 1 µmol/L) using Trizol Reagent (Invitrogen Corporation, Carlsbad, USA) followed by RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and Dnase treatment. RNA samples were evaluated for quantity and quality by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA) and Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Total RNA (10 – 15 µg) was converted into cDNA using amino-allyl dNTP's followed by Cy5/Cy3 (GE Healthcare, USA) labeling using Promega Chipshot Labeling kit as described by manufacturer's protocol. Labeled RNA was hybridized to 34K human 70-mer oligo chip using universal microarray hybridization kit (Corning Incorporated, NY, USA) and scanned using (GeneTAC GT UC scanner, Genomic Solutions Inc., USA). Image analysis and quantification of scanned data was done using GeneTAC Integrator (Genomic solutions Inc., USA) followed by data analysis using GeneSpring GX-9 (Agilent Technologies, Santa Clara, California). Data was log transformed and normalized by Lowess method (36). Log2 fold change in expression for genes were calculated with a fold change of 1.8 fold or greater deviation in expression is considered as differentially regulated and used for further analysis. Unsupervised hierarchical clustering (37) for global expression profile and K-means clustering analyses was done to obtain differentially regulated gene lists patterns (38-40), which were further enriched and grouped for biological annotation using Panther database (41). (http://www.pantherdb.org).

3.6. Gene expression studies using RT-qPCR

For validation of microarray gene expression data, 2 µg of total RNA was converted into cDNA as described by manufacture's protocol (Invitrogen Inc, Carlsbad, CA) and amplified and quantified using Qiagen Quantifast master-mix (Qiagen Inc, Valencia, CA) and Eppendorf Realplex thermal cycler (Eppendorf, Hamburg, Germany). The primer and probe sequences were designed by Primer 3 v.0.4.0 (42) (http://frodo.wi.mit.edu/primer3 web site) using published genetic sequences and provided in Table 1. All gene expression was normalized with GAPDH. Data were analyzed using the realplex software (Eppendorf, USA). The relative expression of each gene was calculated using relative Ct method (43).

3.7. Expression of protein markers

Treated cells or tumor tissues were harvested and washed once with cold phosphate-buffered saline (PBS) and lysed using Cell lysis buffer (CellLytic, Sigma Aldrich, India). Cell lysates were centrifuged for 20 minutes at 13000 rcf , 4°C and supernatants were stored at -70 °C in aliquots. For protein analysis, protein lysates was resolved on a sodium-dodecyl-sulfate (SDS) /12.5 % polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membrane (Millipore, India). Membranes were blocked for 1 hour at room temperature with 5 % non fat dry milk (Bio-Rad, USA), probed overnight at 4°C with primary antibody (Santa Cruz Biotechnology, USA) followed by incubation with HRP-conjugated secondary antibody (Sigma-Aldrich, India) and were detected using chemiluminescent substrate (Pierce Biotech, USA). Immunoprecipitation of Bcr-Abl tyrosine kinase protein was performed for tumors samples both treated and control after tumor homogenization as described earlier (44, 45). Briefly, immune-complexes were harvested from tumors cells by immunoprecipitation with anti-c-Abl (Abcam, UK) antibody and protein A-Sepharose beads. Immune complexes were incubated in kinase buffer containing labeled ATP (BRIT, India), resolved on SDS-PAGE and phosphorylation of Bcr-Abl was analyzed by autoradiography. ImageJ software (46) was used for densitometry analysis.

4. RESULTS

Background: NPB001-05 (International Application PCT/IB2007/050536) is a natural product from leaves of *Piper betle* and found to inhibit Bcr-Abl expressing cells *in-vitro*. NPB001-05 inhibited the proliferation of both wild and clinically relevant imatinib-resistant Bcr-Abl mutant cells in proliferation assays (data not shown). NPB001-05 inhibited both wild type $P210^{Bcr-Abl}$ and $P210^{Bcr-Abl-T315I}$ kinase activity as well as autophosphorylation in time- and dose- dependent manner (data not shown). In this report, we analyzed the *in-vivo* antitumor properties and evaluated the gene expression profile for NPB001-05 and imatinib

Table 1. Primer sequences used for RT-qPCR analysis

Gene	Forward Primer	Reverse Primer
AKT1	TCGGAGACTGACACCAGGTA	CTGGCCGAGTAGGAGAACTG
SKI	GAAAGAGGCCAACGAGTCAC	CTGCAGGTCTTCGATCTGG
PIK3CB	CATCACTCTCTTTGCGCTGA	CCTGAGCGCCTCATCAAAT
DDIT3	CCTGCAAGAGGTCCTGTCTT	TGACCTCTGCTGGTTCTGG
PPP1R15A	GCCCAGAAACCCCTACTCAT	CAGACAGCCAGGAAATGGAC
JUN	AGGAGGAGCCTCAGACAGTG	AGCTTCCTTTTTCGGCACTT
CASP3	GAACTGGACTGTGGCATTGA	TGTCGGCATACTGTTTCAGC
DDX58	CTGCTCTGCAGAAAGTGCAA	TTGAAAACTGCTTTGGCTTG
RAD54L	AGAGCCACAAGAAGGCACTG	GTGCAGTCAGAACCATCAGG
MAN1B1	CAGCAGACAGGCACAACCT	CTAGGCTCGGGCTTCTGAG
BNIP1	CCTCATGGGGATCAGCAG	CTCCCGGCGATTGTATTTT
GAPDH	GCATCCTGGGCTACACTGAG	CCCTGTTGCTGTAGCCAAAT
Pik3cb	GGCATGGGAATCTCTTCATC	GCCTCGTCAAACTTCTGCTT
Akt1	CCCAGGTCACCTCTGAGACT	GCTTCTGGACTCGGCAATG
Jun	ACCACTTGCCCCAACAGAT	CTTCCTTTTCCGGCACTTG
CAsp3	GGGTACGGAGCTGGACTGT	TGCAAAGGGACTGGATGAAC
Ddit3	GGAGGTCCTGTCCTCAGATG	TCCTCCTGGGCCATAGAACT
Gapdh	TGAGGACCAGGTTGTCTCCT	ATGTAGGCCATGAGGTCCAC

Table 2. Acute and chronic dose toxicity of per oral NPB001-05 in Swiss mice

Schedule	NPB001-05 (mg/kg p.o)[3]	Day 25 Survivors/total (n = 10)[4]	Days of deaths[5]
Single dose[1]	3000	3-4/10	2,3,4
	2000	10-10/10	(no deaths)
	1000	10-10/10	(no deaths)
5 dose (daily)[2]	2000	4-6/10	4,5,6,7
	1000	10-10/10	(no deaths)
	500	10-10/10	(no deaths)
	250	10-10/10	(no deaths)

[1]Day 1 (single dose schedule), [2]Day 1-5 (five dose schedule), [3]Injection volumes 10ml/kg of body weight, [4]Range of values in three experiments from single or multiple dose schedules, [5]Dead animals were counted daily, observation until 25 days

4.1. Efficacy/toxicity studies *in-vivo*

The *in-vivo* efficacy of NPB001-05 was studied in xenograft models by subcutaneous administration of Ph^+ CML K562 and $P210^{Bcr\text{-}Abl}$ expressing BaF3 cells. Prior to efficacy, a preliminary toxicity study of orally administered NPB001-05 in non-tumor bearing (normal) mice was conducted using Swiss mice

4.1.1. Per-oral toxicity studies

A preliminary toxicity study was done by administration of a single dose (acute) of the drug at different concentration by oral route in mice. Mice received a single dose of NPB001-05 at randomly chosen doses of 3000-, 2000-, and 1000- mg/kg in 10ml/kg of body-weight. Weights of the mice were recorded on 1-, 5-, 10- and 25- days. It was observed that by day 25, sixty percent mice died in 3000 mg/kg NPB001-05 treated group (Table 2) whereas animals injected with 2000- and 1000- mg/kg NPB001-05 showed no symptoms toxicity or weight loss (Table 2). In case of 3000 mg/kg group, death occurred within 2- 4days after NPB001-05 treatment with the uniform results among experimental replicates. Data from single dose study was used to set subsequent studies with multiple dose administrations. Since 3000 mg/kg NPB001-05 was substantially toxic, lower dose levels were selected for multiple dose regimens. Animals received a single dose of NPB001-05 (2000-, 1000-, 500- and 250- mg/kg) for 5 consecutive days whereas control animals were given water. No toxicity was observed after oral administration of NPB001-05 in case of 1000- and 500- mg/kg group, where as more than or equal to 50 % mice died at 2000 mg/kg group between days 4-7 with extensive (Figure 1A, Table 2) weight loss, a hunched posture and ruffled fur. As noted above, the lower doses (1000- and 500- mg/kg) of NPB001-05 showed no mortality or weight loss as control animals. There fore mice receiving 1000- and 500- mg/kg of NPB001-05 were selected for efficacy studies in SCID mice.

4.1.2. Effect of NPB001-05 on human chronic myelogenous leukemia cells *in-vivo*

Human Ph^+ cell line K562 was used to evaluate the antitumor effect of NPB001-05. Oral administration of NPB001-05 (once daily or twice daily for 500 mg/kg) was initiated on 5th day after tumor inoculation and continued for two weeks. It was observed that NPB001-05 inhibited the growth of tumors in a dose-dependent manner (Figure 2A, 2D) with 82 % and 97 % tumor growth inhibition (TGI) observed at 500 mg/kg o.i.d and b.i.d, respectively. Our data show that TGI was statistically significant at *P* less than 0.005 for 500 mpk at both o.i.d and b.i.d treatment groups. However 250 mg/kg of NPB001-05 did not show significant TGI. Further imatinib dosed in parallel experiments showed statistically significant antitumor activity when administered at a dose of 150 mg/kg o.i.d in K562 xenograft model. In addition, there was no noticeable weight loss for all the dose levels tested for NPB001-05 or imatinib during treatment and/or one week after end of the treatment (Figure 1B).

4.1.3. Effect of NPB001-05 on $P210^{Bcr\text{-}Abl}$ cells derived tumors

We examined the *in-vivo* antitumor effect of NPB001-05, in an established tumor model for wild type and T315I mutant $P210^{Bcr\text{-}Abl}$ expressing BaF3 cells. $BaF3^{Bcr\text{-}Abl}$ and $BaF3^{Bcr\text{-}Abl\ T315I}$ cells were subcutaneously implanted in SCID mice to generate tumors with a mean volume of 100 mm^3 before starting daily treatment of

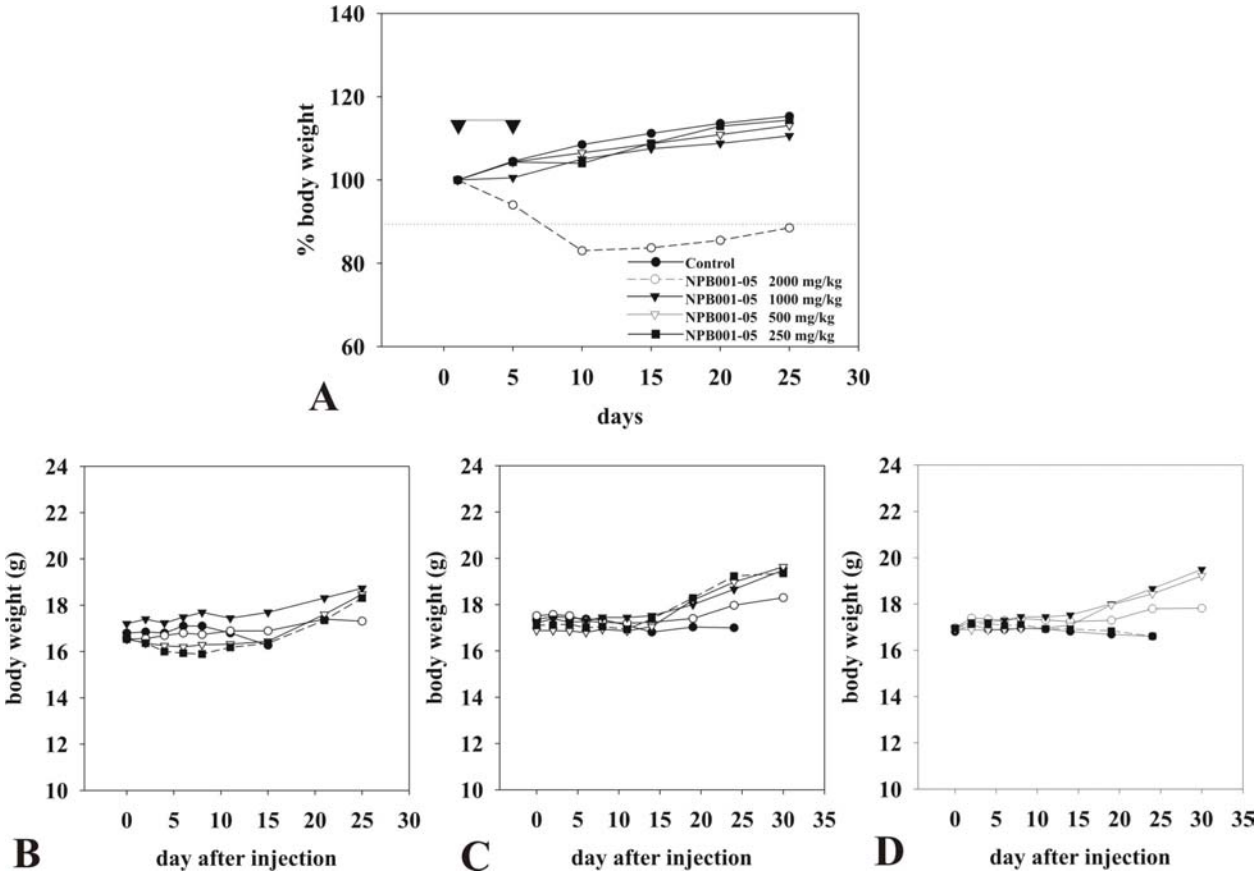

Figure 1. Assessment of toxicity in mice. A) A preliminary toxicity study of per oral NPB001-05 in non-tumor-bearing (normal) Swiss mice was conducted in a five day schedule administration. The mice weights were taken everyday, but represented here for five day period of interval up to 25 days. The graph represent as percent body weight normalized from day 0 considered being 100%. Loss of 10% or more body weight was considered significant and drug induced. Arrows indicated the period of NPB001-05 injection. Error bars are omitted for clarity. Toxicity estimation were performed in tumor bearing SCID mice for the tumor efficacy experiment shown in Figure 1 as B) K562, C) BaF3$^{Bcr\text{-}Abl}$ and D) BaF3$^{Bcr\text{-}Abl\ T315I}$ xenograft models. Mice in treated groups gained weight normally, however the control animals were observed to loose weight due to tumor burden

NPB001-05 and imatinib. On sixth day of tumor induction, mice were randomized into groups (n=10), treatments of NPB001-05 (500 mg/kg -o.i.d and -b.i.d, 250 mg/kg o.i.d) was initiated and continued for 14 days. It was observed that oral gavage feeding of NPB001-05 at 500 mg/kg o.i.d and b.i.d body weight doses for daily up to 2 weeks resulted in a marked time-dependent inhibition in BaF3$^{Bcr\text{-}Abl}$ and BaF3$^{Bcr\text{-}Abl\ T315I}$ tumor xenograft growth (Figure 2B, 2C). Our study showed that NPB001-05 at both doses, 500 mg/kg -o.i.d and -b.i.d treatment showed a statically significant TGI as compared to vehicle treated controls. Moreover, unlike imatinib, NPB001-05 at higher dose showed significant tumor reduction for BaF3P210$^{Bcr\text{-}AblT315I}$ suggesting that mutation T315I is not a limiting factor for the action of NPB001-05. Interestingly, NPB001-05 at a lower dose of 250 mg/kg showed slightly better inhibition (55%) in T315I xenograft model (Figure 2F). On day 24, the TGI due to NPB001-05 (500 mg/kg, o.i.d) was 78 % and 77 % in BaF3$^{Bcr\text{-}Abl}$ and BaF3$^{Bcr\text{-}Abl\ T315I}$ tumor model, respectively, where as imatinib had 74% and 7 % for the same set (Figure 2E, 2F). Further it was also observed that NPB001-05 was safe in mouse models with no significant loss of weight recorded during the study (Figure 1C, 5D).

Our data show that NPB001-05 is efficacious against wild and mutant T315I expressing P210$^{Bcr\text{-}Abl}$ tumors in xenograft models. In all three models (K562, BaF3$^{Bcr\text{-}Abl}$ and BaF3$^{Bcr\text{-}Abl\ T315I}$) used in the current study, there was a dose- dependent response to NPB001-05 exposure, with 500 mg/kg b.i.d was the most efficacious dose.

4.2. Bcr-Abl expression and phosphorylation in tumor samples

To study the direct effect of NPB001-05 on Bcr-Abl tyrosine kinase activity, we examined P210$^{Bcr\text{-}Abl}$ autophosphorylation by using labeled ^{32}P in tumor generated from K562 cells. Tumors treated with NPB001-05 for three days along with controls were used to immunoprecipitate P210$^{Bcr\text{-}Abl}$ followed by kinase reaction. The phosphoprotein were resolved on SDS-PAGE followed by autoradiography. The autoradiogram (Figure 3A) map of gel-purified P210$^{Bcr\text{-}Abl}$ shows a phosphopeptide band representing P210 autophosphorylated *in-vitro*. Control tumors samples showed strong intense band indicating tyrosine kinase activity as compared to NPB001-05 treated

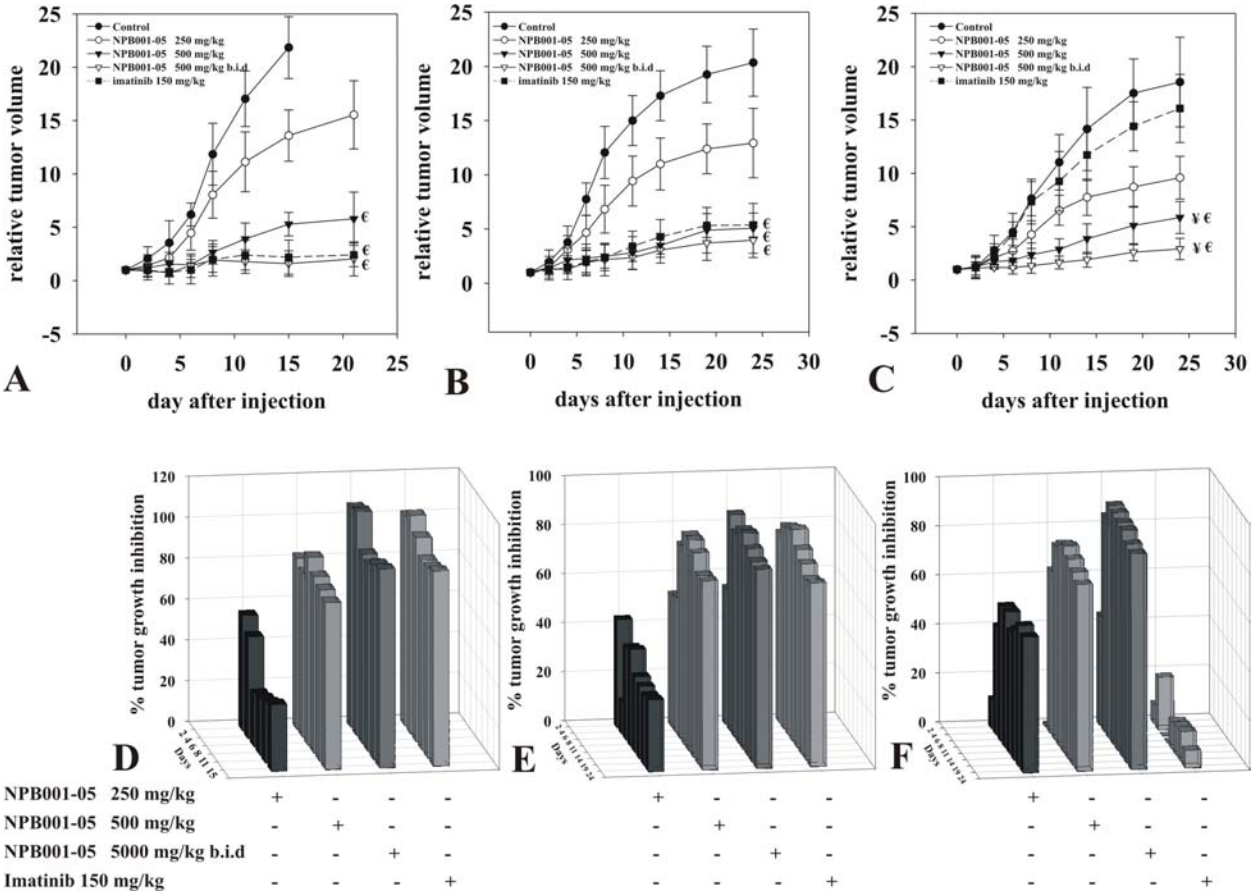

Figure 2. Potential of antitumor effect of NPB001-05 *in-vivo*. Exponentially growing cells were inoculated s.c. into the flank of SCID mice. When tumor volume reached between approx. 100 mm^3, drugs were administered from day 0 to day 14. NPB001-05 was administered in 250 mg/kg, 500 mg/kg once daily and bi-daily, imatinib was given at dose of 150 mg/kg body weight. All dose administration was given per-orally. A representative tumor growth inhibition curves for test drugs in A) K562 xenograft, B) $BaF3^{Bcr\text{-}Abl}$ and C) $BaF3^{Bcr\text{-}Abl\ T315I}$ subcutaneously growing tumor model. Each point represents the mean value of relative tumor volumes (RTVs +/- SD). Statistical analysis was performed using Students t-test comparing RTV means of the control group compared to NPB001-05 or imatinib treated (€, P less than0.005) groups and imatinib treated group with NPB001-05 treatment (¥, P less than0.005). Each treatment group consisted of ten mice, control group was given water. Percentage tumor growth inhibition (%TGI) was calculated as mentioned in material and methods. Data shown as %TGI for D) K562 xenograft, E) BaF3Bcr-Abl and F) BaF3Bcr-Abl T315I xenograft model for a representative set of experiment. NPB001-05 and imatinib treatment highly inhibits the tumor growth increment in K562 and BaF3Bcr-Abl models. Besides, NPB001-05 is found to be active (more than 50% TGI) for all the doses tested in BaF3Bcr-Abl T315I tumor model

tumors where sample showed reduced kinase activity as noted by the band densities (Figure 3B). For same tumor samples Western blotting was performed. It was observed that there was reduction of P210 (Bcr-Abl) protein levels however P110 (c-Abl) expression levels were conserved (Figure 3A). We used anti- c-Abl antibody to detect levels of p120 (c-Abl) protein expression by immunoblots. The c-Abl (unphosphorylated) was detectable in basal levels indicative of oxidative stress in tumor (47). In addition, apoptotic marker cleaved CASP3 showed elevated expression in NPB001-05 treated tumor samples (Figure 3A).

4.3. Transcript profiling of NPB001-05 treated K562 cells *in-vitro* and *in-vivo*

Microarray experimental setup is shown schematically in Figure 4A. Of 34,000 transcripts studied in microarray experiment for *in-vitro* and *in-vivo* tumor models, 13,456 genes passed the filtering criteria that showed (*P* less than 0.001) significant correlation between NPB001-05 and imatinib treatment. It was also observed that, NPB001-05 and imatinib induced global expression profile resulted in two different clusters each specific for *in vitro* or *in vivo* models as seen in the dendrogram (Figure 4B). Additionally gene expression profile for NPB001-05 and imatinib showed significant similarities with both *in vitro* and *in vivo* experimental models.

4.4. Transcriptional profiling of NPB001-05 and imatinib treated K562 cells and analysis of differentially regulated genes

4.4.1. Analysis of similarly expressed genes in NPB001-05 and imatinib treatment

Unsupervised clustering analysis of significantly dysregulated transcripts (Figure 4B) revealed significant similarity in expression patterns in response to NPB001-05

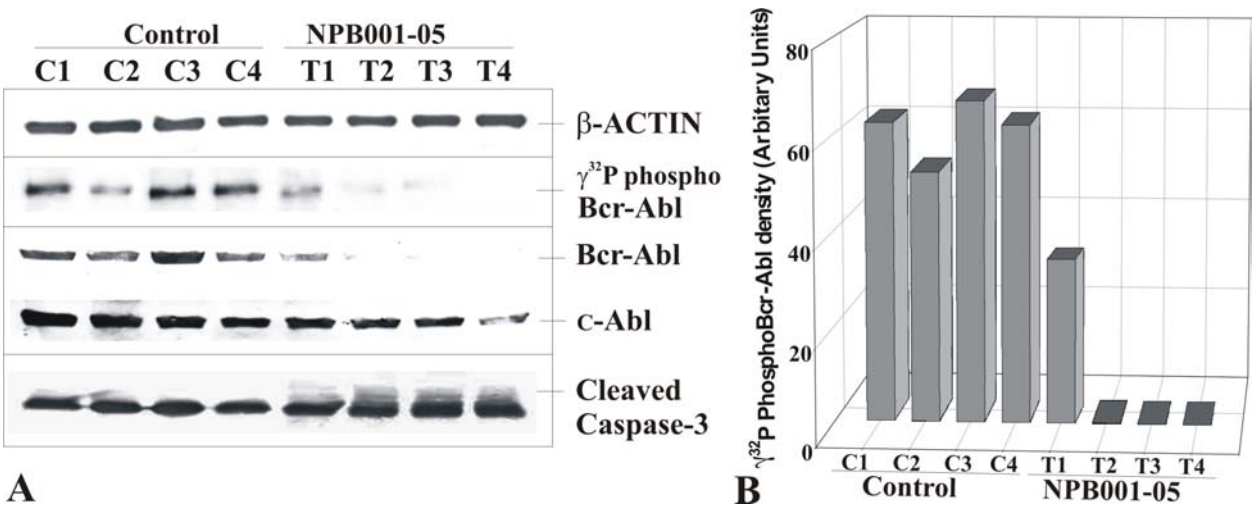

Figure 3. Effect of NPB001-05 on Bcr-abl protein expression and phosphorylation *in-vivo*. K562 xenograft tumors treated with NPB001-05 (500 mg/kg) for 3 days were lysed to extract protein, immunoprecipitate with Bcr-Abl antibody. Identical parallel blots were incubated with antibodies to Bcr-Abl, cleaved caspase3, and beta-actin. A) Inhibitory effect after NPB001-05 treatment was observed for most of the tumor samples, as there was complete to partial loss of kinase activity measured by *in-vitro* kinase reaction using labeled gamma-^{32}P-ATP. Loss of P210$^{Bcr\text{-}Abl}$ protein was associated with induction of caspase-3 in treated tumor samples indicating apoptotic cells. However the level of P110$^{c\text{-}Abl}$ remains unaffected. B) Densitometry for kinase activity measured from *in-vivo* tumor cells. NPB001-05 treated abrogated kinase activity to more than 80% in all the tumor samples compared to the control samples

and imatinib treatment both *in vitro* and *in vivo* models. We identified 284 most consistently but similarly dysregulated genes across all drug treatment conditions (NPB001-05 C1, C2, imatinib C1, and C2, Figure 4D). Further we also identified a set of genes that are differentially dysregulated in response to NPB001-05 and imatinib treatments (Figure 4C, 4D). This gene list was evaluated against Panther database to determine the ontology and biological relevance. Table 3 depicts a list of common signatures for NPB001-05 and imatinib that belong to biological processes such as apoptosis, cell cycle arrest, carbohydrate metabolism, cell proliferation and differentiation, nucleic acid metabolism and signal transduction.

4.4.2. Unique differentially expressed gene in NPB001-05

Global transcriptome analysis of NPB001-05 and imatinib treated K562 reveal that genes involved in apoptotic pathway, mitochondrial-dependent G_1 arrest, mTOR inhibition, SMAD induction and prominently ER stress (Figure 4E) were found to be dysregulated by NPB001-05. For instance twelve members of ER stress induction pathway were present, as well as number of other genes associated with mitochondrial-dependent apoptosis, signal transduction and cell differentiation. Table 4 represents list of genes unique to NPB001-05 signatures enriched for biological processes. It was observed that the greatest number of genes changes was in the ER stress response, suggesting cell death due to DNA damage and apoptosis. Comparison of transcriptional variability indicates fundamental differences between NPB001-05 and imatinib treated cells.

4.4.3. Gene expression validation by RT-qPCR and immunoblotting

We validated 11 dysregulated genes from microarray analysis by RT-qPCR. Four of these genes were significantly under expressed and seven were over expressed in K562 NPB001-05 treated cell line compared to control. Three most significant up-regulated genes and one most down-regulated gene are highlighted in Table 4. After assessment, similar expression levels were detected in the RT-qPCR as compared to microarrays expression values (Figure 3A). Expression of genes identified as being differentially expressed in NPB001-05 and imatinib treatments was examined using RNA from three independent set of experiment consisting of 6- and 12-hours treated K562, BaF3$^{Bcr\text{-}Abl}$ and BaF3$^{Bcr\text{-}Abl\ T315I}$ cells (Figure 5B, 5D). RNA from vehicle treated cells was used as calibrator and expression levels were reported relative to the expression levels of control cells. These qPCR results therefore validate the difference in gene expression revealed by the microarray study

NPB001-05 treatment feature on K562 cells: From microarray and RT-qPCR study, it was observed that predominantly the expression of DDIT3 and a down stream target GADD34 was significantly upregulated in all three cell lines, and was consistent within the experimental replicates (Figure 5B, 5D). These proteins are known to trigger upon ER stress signaling (48). We further tested these markers at protein levels. Levels of DDIT3 protein expression in NPB001-05 treated K562 cells were greater than control as well as imatinib treated cells in 12-, 24, and 48- hours of drug exposure .(Figure 6). GADD34 protein expression levels were slightly higher to the control or imatinib treated cells. Apoptosis scored by expression of

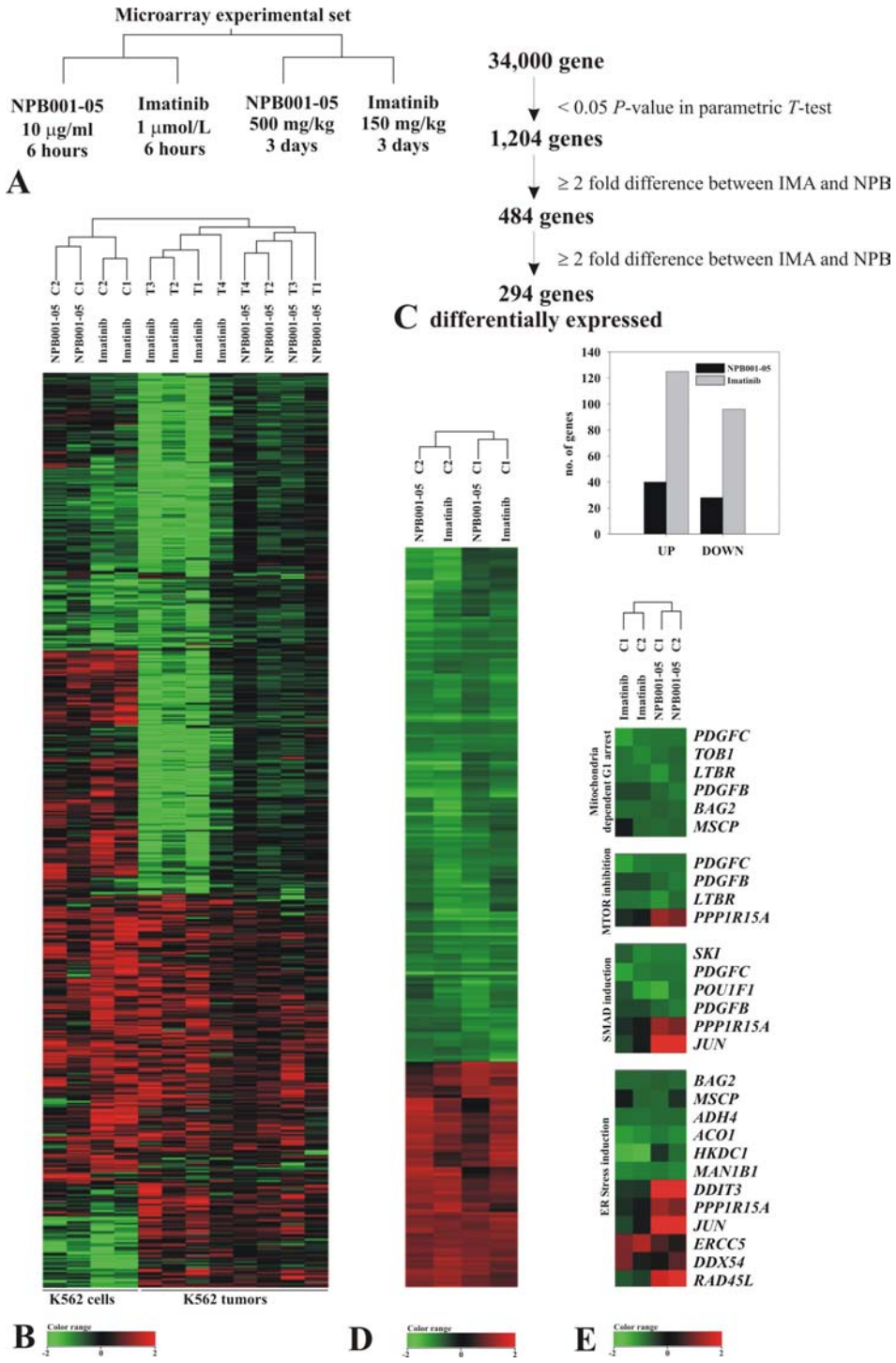

Figure 4. Gene expression of NPB001-05 and imatinib treated K562 cells. A) Experimental overview, a summary of overall approach is shown. B) Hierarchical cluster (global transcriptome) analysis of NPB001-05 and imatinib treated K562 cells *in-vitro* and from tumors. Gene was selected if they showed two fold enrichment (from control). Each column represents microarray profile with a separate probe, and C1, C2 corresponds to *in-vitro* treated sets, T1-T4 is tumor treated with the drugs. Color bars shows the gene expression levels, where red indicates increased expression, green indicates decreased gene expression, and black indicates no change. Intensity of color correlates to the magnitude of change. C) Short listing of differentially expressed genes from K562 treated cells line. D) Gene cluster enriched from (K mean clustering) 284 enriched transcripts, with differentially disregulated clusters common between NPB001-05 and imatinib treatments. E) A supervised hierarchical clustering to the lowest *P*'s comparing the biological significant genes enriched from GO annotation summary

Table 3. Gene signatures showing similarities in expression between NPB001-05 and imatinib

Biological process	Refseq No.	Gene Symbol	Imatinib	NPB001-05
Apoptosis	NM_004282	*BAG2*	-0.8	-1.1
	NM_002342	*LTBR*	-0.9	-1.0
	NM_014314	*DDX58*	1.1	1.2
	NM_018240	*KIRREL*	0.9	0.9
Carbohydrate metabolism	NM_000670	*ADH4*	-0.9	-0.8
	NM_002197	*ACO1*	-1.2	-1.0
	NM_016219	*MAN1B1*	-1.0	-1.0
	NM_025130	*HKDC1*	-0.9	-0.9
Cell adhesion	NM_002886	*RAP2B*	-0.9	-0.8
	NM_005559	*LAMA1*	-0.8	-0.9
	NM_012287	*ACAP2*	-0.9	-0.9
Cell cycle	NM_005749	*TOB1*	-1.0	-0.8
	NM_006520	*DYNLT3*	-1.0	-0.9
	NM_016507	*CDK12*	-0.8	-1.8
	NM_017575	*SMG6*	1.0	0.9
	NM_018042	*SLFN12*	0.9	1.0
Cell proliferation and differentiation	NM_016205	*PDGFC*	-1.1	-0.9
	NM_024019	*NEUROG2*	-1.1	-1.0
	NM_000078	*CETP*	-0.8	-0.9
Nucleoside, nucleotide and nucleic acid metabolism	NM_000306	*POU1F1*	-0.9	-1.1
	NM_001004317	*LIN28B*	-1.1	-0.9
	NM_001358	*DHX15*	-0.9	-0.8
	NM_003096	*SNRPG*	-0.9	-1.2
	NM_004821	*HAND1*	-0.9	-0.9
	NM_015481	*ZNF385A*	-0.9	-0.9
	NM_017755	*NSUN2*	-1.1	-0.8
	NM_021632	*ZNF350*	-1.0	-1.0
	NM_024674	*LIN28A*	-1.2	-1.0
	NM_017838	*NHP2*	0.9	1.1
	NM_080390	*TCEAL2*	1.0	1.0
	NM_138394	*HNRPLL*	1.0	0.9
Oncogenesis	NM_003036	*SKI*	-0.9	-1.0
	NM_006195	*PBX3*	-0.9	-0.8
Protein metabolism and modification	NM_000991	*RPL28*	-0.8	-0.9
	NM_005339	*UBE2K*	-1.0	-1.1
	NM_005726	*TSFM*	-0.9	-0.7
	NM_021821	*MRPS35*	-1.1	-0.8
	NM_004476	*FOLH1*	0.9	0.9
	NM_014369	*PTPN18*	0.9	0.9
	NM_031907	*USP26*	1.0	1.0
	NM_000023	*SGCA*	-0.9	-0.8
	NM_001002925	*OR5AP2*	-0.7	-0.8
	NM_002899	*RBP1*	-0.9	-0.8
	NM_003775	*S1PR4*	-0.8	-0.8
	NM_006320	*PGRMC2*	-1.0	-0.8
	NM_006726	*LRBA*	-0.9	-0.8
	NM_018192	*LEPREL1*	-0.8	-1.0
	NM_018373	*SYNJ2BP*	-0.7	-0.9
	NM_144575	*CAPN13*	-0.8	-0.9
	NM_173492	*PIP5KL1*	-0.9	-0.8
	NM_002663	*PLD2*	0.9	0.9
	NM_006204	*PDE6C*	1.0	0.9
	NM_030891	*LRRC3*	1.0	0.9
Signal transduction	NM_000087	*CNGA1*	-0.8	-0.9
	NM_001044	*SLC6A3*	-0.8	-1.0
	NM_002248	*KCNN1*	-0.9	-0.9
	NM_003040	*SLC4A2*	-0.9	-0.9
	NM_005603	*ATP8B1*	-1.0	-0.9
	NM_022754	*SFXN1*	-0.8	-0.9
	NM_145648	*SLC15A4*	-0.9	-0.8

Values for NPB001-05 and imatinib represent microarray result (average expression ratio from control at $\log_2$ scale). The classification has been done based on Panther data base, only selected genes from selected class has been represented here based on cancer biology.

Caspase-3 protein, was seen to be higher in NPB001-05 and imatinib treated cells for all the time points measured referring cell death (Figure 6). These results confirm the array results of DDIT3 expressing cell at protein level detected by immunoblotting.

Table 4. Gene signatures showing significantly differences in expression identified as unique in NPB001-05 treatment compared to control

Biological process	Refseq No.	Gene Symbol	Imatinib	NPB001-05
Apoptosis	NM_000048	*ASL*	-0.8	1.1
	NM_002228	***JUN***	-0.3	2.2
	NM_004083	***DDIT3***	-0.4	2.1
	NM_014330	***PPP1R15A***	-0.2	1.1
	NM_014685	*HERPUD1*	-0.4	1.7
	NM_031442	*TMEM47*	-0.2	2.5
	NM_033064	*ATCAY*	0.0	0.8
Carbohydrate metabolism	NM_001002021	*PFKL*	-0.2	-1.2
Cell cycle	NM_012310	*KIF4A*	-0.3	-1.0
	NM_015528	*RNF167*	-0.5	-1.2
	NM_001626	***AKT2***	-1.5	-1.7
Chromatin packaging	NM_005321	*HIST1H1E*	-0.4	-1.3
Nucleoside, nucleotide and nucleic acid metabolism	NM_194278	*C14orf43*	-0.4	-1.1
DNA Damage and repair	NM_003579	*RAD54L*	-0.6	1.9
Immunity and defense	NM_000065	*C6*	-0.8	-1.1
	NM_006007	*ZFAND5*	-0.3	0.9
	NM_015055	*SWAP70*	-0.1	0.9
	NM_080612	*GAB3*	-0.7	1.2
mRNA end-processing and stability	NM_015938	*NMD3*	-0.5	-0.9
mRNA splicing	NM_020230	*PPAN*	-0.5	0.8
Signal transduction	NM_015601	*HERC4*	-0.8	0.8
	NM_012373	*OR3A3*	-0.7	-1.1
	NM_000911	*OPRD1*	-0.6	2.1
	NM_016148	*SHANK1*	-0.7	2.5
	NM_080607	*VSTM2L*	-0.2	1.4
Transport	NM_033375	*MYO1C*	0.4	0.9
	NM_016124	*RHD*	0.2	0.8

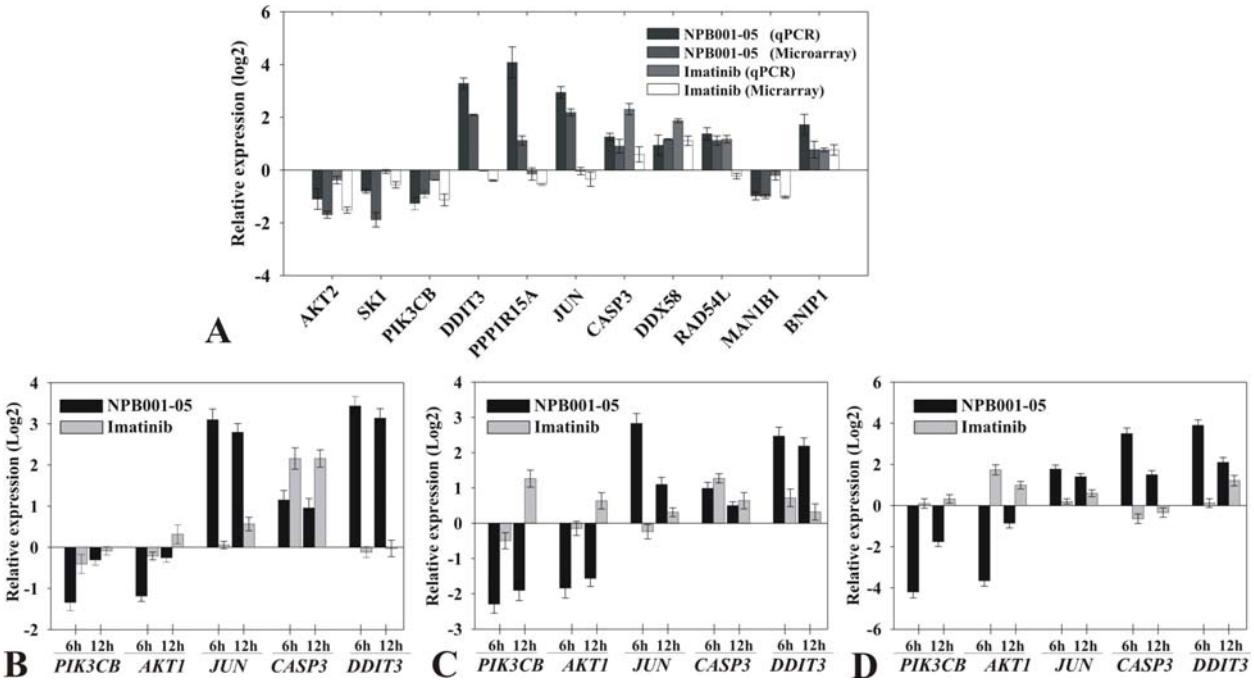

Figure 5. Corroboration by quantitative real-time RT-PCR. A) K562 cells treated with NPB001-05 and imatinib for 6- hours were tested for validation of a subset of eleven differentially expressed genes by quantitative real-time PCR vs DNA array analysis, shows similar level of gene expression from microarray and RT-qPCR analysis. Data is represented as relative expression values in log scale. Meta-analysis of significant differential gene expression signatures was performed in three different cells lines- B) K562, C) $BaF3^{Bcr\text{-}Abl}$ and D) $BaF3^{Bcr\text{-}Abl\ T315I}$, for NPB001-05 and imatinib treatment for 6- and 12- hours *in-vitro*. Relative expression quantities of PIK3CB, AKT1, JUN, CASP3 and DDIT3 were calculated via the delta-Ct method. Mean values and standard deviation from three samples is assessed by qRT-PCR are indicated

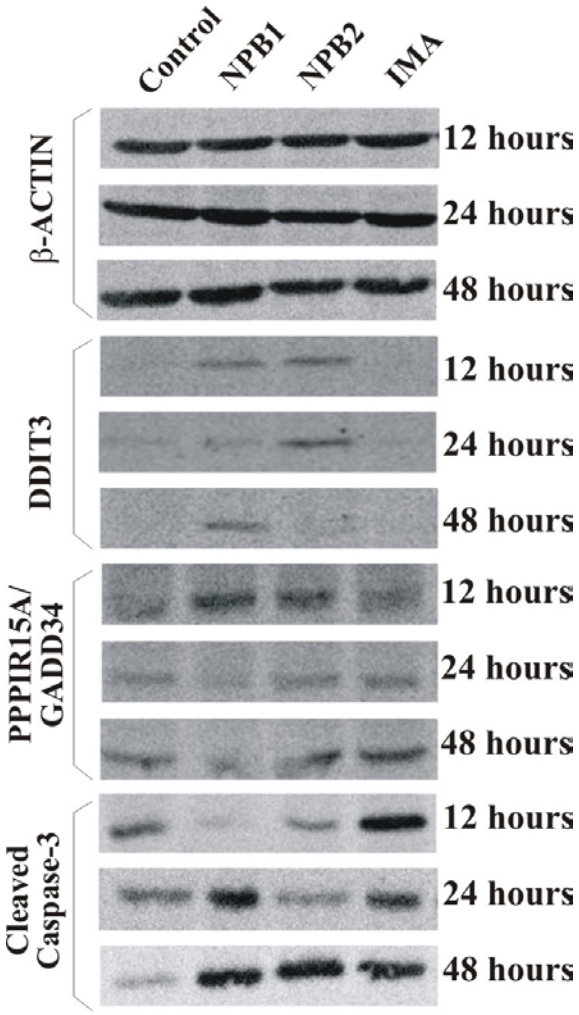

Figure 6. Validations of ER stress response protein by western blot. K562 cells were grown in presence of NPB001-05 (5 and 10 µg/ml, indicated as NPB1 and NPB2, respectively) and imatinib (1 µmol/L) for a period of 12-, 24- and 48-hours. Protein levels of DDIT3, GADD34 and CASP3 were determined by immunoblotting. DDIT3 expression was induced to higher levels exclusively after treatment with NPB001-05, observed immediately after 12- hours and was observed till 48- hours. Downstream target GADD34 was also induced by NPB001-05 treatment. Apoptotic cells were checked using Caspase-3, both NPB001-05 and imatinib were shown to induce activation of CASP3 protein levels indicating cell death

5. DISCUSSION

Targeting Bcr-Abl tyrosine kinase activity is a well established as a therapeutic strategy for CML with significant clinical outcomes. Imatinib, Bcr-Abl tyrosine kinase inhibitor, has considerably changed the landscape of CML treatment; achieving cytogenetic response rates of over 80%, and significant survival of patients. However with growing problem of disease relapse and refractory conditions associated with acquired resistance due to point mutations in the Bcr-Abl gene focus has been shifted to develop novel compounds (49). The imatinib-resistance mutations encode amino acid substitution in the Bcr-Abl kinase domain at residues that are in directly contact to imatinib. That alters the flexibility of Bcr-Abl kinase domain and returns inactive conformation to which imatinib cannot binds (50, 51). NPB001-05 is active as single agent against human CML cells with endogenous expression of the unmutated Bcr-Abl, and notably active against BaF3 cells with ectopic expression of Bcr-Abl or its mutant isoforms including T315I, E255K, E255V and Y253H, as well as against human primary CML cells, as reported by antiproliferative activity (data not shown). Although, second-generation tyrosine kinase inhibitors developed also provide activity against multiple imatinib-resistant Bcr-Abl point mutants but they seems to compromise resistance for many but not all mutations, especially no activity against T315I isoforms. Our results demonstrate that NPB001-05 has preclinical activity in xenograft mouse model of T315I, where imatinib failed to show antitumor activity.

In addition, NPB001-05 has a potent inhibitory activity for Bcr-Abl kinase from wild type and/or T315I mutant Bcr-Abl *in-vitro*. Four tumors samples from SCID mice bearing K562 xenograft also showed the inhibition of autophosphorylation tested after 3-days of treatment with NPB001-05. This gives NPB001-05 a great potential to be developed as a target-specific therapeutic drug for CML. The anticancer properties of *Piper betle* have been widely reported by many research groups. Previously, a report identified a compound (chlorogenic acid) from Piper leafs, that targets myeloid and lymphoid cancer cells but leaves other normal cells unaffected (52). Another study reported that extracts of betel leaves inhibits aspartate aminotransferase and alanine aminotransferase activities in a liver injury in rat models (53), these enzymes are one among target for breast cancer (54) and are associated with high risk of hepatocellular carcinoma (55), that indicates an chemopreventive potential of *Piper betle* against liver fibrosis. Hydroxychavicol, is phenolic components reported from Piper extract, have proven role to play in reducing the risk of oral cancer (56, 57). Significant anticancer activities displayed by NPB001-05 have suggested its potential to be developed as single agent drug and/or neutraceutical.

Our approach to improve drug therapy for CML, focus the identification of natural compounds. Natural products are known for cost effectiveness and importantly for their fewer side effects (58, 59). Current CML therapies, especially with imatinib is associated with several side-effects, prominent one are cardio-toxicity, myelosuppression seen in many patients (60-62). NPB001-05 derived from *Piper betle* is well documented in traditional Indian medicine (Ayurveda) for its medicinal values, it is a part of daily intake in several South Asian countries and is epidemiologically safe.

The expression profiling in CML using microarray chips is shown to have wider coverage of the human genome, and had uncovered several genes which have been confirmed to be important in CML biology (63).

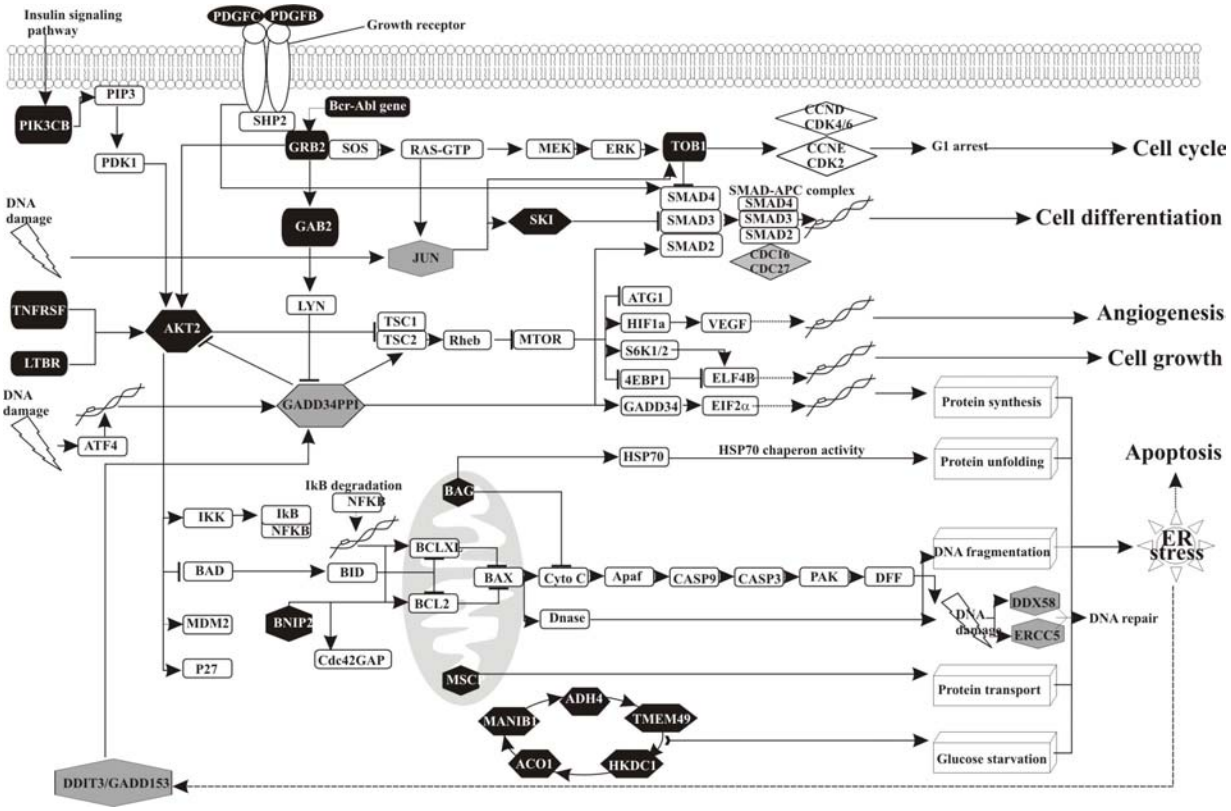

Figure 7. Proposed mechanism of NBP001-05. NBP001-05 acts presumably via five different molecular mechanism-mitochondria-dependent Caspase induction and G1 arrest, mTOR inhibition, SMAD induction and Endoplasmic Reticulum stress. Genes disregulated are represented as grey for upregulated and black colored for down regulated gene sets. Key genes associated to gene ontology are only represented including the significant biological pathways affected.

In an effort to explain the differences and similarities between NPB001-05 and imatinib as inhibitors in CML and to identify transcriptional targets associated with NPB001-05 treatment, a genome-wide analysis was performed with a panel of K562 xenograft and K562 cells treated *in-vitro*. NPB001-05 had significant number of genes with similar expression as to imatinib treated cells or tumor samples. In hierarchical clustering analysis the cells and tumors treated with NPB001-05 and/or imatinib are grouped together, reflecting the similar gene expression profile. Microarray-based expression analyses have been used extensively in the 'discovery phase' of cancer-related biomarkers (64-68). The identification of biomarker for efficient predicting the response to drug is one of the biggest challenges in medicine. The subset of validated genes represents a new group of biomarker for the prediction of NPB001-05 response in CML therapy. The ER stress resulting from a variety of toxic insults leads to apoptosis which largely is regulated by the Bcl-2 family of proteins. Activated caspases causes cell shrinkage and nuclear DNA fragmentation. ER-initiated apoptosis is a response due to over accumulation of unfolded or misfolded proteins, which through GADD34 activation inhibits protein synthesis. ER stress has been previously reported to initiate apoptotic death by imatinib and other tyrosine kinase inhibitors (69). Secondly, c-Jun N-terminal kinase was observed to be activated upon NPB001-05 treatment, which might be crucial for the cell cycle arrest, in addition to cellular differentiation (Figure 7). We show that significant genes in the signatures DDIT3 is directly regulated by NPB001-05 treatment which are a consequence upon ER stress signaling (48).

NPB001-05 is natural product obtained from betel leaves, synthetic efforts are focused in identifying the chemical scaffold which is responsible for the inhibitory activity against Bcr-Abl. In this study we identified and validated a number of differentially expressed genes after NPB001-05 treatment. We reported that DDIT3 represents a novel biomarker for treatment response to NPB001-05. We show the antitumor activity of NPB001-05 in-*vivo* in wild type and T315I mutant Bcr-Abl, which potentiate it to be a single agent therapy for CML. On the basis of its favorable preclinical pharmacological and toxicity profile, NPB001-05 has been selected as a candidate for clinical development as an antitumor agent in CML and imatinib-refractory CML.

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Abbreviations: CML chronic myelogenous leukemia, PLSL Piramal life Sciences Limited, AML Acute myelogenous leukemia, ATCC American Type Culture collection, RPMI-1640 Roswell Park Memorial Institute medium-1640, Ph Philadelphia chromosome, TK tyrosine kinase, DMSO Dimethyl sulfoxide, FBS fetal bovine serum, SDS sodium dodecyl sulfate, PAGE PolyAcrylamide Gel Electrophoresis

Key Words: Chronic myelogenous leukemia, *Piper betle*, imatinib, Bcr-Abl, kinase mutation, T315I

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