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NPB001-05 inhibits Bcr-Abl kinase leading to apoptosis of imatinib-resistant cells

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TABLE OF CONTENTS

1. ABSTRACT

The deregulated activity of the Bcr-Abl tyrosine kinase provides a rational basis for the development therapeutics in all phases of Chronic Myelogenous Leukemia (CML). Although a well studied imatinib therapy has clinical success against CML, resistance to imatinib due to mutations in the kinase domain, especially T315I poses a major problem for the ultimate success of CML therapy by this agent. Herein we describe an NPB001-05, derived from extract of *Piper betle* leafs, which is highly active in specifically inhibiting Bcr-Abl expressing cells. NPB001-05 inhibited the proliferation of BaF3 cells ectopically expressing wild type Bcr-Abl phenotype and 12 different imatinib-resistant mutations of clinical relevance (average IC_{50} 5.7 µg/ml). Moreover, NPB001-05 was highly inhibitory to wild type $P210^{Bcr\text{-}Abl}$ and $P210^{Bcr\text{-}Abl\text{-}T315I}$ kinase activity and abrogated the autophosphorylating enzyme in time- and dose- dependent manner. NPB001-05 was non-toxic on normal cells, but was inhibitory to CML patient derived peripheral blood mononuclear cells. Treatment with NPB001-05 caused apoptosis induction and G_0G_1 cell cycle arrest in both Bcr-Abl wild type and T315I mutant cell lines.

2. INTRODUCTION

Chronic myeloid leukemia (CML) was first discovered in 1960 and was recognized to be distinct from other forms of leukemia with characteristic appearance of a minute chromosome (1) resulting because of reciprocal translocation between chromosome 9 and 22 (2). This chromosome was termed as Philadelphia (Ph) chromosome, and was the reason for oncogenic transformation of myeloid cells, that constitutively express gene fusion product-Bcr-Abl (P210-kD or P185-kD) tyrosine kinase (3, 4) leading to excessive proliferation of myeloid progenitor cells (5).

The clinical course of CML has chronic, accelerated phase and blast crisis. The chronic phase is benign and stable phase, is characterized by expansion of myeloid cell compartments, largely maintaining cellular differentiation function. After a variable length of time, the disease progress to accelerated phase and ultimately to blast crisis, which resembles to an acute leukemia of myeloid or lymphoid, and shows the presence of undifferentiated phenotypes in the peripheral blood (6). Presence of Ph chromosome is detectable throughout the course of the

disease as the tyrosine kinase activity of the Bcr-Abl protein is known to be essential for its transforming abilities (5).

The current treatment modalities in CML ultimately focus on efficacy of therapy as survival. However complete hematological response or complete cytogenetic response has been successfully achieved from allogenic stem cells transplantation and tyrosine kinase inhibitors (7-11). Some conventional chemotherapy with hydoxyurea or busulfan also achieved hematological control but cannot modify the natural disease course (12). Therapy with interferon-alpha and in combination with cytarabine produced cytogenetic remission and prolong survival (13).

Imatinib mesylate has been a remarkable success in the treatment of CML. Imatinib is a rationally-designed agent and has been shown to occupy the ATP-binding site of Bcr-Abl and renders the proteins in its inactive conformation (9, 10, 14). Despite the efficacy of imatinib therapy in CML, the development of resistance continues to challenge the treatment of this disease (15-18). Mechanism of resistance in CML patients treated with imatinib, mostly are mutations within the kinase domain of Bcr-Abl. Importantly, substitution of threonine residue at highly conserved "gatekeeper" residue 315, by hydrophobic amino acid (isoleucine or methionine, as in T315I) is a reoccurring mechanism for resistant in many CML cases and also in other clinically relevant tyrosine kinases (19-21). Such mutation causes steric hindrance leading no access of imatinib to the ATP-binding pocket in Bcr-Abl protein (22, 23). In recent years, several strategies have been pursued to identify treatment option that can override the resistance imposed by the T315I and other Bcr-Abl mutations. Promising are the exploitation of Bcr-Abl kinase sites distant from the ATP-binding pocket and/or to inhibiting signaling pathways downstream from Bcr-Abl.

NPB001-05 is a natural product from the leaves of *Piper betle (Piperaceae)*. These leaves are credited with many medicinally properties according to traditional Ayurvedic medicine. The leaves are well known for their anti-microbial, anti-inflammatory and antifungal properties (24). Moreover they are inhibitory to carcinogen-induced tumors of the oral cavity and mammary tissue (25) and exert chemopreventive effects against lung and fore-stomach tumors (26). In addition, we previously shown a compound (sodium chlorogenic acid) purified from *P. betle* leaves, that inhibit Bcr-Abl kinase and induces apoptosis in CML cell lines (27).

Medicinal importance of betel leaves, prompted us to evaluate the anti-tumor efficacy of this natural product. NPB001-05 shows a selective inhibition of Ph^+ cell lines and is inhibitory for Bcr-Abl with imatinib-resistant mutations. NPB001-05 inhibits phosphorylation and induced apoptosis in wild type Bcr-Abl and Bcr-Abl-T315I expressing cells. We herein summarize the identification and characterization of NPB001-05, inhibitor of Bcr-Abl and clinically relevant imatinib-resistant Bcr-Abl mutant and argue in favor of its potential clinical applicability.

3. MATERIALS AND METHODS

3.1. Plant material, extraction and purification

The betle leaves were collected from Banaras, India. The leaves were dried under shade, and stabilized using the facilities provided by Piramal Life Sciences Limited (PLSL). The leaves were grounded using a grinder (Pharmalab, India) to obtain powder having 6/8-mesh particle size using a mill (Bectochem, Mumbai, India). An extract was prepared taking 100 kg of powdered leaves material in 80 % organic solvents. The resultant semi solid materials was concentrated in vacuum, and named as NPB001-05 (International Application PCT/IB2007/050536). NPB001-05 was stored at room temperature in dark container. Species identification and verification was performed by Dr. Vijay Chauhan.

3.2. Reagents

Stock solutions of 100 mg/ml of the test product NPB001-05 (PLSL, India) was prepared in dimethyl sulfoxide (DMSO) solution. Imatinib mesylate (Natco Pharma Limited, Hyderabad, India) stock solutions were also made in DMSO at a concentration of 100 mmol/L. All stock solutions were preserved at -20°C deep freezer. All experiments were performed with serial dilutions from stock solution in RPMI-1640 medium (Sigma-Aldrich, India), and the concentration of DMSO was kept less than 0.1 %. Control samples were kept with 0.1 % DMSO as vehicle controls.

3.3. Cell lines

The two leukemic Ph chromosome positive (Ph^+) cell lines- K562 (28) (CML, CCL-243) and -KU812 (29) (CML, CCL-2099) and two leukemic Ph chromosome negative (Ph^-) cell lines -HL60 (AML, CCL-240) and -MOLT4 (ALL, CCL-1582) were purchased from the American Type Culture Collection (LGC Promochem, Bangalore, India) and maintained as suspension cultures. In addition, adherent cultures- MCF7 (breast ca., HTB-22), HCT116 (colorectal ca., CCL-247), H460 (lung ca., HTB-177) and PC3 (prostate ca., CRL-1435) were also obtained from ATCC. Cell lines expressing wild type Bcr-Abl and various point mutation of Bcr-Abl kinase domain causing imatinib resistance, [as reported previously (30, 31)] were employed. BaF3 cell lines stably expressing full-length wild-type Bcr-Abl and Bcr-Abl with 12 different kinase domain point mutant (T315I, E255K, E255V, F317L, F359V, H396P, H396R, M244V, M351T, Q252H, Y253F and Y253H) were procured from Dr. Brian Druker (Oregon Health and Science University, Oregon, USA). These lines were grown as suspension cultures. Two primary cultures of human normal lung fibroblast, WI38 (CCL-75), and MRC5 (CCL-171) were purchased from ATCC and were used to measure cytotoxicity of NPB001-05 in normal cells. All cultures were maintained in cultivation medium made of RPMI-1640 (Sigma-Aldrich, India) containing 4 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 100 units/ml of Penicillin-streptomycin (Invitrogen) and supplemented with 10 % fetal bovine serum (Gibco, India). All cell lines were sub-cultivated two to three times in a week and maintained in 5 % CO_2 in a humidified incubator at 37 °C.

3.4. Clinical Samples

Seven CML patients donated their peripheral blood samples at Department of Medical Oncology, Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India, under the supervision of Prof. Dr. Digumarti Raghunadharao. The clinical specimens were collected due approval from the Institutional Ethical Committee (IEC) at NIMS. All 7 CML patients were Ph^+ and 3 specimens were refractory to the Imatinib treatment for last 4 - 15 months. In addition, three human peripheral blood specimens were also collected form normal healthy donors with a prior informed consent. All experiments with human blood were conducted under approval of institutional ethics committee protocols. Peripheral Blood Mononuclear cells (PBMNs) from all blood samples were segregated by standard Ficoll-Hypaque (Sigma-Aldrich, India.) density gradient centrifugation (32) and subsequently grown in RPMI1640 medium containing 4 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 10 % Fetal bovin serum, 100 units/ml of Penicillin-streptomycin and 100 ng /ml GM-CSF (R and D systems, USA) in 5 % CO_2 in a humidified incubator at 37 °C. PBMCs from health donors were blast transformed by addition of phytohemagglutinin (PHA) at concentration of 5 µg /ml for testing them for cytotoxicity assessment.

3.5. Cytotoxicity/proliferation assays

All suspension cultures (K562, KU812, HL60, MOLT4, Ba/F3$^{P210Bcr\text{-}Abl}$ wild type + their 12 point mutant variants and PBMCs) and all adherent cultures (MCF7, HCT116, H460, PC3, WI38 and MRC5) were seeded in 96 well plate (Nunc, India) at a density of 5 x 10^3 in 0.2 ml cultivation medium containing varying concentration of imatinib and NBP001-05. The plates were incubated for 72 hours at 37°C in 5 % CO_2 incubator. The [^{3}H] thymidine uptake assay (33) was performed after 72 hours of drug exposure by addition of 0.25 µCi of [^{3}H] thymidine (Board of Radiation and Isotope Technology, BRIT, India) to each wells and incubating the plates overnight. Adherent cultures were trypsinized to facilitate cell harvesting. All the cells were harvested in a 96 UniFilter plates (Millipore, India) using a Packard FilterMate cell harvester, followed by multiple washing with PBS. Plates were air dried overnight and read on a multi-plate-scintillation counter (TopCount Packard Instruments, USA). Additionally, cytotoxicity of compounds was assayed colorimetrically by addition of 10 µl of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (34) (CCK8, Dojindo Laboratories, Maryland, USA) and further incubating for 3-4 hours followed by reading on a microwell plate reader for absorbance at 450 nm (Molecular Devices, CA, USA). The results after 72- hours drug exposure were used to construct curves and to calculate the cellular IC_{50} using a nonlinear regression method. The average cytotoxicity of drug treated from 3 independent experimental determinations were calculated in comparison with the control set to 100%.

3.6. Cell cycle analysis and Apoptosis assays

Ph^+ were seeded at density of 5 x 10^5 cells in 6-well plates in presence of drugs (NPB001-05: 1, 10, 30 µg / ml and imatinib: 0.1, 0.3 3 µmol/L). After 24-, 48- and 72-hours, cells were harvested by centrifugation and washed 2 times with PBS. For studying the cell cycle, pellet was gradually resuspended in 70 % ice-cold ethanol to facilitate the permeablization. Cell suspensions were fixed for 4 hours at 4-8 °C. Fixed cells were stained with Propidium Iodide (PI, 80 µg/ml, Sigma Aldrich India) in presence of RNase-A (50 µg/ml, Sigma Aldrich, India), and acquired on Becton Dickinson FACS caliber flow cytometer (BD Biosciences, India) to determine the DNA content (35). The relative percentage of cells in G_1, S or G_2/M phases was calculated using BD-CellQuest software, as described previously (36). The number of cells in each cell cycle compartment was expressed as a percentage of the total number of cells present from ≥10,000 events acquired. To study apoptosis, cells were stained with annexin-V (37) (BD Biosciences, India) according to the manufacturer's instructions, and acquired by BD FACS caliber and analyzed using CellQuest software (BD Biosciences). Annexin-V staining was additionally performed with imatinib-resistant mutant cell lines. Light (Zeiss, India) and fluorescent (Nikon, India) microscopy pictures were captured for K562 and Ba/F3$^{Bcr\text{-}Abl\text{-}T315I}$ cells treated with NPB001-05 (10 µg/ml) and imatinib (3 µmol/L).

3.7. *In-vitro* Kinase assays

Exponentially growing K562 and Ba/F3$^{Bcr\text{-}Abl\text{-}T315I}$ cells were treated with NPB001-05 and imatinib at various concentrations for 48- and 72- hours. Following this incubation, the cells were lysed in ice cold cell lysis buffer as described previously (38). The cell lysate was cleared by centrifugation at 10,000 g for 15 min at 4 °C. P210$^{Bcr\text{-}Abl}$ was immunoprecipitated using anti-c-Abl antibody (Abcam, UK) followed by incubation with Protein-A-Sepharose (Pharmacia Biotech, USA) beads overnight at 4°C.The P210$^{Bcr\text{-}abl}$ immune complex was washed thrice with NET-N buffer and twice with kinase buffer (38). Autophosphorylation of Bcr-abl kinase reaction was initiated by addition of 10 µCi [gamma^{32}P]-adenosine triphosphate (BRIT, India) and incubated for 30 minutes at room temperature. Kinase reaction was stopped by the addition of SDS-loading buffer (Fermantas, USA) and heating at 95°C for 5 minutes in a heat block. Phospho-proteins were resolved in SDS-PAGE and detected by autoradiography on a photographic film (Kodak). Densitometric analysis was done using ImageJ tool (39). Other kinase assay performed were Cdk4-CyclinD1 and Cdk2-Cyclin E which were described previously (38). In addition, *in-vitro* kinase assay for c-kit, c-src, PDGFR-beta, Flt3, Jak2, Bcr-Abl and T315I, were performed in the presence of increasing concentrations of NPB001-05 and appropriate substrates using the standard institutional protocol at Kinase-profiler (Upstates, UK).

3.7. Data analysis

Data are expressed as mean +/- SD. Difference in measured variable between the NPB001-05 and control groups or/and imatinib groups were assessed by student's *t*-test. *P* value of less than or equal to 0.05 was considered as statistically significant. Although all experiments comparing NPB001-05 and imatinib were performed in same sets keeping a common control, the graphical representation are kept separate for dose response estimations.

Table 1. Certificate of analysis NPB001-05

Test parameter[1]	Observations	Specifications
Description	Green coloured sticky thick paste	Green coloured sticky thick paste
Odour	Camphoraceous characteristic of herb	Camphoraceous characteristic of herb
Taste	Astringent	Astringent
Loss on drying at 105°C	2.29 % (w/w)	NMT 5 % (w/w)
Total Ash	6.20 % (w/w)	NMT 10 % (w/w)
Acid Insoluble Ash	1.35 % (w/w)	NMT 05 % (w/w)
Lead	Less than 5 ppm	Less than 10 ppm
Arsenic	Less than 1 ppm	Less than 2 ppm
Mercury	Less than 1 ppm	Less than 5 ppm
Cadmium	Less than 0.3 ppm	Less than 0.3 ppm
80% Methanol Soluble Extractives	93.46 % (w/w)	NLT 90 % (w/w)
Residual Solvent Methanol	Less than 1 ppm	NMT 1 ppm
Total Aerobic Bacterial Count	100 cfu/gm	NMT 1000 cfu/gm
Total Yeast, Mold & Fungi	50 cfu/gm	NMT 100 cfu/gm
Escherichia coli	Absent	Should be Absent
Salmonella spp.		
Staphylococcus aureus		
Pseudomonas aeruginosa		
Enterobacteriaceae		

[1] All test procedures followed as per standard operating protocols at PLSL (files not shown)

4. RESULTS

4.1. Discovery and preparation of NPB001-05

NPB001-05 was identified during natural product drug discovery screening program at PLSL. *Piper betle* leaf samples were collected from five geographical locations in India and comparatively studied for the potential cytotoxicity against CML cell lines (data not shown). Betel leaf extract obtained from Banaras, India showed potent cytotoxic activity. NPB001-05 extract was prepared from the same source as exhibited in patent application number 20100028472 (Herbal composition and process for its preparation). NPB001-05 also seen in previous reports (40, 41) was produced in a single batch of approximately 1 kg, specification (CoA) of which is presented in Table 1.

4.2. Effect of NPB001-05 on growth of Bcr-Abl positive and negative cells, primary cultures and normal human peripheral blood mononuclear cells

The potential effect of NPB001-05 treatment on cellular proliferation were evaluated using [^{3}H] thymidine-uptake assay with a panel of human Ph^+ and Ph^- cell lines. NPB001-05, like imatinib, strongly and selectively inhibited the proliferation Bcr-Abl expressing cell lines compared to the other cell lines tested (Figure 1A). The anti-proliferative effect was estimated after 72 hours of treatment produced a dose -dependent inhibition of K562 and KU812 cell lines (IC_{50} values 7.8 and 8.9 µg / ml, respectively). We also performed WST8 cytotoxicity determination with K562, KU812, HL60 and MOLT1 cells. NPB001-05 produced dose-response cytotoxicity, specifically in Bcr-Abl positive cells (Figure 1B). Further, we tested the nature of Bcr-Abl selectivity of NPB001-05 on some malignant cells of different etiology. H460, PC3, HCT116 and MCF7 cells were comparatively resistant for treatment with NPB001-05 (Figure 1C). Additionally to exclude the possibility of toxicity caused on normal cells, we examined the effect of NPB001-05 on non-malignant cells. WI38 and MRC5 cells are normal lung fibroblast and were used to study the cytotoxic effect of NPB001-05 using [^{3}H] thymidine-uptake assay. NPB001-05 was found to be non-cytotoxic to these primary cultures after 72 hours of exposure (Figure 1D) (IC_{50} values more than 100 µg /ml). In addition, NPB001-05 was observed to be non-cytotoxic on three samples of PHA-stimulated and unstimulated cells (Figure 2A-C, Table 2). Collectively, here we studied the efficacy, specificity and cytotoxicity potential for NPB001-05 towards Ph^+ leukemia cells. NPB001-05 decreased viability of Bcr-Abl positive leukemic cells in dose-dependent manner. Table 3 shows the IC_{50} values obtained for the cytotoxicity assays. These results suggest selectivity of NPB001-05 for Bcr-Abl positive cells versus other cancer- and normal- cells.

4.3. NPB001-05 inhibits proliferation of leukemic cell lines independent of Bcr-Abl mutational status

To determine the impact of the Bcr-Abl mutational status on the efficacy of NPB001-05, we treated murine $BaF3^{P210}$ cells, including their 12 imatinib resistant mutants with increasing concentration of NPB001-05. This anti-proliferative assessment was measured via [^{3}H] thymidine-uptake assay. As observed in human leukemic cell lines (K562 and KU812), NPB001-05 not only inhibited cell proliferation of wild type (wt) BaF3 cell expressing Bcr-Abl, but also inhibited Bcr-Abl expressing cells independently of its mutational status, in a dose dependent manner (Figure 3A). The sensitivity of Bcr-Abl mutants to NPB001-05 does not correlate with the degree of resistance to imatinib, for instance inhibition of proliferation by NPB001-05 in highly imatinib-resistant mutants like T315I, E255V, and Y253H was either within or lower in range of wt Bcr-Abl and also low grade imatinib-resistant mutants. However no significant inhibition of proliferation was observed from BaF3 cells grown in presence of NPB001-05. In-contrast a high-level of variation was observed for inhibition of mutant cell line after imatinib treatment (Figure 3B). Table 4 shows the IC_{50} values of NPB001-05 and imatinib in various Bcr-Abl wt and mutant cell lines. The relative coefficient C1, reflects the sensitivity of NPB001-05 on inhibition of proliferation in imatinib-resistant cells versus wt cultures. As shown, it is less than or equal to 1.0 for all the cell lines for NPB001-05 treatment, in contrast to the inhibition with imatinib (more than 1.0). The average IC_{50} values for

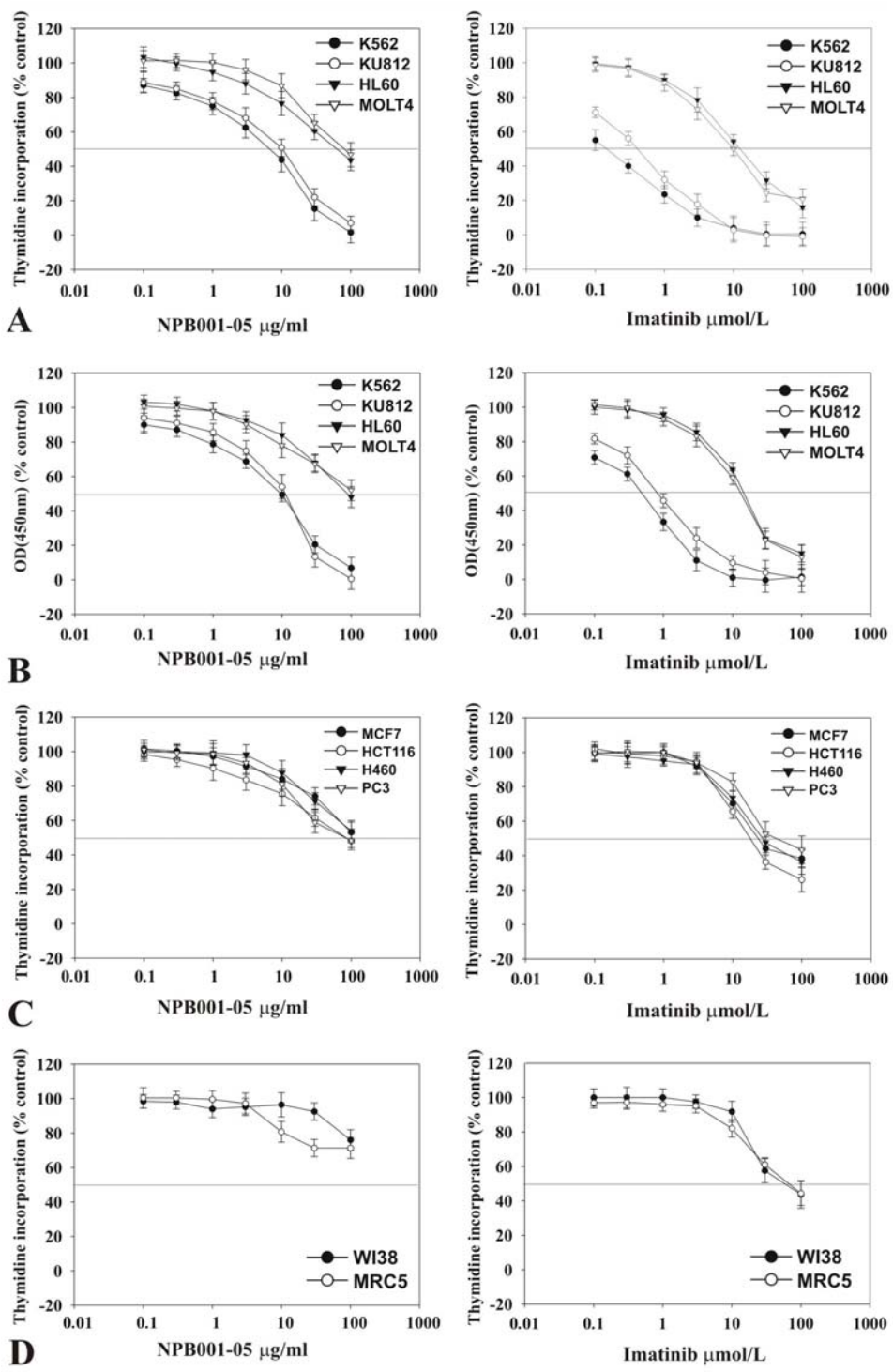

Figure 1. Effect of NPB001-05 and imatinib on cell proliferation. Exponentially growing of CML, non-CML and primary cultures were exposed with escalating concentrations of NPB001-05 and imatinib for 72 hours. Estimation of anti-proliferation/cytotoxicity was performed by [^{3}H] thymidine incorporation assessment and/or WST8 assay as described in material and methods. DMSO-treated control was set to as 100%. Dose-response curves were plotted using Excel spreadsheet for a panel of Ph^+ and Ph^- cell lines estimated via A) [^{3}H] thymidine uptake assay, and B) WST-8 assay. Same concentration of drugs was tested for C) non-leukemic cancer cell line, and D) primary fibroblast cultures, using [^{3}H] thymidine uptake assay. Dotted midline in the graphs represents 50% growth inhibition. The error bars in all plots represents SD, n=3

Table 2. Effect of NPB001-05 and imatinib on stimulated and unstimulated PBMC from healthy donors

Peripheral blood cultures (PBMC)	IC_{50}				Relative coefficient[3] (C2)	
	NPB001-05 µg/ml		Imatinib µmol/L			
	PBMC[1]	PHA-PBMC[2]	PBMC[1]	PHA-PBMC[2]	NPB001-05	Imatinib
donor 1	250.0	235.0	95.0	44.0	0.93	0.45
donor 2	297.0	280.0	71.0	38.0	0.95	0.53
donor 3	290.0	270.0	150.0	61.0	0.94	0.40

[1] unstimulated PBMC, [2] PHA (phytohemagglutinin, 5µg/ml) stimulated PBMC,[3] Relative coefficient (C2) is ratio of stimulated (PBMC[2]) IC_{50} value *vs.* unstimulated (PBMC[1]) IC_{50} value

Table 3. Comparison of NPB001-05 and imatinib on cell proliferation

Cell lines	Cell type	IC_{50} +/- SD	
		NPB001-05 µg/ml	Imatinib µmol/L
Ph chromosome positive leukemia cell lines			
K562	CML, Ph^+	7.8 +/- 0.4	0.3 +/- 0.1
KU812	CML, Ph^+	8.9 +/- 0.6	0.4 +/- 0.1
Ph chromosome negative leukemia cell lines			
HL60	Promyelocytic leukemia	68.0 +/- 7.8	12.0 +/- 0.8
MOLT4	Lymphblastic leukemia	84.0 +/- 11.4	10.0 +/- 0.5
Non-leukemic cell lines			
MCF7	Breast carcinoma	more than 100	21.0 +/- 1.2
HCT116	Colon carcinoma	89.0 +/- 6.9	16.0 +/- 1.5
H460	Lung carcinoma	more than 100	26.0 +/- 2.2
PC3	Prostate carcinoma	85.0 +/- 8.8	41.0 +/- 3.2
Primary cell lines			
WI38	Normal lung fibroblast, primary	more than 100	56.0 +/- 4.2
MRC5	Normal lung fibroblast, primary	more than 100	66.0 +/- 6.7

In-vitro antiproliferative activity performed in CML, non CML and primary cell lines for 72 hours of drug exposure. Dose-response curves were used to calculate IC_{50} values, expressed as mean +/- SD. The antiproliferative activity was estimated by [^{3}H] thymidine uptake assay

Table 4. Effect of NPB001-05 and imatinib on known imatinib-resistant mutants of Bcr-Abl

Ba/F3 cell lines	Bcr-Abl kinase sub-domain	IC_{50}+/- SD		Relative coefficient[5] (C1)	
		NPB001-05 µ g/ml	Imatinib µmol/L	NPB001-05	Imatinib
WT Bcr-Abl[1]	-	7.4 +/- 0.6	0.9 +/- 0.2	1.00	1.00
M244V Bcr-abl[2]	P-loop	5.0 +/- 0.5	4.6 +/- 0.4	0.68	5.11
Q252H Bcr-abl[2]	P-loop	6.6 +/- 0.3	4.9 +/- 0.4	0.89	5.44
Y253F Bcr-abl[2]	P-loop	7.2 +/- 0.4	8.6 +/- 0.5	0.97	9.56
Y253H Bcr-abl[2]	P-loop	5.0 +/- 0.7	14.3 +/- 0.8	0.68	15.89
E255K Bcr-abl[2]	P-loop	5.2 +/- 0.6	10.0 +/- 0.6	0.70	11.11
E255V Bcr-abl[2]	P-loop	5.8 +/- 0.5	13.6 +/- 0.6	0.78	15.11
T315I Bcr-abl[2]	ATP binding site	5.6 +/- 0.5	20.8 +/- 0.9	0.76	23.11
F317L Bcr-abl[2]	ATP binding site	5.0 +/- 0.6	2.4 +/- 0.7	0.68	2.67
M351T Bcr-abl[2]	catalytic domain	5.0 +/- 0.6	2.2 +/- 0.8	0.68	2.44
F359V Bcr-abl[2]	catalytic domain	7.6 +/- 0.7	3.8 +/- 0.9	1.03	4.22
H396P Bcr-abl[2]	A-loop	4.4 +/- 0.4	1.6 +/- 0.2	0.59	1.78
H396R Bcr-abl[2]	A-loop	4.6 +/- 0.4	2.8 +/- 0.2	0.62	3.11
BaF3[3]	-	more than 100.00	16.8 +/- 0.5	-	18.67
Average[4] IC_{50}	-	5.7	7.0	-	-

BaF3 cells engineered to express mutants forms of $P210^{Bcr\text{-}Abl}$ mediating resistance to imatinib were tested for proliferative capability in presence of NPB001-05 and imatinib in varying dose concentrations for 72 hours and tested using [^{3}H] thymidine uptake assay. Dose-response curves were used to calculate IC_{50} values, expressed as mean +/- SD. The average[4] IC_{50} value is from BaF3 cells expressing $P210^{Bcr\text{-}Abl}$, except parent BaF3 cell line, [1] Imatinib sensitive wild type Bcr-Abl, [2] Imatinib resistant Bcr-Abl mutations, [3] Parent cell line, [4] average IC_{50} value from $P210^{Bcr\text{-}Abl}$ cells, [5] Relative coefficient (C1) is ratio between imatinib-resistant[2] IC_{50} value *vs.* IC_{50} value of wild type[1]

NPB001-05 was 5.7 +/- 1.1 µg/ml and imatinib was 7.0 +/- 6.1 µmol/L for all $BaF3P210^{Bcr\text{-}Abl}$ cell lines. These results suggest a high selectivity of NPB001-05 for Bcr-Abl positive cells including the prominent mutants causing imatinib-resistance.

4.4. NPB001-05 inhibits autophosphorylation of wt and resistant Bcr-Abl

We next asked whether the observed inhibition of proliferation caused by NPB001-05 correlate to the degree of Bcr-Abl kinase inhibition. Therefore, we exposed K562 and $BaF3^{Bcr\text{-}AblT315I}$ cells to graded concentrations of NPB001-05 and imatinib for 48- and 72- hours and immunoprecipitated whole cell lysates with Bcr-Abl antibody, and analyzed tyrosine-phosphorylated-Bcr-Abl as a measure of kinase activity. As illustrated in Figure 4A, NPB001-05 inhibited autophosphorylation of Bcr-Abl from Ph^+ cells without affecting protein expression (data not shown). The inhibition of kinase was significant when compared to control for NPB001-05 (10 and 30 µg/ml) and

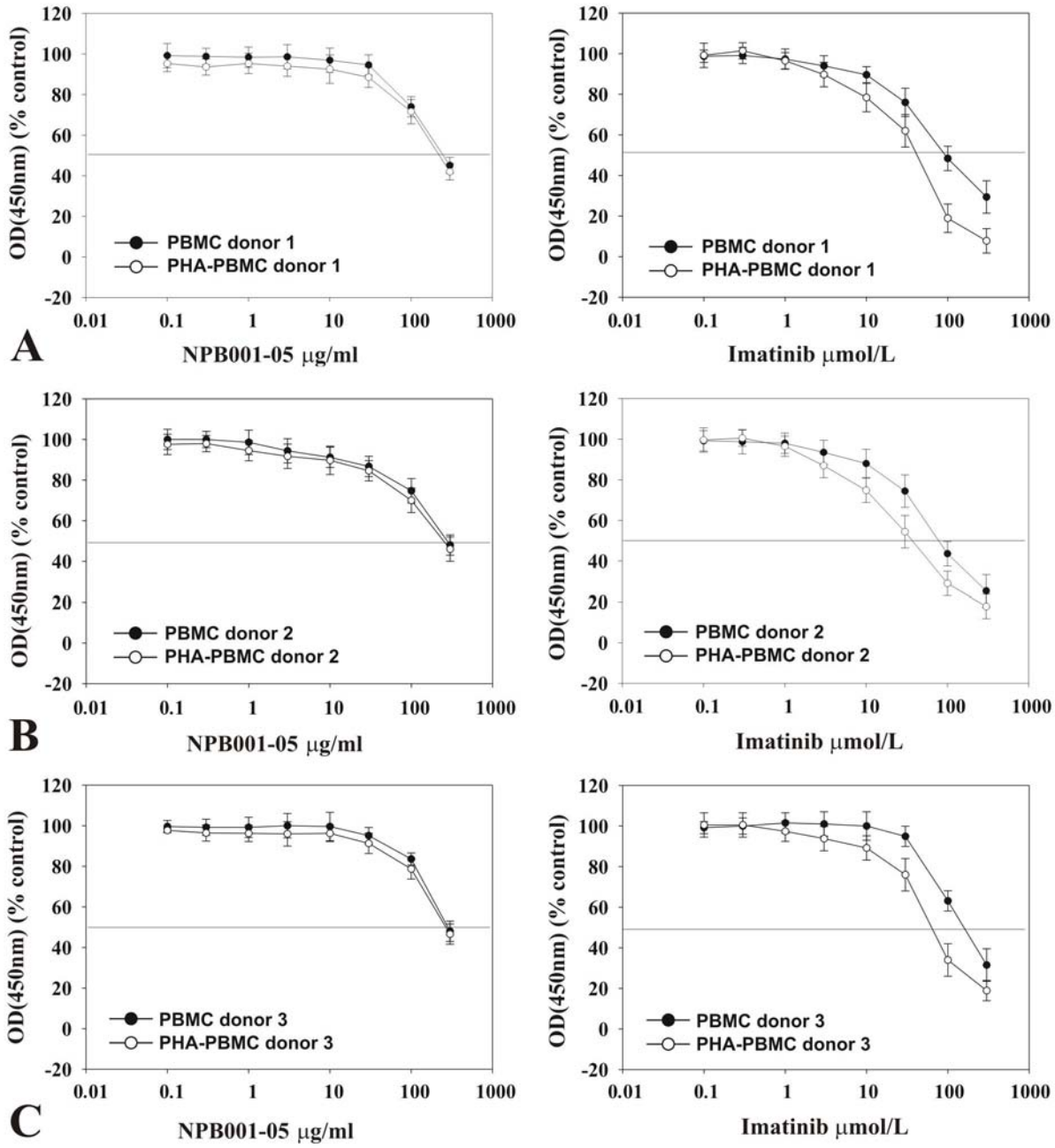

Figure 2. Effect of NPB001-05 and imatinib on peripheral blood mononuclear cell survival. PBMC's were isolated by standard methods of Ficoll-Hypaque from three healthy donors, and cultured in presence or absence PHA (5μg/ml). PBMC's were exposed to varying concentration of NPB001-05 and imatinib for 72 hours, followed by estimation of cytotoxicity by WST-8 assay. Dose response curves for A) donor 1, B) donor 2 and C) donor 3 are represented for unstimulated (PBMC) and stimulated (PHA-PBMC) cells. DMSO (vehicle) wells were set as 100% and the assay was performed in six well replicates represented as SD error bars. The dotted line in the middle indicates 50% cytotoxicity

imatinib (0.5 and 3.0 μmol/L) in K562 cell line (Figure 4B) but only significant for NPB001-05 in BaF3P210$^{Bcr\text{-}Abl T315I}$ cells (Figure 4C). The reduction of kinase activity reflects inhibition of Bcr-Abl phosphorylation, indicating correlation with the anti-proliferative activity of NPB001-05 in Ph^+ cell lines.

4.5. Effect of NPB001-05 on CML clinical samples

Seven CML peripheral blood samples consisting of 70 to 90 % blasts cells were used for testing NPB001-05 action using [^{3}H] thymidine-uptake assay. These primary CML cells were cultured in the presence of GM-CSF overnight, followed by addition of NPB001-05 and

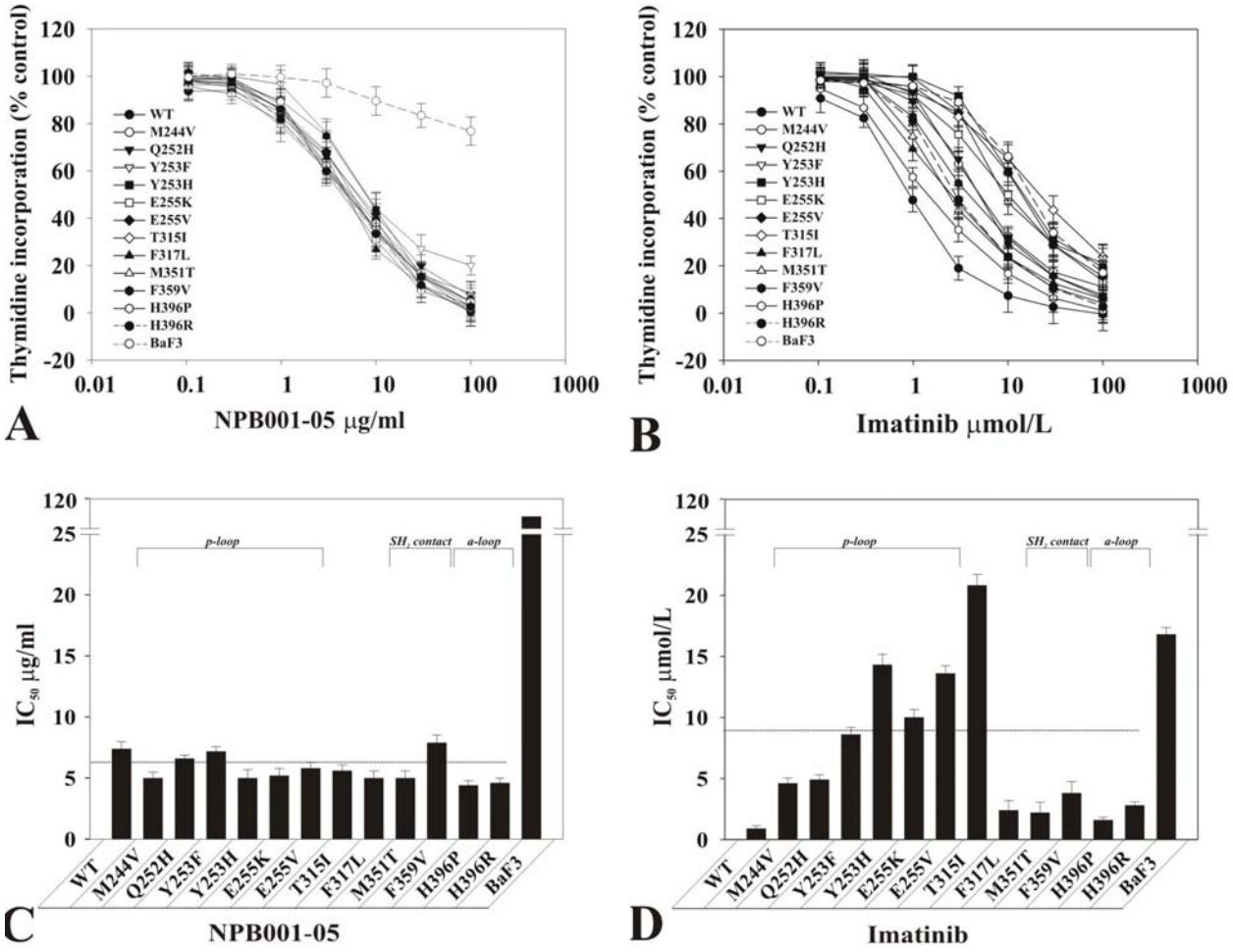

Figure 3. Analysis of NPB001-05 and imatinib in BaF3P210$^{Bcr\text{-}Abl}$ expressing cell lines. BaF3P210$^{Bcr\text{-}Abl}$ wild type and 12 different imatinib-resistant mutant cell lines were treated with seven increasing concentrations of drugs. After 72 hours of treatment, the antiproliferative activity was measured using [^{3}H] thymidine uptake assay as described in materials and methods. Antiproliferative effects were determined by plotting dose-response graphs for treatment with A) NPB001-05, and B) imatinib and IC_{50} values were estimated and plotted as graphical representation for C) NPB001-05, and D) imatinib. Parent BaF3 culture was also used to estimate the drug cytotoxicity. The dotted line mid line in C and D plots represents average IC_{50} value for respective group. Vehicle treated control wells considered as 100%. The error bars represents SD for n=3 sets

imatinib at different concentrations for 72 hours. We then evaluated the influence of NPB001-05 on proliferation of CML-PBMC. All CML-PBMC samples tested were highly responsive to NPB001-05 (Figure 5A). This included three samples which were non-responsive to imatinib treatment although the mutation was not identified for these particular samples (Table 5). Figure 5A,B shows dose response curve for all CML sample tested. Table 5 represents brief information about the patient history including the IC_{50} values for NPB001-05 and imatinib treatment .We observed a dose-dependent decrease of proliferation with NPB001-05 on all the CML patient cultures.

4.6. NPB001-05 blocks cell cycle progressing and induces apoptosis of Bcr-Abl positive cells

We tested NPB001-05 for induction of cell-cycle arrest and apoptosis. Seventy-two hours NPB001-05 treatment results in accumulation of G_0/G_1 sub population and significant cell sub-population showed apoptosis. This effect was seen with wild type and Bcr-Abl^{T315I} cell lines. Propidium iodide staining following 72 hours NPB001-05 treatment showed a dose dependent increase in the proportion of 2N sub population and significant cell population showing apoptosis (less than 2N). Consistent with proliferation assays results, induction of apoptosis were observed (Table 6). Additionally, staining of K562 cells with annexin-V supported apoptotic effect of NPB001-05. Annexin-V staining was performed after 24- and 48- hours of NPB001-05 treatments on K562 and BaF3P210$^{Bcr\text{-}AblT315I}$ cells (Figure 6A, B). In both cell lines, apoptotic cells were detectable as early as 24 hours after treatment with NPB001-05; incremental number of cells population was stained positive for annexin-V by end of 48 hours of drug treatment. These results confirm that the inhibition of proliferation in Ph$^+$ cells was due to the induction of apoptosis caused by treatment of NPB001-05.

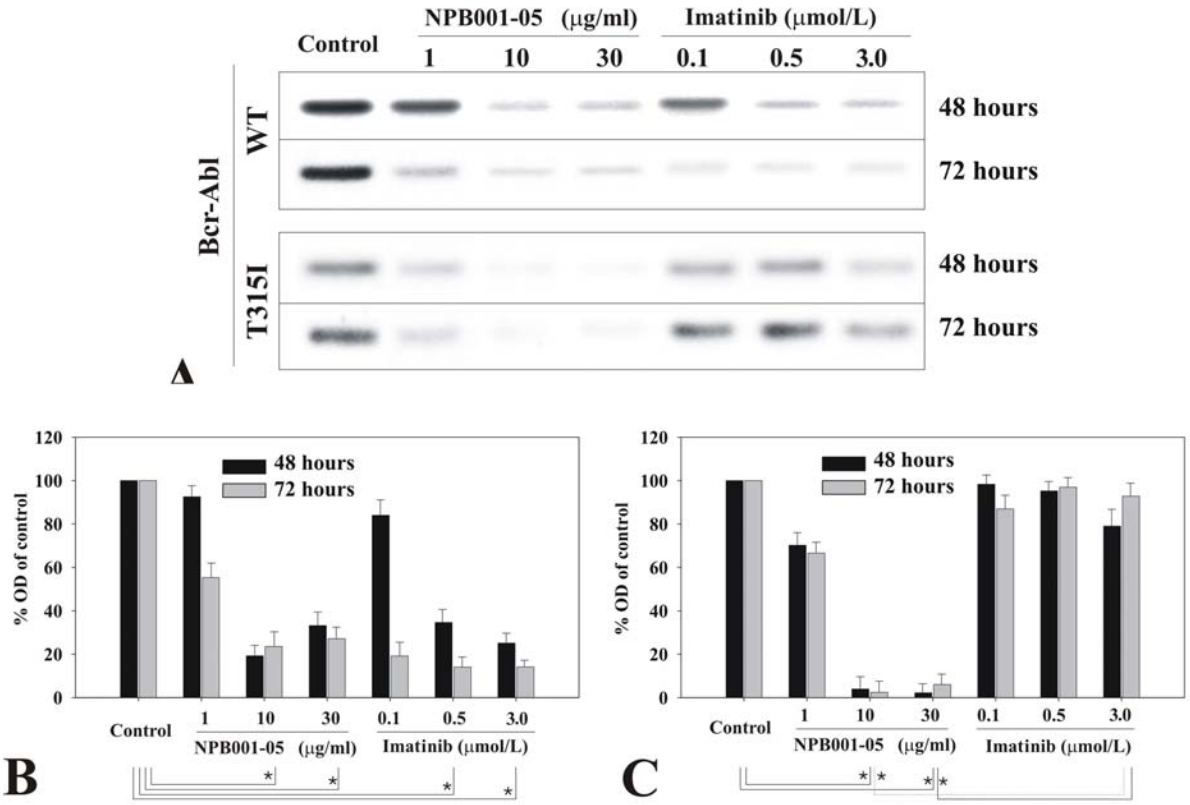

Figure 4. Inhibition of Bcr-Abl tyrosine kinase autophosphorylation. K562 and BaF3P210$^{Bcr\text{-}Abl}$ cells were treated with NPB001-05 and imatinib at indicated concentrations for 48- and 72- hours. P210$^{Bcr\text{-}Abl}$ protein was immune-precipitated from the whole cell lysates after normalization of protein content, followed by kinase assay using gamma ^{32}P-ATP. The phosphoprotein was resolved on SDS-PAGE, and read by autoradiography. A) autoradiogram of P210 indicating the inhibition of autophosphorylation of wild type and T315I mutant Bcr-Abl by NPB001-05 treatment. In contrast, imatinib inhibits only wild type Bcr-Abl kinase activity. Densitometry evaluation of kinase reaction measured from band formed by radiolabeled gamma^{32}P-ATP in B) K562 cells and C) P210$^{Bcr\text{-}AblT315I}$ cells from three separate determinations. Control denotes the vehicle treated sets. Asterix indicate P less than or equal to 0.05

4.7. *In-vitro* profiling of NPB001-05 different kinases

We examined NPB001-05 in *in-vitro* kinase activity for a panel of purified protein tyrosine and serine/threonine kinases. The concentration of compound resulting in 50 % reduction of kinase activity or 50 % reduction in tyrosine phosphorylation of specific tyrosine kinase is reported. Results presented in Table 7, represents the IC_{50} values of various kinase tested. NPB001-05 shows a very potent inhibition of Bcr-Abl and Bcr-Abl^{T315I}, and there was no significant inhibition of other protein kinases tested, except c-kit kinase. Other kinases like PDGFR-beta, Cdk2, Cdk4, c-Src, JAK2 and Flt3 tested were not affected by NPB001-05. Effect of imatinib on same kinase is complied from previous reports (42) (Table 7)

5. DISCUSSION

Imatinib has considerably changed the landscape of CML treatment, achieving cytogenetic response rates of more than 80 %, translating significant survival of patients. However, during the course of treatment with imatinib, this success is compromised to some degree. Firstly because of primary or acquired resistance to imatinib resulting into imatinib-resistance (43), secondly, the limited therapeutic efficacy in advance disease stage (44) and thirdly because of limited effect of drug on immature hematopoietic stem cells leading to minimal residual disease stage (45). Point mutations in the kinase domain Bcr-Abl are the primary cause of resistance leading to decreased or lost sensitivity to imatinib. In past few years, effort to restore target inhibition of mutated Bcr-Abl led to the discovery and development of second generation Bcr-Abl inhibitors, such as dasatinib (46), nilotinib (47), and bosutinib (48). Although they achieved promising clinical outcomes for most mutations, the frequently observed mutant T315I was not effectively targeted by any of these compounds. Hence, Bcr-Abl with T315I gatekeeper mutation is predicted to become the major mechanism of resistance to second-generation Bcr-Abl inhibitors, inviting the need for the development of novel therapeutic strategies to overcome T315I mediated resistance (49-52). One such approach, which has achieved noteworthy success *in-vitro*, is the use of combination of specific kinase inhibitors, with agent's impairing transcription (53) and/ or translation (54, 55), and has proven in decreasing proliferation of CML cell lines and primary CML cells, including those with T315I

Table 5. CML patient characteristics and IC_{50} values for NPB001-05 and imatinib *in-vitro* treatments

Patient ID	Patient code	Age	Sex	Diagnosed in	Ongoing treatment of imatinib	Response to imatinib	Consent on	IC_{50}	
								NPB001-05 μg/ml	Imatinib μmol/L
CML-1	NVS	38	M	1998	-	NR[1]	29 Mar 2006	14.0	7.5
CML-2	NAR	42	F	2006	400 mg/day	R[2]	29 Mar 2006	13.0	0.5
CML-3	VAN	32	F	2002	-	NR[1]	10 Apr 2006	11.0	more than 10.0
CML-4	MB	46	M	1990	600 mg/day	R[2]	12 Apr 2006	6.5	0.2
CML-5	KSR	59	M	2000	400 mg/day	R[2]	12 Apr 2006	11.0	2.0
CML-6	SR	26	F	1998	-	NR[1]	12 Apr 2006	7.5	8.0
CML-7	ASR	20	M	2004	400 mg/day	R[2]	12 Apr 2006	6.0	0.3
Average IC_{50}	-	-	-	-	-	-	-	**9.8 +/- 3.1**	**more than 4.0 +/- 4.2**

[1] NR Non responder, [2] R responder

Table 6. Effect of NPB001-05 and imatinib on cell cycle

Cell lines	Phase percentage	Control	NPB001-05 (μg/ml)			Imatinib (μmol/L)		
			1.0	10.0	30.0	0.1	0.3	3.0
K562	$SubG_0$	3.6	7.0	15.4	33.4	7.7	16.4	40.8
	G_0G_1	37.1	37.3	49.7	44.4	37.1	29.8	25.6
	S	21	14.0	10.2	10.6	19.6	24.1	10.5
	G_2M	37.9	41.4	24.3	11.5	35.1	29.5	22.9
$BaF3P210^{Bcr\text{-}AblT315I}$	$SubG_0$	6.8	13.0	25.4	37.4	6.5	9.2	10.8
	G_0G_1	41.5	35.7	43.3	48.4	34.3	34.2	39.6
	S	20.6	17.2	11.0	5.4	21.6	19.3	13.7
	G_2M	31.1	33.4	19.9	8.7	37.5	37.1	35.7

K562 cells and $BaF3P210^{Bcr\text{-}AblT315I}$ cells were exposed to varying concentrations of NPB001-05 and imatinib for 72 hours. Cells were fixed with ethanol and stained with propidium iodide and acquired on a flow cytometer for DNA content, as percentage of 4 sub-populations as 1) dead cells, $SubG_0$ (less than 2N DNA), 2) G_0G_1 (2N DNA), 3) G_2M phase(4N DNA) and 4) S phase (intermediate between 2N and 4N DNA), from more than or equal to 10,000 cells analyzed. DMSO treated cells indicated as control

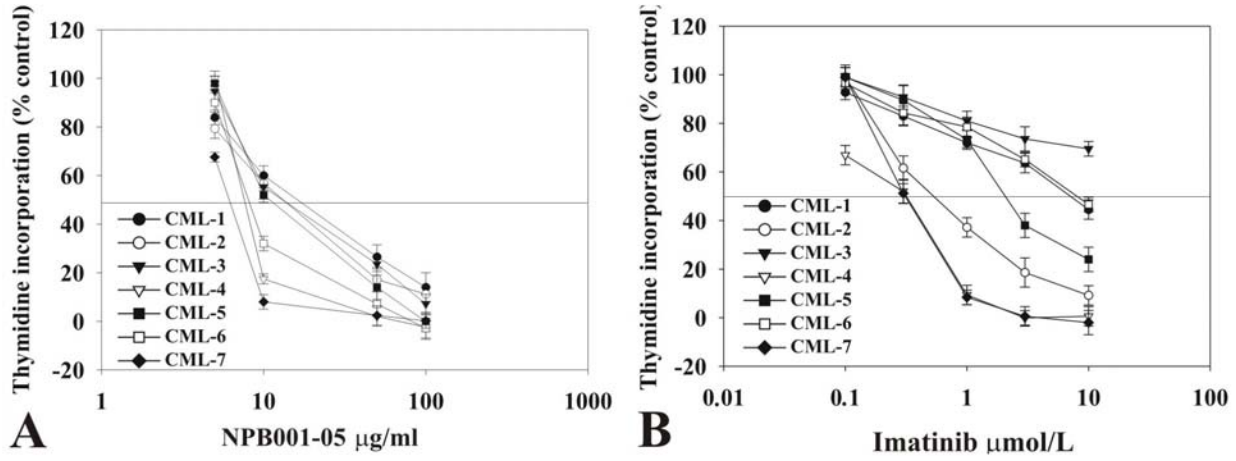

Figure 5. Effect of NPB001-05 and imatinib on mononuclear cells from Bcr-Abl positive CML patients. PBMC's were isolated by Ficoll-Hypaque from seven CML patients and cultured in medium containing GM-CSF, in presence of increasing drug concentrations for 72 hours. Proliferation inhibition was estimated using [^{3}H] thymidine uptake assay. Graphs A) and B) represents the dose response curves for NPB001-05 and imatinib, respectively. DMSO treated wells were measured as 100% as control. The experiment was performed in technical replicate for each concentration comprising six wells seen as SD bars. Dotted line in the middle of graph represents 50% cytotoxicity.

mutation. Current strategies take the advantage for employment of rationally-designed agent's that couples with ATP-binding pocket of Bcr-Abl or utilize Bcr-Abl kinase sites distant from the ATP-binding pocket, resulting the inactivation of Bcr-Abl enzyme. A barely explored field as therapeutic modality in CML is the use of natural products, as small molecules are proven to confer resistant especially in T315I-CML by increasing the Bcr-Abl kinase activity (56). For time immemorial, plants have played a dominant therapeutic role in the treatment of several human ailments. Anticancer activities possessed by many plant materials, whether in form of chemoprevention or as pharmaceutical care has helped overcome cancer and improve survival to some extend (57, 58). Crude or semi-purified plant extracts are compound libraries by itself, various compounds within which might influence different tumor signaling pathways or cell-cycle check points individually or in combination. Embracing the idea of

Table 7. Ability of NPB001-05 and imatinib to inhibit the phosphorylation of Bcr-Abl and other kinase in *in-vitro* kinase assay.

Kinase assay	NPB001-05 IC_{50} (μg/ml)	imatinib IC_{50} (μmol/L)[3]
Bcr-Abl[1]	3.0	0.2
T315I[1]	1.0	more than 10.0
c-Kit[1]	1.0	0.41
PDGFR-beta[1]	more than 100.0	0.38
Flt3[1]	more than 100.0	more than 10
c-src[1]	more than 100.0	more than 100
Cdk2-E/A[2]	more than 100.0	more than 100
Cdk4-D1[2]	more than 100.0	more than 100
JAK2[1]	70.0	more than 100

[1]tested at Kinase-profiler, [2] tested in-house, [3]values are evaluated from a at least 4 different concentrations. Kinase activity was measured with radioactive kinase assay using exogenous peptide substrates at Kinase-profiler[1] and tested in-house[2]. Data on the same kinases for imatinib available from previous literature is also reported

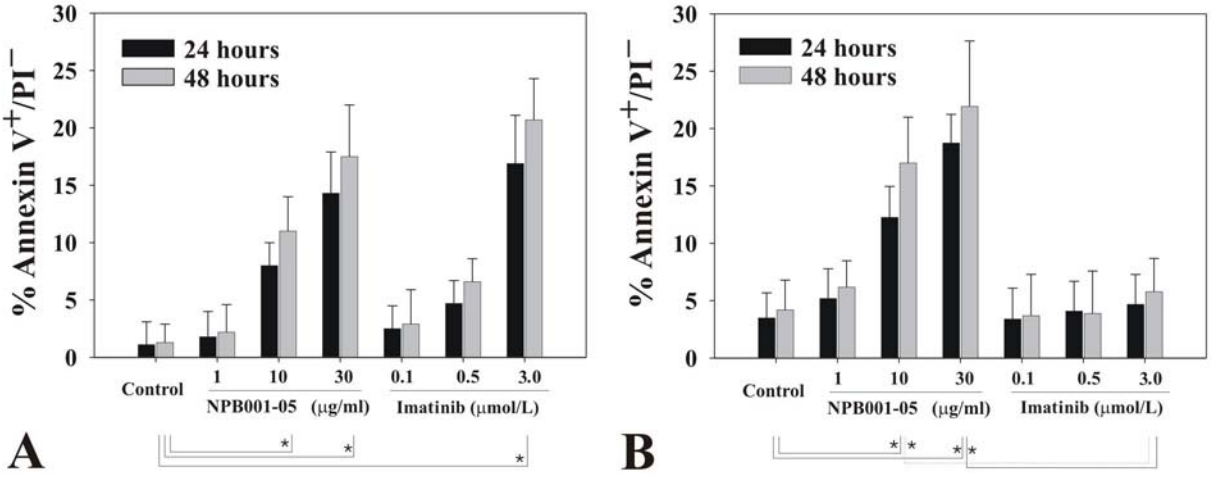

Figure 6. Effect of NPB001-05 and imatinib on induction of apoptosis. K562 and BaF3P210$^{Bcr\text{-}Abl}$ cells were treated with NPB001-05 and imatinib at indicated concentrations for 24- and 48- hours. Apoptotic cell were analyzed using annexin-V binding protocol, and estimating more than or equal to 10,000 events on a flow cytometer. Percent cells stained positive for annexin-V from three independent determinations are plotted for A) K562 cells and B) BaF3P210$^{Bcr\text{-}AblT315I}$ cells. Error bars indicates SD, and DMSO as vehicle stands as control sets. Asterix indicate P less than or equal to 0.05.

using natural products targeting key enzymes simultaneously seems a desirable approach in the treatment of CML.

Here we describe a novel kinase inhibitor NPB001-05 that exhibits inhibitory effect on both Bcr-Abl wild type and various imatinib-resistant Bcr-Abl mutant kinases. Efficacy of NPB001-05 was demonstrated against a two chronic myelogenous leukemia cell lines. Treatment with NPB001-05 significantly decreased the proliferation of K562 and KU812 Bcr-Abl positive human leukemia cell lines (Table3) as well as murine BaF3 cells expressing wild type P210$^{Bcr\text{-}Abl}$ and different imatinib-resistant Bcr-Abl mutants, including T315I (Table 4). NPB001-05, like imatinib, possessed selective inhibition of Ph^+ cancerous cell lines when tested against a panel of Ph^- (HL60 and MOLT4) and solid tumor cell lines (MCF7, H460, HCT116 and PC3). Conversely antiproliferative ability of NPB001-05 did not depend on Bcr-Abl mutational status to the degree of resistance to imatinib. The inhibition of proliferation was within the same ranges, as observed by relative coefficient factor C1 (Table 4), in all mutant conferring resistance to imatinib, including the highly imatinib-resistant T315I, E255V and Y253H. No effect was observed in wild-type BaF3 cells for treatment with NPB001-05 and imatinib (Figure 3 C and D).

Further we explored the effect of NPB001-05 in primary cultures of clinical materials. Efficacy of NPB001-05 was examined in isolated PBMCs from seven CML patients at different stages of diseases. (Table 5). A dose dependent inhibition of cell proliferation was observed in all PBMCs exposed to NPB001-05 (Figure 5A), with an IC_{50} values (9.8 +/- 3.1 μg/ml) close to IC_{50} values (7.8 +/- 0.4 μg/ml) for the *in-vitro* grown CML cell lines. Not much patient history was described pertaining to the acquired resistance, except that most CML-patients were kept on more than or equal to 400 mg/day of imatinib. Three out of seven patients diagnosed not after the year 2002 were resistant to imatinib, were also resistant in *in-vitro* cell proliferation assay, whereas NPB001-05 showed dose dependent inhibition for all the CML-patient cells tested. Additionally, we estimated the action of NPB001-05 on healthy human PBMC donors. NPB001-05 was found to be non-cytotoxic for PBMC isolated from healthy human donors whether grown in presence or absence of mitogen activation. Imatinib in agreement with the previous study (59) under *in-vitro* stimulatory conditions showed a dose-

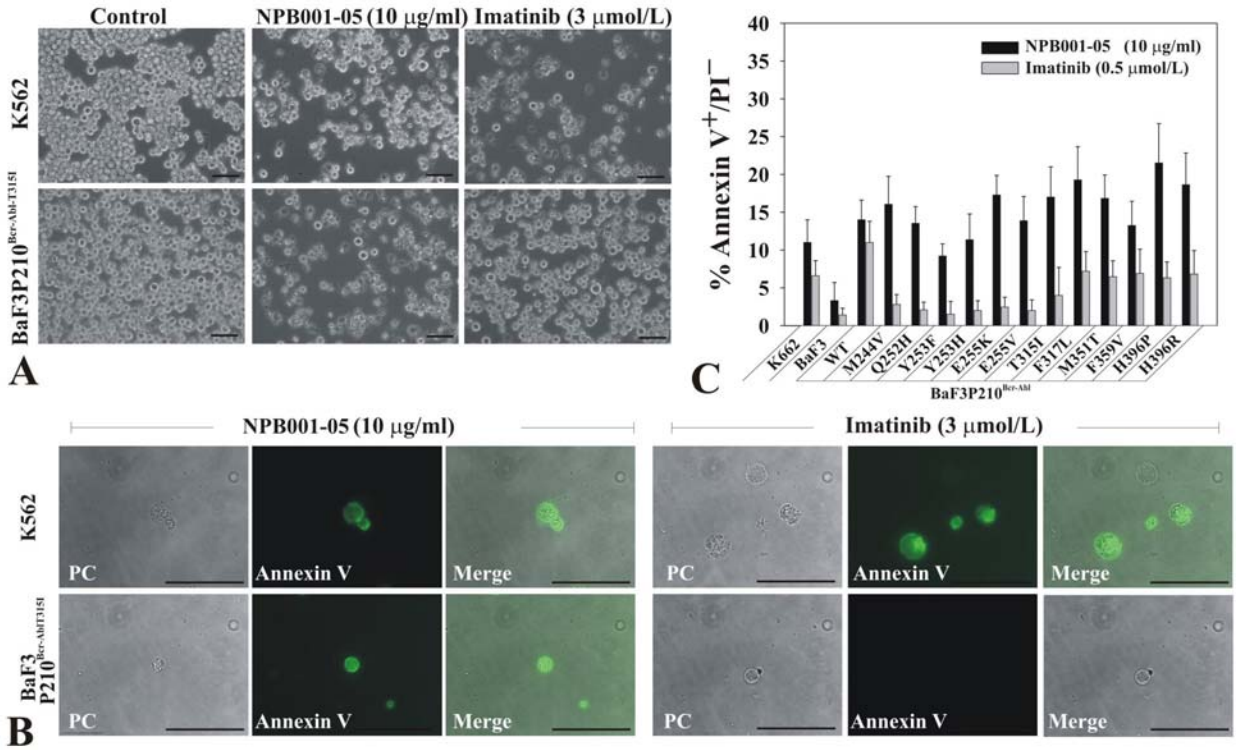

Figure 7. Induction of apoptosis and cell death. K562 and BaF3P210$^{Bcr\text{-}Abl}$ cells, including the 12 variable mutant lines + the parent BaF3 cell line, were treated with NPB001-05 and imatinib for a period of 48 hours. A) Phase contrast microscopic pictures of K562 and BaF3P210$^{Bcr\text{-}Abl}$ cells treated with NPB001-05 and imatinib for 48 hours and B) fluorescence microscopy images from treatments after staining with annexin-V. Scale-bars measures 50μ. C) Flow cytometric analysis of control (DMSO) *vs.* treatment annexin-V binding, represented as cells positively stained for annexin- V, from three separate estimations. Error bar represents SD values.

dependent inhibition of proliferation of PBMC's derived from healthy donors, which is reflected by the relative coefficient factor C2 (Table 2) which in all cases is less than 0.5. Conversely, NPB001-05 was not cytotoxic to stimulated PBMC as seen by the relative coefficient factor C2 which was ≥ 0.9 for all the donors tested. Importantly NPB001-05 was not toxic to the diploid human fibroblast lines WI38 and MRC5, and did not affect their proliferation (Table 3) (60). Taken together, we conclude that NPB001-05 selectively inhibits Bcr-Abl expressing wild type or imatinib-resistant-Bcr-Abl mutant cell lines and primary CML cultures; at the same time NPB001-05 was found to be non-cytotoxic for normal primary culture.

We further elucidated the cytotoxicity induced by NPB001-05 in Bcr-Abl expressing cells and associated it to inhibition of tyrosine kinase phosphorylation and induction of apoptosis. Bcr-Abl protein is a major factor in the pathophysiology of CML, which is mediated by the enhanced tyrosine kinase activity (5, 61). All targeted molecules indentified in treatment of CML were inhibitors of autophosphorylation for Bcr-Abl enzyme (62). A highly significant decrease of autophosphorylation of wild type and Bcr-Abl^{T315I} kinase was observed after treatment with NPB001-05. This effect was time and dose dependent. Interestingly, the decrease of phosphorylation of Bcr-Abl with highly resistant mutant T315I was markedly better than the wild type Bcr-Abl, indicating an effective inhibition of Bcr-Abl carrying this highly resistant mutation (Figure 4B), whereas no inhibition of phosphorylation could be induced by imatinib treatment (Figure 4C). Imatinib induced inhibition of tyrosine kinase was observed only with the wild type Bcr-Abl cells and not with Bcr-Abl^{T315I} mutant cell line. Down modulation of Bcr-Abl kinase activity induces apoptosis and cell death (63). Treatment with NPB001-05 induced apoptosis, as early as 24 hours of drug treatment, and a significant cell population was in this zone after 48 hours, measured by annexin-V staining. After 48 hours of NPB001-05 (10 μg/ml) and imatinib (3 μmol/L) treatments, K562 cells were observed with condensed nuclei and with apoptotic blebs. In BaF3$^{P210Bcr\text{-}AblT315I}$ cells only NPB001-05 showed the presence of apoptotic morphologies (Figure 7A), which was also confirmed from fluorescent microscopy pictures reading annexin-V (FITC) (Figure 7B). Moreover, NPB001-05 induced apoptosis in all Bcr-Abl expressing cells (K562 and BaF3$^{P210\ Bcr\text{-}Abl}$ wild type and all 12 mutant cell lines) tested with annexin-V staining by FACS (Figure 7C), interesting not affecting the parent BaF3 culture. In addition 72 hours of NPB001-05 treatment showed cell cycle arrest at G_0G_1, and an accumulation of apoptotic cells in subG_0 phase. Collectively, we demonstrate that NPB001-05 treatment causes distinct inhibition of Bcr-Abl autophosphorylation, indicating decreased tyrosine kinase

activity and hence induction of apoptosis. NPB001-05 has no effect on some know kinases tested in *in-vitro* kinase assays, like c-Src, JAK2, Cdk4, cdc2, PDGFR-beta and Flt3; however a potent inhibition of Bcr-Abl and Bcr-Abl^{T315I} kinase is confirmed by NPB001-05 treatment. Interestingly, another high level potent inhibition was observed in c-kit kinase assay, however an elaborative *in-vitro* evaluation for the c-kit inhibition has to be perused. Thus, in line with the results of all *in-vitro* experiments, these findings confirms high efficacy of NPB001-05 in CML cells independent of the Bcr-Abl mutational status, including T315I.

In past decade, important implication for new drug development for CML is in focus due to the developing resistance for imatinib and other tyrosine kinase inhibitors. The CML drug pipeline is strongly centered on Bcr-Abl mutation and the inhibition of kinase whose expression is affected by Bcr-Abl (40). The most important Bcr-Abl mutation is undoubtedly T315I, which accounts for 15 % of all mutation-related tyrosine kinase resistance and is not responsive to any approved tyrosine kinase inhibitor (64). We utilized the knowledge from traditional uses as Ayurvedic medicine (41) for the treatment of CML. NPB001-05 is a product from *Piper betle* an ancient medicinally importance herb from Indian-subcontinent. Betel leafs have a proven efficacy as antimicrobial, antioxidant anti-inflammatory and anticancer properties (27, 65-70). The data in this article suggests a prospective use of *Piper betle* (NPB001-05) for the treatment modality in CML. NPB001-05 is extract of betel leafs, with inhibitory potential for Bcr-Abl kinase and largely independent of imatinib-resistant mutation form of Bcr-Abl, including T315I. The results of this study indicate the potential use of NPB001-05 in CML therapy as single agent and argue in favor of potential clinical applicability that will hopefully result in additional major advances in the therapy of 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, PBMC peripheral blood mononuclear cells, Ph Philadelphia chromosome, Ca. Cancer, TK tyrosine kinase, DMSO Dimethyl sulfoxide, FBS fetal bovine serum, GMCSF Granulocyte-macrophage colony-stimulating factor, PHA Phytohaemagglutinin, WST-8 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, PBS Phosphate buffered saline, PI Propidium iodide, FACS Fluorescence-activated cell sorting, CoA certificate of analysis, 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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