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**Research Article**

# DISCOVERY, SYNTHESIS AND PRECLINICAL EVALUATION OF AN-024, A POTENT INHIBITOR OF ACTIVE AND INACTIVE FORMS OF BCR- ABL

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**ABSTRACT**

Chronic Myelogenous Leukemia (CML) and Philadelphia chromosome positive (Ph+) Acute Lymphoblastic Leukemia (ALL) are caused by the *BCR-ABL* oncogene. Imatinib inhibits the tyrosine kinase activity of the *BCR-ABL* protein and is an effective, frontline therapy for chronic-phase CML. However, accelerated or blast-crisis phase CML patients and Ph+ ALL patients often relapse due to drug resistance resulting from the emergence of imatinib-resistant point mutations within the *BCR-ABL* tyrosine kinase domain. This has stimulated the development of new kinase inhibitors that are able to over-ride resistance to Imatinib. The novel *BCR-ABL* inhibitor, AN-024, was designed to fit into the ATP-binding site of the *BCR-ABL* protein with higher affinity than Imatinib. In addition to being more potent than Imatinib (IC50<1 nM) against wild-type *BCR-ABL*, AN-024 is also significantly active against Imatinib-resistant *BCR-ABL* mutants. In preclinical studies, AN-024 demonstrated activity *in vitro* and *in vivo* against wild-type and Imatinib-resistant *BCR-ABL* expressing cells.

## INTRODUCTION

Chronic Myelogenous Leukemia (CML) is associated with the Philadelphia chromosome(Ph), a cytogenic abnormality generated by reciprocal translocation between the BCR gene (Break point Cluster Region) on chromosome 22q and the c-ABL (Abelson Leukemia virus) proto-oncogene on chromosome 9q.[1,2] Imatinib mesylate(STI57I, Gleevec or Glivec; Figure 1) is an Adenosine Triphosphate(ATP) competitive inhibitor of Abl. Several clinical trials revealed that imatinib mesylate induces hematologic remissions in up to 98% of patients, depending on the disease phenotype and stage.[3-7]

Crystallographic studies revealed that STI-571 binds to the kinase domain of c-ABL only when the domain adopts the inactive 'closed' conformation.[8,9]However, despite the good hematological and cytogenic responses obtained, primary refractory disease and secondary resistance still occur with STI-571, particularly in patients with advanced phase disease.[10] Although several mechanisms have been proposed to account for this resistance , including increased expression of BCR-ABL protein, amplification of the BCR-ABL gene and over expression of the multidrug resistance P-glycoprotein, [11-14] point mutations in the BCR-ABL gene itself account for most cases of resistance.

Based on crystallographic studies, it was suggested that the potency and selectivity of Imatinib mesylate could be improved by incorporating alternative binding groups to the N-methyl piperazine moiety of the compound. This led to the development of N-methyl-4-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-3-trifluoromethanesulfonyl-benzamide, AN-024, a high affinity phenylamino pyrimidine based ATP competitive inhibitor. AN024, in contrast to imatinib, binds the kinase domain of ABL kinases in the active and inactive conformations.

### Drug design

AN-024 was developed by modifying the chemical structure of imatinib by using a rational drug design strategy based on the replacement of the methylpiperazinyl group of imatinib and optimization to increase potency against the ABL kinases.

### Imatinib

4-[(4-methylpiperazin-1-yl)methyl]-N-[4-methyl-3-[(4-pyridin-3-ylpyrimidin-2-amino]phenyl]benzamide.

### AN024

3-(Trifluoromethylsulfonyl)-N-[4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]phenyl]benzamide To aid in the design of novel inhibitors, we first analyzed the crystal structure of imatinib in BCR- ABL target (PDB ID – 1IEP).

The key interactions of imatinib are illustrated in **figure-2**

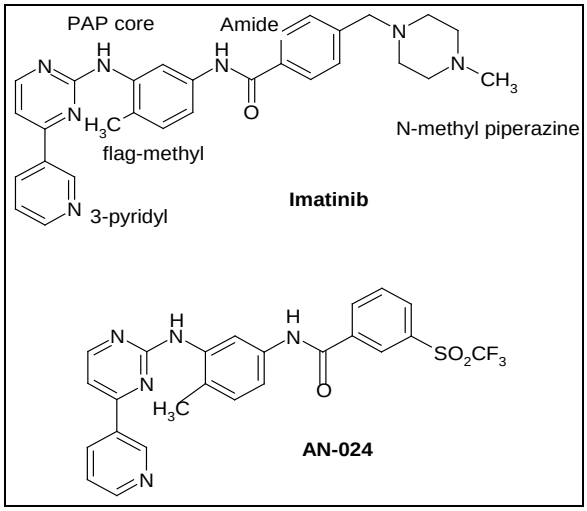



**Figure 1: shows structures of Imatinib and AN-024**

Imatinib binds to ABL domain via six hydrogen bond interactions . The hydrogen bonds involve the pyridine-N and backbone-NH of Met-318, the aminopyrimidine and side chain hydroxyl of Thr-315, the amide-NH and side chain carboxylate of Glu-285, the carbonyl and backbone-NH of Asp-381, the protonated methylpiperazine with the backbone-carbonyl atoms of Ile-360 and His-361. Additionally, a number of Vander Waals interactions contribute to binding.[15] A hydrophobic pocket is formed by amino acid residues Ile-293, Leu-298, Leu-354 and Val-379 around the phenyl ring adjacent to the piperazinyl-methyl group of imatinib.[16]

Molecular docking of AN-024 in the active site of BCR-ABL was carried out using modern docking engine LIGANDFIT available with Discovery Studio 2.1.[17] . AN-024 was manually docked into the binding site. Molecular model overlay confirmed that AN-024 fits in same as imatinib and trifluoromethanesulfonyl group substitution does not affect the major H-bond interaction as present in imatinib

Figure-3 depicts the docking model of ABL in complex with AN024 and imatinib

Analysis indicated the presence of similar H-bond interaction is present in AN-024: the nitrogen of the pyridine with the Met318, the Nitrogen of the aniline group with backbone of Thr315, the amide-NH with the side-chain carboxylate of Glu286, and the amide-

CO with the backbone-NH of Asp381. These hydrogen bonds are complemented by extensive hydrophobic interactions over the whole length of the inhibitor.

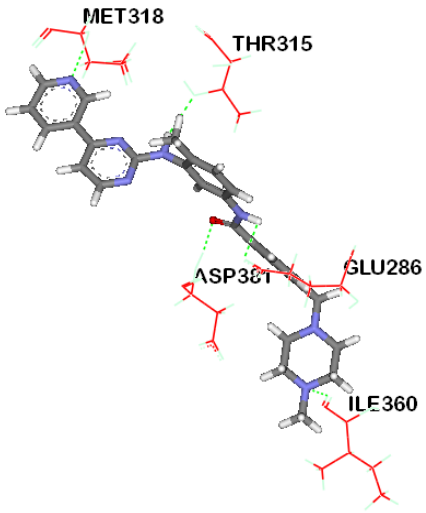



**Fig 2: Imatinib H-bonds represented as green dots docked on PDB ID 1IEP.**

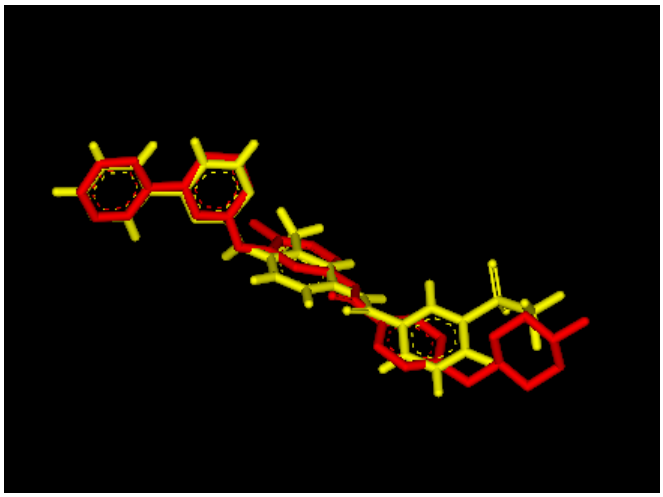


**Fig 3: Overlaid structure of Imatinib (red) with AN-024 (yellow)**

To check the potency of AN-024 further on the active form of BCR-ABL for which imatinib is weak binder, we selected PDB ID – 2GQG Crystal structure bound with dasatinib. Dasatinib is able to bind BCR-ABL with less stringent conformational requirements with respect to imatinib, therefore allowing for efficient inhibition of several, resistance-associated mutant forms of BCR-ABL;( Imatinib forms six hydrogen bonds with the inactive ABL Kinase protein and several other contacts with the active site are mediated through van der waal interactions) which is the same for AN-024 indicated by molecular modeling studies, as they are not forming 6 H-bond with the inactive form of ABL. Our Molecular modeling studies confirmed that AN-024 is even comparable with dasatinib

Figure-**5** depicts the docking model of ABL in complex with AN-024 and dasatinib

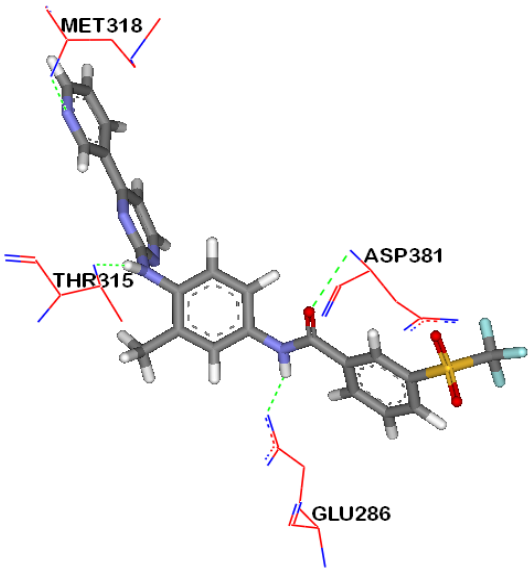



**Fig 4: Interactions of AN024 at the binding site**

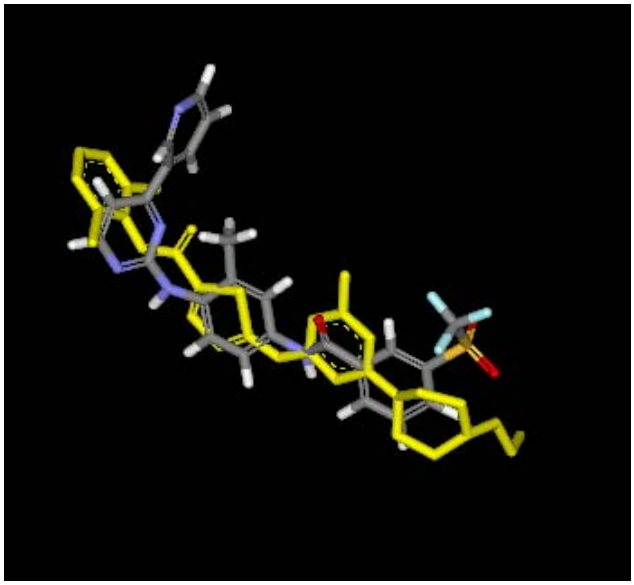


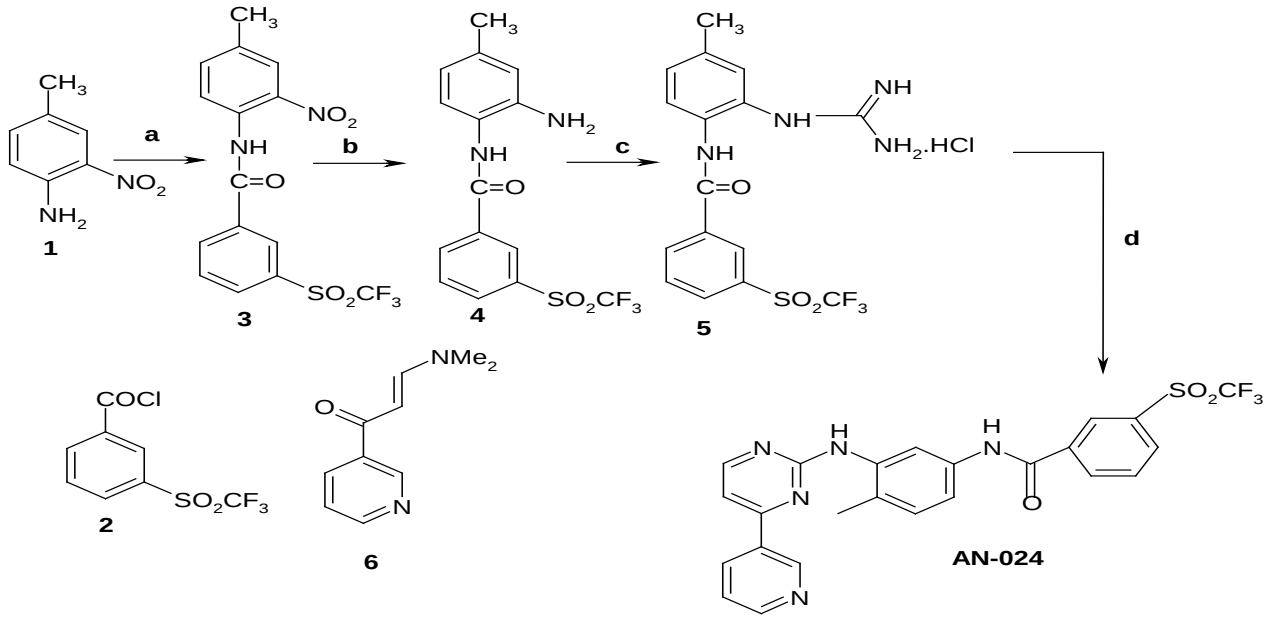
**Fig 5: Docking model of ABL in complex with AN-024 and dasatinib**

**Scoring functions**

The docked conformations were further scored using different scoring functions available with Ligandfit. The Ligand algorithm uses an internal scoring function, Dock Score, to select and return dissimilar poses for each compound. Dock Score is a simple force field based scoring function, which estimates the energy of interaction by summing the ligand/protein interaction energy and the internal energy of the ligand. CFF force field was used to resolve the van der waals parameters for Dock Score. The top Dock Score pose was used for post docking scoring. Scoring was performed using a set of scoring functions as implemented in Ligandfit. These included LigScore1, LigScore2, PLP1, PLP2 and Dock Score available from the docking process. The putative 3D poses and score results were then stored as an SD file.

**Table 1: Scoring table (ligandfit)**

| Molecule | PLP1 | PLP2 | DOCK | LIG2 | H-BONDS |
|---|---|---|---|---|---|
| IMATINIB | 101.81 | 116.42 | 5.16 | 5.16 | IEL360, ASP381, MET318, THR315, GLU286 |
| AN-024 | 115.16 | 112.7 | 102.67 | 6.69 | MET318, GLU286, THR315, ASP381 |




Scheme 1 : Reagents and conditions ; a) **2**, NaOH, Chloroform b) $SnCl_2$ , HCl

c) $CN\text{-}NH_2$ , n-butanol, reflux d) **6,** n-butanol, reflux

**EXPERIMENTAL**

The synthesis of 3-(Trifluoromethylsulfonyl)-N-[4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]phenyl]benzamide, AN-024 is illustrated in Scheme 1.[18]

Benzoylation of 4-methyl-2-nitro-aniline (1) with (3-trifluoromethylsulfonyl)benzoyl chloride (2) at a temperature range 30-40°C in chloroform with the addition of aqueous alkali afforded (3-trifluoromethylsulfonyl)-N-[4-methyl-3- nitro-phenyl]-benzamide(3). Reduction of benzamide 3 with stannous chloride/ conc. HCl at 0-5°C for 3 to 4 hours afforded (3-trifluoromethylsulfonyl)-N-[3-amino-4-methylphenyl]-benzamide 4. Condensation of 4 with cyanamide solution at 90-95°C in n-butanol solvent yielded (3-trifluoromethylsulfonyl)-N-[3-guanidino-4-methyl phenyl]- benzamide (5). Condensation of 5 with 3-dimethylamino-1-pyridin-3-yl-propenone 6 in presence of sodium hydroxide at reflux temperature yielded 3-(trifluoromethylsulfonyl)-N-[4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]phenyl]benzamide(AN-024)

**Pre-clinical evaluation of AN-024**

For *in vivo* experiments involving animals due permission for conduction of these experiments was obtained, from the relevant ethics committees.

**1. Establishment of anti-CML activity of AN-024 in nude mice implanted with k562 cells**

***In vitro* studies with AN-024**

Expression levels of BCR-ABL, Crk and phospho Stat5AY$^{694/699}$ were determined by western blot analysis in K562 cells treated with various concentrations of AN-024 (1, 2.5, 5 or 10µg/ml) for 12h. It was observed that BCR-ABL, Crk and phospho Stat5AY$^{694/699}$ protein levels decreased in a dose dependent manner.

**Western Blot analysis**

K562 cells were treated with 1, 2.5, 5, or 10µg/ml of AN-024. After 12 h, cells were collected and total cell lysates were prepared in extraction buffer containing Tris [0.1 M, pH 7.5], Triton-X114 (1.0%), EDTA (10 mM), aprotinin, and phenylmethylsulphonyl fluoride. 10 µg of protein from these samples were separated under non-reducing conditions by 12% SDS-PAGE and transferred to nitrocellulose membranes . The membranes were probed for 2 h with antibodies against BCR-ABL, Crk and phospho Stat5AY$^{694/699}$ per standard protocols. The membranes were subsequently washed three times with PBS to remove excess primary antibodies, incubated with appropriate HRP conjugated secondary antibodies as required, and then developed according to enhanced chemiluminescence protocol .

**MTT cell growth assay**

K562 cells were treated with various concentrations of AN-024 (1, 2.5, 5, or 10µg/ml) for 24 h, following which MTT assay was performed to determine the proliferative index of the treated cells. The assay was performed per standard protocols according to manufacturer's instructions (Chemicon Temecula, CA). Optical density was measured at 570nm and graphically represented. Absorbance was directly proportional to the number of cells present and the number of viable cells determined.

Figure **6** shows K562 cells treated with various concentrations of AN-024 (1-10µg/ml). Expression levels of BCR-ABL, Crk and

Stat5AY$^{694/699}$ demonstrate that AN024 inhibits BCR-ABL kinase activity in a dose dependent manner.

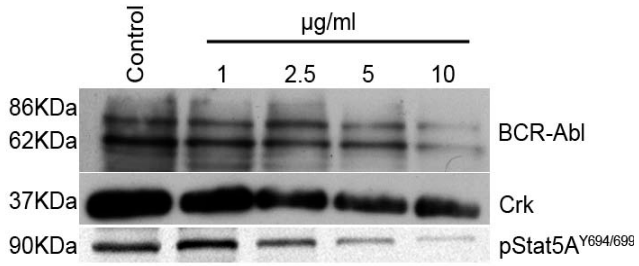



**Figure 6: K562 cells are treated with various concentrations of AN-024 (1-10µg/ml). Cell lysates were collected and western blot analysis was performed as per standard protocols. Expression levels of BCR-ABL, Crk and Stat5AY$^{694/699}$ were determined.**

**2.AN-024 injections of nude mice implanted with K562 cells did not show spleenic enlargement and no Crk expression**

Nude mice implanted with K562 cells were treated with Imatinib or AN-024 via ip injections. Mice were sacrificed after a decrease in LGI was observed and spleen harvested. Spleenic enlargement was observed by gross observation and it was determined that control mice showed enlarged spleen indicative of K562 cellular localization. AN-024-treated cells did not show any spleenic enlargement whereas Imatinib treated mice showed slight spleenic enlargement

Paraffin sections of spleen immuno-probed for the presence of Crk protein showed strong localization of Crk expression accompanied with increased cellular density indicative of K562 localization in control mice.. Mice treated with Imatinib indicated localized expression regions of Crk expression, indicative of K562 cells in the spleen. Control mice also developed random subcutaneous tumors showing Crk expression, indicative of the presence of K562 cells.

From these results it is evident that AN-024 treatment caused the regression of LGI in nude mice. Imatinib treated mice also showed significant reduction in LGI but lagged AN024-treated mice. Both AN024-and Imatinib treated mice showed no abnormal physiological, phenotypic or behavioral abnormality. Control mice showed the presence of random subcutaneous tumors with loss of digits accompanied with reddish spots under the skin and a slight enlargement of the abdomen. From these results, it is evident that AN-024 could be a promising therapeutic drug for the treatment of leukemia.

**3. *In vivo* studies using Baf3 Imatinib resistant murine CML cell lines**

To determine the *in vivo* anti-leukemic activity of AN-024, nude mice were implanted intraperitoneally with Baf3 murine leukemia cells (Wt, T315I, M351T and E225K), and 15 days after implantation the mice were treated with Imatinib (10mg/kg), and AN-024 (20mg/kg) by oral gavage or ip injections. Blood smears were obtained via the tail vein or via the femoral vein every 6th day and blast cells counted and graphically represented. Blood smears of Baf3Wt implanted mice treated with Imatinib (10mg/kg), or AN024 (20mg/kg) were similar to normal controls after 42 days. Blood smears of Baf3M351T implanted mice treated with Imatinib (10mg/kg), or AN-024 (20mg/kg) showed a significant decrease in blast cell count in AN-024 treated mice. Mice treated with an oral dosage of Imatinib did not show a decrease in blast cell count and were similar to untreated controls. Blood smears of Baf3T315I implanted mice treated with Imatinib (10mg/kg), or AN-024 (20mg/kg) showed a significant decrease in blast cell count in AN024 treated mice. Mice treated with oral dosage of Imatinib did not show a decrease in blast cell count and were similar to untreated controls and Baf3M351T implanted mice. Mice implanted with Baf3E255K also behaved similarly to Baf3M351T and Baf3T315I implanted mice.

Nude mice were implanted with Baf3 mutant cells Wt, E255K, T315I, and M351T were treated with Imatinib and AN-024 (orally and ip). Briefly, mice were treated with Imatinib (10mg/kg), and AN-024 (20mg/kg) by oral gavage or ip injections 15 days post-implantation. Abdominal swelling and decrease in activity was monitored daily, blood smear taken via the tail vein or the femoral vein every 6$^{th}$ day and H&E stained as per standard protocols. 42 days after implantation, mice were sacrificed and spleens harvested. Spleenic enlargement was determined and correlated with blood smear blast cell count.

**Results**

**Microscopic determination of blast cell count**

Blood from tail vein or the femoral vein was taken every 6th day from Baf3 cell implanted mice until day 42. On the 15th day post-implantation, the mice were given treatments with Imatinib and AN-024 (orally and ip) as described earlier. It was observed that in Baf3Wt implanted mice, progressive decrease in blast cell count was observed in all treatment conditions. Baf3M351T, T315I and E225K did not respond well to Imatinib treatments. Oral administration of Imatinib had no significant effect in Baf3M351T, T315I and E225K implanted mice. Overall intraperitoneal treatments AN-024 was significantly better at decreasing blast cell count in all Baf3 implanted mice.

AN-024 treatment in Baf3 M351T, T315I and E225K implanted mice showed a significant decrease in blast cell count at day 42; ip-treated mice showed greater regression of blast cells than orally treated mice.

**4. Response of nude mice implanted with k562 normal/luc human leukemic cells with low dose of AN-024 and Imatinib**

**Oral administration of AN-024 and Imatinib**

Nude mice (nu/nu) were implanted with K562 cells (1x106) normal/luc via tail vein. Five mice were used per group and divided into 24 groups + 2 control. All groups were implanted with K562 normal/luc cells. Of the 24 groups, 12 were used for luciferase studies whereas the other 12 were used for blood smear count studies.

Drugs were administered by oral gavage as previously suggested (2% gum acacia and 2% SLS in an aqueous suspension).

**Results**

Oral administration of AN-024 (1, 5, 10 and 20mg/kg) in nude mice implanted with K562 luc human leukemia cells resulted in leukemia regression at higher concentrations. AN-024 concentration at 1mg/kg did not induce a decrease in luciferase expression, whereas 5mg/kg, 10 mg/kg, and 20mg/kg concentrations caused a decrease in luciferase expression.

Oral administration of Imatinib (1, 5, 10 and 20mg/kg) in nude mice implanted with K562 luc human leukemia cells resulted in leukemia regression at higher concentrations. Imatinib administration at 1mg/kg concentration did not induce a decrease in luciferase expression, whereas 10mg/kg and 20mg/kg did show a retardation of luciferase expression.

Leukemia regression was very pronounced in the animals treated with AN-024 and better than Imatinib.

**5. Determination of drug effectiveness ($D_e$) and Drug temporal penetration**

**Determination of drug effectiveness ($D_e$)**

Drug effectiveness was determined using the equation

$$\left[\frac{\sum alive}{\sum luc} - \frac{\sum_{C} alive}{\sum_{C} luc}\right] \div \sum_{C-initial} alive \times 100 = D_e$$

Where
Σalive = total number of mice alive per concentration at the end of experiment times photon count, Σluc = total number of mice alive showing luciferase expression per concentration at end of experiment and c represents control untreated animals times photon count and $\Sigma_{c\text{-}initial}$ represents the initial number of animals in control at start of experiment times photon count. The results were represented graphically as percent drug effectiveness.

**Determination of drug temporal penetrance ($T_p$)**

To determine the temporal penetrance of AN-024 and Imatinib, nude mice were implanted with K562luc cells. The animals were imaged at 6 day intervals post transplantation. Drug treatment (AN0-24 20mg/kg and Imatinib 10mg/kg) was initiated 15 days post implantation by daily ip injections. Drug treatment was stopped on day 35 and animals were imaged till day 45. The temporal penetrance was calculated applying the following equation.

$$\frac{\left(\frac{P}{n}\right)_a - \left(\frac{P}{n}\right)_{ter}}{\left(\frac{P}{n}\right)_a - \left(\frac{P}{n}\right)_b} = T_p$$

Where
P=photon counts at day '*a*', day '*ter*', or day *'b'* where '*a'* is the day when drug treatment was stopped and '*ter'* is the day when the experiment was terminated and *'b'* is the day where P is minimum after day a but before day *'ter'*.

*n*=number of animals alive when P was measured
The larger the value indicates greater effectiveness of the drug after stopping drug treatment, ie the penetrance of the drug over time.

**Determination of $D_e$**

To determine the drug effectiveness at various concentrations we used the equation as described in methods.

**Table 2: shows the drug effectiveness at various concentrations of Imatinib and AN-024 as determined from the *in vivo* studies**

| Drug | 5mg/kg | 5mg/kg | 5mg/kg | 5mg/kg |
|---|---|---|---|---|
| **Imatinib $D_e$** | 10.00 | 32.00 | 13.33 | 8.00 |
| **AN024 $D_e$** | 30.00 | 85.00 | 87.00 | 90.00 |

**Determination of $T_p$**

To determine the temporal penetrance of AN-024 and Imatinib, nude mice were implanted with K562luc cells. The animals were imaged at 6 day intervals post transplantation. Drug treatment (AN-024 20mg/kg and Imatinib 10mg/kg) was initiated 15 days post implantation by daily ip injections. Drug treatment was stopped on day 35 and animals were imaged till day 45, and calculated as described in methods.

By applying the equation for temporal penetrance, it was determined that $T_p$ values for:
AN-024=2.4
Imatinib=0.8

Indicating that AN024 had activity over untreated controls and fared better than imatinib for activity over time after withdrawal of drug treatment

**6. Effect of Dasatinib on Baf3 implanted nude mice when compared to AN-024 and Imatinib**

Previously we had demonstrated the effectiveness of AN024 in the treatment of leukemia when compared to Imatinib by using Baf3 (wt, T315I, M351T and E255K) mutant cell lines. Here, we have used Dasatinib as a control drug to determine the response of Baf3 mutant cells to treatment when compared to AN024 and Imatinib .

**Method**

Nude mice were intraperitoneally implanted with Baf3 mutant cells (Wt, T315I, M351T and E255K). 15 days following implantation, the mice were treated either orally or intra peritoneally with 10mg/kg Dasatinib for 27 days. Blood was drawn from the femoral vein or tail vein every 6th day and blast cell count determined and graphically represented.

**Result**

On the 6th day, blood smears of nude mice implanted with Baf3 mutant cells (wt, T315I, M351T and E255K) showed normal blast cell count, and blast cell count progressively increased as observed on day 12. Dasatinib treatment was started on day 15 post implantation. Dasatinib treatments fared no better than Imatinib. On day 42, the termination of the experiment, mice implanted with Wt cells showed significant response to Dasatinib, indicating that ip treatments were superior to oral. Mice implanted with T315I and M351T cells behaved similar to controls with no significant decrease in blast cell count. Mice implanted with E255K fared only a little better than T315I implanted cells in response to Dasatinib treatment. On the overall Dasatinib and Imatinib treatment only caused retardation in leukemic progress with no significant curative effect. Blast cell count was much lower in the group treated with AN-024 indicating the superiority and the potential of this compound in treating leukemia better than Imatinib and Dasatinib

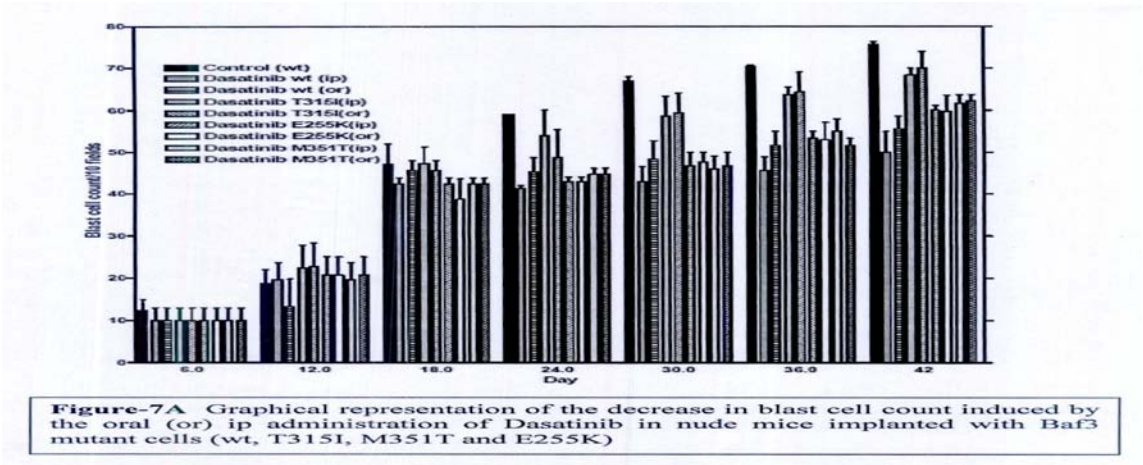



**Figure-7A** Graphical representation of the decrease in blast cell count induced by the oral (or) ip administration of Dasatinib in nude mice implanted with Baf3 mutant cells (wt, T315I, M351T and E255K)

**Fig 7A: shows graphical representation of the decrease in blast cell count induced by the oral (or) ip administration of Dasatinib in nude mice implanted with Baf3 mutant cells (wt, T315I, M351T and E255K)**

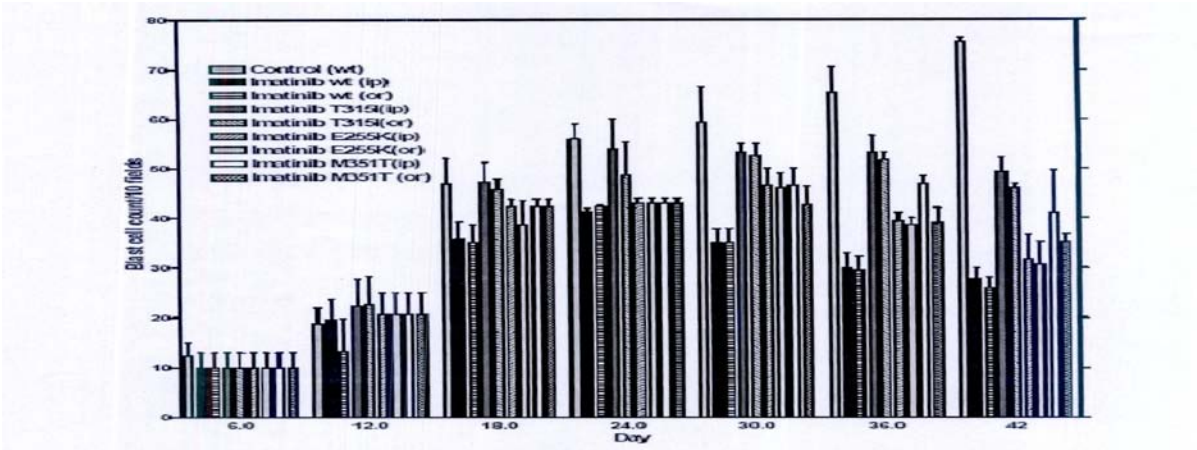



**Figure-7B** Graphical representation of blast cell count in response to oral (or) ip administration of Imatinib in nude mice implanted with Baf3 mutant cells (wt, T315I, M351T and E255K)

**Fig 7B: shows graphical representation of the decrease in blast cell count induced by the oral (or) ip administration of Imatinib in nude mice implanted with Baf3 mutant cells (wt, T315I, M351T and E255K)**

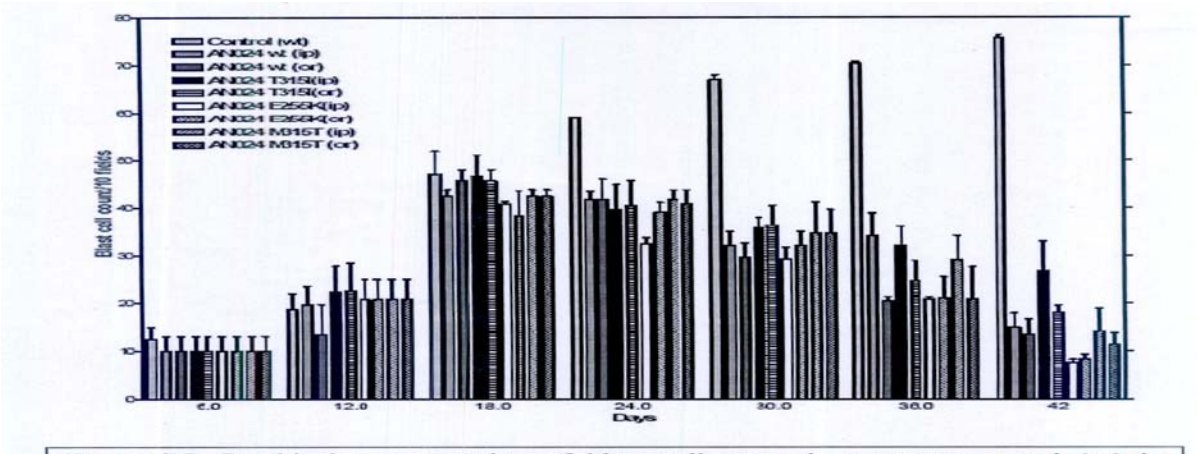



**Figure-7C** Graphical representation of blast cell count in response to oral (or) ip administration of AN-024 in nude mice implanted with Baf3 mutant cells (wt, T315I, M351T and E255K)

**Fig 7C: shows graphical representation of the decrease in blast cell count induced by the oral (or) ip administration of AN-024 in nude mice implanted with Baf3 mutant cells (wt, T315I, M351T and E255K)**

**7. *In vitro* studies on glioma and breast cell lines**

**Material and methods**

To determine the effect of AN-024 and Temozolomide (TMZ) with or without radiation on glioma and breast cancer cells, we treated cells at the specified doses and determined invasion, angiogenesis and changes in certain signaling molecules.

**Matrigel invasion assay**

The *in vitro* invasiveness of 4910, 5310 and U87 glioma cells and MDAMB231 and ZR71 breast cancer cells in the presence of specified concentrations of compounds were assessed using a modified Boyden chamber assay. Cells were treated with these compounds for 48 h. $1x10^6$ cells were suspended in 600µl of serum-free medium supplemented with 0.2% BSA and placed in the upper compartment of the transwell chambers (Corning Costar Fischer Scientific Cat #07-200-158, Pittsburgh PA) coated with Matrigel (0.7 mg/ml). The lower compartment of the chamber was filled with 200 µl of serum- medium and the cells were allowed to migrate for 24 h. After incubation, the cells were fixed and stained with Hema-3 and quantified as previously described (Mohanam et al. 1993). The migrated cells were imaged microscopically to determine the reduction in invasiveness induced by the compounds of this invention.

**Angiogenic assay**

The *in vitro* angiogenesis of 4910, 5310 and U87 glioma cells and MDAMB231 and ZR71 breast cancer cells in the presence of specified concentrations of compounds were determined as follows, cells (2x104/well) were seeded in 8-well chamber slides and were treated with various concentrations of NRC compounds. After a 24 h incubation period, the conditioned media was removed and added to a 4x104 human dermal endothelial cell (monolayer in 8-well chamber slides) and the human dermal endothelial cells were allowed to grow for 72h. Cells were then fixed in 3.7% formaldehyde and stained with H&E and photographed.

**Western blot analysis**

Western blot analysis of 4910, 5310 and U87 glioma cells and MDAMB231 and ZR71 breast cancer cells in the presence of specified concentrations of compounds were assessed as per standard protocols. Cells were treated with AN-024 or Temozolomide at the specified concentrations. 24h after treatment, cells were collected and cell lysates extracted. Equal quantities of proteins were fractionated by SDS-PAGE. The fractionated proteins were blotted on to nylon membranes and immunoprobed for AKT,

ERK and Pi3k. Breast cancer cell protein isolates were additionally immunoprobed for EGFR, ErbB1, ErbB2 and ErbB3.

**Results**

**Matrigel invasion assay**

The *in vitro* invasiveness of 4910, 5310 and U87 glioma cells and MDAMB231 and ZR71 breast cancer cells in the presence of specified concentrations of compounds were assessed using a modified Boyden chamber assay. Cells were treated with these compounds for 48 h. Change in the invasiveness of various cell lines is given in the table 3.

From the invasion assay it is evident that AN-024 was the most effective at inhibiting invasion in a majority of the cells, both with and without radiation

**Angiogenic assay**

From the angiogenesis assay experiments it is observed that AN-024 was most effective at inhibiting angiogenesis.

Temozolomide treatment caused complete inhibition of angiogenesis in ZR-71 cells, whereas in MDA-MB-231 cells only a slight inhibition was observed in control condition with an increase in inhibition after radiation. Glioma xenograft cells 4910 showed significant inhibition of angiogenesis both with and without radiation. In the case of 5310 cells inhibition of angiogenesis was seen in control conditions, whereas angiogenesis was promoted after radiation treatment. U87 glioma cells showed similar inhibition patterns both with and without radiation.

AN-024 treatment caused complete inhibition of angiogenesis in ZR-71 cells, whereas in MDA-MB-231 cells only a slight inhibition was observed in control and radiation treatments. Glioma xenograft cells 4910 showed significant inhibition of angiogenesis both with and without radiation. In the case of 5310 cells inhibition of angiogenesis was seen in control conditions, whereas angiogenesis further inhibited after radiation treatment. U87 glioma cells showed significant retardation in angiogenesis with an increase in inhibition after radiation.

**Table 3: *In vitro* matrigel invasion assay of 4910, 5310 and U87 glioma cells and MDAMB231 and ZR71 breast cancer cells in the presence of specified concentrations of compounds, with and without radiation.**

| Cell line | Drug | -Radiation % Invasion | +Radiation % Invasion | ± Change in invasion after radiation |
|---|---|---|---|---|
| ZR-71 | Temozolomide | 70% | 65% | -5% |
| | AN-024 | 48% | 45% | -3% |
| | | | | - |
| MDA-MB-231 | Temozolomide | 62% | 49% | -13% |
| | AN-024 | 43% | 47% | +4% |
| 4910 | Temozolomide | 95% | 73% | -22% |
| | AN-024 | 56% | 39% | -17% |
| 5310 | Temozolomide | 50% | 63% | +13% |
| | AN-024 | 27% | 32% | +5% |
| U87 | Temozolomide | 90% | 93% | +3% |
| | AN-024 | 53% | 29% | -24% |

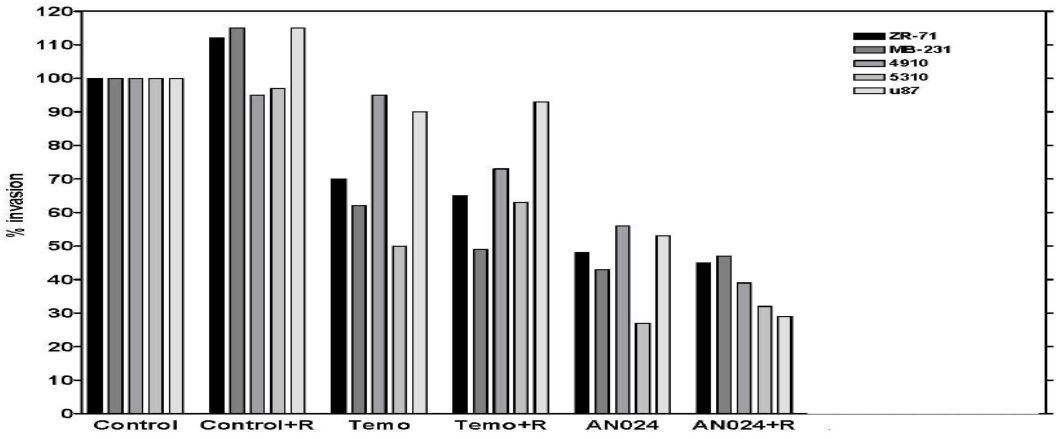



**Figure 8: *In vitro* matrigel invasion assay of 4910, 5310 and U87 glioma cells and MDAMB231 and ZR71 breast cancer cells in the presence of specified concentrations of AN-024, with and without radiation employing temozolomide as standard.**

**Table-4: $ED_{50}$, $LD_{50}$, early cited MTD (Maximum Tolerated Dose) and therapeutic index AN024 in comparison with Imatinib**

| Substance | $LD_{50}$ (po) Mice(mg/Kg) | $ED_{50}$ (po) Mice(mg/Kg) | MTD $LD_{50}/ED_{50}$ | Therapeutic *index (Leukemic mice (K562)) |
|---|---|---|---|---|
| **Imatinib mesylate** | 949 | 12 | 250 | 78.9 |
| **AN-024** | 1440 | 10 | 500 | 144 |

**Western blot analysis**

Western blot analysis of 4910, 5310 and U87 glioma cells and MDAMB231 and ZR71 breast cancer cells in the presence of specified concentrations of compounds of this invention revealed that U87 cells did not show significant change in AKT or PI3k levels both with and without radiation, whereas a slight decrease in ERK levels was observed in AN-024 treated cells and decrease was enhanced after radiation. 4910 cells behaved similar to U87 cells with a decrease in AKT levels in after AN-024 treatment and the decrease in AKT levels was enhanced after radiation. In case of breast cancer cells MDA-MB-231 no significant change in AKT, ERK or PI3k was observed. AN-024 treatment did not show any significant change under unirradiated conditions, whereas after radiation AN-024 treated cells showed a decrease in AKT expression. AN-024 treatment caused decrease in PI3k levels after radiation. Levels of pAKT did not change significantly in any of the treatments with or without radiation, whereas, AN024 showed reduction on pERK levels. Temozolomide treatments both with and without radiation did not show any significant change in pAKT of perk levels.

**8. Leukemic survival study**

K562 luciferase expressing cells were implanted intraperitoneally into nude mice; the mice were scanned using the xenogeny IVIS image station after ip injections of luciferin does determine implantations. Drug treatment was started as in previous studies day 15 after implantation. The animals were treated till day 42, after which drug treatment was stopped and survival of the animals determined as per the animal care regulations. It is observed that control animals developed leukemia and mortality had occurred on day 34 and 35. Drug treatment was withdrawn on day 42 post implantation and survival of animals determined. Animals treated

with AN-024 showed mortality on day 38, the dead animals on further inspection did not reveal spleenic enlargement and cause of death was determined to be other than leukemia, blood smears could not be taken from the dead animal. Of the 10 animals used 8 animals showed no signed of leukemia on day 55. Animals treated with Imatinib showed reoccurrences of leukemic symptoms after treatment withdrawal and showed mortality on day 55, 56, 57 and 58. the surviving animals did show presence of leukemic symptoms.

**9. Studies on $ED_{50}$, $LD_{50}$, MTD and therapeutic index**:

The following **Table-4** summarizes $ED_{50}$, $LD_{50}$, early cited MTD (Maximum Tolerated Dose) and therapeutic index AN-024 in comparison with Imatinib. Standard literature methods were followed for the determinations.[18]

**10. Glioma radiation studies**

Nude mice were intracranially implanted with 4910 human glioma xenograft cells ($1x10^6$ cells) intracranially. Ten days after implantation mice were treated with AN-024 or temozolomide with or without radiation (5Gy/week). The experiment was terminated at day 40 post implantation. From the results it is observed that 100% of control animals developed intracranial tumors and radiation alone had very little effect on tumor size reduction. Animals treated with TMZ alone showed reduction in intracranial tumors with 3 of 10 animals showing complete absence of tumors. Radiation treatment combined with TMZ administration caused a further regression in tumor size with animals showing less symptoms of Intracranial pressure (arched back), in this case 2 of the 10 animals showed no observable intracranial tumor. Animals treated with AN024 without radiation showed presence of intracranial tumors but the tumors were well defined and not showing diffuse edges as seen in controls or TMZ treatments, 3 of 10 animals were cured. After radiation treatments 7 of 10 animals were cured, the animals that showed presence of tumors showed well defined surgically respectable tumors.

**CONCLUSION**

In conclusion, we have designed synthesized AN-024, a phenylamino pyrimidine derivative and evaluated it as BCR-ABL tyrosine kinase inhibitors. AN-024 holds great potential for therapeutic use in the treatment of ph+ leukemias, breast cancers and brain cancers. Our studies shows that AN-024 is highly effective and clearly superior to imatinib, and can eliminate large number of lymphoblastic leukemia cells *in vivo*. AN-024 also showed a potent inhibitory effect against Baf3 murine leukemia cells, T315I, M351T and E225K whose over expression is associated with STI-571 resistance.[19,20] We expect that AN-024 is a potential candidate for proceeding to clinical trials.

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