ORIGINAL ARTICLE

An *In-Silico* and *In-Vitro* Study of Imatinib and Naringin Combination for Inhibiting P-Glycoprotein and Delaying Drug Resistance in Chronic Myeloid Leukemia

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Abstract

Introduction: The present study describes the potential use of naringin (NAR), a flavanone glycoside, in combination with imatinib (IMT), a signal transduction inhibitor used in chronic myeloid leukemia (CML). The study aimed to investigate whether NAR could help overcome the multidrug resistance of IMT by inhibiting the overexpression of the P-glycoprotein gene. **Materials and Methods:** Molecular docking approach is used to investigate the binding affinity of IMT and NAR to human P-glycoprotein. The results showed that IMT had stronger interactions with P-glycoprotein than NAR, indicating better stability with a binding energy of −7.3 kcal/mol compared to −5.6 kcol/mol for NAR. However, NAR demonstrated an excellent affinity toward human P-glycoprotein. **Results:** To investigate the effect of NAR and IMT combination on P-glycoprotein expression, flow cytometry technique on K562 cell lines is used. The results displayed that IMT alone at a concentration of 1 μM had a P-gp expression of 73.6%. Nevertheless, P-gp expression was substantially decreased to 9.7% when coupled with NAR at higher doses (5–30 μM). Moreover, pure NAR alone also showed suppression of P-gp expression. In summary, the study suggests that the combinatorial approach of IMT with NAR could enhance anticancer activity and delay drug resistance by reducing P-gp expression in K562 cell lines. **Conclusion:** These results support the potential use of NAR as an adjuvant therapy to IMT in the management of CML. However, further studies are needed to confirm the efficacy and safety of this approach in clinical settings.

Key words: Chronic myeloid leukemia, imatinib, naringin, docking, P-glycoprotein

INTRODUCTION

Cancer is a cluster of complicated pathways that can lead to the development of malignant tumors with the capacity to spread, including unrestricted cell proliferation, defective cell death, and spatial-temporal alterations in cell physiology.[1] Chronic myeloid leukemia (CML) is a myelo-proliferative disease in which significant increase in granular leucocytes takes place in blood and bone marrow at any stage of the development.[2] According to American Cancer Society, about 8,860 new instances of CML will be detected by 2022, with around 1,220 persons dying from the disease. CML accounts for around 15% of all new instances of leukemia. In the USA, 1 in 526 persons may be detected with CML at any stage of the life.[3] The onset of the CML mostly occurs between the ages of 40–50 years, but at any age, it may show the progression including both sexes. CML begins with any pluripotent stem cell with multiple or single genetic alterations leading to clonal proliferation of altered myeloid progenitors. This will influence all cells generated from this precursor.[4] Out of three phases of the CML, most of the patients are found in chronic phase which is diagnosed by cytogenic and bone marrow sampling. The chronic phase is categorized by slow progression that is easily treatable and maybe maintained for 2–7 years.[5]

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Imatinib (IMT) is first introduced and widely used in CML as signal transduction inhibitor which blocks the BCR-ABL protein in carcinogenesis. IMT inhibits the activity of tyrosine kinase and gets bind to the BCR-ABL kinase preventing phosphate group transfer towards tyrosine and causes activation of phosphorylated protein. This entire scenario leads to the inhibition of proliferation impulses and cell undergoes in to the apoptosis. In December 2002, the Food and Drug Administration approved IMT as the first-line treatment for newly diagnosed CML based on an International Randomized Study (IRIS).[6] However, the development of multidrug resistance (MDR) restricts IMT's usage in drug delivery. The MDR has been associated to over expression of the P-gp gene.[7,8] P-gp-mediated efflux has been identified as a barrier to the brain's uptake of IMT.[8]

P-gp and MRP (MDR-related protein gene) can directly interact with IMT and shows the resistance to the treatment,[9] while similar observations were noted by Mukai *et al.* and IMT was found to be substrate of P-gp.[10] Burger *et al.*, found that IMT is also substrate of breast cancer resistant protein (BCRP) and continuous exposure of the IMT causes the up regulation of P- gp and BCRP leading to the lesser intracellular concentration of IMT.[11] Ferrao *et al.* also believed that the resistance of IMT was attributed to the over expression ofthe P-gp.[12] But according to the Hirayama *et al.*[13] resistance to IMT in K562 was not associated with the BCRP. Considering all these known draw backs of the current therapy, P-gp inhibitors or the molecules which can reduce the P-gp expression in cancer cell lines would be considered in the treatment of the CML.

Natural products are excellent sources for drug discovery as they allow researchers to isolate new molecules that may be used as leads or scaffolds in drug delivery system with great erbiological properties.[14] High consumption of green and other vegetables as well as fruits may lower down the chances of the development of various malignancies, according to scientific findings and traditional wisdom.[15] Flavonoid and its derivatives, fruit-based cancer preventative and therapeutic agents, have demonstrated a notable potential to decrease tumor development and cancer cell proliferation.[16] Flavonoid interacts with several signal transduction path ways in carcinogenesis, increasing apoptosis while inhibiting metastasis, angiogenesis, and proliferation. Natural compounds have been shown excellent potential against cancer with fewer side effects. As a result, use of natural origin products in various cancerous or other treatments is a cost-efficient, accessible, and successful treatment option.[17] Naringin (NAR) is flavonone glycoside having anti- inflammatory, antioxidant, antiviral and antitumor properties.[18] Moreover, it affects and engages several signalling pathways. NAR has been shown to suppress a variety of cancers through regulating a number of cellular signalling cascades, such as oxidative stress processes, angiogenesis, and the prevention of malignant cell proliferation.[19] Several lines of experimental data show that Naringenin or innovative Naringenin formulations might help to prevent malignancies and various cancers including melanoma, breast cancer, and cervical cancer.[20] NAR has been already proven to be effective in the bladder, blood, breast, cervical, lung, and head-neck cancer.[19]

We felt that combining IMT with NAR would be a unique therapeutic strategy for the treatment of CML with improved anticancer activity, given all of the potential advantages of NAR. An exhaustive literature search revealed that the combination of IMT and NAR as a treatment method has yet to be tested. In this study, *in silico* docking and cellular studies were used to establish IMT's improved anticancer efficacy when combined with NAR.

MATERIALS AND METHODS

IMT and NAR were purchased from Acros organics, Belgium. Fetal Bovine Serum, Cell culture medium: RPMI1640 were purchased from Difco; Invitrogen Corp, Burlington, Canada, MTT Reagent and Verapamil from Sigma-Aldrich, D-PBS were purchased from Himedia India. K562 cell lines were purchased from NCCS, Pune, India. DMSO was purchased from Sigma USA. PGP antibody (E-10) FITC was purchased from BS Biosciences. Sodium azide was purchased from Sigma.

Molecular docking study of IMT and NAR

In this study, human P-glycoprotein (PDB ID: 6C0V) was used as the target enzyme while IMT and NAR were used as ligands. The structures of these compounds are presented in Figure 1. The AutoDock Vinaprogramme was used to perform molecular docking experiments on NAR and IMT molecules. All molecules were docked against human P-glycoprotein (PDB ID: 6C0V). The protein P-glycoprotein was obtained from the Protein Data Bank. We utilized grid box settings for human P-glycoprotein docking with grid centre: x = 172.155, y = 188.865, z = 128.374 and grid box size: x = 12, y = 12, z = 22. The grid spacing was set to 1.0Å in this study and generated 20 confirmations for each docking. MGL tools 1.5.6 were used to prepare the input, while AutoDock Tools and Pymol software were used to evaluate the findings.

Estimation of P-Glyco-protein in K562 Cell lines

To induce IMT resistance, the original IMT-sensitive K562 cells were subjected to a stepwise increase in IMT concentration in the culture medium. The concentration was escalated from 1 μM to 5 μM over there months. At each step, the IMT concentration was increased by 0.3 μM and maintained for 10–15 days, depending on the proportion of surviving cells. The resulting K562 IR cell line exhibited resistance to 5 μM IMT. From our previous study results of MTT assay, the IC 50 values of IMT and NAR were 1 μM

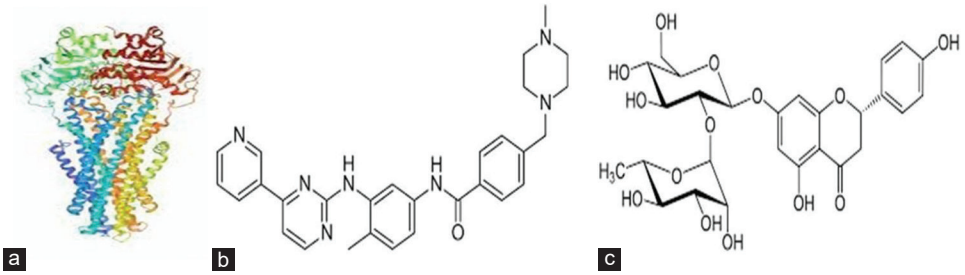

Figure 1: Structures of (a) Human P-glycoprotein (targetenzyme) (PDBID: 6C0V); (b) IMT (ligand) and (c) Naringin (ligand)

and 109 µM, respectively. Hence, the concentrations of IMT were taken <1 µM and NAR concentration at 25, 50 and 100 µM which is also less than the IC50 concentration. This study was conducted in K562 cell lines cultured at density of 1×10^5 cells/2 mL in 6 well plate. These cells were incubated in CO2 incubator at 37°C for period of 24 h in RPMI 1640 supplemented with 10% FBS and Pen-Strep. The cultured cells were separated from spent medium followed by washing with PBS. As per Table 1 the cells were treated with test compounds and controls followed by incubation for 24 h. Lateron these cells were allowed to harvest in polystyrene tube followed by centrifugation at 25°C for 5 minutes. The supernatant was decanted and cells washed twice with PBS. PGP antibody (E-10, 10 µL) and FITC antibodies were mixed properly and incubated at RT for 30 min. Later on these cells were washed with 1× PBS and sodium azide (0.1%). Flow cytometry analysis was performed after the addition of PBS (0.5 mL) with 495 and 519 as excitation emission.

Table 1: The concentrations of the compounds (IMT- Imatinib, Test- Naringin)

S. No.	Sample Name/Code	Concentrations
1.	Verapamil	1 µM
2.	Imatinib	1 µM
3.	Naringin	10 µM
4.	IMT+Test	1 µM+15 µM
5.	IMT+Test	1 µM+20 µM
6.	IMT+Test	1 µM+25 µM
7.	IMT+Test	1 µM+30 µM
8.	Untreated cells	NA

Table 2: Protein-ligand binding interaction energies (in kcal/mol) calculated by using AutoDock vina

Protein name	Imatinib	Naringin
Human P-glycoprotein (6C0V)	−7.3	−5.6

RESULTS AND DISCUSSION

The present research work *in silico* docking and p-glycoprotein expression study in K562 was done with the assumption that NAR would potentially enhance the anticancer activity of IMT using P-glycoprotein inhibition with excellent affinity. We are certain that this innovative combinatorial therapeutic approach will provide fresh perspectives on how to treat CML while delaying the onset of drug resistance.

Human P-glycoprotein docking

One of the most effective ways for determining the binding process and affinity of a ligand with a protein molecule within a receptor is molecular docking.[21] The molecular docking studies can also detect active chemicals that don't fit well in binding sites.[22,23] The docking experiments were used to conclude the binding affinity of NAR and IMT for human P-glycoprotein. The binding affinity of these compounds revealed substantial interaction energies with the active site of human P-gp, as shown in Table 2.

The docking of NAR and IMT compounds was based on the crystal structure of human P-gp (PDB ID: 6C0V). The interactions of NAR and IMT molecules in the active site of human P-gp are depicted in Figures 2 and 3. Hydrophobic interactions and hydrogen bonding stabilize NAR linked to P-gp. Interactions of NAR in the active site of human P-gp are shown in Figure 2. Gly-475, Ser-429, Ser-434, and Gln-438 residues demonstrated hydrogen bond interactions with NAR. NAR is further stabilized by hydrophobic contacts in the active site with Gly-432, Gln-1180, and Ser-1177 residues.

Hydrogen bonds between Gln-438, Arg-905, and Arg-404 residues stabilize the IMT molecule [Figure 3]. It also had hydrophobic interactions with the residues Tyr-401, Val-407, and Gly-432. IMT's robust hydrogen bonding, non-covalent, and hydrophobic interactions with human P-glycoprotein revealed greater stability in contrast to NAR (−5.6 kcal/mol), with a binding energy of −7.3 kcal/mol. It has been determined through docking research that NAR and IMT have strong affinities for human p-glycoprotein.

P-glycoprotein expression study

MDR is still a significant stumbling block to cancer therapy's long-term effectiveness. P-gp is membrane transporter that is responsible for the efflux of the molecules from the cells

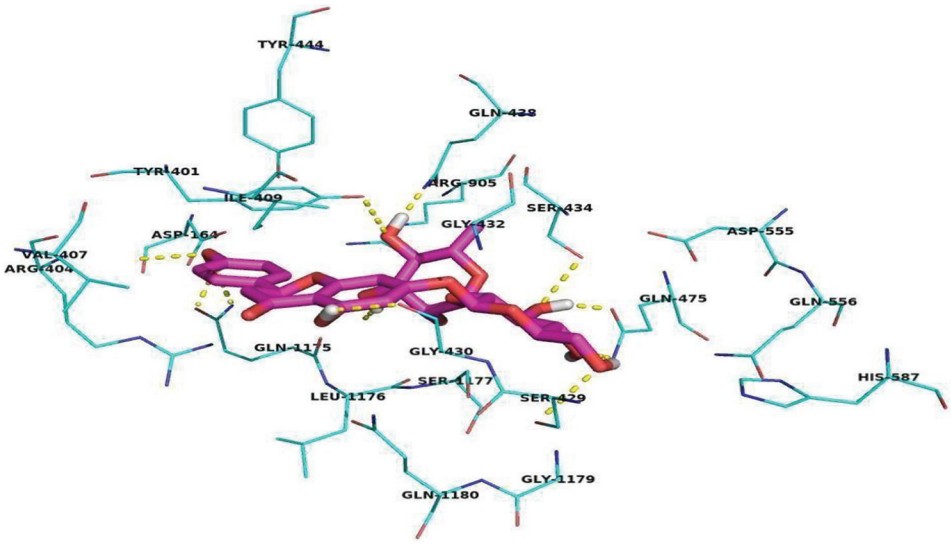

Figure 2: Molecule NAR docked in the active site of the human P-glycoprotein (PDBID: 6C0V). The side chains of proteins and amino acids are displayed inline-style in cyan color, while the side chains of ligand molecules are displayed in pink color stick style

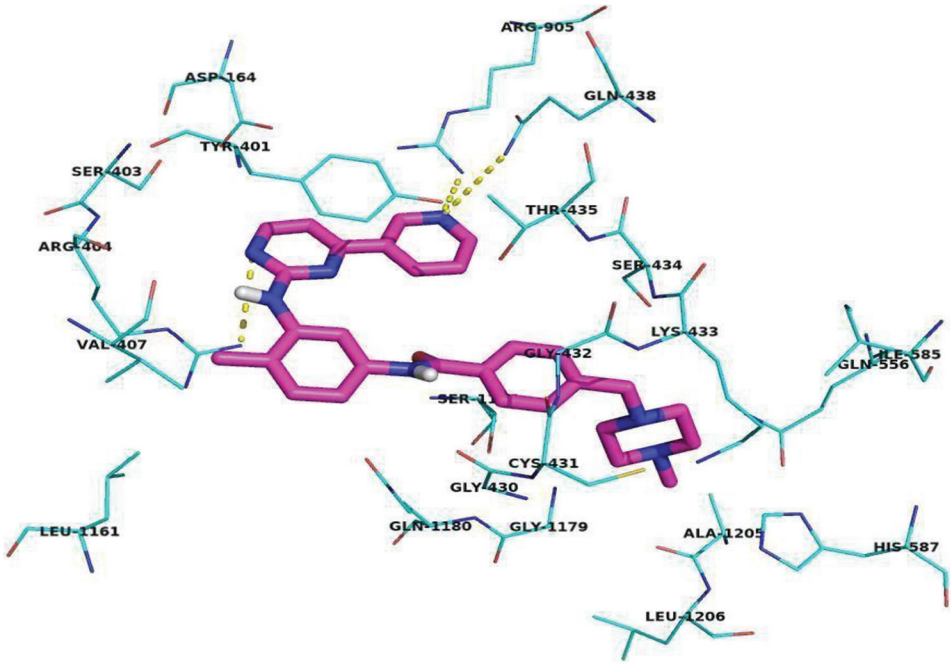

Figure 3: Docking of IMT at active site of the human P-glycoprotein (PDBID: 6C0V). The side chains of proteins and amino acids are depicted in line style in cyan color, while the side chains of ligand molecules are displayed in pink color stick style

reducing chemotherapy efficacy. As an adaptive response to chemotherapy-induced cell death, P-gp expression is increased in cancer cells. Most of the P-gp inhibitors are discovered through preclinical investigations and *in silico* techniques, but only a handful have made it through all the stages of clinical trials.[24] The capacity of NAR in combination with IMT to change the expression pattern of P-gp in cell lines has been investigated in this work. Through this study, it has been revealed that the P-gp over expression is reduced in a dose-dependent manner when compared to untreated K562 cells. Ali *et al.* had previously proven NAR's regulation of anticancer drug-induced P- glycoprotein expression and described an appealing novel agent for cancer cell chemosensitization.[25] In our study, the combination of IMT with NAR has displayed excellent inhibition of P-gp expression in K562cell line.

When IMT was coupled with NAR at concentrations ranging from 25 to 200 M, P-gp expression was significantly decreased to 9.7% from its initial value of 73.6% at 5 M. In our investigation, pure NAR (alone) has likewise shown effective P-gp expression suppression. Percentage of P-gp

expressing K562 cells in performed in triplicate and is presented in Figure 4.

The highest P-gp expression was found in the samples of the untreated cells in flow cytometry analysis. The flow cytometry images of the untreated cells are shown in Figure 5. This group of cells had shown nearly 99.8 % of P-gp expression.

When we analyzed only IMT treated group of the cells, we observed strong expression of P-gp in K562 cells (73.6%). Flow cytometry images of the cells treated with 1 µM are shown in Figure 6. It is a well-known fact that IMT is substrate of P-gp so overexpression was observed in this group of cells.

As expected, the combination of IMT with NAR had displayed excellent inhibition in P-gp expression in K562 cell lines. In our study, NAR had already displayed affinity toward the human P-gp and inhibited the expression. NAR displayed decrease in P-gp expression with respect to dose in comparison to untreated cells. The flow cytometry images of the cells treated with 1 µM of IMT and 30 µM of NAR are shown in Figure 7.

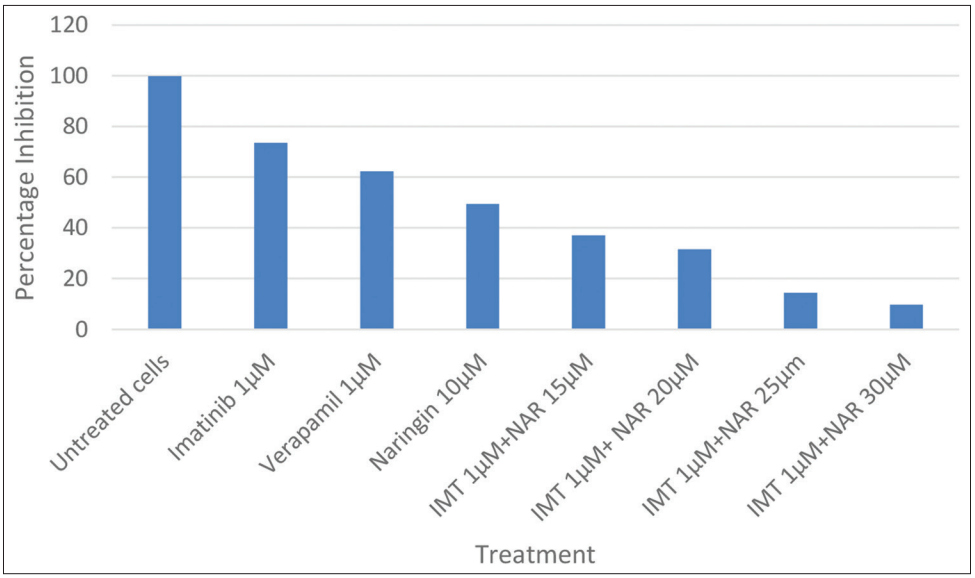

Figure 4: Percentage of P-gp expressing K562 cells-overlay bar graph

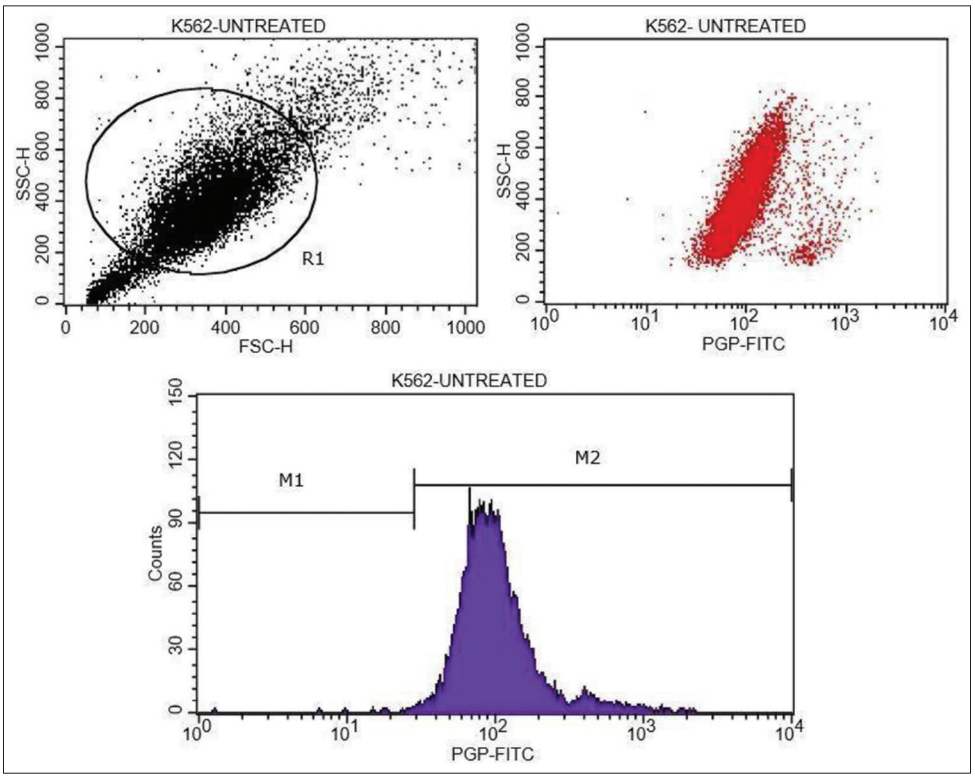

Figure 5: P-gp expression in untreated K562 cell line

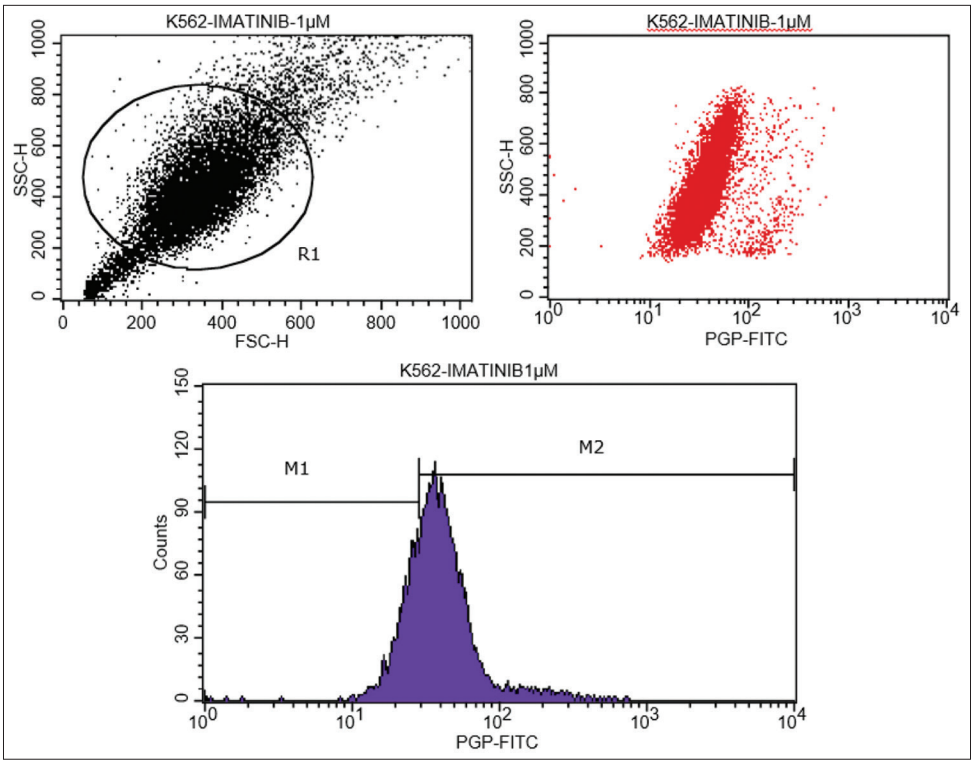

Figure 6: Expression of P-gp in IMT (1 μM) treated K562 cellline

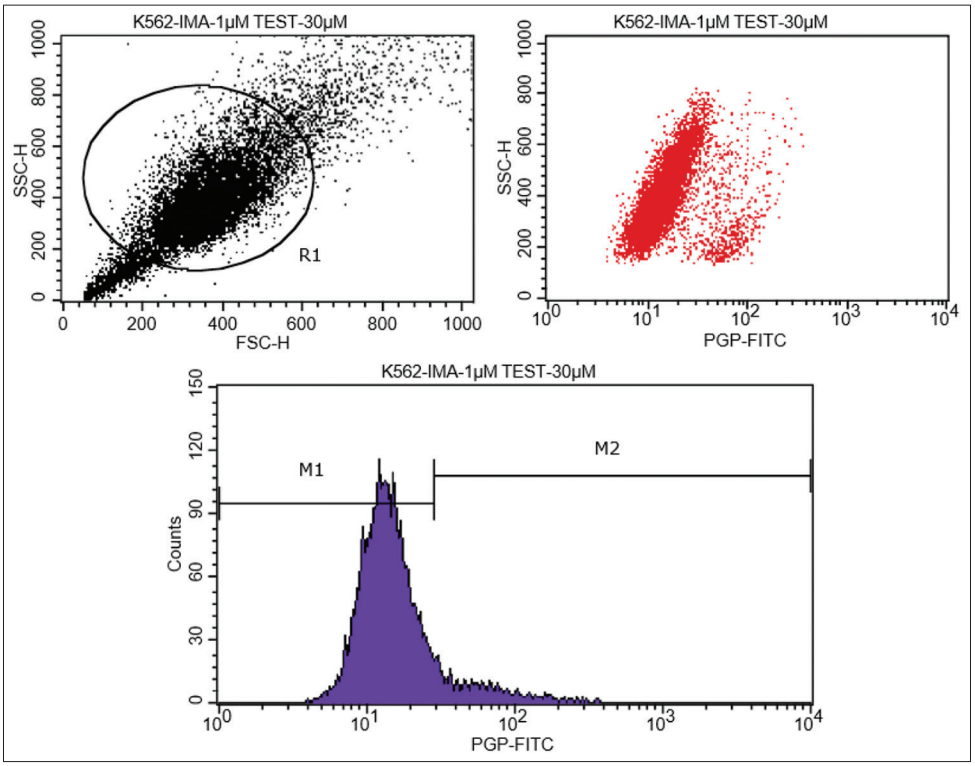

Figure 7: Expression of P-gp in imatinib (1 μM) + NAR (30 μM) treated K562 cell line

The comparative overlay spectra for all the groups displaying P-gp expression are presented in Figure 8. From these overlay spectra, it is clear that the combination of NAR with IMT had helped to reduce the P-gp expression which would be fruitful in the treatment of IMT resistant CML.

P-gp-mediated MDR is found to be major challenge in the treatment of cancer. As a result, most of the present research is focused on identifying molecules which can potentially reverse the MDR avoiding the toxicity.[26] Overexpression of the P-gp efflux pump is a leading cause of chemotherapy

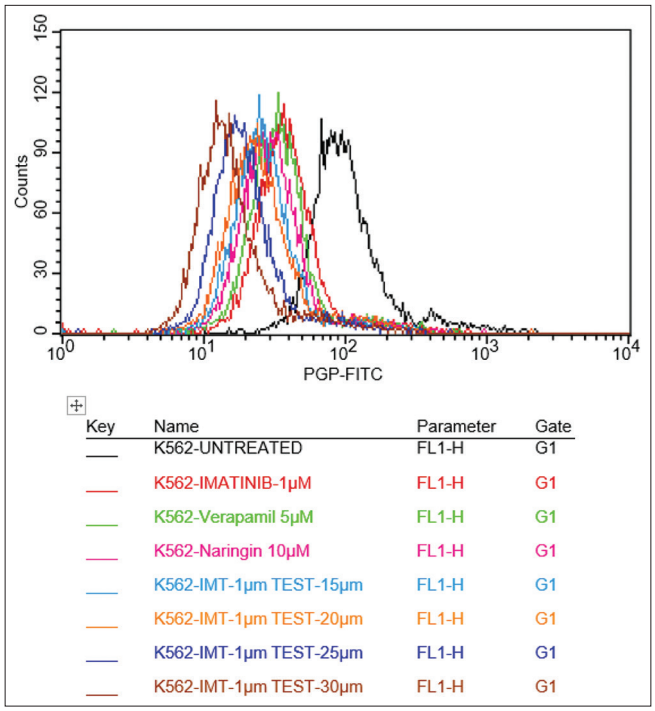

Figure 8: The comparative overlay spectra for all the groups displaying P-gp expression

failure since it causes several anticancer drugs to become resistant to cancer cells.[27] P-gp through its efflux mechanism pumps out the drug molecules from the cancer cells. As a result, P-gp inhibitors must be developed that are both safe and efficient in preventing anticancer treatment resistance caused by P-gp. Flavonoids had previously shown that they inhibitedthe P-gp efflux pump, enabling anticancer drugs to accumulate intracellular in cancer cells.[28]

Most MDR-reversing substances have physical properties in common, including lipophilic characteristics, and charge (positive or neutral) at physiological pH.[29] NAR was found to be a possible chemosensitizing agent because of its hydrophobic nature and aromatic components, which resulted in a considerably stronger suppression of P-gp expression. NAR has already been explored as potential anticancer natural molecule, but its exact mechanism of action is still not fully understood.[30] For the first time, the present work demonstrated the combinatorial approach consisting of IMT with NAR to reduce P-gp expression in K562 cells and by enhancing the anticancer activity.

CONCLUSION

The current research work reveals NAR as a potential P-gp expression inhibitor and could be potentially coupled with IMT to reduce the multiple drug resistance. It was discovered through docking research that NAR exhibited a superb affinity for human p-glycoproteins identical to IMT. The P-gp over expression was found to be reduced in a dose-dependent manner in comparison to the untreated K562 cells. The combination of IMT with NAR would be a unique therapeutic strategy for the treatment of CML with improved anticancer activity and delaying the drug resistance.

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