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Chromen based Novel Molecule from *Wadelia trilobata* Exhibits Molecular Impediment on the Proliferation of Chronic Myeloid Leukemia by Inducing Apoptosis Linked Suppression of STAT5/Bcl-XL Signal Pathway

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Abstract:- Chronic myeloid leukemia (CML) being the first disease identified with the association of chromosomal abnormality referred as Philadelphia condition is also the most widely studied disease among human malignancy. In this study, we characterized the the molecular hindrance property of the novel molecule MPC [7 methoxy 3(4 methoxy 2 methylphenyl) 5 methyl 4H chromen 4 one], extracted from *Wadelia trilobata* against the actively proliferating CML cells. Nuclear staining and flowcytometry analysis revealed the apoptotic activity of MPC. Further studies through immunobloting and real-time PCR, shows down regulation of anti-apoptosis inducing proteins of Bcl2 family specific to CML cells. Molecular docking also displayed that the MPC molecule inhibits JAK-STAT5 pathway where $BclX_L$ is activated transcriptionally through STAT5 for initiating anti-apoptosis and shutdowns the RAS mediated $BclX_L$ assisted anti-apoptotic pathway. In brief this chromen based MPC molecule effectively suppress the proliferation of chronic myeloid leukemia by inducing apoptosis and the molecule promises the effective drug alternate to tyrosine kinase inhibitor (TKI) for treating CML.

Keywords:- *CML; Philadelphia Chromosomes; $BclX_L$ Protein ; Apoptosis ; BCR-ABL Protein ; Tyrosine Kinase.*

I. INTRODUCTION

Chronic myeloid leukemia is developed by the spread of uninhibited proliferative myeloid cells that distorted from the previous progenitors like red blood corpuscles, monocyte, B-lymphoid cells and the megakaryocytes [1]. CML is the prime disease that links the specified genetic aberrative of Philadelphia to the events of pathogenicity [2]. CML is developed due to the translocations of ABL-1 gene in chromosome 9 to chromosome 22 near BCR gene loci and because of this fusion the combitorial gene translates a hybrid protein that switch on and upregulates tyrosine kinase expression along with its signaling which results in uninhibited proliferation and malignant phenotypic character in the cells. The abnormal chromosome is nothing but Philadelphia chromosome [3].

ABL gene with 11 exons in it and the molecular size of 230 kilo bp, usually codes for the enzyme tyrosine kinase of molecular weight 145 kilo Dalton with no specific receptors [4]. The break point in the ABL gene takes place at the 5` region of exon 2 due to which the exon 2 to exon 11 piece of transgene is translocated to the 22nd chromosome in between the exon 12 and 16 that extends to 5.8 Kilo bp extra. Alternative splicing of BCR and ABL genes produce the fusion mRNA transcripts with b2a2 or b3a2 junctions [Figure 1]. This mRNA transcribed from the fusion gene translates to protein of molecular weight 210 kilo Dalton which is nothing but P210 $^{BCR-ABL}$ chimeric Protein. From the *in vivo* experiments and *in vitro* tumor model studies it was evident the main role of chimeric ABL-BCR gene being the arbitrator of the myeloid proliferation and transformation which was also observed in the induction of hematopoietic cell into leukemic cell growth by ABL-BCR gene transcripts [5].

Diverse structural alterations in BCR and ABL assist a leukemogenic modification of ABL-BCR combinations. The coiled motif at the N-terminal region of BCR increases tyrosine kinase activity of ABL and enable its binding to F-actin, in other words though ABL expresses tyrosine kinase, its activation and receptor binding efficiency is mediated by BCR [6]. Another domain of BCR next to coiled motif is a serine - threonine kinase which actives the signaling pathway mediated through $p210^{BCR–ABL}$ and ABL tyrosine kinase [7]. Large number of amino acid sequences is added to SH2 domain of ABL due to the N-terminal attachment of

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BCR with ABL, the binding of amino acids leads to changes adjacent to SH3 domain of ABL and reduces the negative regulatory effect of SH3 domain on the tyrosine kinase function [8]. This results in ABL to express constitutively active tyrosine phosphokinase. Recombination of Bcr-Abl gene is the cause of Philadelphia condition giving three possible forms of chimeric proteins $P210^{BCR-ABL}$ of 210 kDa, $P145^{BCR-ABL}$ of 145 kDa and $P190^{BCR-ABL}$ of 190 kDa. The $p210^{BCR–ABL}$ and $p190^{BCR\ –\ ABL}$ both have increase tyrosine phosphokinase activity in comparison to ABL protein p145 [Figure 1]. Chimeric protein $P210^{BCR-ABL}$ is the major forms that induce CML by Altered adhesion, Mitogenic activation, Inhibition of apoptosis and Degradation of inhibitor proteins which finally turns the myeloid cells to malignant phenotype. The b2a2 or b3a2 recombination form of BRC-ABL chimera in $P210^{BCR–ABL}$ enhances tyrosine kinase activity, and all the domains from the combination works in collaboration for progress of CML. Efforts has been made in developing antibodies like Dasatinib, Bosutinib, Sorafenib, Nilotinib, Ponatinib, Imatinib and Sunitinib which are effectively used as tyrosine kinase Inhibitors for the treatment of CML. But it also clearly indicates the cause of side effects like cardio vascular toxicity [9].

The configuration of $p210^{BCR–ABL}$ permits several protein to protein interactions and influence the involvement in various intracellular signaling pathways [10]. Protein $p210^{BCR–ABL}$ linked to RAS involves in the regulation of cell differentiation and proliferation which is the prominent signaling pathway in the pathogenesis of CML [11]. Downstream signaling events of RAS engage JUN kinase (JNK) pathway also termed as stress-stimulated protein kinase [SAPK] pathway [12]. Jak-Stat mediated pathway came into light by the studies on V-abl-transformed B cells. BCR-ABL protein can directly phosphorylate Stat transcription factors Stat1/Stat5 or also can be activated through phosphorylation of Jak. The activated Stat transcription factors further leads to Mitogenic signaling.

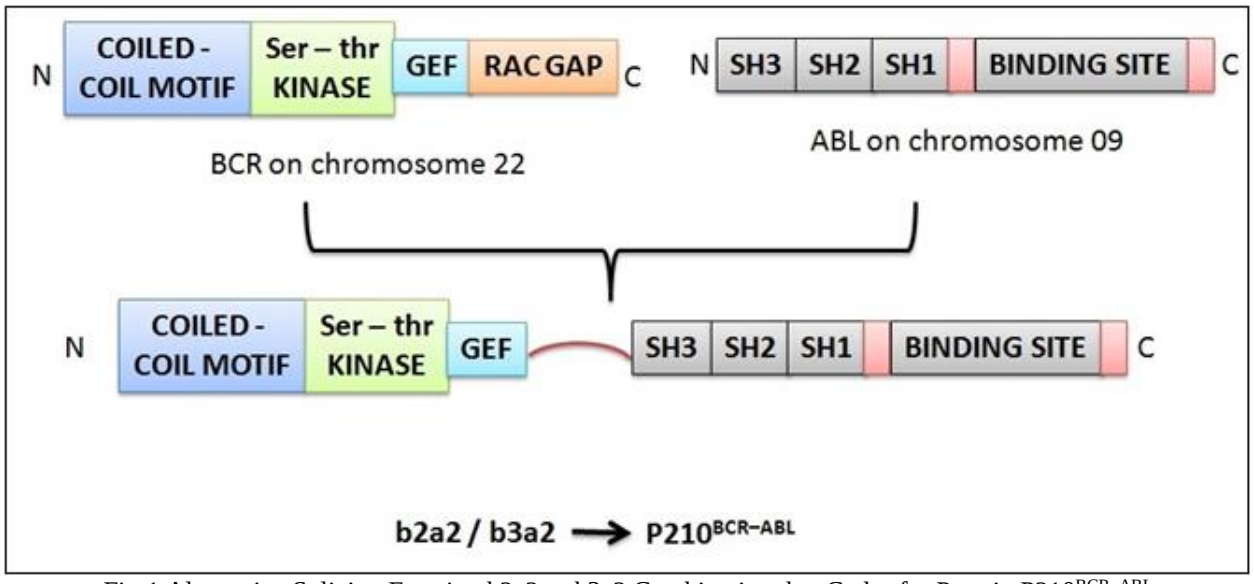

Fig 1 Alternative Splicing Forming b2a2 or b3a2 Combination that Codes for Protein $P210^{BCR–ABL}$

CML cells achieve contact inhibition by reduction of adhesion properties towards stroma cells and extracellular matrix of bone marrow [13, 14] and this is done by the expression of beta1 integrin variant proteins which are adhesion inhibitors, these variants are unique to CML and not found in other progenitors [15]. Stat transcription factor phosporylates to Stat5 and Stat1 has been observed in CML cells. Stat5 being pleotropic favors the CML primarily by exhibiting the inhibition of apoptosis and also activates the transcription of Bcl -X_L [16, 17].

Successive malignancy of CML is by the collaboration of mitogenic activity as well as anti-apoptotic activation. Thus BCR-ABL protein also includes one of its major functions to be an anti-apoptotic. In CML the BCR-ABL protein hurdle cytochrome C release in mitochondria through STAT5 induced activation of Bcl-X_L and there by inhibits the caspase activation [18, 19]. The blockage of cytochrome C release which ultimately leads to caspase inactivation is induced by the activation of Bcl-X_L, the mitochondrial transmembrane protein encoded by Bcl family of genes.

As per the previous studies on *Wadelia trilobata*, revealing the anti-leukemia activity, was the basis for the molecular evaluation, where from a methanolic crude extract a novel molecule MPC [7 methoxy 3(4 methoxy 2 methylphenyl) 5 methyl 4H chromen 4 one] was taken out through series of purification methods along with the stepwise anti-leukemia activity check. The studies tells that initially the methanolic extract showed positive to anti-leukemia and there the journey of purification begins, preliminarily the crude extract is taken to thin layer chromatography where the two bands were segregated and processed for further purification through silica gel chromatography. Out of the two, only one of the bands that showed positive and eminent anti-leukemia activity was selected. The band was subjected to silica gel chromatography, and resulted in five fractions, of which fraction 3 was selected for structural analysis as it showed prominent ant-leukemia and apoptotic activities. Fraction 3

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apoptotic activity was also linked for the cause of megakaryoblastic cell death.

Both anti-leukemia and apoptotic activity were analyzed on MEG 01 cell line, the chronic myeloid leukemia cells and also on the normal HEK-293 cell lines (human embryonic kidney cells). Structure of 7 methoxy 3(4 methoxy 2 methylphenyl) 5 methyl 4H chromen 4 one is obtained by analyzing the fraction 3 sample of silica gel chromatography, through HPLC, mass spectrophotometry and NMR. The separation and selection of MPC molecule, with anti-leukemia and apoptotic activity, which was demonstrated in previous studies [20, 21, 22], was further considered for the analysis of molecular interactions in the current study. MPC selectively induces the apoptosis in CML cells and shows steep reduction in proliferation rate. MPC also down regulates the expression of Bcl-X_L indicating the shutdown of RAS mediated BclXL assisted anti-apoptotic pathway. The present study shows the binding affinity of MPC for STAT5 receptor creating the competition with $P^{210\ BCR\text{-}ABL}$ protein.

II. MATERIALS AND METHODS

➢ *MEG-01 Cell Culture*

MEG 01 cells collected from the bone marrow of male patient with CML positive. The cells exhibit Philadelphia chromosome in its karyotype. It grows in a single suspension with 36 to 48 hours of doubling time. Cells (Sigma Aldrich) with the dilution of 10^6 cells per ml were suspended in 8ml of RPMI 1640 media along with 20% fetal calf serum, 100U/ml of penicillin and 50µg streptomycin supplements. Incubation was done at 37^0 C with 5% CO2 in humidified condition. Under normal cell culture conditions, approximately half of the cultured cells will adhere to the culture flask with extension of pseudopodia. The cells were harvested through centrifugation at 1500rpm for 5min [23].

➢ *Nuclear Staining Assay*

MEG 01 cells grown in 6-well plates (5 X 10^5 cells / well) are treated with 5 µg of MPC molecule for 4 h, whereas untreated cells are kept RPMI 1640 media without addition of MPC. The media was drained out and then the cells were washed in ice cold phosphate-buffered saline once. Then the cells are stained with addition of 1mg/ml acridine orange and ethidium bromide (10 µl) solutions mixture in the ration 1:1. The cells were washed immediately with phosphate-buffered saline and observed under inverted fluorescent microscope [24].

➢ *Flow Cytometry Analysis*

Apoptosis of MEG-01 cells triggered by MPC molecule was studied using BD Annexin V: FITC Apoptosis Detection Kit (BD Bioscience). Fluorescent tagged Annexin V, a protein of 36kDa binds to the phosphatidylserine that has been exposed inside out in the apoptotic cell due to lose in lipid asymmetry of the plasma membrane [25]. Propidium iodide dye is impermeable into viable cells will penetrate into dead cells due to again in permeability of the cell membrane. Conjugate of annexin and propidium iodide are used for the apoptotic detection.

To the MEG-01 cell culture, 5µg of MPC molecule was added and incubated for 4 hours as described in [26]. The cells were then harvested and resuspended at a concentration of 1 × 10^5 cells in 200 µL of Annexin V binding buffer which contains 0.5 mg/ml PI and 2 µL of Annexin V. The content was incubated for 15 min in dark at room temperature. Flow cytometry was set to take the reading by adjusting the dot plots to 520nm for FITC detection and 620nm for propidium iodide.

➢ *Preparation of Cell Lysate*

The protocol was followed as per [27] where the cells was washed with 5 ml of PBS at room temperature and aspirated along with the media, the cell plate is kept on ice and cold PBS 1 ml along with the phosphatase and proteinase inhibitors was add to the plate to incubate for 5 min then the solution was aspirated and the plate is kept on ice again, immediately 1X SDS PAGE sample buffer was add up to 10 to 30 times the volume of cells. After swirling the cells were collected using the cell scraper. The cell suspension was mixed well using the pipette and the cells were taken for centrifuge in microcentrifuge tube. The collected cell sample is heated for 10 min at 95° C, and then chilled quickly on ice; this is done to inactivate the protease and other enzymes. To reduce the viscosity of the sample before loading to the gel the sample was subjected to sonication for 15 to 30 sec followed by the centrifugation at 10000g for 10 min. The supernatant was retained for gel run.

➢ *SDS PAGE and Western Blotting*

The cell lysate from control and MPC treated MEG-01 cells were analyzed for protein contents for SDS-PAGE run and the separated proteins from gel were transferred to Nitrocellulose membrane. The blot was probed with 1:100 dilutions of Bcl-X_L monoclonal antibody (Invitrogen), and then processed for incubation with 1:1000 diluted anti-mouse antibodies that is conjugated with HRP (Sigma, India). Blot was visualized by staining with diaminobenzidine (DAB) - H_2O_2 substrate solution. To justify the cell lysate quantity or concentration loaded to gel, stripping and re-probing is done with the western blot membrane where stripping removes the early probed antibody for Bcl-xL proteins which was achieved by wash with surfactant solution at extreme pH. The re-probing is done for GAPDH protein by addition of 1:100 dilutions of GAPDH monoclonal antibody (Invitrogen), and then processed for incubation with 1:1000 diluted anti-mouse antibodies that is conjugated with HRP (Sigma, India) which is then followed by staining with diaminobenzidine (DAB) - H_2O_2 substrate solution.

➢ *Isolation of RNA and Synthesis of cDNA*

Cultured MEG-01 cells incubated for extra 4 hours with 5µg of MPC molecule was taken as test cell samples, along with the untreated MEG-01 cells were harvested for RNA isolation by using sigma Aldrich RNA isolation kit. As per the protocol 10^8 cells was added to 10ml of solution D. The solution D was earlier prepared by addition of 100µl of beta-mercaptoethanol and 14ml of denaturing solution. The samples were further processed in accordance to the

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isolation kit handbook. The RNA sample thus obtained was measured at absorbance 280, 260 and 230 nm. The samples that showed grater or equal to 1.7 by taking ratio of A260/A280 readings and A260/A230 readings were further taken for cDNA synthesis. The cDNA was synthesised by using Thermo Scientific revertaid first strand cDNA synthesis kit. According to the kit protocol 20µl of reaction mixture was prepared by the addition of 1µg/11µl of isolated total RNA, 1µl of 200U/µl revertaid RT, 1µl of random hexamer primer, 1µl of 20U/µl ribolock RNase inhibitor, 2µl of 10mM dNTP mix, and 4µl of 5x reaction buffer. The sample mixture was then incubated for 10 minutes at 25°C, followed by incubation for 60 minutes at 42°C, and five minutes incubation at 75°C.

➢ *Primer Designing of Target Genes*

Bcl-xL gene which is anti-apoptotic in nature is the target gene in our study along with (GAPDH) glyceral-dehyde-3-phosphate dehydrogenase gene was also chosen as a reference gene. Primer sets of the Bcl-xL and GAPDH genes were designed by using Gene Runner software with reference to the complete gene sequences taken from the NCBI database. After the design the primer sequences were analyzed using basic local alignment search tool for homology check with other genomic regions. The designed primer sequences are shown in Table 1.

Table 1 Primer Sequence of Target Genes

Bcl-xL_Fw	**5` GCTGGTGGTTGACTTTCTCTCC 3`**
Bcl-xL_Rv	5` GGCTTCAGTCCTGTTCTCTTCG 3`
GAPDH_Fw	5` AAGTTCAACGGCACAGTCAAGG 3`
GAPDH_Rv	5` CATACTCAGCACCAGCATCACC 3`

➢ *RT PCR*

For monitoring the step wise amplification process, real time PCR technique is done using Qiagen rotor-gene Q instrument. 25µl of total mixture volume was prepared by adding 1µl of 400nM primers to 12µl of SYBER green reporter dye, 300ng of cDNA and double distilled water. The program cycle was set to 95 °C for 15 minutes followed by 95 °C for 5 seconds, 60°C for 20 seconds along with the ramping analysis of melting curve from 65 °C to 95 with 1 °C rising at every step. The control cDNA templates was diluted by four folds at 2000, 200, 20 and 2 ng to enhance the amplification efficiency of reference and target genes. The slope was obtained by drawing the standard curve considering the input of mean CT versus cDNA concentration. By using the formula E = (10-1/slope) -1 the efficiency of the PCR reaction was calculated.

Levels of expression of the target genes were analyzed by comparative threshold cycle method using the formula $2^{-\Delta\Delta CT}$, where the expression levels of the reference and target genes of the treated samples were compared with the respective expression levels genes in control samples. ΔΔCT refers to [(Ct target - Ct reference) treated - (Ct target- Ct reference) control].

➢ *Statistical Analysis*

All the statistical measures were performed using Graph Pad Prism 5.0 software. Experiments were performed in duplicates and the results were analyzed by t-test, and the data was represented as mean $\pm$ S.D. Significance levels were depicted as * $p \leq 0.05$.

➢ *Molecular docking and virtual screening study*

Proteins receptors JAK1 (PDB id: 5EIE), JAK2 (PDB id: 5USZ), JAK3 (PDB id: 4ZI6) and TYK2 (PDB id: 4OLI) that activate STAT5 were taken as the targets for the binding affinity study with the molecule MPC [7 methoxy 3(4 methoxy 2 methylphenyl) 5 methyl 4H chromen 4 one] in comparison to Bcr-Abl oncoprotein oligomerization domain (PDB id: 1K1L) and the positively binding molecule SOCS (Suppression of cytokine signaling) family Gemfibrozil, a JAK/STAT inhibitor (Drug Bank Accession No DB01241). Protein Data Bank (PDB) the 3-Dimensional structures of the target were taken for analyses and Py - MOL viewer is used for visualization. Co-crystallized ligands and the water molecules were selected and removed from the target. Upon addition of the hydrogen atoms to the structure, minimizations were done through Swiss PDB viewer and Gasteiger atomic partial charges were computed. Geometry optimization for all possible interactions was performed through chimera for the flexible conformations of ligand for the virtual docking screening.

III. RESULTS

➢ *Study of MPC Induced Apoptotic Activity via Nuclear Staining*

Treated and control groups of MEG-01 cells after post harvesting were dual stained in Ethidium bromide and acridine orange. In control no morphological changes are exhibited, the nuclei appeared green and intact in shape Figure 2A. Whereas in the MPC treated MEG-01 cells shows apoptotic inclusion bodies, blebbing of nuclear membrane and crescent shrunken nuclei which are the clear morphological indications of early and late apoptotic stages Figure 2B.

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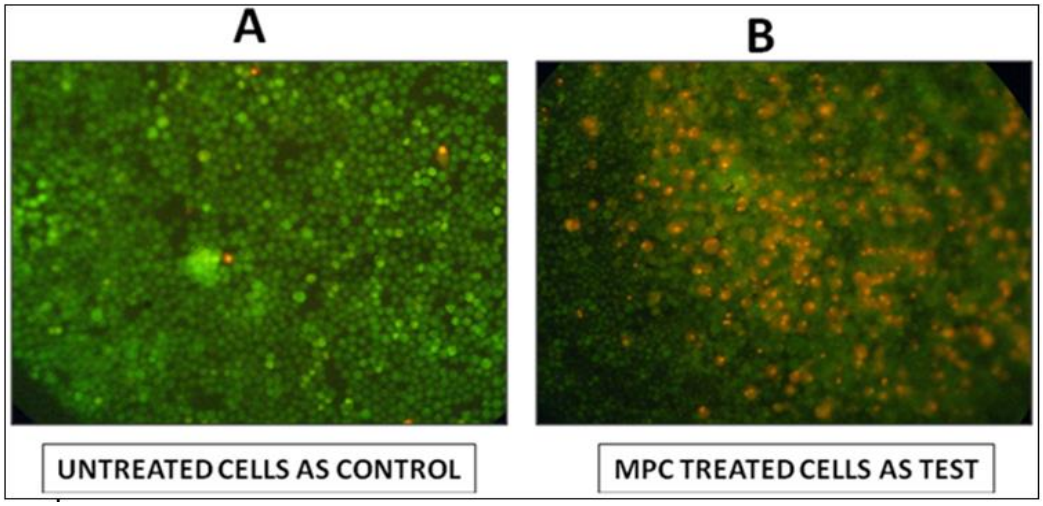

Fig 2 (A - Control) MEG-01 cells cultured in RPMI 1640 media without MPC treatment upon staining with acridine orange and ethidium bromide solutions when observed in fluorescent microscope shows less or nil intake of stain indicating more number of viable cells. (B - Test) MEG-01 cells when treated with MPC followed by staining with acridine orange and ethidium bromide solutions shows more orange spots due to intake of stain indicating the presence of dead and apoptotic cells.

➢ *Flow Cytometry Analysis of Apoptosis by MPC*

In apoptotic cells the translocation of phospholipid phosphatidylserine (PS) from inside out of the membrane attracts calcium dependent phospholipid binding protein - Annexin V (an-V) which has high affinity to PS. Fluorochrome conjugated Annexin V along with the propidium iodide (PI) a vital dye are stained to MPC treated and untreated MEG-01 cells. On comparison with the gated cells the untreated control cells showed no prominent raise of cell percentage with an-V^+/PI^- as well as with an-V^+/PI^+. However in the MPC treated test cells early apoptotic cells which are an-V^+/PI^- were increased by 8.53% out of 11.55% cells, and the late apoptotic cells which are an-V^+/PI^+ were increased by 7.74% out of 10.44% as shown in Figure - 3. This indicates that, in the test cells the translocation of PS precedes the membrane integrity loss, accompanied by later stages of cell death caused due to apoptosis. The gradual increase of the apoptotic cells in the MPC treated MEG-01 cell lines are clarified with annexin V stained cells, that inhibits the uptake of propidium iodide initially. The late apoptotic cells which are positive to both annexin V and propidium iodide were also observed.

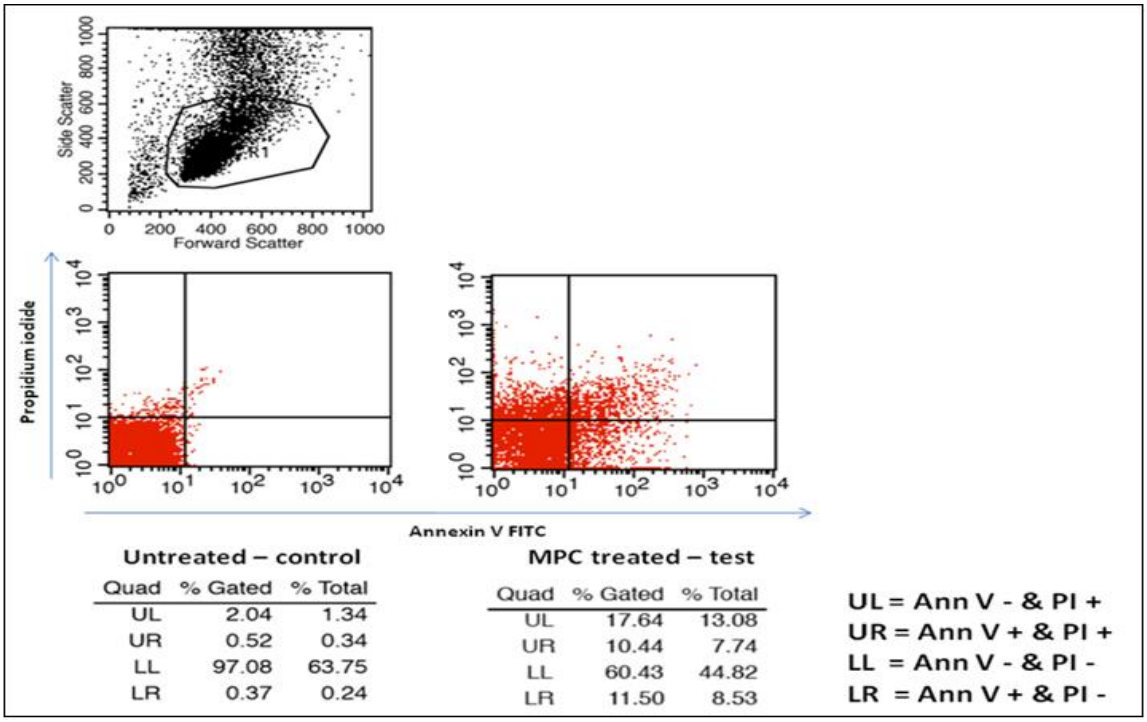

Fig 3 Apoptotic induced change in the plasma membrane. MEG-01 cells that are untreated (showed in left panel) and treated with MPC (showed in the right panel) are subsequently stained with Annexin V – FITC conjugate and Propidium Iodide where the red and far-red fluorescence were analyzed through flow cytometry. Viable cells (LL) are negative for both Annexin V and PI. The cell exhibiting the early stage of apoptosis (LR) binds only to Annexin V excluding PI. At the late stage of apoptosis (UR) they fasten Annexin V-FITC and stain intensively with PI.

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➢ *In Vitro Protein Expression and Estimation of Bcl-X_L through SDS PAGE*

Bcl-X_L, a member protein of Bcl-2 family , works in support of CML as it is an anti-apoptotic in nature, which exactly works by preventing mitochondrial contents release like cytochrome c. One of the major pathways in CML to prevent apoptosis is by over expression and activation of Bcl-X_L protein. Lane 2 of Figure 4 representing the untreated MEG-01, the cell lysate protein shows over expressed protein band of 26kDa of Bcl-X_L gene. Whereas the lane 1 in the same gel representing MPC treated MEG-01 cell lysate shows faint band at 26kDa region indicating the down expression of Bcl-X_L protein.

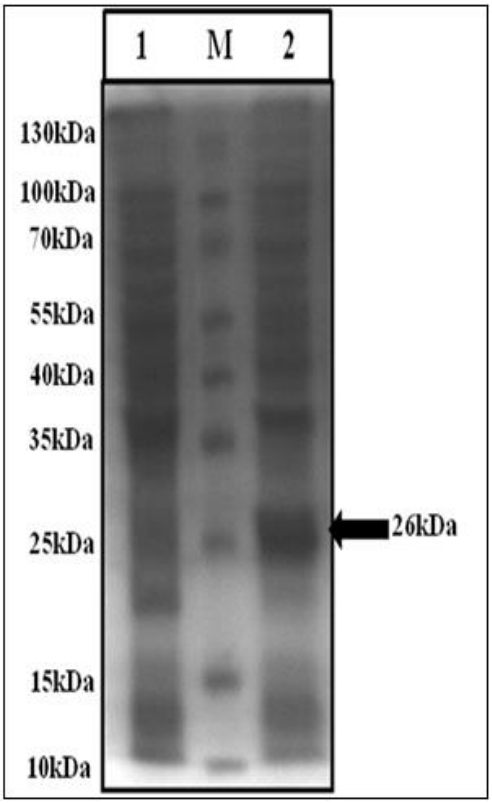

Fig 4 Lysate of MPC treated and untreated MEG-01 cells representing lane 1 and 2 respectively in the 12% poly-acrylamide gel after stained with coomassie brilliant blue, with the molecular marker in place of the lane M. Arrow highlights the 26 kDa expression of BclXL protein in untreated MEG-01 cells of lane 2, the same faintly observed in MPC treated MEG-01 cells of lane

➢ *Immunoblotting of Expressed Bcl-X_L Protein*

To further confirm the under expression of Bcl-X_L protein in the MPC treated MEG-01 cells; the Immunoblotting was done by probing the blot with Bcl-X_L monoclonal antibody and anti-mouse antibodies that is conjugated with HRP. In Figure-4B, the blot evidently exhibit the thick band in lane 2 which is the untreated cell lysate indicating the over expression of Bcl-X_L, and in comparison with lane 2, the band in lane 1 which is the MPC treated cell lysate, is very thin, which convince the under expression of Bcl-X_L protein in MPC treated cell. Chimeric protein $p210^{BCR–ABL}$ in progression of CML increases the regulation of BclX_L, a Bcl2 family protein through P13 kinase dependent manner. Bcl2 family proteins also play an important role in the upstream of caspase activity inhibition. BCR-ABL protein activated by Ras suppresses the growth factors to prevent apoptosis. Mitochondrial cytochrome has dual function as it controls the cellular reactions related to energetic metabolism and also controls the apoptosis. It interacts with the apoptotic protease activating factor and there by initiates the cascades of caspases as it enters the cytoplasm. MPC treated MEG-01 cells shows down regulation of BclX_L which was exposed through SDS PAGE and Western blot (Figure 5) indicating the inhibition of RAS mediated anti-apoptotic pathway through withdrawal of growth factor assisted by Bcl2 family proteins.

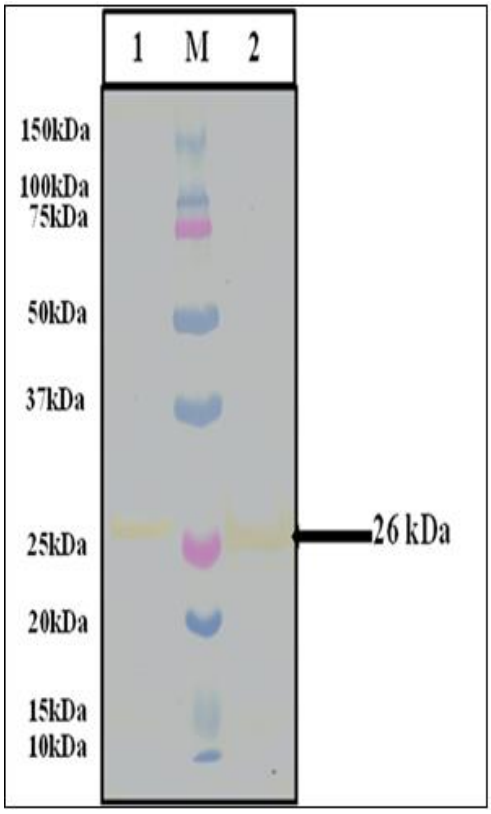

Fig 5 Western blot of MPC treated and untreated MEG-01 cell lysate targeting Bcl-X_L protein expression by tagging with Bcl-X_L monoclonal antibody followed by anti-mouse antibodies that is conjugated with HRP and (DAB) - H_2O_2 substrate wash are represented in lane 1 and 2 respectively along with the colour molecular marker run in lane M.

➢ *Evaluation of GAPDH Protein Expression in Control and Treated Cell Lysate*

In justification to the quantification of cell lysate loaded in the gel the stripping and re-probing the membrane with GAPDH monoclonal antibody and anti-mouse antibodies conjugated with HRP was done and the blot obtained (Figure 6) showed the bands in lane 1 and lane 2 are of similar in size. The lanes 1 and 2 represents treated and untreated cell lysate respectively. The bands of lane 1 and 2 are of 144 kDa. GAPDH, nothing but the house keeping gene expressed protein.

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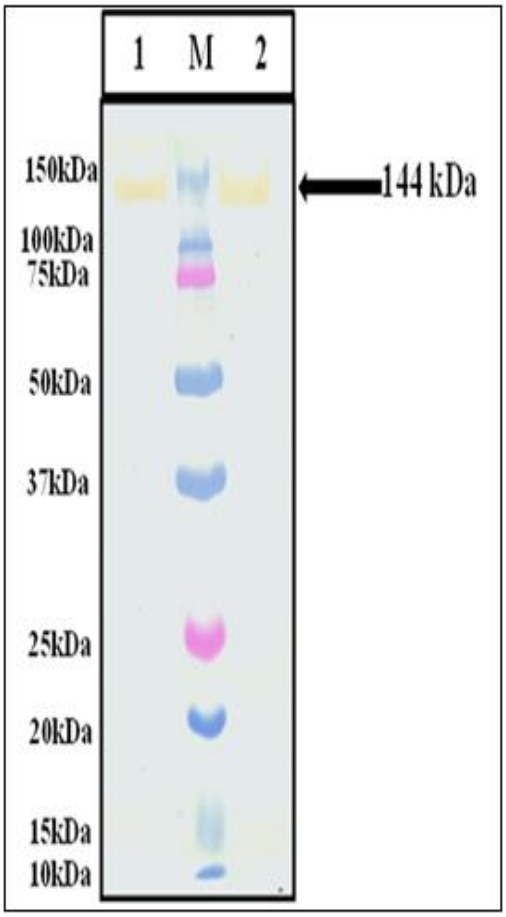

Fig 6 Membrane after stripping, the GAPDH protein was highlighted by GAPDH monoclonal antibody and anti-mouse antibodies that is conjugated with HRP followed by (DAB) - H_2O_2 substrate wash represented in the lane 1 and 2 along with color molecular marker in lane M.

➢ *Real-Time PCR*

PCR efficiency of the genes were calculated by standard curves plotted for the serially diluted DNA of 2000-2ng. The PCR efficiencies obtained were (95.92% for Gapdh and 93.64% for Bcl-xL). Quantitative real time PCR results revealed, significant reduction of around 2 fold in mRNA expression levels of the gene Bcl-xL, in the MCP treated group compared to control group (P value < 0.001) [Figure 7], exhibiting the efficacy of the MPC molecule in manipulating the pathway of anti-apoptosis, targeting CML.

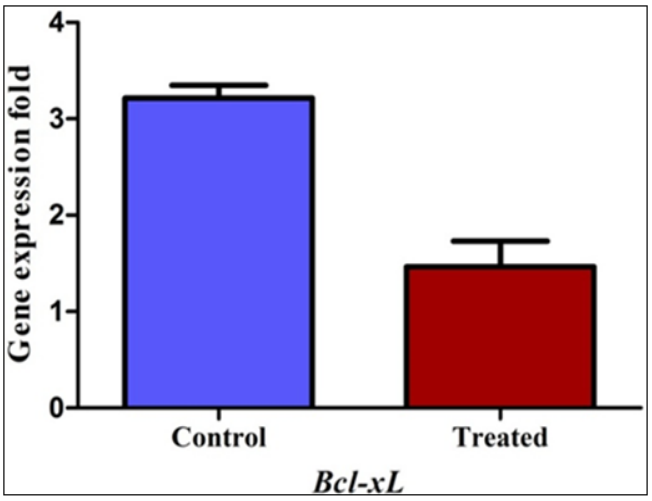

Fig 7 Gene expression study showed significant variation of Bcl-xl mRNA, with MEG-01 cells alone, taken as control in comparison with MPC treated MEG-01 cells as treated group.

➢ *Study of Protein and Molecular Interaction from Molecular Docking and Virtual Screening*

Molecular docking study of receptors JAK1, JAK2, JAK3 and TYK2 binding with MPC in comparison to Bcr-Abl oncoprotein oligomerization domain and SOCS inhibitor Gemfibrozil revealed that the efficacy of binding of MPC is high against Gemfibrozil and almost equal to the binding efficacy of Bcr-Abl oncoprotein oligomerization domain as indicated in the docking [Figure 6] and the hydrogen bond interaction along with the atomic contact energy gliding values as per [Table 1]. MPC is found to be an equal competitor to Bcr-Abl oncoprotein oligomerization domain and more prominent than SOCS inhibitor Gemfibrozil. For receptor TYK2 (PDB id: 4OLI) MPC binds with -205.78 Kcal/mol of energy as compared to Bcr-Abl oncoprotein oligomerization domain which has the binding energy of -223.49 Kilo/mol where as Gemfibrozil has more binding energy of -170.66 to exert in bond formation. MPC binds efficiently than Bcr-Abl oncoprotein oligomerization domain to JAK3 (PDB id: 4ZI6) forming 2 hydrogen bonds with -121.02 Kcal/mol whereas Bcr-Abl oncoprotein oligomerization domain forms one hydrogen bond by consuming -382.76 Kcal/mol of binding energy. Similar way of efficient binding is also seen with JAK2 (PDB id: 5USZ). The bond formations with consumption of binding energy and the amino acids involved in the interactions are evaluated in the Table 2.

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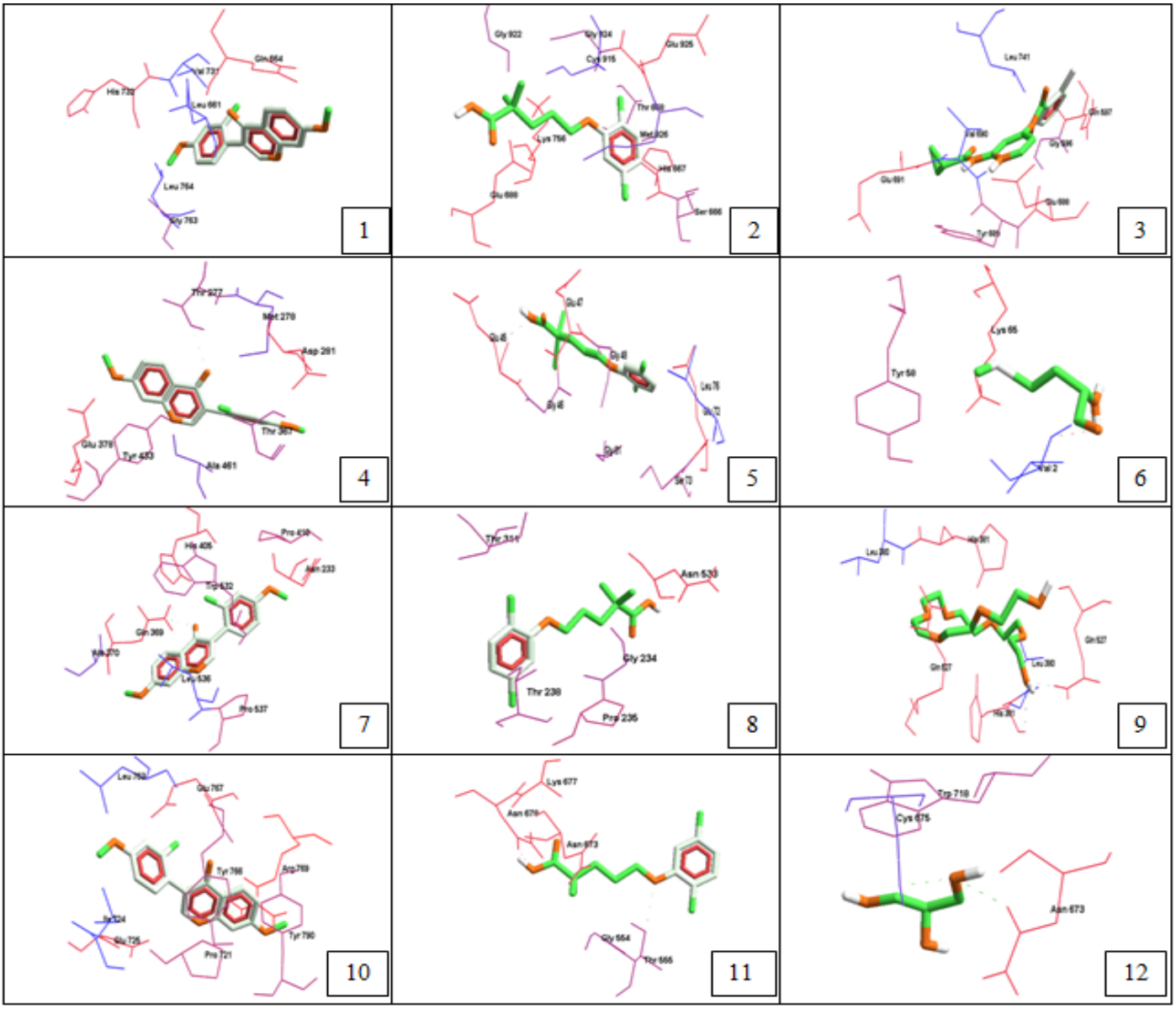

Fig 8 (1) TYK2 Receptor and MPC, (2) TYK2 Receptor and Gemfibrozil, (3) TYK2 Receptor and Bcr-Abl oncoproteins oligomerization domain, (4) JAK3 receptor and MPC, (5) JAK3 receptor and Gemfibrozil, (6) JAK3 receptor and Bcr-Abl oncoproteins oligomerization domain, (7) JAK1 receptor and MPC, (8) JAK1 receptor and Gemfibrozil, (9) JAK1 receptor and Bcr-Abl oncoproteins oligomerization domain, (10) JAK2 JH2 complex and MPC, (11) JAK2 JH2 complex and Gemfibrozil, (12) JAK2 JH2 complex and Bcr-Abl oncoproteins oligomerization domain.

Table 2 Structure-activity relationship (SAR) and binding affinity of Proteins receptors TYK2, JAK3, JAK1 and JAK2 with MPC, the positively binding molecule SOCS (Suppression of cytokine signaling) family Gemfibrozil and Bcr-Abl oncoprotein oligomerization domain

Name of the Biomarker	Name of the Compound	Details of H-bond interaction No. of bond	Atomic contact Energy (ACE) Values	Amino acid residues on docked domains
TYK2 Receptor (4OLI)	**MPC**	0	-205.78	Leu 764, Leu 661, Gln 664, Val 731, His 732, Gly 763
	Gemfibrozil	0	-170.66	Ser 666, His 667, Thr 668, Glu 688, Lys 756, Cys 915, Gly 922, Gly 924, Glu 925, Met 926
	Bcr-Abl oncoproteins oligomerization domain	2	-223.49	Gly 596, Gln 597, Glu 688, Tyr 689, Val 690, Glu 691, Leu 741

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JAK3 Receptor (4ZI6)	**MPC**	2	-121.02	Ala 461, Tyr 433, Thr 277, Met 278, Asp 281, Thr 367, Glu 378
	Gemfibrozil	1	-118.79	Glu 45, Gly 46, Glu 47, Gly 48, Gly 61, Glu 72, Ser 73, Leu 76
	Bcr-Abl oncoproteins oligomerization domain	1	-382.76	Val 2, Lys 65, Tyr 58
JAK1 Receptor (5EIE)	**MPC**	1	-235.74	Asn 233, Gln 369, Ala 370, His 405, Pro 410, Trp 532, Leu 536, Pro 537
	Gemfibrozil	1	-143.09	Asn 533, Gly 234, Pro 235, Thr 238, Thr 311
	Bcr-Abl oncoproteins oligomerization domain	2	-145.51	Leu 380, His 381, Gln 527, Leu 380, Gln 527, His 381
JAK2 JH2 Complex (5USZ)	**MPC**	3	-114.31	Tyr 790, Pro 721, Ile 724, Glu 725, Leu 763, Tyr 766, Glu 767, Arg 769
	Gemfibrozil	2	-102.54	Asn 678, Gly 554, Thr 555, Asn 673, Lys 677
	Bcr-Abl oncoproteins oligomerization domain	2	-221.74	Asn 673, Cys 675, Trp 718

IV. DISCUSSION

At the molecular level the MPC molecule exhibits the competition with Protein $p210^{BCR–ABL}$ in binding to JAK receptors which activates the STAT5 mediated caspase inactivation through $BclX_L$ for anti-apoptosis. The JAN-STAT5 pathway is inhibited by the MPC molecule by binding to JAK receptors which were revealed through structural binding affinity study of molecular docking. Chimeric protein $p210^{BCR–ABL}$ in progression of CML increases the regulation of $BclX_L$, a Bcl 2 family protein through P13 kinase dependent manner. Bcl2 family proteins also play an important role in the upstream of caspase activity inhibition. BCR-ABL protein activated by Ras suppresses the growth factors to prevent apoptosis. Mitochondrial cytochrome has dual function as it controls the cellular reactions related to energetic metabolism and also controls the apoptosis. It interacts with the apoptotic protease activating factor and there by initiates the cascades of caspases as it enters the cytoplasm. MPC treated MEG-01 cells shows down regulation of $BclX_L$ exposed through SDS PAGE and western blot experiments [Figure 4 & Figure 5] indicating the inhibition of RAS mediated anti-apoptotic pathway through withdrawal of growth factor assisted by Bcl2 family proteins. Thereby MPC develops strategy of its style in targeting CML specifically suppressing the molecular efforts of chimeric protein $p210^{BCR–ABL}$ formed by philadelphian chromosome in manipulating the pathway of anti-apoptosis.

V. CONCLUSION

MPC molecule interferes in most the $p210^{BCR–ABL}$ induced several protein-protein interaction to nullify the effects of cell proliferation and anti-apoptotic activities. Down regulation of $BclX_L$ by MPC indicates its specific target of CML cells to undergo apoptosis. It reverse the efforts of $p210^{BCR–ABL}$ induced anti-apoptosis through STAT5 mediated $BclX_L$ up-regulation and activation, whereas the release of cytochrome C is blocked to cease caspase activity. MPC is also an efficient competitor to Bcr-Abl oncoprotein oligomerization domain in binding to tyrosine kinase receptors and thereby reduces the cell proliferation rate. Besides having potential of being alternative therapeutic, MPC can overcome limitations of TKI.

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