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RESEARCH ARTICLE

Determination of the Apoptotic Effect and Molecular Docking of Benzamide Derivative XT5 in K562 Cells

Tulin Ozkan[a,*], Yalda Hekmatshoar[a,*], Tugba Ertan-Bolelli[b], Andry N. Hidayat [c], Meral Beksac[d], Esin Aki-Yalcin[b], Ismail Yalcin[b] and Asuman Sunguroglu[a]

[a]*Department of Medical Biology, School of Medicine, Ankara University, Ankara, Turkey;* [b]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ankara University, Ankara, Turkey;* [c]*Biotechnology Institute, Ankara University, Ankara, Turkey;* [d]*Department of Hematology and Cord Blood Bank, Ankara University, Ankara, Turkey*

Abstract: ***Background*:** The tyrosine kinase inhibitor, imatinib, used as a first line treatment in Chronic Myeloid Leukemia (CML) patients, may lead to resistance and failure to therapy. Novel combinations of imatinib with other drugs is a strategy to improve treatment efficiency.

***Objective*:** In this study, the antileukemic and apoptotic effects of a benzamide derivative XT5 and benzoxazole derivative XT2B and their combination with imatinib were investigated in imatinib-sensitive (K562S) and imatinib-resistant (K562R) CML cells.

***Methods*:** *In vitro* cytotoxicity was determined by MTT assay. Then, apoptotic effect of XT5 on CML cell lines was tested by Annexin V flow cytometry, caspase activation and RT-PCR. Docking calculation was performed using AutoDock Vina in PyMOL environment using AutoDock/Vina plugin for PyMOL.

***Results*:** According to our MTT assay data, XT5 indicated significant antiproliferative effect on cell lines, therefore we investigated apoptotic effects of XT5. Treatment of K562 cell lines with a combination of XT5 and imatinib-XT5 increased cytotoxicity, the Annexin V binding and caspase 3/7 activation. In addition to apoptosis assays, we observed an increase in the expression levels of the pro-apoptotic (BAX, BAD and BIM) genes in XT5 treated K562R and K562S cells. Molecular modelling experiments showed that XT5 showed hydrogen-bonding interactions with important amino acids of BCR-ABL kinase receptor; however XT2B did not show any hydrogen bond interaction.

***Conclusion*:** Our results indicate that XT5 could be a potential candidate to be used as a new anticancer drug and XT5 combination with imatinib as an alternate treatment strategy for overcoming imatinib resistance.

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1. INTRODUCTION

Chronic Myeloid Leukemia (CML) is a myeloproliferative disease associated with reciprocal translocation between chromosomes 9 and 22 (q34; q11) and BCR-ABL fusion gene exhibiting constitutively active tyrosine kinase activity [1].

As CML is caused by BCR-ABL protein, therefore, designing inhibitors that selectively target and inhibit the tyrosine kinase activity of BCR–ABL protein can improve the efficiency of therapy [2]. Imatinib inhibits BCR-ABL tyrosine kinase activity by binding to the ATP-binding site of the protein. Imatinib, is a Tyrosine Kinase Inhibitor (TKI), which is used as a first line treatment in CML. Although imatinib treatment has improved the outcome of CML patients dramatically, imatinib resistance, which leads to treatment failure, also emerged [3]. High dose of imatinib, second generation TKIs(dasatinib and nilotinib) and third-generation TKI (ponatinib) are used to overcome imatinib resistant in CML patients [4, 5]. Imatinib inhibits proliferation and induces apoptosis in CML cell lines(K562) [6].

*Address correspondence to these authors at the Department of Medical Biology, School of Medicine, Ankara University, Ankara, Turkey;
E-mail: tozkan@ankara.edu.tr and Tel: +90 312 595 80 50;
E-mail: hekmatshoar@ankara.edu.tr

Apoptosis or programmed cell death is a process in which cells' chromatin condensation changes, nuclear DNA undergoes fragments, caspases activate and phosphatidylserine exposes the cell surface. These events can be used as biochemical biomarkers of apoptosis [7, 8]. Caspase activation leads to proteolysis of cellular proteins so they play an important role in apoptosis of mammalian cells [9, 10]. Apoptosis is also regulated by pro-apoptotic (Bax, Bim, Bad and Bak) and anti-apoptotic (Bcl-2, Mcl-1 and Bcl-xl) members of Bcl-2 family [11, 12].

Inducing apoptosis in cancer cells would be a useful therapy, as most of the cytotoxic drugs currently in use exhibit apoptotic effect in cancer cells [13]. Recently, the combination therapy was used as an alternative solution in the treatment of blood cancers.

For many years, benzoxazoles and benzamides which are the possible metabolites of benzoxazoles exhibiting various chemotherapeutic activities such as antimicrobial, antiviral and anticancer, have been utilized by many researches [14, 15]. DNA topoisomerases have been considered as important targets for cancer chemotherapy. Our group investigated the inhibitory activities of some benzoxazole derivatives on eukaryotic DNA topoisomerase II in a cell free system, and we pointed out that these compounds exhibited significant DNA topoisomerase II inhibitory activities with an higher potency than the reference drug etoposide [16]. In this study, anticancer activities of a benzamide derivative XT5 and a

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benzoxazole derivative XT2B, which were synthesized by our group before [17, 18], have been tested *in vitro* on human CML cell line, K562.

2. MATERIALS AND METHODS

2.1. Preparation of Compounds

Compounds were dissolved in the DMSO in a stock solution at a concentration of 20mM, stored at -20 °C and protected from light. In each experiment, DMSO concentration never exceeded 0.5 % and this percentage did not interfere with cell growth.

2.2. Cell Culture

K-562 cell line was cultured with 1640 growth medium (Sigma/USA) and L929 cell line with DMEM low glucose, containing 10% fetal bovine serum (FBS)(Sigma/USA), 100 units/ml penisilin and 100g/ml streptomisin (Gibco/USA). In our laboratory, imatinib mesylate resistant cell line (K562R) which grows in the presence of 5μM imatinib, was generated by adding imatinib mesylate by stepwise increasing the concentration during 18 months on imatinib mesylate sensitive K-562 cell lines [19].

2.3. Cell Viability Test/Cytotoxicity Test

MTT assay was performed to assess cell viability [20]. K562S and K562R cells were treated with XT5 and XT2B in the presence and absence of imatinib for indication of IC_{50} values for each component. The cells (4 x 10^4 cells/well) were seeded to 96- well plates. The cell proliferation assay was carried out using the Cell Proliferation Kit I [3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide; (MTT)] (Roche, Germany) as described by the manufacturer using the cells treated at various concentrations (0.1, 1, 5, 10, 20, 40, 60, 80 and 100μM) of compounds XT5 and XT2B for 72h. The spectrophotometric absorbance was measured using a microplate reader (Biotek, USA) at 550 nm, using 690 nm as a reference. Number of viable cells was calculated from untreated cells, and the data were expressed as percent cell viability. All the experiments were conducted in triplicate and DMSO was used as negative control in the corresponding concentrations.

2.4. Flow Cytometric Analysis for Apoptosis Determination

K562S and K562R cells (4 x 10^5 cells/well) were seeded in 6 well- plates. The cells were incubated with compound XT5 (10μM) for 72 h. After incubation period, the cells were collected, washed with PBS twice and resuspended in binding buffer according to the suppliers' instructions (BD Biosciences). 5 μl PE-Annexin V and 5 μl 7AAD were added to 100 μ l of cell suspension. After a brief vortex, the cells were incubated for 15 min at room temperature. After this incubation, 400 μl 1x binding buffer was added to the cells followed by their in flow cytometry (Accuri C6) [21].

2.5. Detection of Caspase 3/7 Activation

Caspase3/7 activation was detected using the Cell Event Caspase-3/7 Green Flow Cytometry Assay Kit as described by the manufacturer's (Thermo Fisher Scientific) protocols. Flow cytometry tubes were prepared; each containing 1 ml of cell suspension at 4×10^5 cells/ml. Following this, 1 μL of Cell Event Caspase-3/7 Green Detection Reagent was then added to each sample and incubated for 30 minutes at 37 °C. The samples were analysed on an Accuri C6 flow cytometer. Data plots of FL-1 and FL-4 were used to show the populations of alive and apoptotic cells with the activated form of caspase 3 and 7 [22].

2.6. Quantitative RT-PCR

Quantitative real-time RT-PCR was used for the detection of apoptosis-related (BAX, BCL-2, BAD, BIM, BCL-XL and MCL1) genes expression levels [23]. K562 cells (4 x 10^5 cells/well) were seeded in six well-plates and incubated with XT5 (10μM) for 72 h. After incubation period, total RNA was extracted from K562 cell line by using the High Pure RNA kit (Roche, Mannheim, Germany) according to the manufacturer's protocols. cDNA was generated from RNA by reverse transcriptase (Transcriptor High Fidelity cDNA Synthesis Kit; Roche). Quantitative real-time RT-PCR was performed using SYBR Green PCR Master Mix (Roche) on LC2480 instrument. mRNA was measured relative to HPRT as an endogenous control. Primer sequences are shown in Table **1**.

2.7. Molecular Docking Studies

BCR-ABL kinase protein used in this study was retrieved from protein data bank with PDB ID 5HU9. Before proceeding to docking study, an amendment was made in the protein was remove water molecules and other non-protein atoms. Docking calculation was performed using AutoDock Vina in PyMOL environment using AutoDock/Vina plugin for PyMOL. Docking calculation was performed on imatinib molecule as control, the known inhibitor for BCR-ABL kinase protein and XT5 molecules as novel molecules. XT2B molecule was also docked with the similar protocol. Visualization of ligand binding was generated using Discovery Studio 3.5 Visualizer. The binding pose of imatinib was also validated with the co-crystalized imatinib and ABL1 kinase structure from PDB databank (PDB ID 2HYY).

2.8. Statistical Analysis

Data were represented as mean ±Standard Deviations (SD) from triplicate experiments performed in a parallel manner. Comparisons between treated and untreated control groups were made by t-tests or ANOVA analysis where applicable. $P < 0.05$ was used as the cutoff for defining statistically significant differences.

Table 1. Primer sequences for Quantitative RT-PCR analysis.

Gene	Forward (5'- 3')	Reverse(5'- 3')
BAD	GATGAGTGACGAGTTTGTGGA	CAAGTTCCGATCCCACCAG
BCL-2	CGCCCTGTGGATGACTGAGT	GGGCCGTACAGTTCCACAA
BIM	ATCTCAGTGCAATGGCTTCC	CATAGTAAGCGTTAAACTCGTCTCC
BAX	GACGGCAACTTCAACTGGG	AGGAGTCTCACCCAACCAC
MCL-1	CGAACCATTAGCAGAAAGTATCAC	GATATGCCAAACCAGCTCCT
BCL-XL [23]	GATCCCCATGGCAGCAGTAAAGCAAG	CCCCATCCCGGAAGAGTTCATTCACT
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT

3. RESULTS

3.1. Cell Viability Test/Cytotoxicity Test

Benzamide derivatives XT5 and XT2B were investigated for their *in vitro* antiproliferative activities in chronic myeloid leukemia cell lines (K562S and K562R) and fibroblast cell line (L929), using MTT assay. Cell viability test results are reported in Fig. (**1**), demonstrating remarkable cytotoxic activity against K562R and K562S cell lines. Viability of cells treated without XT5 was regarded as 100 % and the results demonstrated that XT5 treatment inhibited viability of K562S, K562R+ima and K562R-ima cells between 1µM and 100µM concentrations significantly (p<0.05, p<0,001). XT5 was found to be less cytotoxic to normal fibroblast cells while XT2B was not cytotoxic to all these cell lines.

The IC_{50} values of these compounds are shown in Table **2**. XT5, 4-Ethyl-*N*-(2-hydroxy-4-nitrophenyl) benzamide, showed more antiproliferative activity than XT2B (2-(4-Fluorobenzyl)-5-nitrobenzoxazole). XT5 exhibited the IC_{50} values of 2.89, 3.57, 8.65 and 9.27(µM) in K562S, K562R+ima, K562R-ima and L929 cells, respectively. The IC_{50} values for XT2B in all cell lines were observed to be more than 100 µM.

3.2. Flow Cytometric Analysis for Apoptosis Determination

In this study, flow cytometric analysis with Annexin V-PE/7AAD staining was carried out to explore the effects of XT5 on the apoptosis of K562R and K562S cell lines. The percentage of early apoptotic K562S, K562R+ima and K562R-ima cells (in the lower right quadrant, annexin V^+7AAD^-), incubated with compounds XT5 was dramatically higher (26.64± 4.22, 42.90 ± 1.31, 25.68± 2.46 % respectively) than the control cells (14.34 ± 2.25, 19.11 ± 1.63, 11.71± 0.51 % respectively) at 72 h. The percentage of late apoptotic K562S, K562R+ima and K562R-ima cells (in the

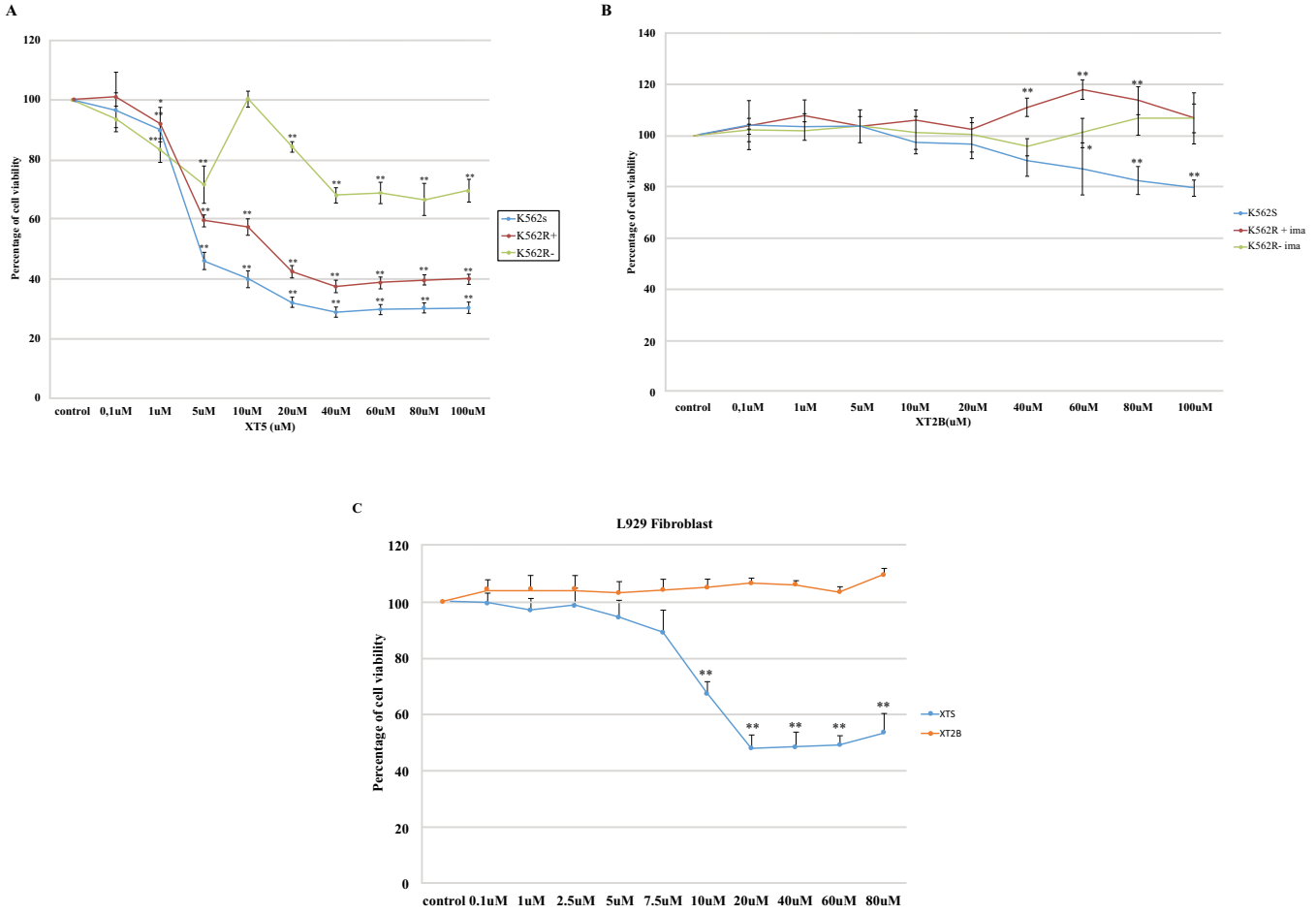

Fig. (1). Effects of XT5 and XT2B on cell viability and growth of cell lines (K562S, K562R+ima, K562R-ima and L929). **A**) MTT assay of CML cells treated with various concentrations of XT5. **B**) MTT assay of CML cells treated with various concentrations of XT2B. **C**) MTT assay L929 fibroblast cells treated with XT5 and XT2B. *p≤ 0,05 ** p≤0,001.

Table 2. Antiproliferative activity after 72h of incubation expressed as IC_{50} value.

Compound	IC_{50}µM			
	K562S	K562R-ima	K562R+ima	L929
XT5	2.89 ±0.30	8.56± 181.2	3.57 ±0.76	9.27 +/- 0.25
XT2B	>100	>100	>100	>100

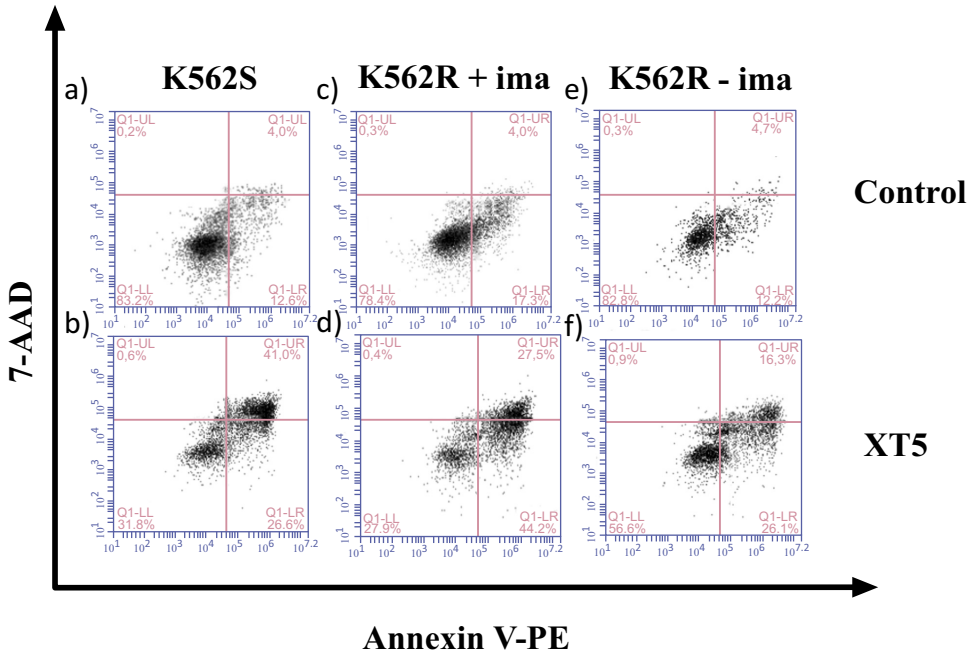

Fig. (2). Flow cytometry results are represented as a) K562S cells without XT5; b) K562S cells incubated with XT5; c) K562R cells with imatinib and without XT5 d) K562R cells incubated with imatinib and XT5; e) K562R cells without imatinib and XT5; f) K562R cells treated only with XT5.

upper right quadrant, annexin V^+ $7AAD^+$) treated with XT5 was significantly higher (37.35 ± 7.49, 22.4 ± 5.13, 15.09 ± 3.28 respectively) than the control cells (4.75 ± 0.68, 8.29 ± 3.81, 4.95 ± 0.47) at 72 h (Figs. **2**, **3**).

3.3. Detection of Caspase 3/7 Activation

According to our annexin V assay, apoptosis was detected in XT5 treated cells. To confirm that XT5 induces apoptosis through caspase activation, we examined caspase 3/7 activation after 72h incubation with XT5. Caspase 3/7 activated in all cell lines K562S (18883.72±191.71) and K562R-ima (21493.52±345.12). The strongest activation was observed in XT5 treated K562R+ima cells (51360.32±3021.50). Detailed results are shown in Figs. (**4**, **5**).

3.4. Quantitative Real-time PCR Analysis

Quantitative analysis of mRNA demonstrated that compound XT5 increased the expression of the proapoptotic genes (BAX, BAD, BIM) in K562S, K562R+ima and K562R-ima cells. Antiapoptotic genes (BCL-2, BCL-XL, MCL-1) overexpressed in XT5 treated K562S and K562R+ima cells, whereas these genes expressions decreased in K562R-ima cells. Although antiapoptotic genes expressions increased, proapototic genes overexpressed more than antiapoptotic genes. XT5 induced higher gene expression ratio of BAX/BCL-2, specially in combination with imatinib (Table **3**) (Fig. **6**).

3.5. Ligand- BCR-ABL Interaction Analysis

In order to understand the binding mechanism of novel molecule XT5 to BCR-ABL kinase to perform their effect against the chronic myeloid leukemia (CML), we applied molecular docking calculation with the high resolution (1.53 Å) crystal structure of ABL1 kinase (PDB ID: 5HU9) as the receptor. The results demonstrated that both of the XT5 binding modes (XT5 pose 1 and pose 2) adopted similar binding mode of co-crystalized ABL1 kinase and its known inhibitor, imatinib (PDB ID: 2HYY). Binding mode of imatinib and ABL1 kinase receptor is known as type II binding mode (DFG-out conformation). According to the docking results, Autodock Vina successfully predicted the true binding mode of imatinib and ABL1 kinase by showing a similar binding interaction of important amino acids (Glu286, Thr315, Met318, Asp381) (Fig. **7A**) for which the docking score was found to be -12.1 Kcal/mol. Superimposition of docked imatinib with ABL1 kinase receptor and it's co-crystalized structure as shown in (Fig. **7B**) with lower RMSD value (0.590 Å), demonstrated that molecular docking calculation conducted by Autodock Vina program was performed well.

Docking result of XT5 pose 1 with the ABL1 kinase receptor showed two interactions (Fig. **8A**) which were represented by hydrogen bond of Asp381 with nitro group of XT5 molecule and Pi-Sigma interaction between Phe382 and nitro phenyl ring of XT5 (Docking score = -9.6 Kcal/mol). According to the docking result (Fig. **8B**) XT5 pose 1 binding mode was pushed deeply to the hydrophobic core of the ABL1 kinase receptor cavity due to ethyl benzamide effect of XT5 molecule (Table **4**).

XT5 pose 2 has a more similar binding mode of imatinib against ABL1 kinase receptor than the XT5 pose 1. XT5 pose 2 has two hydrogen bonding interactions which are represented by two canonical hydrogen bonds formed between the Glu286 located in the c-Helix and Asp381 located in the DFG motif with the amide bond (NHC=O) in the XT5 molecule (Fig. **8C**). This is similar with imatinib which also binds to ABL1 kinase with type II binding mode interaction (Fig. **7B**). In addition, the Pi-sigma bond between the XT5 and Lys271 amino acid is also similar to imatinib interaction.

Docking protocol applied to XT5 molecule was also implemented on XT2B molecule against BCR-ABL kinase receptor. Docking score predicted by Autodock Vina for XT2B pose 1 was observed to be similar to the docking score of XT5 pose 1 (Docking score = -9.6 Kcal/mol). Although XT2B showed similar docking score with XT5 pose 1, the docking result of XT2B did not show any hydrogen bond interactions between XT2B molecule and BCR-ABL kinase receptor (Fig. **9A**). The only possible interactions of XT2B and BCR-ABL kinase receptor on this binding pose were hydrophobic interactions because XT2B molecule docked into deep

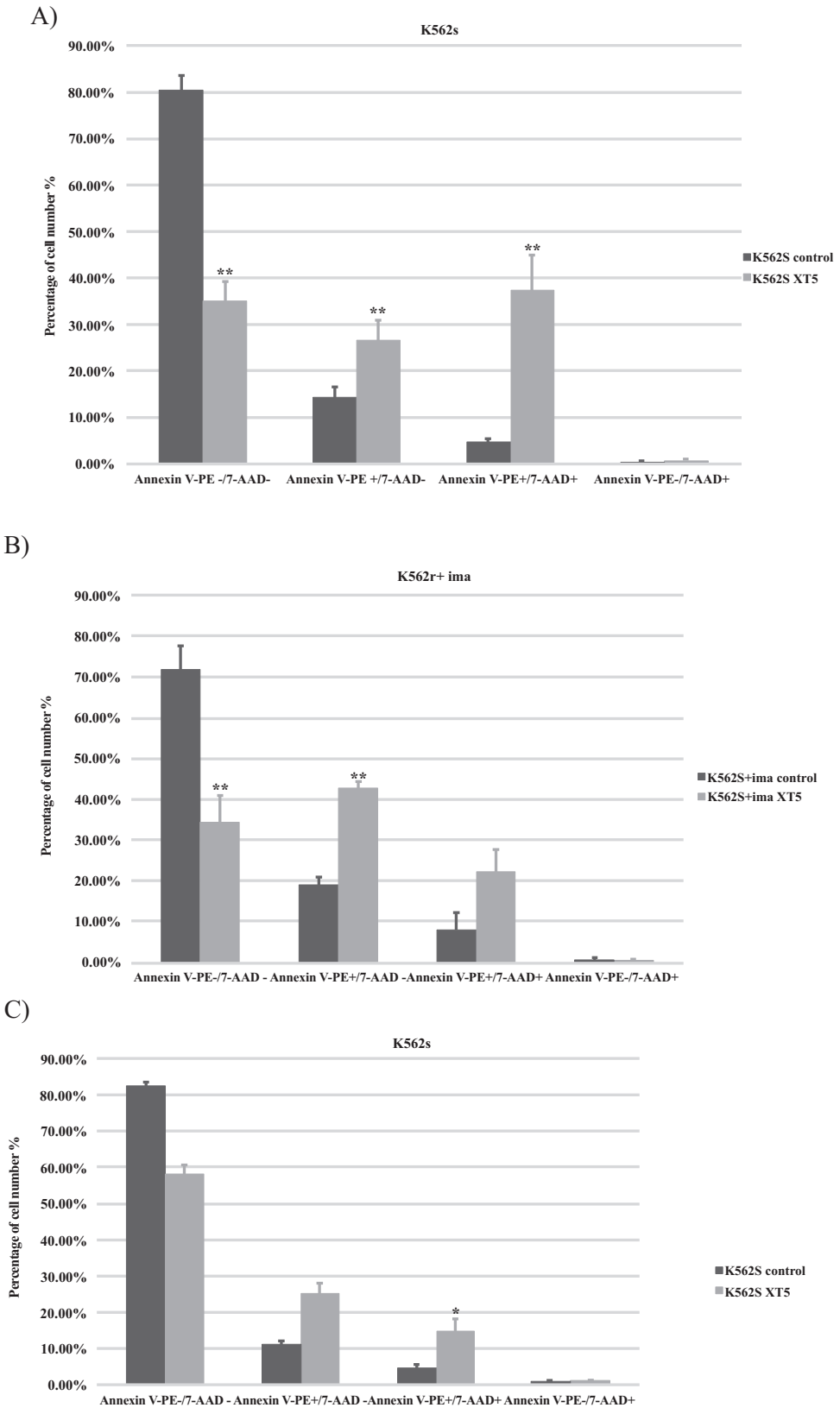

Fig. (3). Flow cytometry results of CML cell lines are shown as bar graphs. **A**) K562S cells with/without XT5; **B**); K562R cells with imatinib and with/without XT5 **C**) K562R cells without imatinib and with/without XT5. Percentage of viable cells (Annexin V-PE −/7-AAD−), early apoptotic cells (Annexin V-PE +/7-AAD−), late apoptotic cells (Annexin V-PE+/7-AAD+), and necrotic cells (Annexin V-PE −/7-AAD+) are shown as means ± SD. *p≤ 0,05 ** p≤0,001.

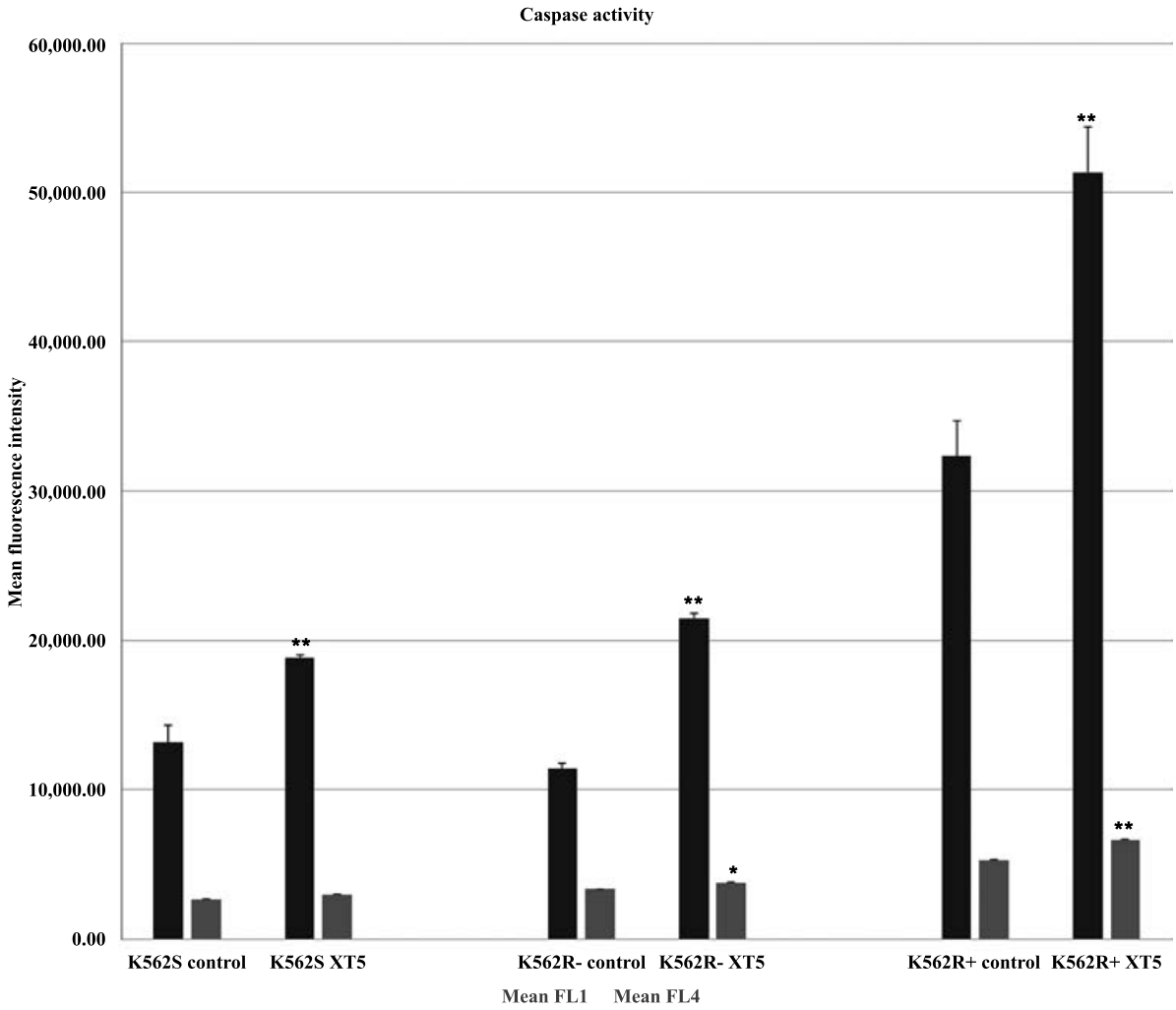

Fig. (4). Caspase 3/7 activation after 72h of incubation with XT5 in K562S, K562R-ima and K562R+ima. *p≤ 0,05 ** p≤0,001.

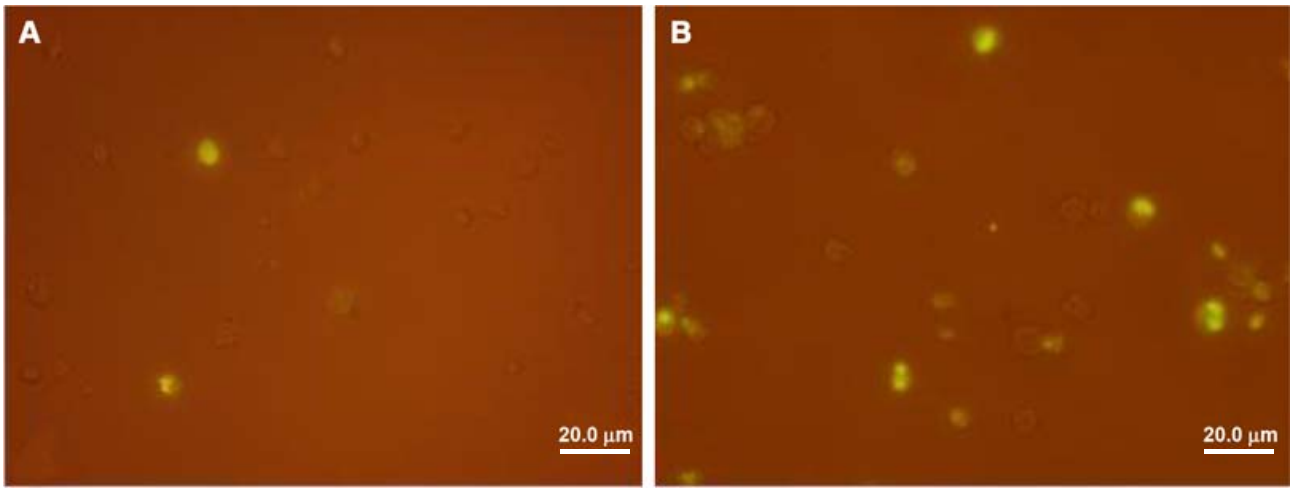

Fig. (5). Fluorescence microscopic examination (magnification 10×40) of CellEvent Caspase-3/7 Green Detection Reagent stained K562R+ima cells. Cells were incubated with imatinib and XT5 for 72 h. **A**) K562R cells with imatinib and without XT5 **B**) K562R cells with imatinib and XT5. These constructs were examined by fluorescent microscopy under fluorescent and active caspase-3/7 were stained green.

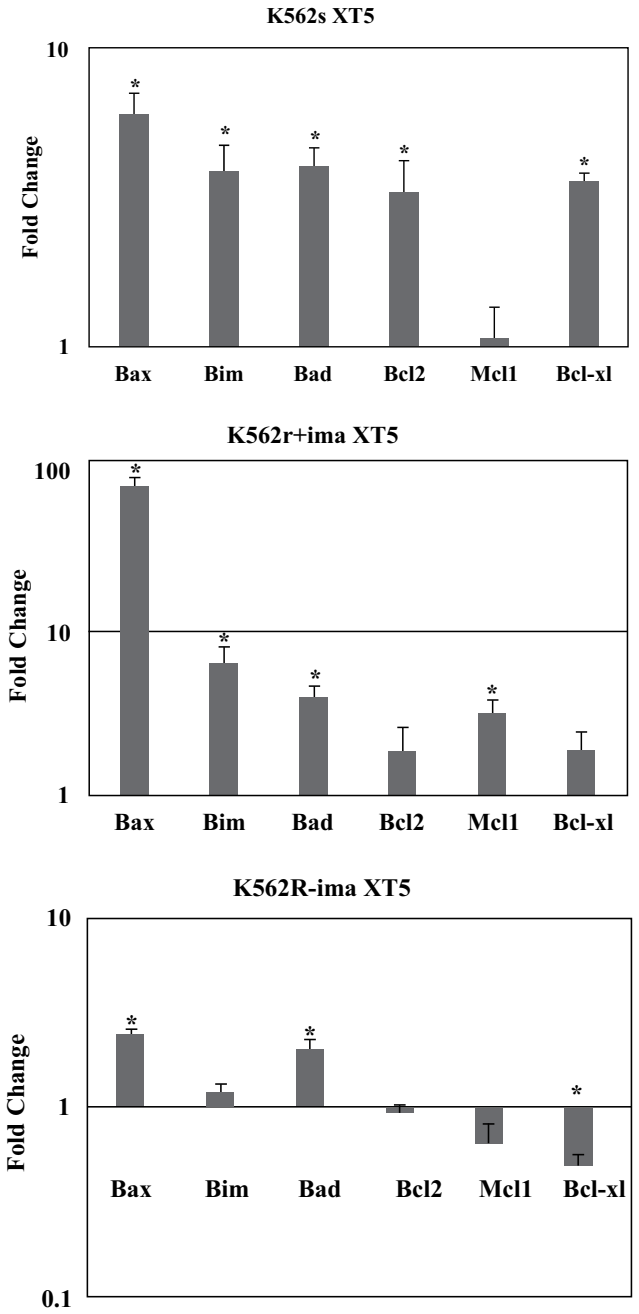

Fig. (6). Expression of apoptosis-related genes BAX, BCL-2, BAD, BIM, BCL-XL and MCL1 after treatment with XT5. Levels of expression are compared with control, and HPRT mRNA was used as an internal control. Experiments were conducted in triplicate. Error bars indicate ± s.d. significantly different from control(*p≤ 0,05 ** p≤0,001).

pocket of the receptor that has high hydrophobicity surface area (Fig. **9B**).

4. DISCUSSION

Imatinib, as a first generation tyrosine kinase inhibitor in CML therapy, has an important role in the inhibition of BCR-ABL tyrosine kinase activity, inducing apoptosis in CML cells. Despite therapeutic success of imatinib, imatinib resistance in CML patients leads to treatment failure.

Table 3. Fold change in expression level of apoptotic genes in K562S, K562R+ima, K562R-ima cells. FC: Fold Change.

Genes			
	K562S	**K562R+ima**	**K562R-ima**
Bax	FC: 6,04 *p: 0,012**	FC:79,30 *p: 0,006**	FC:2,42 *p: 0,003**
Bcl-2	FC:3,29 *p: 0,047**	FC:1,83 *p:0,184*	FC: 0,92 *p: 0,287*
Bcl-XL	FC:3,56 *p: 0,002**	FC:1,88 *p: 0,105*	FC: 0,49 *p: 0,006**
Mcl-1	FC:1,05 *p: 0,778*	FC:3,16 *p:0,030*	FC: 0,64 *p: 0,071*
Bim	FC:3,84 *p: 0,030**	FC:6,48 *p: 0,025**	FC:1,19 *p: 0,116*
Bad	FC:4,03 *p:0,012**	FC:4,49 *p:0,016**	FC:2,02 *p: 0,020**

*p≤ 0,05 ** p≤0,001.

Recently, to increase the anticancer effect of imatinib in the therapy of CML patients, new agents synthesized before [17, 18] were used alone or in combination with imatinib. According to the study by Gosh *et al.*, benzamide induced intrinsic and extrinsic pathway of apoptosis in K562 cells [24]. Ju *et al.* represented that a novel Hsp90 inhibitor BJ-B11, a benzamide molecule induced apoptosis through activation of caspase 9 and caspase 3 and mitochondrial dysfuncion in K562 cells [25]. Mahdavi *et al.* also indicated that spiroquinazolinone benzamide derivatives induced apoptosis in breast cancer [26]. In the present study, we investigated the apoptotic and antiproliferative effect of benzoxazole derivative XT2B and a benzamide derivative XT5 in K562S and K562R cells. In order to determine apoptotic and antiproliferative effects of these compounds, XT5 and XT2B were administered separately as agents and in combination with imatinib. We identified XT5 having significant antiproliferative and apoptotic effects by trigging caspase3/7 activation and overexpression of proapoptotic genes (BAX, BIM, BAD) on chronic myeloid leukemia K562S and K562R alone, being specially more effective when combined with imatinib.

Our results indicated that compounds' function is associated with their structure because of the differences in cytotoxic effects of molecules. The difference between XT5 and XT2B can be attributed to XT5 structure and solubility properties. XT5 as a metabolite of XT2B can be transferred into the cell more than XT2B and XT5, being more soluble in the DMSO, therefore, XT5 shows better antiproliferative activity than XT2B. Moreover, benzamides are known as possible metabolites of benzoxazoles. Therefore, it can be considered that the benzoxazole derivative XT2B could show better antiproliferative activity after biotransformation in the body.

According to our docking data, XT5 molecule could not form four hydrogen bonds with ABL1 kinase receptor as imatinib due to the lack of hydrogen acceptor or donor group within the molecule. The formation of two canonical hydrogen bonds in the DFG motif and c-Helix of the receptor could be effective enough to inhibit the function of ABL1 kinase receptor. XT2B molecule is more likely to not interact or inhibit the function of BCR-ABL kinase receptor due to lack of interactions to important amino acids of BCR-ABL kinase receptor. Based on the structure of XT5, it can be concluded that this novel XT5 molecule could become a candidate molecule for a selective inhibitor against BCR-ABL kinase protein that is

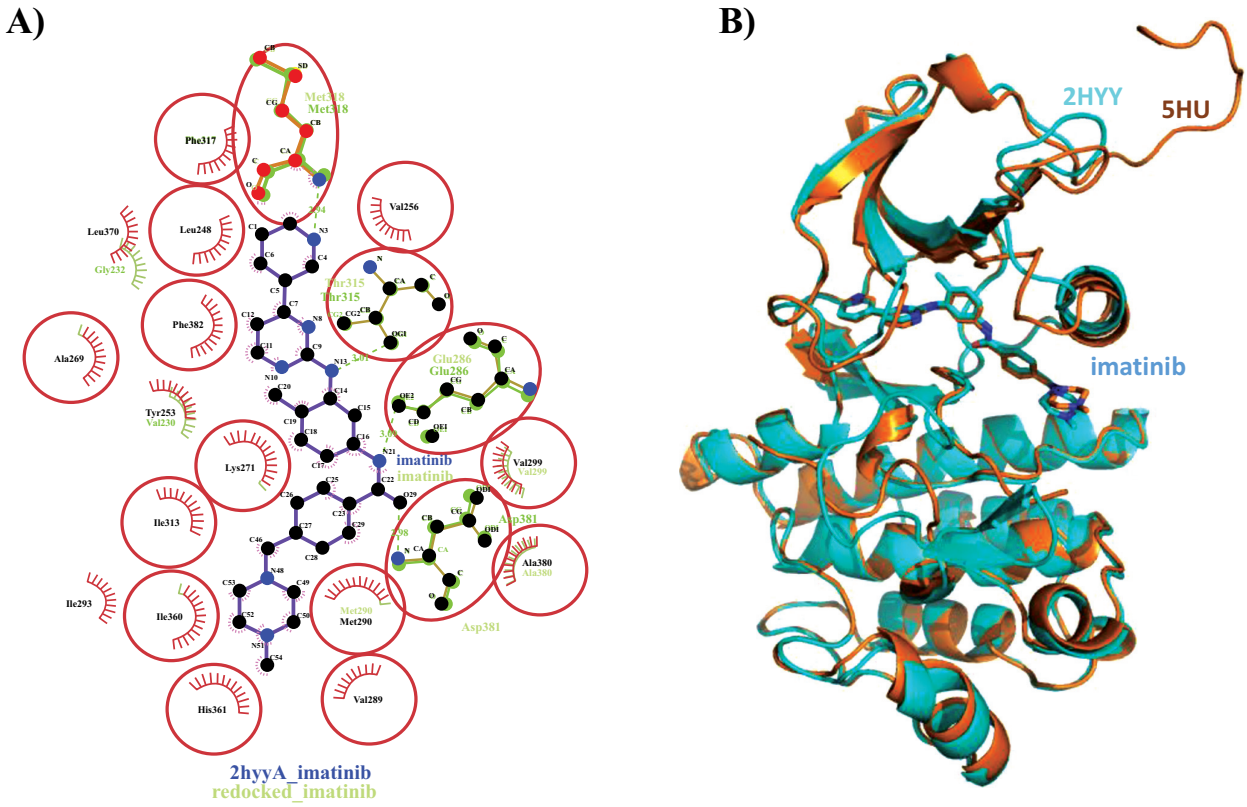

Fig. (7). **A**) Putative interactions between docked imatinib with ABL1 kinase (green) and co-crystalized imatinib with ABL1 kinase (blue). **B**) Superimposition of re-docked imatinib with ABL1 kinase structure (orange) (PDB ID : 5HU9) and co-crystalized imatinib with ABL1 kinase (cyan) (PDB ID : 2HYY). RMSD = 0.590 Å.

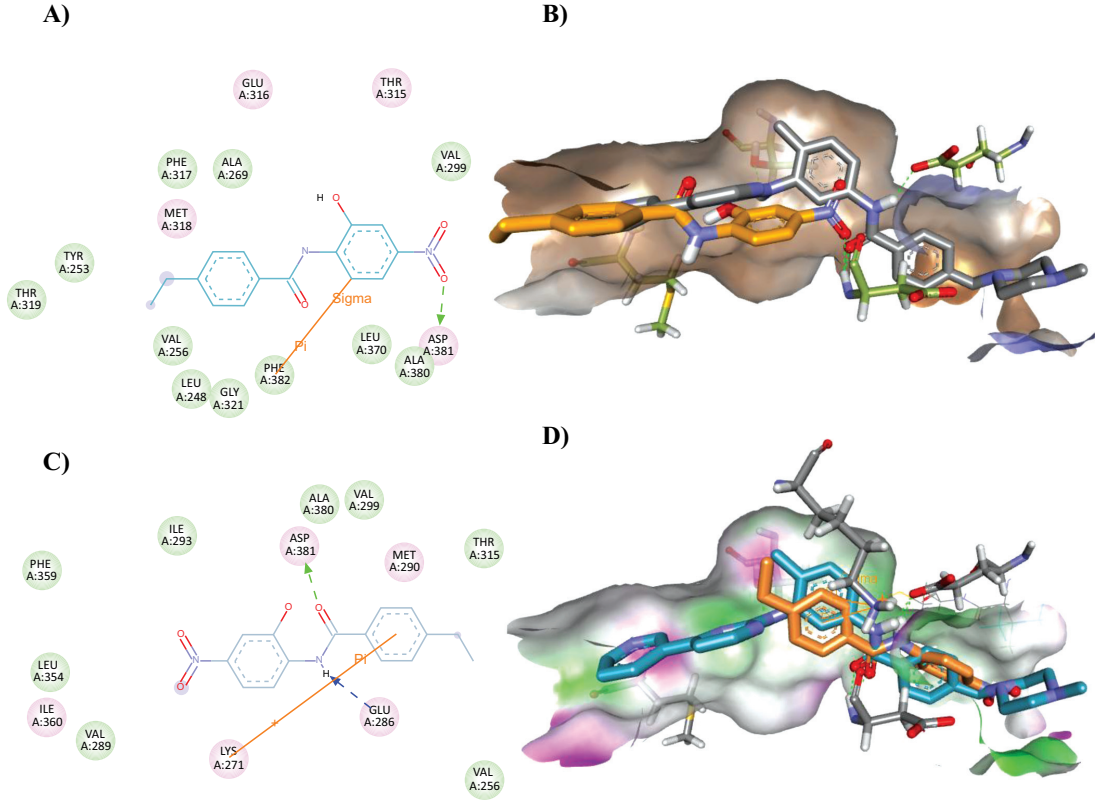

Fig. (8). **A**) Putative interactions of docked XT5 pose 1 and ABL1 Kinase. **B**) Superimposition of XT5 (orange) and imatinib (grey) in the ABL1 kinase structure**.** **C**) Putative interactions of docked XT5 pose 2 and ABL1 Kinase. **D**) Superimposition of XT5 (orange) and imatinib (cyan) in the ABL1 kinase structure.

Table 4. Ligand-BCR-ABL interaction analysis of imatinib, XT5 and XT2B molecules.

Ligand	Docking Score (Kcal/mol)	Amino Acid Interactions	
		Hydrogen Bonds	Other
Imatinib	-12.1	**Glu286**, Thr315, Met318, **Asp381**	**Lys271 (Pi-Sigma)**
XT5 pose 1	-9.6	**Asp381**	Phe382 (Pi-Sigma)
XT5 pose 2	-8.4	**Glu286**, **Asp381**	**Lys271 (Pi-Sigma)**
XT2B pose 1	-9.6	-	-

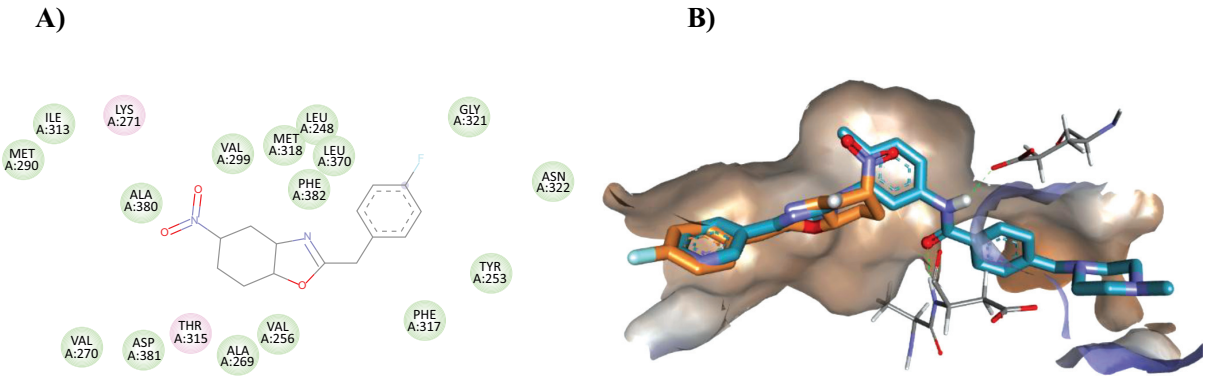

Fig. (9). A) Putative interactions of docked XT2B pose 1 and ABL1 Kinase. **B)** Superimposition of XT2B (orange) and imatinib (cyan) in the ABL1 kinase structure.

related to chronic myeloid leukemia disease. More experiments are required to explore the detailed XT5 mechanism of action.

CONCLUSION

In the present study, we suggested benzamide derivatives XT5 as a new molecule with antiproliferative and apoptotic effects in the imatinib sensitive and imatinib resistant K562 cells. Imatinib resistance is one of the main factors in unsuccessful treatment. These findings may help us design new therapeutic molecules with high cytotoxic activity to induce apoptosis in drug resistant cancer cells.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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