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Evaluation of NAV2729 in combination with imatinib in apoptosis induction in chronic myeloid leukemia cells (K562), an *in vitro* study

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Despite the clinical success of imatinib in chronic myeloid leukemia (CML), drug resistance and adverse effects remain significant challenges, driven in part by tumor-derived extracellular vesicles (TEVs) that promote drug efflux and intercellular survival signaling. While TEV inhibition represents a promising strategy to augment therapy, no studies have yet explored targeting the ARF6-mediated TEV pathway in CML or evaluated its synergy with tyrosine kinase inhibitors. This study addresses this critical gap by investigating the novel combination of imatinib and NAV2729 a selective ARF6 inhibitor known to block TEV release in solid tumors but untested in hematological malignancies to overcome resistance and enhance therapeutic outcomes. The K562 CML cell line was treated with imatinib, NAV2729, or a combination of both for 48 h. Cell viability and metabolic activity were assessed using trypan blue exclusion assay and MTT assay, respectively. Apoptosis was analyzed through Annexin V/PI staining, while gene expression levels were determined using real-time PCR. EV size distribution and concentration were evaluated by dynamic light scattering. The combination treatment demonstrated superior efficacy in reducing cell viability and metabolic activity compared to either drug alone. Furthermore, the combination therapy induced apoptosis by upregulating the anti-apoptotic *BCL-2* and the pro-apoptotic *BAX* gene. Notably, the combined treatment also significantly reduced the number of EVs released by K562 cells. These findings suggest that targeting both CML cells and their secreted EVs with a dual therapeutic approach may enhance the efficacy of Imatinib therapy and potentially overcome drug resistance. Additionally, EVs could serve as valuable biomarkers for monitoring disease progression and treatment response in CML.

Keywords: ARF6 inhibition, Drug resistance, Tyrosine kinase inhibitor, Apoptosis, Tumor microenvironment

Chronic myeloid leukemia (CML) is a hematological malignancy characterized by the dysregulation of hematopoietic stem cells. This aberrant behavior is driven by a chromosomal abnormality that leads to the constitutive activation and phosphorylation of downstream signaling pathways. As a result, the affected cells exhibit altered cell adhesion, impaired apoptosis, and blocked differentiation, culminating in the development of the CML phenotype[1].

Imatinib, a groundbreaking tyrosine kinase inhibitor, was the first FDA-approved drug for the treatment of CML. By targeting the ATP-binding site of the BCR-ABL1 oncoprotein, imatinib effectively inhibits the phosphorylation of essential downstream signaling proteins, thereby disrupting the aberrant cellular proliferation and survival pathways characteristic of CML[2,3]. Consequently, imatinib effectively inhibits cellular proliferation and induces apoptosis in CML cancer cells[4]. Nevertheless, the therapeutic efficacy of imatinib is limited by adverse side effects and the development of drug resistance, particularly in patients who exhibit disease relapse despite initial treatment[4].

Extracellular vesicles (EVs) are membrane-bound vesicles released by cells into various bodily fluids, including blood and urine[5]. EVs are classified based on their size, molecular composition, and cellular origin. Apoptotic bodies represent the largest class of EVs, followed by microvesicles, and exosomes, which are the smallest[6]. Tumor-derived extracellular vesicles (TEVs), with a diameter ranging from 1 to 10 micrometers, are secreted by tumor cells both *in vivo* and *in vitro*[7]. TEVs play a multifaceted role in

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tumor progression, including the transfer of growth factor receptors, enhancement of cell motility, induction of angiogenesis, promotion of drug resistance, and facilitation of tumor invasion, migration, proliferation, and signal transduction[8]. They also modulate the tumor microenvironment, creating a conducive environment for tumor cell growth and survival[9]. Effective cancer therapy relies on maintaining therapeutic drug concentrations within cancer cells. However, TEV release can facilitate drug efflux, thereby diminishing drug retention and reducing tumor sensitivity[10]. Inhibiting EV shedding has been shown to improve drug retention and enhance tumor sensitivity to therapy[11]. Various factors, including ADP ribosylation factor 6 (ARF6), influence EV formation and release[12]. ARF6, a GTP-binding protein belonging to the ARF family, is essential for EV production and release[13]. Consequently, targeting ARF6 with specific inhibitors represents a promising therapeutic strategy to reduce EV-mediated drug resistance and enhance cancer treatment efficacy[14].

NAV2729, a specific ARF6 inhibitor, directly binds to ARF6's GEF-binding site, blocking GTP exchange and subsequent activation of ARF6-mediated pathways[15]. While NAV2729 has demonstrated efficacy in solid tumors by reducing extracellular vesicle (EV) release and enhancing chemotherapeutic retention[16], its application in hematological malignancies remains underexplored. Recent preclinical studies in acute myeloid leukemia (AML) revealed that NAV2729 disrupts sphingolipid homeostasis, inhibits proliferation, and exhibits selective toxicity against leukemic cells while sparing normal hematopoietic cells[17]. However, its role in CML, particularly in combination with TKIs like Imatinib has not been investigated. Critically, ARF6-driven EV secretion is implicated in TKI resistance by facilitating drug efflux and intercellular survival signaling in CML[18], yet no studies have targeted this axis therapeutically.

This study addresses this gap by evaluating NAV2729's novel application in CML. We hypothesize that combining NAV2729 with imatinib will synergistically enhance efficacy by inhibiting EV-mediated drug resistance, disrupting leukemic cell communication, and amplifying apoptosis. Using the K562 CML model, we investigate the combined effects on viability, metabolic activity, apoptosis, and EV dynamics. Our work provides the first evidence that dual targeting of BCR-ABL1 (via imatinib) and ARF6 (via NAV2729) represents a promising strategy to overcome EV-driven resistance and improve outcomes in CML.

Materials and Methods

Cell culture and Drug preparation

The K562 cell line (K562-CCL243), derived from a chronic myeloid leukemia patient, was procured from the Pasteur Institute collection in Tehran, Iran. K562 cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine (Gibco A1049101), 10% fetal bovine serum (Gibco A3160402), and 1% penicillin-streptomycin (Penicillin-Streptomycin Solution 100×, Biowest, L0022). The cells were maintained in a humidified incubator at 37°C with 5% CO_2[19]. A 100 μM stock solution of NAV2729 (Cayman Chemicals, USA) was prepared by dissolving 1 mg of lyophilized powder in 0.5 mL of dimethyl sulfoxide (DMSO) (Merck, CAS 67-68-5). To obtain a working concentration of 5 μM, the stock solution was diluted 1:42 in DMSO[20]. Imatinib (EBEWE Pharma, Austria) stock solutions were prepared according to the manufacturer's instructions and subsequently diluted to the desired concentrations for treatment.

Preliminary dose-response experiments were conducted to determine optimal concentrations of NAV2729 and imatinib. K562 cells were treated with NAV2729 (0-100 μM) and imatinib (0-100 μM) for 24-48 h, and viability was assessed using trypan blue exclusion and MTT assays. Concentrations above 10 μM NAV2729 or 4 μM imatinib resulted in >80% cytotoxicity within 24 h, rendering them unsuitable for prolonged combination studies. Lower doses (NAV2729: 5-10 μM; Imatinib: 2-4 μM) induced sub-maximal cytotoxicity while preserving sufficient cell viability for mechanistic evaluation. The selected doses for combination therapy (5 μM NAV2729 + 4 μM imatinib) were chosen to balance efficacy and tolerability, ensuring synergistic effects could be assessed without overwhelming cytotoxicity.

Trypan blue assay

To assess the impact of NAV2729, imatinib, and their combination on cell viability, trypan blue exclusion assay was employed. K562 cells were seeded in a 6-well plate at a density of 250×10^5 cells/mL and treated with NAV2729 (5 μM), imatinib (4 μM), or a combination of both (5 μM NAV2729 + 4 μM imatinib). After 24 and 48 h of treatment, cells were stained with 0.4% trypan blue solution (Gibco 15250061). Cell viability was determined by manually counting viable (unstained) and non-viable (stained)

cells using a Neubauer chamber under a light microscope[21].

MTT assay

To assess the cytotoxic effects of NAV2729, imatinib, and their combination on K562 cells, an MTT colourimetric assay was performed. K562 cells (7.5×10^3 cells/well) were seeded in a 96-well plate and treated with various concentrations of NAV2729 (5 μM and 10 μM), imatinib (2 μM and 4 μM), or a combination of both (5 μM NAV2729 + 4 μM imatinib) for 48 h. After centrifugation, 100 μL of MTT solution (0.5 mg/mL) was added to each well, and the plate was incubated at 37°C for 4 h. Subsequently, 150 μL of DMSO was added to solubilize the formazan crystals. The absorbance at 570 nm was measured using an ELISA reader. The metabolic activity of treated cells was calculated relative to untreated control cells[21].

Flow cytometry assay

To assess early and late apoptosis, K562 cells were treated with NAV2729 (5 μM), imatinib (4 μM), or a combination of both for 48 h. Following treatment, cells were harvested, stained with FITC Annexin-V Apoptosis Detection Kit II, and analyzed by flow cytometry using FlowJo.7.6.1 software. Cells positive for Annexin-V but negative for propidium iodide (PI) were identified as early apoptotic, while cells positive for both Annexin-V and PI were classified as late apoptotic[22].

Isolation of RNA and cDNA synthesis

Total RNA was isolated from untreated (control) and treated K562 cells (treated with 5 μM NAV2729, 4 μM imatinib, or a combination of both using YTzol Pure RNA (Yekta Tajhiz Azma, YT9066). RNA quantity and quality were assessed using a NanoDrop spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE) and agarose gel electrophoresis, respectively. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, K1622) according to the manufacturer's protocol.

Quantitative real-time PCR

To investigate changes in the mRNA expression of *BAX* and *BCL2* genes, real-time PCR was performed. Real-time PCR reactions were conducted using Real Q Plus 2× Master Mix Green (Amplicon Denmark, A325402), cDNA, gene-specific primers, and nuclease-free water. The reactions were carried out on a RotorGene® Q Real-time PCR System (Qiagen, USA) using a specific thermal cycling profile. Melting curve analysis was performed to confirm the specificity of the PCR products. Relative gene expression levels were calculated using the 2^-ΔΔCt method, with GAPDH serving as the reference gene. The nucleotide sequences of the primers used in real-time PCR are listed in Table 1.

Micro vesicles isolation

EVs were isolated from treated and untreated K562 cells through a series of differential centrifugation steps[22]. Initially, cells were centrifuged at 4000 RPM for 10 min to remove cellular debris. The supernatant was then subjected to a second centrifugation step at the same speed to further eliminate cellular remnants. Subsequently, the supernatant was ultracentrifuged at 20,000 × g for 30 min to pellet the EVs. The EV pellet was resuspended in 1 mL of phosphate-buffered saline (PBS) for subsequent analyses.

Measuring the concentration and size of separated microvesicles

Dynamic light scattering (DLS) is a well-established technique for determining the size distribution of macromolecules and nanoparticles, including extracellular vesicles[23]. In this study, a ZETA SIZER MALVERN instrument was employed to measure the size and concentration of microvesicles. This instrument utilizes a 633 nm laser to illuminate the sample, and the scattered light is detected and analyzed to generate a particle size distribution profile.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated blood using the Ficoll-Paque density gradient centrifugation method. Blood samples were diluted with PBS and centrifuged at 250×g for 35 min. The PBMC layer, located at the interface between the plasma and Ficoll-Paque layers, was carefully collected and washed twice with PBS to remove residual blood cells and platelets[24].

Table 1 — The primer sequences employed in quantitative real-time PCR

Gene	Forward primer	Reverse primer
GAPDH	5'-GAAGGTGAAGGTCGGAGT-3'	5'-GAAGATGGTGATGGGATTTC-3'
Bax	5'-AGGATCGAGCAGGGCGAATG-3'	5'-TCAGCTTCTTGGTGGACGCA-3'
Bcl-2	5'-ATCGCCCTGTGGATGACTGAG-3'	5'-CAGCCAGGAGAAATCAAACAGAG-3'

To assess the cytotoxic effects of NAV2729 on normal cells, PBMCs were seeded in a 96-well plate at a density of (30×10^3 cells/well) cells per well and treated with 5μM NAV2729 for 48 h. Following treatment, cell viability was assessed using the MTT assay, as described in the MTT assay section. This experiment aimed to evaluate the potential selective toxicity of NAV2729 by comparing its effects on normal PBMCs and K562 CML cells.

Statistical analysis

All experiments were performed in triplicate and repeated three times. Data are presented as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism version 9. The effects of treatments on cell viability, as determined by trypan blue exclusion assay, were analyzed using two-way ANOVA. Data from MTT, flow cytometry, quantitative real-time PCR, and DLS were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance was determined at a P value of less than 0.05 (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

Results

Enhanced cytotoxicity of imatinib and NAV2729 in K562 cells

Cell viability was assessed after 24 and 48 h of treatment (Fig. 1). Treatment with imatinib (4 μM) and NAV2729 (5 μM) significantly reduced K562 cell viability in a time-dependent manner. After 24 h, Imatinib reduced viability to 88% and NAV2729 to 81%, compared to the untreated control ($P = 0.7$). At 48 h, cell viability further decreased to 85% with Imatinib and 78% with NAV2729 ($P = 0.2$). The combination treatment (4 μM imatinib + 5 μM NAV2729) resulted in a more pronounced reduction in viability, with 73% at 24 h and 69% at 48 h, showing a statistically significant difference compared to single-drug treatments ($P <0.0001$). These results indicate a synergistic effect of the combination therapy in reducing K562 cell viability over time.

NAV2729 potentiates imatinib-induced cytotoxicity

As illustrated in Fig. 2A &B, imatinib and NAV2729 independently exhibited significant cytotoxic effects on K562 cells, reducing their metabolic activity in a dose-dependent manner after 48 h. Treatment with 2 μM Imatinib reduced metabolic activity to 91% compared to the control ($P \leq 0.05$), while 5 μM NAV2729 decreased it to 86% ($P =0.1$). At higher concentrations, 10 μM NAV2729 and 4 μM imatinib further reduced metabolic activity to 76% and 82%, respectively ($P \leq 0.05$). Notably, the combination treatment (4 μM imatinib + 5 μM NAV2729) led to a more pronounced reduction in metabolic activity, decreasing it to 60%, which was significantly lower than either drug alone ($P <0.01$). This synergistic effect suggests that NAV2729 enhances the cytotoxic potency of Imatinib against K562 cells. The significantly reduced metabolic activity observed in the combination-treated group highlights its potential therapeutic advantage.

NAV2729 does not affect PBMC metabolic activity

To evaluate the selectivity of NAV2729 for cancer cells, PBMCs were treated with a 5 μM concentration of the drug. The results demonstrated that NAV2729 did not exhibit significant cytotoxicity against normal human cells($P =0.4$)., suggesting its potential as a selective anticancer agent.

Gene expression alterations induced by imatinib and NAV2729

Fig. 3A demonstrates that both NAV2729 and imatinib individually significantly increased the expression of the anti-apoptotic *BCL2* gene compared

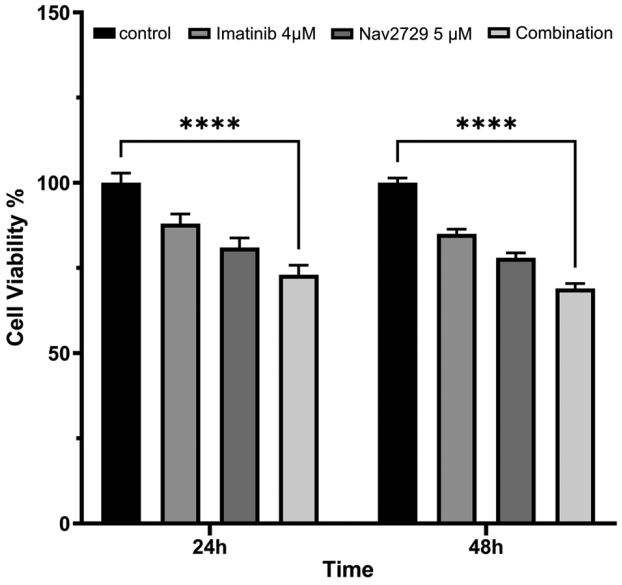

Fig. 1 — Effects of Imatinib, NAV2729 and Their Combination on K562 Cell Viability. K562 cells were treated with imatinib (4 μM), NAV2729 (5 μM), or their combination for 24 and 48 h. Cell viability was assessed using the trypan blue exclusion assay, and data are presented as mean ± SD from three independent experiments performed in triplicate. Single-agent treatments reduced cell viability in a time-dependent manner compared to untreated controls. Moreover, the combination treatment resulted in a further significant decrease in cell viability relative to the individual treatments [**** $P < 0.0001$, two-way ANOVA followed by Tukey's post hoc test]

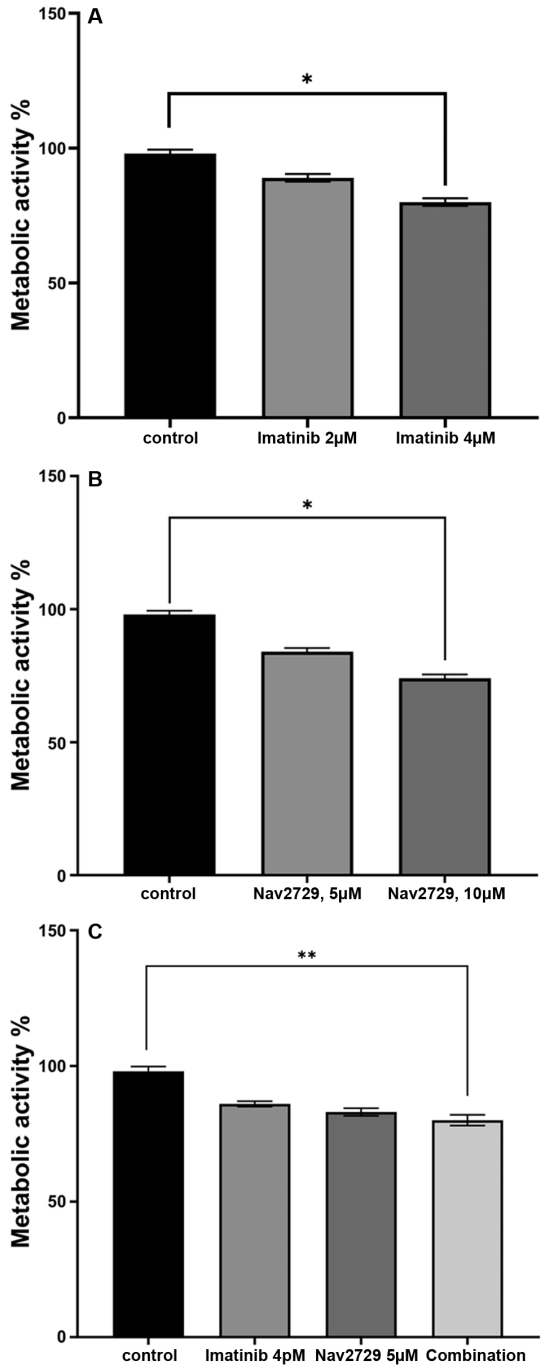

Fig. 2 — Dose-Dependent Effects of Imatinib and NAV2729 on K562 Cellular Metabolic Activity at 48 h. (A & B) display the impact of increasing doses of imatinib and NAV2729, respectively, on the metabolic activity of K562 cells measured 48 h post-treatment. Data are presented as mean ± SD from three independent experiments performed in triplicate. Both agents elicited a dose-dependent reduction in metabolic activity compared to untreated controls (*$P < 0.05$, $P < 0.01$, one-way ANOVA). (C) demonstrates that the combination of imatinib (4 μM) and NAV2729 (5 μM) further reduced metabolic activity relative to the single-agent treatments, and this additional decrease reached statistical significance ($P < 0.01$, one-way ANOVA).

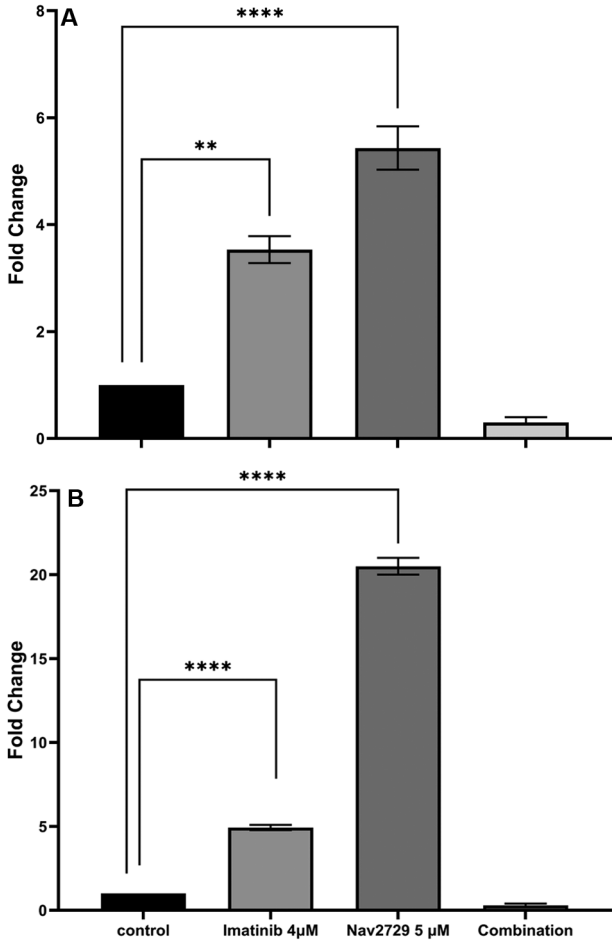

Fig. 3 — Impact of NAV2729, Imatinib, and Their Combination on *BCL-2* and *BAX* Gene Expression in K562 Cells. Gene expression levels were determined by quantitative real-time PCR and are presented as fold change relative to untreated controls (mean ± SD) from three independent experiments performed in triplicate. (A) BCL-2 (anti-apoptotic) expression was significantly upregulated in K562 cells following 48 h treatment with either NAV2729, imatinib, or their combination compared to untreated cells (****$P < 0.0001$, one-way ANOVA). (B) BAX (pro-apoptotic) expression was significantly increased after 48 h of treatment with each agent individually and in combination, with statistical significance indicated as ** $P < 0.01$ and **** $P < 0.0001$ (one-way ANOVA).

to untreated cells. Treatment with 4 μM imatinib led to a 4-fold increase (P <0.0001), while 5 μM NAV2729 resulted in a 20-fold increase (P <0.0001). The combination treatment (4 μM imatinib + 5 μM NAV2729) downregulated *BCL 2* expression, showing a statistically significant increase compared to single-agent treatments (P <0.0001). Conversely, Fig. 3B shows that both NAV2729 and imatinib individually significantly upregulated the expression of the pro-apoptotic *BAX* gene. Treatment with 4 μM imatinib

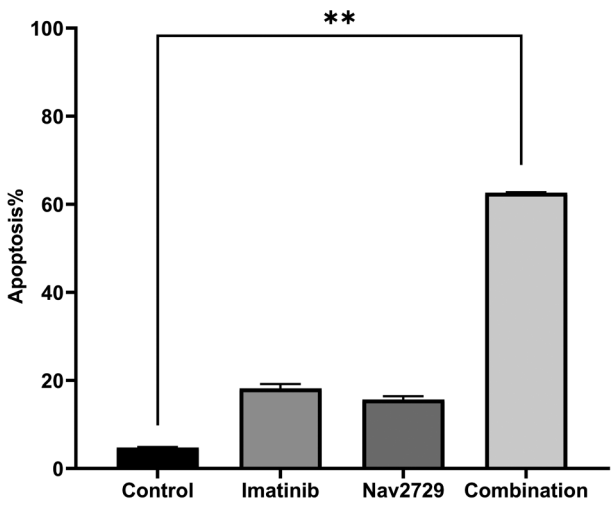

Fig. 4 — Enhanced apoptotic effects of combined imatinib and NAV2729 treatment on K562 Cells. K562 cells were treated with imatinib (4 μM), NAV2729 (5 μM), or their combination for 48 h. Following treatment, cells were stained with Annexin V and analyzed by flow cytometry to assess apoptosis. Data are expressed as mean ± SD from three independent experiments performed in triplicate. The combination treatment significantly increased the percentage of Annexin V positive cells compared to both individual treatments and the untreated control (**$P < 0.01$, one-way ANOVA).

increased *BAX* expression by 5-fold (P <0.0001), while 5 μM NAV2729 induced a 22-fold increase (P <0.0001). However, the combination treatment resulted in a decrease in *BAX* expression compared to single-agent treatments (P <0.0001), suggesting a potential compensatory mechanism in response to enhanced apoptosis.

Increased apoptosis in K562 cells with imatinib and NAV2729

The results of the Annexin V staining assay revealed that the combined treatment with NAV2729 (5 μM) and imatinib (4 μM) significantly increased the proportion of apoptotic cells after 48 h compared to treatment with either drug alone. The combination treatment induced a 62% cell death rate, which was significantly higher than the 15% observed with NAV2729 alone (P =0.3), and the 18% observed with Imatinib alone (P =0.4). The difference between the combination treatment and the single-agent treatments was statistically significant (P <0.001), highlighting the enhanced apoptotic effect of the dual therapy on K562 cells (Fig. 4).

Reduction of extracellular vesicles by combination treatment

The results demonstrated a significant reduction in the percentage of EVs larger than 1000 nm in cells treated with NAV2729, both individually and in

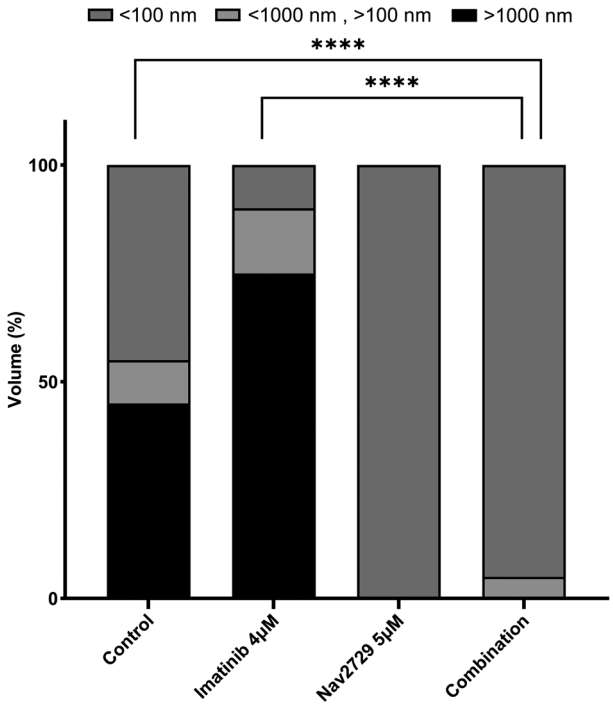

Fig. 5 — Impact of imatinib, NAV2729, and their combination on the size distribution of K562-derived microvesicles. K562 cells were treated with imatinib (4 μM), NAV2729 (5 μM), or their combination for 48 h. Microvesicle size distribution was analyzed and is expressed as the percentage of extracellular vesicles with diameters greater than 1000 nm (mean ± SD) from three independent experiments performed in triplicate. Treatment with the imatinib/NAV2729 combination resulted in a significant reduction in the percentage of vesicles >1000 nm compared to both the imatinib-only group and the untreated control (*** $P < 0.001$, one-way ANOVA).

combination with imatinib, compared to untreated control cells and cells treated with imatinib alone. Specifically, treatment with NAV2729 (5 μM) alone reduced the proportion of large EVs to 48% compared to control cells (P <0.0001). The combination treatment (NAV2729 + imatinib) further decreased the percentage of EVs over 1000 nm to 50%, which was significantly lower than both the control and imatinib-treated groups (P <0.0001). These findings suggest that NAV2729 effectively reduces the release of large EVs, an effect that is further enhanced in combination with Imatinib (Fig. 5).

Discussion

The emergence of tyrosine kinase inhibitors (TKIs) has significantly improved the treatment of CML. By targeting the ATP-binding site of the BCR-ABL1 oncoprotein, TKIs effectively disrupt the aberrant signaling pathways that drive CML cell proliferation and survival[25]. Imatinib is the first TKI approved by

the FDA for the management of CML patients[26]. However, imatinib therapy is often associated with significant adverse effects, including bone and muscle pain, which can adversely impact patients' quality of life, although these side effects are typically mild to moderate in severity[27,28]. Therefore, it is essential to investigate therapies that are more efficacious and also have better long-term survival[29].

Microvesicles (MVs) are critical mediators of intercellular communication, transferring oncogenic cargo (e.g., proteins, nucleic acids) that promote tumor progression, drug resistance, and immune evasion. The inhibition of EV biogenesis or secretion has emerged as a promising strategy to disrupt these pathological processes[30]. ARF6, a small GTPase regulating plasma membrane budding and large oncosome formation, is a key target in this context[14]. NAV2729, an ARF6 inhibitor, blocks EV release by interfering with cytoskeletal remodeling and vesicle scission, thereby attenuating tumor-stroma crosstalk and metastatic signaling[31]. This aligns with studies showing that ARF6 suppression reduces EV-associated integrins and matrix metalloproteinases, impairing invasion and angiogenesis in prostate and breast cancers[32]. In glioblastoma, GW4869 (an exosome secretion inhibitor) sensitizes tumors to temozolomide by blocking EV-mediated transfer of MGMT, a DNA repair enzyme linked to chemoresistance[33]. In ovarian cancer, targeting Rab27a (a regulator of exosome release) reduces EV-driven immunosuppression and enhances T-cell cytotoxicity[34]. In melanoma, combined EV inhibition (via Calpeptin) and BRAF/MEK inhibitors suppress metastatic niche formation by limiting EV-associated PD-L1 and Wnt5a[35]. These findings underscore the versatility of EV-targeted strategies in disrupting oncogenic signaling and overcoming microenvironment-mediated resistance. These findings underscore the versatility of EV-targeted strategies in disrupting oncogenic signaling and overcoming microenvironment-mediated resistance.

In CML, while TKIs like Imatinib target BCR-ABL1 activity, residual EV-mediated survival signals may sustain minimal residual disease (MRD)[36]. EVs from TKI-resistant CML cells transfer BCR-ABL1 transcripts and anti-apoptotic miRNAs (e.g., miR-21, miR-30a) to drug-sensitive cells, propagating resistance[37]. NAV2729, by blocking ARF6-dependent EV release, may counteract this phenomenon. A study demonstrated that Imatinib-resistant CML cells secrete EVs enriched with survivin, which activate PI3K/Akt signaling in recipient cells; combining Imatinib with EV inhibitors reversed this pro-survival effect[38]. Similarly, our data showing reduced EV secretion and enhanced apoptosis with NAV2729/imatinib align with the hypothesis that EV inhibition disrupts compensatory survival pathways.

Extracellular vesicles derived from tumors have significant impacts on disease development and progression[39]. Studies demonstrated that prostate cancer cells secrete large oncosomes, which are bioactive extracellular vesicles enriched with oncogenic and cancer-specific molecules[40,41]. Signaling pathways implicated in the biogenesis of MVs are also involved in the formation of large oncosomes. For example, ARF6, a key regulator of MV formation from the plasma membrane, is also highly expressed in large oncosomes[42]. This study investigated the combined effects of Imatinib and NAV2729, an ARF6 inhibitor. Previous research by Kosgodage *et al*. demonstrated that combining the chemotherapy drug 5-fluorouracil with extracellular vesicle inhibitors, such as chloramidine and bisindolylmaleimide, significantly reduced the viability of PC3 cells by approximately 62% and 59%, respectively[43]. Another study demonstrated the synergistic effect of combining calpeptin, a microvesicle secretion inhibitor, with the chemotherapy drug docetaxel. This combination therapy resulted in a significant increase in intracellular docetaxel concentrations within tumor cells, potentially enhancing its therapeutic efficacy[44]. Additional studies by Pinar Uysal-Onganer *et al*. have demonstrated that inhibiting EV secretion significantly reduces the invasive potential of LN18 cells[45]. In line with these findings, our study also revealed a significant decrease in cell viability, metabolic activity, and an increase in apoptotic cell population in cells treated with the combination of Imatinib and NAV2729 compared to the control group. These results further corroborate the findings of previous studies. While neither NAV2729 nor imatinib alone significantly affected cell death or Annexin V positivity, their combination synergistically increased cell death and Annexin V-positive cell populations. Notably, while extracellular vesicles larger than 1000 nm were detected in both untreated and imatinib-treated cells, they were absent or minimally present in cells treated with NAV2729 alone or in combination with imatinib, suggesting the inhibitory effect of NAV2729 on EV secretion.

The observed modulation of Bcl-2 and Bax in our study reflects the complex interplay between EV inhibition and apoptotic pathways. EVs often carry

anti-apoptotic proteins (e.g., Bcl-2, survivin) that neutralize pro-death signals in recipient cells[46]. Our findings revealed a significant upregulation of both Bcl-2 and Bax mRNA expression in treated cells compared to control cells. While Imatinib and NAV2729 individually induced Bcl-2 expression, the combination treatment led to a significant downregulation of Bcl-2. Conversely, both drugs individually upregulated Bax expression, but the combination treatment resulted in a significant downregulation of Bax. These findings align with previous research by Keeshan *et al*., who demonstrated that combining Imatinib with another cytotoxic drug increased Bax expression and decreased Bcl-2 expression, ultimately inducing apoptosis through the activation of caspase-3 and PRAP pathways and subsequent upregulation of pro-apoptotic genes[47]. Kawano *et al*. investigated the combined effects of a histone deacetylase inhibitor and Imatinib on apoptosis induction. While imatinib alone reduced the expression of both *BAX* and *BCL-2*, the histone deacetylase inhibitor alone had no significant effect on gene expression. However, the combination of both drugs led to a further reduction in the expression of both *BAX* and *BCL-2*, suggesting a synergistic effect on apoptosis induction[48]. Therefore, it is likely that other members of the Bcl-2 family may contribute to apoptosis induction when using combination therapy[49]. Our findings suggest a similar mechanism, whereby the combination treatment may modulate the expression of other Bcl-2 family members. Alternatively, the combination therapy may activate the extrinsic apoptotic pathway rather than the intrinsic pathway.

Conclusion

This study demonstrates that combining the ARF6 inhibitor NAV2729 (5 μM) with imatinib (4 μM) synergistically enhances apoptosis in K562 chronic myeloid leukemia cells, significantly surpassing the effects of monotherapy. NAV2729×s inhibition of ARF6 reduced the release of pro-tumorigenic extracellular vesicles (>1000 nm), likely enhancing Imatinib retention and disrupting intercellular survival signaling. Notably, NAV2729 exhibited selective cytotoxicity against cancer cells while sparing normal PBMCs, underscoring its potential as a safe adjuvant. Paradoxically, the combination downregulated both pro-apoptotic *Bax* and anti-apoptotic *Bcl-2*, suggesting a shift toward extrinsic apoptotic pathways or compensatory regulation by other Bcl-2 family members. These findings highlight the therapeutic advantage of dual targeting blocking EV-mediated resistance while amplifying imatinib's efficacy at sub-maximal doses, which could mitigate adverse effects in patients. Future studies should validate these mechanisms *in vivo* and explore NAV2729's broader impact on EV subtypes and BCR-ABL1 independent resistance pathways.

Limitations of study

One significant limitation of our study is the restricted access to a freshly sourced supply of NAV2729. Due to international sanctions imposed on Iran, along with the low currency value and high inflation rates, repurchasing NAV2729 from a US-based company has proven challenging. This constraint has limited our ability to obtain new batches of the drug, which may affect the reproducibility and consistency of our experimental results. Additionally, the high cost of FBS and cell culture media has prevented us from extending our experiments to multiple cell lines. As a result, the scope and generalizability of our findings are somewhat limited. We acknowledge these economic and political challenges as significant barriers and emphasize the need for alternative funding strategies and local production capabilities in future studies.

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Conflict of interest

The authors declare no conflict of interests.

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