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Biological evaluation of combinations of tyrosine kinase inhibitors with Inecalcitol as novel treatments for human chronic myeloid leukemia

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ABSTRACT

Background: The use of tyrosine kinase inhibitors (TKIs) as a treatment for chronic myeloid leukemia (CML) has improved the natural history of the disease and increased the duration of survival. Tyrosine kinase inhibitors represent the success of target therapies that work on molecular targets, although some patients still have therapy failure. Vitamin D has antiproliferative, pro-apoptotic, and anti-angiogenic effects on cells, therefore it can be considered as a potential cancer preventative and treatment agent. Inecalcitol (TX-522) is the 14-epi-analogue of Calcitriol (1,25(OH)$_2$-vitamin D3), and inhibits cancer cell proliferation more effectively than Calcitriol. This study was conducted to evaluate the antiproliferative and synergistic effects of the anticancer drugs Imatinib and Dasatinib in combinations with Inecalcitol on human chronic myeloid leukemia K-562 cells.

Method: The growth inhibitory activities of Inecalcitol, Imatinib, Dasatinib, and different combinations of one of the two drugs (Imatinib and Dasatinib) with Inecalcitol, were determined *in vitro* using MTT assay against K-562 cell line.

Results: Inecalcitol, Imatinib, and Dasatinib showed potent antiproliferative activities against K-562 cells with GI_{50} values of 5.6 μM, 0.327 μM, and 0.446 nM, respectively. Combinations of Imatinib or Dasatinib with different concentrations of Inecalcitol increased significantly the antiproliferative activities and potencies of both drugs (****$p < 0.0001$), with optimal GI_{50} values of 580 pM (Imatinib) and 0.51 pM (Dasatinib). Furthermore, the combination treatments showed synergistic interaction between the antileukemic drugs and Inecalcitol, with combination indices (CI) < 1.

Conclusion: The study demonstrated that the human chronic myeloid leukemia K-562 cells were subjected to a synergistic growth inhibitory impact when antileukemic drugs (Imatinib or Dasatinib) were combined with Inecalcitol, therefore, it is recommended that these combinations be viewed as promising novel antileukemic medications and used in place of individual medications with lower dosages and negligible side effects in the treatment of CML.

Abbreviations: AML, Acute myeloid leukemia; ANOVA, Analysis of variance; ATP, Adenosine triphosphate; CML, Chronic myeloid leukemia; DMSO, Dimethyl sulfoxide; FDA, Food and drug administration; GI_{50}, 50% Growth inhibition; GSK3, Glycogen synthase kinase 3; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, Optical density; PSA, Prostate-specific antigen; TKIs, Tyrosine kinase inhibitors; VDR, Vitamin D receptor.

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1. Introduction

Chronic myeloid leukemia (CML) is a clonal malignant myeloproliferative disorder caused by a chromosomal translocation between chromosomes 9 and 22 at a very early stem cell level, with the development of Philadelphia chromosome. This translocation of genetic material results in the apposition of *BCR* gene (chromosome 22) and *ABL1* gene (chromosome 9) which results in the development of *BCR-ABL1* fusion oncogene which encodes a fusion protein that contains constitutively active tyrosine kinases (Muselli et al., 2019). The CML represents ~ 20% of all types of leukemia in adults, and the incidence of this disease is 1.6 per 100,000 adults (Cortes, 2004).

The use of tyrosine kinase inhibitors (TKIs) as a treatment for CML has improved the natural history of the disease and increased the duration of survival (Turhan et al., 2015). They represent the success of target therapies that work on molecular targets, although some patients still have therapy failure (Branford et al., 2018). TKIs are the first-line treatment for CML, with the exception of cases of CML diagnosed during pregnancy. Imatinib, Nilotinib, Dasatinib, and Bosutinib are the four TKIs currently approved for CML therapy and almost broadly available, but there are some variations between them in the dosing, indications, and cost (Hochhaus et al., 2020). Imatinib is a well-known safe and efficient first-generation TKI, while Dasatinib, Nilotinib and Bosutinib, which are considered as second-generation TKIs, have lower safety than Imatinib, however they provide a quicker molecular response (Haguet et al., 2020).

Imatinib drug is classified as a tyrosine kinase inhibitor and it enhanced patients' therapeutic prospects, in which 95% of patients have achieved complete hematological remission (CHR) (Muselli et al., 2019). Imatinib was the first TKI to be licensed by the FDA for the treatment of chronic myeloid leukemia-chronic phase (CML-CP) patients (Jabbour and Kantarjian, 2016), and it was originally used as a first-line treatment for CML about ten years ago (Wei et al., 2010). Imatinib (Fig. 1A) binds to *ABL* protein tyrosine kinase-inactive conformation, then blocks the adenosine triphosphate (ATP) binding site and stops the convert of the inactive conformation to its active form (An et al., 2010). Philadelphia-positive (*Ph+*) leukemic cells die apoptotically when *BCR-ABL* catalytic activity is suppressed. Regardless the reduction of *BCR-ABL* activity, Imatinib has only a minor apoptotic effect on CML stem cells (Stagno et al., 2016). The effectiveness of Imatinib drug persisted over time with almost 11 years of following up and its long term use showed no unacceptable or late toxic effects (Hochhaus et al., 2017). The most prevalent mutation in *BCR-ABL* that results in resistance to Imatinib or second-generation TKIs is *T315I*, which also has a poor clinical prognosis (Gao et al., 2023).

Dasatinib (Fig. 1B) is 350 times more effective *in vitro* than Imatinib (Jabbour and Kantarjian, 2018). It inhibits the *BCR-ABL* active and inactive conformations, with the exception of those who have the *T315I* mutation. Dasatinib causes inhibition of other tyrosine kinases such as Fyn, Yes, Src, Lck, and EPH receptors A2 (EPHA2), and therefore causes autophosphorylation and downstream phosphorylation inhibition of other targets that may be considered in mutations in these kinases activation of *BCR-ABL* independent pathways in patients who have Imatinib-intolerant or resistance to CML (An et al., 2010). Based on the Sokal or Hasford risk stratification ratings, Dasatinib has increased efficacy and may be favored in patients with intermediate and high risk (Granatowicz et al., 2015). Dasatinib's CP dosage is 100 mg once a day, and for the advanced phase of CML a 70 mg dosage is used two times a day (Hochhaus et al., 2020). Since Dasatinib has a toxicity associated with pleuropulmonary, respiratory failure and prior or concurrent pleuropulmonary or pericardial disorders are significant contraindications for using it as first-line therapy (Hochhaus et al., 2020).

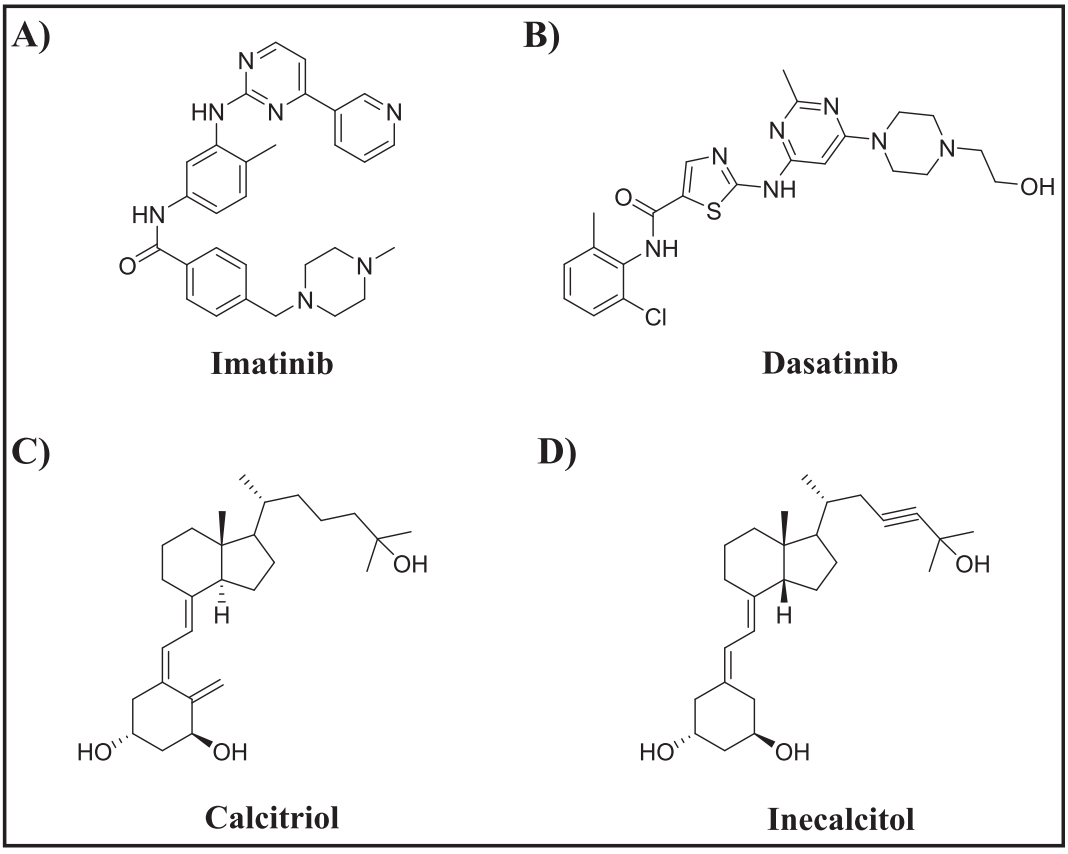

Fig. 1. Structures of some Tyrosine kinase inhibitors; **A)** Imatinib and **B)** Dasatinib. Structures of some vitamin D3 analogues investigated for potential anticancer activity; **C)** Calcitriol and **D)** Inecalcitol.

A mutation within gene encoding of target *BCR-ABL* tyrosine kinase enzyme can generate resistance against the TKI, this resistance is represented by decreasing the inhibitor action of the TKI, without effecting on the enzyme function. Nowadays, more than 100 various mutations of *BCR-ABL1* kinase domain are established to be resistance to Imatinib, although it has less effect than the second generation TKIs, mainly this effect concentrates on the phosphate binding loop (P-loop), activation loop, ATP binding site. This challenge has been overcome by using the second generation of TKIs. Nowadays, for each mutation that is related with TKI resistance, they found a therapeutic TKI for it (Eide and O'Hare, 2015).

Vitamin D is prohormone or precursor of a steroid hormone 1,25-dihydroxyvitamin D3 (Calcitriol; 1,25$(OH)_2$D3), that's also known as Cholecalciferol (Fig. 1C). It is biologically responsible for increasing the absorption of calcium, magnesium, and phosphate which are essential for bone mineralization (Campiotti et al., 2018). Vitamin D has recently been studied for its potential role in the prevention and treatment of a number of diseases, including cancer, autoimmune conditions, and cardiovascular disease. Preclinical evidence is overwhelming in favor of vitamin D's significance in the prevention of cancer due to its effects on cell proliferation, apoptosis, and angiogenesis (Ness et al., 2015). Vitamin D supplementation in the abnormal hematological cells encourages death, causes differentiation, suppresses proliferation, makes tumor cells more susceptible to other anticancer treatments, and lowers the production of pro-inflammatory cytokines (Kulling et al., 2017).

The clinical study made by Seyedalipour *et al.* (Seyedalipour et al., 2017) reported that individuals with acute myeloid leukemia (AML) often have vitamin D insufficiency. Moreover, improved outcomes for AML patients were linked to increased vitamin D levels in these individuals. This prompted scientists to do more study to see if vitamin D may be used with other anticancer medications, such as TKIs, to treat CML. Many studies showed that the combination of vitamin D analogue with an anticancer drug produces a powerful effect *in vitro* (Kulling et al., 2017). Radujkovic *et al.* (Radujkovic et al., 2016) tested the combination of Calcitriol with azacitidine in the acute myeloid leukemia HL-60 and MOLM13 cell lines, the combination caused significantly antiproliferation effect more than either drug alone. Gupta *et al.* (Gupta et al., 2016) reported that several AML cell lines, such as HL-60, OCI-AML3, and Mono-mac3, were able to differentiate when treated with a combination of glycogen synthase kinase 3 (GSK3) inhibitors and a low quantity of Calcitriol. In the HL-60 cell line, this stimulation of differentiation was more potent than Calcitriol alone or GSK3 inhibitors paired with a separate FDA-approved GSK3 inhibitor.

Inecalcitol (known as TX-522 or 19-nor-14-epi23-yne-1,25-$(OH)_2$-vitamin D3) is the 14-epi-analogue of Calcitriol (Fig. 1D) (Verlinden et al., 2000). In a variety of model systems, including those for breast cancer, prostate cancer, and squamous cell cancer, Inecalcitol was shown to be at least ten times more effective than Calcitriol in preventing the development of tumor cells and inducing apoptosis (Verlinden et al., 2000; Okamoto et al., 2012; Ma et al., 2013). The fact that the analogue stimulates higher interaction between vitamin D receptor (VDR) and several of its coactivators, such as SRC-1, TIF2, and DROP205, than Calcitriol, may be the cause of Inecalcitol's enhanced growth inhibitory effect (Eelen et al., 2005). Significantly, Inecalcitol had little to no impact on blood calcium levels at the dosages demonstrated to cause tumor regression in the animal models tested (Okamoto et al., 2012; Ma et al., 2013; Murray et al., 2017).

We describe here the anticancer activity of Inecalcitol against human chronic myeloid leukemia K-562 cell line in comparison to that of the individual TKIs Imatinib and Dasatinib, and the potent antiproliferative and synergistic effects of Imatinib and Dasatinib in separate combinations with Inecalcitol on the same type of leukemia cells.

2. Materials and methods

2.1. Compounds

Imatinib was purchased from TOKYO CHMICAL INDUSTRY CO. (I0906, Tokyo, Japan), while Dasatinib was purchased from SIGMA (SML2589-50MG, United Kingdom), and Inecalcitol was purchased from UFC Biotechnology (I163217-980, Amherst, NY14228, USA). The three compounds were provided as solids and reconstituted with DMSO to yield concentrations of 10 mM.

2.2. Cell Culture

The American Type Culture Collection (ATCC) in Manassas, Virginia, USA, provided the K-562 human chronic myeloid leukemia cell line, which was then frozen in liquid nitrogen. After achieving 70–80% confluency, cells were passaged twice weekly, and modest transit numbers ($\leq$ 20) were used in all tests. To make sure cultures were free of contamination, Lonza MycoAlert™ mycoplasma testing kits were employed in accordance with the manufacturer's recommendations. In addition to 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Biowest, South America Origin), 1% penicillin–streptomycin solution 100X (EuroClone, Italy), and 1% L-glutamine 100X (EuroClone, Italy), cells were sub-cultured in RPMI-1640 medium 1X (EuroClone, Italy). In an incubator with 5% CO_2 that was humidified, cells were kept at 37 °C (NU-5810E, NUAIRE, Plymouth, MN 55447, USA).

2.3. MTT assay (In vitro cell viability)

The method has been described by Saleh *et al.* (Saleh et al., 2017, 2022, 2023; Al-Sammarra'e et al., 2022) and was adapted from Mosmann (1983). The growth inhibitory activities of the experimental compounds were evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay on the human chronic myeloid leukemia K-562 cell line. Cells were seeded into 96-well plates at the density of 17 $\times$ 10^3 per well (180 µL per well). Top stock solutions of Imatinib, Dasatinib and Inecalcitol (10 mM in DMSO) were then freshly prepared. Serial dilutions were made by using RPMI-1640 medium, then added (20 µL per well) to K-562 cells in the test plates. Control wells received vehicle alone (20 µL per well). Final concentrations for Imatinib in the wells were; 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 µM, for Dasatinib were; 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 nM, and for Inecalcitol were; 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 µM. Final DMSO concentration in the wells never exceeded 1%. Vehicle control assays were performed (0.0001–1% DMSO). For combinations with Inecalcitol; Inecalcitol drug (80 µL) was added to the seeded wells before the addition of Imatinib and Dasatinib. Then, serial fresh dilutions of the individual drugs (Imatinib and Dasatinib) were combined immediately and separately with Inecalcitol and evaluated in different trials, using the same final concentrations for Imatinib and Dasatinib in the wells. The final concentrations of Inecalcitol were: 2.7 µM, 5.4 µM, 8.1 µM and 10.8 µM, for 0.5×, 1×, 1.5× and 2×GI_{50} combinations, respectively. Experimental plates were incubated for further 72 h at 37 ºC/5% CO_2. Cell viability was measured at the time when the agent was added (T_0). After 72 h of exposure, 15 µL of MTT solution (Promega, G4102, USA) were added, and the experimental plates were incubated for additional 3 h to allow reduction of MTT by viable cells to insoluble dark purple formazan crystals. Half amount of the supernatant (100 µL) in each well was then aspirated, and the cellular formazan was solubilized by addition of Solubilization solution/Stop mix (Promega, G4101, USA) (100 µL per well). Absorbance (OD) was measured at a wavelength of 550 nm using MULTISKAN GO spectrophotometer plate reader (Thermo Fisher Scientific Oy, FI-01620 Vantaa, Finland). The measured intensity is inversely related to metabolic activity, which is related to the quantity of live cells. Using the Microsoft Excel 2013 software, estimated GI_{50} values – test agent doses that inhibit cell growth by 50% – were

computed. The findings are presented as the average of three separate trials (n = 8 for each trial).

Synergy of the combined treatments was evaluated as described by Sunoqrot *et al.* (Sunoqrot et al., 2023). It was carried out utilizing the MTT assay against the human leukemia K-562 cell line. Based on Chou and Talalay's median-effect study (Chou, 2006), the combination index (CI) was calculated using CompuSyn software (version 1.0) to measure the degree of synergism (CI = 1, CI > 1 and CI < 1 denote additive, antagonistic, and synergistic effects, respectively).

2.4. Statistical analysis

The Figures are exemplary experiments that were carried out at least for a total of three times. To assess statistical significance, one-way ANOVA analysis was utilized (Kim, 2017). Dunnett's multiple comparison test was used to evaluate the levels of significance (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ and ****$p < 0.00005$ compared to control (drug alone)) (McHugh, 2011; Jaki and Hothorn, 2013).

3. Results

3.1. Growth inhibitory effect of Imatinib, Dasatinib and Inecalcitol on human chronic myeloid leukemia K-562 cell line

The growth inhibitory effect of Imatinib, Dasatinib and Inecalcitol were estimated *in vitro* against human chronic myeloid leukemia cell line K-562 by using the MTT assay (Saleh et al., 2017, 2022, 2023; Mosmann, 1983; Denizot and Lang, 1986). After 72 h of exposure of cells to Imatinib, Dasatinib and Inecalcitol, concentrations at which 50% of cell growth is inhibited (GI_{50}) were determined from dose-response curves, and are shown in Table 1.

All the tested compounds showed a potent cellular growth inhibitory activity against human chronic myeloid leukemia K-562 cell line in the concentration ranges (0.01–100 μM), (0.001–10 μM) and (0.001–10 nM) used for Inecalcitol, Imatinib and Dasatinib, respectively. The highest potency against the human leukemia cells is shown by Dasatinib scoring the smallest picomolar GI_{50} value of 446 pM, followed by Imatinib (GI_{50} = 327 nM), while Inecalcitol exhibits the least potent growth inhibitory activity with GI_{50} value of 5.6 μM. The dose–response curves are shown in Fig. 2.

3.2. Combined effect of Imatinib with Inecalcitol on human chronic myeloid leukemia K-562 cells

The growth inhibitory activity of Imatinib combined with various concentrations of Inecalcitol against human chronic myeloid leukemia K-562 cell line was assessed. Table 2 presents the estimated GI_{50} values of Imatinib in combinations with 0.5×, 1×, 1.5× and 2×GI_{50} of Inecalcitol against K-562 cells after 72 h treatment. Fig. 3 shows the dose–response curves from which GI_{50} values were calculated.

The results reveal that when Imatinib is combined with different concentrations of Inecalcitol, its potent antiproliferative activity against K-562 cell line becomes significantly greater than that of Imatinib alone. Imatinib in combinations with Inecalcitol (0.5×, 1×, 1.5× and 2×GI_{50}), exhibits more significant growth inhibitory activity than Imatinib alone (GI_{50} = 327 nM) with 1.4-fold (GI_{50} = 229 nM) 11.7-fold (GI_{50} = 29 nM), 409-fold (GI_{50} = 0.8 nM) and 564-fold (GI_{50} = 0.58 nM), respectively, reveling that as the concentration of Inecalcitol increases in the

Table 1
Growth inhibitory activities of Imatinib, Dasatinib and Inecalcitol against human chronic myeloid leukemia K-562 cell line.

Ligand	GI_{50} (Mean ± S.D.)*
	K-562 Cell Line
Inecalcitol	5.6 ± 0.3 μM
Imatinib	327 ± 56 nM
Dasatinib	446 ± 97 pM

* Mean ± standard deviation of at least three independent experiments (n = 8 per trial).

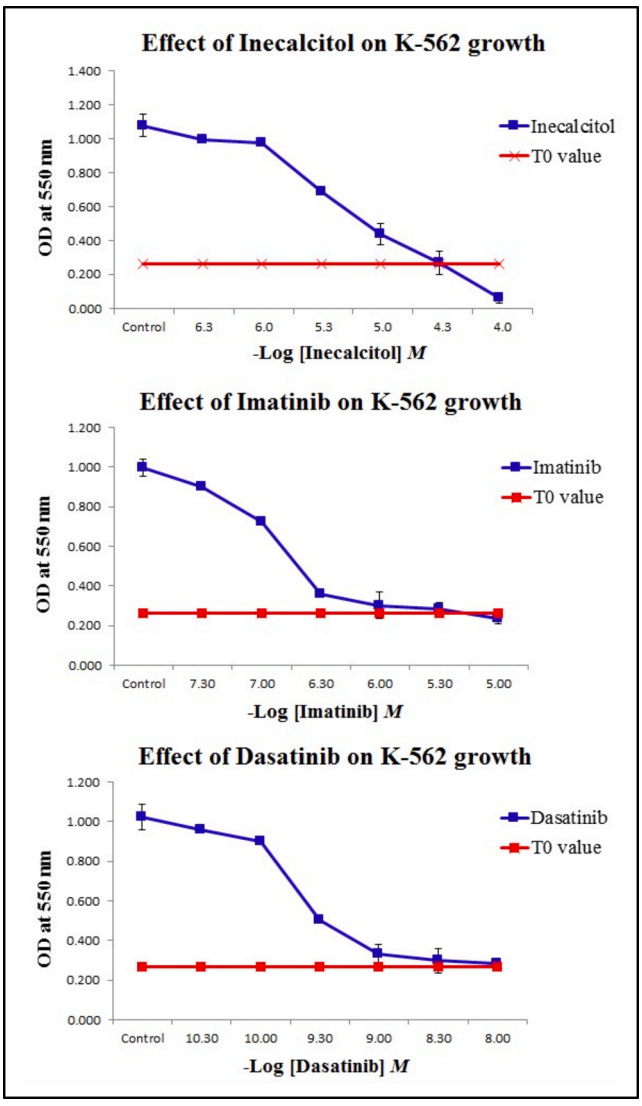

Fig. 2. Dose-response curves that show the growth inhibitory effects of Inecalcitol, Imatinib and Dasatinib against leukemia K-562 cell line. Values are mean ± SD, n = 8, graphs representative of experiments performed on at least three separate trials.

Table 2
Growth inhibitory activities of combinations of Imatinib with 0.5×, 1×, 1.5× and 2×GI_{50} of Inecalcitol against human chronic myeloid leukemia K-562 cell line. GI_{50} values are represented as mean ± standard deviation of ≥ 2 independent experiments (n = 8 per trial).

Ligand	GI_{50} (nM) (Mean ± S.D.)
	K-562 Cell Line
Imatinib	327 ± 56
Imatinib + Inecalcitol (0.5×GI_{50})	229 ± 19
Imatinib + Inecalcitol (1×GI_{50})	29 ± 4
Imatinib + Inecalcitol (1.5×GI_{50})	0.8 ± 0.1
Imatinib + Inecalcitol (2×GI_{50})	0.58 ± 0.05

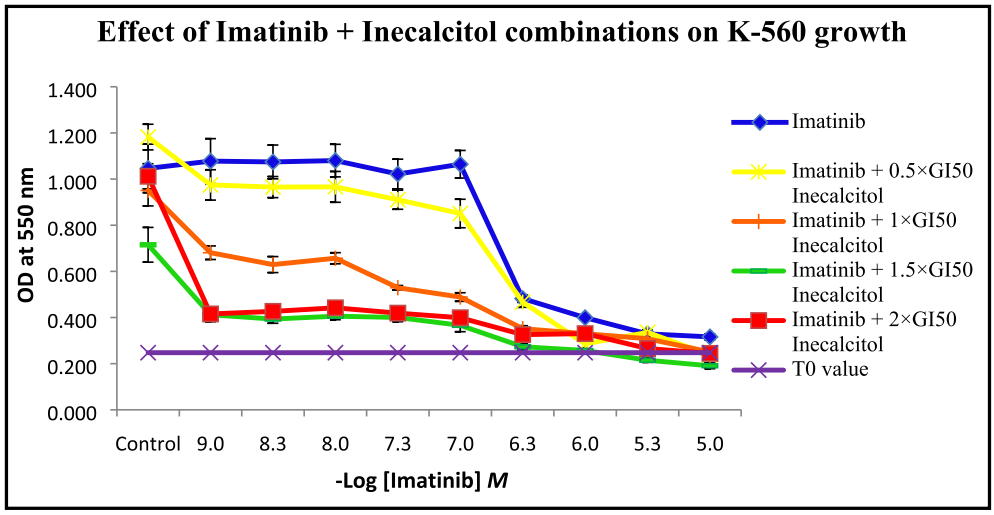

Fig. 3. Dose-response curves that show the difference in cytotoxic activities between Imatinib alone and Imatinib combined with Inecalcitol (0.5×, 1×, 1.5× and 2×GI_{50}) against human chronic myeloid leukemia K-562 cell line. Values are mean ± SD, n = 8, graphs representative of experiments performed on at least two separate occasions.

combinations, the potency of Imatinib increases significantly against K-562 cell line.

One-way ANOVA showed a significant difference among all groups treated with the combinations of Imatinib and Inecalcitol, and those with Imatinib alone (****$p < 0.0001$). Dunnett's multiple comparison test revealed that all the combinations of Imatinib with 0.5×, 1×, 1.5 and 2×GI_{50} of Inecalcitol showed a significant statistical decrease in GI_{50} values in comparison to the cells treated with Imatinib alone (**$p < 0.001$, ****$p < 0.0001$, ****$p < 0.0001$ and ****$p < 0.0001$), respectively (Fig. 4).

The synergy study was determined using CompuSyn software. Synergistic interactions were observed between Imatinib and Inecalcitol (0.5×, 1×, 1.5×, and 2×GI_{50}) combinations in the serial concentrations of 0.001–1 μM with combination indices (CI) < 1. However, with Imatinibs' concentrations of 5 μM and 10 μM in all combinations with Inecalcitol, antagonistic activity was observed (CI > 1), as shown in Table 3. CompuSyn Reports for the combination treatments of Imatinib

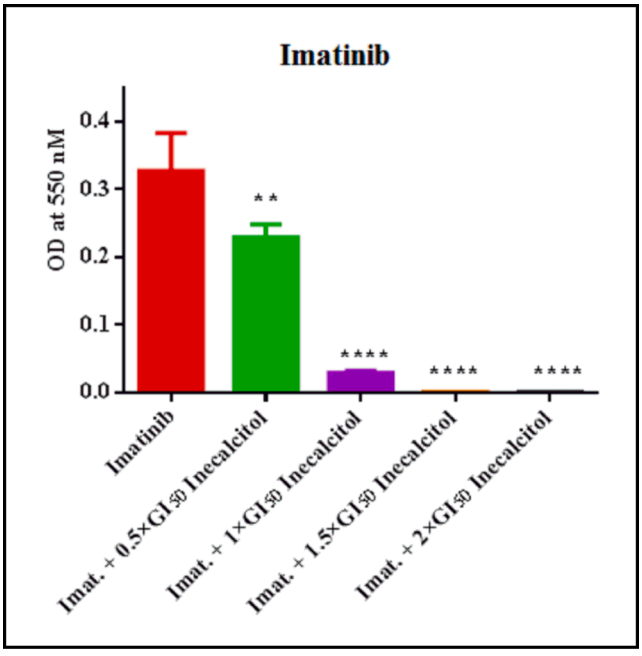

Fig. 4. A statistical analysis of MTT assay of human chronic myeloid leukemia K-562 cells treated with Imatinib combined with Inecalcitol (0.5×, 1×, 1.5×, and 2×GI_{50}) compared to the cells treated with Imatinib alone for 72 h.

Table 3
Combination indices (CI) for non-constant combinations of Imatinib with 0.5×, 1×, 1.5× and 2×GI_{50} of Inecalcitol against human chronic myeloid leukemia K-562 cell line.

[Imatinib] (μM)	[Inecalcitol] (0.5×GI_{50}) (μM)	Effect	CI
0.001	2.8	0.8606	0.91139
0.005	2.8	0.847	0.84315
0.01	2.8	0.842	0.8368
0.05	2.8	0.7639	0.63121
0.1	2.8	0.7128	0.58809
0.5	2.8	0.39	0.33302
1.0	2.8	0.247	0.27242
5.0	2.8	0.284	1.37973
10.0	2.8	0.2028	1.65061
[Imatinib] (μM)	**[Inecalcitol] (1×GI_{50}) (μM)**	**Effect**	**CI**
0.001	5.6	0.6919	0.71399
0.005	5.6	0.66186	0.63494
0.01	5.6	0.68039	0.6946
0.05	5.6	0.57933	0.50502
0.1	5.6	0.52671	0.45402
0.5	5.6	0.36699	0.40271
1.0	5.6	0.33799	0.5269
5.0	5.6	0.29701	1.55446
10.0	5.6	0.26269	2.45391
[Imatinib] (μM)	**[Inecalcitol] (1.5×GI_{50}) (μM)**	**Effect**	**CI**
0.001	8.4	0.57579	0.67211
0.005	8.4	0.54400	0.60012
0.01	8.4	0.58338	0.70119
0.05	8.4	0.53746	0.62417
0.1	8.4	0.52445	0.63478
0.5	8.4	0.40222	0.58526
1.0	8.4	0.40045	0.81331
5.0	8.4	0.31993	1.83086
10.0	8.4	0.28902	2.90797
[Imatinib] (μM)	**[Inecalcitol] (2×GI_{50}) (μM)**	**Effect**	**CI**
0.001	11.2	0.48872	0.64798
0.005	11.2	0.48665	0.6458
0.01	11.2	0.51754	0.72785
0.05	11.2	0.44504	0.57884
0.1	11.2	0.47711	0.68563
0.5	11.2	0.41957	0.75357
1.0	11.2	0.41240	0.97738
5.0	11.2	0.34402	2.15133
10.0	11.2	0.31196	3.35644

with Inecalcitol can be found in the Supplementary Materials (File S1).

3.3. Combined effect of Dasatinib with Inecalcitol on human chronic myeloid leukemia K-562 cells

Dasatinib was combined with different concentrations of Inecalcitol, and its growth inhibitory activity in each combination against human chronic myeloid leukemia K-562 cell line was investigated. The estimated GI_{50} values were calculated from dose-response curves (Fig. 5) after 72 h exposure of K-562 cells to Dasatinib in combinations with 0.5×, 1× and 1.5×GI_{50} of Inecalcitol, and are presented in Table 4.

The results show a significant increase in the potency of Dasatinib against chronic myeloid leukemia cell line, as the concentration of Inecalcitol is increased, that reaches to the lowest GI_{50} value of 0.51 pM. Compared to the uncombined drug (GI_{50} = 446 pM), the growth inhibitory activity of Dasatinib combined with 0.5×, 1× and 1.5×GI_{50} of Inecalcitol against K-562 cells became has been increased significantly, with GI_{50} values of 177, 7.0, 0.51 pM, and 2.5-, 64-, 875-folds, respectively.

One-way ANOVA analysis showed that the difference between Dasatinib alone and Dasatinib combined with different concentrations of Inecalcitol is significant (****$p < 0.0001$) as shown in Fig. 6. Dunnett's – post analysis test exhibited a significant statistical decrease in GI_{50} values shown by all the combinations of Dasatinib with Inecalcitol (0.5×, 1× and 1.5×GI_{50}) compared to the cells treated with Dasatinib only (**$p < 0.001$, ***$p < 0.0001$ and ****$p < 0.0001$), respectively.

CI values indicated the synergistic activity with Inecalcitol (0.5×GI_{50}) at the concentration serial (0.1–10 nM) (CI < 1). However, concentrations of 0.001–0.05 nM showed antagonistic activity (CI > 1). Similar Data was shown in the combination of Dasatinib with Inecalcitol (1.0×GI_{50}), in which synergistic activity was discovered in the serial concentrations (0.01–10 nM) (CI < 1). Also, antagonistic activity was noticed at the concentrations of 0.001 and 0.005 nM (CI > 1). Furthermore, the synergistic activity was concord between Dasatinib and Inecalcitol (1.5×GI_{50}) in all different concentrations (CI < 1), as shown in Table 5. CompuSyn Reports for the combination treatments of Dasatinib with Inecalcitol can be found in the Supplementary Materials (File S2).

Table 4
Growth inhibitory activities of combinations of Dasatinib with 0.5×, 1× and 1.5×GI_{50} of Inecalcitol against human chronic myeloid leukemia K-562 cell line. GI_{50} values are represented as mean ± standard deviation of ≥ 2 independent experiments (n = 8 per trial).

Ligand	GI_{50} (pM) (Mean ± S.D.)
	K-562 Cell Line
Dasatinib	446 ± 97
Dasatinib + Inecalcitol (0.5×GI_{50})	177 ± 77
Dasatinib + Inecalcitol (1×GI_{50})	7.0 ± 1.5
Dasatinib + Inecalcitol (1.5×GI_{50})	0.51 ± 0.07

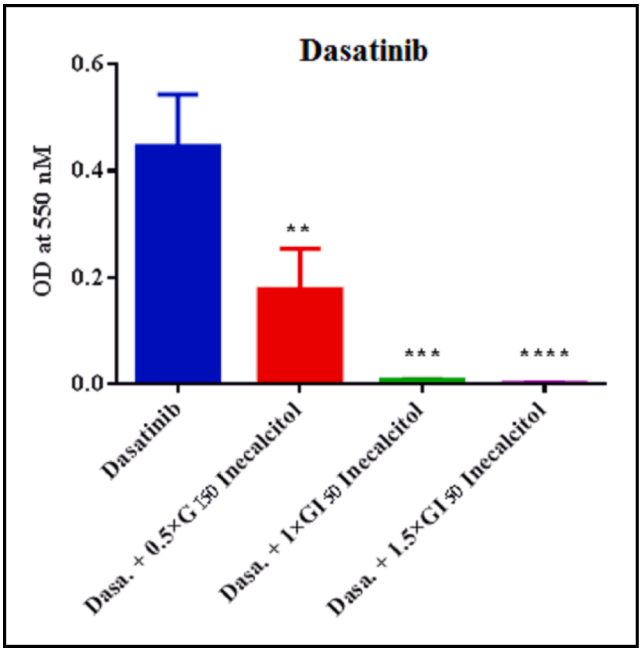

Fig. 6. A statistical analysis of MTT assay of human chronic myeloid leukemia K-562 cells treated with Dasatinib combined with 0.5×, 1× and 1.5×GI_{50} of Inecalcitol compared to the cells treated with Imatinib alone for 72 h.

4. Discussion

Vitamin D is a steroid hormone that has an impact on several biological processes, its insufficiency appears to raise the chance of developing a variety of malignancies in individuals. Strong

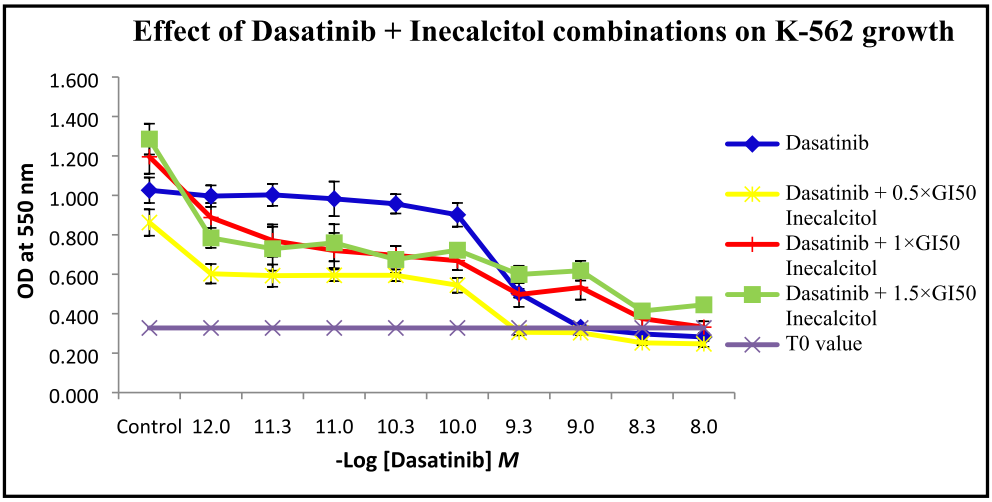

Fig. 5. Dose-response curves that show the difference in cytotoxic activities between Dasatinib alone and combinations of Dasatinib with 0.5×, 1× and 1.5×GI_{50} of Inecalcitol against human chronic myeloid leukemia K-562 cell line. Values are mean ± SD, n = 8, graphs representative of experiments performed on at least two separate occasions.

Table 5
Combination indices (CI) for non-constant combinations of Dasatinib with 0.5×, 1× and 1.5×GI_{50} of Inecalcitol against human chronic myeloid leukemia K-562 cell line.

[Dasatinib] (nM)	[Inecalcitol] (0.5×GI_{50}) (nM)	Effect	CI
0.001	2800.0	0.86113	1.24852
0.005	2800.0	0.89933	3.15747
0.01	2800.0	0.85980	1.43119
0.05	2800.0	0.85907	2.26277
0.1	2800.0	0.80163	0.91447
0.5	2800.0	0.70812	0.47525
1.0	2800.0	0.61217	0.2893
5.0	2800.0	0.62482	0.32209
10.0	2800.0	0.58018	0.25971
Dasatinib (nM)	**Inecalcitol (1×GI_{50}) (nM)**	**Effect**	**CI**
0.001	5600.0	0.75270	1.15127
0.005	5600.0	0.72838	1.0067
0.01	5600.0	0.64066	0.65253
0.05	5600.0	0.67023	0.7505
0.1	5600.0	0.58077	0.49932
0.5	5600.0	0.54414	0.42643
1.0	5600.0	0.54314	0.42481
5.0	5600.0	0.54770	0.435
10.0	5600.0	0.50722	0.36573
Dasatinib (nM)	**Inecalcitol (1.5×GI_{50}) (nM)**	**Effect**	**CI**
0.001	8400.0	0.44567	0.42031
0.005	8400.0	0.50637	0.54436
0.01	8400.0	0.45873	0.4445
0.05	8400.0	0.53208	0.60726
0.1	8400.0	0.46416	0.45494
0.5	8400.0	0.51770	0.57131
1.0	8400.0	0.42628	0.38658
5.0	8400.0	0.50759	0.54796
10.0	8400.0	0.42236	0.38019

epidemiological and experimental data support the role of vitamin D in cancer prevention and treatment for several types of malignancies (Vuolo et al., 2012; Ma et al., 2016).

In line with the previous studies, this study was helpful in identifying a potent growth inhibitory activity against the human chronic myeloid leukemia K-562 cell line by using different concentrations of Inecalcitol drug, the concentration that can inhibit the K-562 cell line, ranged between 0.01 μM and 100 μM for Inecalcitol drug, as shown in Fig. 2 and Table 1. The study of Duffy *et al.* (Duffy et al., 2017) has highlighted the use of Inecalcitol against different types of cancer, and they stated that administration of 80 μg of Inecalcitol daily for three days decreased tumor growth. Similarly, giving 1300 μg/kg Inecalcitol three times a week to a model that had prostate cancer stopped tumor growth, while in the squamous cancer model, Inecalcitol appears to mediate apoptosis. Those results support the findings of this study that Inecalcitol has a potent antiproliferative activity against human chronic myeloid leukemia K-562 cells. The growth inhibitory activity of Inecalcitol can be explained by that vitamin D has anticancer properties, in which apoptosis is the key component of 1,25D3-mediated cell death. 1,25D3 causes apoptosis in a variety of malignancies in diverse ways, mostly through intrinsic apoptotic mechanisms. The mechanisms appear to differ depending on the tumor type and the type of cell. Together with its ability to prevent apoptosis in some normal cells, 1,25D3 appears to be a promising chemotherapeutic drug (Ma et al., 2016).

Other study showed that the potent growth inhibitory effect on cancer can be attributed to many theories that have been offered to explain vitamin D anticancer properties. Vitamin D has been shown to affect the entire carcinogenesis process, from initiation to metastasis and cell-microenvironment interactions. Regulation of cell activities including proliferation, differentiation, apoptosis, autophagy, and epithelial-mesenchymal transition (EMT), as well as modification of cell-microenvironment interactions like angiogenesis, antioxidants, inflammation, and the immune system, are among these mechanisms (Jeon and Shin, 2018).

From Table 2, it was demonstrated that there is a potent inhibition of human chronic myeloid leukemia K-562 cell line by using Imatinib drug in different concentrations, the concentration that causes K-562 inhibition ranged between 0.001 μM and 10 μM, as shown in Fig. 3. Imatinib is a *BCR-ABL* tyrosine kinase inhibitor. In the last few years, the use of Imatinib for CML therapy has increased treatment efficacy and allowed CML patients to live near-normal lives. Resistance to Imatinib therapy evolves, resulting in relapsing and failure, making CML treatment more difficult (Ozkan et al., 2021). *BCR-ABL* kinase is targeted by Imatinib drug. Imatinib inhibits ATP dephosphorylation by binding to the inactive conformation of the *BCR-ABL* fusion protein. The conformational modifications required for the oncoprotein's kinase activity are subsequently inhibited. Inhibition of *BCR-ABL* catalytic activity causes *Ph*+ leukemic cells death by triggering proapoptotic signalling pathways (Gajski et al., 2019). The study done by Danışman Kalındemirtaş *et al.* (Kalındemirtaş et al., 2019) has highlighted that the effective concentration for Imatinib to inhibit K-562 cell line was 5 μM, which showed similarity to the findings of this study.

Also in this study, Dasatinib drug in different concentrations, showed a potent growth inhibitory effect on human chronic myeloid leukemia (K-562) cell line, the range of concentration that caused inhibition on K-562 cell line was between 0.001 nM and 10 nM as shown in and Table 4 and Fig. 5. *BCR-ABL1*, *c-KIT*, *EPHA2*, platelet-derived growth factor receptor-b, and the SRC family of kinases (e.g., SRC, LCK, YES, FYN) are all inhibited by Dasatinib at nanomolar concentrations. Dasatinib is structurally different from Imatinib and inhibits wild-type *BCR-ABL1 in vitro*, Dasatinib is 325 times more effective than Imatinib. Imatinib exclusively binds to the inactive conformation of the *BCR-ABL1* kinase domain; the most typical route of developing Imatinib resistance is point mutations in *BCR-ABL1*, that destabilizing the inactive conformation of the *BCR-ABL1* kinase domain. Dasatinib, on the other hand, binds to the *BCR-ABL1* kinase domain in both conformations that are inactive and active (Keating, 2017). *T315I* was the only completely insensitive *BCR-ABL1* mutant discovered, while *F317L*, *V299L*, and *T315A* mutations showed lower sensitivity to Dasatinib drug (Shoumariyeh and Bubnoff, 2014). A study by Deguchi *et al.* (Deguchi et al., 2008) demonstrates that the concentration that inhibits proliferation of K-562 cell line is 1.5 nM, in second generation which corresponds with the findings of this study.

This study also revealed a significant increase in growth inhibition effect (****$p < 0.0001$) on the human chronic myeloid leukemia K-562 cells when the Imatinib was combined with different concentrations of Inecalcitol. All the combination groups, in which 0.5×, 1×, 1.5× and 2×GI_{50} of Inecalcitol were combined with Imatinib, revealed a significant increase in Imatinib's growth inhibitory effect (**$p < 0.001$, ****$p < 0.0001$, ****$p < 0.0001$ and ****$p < 0.0001$) respectively, on K-562 cell line compared to the inhibited cells treated with Imatinib alone, as shown in Fig. 4. The estimated optimal combination that greatly potentiated the growth inhibitory activity of Imatinib ($GI_{50} = 0.58$ nM) with 564-fold, and caused the highest significant increase in growth inhibition effect on K-562 cells (Table 2) (Fig. 3) was the combination of Imatinib with 2×GI_{50} of Inecalcitol, which indicated synergistic effect with CI < 1 (Table 3). Vitamin D has been demonstrated to not only enhance the effectiveness of established cancer treatments, but also to help overcome the molecular pathways that cause medication resistance, in which it can promote tumor spreading (Negri et al., 2020; Ito et al., 2022). A study by Medioni *et al.* (Medioni et al., 2014) have suggested that using high dose of antiproliferative Inecalcitol combined with docetaxel daily, showed to be safe and lead to an encouraging prostate-specific antigen (PSA) response, during 3 months 85 % of patients showed > 30 % PSA decline, while 76 % of patients showed > 50 % PSA decline at any time within the study. The findings of the previous study are similar to the results of our study, in which combination of Inecalcitol with Imatinib potentiates the effect of Imatinib dose, so that we can decrease the dose of Imatinib and possibly reduce its negative effects. Also the growth inhibitory activity of Imatinib combined with

$2\times GI_{50}$ Inecalcitol (580 pM) became nearly the same as that of Dasatinib alone (446 pM) against K-562 cell line (Figs. 3 and 5).

The use of Dasatinib in combination with different concentrations of Inecalcitol revealed a significant increase in growth inhibition (****$p <$ 0.0001) of human chronic myeloid leukemia K-562 cells. Dasatinib was combined with 0.5×, 1× and $1.5\times GI_{50}$ of Inecalcitol, and the result of all the combination groups showed a significant increasing in Dasatinib's growth inhibitory activity (**$p < 0.001$, ***$p < 0.0001$ and ****$p <$ 0.0001) respectively, against K-562 cell line in comparison to the inhibited cells treated with Dasatinib alone, as shown in Fig. 6. Combination of Dasatinib with Inecalcitol ($1.5\times GI_{50}$) was the estimated optimal composition that tremendously potentiated Dasatinib's growth inhibitory activity ($GI_{50} = 0.51$ pM) to become 875 times its activity alone ($GI_{50} = 446$ pM), caused the highest significant increase in growth inhibition effect on K-562 cells (Table 4) (Fig. 5), and showed synergistic activity with CI < 1, as shown in Table 5. Such combination can be considered as a promising novel antileukemic drug to be used instead of Dasatinib alone in treatment of CML with lower dose and potential minor side effects. Those results are similar to the previous study that was done by Murray *et al.* (Murray et al., 2017), in which Inecalcitol was used to suppress the proliferation of breast cancer cells particularly those that express Vitamin D receptor. Vitamin D receptor (VDR) is a steroid hormone transcriptional regulator that belongs to the nuclear family. The binding of the active form of vitamin D, that is recognized as Calcitriol or $1,25(OH)_2$-vitamin D3, activates vitamin D receptor (Murray et al., 2017). Ligand binding causes retinoid X receptor (RXR) heterodimerization, which is followed by binding to vitamin D response regions on DNA. The Calcitriol-bound VDR/RXR complex appears to influence gene expression after the recruitment of several transcriptional regulatory proteins such as nuclear receptor co-activators (Murray et al., 2017). VDR demonstrated to be a transcriptional activator and inhibitor, meaning that its target genes can be upregulated or downregulated (Murray et al., 2017). The combined treatment of Dasatinib with Inecalcitol can be used to potentiate the Dasatinib effect to lower the administration dose to the patients for the treatment of human chronic myeloid leukemia, and therefore may reduce the major side effects of Dasatinib.

This is a novel study (to our knowledge, no previous studies have shown such novel results), as it gives a deeper insight for using the antileukemic drugs (Imatinib and Dasatinib) in separate combinations with Inecalcitol (Vitamin D_3 analogue), which potentiate the drugs' anticancer activity, lower their administration doses and may reduce the serious side effects.

5. Conclusion

In conclusion, we present a novel study that describes the potent antiproliferative activity of Inecalcitol against human chronic myeloid leukemia K-562 cell line, and demonstrates its synergic anticancer effect in different combinations with the antileukemic TKIs Imatinib and Dasatinib drugs on the same type of leukemia cells. MTT assays proved that the potent growth inhibitory activity of Imatinib and Dasatinib against K-562 cell line is increased significantly when both drugs are combined separately with different concentrations of Inecalcitol. Furthermore, Imatinib's antiproliferative activity is potentiated ($GI_{50} =$ 0.58 nM) when is combined with $2\times GI_{50}$ of Inecalcitol, to become greater than that of Imatinib alone ($GI_{50} = 327$ nM), with 564-fold, therefore reaches nearly the same activity level of Dasatinib drug (0.446 nM). This synergic effect makes such combination a promising alternative drug to Dasatinib in CML therapy, with possible lower side effects. While synergic effect resulting from the combination of Dasatinib with Inecalcitol ($1.5\times GI_{50}$) potentiates and increases the potent growth inhibitory activity of Dasatinib ($GI_{50} = 0.51$ pM) with 875-folds, making such combination a promising potent novel antileukemic drug, with potential minor side effects, for the treatment of CML. For the future work, screening will be repeated to evaluate the antiproliferative and synergistic effect of our combined treatments against resistant human leukaemia cell lines, such as CCRF-CEM and MOLT-4. Selectivity indices (SI) for the treatment combinations will be determined by testing them against fibroblast normal cell line. Furthermore, the mechanism of action and pathway for the combined treatments in different types of leukaemia cells will be investigated *in vitro*, via metabolomics, lipidomics, proteomics, cell cycle analysis, apoptosis, and Western blot analysis.

CRediT authorship contribution statement

Luma Al-Ali: Investigation, Data curation and Writing – original draft. **Raad J. Al-Ani:** Conceptualization, Project administration, Supervision, Funding acquisition, Resources, Writing – original draft and Writing – review & editing. **Maysaa M. Saleh:** Conceptualization, Project administration, Supervision, Investigation, Methodology, Formal analysis, Software, Funding acquisition, Resources, Writing – original draft and Writing – review & editing. **Alaa M. Hammad:** Software, Formal analysis and Writing – review & editing. **Duaa A. Abuarqoub:** Supervision, Formal analysis and Writing – original draft. **Bashaer Abu-Irmaileh:** Data curation. **Abdallah Y. Naser:** Writing – original draft, Writing – review & editing. **Manal M. Najdawi:** Resources. **Manal M. Abbas:** Resources. **Jamal Alyoussef Alkrad:** Formal analysis, Writing – review & editing. All the authors reviewed the results, read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

File **S1**: Synergy CompuSyn Reports for the combination treatments of Imatinib with **(A)** Inecalcitol ($0.5\times GI_{50}$), **(B)** Inecalcitol ($1\times GI_{50}$), **(C)** Inecalcitol ($1.5\times GI_{50}$) and **(D)** Inecalcitol ($2\times GI_{50}$). File **S2**: Synergy CompuSyn Reports for the combination treatments of Dasatinib with **(A)** Inecalcitol ($0.5\times GI_{50}$), **(B)** Inecalcitol ($1\times GI_{50}$) and **(C)** Inecalcitol ($1.5\times GI_{50}$).

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