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Effects of Nicotinamide and Nilotinib on Telomerase Activity and Telomere Length in K562 Myeloid Cell Line

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Abstract
Chronic myelogenous leukaemia (CML) has continued to exist as a challenging disease even though advanced tyrosine kinase inhibitor therapy has been introduced. We study the effect of nicotinamide on telomerase activity, telomere length, and TERT expression in the K562 myeloid cell line as an approach to enhance the existing therapy for CML. However, the role of nicotinamide in tumorigenesis is controversial. We hypothesised that nicotinamide would enhance the effects of Nilotinib on K562 cells, hence reducing tumour growth and/or promoting tumour cell death. K562 cells were treated with nicotinamide, nilotinib and a combination of both for IC50 assay. The treated cells later undergo telomerase activity assay and telomere length analysis. Finally, the expression of the TERT gene using a probe-based qPCR method. This study has shown the effect of nicotinamide, nilotinib and both substances in exhibiting the anti-proliferation ability on the K562 cell line after 48 hours. The implicated mechanism involved in inducing such an effect is not yet clear. All treated samples exposed to all treatments have been assessed as telomerase-positive, suggesting that these treatments were most likely unable to repress telomerase activity in K562 cells. Except for nicotinamide, which slightly reduces telomerase activity and shortens telomeres, all treatment groups result in longer telomere lengths. The expression of TERT in this study suggests that the effect of these substances on telomerase activity is necessarily dependent on their impact on TERT expression. However, no study has been done to investigate the effect of nicotinamide in combination with nilotinib on telomerase and telomere regulation, and thus, further investigation on these substances is warranted.

Keywords:
Nicotinamide, Nilotinib, Telomerase, Telomere, CML

Introduction

Blast crisis remains the main challenge in CML management due to the more aggressive condition and genetic instability. Even the current treatment of CML could not improve the overall survival rate [1]. Therefore, it is necessary to determine another innovative treatment and identify new pharmacological options to treat CML patients during a blast crisis.

Nilotinib is used to treat CML patients in a more advanced phase of the disease and patients who have developed resistance from previous treatment [2]. Combination therapy is a keystone of cancer therapy where two or more therapeutic agents are combined to create a new or improved treatment for a specific disease [3]. Novel approaches are required to avoid relying solely on a single treatment agent. This study will be focused on the effect on telomerase activity and telomere length after treatment with nicotinamide and/or nilotinib.

Nicotinamide, an active form of vitamin B3, is an amide derivative of water-soluble niacin, often known as a nicotinic acid amide or niacinamide. Research on animal models suggests that nicotinamide may influence tumours by promoting carcinogenesis in a dose-dependent approach, depending on the organ involved [4]. In previous studies, several cancer cell lines have also responded favourably to nicotinamide in suppressing cell proliferation [5–7]. Nicotinamide also improves the replicative lifespan of normal human cells through its influence on telomere length [8,9].

Telomeres are repetitive segments of the DNA sequence at each end of the chromosome. Its purpose is to maintain chromosome stability and prevent chromosome degeneration or fusion with nearby chromosomes [10]. Many epidemiological studies also have found a correlation between telomere length and cancer risk [11,12]. The amplified telomerase activity in several cancers has also been found to prevent telomere shortening, enabling the cancer cells to multiply beyond their usual limits [13,14]. Telomere and telomerase, the enzymes stabilising telomere length, play a role in forming a cancerous human tumour. We hypothesised that nicotinamide might affect telomere and telomerase activity in the K562 myeloid cell.

This research aims to study the potential of nicotinamide as a supplementary agent in enhancing the sensitivity of nilotinib in treatment to inhibit tumour growth or promote tumour cell death through their effects on telomerase activity, telomere length and gene expression of telomerase component (TERT) in K562 myeloid cell line. Understanding the role and potential of nicotinamide and/or nilotinib in telomere and enzyme telomerase might improve the treatment approach for CML for better management of this disease.

Methodology

Cell Culture

K562 cells (ATCC® CCL-243™) were cultured in RPMI 1640 medium with L-glutamine, supplemented with 10% of fetal bovine serum (FBS) and 0.5% penicillin-streptomycin (Pen-Strep) which were purchased from Invitrogen, USA. Cells were incubated at 37°C and 5% CO_2 in a humidified incubator (Heraeus, UK) and monitored daily. Subculturing of cell culture was done to the extracted and harvested cells and proceeded to cell counting using 0.4% of Trypan blue (Invitrogen, USA) in a ratio of 1:1 and counted by using a hemocytometer (Hirschman, German).

Dilution of nicotinamide and nilotinib

To achieve the appropriate concentration of nicotinamide (Sigma Aldrich, USA), a concentration dilution procedure was used to prepare five points of concentration dilution of 0.02 M, 0.04 M, 0.06 M, 0.08 M, and

0.1 M. While serial dilution of Nilotinib (Qiagen, Germany) was done to obtain five different concentrations which are 5.0 x 10^{-9} M, 1.0 x 10^{-8} M, 2.0 x 10^{-8} M, 4.0 x 10^{-8} M, and 8.0 x 10^{-8} M.

Determination of IC50 value of nicotinamide and nilotinib

Cells were counted microscopically and adjusted to 5×10^3 cells/µL followed by seeding with 100 µl of cell suspension into the 96-well cell culture plate with v-bottom (NEST, China). The cell suspension was pre-incubated for 24 hours before each well plate was replaced with cell culture media containing the desired concentration of nicotinamide and nilotinib. The plate was incubated for further treatment for 48 hours. Cell Count Reagent (Nacalai Tesque, Japan) was used to detect the absorbance reading of cells at 450 nm by using the Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, USA). The IC50 value is obtained using the Graphpad Prism 8 software.

Each IC50 concentration for nicotinamide and nilotinib was determined separately. Before all the steps were repeated for combination treatment, both IC50 concentrations of nicotinamide and nilotinib were mixed to determine the combination treatment's cell viability.

Telomerase activity assay

TeloTAGGG telomerase PCR ELISA kit (Sigma Aldrich, USA) was utilised to detect the telomerase activity of the K562 cell line. The kit is based on the Telomeric Repeat Amplification Protocol (TRAP) assay with a non-radioactive ELISA detection. Cells were processed according to the manufacturer's protocol, where the cells were lysed before proceeding to TRAP assay using the GeneAmp PCR System 9700 machine (Applied Biosystems, USA). The absorbance reading of the cells was immediately measured using the ELISA reader at 450 nm.

Genomic DNA analysis

According to the manufacturer's protocol, DNA was extracted using GeneAll® Exgene™ Cell SV mini kit (GeneAll, South Korea). DNA quality was determined by loading the extracted DNA on 2% agarose gel electrophoresis. High-quality DNA was shown as a bright and strong band without any smear effect at the top of the agarose gel. All DNA samples were then used in the telomere length analysis.

Telomere length analysis

The method used to measure and analyse telomere length is quantitative real-time PCR (qPCR), which introduces an oligomer standard using the SYBR Green-based method. Advanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) was used with standard and primer supplied by Integrated DNA Technologies, USA. The standards, samples, and controls were amplified using Stratagene Mx3000P qPCR System (Agilent Technologies, USA). The standard and primer sequences are as in the supplementary data (Table 1).

The telomere standard curve was constructed by serial dilution of standard telomere containing only TTAGGG repeats to measure the telomeric sequence per sample in kilobase (kb). Plasmid DNA pBR322 was used, and 20 ng of pBR322 was added to each of the dilutions to maintain a constant total DNA per reaction. Telomere standard was adjusted to 1 x 10^{-3} µM (26.67 pg/µL) and used as the starting concentration of the ten-folded dilution with five different concentrations of 1 x 10^{-7} µM, 1 x 10^{-6} µM, 1 x 10^{-5} µM, 1 x 10^{-4} µM, and 1 x 10^{-3} µM. As a control by the amplification for every sample tested, a single copy gene was used to determine genome copies per sample. 36B4 was used as the human single-copy gene, which encodes the acidic ribosomal phosphoprotein.

Amplification, standard curve, and melt curve graph were constructed and obtained by using MxPro qPCR software. Relative quantitation of all samples was determined by comparison with the standard curve derived from the amplification of C_t versus log of standard samples. Samples from all group treatment were calculated for total telomere length in kb per human diploid genome.

RNA analysis and reverse transcriptase PCR (RT-PCR)

RNA was extracted using innuPREP RNA Mini Kit (Analytik Jena, Germany) per the manufacturer's instructions. First-strand cDNA was synthesised using SuperScript™ IV First-Strand Synthesis System (Invitrogen, USA). All cDNA samples were then used in the gene expression analysis.

Gene expression analysis

The gene expression level was analysed using a probe-based method system that used primer, probe, and master mix of SensiFAST™ Probe Lo-ROX kit (Bioline, USA). Three sets of primers and probes were used: the target gene TERT and two housekeeping genes PPIB and PGK1. Two housekeeping genes were used as the normaliser gene between the gene from the treatment group and the untreated control group. The amplification was performed using the Stratagene Mx3000P qPCR system (Agilent, USA). A comparative qPCR quantification method was used to distinguish the gene expression differences between the treatment and untreated control groups. The primer and probe sequences used for qPCR analysis of gene expressions were as follows: TERT forward, 5'-GTCCAGACTCCGCTTCATCC-3' and reverse, 5'-CAGGCCCTGTGGATATCGTC-3'; TERT probe, 5'-GACGGGCTGCTCCTGCGTTT-3'.

A comparative qPCR quantification method was used to distinguish the gene expression differences between all treatment groups and the untreated control group. The expression level of the TERT gene was assessed for upregulation or downregulation compared to the untreated sample as a control.

Statistical analysis

All the assays and analyses were carried out in triplicates, and data were calculated as mean ± standard deviation (SD). Statistical differences were evaluated using the Paired t-test to compare the gene expression difference between all groups for gene expression analysis. All data were analysed and generated using GraphPad Prism 8 software. The value of $p < 0.05$ was considered statistically significant.

Results

The half-maximal inhibitory concentration of nicotinamide and nilotinib

Five nicotinamide and nilotinib dilution points have been prepared to construct a dose-response cell viability curve on the K562 cell line. The cytotoxicity effect of nicotinamide and nilotinib increased with the concentration (Figure 1). The proliferation of the K562 cell line was inhibited dose-dependently.

The cytotoxicity effect of nicotinamide increased with the concentration whereby the highest concentration of 0.1 M was shown to inhibit more than 80% of viable cells with 16.56% cell viability, and the lowest concentration of 0.02 M was shown to inhibit less than 5% of viable cell with 95.98% cell viability.

The cytotoxicity effect of nilotinib increased with the concentration whereby the highest concentration of 8.0×10^{-8} M was shown to inhibit more than 60% of viable cells with 33.83% cell viability, and the lowest concentration of 5.0×10^{-9} M was shown to inhibit less than 10% of viable cells with 92.05% cell viability.

Following 48 hours of cell treatment, a 50% reduction (IC50) of cell viability of the K562 cell line by nicotinamide and nilotinib was determined. The graph was plotted with concentrations against cell

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viability. IC50 of nicotinamide and nilotinib was determined at 0.03433 M and 8.788 x 10^{-9} M, respectively. As the concentration of both substances increases where at the highest point of concentration used, cell inhibition efficacy decreases by the plateau phase of the drug-response curve.

At the lowest concentrations of nicotinamide, it efficiently inhibited the proliferation of K562 cells, with more than 15% to 96% cell viability, compared to the concentrations of nilotinib, with more than 32% to 93%.

A combination treatment of nicotinamide and nilotinib was prepared by mixing both IC50 concentrations of nicotinamide and nilotinib on the K562 cell line. Cell viability of combination treatment has shown to be higher than 60% (with 69.57% of cell viability) compared to 50% of cell inhibition.

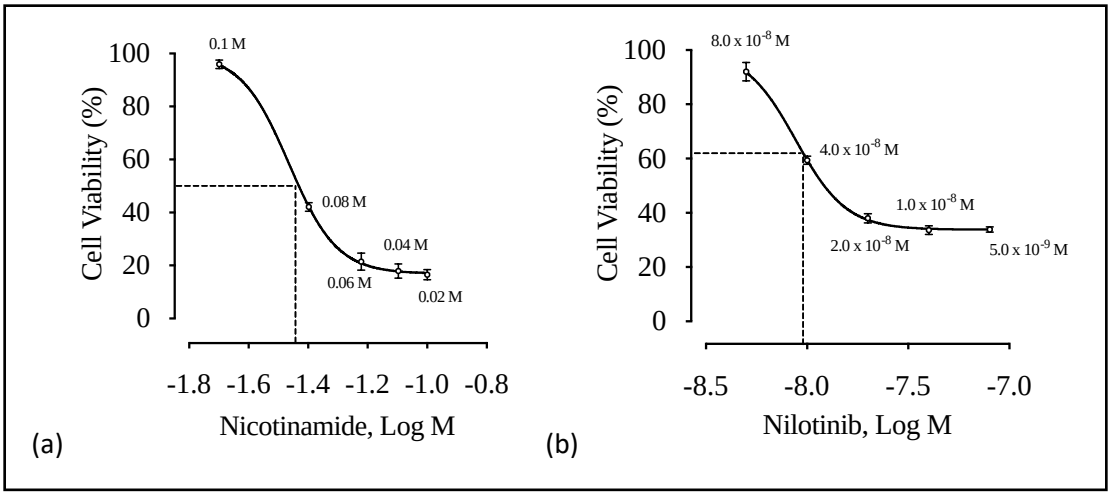

Figure 1: Dose-response curves generated by GraphPad Prism 8 were used to evaluate IC50 values of (a) nicotinamide and (b) nilotinib in the K562 cell line after 48 hours of cell treatment.

Telomerase activity

K562 cell lines were exposed to the cell treatment with nicotinamide, nilotinib, and a combination of both substances before telomerase activity was assessed using the TeloTAGGG Telomerase PCR ELISA kit. There is no significant difference in telomerase activity following treatment with either of these substances compared to the untreated control group. Telomerase activity of negative control, which has been treated with heat treatment and positive-telomerase control provided by the manufacturer, has shown to be in the acceptable range according to the manufacturer's protocol with 0.084 and 4.209 $A_{450\ nm}–A_{690\ nm}$, respectively. The absorbance value of negative control should be lower than 0.25 $A_{450\ nm}–A_{690\ nm}$, while the absorbance value of positive-telomerase control should be higher than 1.5 $A_{450\ nm}–A_{690\ nm}$.

Nicotinamide has been shown to slightly decrease the telomerase activity with 3.96 $A_{450\ nm}–A_{690\ nm}$, and both nilotinib and combination treatment have been shown to slightly increase the telomerase activity in the K562 cell line with 4.63 and 4.72 $A_{450\ nm}–A_{690\ nm}$ compared to the untreated control group with 4.24 $A_{450\ nm}–A_{690\ nm}$ as shown in Table 1 and Figure 2. All samples were determined as telomerase-positive, where the absorbance value of telomerase activity is more than 0.2 $A_{450\ nm}–A_{690\ nm}$.

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Table 1: Telomerase activity in the presence of nicotinamide and nilotinib in the K562 cell line.

Treatment	Mean (SD)		Mean Difference (95% CI)	t-test (df)	p-value
	Control (n=3)	Treatment (n=3)			
Nicotinamide	4.24 (0.01)	3.96 (0.02)	-0.28 (-0.35, -0.21)	16.17 (2)	.0038**
Nilotinib		4.63 (0.04)	0.39 (0.32, 0.46)	25.53 (2)	.0015**
Combination		**4.72 (0.02)**	**0.48 (0.46, 0.50)**	**83.14 (2)**	**.0001*** **

Paired T-test is used to compare treated groups with untreated with p <.01 is considered statistically significant compared to the untreated control group (**), and p <.001 is considered statistically significant compared to the untreated control group (***).

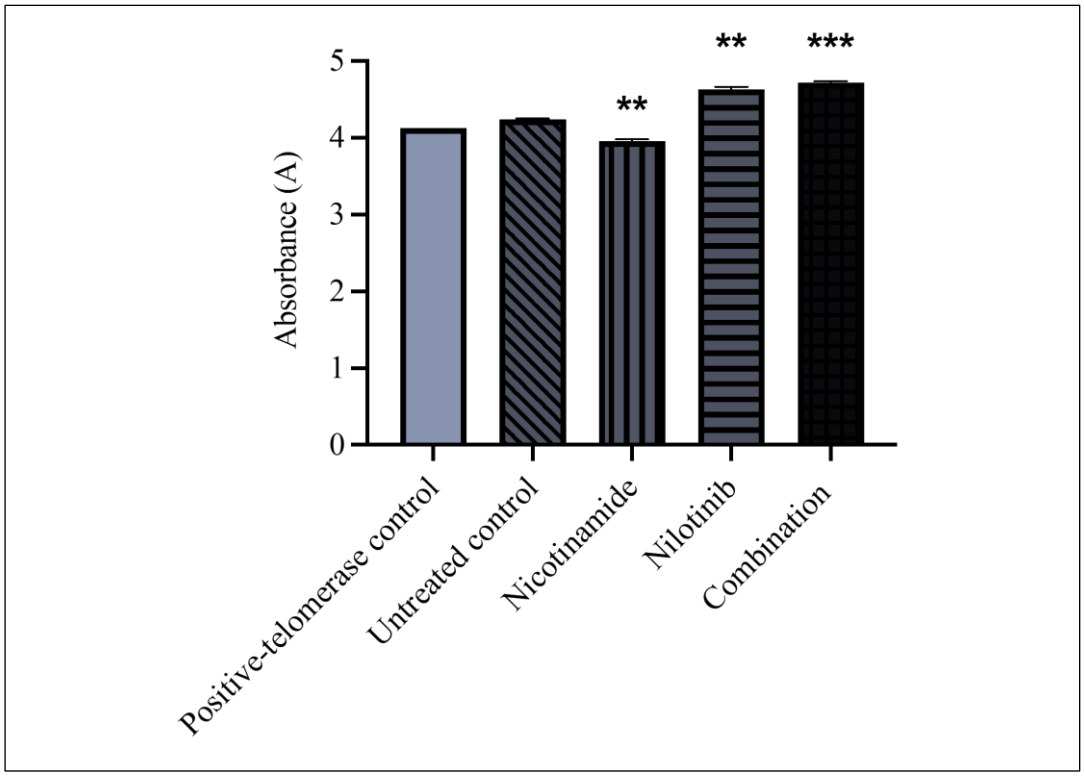

Figure 2: Quantification of telomerase activity on the K562 cell line following 48 hours of treatment with nicotinamide, nilotinib, and combination treatment. Paired T-test is used to compare treated groups with untreated with p <.01 is considered statistically significant compared to the untreated control group (**), and p <.001 is considered statistically significant compared to the untreated control group (***).

Telomere length analysis using qPCR by SYBR green-based method

The protocol to measure telomere length using qPCR was based on the modification by O'Callaghan and Fenech [15] from the qPCR method by Cawthon [16]. A linear range of reaction was observed in the standard

curve by both standards, with PCR efficiency falling within the acceptable range of overall efficiency with a 90% to 110% range. Both standards' melting or dissociation curves result in a single peak generally assessed as a pure single amplicon. All the samples from all group treatments fell within the linear range of the standard curve of both the telomere standard and SCG standard. The data was analysed using the total telomere length in kb per human diploid genome of all treatment groups compared with the untreated control group. Cell treated with nicotinamide and a combination of both nicotinamide and nilotinib showed a decrease in the total number of telomere length compared to the untreated control group of (833.84 total telomeric lengths in kb/human diploid) with 672.79 and 820.03 total telomeric lengths in kb/human diploid, respectively. Total telomere length in nilotinib treatment has increased compared to the untreated control group, with 1141.63 total telomeric lengths in kb/human diploid. The summary of data is in the supplementary data (Table 2 and Figure 3).

Effect of nicotinamide, Nilotinib, and combination of both substances on gene expression of *TERT*

TERT gene expression was shown to be downregulated in the exposure of nicotinamide with a relative expression level of 0.56-fold and upregulated in the exposure of nilotinib and combination treatment with a relative expression level of 1.42-fold and 1.02-fold, respectively. The values were compared with the untreated control group of untreated cells at 1.00-fold. The *TERT* expression level was significantly downregulated with a p-value of 0.0004 with the treatment by nicotinamide. In contrast, combination treatment has shown that the expression is insignificant, with a p-value of more than 0.05 (Figure 4 and Table 3).

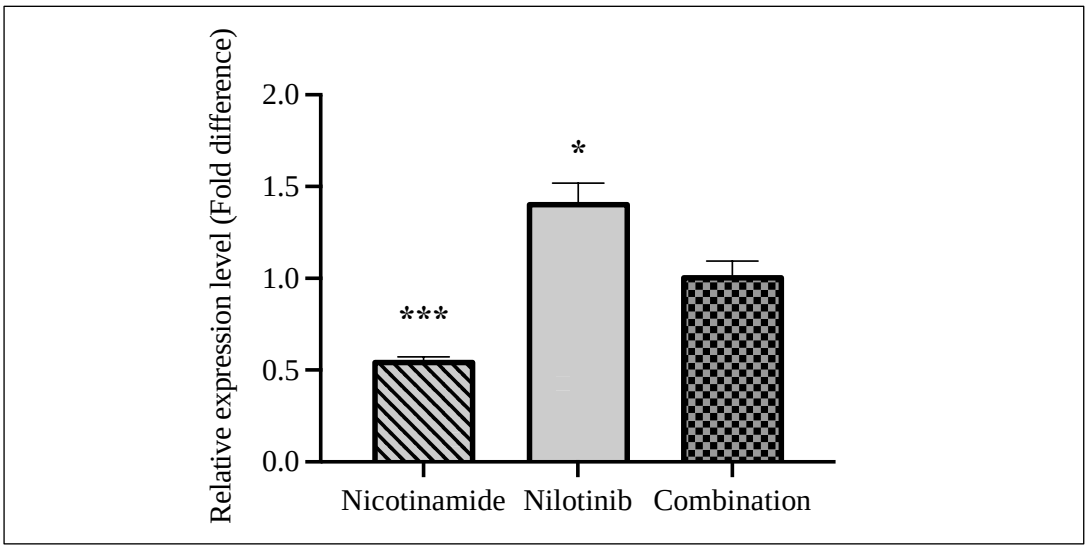

Figure 4: Relative expression level (fold difference) of *TERT* in the K562 cell line after being treated with nicotinamide, nilotinib, and a combination of both substances. Paired T-test is used to compare treated groups with untreated with p <.05 is considered as statistically significant compared to the untreated control group (*) and p <.001 is considered as statistically significant compared to the untreated control group (***).

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Table 3: Summary of association effect of nicotinamide, Nilotinib, and combination treatment on telomerase activity, telomere length, and *TERT* expression.

Type of analysis	**Treatment**			
	Untreated control	Nicotinamide	Nilotinib	Combination
Telomerase activity	4.24 (Positive)	3.96 (Positive)	4.63 (Positive)	4.72 (Positive)
***TERT* expression (Fold-change)**	1.00	0.56 (Downregulated)	1.42 (Upregulated)	1.02 (Upregulated)
Telomere length (kb)	833.84	672.79	1141.63	820.03

Discussion

The cytotoxic effect of nicotinamide, nilotinib, and a combination of both treatments on the K562 cell line

In this study, nicotinamide has been shown to inhibit the proliferation of the K562 cell line by 5% to 80% with an IC50 value of 34.33 mM. Based on the previous study, nicotinamide has displayed an anti-proliferation effect in the K562 cell line [17], inhibitory effects in pancreatic cancer [18], prostate carcinoma [19], and higher cytotoxic activity on K562, BL2, and primary leukaemia cell [20].

A relatively high concentration of nicotinamide is required for the initiation of the anti-proliferation effect in the K562 cell line, probably due to the structure of the substances that reduced in cell membrane permeability of a nicotinic acid-related compound through the cell membrane, high water solubility, and electric charges of these substances [17].

Nilotinib has been developed with more efficient suppression capability and increased specificity and potency toward CML cells [21]. Nilotinib has been granted by the US FDA to be used for patients with chronic or accelerated phases of CML based on the result of the clinical studies [22]. This study also showed the anti-proliferation effect of nilotinib in the K562 cell line by 10% to 60% with an IC50 value of 8.788×10^{-6} mM. This concentration agrees with the previously published data where nilotinib can reduce 50% of cell viability at a range of less than 30×10^{-6} mM [23,24].

Nilotinib has shown an inhibitory effect in low concentrations by inhibiting MDM2 expression in ALL cells [25], suppressing the proliferation in the cell isolated from the CML blast crisis patient [26] and HL60 cell [24]. Many therapeutic effects of nilotinib have been reported, and many studies have been done to provide evidence of the efficiency of Nilotinib in CML and other types of diseases [27,28].

The ratio of the IC50 value of nicotinamide compared to the IC50 value of nilotinib may represent a therapeutic potential of nilotinib as an anticancer drug and nicotinamide as the supplementary agent for CML patients. The efficiency of nicotinamide may not be as acute as the targeted enzyme drug. Still, the effect of nicotinamide leads to an elevated level of NAD+, which plays a critical role in health and longevity

[29]. With a much higher binding site affinity, nilotinib has demonstrated anticancer activity in CML by inhibiting cellular proliferation more potently than imatinib [30] and administered to CML patients [31,32].

Nilotinib alone could significantly affect cell proliferation of the K562 cell line. However, to avoid drug resistance, a combination of drugs may be required [33,34]. Observation in combination treatment by both IC50 values of nicotinamide and nilotinib in cell proliferation of the K562 cell line showed increased cell viability compared to 50% of cell inhibition. Combination treatment is generally considered antagonistic when the combined effect is reduced compared to the effect of each substance [35]. Therefore, a combination index (CI) is required to determine the degree of interaction and link relationship between two substances.

Based on the IC50 value of both substances, it has been proven that the potency of nilotinib potently inhibits the cell proliferation of the K562 cell line compared to nicotinamide due to the lower concentration required to achieve cell inhibition. However, nicotinamide could efficiently inhibit the K562 cell proliferation with more than 15% to 96% of cell viability compared to nilotinib with more than 32% to 93%. The highest concentration of both substances showed a plateau phase of the drug-response curve, indicating the maximal efficacy of the drug [36]. The target protein involved in inhibiting cell proliferation by both substances may differ, leading to different efficacies of nicotinamide and nilotinib. Variation in drug response arises from the pharmacodynamics of the drugs, where there is variation in chemical structure and the interaction with the targeted proteins for the drug action in the cells [37].

Correlation of telomerase activity, telomere length, and TERT expression with exposure of nicotinamide, Nilotinib, and combination of both substances in K562 cell line

Given that telomere length and telomerase activity are crucial in developing tumours, their relationship and interaction with various types of drugs have been extensively investigated [38]. Compared to somatic cell populations, CML cell has significantly higher telomerase activity [39]. Since higher telomerase activity has been reported in CML cells, this study examines the ability of nicotinamide to operate as a supplementary agent and nilotinib as a second-generation treatment of CML in K562 cells. All the samples exposed by nicotinamide and nilotinib have been assessed as telomerase-positive with mean differences ranging from -0.28 to 0.48, suggesting that all treatments most likely could not repress telomerase enzyme activity in the K562 cell line. Nicotinamide showed a slight reduction effect on telomerase activity compared to nilotinib and combination treatment, where both showed an increment of telomerase activity.

Nevertheless, a study by Shapira et al. [24] showed that nilotinib had inhibited 90% of telomerase activity with a concentration of 24×10^{-6} mM. They also suggested that the effect of nilotinib on telomerase activity is not dependent on its relationship with BCR-ABL. Their result was consistent with the *TERT* expression, where nilotinib downregulates *TERT* expression and reduces telomerase activity. Contrary to this study, we observed that *TERT* expression was upregulated with nilotinib and combination treatment compared to the untreated control group. At the same time, it correlated with the telomerase activity that has been increased in the exposure to these treatments. In contrast, nicotinamide was able to downregulate the *TERT* expression associated with its effect on telomerase activity.

It is worth noting that the concentration of nilotinib used in this study differs from the concentration used in the study conducted by Shapira et al. In their study, a higher concentration of nilotinib was used, which resulted in the inhibition of telomerase activity. In contrast, the concentration used in this study did not demonstrate significant telomerase inhibition, even though it did reduce cell viability by half. This suggests that the effect of nilotinib on telomerase activity may be dose-dependent, with higher concentrations inducing greater inhibition. It is possible that the results of the study by Shapira and colleagues were

influenced by a more complex drug effect, leading to telomerase activity inhibition. Further research is needed to determine the exact mechanism of this phenomenon.

Several studies have reported that telomerase activity was closely associated with *TERT* expression in a different type of cancer cell [40,41]. Tumour cells express the *TERT*, one of the catalytic subunits of the telomerase enzyme, where high or detectable telomerase activity can be measured [42]. Transduction of *TERT* has prevented the telomere shortening but showed no effect on the growth of culture from distal tibial chondrocyte patients [43]. Nicotinamide has been reported to maintain the telomere length in normal human fibroblasts while the rate of telomere shortening was reduced [8]. Expression of the *TERT* via telomerase activity influences and impacts telomere length and may be a helpful marker for various cancer therapies [44]. No other published data or studies have been done to provide evidence regarding the ability of nicotinamide and nilotinib to affect telomerase activity in the K562 cell line, including its molecular mechanism.

To conclude, the findings of this study suggest that individual treatment of K562 cells with nilotinib and nicotinamide would be more cytotoxic to the cells than the combination treatment. In addition, nilotinib and nicotinamide individually may affect the cell's TERT gene expression, telomerase activity, and telomere length differently. The current study is limited to one type of cell line. Other types of cell lines may be needed for future investigation to determine the further potential of nicotinamide as the supplementary agent in cancer therapy. Other mechanisms or pathways may inhibit K562 cells by nicotinamide, nilotinib, and a combination of both substances. Thus, further study is required to evaluate the mode of action of these substances through a comprehensive fundamental study.

Conclusion

We hypothesised that nicotinamide would enhance the effects of nilotinib on K562 cells, hence reducing tumour growth and/or promoting tumour cell death. This study has shown that nicotinamide, nilotinib, and a combination of both substances exhibit the anti-proliferation ability on the K562 cell line after 48 hours. These data are aligned with published data indicating the consistency of both substances in inhibiting tumour growth and promoting cell death. However, the efficacy of the combination treatment is no more significant than cells solely treated with nicotinamide and nilotinib, where the combination has higher cell viability compared to the 50% inhibition.

This study finds that cells treated solely with nicotinamide and nilotinib have seen a significant decrease and increase, respectively, in telomerase activity, *TERT* expression, and telomere length compared to untreated controls. The combination treatment had shown a significant increase in telomerase activity. However, the difference in *TERT* expression and telomere length was not significant compared to untreated controls.

In conclusion, nicotinamide and nilotinib have a better cytotoxic effect individually when compared to a combination of both. The effects of both nilotinib and nicotinamide on the *TERT* gene expression, telomere length and telomerase activity indicate that a more complex mechanism may be in place. The mechanism involved is not yet clear, and more studies are needed.

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