Original article

Protective effect of Bosutinib with caspase inhibitors on human K562 cells

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Abstract

Introduction: Cancer therapy has become increasingly focused on molecularly targeted medications. Despite the fact that multi-cytotoxic medication regimens have proven to be highly effective, many investigations in targeted treatments have focused on a single agent. The precise molecular mechanism of action of second-generation BCR-ABL tyrosine kinase inhibitors, which includes different targets and pathways, can help rationalize therapy in chronic myelogenous leukemia (CML) and other diseases affected by BCR-ABL tyrosine kinase inhibitors (TKIs).

Aim: The purpose of this study was to analyze if bosutinib (BOS) combined with Boc-D-FMK effectively suppressed proliferation and induced apoptosis in K562 cells to a lesser extent, implying that bosutinib is an effective leukemia treatment and that its combination with Boc-D-FMK is a mild chemotherapeutic agent against leukemia.

Methods: In this study, bosutinib was obtained together with other materials to perform a cell culture experiment with human cell lines, as well as additional drug treatment. Furthermore, cell viability (MTT assay) and flow cryometry such as viability and cell cycle assays are performed. The target profile of the dual SRC/ABL inhibitor bosutinib was studied in this study as a first kinase inhibitor to target K562 cells, which has recently been linked to the proliferation of myelogenous leukaemia cells, these results suggest the effectiveness of inhibitory activity on cell viability/proliferation, alone generated a potent value of 250 nM (39.27 ± 1.17) for 48 h as optimal dose.

Results: The cytotoxic effect of bosutinib on the K562 cell line was assessed in vitro using the MTT assay, and the cytotoxicity was further clarified using cell viability and cell cycle assays. Guava Cell Assay software validated the activation of apoptosis. Sub-G1, G0/G1, S, and G2/M phases are depicted. Cell cycle research revealed that K562 cells treated with bosutinib accumulated much more in the sub-G1 phase, which was later validated by a drop peak at the G2/M phase.

Conclusion: In conclusion, the nature of bosutinib’s reduction of cancer cell growth may open the door to future research into the development of green synthesis medicines, particularly for cancer treatment.

1. Introduction

Bosutinib (SKI-606) is a second-generation tyrosine kinase inhibitors (TKIs) that inhibits the majority of imatinib-resistant BCR-ABL mutations (Gambacorti-Passerini et al., 2020). Chronic myelogenous leukemia (CML) is a hematopoietic stem cell malignancy that results from the t(9;22)(q34;q11) balanced reciprocal translocation of the Philadelphia (Ph) chromosome. This results in the production of the BCR-ABL oncogenic fusion gene, which produces the chimeric BCR-ABL protein with constitutive kinase activity (Lei et al., 2021). CML is a clonal myeloproliferative malignancy that develops in the stem cell compartment of the bone marrow and progresses through the three stages of the haemopoietic illness to acquired CML (Jiang et al., 2021). As a result, BA-harboring hematopoietic stem cells may contribute more myeloid cells to the peripheral blood than total blood myeloid cells. The TKIs are mostly used to treat CML. In the majority of cases, however, advanced-stage CML patients have difficulties with these
inhibitors. There still needs to be an extensive examination of the molecular etiology and potential treatment targets of CML (Chen et al., 2021). In terms of the CML condition in general, it is estimated that roughly 40% of CML patients are 60 years of age or older. Furthermore, 2.5 cases per million children, adolescents, and young adults are expected to acquire CML each year. Overall, the rise in CML occurrence can be attributed to therapeutic advances, with CML patients having the same life expectancy as the general population (Smith et al., 2021). Among the majority of individuals with CML, the BCR breakpoint cluster region contains the breakpoint in the BCR gene, producing two fusion transcripts e13a2 or e14a2. E13a2 encodes for a 210 kD protein, and e14a2 for a 245 kD protein. While this occurs seldom, it does happen when the breakpoint takes place within the smaller breakpoint region and leads to the e1a2 transcript, which encodes a 190 kDa protein (Verrou et al., 2021). In recent decades, a more targeted technique utilizing TKIs has been used in the treatment of newly diagnosed patients with chronic-phase CML. BCR-ABL kinase-activated downstream signaling is halted by the action of TKIs, which bind to the BCR-ABL kinase and interrupt the regulatory and constitutively active kinase-activated downstream signaling.

Imatinib (formerly known as ST571/CGP57158B) was the first TKI to receive approval for the treatment and outcome of CML patients. A landmark International Randomized Study of Interferon Plus Cytarabine vs ST571 compared the effectiveness and tolerability of imatinib to the combination of interferon-α plus cytarabine, which had been ineffective in doing so. CML patients who were randomly assigned to receive imatinib plus another combination therapy had significantly better progression-free survival (PFS: 97% vs 80%; P < 0.001) and estimated rates of independence from progression to accelerated or blast phase illness (99% vs 93%; P < 0.001). Monitoring techniques and definitions of response were devised after long-term follow-up of participants in the IRIS trial and applied to patients diagnosed with CML (Vigil et al., 2011).

Bosutinib and dasatinib are TKIs that target a number of kinases, including SRC and ABL kinases by both competitive and noncompetitive mechanisms, as well as TEC family kinases, such as BTK, has been demonstrated to have immunomodulatory effects (Rensing Rix et al., 2009).

Methyl vanillate was used as the starting compound to synthesize an inhibitor of the Src kinase family, a low-weight (548.46 kDa) small molecule inhibitor, which is orally accessible. In vitro and in vivo studies, bosutinib was found to be a powerful inhibitor of CML cell proliferation, with positive outcomes shown in the ongoing phase I/II clinical trials for treating CML patients with an intolerance or resistance to inhibitor imatinib (Keller et al., 2010). For Philadelphia chromosome-positive CML, the US Food and Drug Administration (FDA) has approved bosutinib as a first-line treatment (Keller-von Amsberg and Schafhausen, 2013).

Boc-D-FMK (Boc-Asp (OMe)-fluoromethyl ketone) was tested as a caspase inhibitor in chorion cells to analyze how it affected LDH leakage, apoptosis, and viral growth (Uchide et al., 2009). Boc-D-FMK completely inhibited all apoptotic biochemical and ultrastructural changes. Boc-D-FMK is a two-cell permeable, broad-spectrum caspase inhibitor that reduces apoptosis caused by a variety of stressors (Segovia and Berges, 2009). Taken together, our findings suggest that the effect of bosutinib co-treated with Boc-D-FMK effectively suppressed proliferation and induced apoptosis in K562 cells to a lesser extent, implying that bosutinib is an efficient drug used for leukemia, and that its combination with Boc-D-FMK is a mild chemotherapeutic agent against leukemia.

The purpose of this study was to analyze if bosutinib combined with Boc-D-FMK effectively suppressed proliferation and induced apoptosis in K562 cells to a lesser extent, implying that bosutinib is an effective leukemia treatment and that its combination with Boc-D-FMK is a mild chemotherapeutic agent against leukemia.

2. Materials and methods

2.1. Kinase and caspase inhibitors

Bosutinib (Cat. No. HY-10158), was purchased from MedChem-Express (LLC, Princeton, NJ, USA). These stock solutions were produced with bosutinib at the 150 μM concentration and stored in 10 μl portions at −20 °C. Because of this, final working doses for in vitro studies were obtained by repeated dilution of aliquots, as indicated by text. (Boc-Asp (OMe)-fluoromethyl ketone) Boc-D-FMK (Cat. No. HY-13229), was purchased from MedChemExpress (LLC, Princeton, NJ, USA). DMSO was used as a stock solution, which contained 20 mM of the compound. The 20 μM was utilized (1 μl/ml) and was stable for six months at −20 °C.

2.2. Reagents

Propidium iodide (PI) (Cat. No. P4170) was obtained from Merck Life Science UK Limited, UK. Ribonuclease A solution (RNase A) (Cat. No.19101) was obtained from Qiagen, Valencia, CA. It is provided in a solution containing 50% glycerol and 10 mM Tris-HCl, PH 8.0, and is preserved at −20 °C. Millipore, Watford, UK, provided the ViaCount reagent kit.

2.3. Culturing cellular lines and conditions

Human leukemic cells K562 with BCR-ABL were acquired from the ATCC (Manassas, VA). Normal culture flasks with PRMI-1640 growth media containing 1% penicillin/streptomycin and 2 mM L-glutamine and supplemented with 10% (v/v) (Heat inactivated FBS) were used to maintain K562 cells. They were kept at 37 °C in a humidified environment with 5% CO2. Every two days, a ritual called a passage was held. Approximately one million cells per milliliter were planted. Three times a week, the cells were washed twice with PBS, trypsinized with 0.1% of trypsin, 0.1% EDTA, and passed at 1:3 ratio.

2.4. Drug treatment

To determine the concentration of bosutinib that inhibited cell proliferation by 50% (IC50). 5 × 105 cells/well were seeded in a 96-well microtiter plates and incubated for 24 h in a humidified incubator of 5% CO2. Cells were treated the following day with the bosutinib at varied doses (50, 100, 150, 200, 250, 500 nM) and cultivated the cells for (6, 12, 24, 48, 72 h), to determine cytotoxicity in an end-point assay. Cells were compared with a negative control that was incubated in the absence of inducing agents. For cell viability and cell cycle assays, 250 nM of bosutinib with or without Boc-D-FMK at 5 μM was used for 48 h on the cells.

2.5. Measurement of cell viability (MTT assay)

The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay was used to investigate the effect of tyrosine kinase inhibitors (TKIs), bosutinib on CML cellular growth. Briefly, K562 cells (1 × 105 cells/well) were cultured on 96-well plates in 0.2 ml of medium/well. Each well was loaded with 10 μl (5 mg/ml) of the solution to which MTT was added and placed in a 5% CO2 incubator at 37 °C for 4 h for testing for cytotoxicity. The formazan crystals in the wells were dissolved by combining the cells with 100 μl of the detergent reagent of DMSO. The optical density (OD) values were read from a microplate reader (Thermo, Multiskan EX, UK) at an absorption wavelength of 570 nm to determine cell proliferation. The means of triplicate culture readings were compared to controls and calculated as percentage of the mean.
(taken as 100%). For following experiments, the cell viability was plotted on a graph and the IC<sub>50</sub> was computed based on this data. Concentrations were tested and a 50% reduction of cell viability (IC<sub>50</sub>) was calculated graphically. Concentration of the drug in the X-axis and relative cell viability in the Y-axis were used to construct the standard graph.

2.6. Flow cytometry (Viability assay)

The ViaCount Assay was used to obtain viable cell counts (Guava Technologies). K562 cells (about 3 x 10<sup>4</sup>) were seeded and plated on 24-well plates with 2 ml of growth media, then cultured for 24 h in a humidified incubator with 5% CO<sub>2</sub>. Following the incubation period, stained cell samples were generated by harvesting and combining 25 µl of cells with 225 µl of Guava ViaCount<sup>TM</sup> Reagent and incubated in a 96-well plate for 5 min at room temperature, as directed by the manufacturer. To guarantee high specificity, cytometer acquisition was performed at 100 µl/min on a total of 100,000 events/run. The cytometer’s voltage was always calibrated using an unstained control sample. The Guava ViaCount software was used to calculate cell viability percentages from histograms.

2.7. Flow cytometry (cell cycle assay)

Flow cytometry was used to perform cell cycle analysis on PI-stained cells, and the results were reported in flow cytometry and cell cycle analysis on PI-stained cells. Briefly, K562 cells were plated and incubated for 24 h. After being exposed to 250 nM of bosutinib with or without 5 µM of Boc-D-FMK for 48 h, the cells were collected, fixed and permeabilized with ice cold 70% ethanol and stored overnight at 4 °C. Cells were first washed in PBS and then plated on 96-well plates containing 100 µl of cells and PBS. The staining solution also included 25 µl of PI staining buffer (a solution of 1 mg/ml PI in PBS and 1 mg/ml Ribonuclease A (RNase A) in 1% Triton/PBS at a ratio of 1:9). Samples were incubated at room temperature in the dark with the shield to minimize light exposure for 30 min. To ensure high quality samples, samples were collected on the Guava Cell cycle (Guava Technologies) equipment. In order to ensure high specificity, cytometer acquisition was done at 100 µl/min, using a total of 100,000 events/run. Because the cytometer is able to calibrate its voltage using the unstained, control sample, the cytometer’s voltage calibration is always performed. Using the Guava Cell Cycle Analysis software, the percentages of the cell population in the G0/G1, S, and G2/M phases were determined by using histograms.

3. Results

3.1. Bosutinib inhibits CML cell proliferation in a dose-dependent manner

The cytotoxicity assessment of bosutinib was evaluated by MTT assay in CML cells by monitoring the IC<sub>50</sub> values, the suboptimal experimental parameters on dose response was estimated in K562 cell line treated with bosutinib with different concentrations (50, 100, 150, 200, 250, 500 nM) and cultured for (6, 12, 24, 48, 72 h). Table 1 and Fig. 1 show the dose–response curve, which was generated using ggplot2 (version 3.2.1). Bosutinib treated cells led to growth inhibition in dose dependent manner (Fig. 1). Among 7 evaluated concentrations, 250 nM of bosutinib met this criterion and had its IC<sub>50</sub> values determined after time course of incubation. As it shows in Table 1, the bosutinib showed different cytotoxicity values. The IC<sub>50</sub> values for bosutinib treatment for 6, 12, 24, 48 and 72 h post-treatment increased with increasing doses of untreated viable cells. (IC<sub>50</sub> = 32.40 for 50 nM, 37.26 for 100 nM, 41.19 for 150 nM, 51.67 for 200 nM, 60.40 for 250 nM and 96.78 for 250 nM).

3.2. Bosutinib and Boc-D-FMK inhibition affects K562 cell viability

Cell viability analysis was conducted to investigate the mechanism of bosutinib effect on the K562 viability. K562 cell populations harvested after 48 h of incubation with doses of different treatments with the Guava ViaCount flow cytometer; 5 µM of Boc-D-FMK, 250 nM of BOS, and BOS/Boc-D-FMK. The Guava ViaCount software was used to compute cell viability percentages from histograms. The results showed that the bosutinib in the presence or absence of Boc-D-FMK significantly decreased cell viability (Fig. 2). After 48 h incubation, the cytotoxic behavior of the different treatments observed in Fig. 2A, showed loss of cell viability, the rates of apoptosis in cell lines treated with (250 nM BOS alone or in combination with 5 µM of Boc-D-FMK) showed increased remarkably compared to untreated cells.

At the IC<sub>50</sub> concentrations tested, bosutinib in the presence or absence of Boc-D-FMK increased the number of cells in apoptosis. Bosutinib alone significantly increased the number of cells in early apoptosis (P < 0.001), where 34.1% of the cells were in early apoptosis. The combination of BOS/Boc-D-FMK significantly induced cell death following 48 h exposure to IC<sub>50</sub> concentration in K562 cell line (42.72%, P < 0.001).

Untreated control: Cell viability % (84.41 ± 2.92%), Boc-D-FMK (81.67 ± 3.94%), BOS (62.78 ± 3.95%) and BOS + Boc-D-FMK (54.02 ± 3.25%), hence decreased percentage of viable cells were seen with 250 nM BOS alone or in combination with 5 µM Boc-D-FMK, co-treatment of BOS with Boc-D-FMK induced more apoptosis (P < 0.001).

3.3. K562 cells in G1 are arrested by combining bosutinib and Boc-D-FMK, depending on the drug concentration

In order to improve the study of the mechanism of death induction by bosutinib in K562 cells, flow cytometry was used to assess the effect of the bosutinib combining with Boc-D-FMK on cell cycle progression based on the DNA content of propidium iodide-labelled cells (Fig. 3).

In Fig. 3A, Cell cycle arrest and apoptosis assay, using the Guava Cell Cycle Analysis software, based on histograms, it was possible to estimate the percentages of cells in G0/G1, S, and G2/M phases. Fig. 3B shows the cell cycle phase distribution after 48 h; as a percentage, three separate experiments were used to calculate the average percentage of cell cycle stages mean ± SEM.

Bosutinib produced cell cycle arrest and death in CML cells based on the BOS/Boc-D-FMK values obtained. After 48 h incubation without treatment, (44.94 ± 2.02%) of the cells were in the G1 phase. At the concentration of 5 µM of Boc-D-FMK, the population in the G1 phase slightly increased to (48.66 ± 1.86%). Treatment with 250 nM of BOS alone and in combination with 5 µM Boc-D-FMK significantly increased the percentage of cells in the G1 phase of cell cycle by (74.27 ± 1.54; 71.31 ± 1.56%) respectively, when compared to non-treated (control) cells. There was a highly significant decrease of cells when treated with bosutinib alone and in combination with Boc-D-FMK in both G1 and G2/M phase. The decrease peak of BOS and BOS/Boc-D-FMK was significantly shown in Fig. 3A. No effect on cell cycle arrest was observed in cells treated with 5 µM of Boc-D-FMK at 48 h in all cell cycle phases. These results indicate that bosutinib alone or in combination with Boc-D-FMK causes cell cycle arrest in G1 phase.
4. Discussion

CML is a clonal stem cell disorder that is caused by the onco-
genic fusion protein BCR-ABL. As a result of the BCR-ABL TKIs, CML patients now have a life expectancy comparable to that of the general population. Approximately half of patients who obtained a profound molecular response can now achieve and retain a molecular remission after stopping the TKIs (Ureshino et al., 2020).

Table 1
Mean and IC_{50} values of bosutinib determined with the MTT assay after time course incubation in K562 cells.

|          | 6 hrs Mean nM ± SD | 12 hrs Mean nM ± SD | 24 hrs Mean nM ± SD | 48 hrs Mean nM ± SD | 72 hrs Mean nM ± SD | IC_{50} nM |
|----------|---------------------|----------------------|---------------------|---------------------|---------------------|-------------|
| 50 nM    | 94.58 ± 1.70        | 91.87 ± 0.69         | 83.32 ± 0.77        | 76.93 ± 1.31        | 74.40 ± 0.34        | 32.40       |
| 100 nM   | 94.58 ± 3.70        | 89.56 ± 2.70         | 76.29 ± 1.58        | 65.50 ± 1.51        | 61.73 ± 0.34        | 37.26       |
| 150 nM   | 90.69 ± 1.12        | 86.84 ± 0.84         | 64.31 ± 0.87        | 58.98 ± 0.71        | 56.18 ± 0.26        | 41.19       |
| 200 nM   | 90.69 ± 1.12        | 79.51 ± 1.73         | 53.79 ± 1.18        | 47.12 ± 1.59        | 40.62 ± 0.97        | 51.67       |
| 250 nM   | 86.13 ± 1.81        | 76.38 ± 1.98         | 43.99 ± 1.27        | 39.27 ± 1.17        | 33.99 ± 0.84        | 60.40       |
| 500 nM   | 72.12 ± 1.94        | 39.67 ± 1.19         | 31.06 ± 1.51        | 22.68 ± 1.23        | 21.04 ± 0.88        | 96.78       |

In Fig. 1, cell viability generated after optimization the result of bosutinib on CML cell viability assay for different time period representative dot plot of viability. According to tradition, IC_{50} is used in cell-based cytotoxicity investigations in order to measure drug potency to quantify medication efficacy in cell-based cytotoxicity studies, IC_{50} is often utilized. New important experimental parameters were identified such as control for solvent concentration and medication storage that are matched. According to the IC_{50} values of bosutinib (60.40), at 250 nM for 48 h was taken as an optimal dose. Using Quality Control Metrics, on the other hand, increases the chances of identifying hits inside studies. A careful examination of assay optimization and potency estimation may also aid to boost the success rates of cancer therapy candidates that approach clinical trials during their preclinical drug screening phase.

When comparing this sensitivity to that of other studies, it’s worth mentioning with other cell lines were bosutinib is sensitive towards neuroblastoma IMR-32 cells. The SK-N-AS cell, on the other hand, displayed resistance to bosutinib treatment with an IC_{50} of 11.26 μg (Bieerkehazhi et al., 2017). Bosutinib also inhibited to high degree in epidermal growth factor with an IC_{50} of 53 nM than; dasatinib 322 nM (Remsing Rix et al., 2009). However; bosutinib with /C20 l showed little inhibitory activity on cell viability, but with the combination of dasatinib with IC_{50} values of <100 nM enhanced cell proliferation in four cell lines; MDA-MB-231, HCC1806, HCC1143 and Hs578T (Tarpley et al., 2014). As a first-line treatment for chronic-phase CML; bosutinib was also effective (Cortes et al., 2018). Suggesting bosutinib could be viable candidate for leukemia treatment when used with further drug combination.
In addition, Guava ViaCount flow cytometry's examination of cell viability confirms the MTT assay's findings regarding the cytotoxic effects of different treatments. Viability was determined by flow cytometry after the dose-dependent MTT assay, followed by cell cycle arrest and apoptosis assays in CML cells with flow cytometry. The percentage of viable cells determined by flow cytometry gradually reduced with the treatment of bosutinib/Boc-D-FMK (57.2%) and with increased apoptotic cell death (42.7%). Bosutinib drug showed viable cell (65.90%) with apoptotic cells (34.1%) with incubation time for 48 h. Similarly, bosutinib drug showed enhanced cell proliferation in combination of dasatinib in TNBC cell lines (Tarpley et al., 2014), showed viable cell. Alone Boc-D-FMK viable cells (84.80%) with apoptotic cell (15.2%) was observed, in previous studies necroptosis was seen in Boc-D-FMK with Zvad-fmk in L929 cells (Wu et al., 2011). According to the IC₅₀ values, K562 cell lines are more sensitive to bosutinib used in combination with Boc-D-FMK at the indicated concentration for 48 h (Fig. 2B). Using histograms and the Guava ViaCount software, we calculated the percentages of cell viability. This was determined by using the t-test for statistical significance.
The cell cycle analysis by flow cytometry were described as the results of experiments to evaluate K562 cell lines treated with various treatments that interfere with the cell cycle, Using the Guava Cell Cycle Analysis software, the percentages of the cell population in sub-G1, G0/G1, S, and G2/M phases were estimated from histograms. According to this study, the G1 population increased while the cell population decreased during the S and G2 phases. Thus, the therapy may interfere with cell proliferation and cell cycle dynamics, according to this study’s findings. These cells induce morphological alterations leading to cell death and a large rise in the sub-G1 population in DNA histogram, indicating apoptosis with involvement of intrinsic pathways when exposed to BOS followed by BOS/Boc-D-FMK. A high apoptotic peak was identified in BOS followed by BOS/Boc-D-FMK compared to control and Boc-D-FMK. As a result of cell cycle arrest in the G0/G1 phase, the percentage of cells in the S and G2/M phases decreased. The BOS, BOS/Boc-D-FMK, Boc-D-FMK, and untreated control K652 cells showed the largest decreases in cell population percentages.

There are a number of proteins involved in cell cycle progression, including cyclins, CDKs, E3 ubiquitin ligases, CDK activating kinase (CDAK) and CDK inhibitors (Ding et al., 2020). As a result of apoptosis, DNA fragmentation occurs in the inter-nucleosomal region. Due to the removal of small DNA fragments during cell labeling in aqueous solutions, cells that are apoptotic can be recognized on DNA content frequency histograms as cells with a fractional DNA content (“sub-G1”) (Kajstura et al., 2007).

Bosutinib, when used alone or in combination with other chemotherapeutic drugs like vincristine, may be a successful therapeutic option for people with non-Hodgkin lymphoma (Bieerkehazhi et al., 2017). As soon as 24 h had passed, Boc-D-FMK revealed cytoplasmic blebbing (Segovia and Berges, 2009). CML K562 cancer cells were found to be less proliferative when exposed to bosutinib, resulting in a cell cycle arrest at G2/M phase and apoptotic cell death.

5. Conclusion

The conclusion of this study is that bosutinib (originally developed as an SRC inhibitor), which is now being studied in clinical trials for the treatment of CML patients, is effective. The K562 cell lines were shown to be cytotoxic towards bosutinib and have also been determined to be apoptotic, as demonstrated by cell viability and cell arrest assays. This study also concludes that bosutinib induces apoptosis (an indication of apoptosis-inducing apoptosis) during the cell cycle’s G2/M phase. Several experiments have shown that the bosutinib medicine is apoptogenic. Bosutinib has the potential to be developed for the treatment of leukemias. A greater understanding of the mechanisms of action will help in the generation of critical information for use in cancer prevention, cancer therapy, and other disorders. Future studies are advised to expand the current study for better understand the efficacy of bosutinib.

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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Declarations

Author declare that all works are original, and this manuscript has not been published in any other journal.

Data Availability

All data sheet available on request from corresponding author.

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