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ORIGINAL PAPER

Therapeutic natural compounds Enzastaurin and Palbociclib inhibit MASTL kinase activity preventing breast cancer cell proliferation

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Abstract

Microtubule-associated serine/threonine kinase-like (MASTL) regulates mitotic progression and is an attractive target for the development of new anticancer drugs. In this study, novel inhibitory molecules were screened against MASTL kinase, a protein involved in cell proliferation in breast cancer. Natural source-derived drugs Enzastaurin and Palbociclib were selected to identify their role as MASTL kinase inhibitors. Cytotoxic activity, kinase activity, and other cell-based assays of Enzastaurin and Palbociclib were evaluated on human breast cancer (MCF-7) cells. The potential natural compounds caused cytotoxicity in MCF-7 cells in a dose- and time-dependent manner. Further analysis by Annexin V and PI staining indicated that both drugs are potent inducers of apoptosis. Enzastaurin induced G2/M phase arrest, while Palbociclib caused G1 arrest. MASTL kinase activity was significantly abrogated with both the compounds showing EC_{50} values of 17.13 µM and 10.51 µM, respectively. Taken together, these data strongly suggest that Enzastaurin and Palbociclib possess the ability to inhibit MASTL kinase activity and induce cell death in breast cancer cells, thus exhibiting significant therapeutic potential.

Keywords Breast cancer · Microtubule-associated serine/threonine kinase (MASTL) · Drug discovery · Therapeutics · In vitro kinase assay

Abbreviations

MASTL	Microtubule-associated serine/threonine kinase
GWL	Great wall kinase
ENSA	Endosulfine Alpha
CDK1	Cyclin-dependent kinase 1
PI	Propidium iodide
IC_{50}	Inhibitory concentration
EC_{50}	Effective concentration

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Introduction

The Serine/Threonine Protein Kinase subfamily which includes AGC kinases and Microtubule-associated serine/threonine kinase (MASTL) or Great wall kinase (GWL) is a member of the AGC family of kinases. The huge number of proteins that AGC kinases can phosphorylate demonstrates their importance in a variety of cellular activities. Serine/threonine kinases are essential regulators of cell adhesion and contraction, which are important for cancer growth and metastasis [1]. Many human disorders, including cancer, are caused by mutations or dysregulation of AGC kinases, which mediate a wide range of critical cellular processes [2].

MASTL is a unique AGC kinase that lacks a hydrophobic motif, unlike most AGC kinases, despite the presence of a hydrophobic pocket that specifies its particular method of regulation [3]. It features a unique T-loop region with insertion of roughly 500 amino acids. It has received far less attention as compared to other AGC kinases. Furthermore, in immortalized normal breast epithelial cells, overexpression

of MASTL slows cell cycle progression, causes abnormal cell division, DNA damage response, affects migration, the actin cytoskeleton, and cell–cell junctions, tumor resistance in response to anticancer treatments, and leads to enhanced invasion and metastasis in vitro and in vivo, leading to cancer development [4]. Additionally, MASTL triggered mitotic cell death in a variety of cancer cells, while normal cells were less affected [5]. It is known to be a key player in the cell division, growth, metabolism, and differentiation. It accelerates the cell cycle progression by phosphorylating Endosulfine Alpha (ENSA) and Arpp19, which limits PP2A-B55 phosphatase activity and hence maintains the phosphorylated status of Cyclin-dependent kinase 1 (CDK1) substrates [6]. MASTL is phosphorylated during mitosis which is an essential requirement for its activation [7, 8]. MASTL inhibition of PP2A-B55 is essential to sustain the mitotic state during cell division, whereas MASTL inactivation and PP2A reactivation are required for mitotic departure [9]. An overview of MASTL functioning in breast cancer cells is depicted in Fig. 1. In vitro and in vivo studies in breast cancer found that inhibiting MASTL reduced tumor development and metastasis. These findings suggest that MASTL is a new breast cancer oncogene that may overcome contact inhibition, invasion, and chromosomal instability [10]. Overall, the evidence revealed that MASTL can be a promising target for selective anticancer treatment [11].

Researchers have focused on the role of naturally originating drugs in anticancer treatment due to the limited number of adverse effects and the wide range of targets of naturally derived components [12]. Till date, a few studies have reported MASTL inhibitors, like GK-1, MKI-1, MKI-2, and a thienopyrimidinone-based tricyclic derivative. These have been identified and validated using virtual screening and in vitro analysis GKI-1 inhibited MASTL in vitro and interrupted mitotic events by lowering phosphorylated ENSA with µM range potency in HeLa cells. Moreover, in other breast cancer cells, GKI-1 exhibited negligible anticancer effect [13]. In in vitro and in vivo models of breast cancer, MKI-1 was found to have anticancer and radiosensitizer properties. MKI-1 demonstrated µM range potency and efficacy for MASTL inhibition. In addition, MKI-1 reduced the amount of c-Myc protein in breast cancer cells through increasing PP2A activity [14]. Recently, it was found that MKI-2 decreased recombinant MASTL activity and induced mitotic catastrophe in breast cancer cells through modulating the MASTL-PP2A axis. In mouse oocytes that were employed as a model to validate MKI-2 activity, the MKI-2 treatment displayed phenocopies with MASTL-null oocytes. MKI-2 inhibited MASTL in breast cancer cells with potency and effectiveness in the nM range [15]. All these identified MASTL inhibitors are synthetic and their toxicity profiles and long-term effects are yet to be studied. Our group has also previously identified various compounds from both natural and synthetic sources, ZINC85597499 and ZINC53845290 using virtual screening that proved to be significant leads for further experimental validation [16]. Recent probe-based approaches were used for understanding the effect of Palbociclib in breast cancer cells. It was observed that Palbociclib involved only Cdk4 or Cdk6 in sensitive cells with no effect in the resistant cells. Further, Palbociclib incubation for 24 h affected many other kinases involved in cell cycle progression [17].

The present study is focused on the identification of new MASTL inhibitors from natural sources. The Enzastaurin and Palbociclib were some of the leads obtained from our previous in silico work that are from natural source and have been validated as MASTL kinase inhibitors using in vitro kinase assay in the present study. These compounds will

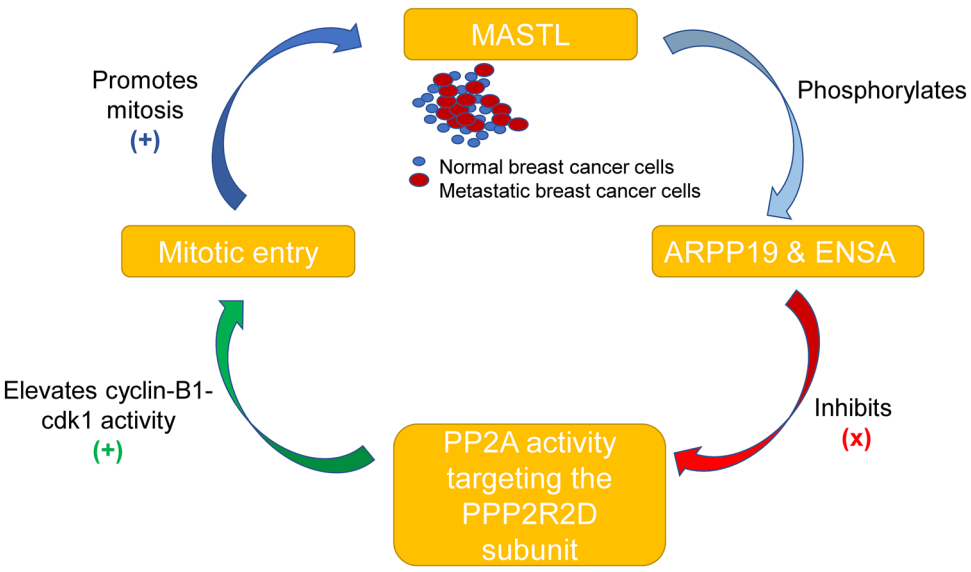

Fig. 1 Overview of MASTL functioning in breast cancer cells

be less toxic to the normal cells. These compounds demonstrated an anti-proliferative effect on breast cancer cells. Both the compounds exhibited potency and inhibition efficacy in the μM range (1.56 to 100 μM). In addition, we investigated the influence on cell cycle progression and apoptosis as an anti-proliferative effect was observed in MCF-7 cells treated with these natural products. Cell cycle study revealed that the Enzastaurin and Palbociclib are capable of arresting the cells in G2/M and G1 phases, respectively. Thus, in the present study we have identified naturally derived compounds Enzastaurin and Palbociclib as novel MASTL kinase inhibitors with significant antitumor effect as potential therapeutic leads against breast cancer that can further be validated in animal models.

Methods

Chemicals

The compounds, namely, Enzastaurin and Palbociclib were purchased commercially from Cayman chemicals. The physicochemical properties of the naturally derived compounds Enzastaurin and Palbociclib were calculated using the Swiss ADME server [18].

Cell culture

MCF-7 cell line purchased from NCCS, Pune was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution. The cell line was maintained in a water-jacketed CO_2 incubator (Forma series 2, Thermo Fisher, India) at 37 °C and 5% CO_2. To ensure culture was free from mycoplasma contamination, cells were tested and authenticated. The cells were sub-cultured routinely, every three to four days [19].

Luminescence-based in vitro kinase assay

MASTL kinase enzyme system kit manufactured by Promega Corporation was used to perform this assay and protocol was followed as per the manufacturer's specifications. The MASTL kinase inhibitor, Enzastaurin (1.56 to 50 μM) and Palbociclib (1.56 to 50 μM) along with MASTL kinase (25 ng), substrate (0.1 μg/μl), and ATP (10 μM) were diluted separately in 1X kinase buffer. All the solutions were mixed in a 96-well plate, solid white, round bottom, and low volume (Corning, India). After incubating the mixture at room temperature for 1 h, 25 μl of ADP-Glo Reagent were added and incubated at room temperature for 40 min to terminate the kinase reaction. This was followed by adding 50 μl of Kinase detection reagent to convert ADP to ATP. The solution was further incubated at room temperature for 30 min. After incubation, the newly synthesized ATP was measured as luminescence value in a multimode microplate reader (Spark, Tecan Lifesciences) [20].

Cell viability assay

MCF-7 (1×10^4) cells at 70–80% confluency was plated in triplicates in 96-well plate (Costar, Corning, India). Cells were allowed to adhere for 24 h in 100 μl of DMEM per well. Cells were treated with Enzastaurin and Palbociclib in a dose-dependent manner (1.56 to 25 μM) for 24 h in CO_2 incubator. Cells without any drug treatment was used as control. After 24 h of incubation, complete media were removed and cells were washed with 1X PBS. 20 μl MTT (5 mg/ml) was added and further incubated for 2 h [21]. MTT was aspirated and formazan produced was dissolved in 100 μl of 0.1% DMSO. OD (optical density) values were taken at 570 nm (630 nm as a reference wavelength) using DMSO as blank.

Annexin V/Propidium iodide apoptosis assay

MCF-7 (2×10^5) cells were seeded per well in a 6-well plate in 2 ml media (in triplicates) overnight. Cells were treated with Enzastaurin (6.25 to 25 μM) and Palbociclib (3.12 to 12.5 μM) in a dose-dependent manner for 24 h. The assay was performed using the Annexin V/Propidium iodide apoptosis assay kit (V13241, Invitrogen) according to the manufacturer's protocol [22]. Flow cytometry was performed on a BD FACS Lyric flow cytometer (BD Biosciences, India). Data were generated for 10,000 events per sample and analyzed using BD FACS Suite software (BD Biosciences, India) [23].

Cell cycle assay

MCF-7 (2×10^5/2 ml) cells plated in a 6-well plate were treated with Enzastaurin (6.25 to 25 μM) and Palbociclib (3.12 to 12.5 μM) for 24 h. Cells were washed in chilled1X PBS after trypsinization. Cells were fixed in 70% ethanol for 30 min at 4 °C. The centrifuged cell pellet after incubation was suspended in 1X PBS. Further, cell pellet was resuspended in 50 μg/ml of propidium iodide and 10 μg/ml of RNAse A prepared in 1X PBS. The cells were incubated in dark for 30 min before data acquisition [24]. Further, samples were examined on a BD FACS Lyric flow cytometer (BD Biosciences, India). Data analysis was performed using BD FACS Suite software (BD Biosciences, India) with 10,000 events per sample.

The raw data of apoptosis and cell cycle analysis are provided as Supplementary Figs. 1 and 2.

Results

MASTL kinase assay of novel inhibitors

The effects of natural drugs on MASTL activity were investigated by in vitro kinase assay using ADP-Glo luminescent assay kit. A significant decrease was observed in the luminescence values of MASTL kinase activity with an increase in the concentration of drugs as shown in Fig. 2a.

The luminescent signal generated was correlated with the kinase activity. Incubation of MASTL kinase with 50 µM concentration of Enzastaurin and Palbociclib, resulted in 66% and 71% of inhibition. This clearly reflects that MASTL kinase activity decreased with an increase in the concentration of both the compounds as compared to control.

The EC_{50} (effective concentration) value for inhibitors was calculated from the percent (%) kinase activity values using the GraphPad Prism software. Thus, the luminescence-based in vitro kinase assay established EC_{50} for Enzastaurin and Palbociclib as 17.13 µM and 10.51 µM, respectively (Fig. 2b). This signifies that Palbociclib exhibits a better inhibitory kinase activity against MASTL.

Enzastaurin and Palbociclib inhibited proliferation of MCF-7 cells

The cytotoxic effect of Enzastaurin and Palbociclib inhibitors from natural sources on MCF-7 cells was monitored by MTT assay. Cells treated with compounds for 48 h in a dose-dependent manner showed a decrease in the percentage of cell viability (Fig. 3a). The cell viability decreased from 94.4% at 1.56 µM to 44.2% at 25 µM of Enzastaurin as compared to control (without drug treatment). On the other hand, the percentage of cell viability in Palbociclib-treated cells decreased from 93.7% at 1.56 µM to 4.4% at 25 µM. The IC_{50} value of Enzastaurin and Palbociclib was 19.18 µM and 5.22 µM, respectively (Fig. 3b and c). IC_{50} dose and sublethal doses for both the compounds were selected for further experimentation.

Enzastaurin and Palbociclib induced apoptotic cell death in MCF-7 cells

The effect of Serine/Threonine kinase inhibitors on apoptosis induction in MCF-7 cells was examined. MCF-7 cells were exposed to various concentrations of the compounds for 24 h and apoptosis was detected. Flow cytometric analysis with Annexin V and PI fluorescent staining was used to quantify the induction of apoptosis by natural inhibitors.

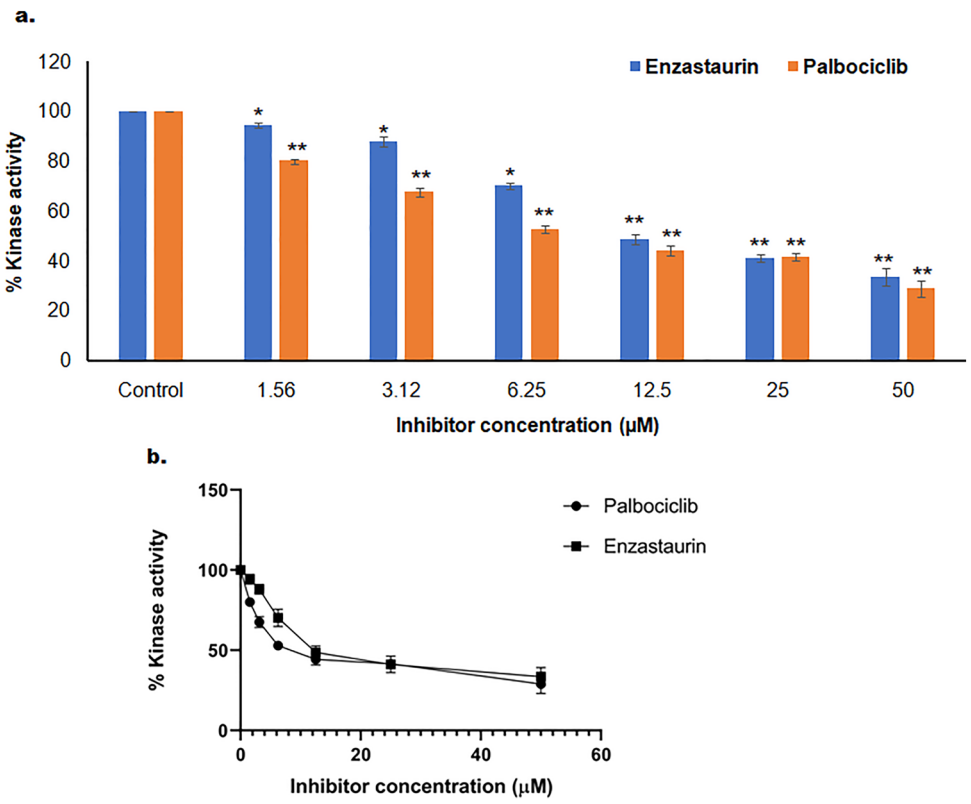

Fig. 2 In vitro MASTL kinase activity. **a** % inhibition of MASTL kinase activity in presence of inhibitors. **b** EC_{50} of Enzastaurin and Palbociclib against MASTL is 17.13 µM and 10.51 µM, respectively. Statistical significance *($p < 0.05$) and **($p < 0.001$) compared to control

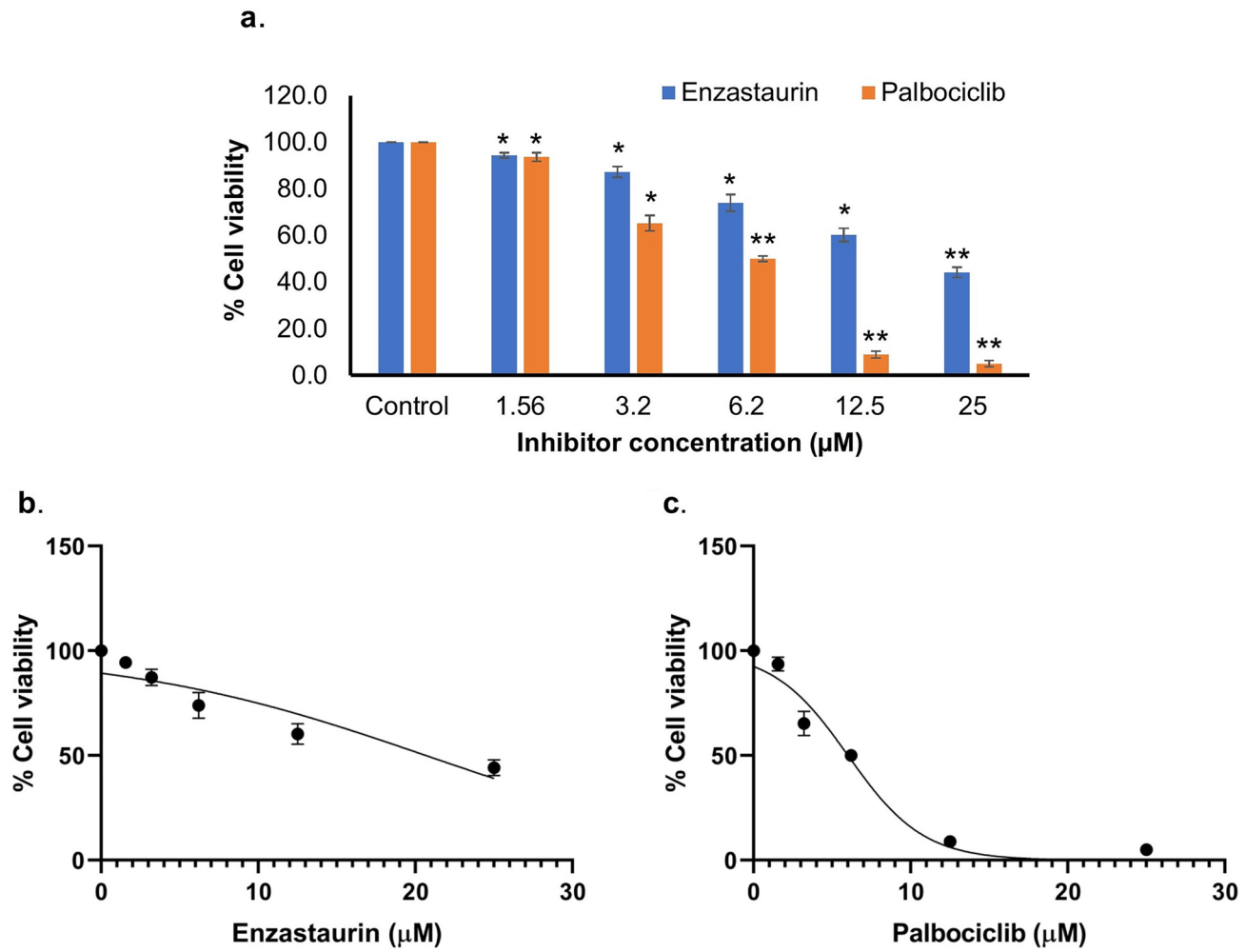

Fig. 3 MASTL inhibition induces cell death in breast cancer cells. **a** MCF-7 cells proliferation was suppressed by inhibitors in a dose-dependent manner as measured by MTT assay. **b** IC_{50} value of Enzastaurin—19.18 μM. **c** IC_{50} of Palbociclib—5.22 μM, respectively, was calculated using GraphPad prism software [24]. Statistical significance *($p < 0.05$) and **($p < 0.001$) compared to control

The number of apoptotic cells increased in a dose-dependent manner from 13.6% at 6.25 μM to 45.5% at 25 μM upon Enzastaurin treatment as compared to control cells. A similar increase in apoptotic cells was observed with Palbociclib, but the effect was less prominent in comparison to Enzastaurin, whereby 23.3% apoptosis was seen with 12.5 μM Palbociclib as depicted in Fig. 4. This signifies that the natural compounds are capable of inducing apoptosis in MCF-7 cells in a dose-dependent manner.

Newly identified natural compounds induce cell cycle arrest in MCF-7 cells

To evaluate the effect of natural compounds on cellular proliferation, the cell cycle distribution of MCF-7 cells was assessed. MCF-7 cells treated with Enzastaurin exhibited a significant increase in G2/M phase population depicting arrest in the G2/M phase, as revealed by cell cycle analysis, while Palbociclib arrested cells in G1 phase (Fig. 5a and b). Approximately, 24% of cells were arrested in G2/M at 25 μM Enzastaurin and 55% in G1 with 12.5 μM Palbociclib. Interestingly, Palbociclib exhibits a better inhibitory activity than Enzastaurin by inhibiting the cell cycle progression at the initial checkpoint itself.

Physiochemical properties of Enzastaurin and Palbociclib

The chemical Enzastaurin has a molecular weight of 515.6 g/mol, while Palbociclib has 447.53 g/mol of molecular weight. The chemical structure of Enzastaurin is depicted in Supplementary Fig. 3 and Palbociclib in Supplementary Fig. 4, respectively.

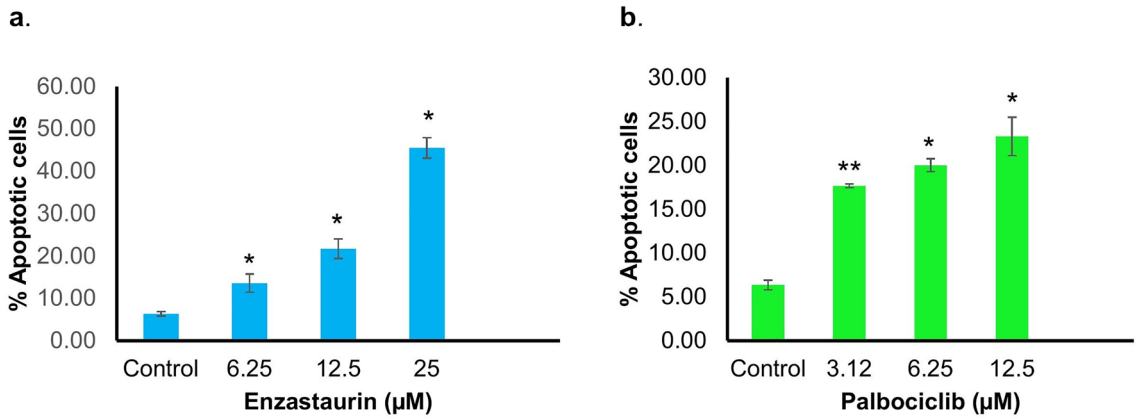

Fig. 4 Natural drugs induced dose-dependent apoptosis in MCF-7 cells. **a** Enzastaurin-treated and **b** Palbociclib-treated cells. Statistical significance *($p < 0.05$) and **($p < 0.001$) compared to control

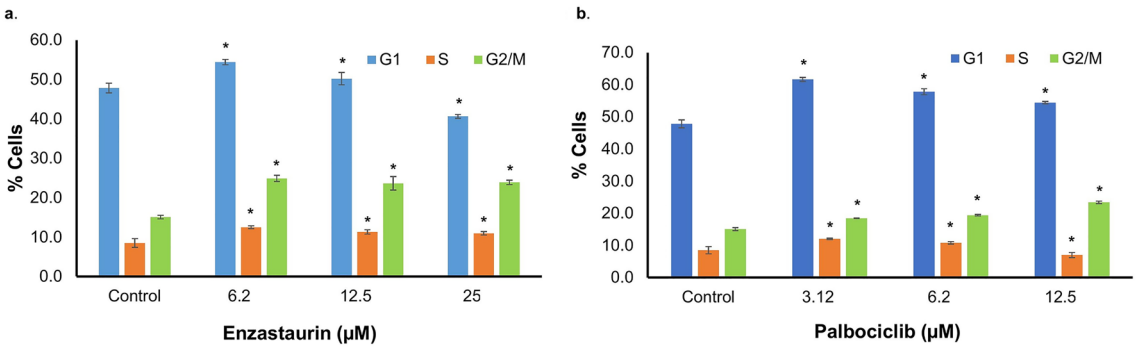

Fig. 5 The regulatory effect of natural inhibitors on cell cycle distribution in MCF-7 cells. **a** Enzastaurin and **b** Palbociclib inhibited cell cycle progression in MCF-7 cells. Statistical significance *($p < 0.05$) compared to control

An important property, the Molar refractivity (m^3/mol) of drugs was estimated to be 160.96 for Enzastaurin and 136.03 for Palbociclib. The available H-bond acceptor atoms in Enzastaurin are 4 and Palbociclib is 6. In addition, the H-bond donor atoms in Enzastaurin and Palbociclib are 1 and 2. The number of rotatable bonds in both compounds is 5. Further, the polar surface area ($Å^2$) of Enzastaurin was 72.16 and 105.04 for Palbociclib, respectively. The Lipophilicity (consensus logP) for Enzastaurin is 3.58, while Palbociclib shows 2.39.

Water Solubility properties of Enzastaurin calculated is ESOL − 5.48, solubility of 1.71e−03 mg/ml; 3.31e−06 mol/l; and of moderately soluble class; Ali − 4.89, solubility of $6.71e^{-02}$ mg/ml; 1.30e−05 mol/l; and of moderately soluble class; and SILICOS-IT − 9.18, solubility of 3.42e−07 mg/ml; 6.64e-10 mol/l; and of poorly soluble class. While for Palbociclib the water Solubility properties computed is ESOL − 3.78, solubility of 7.36e−02 mg/ml; 1.65e−04 mol/l; and of soluble class; Ali − 3.64, solubility of $1.04e^{-01}$ mg/ml; 2.32e−04 mol/l; and of soluble class; and SILICOS-IT − 6.49, solubility of $1.45e^{-04}$ mg/ml; 3.24e−07 mol/l; and of poorly soluble class.

Both the compounds exhibit drug-like tendency. The detailed physicochemical properties of these compounds are summarized in Supplementary Table 1.

Discussion

Studies investigated MASTL to be an important drug target for anticancer treatment due to its multifarious roles, such as cellular transformation, metastasis, chromosomal instability, and the DNA damage response in various cancers [10, 11, 25]. MASTL is an important therapeutic drug target in breast cancer. Till date, not much work has been done for the identification of inhibitors targeting MASTL. This is the first study where compounds from natural origin, namely, Enzastaurin and Palbociclib have been validated as MASTL inhibitors. Recent research to unravel the role of Spatiotemporal regulation in cell cycle progression has also emphasized the role of MASTL wherein a conserved MASTL (also known

as Gwl)–Endosulfine (Endos) and PP2A in vertebrates regulates mitotic entry. MASTL relocation to cytoplasm during prophase is responsible for phosphorylating Endos which thereafter inhibits PP2A-B55 before nuclear envelope breakdown thus ensuring correct mitotic entry [26].

Role of MASTL has also been now established as crucial for hepatocarcinogenesis. Liver cancer cell proliferation was mediated by TNF-α and IL-6 resulting in H3K4 trimethylation that causes activation of NF-κB pathway which thereby induces *MASTL* transcription [27].

MASTL is reported to be upregulated in several cancer cell lines, including breast cancer cells [4, 27–30]. MASTL upregulation is also strongly correlated with poor survival in breast cancer patients [31]. Furthermore, MASTL depletion induced cell death in MCF-7 breast cancer cells in response to irradiation [32]. These studies suggest the potential role of MASTL in cancer cells survival and proliferation. Hence, identifying the new natural inhibitors against MASTL will be an attractive strategy for cancer therapy. *In silico* screening and in vitro analyses have previously revealed GK-1, MKI-1, and MKI-2 as potential MASTL inhibitors. GK-1 and MKI-1 showed potency in μM range, while the inhibitory potential of MKI-2 was in nM range [13–15]. Previously, our group has also computationally identified and validated different inhibitors, ZINC85597499 and ZINC53845290, against this protein from both the natural and synthetic origin [16]. Till date, no natural MASTL inhibitor with antitumor activity has been discovered. Thus, in the current research, compounds of natural origin were chosen to target MASTL protein in the cancerous cells. Enzastaurin and Palbociclib were explored as MASTL inhibitors with anticancer activity against breast cancer cells. Enzastaurin and Palbociclib showed MASTL kinase inhibition in a dose-dependent manner with EC_{50} value of 17.13 μM and 10.51 μM, respectively. Further, we investigated the cytotoxic activities of these natural products on MCF-7 cell proliferation and found the IC_{50} values of Enzastaurin and Palbociclib to be 19.18 μM and 5.22 μM.

The compounds caused MCF-7 cells to arrest in different phases of cell cycle. Palbociclib, a mimic of natural product [33] remarkably, arrested cells in the G1 phase and on the other hand, Enzastaurin, an analog of Staurosporine isolated from Streptomyces sp. [34], showed G2/M phase arrest. Interestingly, Palbociclib significantly slowed cell cycle advancement in the G1 phase by suppressing the progression at the initial checkpoint. Further, our data showed that drugs were effective in inducing apoptosis in a dose-dependent way with respect to the control cells showing 45.5% and 23.3% of apoptotic cells at the highest dosage of Enzastaurin (25 μM) and Palbociclib (12.5 μM).

Therefore, our data strongly indicate Enzastaurin and Palbociclib as new natural inhibitors of MASTL kinase. In addition, we also observed anticancer activity with both the compounds as they inhibited cell proliferation and induced apoptosis in breast cancer cells. Further, animal-based studies with these compounds will provide mechanistic insights and establish these compounds as promising leads for breast cancer treatment.

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Author contributions GM contributed to conceptualization, methodology, and manuscript compilation; AP contributed to data curation, methodology, and manuscript compilation; JR contributed to methodology and manuscript compilation; AK contributed to software and data curation; Harpreet Singh performed data analysis; ANB performed data analysis.

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Data availability Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Conflict of interest There are no potential conflicts of interest declared by the authors. The authors have no relevant financial or non-financial interests to disclose.

Ethical approval The manuscript does not contain clinical studies or patient data.

Consent to participate Not applicable.

Consent to publish Not applicable.

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