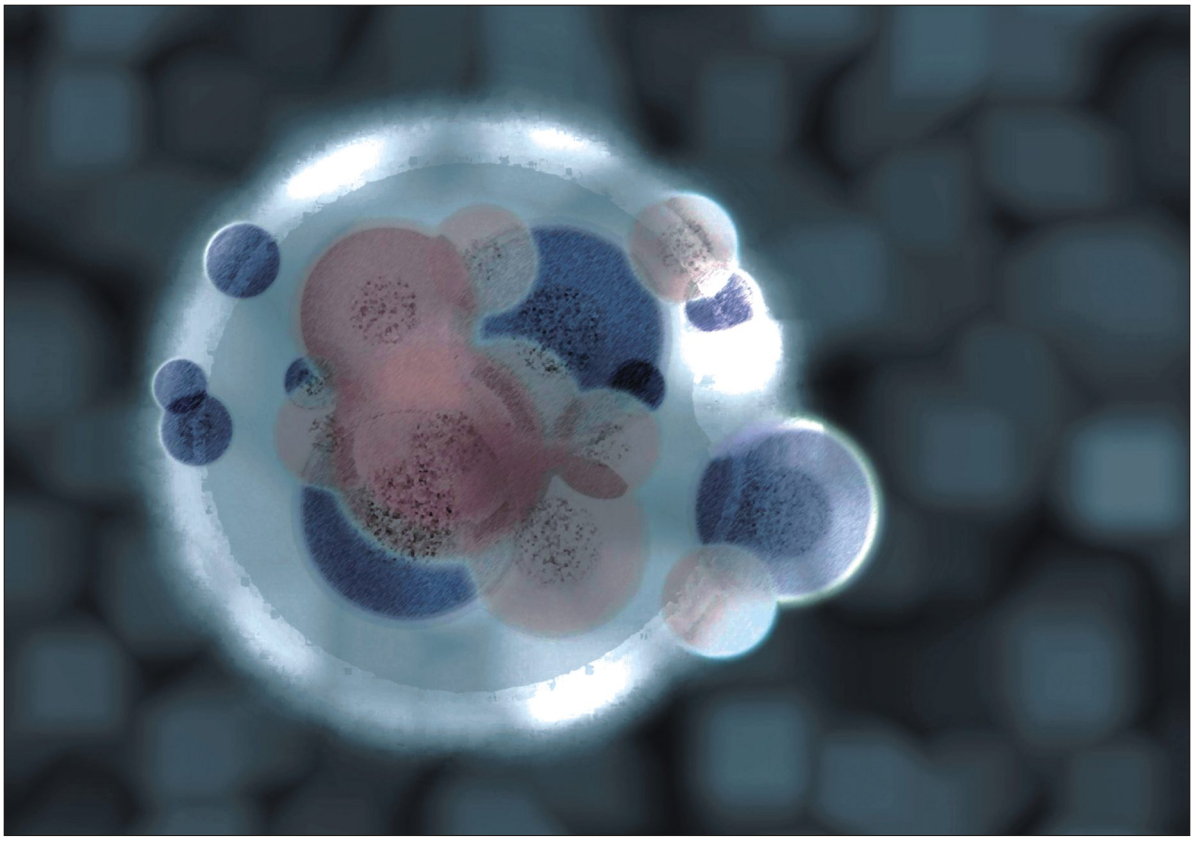

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Short Communication
Assessment of the Inhibitory Mechanism of Action for a Yeast Cell-based Screening System Targeting Glycogen Synthase Kinase-3β (GSK-3β)

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Abstract

Background and Objective: Glycogen Synthase Kinase-3 (GSK-3) is one of the prior targets for drug discovery due to its involvement in many cell signaling and metabolism. It has been implicated in several critical diseases such as diabetes, Alzheimer's disease, cancer and inflammation. To date, many GSK-3 inhibitors have been identified and classified into different type such as inorganic atom, ATP competitive and non-ATP competitive types. Many laboratories worldwide are still actively screening for bioactive compounds for GSK-3 inhibitory activity using diverse screening systems. This study assessed an assay developed using a yeast cell-based system specifically targeting GSK-3β for preliminary screening and cost effectiveness. **Methodology:** In this study, the GSK-3 homologues in yeast (*MCK1*, *MDS1*, *MRK1* and *YOL128C*) were knocked out and inserted with mammalian GSK-3β. In order to determine the inhibitory mechanism, known GSK-3β inhibitors were tested and evaluated. **Results:** The GSK-3β inhibitor I and staurosporine showed inhibition on GSK-3β activity at a concentration of 1 and 20 μg $disc^{-1}$, respectively. Other known inhibitors, such as indirubin-3'-monoxime, kenpaullone, GSK-3 inhibitor IV and enzastaurin showed no detectable inhibition in this study. **Conclusion:** The GSK-3β inhibitor I and staurosporine interacted with the same amino acid on GSK-3β which is Cys199 while other inhibitors have no interactions with Cys199 as reported in docking study. This study suggests that this yeast cell-based system can be used to screen GSK-3β inhibitors that is targeting on Cys199 residue.

Key words: Cys199 residue, glycogen synthase kinase-3β, GSK-3 inhibitors, yeast cell-based system, GSK-3β inhibitor I, staurosporine

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Glycogen Synthase Kinase-3 (GSK-3) is a serine-threonine kinase that has a diverse array of substrates ranging from transcription factors, structural proteins as well as signaling and metabolic proteins[1]. The association of GSK-3 with different complex proteins underlies distinct signaling pathways that result in different events[2]. Consequently, GSK-3 is linked to the pathogenesis of several diseases like diabetes, cancer, inflammation, Alzheimer's and bipolar disorder. Thus, the GSK-3 protein is a favorable therapeutic target in drug discovery[3,4].

Inhibitors of GSK-3β are also diverse and include metal ions, natural products, synthetic molecules and peptides. For instance, lithium, zinc and beryllium are small metal cations reported to inhibit GSK-3 activity[5,6]. Drugs, such as indirubins and manzamine A are isolated from plant and marine organisms, respectively[7,8]. Meanwhile, there are innumerable anti-GSK-3 compounds produced through organic synthesis such as thiadiazolidinones[9], paullones[10] and small molecule inhibitors. Another molecule interacting with GSK-3 is GBP-a maternal XGSK-3-binding protein that is similar to a T-cell proto-oncogene. This peptide has more selective inhibition of GSK-3[11]. All of these inhibitors can inhibit GSK-3 activity at specific sites on GSK-3. For example, manzamine A inhibits GSK-3β by phosphorylation at Ser9 and alsterpaullone and AR-A014418 bind to the ATP site of GSK-3[12]. However, some of these compounds have unfavorable targets on other protein kinases and offer adverse effects[13,14]. Hence, more potential inhibitors that correspond to an efficient pathway for providing promising drugs for therapeutic intervention are still actively under development.

The interaction between inhibitors and GSK-3 can be studied via the crystal structure. This provides detailed information on the design of new anti-GSK-3 species based on the inhibitor selectivity towards GSK-3 active sites[15,16]. Furthermore, many assays have been developed to determine the biological activity and target specificity of these inhibitors towards the protein. For example, the Kinase-Glo™ system (Promega, Madison, WI) is a high throughput luminescent assay that provides sensitive and reproducible information[17]. Cell-based assays can measure Tau phosphorylation at Ser396 to measure the cellular activity of GSK-3 inhibitors[18]. However, these assays are costly to be used for preliminary screening. A simple and affordable preliminary screening assay for GSK-3β inhibition using yeast as a cell-based system is reported here. In this study, the following GSK-3 homologues in yeast were knocked out: *MCK1*, *MDS1*, *MRK1* and *YOL128C*. Andoh *et al.*[19] reported that when these four genes were disrupted, the yeast was sensitive to the growth temperature of 37°C. However, this phenotype is complemented by the expression of mammalian GSK-3β in the yeast cell. This system was used to successfully screen for GSK-3β inhibitors from an organic extract of *Streptomyces* sp., H7667[20] and several compounds exhibited inhibitory activity to the protein in the assay. However, the mechanism of inhibition in this study is unknown. Therefore, this study assesses the system by investigating the possible mode of inhibition and evaluates the inhibitory activity of different known inhibitors such as indirubin-3'-monoxime, kenpaullone, GSK-3 inhibitor IV (SB-216763), enzastaurin, staurosporine, lithium chloride and GSK-3β inhibitor I (TDZD-8).

MATERIALS AND METHODS

Preparation of GSK-3 inhibitor solutions: The GSK-3 inhibitors including indirubin-3'-monoxime, kenpaullone (9-bromo-7, 12-dihydroindolo [3, 2-d] [1] benzazepin-6(5H) -one), GSK-3 inhibitor IV (SB-216763) and GSK-3β inhibitor I (*TDZD*-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione) were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Enzastaurin was purchased from Chemtron Biotechnology, Malaysia. Lithium chloride was purchased from R and M Chemicals (Essex, U. K.). Staurosporine from *Streptomyces* sp., was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of these inhibitors were prepared in dimethyl sulfoxide (DMSO) except for lithium chloride that was dissolved in distilled water before use.

Glycogen synthase kinase-3β inhibitory assay: Yeast mutant with Δ*mck1*, *Δmds1*, *Δmrk1* and Δ*yol128c* complemented with human GSK-3β cells was grown in 5 mL SC minus uracil broth at 37°C for 48 h. The construction of the yeast mutant was reported previously by Andoh *et al.*[19]. To prepare the medium for the assay, the yeast cells were plated on SC minus uracil agar with different concentrations of inhibitors on a 6 mm paper disc on the agar (Whatman No. 3). The cultures were incubated at 25 and 37°C for 5 days. When inhibition zone was observed it was scored as positive (+). Crude extracts of *Streptomyces* H7667 were used as the positive control and DMSO as the negative control.

RESULTS

The four homologous GSK-3β genes *MCK1*, *MDS1*, *MRK1* and *YOL128C* were knocked out in the yeast used in this study. This yeast mutant was temperature sensitive where growth was not observed when incubated at 37°C. However, this phenotype is recovered when the yeast mutant was complemented with human GSK-3β gene. Therefore,

inhibition zone of yeast mutant (score as positive) can be observed at 37°C but not at 25°C in the presence of inhibitor indicating inhibition of GSK-3β genes. In this study, inhibition zone of yeast was compared between 25 and 37°C after 5 days of incubation. Of all the GSK-3 inhibitors tested in this study, GSK-3β inhibitor I (TDZD-8) and staurosporine showed inhibitory activity at 1 and 20 μg disc^{-1}, respectively (Table 1). Meanwhile, inhibition by lithium chloride was only observed at high concentrations was reached indicating low inhibitory efficiency. Other known inhibitors, such as indirubin-3'-monoxime, kenpaullone, GSK-3 inhibitor IV and enzastaurin tested showed no detectable inhibition zone (Fig. 1). This finding showed that the yeast cell-based system tested is specific, targeting inhibitors in the same group as GSK-3β inhibitor I (TDZD-8) and staurosporine.

Table 1: Screening result of GSK-3 inhibitor in glycogen synthase kinase-3β inhibitory assay

	Concentration (μg disc^{-1})						
Inhibitors	1	2.5	5	20	80	120	Status
GSK-3 inhibitor I (TDZD-8)	+	+	+	0	0	0	Active
Indirubin-3-monoxime	0	0	0	_	_	_	Not active
Kenpaullone	0	0	0	_	_	_	Not active
GSK-3 inhibitor IV (SB-216763)	0	0	0	_	_	_	Not active
Staurosporine	0	0	0	+	+	+	Active
Enzastaurin	0	0	0	0	_	_	Not active

Guide for symbol, 0: Not tested, +: Detectable inhibition zone, -: No detectable inhibition

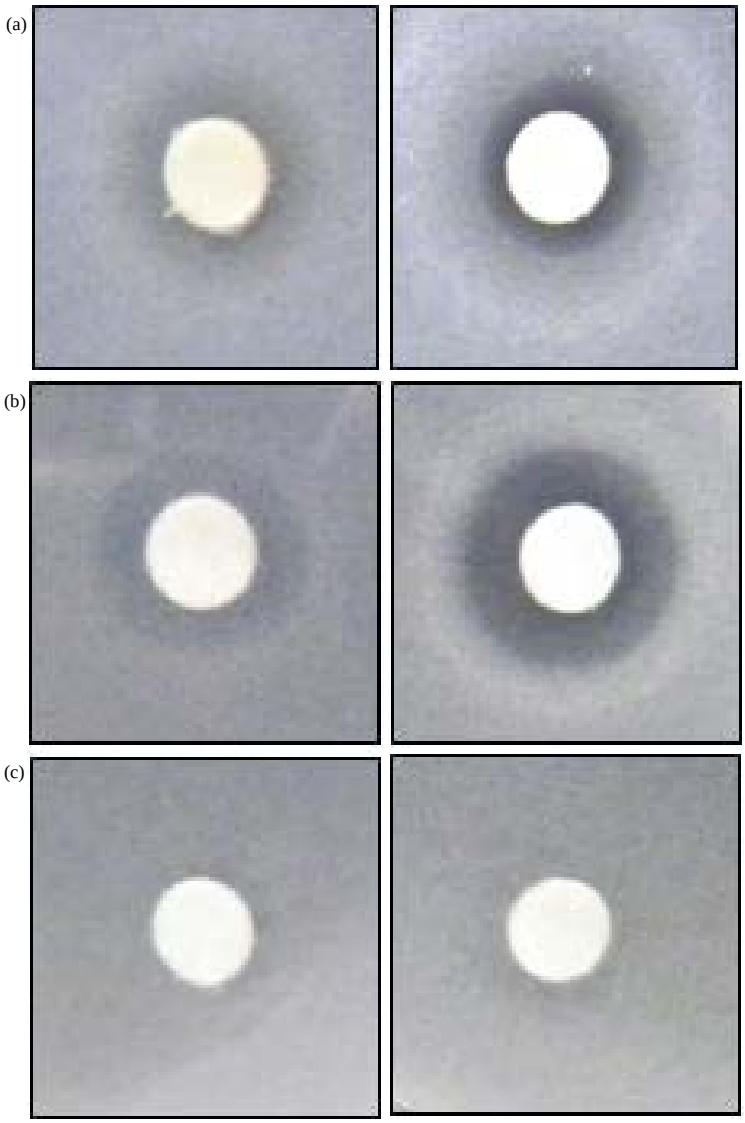

Fig. 1(a-c): Growth inhibition of yeast mutant carrying human GSK-3β by, (a) GSK-3β inhibitor I (TDZD-8) at 1 μg disc^{-1}, (b) Staurosporine at 20 μg disc^{-1} and (c) Others inhibitor at 120 μg disc^{-1}. Left: 25°C, right: 37°C

DISCUSSION

The GSK-3β inhibitor I (TDZD-8) and staurosporine have been used in previous studies by Cheenpracha *et al.*[20] as a positive controls, but their mechanism of action to GSK-3β in yeast cells is not known. Thus, this study tested known inhibitors, such as indirubin-3'-monoxime, kenpaullone, GSK-3 inhibitor IV (SB-216763), enzastaurin, staurosporine, lithium chloride and TDZD-8 to evaluate their inhibitory mechanism of action exerted against GSK-3β.

The GSK-3 inhibitors that are ATP competitive tested in this study include indirubin-3'-monoxime, kenpaullone, GSK-3 inhibitor IV (SB-216763), enzastaurin and staurosporine. The indirubin-3'-moxime, kenpaullone, GSK-3 inhibitor IV and enzastaurin are reported to decrease phosphorylation of Tyr276 (GSK-3α) and Tyr216 (GSK-3β), which is located on the 'Activation loop' of GSK-3β[21-23]. Staurosporine isolated from *Streptomyces staurosporeus* binds to the ATP binding site of GSK-3[15,24]. Staurosporine prevents phosphorylation at Tyr216 when incubated alone with GSK-3[25].

The non-ATP competitive inhibitors tested in this study are lithium chloride and TDZD-8. Both inhibitors inhibit GSK-3β activity via phosphorylation at Ser9[26,27]. Lithium chloride also causes direct inhibition by binding to the magnesium site on GSK-3β and TDZD-8 has better selectivity because it binds outside the ATP kinase pocket[11].

The GSK-3β inhibitor I (TDZD-8), staurosporine and lithium chloride showed detectable inhibition. However, only TDZD-8 and staurosporine showed significant inhibition at 37°C compared to 25°C when tested at 1 and 20 μg $disc^{-1}$, respectively. Meanwhile, the inhibitory activity of lithium chloride was insignificant because the inhibition zone was only detectable at high concentrations.

The mechanism of action for this assay system was then evaluated by comparing the interaction between the known inhibitors and GSK-3β based on the reported docking study. The mechanisms by which GSK-3β inhibitor I (TDZD-8) and staurosporine inhibit GSK-3β activity are different. However, there is one residue on GSK-3β that interacts with both TDZD-8 and staurosporine, which is Cys199[11,28]. Indirubin-3'-monoxime has no interaction with Cys199[15]. Meanwhile, there is limited information on the interaction with Cys199 for kenpaullone, SB-216763 and enzastaurin[24]. Both TDZD-8 and staurosporine inhibitors were positive against GSK-3 activity and showed significant inhibition when tested using the yeast cell-based screening system. Thus, the possible target site of this screening system is the Cys199 residue of GSK-3β.

Targeting Cys199 residue by inhibitors can inhibit GSK-3β[29]. This residue is located at the entrance of the ATP site and not in the ATP pocket[12,30]. The effect of targeting the Cys199 residue has been implicated at low concentrations of inhibitor. This indicates the inhibition efficiency. Therefore, screening inhibitors using this assay system in the preliminary phase is a promising approach for discovering GSK-3β inhibitors that target Cys199. Nevertheless, further tests, such as using a Cys199 mutant and structure modeling are necessary to further confirm the mechanism of inhibition.

CONCLUSION

In conclusion, this study suggests that the yeast cell-based screening system is easy to perform and can be applied as a low cost approach to the preliminary screening of GSK-3β inhibitors.

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