A new biological activity of 6-(methylsulfinyl)hexyl isothiocyanate derived from Wasabia japonica was discovered as an inhibitor of glycogen synthase kinase-3β. The most potent isothiocyanate, 9-(methylsulfinyl)hexyl isothiocyanate, inhibited glycogen synthase kinase-3β at a Ki value of 10.5 μM and showed ATP competitive inhibition. The structure-activity relationship revealed an inhibitory potency of methylsulfinyl isothiocyanate dependent on the alkyl chain length and the sulfoxide, sulfone, and/or the isothiocyanate moiety.

Key words: wasabi; Wasabia japonica Matsum; methylsulfinyl isothiocyanate; GSK-3β

Wasabia (Wasabia japonica Matsum) is a member of the Brassicaceae family of vegetables, and its rhizome is a very popular pungent spice in Japan. The active compound of wasabi responsible for growth suppression of human cancer cell lines such as breast cancer and melanoma has been identified as 6-(methylsulfinyl)hexyl isothiocyanate (6-MSITC).1 It was also reported that 6-MSITC suppressed inducible nitric oxide synthase and lipopolysaccharide-induced cyclooxygenase-2 transcription in mouse macrophages, and that the anti-inflammatorv activities were dependent on the alkyl chain length of MSITC.2,3 These results indicate that 6-MSITC has a variety of biological activities and is a potent chemopreventive compound in wasabi.

Glycogen synthase kinase-3β (GSK-3β) is a ubiquitous serine/threonine kinase that inactivates glycogen synthase through phosphorylation of it. It is involved in the molecular pathogenesis of human diseases such as type-2 diabetes and Alzheimer’s disease.4–6 It has been reported that several synthetic small-molecule inhibitors of GSK-3β showed anti-type-2 diabetes, anti-Alzheimer’s disease, anti-cancer, and anti-inflammation effects.7 Although curcumin, the major constituent of the spice turmeric is recognized as an inhibitor of GSK-3β in vitro and in vivo,8 there is no other study of the inhibitor in food ingredients.

To detect the GSK-3β inhibitor in our MeOH extract of food ingredients, we used a unique phenotypic screening system that evaluated the growth zone of a mutant yeast, Saccharomyces cerevisiae (zds1Δ erg3Δ pdr1Δ pdr3Δ), caused by inhibition of Ca2+-signal transduction.9,10 The Ca2+-signaling pathways for yeast growth regulation are composed of several signaling molecules, including the Ca2+ channel, calcineurin, and Mck1 GSK-3.9,10 The MeOH extract of wasabi showed a faint growth zone around an inhibition zone to the mutant yeast, and we identified 6-MSITC as an active compound. That phenotype suggested to us that 6-MSITC has inhibitory activity for GSK-3β.11 Here, we describe the inhibitory activity of 6-MSITC and related compounds against human GSK-3β, one of the molecular targets in the mutant yeast.

MeOH extracts from the rhizome of Wasabia japonica grown in Miyamori Village in Japan (now Tono City) and commercial 6-MSITC (LKT Laboratories, St. Paul, MN) showed a faint growth zone around an inhibitory zone on Ca2+-signal transduction inhibitor screening (400 μg/disc and 4 μg/disc respectively). Figure 1 shows a typical screening phenotype of 6-MSITC and related compounds to the mutant yeast with 0.3 M CaCl2, Alkyl isothiocyanates, 6-, 7-, 8-, 9-MSITCs, and 6-(methylsulfonyl)hexyl isothiocyanate (6-MSOITC) (LKT Laboratories), containing a sulfoxide or a sulfone moiety, also showed a faint growth zone around an inhibitory zone (4 μg/disc). The potency of activity was observed to be proportional to the length of the alkyl chain as defined by the diameter of the growth zone: 9-MSITC 27.9 mm (Fig. 1, no. 1), 8-MSITC 25.2 mm (Fig. 1, no. 2), 7-MSITC 23.4 mm (Fig. 1, no. 3), 6-MSITC 19.4 mm (Fig. 1, no. 4), 6-MSOITC 19.3 mm (Fig. 1, no. 5). In contrast, 6-(methylthio)hexyl isothiocyanate (6-MTITC) (LKT Laboratories), not containing a sulfoxide or a sulfone moiety, did not show a faint growth zone or an inhibitory zone (Fig. 1, no. 6). Thus the alkyl chain length and sulfoxide or sulfone moiety in the MSITCs was important for activity against the mutant yeast.

MSITCs showed a phenotype similar to the GSK-3β specific inhibitor GSK-3β inhibitor-I (Merk Calbiochem, Darmstadt, Germany) against the mutant yeast on the plate.11 Hence, the inhibition activity of MSITCs on GSK-3β was examined directly by luminescent kinase assay. GSK-3β assays were performed using the Kinase-Glo® plus luminescent kinase assay platform (Promega, Madison, WI) in a white 96-well plate, and GSK-3β inhibitor-I was used as positive control. Phospho-
glycogen synthase peptide-2 (Millipore, Billerica, MA) (20 μM) was mixed with recombinant human GSK-3β (Millipore) (0.022 unit) in a total volume of 50 μL of assay buffer (8 mM 3-morpholinopropanesulfonic acid (MOPS)-KOH, Dojindo Laboratories, Kumamoto, Japan, pH 7.0, 0.2 mM EDTA, 5 μM ATP, and 10 mM MgCl2) in the presence and the absence of inhibitors (2 μL of DMSO). After 3 h of incubation at 30°C, the enzymatic reaction was stopped by the addition of 50 μL of Kinase-Glo reagent. Glow-type luminescence was measured after 10 min using a Powerscan HT spectrophotometer (Dainippon Sumitomo Pharma, Osaka, Japan). GSK-3β activity is proportional to the difference between total ATP and consumed ATP. The inhibitory activities of the tested compounds were calculated on the basis of the activity with and without them.

Alkyl isothiocyanates, 6-, 7-, 8-, 9-MSITCs, and 6-MSOITC showed inhibitory activity against GSK-3β in proportion to the alkyl chain length of the molecule (Fig. 2A). 6-MSITC showed an IC50 value of 70.3 μM against GSK-3β, and the most potent GSK-3β inhibitor

<table>
<thead>
<tr>
<th>Isothiocyanate</th>
<th>GSK-3β inhibition (IC50, μM)</th>
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<tbody>
<tr>
<td>9-MSITC</td>
<td>18.6</td>
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<tr>
<td>8-MSITC</td>
<td>21.5</td>
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<tr>
<td>7-MSITC</td>
<td>40.0</td>
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<tr>
<td>6-MSOITC</td>
<td>54.8</td>
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<td>6-MSITC</td>
<td>70.3</td>
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<td>6-MTITC</td>
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<tr>
<td>4-MSITC</td>
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Fig. 2. GSK-3β Inhibition by Isothiocyanates.
A. GSK-3β inhibition activity of 6-MSITC and related isothiocyanates. 6-MSITC and related isothiocyanates were dissolved in DMSO and then added to the GSK-3β reaction. The GSK-3β reaction was performed as described in the text using recombinant human GSK-3β and a peptide substrate in a luminescent kinase assay. The IC50 values are calculated assuming an average value of n = 2. B. Lineweaver-Burk plot of 9-MSITC against GSK-3β. Kinetic analysis was performed using four 9-MSITC concentrations, 10 (○), 7.5 (●), 2.5 (△) and 0 (▲) μM, and three ATP concentrations, 5, 10, and 20 μM. The GSK-3β reaction was performed as described in the text.
of the isothiocyanates was 9-MSITC (IC\textsubscript{50} = 18.6 μM). The positive control, GSK-3β inhibitor-I, inhibited GSK-3β with an IC\textsubscript{50} value of 95.6 μM. In contrast, 6-MTITC and 4-MSITC (LKT Laboratories) did not show inhibitory activity against GSK-3β even at 200 μM. 9-MSOITC and 9-MTITC also showed inhibition activity against GSK-3β, with IC\textsubscript{50} values of 19.5 and 133.7 μM respectively. Thus alkyl chain length, and the sulfoxide or sulfone moiety in MITCs was important to GSK-3β inhibitory activity.

The isothiocyanate having the longest alkyl chain in our study, 9-MSITC, inhibited GSK-3β most potently of the nine related compounds, with an IC\textsubscript{50} value of 18.6 μM. Hence, we determined the inhibition mechanism of 9-MSITC against GSK-3β at different concentrations of ATP and 9-MSITC. Lineweaver-Burk plot analysis of 9-MSITC showed competitive ATP binding to GSK-3β (Fig. 2B). The inhibition constant (K\textsubscript{i} value) was determined by Dixon plot at 10.5 μM (data not shown). This suggests that MSITCs inhibits GSK-3β by binding at the ATP binding site of GSK-3β.

To determine whether the isothiocyanate group is important for inhibitory activity toward GSK-3β, 6-MSITC/\textit{N}-acetyl-l-cysteine methyl ester (NACM) conjugate was examined for inhibition of GSK-3β activity. The isothiocyanate moiety in 6-MSITC is an electrophilic group that can react with the nucleophilic group of thiol in NAC.\textsuperscript{12} 6-MSITC (15 mM) was incubated with 30 mM NACM (Sigma, St. Louis, MO) in 1 mL of 40 mM MOPS-KOH buffer (pH 7.0) at 37 °C. The reaction mixture was incubated at 30 μM NACM (Sigma, St. Louis, MO) in 1 mL of 40 mM MOPS-KOH buffer (pH 7.0) at 37 °C. The reaction was monitored by analytical HPLC (column, Capcell pak C18, 4.6 mm i.d. × 150 mm, Shiseido; mobile phase, MeOH and water = 30:70 v/v; flow rate, 1.0 mL/min) equipped with an 880-PU pump and a MD-910 multi-channel detector (Jasco, Tokyo).

Figure 3A shows a typical chromatogram of the reaction between NACM and 6-MSITC on 0 h and 1 h of reaction. As in the literature, free peaks of NAC and 6-MSITC decreased at once, and a new peak appeared as the 6-MSITC/NAC conjugate between the retention times of NAC and 6-MSITC.\textsuperscript{12} Similarly, the mixture of 6-MSITC and NACM also changed to the conjugate (retention time, 23.4 min) at once (Fig. 3A), and after 24 h the reaction product was subjected directly to HPLC (column, Capcell pak C18, 10 mm i.d. × 250 mm, Shiseido; mobile phase, MeOH and water = 35:65 v/v; flow rate, 3.0 mL/min) for the purification of 6-MSITC/NACM conjugate, and was isolated as a single peak with a retention time of 33 min (2.4 mg).

The molecular formula of the 6-MSITC/NACM conjugate was judged by HR-FAB-MS (m/z (M + H)\textsuperscript{+}: Calcd. for C\textsubscript{12}H\textsubscript{27}N\textsubscript{2}O\textsubscript{12}S\textsubscript{7}: 383.1132, Found: 383.1143). Figure 3B shows the LC/APCI-MS spectra of the 6-MSITC/NACM conjugate with a tentative structure for it. The protonated molecule peaks of the 6-MSITC, NACM, and 6-MSITC/NAC conjugate were observed at m/z of 206, 178, and 383 respectively. The fragment ion peak at an m/z of 319 was probably formed by cleavage at the dotted line (Fig. 3B). This indicates that the isothiocyanate group was masked by a thiol moiety of NACM in the 6-MSITC/NACM conjugate (Fig. 3B). The 6-MSITC/NACM conjugate inhibited GSK-3β with an IC\textsubscript{50} value of 312.0 μM, and was 4.4 times less potent than 6-MSITC. Additionally, the 6-MSITC/NACM conjugate did not show a growth zone or an inhibitory zone against the mutant yeast at 20 μg/disc (data not shown). Therefore, the isothiocyanate group of MITCs is important for the inhibition of GSK-3β.

MSITCs, including 6-MSITC, are inhibitors of GSK-3β that compete with ATP for binding. In addition, the alkyl chain length, sulfoxide, sulfone, and isothiocyanate structures appear to be important to their inhibitory activities on GSK-3β. Alkyl isothiocyanates (2-, 4-, 6-, and 8-MSITCs) inhibit epidermal growth factor receptor kinase but not protein kinase C, and calmodulin kinase III.\textsuperscript{13} Therefore, a variety of biological activities of MSITCs may be involved in the inhibition of epidermal growth factor receptor kinase and GSK-3β. In Saccharomyces cerevisiae, there are four genes, MCK1, MDS1/RIM11, MRK1, and YOL128c, that encode homologs of mammalian GSK-3. A gsk-3 null mutant in which these four genes are disrupted show growth defects under NaCl salt stress condition.\textsuperscript{14} Because Ca\textsuperscript{2+}-signal transduction inhibitor screening is...
done under CaCl$_2$ stress conditions for the mutant yeast, non-specific GSK-3 inhibitors might show an inhibitory zone stronger than the growth zone.

Glucose-6-phosphatase and phosphoenolpyruvate carboxykinase are key enzymes with important roles in the rate-controlling steps of hepatic gluconeogenesis. They are suppressed by GSK-3β inhibitors such as lithium and SB216763.$^{15}$ The phenotypic screening system for the Ca$^{2+}$-signal transduction inhibitor using the mutant yeast is a simple, powerful system for the identification and characterization of GSK-3β inhibitors. We propose that 6-MSITC and/or food ingredients including it might be useful functional compounds for the treatment of type-2 diabetes. Moreover, isothiocyanate derivatives are good lead compounds as GSK-3β inhibitors.

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