Biochemical Characterization of Novel Lignans Isolated from the Wood of *Taxus yunnanensis* as Effective Stimulators for Glycogen Synthase Kinase-3 β and the Phosphorylation of Basic Brain Proteins by the Kinase *in Vitro*

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The stimulatory and inhibitory effects of several compounds and lignans isolated from the water extract of *Taxus yunnanensis* on the phosphorylation of three functional brain proteins (bovine myelin basic protein (bMBP), recombinant human tau protein (rhTP) and rat collapsin response mediator protein-2 (rCRMP-2)) by glycogen synthase kinase-3 β (GSK-3 β) were quantitatively compared *in vitro*, using (–)-epigallocatechin-3-gallate [(–)EGCG] as a positive control. We found that (i) three selected *Taxus* lignans [(3S,4R)-4'-hydroxy-6,3'-dimethoxyisoflavan-4-ol, (7R)-7-hydroxytaxiresinol and tanegool] highly stimulated the autophosphorylation of GSK-3 β and the GSK-3 β -mediated phosphorylation of two basic brain proteins [bMBP (pI =11.3) and rhTP (pI =8.2)], but inhibited dose-dependently the phosphorylation of an acidic protein (rCRMP-2, pI =6.0) by the kinase; (ii) these three *Taxus* lignans showed binding-affinities with bMBP as well as rhTP, but had low affinities with rCRMP-2; (iii) the binding of tanegool and (7R)-7-hydroxytaxiresinol to these two basic proteins induced their novel potent phosphorylation sites for GSK-3 β ; and (iv) these three *Taxus* lignans, but not EGCG, induced Tyr-phosphorylation of GSK-3 β *in vitro*. These results provided here suggest that (i) these three *Taxus* lignans act as novel effective activators for GSK-3 β and the GSK-3 β -mediated phosphorylation of their binding basic proteins (rhTP and bMBP); and (ii) tanegool (IC_{50} =1 μ M) is an effective inhibitor for the phosphorylation of rCRMP-2 by the kinase *in vitro*.

Key words *Taxus* lignan; glycogen synthase kinase-3 β ; stimulatory effect; protein phosphorylation

Recently, we reported that (i) plant polyphenol epigallocatechin-3-gallate (EGCG), not quercetin and luteolin, highly stimulates the phosphorylation of human recombinant tau protein (hrTP, pI =8.2) and bovine myelin basic protein (bMBP, pI =11.3) by glycogen synthase kinase-3 β (GSK-3 β); (ii) EGCG has a binding-affinity with rhTP as well as bMBP; and (iii) the direct binding of EGCG to these two basic brain proteins (bMBP and rhTP) induces their novel potent phosphorylation sites for GSK-3 β , and leads to highly stimulate their phosphorylation by the kinase *in vitro*.¹⁾ Also, we previously reported that phosphatidylinositol (PI) and two sulfated lipids [sulfatide and heparin (SH)] function as effective stimulators for the autophosphorylation of GSK-3 β and the GSK-3 β -mediated phosphorylation of SH-binding proteins *in vitro*.²⁾ From these previous reports,^{1,2)} we conclude that both EGCG and SH may function as effective stimulators for the GSK-3 β -mediated phosphorylation of these two basic brain proteins (bMBP and rhTP), containing multiple potent phosphorylation sites for the kinase *in vitro*.^{1,2)}

Glycogen synthase kinase 3 (GSK-3) is a constitutively active, proline-directed serine (Ser)/threonine (Thr) protein kinase encoded by two isoforms [GSK-3 α (approx. 51 kDa) and GSK-3 β (approx. 47 kDa, pI =8.98)], which possess similar biochemical characteristics and substrate specificities.^{3–5)} GSK-3 is originally identified as a protein kinase that preferentially phosphorylates the rate-limiting enzyme, glycogen synthase (GS), in glycogen synthesis.^{6,7)} Further detailed characterization of GSK-3 β revealed that (i) the kinase has a wide array of substrates, including cytoplasmic enzymes, nuclear transcriptional factors and signal-mediating molecules; (ii) the

phosphorylation of Tyr-216 on GSK-3 β is essential for the kinase activity;⁸⁾ and (iii) GSK-3 β plays an important physiological role in the regulation of numerous signaling pathways involved in cell differentiation and the morphological development of neurons.⁹⁾

Taxus yunnanensis CHENG *et* L.K. FU, which belongs to the plant family *Taxaceae*, is widely distributed in Yunnan Province of the People's Republic of China and is commonly known as “Yunnan Hongdoushan.” The wood of *Taxus yunnanensis* has been used in traditional Chinese medicine by several ethnic societies in the Yunnan Province for the treatment of kidney problems and diabetic conditions. The Kadota research group (Toyama Univ., Toyama, Japan) has characterized and identified many novel active compounds isolated from the water-extract of *Taxus yunnanensis*.^{10–16)} Recently, they reported a novel isoflavans [compound 1 (A)], a novel degraded compound 2 (B) from compound 4 (isotaxiresinol)] and a novel lignan compound 3 (C), which significantly inhibit the activity of cytochrome P450-3A4 *in vitro*.¹⁷⁾ Compound 6 (E) is identified as a known lignin of tanegool.¹⁸⁾ Since several *Taxus* compounds have been characterized to exhibit antioxidant, anti-inflammatory and neuroprotective effects,^{10–16)} we preliminarily tested the effects of at least 15 *Taxus* compounds, including five novel *Taxus* lignans (compounds A–E) on the GSK-3 β -mediated phosphorylation of three brain proteins [bMBP, rhTP and rat collapsin response mediator protein-2 (rCRMP-2, pI =6.0)] *in vitro*. By our preliminary screening, five *Taxus* compounds (A–E) exhibiting significant stimulatory effects on the GSK-3 β -mediated phosphorylation of two basic proteins (bMBP and rhTP) were selected and fur-

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ther biochemically characterized *in vitro*.

The present study has been carried out to (i) determine the effects of two novel *Taxus* lignans (compounds A and C) and compound E (tanegool), in comparison with EGCG, on the autophosphorylation of GSK-3 β and the phosphorylation of two basic proteins (bMBP and rhTP) and an acidic protein (rCRMP-2) by the kinase; (ii) characterize the *Taxus* compound-induced novel potent phosphorylation sites for GSK-3 β on these two basic proteins; and (iii) determine the autophosphorylation kinetics of GSK-3 β in the presence or absence of tanegool *in vitro*.

MATERIALS AND METHODS

Chemicals [γ - 32 P]ATP (111 TBq/mmol) was obtained from Perkin Elmer Inc. (MA02451, U.S.A.); dithiothreitol (DTT) from Wako (Osaka, Japan); ECL detection reagent from GE healthcare UK Ltd. (Amersham Place, U.K.); (-)epigallocatechin gallate [(−)EGCG], bovine serum albumin (BSA) from Sigma Aldrich (St. Louis, U.S.A.); trypsin from Promega Co. (Madison, U.S.A.); and tyrosine-kinase inhibitor (PP2) from Alexis Biochemicals (New York, U.S.A.).

Protein Kinases and Two Effective Substrate Proteins (bMBP and rhTP) for GSK-3 β Rabbit GSK-3 β (rabbit muscle, specific activity: 14629 units/mg) and purified bovine myelin basic protein (bMBP) were obtained from Sigma Aldrich (St. Louis, U.S.A.); and recombinant human tau protein (rhTP) from Calbiochem (San Diego, U.S.A.). Rat recombinant CK1 δ (specific activity: 17500 units/mg) was obtained from Promega Co. (Madison, U.S.A.).

Extraction, Isolation and Identification of *Taxus* Compounds The wood of *Taxus yunnanensis* was chopped into small pieces and crushed into a powder. The dried wood powder (approx. 75 g) was extracted with H₂O under reflux for 45 min to yield the H₂O extract (approx. 51.0 g). The residue was further extracted with H₂O/MeOH and MeOH to give the H₂O/MeOH (1:1) and the MeOH extracts (approx. 7.5 g each), respectively. Three partially purified fractions (F-I, F-II and F-III), containing novel compounds (A–D) and tanegool, were prepared from the aqueous extract of *Taxus yunnanensis*. Finally, the chemical structures of at least 12 compounds (1–12) from the three *Taxus* fractions were identified.¹⁷⁾ From our preliminary experiments using *in vitro* kinase assay, at least five effective compounds: compound 1 (A) identified as a novel isoflavan [(3*S*,4*R*)-4'-hydroxy-6,3'-dimethoxyisoflavan-4-ol] degraded from isotaxiresinol, compound 2 (B) as a novel lignan, [2,3-bis(hydroxymethyl)-7-hydroxy-6-methoxy-tetralone] degraded from compound 4 (isotaxiresinol), compound 3 (C) as a novel lignan [(7*R*)-7-hydroxytaxiresinol],¹⁷⁾ compound 6 (E) as a known lignan (tanegool),¹⁸⁾ and compound 12 (D),¹⁹⁾ as a known α -conidendrin, were selected from 15 *Taxus* compounds, including secoisolariciresinol, isotaxiresinol and taxiresinol.

Purification of CRMP-2 from Rat Brain as an Effective Substrate for GSK-3 β CRMP-2 was highly purified from the extract of rat brain by means of successive DEAE-cellulose column chromatography, gel filtration of a SDX 200pg column and heparin-affinity column chromatography. Finally, CRMP-2 (70 kDa polypeptide, p70) in the heparin-affinity fraction was purified by Mono Q column chromatography as a single polypeptide, as previously reported.¹⁾

Assay for GSK-3 β Activity *in Vitro* Three protein substrates (bMBP, rhTP and rCRMP: approx. 2 μ g each) were separately incubated for 30 min at 30°C in the reaction mixtures (50 μ L) comprising GSK-3 β (approx. 50 ng), 40 mM Tris–HCl (pH 7.6), 2 mM DTT, 3 mM Mn²⁺ and 5 μ M [γ - 32 P]ATP (500 cpm/pmol) in the presence or absence of the indicated *Taxus* compounds. 32 P-Labeled substrate proteins in the reaction mixtures were separately detected by autoradiography after protein analysis by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein phosphorylation by GSK-3 β was terminated by the addition of 0.5 mL 20% trichloroacetic acid (TCA) and 0.25 mL 0.1 M sodium pyrophosphate containing bovine serum albumin (BSA) (1 mg/mL) and 10 mM ethylene diaminetetraacetic acid (EDTA). The TCA-insoluble precipitates were trapped on a glass membrane filter (Advantec GF/75), and then washed successively with 2% TCA and 100% ethanol. After drying, the 32 P-radioactivity on the membrane filter was measured with a liquid scintillation spectrophotometer, as described in our previous reports.^{1,2)}

Phosphopeptide Mapping Briefly, either bMBP or rhTP (approx. 2 μ g) in reaction mixtures (50 μ L) comprising 40 mM Tris–HCl (pH 7.6), 2 mM DTT, 3 mM Mn²⁺, 5 μ M [γ - 32 P]ATP (500 cpm/pmol) and GSK-3 β (approx. 50 ng) in the absence or presence of one of three *Taxus* compounds (A, C and E) were incubated with GSK-3 β (approx. 50 ng) for 120 min at 30°C. 32 P-Labeled bMBP or rhTP digested by trypsin in the reaction mixtures was analyzed by TLC electrophoresis under the conditions of 40 mA for 60 min (first dimension) and chromatography (second dimension). Separated 32 P-labeled fragments cleaved from bMBP or rhTP were detected by autoradiography.^{2,20)}

Measurement of the Binding Affinities of Three *Taxus* Lignans with Three Substrate Proteins by a Quartz Crystal Microbalance (QCM) The binding affinities of three substrate proteins (bMBP, rhTP and rCRMP-2) with one of three *Taxus* compounds (A, C and E) in comparison with EGCG were separately determined with a QCM (Initium, Tokyo, Japan) using BSA as a negative control, as described in our previous reports.^{2,20)}

RESULTS

The Stimulatory Effects of Three Selected *Taxus* Compounds (A, C and E) on the GSK-3 β -Mediated Phosphorylation of Three Brain Proteins *in Vitro* To determine the stimulatory effects of three selected *Taxus* compounds (A, C and E) on the GSK-3 β -mediated phosphorylation of rhTP, this protein substrate (approx. 3 μ g) was incubated for 30 min at 30°C with GSK-3 β (approx. 50 ng) and 5 μ M [γ - 32 P]-ATP (500 cpm/pmol) in the presence of the indicated concentrations of one of the three *Taxus* compounds *in vitro*. 32 P-Labeled rhTP in the reaction mixtures was detected by autoradiography after SDS-PAGE. As shown in Fig. 2, it was found that (i) these three *Taxus* compounds highly stimulated the GSK-3 β -mediated phosphorylation of rhTP; and (ii) the maximum phosphorylation of rhTP by GSK-3 β was observed when incubated with these three *Taxus* compounds at approx. 10 μ M. Under the same assay conditions, taxiresinol, but not isotaxiresinol and secoisolariciresinol, significantly stimulated the GSK-3 β -mediated phosphorylation of rhTP *in vitro* (data not shown). In the presence of these three *Taxus* compounds

Fig. 1. The Chemical Structures of Five Selected *Taxus* Compounds (A–E) and (–)EGCG

Fig. 2. The Stimulatory Effects of Three Selected *Taxus* Compounds (A, C, E) on the GSK-3 β -Mediated Phosphorylation of rhTP *in Vitro*

After incubation for 30 min at 30°C in the presence of the indicated concentrations of three selected *Taxus* compounds (A, C, E), 32 P-labeled rhTP in the reaction mixtures was detected by autoradiography after SDS-PAGE. The rate 1 represents the phosphorylation of rhTP by GSK-3 β in the absence of these three *Taxus* compounds *in vitro*. Compound A (●); compound C (Δ); and compound E (○).

(A, C and E) at 10 μ M, GSK-3 β preferentially phosphorylated Ser-residues rather than Thr-residues on rhTP (Fig. 3).

The stimulatory and inhibitory effects of these three *Taxus* compounds on the GSK-3 β -mediated phosphorylation of bMBP ($pI=11.3$) and rCRMP-2 ($pI=6.0$) were separately determined *in vitro*. As shown in Fig. 4, it was found that (i) compound E stimulated 5.3-fold the GSK-3 β -mediated phosphorylation of bMBP, as compared without the *Taxus* compound; (ii) the stimulatory effect of compound C on the phosphorylation of bMBP by the kinase was similar to that observed with EGCG; and (iii) this phosphorylation was significantly stimulated by compound A *in vitro*. In the case of rCRMP-2, two *Taxus* compounds (A and C) at 10 μ M inhibited more than 90% of the GSK-3 β -mediated CRMP-2 phosphorylation (Fig. 5). The IC_{50} (approx. 1 μ M) of compound E on the

GSK-3 β -mediated phosphorylation of CRMP-2 was about 5-fold higher than that (approx. 0.2 μ M) of EGCG *in vitro*. The IC_{50} of taxiresinol on the GSK-3 β -mediated phosphorylation of rCRMP-2 was similar to that (IC_{50} =approx. 3 μ M) of compound A *in vitro*. These results show that compound E among tested *Taxus* compounds acts as an effective stimulator for the GSK-3 β -mediated phosphorylation of two basic proteins (rhTP and bMBP) and a potent inhibitor for the phosphorylation of an acidic protein (rCRMP-2) by the kinase *in vitro*.

The Stimulatory Effects of Three *Taxus* Compounds on the Autophosphorylation of GSK-3 β *in Vitro* To determine the stimulatory effects of the three *Taxus* compounds and EGCG on the autophosphorylation of GSK-3 β *in vitro*, the kinase was directly incubated with 5 μ M [γ - 32 P]ATP (500 cpm/pmol) in the presence of one of the three *Taxus* compounds or EGCG. As shown in Fig. 6, it was found that (i) compound E at 3 μ M increased approx. 6.3-fold the autophosphorylation of GSK-3 β ; and (ii) compounds A at 10 μ M and compound C at 3 μ M also highly stimulated the autophosphorylation of GSK-3 β , but not significantly with 1 μ M EGCG. In the presence of 3 μ M compound E, Thr-residues in comparison with Ser-residues on GSK-3 β were preferentially phosphorylated (Fig. 7). Interestingly, significant Tyr-phosphorylation of GSK-3 β was detected when incubated with 3 μ M compound E (Fig. 7). Similar Tyr-phosphorylation of GSK-3 β was observed with the other two *Taxus* compounds (A and C), but not with EGCG. As expected, PP2 (Tyr-kinase inhibitor) at 2 μ M inhibited approx. 90% of the compound E-induced high autophosphorylation of GSK-3 β *in vitro*. These results clearly show that the direct binding of these three *Taxus* compounds, not EGCG, to GSK-3 β may induce the high autophosphorylation and Tyr-phosphorylation by the kinase *in vitro*.

The autophosphorylation kinetics of GSK-3 β were determined in the induce Tyr-phosphorylation of the kinase presence or absence of either compound E (3 μ M) or 1 μ M EGCG *in vitro*. No significant change was observed the apparent K_m value (control: 0.70 μ M) of ATP for GSK-3 β in the autophosphorylation of the kinase in the presence of 3 μ M compound E, whereas the V_{max} value of ATP for GSK-3 β shifted to 4.2 from 0.47 μ mol/mg/min (Fig. 8). In contrast, the apparent V_{max} value of ATP for GSK-3 β shifted to 1.0 from 0.45 μ mol/mg/min when incubated with 1 μ M EGCG (Fig. 8). These results

Fig. 3. Detection of Phosphoamino Acids on rhTP Fully Phosphorylated by GSK-3 β in the Presence of Three *Taxus* Compounds (A, C, E) *in Vitro*

The ^{32}P -Labeled phosphoamino acids of rhTP fully phosphorylated by GSK-3 β in the absence or presence of either compound A, compound C or compound E at $10\mu\text{M}$ were separately detected by autoradiography after two-dimensional TLC electrophoresis, as described in Materials and Methods.

Fig. 4. The Stimulatory Effects of Three *Taxus* Compounds (A, C, E) and EGCG on the GSK-3 β -Mediated Phosphorylation of bMBP *in Vitro*

After preincubation for 10 min at 30°C with GSK-3 β (approx. 50 ng) and $5\mu\text{M}$ [$\gamma\text{-}^{32}\text{P}$]ATP (500 cpm/pmol) with the indicated concentrations of one of three *Taxus* compounds, bMBP was added to the reaction mixtures, and then further incubated for 30 min at 30°C . ^{32}P -Labeled bMBP in the reaction mixtures was detected by autoradiography after SDS-PAGE. The rate 1 represents the phosphorylation of bMBP by GSK-3 β in the absence of these three *Taxus* compounds *in vitro*. Compound A at $10\mu\text{M}$; $10\mu\text{M}$ compound C; $10\mu\text{M}$ compound E and $3\mu\text{M}$ EGCG.

show that the direct binding of compound E to GSK-3 β highly stimulates the autophosphorylation of the kinase and an increase in the V_{max} value of ATP for the kinase *in vitro*.

Detection of the *Taxus* Compound-Induced Novel Potent Phosphorylation Sites for GSK-3 β on rhTP and bMBP *in Vitro* To further characterize the *Taxus* compound-induced high phosphorylation of rhTP by GSK-3 β *in vitro*, phosphopeptide-mapping was performed after rhTP was incubated for 120 min at 30°C with GSK-3 β (approx. 50 ng) and $5\mu\text{M}$ [$\gamma\text{-}^{32}\text{P}$]ATP (500 cpm/pmol) in the absence or presence of one of the three *Taxus* compounds or EGCG. ^{32}P -Fragments degraded

from ^{32}P -labeled rhTP were subjected to SDS-PAGE, after exhaustive in gel digestion of the rhTP-band with trypsin. Autoradiography (Fig. 9) detected four phosphorylated fragments (spots 1–4) and seven minor spots (spots: a–g), which were clearly different from the phosphopeptide pattern observed with EGCG, when the phosphorylation of rhTP by GSK-3 β was carried out in the presence of one of these three *Taxus* compounds at $10\mu\text{M}$ *in vitro*. Similar analysis of bMBP fully phosphorylated by GSK-3 β in the absence or presence of these *Taxus* compounds was carried out. Autoradiography (Fig. 10) detected three phosphorylated fragments (spots: 1–3) degraded from bMBP fully phosphorylated without these *Taxus* compounds *in vitro*. In contrast, three major spots and two minor spots (a and b) were detected when full phosphorylation of bMBP by GSK-3 β was carried out in the presence of either compound A or compound C at $10\mu\text{M}$ (Fig. 10). Furthermore, three major spots and three-minor spots (a, b and c) were detected when bMBP was fully phosphorylated by GSK-3 β in the presence of $10\mu\text{M}$ compound E *in vitro* (Fig. 10). These results show that (i) the direct incubation of two basic proteins (rhTP and bMBP) with these three *Taxus* compounds (A, C and E) may induce their novel potent phosphorylation sites for GSK-3 β ; and (ii) these *Taxus* compound-induced potent phosphorylation sites for GSK-3 β are different from those observed with EGCG *in vitro*.

The Binding Affinities of the Three *Taxus* Compounds with bMBP, rhTP and rCRMP-2 *in Vitro* The above results (Figs. 9, 10) suggest that the direct bindings of these three *Taxus* compounds to two basic brain proteins (bMBP and rhTP) result in the induction of their high phosphorylation by GSK-3 β *in vitro*. To confirm this, the binding affinities of these three *Taxus* compounds with three brain proteins (rhTP, bMBP and rCRMP-2) and EGCG were separately measured by a QCM. As expected, these three *Taxus* compounds directly bound to bMBP and rhTP, while they showed low binding affinities with rCRMP-2 (Fig. 11). EGCG had binding affinities with these two basic proteins, as previously reported.¹⁾ However, the binding abilities of these three *Taxus* compounds with three protein substrates (bMBP, rhTP and rCRMP-2) did

Fig. 5. The Inhibitory Effects of Three *Taxus* Compounds (A, C, E) and EGCG on the GSK-3 β -Mediated Phosphorylation of rCRMP-2 *in Vitro*

After incubation for 30 min at 30°C in the presence of the indicated concentrations of one of the three *Taxus* compounds (A, C, E) and EGCG, 32 P-labeled rCRMP-2 in the reaction mixtures was detected by autoradiography after SDS-PAGE. The rate 1 represents the phosphorylation of rCRMP-2 by GSK-3 β in the absence of these *Taxus* compounds and EGCG *in vitro*.

Fig. 6. The Stimulatory Effects of Three *Taxus* Compounds and EGCG on the Autophosphorylation of GSK-3 β *in Vitro*

GSK-3 β (approx. 50 ng) was directly incubated for 30 min at 30°C with 5 μ M [γ - 32 P]ATP (500 cpm/pmol) in the presence of the indicated concentrations of one of the three *Taxus* compounds (A, C, E) or EGCG. 32 P-Labelled GSK-3 β in the reaction mixtures was detected by autoradiography after SDS-PAGE. The rate 1 represents the autophosphorylation of GSK-3 β in the absence of these three *Taxus* compounds *in vitro*. Compound A at 10 μ M; 3 μ M compound C; 3 μ M compound E and 1 μ M EGCG.

not correspond to their stimulatory and inhibitory effects on the GSK-3 β -mediated phosphorylation of these three protein substrates *in vitro*.

The High Stimulatory Effect of Compound E on the GSK-3 β -Mediated Phosphorylation of rhTP Primed by CK1 δ *in Vitro* Previously, we reported that rhTP primed by casein kinase 1 δ (CK1 δ) functions as an effective phosphate acceptor for GSK-3 β *in vitro*.²¹⁾ The stimulatory effect of compound E on the GSK-3 β -mediated phosphorylation of unprimed rhTP and rhTP primed by CK1 δ were compared *in vitro*. Compound E at 10 μ M stimulated about 1.9-fold the GSK-3 β -mediated phosphorylation of primed by CK1 δ (lane 4) in comparison with unprimed rhTP (lane 3, Fig. 12). Under the same experimental conditions, similar stimulatory effects of other two *Taxus* compounds (A and C), but not with EGCG, on the GSK-3 β -mediated phosphorylation of rhTP primed by CK1 δ were observed. However, no significant effects of these three *Taxus* compounds on the CK1 δ -mediated phosphorylation of rhTP and α -casein were observed *in vitro* (data not shown). These results show that (i) rhTP primed by CK1 δ functions as an effective substrate for GSK-3 β ; and (ii) compound E highly stimulates the GSK-3 β -mediated phosphorylation of rhTP by primed by CK1 δ *in vitro*.

Fig. 7. Detection of Tyr-Phosphorylation on GSK-3 β Autophosphorylated in the Presence of Compound E or EGCG *in Vitro*

GSK-3 β (approx. 50 ng) was directly incubated for 30 min at 30°C with 5 μ M [γ - 32 P]ATP (500 cpm/pmol) in the absence or presence of either 3 μ M compound E or 1 μ M EGCG. 32 P-Labelled phosphoamino acids of fully autophosphorylated GSK-3 β were detected, as described in the legend of Fig. 3.

Fig. 8. Autophosphorylation Kinetics of GSK-3 β in the Presence of Compound E *in Vitro*

The autophosphorylation kinetics for GSK-3 β were determined by its incubation for 30 min at 30°C with the indicated concentrations of [γ - 32 P]ATP in the presence or absence of either 3 μ M compound E or 1 μ M EGCG *in vitro*. 32 P-Labelled GSK-3 β in the reaction mixtures was determined by the membrane filter method, as described in the Materials and Methods. Lineweaver-Burk plots of ATP for GSK-3 β in the absence (○) or presence of either 3 μ M compound E (●) or 1 μ M EGCG (Δ).

DISCUSSION

The present *in vitro* biochemical characterization revealed that three selected *Taxus* lignans [(3*S*,4*R*)-4'-hydroxy-6,3'-dimethoxyisoflavan-4-ol (compound A), (7*R*)-7-hydroxytaxiresinol (compound C) and tanegool (compound E)] functioned as effective activators for GSK-3 β and high stimulators for the GSK-3 β -mediated phosphorylation of two basic brain proteins (bMBP and rhTP), but not for an acidic protein (rCRMP-2). This conclusion is supported by evidence that (i) these three *Taxus* lignans induce the high autophosphorylation of GSK-3 β (Fig. 6) and the GSK-3 β -mediated high phosphory-

lation of these two basic proteins (Figs. 2, 4), but inhibit dose-dependently the phosphorylation of rCRMP-2 by the kinase (Fig. 5); (ii) the direct bindings of these three *Taxus* lignans to rhTP and bMBP result in the induction of novel potent phosphorylation sites for the kinase on these two basic proteins (Figs. 9, 10); and (iii) these three lignans have binding affinities with bMBP and rhTP, but low binding affinities with rCRMP-2 *in vitro* (Fig. 11). Similar stimulatory and inhibitory effects on the GSK-3 β -mediated phosphorylation of bMBP (Fig. 4) and rCRMP-2 (Fig. 5), respectively, were observed with EGCG *in vitro*. However, the stimulatory effects of two *Taxus* lignans [tanegool and (7*R*)-7-hydroxytaxiresinol] on the autophosphorylation of GSK-3 β are different from that observed with EGCG, because these two lignans, but not EGCG, cause the high autophosphorylation of GSK-3 β through preferential phosphorylation of Thr-residues and the induction of Tyr-phosphorylation on the kinase *in vitro* (Fig. 7). Since it has been reported that the intramolecular autophosphorylation at Tyr-216 on GSK-3 β is required for an activation of the kinase *in vitro*,^{22,23} both tanegool and (7*R*)-7-hydroxytaxiresinol may function as effective activators for GSK-3 β through the induction of Tyr-phosphorylation and a significant increase in the V_{max} value of ATP for the kinase *in vitro*.

Earlier studies concerning the biochemical characterization of high TP phosphorylation have revealed that (i) GSK-3 β is characterized as a protein kinase responsible for hyperphosphorylation of TP in an early stage of human neurodegenerative disorder Alzheimer's disease (AD)²³; (ii) two CK1 isoforms (α and δ) are tightly associated with neurofibrillary lesions²⁴; (iii) cellular CK1 δ is highly elevated in AD brain²⁵; and (iv) the GSK-3 β -mediated high phosphorylation of TP induces synapse loss and neuronal death through the formation of neurofibrillary tangle with highly phosphorylated TP.²⁶ As expected, we confirmed that two *Taxus* lignans [tanegool and (7*R*)-7-hydroxytaxiresinol] significantly stimulated the GSK-3 β -mediated phosphorylation of rhTP primed by CK1 δ

Fig. 9. Peptide Mapping of rhTP Fully Phosphorylated by GSK-3 β in the Presence of One of the Three *Taxus* Compounds or EGCG *in Vitro*

After hrTP (approx. 2 μ g) was incubated for 120 min at 30°C with GSK-3 β (approx. 50 ng) and 5 μ M [γ - 32 P]ATP (500 cpm/pmol) in the presence of either 10 μ M compound A, 10 μ M compound C, 10 μ M compound E or 3 μ M EGCG, 32 P-labelled rhTP was digested with trypsin in the gel, the resulting fragments were analyzed and detected, as described in the legend of Fig. 3.

Fig. 10. Peptide Mapping of bMBP Fully Phosphorylated by GSK-3 β in the Presence of One of the Three *Taxus* Compounds *in Vitro*

After bMBP (approx. 2 μ g) was incubated for 120 min at 30°C with GSK-3 β (approx. 50 ng) and 5 μ M [γ - 32 P]ATP (500 cpm/pmol) in the presence of either 10 μ M compound A, 10 μ M compound C, 10 μ M compound E or 3 μ M EGCG, 32 P-labelled bMBP was digested with trypsin in the gel, and the resulting fragments were detected, as described in the legend of Fig. 9.

Fig. 11. Binding Affinities of the Three *Taxus* Compounds with Three Substrate Proteins (bMBP, rhTP and rCRMP-2) *in Vitro*

The binding affinities of two basic proteins [rhTP (a) and bMBP (b)], an acidic protein [rCRMP-2 (c)] and BSA (control protein) with one of three *Taxus* compounds (A, C, E) at 10 μ M in comparison with 3 μ M EGCG were separately determined at 30°C, using a QCM in 8 mL of 40 mM Tris-HCl (pH 7.6).

in vitro (Fig. 12). Further analytical studies on the GSK-3 β -mediated high phosphorylation of rhTP primed by at least two TP-kinases (CK1 δ and novel PKC δ (nPKC δ)), using these two *Taxus* ligands and EGCG as selective high stimulators for GSK-3 β , may provide direct evidence to explain the biochemical process and the regulatory mechanism in the GSK-3 β -mediated hyperphosphorylation of TP primed by these two TP-kinases in an early stage of AD.

The binding abilities of these three *Taxus* compounds with three brain proteins (bMBP, rhTP and rCRMP-2) (Fig. 11) did not correspond their stimulatory and inhibitory effects on the GSK-3 β -mediated phosphorylation of these substrate proteins *in vitro* (Figs. 2, 4, 5). The findings that these three *Taxus*

compounds induce the novel potent phosphorylation sites for GSK-3 β on these two basic brain proteins (Figs. 9, 10), suggest that the direct binding of these *Taxus* compounds to them may induce the conformational changes, which presumably expose the novel potent phosphorylation sites for GSK-3 β *in vitro*. In contrast, tanegool at a low dose (IC₅₀=approx. 1 μ M) inhibited the GSK-3 β -mediated phosphorylation of rCRMP-2 (Fig. 5), which had a low binding affinity with the *Taxus* compound *in vitro* (Fig. 11). Since tanegool reduces the apparent K_m values of two substrates (rCRMP-2 and ATP) for GSK-3 β (data not shown), it seems like that the direct binding of tanegool to GSK-3 β may inhibit the enzymatic reaction of the kinase with its two substrates (rCRMP-2 and ATP) *in vitro*. In the case of

Fig. 12. The High Substrate Activity of rhTP Primed by CK1 δ for GSK-3 β in the Presence of Compound E *in Vitro*

(a) rhTP (approx. 1 μ g) previously fully phosphorylated by CK1 δ (approx. 5 units) for 120 min at 30°C using cold ATP was used as a phosphate acceptor for GSK-3 β *in vitro*. Either unprimed rhTP (lanes 1 and 2) or primed rhTP (lanes 3 and 4) was further incubated for 20 min at 30°C with GSK-3 β (approx. 50 ng) and 5 μ M [γ - 32 P]ATP (500 cpm/pmol) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 μ M compound E. This phosphorylation was arrested by the addition of SDS-sample buffer, 32 P-labelled unprimed rhTP or primed rhTP in the reaction mixtures was detected by autoradiography after SDS-PAGE. (b) Mean values obtained from the autoradiogram (Fig. 12a) by scanning with a spectrophotometer. The rate 1 represents the GSK-3 β -mediated phosphorylation of unprimed rhTP (lane 1) and primed rhTP (lane 3) in the absence of compound E *in vitro*. ** p <0.01, primed rhTP in the absence of compound E; *** p <0.01, unprimed rhTP (lane 2) compared with primed rhTP (lane 4).

EGCG, this polyphenol compound may preferentially bind to the domains, containing the potent phosphorylation sites for GSK-3 β on rCRMP-2, as previously reported.¹⁾ However, the physiological significance of the EGCG-induced selective inhibition for the GSK-3 β -mediated phosphorylation of CRMP-2 at the cellular level is not yet elucidated in present.

To clearly understand the biological significance of three effective *Taxus* lignans [(3S,4R)-4'-hydroxy-6,3'-dimethoxyisoflavan-4-ol, (7R)-7-hydroxytaxiresinol and tanegool] on the GSK-3 β -mediated high phosphorylation of two basic proteins (MBP and TP) and an acidic protein (CRMP-2), further analytical studies are required to (i) determine the novel consensus *Taxus* lignan-binding domains, containing at least one potent phosphorylation site (Ser-residue) for GSK-3 β on TP and MBP; (ii) characterize novel *Taxus* lignans as potent selective inhibitors for the GSK-3 β -mediated phosphorylation of CRMP-2 (a microtubule-binding protein involved in the axon outgrowth of neurons) in normal neuron; (iii) detect their binding novel functional proteins preferentially phosphorylated by GSK-3 β in the brain of AD model mouse; and (iv) elucidate the novel effects of these three *Taxus* lignans on the GSK-3 β -mediated regulation of TP with its associated proteins, containing multiple potent phosphorylation sites for CK1 δ and nPKC δ , in neuronal cell function.^{21,27)} After these analytical studies, it should be possible to (i) explain the biological and pharmacological significances of novel *Taxus* lignans on the GSK-3 β -mediated regulation of TP with its associated functional proteins²¹⁾ and a native novel substrate β -adducin (80 kDa, pI =5.67)²⁸⁾ involved in cell differentiation and morphological development of neuron; and (ii) elucidate the biochemical mechanisms of anti-inflammatory,¹⁰⁾ anti-proliferation of cancer cell¹⁷⁾ and neuroprotective effects¹⁷⁾ in-

duced by these *Taxus* lignans at the cellular level.

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