Characterization of (–)-Matairesinol as a Potent Inhibitor of Casein Kinase I *in Vitro*

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The inhibitory effects of (–)-matairesinol (MTS) isolated from *Thujopsis dolabrata* var. *hondai* on the activities of four distinct Ser/Thr-protein kinases [two casein kinases (CK-I and CK-II), A-kinase and C-kinase] were determined *in vitro*. It was found that (i) MTS inhibits the activities of CK-I and C-kinase α (ID_{50} =approx. 10 μ M) in a dose-depended manner, but high doses are required to inhibit A-kinase activity (ID_{50} =approx. 90 μ M); (ii) the autophosphorylation of CK-I is more sensitive to MTS (ID_{50} =approx. 0.2 μ M); (iii) MTS inhibits CK-I activity in a manner similar to that observed with CK-I-7 (a CK-I inhibitor); and (iv) the compound inhibits CK-I activity by affecting ATP binding in a mixed type manner. These results indicate that MTS is a potent CK-I inhibitor *in vitro*.

Key words casein kinase I; (–)-matairesinol; potent CK-I inhibitor; *Thujopsis dolabrata* var. *hondai*

Recently, (–)-matairesinol (MTS, see Fig. 1) isolated from *Symplocos setchuensis*, was reported to inhibit HIV replication in H9 lymphocyte cells, and its derivatives exhibit more potent inhibition of the virus *in vitro*.^{1,2)} We have been studying the mechanical actions of several natural anti-viral substances, including glycyrrhizin (GL)^{3,4)} and anti-oxidant polyphenol containing compounds,^{5,6)} in the *in vitro* assay systems for several distinct protein kinases, such as casein kinases I and II (CK-I and CK-II), cAMP-dependent protein kinase (A-kinase) and Ca^{2+} - and phospholipid-dependent protein kinase (C-kinase). Recently, we reported that (i) high mobility group protein 1 (HMG1) is a cholesterol-3-sulfate (CH-3S)-binding protein; and (ii) CH-3S acts as an effector of the phosphorylation of HMG1 by CK-I *in vitro*.^{7,8)} Since the physiological activity of MTS *in vitro* has been not yet elucidated, the present study was carried out to determine its inhibitory effects on the activities of four distinct Ser/Thr-protein kinases (CK-I, CK-II, A-kinase and C-kinase) *in vitro*. Here, we describe (i) biochemical characterization of MTS as a potent inhibitor of CK-I *in vitro*; (ii) the inhibitory kinetics of MTS on the CK-I-mediated phosphorylation of α -casein or HMG1 *in vitro*; and (iii) the MTS-induced selective inhibition of the CK-I-mediated phosphorylation of cellular proteins in the partially purified heparin fraction containing two casein kinases (CK-I and CK-II) from rat liver *in vitro*.

MATERIALS AND METHODS

Chemicals [γ -³²P]ATP (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Arlington Heights, U.S.A.); dithiothreitol (DTT) and a silver staining kit from Wako Pure Chemical Ind. (Osaka, Japan); CH-3S (5-cholesten-3 β -ol sulfate) from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan); myelin basic protein (MBP, a protein substrate of C-kinase η), CK-I-7 [*N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide (a specific CK-I inhibitor)] from Seikagaku Kogyo (Tokyo, Japan); and α -casein, histone IIIS (calf thymus), actin (rabbit skeletal muscle), poly-L-arginine (poly-Arg, about 100 residues), phosphatidylserine (PS) and

Fig. 1. The Chemical Structure of MTS

diacylglycerol (DG) from Sigma Chemical (St. Louis, U.S.A.).

Extraction of MTS Sawdust of *Thujopsis dolabrata* SIEB. *et* ZUCC. var. *hondai* MAKINO (5.8 kg) was successively extracted three times (3 d each) with *n*-hexane, chloroform and methanol at room temperature. After filtration, the solvent was evaporated under reduced pressure to afford a *n*-hexane extract (91 g), a chloroform extract (109 g) and a methanol extract (54 g). The chloroform extract (109 g) was dissolved in chloroform (1.0 l), and then extracted with 1% NaOH (1.5 l). The aqueous layer was separated and acidified with HCl, followed by extraction with chloroform. The chloroform layers were combined and washed with NaCl saturated water (1.0 l). The concentrated chloroform layer was used as an acidic part of the chloroform extract (14.0 g).

Isolation and Identification of MTS The acidic fraction of the chloroform extract of *T. dolabrata* var. *hondai* (2.8 g) was chromatographed over Florisil (100–200 mesh, Wako Chemicals U.S.A., Inc.) with guidance by positive reaction with $FeCl_3$, using solvents of increasing polarity from chloroform through methanol to give 6 fractions (Fr. I-1 to Fr. I-6). Fr. I-1 (835 mg) was further chromatographed over Florisil, eluting with chloroform/ethyl acetate (50 : 1 to 1 : 1), to give 11 fractions (Fr. II-1 to Fr. II-11). Fr. II-11 (220 mg) was successively subjected to Florisil column chromatography (chloroform/methanol, 10 : 1) and preparative TLC (benzene/ethyl acetate, 2 : 1) to give MTS (20 mg) with a yield of 0.0017%. The spectra of IR, UV, MS, ¹H- and ¹³C-NMR, and [α]_D of the purified compound corresponded exactly to those reported for MTS.⁹⁾ This is the first report of the isolation of MTS from *T. dolabrata* var. *hondai*.

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Protein Kinases Rat liver CK-I (specific activity: 17500 units/mg) was obtained from Promega Co. (Madison, U.S.A.); bovine heart type I A-kinase (specific activity: 400×10^3 units/mg protein) from Sigma Chemical (St. Louis, U.S.A.); rat liver C-kinase α (specific activity: $1-2 \mu\text{mol}$ phosphate/min/mg) purified from Molecular Probes (Oregon, U.S.A.); recombinant human C-kinase η (rhC-kinase η , specific activity: 0.9 mmol phosphate/min/mg protein) and recombinant human CK-II (rhCK-II, specific activity 400×10^3 units/mg protein) from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, U.S.A.).

Assay for the Activity of A-Kinase The activity (phosphorylation of histone H2B) of purified A-kinase (bovine heart) was assayed in the presence of $2 \mu\text{M}$ cAMP, as reported previously.⁶⁾

Assays for the Activities of CK-I and CK-II The activity (phosphorylation of α -casein or HMG1) of CK-II was assayed in reaction mixtures comprising 40 mM Tris-HCl (pH 7.4), CK-II (approx. 10 ng), $10 \mu\text{M}$ [γ - ^{32}P]ATP (1000 cpm/pmol), 10 mM Mg^{2+} , 2 mM DTT, poly-Arg (a CK-II activator, $0.5 \mu\text{g}$) and α -casein or HMG1 ($4 \mu\text{g}$). Phosphorylation of HMG1 by CK-I was carried out in the presence of $3 \mu\text{M}$ CH-3S (an activator of the CK-I-mediated phosphorylation of HMG1 *in vitro*).⁷⁾ After incubation for 30 min at 30°C , ^{32}P -phosphorylated α -casein or HMG1 (p31) in the reaction mixtures was detected directly by autoradiography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as reported previously.⁴⁻⁷⁾

Assay for the Activities of C-Kinase α and C-Kinase η The activity (phosphorylation of histone H1S) of C-kinase α was assayed in the presence of C-kinase activators (1 mM Ca^{2+} , $25 \mu\text{M}$ PS and $3 \mu\text{M}$ DG). Phosphorylation of MBP by C-kinase η was carried out in the presence of 1 mM Ca^{2+} . After the incubation for 30 min at 30°C with the indicated doses of MTS, the ^{32}P -phosphorylated histone H1S or MBP (p21) in the reaction mixtures was detected by SDS-PAGE followed by autoradiography, as previously reported.⁸⁾

RESULTS AND DISCUSSION

Inhibitory Effects of MTS on the Activities of Four Distinct Protein Kinases *in Vitro* The inhibitory effects of MTS on the activities of CK-I, CK-II, A-kinase and C-kinase α were examined *in vitro*. As shown in Fig. 2A, MTS inhibited CK-I activity (phosphorylation of α -casein) in a dose dependent manner (ID_{50} =approx. $10 \mu\text{M}$), but did not affect CK-II activity (phosphorylation of α -casein) even at 1 mM . The activity (phosphorylation of histone H1S) of C-kinase α was inhibited in a similar fashion to CK-I activity (ID_{50} =approx. $10 \mu\text{M}$), but required high doses to inhibit A-kinase activity (ID_{50} =approx. $90 \mu\text{M}$). Similar inhibitory kinetics of MTS on CK-I activity were obtained when HMG1 was used as a substrate instead of α -casein (Fig. 2B). These results show that MTS inhibits the activities of CK-I and C-kinase α *in vitro*.

To test the direct effects of MTS on the autophosphorylation of CK-I *in vitro*, CK-I was incubated with $10 \mu\text{M}$ [γ - ^{32}P]ATP (1000 cpm/pmol) in the presence or absence of various doses of MTS. About 80% of the autophosphorylation of CK-I was inhibited at $1 \mu\text{M}$ and this inhibition was maintained above $1 \mu\text{M}$ (Fig. 3). The 50% inhibition dose (ID_{50}) of

Fig. 2. [A] The Inhibitory Effects of MTS on the Activities of Four Distinct Protein Kinases *in Vitro*

Four distinct Ser/Thr-protein kinases (CK-I, CK-II, A-kinase and C-kinase α) were separately assayed in the presence of the indicated doses of MTS, as described in Materials and Methods. ^{32}P -Phosphorylated proteins (α -casein, HMG1 or histone H1S) in the reaction mixtures were detected by SDS-PAGE followed by autoradiography. The autoradiogram was scanned by a spectrophotometer. 100% represents protein phosphorylation by each protein kinase in the absence of MTS. CK-I (●), CK-II (■), A-kinase (○), and C-kinase α (▲).

[B] The Inhibitory Effect of MTS on CK-I Activity Using Two Distinct Protein Substrates *in Vitro*

CK-I was assayed in the presence of the indicated doses of MTS using α -casein or HMG1. ^{32}P -Phosphorylated α -casein (●) or HMG1 (○) in the reaction mixtures was detected by SDS-PAGE followed by autoradiography.

Fig. 3. The Inhibitory Effect of MTS on the Autophosphorylation of CK-I and CK-I Activity *in Vitro*

CK-I was incubated with $10 \mu\text{M}$ [γ - ^{32}P]ATP (1000 cpm/pmol) in reaction mixtures comprising 40 mM Tris-HCl (pH 7.4), CK-I (approx. 10 ng), 10 mM Mg^{2+} and 2 mM DTT, and incubated for 30 min at 30°C in the presence of the indicated doses of MTS. ^{32}P -Labeled CK-I (an autophosphorylated form of CK-I, p37) in the reaction mixtures was detected by SDS-PAGE followed by the autoradiography. The autoradiogram was scanned by a spectrophotometer. 100% represents autophosphorylation of CK-I in the absence of MTS. Autophosphorylation of CK-I (○) and the CK-I-mediated phosphorylation of α -casein (●).

MTS on the autophosphorylation of CK-I was about one fiftieth the concentration of that required to inhibit CK-I activity on α -casein (Fig. 3). Under the same experimental conditions, a similar inhibition of MTS on CK-I activity was obtained when skeletal muscle actin (rabbit) instead of α -casein was used as a substrate for CK-I (data not shown). These results suggest that MTS directly binds to CK-I and results in the inhibition of its autophosphorylation *in vitro*. In contrast, the ID_{50} of MTS on the autophosphorylation of C-kinase α was found to be approx. $10 \mu\text{M}$, which is about the same dose

Fig. 4. The Inhibitory Effects of MTS and CK-I-7 on the Activities of CK-I and CK-II *in Vitro*

The inhibitory effects of MTS and CK-I-7 on the activities of CK-I and CK-II were separately determined in the presence of the indicated doses, as described in Fig. 2A. 32 P-labeled α -casein in the reaction mixtures was detected by SDS-PAGE followed by autoradiography. The autoradiogram was scanned by a spectrophotometer. 100% represents protein phosphorylation by each protein kinase in the absence of the two treatments. The inhibitory effect of CK-I-7 on the activities of CK-I (○) and CK-II (□); and the inhibitory effect of MTS on the activities of CK-I (●) and CK-II (■).

as the inhibitory effect on the activity of C-kinase α (Fig. 2A). Similar inhibitory effects of MTS on the activity (phosphorylation of MBP) of C-kinase η and its autophosphorylation *in vitro* were observed (data not shown).

Characterization of MTS as a Selective CK-I Inhibitor *in Vitro* To characterize the selective effect of MTS on the autophosphorylation of CK-I and the CK-I-mediated phosphorylation of α -casein, two inhibitors (MTS and CK-I-7) were compared. The inhibitory kinetics of MTS on CK-I activity were similar to those observed with CK-I-7 (ID_{50} = approx. 10 μ M) (Fig. 4). Although CK-I-7 at 1 mM inhibited about 50% of CK-II activity, there was no obvious effect of MTS on CK-II activity even at 1 mM. These results show that the sensitivity of CK-II activity to MTS is different from that to CK-I-7 up at 1 mM *in vitro*.

Inhibitory Kinetics of MTS on CK-I Activity *in Vitro*

The inhibitory kinetics of MTS on CK-I activity were determined using α -casein as a substrate. The V_{max} value shifted from 45 to 31 nmol/mg/min when MTS (30 μ M) was added to the reaction mixtures (Fig. 5). In contrast, the K_m value (1.14 μ M) for ATP shifted to 2.06 μ M under the given experimental conditions. Similar inhibitory kinetics of MTS on the phosphorylation of HMG1 by CK-I were observed (data not shown). These results show that MTS inhibits protein phosphorylation by CK-I by affecting ATP binding in a mixed type manner.

In the present study, MTS has been characterized as a potent CK-I inhibitor *in vitro*, because (i) MTS inhibits selectively CK-I activity (ID_{50} = approx. 10 μ M) (Fig. 2A) by affecting ATP binding in a mixed type manner (Fig. 5); and (ii) at a low dosage (ID_{50} = approx. 0.2 μ M), MTS inhibits the autophosphorylation of CK-I *in vitro* (Fig. 3). Although CK-I-7 at 1 mM inhibited about 50% of CK-II activity, there was no such effect of MTS on CK-II activity (Fig. 4). These results suggest that MTS acts as a selective CK-I inhibitor more than CK-I-7 at above 1 mM *in vitro*. Therefore, it is possible to speculate that MTS may inhibit the replication of human immunodeficiency virus (HIV) in H9 lymphocyte cells through its selective inhibition of the CK-I-mediated phos-

Fig. 5. The Inhibitory Kinetics of MTS on the Phosphorylation of α -Casein by CK-I *in Vitro*

After incubation (30 min at 30 °C), the radioactivity of 32 P-labeled α -casein on a glass filter was measured with a scintillation spectrometer, as reported previously.¹⁰⁾ The K_m and V_{max} values are represented on Lineweaver-Burk plots. The phosphorylation of α -casein by CK-I was determined in the absence (○) or presence of 30 μ M MTS (●).

phorylation of viral and cellular proteins involved in HIV replication in HIV-infected cells.

The CK-I family consists of multiple isoforms encoded by seven distinct genes (α , β , δ , ϵ , and γ 1–3) in various mammalian cells.¹¹⁾ Recently, IC261 (3[2,4,6-trimethoxyphenyl]-methylidenyl]-indolin-2-one) has been characterized as a specific inhibitor of both CK-I δ and CK-I ϵ , and it has been demonstrated that this compound inhibits the activities of these two CK-I isoforms (ID_{50} = approx. 1 μ M) in an ATP-competitive manner *in vitro*.¹²⁾ Furthermore, it has been reported that IC261 specifically inhibits the CK-I δ - and CK-I ϵ -mediated signal transduction involved in cell division, possibly through the regulation of centrosome or spindle function during mitosis.¹³⁾ Although there is no structural similarity between MTS and IC261, it will be interesting (i) to examine the *in vitro* phosphorylation of cellular proteins by the MTS-sensitive CK-I isoforms, which are implicated in triggering the progression of HIV replication and in CK-I-regulated biological events, such as DNA replication, DNA repair, mitosis,^{14–16)} and Wnt signaling¹⁷⁾; and (ii) to identify the functional mediators, such as p45, specifically phosphorylated by the MTS-sensitive CK-I isoforms at the cellular level.

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