A Novel, Stable, Estradiol-Stimulating, Osteogenic Yam Protein with Potential for the Treatment of Menopausal Syndrome

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A novel, stable, estradiol-stimulating, osteogenic Yam protein with potential for the treatment of menopausal syndrome

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A novel protein, designated as DOI, isolated from the Chinese yam (Dioscorea opposita Thunb.) could be the first protein drug for the treatment of menopausal syndrome and an alternative to hormone replacement therapy (HRT), which is known to have undesirable side effects. DOI is an acid- and thermo-stable protein with a distinctive N-terminal sequence Gly-Ile-Gly-Lys-Ile-Thr-Thr-Tyr-Trp-Gly-Gln-Tyr-Ser-Asp-Glu-Pro-Ser-Leu-Thr-Glu. DOI was found to stimulate estradiol biosynthesis in rat ovarian granulosa cells; induce estradiol and progesterone secretion in 16- to 18-month-old female Sprague Dawley rats; upregulate expressions of follicle-stimulating hormone receptor and ovarian aromatase; counteract the progression of osteoporosis and augment bone mineral density; and improve cognitive functioning by upregulating protein expressions of brain-derived neurotrophic factor and TrkB receptors in the prefrontal cortex. Furthermore, DOI did not stimulate the proliferation of breast cancer and ovarian cancer cells, which suggest it could be a more efficacious and safer alternative to HRT.

• Menopause is the period during which the level of estrogen secreted by the ovaries gradually declines and patients experience osteoporosis, cognitive decline, hot flush, mood disorder, night sweat, depression, etc [1].

• The current conventional medical treatment to relieve menopausal syndrome is hormone replacement therapy (HRT). However, various studies showed that HRT might increase the incidence of breast cancer and ovarian cancer.

• A potential safer alternative for menopause is desirable.
• Chinese yam (the tuber of *Dioscorea opposita*), is a well-known edible and medicinal herb for treating menopausal syndrome in Chinese medicine.

• In Clinical study, Chinese yam extract improves the status of sex hormones (E$_2$ & P), lipids, and antioxidants in menopausal women [2-4].

• However, the estrogenic properties of bioactive component in Chinese yam have not been reported yet.
1. What is the **estrogenic component** contained in *Dioscorea* tubers for treating menopause?
2. What is its chemical and biological characters?
3. What is its action mechanism?

(Based on our pilot study, **total protein extract** of *Dioscorea opposita* tuber possessed estrogen-stimulating effect on rat ovarian granulosa cell.)

- Thus, we hypothesize that
- Protein isolated from *Dioscorea opposita* tuber could increase estradiol biosynthesis *in vitro* and *in vivo*: implication for improving the menopausal status.
Microscopic Authentication (Paraffin Section and Powder Section)

Table 2. Comparison of rhizomes of four <i>Dioscorea</i> species in microscopic authentication—powder sections (ZEISS 200X)

<table>
<thead>
<tr>
<th>Contents</th>
<th>DA</th>
<th>DZ</th>
<th>DH</th>
<th>DO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessels</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>Starch granules</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td>Mucous cells and raphides of calcium oxalate</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
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<tr>
<td>Thick-walled cells</td>
<td>--</td>
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<tr>
<td>Resin</td>
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</tr>
</tbody>
</table>

DA, DZ, DH, and DO represent <i>D. alata</i> L., <i>D. zingiberensis</i> C.H. Wright, <i>D. colletti</i> var. hypoglauca (Palib.) S.J. Pel & C.T. Ting, and <i>D. oppositifolia</i> L., respectively

-- not detected

Fig. 1. Paraffin sections of rhizomes of four <i>Dioscorea</i> rhizome (ZEISS 100X). The major features of a DA rhizome includes several layers of suberized cells followed by cortex which contains mucous cells and raphides of calcium oxalate, resin ducts, and starch granules; b DZ rhizome includes several layers of suberized cells followed by cortex which contains resin ducts, vascular bundles, and starch granules; c DH rhizome includes several layers of suberized cells, followed by cortex which contains mucous cells and raphides of calcium oxalate, amphivasal vascular bundles, and plenty of starch granules; d DO rhizome includes two layers of suberized cells, followed by cortex which contains resin ducts, mucous cells, and raphides of calcium oxalate and starch granules. DA, DZ, DH, and DO represent <i>D. alata</i> L., <i>D. zingiberensis</i> C.H. Wright, <i>D. colletti</i> var. hypoglauca (Palib.) S.J. Pel & C.T. Ting, and <i>D. oppositifolia</i> L., respectively.
Rhizome Proteins Isolation by DOI-affinity Antibody Column

(c) Antibody-affinity chromatography

Fig. 4  a The total protein extracts and b DOI-like proteins of rhizomes of four different Dioscorea species were visualized on SDS-PAGE gel after silver staining. DA, DZ, DH, and DO represent D. alata L., D. zingiberensis C.H. Wright, D. colletii var. hypoglauca (Palib.) S.J. Pei & C.T. Ting, and D. oppositifolia L., respectively.
Measurement of 17β-estradiol Levels in Cell Culture Medium

Fig. 2  Effect of estrogen-stimulating DOI-like proteins purified from the total protein extracts of four different *Dioscorea* species on E₂ secretion/cellular protein level in ovarian granulosa cells after 12 h of treatment. Data are presented as mean ± SEM of three replicate cultures. The statistical significance of difference between control and the DOI-like protein-treated groups was analyzed by Tukey test (*n* = 3). *Asterisks* indicate statistically significant difference compared with control group (**p < 0.01, ***p < 0.001*)
Results (1)
DOI isolation from *Dioscorea opposita*

Tuber of *Dioscorea opposita* → Protein extraction → FPLC → SDS-PAGE

**Purification of DOI**
- on HiPrep 16/10 DEAE FF column.
- on HiPrep 16/10 Phenyl FF (high sub) column.
- on Superdex 75 10/300 GL column.

**MS**
- Mass spectrometry analysis of DOI
- *DOI* → 33.5 kDa

**SDS-PAGE**
- *DOI* → 33.5 kDa
- 34 kDa
- 26 kDa
Result (2): *In vitro* study --- DOI

2.1. *In vitro* study-- DOI increases estradiol biosynthesis and up-regulates aromatase and FSHR in ovarian granulosa cells

Isolation of ovarian granulosa cell → Primary cell culture of ovarian granulosa cell → E2 detection in cell culture medium → FSHR and Aromatase protein levels detection

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Results are expressed as means ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control group (un-paired t-test).
2.2. In vitro study--DOI increases E$_2$ level under 60 °C and 80°C, it is an acid stable protein

Isolation of ovarian granulosa cell → Primary cell culture of ovarian granulosa cell → E$_2$ detection
2.3. DOI does not stimulate proliferation of MCF-7 cells (breast cancer) and OVCA-429 cells (ovarian cancer), it showed tissue-specific protein expression of aromatase in the ovary, but not in the breast in vivo.

**Result (2): In vitro study and in vivo study --- DOI and aromatase**

Results are expressed as means ± SEM, n=3. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control group (un-paired t-test).
Result (3): *In vivo* study --- DOI

3.1. *In vivo* study--DOI increases serum estradiol level, progesterone level and protein levels of aromatase and FSHR in ovaries of aged female SD-rats (16-18 month-old)

Intraperitoneal injection of DOI to SD rat

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**E2 detection**

**FSHR and Aromatase detection**

By Western Blotting analysis

Results are expressed as means ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control group (One-way ANOVA followed by Dunnett’s Multiple Comparison Test). Ctl: control group received daily intraperitoneal injections of PBS; Premarin: positive control group received daily Premarin (12.4 mg/kg) by oral administration; DOI group: DOI-treated groups received daily intraperitoneal injections of DOI (2.5, 5, and 10 mg/kg).
3.2. *In vivo* study--DOI increases mRNA levels of CYP-19 (aromatase) and FSHR in ovaries of aged female SD-rats (16-18 month-old)

Intraperitoneal injection of DOI to SD rat

**RT-PCR**

Results are expressed as means ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control group (One-way ANOVA followed by Dunnett’s Multiple Comparison Test). Ctl: control group received daily intraperitoneal injections of PBS; Premarin: positive control group received daily Premarin (12.4 mg/kg) by oral administration; DOI group: DOI-treated groups received daily intraperitoneal injections of DOI (2.5, 5, and 10 mg/kg).
3.3. DOI increases apparent trabecular bone mineral density (tBMD)

Intraperitoneal injection of DOI to SD rat

Result (3): *In vivo* study --- DOI

Micro-CT

**In vivo** study --- DOI

Results are expressed as means ± SEM, n=6
* p <0.05, compared with control group by un-paired t-test.
Result (3): In vivo study --- Cognitive function deterioration accompanied

- Cognitive function deterioration is a common disorder in old age accompanied with menopause.
- It was reported that the ability of spatial learning and memory could be improved by adjusting the brain derived nerve growth factor (BDNF) in the hippocampus and the BDNF-TrkB pathway[10].
Result (3): *In vivo* study --- DOI

3.4. DOI increases BDNF protein expression in hippocampus and prefrontal cortex and increases TrkB receptor protein expression in prefrontal cortex

Intraperitoneal injection of DOI to SD rat

BDNF detection

TrkB gp145 detection

Results are expressed as means ± SEM (n = 6). *p < 0.05, **p < 0.01 compared with the control group (un-paired t-test).
Discussion and Summary (1)

1. The novel DOI protein isolated from *Dioscorea opposita* stimulated estradiol secretion by upregulating the expression of ovarian FSHR and aromatase both *in vitro* and *in vivo*.

2. DOI also induced the secretion of estradiol and progesterone in aged female SD rats.

3. More importantly, DOI has tissue-specific bioactivity as it could upregulate protein expression levels of ovarian aromatase but not breast aromatase. These results suggest that DOI could be a more efficacious and safer alternative to HRT for the treatment of menopausal syndrome.

4. Apart from improving the hormonal status, DOI could be beneficial for menopausal osteoporosis and improve cognitive functioning.
A bioactive protein that increases estrogen and progesterone biosynthesis, has been isolated from Chinese Yam by our research team, for treating conditions resulting from low serum estrogen and progesterone levels including osteoporosis, menopausal syndrome and cognitive function deterioration accompanied. US Patent has been obtained [Novel Bioactive Protein Isolated from Chinese Yam (U.S. Patent No.: US9273105B2; 1st Mar 2016)].

This study will provide a better treatment plan and concept in dealing with the medical conditions of menopausal syndrome and should benefit the good sake of health for both the community of Hong Kong and even worldwide.
References


8. Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS. Follicle-Stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. Molecular Endocrinology Baltimore, Md 2000;14:1283-1300


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