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Melanin-concentrating Hormone-1 Receptor (MCH-1) Antagonism of the Leaves Extract from *Morus alba*

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Abstract – The present study was performed to investigate the binding affinity of the ethanol extract from the leaves of *Morus alba* (EMA) and some EMA related plant materials (EMA-D, EMA-DM) for melanin-concentrating hormone-1 receptor (MCH-1) and also to examine the antagonistic effect of them for the recombinant MCH-1 receptor expressed in CHO cells. EMA, dichloromethane fraction (EMA-D) and EMA-DM exhibited high affinity for mammalian MCH receptor in receptor binding assays (IC_{50} value: 2.3, 1.6 and 1.0 μg/ml, respectively). Other plant materials (MMA-D, MMA-DM) obtained from methanol extracts from the leaves of *Morus alba* (MMA) also exhibited high affinity for mammalian MCH receptor, even though the IC_{50} values of them were lower than those of EMA-D and EMA-DM. In Chinese hamster ovary (CHO) cells expressing human MCH-1, EMA-DM and EMA-D significantly inhibited MCH-induced intracellular Ca^{2+} increase (IC_{50} values: 16.5 and 22.7 μg/ml, respectively). These results clearly indicate that the ethanol extract from the leaves of *Morus alba* (EMA) and some EMA related plant materials (EMA-D, EMA-DM) are novel selective MCH-1 receptor antagonist, respectively.

Keywords – *Morus alba*, Moraceae, Melanin-concentrating hormone, MCH-1 receptor antagonist

Introduction

Obesity is the principal risk factor of metabolic syndrome, and is developed when the energy intake exceeds the energy expenditure (Lebovitz, 2003; Lubrano *et al.*, 2004). Recent pharmacological approaches to induce weight loss have included modulation of several metabolic processes. One of the most promising pharmacological targets in these endeavors is the melanin-concentrating hormone (MCH; Pissios and Maratos-Flier, 2003). There are a lot of scientific data to support a role for MCH in the regulation of food intake, body weight, and energy balance (Shearman *et al.*, 2003). Recently, many pharmaceutical companies make every effort to discover novel melanin-concentrating hormone receptor subtype-1 (MCH-1) antagonists for the treatment of obesity and metabolic syndrome, evidenced by the increased number of patents describing MCH-1 antagonists (Kowlaski & McBriar, 2004).

Over the last decade, interest by the general public to prevent and heal of diet and metabolic disorders by using dietary phytomedicine has risen exponentially, due to its low toxicity and good therapeutically performance. The leaves of mulberry, *Morus alba* (Moraceae) is one of the resources commonly used for sericulture in the world and have been traditionally administered as natural therapeutic agent for the alleviating dropsy and diabetes. In addition, Mulberry leaf apart from its use as animal and insect feed, have widely consumed in Korea, Japan and Chile as antihyperglycemic nutraceutical foods for patients with diabetes mellitus (Jang *et al.*, 2002). Recently, many studies showed that leaves extracts of *Morus alba* exhibited several pharmacological effects including regulation adipocytokine in db/db mice (sugimoto *et al.*, 2008), anti-atherogenic effect in apolipoprotein E-deficient mice (Harauma *et al.*, 2007) and biphasic effects on mice in chronic forced swimming model (Sattayasai *et al.*, 2008). However, to our best knowledge, the MCH-1 receptor antagonistic effects of leaves extracts of *Morus alba* have not been examined previously.

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In our previous efforts using the binding assay to discover a novel MCH-1 receptor antagonist from natural resources, we found that leaves extracts of *Morus alba* (EMA) binds with high affinity to the MCH-1 receptor. Therefore, the purpose of present study was to investigate whether EMA might possess some potent MCH-1 receptor antagonists, which could be employed as promising natural crude drug for the treatment of obesity.

Experimental

Plant materials – The leaves of *Morus alba* were collected at Jeonbuk Province, Korea on May in 2006, and identified by Prof. K. Bae, Chungnam National University. A voucher specimen was deposited at the herbarium of Korea Research Institute of Chemical Technology, Korea (KR-0162). The dried leaves (1.0 kg) were soaked in 20 liter of ethanol at room temperature for 7 days. Then, it filtered on cotton ball and the filtrate was subjected to evaporation at 40°C under reduced pressure on a rotary evaporator to afford 205 g of dark syrupy residues, *i.e.*, the crude ethanol extract of *Morus alba* leaves (EMA). EMA was suspended in 1 liter of water, and then partitioned with equal volume of dichloromethane. The dichloromethane layer was concentrated to dryness under reduced pressure at 40°C to give 33 g of dichloromethane fraction (EMA-D). The remained water layer was further extracted with the equal volume of ethylacetate and *n*-butanol sequentially, which finally afforded the 0.6 g of ethylacetate fraction (EMA-E), 21 g of *n*-butanol fraction (EMA-B) and 151 g of remained water fraction (EMA-W). By the assessment of each solvent fraction, dichloromethane fraction (EMA-D) exhibited potent inhibitory effect on binding of Eu-MCH to the human recombinant MCH-1 receptor. Thus, dichloromethane fraction (EMA-D) was suspended in 1 liter of 98% methanol, and then extracted with equal volume of *n*-hexane to afford 27 g of dichloromethane-hexane fraction (EMA-DH) and 6 g of dichloromethane-methanol fraction (EMA-DM), respectively. According to the same extraction protocol described above, 270 g of methanol extract (MMA) was prepared from 1.0 kg of dried *Morus alba* leaves. Subsequently, the 64 g of dichloromethane fraction (MMA-D), 2 g of ethylacetate fraction (MMA-E), 35 g of *n*-butanol fraction (MMA-B) and 165 g water fraction (MMA-W) were also prepared.

Europium (Eu)-ligand receptor binding assay – For Europium (Eu)-ligand receptor binding assay, the mammalian MCH was labeled with europium at N1 position (Eu-MCH) by the Wallac labeling service (PerkinElmer Oy, Turku, Finland). This mammalian MCH is a 19-amino acid peptide that is fully conserved in human, mouse and rat. Receptor binding assays with Eu-MCH were performed in 96-well AcroWell™ plates as previously reported (Lee *et al.*, 2006). The assay buffer contained 25 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% bovine serum albumin pH 7.4. Non-specific Eu-MCH binding was determined experimentally by the presence of 0.5 μM unlabeled MCH (human). After incubation at room temperature for 90 min, the incubation mixtures were filtered in the automatic vacuum filtration system through filter plates (Korean patent application number: 04-0050343) and rapidly washed three times with 300 μl of ice-cold 25 mM HEPES buffer (pH 7.4). The europium was dissociated from the bound ligand by the addition of 150 μl of DELFIA enhancement solution (PerkinElmer Oy, Turku, Finland) and incubated for 10 min with shaking. Dissociated europium created highly fluorescent complexes, which were measured in a multi-label counter with a TRF option (Victor II, PerkinElmer Oy, Turku, Finland). The extracts of *Morus alba* leaves and their fractions were treated 5 min before the initiation of fluorescence measurements. The counter setting was 340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm. GW803430 used as a reference compound has been undergoing Phase I clinical development as MCH-1 receptor antagonist (Hertzog *et al.*, 2006).

Functional assay measuring MCH-1 receptor activity – The antagonistic activity was determined by evaluating the inhibitory effect on MCH-1 receptor-coupled calcium transients in response to 100 nM MCH-1 in Chinese hamster ovary (CHO) cells expressing human MCH-1 receptor by a benchtop scanning fluorometer (FlexStation II, Molecular Devices, Sunnyvale, CA, USA). CHO cells were purchased from American Tissue Culture Collections (Manasa, VA, USA. cDNA for human MCH-1 receptor (SLC1/GPR24, GenBank Acc# AY562945) and human G protein a16 (GenBank Acc# AF493904) in pcDNA3.1$^+$ were transfected with Lipofectamine 2000 according to manufacturer's instructions. To obtain reproducible Ca^{2+} responses, cells were split every 3 days before they became confluent at a culture condition of Ham's F12 with 10% fetal bovine serum (Gibco BRL, Rockville, MD, USA), and discarded after 2 - 3 months of continuous growth with splitting to prepare new cells from a frozen stock. Before the day of experiments, CHO-MCH-1 receptor cells at optimum growth were transferred onto black sided and clear bottomed 96 well plates for FlexStation assays (8,000-10,000 cells/well). After overnight incubation, the homo-

geneous fluorescence calcium assay dye (Calcium assay kit, BD Biosciences, San Jose, CA, USA) in Hank's balanced salt solution (HBSS) with 20 mM of HEPES and 2.5 mM of probenecid at pH 7.4 was loaded according to the manufacturer's instructions. The plates were then incubated at 37°C for 60 min before being placed in the FlexStation II (MDS Analytical Technologies, Sunnyvale, CA, USA). Fluorescence signals were measured every 1.52 s for the total of 120 s time frame, and 50 μl of agonist (100 nM of MCH) or dye buffer (HBSS with 20 mM of HEPES, pH 7.4) was added to each well in column-wise after 20 s of the equilibration period. The fractions or vehicle (0.5% DMSO) were treated 5 min before the initiation of fluorescence measurements. The filter settings for fluorescence measurements for FlexStation II were 485 nm for excitation and 525 nm for emission with cutoff at 515 nm. The real time kinetics was measured for 60 s to observe calcium changes. The maximum change in fluorescence over baseline was used to determine the agonist response. GW803430 was used as reference compound.

Statistical analysis – The concentration-response curves and IC_{50} were analyzed by nonlinear regression (GraphPad Prism 3.0, La Jolla, CA, USA). All values are expressed as mean ± S.D. and Data represent mean value obtained from three or four separate experiments.

Results and Discussion

Europium (Eu)-ligand receptor binding assay – The binding affinity of plant materials prepared from leaves extracts of *Morus alba* on the MCH-1 receptor was determined using Eu-MCH in a competition binding experiment. We previously reported that the europium-ligand receptor binding assay, together with the time-resolved fluorescence ligand and the AcroWell™ 96-well filter plate, is capable of screening human MCH-1 receptor antagonists in high throughput format (Lee *et al.*, 2006) and this binding assay was verified with GW803430 as reference compound (IC_{50} value: 4.1 ng/ml). By using the europium-ligand receptor binding assay, we found that ethanol extract of *Morus alba* leaves (EMA) binds with high affinity to the human recombinant MCH-1 receptor. The IC_{50} value (i.e. the concentration that causes 50% inhibition of specific binding between Eu-MCH and receptor) of EMA was 2.3 ± 1.0 μg/ml. Other plant materials derived from EMA, EMA-D and EMA-DM showed more potent binding affinity (IC_{50} value: 1.6 ± 0.8 and 1.0 ± 0.5 μg/ml, respectively) than EMA (Fig. 1). On the other hand, EMA-E and EMA-DH showed relatively lower activities (IC_{50} values: 6.7 ± 1.8 and 17.1 ± 9.9 μg/ml, respectively), whereas EMA-W and EMA-B exhibited poor inhibition (Fig. 1).

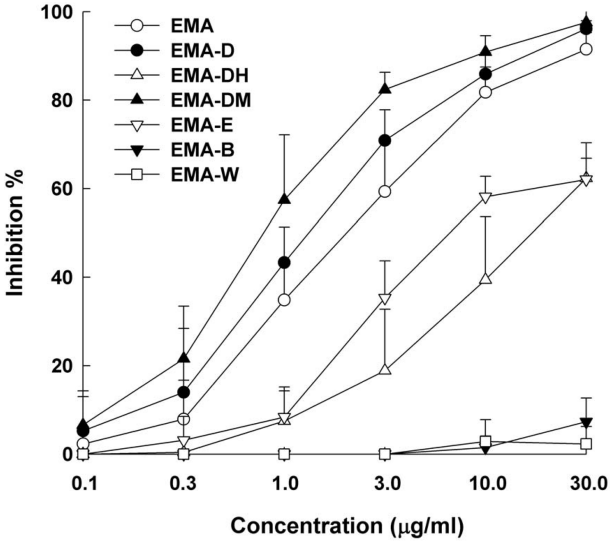

Fig. 1. Concentration-response curves of various plant materials derived from ethanol extract of *Morus alba* leaves (EMA) for human melanin-concentrating hormone subtype 1 (MCH-1) receptor binding assay. Binding affinity for MCH-1 receptor was determined by competitive binding with Eu-MCH. Data represent mean value obtained from three separate experiments.

The other plant materials MMA-D and MMA-E derived from MMA (IC_{50} value: 4.0 ± 1.0 μg/ml) also demonstrated high binding affinity to MCH-1 even although the binding potency of them were lower than those of corresponding plant materials derived from EMA (Fig. 2). These results suggested that the proportion of active components with MCH-1 receptor antagonism in EMA might be different from those in MMA.

Functional assay measuring MCH-1 receptor activity – The plant materials derived from EMA, which showed good binding affinity to MCH-1 receptor, were evaluated for antagonistic activity to the receptor. To determine the antagonist activity, a functional assay was performed by way of examining the intracellular Ca^{2+} content in Chinese hamster ovary (CHO) cells expressing human MCH-1 receptor. These *in vitro* functional assays by using intracellular calcium signaling are well established as an efficient system for the characterization of GPCRs coupled through Gq to the mobilization of intracellular calcium with fluorescent indicator dyes (Zang *et al.*, 2003). Addition of MCH (100 nM) stimulates a 4-fold increase of intracellular Ca^{2+} levels in CHO-MCH-1 receptor cells (Fig. 3A) and the GW803430 which is reference compound of MCH-1 receptor antagonist,

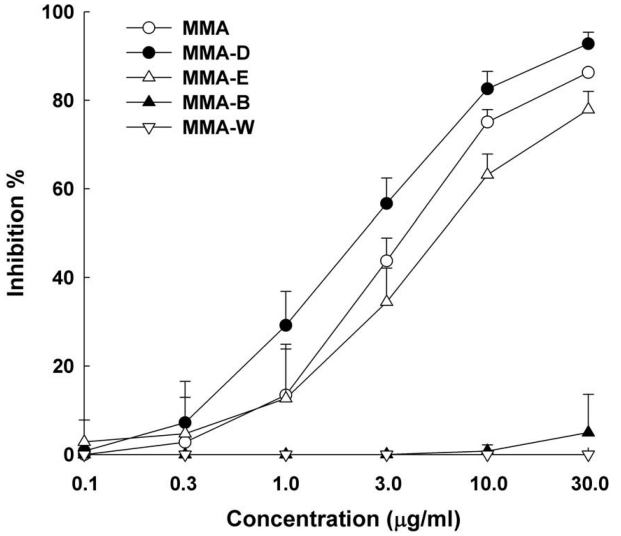

Fig. 2. Concentration-response curves of various plant materials derived from methanol extract of *Morus alba* leaves (MMA) for human melanin-concentrating hormone subtype 1 (MCH-1) receptor binding assay. Binding affinity for MCH-1 receptor was determined by competitive binding with Eu-MCH. Data represent mean value obtained from three separate experiments.

exhibited antagonistic activity (the IC_{50} value: 11.7 ± 1.6 ng/ml).

The EMA-DM, EMA-D, and EMA-E significantly decreased the intracellular Ca^{2+} levels of CHO-MCH-1 receptor cells, in a concentration-dependent manner (Fig. 3B). The IC_{50} value (i.e. the concentration that produces 50% inhibition of the MCH-induced intracellular Ca^{2+} increase) was calculated as 16.5 ± 2.6, 22.7 ± 3.6 and 22.0 ± 2.3 μg/ml, respectively. On the other hand, other plant materials such as EMA-W and EMA-B fractions did not exhibit any functional antagonistic effect. These results suggested that EMA-DM, EMA-E and EMA-D might be functional antagonists for the MCH-1 receptor and inhibit the signaling pathways in intracellular calcium response to MCH-1 by binding MCH-1 receptor.

Conclusion

The present study revealed that the leaves extract of *Morus alba* (EMA) and some related plant materials derived from EMA, especially the dichloromethane soluble fraction (EMA-D) of EMA, and EMA-DM exhibited potent binding affinity to the MCH-1 receptor and significantly decreased the MCH-1 induced intracellular calcium content in CHO-MCH-1 receptor cells. These results suggest that the EMA-D and EMA-DM might be employed as a promising natural therapeutic agent with MCH-1 receptor antagonistic

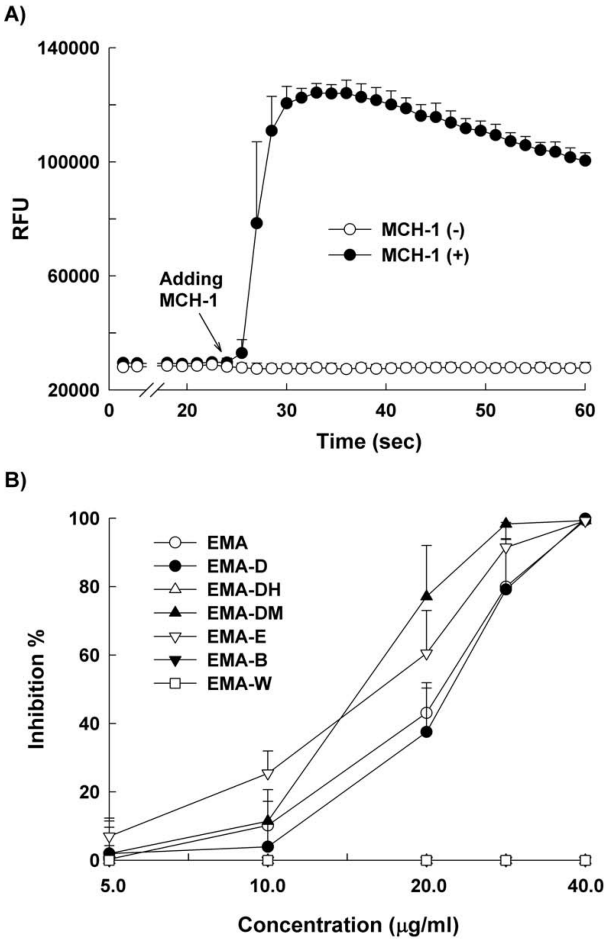

Fig. 3. A) Calcium responses in CHO cells stably expressing MCH-1 receptor following stimulation with 100 nM MCH-1 or buffer. B) Antagonist activity of various plant materials derived from ethanol extract of *Morus alba* leaves (EMA) for human melanin-concentrating hormone subtype 1 (MCH-1) receptor. Antagonist activity was determined by evaluating the content of intracellular Ca^{2+} induced by 100 nM MCH in CHO-MCH-1 receptor cells. Data represent mean value obtained from four separate experiments.

activity as well as a natural crude drug for treatment of obesity. In future, more *in vivo* studies would be required not only to elucidate the underlying mechanisms responsible for these responses, but also to identify and characterize active components of *Morus alba* responsible for MCH-1 receptor antagonism.

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