Introduction

Type I allergic disorders, including allergic rhinitis, atopic dermatitis, and food allergies, are widespread illnesses, particularly in developed countries. The allergic responses are generally associated with the expansion of the T helper 2 cell subset. Some cytokines, such as interleukin-4 (IL-4), produced by T helper 2 cells cause isotype switching of B cells to produce immunoglobulin E (IgE) antibodies against environmental allergens (1). Most allergy patients are atopic and are thus genetically predisposed to produce IgE in the body. Under atopic conditions, mast cells are the major cells sensitized by IgE through the IgE high-affinity receptor (FceRI) on their membrane (2). After receptor aggregation, mast cells secrete histamine, eicosanoids, and various inflammatory cytokines to induce allergic responses (3, 4). Therefore, mast cells play multiple roles in acute and chronic allergic inflammation and are considered an attractive therapeutic target in the treatment of allergic diseases (5, 6).

The aggregation of FceRI by an antigen leads to the activation of Lyn or other Src-family kinases, including Fyn. This results in the stimulation of Syk through the phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) of FceRI or in the direct phosphorylation of Syk (7). Activated Syk subsequently
stimulates many downstream signaling molecules such as the adaptor protein linker for the activation of T cells (LAT), the 76-kDa Src homology 2 domain–containing leukocyte protein (SLP-76), Vav, phospholipase C-γ (PLC-γ), phosphoinositide 3-kinase (PI3K), and mitogen-activated protein (MAP) kinases (8). These pathways are essential for the degranulation of mast cells and secretion of inflammatory cytokines and eicosanoids (4). Recently, numerous molecules, including initial signaling molecules such as Src-family kinases and Syk, have been suggested to be excellent therapeutic targets in the treatment of allergic diseases (9, 10).

In this study, we determined the effect of 3-butyl-1-chloro-8-(2-methoxycarbonyl)phenyl-5H-imidazo[1,5-b][1,2]isoquinolin-10-one (U63A05) on mast cell activation and on passive cutaneous anaphylaxis (PCA) in mice. U63A05 suppressed degranulation and cytokine secretion in antigen-stimulated mast cells as well as antigen-induced PCA in mice. U63A05 inhibited the phosphorylation of Syk and its downstream signaling molecules. These findings strongly suggest that the anti-allergic effect of U63A05 is closely associated with Syk inhibition in mast cells.

Materials and Methods

Reagents

2,4-Dinitrophenyl (DNP)-specific IgE, DNP-conjugated bovine serum albumin (DNP-BSA, antigen), cetirizine, and Evans blue were obtained from Sigma-Aldrich (St. Louis, MO, USA). PP2 and thapsigargin (Tg) were obtained from Calbiochem (La Jolla, CA, USA). Antibodies against the phosphorylated forms of Erk1/2, p38, c-Jun N-terminal kinase (JNK), Akt, Syk (Y346 in mice), LAT (Y191 in humans), and PLCγ were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Antibodies against Syk and actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The antibody (4G10) against phosphotyrosine (pY) and those against LAT and SLP-76 were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Enzyme-linked immunosorbant assay (ELISA) kits for assessing TNF-α and IL-4 activities in media were obtained from Invitrogen-Biosource Cytokine & Signaling (Camarillo, CA, USA). Cell culture media and other components were obtained from Gibco/Life Technologies Inc. (Rockville, MD, USA).

Generation of antigen-induced PCA in mice

Balb/c mice (male, 4 weeks of age) were housed in the animal housing facilities at the College of Medicine, Konkuk University. The facility was maintained at 22 ± 1°C and with a relative humidity of 55 ± 10%. After a 1-week acclimatization, PCA was induced in the mice according to a previously described method (11). Briefly, to prime FcεRI receptors on mast cells located in the ear, DNP-specific IgE (0.5 μg per mouse ear) was intradermally injected into the left ear. The same volume of 1 × phosphate-buffered saline (PBS) was injected into the right ear as a control. After 24 h, an antigen solution (250 μg dissolved in 250 μl of PBS containing 4% Evans blue) was injected into the mouse caudal vein. To assess the effect of U63A05 or cetirizine, these compounds were orally administered 1 h before antigen administration. Mice were killed 1 h after treatment with antigen, the ears were excised, and the dye extracted in order to measure the amount that had been extravasated into the ear by the antigen. Dye from the ear was extracted overnight in 700 μl of formamide at 63°C. Dye absorbance was measured at 620 nm. The animal study was performed using a protocol pre-approved by the Institutional Animal Care and Use Committee (IACUC) at Konkuk University.

Preparation of bone marrow–derived mast cells and rat basophilic leukemia-2H3 mast cells

Rat basophilic leukemia (RBL)-2H3 cells were cultured in minimal essential medium (MEM) containing Earle’s salts and supplemented with glutamine, antibiotics, and 15% fetal bovine serum (FBS). Bone marrow cells were isolated from the femur and tibia of mouse hind legs. Cells were differentiated to bone marrow–derived mast cells (BMMCs) using a reported protocol with minor modification (12). Briefly, bone marrow cells were cultured for 4 weeks in Roswell Park Memorial Institute (RPMI)-1640 medium containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% FBS in the presence of 10 ng/ml of IL-3. The resulting cells were used for our study.

Assessment of degranulation in mast cells

To measure the degranulation of mast cells, hexosaminidase activity was assayed in the culture media and in cell lysates, as previously reported (13). Briefly, mast cells (2 × 10^5 cells per well for RBL-2H3 cells and per microtube for BMMCs) were cultured overnight in complete medium containing 50 ng/ml of IgE. Cultures were washed and buffered solution was added (0.2 ml/well), as required. Experiments were performed in 1,4-piperazinediethanesulfonic acid (PIPES)-buffered medium [25 mM PIPES (pH 7.2), 159 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 1 mM CaCl₂, 5.6 mM glucose, and 0.1% fatty acid-free fraction V from bovine serum] for RBL-2H3 cells or in Tyrode buffer [20 mM HEPES (pH 7.4), 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.05% bovine serum albu-
min (BSA)] for BMMCs. Cells were stimulated with 25 ng/ml of antigen for 10 min with or without U63A05 or PP2. Degranulation was determined by measuring the release of $\beta$-hexosaminidase, a granule marker, using p-nitrophenyl-N-acetyl-$\beta$-d-glucosaminide as a substrate. The optical density (OD) of p-nitrophenol in solution was analyzed at 405 nm. Values are expressed as a percentage of the intracellular $\beta$-hexosaminidase released into the medium, which was calculated by using the following formula:

$$\text{OD (culture supernatant)} = \frac{\text{OD (culture supernatant)}}{\text{OD (cell lysate)}} \times 100$$

**Immunoprecipitation and immunoblotting analysis**

Immunoprecipitation and western blot analysis were performed as described previously (11). Mast cells were washed twice with ice-cold 1 × PBS after stimulation with antigen and then lysed for 30 min in lysis buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 60 mM octyl $\beta$-glucoside, 10 mM NaF, 1 mM Na$_2$VO$_4$, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenyl phosphate, 0.7 μg/ml pepstatin, and a protease inhibitor cocktail tablet]. To immunoprecipitate target proteins, lysates were incubated with 50 μl protein G–agarose and gently rocked for 1 h to remove nonspecifically bound protein. After centrifugation to pellet the agarose gel, lysates were used for immunoprecipitation experiments. Target proteins were incubated overnight with specific antibodies and precipitated with protein G–agarose. The agarose was washed five times with lysis buffer, and then the precipitated proteins were denatured by boiling for 5 min in 2 × Laemmli buffer and then subjected to western blot analysis. Briefly, precipitated proteins or whole cell lysates were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Target proteins on the membranes were probed with specific antibodies and peroxidase-labeled secondary antibody. Protein bands were detected using chemiluminescence reagents according to the manufacturer’s protocol.

**Reverse transcriptase–polymerase chain reaction**

To assess the expression levels of TNF-$\alpha$ and IL-4 mRNAs in antigen-stimulated mast cells, the reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described previously (11). Briefly, total RNA was extracted from RBL-2H3 cells or BMMCs using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed using the Superscript first-strand synthesis system (Invitrogen). The PCR included 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s. The following specific primers were used: rat TNF-$\alpha$ forward, 5′-CACCACGCTCTTCTG TCTACTGAAC-3′; rat TNF-$\alpha$ reverse, 5′-CAGGACTC GTGATGTCCTAGTACTT-3′; rat IL-4 forward, 5′-AC CTTGCTGTGACCCCTGTC-3′; rat IL-4 reverse, 5′-TT GTGAGGCTGGAAGTACTT-3′; mouse TNF-$\alpha$ forward, 5′-ATGAGCAACAGAAAGCATGATCC-3′; mouse TNF-$\alpha$ reverse, 5′-GAGGCCATTTGGGAAGACT TCTC-3′; mouse IL-4 forward, 5′-AGGAGAAGGGGA CGCCATGCACAGGA-3′; mouse IL-4 reverse, 5′-ATCG AAAAGCCCGAAGAGTCTTCG-3′; mouse GAPDH forward, 5′-TGAGCGTGGCGGGAGAAA-3′; and mouse GAPDH reverse, 5′-AGTGTAAGCCAGATGC CCTTTCAG-3′.

**ELISA**

RBL-2H3 cells or BMMCs were incubated with 50 ng/ml DNP-specific IgE overnight, and the primed cells were stimulated with 25 ng/ml antigen for 4 h, with or without U63A05 or PP2. The level of TNF-$\alpha$ or IL-4 in the culture media was then determined using ELISA kits (Invitrogen-Biosource Cytokine & Signaling).

**Statistical analyses**

Results are expressed as the mean ± S.E.M. from three or more independent experiments. Statistical analyses were performed by one-way analysis of variance (ANOVA) and Dunnett’s test. All statistical calculations ($^*P < 0.05$ and $^{**}P < 0.01$) were performed with SigmaStat software (Systat Software, Inc., Point Richmond, CA, USA).

**Results**

**Synthesis of U63A05**

The synthesis of U63A05 (compound 1) was performed according to previously published methods (14). As shown in Fig. 1, imidazole aldehyde compound 2 was alkylated with biphenyl bromo compound 3 in the presence of K$_2$CO$_3$ to yield the N-alkylated compound 4. The aldehyde moiety of compound 4 was converted to a carboxylic acid group to obtain imidazole carboxylic acid compound 5. Conversion of the carboxylic acid group of compound 5 to the corresponding acid chloride with oxalyl chloride followed by intramolecular Friedel–Craft acylation with AlCl$_3$ produced the desired imidazo[1,5-b] isoquinolinone derivative (compound 1).
Effect of U63A05 on degranulation in antigen-stimulated mast cells

Degranulation and secretion of inflammatory mediators are principal features of FceRI-induced mast cell activation and allergic responses. In this study, two types of mast cells, RBL-2H3 cells and BMMCs, were used to assess the effect of U63A05 on degranulation. In the antigen-stimulated mast cells without inhibitors, 50.1 ± 2.3% and 45.8 ± 2.2% of β-hexosaminidase was released into the medium from RBL-2H3 cells and BMMCs, respectively. Degranulation was significantly inhibited by U63A05 in a dose-dependent manner: IC50 values for RBL-2H3 and BMMCs were approximately 4.1 and 4.8 μM, respectively. At a dose of 10 μM, the effects were similar to those of the common Src-family kinase inhibitor PP2 (Fig. 2A). There was no effect on cell viability in

Fig. 1. Structure and synthesis of U63A05. Reactions were performed using the following reagents and conditions: a) K2CO3, DMF, RT, 12 h; b) KMnO4, KH2PO4, t-butanol-H2O, RT, 1 h; c) (COCl)2, CH2Cl2, RT, 1 h; and then AlCl3, CS2, reflux, 6 h.

Fig. 2. Effect of U63A05 on the degranulation of mast cells and its reversibility. A) RBL-2H3 cells (2 × 10⁵ cells/well) and BMMCs (2 × 10⁵ cells/tube) were stimulated for 10 min with 25 ng/ml antigen (Ag) after incubation with 50 ng/ml DNP-specific IgE overnight. Cells were stimulated by antigen with or without U63A05 or PP2, a typical Src-family kinase inhibitor. B) RBL-2H3 cells (2 × 10⁵ cells/well) were washed five times with PIPES buffer after incubating with 10 μM U63A05 for 1 h. Degranulation was measured by assessing hexosaminidase activity in the media or cell lysates. Values are expressed as a percentage of the intracellular β-hexosaminidase released into the medium (mean ± S.E.M. of the values from three independent experiments). Significant differences are indicated as follows: *P < 0.05 or **P < 0.01, compared with Ag-stimulated degranulation without inhibitors (A) or without wash (B).
the range of concentration of U63A05 (data not shown). However, these effects disappeared when the mast cells were washed with PIPES buffer after a 1-h treatment with 10 μM U63A05 (Fig. 2B), indicating that the activity of U63A05 is reversible.

Effect of U63A05 on the expression and secretion of TNF-α and IL-4

Several cytokines, including TNF-α and IL-4, play pivotal roles in mediating delayed types of hypersensitivity in the allergic process (15). We examined whether the expressions of TNF-α and IL-4 are suppressed by U63A05 in antigen-stimulated mast cells. As shown in Fig. 3, both the secretion and expression of TNF-α and IL-4 from antigen-stimulated mast cells (RBL-2H3 and BMMCs) were dose-dependently inhibited by U63A05. This inhibitory effect on cytokine secretion was evident at doses as low as 1 μM and was almost completely blocked at 10 μM (Fig. 3: B and C).

Action mechanism of U63A05 in mast cells

Next, we determined the mechanism of U63A05-mediated inhibition of mast cell activation. We first investigated whether U63A05 inhibited degranulation in mast cells by using Tg, a common calcium inducer in mast cells (16). As shown in Fig. 4, Tg-stimulated degranulation was not inhibited by U63A05 in mast cells, indicating that the target of U63A05 is a molecule located in the upper signaling pathway that does not inhibit the calcium-dependent signals in Tg-stimulated mast cells.

We accordingly examined whether U63A05 inhibited FceRI-associated proximal signaling molecules such as Syk, LAT, and SLP-76 in antigen-stimulated mast cells. Notably, U63A05 suppressed Syk in a dose-dependent manner (Fig. 5). The downstream signaling molecules of Syk, LAT, and SLP76 were also inhibited by U63A05.

Fig. 3. Effect of U63A05 on the expression and secretion of TNF-α and IL-4 in antigen-stimulated mast cells. A) IgE-primed RBL-2H3 cells (1 × 10^6 cells/well) or BMMCs (1 × 10^6 cells/well) were stimulated with 25 ng/ml antigen (Ag) for 15 min with or without U63A05, and total RNA was isolated and reverse-transcribed. The polymerase chain reaction was performed using TNF-α or IL-4-specific primers as described in the Materials and Methods. B, C) IgE-primed RBL-2H3 cells or BMMCs were stimulated with 25 ng/ml antigen overnight with or without U63A05. Next, the amounts of TNF-α and IL-4 in the medium were assessed using ELISA assay kits. Values are shown as the mean ± S.E.M. from three independent experiments. Significant differences are indicated as follows: *P < 0.05 or **P < 0.01, compared with Ag-stimulated release of TNF-α or IL-4 without inhibitors. PP2 is a general Src-family kinase inhibitor.

Fig. 4. Effect of U63A05 on thapsigargin (Tg)-stimulated degranulation in mast cells. RBL-2H3 cells (2 × 10^5 cells/well) were stimulated with 300 nM Tg for 10 min with or without U63A05. Degranulation was measured by assessing hexosaminidase activity in the medium or cell lysates. Values are expressed as a percentage of the intracellular β-hexosaminidase released into the medium (mean ± S.E.M. of the values from three independent experiments). Significant differences in release are indicated as follows: **P < 0.01, compared with Ag-stimulated degranulation without inhibitors.
Anti-allergic Effect of U63A05

The inhibition was evident at doses as low as 1 μM, and near complete inhibition was induced at 10 μM (Fig. 5A). It is generally accepted that Lyn initially phosphorylates the tyrosine 346 residue (tyr346) of murine Syk (tyr352 in humans) in antigen-stimulated mast cells (17, 18). Thus, we analyzed whether phosphorylation of the tyrosine 346 of Syk is inhibited by U63A05. We found that the phosphorylation of this residue was indeed inhibited by U63A05 (Fig. 5B).

Next, we investigated whether U63A05 inhibited other downstream signaling molecules, including PLC\(_{\gamma}\), a critical molecule for calcium signaling, and Akt, a surrogate molecule that indicates the activation of PI3K. Both signaling molecules are crucial for mast cell activation (8). We found that the phosphorylations of PLC\(_{\gamma}\) and Akt were inhibited in a dose-dependent manner (Fig. 5B).

Effect of U63A05 on the activation of MAP kinases

MAP kinases play a pivotal role in the expression and secretion of inflammatory cytokines in antigen-stimulated mast cells (19, 20). Three typical MAP kinases, Erk1/2, p38, and JNK, were suppressed by U63A05 in a dose-dependent manner; and their activations were almost completely blocked at a dose of 10 μM, which is comparable to the dose required for the inhibition of cytokine production (Fig. 5B).

Effect of U63A05 on PCA in mice

The above results led us to attempt an experiment using an IgE-dependent type I hypersensitive animal model, PCA. PCA was successfully induced by injecting DNP-specific IgE and antigen as described in the Materials and Methods section (Fig. 6A). The antigen-induced anaphylactic response was suppressed by U63A05 in a dose-dependent manner (Fig. 6B; ED\(_{50}\), 26.3 mg/kg). The
effect was comparable to that of 50 mg/kg cetirizine, an anti-histamine drug (Fig. 6B).

Discussion

Accumulating evidence supports the notion that allergic disorders such as allergic rhinitis, atopic dermatitis, allergic asthma, and some food allergies are associated with mast cells under atopic conditions; and they are characterized by the high production of antigen-specific IgE and the expansion of antigen-specific T cell populations (5). In allergic conditions, mast cells are sensitized by IgE and are subsequently stimulated by an antigen. These cells eventually secrete various allergic inflammatory mediators such as histamine, cytokines, and eicosanoids (3, 21).

Allergen avoidance, allergen-specific immunotherapy, and antagonizing the action of mediators (antihistamines, pseudoephedrine, anti-cysteiny1 leukotrienes, and corticosteroids) have been clinically employed to treat allergic disorders. However, these treatments typically have limitations with respect to efficacy and some minor or severe side effects (10). Thus, additional remedies are being investigated. Among these, inhibition of IgE and FcεRI-signaling molecules in mast cells is a promising approach for the treatment of allergic disorders. Administration of the anti-IgE antibody omalizumab is clinically efficacious in patients with allergic asthma and allergic rhinitis (22, 23); however, clinical therapy using omalizumab has certain limitations, including its high cost and partial efficacy (24).

Alternatively, inhibition of intracellular signaling events in mast cells may downregulate the secretion of allergic mediators from antigen-stimulated mast cells. Upon cross-linking of FcεRI onto mast cells, Src-family kinases such as Lyn and Fyn are initially activated. They can subsequently phosphorylate the ITAM motif of the FcεRI β- and γ-subunits and Syk, which is a pivotal signaling molecule that directly or indirectly activates the downstream signaling molecules, such as LAT, SLP-76, PLCγ, and other molecules, to activate mast cells. Therefore, the release of allergic mediators is dominantly regulated by the Src-family kinases and Syk, suggesting that such tyrosine kinases are good targets for suppressing mast cell activation (9, 25). Recently, morin, a flavonoid, was reported to suppress PCA in mice through the primary inhibition of Fyn in mast cells (11). Clinical investigations using the reversible Syk inhibitor R112 and other Syk inhibitors are also underway in order to confirm its efficacy against allergic disorders (26). U63A05
also reversibly inhibited Syk activation in antigen-stimulated mast cells and eventually suppressed the same antigen-induced PCA in mice (Fig. 6).

U63A05 does not, however, inhibit degranulation in Tg-stimulated mast cells, suggesting that the activity of U63A05 is limited to receptor-proximal signaling molecules. At present, several Src-family kinases, including Lyn, Fyn, and Hck, are known to have individual signaling functions in mast cells. Although Lyn can initially stimulate the FcεRI proximal signals in mast cells, it is usually recognized as a negative regulator in antigen-stimulated mast cells, particularly at high antigen concentrations (27). In contrast, the positive regulators Fyn (28) and Hck (29) activate the Gab2/PI3K pathway, which is also necessary for degranulation, synthesis, and release of leukotrienes, and production of cytokines. On the basis of these and our findings, we further examined whether U63A05 inhibited these Src-family kinases in antigen-stimulated mast cells. U63A05 inhibited the phosphorylation of tyr346 of Syk, which is a potential phosphorylation site for Lyn and other Src-family kinases (17, 18), indicating that U63A05 inhibits the Src-family kinases. Next, in order to determine the direct effect of U63A05 on Src-family kinases, we examined whether U63A05 inhibited the activity of positive Src-family kinases of mast cells, that is, Fyn and Hck, in vitro. The activities of Fyn and Hck were partially, but significantly, inhibited by U63A05 (approximately 23.5% for Fyn and 33.5% for Hck at 10 μM) (data not shown). These results strongly suggest that U63A05 inhibits the activation of Syk in mast cells through the inhibition of Src-family kinases (Fig. 6C). However, our data do not completely rule out the possibility that U63A05 has other mechanisms of action whereby it inhibits as yet unidentified IgE-receptor–associated molecules to stimulate Syk activation in mast cells. Further studies are necessary in order to answer this question.

We were slightly puzzled by the results with PP2, a general Src-family kinase inhibitor, because the Tg-stimulated degranulation in mast cells was inhibited by PP2 (Fig. 4). Accumulating evidence suggest that several isoforms of Src-family kinase, including Lyn, Fyn, c-Src, Hck, Fgr, and Yes, are expressed in mast cells (8, 30). The roles of Lyn, Hck, and Fyn are closely associated with the FcεRI receptor (28, 29). Recently, other Src-family kinases, Fgr in plasma membrane and Yes in intracellular granules, were reported (30). Therefore, it is suggested that other Src-family kinases, including Yes, are not directly associated with the receptor. Of note, Yes is critical for degranulation in mast cells (our unpublished observation). In this context, the effect of PP2 in mast cells was primarily mediated via the inhibition of the receptor-associated Src-family kinases (Lyn and/or Fyn).

In addition, other Src-family kinases, such as Yes, may also be inhibited by PP2. Therefore, it is suggested that the downstream Src-family kinases, including Yes, may also be inhibited by PP2 in Tg-stimulated mast cells. However, further study is necessary to elucidate the exact mechanism of PP2 action.

The PCA animal model is generally used to evaluate local IgE-mediated allergic responses in mice (31, 32). U63A05 significantly suppressed the PCA reaction in a dose-dependent manner (Fig. 6). The ED50 value for this inhibition was 26.3 mg/kg, indicating that the efficacy of U63A05 was comparable to that achieved using 50 mg/kg cetirizine, a second-generation antihistamine drug. Because cetirizine is similar to hydroxyzine and is a derivative of piperazine, several adverse reactions and properties can be attributed to other piperazine derivatives. U63A05, on the other hand, is an imidazo[1,5-b]isoquinolinone derivative (Fig. 1). The structural dissimilarity and our results suggest that this compound may be a new drug candidate for the effective treatment of IgE-mediated allergic diseases. Accordingly, further studies should be conducted in order to determine U63A05 toxicity and pharmacokinetics.

In conclusion, our data clearly show that U63A05 suppresses antigen-stimulated mast cells and subsequently IgE-mediated anaphylaxis in mice by inhibiting the activation of Syk in mast cells. Further investigation is warranted on U63A05 as a starting molecule for the development of a therapeutic agent to treat IgE-mediated allergic diseases.

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References