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[4-t-Butylphenyl]-N-(4-imidazol-1-yl phenyl)sulfonamide (ISCK03) inhibits SCF/c-kit signaling in 501mel human melanoma cells and abolishes melanin production in mice and brownish guinea pigs

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ABSTRACT

It is well known that c-kit is related to pigmentation as well as to the oncology target protein. The objective of this study was to discover a skin-whitening agent that regulates c-kit activity. We have developed a high-throughput screening system using recombinant human c-kit protein. Approximately 10,000 synthetic compounds were screened for their effect on c-kit activity. Phenyl-imidazole sulfonamide derivatives showed inhibitory activity on c-kit phosphorylation *in vitro*. The effects of one derivative, [4-*t*-butylphenyl]-*N*-(4-imidazol-1-yl phenyl)sulfonamide (ISCK03), on stem-cell factor (SCF)/c-kit cellular signaling in 501mel human melanoma cells were examined further. Pretreatment of 501mel cells with ISCK03 inhibited SCF-induced c-kit phosphorylation dose dependently. ISCK03 also inhibited p44/42 ERK mitogen-activated protein kinase (MAPK) phosphorylation, which is known to be involved in SCF/c-kit downstream signaling. However ISCK03 did not inhibit hepatocyte growth factor (HGF)-induced phosphorylation of p44/42 ERK proteins. To determine the *in vivo* potency of ISCK03, it was orally administered to depilated C57BL/6 mice. Interestingly, oral administration of ISCK03 induced the dose-dependent depigmentation of newly regrown hair, and this was reversed with cessation of ISCK03 treatment. Finally, to investigate whether the inhibitory effect of ISCK03 on SCF/c-kit signaling abolished UV-induced pigmentation, ISCK03 was applied to UV-induced pigmented spots on brownish guinea pig skin. The topical application of ISCK03 promoted the depigmentation of UV-induced hyperpigmented spots. Fontana–Masson staining analysis showed epidermal melanin was diminished in spots treated with ISCK03. These results indicate that phenyl-imidazole sulfonamide derivatives are potent c-kit inhibitors and might be used as skin-whitening agents.

1. Introduction

The biological activities of stem-cell factor (SCF) are induced through its receptor, c-kit, which is expressed on melanocytes in the skin. SCF and c-kit are encoded by the steel locus (*sl*) and the dominant white-spotting (W) locus in mice, respectively [1,2]. SCF, a c-kit ligand, stimulates melanocyte migration, proliferation, and differentiation. SCF can be produced in

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keratinocytes, fibroblasts, and endothelial cells of the skin. There are two types of SCF, a soluble form and membrane-bound form. The latter is necessary for a normal melanocyte phenotype in human skin [3]. c-kit belongs to the platelet-derived growth factor (PDGF) family of receptor tyrosine kinases (RTKs). Binding of SCF to the extracellular domain of c-kit induces dimerization of the receptors, activation of the tyrosine kinase, and subsequent auto-phosphorylation [4]. The intracellular signaling downstream of SCF/c-kit plays a critical role in the development of a variety of mammalian cells, including melanocytes, mast cells, hematopoietic progenitor cells, primordial germ cells, and intestinal cells of Cajal [5–7].

It has been reported that the SCF/c-kit signaling is related to melanocyte development and pigmentation. For example, oral administration of the multitargeted tyrosine-kinase inhibitor, SU11248, induces dose-dependent depigmentation of newly regrown hair in depilated C57BL/6 mice [8]. It has been demonstrated that ACK45, a c-kit neutralizing monoclonal antibody, prevents pigmentation of the hair shaft during the anagen cycle of hair follicle regeneration [9]. In addition to these animal model data, clinical studies have shown the importance of the SCF/c-kit in human pigmentation. Piebaldism, a rare autosomal disorder causing depigmented patches and hair, is due to an absence of melanocytes in affected skin and hair follicles as a result of mutations in the *KIT* proto-oncogene [10,11]. Treatment with imatinib mesilate, a selective inhibitor of several tyrosine kinases, including c-kit, Bcr-Abl, and PDGF receptor β, induced hypopigmentation in an African patient [12,13]. Hattori et al. [14] have reported that epidermal SCF is over-expressed in lentigo senilis and that it is associated with the mechanism of hyper-pigmentation in epidermal lentigo senilis lesions. Recently, we have found that SCF/c-kit proteins are over-expressed in melasma. In that report we suggested that the increased expression of SCF in the dermis and of c-kit in the epidermis play an important role in the mechanism of hyperpigmentation in melasma [15]. These previous studies suggest that inhibition of the SCF or c-kit protein could be a potent target for regulation of pigmentation.

To date, numerous approaches have been applied to the development of potential skin-whitening agents. In this study, we examined synthetic molecules as potential c-kit inhibitors that might induce depigmentation in an *in vivo* model. To find effective c-kit inhibitors, we screened about 10,000 synthetic compounds from chemical libraries. In particular, [4-*t*-butyl-phenyl]-*N*-(4-imidazol-1-yl phenyl)sulfonamide (ISCK03) had the highest activity *in vitro*. Results presented here demonstrate that ISCK03 also inhibited c-kit activity *in vivo*, and finally induced depigmentation in an animal model. We suggest that ISCK03 is a potent c-kit inhibitor and may be useful for treating hyperpigmented spots that are partially or completely caused by effects on c-kit expression or activity.

2. Materials and methods

2.1. Cell cultures and compounds

501mel human melanoma cells were purchased from Dr. Ruth Halaban (Yale University, Boston, USA) and were maintained in Opti-MEM (GibcoBRL) medium, containing 7% fetal calf serum (FCS) supplemented with 50 U/ml penicillin and 50 μg/ml at 37 °C in a humidified atmosphere of 5% CO_2. HM3KO, a pigmented melanoma cell line, established by Ohashi et al. [16] from metastatic melanoma cells of peritoneal fluids, was kindly supplied by Dr. Y. Funasaka (Kobe University School of Medicine, Kobe, Japan). HM3KO cells were grown in Minimal Essential Medium (GibcoBRL), containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Chemical libraries were purchased from Korea Chemical Bank (Daejeon, Korea).

2.2. In vitro c-kit phosphorylation assay

High-throughput screening (HTS) was performed with a Panvera Z-Lyte™ biochemical assay kit (Invitrogen) and a recombinant human c-kit protein, which we have developed (Neurogenex, Korea). The whole procedures were followed the manufacturer's instructions except that reaction conditions (for example, kinase protein concentration, reaction time, etc.) were modified and optimized for the c-kit activity assay. Briefly, ATP was dispensed into 384-well plates, chemical compounds were added by replicative plate (96 thick/short, Genetix), and recombinant human c-kit protein was added for the kinase reaction. Following a 45-min incubation at 37 °C, the development reaction was carried out for 40 min at room temperature. After the reaction was stopped, the coumrain and fluorescein fluorescence-emission signals were detected.

2.3. Cell growth assay

The effects of chemicals on the growth of 501mel cells were measured using the MTT assay [17]. Briefly, MTT (50 μl of 2 mg/ml) dissolved in Opti-MEM was added to 501mel cells, and the cells were incubated for 3 h in a 37 °C-incubator. The medium was removed and formazan crystals of these cells were solubilized in 200 μl DMSO by gentle shaking for 10 min. The amount of formazan was quantified in an ELISA reader at 540 nm.

2.4. Stimulation with SCF and detection of proteins by Western blot analysis

501mel human melanoma cells were stimulated for 10 min with 50 ng/ml recombinant human SCF (R&D Systems). To determine the inhibitory effects of ISCK03, the cells were pretreated with various concentrations of ISCK03 for 2 h prior to SCF stimulation. After 10 min of stimulation, cells were harvested for whole-protein preparation. Whole-cell lysates were resolved by 10% SDS-PAGE. After Western blotting, membranes were probed with appropriate primary antibodies, detected using peroxidase-conjugated anti-rabbit or anti-mouse antibody, and visualized by ECL solution (Amersham). The primary antibodies were anti-phosphorylated c-kit (Zymed), anti-c-kit (Santa Cruz), and anti-phosphorylated and non-phosphorylated ERK (Cell Signal). Hepatocyte growth factor was purchased from R&D Systems.

2.5. Oral administration and induction of hair depigmentation

Female C57BL/6 mice (6-week-old) were purchased from Orient CO.LTD (Korea). Animals received sterile rodent chow and water *ad libitum* and were kept under a 12-h light/dark cycle. To induce the hair cycle, depilation of skin on the back of the mice was performed as described previously [8,18]. Briefly, the hair was removed from anesthetized mice. The rat anti-mouse c-kit-neutralizing monoclonal antibody ACK2 [7] was purchased from eBioscience (San Diego, CA) and administered intraperitoneally (50 μg) every day. ISCK03 was administered orally once a day. On days 21–28, animals were sacrificed or analyzed for repigmentation of the newly regrown hair shaft. Skin was harvested and fixed in paraffin or frozen for immunohistochemical analyses.

2.6. In vivo UV-induced pigmentation model and topical treatment

Hyperpigmentation was induced on the backs of brown guinea pigs weighing about 500 g each, by a modification of the UV method described by Yokota et al. [19] and Lim et al. [20]. Guinea pigs were anesthetized with pentobarbital (35 mg/kg), and four separate areas (1-cm-diameter circles) on the back of each animal were shaved and exposed to UV from three TL20W/09UV Philips (Koninklijke Philips Electronics NV, Eindhoven, The Netherlands) UVA lamps and seven TL 20W/12UV Philips UVB lamps. The total UV-irradiation energy dose was 250 mJ/cm^2 per exposure period. Groups of five animals were used for each experiment. Animals were exposed to UV irradiation once a week for three consecutive weeks. After the last UV irradiation, ISCK03 treatment was initiated. ISCK03 was dissolved in PG:EtOH:water (5:3:2) at concentrations of 0.1% and 0.3%, and topically administered twice per day for 5 weeks.

3. Results

3.1. ISCK03 inhibits c-kit phosphorylation in vitro and SCF-induced c-kit phosphorylation in 501mel human melanoma cells

We developed a recombinant human c-kit protein and used it for *in vitro* c-kit kinase-activity assays. Nearly 10,000 compounds were assayed by HTS, as described in Section 2. Phenyl-imidazole sulfonamide derivatives showed inhibitory effects on c-kit phosphorylation *in vitro*. Six additional phenyl-imidazole sulfonamide derivatives were synthesized with a few side chain modifications. The chemical structures of four of these are shown in Fig. 1A. [4-t-butylphenyl]-N-(4-imidazol-1-yl phenyl)sulfonamide, called ISCK03, showed the most potent inhibition of c-kit kinase activity *in vitro* (Fig. 1B).

Recombinant human SCF induced c-kit phosphorylation at early time points (within 10 min), and 50 ng/ml SCF fully stimulated c-kit phosphorylation in 501mel cells (data not shown). No significant c-kit phosphorylation was observed in the absence of SCF. Pretreatment with ISCK03 inhibited SCF-induced c-kit phosphorylation in a dose-dependent manner in

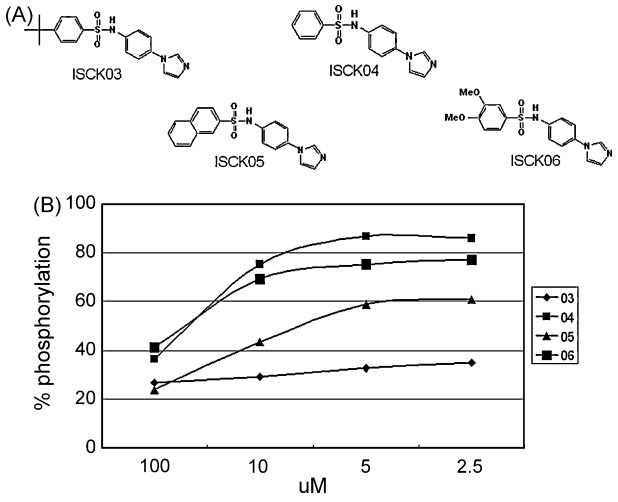

Fig. 1 – Dose-dependent inhibition of c-kit phosphorylation by the ISCK series *in vitro*. (A) Structures of ISCK compounds. (B) The detection system for *in vitro* c-kit activity was developed as described in Section 2. The ISCK compounds were added at the indicated doses. The inhibitory effect on c-kit phosphorylation was different for each compound. Representative results from one of two independent experiments are shown.

501mel cells (Fig. 2), and also in the human melanoma cell line HM3KO (data not shown). Complete inhibition was observed with 1 and 5 μM ISCK03.

To determine any cytotoxic effects of ISCK03 on 501mel cells, MTT assays were performed with various doses of ISCK03. 501mel cells were cultured with SCF alone (50 ng/ml) or SCF with ISCK03 for 48 h. As shown in Fig. 3, slightly increased cell proliferation was observed with SCF treatment alone. The proliferation of co-treated cells was not affected at the tested doses of ISCK03.

3.2. ISCK03 inhibits SCF-induced p44/42 ERK phosphorylation in 501mel human melanoma cells

Autophosphorylation of c-kit occurs when SCF binds to c-kit protein on the cell surface [4]. It is well known that the events

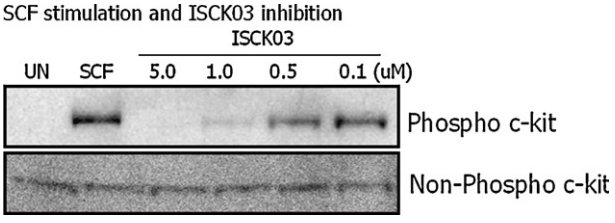

Fig. 2 – Inhibition of c-kit phosphorylation by ISCK03 in 501mel melanoma cells. Cells were pretreated with 5.0, 1.0, 0.5, and 0.1 μM ISCK03 for 2 h and c-kit phosphorylation was stimulated with rhSCF (50 ng/ml) for 10 min, followed by western blot analysis. ISCK03 inhibited SCF-induced c-kit phosphorylation in a dose-dependent manner. Representative results from one of three independent experiments are shown.

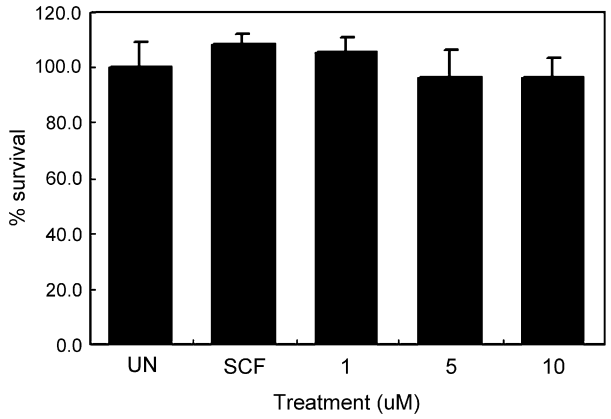

Fig. 3 – Effect of ISCK03 on SCF-dependent proliferation of 501mel cells. Cells were plated in a 24-well plate at a concentration of 30,000 cells/well and grown in Opti-MEM supplemented with 7% FCS, with or without rhSCF (50 ng/ml). ISCK03 was added simultaneously with rhSCF. Proliferation at 48 h was measured with an MTT-based assay system. Results are expressed as a percentage of the maximal proliferation (untreated). Representative results from one of two independent experiments are shown.

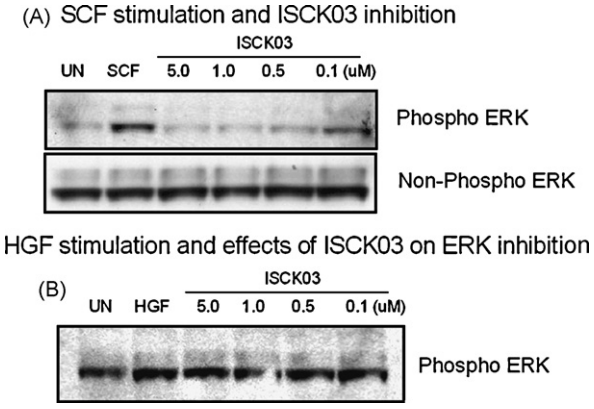

Fig. 4 – Effect of ISCK03 on c-kit-related signal transduction in 501mel melanoma cells. (A) Inhibition of ERK phosphorylation by ISCK03 in 501mel melanoma cells. Cells were pretreated with 5.0, 1.0, 0.5, and 0.1 μM ISCK03 for 2 h, followed by treatment with rhSCF (50 ng/ml) for 10 min to stimulate ERK phosphorylation. Western blot analysis was performed. ISCK03 inhibited SCF-induced ERK phosphorylation in a dose-dependent manner. Representative results from one of three independent experiments are shown. (B) HGF-stimulated ERK phosphorylation was not affected by ISCK03 pretreatment. The same experiments as in (A) were performed using HGF instead of rhSCF. Representative results from one of two independent experiments are shown.

downstream of c-kit phosphorylation are related to p44/42 ERK mitogen-activated protein kinase (MAPK), a common signaling pathway in melanocytes [21,22], endothelial cells [23], and hematopoietic cells [24]. SCF treatment induced p44/42 protein phosphorylation, which was inhibited by ISCK03 treatment in a dose-dependent manner (Fig. 4A), consistent with our previous results (Fig. 1). Total ERK expression was not altered by treatment with ISCK03 (Fig. 4A). To investigate the specificity of ISCK03 inhibition of SCF/c-kit-induced ERK activation, 501mel cells were treated with hepatocyte growth factor (HGF). HGF plays an essential role in melanocyte maintenance, as do SCF and endothelins. c-Met and c-kit are tyrosine kinase receptors and have been reported to regulate proliferation, differentiation, and pigmentation of melanocytes [25–27]. Interestingly, ISCK03 did not inhibit HGF-induced phosphorylation of ERK at concentrations of 0.1–5 μM (Fig. 4B). Thus, pretreatment with ISCK03 affected SCF-dependent, but not HGF-dependent, activation of ERK.

3.3. *Oral administration of ISCK03 induces hair depigmentation*

To clarify the activity of ISCK03 on c-kit *in vivo*, we used the same experimental system as in a previous study [8]. For the positive control, 50 μg of the c-kit-neutralizing antibody ACK2 was administered intraperitoneally once daily. As shown in Fig. 5A, ACK2 treated mice ($n = 3$) showed hair depigmentation. Once-daily oral administration of ISCK03 (500, 250, or 125 mg/kg) had a dramatic dose-dependent effect on hair depigmentation (Fig. 5A). In particular, the regrown hair of mice treated with 500 mg/kg ISCK03 was as completely whitened as that of the ACK2-treated mice. A mouse from the 500 mg/kg-dose group was removed from ISCK03 treatment and its depigmented hair was replucked. By day 42, complete repigmentation had occurred (Fig. 5B). This result demonstrated the reversibility of the ISCK03 effect.

3.4. *ISCK03 treatment abolishes UV-induced hyperpigmentation in brownish guinea pigs*

The UV-induced pigmentation model was established as described in Section 2. It was previously reported that c-kit protein is expressed in the epidermis of the brownish guinea pig, and that the c-kit-inhibitory antibody, ACK2, abolishes UVB-induced pigmentation on the dorsal skin of brownish guinea pigs [28]. ISCK03 was applied topically to hyperpigmented spots twice per day for 5 weeks ($n = 5$). As shown in Fig. 6, as little as 0.1% ISCK03 in PG:EtOH:water (5:3:2) promoted depigmentation of UV-induced spots, as compared with vehicle (VH) treatment alone. Tissue biopsies were analyzed by Fontana-Masson staining and epidermal melanin was found to be decreased in the ISCK03-treated pigment spots.

4. Discussion

As mentioned above, it is well known that the deregulation of SCF or c-kit is involved in pigmentation defects. Genetic and experimental evidence has demonstrated the importance of SCF/c-kit signaling in pigmentation processes. For example, human piebaldism, a disorder presenting at birth with amelnotic patches on ventral and/or acral skin surfaces, is caused by c-kit gene mutations [29]. Recent studies have

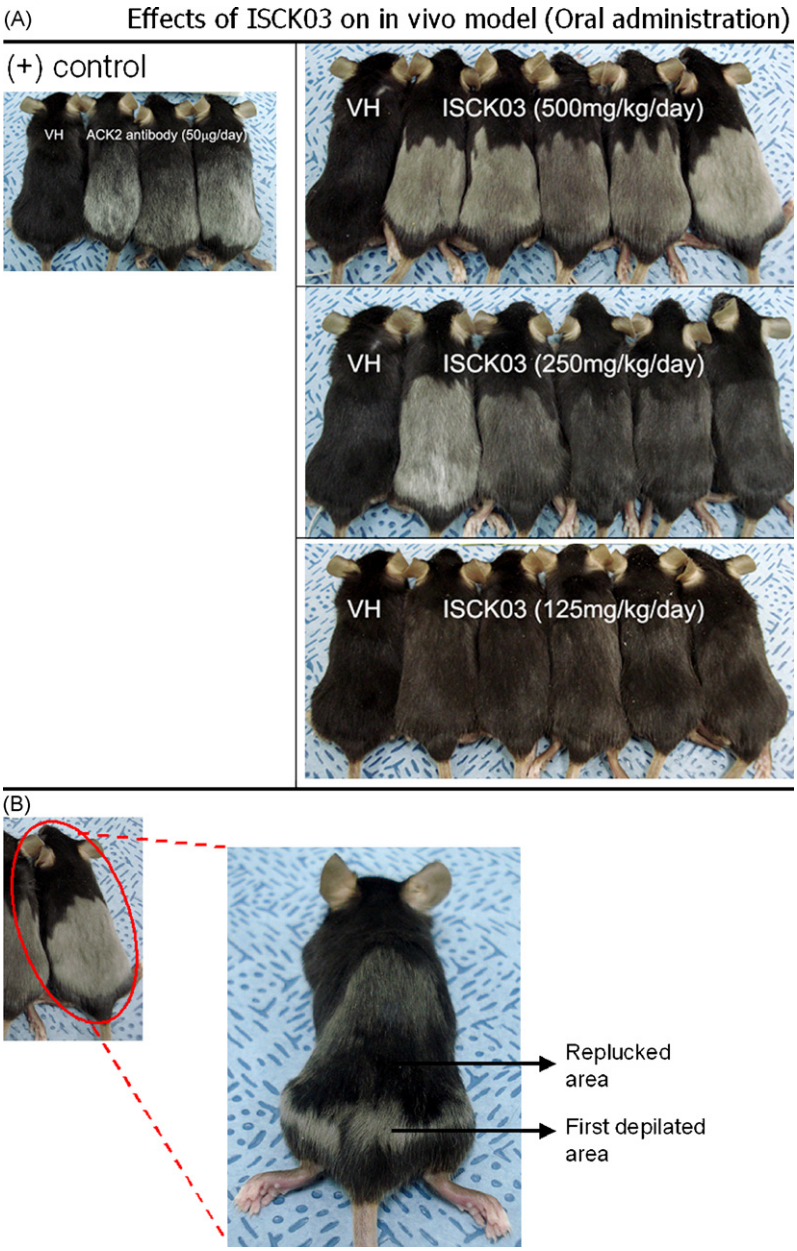

Fig. 5 – Systemic administration of ISCK03 induces hair depigmentation in C57BL/6 mice. (A) Daily oral administration of ISCK03 at 500, 250, and 125 mg/kg inhibited hair pigmentation in a dose-dependent manner. The c-kit-neutralizing antibody ACK2 was administered subcutaneously at 50 μg/day as a positive control. (B) Reversibility of ISCK03-induced hair depigmentation. On day 28, one mouse was removed from the 500 mg/kg-treatment group. On day 31, this mouse was depilated again without ISCK03 treatment. Complete repigmentation had occurred by day 42.

shown that SCF/c-kit proteins are over-expressed in melasma. These findings suggest that the increased expression of SCF in the dermis and of c-kit in the epidermis may be related to hyperpigmentation in melasma [15]. Thus, it is assumed that SCF or c-kit signaling could be good targets for the regulation of skin pigmentation.

In this study, for the first time, we have characterized phenyl-imidazole sulfonamide derivatives as potent c-kit inhibitors. We show that [4-*t*-butylphenyl]-*N*-(4-imidazol-1-yl phenyl)sulfonamide, called ISCK03, shows the most potent inhibitory effect on c-kit kinase activity *in vitro*. Its inhibitory activity on c-kit protein has been investigated in cellular assays and *in vivo* animal models. Pretreatment with ISCK03 inhibits SCF-stimulated c-kit protein phosphorylation, as well as its downstream signaling pathways such as ERK. Our animal studies show promising results for c-kit inhibition by ISCK03. Oral administration of ISCK03 induces complete depigmentation of regrown hair in C57BL/6 mice at a once-daily dose of 500 mg/kg. In addition, ISCK03 applied topically to UV-induced hyperpigmented spots, diminishes epidermal melanin production as compared with the vehicle treatment alone.

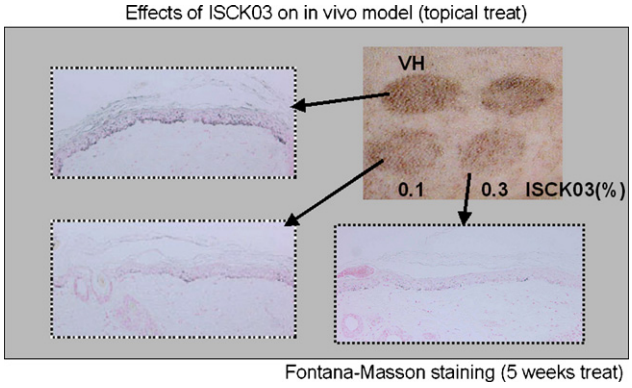

Fig. 6 – Depigmentation of UV-induced pigmentation by topical application of ISCK03. Brownish guinea pigs were exposed to UV lights (UVA + UVB) once per week for three consecutive weeks and then treated with topical ISCK03 for 5 weeks, as described in Section 2. Tissue biopsies were subjected to Fontana–Masson staining following animal sacrifice.

We have used recombinant c-kit protein for *in vitro* high-throughput screening. The usual screening system for large batches of chemicals has a chance of yielding false-positive results. The selectivity of ISCK03 for c-kit protein is an important issue and is confirmed by our cell-based assay. When SCF stimulates c-kit protein in melanocytes, c-kit is auto-phosphorylated and transmits signals downstream [4,21,22]. As we expected, SCF-induced ERK phosphorylation was inhibited by ISCK03, and this inhibition is consistent with the inhibition of c-kit phosphorylation. Other studies have examined c-Met, a member of the receptor tyrosine kinase family that is expressed in melanocytes [30]. SCF and HGF have also been identified as intrinsic melanogenic cytokines in dermatofibromas where hyperpigmentation occurs [31]. On binding HGF, c-Met is activated via auto-phosphorylation [32] and a number of common signaling pathways are induced downstream of c-Met, including ERK activation [33]. Interestingly, HGF-induced ERK phosphorylation was not affected by ISCK03 in 501mel cells, even at a concentration of 5 μM. These results demonstrate that ISCK03 specifically affects the SCF/c-kit signaling pathways.

Following our investigation of the activity and selectivity of ISCK03 on c-kit protein inhibition in cellular systems, ISCK03 was administered orally to depilated C57BL/6 mice. It has been reported that it is feasible to use hair depigmentation as a biological readout for c-kit inhibition in preclinical drug discovery [8]. Here, complete depigmentation was observed with the daily administration of ISCK03 at 500 mg/kg. Interestingly, we observed slower hair regrowth with higher doses of ISCK03. In contrast, mice treated with 125 mg/kg ISCK03 showed nearly the same hair regrowth rate as those treated with the vehicle alone. It is well known that SCF and c-kit signaling is required for maintenance of hair follicle melanocytes [7]. In addition, SCF/c-kit signaling is required for the generation and migration of functional melanocytes during each new hair-growth cycle [9]. Thus, the inhibition of c-kit by ISCK03 can explain the attenuation of new hair growth in the anagen state. Although we have shown that ISCK03 has a specific inhibitory effect on c-kit in melanocytes, we cannot completely exclude the possibility that ISCK03 may affect another receptor tyrosine kinase in the metabolic pathway or in other cells that are involved in hair growth processes.

In our experiments, skin pigmentation of brownish guinea pigs was induced by UVA + UVB, as described in Section 2, instead of UVB alone. Previous studies have shown that UVB light up-regulates the transcription and protein expression of SCF and c-kit and that injection of ACK2 abolishes UVB-induced pigmentation in brownish guinea pigs [28]. Since our purpose is the development of a skin-applicable agent, ISCK03 potency was investigated by topical application to the dorsal skin of brownish guinea pigs. ISCK03 treatment diminished UV-induced pigment spots as compared with vehicle treatment alone. Although its effect on UV-induced depigmentation was not as dramatic as with the mouse hair depigmentation, this result nevertheless demonstrates the inhibitory potency of ISCK03 against c-kit.

Taken together, we have demonstrated that phenyl-imidazole sulfonamide derivatives are potent inhibitors of c-kit protein activity. ISCK03, one of the most potent derivatives, inhibits c-kit protein phosphorylation *in vitro* and in cells. In addition, ISCK03 induces depigmentation in mice and in the brownish guinea pig model. These results strongly suggest that ISCK03 could be useful as a treatment for hyperpigmentation that is partially or completely caused by c-kit expression or activity.

Acknowledgement

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