In pharmaceutical development, the subcutaneous (SC) route is often selected for its slow absorption rate\(^1\) and easy injectability for patients. Depending on the physicochemical properties of the active pharmaceutical ingredient (API), additional excipients may be required to improve the stability and solubility of the API\(^2\). It is essential to optimize the API and excipient composition to minimize the risk of local irritation, especially for formulations requiring daily dosing, because medication that causes irritation should not be subcutaneously administered\(^3\); adverse reactions, such as tissue necrosis or abscess, may occur at injection sites, and this could be a critical issue for subjects and patients. There are a limited number of excipients registered in the FDA Inactive Ingredient Database that can be used in formulations for SC administration\(^3\). In addition, there is relatively little information about the non-clinical safety of excipients administered subcutaneously compared with the information available for excipients administered via other routes, such as the oral and intravenous routes\(^4\). Thus, early safety evaluation of candidate excipients is important for the development of safer formulations. In this study, we conducted in vivo screening of novel excipients (in single doses formulations), such as surfactants, polymers, and lipids, in rats to rank candidate excipients based on local tolerability. In addition to the candidate excipient formulations, we prepared two vehicles containing marketed SC products, insulin glulisine (Apidra\(^®\); Sanofi-Aventis U.S. LLC, Bridgewater, NJ, USA)\(^5\) and liraglutide (Victoza\(^®\); Novo Nordisk A/S, Bagsvaerd, Denmark)\(^6\), according to the label information, to use as references for measuring the significance of histopathological findings. Additionally, using vehicles of marketed SC products as references helped determine the developability of the novel excipients.

The care and use of the animals and experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of BoZo Research Center Inc. (approval No. G190297). In this study, we used 90 6-week-old male Sprague-Dawley rats (Crl:CD(SD);
Charles River Laboratories Japan, Inc., Kanagawa, Japan). Each dose group was assigned two rats for local tolerability screening. The rats were housed in a solid-floor plastic cage (W 440 × D 275 × H 280 mm; Hannyu Co., Saitama, Japan) with bedding (ALPHA-dri, Shepherd Specialty Papers, Inc., Watertown, TN, USA) under the following environmental conditions: the temperature was maintained between 20 °C and 26 °C, relative humidity ranged from 30% to 70%, the rate of air exchange was between 10 and 15 times/h, and a 12-h light/dark cycle was implemented (lights on from 7:00 a.m. to 7:00 p.m., 300 lux or less). The rats were allowed free access to pelleted diets (γ-irradiated CR-LPF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Appropriate environmental enrichment was provided to the rats in accordance with the guidelines of the IACUC. The data in this report was derived from three separate toxicity studies that had identical study procedures.

For this screening study, we produced 45 formulations (including two reference vehicles and a negative control mentioned below) using 24 excipients, including novel surfactants, polymers, and lipids, for SC formulation development, as shown in Table 1. Each excipient was prepared at a concentration assumed to confer clinical benefits, such as improvement of stability or solubility of the drug load. To rule out the possibility of local irritation due to physicochemical properties, most of the formulations were adjusted to a pH between 5.5 and 8.5 using acid or base, and physiological osmolality was adjusted using phosphate-buffered saline or mannitol. Please note that pH of polyvinyl alcohol at 4.6% w/v was 5.3, which can be considered to be within the injectable pH range of saline. The pH values of the following formulations were outside the pH range of 5.5–8.5: gentisic acid at 0.2% w/v (pH 3.2), benzoic acid at 0.2% w/v (pH 4.0), levulinic acid at 0.2% w/v (pH 4.4), and oleyl sarcosine at 1% / Transcutol at 10% (pH 4.7). These pH conditions were selected to maintain the native charged state of the main formulation component (API), which would improve the physicochemical properties of potential APIs (solubilization through co-crystal/co-amorphous formation or molecular assemblies). Several excipient formulations had a measured osmolality that was over 600 mOsm/kg, which is reported to be the threshold for pain in humans. These included: trimethyl glycine at 8% (740 mOsm/kg), glycine at 5% (609 mOsm/kg), and sucrose at 20% (657 mOsm/kg). We were unable to measure the osmotic pressure of Transcutol and oleyl sarcosine/Transcutol formulations owing to tech-

### Table 1. Test Article Information and Local Tolerability Data

| Test vehicle | CAS No. | Composition | pH | Osmolality (mOsm/kg) | Clinical sign | Necropsy | Histopathology |
|--------------|---------|-------------|----|---------------------|---------------|----------|----------------|
| Saline (negative control) | 7647-14-5 | Aqueous solution containing metacresol (3.15 mg/mL), tromethamine (6 mg/mL), sodium chloride (5 mg/mL) and polysorbate 20 (0.01 mg/mL) | 4.5–8 | 285 | - | - | Deg/Nec (+), Mono (-) |
| Vehicle of apida | NA | Aqueous solution containing disodium phosphate dihydrate (1.42 mg/mL), propylene glycol (14 mg/mL) and phenol (5.5 mg/mL) | 8.1 | 274 (0.9) | - | - | Deg/Nec (+), Mix (+), Mono (+), RegM (+) |
| Vehicle of Victoza | NA | Aqueous solution containing mannitol (5%) | 5.2 | 392 (1.4) | E (±, 2d) | - | Deg/Nec (+), Mix (+), Mono (+), T (+) |
| HPC (4.6%) | 9004-64-2 | Dissolved in mannitol (5%) solution | 6.8 | 421 (1.5) | E (±, +, 1h to 8h) | - | Mono (+) |
| Poloxamer 188 (6.54%) | 106392-12-5 | Dissolved in PBS | 7.1 | 291 (1.0) | - | - | Mono (+) |
| Poloxamer 188 (6.54%) | 106392-12-5 | Dissolved in PBS | 7.0 | 328 (1.2) | E (+, 1h) | - | Deg/Nec (+), Mono (+) |
| Poloxamer 188 (14%) | 106392-12-5 | Dissolved in mannitol (1.6%) solution | 6.4 | 377 (1.3) | E (+, 1h) | - | Deg/Nec (+), Mono (+) |
| Hydroxypropyl methylcellulose (HPMC) (4.6%) | 9004-65-3 | Dissolved in PBS | 7.0 | 328 (1.2) | E (±, +, 1h to 8h) | - | Deg/Nec (+), Mono (+) |
| HPC (14%) | 9004-64-2 | Dissolved in PBS | 5.3 | 343 (1.2) | E (±, +, 1h to 8h) | - | Deg/Nec (+), Mix (+), CelD, C (+) |
| Kollidon VA64 (6.5%) | 25086-89-9 | Dissolved in PBS | 7.1 | 503 (1.8) | E (±, +, 1h to 8h) | - | Deg/Nec (+), Mono (+), C (+) |
| Poloxamer 407 (4.6%) | 977057-91-2 | Dissolved in PBS | 7.0 | 316 (1.1) | E (±, 1h to 4h), DRD (1h to 2h) | - | Deg/Nec (+), Mono (+), C (+) |
| Poloxamer 407 (4.6%) | 977057-91-2 | Dissolved in mannitol (1.7%) solution | 6.2 | 381 (1.3) | E (±, 1h to 2h) | - | Deg/Nec (+), Mono (+) |
| Dioctyl sodium sulfosuccinate (DOSS) (0.18%) | 577-11-7 | Dissolved in mannitol (5%) solution | 6.5 | 300 (1.1) | E (±, +, 1h to 4h), DRD (1h to 2d) | C, DRF | Deg/Nec (+), Mono (+), RegM (+), T (+), H (+), C (+) |
| Test vehicle | CAS No. | Composition | pH | Osmolality (mOsm/kg) | Clinical sign | Necropsy | Histopathology |
|--------------|---------|-------------|----|----------------------|---------------|----------|----------------|
| DOSS (0.54%) | 577-11-7 | Dissolved in mannitol (5%) solution | 6.9 | 303 (1.1) | E (± to +, 1h to 4h), WD (1h to 2h), DRD (8h), C (1d to 2d) | C, DRF | Deg/Nec (++), Mix (+) |
| Sodium dodecyl sulfate (SDS) (0.18%) | 151-21-3 | Dissolved in PBS | 7.0 | 269 (0.9) | E (±, 1h to 2h), DRD (1h to 8h), C (1d to 2d) | C, DRF | Deg/Nec (+), Mono (+), CelD |
| Kolliphor HS15 (0.18%) | 70142-34-6 | Dissolved in PBS | 7.4± | 287 (1.0) | - | - | Deg/Nec (+), Mono (+) |
| Kolliphor HS15 (0.54%) | 70142-34-6 | Dissolved in PBS | 7.4± | 289 (1.0) | - | - | Deg/Nec (+), Mono (+) |
| Kolliphor HS15 (3%) | 70142-34-6 | Dissolved in PBS | 7.4± | 299 (1.0) | - | - | Deg/Nec (+), Mono (+), RegM (+) |
| Deoxycholate Sodium (DS) (0.18%) | 302-95-4 | Dissolved in mannitol (5%) solution | 7.3 | 298 (1.0) | E (± to +, 1h to 4h) | - | Deg/Nec (+), Mono (+), RegM (+) |
| DS (0.54%) | 302-95-4 | Dissolved in mannitol (5%) solution | 7.5 | 305 (1.0) | E (± to +, 1h to 4h), DRD (1h to 2d) | C, DRF | Deg/Nec (+), Mix (+) |
| Ployisorbate 80 (0.54%) | 9005-65-6 | Dissolved in PBS | 7.4± | 289 (1.0) | - | - | Deg/Nec (+), Mono (+) |
| Ployisorbate 80 (2.5%) | 9005-65-6 | Dissolved in PBS | 7.4± | 300 (1.1) | - | - | Deg/Nec (+), Mono (+) |
| Kolliphor EL (0.84%) | 61791-12-6 | Dissolved in PBS | 7.4± | 287 (1.0) | - | - | Mono (+) |
| Kolliphor EL (0.12%) | 61791-12-6 | Dissolved in PBS | 7.4± | 288 (1.0) | - | - | Mono (+) |
| Vitamin E TPGS (0.18%) | 9002-96-4 | Dissolved in PBS | 7.4± | 287 (1.0) | - | - | Mono (+) |
| Vitamin E TPGS (0.54%) | 9002-96-4 | Dissolved in PBS | 7.4± | 288 (1.0) | - | - | Mono (+) |
| Vitamin E TPGS (3%) | 9002-96-4 | Dissolved in PBS | 7.4± | 296 (1.0) | E (± to +, 1h to 4h), E (± to +, 1h to 1d) | - | Deg/Nec (+), Mix (+) |
| Methylcellulose (MC)(0.5%) | 9004-67-5 | Dissolved in PBS | 7.1 | 293 (1.0) | - | - | Deg/Nec (+), Mono (+) |
| MC (1.5%) | 9004-67-5 | Dissolved in PBS | 7.1 | 299 (1.0) | - | - | Deg/Nec (+), Mono (+) |
| Transcutol (10%) | 111-90-0 | Dissolved in PBS | 7.2 | NA | - | - | Mono (+) |
| Transcutol (30%) | 111-90-0 | Dissolved in PBS | 7.5 | NA | E (±, 1h), DRD (1h to 8h), BD (1d to 2d) | BF | Deg/Nec (+), Mix (+), CelD, RegM (+), T (+), H (+), C (+) |
| Benzoic (0.2%) | 65-85-0 | Dissolved in Sucrose (5%) and HEPES (10 mM) solution | 4.0 | 284 (1.0) | - | - | Mono (+) |
| Levulinic (0.2%) | 123-76-2 | Dissolved in Sucrose (5%) and HEPES (10 mM) solution | 4.4 | 291 (1.0) | - | - | Mono (+) |
| Gentisic (0.2%) | 490-79-9 | Dissolved in Sucrose (5%) and HEPES (10 mM) solution | 3.2 | 282 (1.0) | E (±, 1h) | - | Deg/Nec (+), Mono (+) |
| 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Polyethylene glycol 5000 (PEG5k-DSPE) (0.4%) | NA 3 | Dissolved in Sucrose (5%) and HEPES (10 mM) solution | 7.4 | 294 (1.0) | E (±, 1h to 4h) | - | Mono (+) |
| Oleyl sarcosine (0.1%) / transcutol (10%) | 110-25-8, 110-90-0 | Dissolved in Sucrose (5%) and HEPES (10 mM) solution | 7.1 | NA | E (± to +, 1h to 4h) | - | Deg/Nec (+), Mix (+), Mono (+) |
| Oleyl sarcosine (1%) / transcutol (10%) | 110-25-8, 110-90-0 | Dissolved in Sucrose (5%) and HEPES (10 mM) solution | 4.7 | NA | E (± to +, 1h to 8h) | - | RegM (+), Mono (+) |
| Trimethyl gly cine (3%) | 107-43-7 | Dissolved in distilled water | 6.4 | 254 (0.9) | E (± to +, 1h to 2h) | - | C (+) |
| Trimethyl gly cine (8%) | 107-43-7 | Dissolved in distilled water | 6.5 | 740 (2.6) | E (±, 1h) | - | Mono (+) |
| Glycine (2%) | 56-40-6 | Dissolved in distilled water | 6.1 | 258 (0.9) | - | - | Mono (+) |
| Glycine (5%) | 56-40-6 | Dissolved in distilled water | 6.1 | 609 (2.1) | E (±, 1 to 2h) | - | - |
| Sucrose (20%) | 57-50-1 | Dissolved in distilled water | 6.2 | 657 (2.3) | E (±, 1h to 2h) | - | - |

Test vehicles shown in bold text were less irritative than the other ones with degeneration/necrosis.

*1: Specification value according to The Japanese pharmacoepia. pH was not measured in this study. *2: estimated value because pH was not measured. *3: Product Name: SUNBRIGHT DSPE-050CN, NA: not applicable, PBS: phosphate-buffered saline, HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Osmolarity: Values in parentheses: the ratio to the Physiological saline (285 mOsm/kg, as theoretical value).

Abbreviations for clinical sign. E: Edema; Er: Erythema; DRD: dark red discoloration; BD: Black discoloration; WD: White discoloration; C: Crust.
Draize score of edema, −: no edema, ± : very slight edema (barely perceptible), +: slight edema (edges of area well defined by definite raising), ++: moderate edema (raised more than 1 mm and extending beyond area of exposure).

Abbreviations for necropsy. C: Crust (epidermis), DRF: Dark red focus (sc), BF: Black focus (epidermis and sc).

Abbreviations for histopathology. Deg/Neg: Degeneration/necrosis, epidermis/dermis/subcutis, Mix: Inflammatory cell infiltration, mixed, dermis/subcutis, Mono: Inflammatory cell infiltration, mononuclear cell, dermis/subcutis, CelD: Retention, cell debris (non-graded finding), granulocyte, RegM: Regeneration, muscle fiber, subcutis, T: Thrombus, subcutis, H: Hemorrhage, subcutis, C: Crust, epidermis.

Histopathological grades. −: Not remarkable, ± : minimal (sparse or focal finding), +: mild (multifocal or diffuse finding), ++: moderate (multifocal or diffuse finding extended to adjacent tissue such as from subcutis to dermis).
nical limitations. The reference SC vehicles were prepared using the description on the information labels of Apidra® and Victoza®, and the osmotic pressure and pH were measured. Physiological saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan), the specification of which was according to the Japanese Pharmacopoeia, was used as a negative control. The EMA guidelines recommend a maximum applicable volume for SC tolerability evaluation, and the dose volume in this study was set at 1 mL/site/animal, approximately 5 mL/kg for 6-week-old male rats (the body weight range at dosing: 154–225 g), which is the administration volume commonly used in SC toxicity studies. Each test article was administered subcutaneously via the clipped abdominal skin (outside the median) using a needle (25G or 27G). We chose the abdominal skin as the injection site, rather than the dorsal skin, to minimize diffusion of the injected liquid into the mobile SC connective tissue of the dorsal skin. To evaluate skin irritation reactions, erythema, and edema (dermal swelling indicating fluid retention) at the injection site (abdominal skin), the Draize test was used to score the reactions. Observations were performed five times on the day of dosing: at predose, 1, 2, 4, and 8 h post dose; and once a day on days 1 and 2 after dosing. Following clinical observations 2 days after dosing, the animals were euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia. Thereafter, macroscopic examination of the injection site was conducted. The abdominal skin (including injection site) was dissected, fixed, and preserved in phosphate buffered 10% formalin. The abdominal skins from all the rats, which were trimmed to include both the injection site and intact area, was embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. Each microscopic finding was graded as follows: a sparse or focal finding in one region of the subcutis, dermis, or epidermis was graded as "minimal"; a multifocal or diffuse finding in one region of the subcutis, dermis, or epidermis was graded as "mild"; and a multifocal or diffuse finding extending to adjacent tissues, such as findings extending from the subcutis to the dermis and/or epidermis, was graded as "moderate". In this study, we chose only one timepoint, day 2 post dose, for pathological examination to observe the acute phase effect after a single dose. However, the dose regimen or timing of the terminal necropsy could be arranged with flexibility depending on the intended clinical dose regimen or study purpose, such as the evaluation of repeat dose or recovery.

The results of skin observations, necropsy, and histopathology of all the rats are summarized in Table 1; additionally, we listed findings observed in at least one rat from each dose group. If both rats in the group had similar findings, but one of the rats was more severely affected than the other, the more severe grade was recorded in the table.

During the clinical observations, edema was observed after the administration of various formulations. The formulations included both irritative and non-irritative formulations (described in the next paragraph), which indicated that external edema was not evidence of SC irritation, especially when observed at earlier time points, such as 1 to 8 h after dosing. On the other hand, external black or dark red discoloration and crust formation, most of which were observed at later time points, such as one to two days after dosing, were observed only in the irritative formulations. In addition, macroscopic findings of crust formation and black focus in the epidermis and/or subcutis were observed during necropsy of the rats that had received the irritative formulations. Dark red foci in the subcutis, which were noted with several formulations, seemed to be related to microscopic hemorrhage.

Twenty-eight out of forty-four excipient formulations that showed necrosis/degeneration, indicating tissue injury, were considered irritative and poorly tolerated as single SC injections (examples represented in Fig. 1). In these 28 formulations, other histopathological findings, such as mononuclear/mixed cell infiltration, granulocytic cell debris, regeneration of muscle fibers, thrombus, hemorrhage, and/or crust formation were observed. Two formulations (HPC at 14% and vitamin E TPGS at 0.18%) showed only mild mononuclear cell infiltration, which was considered a less irritative test-article-related finding owing to a lack of apparent tissue injury. Although minimal mononuclear cell infiltration, hemorrhage, and/or crust formation were observed after administration of trimethyl glycine at 3% and 8%, glycine at 2%, 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Polyethylene glycol 5000 (PEG5k-DSP) at 0.4%, Poloxamer 188 at 0.54%, Polylip o 0.04% and 0.12%, Transectol at 10%, benzoic acid at 0.2%, and levulinic acid at 0.2%, they were considered to be spontaneous or SC injection procedure-related findings. In addition to these formulations, sucrose and glycine at 5% had no microscopic findings, and these formulations were well tolerated.

To avoid the risk of tissue damage, a pH range of 5.5–8.5 is recommended. Low pH values (<5.5) could be related to the SC irritation observed after the administration of some formulations, such as polyvinyl alcohol at 4.6%, gentisic at 0.2%, and oleoyl sarcosine at 1% / Transcutol at 10%; however, no irritative findings were noted with benzoic acid at 0.2% and levulinic acid at 0.2%, which had low pH values. Furthermore, a correlation between the degree of hypertonicity and the sensation of pain in humans was reported, and an upper limit of 600 mOsm/kg was proposed to minimize the risk of pain. In this evaluation, 3 formulations, trimethyl glycine at 8%, glycine at 5%, and sucrose at 20% did not appear to cause any irritation despite having high osmolality values (>600 mOsm/kg).

In this screening assessment, vehicles of marketed products (Apidra® and Victoza®), which are subcutaneously administered daily in clinical settings, were used as reference vehicles. Clinical local tolerability data of the reference vehicles is not available; however, they showed minimal to mild tissue degeneration or necrosis in rats (Fig. 1). Although irritative candidate novel excipients should be deprioritized for safer clinical formulation development, comparison of tolerability profiles of these excipients with those of marketed reference vehicles, in a risk-benefit analy-
sis, could support their use. For example, the developability of novel excipients could be considered if their tolerability is similar to that of the reference vehicles, and the benefits of the excipients are significant for patients; such benefits include improvement of API stability and solubility for better pharmacodynamic and pharmacokinetic profiles.

In conclusion, evaluation of the SC tolerability of novel excipients, using a single-dose, in rat models identified a useful way to rank the safety of novel candidate formulations at the early stage of development.

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