(±)-*cis*-2-Methylspiro[1,3-oxathiolane-5,3′-quinuclidine] Hydrochloride, Hemihydrate (SNI-2011, Cevimeline Hydrochloride) Induces Saliva and Tear Secretions in Rats and Mice: The Role of Muscarinic Acetylcholine Receptors

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ABSTRACT—We investigated effects of (±)-*cis*-2-methylspiro[1,3-oxathiolane-5,3′-quinuclidine] hydrochloride, hemihydrate (SNI-2011, cevimeline hydrochloride), a rigid analogue of acetylcholine, on saliva and tear secretions in rats and mice to evaluate its therapeutical efficacy for xerostomia and xerophthalmia in patients with Sjögren's syndrome and X-ray exposure in the head and neck. Intraduodenal administrations of SNI-2011 increased saliva secretion in a dose-dependent manner at doses ranging from 3 to 30 mg/kg in normal rats and mice, two strains of autoimmune disease mice and X-irradiated saliva secretion defective rats. The salivation elicited by SNI-2011 was completely inhibited by atropine. A similar atropine-sensitive response was observed in tear secretion. In rat submandibular/sublingual gland membranes, [^{3}H]quinuclidinyl benzilate (QNB) binding was saturable, and Scatchard plot analysis revealed a single population of binding sites with a K_d of 22 pM and a maximal binding capacity of 60 fmol/mg protein. The competitive inhibition curve of the [^{3}H]QNB binding by SNI-2011 was obtained, and its dissociation constant value calculated from IC_{50} was 1–2 μM. These results suggest that SNI-2011 increases saliva and tear secretions through a direct stimulation to muscarinic receptors in salivary and lacrimal glands, and they suggest that SNI-2011 should be beneficial to patients with Sjögren's syndrome and X-ray exposure in the head and neck.

Keywords: SNI-2011, Muscarinic agonist, Sjögren's syndrome, Salivary gland, Lacrimal gland

Sjögren's syndrome is an autoimmune disease characterized by inflammations in the exocrinal glands, especially in the salivary and lacrimal glands (1). Although many patients are enduring xerostomia (dry mouth) and xerophthalmia (dry eyes), only an artificial saliva and/or tears are used for temporal relief for these sicca symptoms (2). Xerostomia is also caused as a side effect of the X-ray irradiation therapy to head and neck cancer (3–5). Since the muscarinic acetylcholine receptor (muscarinic receptor) mediates serous-saliva secretion (6, 7) and tear secretions (8), muscarinic agonists should be beneficial to xerostomia and/or xerophthalmia in Sjögren's syndrome patients and patients with X-irradiation therapy in the head and neck.

SNI-2011 (cevimeline hydrochloride), a rigid analogue of acetylcholine, (±)-*cis*-2-methylspiro[1,3-oxathiolane-5,3′-quinuclidine] hydrochloride, hemihydrate, has been proposed as a muscarinic agonist (9). Several reports showed that SNI-2011 induced saliva secretion in normal rats, Sjögren's syndrome model mice (i.e., MRL/lpr mice) and dogs by intravenous, intraperitoneal and/or intraduodenal (i.d.) administrations (10–12). However, details of its activity to induce saliva and tear secretion and its binding to muscarinic receptors in salivary glands have not been reported. Accordingly, we investigated pharmacological profiles of SNI-2011 on saliva and tear secretions such as the correlation between saliva secretion and plasma concentration of SNI-2011, binding property for muscarinic receptor in salivary glands, capability of tolerance in its efficacy by repeated administration, as well as its activity to induce saliva and/or tear secretion in normal and Sjögren's syndrome model animals and X-irradiated rats.

MATERIALS AND METHODS

Determination of saliva and tear secretions in rats and mice

The following animals were used to evaluate effects of SNI-2011 on saliva and/or tear secretions. Male Wistar rats (8-week-old; Charles River Japan, Yokohama) and female ICR mice (36-week-old, Charles River Japan) were used as normal animals. Female MRL/MpJUmm Crj-lpr /lpr (MRL/lpr) mice (10-week-old, Charles River Japan) and female IQI/Jcl (IQI) mice (36-week-old; Clea Japan, Tokyo) were used as models of autoimmune disease. For a salivary secretion defective model, salivary glands of male Wistar rats (8-week-old, Charles River Japan) was irradiated with X-ray at a dose of 35 Gy (approximately 0.5 Gy/min, filtered through 0.1 mm Cu and 0.5 mm Al; MBR-1505R; Hitachi Medico, Tokyo) according to the method of Takei et al. (13).

Each rat or mouse was anesthetized with pentobarbital sodium (50 mg/kg, i.p. or 20 mg/kg, i.p. + 60 mg/kg, s.c.) and placed on a heating pad. The trachea was cannulated with a polyethylene tube. Saliva was collected from the floor of the mouth with a cotton ball, and the weight of saliva was measured over an interval of 5, 10 or 15 min for 60 or 90 min. As for the X-irradiated rats, measurements of saliva were carried out 7 days after the irradiation. SNI-2011 (3, 10 or 30 mg/kg; Snow Brand Milk Products, Tokyo) was dissolved in distilled water or physiological saline and administered to the duodenum at a volume of 1 ml/kg. Tear secretion was measured with Schirmer strip (1 mm in width; Showa Yakuhin Kako, Tokyo) inserted into the inner aspect of an eyelid. Soaked length of the strip paper by tears during 90 min was determined as the amount of tears secreted. To clarify the inhibitory effect of a muscarinic receptor antagonist on the action of SNI-2011, atropine methyl bromide (0.1 mg/kg; Sigma, St. Louis, MO, USA) was injected intravenously 10 min before the administration of SNI-2011.

Determination of plasma concentration of SNI-2011

Plasma concentration of SNI-2011 free base was determined in male Wistar rats 15 and 60 min after intraduodenal administration by gas chromatography-mass spectrometry (GC-MS) analysis. A 200 μl-aliquot of plasma, 100 μl of deuterated SNI-2011 (d_4-SNI-2011) solution (500 ng/ml), and 100 μl of 1 N NaOH were placed in a glass centrifuge tube. The mixture was shaken with 5.5 ml of *n*-hexane for 10 min and then centrifuged at 2000 rpm for 5 min. The organic layer was evaporated to dryness under a stream of nitrogen at 40°C. The residue was reconstituted in 35 μl of methanol, and 3 μl of this solution was analyzed in a GC-MS system consisting of HP5890 Series II gas chromatograph (Hewlett Packard, Avondale, PA, USA) and JMS-AX505WA mass spectrometer (JEOL, Tokyo) with a Durabond-17 capillary column (15 m × 0.25 mm in internal diameter, coating thickness of 0.25 μm; J&W Scientific, Folsom, CA, USA). The injection port temperature was 280°C. The oven temperature was held at 100°C for 1 min, increased to 200°C at a rate of 40°C/min, and then increased to 280°C at a rate of 50°C/min and held for 2 min. The mass spectrometer was operated at an ionization energy of 70 eV with an emission current of 300 μA in the positive electron impact mode. Selected ions for determination were monitored at m/z 199 for SNI-2011 and m/z 203 for d_4-SNI-2011.

Preparations of salivary gland membrane fraction

Male Sprague-Dawley rats (7-week-old, Charles River Japan) were killed by decapitation, and the submandibular /sublingual glands were dissected in 10 mM Na^+-K^+ phosphate buffer (pH 7.4) and homogenized in a Polytron (setting 6, 4 × 20 sec bursts; Kinematica, Lucern, Switzerland). The homogenates were passed through a cheesecloth and centrifuged at 30000 × *g* for 30 min. The pellets were homogenized in buffer in a Potter-Elvehjem homogenizer and centrifuged at 30000 × *g* for 30 min. The final membrane pellet was resuspended in a tenfold volume of Na^+-K^+ phosphate buffer (pH 7.4) and stored at −80°C until required.

Receptor binding assay

The [^{3}H]quinuclidinyl benzilate (QNB) binding assay was carried out according to the method of Watson et al. (14). Routinely, the membrane suspension was incubated with 0.2 nM [^{3}H]QNB (43 Ci/mmol; NEN, Boston, MA, USA) in 10 mM Na^+-K^+ phosphate buffer (pH 7.4) for 120 min at 30°C. The reaction was terminated by addition of 3 ml of ice-cold stop solution (10 mM Na^+-K^+ phosphate buffer/pH 7.4) and filtration under negative pressure on a glass fiber filter (Whatman GF/B). The tube and filter were rapidly rinsed 3 times with 3 ml of stop solution, and filters were removed and dissolved in scintillation cocktail (Emulsifier-Scintilator 299; Packard Instrument, Meriden, CT, USA). The radioactivity on the filter was counted with a liquid scintillation counter (counting efficiency of 46%; LS 7800; Beckman, Fullerton, CA, USA). Non-specific binding was determined in the presence of 1 μM atropine sulfate (Sigma). Protein concentration was determined according to the method of Lowry et al. (15) with bovine serum albumin as standard.

The dissociation constant (K_d) for [^{3}H]QNB binding was obtained from the results of saturation experiments by Scatchard plot analysis. The dissociation constant of non-labeled muscarinic ligand (K_i) was calculated as

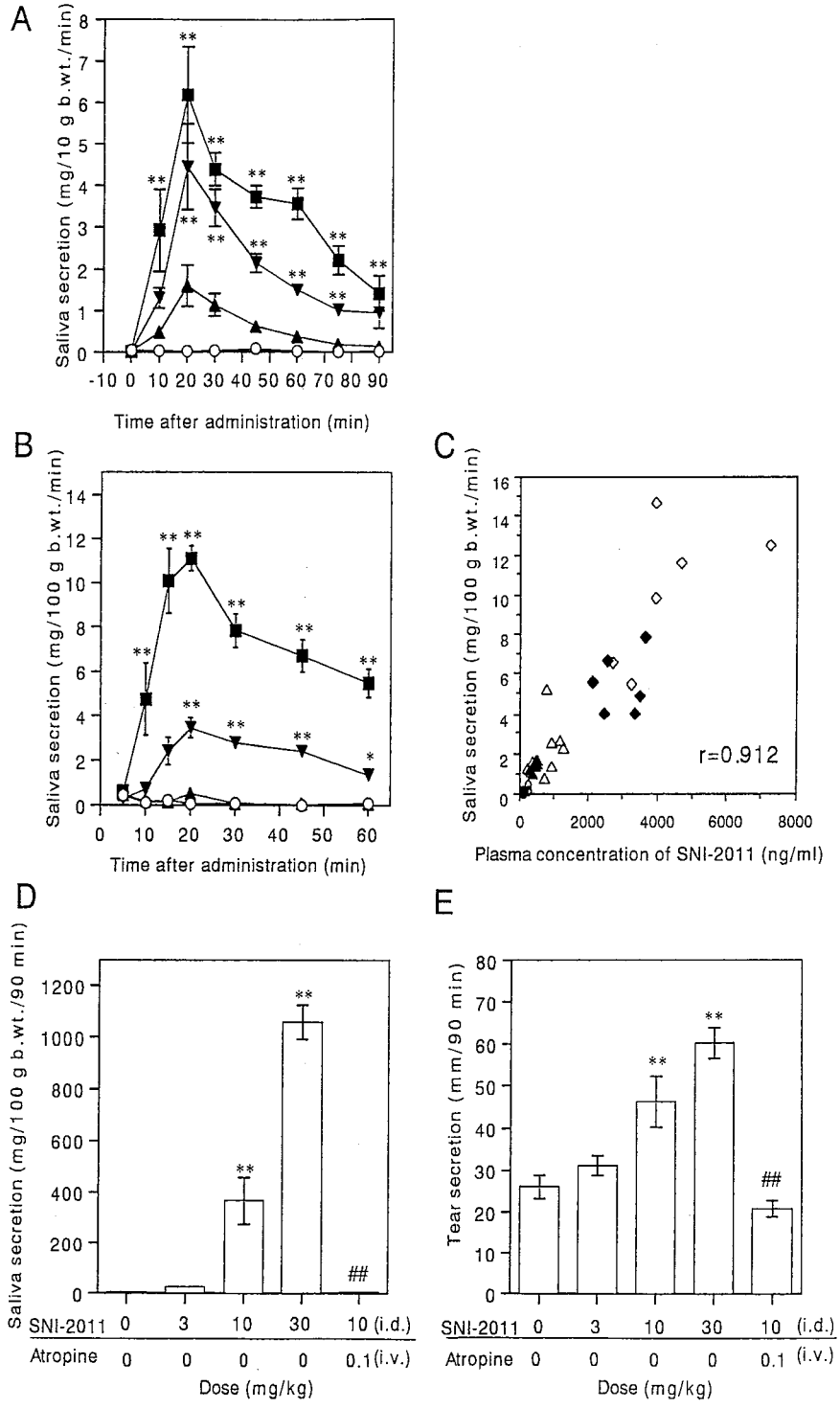


Fig. 1. Effects of intraduodenal administrations of SNI-2011 on saliva and tear secretions in normal mice and rats. A: Saliva secretions in ICR mice. B: Saliva secretions in Wistar rats. Values represent means±S.E.M. (n=6). ○: vehicle, ▲: 3 mg/kg, ▼: 10 mg/kg, ■: 30 mg/kg. *P<0.05, **P<0.01 vs vehicle group (one-way ANOVA followed by Dunnett's multiple comparison). C: Correlation of plasma concentration of SNI-2011 and saliva secretion in Wistar rats. Values are the data from each rat at each measuring point. ○: 3 mg/kg, 15 min after administration; ●: 3 mg/kg, 60 min after administration; △: 10 mg/kg, 15 min after administration; ▲: 10 mg/kg, 60 min after administration; ◇: 30 mg/kg, 15 min after administration; ◆: 30 mg/kg, 60 min after administration. D and E: SNI-2011-induced saliva and tear secretions, respectively, during 90 min and inhibitory effects of atropine in Wistar rats. Values represent means±S.E.M. (n=6). **P<0.01 vs vehicle group, ##P<0.01 vs SNI-2011 at 10 mg/kg without atropine (one-way ANOVA followed by Tukey compromise multiple comparison).

$K_i = IC_{50} / \{1 + (C/K_d)\}$, followed by Hill analysis (IC_{50}: concentration of non-labeled ligand that caused half-maximal displacement of [^{3}H]QNB binding, C: concentration of [^{3}H]QNB). The appropriate concentration of the following muscarinic ligands were incubated with [^{3}H]QNB: SNI-2011; arecoline hydrochloride (Nacalai Tesque, Kyoto); bethanechol chloride, oxotremorine sesquifumalate and pilocarpine hydrochloride (Sigma).

Repeated administration of SNI-2011 in rats

SNI-2011 (10 mg/kg) or vehicle was orally administered to male Wistar rats (8-week-old) twice daily for 2 or 4 weeks. On the day after the last administration, SNI-2011 (10 mg/kg) was administered intraduodenally to both the SNI-2011-treated group and the vehicle-treated group, and saliva secretion was measured. After the measurement of saliva secretion, rats were sacrificed with an overdose of pentobarbital sodium and then submandibular/sublingual glands were removed. [^{3}H]QNB binding in salivary gland membranes was assayed with the method described above. Naive rats that were given neither SNI-2011 nor vehicle orally were also tested for SNI-2011-induced saliva secretion and [^{3}H]QNB binding in salivary glands.

RESULTS

Effects of SNI-2011 on saliva and tear secretions in rats and mice

SNI-2011 dose-dependently induced saliva secretion at doses of 3 mg/kg, i.d. and higher in normal mice (Fig. 1A) and at 10 mg/kg, i.d. and higher in normal rats (Fig. 1: B and D). The amount of saliva started to increase within 10 min after the administration and reached the maximum at approximately 20 min and thereafter decreased gradually. The secreted saliva amount was positively correlated with the plasma concentration of SNI-2011 with a correlation coefficient of 0.912 (Fig. 1C). SNI-2011 also induced tear secretion dose-dependently at 10 and 30 mg/kg (Fig. 1E). The time course of tear secretion was similar to that of saliva secretion (data not shown). The stimulating effects of SNI-2011 on saliva and tear secretions were completely inhibited by pretreatment with atropine (Fig. 1: D and E).

In autoimmune disease models such as MRL/lpr and IQI mice, SNI-2011 (3–30 mg/kg, i.d.) dose-dependently increased the amount of saliva (Fig. 2). The amount of saliva started to increase within 10 min after the administration and reached the maximum at approximately 20 min and thereafter decreased gradually. SNI-2011 also stimulated saliva secretion in X-ray (35 Gy) irradiated rats in a dose dependent manner (Fig. 3). However, the amount of saliva secreted after SNI-2011 at 10 mg/kg was less in X-irradiated rats (129±52 mg/100 *g* b.wt./90 min, mean±S.E.M.) than in normal control rats (230±33).

Effects of SNI-2011 and classical muscarinic agonists on [^{3}H]QNB binding in rat salivary gland membranes

To assess the possible contribution of muscarinic receptors in salivary glands to the stimulation of saliva secretion elicited by SNI-2011, we tested the effects of SNI-2011 on [^{3}H]QNB binding in submandibular/sublin-

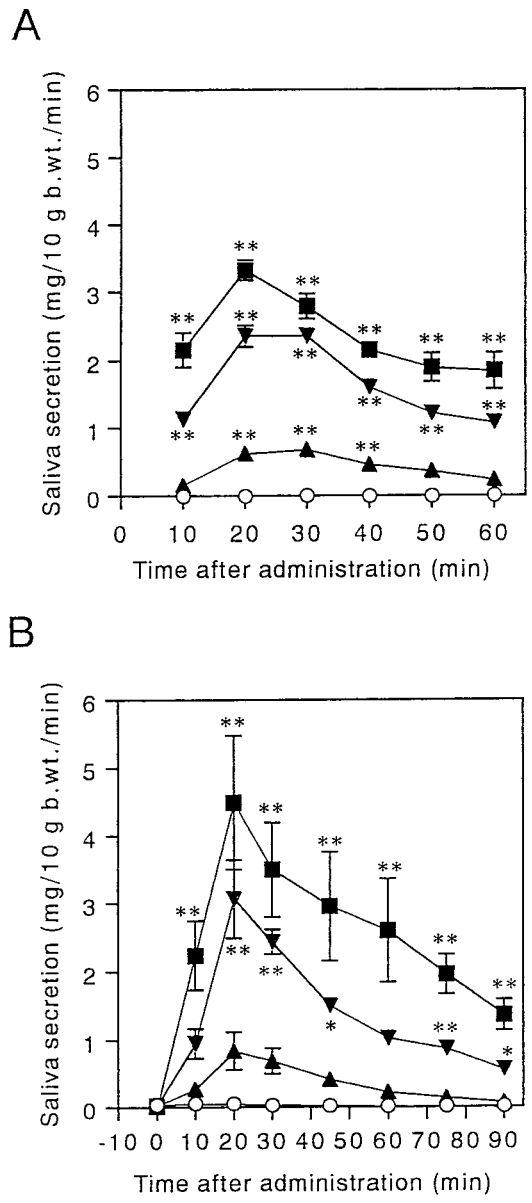
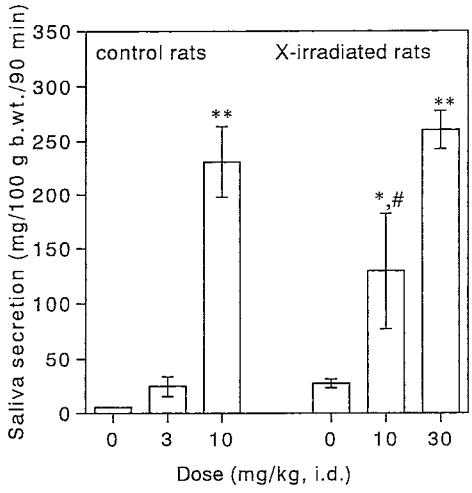


Fig. 2. Effects of intraduodenal administrations of SNI-2011 on saliva secretions in autoimmune disease mice. A: MRL/lpr mice. B: IQI mice. Values represent means±S.E.M. (n=6). ○: vehicle, ▲: 3 mg/kg, ▼: 10 mg/kg, ■: 30 mg/kg. *P<0.05, **P<0.01 vs vehicle group (one-way ANOVA followed by Dunnett's multiple comparison).



gual gland membranes. In rat salivary gland membrane fraction, specific [^{3}H]QNB binding was concentration-dependent and saturable (Fig. 4A). As shown in Fig. 4B, Scatchard plot analysis of the saturation curve revealed a single component of binding with a K_d of 22±1.5 pM and a maximal binding capacity (B_{max}) of 60±1.6 fmol/mg protein.

Fig. 3. Effects of intraduodenal administrations of SNI-2011 on saliva secretions in X-irradiated rats and normal control rats. Values represent means±S.E.M. (n=6). *P<0.05, **P<0.01 vs each vehicle group; #P<0.05 vs SNI-2011 (10 mg/kg)-treated control group (one-way ANOVA followed by Tukey compromise multiple comparison).

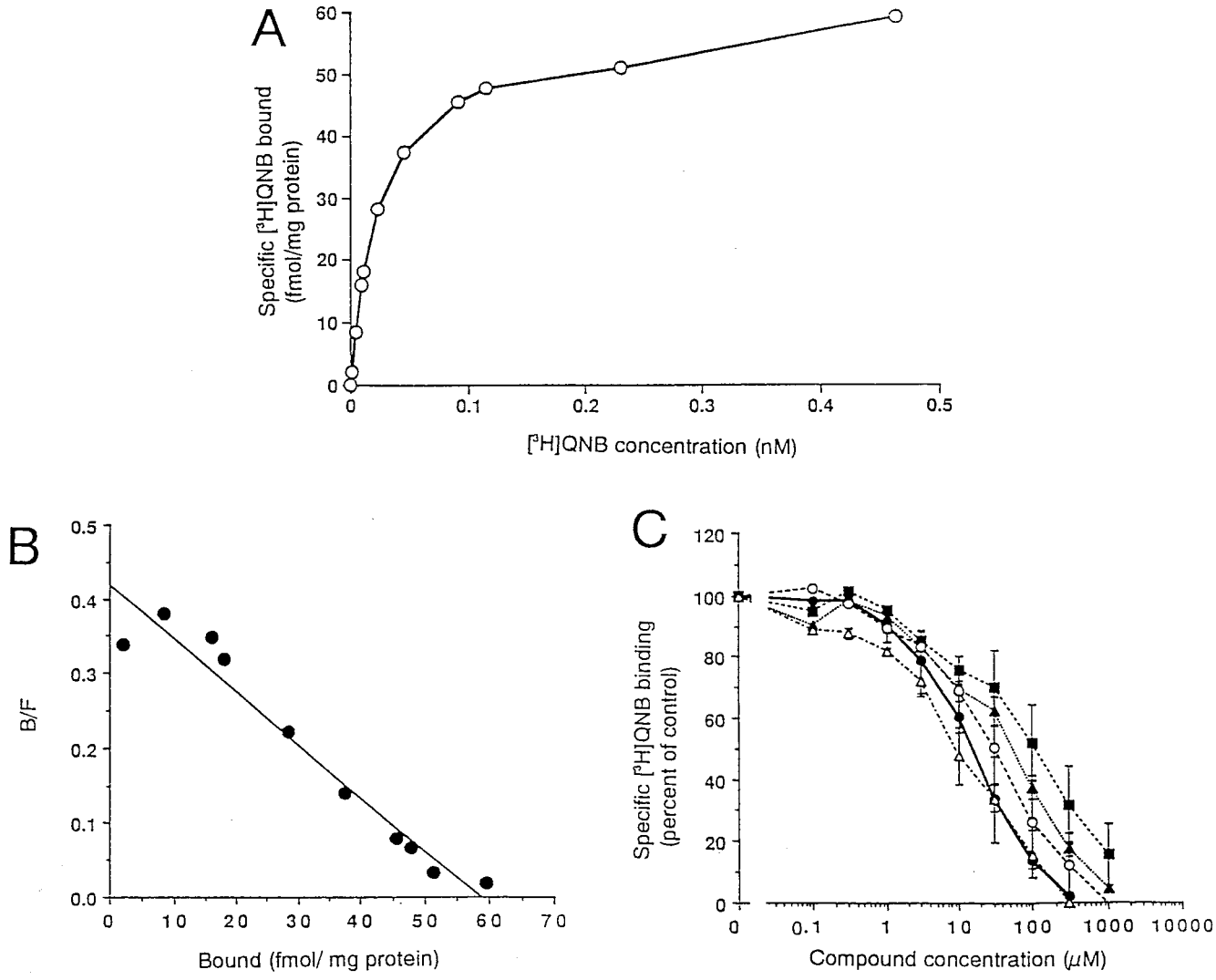


Fig. 4. Effect of SNI-2011 on [^{3}H]QNB binding in rat salivary gland membranes. A: Saturation curve of [^{3}H]QNB binding. B: Scatchard plot of [^{3}H]QNB binding. C: Competitive inhibition of [^{3}H]QNB binding by SNI-2011 and muscarinic agonists. Values represent means±S.E.M. (n=3). ●: SNI-2011, ▲: Arecoline, ■: Bethanechol, ○: Pilocarpine, △: Oxotremorine.

Table 1. Abilities of SNI-2011 and muscarinic agonists to compete with [^{3}H]QNB binding to rat submandibular/sublingual gland membranes

Agonist	[^{3}H]QNB binding (K_i, μM)
SNI-2011	1.2±0.3
Arecoline	3.2±0.5
Bethanechol	22±19
Oxotremorine	1.4±1.6
Pilocarpine	4.6±3.1

Values represent means±S.E.M. (n=3).

To investigate the ability of SNI-2011 to compete with [^{3}H]QNB binding to rat salivary gland membrane fraction, we compared SNI-2011 with classical muscarinic agonists (Fig. 4C). The competitive inhibition curve of the binding by SNI-2011 was obtained and its IC_{50} was about 14 μM. [^{3}H]QNB binding was also inhibited in a concentration-dependent manner by four muscarinic agonists, arecoline, bethanechol, oxotremorine and pilocarpine (Fig. 4C). As shown in Table 1, these agonists exhibited the following rank of order of inhibiting activities and K_i values: SNI-2011 (1.2±0.3 μM) ≒ oxotremorine (1.4±1.6 μM) > arecoline (3.2±0.5 μM) ≒ pilocarpine (4.6±3.1 μM) ≫ bethanechol (22±19 μM).

Effects of repeated administration of SNI-2011 on salivary gland

The amount of saliva induced by SNI-2011 (10 mg/kg, i.d.) was not significantly different between the SNI-2011 (10 mg/kg, p.o., 2 times/day)-treated group and the vehicle-treated group at the 2- and 4-week administration periods (Table 2). There was no significant difference in the [^{3}H]QNB binding to rat salivary gland membrane fraction between the vehicle- and SNI-2011-treated groups (Table 2).

DISCUSSION

The present results show that intraduodenal administration of SNI-2011 enhances saliva secretion in normal mice and rats. Similar findings were reported in studies examining the effects on saliva secretion after intravenous injection in normal rats (10) and intraperitoneal injection in MRL/lpr mice (11). The effective dose of SNI-2011 in these studies (3−10 mg/kg) were comparable to those in the present report (3 or 10 mg/kg), suggesting that SNI-2011 is easily absorbed through the small intestine and that it is effective by oral administration. Recently Masunaga et al. (12) also reported that intraduodenal administration of SNI-2011 enhanced saliva secretion at relatively high doses (20−40 mg/kg). The present results revealed that an increase in saliva secretion was caused by a lower dose and it correlated with the plasma concentration.

Several lines of evidence indicate that MRL/lpr mice (16, 17) and IQI mice (18, 19) are considered model animals of Sjögren's syndrome. Since histopathological degenerations in these strains, such as mononuclear cells and/or lymphoid cells infiltrations in salivary glands and lacrimal glands, were reported to be more prominent in females than in males (16−19), we used female mice in this study. On the other hand, X-ray irradiation therapy in patients with head and neck cancer frequently causes xerostomia (3−5). X-irradiation also reportedly induced a defect in saliva secretion in rats (13, 20−22). Takei et al. showed that X-irradiation at doses of 35 and 50 Gy significantly decreased pilocarpine-induced saliva secretion from one day through 50 days after irradiation (13). Our present study was carried out under similar experimental conditions (salivation was induced by SNI-2011, muscarinic agonist, at 7 days after irradiation at 35 Gy) and corresponding results were observed. That is, the amount of saliva induced by SNI-2011 at 10 mg/kg, i.d. in X-irradiated rats was less than that in control rats, suggesting that the secretory function of the salivary gland was damaged by X-irradiation. We also observed histopathol-

Table 2. Effects of repeated administration of SNI-2011 on saliva secretion and [^{3}H]QNB binding in submandibular/sublingual gland membrances in rats

Group	Amount of saliva (mg/100 g b.wt./60 min)			Specific [^{3}H]QNB binding (fmol/mg protein)		
	Administration periods (week)			Administration periods (week)		
	0	2	4	0	2	4
Non-treated	206.7±39.4			21.8±1.2		
Vehicle (10 mg/kg×2/day, p.o.)		374.2±59.3	236.6±56.0		17.0±1.1	15.0±0.9
SNI-2011 (10 mg/kg×2/day, p.o.)		268.0±82.8	229.4±26.1		17.1±1.0	15.5±0.7

Salivation was induced by single intraduodenal administration of SNI-2011 (10 mg/kg) in each animal. Values represent means±S.E.M. (n=6).

ogically that more than half of these X-irradiated rats show the atrophy of aciner cells in the parotid gland, but not in the submandibular and sublingual glands (data not shown). These observation may correlated with higher sensitivity for irradiation in parotid gland (23, 24). In our present study, intraduodenal administration of SNI-2011 enhanced saliva secretion in MRL/lrp mice, IQI mice and X-irradiated rats. Thus, SNI-2011 may prove useful for xerostomia in patients with Sjögren's syndrome and in patients with X-ray exposure in the head and neck.

SNI-2011 had a high affinity for [^{3}H]QNB binding in rat submandibular/sublingual gland membranes with a K_i of approximately 1 μM. This K_i value was comparable to that of oxotremorine. This present result suggests that SNI-2011 has the ability to bind to muscarinic receptors of rat salivary glands (M_3 subtype). Indeed, a recent report has shown that the SNI-2011-mediated sialogogic response displays a high sensitivity to the M_3-selective antagonist 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine), but a low sensitivity to the M_1-selective antagonist pirenzepine (10). In addition, SNI-2011 has also been reported to show an agonistic action on the M_3 receptor of guinea pig ileum (9). Thus, it is conceivable that the direct stimulation of the M_3 receptor in salivary glands reflects the sialogogic effects of SNI-2011 in vivo.

SNI-2011 also enhanced tear secretion in normal rats. It is reported that tear secretion is mediated mainly by M_3 receptor (8). Since the stimulating effects of SNI-2011 on tear secretion, as well as saliva secretion, were inhibited by atropine, it is suggested that SNI-2011 also stimulates M_3 receptors in lacrimal glands to increase tear secretion.

It is well known that long term stimulation to receptors results in a decrease in the number of the receptors, which may cause tolerance to drug effects. As for muscarinic receptors, subcutaneous injections for 2 weeks of diisopropyl fluroposphate, a choline esterase inhibitor, reduce the number of central and peripheral muscarinic receptors in rats (25, 26). Repeated oral administrations of SNI-2011 (10 mg/kg, 2 times/day for 4 weeks) did not alter the function of saliva secretion and [^{3}H]QNB binding in salivary glands. An intermittent stimulation of muscarinic receptors may not cause down regulation in the receptors at the dose that moderately increases saliva secretion. Consequently, repeated administrations of SNI-2011 at the effective dose is considered to induce no tolerance in its sialogogic to the effects.

Sjögren's syndrome is an autoimmune disease characterized by sicca symptoms and complications of mucousal dryness associated with the inflammations in exocrinal glands (1), and its exocrinopathy is suggested to be initiated by α-fodlin as an autoantigen (27). Xerostomia is also observed as a side effect of X-ray irradiation therapy to head and neck cancer (3–5). To alleviate the dryness of mouse and/or eyes in these patients is thought to be important for their quality of life. The present study showed that SNI-2011 enhances saliva and tear secretions by direct stimulation of muscarinic receptors in salivary and lacrimal glands in model animals of Sjögren's syndrome and X-ray induced xerostomia, and that it does not cause the tolerance in its efficacy on repeated oral administration in rats. These findings suggest that SNI-2011 is useful for managing xerostomia and xerophthalmia in Sjögren's syndrome patients and X-irradiation associated xerostomia.

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