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Photodegradation products of propranolol: The structures and pharmacological studies

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Abstract

Recently, single-dose drug packaging systems, allowing the administration of multiple drugs in a single pill, have become popular for the convenience of the patient. The quality of drugs and an accurate measurement of their photostabilities within this system, however, have not been carefully addressed. Drugs that are unstable in light should be carefully handled to protect their potency and ensure their safety. Propranolol (**1**), a β-adrenergic receptor antagonist, is widely used for angina pectoris, arrhythmia, and hypertension. Due to its naphthalene skeleton, this drug may be both light unstable and a photosensitizing agent. In this study, we isolated three photodegraded products of propranolol (**1**): 1-naphthol (**2**), *N*-acetylpropranolol (**3**), and *N*-formylpropranolol (**4**). The structures of these compounds were determined by spectroscopic methods and chemical syntheses. We also examined the acute toxicities of these substances in mice and their binding to β-adrenergic receptors using rat cerebellum cortex membranes. Although the photoproducts isolated in this study did not exhibit any acute toxicity or significant binding to β-adrenergic receptors, these results serve as a warning to single-dose packaging systems, as propranolol (**1**) must be handled carefully to protect the compound from light-induced degradation.

Keywords: Propranolol; Photodegradation; β-adrenergic receptor; Receptor binding

Introduction

Drug stability research is critical in pharmaceutical studies as the increased degradation decreases the potency of the drug and can create compounds with undesirable pharmacological effects (Andrisano et al., 1999). Moisture, temperature, and light can all alter drug quality. Currently, several classes of drugs, including dihydropyridines (nifedipine (Berson and Brown, 1995; Grundy et al., 1994; Pietta et al., 1981; Shamsipur et al., 2003; Suzuki et al., 1985), nisoldipine (Marinkovic et al., 2003), nitrendipine (Marciniec and Ogrodowczyk, 2003) etc.), azulene sulfonate (Comtet and Mettee, 1970; Olmsted, 1969), and new quinolones (norfloxacin (Cordoba-Borrego et al., 1999), levofloxacin (Yoshida et al., 1993), and lomefloxacin (Matsumoto et al., 1992), etc.), are known to be unstable under light.

In Japan, drug photostabilities are typically examined according to the 'Drug Approval and Licensing Procedures in Japan'. If the drug does not meet the criteria outlined in these procedures, then a form of photoprotection media must be provided on the surface of the drug or its packaging to guarantee photostability. A single-dose packaging system, in which multiple drugs are administered in one dose, has become increasingly popular for the added convenience of patients. While this system facilitates patient compliance with the proper dosages of multiple drugs, neither the quality of drugs in this format or the resultant photostability has been adequately tested. Therefore, drugs that are unstable towards light should be carefully handled to protect their potency and safety.

Propranolol (**1**, Fig. 1) is a β-adrenergic antagonist that is widely used for the treatment of angina pectoris, arrhythmia, and the hypertension (Hoffman and Lefkowitz, 1996). The

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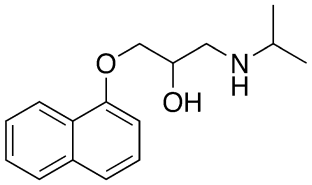

Fig. 1. Structure of propranolol (**1**).

usual dose, which ranges from 30 to 60 mg daily, can be in creased to 120 mg daily. In many Japanese hospitals, both the prescription and single-dose packaging forms of propranolol (**1**) are dispensed in small packages or are ground to adjust the dose and/or aid in swallowing. The photostability of these various pharmaceutical forms, including the naked form, has not been reported. Propranolol (**1**), however, may be both a photosensitizing agent and light-unstable, similar to other drugs that have chromophoric structures containing a naphthalene skeleton.

In this study, we examined the structures of the decomposition products of propranolol (**1**), determining the LD_{50} for each product in mice. In addition, we measured the potency of each compound to inhibit the specific binding of [^{3}H]-dihydroalprenolol hydrochloride ([^{3}H]DHA) to β-adrenergic receptors.

Materials and methods

General

All solvents were solvent grade. Wako gel C-200 (70–150 μm, Wako pure chemicals) and Alumina activated 200 (abt. 200 mesh, nacalai tesque) were used for column chromatography. Precoated Kieselgel 60 F_{254} plate (0.25 mm, Merck) and precoated aluminium oxide 60 F_{254} neutral plate (0.2 mm, Merck) were used for TLC (thin layer chromatography) analysis and the spots were detected by the absorbance of ultra violet (UV) light at 254 nm spraying with Dragendorff's reagent. ^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra were measured using JEOL JNM 400 (400 MHz), and JEOL JNM 600 (600 MHz) spectrometers. Chemical shifts (δ) are reported as ppm downfield from tetramethylsilane (TMS), and coupling constants are given in Hz. Multiplicity is indicated as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quintet), and m (multiplet), etc. Mass spectra were recorded on JEOL JMS DX-303 and JMA-DA 5000 spectrometers.

Photostability test of propranolol

Pharmaceutical preparations of propranolol in variety of packages and dosages (A: whole tablet covered with aluminum foil (control); B: whole tablet in a press-through packaging; C: whole tablet in paraffin paper; D: half tablet in paraffin paper; E: ground form in paraffin paper) were kept under scattered light (av. 8400 lx at 1:00 pm) for 28 days. To exclude the influence of moisture or oxygen on propranolol (**1**) stability, both the tablet and ground forms of the drug on Petri dishes without paraffin paper were kept in a closed cabinet in the same room for the same period. During the experimental period, the production of photodegradtion products was monitored by TLC analyses ($CHCl_3$–MeOH (22:3 *v*/*v*)).

Isolation and characterization of photoproducts

Propranolol hydrochloride (2.0 g, 6.76 mmol) was treated with an aqueous solution of 10% NH_4OH, then extracted with $CHCl_3$. The organic phase was concentrated in vacuo. The resulting solid was recrystalized with cyclohexane to produce propranolol (**1**, 1.70 g, 6.53 mmol, 97%).

Propranolol (**1**, 1.70 g, 6.53 mmol) was irradiated with 95,000 lx (40 cm far light from a halogen lamp (National JD110V250W/E)) under an Ar atmosphere. After five days of irradiation, column chromatography of a portion of the resulting solid (71.1 mg) on silica gel (10 g) [*n*-hexane-AcOEt (9:1, 17:3, 7:3, 3:2, 1:1, 2:3 *v*/*v*), and MeOH] yielded five fractions (fraction A to E). Fraction A, eluted in *n*-hexane-AcOEt (9:1 *v*/*v*), exhibited a single spot on TLC, which was confirmed to be 1-naphthol (**2**, 2.1 mg, 6.1%). Fraction D (25.2 mg), eluted in *n*-hexane-AcOEt (3:2 to 2:3 *v*/*v*), was subjected to alumina column chromatography using *n*-hexane-AcOEt (13:7, 3:2, 11:9, 1:1, 2:3, 3:7) as the eluting solvent, identifying both *N*-acetylpropranolol (**3**, 2.0 mg, 2.8%) and *N*-formylpropranolol (**4**, 16.5 mg, 24%). Propranolol (**1**) was also recovered in this fraction (14.9 mg, 21%).

*N-[2-hydroxy-3-(1-naphthalenyloxy)propyl]-N-(1-methylethyl)acetamide (N-acetylpropranolol (**3**))*: While the properties of this compound has been reported in previous papers (Chiou et al., 1997; Dewar et al., 1982; Nelson and Walker, 1978), we include the spectral data here. Colorless oil. ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 1.22 (d, 3H, J=6.6 Hz, –CH(CH_3)$_2$), 1.36 (d, 3H, J=6.6 Hz, –CH(CH_3)$_2$), 2.27 (s, 3H, –C(=O)–CH_3), 3.54 (dd, 1H, J=1.5, 14.6 Hz, CH(OH)–CH_2–N), 3.75 (dd, 1H, J=8.4, 14.6 Hz, CH(OH)–CH_2–N), 4.04 (t, 1H, J=8.4 Hz, O–CH_2–CH(OH)), 4.10 (quint., 1H, J=6.6 Hz, N–CH(CH_3)$_2$), 4.18–4.23 (m, 1H, CH_2–CH(OH)–CH_2), 4.24 (dd, 1H, J=4.0, 8.4 Hz, O–CH_2–CH(OH)), 5.67 (s, 1H, –O*H*), 6.86 (d, 1H, J=7.7 Hz, aromH$_2$), 7.38 (t, 1H, J=7.7 Hz, aromH$_3$), 7.44–7.49 (m, 3H, aromH$_4$, aromH$_6$, aromH$_7$), 7.81 (d, 1H, J=7.3 Hz, aromH$_5$), 8.21 (d, 1H, J=8.1 Hz, aromH$_8$). ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 20.63 (q, –CH(CH_3)$_2$), 21.36 (q, –CH(CH_3)$_2$), 21.87 (q, –C(=O)–CH_3), 46.14 (t, CH(OH)–CH_2–N), 50.27 (d, N–CH(CH_3)$_2$), 69.81 (t, O–CH_2–CH(OH)), 72.40 (d, CH_2–CH(OH)–CH_2), 104.84 (d, aromC$_2$), 120.65 (d, aromC$_4$), 121.55 (d, aromC$_8$), 125.24 (d, aromC$_3$), 125.40 (s, C$_{8a}$), 125.95 (d, C$_7$), 126.39 (d, C$_6$), 127.66 (d, C$_5$), 134.52 (s, C$_{4a}$), 154.07 (s, C$_1$), 173.86 (s, –C(=O)–CH_3). MS *m*/*z*: 301 (M^+), 256 (M^+-Ac-2), 158 (M^+-naphthalene oxide, 100%), 116 (M^+-naphthalene oxide-Ac), 43 (Ac). High-resolution MS calcd for $C_{18}H_{23}O_3N$ (M^+): 301.1672. Found: 301.1678.

*N-[2-hydroxy-3-(1-naphthalenyloxy)propyl]-N-(1-methylethyl)formamide (N-formylpropranolol (**4**))*: Colorless oil. ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 1.25 (d, 3H, J=7.0 Hz, –CH(CH_3)$_2$), 1.33 (d, 3H, J=7.0 Hz, –CH(CH_3)$_2$), 3.59 (dd, 1H, J=2.2, 14.3 Hz, CH(OH)–CH_2–N), 3.65 (dd, 1H, J=7.7, 14.3 Hz, CH(OH)–CH_2–N), 3.87 (sept., 1H, J=6.6 Hz, N–CH(CH_3)$_2$), 4.06 (dd, 1H, J=7.7, 9.5 Hz, O–CH_2–CH(OH)), 4.21 (dd, 1H, J=4.8, 9.5 Hz, O–CH_2–CH(OH)), 4.25–4.28 (m, 1H, CH_2–CH(OH)–CH_2), 4.90 (d, 1H, J=3.3 Hz, –OH), 6.85 (d, 1H, J=7.7 Hz, aromH$_2$), 7.38 (t, 1H, J=7.7 Hz, aromH$_3$), 7.45 (d, 1H, J=7.7 Hz, aromH$_4$), 7.47–7.52 (m, 2H, aromH$_6$, aromH$_7$), 7.80 (dd, 1H, J=7.4, 1.8 Hz, aromH$_5$), 8.20 (dd, 1H, J=7.7, 1.5 Hz, aromH$_8$), 8.26 (s, 1H, –C(=O)–H). ^{13}C–NMR (150 MHz, $CDCl_3$) δ: 20.80 (q, –CH(CH_3)$_2$), 21.82 (q, –CH(CH_3)$_2$), 46.09 (t, CH(OH)–CH_2–N), 51.14 (d, N–CH(CH_3)$_2$), 69.52 (t, O–CH_2–CH(OH)), 71.34 (d, CH_2–CH(OH)–CH_2), 104.91 (d, aromC$_2$), 120.78 (d, aromC$_4$), 121.49 (d, aromC$_8$), 125.31 (d, aromC$_3$), 125.37 (s, C$_{8a}$), 125.92 (d, C$_7$), 126.43 (d, C$_6$), 127.66 (d, C$_5$), 134.52 (d, C$_{4a}$), 153.94 (s, C$_1$), 165.03 (s, –C(=O)–H). MS m/z: 287 (M^+), 256 (M^+-Ac-2), 144 (M^+-naphthalene oxide, 100%), 115, 100, 58. High-resolution MS calcd for $C_{17}H_{21}O_3N$ (M^+): 287.1516. Found: 287.1519. All spectral data are consistent with previously reported results (Chen et al., 1993).

Synthesis of photoproducts

*N-[2-(acetyloxy)-3-(1-naphthalenyloxy)propyl]-N-(1-methylethyl)acetamide (N,O-diacetylpropranolol (**5**))*

A mixture of propranolol hydrochloride (3.0 g, 10.1 mmol), triethylamine (Et_3N) (7.1 ml, 51.0 mmol), and acetic anhydrate (Ac_2O) (2.9 ml, 30 mmol) in $CHCl_3$ was stirred at room temperature for 20 h. The resulting solution was washed sequentially with a saturated solution of aqueous NH_4Cl, a saturated solution of aqueous $NaHCO_3$, and brine. Samples were then dried over $MgSO_4$ and concentrated in vacuo. Purification by column chromatography on silica gel ($CHCl_3$) gave a solid. After recrystalization in benzene-*n*-hexane, compound **5** (2.99 g, 87%) was obtained as a colorless crystal. While this compound has already been reported in previous reports (Chen et al., 1993; Nelson and Walker, 1978), we could not found its detailed spectral data; thus, we detail the spectra here. This compound was obtained as a 3:1 mixture of unseparable rotamer or isomer. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.21–1.26 (m, 3.75H, –CH(CH_3)$_2$), 1.30 (d, 2.25H, J=6.8 Hz, –CH(CH_3)$_2$), 2.08 (s, 2.25H, –C(=O)–CH_3), 2.11 (s, 0.75H, –C(=O)–CH_3), 2.15 (s, 2.25H, –C(=O)–CH_3), 2.23 (s, 0.75H, –C(=O)–CH_3), 3.41 (dd, 0.75H, J=6.8, 14.4 Hz, CH(OAc)–CH_2–N), 3.64 (dd, 0.25H, J=3.2, 15.9 Hz, CH(OAc)–CH_2–N), 3.82 (dd, 1H, J=5.4, 14.4 Hz, CH(OAc)–CH_2–N), 4.01–4.07 (m, 0.75H, N–CH(CH_3)$_2$), 4.23–4.33 (m, 2H, O–CH_2–CH(OAc)), 4.46–4.53 (m, 0.25H, N–CH(CH_3)$_2$), 5.45–5.51 (m, 0.25H, CH_2–CH(OAc)–CH_2), 5.57–5.63 (m, 0.75H, CH_2–CH(OAc)–CH_2), 6.80 (d, 1H, J=7.6 Hz, aromH$_2$), 7.35 (t, 1H, J=8.3 Hz, aromH$_3$), 7.42 (d, 1H, J=8.3 Hz, aromH$_4$), 7.45–7.57 (m, 2H, aromH$_6$, aromH$_7$), 7.77–7.82 (m, 1H, aromH$_5$), 8.18–8.23 (m, 1H, aromH$_8$). ^{13}C-NMR (100 MHz, $CDCl_3$). As the ^{13}C-NMR spectra in the region of aromatic carbons, we could not assign all of the component forms. δ: 20.21 (q, minor), 20.65 (q, minor), 20.98 (q, major and minor), 21.14 (q, major), 21.73 (q, major), 22.12 (q, major), 22.76 (q, minor), 41.42 (t, CH(OAc)–CH_2–N, major), 45.52 (t, CH(OAc)–CH_2–N, minor), 47.25 (d, N–CH(CH_3)$_2$, minor), 49.52 (d, N–CH(CH_3)$_2$, major), 66.95 (t, O–CH_2–CH(OAc), minor), 68.43 (t, O–CH_2–CH(OAc), major), 70.97 (d, CH_2–CH(OAc)–CH_2, minor), 71.64 (d, CH_2–CH(OAc)–CH_2, major), 104.83 (d, minor), 104.93 (d, major), 120.61 (d, major), 121.22 (d, minor), 121.44 (d, minor), 121.86 (d, major), 125.27 (d, major), 125.38 (s, minor), 125.57 (s, major), 125.71 (d, minor), 125.85 (d, major), 126.38 (d, major), 126.62 (d, minor), 127.46 (d, major), 127.67 (d, minor), 128.32 (d, minor), 134.48 (s, major), 134.55 (s, minor), 153.74 (s, minor), 154.28 (s, major), 170.19 (s, minor), 170.52 (s, major), 171.28 (s, major and minor). MS m/z: 343 (M^+), 200 (100%), 158 (M^+-naphthalene oxide-Ac), 115 (M^+-naphthalene oxide-2Ac), 43 (Ac). High-resolution MS calcd for $C_{20}H_{25}O_4N$ (M^+): 343.1783. Found: 343.1779.

*N-[2-hydroxy-3-(1-naphthalenyloxy)propyl]-N-(1-methylethyl)acetamide (N-acetylpropranolol (**3**)*

A mixture of compound **5** (2.1 g, 6.0 mmol) and K_2CO_3 (2.5 g, 18.1 mmol) in MeOH (60 ml) was stirred for 2 h at room temperature. The resulting solution was concentrated in vacuo. Purification by column chromatography on silica gel ($CHCl_3$) gave a title compound **3** (1.4 g, 78%) as a colorless oil. All spectral data were agreed with those recorded for the photoproduct.

*N-[2-hydroxy-3-(1-naphthalenyloxy)propyl]-N-(1-methylethyl)formamide (N-formylpropranolol (**4**))*

*N-[2-(formyloxy)-3-(1-naphthalenyloxy)propyl]-N-(1-methylethyl)formamide (N,O-diformylpropranolol (**6**))*

To a solution of propranolol (2.7 g, 10.4 mmol) in formic acid (26 ml) was added Ac_2O (8.7 ml) dropwise at 55 °C and stirred for 24 h. After the addition of 10 ml of ice/water , the solution was concentrated in vacuo. Purification by column chromatography on silica gel (AcOEt-*n*-hexane, 1:3 *v*/*v*) gave title compound **4** (318 mg, 11%) and title compound **6** (118 mg, 3.7%) as colorless oils. Compound **4**: All spectral data were agreed with those observed for the photoproduct. Compound **6**: While this compound has been previously described (Chen et al., 1993; Chen, 1994), we could not find any description of the spectral data, thus, we detail the results here. This compound was obtained as a 10:3 mixture of an unseparable rotamer or isomer. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.27 (d, 0.69H, J=6.8 Hz, –CH(CH_3)$_2$), 1.28 (d, 0.69H, J=6.8 Hz, –CH(CH_3)$_2$), 1.31 (d, 2.31H, J=6.9 Hz, –CH(CH_3)$_2$), 1.35 (d, 2.31H, J=6.8 Hz, –CH(CH_3)$_2$), 3.59 (dd, 0.77H, J=7.0, 14.4 Hz, –CH(*O*-formyl)–CH_2–N), 3.65 (dd, 0.23H, J=8.3, 15.4 Hz, –CH(*O*-formyl)-CH_2-N), 3.72–3.86 (m, 1.23H, –CH(*O*-formyl)–CH_2–N, N–CH(CH_3)$_2$), 3.79 (dd, 0.77H, J=5.4, 14.4 Hz, –CH(*O*-formyl)–CH_2–N), 4.27–4.33 (m, 1.77H, O–CH_2–CH(*O*-formyl)), 4.46 (sept., 0.23H, J=6.6 Hz, O–CH_2–CH(*O*-formyl)), 5.51–5.59 (m, 0.23H, CH_2–CH(*O*-formyl)–CH_2), 5.69–5.78 (m, 0.77H, CH_2–CH(*O*-formyl)–CH_2), 6.79 (d, 1H, J=7.6 Hz, aromH$_2$), 7.36

Table 1
Color change observations for propranolol tablets in various forms under natural light[a]

Form	Days					
	3	7	10	14	21	28
A	–	–	–	–	–	–
B	–	–	–	–	–	–
C	–	±[b]	+[c]	++	+++[d]	+++
D	–	±[b]	+[c]	++	+++[d]	+++
E	±[b]	+[c]	++	+++[d]	+++	+++

[a] Pharmaceutical preparations of propranolol in various storage conditions and dosages (A: whole tablet covered with aluminum foil (control); B: whole tablet in press-through packaging; C: whole tablet in paraffin paper; D: half tablet in paraffin paper; E: ground form in paraffin paper) were kept under the scattered light (av. 8400 lx at 1:00 pm) in the room for 28 days.
[b] Slightly colored.
[c] Browned.
[d] Blackened.

(t, 0.77H, J=8.3 Hz, aromH$_3$), 7.35–7.39 (m, 0.23H, aromH$_3$), 7.44–7.52 (m, 3H, aromH$_4$, aromH$_6$, aromH$_7$), 7.80 (d, 0.77H, J=9.3, aromH$_5$), 7.82–7.90 (m, 0.23H, aromH$_5$), 8.14–8.21 (m, 2.23H, aromH$_8$, –C(=O)–H), 8.28 (s, 0.77H, –C(=O)–H). ^{13}C-NMR (100 MHz, CDCl$_3$) As the ^{13}C-NMR spectrum of isomers of this compound was overlapped in the region of aromatic carbons, we could not assign all of the component forms. δ: 20.23 (q, minor), 20.37 (q, minor), 22.16 (q, major), 22.36 (q, major), 41.81 (t, CH(OH)–CH$_2$–N, major), 44.56 (t, CH(OH)–CH$_2$–N, minor), 45.02 (d, N-CH(CH$_3$)$_2$, minor), 50.65 (d, N-CH(CH$_3$)$_2$, major), 66.31 (t, O–CH$_2$–CH(O-formyl), minor), 67.77 (t, O–CH$_2$–CH(O-formyl), major), 70.34 (d, CH$_2$–CH(O-formyl)–CH$_2$, minor), 70.61 (d, CH$_2$–CH(O-formyl)–CH$_2$, major), 104.88 (d), 121.01 (d), 121.43 (d), 121.45 (d), 121.76 (d), 125.45 (d), 125.65 (d), 125.67 (s), 125.75 (s), 126.53 (d), 126.69 (d), 127.53 (d), 127.69 (d), 134.52 (s), 134.58 (s), 153.42 (s, minor), 153.91 (s, major), 160.06 (s, minor), 160.44 (s, major), 163.17 (s, major), 163.82 (s, minor). MS m/z: 315 (M$^+$), 172 (100%), 144, 115, 58, 43. High-resolution MS calcd for $C_{18}H_{21}O_4N$ (M$^+$): 315.1470. Found: 315.1478.

Acute toxicity test

Male mice of ddY strain (21–25 g) were purchased from Nihon SLC Co. (Hamamatsu, Japan). Mice were housed in groups of 10 per cage (30 × 30 × 16 cm) in an air conditioned room (ambient temperature 22±2 °C and 55±5% relative humidity) under a 12 h light cycle. Animals were allowed to ingest food (F-2 obtained from Funabashi Farm Co., Funabashi, Japan) and water ad libitum. Samples, suspended in physiological saline containing 2% arabia gum, were administered intraperitoneally (10 ml/kg body weight). Ten mice comprised a group that received the same dose. LD_{50} values were estimated according to the Litchfield-Wilcoxon method (Litchfield and Wilcoxon, 1949).

Receptor-binding assay

Assays measuring the receptor binding of the propranolol derivatives were performed as previously reported (Malinowska et al., 2003) with the following minor modification. Cerebral cortex membranes from male Wistar rats (180–200 g) were homogenized using a Potter homogenizer (10 strokes, 1100 rpm) in 25 volumes of ice-cold Tris–HCl buffer (10 mM Tris, pH 7.5; 0.25 M sucrose; 2 mM EGTA; 2 mM $MgCl_2$). Homogenates were then centrifuged at 1000 × g for 10 min at 4 °C. Supernatants were centrifuged at 35,000 × g for 20 min at 4 °C, then re-centrifuged (35,000 × g for 20 min at 4 °C) twice in 15 ml of Tris–HCl buffer (50 mM Tris, pH 7.5; 5 mM EDTA). The resulting pellet was resuspended in Tris–HCl buffer and stored at −80 °C until use.

Ligand binding assays were performed to determine receptor specificity. A solution containing of 0.1 ml of 0.4 mg/ml cerebral cortex membrane, 10 μl 8 nM [^{3}H]DHA, and 5 μL of varying concentrationsof propranolol (**1**) or its derivatives (**2**–**6**) (eight concentrations ranging from 1.0 pM to 10 μM). The mixture was incubated for 30 min at 30 °C. To terminate the reaction, 0.5 ml of ice-cold Tris–HCl buffer was added. The solution was then filtrated through 0.3% polyethyleneimine-pretreated Whatman GF/C filters. After washing three times with 2 ml ice-cold Tris–HCl buffer, the filters were transferred into a vial. Three milliliters crea-sol I (Nacalai Tesque, Japan) was added to a vial as a scintillator; the radioactivity was measured with a liquid scintillation counter (LS7800, Beckman). To determine non-specific binding, 10 μM of propranolol alone was used (7% for 8 nM [^{3}H]DHA). Protein concentrations were assayed by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

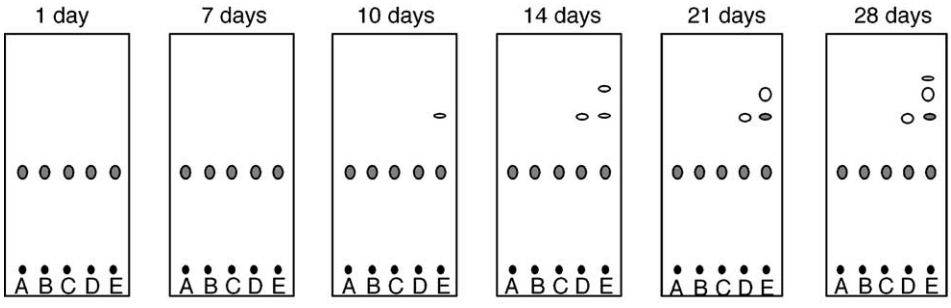

Fig. 2. TLC analyses of the photodegration products of propranolol (**1**). A: whole tablet covered with aluminum foil (control); B: whole tablet in press-through packaging; C: whole tablet in paraffin paper; D: half tablet in paraffin paper; E: ground form in paraffin paper. Each TLC was developed with CHCl$_3$–MeOH (22 : 3 v/v). ● spots colored by Dragendorff's reagent. ○: spots absorbed UV light at 254 nm.

Results

Photostability test of propranolol

The preparations of propranolol used for photostability testing, with the exception of the control samples, the pharmaceutical preparations in press-through packages, and the samples stored in the dark, were colored as summarized in Table 1. Whole tablets, half tablets, and ground tablets stored in paraffin paper tended to turn gradually from yellow to brown, and finally to black. Within 3 weeks, all drug forms kept in paraffin paper were black. The rate of discoloration for the ground tablet was much faster than the other forms; the ground tablet turned black after only 2 weeks, while the other pharmaceutical preparations stored in paraffin paper blackened within 3 weeks.

TLC analyses monitored the production of the photodegration products (Fig. 2). Whole tablets covered with an aluminum foil (condition A) served as a control. These results demonstrated that irradiation with light for approximately 10 days was sufficient to produce a photoproduct (condition E). This spot was detected by Dragendorff's reagent after irradiating for 21 days. The number of photoproducts (spots) increased with time; two and three spots were detected after 14 and 28 days, respectively (condition E).

Structure analysis

We used the free base, a relatively unstable form of propranolol, for the structure analyses of photoproducs, because the rate of photoproduct formation from the pharmaceutical preparations was too slow to isolate sufficient quantity of photoproducts for a structural determination. After a 5-day experiment, the photo-irradiated products derived from propranolol (**1**) were repeatedly subjected to column chromatography to isolate 1-naphthol (**2**), *N*-acetylpropranolol (**3**), and *N*-formylpropranolol (**4**) in pure forms (Fig. 3). 1-Naphthol (**2**), *N*-acetylpropranolol (**3**), and *N*-formylpropranolol (**4**) corresponded to the first, second, and third spots in the TLC analyses, respectively (see Fig. 2, lane E of 21 day plate beginning at the top).

Compound **2** was identified by comparison of the acquired spectroscopic data with those of known samples.

The mass spectrum of compound **3** exhibited a molecular ion peak $[M]^+$ at 301 which is consistent with a molecular formula of $C_{18}H_{23}O_3N$. The ^1H and ^{13}C NMR signals for compound **3** exhibited an acetyl group bound to propranolol (**1**). The chemical shifts of the methylene and methyne groups next to the amino group were downshifted relative to propranolol (**1**), with methylene shifting from δ_H 2.85 to 3.54 and 3.00 to 3.75 and methyne moving from δ_H 2.85 to 4.10. These findings suggested that acetylation of the propranolol (**1**) amino group produced compound **3**. Correlation between these ^1H and ^{13}C NMR signals was unambiguously confirmed by the HMQC (^1H-detected multiple quantum coherence) spectrum. In addition, the HMBC (^1H-detected multiple bond heteronuclear multiple quantum coherence) spectrum of compound **3** clarified the connectivities between the functional groups, allowing us to propose the structure for *N*-acetylpropranolol (**3**) detailed in Fig. 4.

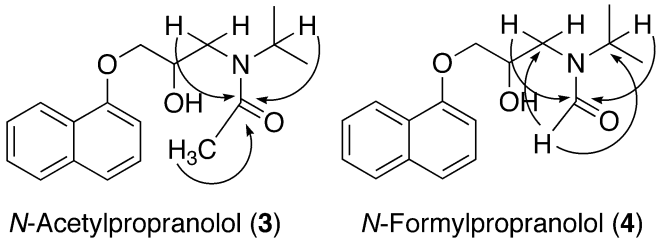

Fig. 4. Correlations between the acetyl or formyl groups and the propranolol structure from the HMBC spectrum.

The structure of compound **4** was elucidated in the same manner as that performed for compound **3**. Briefly, the mass spectrum of **4** exhibited a molecular ion peak $[M]^+$ at 287, consistent with a molecular formula of $C_{17}H_{21}O_3N$. The ^1H and ^{13}C NMR signals of compound **4** displayed a formyl group bound to propranolol (**1**). Correlation between these ^1H and ^{13}C NMR signals was unambiguously confirmed by the HMQC spectrum. The HMBC spectrum of compound **4** clarified the connectivities of the functional groups, leading to proposal for the structure for *N*-formylpropranolol (**4**). Fig. 4 demonstrated the important correlations in HMBC spectrum.

Chemical syntheses

To generate the compounds in substantial quantity for pharmacological studies and for use as standards in spectrographic analyses, we synthesized *N*-acetyl and *N*-formylpropranolol (**3** and **4**) according to standard methods (Fig. 5). Briefly, acetylation of propranolol (**1**), which gave diacetate (**5**), was followed by ester-selective solvolysis to give *N*-acetylpropranolol (**3**). Formylation of propranolol (**1**) using a

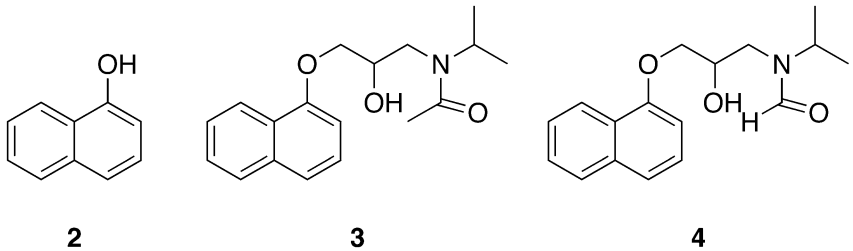

Fig. 3. Structures of photoproducts (1-naphthol (**2**), *N*-acetylpropranolol (**3**), and *N*-formylpropranolol (**4**)).

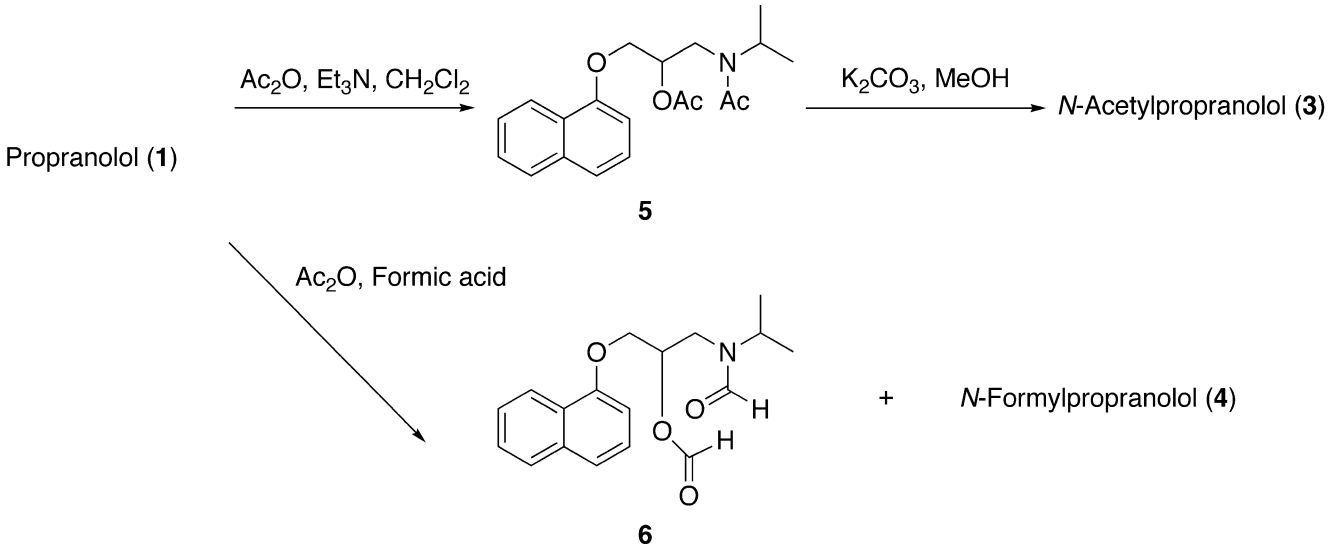

Fig. 5. Chemical syntheses of *N*-acetyl and *N*-formylpropranolol (**3** and **4**).

mixed anhydride yielded both *N*-formyl- and *N*,*O*-diformylpropranolol (**4** and **6**).

Acute toxicity test

The acute toxicities (LD_{50}) of propranolol (**1**), the photoproducts of propranolol (**2**, **3**, and **4**), and their derivatives (**5** and **6**) in mice were determined using the Litchfield-Wilcoxon method (Litchfield and Wilcoxon, 1949) (Table 2). The results demonstrated that both the photoproducts (**2**, **3**, and **4**) and their derivatives (**5** and **6**) do not display acute toxicity even at intraperitoneal injections of 1000 mg/kg. In contrast, propranolol (**1**) is relatively toxic at these concentrations.

Receptor-binding assay

We examined β-adrenergic receptor binding for compounds **1** to **6** using rat cerebellum cortex membranes according to the procedure outlined by reported method (Malinowska et al., 2003). Binding was evaluated by the competition of these compounds with [^{3}H]DHA.

Fig. 6 and Table 3 describes the results. According to the IC_{50} values, of the tested propranolol derivatives, propranolol (**1**), the parental compound, exhibited the highest binding affinity to the β-adrenergic receptor in low concentrations (about 47 pM); the photoproducts (**2**, **3**, and **4**) and their analogues (**5** and **6**) demonstrated moderate activities. The greater the masking of the *N*- or *O*-functional groups, the lower the affinity to the β-adrenergic receptor.

Table 2
Acute toxicities of the propranolol derivatives[a]

Sample	LD_{50} (mg/kg)
Propranolol (**1**) Hydrochloride	580
Compound **2**	2500
Compound **3**	>1000
Compound **4**	>1000
Compound **5**	>1000
Compound **6**	>1000

[a] Samples, suspended in physiological saline containing 2% arabia gum, were administered intraperitoneally (10 ml/kg body weight) to male mice of the dd strain (21–25 g). Ten mice comprised a group receiving the same dose. The LD_{50} values were estimated according to the Litchfield-Wilcoxon method.

Discussion

Pharmacopoeias directs that propranolol must be protected from light. In a pharmaceutical interview, commercial manufacturers described that the powder gradually turned black, but assured that the quality of the base drug did not decrease when propranolol was kept under indoor diffused light at room temperature.

We confirmed that every pharmaceutical preparation of propranolol, with the exception of the control sample kept in

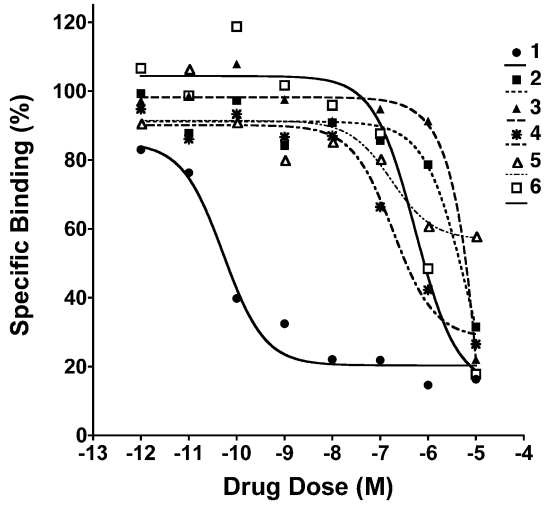

Fig. 6. Inhibition of the specific [^{3}H]DHA binding by propranolol analogues (**1**–**6**) to rat cerebral cortex membrane. Displacement curves were obtained using a variety of concentrations of propranolol analogues (**1**–**6**) in the presence of 8 nM of [^{3}H]DHA. Each point represents the mean of three duplicate experiments.

Table 3
Affinities for the β-adrenergic receptor binding site for propranolol, its photoproducts and their analogues[a]

Sample	IC_{50} (M)[b]
Propranolol (**1**)	4.7×10^{-11}
Compound **2**	4.8×10^{-4}
Compound **3**	2.2×10^{-5}
Compound **4**	2.7×10^{-7}
Compound **5**	1.6×10^{-6}
Compound **6**	8.3×10^{-3}

[a] Ligand binding assays to determine the receptor specificity were performed using a solution containing cerebral cortex membrane, [^{3}H]DHA, and eight varying concentrations of samples ranging from 1.0 pM to 10 μM. The mixture was then incubated for 30 min at 30 °C. After the addition of ice-cold Tris–HCl buffer to terminate the reaction, the solution was filtrated. Filters were washed with ice-cold Tris–HCl buffer, then transferred into a vial. Crea-sol I was added to a vials as a scintillator; the radioactivity was measured with the liquid scintillation counter.
[b] Concentration that gives a half-maximal effect.

aluminium foil, the pharmaceutical preparations in press-through packaging, and the samples maintained in the dark, was discolored by natural light. TLC analyses confirmed the presence of degradation products. As the only difference between these conditions is irradiation with light, the color-change and formation of degradation products depended on light, not on temperature or moisture. The appearance and contents of propranolol tablets kept in the press-through packaging was unchanged demonstrating that the press-through packaging had been modified to protect the drug from light. The rate of degradation of the ground tablets was much faster than that seen for the other forms, likely due to the increased surface area irradiated.

Several photodegradation products of propranolol have been previously reported, including naphthalene, 1,4-naphthoquinone (Salomies, 1987), and 6-hydroxy-1,4-naphthoquinone (Sortino et al., 2002). In this study, we could not detect any of these products even after irradiating the drug with light for five days. Three other photoproducts were isolated, however, we used spectroscopic methods to characterize their structures as 1-naphthol (**2**), *N*-acetylpropranolol (**3**), and *N*-formylpropranolol (**4**). These photodegradation products are different from those reported in other studies, because the drug was irradiated under different conditions. Salomies oxidized propranolol (**1**) with NBS, a versatile oxidizing agent, under both acidic and neutral conditions concurrent with irradiation under a mercury lamp. In contrast, Sortino et al. irradiated air-equilibrated solutions in phosphate buffer using phosphor lamps emitting light in the 310–390 range. The mechanism whereby these 1,4-naphthoquinones were produced was proposed as depicted in Fig. 7. The reaction proceeds via a reversible [2+4] cycloaddition of $^1O_2$ to the naphthalene skeleton, leading to the formation of 1,4-endoperoxides (**7**) (Sortino et al., 2002). This step is strongly enhanced in water-based solution (Aubry et al., 1995; Hart and Oku, 1972). The acetal structure of the intermediate (**7**) is hydrolized in aqueous medium (Pierlot et al., 1996) to give 1,4-naphthoquinone (**8**) and the remaining side chain (**9**).

As the propranolol (**1**) used in this study was in a solid state, the hydrolysis of the acetal (**7**) did not appear to proceed. Thus, the C–O bond of the naphthyl ether was cleaved by a thermally activated crossing of the bonding S_1 ($\pi\pi^*$) and T_1($\pi\pi^*$) states into $\pi\sigma^*$ states (Pohlers et al., 1996), generating 1-naphthol (**2**) and the remaining side chain (**9**). Although the origin of the

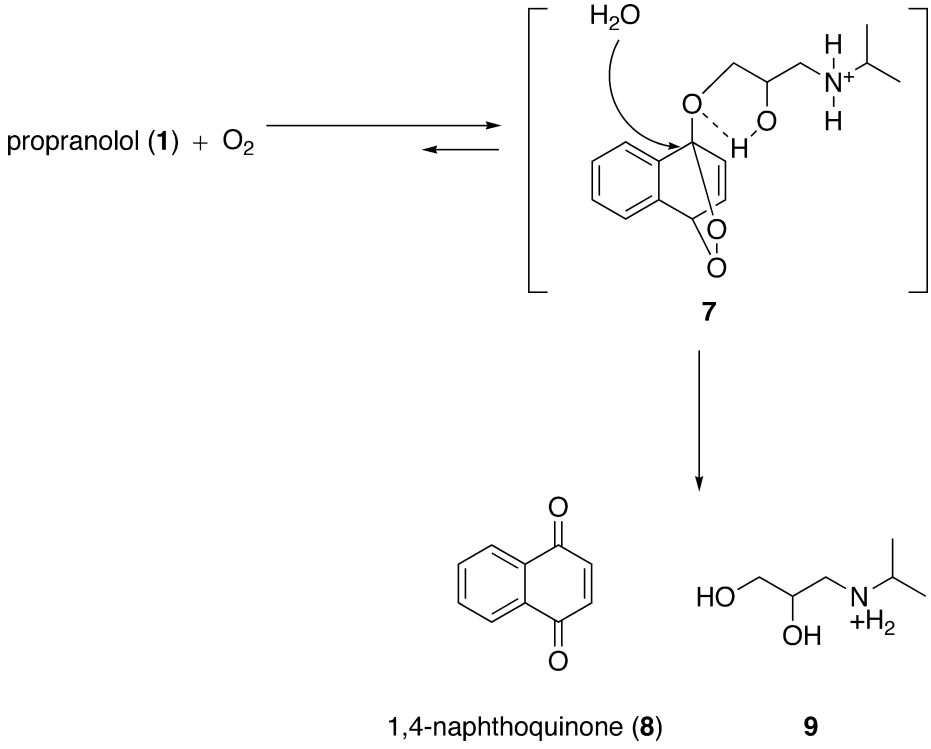

Fig. 7. The mechanism proposed for the formation of 1,4-naphthoquinone (**8**) following exposure of propranolol (**1**) in the aqueous solution to light.

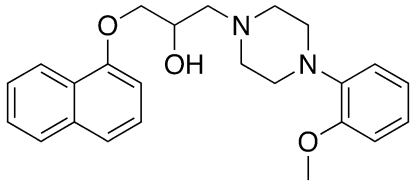

Fig. 8. Structure of naftpidil (**10**).

acetyl and formyl moieties appears to derive from the side chain (**9**), additional experiments (photo irradiation of D-labeled propranolol (**1**)) will be required to clarify this mechanism. In addition, the irradiation of propranolol (**1**) in a solution state with light was not suitable for the purpose of our research.

We synthesized substantial quantities of *N*-acetyl- and *N*-formylpropranolol (**3** and **4**) for pharmacological studies and for use as a standard in spectrographic studies. Although the photoproducts obtained this study are known compounds, their pharmacological properties had not yet been investigated.

We subjected the photoproducts (**2**, **3**, and **4**) and their synthesized analogues (**5** and **6**) to biological studies. In an acute toxicity test, all of the degradation products exhibited low toxicities, in comparison to the parental compound propranolol (**1**). These results may indicate that the free amino and hydroxyl groups of the propranolol (**1**) side chain are important for the toxic activity.

Next, we examined the binding of the propranolol derivatives to β-adrenergic receptors in rat cerebral cortex membranes. These substances exhibited low affinities to the β-adrenergic receptor in comparison to propranolol (**1**). 1-Naphthol (**2**), which only shares the naphthalene moiety with propranolol (**1**), demonstrated a markedly lower affinity (IC_{50}: 4.8×10^{-4} M). These results suggested that the propranolol (**1**) side chain is important for high binding affinity. Both *N*-acetyl- and *N*-formylpropranolol (**3** and **4**), each of which contains a masked secondary amino group, exhibited receptor binding affinities of 2.2×10^{-5} and 2.7×10^{-7} M, respectively, values significantly lower than the parental compound. These β-adrenergic receptor binding affinities indicated that the free amino group is likely also critical for the activity. *N*,*O*-diacetyl- and diformylpropranolol (**5** and **6**), both of which have masked amino and hydroxyl groups, exhibit IC_{50} values of 1.6×10^{-6} and 8.3×10^{-3} M, respectively. As both of these values are of a lower affinity than propranolol (**1**), these results may indicate that the region of propranolol (**1**) side chain with the free amino and hydroxyl groups is critical for high affinity binding to the β-adrenergic receptor. Of the acetyl derivatives, the IC_{50} value for *N*,*O*-diacetate (**5**) was lower than that for *N*-acetate (**3**), which is opposite to the results observed for the formyl derivatives; the IC_{50} values for *N*,*O*-diformate (**6**) was higher than that seen for *N*-formate (**4**). As the sigmoidal pattern of *N*,*O*-diacetate (**5**) differed from that observed for other derivatives (**2**, **3**, **4** and **6**), it is possible that compound **5** binds the β-adrenergic receptor in a different manner.

Currently, single-dose packaging systems and ground pharmaceutics are becoming increasingly popular; as this study demonstrated, however, removing the drug from its press—through packaging or grinding the drug results in the photodegradation of propranolol. These administration practices jeopardize the measures taken to protect the drug from light-mediated degradation. Although the photoproducts obtained in this study did not exhibit any significant acute toxicities and or high binding affinities to the β-adrenergic receptor, the metabolic and biologic activities of these compounds are still uncertain. In addition, there are several drugs with structures similar to propranolol (**1**) such as naftopidil (**10**, Fig. 8), a α_1-adrenergic receptor blocker, that may also decompose upon exposure to light; these photoproducts may be biologically active compounds. This study serves as a warning to all medical workers to be more vigilant in managing supplies, especially the storing of pharmaceutics.

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