**Regular Article**

**Metabolism of Butyrylfentanyl in Fresh Human Hepatocytes: Chemical Synthesis of Authentic Metabolite Standards for Definitive Identification**

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The metabolism of butyrylfentanyl, a new designer drug, was investigated using fresh human hepatocytes isolated from a liver-humanized mouse model. In the culture medium of hepatocytes incubated with butyrylfentanyl, the desphenethylated metabolite (nor-butyrylfentanyl), \(\omega\)-hydroxy-butyrylfentanyl, \((\omega\text{-1})\)-hydroxy-butyrylfentanyl, 4'-hydroxy-butyrylfentanyl, \(\beta\)-hydroxy-butryrylfentanyl, 4'-hydroxy-3'-methoxy-butyrylfentanyl, and \(\omega\)-carboxy-fentanyl were identified as the metabolites of butyrylfentanyl. Each metabolite was definitively identified by comparing the analytical data with those of authentic standards. The amount of the main metabolite, nor-butyrylfentanyl, reached 37% of the initial amount of butyrylfentanyl at 48h. \(\omega\)-Hydroxy-butyrylfentanyl and \((\omega\text{-1})\)-hydroxy-butyrylfentanyl, formed by hydroxylation at the \(N\)-butyryl group of butyrylfentanyl, were the second and third largest metabolites, respectively. The majority of 4'-hydroxy-butyrylfentanyl and 4'-hydroxy-3'-methoxy-butyrylfentanyl was considered to be conjugated. CYP reaction phenotyping for butyrylfentanyl using human liver microsomes and various anti-CYP antibodies revealed that CYP3A4 was involved in the formation of nor-butyrylfentanyl, \((\omega\text{-1})\)-hydroxy-butyrylfentanyl, and \(\beta\)-hydroxy-butryrylfentanyl. In contrast, CYP2D6 was involved in the formation of \(\omega\)-hydroxy-butyrylfentanyl.

**Key words** butyrylfentanyl; metabolism; hepatocyte; CYP reaction phenotyping

**INTRODUCTION**

In recent years, the abuse of opioid drugs, especially fentanyl analogs, has become a serious problem, particularly in North America and Europe, where many opioid-related overdose deaths have been reported.1,2) Butyrylfentanyl is a new designer drug chemically similar to fentanyl, with an \(N\)-butyryl group replacing the \(N\)-propionyl group of fentanyl (Fig. 1). The pharmacological activity of butyrylfentanyl is reportedly 13% that of fentanyl.3) In the United States, seven butyrylfentanyl cases were reported for the first time in 2014, and the number of reports increased to 81 in 2015.4) Butyrylfentanyl is used as a substitute for heroin or prescription opioids; however, the identity, purity, and quantity of illicit butyrylfentanyl is uncertain and inconsistent, posing serious adverse health risks to abusers. To avoid the imminent risks caused by butyrylfentanyl, the United States Drug Enforcement Administration designated this drug as a schedule I substance under the Controlled Substances Act in 2016.5) In addition, they analyzed authentic blood and urine samples, and they concluded that hydroxylation of the butanamide side chain followed by oxidation to the carboxylic acid was the major metabolic pathway of butyrylfentanyl in vivo. Staeheli et al. analyzed blood, urine, and organ samples from a fatal case of butyrylfentanyl poisoning, and they also reported that carboxy- and hydroxy-butyrylfentanyl were the most abundant metabolites of butyrylfentanyl.6) However, they did not use authentic standards to identify and quantitate the metabolites of butyrylfentanyl; therefore, definitive identification and accurate quantitation of the metabolites of butyrylfentanyl has not been accomplished yet.

Several studies on the metabolism of butyrylfentanyl have been published. Steuer et al. investigated the metabolism of butyrylfentanyl using human liver microsomes and recombinant CYP, and revealed that butyrylfentanyl underwent hydroxylation at the butanamide side chain and aromatic ring and \(N\)-dealkylation, and that CYP3A4 and CYP2D6 were involved in these reactions.7) In addition, they analyzed authentic blood and urine samples, and they concluded that hydroxylation of the butanamide side chain followed by oxidation to the carboxylic acid was the major metabolic pathway of butyrylfentanyl in vivo. Staeheli et al. analyzed blood, urine, and organ samples from a fatal case of butyrylfentanyl poisoning, and they also reported that carboxy- and hydroxy-butyrylfentanyl were the most abundant metabolites of butyrylfentanyl.6) However, they did not use authentic standards to identify and quantitate the metabolites of butyrylfentanyl; therefore, definitive identification and accurate quantitation of the metabolites of butyrylfentanyl has not been accomplished yet.

In this study, we investigated the metabolism of butyrylfentanyl using fresh human hepatocytes isolated from a liver-humanized mouse model (PXB-cells)7) and synthesized the putative metabolites chemically to identify the metabolites. In addition, we performed CYP reaction phenotyping using human liver microsomes and anti-CYP antibodies to clarify which CYP isoform is involved in the metabolism of butyrylfentanyl.

**MATERIALS AND METHODS**

**Chemicals and Reagents** Authentic standards of butyrylfentanyl, thiofentanyl, and the metabolites of butyrylfentanyl were synthesized in our laboratory. PXB-cells (seeded in a 24-well plate, 2.1 × 10^5 cells/cm^2) and the culture medium for PXB-cells were purchased from PhoenixBio (Higashihiro-
Mass spectrometer (LCQ FLEET, Thermo Fisher Scientific, Japan) using tetramethylsilane as an internal standard (IS). An NMR spectrometer (JNM-ECA600, JEOL, Akishima, Japan); 624 Vol. 42, No. 4 (2019)

**Synthesis of Authentic Standards of Butyrylfentanyl and Its Metabolites**

All synthesized standards were confirmed by positive electrospray ionization (ESI) mass spectrometry and 1H-NMR. ESI MS were obtained from an ion trap mass spectrometer (LCQ FLEET, Thermo Fisher Scientific, Waltham, MA, U.S.A.). 1H-NMR spectra were measured on an NMR spectrometer (JNM-ECA600, JEOL, Akishima, Japan) using tetramethylsilane as an internal standard (IS).

**N-Phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]-butanamide (Butyrylfentanyl)**

Butyrylfentanyl was synthesized according to the method of Siegfried. Briefly, 1-(2-phenylethyl)-4-piperidone was condensed with aniline in the presence of 4A molecular sieves. The product was reduced using sodium borohydride to obtain N-phenyl-1-(2-phenylethyl)-4-piperidinamine (despropionylfentanyl). Despropionylfentanyl was butyrylated using butyric anhydride to give butyrylfentanyl. The butyrylfentanyl free base was converted to the hydrochloride salt by the addition of concentrated hydrochloric acid–methanol (1:9), then the hydrochloride salt was precipitated by the addition of diethyl ether to obtain butyrylfentanyl hydrochloride as a white powder.

Butyrylfentanyl (Free Base)

1H-NMR (CDCl3) δ: 0.80 (3H, t, J = 7.2 Hz), 1.43 (2H, qd, J = 3.0, 12.0 Hz), 1.52–1.61 (2H, m), 1.81 (2H, d, J = 10.8 Hz), 1.89 (2H, t, J = 7.5 Hz), 2.16 (2H, t, J = 11.4 Hz), 2.50–2.58 (2H, m), 2.70–2.77 (2H, m), 3.00 (2H, d, J = 11.4 Hz), 4.66–4.73 (1H, m).

Butyrylfentanyl Hydrochloride

1H-NMR (CD3OD) δ: 7.23–7.27 (5H, m), 7.30–7.34 (2H, m), 7.48–7.55 (3H, m).

**N-Phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]-butanamide (Nor-butyrylfentanyl)**

1-Benzyl-4-piperidone was condensed with aniline and was reduced using sodium borohydride as described above to give N-phenyl-1-benzylpiperidin-4-amine. N-Phenyl-1-benzylpiperidin-4-amine (0.319 g), butyric anhydride (1.25g), and triethylamine (0.34g) in ethyl acetate (10mL) were refluxed for 2h. The solvent was removed under vacuum, water was added to the residue, and the solution was basified with sodium hydroxide solution then extracted with chloroform. The extract was evaporated to dryness under vacuum and the residue was purified by flash chromatography (column, silica gel 12g; solvent, chloroform–methanol). The purified product was dissolved in methanol, acidified with concentrated hydrochloric acid, and then stirred with palladium-activated carbon (20mg, Pd 5%) under a hydrogen atmosphere for 7d. Water was added to the reaction mixture and the solution was basified with 28% ammonium hydroxide solution then extracted with chloroform–2-propanol (3:1). The solvent was evaporated to dryness under vacuum and the residue was purified by flash chromatography (column, silica gel 4g; solvent, chloroform–methanol). The amine product was converted to the hydrochloride salt as described above to obtain N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]-4-hydroxybutanamide (ω-hydroxy-butyrylfentanyl) hydrochloride (11.2mg) as a white solid.

ω-Hydroxy-butyrylfentanyl (Free Base)

1H-NMR (CDCl3) δ: 1.44 (2H, qd, J = 3.5, 12.2Hz), 1.75–1.84 (4H, m), 2.10 (2H, t, J = 6.6 Hz), 2.16 (2H, t, J = 11.1 Hz), 2.51–2.57 (2H, m), 2.70–2.75 (2H, m), 3.00 (2H, d, J = 11.4 Hz), 3.58–3.63 (2H, m), 4.63–4.71 (1H, m).

ω-Hydroxy-butyrylfentanyl Hydrochloride

1H-NMR (CD3OD) δ: 7.23–7.29 (5H, m), 7.30–7.34 (2H, m), 7.47–7.55 (3H, m).

**N-Phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]-3-hydroxybutanamide (ω-1-Hydroxy-butyrylfentanyl)**

Despropionylfentanyl (100mg), 3-butenolic acid (72mg), HATU (180mg), and triethylamine (100µl) in acetonitrile (6mL) were refluxed for 20h. The solvent was evaporated under vacuum, and then the residue was purified by flash chromatography (column, silica gel 12g; solvent, chloroform–ethyl acetate). The purified product (44mg) dissolved in tetrahydrofuran (1mL) was added to a solution of mercury(II) acetate (120mg) in 10% acetic acid (1mL), and the solution was heated at 90°C for 20h. After the reaction was complete, 0.5M sodium borohydride (0.4mL) in 3M sodium hydroxide solution was added, and then sodium chloride was added until the solution separated into two layers. The tetrahydrofuran layer was transferred to a separating funnel, water was added, and the solution was basified with 28% ammonium hydroxide solution and extracted with chloroform. The solvent was evaporated to dryness under vacuum, and then the residue was purified by preparative TLC (plate, silica gel; solvent, ethyl acetate). The amine product was converted to the hydrochloride salt as described above to obtain N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]-3-hydroxybutanamide (ω-1-hydroxy-butyrylfentanyl) hydrochloride (14.3mg) as a white solid.

ω-1-Hydroxy-butyrylfentanyl (Free Base)

1H-NMR (CDCl3) δ: 1.04 (3H, d, J = 6.6Hz), 1.40–1.49 (2H, m), 1.78–1.84 (2H, m), 1.96 (1H, dd, J = 9.6, 16.8Hz), 2.05 (1H, dd, J = 2.7, 16.5Hz), 2.16 (2H, t, J = 12.0Hz), 2.51–2.56 (2H,
(ω)-1-Hydroxy-butylfentanyl Hydrochloride

N-Phenyl-N-[1-(2-hydroxy-2-phenylethyl)-4-piperidinyl]-butanamide (β-Hydroxy-butylfentanyl) A mixture of nor-butylfentanyl hydrochloride (40 mg), phenacyl chloride (30 mg), and sodium bicarbonate (16 mg) in water–2-butanone (1.2 mL, 1:5) was heated at 90°C for 3 h. Water was added to the reaction mixture, and the solution was basified with 28% ammonium hydroxide solution then extracted with ethyl acetate. The solvent was evaporated to dryness under vacuum and the residue was dissolved in methanol (3 mL). Sodium borohydride (30 mg) was added to the solution and it was stirred for 10 min. Water was added to the reaction mixture, the solution was basified with 28% ammonium hydroxide solution, and then extracted with chloroform. The solvent was evaporated to dryness under vacuum and the residue was purified by preparative TLC (plate, silica gel; solvent, ethyl acetate). The amine product was converted to the hydrochloride salt as described above to obtain N-phenyl-N-[1-(2-hydroxy-2-phenylethyl)-4-piperidinyl]butanamide (β-hydroxy-butylfentanyl) hydrochloride (27.1 mg) as a white powder.

β-Hydroxy-butylfentanyl Hydrochloride

N-Phenyl-N-[1-[2-(4-hydroxy-3-methoxyphenyl)ethyl]-4-piperidinyl]butanamide (4'-Hydroxy-butylfentanyl) A mixture of nor-butylfentanyl hydrochloride (40 mg), 2-(4-benzoyloxyphenyl)ethyl methanesulfonate (92 mg), and potassium carbonate (90 mg) in 2-butanol (4 mL) was heated at 90°C for 20 h. 2-(4-Benzoyloxyphenyl)ethyl methanesulfonate was prepared from 2-(4-benzoyloxyphenyl)ethanol by a previously reported method. Water was added to the reaction mixture and the mixture was extracted with chloroform. The solvent was evaporated to dryness under vacuum, and then the residue was dissolved in 0.1 M hydrochloric acid (2 mL) and refluxed for 2 h. The reaction mixture was basified with 10 M sodium hydroxide solution, and then washed with chloroform. The aqueous layer was acidified with 3 M hydrochloric acid then the pH was adjusted to 9 with 28% ammonium hydroxide solution before extracting with chloroform–2-propanol (3:1). The organic layer was evaporated to dryness under vacuum, and then the residue was purified by flash chromatography (column, silica gel 4 g; solvent, chloroform-methanol) to obtain N-phenyl-N-[1-[2-(4-hydroxy-2-phenylethyl)-4-piperidinyl]-3-carboxypropanamide (ω-Carboxy-fentanyl) A mixture of depropionylfentanyl (50 mg), methyl 4-chloro-4-oxobutyrate (40 mg), and triethylamine (30 μL) in dichloromethane (2 mL) was stirred on ice for 1 h. Zero point one molar hydrochloric acid was added to the reaction mixture, and the mixture was extracted with chloroform. The solvent was evaporated to dryness under vacuum, and the residue was dissolved in 0.1 M hydrochloric acid (2 mL) and refluxed for 2 h. The reaction mixture was basified with 10 M sodium hydroxide solution, and then washed with chloroform. The aqueous layer was acidified with 3 M hydrochloric acid then the pH was adjusted to 9 with 28% ammonium hydroxide solution before extracting with chloroform–2-propanol (3:1). The organic layer was evaporated to dryness under vacuum, and then the residue was purified by flash chromatography (column, silica gel 4 g; solvent, chloroform-methanol) to obtain N-phenyl-N-[1-[2-(4-hydroxy-2-phenylethyl)-4-piperidinyl]-3-carboxypropanamide (ω-carboxy-fentanyl) (39.7 mg) as a clear oil.

ω-Carboxy-fentanyl (Free Base)

4'-Hydroxy-butylfentanyl Hydrochloride

N-Phenyl-N-[1-[2-(4-hydroxy-3-methoxyphenyl)ethyl]-4-piperidinyl]butanamide (4'-Hydroxy-3'-methoxy-butyrylfentanyl) A sample of 4'-hydroxy-3'-methoxy-butyrylfentanyl hydrochloride (9.8 mg) was prepared from nor-butylfentanyl hydrochloride (40 mg) by the method used for the synthesis of 4'-hydroxy-butylfentanyl.

4'-Hydroxy-3'-methoxy-butyrylfentanyl Hydrochloride

β-Hydroxy-butyrylfentanyl Hydrochloride

N-Phenyl-N-[1-[2-(4-hydroxy-2-phenylethyl)-4-piperidinyl]-3-carboxypropanamide (ω-Carboxy-fentanyl) A mixture of depropionylfentanyl (50 mg), methyl 4-chloro-4-oxobutyrate (40 mg), and triethylamine (30 μL) in dichloromethane (2 mL) was stirred on ice for 1 h. Zero point one molar hydrochloric acid was added to the reaction mixture, and the mixture was extracted with chloroform. The solvent was evaporated to dryness under vacuum, and the residue was dissolved in 0.1 M hydrochloric acid (2 mL) and refluxed for 2 h. The reaction mixture was basified with 10 M sodium hydroxide solution, and then washed with chloroform. The aqueous layer was acidified with 3 M hydrochloric acid then the pH was adjusted to 9 with 28% ammonium hydroxide solution before extracting with chloroform–2-propanol (3:1). The organic layer was evaporated to dryness under vacuum, and then the residue was purified by flash chromatography (column, silica gel 4 g; solvent, chloroform-methanol) to obtain N-phenyl-N-[1-[2-(4-hydroxy-2-phenylethyl)-4-piperidinyl]-3-carboxypropanamide (ω-carboxy-fentanyl) (39.7 mg) as a clear oil.

ω-Carboxy-fentanyl (Free Base)

4'-Hydroxy-butyrylfentanyl Hydrochloride

N-Phenyl-N-[1-[2-(4-hydroxy-2-phenylethyl)-4-piperidinyl]-3-carboxypropanamide (ω-Carboxy-fentanyl) A mixture of depropionylfentanyl (50 mg), methyl 4-chloro-4-oxobutyrate (40 mg), and triethylamine (30 μL) in dichloromethane (2 mL) was stirred on ice for 1 h. Zero point one molar hydrochloric acid was added to the reaction mixture, and the mixture was extracted with chloroform. The solvent was evaporated to dryness under vacuum, and the residue was dissolved in 0.1 M hydrochloric acid (2 mL) and refluxed for 2 h. The reaction mixture was basified with 10 M sodium hydroxide solution, and then washed with chloroform. The aqueous layer was acidified with 3 M hydrochloric acid then the pH was adjusted to 9 with 28% ammonium hydroxide solution before extracting with chloroform–2-propanol (3:1). The organic layer was evaporated to dryness under vacuum, and then the residue was purified by flash chromatography (column, silica gel 4 g; solvent, chloroform-methanol) to obtain N-phenyl-N-[1-[2-(4-hydroxy-2-phenylethyl)-4-piperidinyl]-3-carboxypropanamide (ω-carboxy-fentanyl) (39.7 mg) as a clear oil.

ω-Carboxy-fentanyl (Free Base)

N-Phenyl-N-[1-[2-(4-hydroxy-2-phenylethyl)-4-piperidinyl]-3-carboxypropanamide (ω-Carboxy-fentanyl) A mixture of depropionylfentanyl (50 mg), methyl 4-chloro-4-oxobutyrate (40 mg), and triethylamine (30 μL) in dichloromethane (2 mL) was stirred on ice for 1 h. Zero point one molar hydrochloric acid was added to the reaction mixture, and the mixture was extracted with chloroform. The solvent was evaporated to dryness under vacuum, and the residue was dissolved in 0.1 M hydrochloric acid (2 mL) and refluxed for 2 h. The reaction mixture was basified with 10 M sodium hydroxide solution, and then washed with chloroform. The aqueous layer was acidified with 3 M hydrochloric acid then the pH was adjusted to 9 with 28% ammonium hydroxide solution before extracting with chloroform–2-propanol (3:1). The organic layer was evaporated to dryness under vacuum, and then the residue was purified by flash chromatography (column, silica gel 4 g; solvent, chloroform-methanol) to obtain N-phenyl-N-[1-[2-(4-hydroxy-2-phenylethyl)-4-piperidinyl]-3-carboxypropanamide (ω-carboxy-fentanyl) (39.7 mg) as a clear oil.

ω-Carboxy-fentanyl (Free Base)

4'-Hydroxy-butyrylfentanyl Hydrochloride

N-Phenyl-N-[1-[2-(4-hydroxy-2-phenylethyl)-4-piperidinyl]-3-carboxypropanamide (ω-Carboxy-fentanyl) A mixture of depropionylfentanyl (50 mg), methyl 4-chloro-4-oxobutyrate (40 mg), and triethylamine (30 μL) in dichloromethane (2 mL) was stirred on ice for 1 h. Zero point one molar hydrochloric acid was added to the reaction mixture, and the mixture was extracted with chloroform. The solvent was evaporated to dryness under vacuum, and the residue was dissolved in 0.1 M hydrochloric acid (2 mL) and refluxed for 2 h. The reaction mixture was basified with 10 M sodium hydroxide solution, and then washed with chloroform. The aqueous layer was acidified with 3 M hydrochloric acid then the pH was adjusted to 9 with 28% ammonium hydroxide solution before extracting with chloroform–2-propanol (3:1). The organic layer was evaporated to dryness under vacuum, and then the residue was purified by flash chromatography (column, silica gel 4 g; solvent, chloroform-methanol) to obtain N-phenyl-N-[1-[2-(4-hydroxy-2-phenylethyl)-4-piperidinyl]-3-carboxypropanamide (ω-carboxy-fentanyl) (39.7 mg) as a clear oil.

ω-Carboxy-fentanyl (Free Base)
mass spectrometer. The conditions were as follows: column, CORTECS C18 (2.1×50 mm; particle diameter, 2.7 µm; Waters, Milford, MA, U.S.A.) maintained at 40°C; mobile phase composition, 0.1% formic acid (A) and methanol (B); linear gradient mode, 20% B for 1 min, 20 to 80% B over 8 min, 80% B for 2 min, and 80 to 20 B over 0.1 min; flow rate, 0.2 mL/min; MS interface, positive ESI; analysis mode, scan (m/z 100–500) and product ion analysis (normalized collision energy, 35%; precursor ions, protonated molecule of each compound).

Quantitation of the Metabolites A sample of the culture medium (25 µL) was hydrolyzed as described above. The IS (10 µL) solution (thiofentanyl hydrochloride, 50 ng/10 µL water) and acetonitrile (250 µL) were added to the reaction mixture and the mixture was vortexed for 5 s. After centrifugation at 10000 × g for 5 min, the supernatant (50 µL) was mixed with 0.1% formic acid (200 µL), and centrifuged at 10000 × g for 5 min. The supernatant was analyzed by LC/MS using an LC system (NANOSPACE SI-2, Shiseido, Tokyo, Japan) connected to a triple quadrupole mass spectrometer (TSQ Quantum, Thermo Fisher Scientific). The column, mobile phase composition, flow rate, and MS interface were the same as for the identification of the metabolites. The analysis mode was selected reaction monitoring (SRM). The SRM parameters are listed in Table 1.

| Compound                      | Monitoring ion (m/z) | Collision energy (eV) |
|-------------------------------|----------------------|-----------------------|
|                               | Precursor (M + H⁺)   | Product               |
| Butyrylfentanyl               | 351.1                | 188.2                 | 23                    |
| Nor-butyrylfentanyl           | 247.1                | 84.2                  | 18                    |
| α-Hydroxy-butyrylfentanyl     | 367.1                | 188.2                 | 23                    |
| (α-1)-Hydroxy-butyrylfentanyl | 367.1                | 188.2                 | 23                    |
| 4'-Hydroxy-butyrylfentanyl    | 367.1                | 121.1                 | 33                    |
| β-Hydroxy-butyrylfentanyl     | 367.1                | 204.2                 | 21                    |
| 4'-Hydroxy-3'-methoxy-butyrylfentanyl | 397.1            | 151.2                 | 32                    |
| α-Carboxy-fentanyl            | 381.1                | 188.2                 | 24                    |
| Thiofentanyl (IS)             | 343.1                | 111.1                 | 33                    |

* Triple quadrupole mass spectrometer was used for quantitative analysis.

CYP Reaction Phenotyping CYP reaction phenotyping was performed according to the protocol provided by the manufacturer. Briefly, 0.1 M potassium phosphate buffer (KPi, pH 7.4), human liver microsomes, and an anti-CYP antibody were mixed in a test tube and incubated at 37°C for 3 min. As a control, preimmune rabbit IgG was used instead of the anti-CYP antibody. After standing at room temperature for 10 min, 1 M KPi, water, butyrylfentanyl hydrochloride solution, and a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (mixture of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADPH in water) were added to the test tube and incubated at 37°C for 3 min.

Fig. 2. TIC and EICs Obtained from the Medium of the Hepatocytes Cultured with Butyrylfentanyl for 48 h

Fig. 3. Product Ion Spectra of Authentic Standards of Butyrylfentanyl and Its Metabolites Obtained by the Ion Trap Mass Spectrometer

1. butyrylfentanyl; 2. nor-butyrylfentanyl; 3. α-hydroxy-butyrylfentanyl; 4. (α-1)-hydroxy-butyrylfentanyl; 5. 4'-hydroxy-butyrylfentanyl; 6. β-hydroxy-butyrylfentanyl; 7. α-carboxy-fentanyl; 8. 4'-hydroxy-3'-methoxybutyrylfentanyl.
30 min. The volume of each reaction mixture was 200 µL. The final concentrations of the components in the reaction mixture were as follows: butyrylfentanyl 50 µM, KPi 0.1 M, CYP 0.1 µM, glucose-6-phosphate 10 mM, glucose-6-phosphate dehydrogenase 1 U/mL, NADP⁺ 0.5 mM, and anti-CYP antibody 0.075–0.75 mg/mL. After incubation, IS solution (10 µL; thiofentanyl hydrochloride, 50 ng/10 µL water) and acetonitrile (0.8 mL) were added and the mixture was vortexed for 5 s. A portion of the supernatant was diluted five times with 0.1% formic acid and centrifuged at 10000 × g for 5 min. The supernatant was analyzed using the LC-triple quadrupole mass spectrometer as described above to quantitate the metabolites.

RESULTS AND DISCUSSION

Identification of the Metabolites Because fresh human hepatocytes isolated from a liver-humanized mouse model (PXB-cells) have high activities of phase I and II drug-metabolizing enzymes, we used these cells in studying the metabolism of butyrylfentanyl. The total ion current chromatogram (TIC) and extracted ion chromatograms (EICs) obtained from the medium of hepatocytes cultured with butyrylfentanyl for 48 h are shown in Fig. 2. Unmetabolized butyrylfentanyl (peak 1) and seven metabolites (peak 2, nor-butyrylfentanyl; peak 3, ω-hydroxy-butyrylfentanyl; peak 4, (ω-1)-hydroxy-butyrylfentanyl; peak 5, 4'-hydroxy-butyrylfentanyl; peak 6, β-hydroxy-butyrylfentanyl; peak 7, ω-carboxy-fentanyl; peak 8, 4'-hydroxy-3'-methoxy-butyrylfentanyl) were detected in the EICs. All metabolites were confirmed by comparing their retention times and mass spectra with those of the authentic standards. The product ion spectra of the compounds are shown in Fig. 3. Among the hydroxylated metabolites (peaks 3–6), the spectra of ω-hydroxy-butyrylfentanyl and (ω-1)-hydroxy-butyrylfentanyl (peaks 3, 4) were similar to each other, whereas those of 4'-hydroxy-butyrylfentanyl and β-hydroxy-butyrylfentanyl (peaks 5 and 6) were easily distinguished from the other hydroxylated metabolites. The proposed metabolic pathways for butyrylfentanyl are shown in Fig. 4.

CYP Reaction Phenotyping To clarify which isof orm of CYP was involved in the formation of each metabolite of butyrylfentanyl, CYP reaction phenotyping was performed. Figure 5 shows the inhibition of the formation of each metabolite of butyrylfentanyl by various anti-CYP antibodies. None of the five metabolites in Fig. 5 were formed without NADPH, indicating that these metabolites were formed by NADPH-dependent enzyme(s) (data not shown). The formation of nor-butyrylfentanyl and β-hydroxy-butyrylfentanyl were markedly inhibited by the anti-CYP3A4 antibody, indicating that CYP3A4 was involved in the majority of the formation of these metabolites. In contrast, the formation of (ω-1)-hydroxy-butyrylfentanyl was inhibited by half by the anti-CYP3A4 antibody, implying that CYP3A4 plus another NADPH-dependent enzyme (possibly another CYP) produced this metabolite. In contrast, the formation of ω-hydroxy-butyrylfentanyl was inhibited by the anti-CYP2D6 antibody, instead of the anti-CYP3A4 antibody. The production of 4'-hydroxy-butyrylfentanyl was not inhibited by any of the anti-CYP-antibodies tested. According to our previous study, 4'-hydroxy-fentanyl is formed from fentanyl by CYP2D6. A different enzyme may be involved in the 4'-hydroxylation of butyrylfentanyl. Steuer et al. reported that CYP3A4 and CYP2D6 are involved in the formation of nor-butyrylfentanyl and ω-hydroxy-butyrylfentanyl, respectively, and their results are consistent with our results.

Metabolite Profile of Butyrylfentanyl in PXB-Cells The metabolite profile of butyrylfentanyl in PXB-cells is shown in Fig. 6. After 24 and 48 h incubation of butyrylfentanyl with PXB-cells, 23 and 13% of butyrylfentanyl remained in the culture medium, respectively. The amount of the main metabolite of butyrylfentanyl formed by PXB-cells, nor-butyrylfentanyl, reached 37% of the initial amount of butyrylfentanyl at 48 h. The second and third largest metabolites were (ω-1)-hydroxy-butyrylfentanyl and ω-hydroxy-butyrylfentanyl,

![Fig. 4. Proposed Metabolic Pathways for Butyrylfentanyl](image)
respectively, and 4'-hydroxy-butyrylfentanyl, β-hydroxy-butyrylfentanyl, ω-carboxy-fentanyl, and 4'-hydroxy-3'-methoxy-butyrylfentanyl were the minor metabolites. The amount of each metabolite increased with the incubation time (24 h < 48 h), except for β-hydroxy-butyrylfentanyl; this metabolite might undergo further metabolism, such as hydroxylation and/or N-dealkylation, in PXB-cells.

Steuer et al.⁵ and Staeheli et al.⁶ reported that ω-hydroxy-butyrylfentanyl and ω-carboxy-fentanyl are the major metabolites of butyrylfentanyl in biological specimens, such as blood and urine, obtained from butyrylfentanyl users, whereas nor-butyrylfentanyl is the minor metabolite. The detected amounts of nor-butyrylfentanyl and ω-carboxy-fentanyl in our in vitro results were not consistent with their in vivo results. ω-Carboxy-fentanyl was formed from ω-hydroxy-butyrylfentanyl by further oxidation via an aldehyde; the candidate enzymes involved in these reactions are alcohol dehydrogenase and aldehyde dehydrogenase. The low activity of these enzymes in PXB-cells may explain why only small amounts of ω-carboxy-fentanyl were formed in the PXB-cells. It is unclear what made such difference in the amount of nor-butyrylfentanyl between in PXB-cells and in vivo. One possibility is that the activity of CYP2D6 was relatively lower in PXB-cells than in vivo, resulted in producing less amount of CYP2D6-mediated metabolite (ω-hydroxy-butyrylfentanyl) and more amounts of CYP3A4-mediated metabolites (nor-
butyrylfentanyl, (ω-1)-hydroxy-butyrylfentanyl and β-hydroxy-butyrylfentanyl) in PXB-cells. To investigate the relationship of the length of the N-acyl group of fentanyl analogs with their metabolite formation patterns, the EICs for the monohydroxylated metabolites of acetylfentanyl, fentanyl, and butyrylfentanyl were compared (Fig. 7, data for acetylfentanyl and fentanyl are from our previous study10). The peaks indicated by arrows are the metabolites hydroxylated at the N-acyl group. Figure 7 clearly indicates that the longer the N-acyl group (acetyl < propionyl < butyryl), the more amount of N-acyl group-hydroxylated metabolites were formed.

The hydroxylated and carboxylated metabolites of butyrylfentanyl have the potential to be conjugated. To clarify whether these metabolites are conjugated, the amount of each metabolite in the hydrolyzed culture medium was compared with that in the untreated medium. Figure 8 shows the relative amount of each metabolite, where the amount of each metabolite in the hydrolyzed culture medium is expressed as 100%. The amounts of 4'-hydroxy-butyrylfentanyl and 4'-hydroxy-3'-methoxy-butyrylfentanyl drastically decreased when the hydrolysis step was omitted, indicating that most of these metabolites were conjugated with glucuronic acid or sulfate. The amount slightly decreased in case of β-hydroxy-butyrylfentanyl, however, it was not sure whether this metabolite was conjugated or not. A significant decrease was not observed for the other metabolites, which are not suitable for conjugation. These results are consistent with the results for fentanyl and acetylfentanyl.10

CONCLUSION

In this study, the metabolism of butyrylfentanyl was investigated using fresh human hepatocytes, and the putative metabolites were chemically synthesized. Seven metabolites of butyrylfentanyl were identified using authentic standards. In particular, ω-hydroxy- and (ω-1)-hydroxy-metabolites of
butyrylfentanyl were definitively identified in our study; these metabolites were hardly distinguished only by their mass spectra. In addition, the authentic standards of the metabolites made accurate quantification possible. Furthermore, it was revealed that CYP3A4 and CYP2D6 played a key role in the metabolism of butyrylfentanyl.

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Conflict of Interest The authors declare no conflict of interest.

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