Studies on 2′,3′-Dideoxy-2′,3′-didehydropyrimidine Nucleosides. II. N_4-Benzoyl-2′,3′-dideoxy-2′,3′-didehydrocytidine as a Prodrug of 2′,3′-Dideoxy-2′,3′-didehydrocytidine (DDCN)

Takeo KAWAGUCHI,*[,a] Kozo ISHIKAWA,[a] Toshinobu SEKI,[a] Kazuhiko JUNI,[a] Shoji FUKUSHIMA,[b] and Masahiro NAKANO[b]

Faculty of Pharmaceutical Sciences, Josai University,[a] 1–1 Keyakidai, Sakado, Saitama 350–02, Japan and Department of Pharmacy, Kumamoto University Hospital,[b] 1–1–1 Honjo, Kumamoto 860, Japan. *Received February 22, 1989*

N_4-Benzoyl-2′,3′-dideoxy-2′,3′-didehydrocytidine (Bz-DDCN) was synthesized as a novel prodrug of 2′,3′-dideoxy-2′,3′-didehydrocytidine (DDCN), which is a reverse transcriptase inhibitor and is considered to be a potential anti-acquired immunodeficiency syndrome agent. Chemical and enzymatic regeneration of DDCN from the prodrug has been investigated in both *in vitro* and *in vivo* experiments. Bz-DDCN regenerated DDCN under basic conditions (>pH 8), while cleavage of the *N*-glycosidic linkage and production of N_4-benzoylcytosine were observed under acidic conditions (<pH 6). DDCN was enzymatically regenerated from the prodrug in the presence of several enzyme preparations, including human plasma. DDCN and Bz-DDCN were intravenously administered to mice and the plasma concentrations of DDCN and the prodrug were measured. Though DDCN levels following direct DDCN administration decreased exponentially with a half-life of 14.5 min, the plasma levels of DDCN following the prodrug administration were sustained above 2 μM for over 3 h.

Keywords 2′,3′-dideoxy-2′,3′-didehydrocytidine; prodrug; DDCN; Bz-DDCN; reverse transcriptase inhibitor; AIDS

2′,3′-Dideoxy-2′,3′-didehydrocytidine (DDCN) is a strong inhibitor of the reverse transcriptase from the human immunodeficiency virus (HIV) and is considered to be a potential anti-acquired immunodeficiency syndrome (AIDS) agent.[1,2] Though several inhibitors of the reverse transcriptase have been used in the treatment of AIDS patients, some severe side effects have been reported.[3,4] Since these inhibitors of the reverse transcriptase work as metabolic antagonists, or their anti-viral effects can be time-dependent, an adequate inhibitor concentration in a body should be maintained to achieve the anticipated anti-viral effect, and to avoid undesirable side-effects such as bone marrow toxicity, which seems to be attributable to an excessive plasma concentration of the drugs.

N_4-Benzoyl-2′,3′-dideoxy-2′,3′-didehydrocytidine (Bz-DDCN) was synthesized as a prodrug of DDCN from which DDCN would be released enzymatically to sustain an appropriate DDCN level in the body. In this report, chemical and enzymatic regeneration of DDCN from Bz-DDCN has been investigated in both *in vitro* and *in vivo* experiments.

Experimental

General Experimental Procedures Melting points were determined on a Yanagimoto MP-S3 micro melting point apparatus and are recorded uncorrected. Ultraviolet (UV) absorption spectra were taken on a Shimadzu 265 UV-VIS spectrophotometer. Mass spectra (MS) were taken on a JEOL JMS DX-300 mass spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained on a JEOL JNM GX-270 FT NMR spectrometer at 270 MHz and chemical shifts are given in δ (ppm) with tetramethylsilane (TMS) as an internal standard. A high performance liquid chromatography (HPLC) system consisting of a pump (LC-6A, Shimadzu), a variable-wavelength detector (SPD-6A, Shimadzu), and a 20-μl fixed loop injector (model 7125, Rheodyne) were used. The pH values of the buffer were read with a pH meter (model PH51, Yokokawa-Hokushin Electric) at 40 °C.

Materials 2′-Deoxycytidine hydrochloride was purchased from Wako Pure Chemical Industries (Osaka, Japan). Dimethyl sulfoxide was of silylation grade and was purchased from Pierce Chemical (Rockford, IL). Acetonitrile, chloroform, and *n*-hexane were of HPLC grade and were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were reagent grade products obtained from commercial sources and were used without further purification.

Synthesis DDCN was synthesized from 2′-deoxycytidine according to the procedure reported by Horwitz *et al.*[5,6] Briefly, N_4-benzoyl-2-deoxycytidine was treated with 2 eq of methanesulfonyl chloride to give the 3′,5′-di-*O*-mesyl derivative. The cyclization of the dimesyl derivative by the action of aqueous sodium hydroxide gave 1-(2-deoxy-3,5-epoxy-β-D-*threo*-pentofuranosyl)cytosine. The decyclization of the oxetane ring in the latter compound was effected by potassium *tert*-butoxide in dimethyl sulfoxide, affording DDCN, which was identified on the basis of its melting point (163—164 °C), and NMR and MS. The 4-amino position of DDCN was selectively acylated with benzoic anhydride in water–dioxane (1 : 3) mixture according to the method reported by Akiyama *et al.*[7] to give Bz-DDCN in a 64% yield based on DDCN. Stock solutions of DDCN and Bz-DDCN were prepared in methanol to give a concentration of 1.6 mM and were stored at −20 °C.

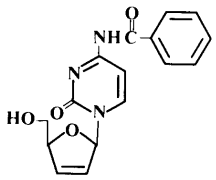

Fig. 1. Chemical Structure of Bz-DDCN

Bz-DDCN mp 155—156 °C. UV (EtOH) λ_{max}: 260 (ε 21000), 308 (ε 8200). NMR ($CDCl_3$) δ: 3.82—3.92 (m, 2H, H-5′), 5.03 (br, 1H, H-4′), 6.03 (d, 1H, J=5.9 Hz, H-2′), 6.31 (ddd, 1H, J=5.9 Hz and 2×1, 47 Hz, H-3′), 7.03—7.04 (m, 1H, H-1′), 7.49—7.99 (m, 5H, benzoyl), 7.60 (d, 1H, J=7.7 Hz, H-5), 8.29 (d, 1H, J=7.7 Hz, H-6).

Buffers Buffer solutions were prepared with glycine–sodium hydroxide for the pH values of 10.0 and 11.0, boric acid–sodium hydroxide for pH 8.0 and 9.0, disodium hydrogen phosphate–sodium dihydrogen phosphate for pH 6.0 and 7.0, acetic acid–sodium acetate for pH 4.0 and 5.0, and glycine–HCl for pH 3.5. The buffer concentration range was 0.02—0.03 M. All the buffers were adjusted to a constant ionic strength of 0.02 with sodium chloride. The effect of varying the buffer concentrations (0.02—0.12 M) on the chemical degradation rates at constant pH values (pH 3.5, 4.0, 5.0, 6.0, 8.0, and 10.0) were evaluated. Since no buffer catalysis was noted at any pH value, no further studies on the effect of buffers were done.

HPLC Analysis For HPLC analysis of DDCN and Bz-DDCN, a mixture of acetonitrile and 0.02 M Tris–HCl buffer, pH 8.0, (3 : 97 and 26 : 74 for DDCN and Bz-DDCN, respectively) was used as a mobile phase on a C18 reversed-phase (Superspher RP-18, Merck-Kanto Chemicals) 300 × 4.6 mm column with a flow rate of 1.0 ml/min. The wavelength of the spectrophotometer was set at 269 or 260 nm for DDCN or Bz-DDCN, respectively.

Stability Study The chemical stabilities of DDCN and Bz-DDCN were measured at 40 °C. Each reaction was initiated by adding a stock solution (20 μl) to 2 ml of preheated solution in a glass tube with a screw cap. A

25 μl portion of the reaction mixture was periodically withdrawn and analyzed by HPLC.

Partition Coefficient Measurement Apparent partition coefficients of DDCN and Bz-DDCN were determined in a chloroform/0.1 M phosphate buffer (pH 7.0) system at 25 °C.

Preparation of Enzyme Systems Male Sprague Dawley rats (250—285 g) were used to obtain blood. Male ddY mice (25—28 g) were sacrificed to obtain blood, livers, and kidneys. Human blood was collected from three volunteers with a heparinized syringe. The blood was centrifuged at 1000 × *g* for 15 min, and the resulting plasma was stored at −40 °C until use. The tissues were homogenized with pH 7.0 isotonic phosphate buffer (0.1 M) containing 0.19 M sucrose at 0 °C to give a concentration of 4.0 w/v%. Portions (1 ml) of the homogenates were transferred to small glass tubes and stored at −80 °C until use. No decrease in the enzyme activity of the stored samples was observed over a period of 6 weeks.

Enzymatic Regeneration of DDCN Enzymatic regeneration rates of DDCN from Bz-DDCN were measured in the presence of the plasmas (mouse, rat and human), and mouse liver and kidney homogenates. The experiments were performed at 37 °C and initiated by adding the stock solution (10 μl) to one of the diluted enzyme preparations (1.0 ml) with isotonic phosphate buffer (pH 7.0) to give a final Bz-DDCN concentration of 0.016 or 0.16 mM. A portion (200 μl) of the reaction mixture was periodically withdrawn and immediately mixed with 400 μl of acetonitrile for deproteinization. The mixture was centrifuged for 10 min at 1000 × *g* and a supernatant fluid was obtained. The decrease in concentration of Bz-DDCN and the increase in concentration of DDCN were followed by HPLC analysis of the resulting supernatant samples. The enzymatic reaction was not saturated at a higher substrate concentration (0.16 mM).

***In Vivo* Study** Each of the five male ddY mice (25—27 g) in each group was used in the test. DDCN and Bz-DDCN were dissolved in saline containing 0.1% Tween 80 to give a concentration of 14 mM. Then 0.1 ml of the solution was administered *via* the mouse tail vein. Blood was collected from each of the five mice into heparinized glass tubes by decapitation at specified times after administration. The blood was centrifuged at 1000 × *g* for 15 min and the resulting plasma was stored at −40 °C until HPLC analysis. A 300 μl aliquot of the plasma was mixed with 300 μl of acetonitrile for deproteinization and the mixture was centrifuged for 10 min at 1000 × *g*. The resulting supernatant fluid was analyzed by HPLC. The calibration curves for DDCN and Bz-DDCN in mouse plasma were obtained from the absolute peak areas of standard plasma samples (0.5—50 μM), and were linear over the concentration range of 1.0—50 μM ($r^2 = 0.997$).

Results and Discussion

Figure 2 shows the log rate–pH profiles for the degradation of DDCN and Bz-DDCN in the buffer at 40 °C. The degradation products of DDCN and Bz-DDCN under acidic conditions (<pH 6) were identified as cytosine and N_4-benzoylcytosine, respectively. The faster cleavage rates of the *N*-glycosidic linkage on Bz-DDCN than those of DDCN under acidic conditions could be attributed to the electron withdrawing effect of the benzoyl moiety. Though DDCN was stable under basic conditions (>pH 8), Bz-DDCN was deacylated and released DDCN.

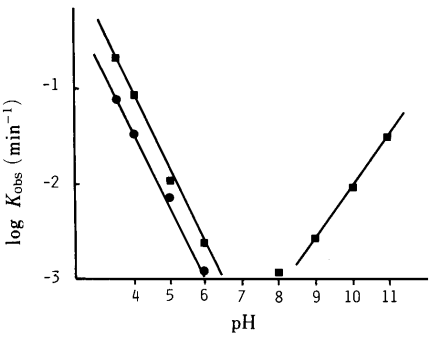

Fig. 2. The pH-Rate Profiles for the Degradation of DDCN (●) and Bz-DDCN (■) under Acidic Conditions and Deacylation of Bz-DDCN under Basic Conditions at 40 °C

First-order regeneration of DDCN from Bz-DDCN was observed in the presence of the plasma. Typical time courses of disappearance of Bz-DDCN and appearance of DDCN in the mouse plasma are shown in Fig. 3. The slope of the semilogarithmic plot of 0.016 mM minus regenerated DDCN concentration, which was consistent with Bz-DDCN concentration, against time gave the pseudo-first-order rate constant. The values of the constant in the 20 v/v% plasmas of human, rat and mouse were 0.53, 1.64 and 1.57×10^{-3} min^{-1}, respectively. Enzymatic regeneration of DDCN also occurred in the mouse tissue homogenates; the pseudo-first-order rate constants in the 4.0 w/v% liver and kidney homogenates were 1.61 and 0.61×10^{-3} min^{-1}, respectively. Though these constants include the chemical reaction, the contribution of the chemical reaction is limited at this pH (1×10^{-4} min^{-1} from Fig. 2).

Plasma concentrations of DDCN and Bz-DDCN following intravenous administration of DDCN or Bz-DDCN are shown in Fig. 4. DDCN administered intravenously was rapidly eliminated from plasma with a half-life of 14.5 min; the plasma concentration of DDCN at 3 h after administration of DDCN was undetectable (<0.5 μM). Though the elimination of Bz-DDCN was faster than that of DDCN, DDCN levels in plasma following Bz-DDCN administration were more sustained than those after direct administration of DDCN. Since Bz-DDCN shows a higher par-

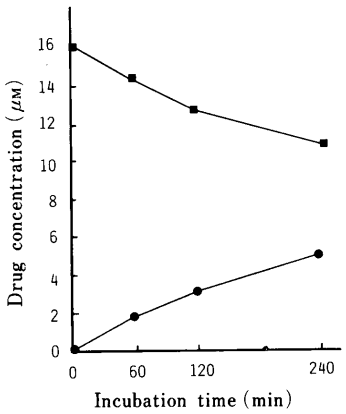

Fig. 3. Disappearance of Bz-DDCN and Appearance of DDCN in 20% Mouse Plasma at 37 °C

(●), DDCN; (■), Bz-DDCN.

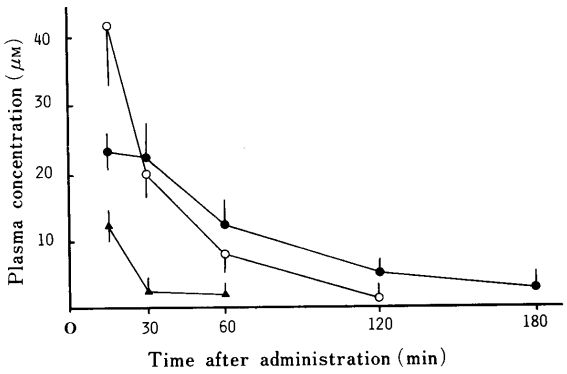

Fig. 4. Plasma Concentrations of DDCN and Bz-DDCN

(○), DDCN following DDCN administration (1.4 μmol/mouse); (●), DDCN following Bz-DDCN administration (1.4 μmol/mouse); (▲), Bz-DDCN following Bz-DDCN administration (1.4 μmol/mouse). Each point represents the mean and S.E. of five mice.

tition coefficient ($PC=2.5$) compared with DDCN ($PC=1.6\times10^{-2}$), the rapid elimination of Bz-DDCN may partly be attributed to a favorable distribution to some tissues, and the distributed prodrug may act as a DDCN precursor for slow and sustained release of DDCN.

Although Bz-DDCN is unstable under acidic conditions and its retention *in vivo* still seems to be inadequate, modification of the acyl moiety and/or alteration of the position of acylation (*e.g.*, the 5′-hydroxy group can be acylated) may improve the above characteristics.

References

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