Pharmacokinetic Properties of Orally Administered 4’-Cyano-2’-deoxyguanosine, a Novel Nucleoside Analog Inhibitor of the Hepatitis B Virus, in Viral Liver Injury Model Rats

Mai Hashimoto, Kazuaki Taguchi, Shuhei Imoto, Keishi Yamasaki, Hiroaki Mitsuya, and Masaki Otagiri

INTRODUCTION

Current pharmacotherapy based on nucleoside analog (NA) preparations, including entecavir (ETV) and adefovir dipivoxil (ADV), is used to control the progression of chronic hepatitis B to cirrhosis and hepatocellular carcinoma with mortality being improved. However, over 200 million people still suffer from Hepatitis B virus (HBV) infections and about one million individuals die annually, worldwide, due to the fact that resistant HBVs against marketed NA preparations, such as ADV-resistant HBV (HBV<sub>ADV</sub>) and ETV-resistant HBV (HBV<sub>ETV</sub>), appear during long-term their use. Therefore, the development of novel NAs for the treatment of HBV is an urgent issue in the field of hepatology with the goal of neutralizing resistant HBV against currently marketed NA preparations.

Our group has searched for novel NAs with antiviral activity against not only wild-type HBV but also HBV that is resistant to the currently marketed NA preparations. As a result of screening of over 100 NAs in a real-time HBV-PCR assay, we identified a candidate compound, 4’-cyano-2’-deoxyguanosine (CdG, Chart 1), which showed superior antiviral activity against ADV-resistant HBV (HBV<sub>ADV</sub> and ETV-resistant HBV (HBV<sub>ETV</sub>) to ADV and ETV, respectively. In addition, a study of the pharmacokinetics of CdG in healthy rats showed that CdG possessed favorable pharmacokinetic properties for use as an oral NA preparation against HBV infections with good bioavailability and a high distribution to the liver. However, there is a concern that liver pathology may influence the pharmacokinetic characteristics of CdG, resulting from an alteration in the distribution to the liver. Thus, clarifying the pharmacokinetics of orally administered CdG under conditions of liver pathology would further provide useful information for the development of CdG as an oral NA preparation with the goal of treating HBV infections.

In this study, we report on an investigation of the pharmacokinetic properties of CdG under liver pathological conditions, in particular, in a viral liver injury (VLI) model of viral liver injury induced by treatment with concanavalin A. Following an intravenous administration of CdG at a dose of 1 mg/kg, the plasma concentration profile of CdG in VLI model rats was found to be similar to that of healthy rats with no significant difference in kinetic parameters. However, when CdG was orally administered at a dose of 1 mg/kg, the maximum blood concentration was much lower in VLI model rats than in healthy rats. Interestingly, the amount of residual food in the stomachs in VLI model rats was significantly larger than that in healthy rats, indicating that the adsorption of CdG in the gastrointestinal tract was inhibited in the presence of food as well as other marketed nucleoside analogs. As observed in healthy rats, CdG was largely distributed to the liver compared to the kidney in the VLI model. These results suggest that liver pathology has only a minor effect on the pharmacokinetic properties of CdG, but the influence of food on CdG absorption needs to be considered.

Key words: nucleoside analog; hepatitis B; disposition; acute liver injury; food–drug interaction

INTRODUCTION

Current pharmacotherapy based on nucleoside analog (NA) preparations, including entecavir (ETV) and adefovir dipivoxil (ADV), is used to control the progression of chronic hepatitis B to cirrhosis and hepatocellular carcinoma with mortality being improved. However, over 200 million people still suffer from Hepatitis B virus (HBV) infections and about one million individuals die annually, worldwide, due to the fact that resistant HBVs against marketed NA preparations, such as ADV-resistant HBV (HBV<sub>ADV</sub>) and ETV-resistant HBV (HBV<sub>ETV</sub>), appear during long-term their use. Therefore, the development of novel NAs for the treatment of HBV is an urgent issue in the field of hepatology with the goal of neutralizing resistant HBV against currently marketed NA preparations.

Our group has searched for novel NAs with antiviral activity against not only wild-type HBV but also HBV that is resistant to the currently marketed NA preparations. As a result of screening of over 100 NAs in a real-time HBV-PCR assay, we identified a candidate compound, 4’-cyano-2’-deoxyguanosine (CdG, Chart 1), which showed superior antiviral activity against ADV-resistant HBV (HBV<sub>ADV</sub> and ETV-resistant HBV (HBV<sub>ETV</sub>) to ADV and ETV, respectively. In addition, a study of the pharmacokinetics of CdG in healthy rats showed that CdG possessed favorable pharmacokinetic properties for use as an oral NA preparation against HBV infections with good bioavailability and a high distribution to the liver. However, there is a concern that liver pathology may influence the pharmacokinetic characteristics of CdG, resulting from an alteration in the distribution to the liver. Thus, clarifying the pharmacokinetics of orally administered CdG under conditions of liver pathology would further provide useful information for the development of CdG as an oral NA preparation with the goal of treating HBV infections.

In this study, we report on an investigation of the pharmacokinetic properties of CdG under liver pathological

INTRODUCTION

Current pharmacotherapy based on nucleoside analog (NA) preparations, including entecavir (ETV) and adefovir dipivoxil (ADV), is used to control the progression of chronic hepatitis B to cirrhosis and hepatocellular carcinoma with mortality being improved. However, over 200 million people still suffer from Hepatitis B virus (HBV) infections and about one million individuals die annually, worldwide, due to the fact that resistant HBVs against marketed NA preparations, such as ADV-resistant HBV (HBV<sub>ADV</sub>) and ETV-resistant HBV (HBV<sub>ETV</sub>), appear during long-term their use. Therefore, the development of novel NAs for the treatment of HBV is an urgent issue in the field of hepatology with the goal of neutralizing resistant HBV against currently marketed NA preparations.

Our group has searched for novel NAs with antiviral activity against not only wild-type HBV but also HBV that is resistant to the currently marketed NA preparations. As a result of screening of over 100 NAs in a real-time HBV-PCR assay, we identified a candidate compound, 4’-cyano-2’-deoxyguanosine (CdG, Chart 1), which showed superior antiviral activity against ADV-resistant HBV (HBV<sub>ADV</sub> and ETV-resistant HBV (HBV<sub>ETV</sub>) to ADV and ETV, respectively. In addition, a study of the pharmacokinetics of CdG in healthy rats showed that CdG possessed favorable pharmacokinetic properties for use as an oral NA preparation against HBV infections with good bioavailability and a high distribution to the liver. However, there is a concern that liver pathology may influence the pharmacokinetic characteristics of CdG, resulting from an alteration in the distribution to the liver. Thus, clarifying the pharmacokinetics of orally administered CdG under conditions of liver pathology would further provide useful information for the development of CdG as an oral NA preparation with the goal of treating HBV infections.

In this study, we report on an investigation of the pharmacokinetic properties of CdG under liver pathological

INTRODUCTION

Current pharmacotherapy based on nucleoside analog (NA) preparations, including entecavir (ETV) and adefovir dipivoxil (ADV), is used to control the progression of chronic hepatitis B to cirrhosis and hepatocellular carcinoma with mortality being improved. However, over 200 million people still suffer from Hepatitis B virus (HBV) infections and about one million individuals die annually, worldwide, due to the fact that resistant HBVs against marketed NA preparations, such as ADV-resistant HBV (HBV<sub>ADV</sub>) and ETV-resistant HBV (HBV<sub>ETV</sub>), appear during long-term their use. Therefore, the development of novel NAs for the treatment of HBV is an urgent issue in the field of hepatology with the goal of neutralizing resistant HBV against currently marketed NA preparations.

Our group has searched for novel NAs with antiviral activity against not only wild-type HBV but also HBV that is resistant to the currently marketed NA preparations. As a result of screening of over 100 NAs in a real-time HBV-PCR assay, we identified a candidate compound, 4’-cyano-2’-deoxyguanosine (CdG, Chart 1), which showed superior antiviral activity against ADV-resistant HBV (HBV<sub>ADV</sub> and ETV-resistant HBV (HBV<sub>ETV</sub>) to ADV and ETV, respectively. In addition, a study of the pharmacokinetics of CdG in healthy rats showed that CdG possessed favorable pharmacokinetic properties for use as an oral NA preparation against HBV infections with good bioavailability and a high distribution to the liver. However, there is a concern that liver pathology may influence the pharmacokinetic characteristics of CdG, resulting from an alteration in the distribution to the liver. Thus, clarifying the pharmacokinetics of orally administered CdG under conditions of liver pathology would further provide useful information for the development of CdG as an oral NA preparation with the goal of treating HBV infections.

In this study, we report on an investigation of the pharmacokinetic properties of CdG under liver pathological

INTRODUCTION

Current pharmacotherapy based on nucleoside analog (NA) preparations, including entecavir (ETV) and adefovir dipivoxil (ADV), is used to control the progression of chronic hepatitis B to cirrhosis and hepatocellular carcinoma with mortality being improved. However, over 200 million people still suffer from Hepatitis B virus (HBV) infections and about one million individuals die annually, worldwide, due to the fact that resistant HBVs against marketed NA preparations, such as ADV-resistant HBV (HBV<sub>ADV</sub>) and ETV-resistant HBV (HBV<sub>ETV</sub>), appear during long-term their use. Therefore, the development of novel NAs for the treatment of HBV is an urgent issue in the field of hepatology with the goal of neutralizing resistant HBV against currently marketed NA preparations.

Our group has searched for novel NAs with antiviral activity against not only wild-type HBV but also HBV that is resistant to the currently marketed NA preparations. As a result of screening of over 100 NAs in a real-time HBV-PCR assay, we identified a candidate compound, 4’-cyano-2’-deoxyguanosine (CdG, Chart 1), which showed superior antiviral activity against ADV-resistant HBV (HBV<sub>ADV</sub> and ETV-resistant HBV (HBV<sub>ETV</sub>) to ADV and ETV, respectively. In addition, a study of the pharmacokinetics of CdG in healthy rats showed that CdG possessed favorable pharmacokinetic properties for use as an oral NA preparation against HBV infections with good bioavailability and a high distribution to the liver. However, there is a concern that liver pathology may influence the pharmacokinetic characteristics of CdG, resulting from an alteration in the distribution to the liver. Thus, clarifying the pharmacokinetics of orally administered CdG under conditions of liver pathology would further provide useful information for the development of CdG as an oral NA preparation with the goal of treating HBV infections.

In this study, we report on an investigation of the pharmacokinetic properties of CdG under liver pathological
conditions and compared the findings with results that were obtained under healthy conditions that were reported in a previous study. For this purpose, we used viral liver injury (VLI) model rats in which the injury was induced by the intravenous administering concanavalin A (ConA), which have been widely used as model animals in studies of VLI.

MATERIALS AND METHODS

Animals and Ethics  Male Sprague-Dawley rats (170–200 g, SLC Co., Ltd., Shizuoka, Japan) were housed in temperature-controlled conventional room with a 12 h dark/light cycle. All care and the experimental procedures of rats were carried out according to National Institutes of Health guidelines with approval by the Sojo University Animal Care and Use committee (Permit No.: 2018-P-015, 2019-P-010).

Preparation of ConA-Induced VLI Model Rats  VLI model rats (n = 4) were prepared by a single intravenous injection of ConA dissolved in saline at a dose of 20 mg/kg (4 mL/kg) via the tail vein as previously reported. Control rats intravenously administered saline (4 mL/kg) via the tail vein. At pre-determined times before and after the saline or ConA administration (0, 3, 6, 9, 12 and 24 h), about 250 µL blood samples were collected from the femoral vein under isoflurane anesthesia. Plasma samples were then prepared by centrifugation (3000 rpm, 10 min) and stored at −80°C.

Pharmacokinetic Study  After intravenous administration of ConA, rats for oral pharmacokinetic study were fasted until they were sacrificed. At 12 h after administration, the stomach was collected after euthanization, and the residual food in stomach was then weighed.

Pharmacokinetic Study  After intravenous administration of ConA, rats for oral pharmacokinetic study were fasted for 12 h before oral administration of CdG. CdG was synthesized as reported previously. Blood samples (250 µL) were collected from the femoral vein using a heparinized syringe at 3, 15, 30, 45, 60, 90 and 180 min after intravenous administration and at 5, 15, 30, 45, 60, 90 and 180 min after oral administration. The blood samples were centrifuged at 3000 rpm for 10 min to obtain plasma. After the last sampling of blood, all rats were euthanized, and the kidneys and liver were then removed from the rats that have been orally administered CdG. The concentration of CdG in plasma and organs were quantitated using LC-MS as reported previously.

The LC-MS system was consisted of a Waters ACQUITY ultra performance liquid chromatography (UPLC) system with a time-of-flight (TOF)-MS system (Xevo® G2-S Tof, Waters, flow rate: 0.4 mL/min) and a ACQUITY UPLC BEH Phenyl column (2.1 × 100 mm, 1.7 µm particle size, Waters, 40°C). The lower limit of quantification of CdG was 15 ng/mL in this established method.

Food Content in Stomach  Sprague-Dawley rats (n = 4/each group) were intravenously injected with either saline (4 mL/kg) or CdG dissolved in saline at a dose of 20 mg/kg (4 mL/kg) via the tail vein under isoflurane anesthesia, and were fasted until they were sacrificed. At 12 h after administration, the stomach was collected after euthanization, and the residual food in stomach was then weighed.

Data Analysis  All data are expressed as the mean ± standard deviation (S.D.). Statistical differences between groups were analyzed using two-way ANOVA followed by the unpaired t-test. A probability value of p < 0.05 was considered significant. Each pharmacokinetic parameter was calculated by noncompartment model using the moment analysis program. The area under the concentration–time curve (AUC) was calculated by the integration of blood concentration from time 0 to infinity.

RESULTS AND DISCUSSION

Assessment of VLI Model Rats Induced by ConA  Since ConA-induced VLI model animals have been widely used in drug discovery development as VLI, we selected this model for the purpose of analyzing the pharmacokinetics of CdG, a novel NA against HBV infections, in this study. Before investigating the pharmacokinetics of CdG in the ConA-induced VLI model rats, we assessed the time-dependent progression of hepatic injury for this model. As a result of ConA administration (20 mg/kg, intravenously (i.v.)), the AST and ALT levels in plasma significantly increased starting a 3 h after administration and reached a plateau at 12 h after the administration (Fig. 1). Based on these results, the following pharmacokinetic studies of CdG were carried out at 12 h after the administration of ConA.

Pharmacokinetic Studies of CdG Using the ConA-Induced VLI Model  We first monitored the plasma concentration profile of CdG after intravenous administration to the ConA-induced VLI model. The findings showed that
CdG was rapidly cleared from the blood circulation and reached below detection limit at 3 h after administration (Fig. 2). Accompanied with the plasma concentration profile, the pharmacokinetic parameters of ConA-induced VLI model rats, as shown in Table 1, were not significantly different when compared to those of healthy rats that have been intravenously administered CdG at doses of 1 mg/kg (half-life ($t_{1/2}$): 0.45 ± 0.09 h, area under the concentration–time curve ($AUC_\infty$): 122.8 ± 28.1 h·ng/mL, clearance (CL): 8.48 ± 2.21L/h/kg, distribution volume ($V_{dss}$): 3.87 ± 0.27 L/kg for healthy rats$^6$).

We previously reported that (i) approximately 50% of CdG intravenously administered to healthy rats was excreted into the urine, but not into the feces, in an unchanged form and (ii) over 95% of CdG was remained unchanged form when mixed with human and rat liver microsomes in vitro.$^6$ These facts indicate that a hepatocellular injury would have a negligible effect on the disposition of CdG, especially regarding its metabolism and excretion. It therefore appears that the pharmacokinetics of CdG in ConA-induced VLI model rats is comparable to that of healthy rats after intravenous administration.

We next investigated the pharmacokinetics characteristics of CdG in ConA-induced VLI model rats after oral administration. Figure 3A provides information on the plasma concentration profiles for CdG after oral administration, and the pharmacokinetic parameters calculated from plasma concentration curve are listed in Table 1. As in the case of intravenous administration, CdG was rapidly cleared from the blood circulation after being rapidly absorbed with no significant difference in $t_{1/2}$ and time to reach peak plasma concentration ($t_{max}$) between the groups ($t_{1/2}$: 0.73 ± 0.12 h,$^6$ 0.80 ± 0.04 h, $t_{max}$: 0.75 ± 0.00 h,$^6$ 0.58 ± 0.12 h, for healthy and ConA-induced VLI model rats, respectively). However, the values for the maximum blood concentration ($C_{max}$) and $AUC_\infty$ in the ConA-induced VLI model rat were significantly decreased compared to those of healthy rats that have been orally administered CdG at doses of 1 mg/kg ($C_{max}$: 53.8 ± 8.6,$^6$ 19.8 ± 1.7 ng/mL, $p < 0.01$, $AUC_\infty$: 90.1 ± 5.7,$^6$ 32.2 ± 1.6 h·ng/mL, $p < 0.01$, for healthy and ConA-induced VLI model rats, respectively). These decreases in pharmacokinetic parameters strongly suggest that inhibited absorption might have occurred in the gastrointestinal tract after the oral administration of CdG in the ConA-induced VLI model rat. According to recommendations in the package of ETV, it recommends taking ETV on at least 2 h after a meal or before the next meal due to fact that food consumption dramatically reduced the absorption of ETV.$^{11}$ Biopharmaceutical classification systems (BCS) are often used to classify drug–meal interaction based on drug water

### Table 1. Pharmacokinetic Parameters of CdG after Intravenous and Oral Administration at a Dose of 1 mg/kg in ConA-Induced VLI Model Rats

| Parameter | Intravenous | Oral |
|-----------|-------------|------|
| $t_{1/2}$ (h) | 0.44 ± 0.04 | 0.80 ± 0.04 |
| $AUC_\infty$ (h·ng/mL) | 114.2 ± 8.3 | 32.2 ± 1.6 |
| CL (L/h/kg) | 8.80 ± 0.61 | — |
| CL/F (L/h/kg) | — | 31.1 ± 0.1 |
| $t_{max}$ (h) | — | 0.58 ± 0.12 |
| $V_{dss}$ (L/kg) | 4.81 ± 0.13 | — |

$t_{1/2}$: half-life, $AUC$: area under the concentration–time curve, CL: clearance, F: bioavailability, $t_{max}$: time to reach peak plasma concentration, $V_{dss}$: distribution volume. Each value represents the mean ± S.D. ($n = 4$).
solubility and membrane permeability, and their contribution to variability in absorption.\(^2\) Drugs in the BCSIII category, which is classified into ETV and CdG, have low membrane permeability and may be differently absorbed depending on the site of the gastrointestinal tract. Therefore, food in the gastrointestinal tract may have had an effect on the absorption of CdG in the ConA-induced VLI model rat. Thus, we examined the food content in stomachs of healthy and ConA-induced VLI model rats at 12 h after ConA administration with 12 h fasting. As a result, the residual food in the stomachs of the ConA-induced VLI model rat was 13 times heavier than that of healthy rats (Fig. 3B), suggesting the possibility that residual food inhibits the absorption of CdG. Apart from this, the functional depression of digestive tract, such as a diminished peristalsis, by ConA administration may reduce passage of CdG food from stomach to duodena, resulting in the inhibition of CdG absorption. These results indicate that residual food in the stomach or a diminished peristalsis by ConA, rather than liver pathology, influence the pharmacokinetic characteristics of the drug, especially the absorption of CdG.

Finally, the amount of CdG in the liver and kidney was also evaluated at 3 h after the oral administration of CdG. As was found in the case of healthy rats, CdG was largely distributed to the liver compared to the kidney (Fig. 4). However, accompanied with the reduction of absorption, the distribution of CdG to the both organs in ConA-induced VLI model rats were significantly lower than those in healthy rats (Liver: 5394.1 ± 748.2 ng/g of tissue,\(^6\) 1946.5 ± 522 ng/g of tissue, \(p < 0.01\), Kidney: 2173.6 ± 312.1 ng/g of tissue,\(^6\) 370 ± 147.5 ng/g of tissue, \(p < 0.01\), for healthy and ConA-induced VLI model rats, respectively).

CONCLUSION

The results obtained in this study indicate that liver pathology has a negligible effect on the pharmacokinetics of CdG. In addition, it was collaterally presumed that food consumption inhibits the absorption of CdG after oral administration. Further studies will clearly be needed to accumulate additional evidence regarding the factors that influence the pharmacokinetics of CdG, such as other disorders and food type, if CdG is to be used as a novel oral NA preparation against HBV infections.

Acknowledgments This work was supported by the Japan Agency for Medical Research and Development (AMED) [Research on the innovative development and the practical application of new drugs for hepatitis B].

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Zoulim F. Hepatitis B virus resistance to antiviral drugs: where are we going? Liver Int., 31 (Suppl. 1), 111–116 (2011).
2) “WHO|Hepatitis B.”: http://www.who.int/mediacentre/factsheets/fs204/en/, accessed 20 April, 2020.
3) Roth GA, Abate D, Abate KH, et al. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet, 392, 1736–1788 (2018).
4) Takamatsu Y, Tanaka Y, Kohgo S, Murakami S, Singh K, Das D, Venzon DJ, Amano M, Higashi-Kuwata N, Aoki M, Delino NS, Hayashi S, Takahashi S, Sukenaga Y, Haraguchi K, Sarafianos SG, Maeda K, Mitsu H. 4'-Modified nucleoside analogs; potent inhibitors active against entecavir-resistant hepatitis B virus. Hepatology, 62, 1024–1036 (2015).
5) Kohgo S, Yamada K, Kitano K, Iwai Y, Sakata S, Ashida N, Hayakawa H, Nameki D, Kodama E, Matsuoka M, Mitsu H, Ohri H. Design, efficient synthesis, and anti-HIV activity of 4'-C-cyano- and 4'-C-cyano-2'-deoxy purine nucleosides. Nucleosides Nucleotides Nucleic Acids, 23, 671–690 (2004).
6) Hashimoto M, Taguchi K, Ishiguro T, Kohgo S, Imoto S, Yamasaki K, Mitsu H, Otad M. Pharmacokinetics studies of 4'-cyano-2'-deoxyguanosine, a potent inhibitor of the hepatitis B virus, in rats. J. Pharm. Pharmacol., 70, 723–731 (2018).
7) Rehermann B, Fowler P, Sidney J, Person J, Redeker A, Brown M, Moss B, Sette A, Chisari FV. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J. Exp. Med., 181, 1047–1058 (1995).
8) Maeda H, Hirata K, Watanabe H, Ishima Y, Chuang V, Ta- guchi K, Ichina K, Kinoshita M, Tanaka M, Sasaki Y, Otad M, Maruyama T. Polythiol-containing, recombinant mannosylated-albumin is a superior CD68 + CD206 + Kupffer cell-targeted nanoadinont for treatment of two acute hepatitis models. J. Pharmacol. Exp. Ther., 352, 244–257 (2015).
9) Moriyama Y, Saito S, Kobayashi S, Ogihara R, Koto D, Kitamura A, Matsuishi T, Nishuara M, Murase K. Evaluation of concanavalin a-induced acute liver injury in rats using an empirical mathematical model and dynamic contrast-enhanced MR imaging with Gd-EOB-DTPA. Magn. Reson. Med. Sci., 11, 53–60 (2012).
10) Tabata K, Yamaoka K, Kaibara A, Suzuki S, Terakawa M, Hata I. Moment analysis program available on microsoft excel. Drug Metab. Pharmacokinet., 14, 286–293 (1999).
11) “Package Inserts of Baraclude ® (Entecavir) Tablets.”: https://pack ageinserts.bms.com/pi/pi_baracle ud.pdf, accessed 24 March, 2020.
12) Fleisher D, Li C, Zhou Y, Pao LH, Karam A. Drug, meal and formulation interactions influencing drug absorption after oral administration. Clin. Pharmacokinet., 36, 233–254 (1999).