RESEARCH ARTICLE

Genetic Evidence for Genotoxic Effect of Entecavir, an Anti-Hepatitis B Virus Nucleotide Analog

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Abstract

Nucleoside analogues (NAs) have been the most frequently used treatment option for chronic hepatitis B patients. However, they may have genotoxic potentials due to their interference with nucleic acid metabolism. Entecavir, a deoxyguanosine analog, is one of the most widely used oral antiviral NAs against hepatitis B virus. It has reported that entecavir gave positive responses in both genotoxicity and carcinogenicity assays. However the genotoxic mechanism of entecavir remains elusive. To evaluate the genotoxic mechanisms, we analyzed the effect of entecavir on a panel of chicken DT40 B-lymphocyte isogenic mutant cell line deficient in DNA repair and damage tolerance pathways. Our results showed that Parp1-/- mutant cells defective in single-strand break (SSB) repair were the most sensitive to entecavir. Brca1-/-, Ubc13-/- and translesion-DNA-synthesis deficient cells including Rad18-/- and Rev3-/- were hypersensitive to entecavir. XPA-/- mutant deficient in nucleotide excision repair was also slightly sensitive to entecavir. γ-H2AX foci forming assay confirmed the existence of DNA damage by entecavir in Parp1-/-, Brca1-/-, Ubc13-/- and Rad18-/- mutants. Karotype assay further showed entecavir-induced chromosomal aberrations, especially the chromosome gaps in Parp1-/-, Brca1-/-, Rad18-/- and Rev3-/- cells when compared with wild-type cells. These genetic comprehensive studies clearly identified the genotoxic potentials of entecavir and suggested that SSB and postreplication repair pathways may suppress entecavir-induced genotoxicity.

Introduction

Chronic infection with hepatitis B virus (HBV) remains a major global health problem. Currently, the number of persons infected with HBV is approximately 2 billion, and over
400 million are suffering from chronic hepatitis B (CHB) worldwide [1]. Nucleoside analogues (NAs) have been the most frequently used treatment option for CHB patients due to their effects on inhibiting replication of hepatitis B virus [2]. The majority of CHB patients need long-term treatment with NAs [3, 4]. Entecavir, a carbocyclic 2'-deoxyguanosine analog, possesses potent and selective anti-hepatitis B virus (anti-HBV) activity. Entecavir induces a rapid biochemical and virologic response in CHB patients and has a high genetic barrier to resistance [5, 6]. These characteristics make it recommended as a first-line antiviral therapy for patients with CHB by international guidelines [7–9]. Unfortunately, the US prescribing information sheet and European centralized procedure (CP) indicate that entecavir is carcinogenic in primary human lymphocytes and induces lung, vascular, brain, liver and skin tumors in mice and rats [10–12]. Recently, Brown et al. reported that entecavir can be incorporated and embedded into the human genome via primer extension or subsequent ligation and that may contribute to a putative mechanism of carcinogenicity [13]. However, further studies remain to be done to gain a better understanding of the genotoxicity mechanisms of entecavir.

DNA damage occurs daily with physical and chemical mutagens. In response to it, cells have evolved specific methods of repairing the damages, including base excision repair (BER), nucleotide excision repair (NER), single-strand break (SSB) repair and double-strand break repairs consist of nonhomologous end joining (NHEJ) repair and homologous recombination (HR) [14]. DNA lesions that remain unrepaired before entering S phase often cause the collapse of DNA replication, leading to chromosomal breaks in mitotic cells and subsequent cell death [15]. To restart blocked DNA replication forks, cells have evolved postreplication repair (PRR), including HR and translesion DNA synthesis (TLS) pathways [16]. TLS pathways release the replication block by filling a daughter strand gap, employing a number of DNA polymerases, including Rad18, Rad6 and Polζ [17], whereas HR relies on recombination processes [18]. Both of Brca1 and Ubc13 play critical roles in PRR [19, 20].

DT40 cells have been a favorable tool for studying the DNA repair pathways due to its high-frequency gene targeting [21, 22]. Previously, we had generated a panel of DNA-repair deficient DT40 clones which were defective in BER, NER, SSB, NHEJ, HR and TLS respectively (Table 1). Due to the defective function on DNA repairs, these mutant clones are also sensitive to different genotoxic chemicals [23–25]. The characteristics of DNA-repair deficient DT40 clones are advantageous and useful for investigating the mechanisms of chemical genotoxicity [15, 26]. In this study, to explore the underlying mechanisms that suppress entecavir-induced genotoxicity, we performed comprehensive analyses of the genotoxicity with a panel of DT40 DNA repair mutants (Table 1).

**Results**

**Mutant cells defective in DNA repair pathways were sensitive to entecavir**

To study the genotoxicity of entecavir, we evaluated the effects of entecavir on a panel of gene disrupted clones (Table 1) by MTT assay. Camptothecin (CPT), a topoisomerase I poison, was selected as a positive control. We continuously exposed WT and mutant cells to entecavir or CPT at various concentrations for 72h. The results indicated that entecavir inhibited the growth of DT40 cells in a dose-dependent manner. As shown in Fig 1, Parp1−/− cells defective in DNA SSB exhibited the hypersensitivity to entecavir. Ubc13 deficient cells and TLS-deficient clones, both Rad18−/− and Rev3−/−, were sensitive to entecavir. To investigate the two major double-strand break repair pathways, HR and NHEJ, Brca1−/−, Brca2−/−, Xrcc2−/− and Ku70−/− were analyzed. Only Brca1−/− cells manifested significant sensitivity to entecavir. Xrcc2−/− cells were even slightly resistant to entecavir. The other DNA repair gene deficient cells, including XPA−/−...
cells were also sensitive to entecavir, but Polβ−/−, Fen1−/− and CtIP (S332A−/−) cells were not. CPT can induce DNA damage by inhibiting the ligation of SSBs that are formed during the normal functioning of topoisomerase I [38]. Unrepaired SSBs are converted to double-strand breaks upon replication. It has been shown that CPT induced double-strand breaks are mainly repaired by HR in DT40 cells [39]. As shown in Fig 1B and S1 Fig, Parp1−/−, Rad18−/−, Ubc13−/−, CtIP (S332A−/−), Brca1−/− and Brca2−/− cells were hypersensitive to CPT. In contrast, Polβ−/− and Ku70−/− were resistant to CPT, as previously reported [39]. This observation indicated that entecavir may exert potential genotoxic mechanisms which mainly associate with SSB repair and PRR, but not a double-strand break repair.

Entecavir induced the accumulation of γ-H2AX in nuclei of DT40 cells

To investigate entecavir-induced damages responses, we determined the number of γ-H2AX foci, a sensitive molecular marker of DNA damage in nuclear DNA [40]. The immunofluorescence assay was conducted using WT, Parp1−/−, Rad18−/− and Brca1−/− cells for entecavir. Six hours after exposure to 100nM entecavir, Parp1−/−, Rad18−/− and Brca1−/− exhibit more numbers of γ-H2AX foci when compared with WT cells (Fig 2A and 2B). The increased accumulation of γ-H2AX in nuclei of Parp1−/−, Rad18−/− and Brca1−/− cells suggested increased DNA damages, which is consistent with hypersensitivity of these cells to entecavir.

DNA repair-deficient cells showed a marked increase in entecavir-induced chromosome breaks

To further investigate entecavir-induced DNA damages, we measured cytologically detectable chromosomal aberration in chromosome spreads. WT, Parp1−/−, Rad18−/−, Brca1−/− and Rev3−/− cells were exposed to entecavir 200nM from 3 to 24 hours (Figs 3 and 4). Interestingly, WT,
Fig 1. Mutant cells defective in DNA repair pathways were sensitive to entecavir. (A) The X-axis represents the concentration of entecavir and the Y-axis represents the relative number of surviving cells at 72 hours. Survival data were log-transformed giving approximate normality. Analysis of covariance (ANCOVA) was used to test for differences in the linear dose-response curves between wild-type and a series of mutant cells. A p-value < 0.05 was considered to be significant. (B) Relative IC50 values of cell survival results in wild-type and their mutants exposed to entecavir or CPT. Each IC50 value was calculated from results of cell survival data shown in Fig 1A and S1 Fig. Relative IC50 values were normalized according to the IC50 value of parental wild-type cells. The IC50 was calculated by SPSS software version13.0. Data shown are the means of three experiments. Values shown are mean ± SD.

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Fig 2. Entecavir induced the accumulation of γ-H2AX in nuclei of DT40 cells. (A) Immuno-staining of wild-type (WT) and mutant DT40 clones using anti-γ-H2AX antibody and DAPI. Cells were fixed 6 hours after treated with entecavir 100nM. ETV, entecavir. (B) Quantification of γ-H2AX foci in individual cells of...
Rad18−/−, Rev3−/−, Parp1−/− and Brca1−/− cells demonstrated a monophasic pattern of induced chromosome breaks; the peaks were detectable at 12, 12, 15, 15 and 16 hours respectively (Fig 3). The peaks were significantly higher in DNA repair-deficient cells than in WT cells. Remarkably, the number of chromosome gap was higher than that of chromosome break in both WT and DNA repair-deficient cells. Entecavir mainly induced chromosome gap, but not break in metaphase, further suggesting its action for SSB, but not double-strand break. The increased chromosomal aberrations in Parp1−/−, Brca1−/−, Rad18−/− and Rev3−/− when compared with WT just reflected these genes have critical role in preventing entecavir-induced chromosomal aberrations.

**Discussion**

Entecavir, a carbocyclic 2′-deoxyguanosine analog, was widely used for HBV clinical therapies by inhibiting the HBV polymerase, competing with dGTP. In this study, we used the concentration of entecavir from 4 to 64 nM, which was based upon the maximal clinical exposure concentration 30nM [41, 42], to analyze the sensitivity of a panel of DNA repair deficient DT40 cells to entecavir. These cells include SSB repair mutant Parp1−/−, BER repair mutant Polβ−/−, NER mutant XPA−/−, HR repair mutants Brca1−/−, Brca2−/−, Xrcc2−/− and CtIP (S332A−/−), NHEJ repair mutant Ku70−/−, PRR mutants Ubc13−/−, Rad18−/− and Rev3−/− as well as flap structure-specific endonuclease 1 mutant Fen1−/−. Results showed that the SSB repair mutant of Parp1−/−, PRR mutants Rad18−/−, Rev3−/−, Ubc13−/− and Brca1−/− cells were significant sensitive to entecavir. At the same time, we found that the sensitivities of Parp1−/−, Rad18−/−, Ubc13−/− and Brca1−/− cells to entecavir were similar to CPT. In contrast, Brca2−/− and CtIP (S332A−/−) were hypersensitive to CPT, not entecavir. Further immunofluorescent analysis indicated that the number of γ-H2AX foci was significantly increased in SSB repair mutant Parp1−/− and TLS mutant Rad18−/− cells. Chromosomal aberration assay also proved that the number of chromosome gap was significantly increased in SSB repair mutant Parp1−/− and PRR mutants, Brca1−/−, Rad18−/− and Rev3−/− compared with WT. The data strongly suggest that entecavir is genotoxic and two DNA repair pathways, SSB repair and PRR, are responsive to suppress the genotoxicity.

SSBs in DNA are often raised by loss of a single nucleotide and by damaged 5′- and/or 3′- termini at the site of the break [43]. A multitude of factors trigger SSBs. Erroneous incorporation of ribonucleotides into DNA is the commonest sources of endogenous SSBs [44]. Parp1 is a sensor protein, which plays an important role in DNA SSB detection [43, 45]. In the current study, we found that Parp1−/− cells exhibited the hypersensitive to entecavir and manifested significantly increase in the number of γ-H2AX foci and chromosomal aberrations compared with WT, suggesting that entecavir may induce SSBs. As Parp1 also functions in BER, we examined the sensitivity of BER deficient cells Polβ−/−, and results showed Polβ−/− cells were not significantly sensitive to entecavir. But we found the NER deficient cells XPA−/− were slightly sensitive to entecavir.

We also examined Brca1−/−, Brca2−/−, Xrcc2−/−, CtIP(S332A−/−) and Ku70−/− cells, which respectively defective in HR and NHEJ, two major pathways for double strand breaks repair [37], and only Brca1−/− cells showed sensitivity to entecavir. We speculate that double strand breaks might not be the majority of entecavir-induced DNA damages. Recent studies had proved that besides the function on HR for double strand breaks repair, Brca1 could directly recruits trans lesion polymerases, such as Polβ and Rev1, to the lesions through protein-protein interactions, suggesting its critical role in PRR [19]. Currently, we found Rev3−/− and Rad18−/− were also...
sensitive to entecavir and had increased entecavir-induced chromosomal aberrations (Fig 3). Both Rad18 and Rev3 play critical role in PRR pathway. Studies indicated that Rad18 forms a complex with Rad6 to promote PCNA mono-ubiquitination, which is a crucial step in PRR pathway [16], whereas Rev3 gene encodes the catalytic subunit of DNA Polξ, which is involved in TLS, one of PRR pathway [27]. Furthermore, Ubc13, a K63-linked E2 Ub-conjugating enzyme, have been proved to function on both HR and error-free PRR [29, 20]. Results showed cells deficient in Ubc13 were also sensitive to entecavir. Above all, we hypothesize that entecavir induces DNA damage, which may collapse the replication forks and PRR pathway might release the replication fork stall.

Entecavir was metabolized by phosphorylation to triphosphate (TP) form in mammalian cells by cellular enzymes to inhibit HBV DNA replication [46]. The mechanism for chain termination by entecavir is likely to involve incorporation and abortive extension of ETV-containing HBV DNA [47]. Some studies reported that entecavir displays no interaction with host polymerase and failed to be incorporated into human DNA [41]. Nonetheless, the recent study by Brown et al. showed that entecavir can be incorporated and embedded into the human genome via primer extension with human X or Y polymerases or subsequent ligation [13]. One possible model that could explain our data is shown in Fig 5. The triphosphate of entecavir is incorporated into DNA strand by host replication or repair polymerases, which blocking
extension of the nascent strand and inducing DNA SSB and Parp1 dependent repair. The entecavir-induced DNA lesions could also be repaired by PRR to avoid the replication fork collapse and chromosomal breaks when cells enter into S phase.

NAs have been shown effective inhibition of HBV replication, which delay the progression of liver cirrhosis, reduce the incidence of HBV related liver cancer, above all, increase the life span of the patients [2]. Until now, most current guidelines recommended that a long-term treatment with NAs is essential to majority CHB patients, even a life-long therapy for CHB with cirrhosis. And entecavir is one of the first-line therapies. Especially in those with decompensated liver disease, undergoing immunosuppressive treatment or with contraindications, and those unwilling to receive Peg-IFN, entecavir or tenofovir is the only therapeutic options in patients [4]. However, long-term safety data are still lacking for NAs, including entecavir [3, 7]. Some studies demonstrated entecavir was clastogenic at 36 μM in primary human lymphocytes [10]. Considering that entecavir inhibited HBV DNA synthesis in the nanomolar range [42], so they thought it’s safe to humans. But in our study, entecavir induced DNA damage at nanomolar in DT40 cells, especially in the more sensitive DNA repair deficient cells. So we think it is necessary to monitor the genotoxicity of NAs, especially entecavir, and to restrict treatment period.

Much work remains to be done to gain a better understanding of the mechanism of genotoxicity of entecavir. A better understanding of entecavir-induced genotoxicity may contribute to development of new drugs for the treatment or prevention of chronic hepatitis B with higher therapeutic efficacy and less genotoxicity.

Materials and Methods

Chemicals

Entecavir was obtained from Sigma-Aldrich (St. Louis, MO, USA). CPT was purchased from Shanghai standard Biotech Co., Ltd. Stock solution of entecavir (100 μM) and CPT (100 μM)
were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C in aliquots until use. Pharmaceuticals were dissolved using DMSO and maximum volume of the solvent did not exceed 0.1% of the culture medium.

**Cell lines and cell culture**

Cell lines used in this study are listed in Table 1. Cells were cultured as described before [37]. The DT40 cell lines were cultured in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 1% chicken serum, 1% penicillin streptomycin (Gibco) and 50 μM β-mercaptoethanol (Gibco) at 39.5°C in a humidified atmosphere of 5% CO₂ (Sanyo, Osaka, Japan).

**MTT assay**

The cytotoxicity of entecavir or CPT on DT40 cell lines was determined by the MTT assay [48, 49]. Cells were seeded in 96-well plates (Costar Corning, Rochester, NY). Cells were treated during...
with entecavir or CPT at various concentrations, and carrier DMSO (< 0.1%) was used as a control, 3 wells were included in each concentration. After 72h, the cells were treated with 20 μl of 5 mg/ml MTT (Amresco, USA) and the resulting formazan crystals were dissolved in 50 μl of 20% SDS (pH4.7) over night. The absorbance at 570 nm was measured using wells without cells as blanks. All experiments were performed in triplicate. The 50% inhibiting concentration (IC50) was calculated by SPSS software version 13.0.

Chromosomal aberrations analysis

Karyotype analysis was done as previously described [50]. Briefly, cells were treated with entecavir in the complete medium. To arrest cells in metaphase, 0.1% colcemid (GIBCO-BRL, Grand Island, NY, USA) was added 3h before harvest. Then, cells were resuspended in 1 ml of 75 mM KCl for 15 min at room temperature, and fixed in 5 ml of Carnoy’s solution (mixture of methanol and acetic acid, 3:1). The cells suspension was dropped onto ethanol-cleaned slides and dried by a flame. The slides were stained with 5% Giemsa solution for 7 min, and dried after being rinsed carefully with water. The chromosomal aberrations were observed under a light microscope (with 1000× magnification). All experiments were performed in triplicate. Data are derived from 50 metaphase cells for each treatment. The scoring criteria were essentially the same as those of ISCN [51]. According to ISCN, a break is defined as a discontinuity of a chromosome that shows a clear misalignment of the distal fragment of a broken chromosome. A gap is defined as a clear non-staining region on a chromosome [50]. Chromosome gaps and breaks were both sister chromatids of a single chromosome broken at the same locus, whereas chromatid gaps and breaks were a single chromatid broken.

Immunofluorescent

Experimental condition for immunofluorescent analysis is described previously [52]. Briefly, DT40 cells (10^5 cells) were harvested on a slide glass after treated with entecavir for different hours. Cells were fixed with 3% formaldehyde for 10 min at room temperature and then washed with PBS. For permeabilisation, cells were incubated with 0.1% NP-40 for 15 min at room temperature and washed again with PBS. After blocking with 3% BSA, fixed cells were treated with specific antibodies. The cells were incubated with anti-phospho-Histone H2AX (Ser139) mouse monoclonal antibody at a dilution of 1:500 (Millipore, Billerica, MA, USA). Following another washing step with PBS, cells were incubated for 1h with a secondary Alexa Fluor 488-conjugated anti-mouse antibody (1:1000; Beyotime, Wuhan, China).

Supporting Information

S1 Fig. Sensitivity of wild-type (WT) and isogenic DNA-repair deficient DT40 clones to entecavir or CPT. Cellular sensitivities to entecavir (A) or CPT (B and C) were analyzed using the same method as in Fig 1. (TIF)

Author Contributions

Conceived and designed the experiments: YQ XW. Performed the experiments: LJ YL. Analyzed the data: LJ YQ XW ST FH. Contributed reagents/materials/analysis tools: LJ ST YQ XW. Wrote the paper: LJ XW YQ ST FH XH.
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