Entecavir Patent Evaluation & Genotoxicity

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Abstract
Entecavir is an oral antiviral drug used in the treatment of hepatitis B infection. Entecavir is a guanosine nucleoside analogue with selective activity against Hepatitis B Virus (HBV), which inhibits reverse transcription. The initial patent on entecavir expired in South Africa in 2011 ZA 1991/07894. Entecavir, a deoxyguanosine analog, is one of the most widely used oral antiviral NAs against hepatitis B virus [1]. 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−/−, Rad18−/− and Brca1−/− mutants. Karyotype 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.

Keywords: ANCOVA; Antiviral drug; CPT; Genotoxicity; Hepatitis; HBV; Patent evaluation; BMS; γ-H2AX

Introduction
Hepatitis B disease is a major health problem in the world, today received FDA approval for the treatment of many nucleoside analog drugs are used. Entecavir in our study is one of them, there is much work related to genotoxicity. To determine the entecavir the genotoxic effects in our study, people in peripheral lymphocyte culture medium entecavir three different concentrations (1.66mg/mL, 3.33mg/mL and 6.66mg/mL) for 24 and 48 hours were treated. In our study, 6.66mg/mL concentrations of entecavir in an increased number of cells with chromosomal abnormalities and structural chromosomal abnormalities in 48 hours of application and the increase was found to be significant when analyzed statistically. Additionally, the Mitotic Index (MI) has fallen from 24 and 48 hours practice and it was determined that this decline was greater in the 24-hour application. Further more entecavir on the micronucleus formation was determined as well as control show a different effect. In addition, the nuclear fission index of entecavir just 24 hours of application (NBU) in it was determined that significantly lowers. As a result [2-4], entecavir in high doses to cause genotoxicity of long-term use and entecavir administered first 24 hours cytotoxic effect that and the mitotic activity in the subsequent process of the cells was observed that continued division by winning again.

As per the results of Entecavir is a guanosine analogue with activity against hepatitis B virus (Ref: Celen MK, Dal T, Ayaz C, Bayan K, Mert D, Devecil O, Oruc EK). The aim of this 4-year trial was to evaluate entecavir treatment in nucleus (t) ide- naïve HBsAg-positive chronic hepatitis B patients. Forty-nine patients received entecavir and nine of them withdrew from the trial at the end of week 96. The initial mean value of alanine aminotransferase was 79.4±41.5 IU/L, and at the end of the 4-year study period, 90% of patients had alanine aminotransferase values within the normal range. At week 96, 91.7% of patients had HBV DNA<300 copies [5]; at month 48, 90% of patients had HBV DNA<50 IU/mL. HBsAg loss was recorded in 7.1% of patients at week 96 and in 12.5% at month 48. The rate of HBsAg seroconversion was 4.8% at week 96 and 7.5% at month 48. The rate of HBsAg seroconversion was 2.1% at week 96 and 2.5% at month 48. Entecavir as a potent and safe agent leading to continuous viral suppression proved to be safe and well tolerated therapy [6].

The initial patent on entecavir expired in South Africa in 2011 ZA 1991/07894. Current status available on: http://patentssearch.cipc.co.za/ [7] which should have permitted lower-cost generic competitors to enter the market. However, South Africa granted BMS three additional patents on entecavir that only expire between 2022 and 2026. Two of these patents have lapsed-meaning BMS has not paid the renewal fees, and they cannot be enforced-while one patent covering a lower dosage form of entecavir remains in force. This patent is currently under litigation in India Basheer S. BMS Hepatitis Patent Invalidated: A Viral Effect for India? http://spicyip.com/2013/02/bms-hepatitis-patent-invalidated-viral.html but because it is in force in South Africa, generic suppliers may be discouraged from bringing their low-dose products to market. A more recent patent on entecavir has not yet been received or processed by the patents office, but it could...
be filed up until the end of 2014 patent number: WO/2013/177672. Current status available on patentscope.wipo.int. This patent covers the manufacturing process of entecavir, and is an example of patent evergreening—where companies file patents on minor changes to an existing drug to maintain patent protection and block competition [8]. The same patent was recently overturned in the United States for failing to meet the criteria of inventive step. However, in South Africa, since no examination of patent applications occurs, if the patent is filed, it is likely to be granted to BMS. So long as BMS maintains a monopoly on entecavir in South Africa, the price is likely to remain high, and entecavir will remain out of reach for those who need it. But the crystalline forms of entecavir and its performances are not researched and reported in the above-mentioned patent. Entecavir also helps to prevent the hepatitis B virus from multiplying and infecting new liver cells, is also indicated for the treatment of chronic hepatitis B in adults with HIV/AIDS infection [9-13].

To study the genotoxicity of entecavir, we evaluated the effects of entecavir on a panel of gene disrupted clones below 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 [14-16]. The results indicated that entecavir inhibited the growth of DT40 cells in a dose-dependent manner. As shown in figure 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>References</th>
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<tbody>
<tr>
<td>Rev3</td>
<td>TLS, HR (Catalytic submit of polξ)</td>
<td>[17]</td>
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<tr>
<td>XPA</td>
<td>An initial step of nucleotide excision repair</td>
<td>[18]</td>
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<tr>
<td>UBC13</td>
<td>Ubc13 is related to the initial step of HR and postreplication repair</td>
<td>[14,19]</td>
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<td>Purp1</td>
<td>Poly (ADP) ribosylation, related to single-strand break and base excision repair</td>
<td>[15]</td>
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<tr>
<td>Brca1</td>
<td>HR</td>
<td>[16]</td>
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<tr>
<td>Brca2</td>
<td>HR</td>
<td>[20]</td>
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<tr>
<td>Rad18</td>
<td>TLS</td>
<td>[18]</td>
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<tr>
<td>Polβ</td>
<td>Base excision repair</td>
<td>[21]</td>
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<tr>
<td>Fen1</td>
<td>Base excision repair, processing 5’ flap in long-patch and lagging stand DNA replication</td>
<td>[22]</td>
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<tr>
<td>Xrcc2</td>
<td>Rad51 paralog, homologous recombination, promotion of Rad51 assembly</td>
<td>[23]</td>
</tr>
<tr>
<td>CtIP (S332A-)</td>
<td>Eliminating covalently bound polypeptides from DSBs</td>
<td>[24]</td>
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<tr>
<td>Ku70</td>
<td>Initial Step for NHEJ defective DSB repair</td>
<td>[25]</td>
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Table 1: Effects of entecavir on a panel of gene disrupted clones.

Note: DSB: Double-Strand Break; HR: Homologous Recombination; NHEJ: Nonhomologous End Joining Repair; TLS: Translesion DNA Synthesis.

(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 figure 1, [26-30], relative IC50 values were normalized according to the IC50 value of parental wild-type cells.

Figure 1: Mutant cells defective in DNA repair pathways were sensitive to entecavir.

The IC50 [14-16,20,21] was calculated by SPSS software version 13.0. Data shown are the means of three experiments. Values shown are mean±SD. 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 [31,32], 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 [33]. 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. 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. As shown in figure 2.

Figure 2: Entecavir induced the accumulation of γ-H2AX in nuclei of DT40 cells.
Entecavir is an oral antiviral drug used in the treatment of Hepatitis B virus infection because of their respective limitation. Entecavir, BMS-200475, SQ-34676 \((1S,3R,4S)-9\)-(4-Hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl) guanine CAS-42217-69-4, is a deoxyguanosine \(\text{dN}\) analogue that inhibits reverse transcription, DNA replication, and transcription in the viral replication process. Entecavir induces the accumulation of \(\gamma\)-H2AX in nuclei of DT40 cells.

To investigate entecavir-induced damages responses, we determined the number of \(\gamma\)-H2AX foci, a sensitive molecular marker of DNA damage in nuclear DNA. The immunofluorescence assay was conducted using WT, Parp1\(^{-/-}\), Rad18\(^{-/-}\) and Brca1\(^{-/-}\) and Brca2\(^{-/-}\) cells for entecavir. Six hours after exposure to 100\(\mu\)M [25,36] entecavir, Parp1\(^{-/-}\), Rad18\(^{-/-}\) and Brca1\(^{-/-}\) exhibit more numbers of \(\gamma\)-H2AX foci when compared with WT cells figure 1 and 2. The increased accumulation of \(\gamma\)-H2AX in nuclei of Parp1\(^{-/-}\), Rad18\(^{-/-}\) and Brca1\(^{-/-}\) cells suggested increased DNA damages, which is consistent with hypersensitivity of these cells to entecavir. This research article describes 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. 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. The majority of CHB patients need long-term treatment with NAs Entecavir, a carbocyclic nucleoside analog, possesses potent and selective activity. Entecavir induces a rapid biochemical and virologic response [37], in CHB patients and has a high genetic barrier to resistance. These characteristics make it recommended as a first-line antiviral therapy for patients with CHB by international guidelines. 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 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. However, further studies remain to be done to gain a better understanding of the genotoxicity mechanisms of entecavir [27,28].

**Patent information**

Bristol-Myers Squibb was the original patent holder for Baraclude, the brand name of entecavir in the US and Canada. The drug patent expiration for Baraclude was in 2015. On August 26, 2014, Teva pharmaceuticals USA gained FDA approval for generic equivalents of Baraclude 0.5mg and 1mg tablets; Hetero Labs received such approval on August 21, 2015; and Aurobindo Pharma on August 26, 2015. Chronic hepatitis B virus infection is one of the most severe liver diseases in morbidity and death rate in the worldwide range. At present, pharmaceuticals for treating Chronic Hepatitis B (CHB) virus infection are classified to interferon \(\alpha\) and nucleoside/nucleotide analogue, i.e., Lamivudine and Adefovir. However, these pharmaceuticals can not meet needs for doctors and patients in treating chronic hepatitis B virus infection because of their respective limitation. Entecavir (ETV) [17,23,29,30,38], is referred to as a 2′-cyclopentyl deoxyguanosine (BMS2000475) which belongs to analogues of Guanine nucleotide and is phosphorylated to form an active triple phosphate in vivo. The triple phosphate of entecavir inhibits HBV polymerase by

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\text{C}_3\text{H}_5\text{N}_3\text{O}_4\text{O}_3. \text{ Entecavir is a white to off-white powder [35]. It is slightly soluble in water (2.4mg/mL), and the pH of the saturated solution in water is 7.9 at 25°C±0.5°C. Baraclude film-coated tablets are available for oral administration in strengths of 0.5mg and 1mg of Entecavir.}
\]
Entecavir was successfully developed by Bristol-Myers Squibb Co. of USA first and the trademark of the product formulation is Baraclude™, including two types of formulations of tablet and oral solution having 0.5mg and 1mg of dosage. Chinese patent No. CN1310999 made by COLONNO, Richard J et al., discloses a low concentration of entecavir 0.005mg. Chinese patent No. CN11329379 made by COLONNO, Richard J et al., discloses a low concentration of entecavir 0.005mg. The chemical name of entecavir is 2'-deoxyguanosine-5'-triphosphate as a nature substrate of HBV polymerase, so as to achieve the purpose of effectively treating chronic hepatitis B virus infection and have strong anti-HBV effects [18].

Entecavir scale up process

To further investigate entecavir-induced DNA damages, we measured cytologically detectable chromosomal aberration in chromosome spreads [39,40]. WT, Parp1−/−, Rad18−/−, Brca1−/− and Rev3−/− cells were exposed to entecavir 200nM from 3 to 24 hours (Figure 3 and 4). Interestingly, WT, Rad18−/−, Rev3−/−, Parp1−/− and Brca1−/− cells demonstrated a monophasic pattern of induced chromosome breaks; the peaks were detectable at 12, 12, 15 and 16 hours respectively (Figure 3). The peaks were significantly higher in DNA[41] 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 double-strand break. The increased chromosomal aberrations in Parp1−/−, Brca1−/−, Rad18−/− and Rev3−/− when compared with WT [42], just reflected these genes have critical role in preventing entecavir-induced chromosome aberrations.

Increased frequency of Chromosomal Aberrations (CAs) in DNA repair-deficient cells and WT treated with entecavir (200nM) from 3 hours to 24 hours. Data are derived from 50 metaphase cells for each treatment. The experiments were independently repeated three times for statistical analysis. Values shown are mean±SD. *P<0.05 compared to WT. The differences between the WT and DNA [39,40,43], repair-deficient cell lines were tested for statistical significance using t-test.

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

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As shown in scheme 1, compound 3 was prepared as a single diastereomer from 3kg of 92% ee (S)-(+)-carvone via a two-step
different techniques listed in Table 1. The work also includes the methodology development and the complete validation [49] as per ICH guidelines. Hitherto, there is no article for the quantification and determination of diastereomeric impurities of Entecavir in drug substances and drug products. This is a novel and sensitive method for Entecavir, a carbocyclic 2’-deoxyxanosine 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 64nM, which was based upon the maximal clinical exposure concentration 30nM [50,51], to analyze the sensitivity of a panel of DNA repair deficient DT40 cells to entecavir. These cells include SSB repair deficient Parp1−/−, BER repair deficient Polβ−/−, NER mutant XPA−/−, HR repair mutants Brca1−/−, Brca2−/−, Xrcc2−/− and Cip1/S532A−/−, NHEJ repair mutant Ku70−/−, PRR mutants Ubc13−/−, Rad18−/− and Rev3−/− as well as flap structure-specific endonuclease 1 mutant Fem1−/−. 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 Cip1/S532A−/− were hypersensitive to CPT, not entecavir. Further immunofluorescent 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 [52].

Figure 3: DNA repair-deficient cells showed a marked increase in entecavir-induced chromosome breaks.

transformation including a stereoselective epoxidation and chlorohydrin formation from the newly formed epoxide. Tosylation of the sec-hydroxyl group of compound 3 afforded 4.25kg of product 4 (60% yield over 3 steps) in 100% ee after recrystallization from MeOH. This ultra-pure intermediate was then reacted with mCPBA to afford epoxide 5, which was converted into diol 6 after treatment with dilute aqueous sulfuric acid. Protection of the diol with dithioxypropano afforded 3.4kg of intermediate 7 (67% over 3 steps). This product was treated with sodium methoxide in methanol to initially provide the cis-substituted Favorzki rearrangement product 8a, which upon isomerization gave the thermodynamically more stable cyclopentanecarboxylate 8 under the reaction conditions, though the epimerization was incomplete even after being stirred for 24 hours (50g scale) at room temperature. Fortunately, the problem was solved by using Methyl t-Butyl Ether (MTBE)/methanol as the solvent and the reaction was complete in less than 17 hours (50g scale). Effective chromatographic separation was achieved on a C18 stationary phase (150×4.6mm, 3.5microns particles) with the economical and simple mobile phase combination delivered in an isocratic mode at a flow rate of 1.0mlmin−1 at 254nm. In the developed method, the resolution between Entecavir and its diastereomeric impurities was found to be greater than 2.0. regression analysis shows an r2 value (correlation coefficient) greater than 0.999 for Entecavir and for its diastereomeric impurities. This method was capable to detect Entecavir and its diastereomeric impurities at a level below 0.009% with respect to test concentration of 500µgml−1 for a 20µL injection volume. The method has shown good, consistent recoveries for diastereomeric impurities (95-105%). The test solution was found to be stable in the diluent for 48h. The drug was subjected to stress conditions. The mass balance was found close to 99.5%. Entecavir, HPLC, RP-LC, LC development, validation, diastereomers and several methods have been developed for the determination of Entecavir and its enantiomers by HPLC, LCMS techniques [27,28]. Entecavir is an oral antiviral drug used in the treatment of Hepatitis B Virus (HBV) infection. Entecavir is a reverse transcriptase inhibitor. It prevents the hepatitis B virus from multiplying and reduces the amount of virus in the body. More specifically, it is a deoxyxanosine analogue that inhibits reverse transcription, DNA replication and transcription in the viral replication process. Entecavir belonging to the chemical class of purine derivatives and chemically it is 2-amino-9-{[(1S, 3R, 4S)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-3H-purin-6-one with molecular formula C19H15N3O4. Entecavir is a white to off-white powder. It is slightly soluble in water (2.4mg/mL), and the pH of the saturated solution in water is 7.9 at 25°C±0.5°C. Baraclude film-coated tablets are available for oral administration in strengths of 0.5mg and 1mg of Entecavir. The methods of [1,7] describes about the determination of Entecavir in tablet dosage form by LC. The LC method of [44,45] defines about the estimation of Entecavir in bulk as well as pharmaceutical dosage forms. The method of [9,10,46] explains about the determination of Entecavir using LC-MS techniques in drugs and plasma. The method of [11,12] clarifies, the determination of Entecavir by spectrophotometric procedure. The approaches of [13] states that the optical isomer of Entecavir through enantiospecific HPLC. The tactics of [47] enlightens the determination of related compounds of Entecavir by LC. The method of [48] instructs about the determination of Entecavir by voltammetry in formulated dosage forms. This research article describes a simple, sensitive and cost effective mobile phase method for determination/quantitation of diastereomeric impurities of Entecavir in drug substances as well as in drug products. Comparison of
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. A multitude of factors trigger SSBs. Erroaneous incorporation of ribonucleotides into DNA is the commonest source of endogenous SSBs. Parp1 is a sensor protein, which plays an important role in DNA SSB detection. In the current study, we found that Parp1+ cells exhibited the hypersensitive response 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 [53], which respectively defective in HR and NHEJ, two major pathways for double strand breaks repair, 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 translesion polymerases, such as Polη and Rev1, to the lesions through protein-protein interactions, suggesting its critical role in PRR [54-56]. Currently, we found Rev3− and Rad18− were also sensitive to entecavir and had increased entecavir-induced chromosomal aberrations (Figure 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, where as Rev3 gene encodes the catalytic subunit of DNA Polζ, which is involved in TLS, one of PRR pathway [54, 57-59]. Furthermore, Ubc13, a K63-linked E2 Ub-conjugating enzyme, have been proved to function on both HR and error-free PRR. 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 (Figure 4).

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 [36,62,63].
Figure 6: Sensitivity of Wild-Type (WT) and isogenic DNA-repair deficient DT40 clones to entecavir or CPT.

Conclusion

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. Some data already published evaluate the efficacy and safety of Entecavir (ETV) among Chronic Hepatitis B (CHB) nucleos (t) ide-naive Egyptian patients results ETV proved to have a potent antiviral efficacy and safety in nucleoside/tide-naive Egyptian patients. Rate of HBV DNA undetectability was higher in patients above 40 years of age and in patients who initially had a low viral load. ETV was well tolerated during the treatment period with a good overall safety profile. Ref: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5916638

References


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