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# Mitomycin, 5-fluorouracil, leflunomide, and mycophenolic acid directly promote hepatitis B virus replication and expression in vitro

Jie Ruan<sup>1,2</sup>, Shuo Sun<sup>1</sup>, Xin Cheng<sup>1</sup>, Pengyu Han<sup>1</sup>, Yinge Zhang<sup>1</sup> and Dianxing Sun<sup>1\*</sup>

## Abstract

**Background:** Reactivation of hepatitis B virus is a common complication that occurs in patients with hepatitis B virus (HBV) infection who have received cytotoxic chemotherapy or immunosuppressive therapy. This clinical phenomenon not only occurs in overt HBV infection patients but also occurs in patients with resolved HBV infection. Previous research has confirmed that epirubicin and dexamethasone can stimulate HBV replication and expression directly rather than indirectly through immunosuppression. Mitomycin and 5-fluorouracil are currently used as cytotoxic chemotherapy drugs for cancer patients. Leflunomide and mycophenolic acid are regarded as immunosuppressants for autoimmune diseases, and numerous clinical studies have reported that these drugs can reactivate HBV replication. In this study, we aimed to investigate whether mitomycin, 5-fluorouracil, leflunomide and mycophenolic acid induce HBV reactivation directly rather than indirectly through immunosuppression.

**Methods:** To observe the effect of mitomycin, 5-fluorouracil, leflunomide and mycophenolic acid on HBV replication and expression, we employed HepG2.2.15 and HBV-NLuc-35 cells as a cell model. Next, by native agarose gel electrophoresis (NAGE), quantitative PCR (qPCR), luciferase assay and HBV e antigen (HBeAg) enzyme-linked immunosorbent assay (ELISA) we detected changes in HBV replication and expression induced by these drugs. We also investigated whether lamivudine could inhibit the observed phenotype. SPSS 18.0 software was employed for statistical analysis, One-way ANOVA was used to compare multiple groups.

**Results:** Expression of HBV capsids and HBeAg in HepG2.2.15 cells was increased by increasing concentration of mitomycin, 5-fluorouracil, leflunomide, and mycophenolic acid. This phenomenon was also demonstrated in HBV-NLuc-35 cells, and the expression of capsids and luciferase activity increased in the same concentration-dependent manner. Replication levels of intracellular capsid DNA and extracellular HBV DNA in HepG2.2.15 cells gradually increased in a dose-dependent manner. In addition, although epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide and mycophenolic acid enhanced HBV replication, lamivudine inhibited this process.

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\* Correspondence: [sundianxing@hotmail.com](mailto:sundianxing@hotmail.com)

<sup>1</sup>The Liver Disease Diagnosis and Treatment Center of PLA, Bethune International Peace Hospital, Zhongshanxi street, Shijiazhuang 050082, Hebei Province, China

Full list of author information is available at the end of the article



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**Conclusion:** Our study confirmed that mitomycin, 5-fluorouracil, leflunomide and mycophenolic acid directly upregulated HBV replication and expression in vitro. This effect was investigated not only in HepG2.2.15 cells but also in the HBV-NLuc-35 replication system. Moreover, this effect could be prevented by nucleoside analogs, such as lamivudine (LAM). Thus, for patients with HBV infection, prophylactic antiviral therapy is necessary before receiving cytotoxic chemotherapy or immunosuppressive therapy.

**Keywords:** Reactivation of hepatitis B virus; Mitomycin, 5-fluorouracil, Leflunomide, Mycophenolic acid, Lamivudine

## Background

Reactivation of hepatitis B is a common complication that occurs in patients with HBV infection who receive cytotoxic chemotherapy or immunosuppressive therapy [1–4]. The first case of hepatitis B reactivation was described in 1975 [5, 6], and the studies reported that the patient was HBV surface antigen positive and received cytotoxic chemotherapy for cancer. The sign of hepatitis B reactivation is the reoccurrence of HBV DNA in the serum of patients with cured or inactive infection, often accompanied by inflammation activity in the liver and could occur spontaneously but mostly occurs during or after cytotoxic chemotherapy or immunosuppressive therapy, which could lead to acute hepatitis, liver failure, or even death [7, 8]. Nevertheless, most cases are not clinically significant or are not diagnosed until the infection has developed into active hepatitis, which leads to the interruption of cytotoxic chemotherapy or immunosuppressive therapy and a poor prognosis [9, 10].

HBV reactivation starts with an enhancement of HBV replication and occurs shortly after cytotoxic chemotherapy or immunosuppressive therapy [11, 12], and the mechanism of this phenomenon has not been determined thus far. Previous research had confirmed that dexamethasone [13] and epirubicin [14, 15] can increase HBV replication and expression in vitro, which suggests that dexamethasone and epirubicin could stimulate viral replication and expression directly rather than indirectly through immunosuppression. Numerous clinical studies have reported that mitomycin [1], 5-fluorouracil [1, 16], leflunomide [17], and mycophenolic acid [18] treatment can reactivate HBV replication. In this study, we aimed to investigate whether mitomycin, 5-fluorouracil, leflunomide and mycophenolic acid induce HBV replication in the same manner; thus, we employed HepG2.2.15 cells as a cell model [19]. Moreover, we also investigated the effect of these cytotoxic chemotherapy drugs and immunosuppressants on the new HBV replication system HBV-NLuc-35 cells [20], which are generated by transfecting the pTRE-sNLuc vector into HepG2 TA-7 cells [21, 22] and could stably secrete secNLuc recombinant HBV particles, detected the level of secNLuc expression to evaluate HBV replication and expression.

## Methods

### Drugs, cell culture and toxicity test

Epirubicin, mitomycin (Hisun, China), 5-fluorouracil (Shanghai Xudong Haipu Pharmaceutical Co. Ltd., China), dexamethasone (Solarbio, China), lamivudine (Xinjialin Biotech Ltd.), were diluted by double distilled water and stored at 4 °C; leflunomide (Sangon Biotech, China), mycophenolic acid (BioDee, China), were diluted in dimethyl sulfoxide and stored at 4 °C; and HepG2.2.15 and HBV-NLuc-35 cells were cultured as previously described [21]. For detecting the cell proliferation toxicity of drugs, HepG2.2.15 cells were treated with increasing concentrations of epirubicin (0, 0.1, 0.5, 1, 2.5 and 5 μM), mitomycin (0, 0.5, 1, 2.5, 5 and 10 μM), 5-fluorouracil (0, 5, 10, 50, 100 and 150 μM) for 16 h, and increasing concentrations of dexamethasone (0, 0.1, 1, 10, 50 and 100 μM), leflunomide (0, 10, 50, 100, 200 and 300 μM), and mycophenolic acid (0, 3, 15, 30, 75 and 150 μM) for 5 days. In addition, the cell proliferation toxicity test of HBV-NLuc-35 cells under different concentrations of Mitomycin treatment was the same as described for HepG2.2.15 cells. Next, the cell proliferation toxicity test was performed by using the CCK-8 reagent as recommended by the manufacturer (Dojindo, Japan).

### Native agarose gel electrophoresis (NAGE), quantitative PCR (qPCR)

Cytoplasmic intact capsids were detected by anti-HBV core protein antibody after native agarose gel electrophoresis (NAGE) as previously described [23]. Moreover, capsid DNA was detected by molecular hybridization with the  $\alpha$ -<sup>32</sup>P-labeled HBV DNA probe [24]. HBV DNA copies in the supernatant were detected by a commercial HBV DNA kit (Kehua, Shanghai, China) on a SLAN™ Real-Time PCR system (Hongshi, Shanghai, China).

### Luciferase activity, HBeAg ELISA

Luciferase activity of the culture supernatant was detected by the Nano-Glo™ luciferase assay reagent as recommended by the manufacturer (Promega). HBeAg in culture supernatants was assayed by commercial ELISA kits (Chemclin Biotech Co. Ltd., Beijing, China).

### Drug susceptibility assays

HepG2.2.15 and HBV-NLuc-35 cells were seeded in 96-well plates at a density of  $2 \times 10^4$  cells/well. For observing the effect of the drugs, the cells were incubated with increasing concentrations of epirubicin, mitomycin, and 5-fluorouracil for 16 h and treated with increasing concentrations of dexamethasone, leflunomide, and mycophenolic acid for 5 days. Intact capsids and capsid DNA of cytoplasmic lysates were analyzed by NAGE. Luciferase, HBeAg and HBV DNA in the cell supernatant were assessed by the Nano-Glo™ luciferase assay reagent, ELISA, and qPCR, respectively. For observing lamivudine sensitivity, HepG2.2.15 cells were treated with increasing concentrations of lamivudine (0, 1, 10, 20 and 50  $\mu\text{M}$ ) for 24 h and then treated with the following drugs for 16 h: 1  $\mu\text{M}$  epirubicin, 1  $\mu\text{M}$  mitomycin, or 100  $\mu\text{M}$  5-fluorouracil or were similarly treated with the following drugs for 5 days: 10  $\mu\text{M}$  dexamethasone, 50  $\mu\text{M}$  leflunomide, or 30  $\mu\text{M}$  mycophenolic acid. Then, cell media was replaced with drug-free culture media, followed by incubation with increasing concentrations of lamivudine for 24 h. Subsequently, the cytoplasmic lysates were analyzed for intracellular capsid DNA by NAGE, and HBV DNA in the culture supernatant was detected by qPCR.

### Statistical analysis

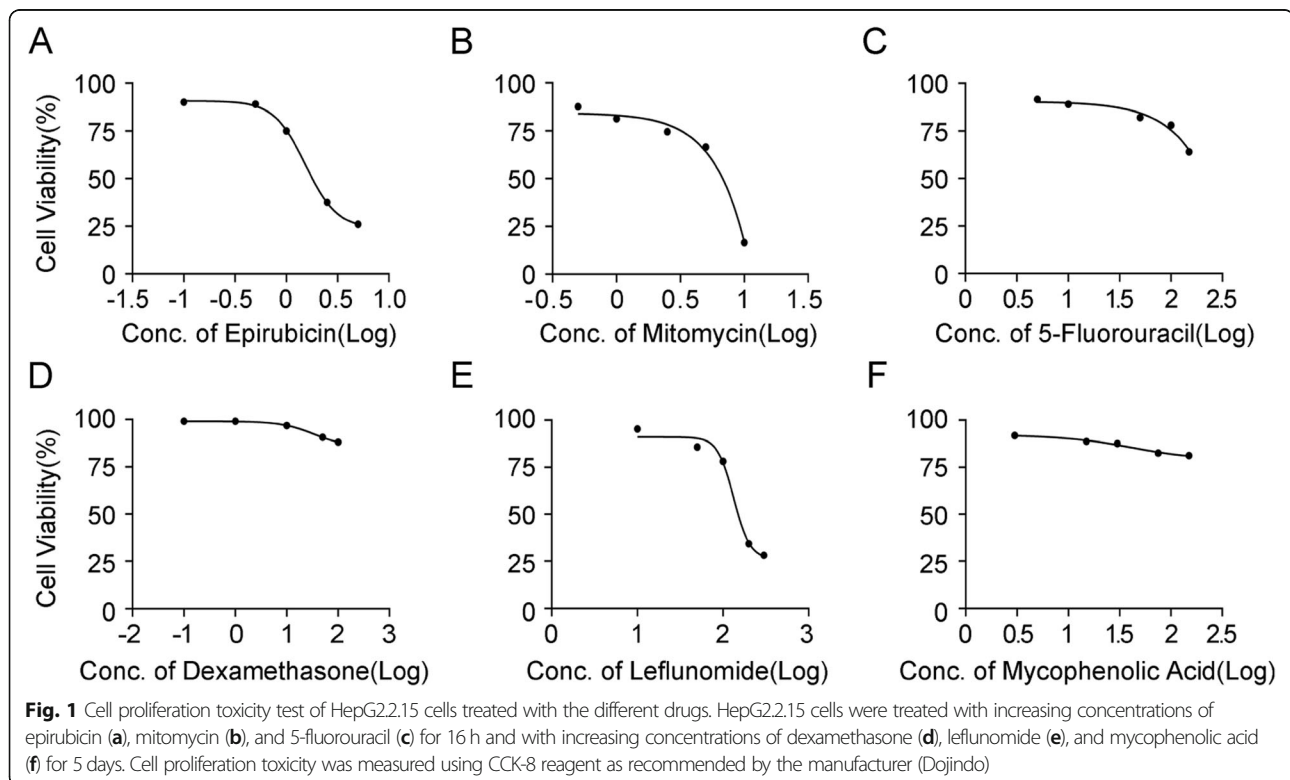
SPSS 18.0 software was employed for statistical analysis. We calculated the median lethal dose ( $\text{LD}_{50}$ ) for drugs

with modules in the SPSS 18.0 package, and the 95% CI of  $\text{LD}_{50}$  was obtained at the same time. One-way ANOVA was used to compare multiple groups, and all data were expressed as the mean with standard deviation (SD).  $P < 0.05$  was regarded as statistically significant.

## Results

### Cell proliferation toxicity test

We used the CCK-8 reagent to detect the cell proliferation toxicity of drugs in HepG2.2.15 cells which treated with different concentrations of epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid at the indicated times. The results showed that the proliferation activity of HepG2.2.15 cells was reduced below 50% with concentrations of epirubicin up to 2.5  $\mu\text{M}$  (Fig. 1a). The  $\text{LD}_{50}$  was 2.085  $\mu\text{M}$ , and the 95% CI of the  $\text{LD}_{50}$  was 1.3–3.394  $\mu\text{M}$ . The same result was observed for mitomycin up to 10  $\mu\text{M}$  (Fig. 1b), leflunomide up to 200  $\mu\text{M}$  (Fig. 1e). The  $\text{LD}_{50}$  of these drugs were 5.405  $\mu\text{M}$  and 172.2  $\mu\text{M}$ , and the 95% CIs of the  $\text{LD}_{50}$  were 2.507–12.43  $\mu\text{M}$  and 89.99–341.7  $\mu\text{M}$ , respectively. Regarding 5-fluorouracil (Fig. 1c), dexamethasone (Fig. 1d) and mycophenolic acid (Fig. 1f), when concentrations up to 150  $\mu\text{M}$ , 100  $\mu\text{M}$  and 150  $\mu\text{M}$ , respectively, were used, the proliferation activity of HepG2.2.15 cells was decreased but above 50%. The  $\text{LD}_{50}$  of these drugs were 276  $\mu\text{M}$ , 641.9  $\mu\text{M}$  and 459.1  $\mu\text{M}$ , and the 95% CIs of the  $\text{LD}_{50}$  were (176.4–480.5)  $\mu\text{M}$ , (471.0–964.4)  $\mu\text{M}$  and (219.3–1877)  $\mu\text{M}$ , respectively. Hence, for further study, we

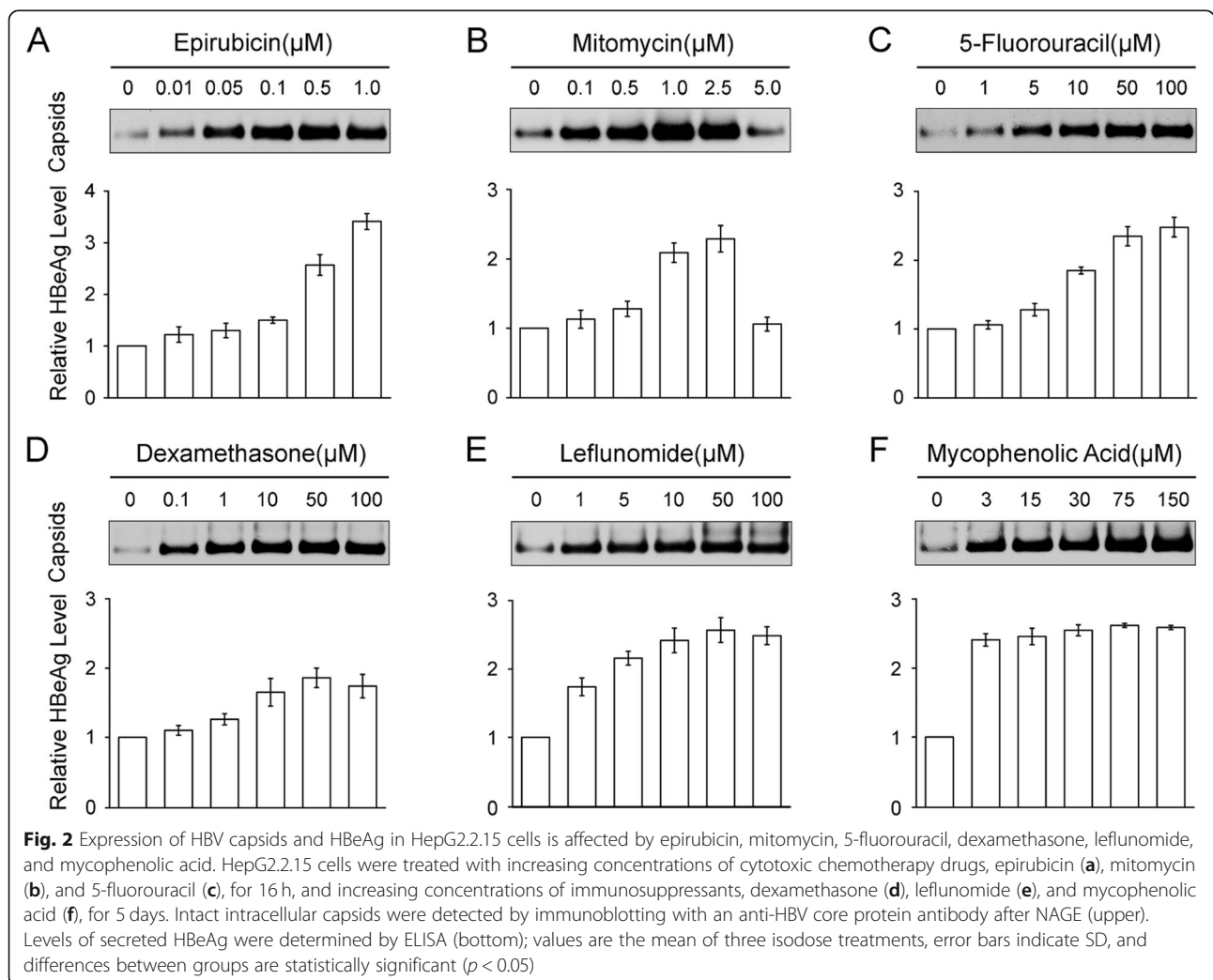


chose the following lower toxicity concentrations of the drugs: epirubicin (0, 0.01, 0.05, 0.1, 0.5 and 1  $\mu$ M), mitomycin (0, 0.1, 0.5, 1, 2.5 and 5  $\mu$ M), 5-fluorouracil (0, 1, 5, 10, 50 and 100  $\mu$ M), dexamethasone (0, 0.1, 1, 10, 50 and 100  $\mu$ M), leflunomide (0, 1, 5, 10, 50 and 100  $\mu$ M), and mycophenolic acid (0, 3, 15, 30, 75 and 150  $\mu$ M).

**Concentration-dependent expression of HBV antigens in HepG2.2.15 cells stimulated by epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid**

To test if HBV antigen expression was affected by epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid, we employed HepG2.2.15 cells as a cell model, and treated the cells with various doses of drugs. According to the cell proliferation toxicity test results, we selected a safe dose and exposed HepG2.2.15 cells to increasing concentrations of drugs to observe the effect. Following treatment with increasing concentrations of drugs, the formation of

HBV capsids in inside of cells was upregulated in a dose-dependent manner. NAGE results showed that capsid signals from HepG2.2.15 cells treated with gradually increasing concentrations, increased with epirubicin up to 1  $\mu$ M (Fig. 2a, upper), mitomycin up to 2.5  $\mu$ M (Fig. 2b, upper), 5-fluorouracil up to 100  $\mu$ M (Fig. 2c, upper), dexamethasone up to 50  $\mu$ M (Fig. 2d, upper), leflunomide up to 50  $\mu$ M (Fig. 2e, upper), and mycophenolic acid up to 150  $\mu$ M (Fig. 2f, upper). The comparable HBeAg ELISA results revealed that the level of HBeAg in the culture supernatant was over 3 times that of the untreated control when the concentration of epirubicin increased to 1  $\mu$ M (Fig. 2a, bottom). In addition, when mitomycin increased to 2.5  $\mu$ M (Fig. 2b, bottom), 5-fluorouracil to 100  $\mu$ M (Fig. 2c, bottom), dexamethasone to 50  $\mu$ M (Fig. 2d, bottom), leflunomide to 50  $\mu$ M (Fig. 2e, bottom), and mycophenolic acid to 150  $\mu$ M (Fig. 2f, bottom), the level of HBeAg was over 2 times that of the untreated control. Notably, when the concentration of mitomycin was up to 5  $\mu$ M, both the

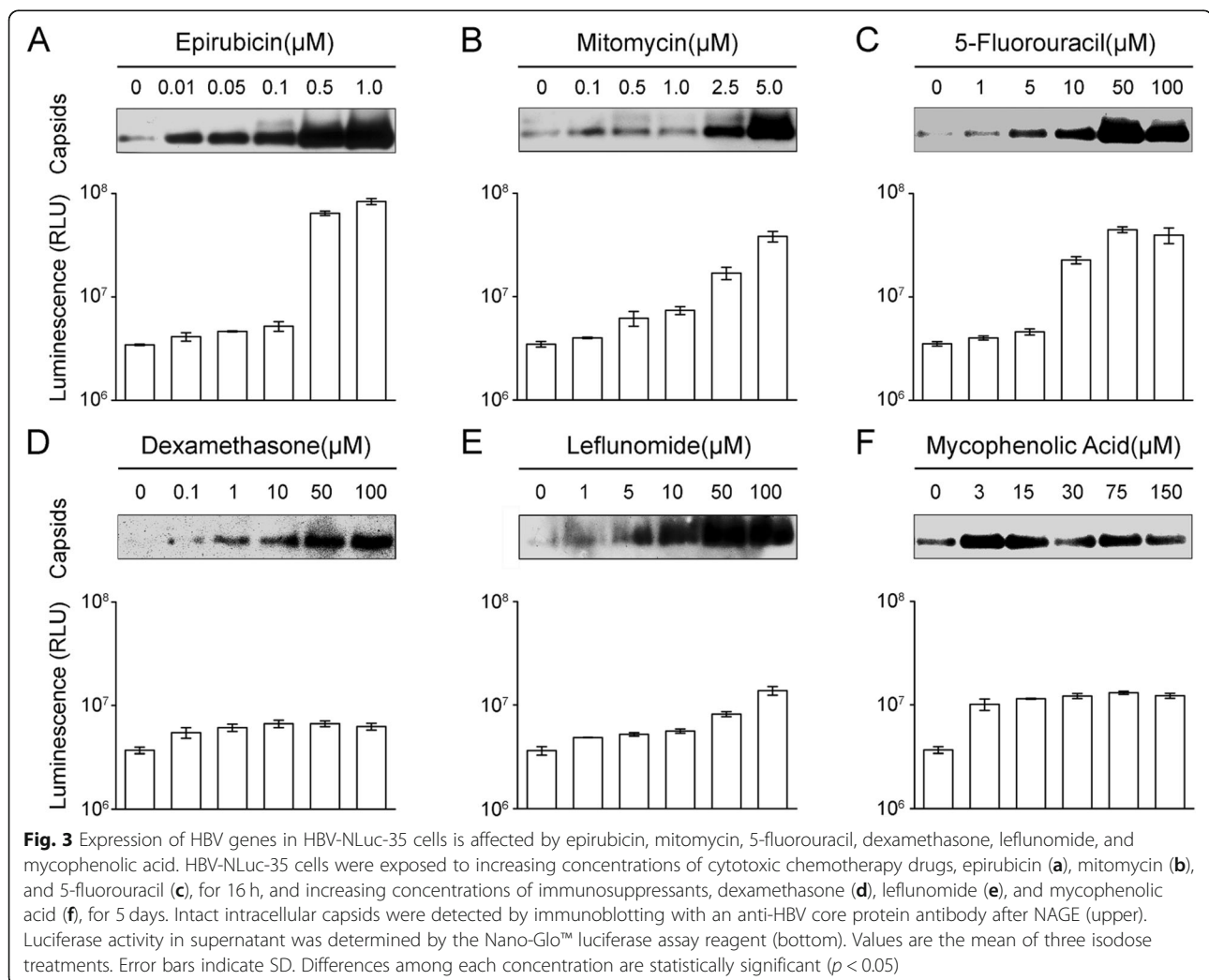


expression level of HBV capsids and HBeAg declined because HepG2.2.15 cells were more sensitive to toxicity at this concentration. Altogether, expression of HBV capsids and HBeAg increased in HepG2.2.15 cells stimulated with increasing concentrations of epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid.

### Concentration-dependent expression of HBV genes in HBV-NLuc-35 cells induced by epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid

To investigate whether this effect was similar in other HBV replication and expression cell lines, we employed HBV-NLuc-35 cells. These cells were exposed to increasing doses of epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid in the same as with the HepG2.2.15 cells. The formation of HBV capsids in inside of cells was detected by NAGE,

and the results showed that capsids signals from HBV-NLuc-35 cells also gradually increased with increasing concentrations (Fig. 3, upper). However, one difference was that when the concentration of Mitomycin increased to 5  $\mu\text{M}$ , the capsid signals from HBV-NLuc-35 cells was stronger compared with the weaker signals from HepG2.2.15 cells, the reason was that HBV-NLuc-35 cells were more tolerant than HepG2.2.15 cells at this concentration (Supplemental figure). Moreover, we also detected the luciferase level in the cell supernatant, and an identical result was observed (Fig. 3, bottom). The luciferase level was up to  $10^8$  RLU when the concentration of epirubicin increased to 1  $\mu\text{M}$  compared with the lower  $10^6$  RLU in the negative control group (Fig. 3a, bottom). In addition, when mitomycin was up to 5  $\mu\text{M}$  (Fig. 3b, bottom), 5-fluorouracil to 50  $\mu\text{M}$  (Fig. 3c, bottom), leflunomide to 50  $\mu\text{M}$  (Fig. 3e, bottom), and mycophenolic acid to 3  $\mu\text{M}$  (Fig. 3f, bottom), the luciferase level increased to over  $10^7$  RLU compared to the baseline  $10^6$

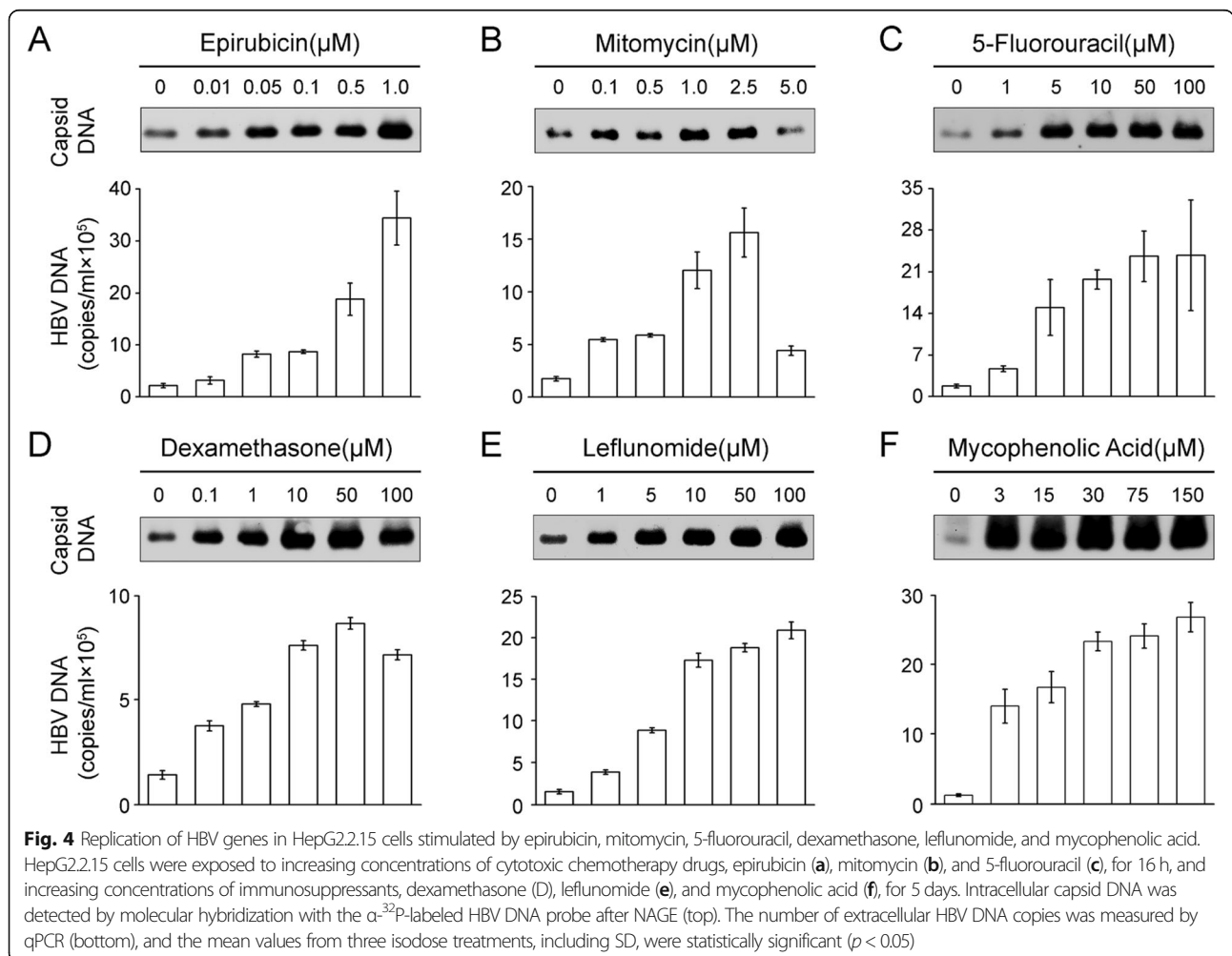


RLU. The effect of dexamethasone was relatively weaker, and the luciferase level was under  $10^7$  RLU but over baseline  $10^6$  RLU when dexamethasone was up to  $50 \mu\text{M}$  (Fig. 3d, bottom). These data demonstrated that treatment with epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid promoted HBV gene expression not only in HepG2.2.15 cells but also in another HBV replication and expression cell line, HBV-NLuc-35 cells.

**Epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid dose-dependently enhance HBV DNA replication in HepG2.2.15 cells**

The effect of epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide and mycophenolic acid on HBV replication was examined after treatment with increasing doses of these drugs in HepG2.2.15 cells. The intracellular capsid DNA was analyzed by Native Southern blotting using radioactively labeled HBV DNA as a probe. The results revealed that capsid DNA signals were gradually enhanced with increasing doses and were stronger

at  $1 \mu\text{M}$  epirubicin (Fig. 4a, upper),  $2.5 \mu\text{M}$  mitomycin (Fig. 4b, upper),  $100 \mu\text{M}$  5-fluorouracil (Fig. 4c, upper),  $50 \mu\text{M}$  dexamethasone (Fig. 4d, upper),  $100 \mu\text{M}$  leflunomide (Fig. 4e, upper) and  $150 \mu\text{M}$  mycophenolic acid (Fig. 4f, upper). For comparison, extracellular HBV DNA was detected by qPCR, revealing that HBV DNA copies in the supernatant gradually increased in a dose-dependent manner (Fig. 4, bottom). When the concentration of epirubicin was up to  $1 \mu\text{M}$ , the cells produced as many as  $3 \times 10^6$  copies/ml of virions compared with  $10^5$  copies/ml in the untreated cells (Fig. 4a, bottom). A similar result was observed with mitomycin up to  $2.5 \mu\text{M}$  (Fig. 4b, bottom), 5-fluorouracil up to  $100 \mu\text{M}$  (Fig. 4c, bottom), leflunomide up to  $100 \mu\text{M}$  (Fig. 4e, bottom) and mycophenolic acid up to  $150 \mu\text{M}$  (Fig. 4f, bottom). In addition, the effect of dexamethasone was weaker which only produced approximately  $8 \times 10^5$  copies/ml of virions with concentrations up to  $50 \mu\text{M}$  (Fig. 4d, bottom). Notably, when the concentration of mitomycin was up to  $5 \mu\text{M}$ , the replication level of both intracellular and extracellular HBV DNA significantly



declined. A reasonable explanation was that the cell proliferation toxicity of 5  $\mu\text{M}$  mitomycin was strong enough to affect the growth of HepG2.2.15 cells. These data confirmed that epirubicin, mitomycin, 5-fluorouracil, leflunomide, mycophenolic acid, and dexamethasone could stimulate HBV replication in vitro.

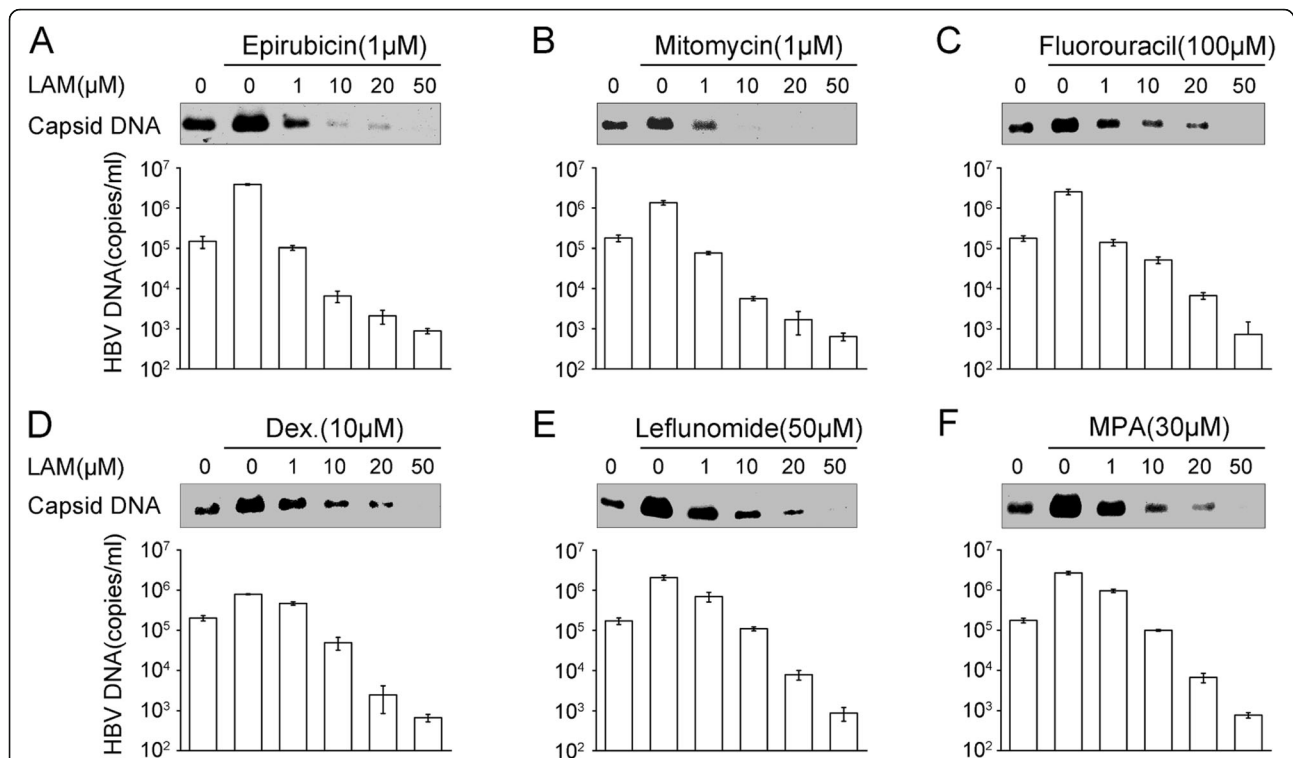
#### Lamivudine inhibits the reactivation of HBV replication induced by epirubicin, mitomycin, 5-fluorouracil, leflunomide, mycophenolic acid and dexamethasone

In this study, we observed the effect of lamivudine (LAM) on HBV replication induced by epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid, the LAM EC<sub>50</sub> value was determined in previous research [21]. Native Southern blotting showed that intracellular capsid DNA signals were stronger in drug-treated cells with the absence of LAM, while almost no capsid DNA signals were detectable at the 50  $\mu\text{M}$  LAM concentration after treating with 1  $\mu\text{M}$  epirubicin (Fig. 5a, upper), 1  $\mu\text{M}$  mitomycin (Fig. 5b, upper), 100  $\mu\text{M}$  5-fluorouracil (Fig. 5c, upper), 10  $\mu\text{M}$  dexamethasone (Fig. 5d, upper), 50  $\mu\text{M}$  leflunomide (Fig. 5e, upper), or 30  $\mu\text{M}$  mycophenolic acid (Fig. 5f,

upper). In addition, untreated HepG2.2.15 cells produced approximately  $10^5$  copies/ml of virions. However, the concentration of LAM up to 50  $\mu\text{M}$  decreased HBV copy numbers to the lower limit of detection compared with the HBV level above  $10^6$  copies/ml in 1  $\mu\text{M}$  epirubicin (Fig. 5a, bottom), 1  $\mu\text{M}$  mitomycin (Fig. 5b, bottom), 100  $\mu\text{M}$  5-fluorouracil (Fig. 5c, bottom), 10  $\mu\text{M}$  dexamethasone (Fig. 5d, bottom), 50  $\mu\text{M}$  leflunomide (Fig. 5e, bottom) and 30  $\mu\text{M}$  mycophenolic acid (Fig. 5f, bottom). These results confirmed that epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid could enhance HBV DNA replication; however, this activation effect could not lower lamivudine sensitivity.

#### Discussion

Reactivation of hepatitis B is a common complication in HBV infection patients undergoing cytotoxic chemotherapy or immunosuppressive therapy for cancer [2, 4], autoimmune diseases [7, 25] and organ transplantation [26]. Several clinical studies have reported that chemotherapy or immunosuppressive therapy, including epirubicin [16, 27, 28], mitomycin [1], 5-fluorouracil [1, 16],



**Fig. 5** Lamivudine susceptibility assay. Lamivudine inhibits the activation of HBV replication in HepG2.2.15 cells treated with epirubicin, mitomycin, 5-fluorouracil, dexamethasone, leflunomide, and mycophenolic acid. HepG2.2.15 cells were treated with increasing concentrations of lamivudine for 24 h and then treated with 1  $\mu\text{M}$  epirubicin (a), 1  $\mu\text{M}$  mitomycin (b), or 100  $\mu\text{M}$  5-fluorouracil (c), for 16 h, or 10  $\mu\text{M}$  dexamethasone (d), 50  $\mu\text{M}$  leflunomide (e), or 30  $\mu\text{M}$  mycophenolic acid (f), for 5 days. After incubation with increasing concentrations of lamivudine for 24 h, the cells were cultured in fresh media without drugs. Subsequently, intracellular capsid DNA was detected by molecular hybridization with the  $\alpha$ - $^{32}\text{P}$ -labeled HBV DNA probe after NAGE (top). The number of extracellular HBV DNA copies was measured by qPCR (bottom), and the mean values from three isodose treatments, including SD, were statistically significant ( $p < 0.05$ )

leflunomide [17], mycophenolic acid [18], and dexamethasone [13] treatment, could reactivate HBV replication.

Epirubicin, mitomycin, and 5-fluorouracil are currently used as cytotoxic chemotherapy drugs for cancer patients, such as those with hematological malignancies [29, 30], breast cancer [29, 30], digestive cancer [31], gastrointestinal cancer [32], and lung cancer [33]. In the clinic, cancer patients undergoing chemotherapy, including mitomycin treatment [1] or 5-fluorouracil treatment [1, 16], are at an increased risk of HBV reactivation. The effects of epirubicin on enhancing HBV replication have been reported [14]; however, the effects of mitomycin and 5-fluorouracil on HBV replication have not been investigated. HepG2.2.15 cells [19] were widely used to research HBV replication and expression because these cells can stably secrete HBV viral particles. HBV-NLuc-35 cells [20], which stem from HepG2 TA-7 cells transfected with the pTRE-sNLuc vector. In terms of pTRE-sNLuc vector, the secNLuc reporter gene binds downstream of the HBV C open reading frame (ORF) and upstream of the HBV P ORF. More importantly, the transcription product of the secNLuc gene is fused to recombinant HBV pregenomic RNA (pgRNA), which can be used to detect the expression and replication of HBV genes. By NAGE and Native Southern blotting, we observed that mitomycin and 5-fluorouracil could directly induce HBV replication and expression in vitro, rather than indirectly by affecting immunosuppression. This phenomenon was further confirmed by HBeAg ELISA, luciferase assay, and qPCR results. Different from epirubicin and 5-fluorouracil in concentration-dependent manner, when the concentration of mitomycin increased to 5  $\mu$ M, the capsid signals from HBV-NLuc-35 cells was stronger compared with the weaker signals from HepG2.2.15 cells. By comparing HepG2.2.15 with HBV-NLuc-35 cells for their cell culturing status under different concentrations of Mitomycin treatment, the LD<sub>50</sub> of Mitomycin in HepG2.2.15 cells was below HBV-NLuc-35, it was indicated that HepG2.2.15 cells were more sensitive to toxicity of Mitomycin than HBV-NLuc-35, consequently, producing different results in both cells. Previous study confirmed that mitomycin can strikingly activated HepG2 cells apoptosis [34]. Thus, we think that the type of HepG2.2.15 cells damage which mitomycin induced was apoptosis. In our study, in spite of plenty of HepG2.2.15 cells apoptosis, the level of HBV expression and replication of residual cells was the same as mitomycin-free according to HBeAg ELISA and qPCR results, supported that mitomycin can induce HBV replication and expression in concentration-dependent manner.

Leflunomide, mycophenolic acid and dexamethasone were regarded as immunosuppressants that could be

used as therapy for autoimmune diseases, such as rheumatoid arthritis [35] and acute rejection of transplanted organs [36]. There was a report that dexamethasone could trigger the severity of hepatitis B virus-related chronic hepatitis in humans and could directly stimulate hepatitis B virus gene replication in vitro [13]. Interestingly, leflunomide has been reported to inhibit the replication of several viruses, such as human polyomavirus type [37, 38], human cytomegalovirus [39], herpes simplex virus type-1 [40], human immunodeficiency virus-1 [41] and respiratory syncytial virus [42], showing broad antiviral activities. However, in the clinic, there is a high risk of hepatitis B reactivation in rheumatoid arthritis patients with HBV infection after receiving leflunomide treatment [17]. Additionally, our study confirmed that leflunomide could significantly stimulate HBV gene expression and replication in vitro. This effect was not only observed in HepG2.2.15 cells but also in HBV-NLuc-35 cells. Mycophenolic acid is a highly effective immunosuppressant that is widely used to treat the rejection of solid organ transplants. As such, several reports have indicated that mycophenolic acid has antiviral activities against dengue virus [43], avian reovirus [44], yellow fever virus [45], and West Nile virus [46], especially hepatitis C virus [47–49] and hepatitis E virus [50]. However, a few studies have reported that the effect of mycophenolic acid on HBV replication ranges from weak suppression to no effect [51, 52]. In this study, we clearly demonstrated that mycophenolic acid could enhance HBV gene expression and replication in vitro, rather than have no effect or antiviral activities.

There were several published researches attempting to explore the molecular mechanism of hepatitis B virus reactivation. Hsu CH suspected that cytotoxic chemotherapy drug induced HBV reactivation due to interfere the normal cell cycle process and block at G2/M –phase [15]. Chen YF demonstrated that cytotoxic chemotherapy drug elevated the expression of the cell cycle regulator p21 (Waf1/Cip1) and CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ) contributed to the reactivation of HBV [14], which could interactive with responsive sites on the HBV promoters and effect the transcription of HBV pgRNA. Moller Rechtman M indicated that peroxisome proliferator-activated receptor-gamma coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) play a central mediator in HBV reactivation induced by cytotoxic chemotherapy drugs, which strongly coactivate for HBV transcription [53]. Huang W showed that immunosuppressant could activate cellular autophagy to induce HBV reactivation [54]. Hoppe-Seyler K proved that immunosuppressant induced HBV reactivation was closely linked with increasing of mitogen-activated protein kinase p38 phosphorylation, which was crucial for pgRNA increasing [55]. HBV pgRNA plays an important role in the HBV life cycle, it serves as mRNA encoding the core protein



(HBcAg) and the viral reverse transcriptase, moreover, it can be packaged into progeny capsids and converted into relaxed circular DNA (RC-DNA) [56]. A new explanation for HBV reactivation is that accumulation of HBV Pol promotes the activity of the viral polymerase [57]. Thus, we speculate that different drugs might have different molecular mechanisms of HBV reactivation, pgRNA might play an important role in this procedure.

The reactivation of hepatitis B occurs not only in overt HBV infected patients who are HBV surface antigen positive (HBsAg+) and receive cytotoxic chemotherapy or immunosuppressive therapy [26] but also in patients with resolved infection that are HBV surface antigen negative (HBsAg-), HBV core antibody positive (HBcAb+), and positive or negative for antibodies against HBV surface antigen (HBsAb+) [58, 59]; consequently, we should pay close attention to this phenomenon in the clinic. Several studies have reported that HBV could maintain a lower level of replication ability in both hepatocytes and peripheral blood mononuclear cells in resolved HBV infect patients [60, 61] and that cccDNA is very difficult to eliminate, even though when undetectable in the serum. This is an explanation why HBV reactivation can occur in patients with resolved HBV infection.

In this study, we observed that LAM could counteract the reactivation of HBV replication induced by epirubicin, mitomycin, 5-fluorouracil, leflunomide, mycophenolic acid and dexamethasone in vitro. LAM (nucleoside analog, NA), as a well-characterized antiviral drug, is used to treat chronic HBV infection [62, 63]. Thus, in the clinic, it is necessary that patients with overt HBV infection receive antiviral therapy before receiving cytotoxic chemotherapy or immunosuppressive therapy [10]. Most importantly, HBV reactivation can occur in patients with resolved HBV infection as well; however, the risk is lower [64]. At present, the controversy continues, and it is not unequivocally agreed whether prophylactic antiviral therapy should be prescribed to patients who are HBsAg negative and HBcAb positive with or without HBsAb positivity. Given our research, cytotoxic chemotherapy drugs and immunosuppressants could directly stimulate the replication and expression of HBV rather than indirectly through immunosuppression. And, given that cccDNA is difficult to eliminate, closely monitoring changes in HBV serological markers to prevent HBV reactivation is necessary when patients with resolved HBV infection receive cytotoxic chemotherapy or immunosuppressive therapy. Hence, screening the most sensitive serological marker is important for monitoring HBV reactivation, and based on our research, HBV pgRNA may be a promising candidate.

## Conclusions

In our study, we confirmed that mitomycin, 5-fluorouracil, leflunomide and mycophenolic acid can directly upregulated HBV replication and expression in vitro rather than indirectly through immunosuppression. Moreover, this effect could be prevented by nucleoside analogs, such as lamivudine (LAM). Thus, HBV reactivation is a serious but avoidable complication of receiving cytotoxic chemotherapy or immunosuppressive therapy and could be prevented with the use of prophylactic antiviral therapy.

## Supplementary information

**Supplementary information** accompanies this paper at <https://doi.org/10.1186/s12985-020-01339-5>.

**Additional file 1: Figure S1.** Cell proliferation toxicity test of HepG2.2.15 and HBV-Nluc-35 cells treated with the different concentrations of Mitomycin. For comparing HepG2.2.15 (bottom) with HBV-Nluc-35 (top) cells for their cell culturing status under different concentrations of Mitomycin, both cells were treated with increasing concentrations of mitomycin for 16 h. Cell proliferation toxicity was measured using CCK-8 reagent as recommended by the manufacturer (Dojindo). For HBV-Nluc-35 cells, the LD<sub>50</sub> of Mitomycin was 8.562 μM, and the 95% CI of the LD<sub>50</sub> was 5.71–34.98 μM. It was indicated that HepG2.2.15 cells were more sensitive to toxicity of Mitomycin than HBV-Nluc-35.

## Abbreviations

HBV: Hepatitis B virus; HBsAg: HBV surface antigen; HBcAb: HBV core antibody; HBeAg: HBV e antigen; RC-DNA: Relaxed circular DNA; cccDNA: Covalently closed circular DNA; pgRNA: Pregenomic RNA; ORF: Open reading frame; LAM: Lamivudine; NAGE: Native agarose gel electrophoresis; qPCR: Quantitative PCR; ELISA: Enzyme-linked immunosorbent assay; LD<sub>50</sub>: Median lethal dose; EC50: 50% effective concentration

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## Authors' contributions

DS performed the methodology and project application; JR, SS, XC, and DS designed and coordinated the research; JR, SS, PH and YZ performed the majority of the experiments and analyzed the data; JR and DS wrote the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

Not applicable.

## Ethics approval and consent to participate

Because no animals and patients were involved in this study, the Ethics approval not applicable in this section.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## Author details

<sup>1</sup>The Liver Disease Diagnosis and Treatment Center of PLA, Bethune International Peace Hospital, Zhongshanxi street, Shijiazhuang 050082, Hebei

Province, China. <sup>2</sup>Department of Infection and Liver Disease, Shaanxi University of Chinese Medicine, Xianyang 712000, Shaanxi Province, China.

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