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Effect of Nuclear Export Inhibitor Leptomycin B on the Intracellular Localization of HBV Core Protein into Hepatocytes Cell Line Huh-7 and HepG2 Cells

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Abstract

Leptomycin B (LMB) was originally discovered as a potent anti-fungal antibiotic from *Streptomyces* species. The cellular target of LMB has been identified as the nuclear export receptor CRM-1 or exportin-1, which is involved in nuclear trafficking of cellular RNAs or proteins containing the nuclear export sequence (NES). CRM-1 is the main mediator of nuclear export in many cell types including hepatocyte cell lines. The ability of LMB to inhibit nuclear export has made it a useful tool in the study of the intracellular localization of many regulatory proteins. In this study, we evaluated the effect of nuclear export inhibitor LMB treatment on the intracellular localization of HBV core protein into the hepatocyte cell lines, Huh-7 and HepG2 cells. We also reported the quantification of the distribution of EGFP-Core fusion protein with redundant core NLS as well as SV-40 NLS into cell compartments. Results shown that in Huh-7 cells treatment of LMB caused retention of EGFP-Core fusion protein into the nucleus, so increased the nuclear localization of EGFP-Core and all variants. In HepG2 cells, although not significantly, treatment of LMB increased a number of nuclear localization in all EGFP-Core constructions, even the nuclear localization in HepG2 cells is not so high as in Huh-7 cells.

Keywords: Leptomycin B, HBV, core protein, intracellular localization, NLS, Huh-7, HepG2 cell

Introduction

Hepatitis B virus (HBV) is a major human pathogen causing acute and chronic hepatitis or liver inflammation. HBV is the prototype of a family of hepatotrophic, enveloped DNA viruses with a very narrow host range, referred to as *hepadnaviridae*. Virus particle is spheric which has a diameter of 42 nm and consists of an envelope carrying three surface proteins. It surrounds an icosahedral capsid enclosing an open circular with partially double stranded DNA (3.2-kb) as well as the viral DNA polymerase. HBV capsid has a diameter of 30 nm and is formed by multiple copies of one species of core protein (Nassal, 1996). HBV capsid is known as HBV core protein consists of 185 amino acids (aa) for genotype A and forms dimers which self-assemble in heterologous expression systems into shells of T=3 and T=4 symmetry (Zlotnick *et al*., 1996). The C-terminal of HBV core protein which consist of 30 aa are very rich in arginine residues and probably bind to the encapsidated viral nucleic acid. The N-terminal 155 aa are sufficient for capsid

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formation and referred to as the assembly domain (Gallina *et al*., 1989).

During infection the nucleocapsid of HBV is released from the incoming virus into the cytoplasm. Then the viral DNA genome is transported into the nucleus and repaired to give a *circular covalently closed* DNA (cccDNA), called as episome. The cccDNA serves as the template for transcription by host factors. The viral DNA genome is then synthesized in the lumen of the capsid by reverse transcription of the 3.5-kb RNA followed by second-strand DNA synthesis. The nucleocapsid can follow two different pathways. It can stay within the cell, in which case its genome contributes to the intracellular amplification of the viral episomes (Tuttleman *et al*., 1986).

Leptomycin B or LMB was originally discovered as a potent anti-fungal antibiotic from *Streptomyces* species (Hamamoto *et al*., 1983). The cellular target of LMB has been identified as the nuclear export receptor CRM-1 or exportin-1 (Nishi *et al*., 1994), which is involved in nuclear trafficking of cellular RNAs or proteins containing the nuclear export sequence (NES) (Fornerod., 1997). LMB is shown to inhibit nuclear export of RNAs such as cfos (Brennan *et al*., 2000) or of the NES-containing proteins such as α-catenin (Giannini *et al*., 2004) or inhibitor of κB-alpha (IκB-α) (Huang *et al*., 2000). LMB was also shown to inhibit mRNA nuclear export of cyclooxygenase-2 (COX-2), thus down-regulating the expression of COX-2, an inflammatory enzyme producing prostaglandins, in MDA-MB-231 cells (Jang *et al*., 2003).

Structure of the LMB is an unsaturated, branched-chain fatty acid which is an important tool in the study of nuclear export (Figure 1). LMB inhibits nucleo-cytoplasmic translocation of molecules such as the HIV-1 Rev protein and Rev-dependent export of mRNA. Some cellular proteins such as actin, c-Abl, cyclin B1, p53, Iκb, MPF and PKA are also reported can be influenced by Leptomycin B (Ullman, *et al*., 1997; Wolff, *et al*., 1997; Fukuda, *et al*., 1997). Some research reported that LMB causes

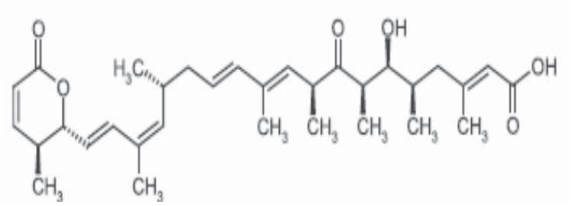

Figure 1. Structure of nuclear export inhibitor Leptomycin B (LMB).

G1 cell cycle arrest in mammalian cells and it is a potent anti-tumor agent against murine experimental tumors (Yoshida *et al*., 1990; Komiyama *et al*, 1985).

In this study, we evaluated the effect of nuclear export inhibitor LMB treatment on the intracellular localization of HBV core protein into the hepatocyte cell lines. We also reported the quantification of the distribution of EGFP-Core fusion protein with redundant core NLS as well as SV-40 NLS into cell compartment of Huh-7 and HepG2 cells.

Materials and Methods

Main materials of this work consists of green fluorescence protein encoding DNA plasmid pEGF-C3 as plasmid control, HBV core fusion protein encoding DNA plasmid with redundant NLS of HBV core (pEGFP-Core 1, 2 and 3 NLS) and HBV core fusion protein encoding DNA plasmid with redundant NLS of SV-40 (pEGFP-Core 2 and 3 NLS of SV-40). All plasmids have been prepared as described by Haryanto and Kann (2006) and Haryanto *et al*. (2007), HBV core protein nuclear export inhibitor Leptomycin B (SIGMA), transfection agent Tfx-20 (PROMEGA), hepatocyte cell lines Huh-7 and HepG2 cells, DMEM medium (GIBCO-BRL) and Fetal Calf Serum or FCS (GIBCO-BRL).

Plasmid desain for EGFP-core fusion protein and its variants

Plasmid pEGFP-C3 is a original plasmid and it used as control plasmid. Construction of recombinant plasmid pEGFP-Core 1, 2, 3 NLS and pEGFP-Core 2 and 3 NLS of

SV-40 were designed by using synthetic oligonucleotides as described by Haryanto and Kann (2006); and Haryanto *et al.* (2007).

Preparation of Huh-7 and HepG2 cell lines

Huh-7 and HepG2 cells were grown onto collagenized cover slips 24 h before transfection into 24 well plate. Then the cells were incubated over night at 37°C in incubator CO_2 until 75-80% confluent before transfected by recombinant plasmids.

Transfection and treatment of Leptomycin B

All recombinant DNA plasmid were transfected into Huh-7 and HepG2 cells in 24 well plate. Each transfection reaction, which consists of 5 µl DNA plasmid (200 ng/µl), 5 µl Tfx-20 and 300 µl FCS free medium, was then mixed gently and incubated at room temperature for 5-10 min. The 24 well plate were took out from CO_2 incubator and changed the 10% FCS containing medium with FCS free medium. The 24 well plate was returned to the CO_2 incubator and continued the incubation for the appropriate length of time before transfection. Transfection was done, by replacing FCS free medium with the mixture of DNA/Tfx-20 reagent/FCS free medium 310 µl per well. The 24 well plate was incubated in the CO_2 incubator at 37 °C for 1 h. During incubation the 10% FCS containing medium was warmed at 37°C in the waterbath and Leptomycin B in final concentration of 10 nM/ml was directly added in DMEM medium. Then the 24 well plate was incubated into the CO_2 incubator at 37°C over night.

Indirect immunostaining and confocal laser microscopy

Transfected Huh-7 and HepG2 cells were immunostained with mouse monoclonal antibody 414 anti NPC (1:500) as primary antibody and labeled with secondary antibody anti mouse, which marked texas red dye (1:100). Then the intracellular localization of EGFP-Core fusion protein determined under confocal laser microscope as described before by Haryanto (2006).

Quantification of intracellular localization

The HBV core fusion protein which found localized in the cell compartment, was quantified manually using confocal laser scanning microscope. The Amount of HBV core protein that distributed in the cytoplasm, nucleus or both of cell compartment were quantified in the absolute and relative values.

Results and Discussion

Leptomycin B (LMB) is known as a nuclear export inhibitor. It works to prevent the export of proteins from the nucleus to the cytoplasm, because of its ability to interact with and impair the function of the nuclear export factor CRM-1 (Kudo *et al*, 1999). In other study, LMB is reported as a very potent inducer of the p53 response, which does not act directly through

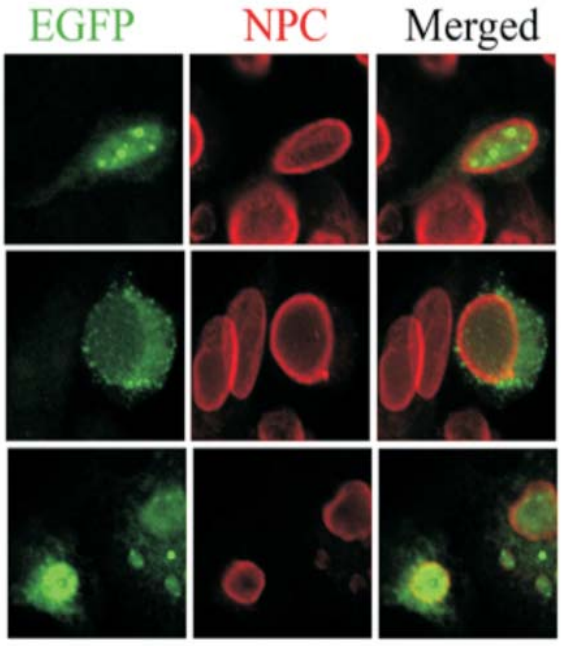

Figure 2. Intracelullar localization of EGFP-Core fusion protein in Huh-7 cells. The fusion protein found localized in the nucleus (upper row), cytoplasm middle row, and both nucleus and cytoplasm (lower row). The fusion proteins are shown As green fluorescence and the nuclear pore complexes (NPC) in red.

the DNA damage pathway (Lain *et al,* 1999). In this research, we study the Intracellular localization study of HBV core protein as EGFP-Core fusion protein with single, double and triple HBV core NLS (EGFP-Core 1, 2 and 3 NLS) as well as with single double and triple SV-40 NLS (EGFP-Core 1, 2 and 3 SV-40) which treated using nuclear export inhibitor LMB. The intracellular localisation of EGFP-Core and its variants with HBV core and SV-40 NLS in Huh-7 and HepG2 cells more detail depicted in Figure 2.

Quantification of the distribution of EGFP-Core fusion proteins into cells compartment with cytoplasmic and nuclear localization into Huh-7 cell can be seen more detail in Table 1. In the control group without LMB treatment (Figure 2) and the data in Table 1 shown below indicate that the intracellular localization of EGFP-Core fusion protein and its variants are dominantly found into the nucleus of Huh-7 cells. Treatment using LMB increased nuclear localization, because the LMB inhibit specifically the nuclear export of EGFP-Core fusion protein (Wolff *et al*., 1997). In the nucleus, LMB interferes with the binding of the leuice-rich Rev types nuclear export sequence (NES) to export receptor exportin or CRM 1 (Fornerod *et al*., 1997; Ossareh-Nazari *et al*., 1997).

Table 1 shown that treatment of LMB as nuclear export inhibitor in Huh-7 cells caused retention of EGFP-Core fusion protein into the nucleus. This nuclear localization increase in all of EGFP-Core constructions. LMB treatment increase the nuclear localization of EGFP-Core and all variants, because it is a potent, specific inhibitor of nuclear export protein (NES). The cellular target of LMB has been identified as the nuclear export receptor CRM-1 or Exportin-1. CRM-1 is responsible for intracellular transport mediated by the nuclear export signal (Fukuda *et al*., 1997). It shown that LMB has been an important tool in the elucidation of the role of CRM-1 in the export process. The inhibition of growth of fission yeast and mammalian cells by LMB was shown to be due to the inhibitory effect of LMB on CRM-1 mediated processes (Yoshida *et al*., 1990; Nishi *et al.,* 1994).

In other work, the intracellular localization of EGFP-C3 as control, EGFP-Core fusion protein and its variants in compartment cell of HepG2 cells with cytoplasmic and nuclear localization shown more detail in Table 2.

Table 2 shown that intracellular localization of EGFP-C3 and EGFP-Core fusion protein and its variants in HepG2 cells are totally different. It shown only EGFP-C3 as control protein predominantly found local-

Table 1. Intracellular localization of fusion protein EGFP-C3, EGFP-Core fusiuon protein and its variants in Leptomycin B untreated and treated into Huh-7 cells.

No	Sample	Treatment	Cytoplasm	Nucleus	Both	Total
1.	**pEGFP-C3**	No	11%	89%	0%	100%
		Leptomycin B	5%	94%	1%	100%
2.	**pEGFP-Core 1 NLS**	No	25%	75%	0%	100%
		Leptomycin B	9%	91%	0%	100%
3.	**pEGFP-Core 2 NLS**	No	18%	82%	0%	100%
		Leptomycin B	12%	88%	0%	100%
4.	**pEGFP-Core 3 NLS**	No	9%	90%	1%	100%
		Leptomycin B	5%	94%	1%	100%
5.	**pEGFP-Core 2 NLS of SV40**	No	23%	77%	0%	100%
		Leptomycin B	12%	88%	0%	100%
6.	**pEGFP-Core 3 NLS of SV-40**	No	18%	82%	0%	100%
		Leptomycin B	6%	94%	0%	100%

Tabel 2. Intracellular localization of fusion protein EGFP-C3, EGFP-Core fusion protein and its variants in Leptomycin B untreated and treated into HepG2 cells.

No	Sample	Treatment	Cytoplasm	Nucleus	Both	Total
1.	**pEGFP-C3**	No	16%	84%	0%	100%
		Leptomycin B	15%	85%	0%	100%
2.	**pEGFP-Core 1 NLS**	No	96%	3%	1%	100%
		Leptomycin B	86%	14%	0%	100%
3.	**pEGFP-Core 2 NLS**	No	92%	8%	0%	100%
		Leptomycin B	90%	10%	0%	100%
4.	**pEGFP-Core 3 NLS**	No	88%	12%	0%	100%
		Leptomycin B	87%	13%	0%	100%
5.	**pEGFP-Core 2 NLS of SV40**	No	90%	9%	1%	100%
		Leptomycin B	89%	11%	0%	100%
6.	**pEGFP-Core 3 NLS of SV-40**	No	85%	15%	0%	100%
		Leptomycin B	80%	20%	0%	100%

ized into the nucleus, whereas EGFP-Core 1, 2, 3 NLS and EGP-Core 1, 2, 3 NLS of SV-40 fusion proteins majority found localized in cytoplasm. Although not significantly, treatment with LMB increase a number of nuclear localization in all EGFP-Core constructions. The nuclear localization in HepG2 cell is not so high as in Huh-7, however treatment with LMB also increase the nuclear localization. It indicated that LMB works to inhibit CRM-1 interaction with the NES of EGFP-Core fusion protein. LMB binds covalently to the CRM-1 protein. As known that CRM-1 is a karyopherin specific for nuclear export (exportin). This CRM-1 protein directly binds protein that contain a leucine-rich NES (Fornerod *et al*., 1997; Fukuda *et al*., 1997). In addition, the export mechanism appears to involve CRM-1 binding to both Ran-GTP and nucleoporins (Floer and Blobel, 1999).

LMB is thought to function primarily as an inhibitor of the export of proteins from the nucleus to the cytoplasm because of its ability to interact with and impair the function of the nuclear export factor CRM-1 (Kudo *et al.*, 1999). Although other effects of LMB that could lead to a DNA damage response cannot be excluded, LMB is likely to be a very potent inducer of the p53 response, which does not act directly through the DNA damage pathway (Lain *et al*, 1999). It state that both in Huh-7 and HepG2 cells treatment of LMB caused retention of EGFP-Core fusion protein and its variant into the nucleus so the nuclear localization tend to increase after treatment. It indicated clearly that the cellular effects of LMB are due to inhibition of the nuclear export from nucleus to cytoplasm cell because of LMB binding covalently to the CRM-1 protein. This binding occurs in its conserved central region at a critical cysteine residue and prevents formation of the complex between CRM-1 and the NES of cargo proteins (Kudo *et al*., 1998; Kudo *et al.*, 1999).

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