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Effect of the HBV Capsid Assembly Inhibitor Bayer 41-4109 on the Intracellular Localization of EGFP-Core Fusion Proteins

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Abstract

Bayer 41-4109 is heteroarylpyrimidine (HAP) which has been identified as potent of HBV capsid assembly inhibitor. The present study was to study effect of Bayer 41-4109 treatment on the intracellular localization of EGFP-Core fusion proteins into HepG2 cells. Three recombinant plasmids of pEGFP-Core with single, double and triple NLS of HBV core (EGFP-Core 1C, 2C and 3C) and two recombinant plasmids with single and triple NLS of SV-40 (EGFP-Core 1 and 3 SV-40) were used in this work. After transient transfected into HepG2 cells and treated with Bayer 41-4109, the intracellular localization of expressed fusion proteins from all plasmid constructions were determined and quantified under confocal laser microscope. Results shown that Bayer 41-4109 treatment in HepG2 cells inhibited the nuclear localization of EGFP-Core with single of triple HBV core NLS. As well as the constructions of expressed fusion protein with single and triple SV-40 NLS (EGFP-Core 1 and 3 SV-40 NLS) showed decreasing the nuclear localization after treated with Bayer 41-4109, even not as strong as EGFP-Core 1C and 3C NLS. Bayer 41-4109 has been identified as a potent inhibitors of HBV replication which has multiple effects on HBV capsid assembly. It may inhibit virus replication by inducing assembly inappropriately and by misdirecting assembly decreasing the stability of normal capsids.

Keywords: HBV capsid, Bayer 41-4109, EGFP-Core fusion protein, HepG2 cell

Introduction

Hepatitis B virus (HBV) infects chronically more than 400 million people worldwide. Chronic HBV infections the major risk factor for liver cancer called hepatocellular carcinoma or HCC (Hollinger, 1996). HBV is an enveloped DNA virus with an icosahedral capsid or core. The HBV capsid is assembled in the cytoplasm from core protein, viral pregenome RNA, viral reverse transcriptase and few host proteins. The core protein plays an important roles in viral DNA synthesis from pregenome and intracellular tracfficking in the nucleus (Ganem and Schneider, 2001).

HBV persistence and transmission require HBV replication, which depends on the assembly of a core particle composed of capsid protein (Cp), polymerase, and pregenomic RNA. Reverse transcription to produce infectious DNA-containing par-

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ticles occurs solely within the core residing in the cytoplasm (Ganem and Schneider, 2001, Seeger and Mason, 2000). HBV core assembly is likely to be a high value target for therapeutics (Zlotnick and Stray, 2003).

Approval treatments for chronic HBV are IFN-a and the polymerase inhibitors lamivudine or adefovir (Karayiannis 2004, Wands, 2004). IFN-a therapy has limited efficacy (Brunetto *et al*., 1993). Resistant viruses arise during treatment with polymerase inhibitors (Ling *et al*., 1996, Villeneue *et al*., 2003), with rates for lamivudine resistance approaching 50% (Karayiannis, *et al*., 2004, Liaw *et al*., 2004). The most of antiviral strategy against HBV is to attack the reverse transcriptase. An alternative therapeutic strategy would be targeting capsid assembly (Zlotnick *et al*., 2002).

Heteroaryldihydropyrimidines (HAPs), first identified by scientists at Bayer company as having anti-HBV activity in a cell culture-based screen, act in a capsid protein-specific manner (Deres *et al*., 2003; Stray *et al*., 2005). HAP as a novel class of HBV inhibitors in tissue culture and animal models, effective at nanomolar concentration (Weber *et al*., 2002). The mechanism of HAP activity has been studied in vitro using the 149-residue assembly domain of the HBV capsid protein (Hacker *et al*., 2003; Stray *et al*., 2005).

Bayer 41-4109 is also referred to as heteroaryldihydropyrimidine or HAP. This compound has been tested in cell culture and animal model (Weber *et al*., 2002), and on HBV capsid assembly in vitro. Bayer 41-4109 is discovered as highly potent non-nucleosidic inhibitors of HBV replication both in vitro and in vivo (Weber *et al*., 2002). As another antiviral, HAP work to prevent the proper formation of HBV core particles (nucleocapsid), which are the site of viral DNA replication (King *et al*., 1998). Core particles are stable and they have high molecular weight aggregates assembled from HBV core protein subunits (Nassal, 1992).

The chemical structure of Bayer 41-4109 that used in this work more detail could be shown in figure 1. The inhibitory concentration of Bayer 41-4109 needed to decrease HBV genome replication by 50% (IC_{50}) in stably transfected HepG2.2.15 cells or HBV-producing hepatoma cells (Sells *et al*., 1987).

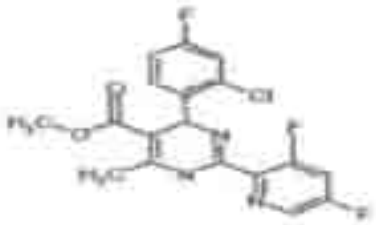


Figure 1. Structures of Bayer 41-4109 is a heteroaryldihydropyrimidine or HAP according to Weber et al., 2002. Structure of Bayer 41-4109 is as a pure (-) enantiomer or R enantiomer.

Here, we report the of HBV capsid assembly inhibitor Bayer 41-4109 as a heteroaryldihydropyrimidine (HAP) on the intracellular localization of EGFP-Core fusion proteins. We also reported the quantification of EGFP-Core fusion protein with redundant single and triple NLS of HBV core and SV-40 which expressed as fusion protein in the cell compartments.

Materials and Methods

Materials of this study are HBV core fusion protein encoding DNA plasmid with redundant NLS of HBV core and SV-40 which have been prepared by Haryanto and Kann (2006), HBV capsid assembly inhibitor Bayer 41-4109 (Bayer AG), transfection agent Tfx-20 (PROMEGA), hepatocyte cell line HepG2, DMEM medium (GIBCO-BRL) and Fetal Calf Serum or FCS (GIBCO-BRL).

Design of EGFP-Core Fusion Protein Encoding Plasmids with redundant NLS of HBV core and SV-40.

The recombinant plasmid pEGFP-Core with single, double and triple NLS of HBV core (EGFP-Core 1C, 2C and 3C) as well as

the recombinant plasmid with double and triple NLS of SV-40 (EGFP-Core 2 and 3 SV-40) were designed by Haryanto et al, (2007), which used as original recombinant DNA plasmid.

Preparation of hepatocyte cell line HepG2 for Transfection

In 24 well plat, HepG2 cells was grown onto collagenized cover slips one day before transfection. The cell lines were incubated over night at 37°C in incubator CO_2 until 75-80% confluent.

Transfection and treatment of Bayer 41-4109

All construction of recombinant DNA plasmid were transfected into HepG2 cells. The transfection reaction consist of 5 ìl DNA plasmid (200 ng/ìl), 5 ìl Tfx-20 and 300 ìl serum free medium. Then it was mixed gently and incubated at room temperature for 5-10 minutes. While, 24 well plat were took out from CO_2 incubator and changed the 10% FCS containing medium with serum free medium. 24 well plat was returned to the CO_2 incubator and continued the incubation for the appropriate length of time before transfection. Transfection was done, by replacing serum free medium with the mixture of DNA/Tfx-20 reagent/FCS free medium 310 ìl per well. The 24 well plate was incubated in the CO_2 incubator at 37 °C for 1 hour. During incubation the 10% FCS containing medium was warmed at 37°C in the waterbath and Bayer 41-4109 in final concentration of 10 nM/ml was directly added in DMEM medium. Then the well plat was incubated into the CO_2 incubator at 37°C over night.

Indirect immunostaining and confocal laser microscopy.

The transfected cells was immune stained 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 compartment of HepG2 cells, was quantified manually using confocal laser scann 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

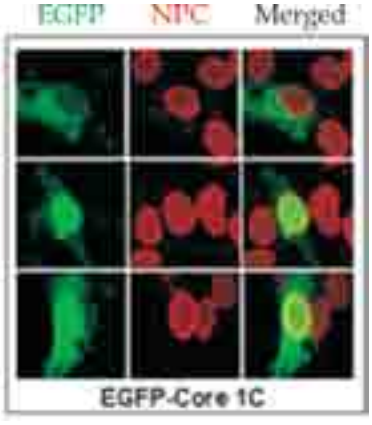
Recently, an assembly inhibitor of HBV capsids was found (Deres *et al*., 2003). This inhibitor interacts with capsid formation. However, the site of interaction and the stage at which assembly is blocked dimerization of the capsid protein monomers or the trimerization of the dimers is yet unclear. Mature HBV particles consists of a 3,5 kb partially single stranded circular genome and a single copy of the viral reverse transcriptase (RT) encapsidated in an icosahedral protein composed of 120 HBV capsid protein or Cp dimmers, host chaperons and kinases may also be incorporated (Seeger and Mason, 2000).

First, the HBV genome was transfected into HuH-7 cells as described before. Twenty four hours after transfection, the inhibitor was added to one sample whereas a second one remained untreated. The successful inhibitor treatment was determined by real time PCR. Supernatant of cell culture was analyzed 48 hours post transfection. Unencapsidated viral DNA, as it may be have been derived from transfected genomes sticking to the dish or from lysed cells, was degraded prior to purification of viral DNA .

Real time PCR was done in triplets showing the addition of the inhibitor reduced the number of encapsidated viral genomes to 60%. This inhibition rate was significantly smaller than the one published for HepG2.2.15 cells in which virus secretion was reduced six fold (Deres *et al.*, 2003). This difference may either mean that the cell type or may play a role, for instance that HuH-7 can export the inhibitor more efficiently than HepG2 cells. However, since the transfected HBV genome is genotype A and HepG2.2.15 cells express HBV of genotype D a genotype-specific binding of the inhibitor could not be excluded.

Then the effect of the inhibitor on localization of the fusion proteins was investigated. The experimental procedure was done as described before but the inhibitor was added after over night incubation following transfection. NPCs were stained after further 24 hours of incubation. As decribed before that structure of Bayer 41-4109 is an (-) enantiomer, binding of Bayer 41-4109 to HBV core protein the is stereospecific, with only the (-) enantiomer capable of binding to HBV core particles (Deres *et al.*, 2003).

Figure 2 and 3 showed a changed distribution pattern within the nucleus. In contrast enrichment in defined areas the nuclear fluorescence filled the entire karyoplasm with the exception of some nucleoli-shaped areas.



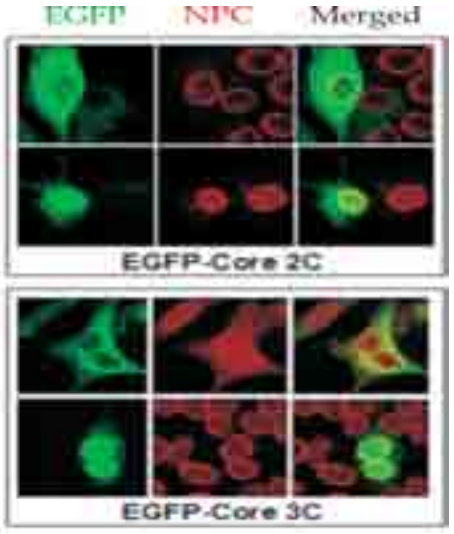


Figure 2. Localization of EGFP-Core 1C, 2C, 3C in HepG2 cells. The fusion protein localized in cytoplasm (upper row), nucleus (middle row) and both cytoplasm and nucleus (lower row) in EGFP-Core 1C and 2 SV40 NLS. In EGFP-Core 2C, 3C and 3 SV40 NLS localized only in cytoplasm (upper row), nucleus (upper row). The fusion proteins are shown as green fluorescence, the nuclear pore complexes in red.

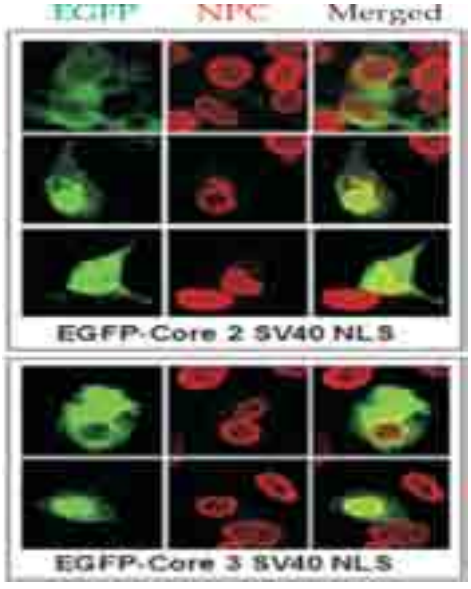


Figure 3. Localization of EGFP-Core 2 SV-40 NLS and 3 SV-40 NLS in HepG2 cells. The fusion protein localized in cytoplasm (upper row), nucleus (middle row) and both cytoplasm and nucleus (lower row) in EGFP-Core 1C and 2 SV40 NLS. In EGFP-Core 2C, 3C and 3 SV40 NLS localized only in cytoplasm (upper row), nucleus (upper row). The fusion proteins are shown as green fluorescence, the nuclear pore complexes in red.

When quantifying the number of cells with cytoplasmic and nuclear localization of fluorescence fusion protein (see more detail in the Table) a striking difference for the EGFP-core fusion proteins compared to capsid localization was observed. While EGFP-core 1C showed a nuclear localization in 52% of the untreated cells, Bayer 41-4109 treated cells showed a nuclear localization in only 11%. The same ratio or even more pronounced was observed for EGFP-Core 3C (57% versus 4%).

Table. Localization of fusion protein EGFP-Core 1C, 3C and EGFP-Core 2, 3 SV40 NLS fusion protein in Bayer 41-4109-treated and untreated Hep G2 cells.

Expressed Protein	Inhibitor	Cytoplasm	Nucleus	Both	Total
EGFP-Core 1C	No treatment	48%	52%	0%	100%
	Bayer 41-4109	88%	11%	1%	100%
EGFP-Core 3C	No treatment	42%	57%	1%	100%
	Bayer 41-4109	96%	4%	0%	100%
EGFP-Core 1 SV-40 NLS	No treatment	20%	80%	0%	100%
	Bayer 41-4109	29%	71%	0%	100%
EGFP-Core 3 SV-40 NLS	No treatment	11%	88%	1%	100%
	Bayer 41-4109	21%	79%	0%	100%

In Table shown that Bayer 41-4109 treatment in HepG2 cells inhibited the nuclear localization of EGFP-Core with single of triple HBV core NLS. As well as the constructions of expressed fusion protein with single and triple SV-40 NLS (EGFP-Core 2 and 3 SV-40 NLS) showed decreasing the nuclear localization after treated with Bayer 41-4109, even not as strong as EGFP-Core 1C and 3C NLS. It shown that Bayer 41-4109 which is a member of a class of HAP has been identified as a potent inhibitors of HBV replication. Bayer 41-4109 has multiple effects on HBV capsid assembly. It may inhibit virus replication by inducing assembly inappropriately and by misdirecting assembly decreasing the stability of normal capsids (Stray and Zlotnick, 2006).

Apparently, dimerization increases the nuclear transport capacity of the fusion proteins. The effect of dimerization was much more pronounced when adding an additional NLS to the fusion protein. In contrast, the effect of the inhibitor on EGFP-Core SV40 NLS protein localization was not significant. Although the percentage of cells with cytoplasmic fluorescence increased in the inhibitor treated cells for both EGFP-Core 1 SV40 NLS and EGFP-Core 3 SV40 NLS, this difference was only marginal and with 10% not above the variation occurring between different transfections. The difference between the EGFP-Core 1, 3C and EGFP-Core 1, 3 SV40 NLS may imply that the inhibitor interacts with the C-terminus of the capsid protein preventing binding of importin a to the NLS. This area overlaps with the nucleic acid binding domain (Hatton *et al*., 1992) of the capsid protein that stabilizes the capsid. As it was shown previously for DHBV capsids, mutations within this domain destabilize the viral capsid. It must be thus assumed that the described effect on capsid formation by Deres *et al*., (2003) is not necessarily a direct inhibition of assembly but may cause a reduced stability.

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