VIRIDIS BioPharma

Report prepared for
American Biotech Laboratory

Viricidal Activity of ASAP
against Hepatitis B Virus
&
Cytotoxicity of ASAP
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Introduction & Purpose

Silver in its active form is reported to kill microorganisms instantly by blocking the respiratory enzyme system while having no negative effect on human cells. Silver's antiviral activity is not known through scientific tests. VIRIDIS BioPharma, has demonstrated viricidal activity of ASAP and communicated to Mr. William Moeller, American Biotech Laboratory, on 26th November 2002 . (See Appendix A). This study has led to the current study determining ASAP cytotoxicity and antiviral activity against Hepatitis B.

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(Registered under Societies Registration Act. 1860)

Report of ***invitro*** evaluation of Anti HBV activity of samples received from VIRIDIS BIOPHARMA PVT.LTD.

	Sample	DNA polymerase Inhibition	Reverse Transcriptase Inhibition
	ASAP-10	77.73%	89.52%
	ASAP-14	65.6%	86.93%
	ASAP-22	60.89%	84.46%
ROL	Lamuvidine (Pol.)	31.33%	--
ROL	AZT (RT.)	--	18.06%

References for methodology:

1. Effects of an extract from Phyllanthus niruri on Hepatitis B and woodchuck hepatitis viruses: in vivo and in vitro studies: Venkateshwaran P.S., Millman I., Blumberg B. S., Proc. Natl. Acad. Sci. USA, January 1987, 84: 274-278

2. Phyllanthus amarus down-regulates hepatitis B virus mRNA transcription and replication: Lee C.D., Ott M., Thyagarajan S. P., et.al., Eur. J. Clin. Invest. 1996, 26: 1069-1076

RAD

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1. ASAP Antiviral Activity in Hepatitis B:

Viricidal activity of ASAP solutions is determined through the ability of ASAP to inhibit:

A) DNA Polymerase activity

B) Reverse Transcriptase activity.

Appendix B is a ready reference background.

2. Test Procedure for DNA Polymerase (DNAp) Inhibition

DNA Polymerase:-

DNA polymerase protein (DNAp) is 90 kd in size and has RNA & DNA dependant polymerase activity. DNAp plays a key role in Hepatitis B Virus (HBV) genome generation as well as pgRNA encapsulation. DNAp is packaged together with pgRNA within HBV nucleocapsids.

This enzyme can be detected in the bloodstream soon after initial infection by hepatitis B at about the same time as HBV DNA. i.e. generally within a 1 week or so after infection.

2.1 Aim

To detect percentage inhibition of DNA polymerase by ASAP solution using liquid scintillation counter.

2.2 Principle

Hepatitis B viral extracts from human subjects are incubated with radiolabelled nucleotides and an active inhibitor. Percent inhibition is calculated based

on amount of *de novo* viral nucleic acid synthesized with respect to Lamivudine as a positive & Phosphate buffer saline (PBS) as a negative controls.

2.3 Equipment

1. Liquid Scintillation Counter (Blue Star) (Refer to Appendix C)
2. Incubator
3. Mettler analytical balance

2.4 Material

1. Micropipette
2. Sterile micropipettar tips
3. Ionic paper (DEAE)
4. Sterile eppendoff tubes
5. Nucleotides; dATP, dGTP, dCTP, (^{3}H) dTTP, (Radioactive nucleotide)
6. Lamivudine, (3mg / ml)
7. Test sample,
8. EDTA,
9. Trichloroacetic acid (TCA)

2.5 Test Organism

The isolated Hepatitis B Virus is freshly obtained from a person suffering from Hepatitis B infection and was taken up by Haffkine Institute, Mumbai (WHO certified testing laboratory).

2.6 Procedure

Isolated Hepatitis B virus was lysed to extract free polymerase enzyme, which is free from contaminating enzymes. 25µl of virus extract was added to 25µl of reaction mixture having a mixture of dATP, dGTP, dCTP & (^{3}H) dTTP nucleotides. To this reaction mixture 3µl of active inhibitor was added and mixture was incubated at 37^{0}C for 2 hrs. For a negative control viral suspension was mixed with 3µl of phosphate buffer saline (PBS) instead of inhibitor. Lamivudine (3mg/ml), a well known DNAp inhibitor(3µl) was added as a positive control. The reaction was stopped by adding 25µl EDTA and 25µl TCA. The reaction mixture was then spotted on ionic paper (DEAE paper). The paper was washed thrice with TCA and then with alcohol. Filter paper was air dried and put in scintillation vial having scintillation cocktail. Radioactivity was then measured with the help of a liquid scintillation counter (Blue Star).

Note: Blank ASAP was run through the complete procedure without viral load, to check its ionic interference in the scintillation counter method.