VIRIDIS BioPharma

Report prepared for
American Biotech Laboratory

Viricidal Activity of ASAP
against Hepatitis B Virus
&
Cytotoxicity of ASAP
March 2003 – June 2003

By:
VIRIDIS BioPharma Pvt. Ltd.
6/312, Jogani Indl. Complex,
V. N. Purav Marg, Chunabhatti,
Mumbai - 400 022 India
Tel.: 2524 3020 Fax: 2523 5952
viridis@vsnl.com www.viridisbiopharma.com

Table of Contents

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.

हाफकिन प्रशिक्षण, संशोधन व चांचणी संस्था
(सोसायटीज रजिस्ट्रेशन ॲक्ट १८६० अधिनियमान्वये नोंदणी कृत)
Haffkine Institute For Training, Research & Testing
(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

Dr. (Mrs.) R.A.Deshmukh
Head, Department of Virology

Place: Mumbai
Date: 12/05/2003

ता :
ाचार्य दोंदे मार्ग, परळ,
बई - ४०० ०१२ (भारत)
दूरध्वनी/Tel 4160947, 4160961, 4160962
4161785, 4113037
फॅक्स/Fax 4161787; तार/Telegram Research, Mumbai
ई.मेल/E mail [redacted]
Address :
Achyarya Donde M
Parel,
Mumbai 400 012 (I

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.

Schematic Representation

25µl of virus extract + 25µl of reaction mixture + 3µl of active inhibitor

↓

Incubate at 37^0C for 2 hrs.

↓

Adddition of 25µl EDTA + 25µl TCA

↓

Spot on ionic paper (DEAE)

↓

Wash the paper thrice with TCA.

↓

Wash once with alcohol

↓

Air dry

↓

Check for radioactivity.

Reference :

"Effect of an extract from *Phyllanthus niruri* on hepatites B and woodchuick hapetitis virus: *In vitro* and *in vivo* studies.", P. S. Venkateswaran, I. Millman, and B. S. Blumberg, Proc. Natl. Acad. Sci., USA, Jan 1987, Volume 84, Pg 274-278. (refer to Appendix D).

VIRIDIS

VIRIDIS BioPharma Pvt. Ltd., 6/10 Jogani Industrial Complex, V. N. Purav Marg. Chunabhatti, Mumbai – 400 022 India.
Tel.: 2524 3020. Fax: (91-22) 2523 5952. E - mail: viridis@vsnl.com www.viridisbiopharma.com

DNA Polymerase (DNAp) Inhibition Test Results

Sample Source American Biotech Lab

Sample Description ASAP 10 ppm (Lot # 02198)
ASAP 14 ppm + 1.5% H_2O_2 (No. Lot #)
ASAP 22 ppm (Lot # 02193)

Date of Analysis 5th May 2003

Sample	***% Inhibition***
ASAP-10	**77.73%**
ASAP-14 + 1.5% H_2O_2	**65.6%**
ASAP-22	**60.89%**
+ve control, Lamivudine*	**31.33%**
-ve control, Phosphate Buffer Saline (PBS)	**0%**

* Refer Appendix E & F

Kp Rane
Quality Assurance

2.7 Conclusion:

Test results suggest that ASAP solutions hold promising inhibiting activity against DNA Polymerase and hence HBV.

3. Test Procedure for Reverse Transcriptase (RT) Inhibition

Reverse Transcriptase:-

Reverse transcriptase was discovered in 1970 by two independent groups, a Baltimore group and the Termin and Mizutani group. It is an essential for the processes of retrovirus replication and has therefore become an important target for drug inhibition. Reverse transcription begins working in the virion with the synthesis of a minus strand of DNA (DNA polymerase activity).

3.1 Aim

To detect percentage inhibition of reverse transcriptase by ASAP solution using liquid scintillation.

3.2 Principle

Viral extracts are incubated with radiolabelled nucleotides and an active inhibitor. Percent inhibition is calculated based on amount of viral nucleic acid synthesized with respect to positive & negative controls.

3.3 Equipment

1. Liquid Scintillation Counter (Blue Star)
2. Incubator
3. Mettler analytical balance

3.4 Material

1. Micropipette
2. Sterile micropipettar tips
3. Ionic paper (DEAE)
4. Sterile eppendoff tubes
5. Azidothymidine(AZT - 0.625µg/ml)
6. Test sample,
7. EDTA,
8. Trichloroacetic acid (TCA)

3.5 Test Viral Preparation

Commercial Viral Enzyme Preparation of Moloney Murine Leukaemia Virus Reverse Transcriptase (MoMuLV) having Poly(A)dT (Primer for RT) & mixture of dATP, dGTP, dCTP & (^{3}H) dTTP nucleotides.

3.6 Procedure

Add 50µl of commerical preparation of MoMuLV RT having Poly(A)dT (Primer for RT) & 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 instead of inhibitor. AZT(0.625µg/ml), a well known RT inhibitor(3µl) was added as a positive control. The reaction was stoped 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.

Schematic Representation

50µl commerical preparation of MoMuLV RT + 3µl of active inhibitor

↓

Incubate at 37^0C for 2 hrs.

↓

Adddition of 25µl EDTA + 25µl TCA

↓

Spot on ionic paper (DEAE)

↓

Wash the paper thrice with TCA.

↓

Wash once with alcohol

↓

Air dry

↓

Check for radioactivity.

Reference :

"Phyllanthus amarus down-regulates hepatites B virus mRNA transcription and replication", C. D. Lee, M. Ott., S. P. Thyagarajan, D. A. Shafritz, R. D. Burk & S. Gupta, European Journal of Clinical Investigation (1996) 26, 1069-1076 (Refer to Appendix G).

VIRIDIS

VIRIDIS BioPharma Pvt. Ltd., 6/10 Jogani Industrial Complex, V. N. Purav Marg, Chunabhatti, Mumbai – 400 022 India.
Tel.: 2524 3020. Fax: (91-22) 2523 5952. E - mail: viridis@vsnl.com www.viridisbiopharma.com

Reverse Transcriptase (RT) Inhibition Test Results

Sample Source American Biotech Lab

Sample Description ASAP 10 ppm (Lot # 02198)
ASAP 14 ppm + 1.5% H_2O_2 (No Lot #)
ASAP 22 ppm (Lot # 02193)

Date of Analysis 5th May 2003

Sample	***% Inhibition***
ASAP-10	**89.52%**
ASAP-14 + 1.5% H_2O_2	**86.93%**
ASAP-22	**84.46%**
+ve control, AZT*	**18.06%**
-ve control, Phosphate Buffer Saline (PBS)	**0%**

* Refer Appendix H

KP Rane
Quality Assurance

3.7 Conclusion:

The test results suggest that ASAP solution hold promising inhibiting activity against Reverse Transcriptase and hence against HBV.

4 Test Procedure for Cytotoxicity

Cytotoxicity:-

Cytotoxicity test was carried out to evaluate toxic effects of ASAP in an *in vitro* system using two models Vero cells (African Green Monkey Cell line) and Hep2 (Human epithelial cell)

4.1 Aim

This Assay Method describes an *in vitro* test method for assaying cytotoxicity of ASAP solution.

4.2 Principle

Two freshly prepared cell cultures; Vero & Hep2 were inoculated with ASAP solutions and incubated under CO_2 environment. Cytopathic effects, if any are observed under inverted microscope. Cell culture with the addition of Phosphate Buffer Saline (PBS) served as reference control.

4.3 Equipment

1. Incubator having 5% CO_2 environment
2. Inverted microscope
3. Syringe
4. Microtitre wells
5. Autoclave

4.4 Materials

1. Fetal Calf serum
2. Vero cell line (African Green Monkey Cell line)
3. Hep2 (Human epithelial cell)
4. Minimum essential medium

   9.61 g MEM 16 with Earle's salts

   2.2 g sodium bicarbonate ($NaHCO_3$)17

   Dissolve reagents in above two materials in 900 ml deionized water (DW).

   Add 5.0 g lactalbumin hydrolysate or edamin 18 to 10 ml DW, and heat to 60° ± 2°C until dissolved. Add to above 900ml solution with constant mixing.

4.5 Procedure

Cells are prepared from healthy, confluent Vero cells and Hep2 cells that are maintained by passing every 3 to 4 days. One day prior to test initiation, using a self-refilling repetitive syringe, cells suspended in Growth Medium are dispensed into wells. Incubate at 37° ± 2°C in a 5% CO_2 incubator for 72 ± 12 hr.

100 µl of each substance to be tested was introduced into wells in triplicates. 100µl of PBS served as positive control. The cell lines were reincubated at 37° ± 2°C in a 5% CO_2 incubator for 72 ± 12 hr. Every 24 hrs wells were examined under high power of an inverted microscop to check for cytopathic effect (CPE).

Schematic Representation

Cultivate the susceptible cell lines (Vero, Hep2) in Minimum essential medium containing 10% Fetal Calf serum

↓

Monolayer of a susceptible cell line

↓

Inoculate 0.1 ml of the sample in the required number of wells.

↓

Inoculate sterile 0.1 ml PBS into one of the wells as control

↓

Incubate at 37^0C, under 5% CO_2 for 72 hrs

↓

Observre under high power for cytotoxicity (Cytopathic effect)

VIRIDIS

VIRIDIS BioPharma Pvt. Ltd., 6/10 Jogani Industrial Complex, V. N. Purav Marg, Chunabhatti, Mumbai – 400 022 India.
Tel.: 2524 3020. Fax: (91-22) 2523 5952. E - mail: viridis@vsnl.com www.viridisbiopharma.com

Cytotoxicity Test Results

Sample Source American Biotech Lab

Sample Description ASAP 10 ppm (Lot # 02198)
ASAP 14 ppm + 1.5% H_2O_2 (No lot #)
ASAP 22 ppm (Lot # 02193)

Date of Analysis 5th May 2003

Sample	***Vero cell line***	***Hep2 cell line***
ASAP-10	**No CPE**	**No CPE**
ASAP-14 + 1.5% H_2O_2	**CPE - positive**	**CPE - positive**
ASAP-22	**No CPE**	**No CPE**
PBS; (Control)	**No CPE**	**No CPE**

CPE - Cytopathic Effect

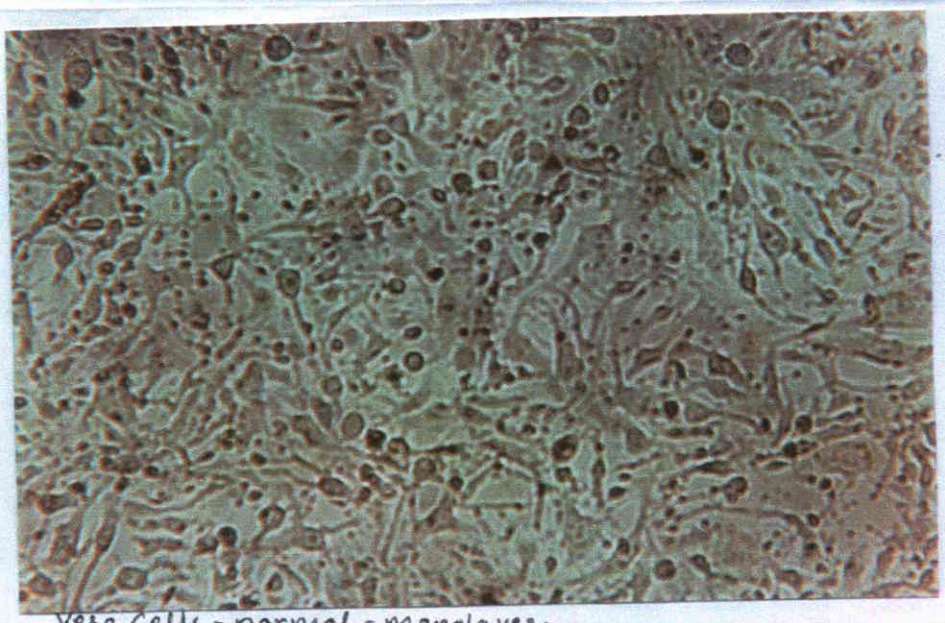
Quality Assurance

4.6 Conclusion:

Distinct cytopathic effect were observed in cell lines with ASAP-14. In fact, on addition of 100µl to the wells total bleaching of the vital indicator occurred. Cytopathic effects were noted as follows:

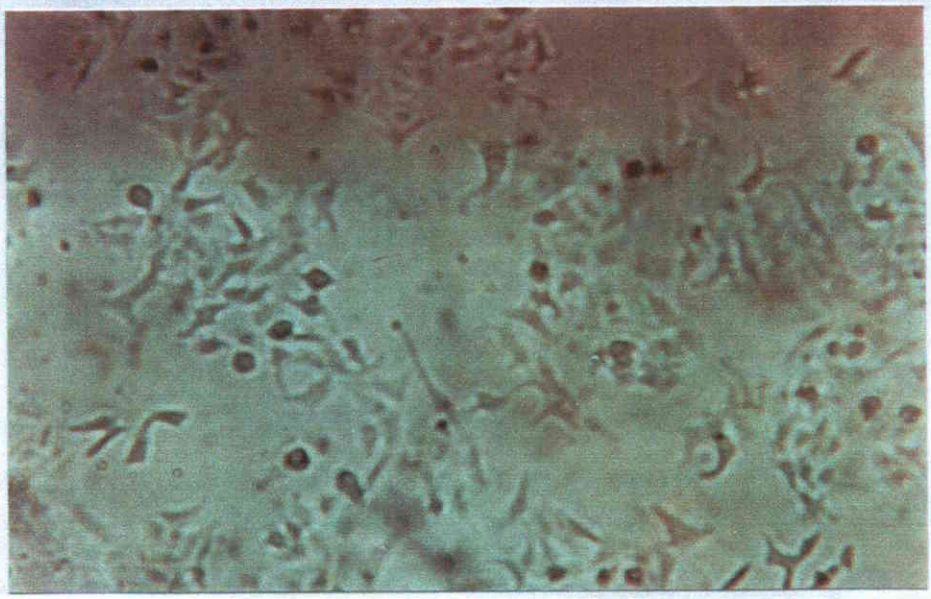
1. Rounding of cells
2. Granulation of cell cytoplasm
3. Detachment of cell monolayer from well surface.

The above can be easily seen in the attached photomicrographs (Pg. 22 & 23).
Cell lines treated with ASAP-10 & 22 were indistinguishable from the control indicating no cytotoxicity. ASAP 14 ppm displayed cytotoxicity which is likely due to H_2O_2.



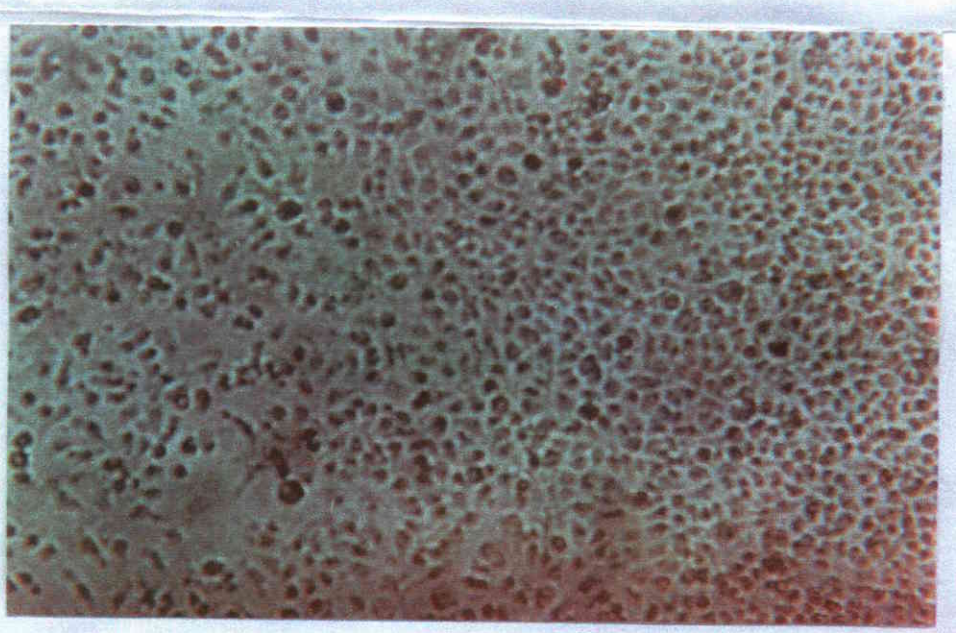
Vero Cells, normal - monolayer.

Reference Control: Vero Cell line (African Green Monkey Cell line) without any CPE



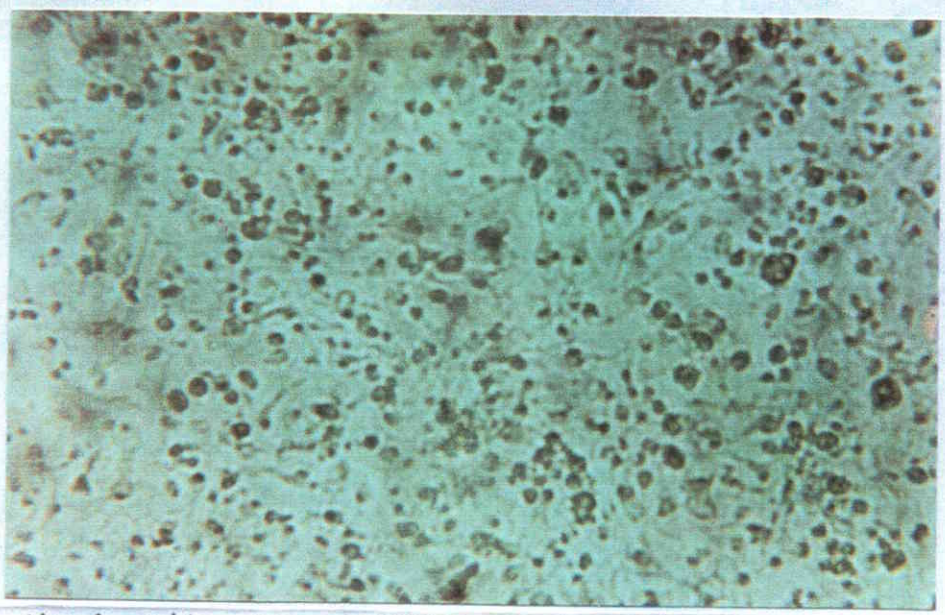
Vero Cells
CPE - POSITIVE
Cells detached from the surface.

Vero Cell line (African Green Monkey Cell line) having 14 ppm ASAP + 1.5% H_2O_2 showing CPE i.e. detachment of cells from the surface.



Hep2 cells, normal - monolayer

Reference Control: Human Epithelial Cell line (Monolayer) without any CPE.



Hep 2 cells, CPE-POSITIVE, Granulation in cytoplasm.

Human Epithelial Cell line with 14 ppm ASAP + 1.5% H_2O_2 showing CPE i.e. granulation in cytoplasm

5 Revalidation

The results of ASAP antiviral activity in this study were thought to be unusual. Hence, all the tests were repeated beyond the usual repeat. The results have been incorporated in the previous sections and were no different then the original tests activities.

Appendix A

Sample Source : Amreican Biotech Laboratories

Sample Identification : ASAP 22 (Lot # 02193)

Date of Analysis : 29th October 2002

Report issue date : 26th November 2002

Total No. of pages : Two

Antiviral Activity of ASAP Solution

Aim : To check antiviral activity of ASAP Solution (22 ppm) using a bacteriophage model.

Culture Used : T – even phage
E.coli host

Method :

Phage virulence

Virulence of Phage was activated by performing 3 successive transfers in the host and extracting with chloroform. The virulence of the Phage was checked by spotting on *E.coli* (host) lawn and checking for zone of lysis.

Experimental conditions

10 ml of ASAP solution was challenged with 1 ml of Phage suspension (10^9 Phage particles). Similarly a negative control was run using 10 ml saline in lieu of ASAP solution. 20 µl aliquots were withdrawn from 0 hour onwards at 30 minutes intervals and presence of Phage was determined using the host indicator system. Results are as per Table-1.

TABLE-1:

Sr. No.	Exposure Time (Hrs.)	Test		Control	
		Zone of Lysis	Presence of Phage	Zone of Lysis	Presence of Phage
1.	0	+++	Present	+++	Present
2.	0.5	+++	Present	+++	Present
3.	1	++	Present	+++	Present
4.	1.5	+	Present	+++	Present
5.	2	2 viral particles	Present	+++	Present
6.	2.5	-	Absent	+++	Present

Conclusion : The ASAP solution showed virucidal activity completely eliminating all viral particles in a period of 2.5 hours. The negative control samples showed presence of Phage after 2.5 hours under similar conditions. Though silver is postulated to exert anti-microbial activity by uncoupling the ETC mechanism in prokaryotes, it is acting through a different mechanism in this case possibly through precipitation of the viral proteins. These results could be extrapolated and it would be interesting to determine antiviral activity against known pathogenic animal viruses using a tissue culture model.

Appendix B

What is Hepatitis B Virus?

Hepatitis B is a DNA Virus of the hepadnaviridae family of viruses. It is a 3.2-kb DNA virus, replicating almost exclusively in the liver cells (hepatocytes). Replication involves two main enzymes; DNA polymerase & reverse transcriptase. The infectious particle consists of an inner core plus an outer surface coat.

To aid visualisation a conceptual and a real picture is shown below:

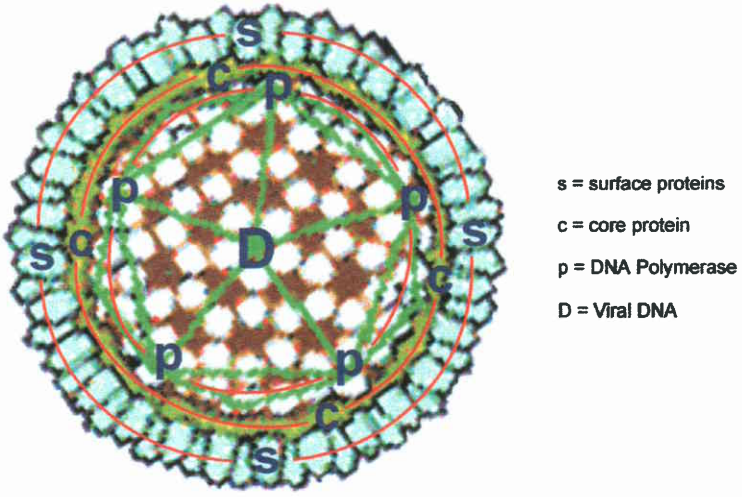


Fig. 1 - Hepatitis B Virus (HBV).

In real life (Fig 1.) the virus is a spherical particle with a diameter of 42nm (1nm = 0.000000001 metres) and is composed as follows. There is an outer shell (or envelope) composed of several proteins known collectively as HBs or surface proteins (indicated by 's' in Fig 1.). This outer shell is frequently referred to as the surface coat. The outer surface coat surrounds an inner protein shell, composed of HBc protein (shown as 'c' in Fig 1). This inner shell is referred to as the core particle or capsid. Finally the core particle surrounds the viral DNA ('D') and an enzyme DNA Polymerase ('p').

How does the virus replicate?

When the virus enters the body of a new host it's initial response, if it's gets past the immune system, is to infect a liver cell. To do this the virus attaches to a liver cells membrane and the core particle enters the liver cell. The core particle then releases it's contents of DNA and DNA polymerase into the liver cell nucleus. In the liver cell virus replicates via reverse trancsription and translation process which involves reverse trancriptase and DNA polymerase enzymes. Thus DNA polymerase causes the liver cell to make copies of hepatitis B DNA. These copies of the virus are released from the liver cell membrane into the blood stream and from there can infect other liver cells and thus replicate effectively. The incubation of the Hepatitis B Virus (hepatitis B) is about 6 to 25 weeks (i.e. before physical and generally detectable histological or physical symptoms occur) however there are several biochemical and histological changes that occur in stages after infection with the hepatitis B virus.

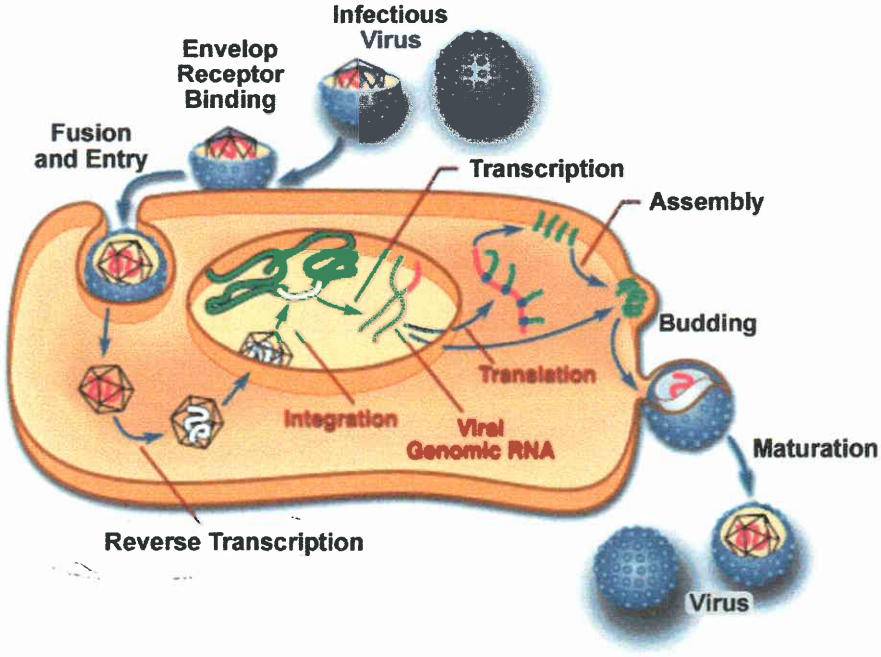
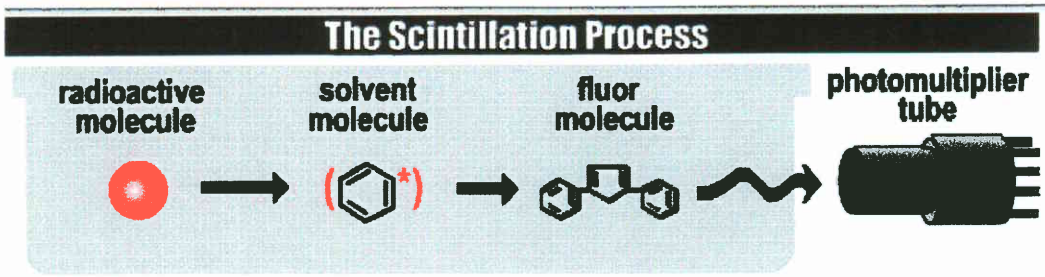


Fig. 2 - Diagramatic Representation of HBV Replication

Key Targets : Reverse Trancsription (reverse transcriptase)
Viral DNA integration (DNA polymerase)

Appendix C

Liquid Scintillation Counting: an Overview



The process of liquid scintillation counting is relatively simple. The beta decay electron emitted by the radioactive isotope in the sample excites the solvent molecule, which in turn transfers the energy to the solute, or fluor. The energy emission of the solute (the light photon) is converted into an electrical signal by a photomultiplier tube.

The process of liquid scintillation involves the detection of beta decay within a sample via capture of beta emissions in a system of organic solvents and solutes referred to as the scintillation cocktail. This mixture is designed to capture the beta emission and transform it into a photon emission which can be detected via a photomultiplier tube within a scintillation counter. The cocktail must also act as a solubilizing agent, keeping a uniform suspension of the sample.

The solvent is the first compound in the scintillation cocktail to capture the energy of the beta particle. The solvent molecule achieves an excited state, and the excess energy is transferred from solvent molecule to solvent molecule. The solvent remains in the excited stated for an extended period of time, decaying into the ground state without the emission of light. The solute then absorbs the exitation energy of the solvent, and quickly returns to the ground state by emitting light. If a secondary solute is used, that solute absorbs the signal of the first solute and emits a second burst of light at a longer wavelength.

Reference:

http://laxmi.nuc.ucla.edu:8248/M248_99/autorad/Scint/scint1.html