THE ANTIMICROBIAL COMPOUND, K-21 INHIBITS REPLICATION OF ENVELOPED & NON-ENVELOPED DNA & RNA VIRUSES

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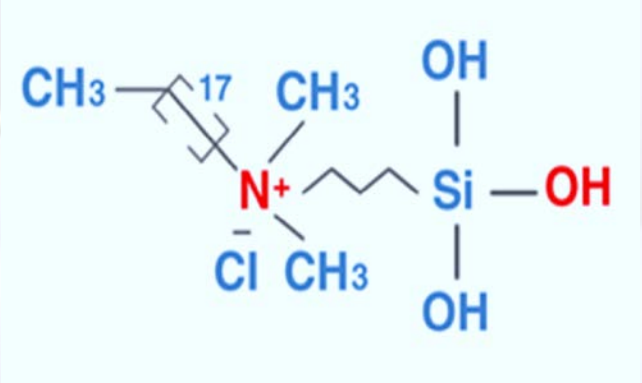
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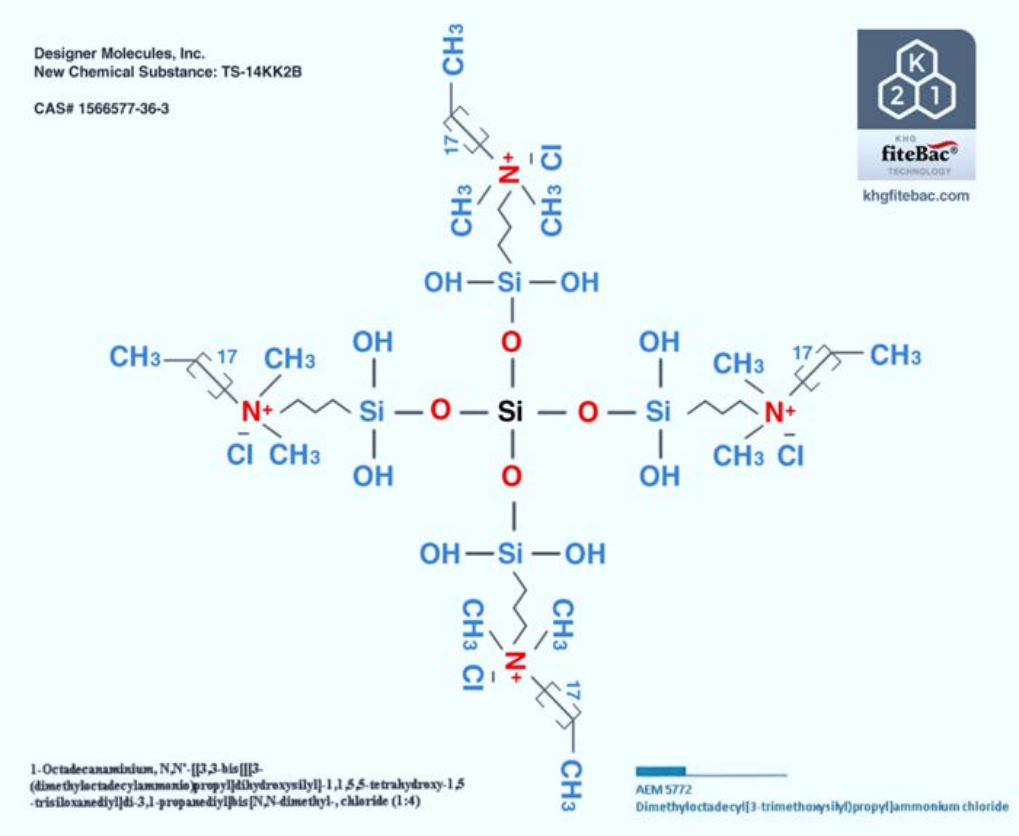
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K-21 STRUCTURE

INTRODUCTION:

QAC





Quaternary Ammonium Compounds (QACs) DC5700 of Dow Corning molecule is shown to possess mild antimicrobial activity, and this molecule is the precursor of K-21 molecule. K-21 is just four arms of SiQAC (DC5700) on silica core, which is less toxic than QAC. Tuladhar et al., 2012 tested QACs to assess viralcidal affects on two human RNA Viruses, one enveloped (Influenza A Virus) and other non-enveloped (Polio Virus). Their results showed that according to their assay SiQACs had no viralcidal affects on Polio Virus on glass and plastic surfaces, since Polio Virus was high, with less than 1 Log10 decay in 10 days. On the other hand, Influenza A Virus showed fast decay the first 24 hours, and then slower decay up to 5 days and again faster decay after 5 days. They indicated that reduction in influenza virus by QACs is not the result of a profound effect on viral genome, but a primary effect on viral envelope. Based on these findings we tested the anti-viral effects of K-21 on Bacteriophage MS2, a surrogate of Polio Viruses, the host cells for which is E. coli, and also tested K-21 anti-viral activity against Influenza A Virus, and a single stranded RNA non-enveloped feline Norovirus. Noroviruses belong to the family caliciviridae. Noroviruses are major cause of non-bacterial epidemic gastro-enteritis which could be a community wide breakout, and in children known to lead to severe diarrhea. Our study also included testing K-21 *in vitro* on 3 human herpesviruses i.e. HSV-1, HHV-6A, and HHV-7 which are highly neurotropic and are shed in the oral and nasal cavities, and contribute to many important human diseases. Thus to prevent these viruses and infection would have important implication as therapeutic

IN VITRO INHIBITION OF VARIOUS MICRO-ORGANISMS BY NOVEL MOLECULE K-21

- **E. Coli (Antibiotic Resistant Strain)**
- **Staphylococcus Aureus (Antibiotic Resistant Strain)**
- **MTB-Mycobacterium tuberculosis (Drug Resistant Strain)**
- **Chlamydia Trachomatis**
- **Klebsiella Pneumoniae (Drug Resistant Strain) Isolated from Zambian Neonates**
- **Klebsiella Oxytoca (Drug Resistant Strain) Isolated from Zambian Neonates**
- **Porphyromonas Gingivalis**
- **MRSA-*Methicillin-Resistant Staphylococcus Aureus***
- ***CRE-Carbapenem-Resistant Enterobacteriaceae***
- **Fungal Species**
- **Ruptured Clostridium Difficile Spores**

(Un-published data)

MATERIALS & METHODS

Two preparations of K-21 were tested on RNA viruses with EPA approved protocol for testing hand gels, antimicrobial spray on germicidals in which the contact time of test compound is 10 minutes. K-21 in 50% ethanol was dissolved in ethylene glycol and then in water to obtain a clear solution (Lina Niu's protocol). The second formula, K-21 from 50% ethanol was dissolved in acetone then in distilled water to obtain solution, which was slightly milky, but got cleared on heating up 80^{o}c and it still retained antiviral activity (Bhupesh Prusty formula). The working dilution used was 0.025% and was not toxic to cells used to assay HSV-1, HHV-6A, Influenza Norovirus

DIAGRAM OF THE PROCEDURE



SUMMARY OF THE PROCEDURE FOR ENVELOPED AND NON-ENVELOPED RNA VIRUSES

- Stock virus is thawed and may be supplemented with an organic soil load, if requested
- Sterile glass **Petri dish carriers (100 x 15 mm) are inoculated** with a volume of virus suspension containing an adequate titer to recover a minimum of 4-Log_{10} infectious viruses per carrier. A sufficient number of test and control carriers are prepared
- **Inoculated carriers are dried** at room temperature under laminar flow conditions
- **The test substance is prepared** according to the Study Sponsor's instructions as requested, and applied to the test carriers using a spray device. The distance, angle, and number of sprays applied are recorded
- The treated **carriers are held for the predetermined contact** time(s), and **then neutralized** in a manner appropriate for the test substance (e.g. dilution and/or gel filtration)
- **The control carrier is harvested using an equivalent volume cell culture** medium or other suitable buffer
- **Following neutralization** of test and control carriers, the **viral suspensions are quantified** to determine the levels of infectious virus using standard cell culture (e.g. $TCID_{50}$) or plaque assay techniques
- **Assay trays/plates are incubated for the period most suitable** for the virus-host cell system (e.g. 7 days)
- **After the incubation period, the assay is scored for the presence/absence of test virus and cytotoxic effects.** The appropriate calculations are performed (e.g. Spearman-Karber) to determine viral titers and levels of test substance cytotoxicity, where applicable
- **Log_{10} and percent and percent reductions are computed** for viral films exposed to the test product relative to the titer obtained for the study control carrier(s), and reported to the Study Sponsor

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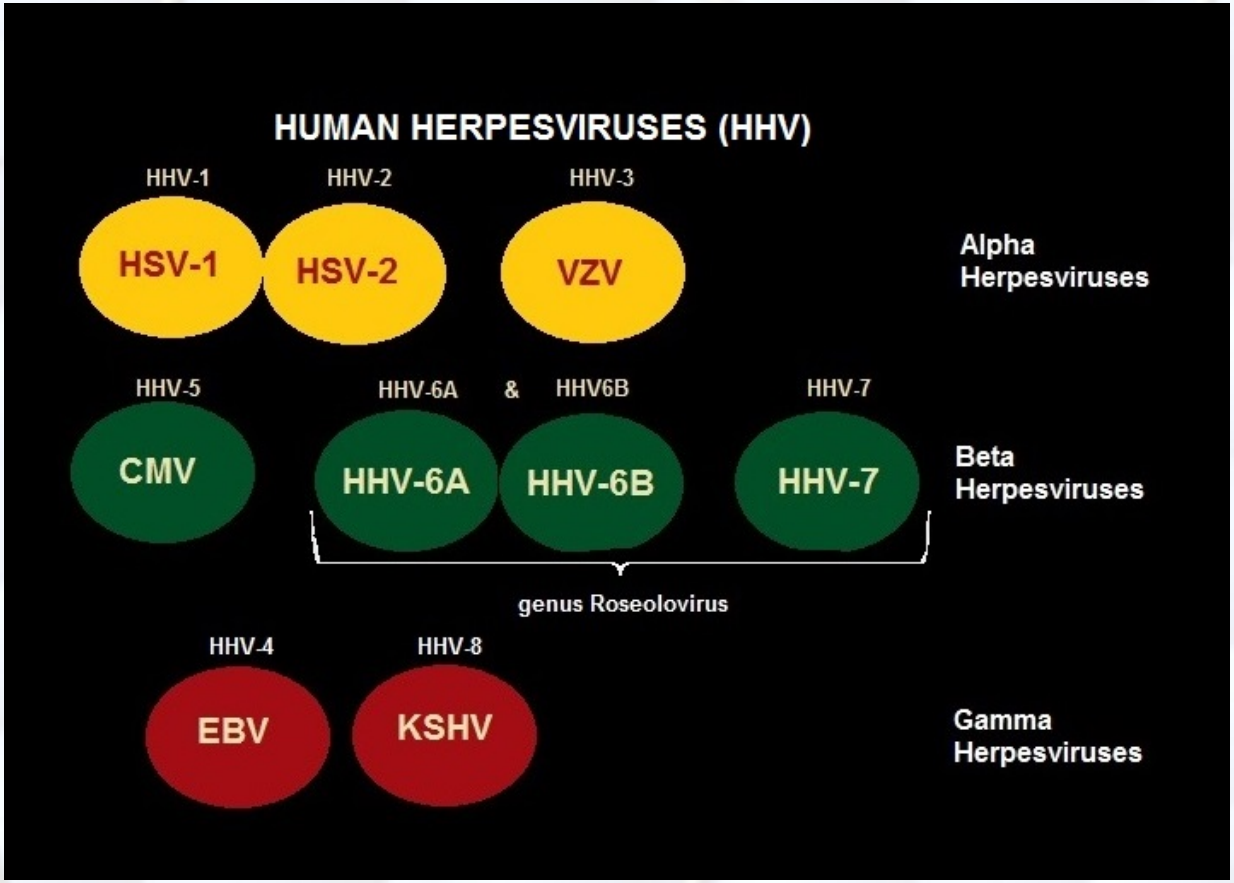
PASSING CRITERIA

AOAC International has defined the passing criteria for the Germicidal Spray test for viruses as:

1. Complete inactivation of the test virus at all dilutions

2. If cytotoxicity is observed, a $\geq$3-Log_{10} reduction in viral titer is observed past the level of cytotoxicity relative to the virus control

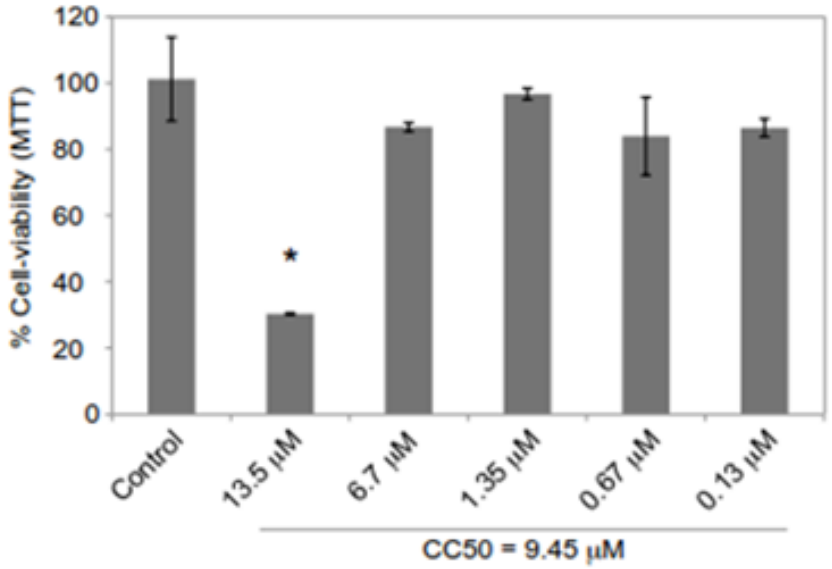
NOMENCLATURE OF HUMAN HERPES VIRUSES (AS OF 2013)



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CYTOTOXICITY OF K-21 IN HUMAN FORESKIN FIBROBLASTS



Cell viability (MTT) assay in primary human foreskin fibroblasts (HFFs) to determine cytotoxic dose (CC50) of K21. HFFs were seeded into 96-well plates (10^3 cells/well). At 24 h in culture with varying amounts of K21, cells were analyzed for viability by MTT assay. Results show the mean % cell viability relative to solvent control treated cells (Control) from two repeated experiments performed in triplicate. *$p<0.05$. CC50 value was calculated to be 9.45 µM from the calculated slope equation.

K-21 INHIBITS HSV-1 ENTRY IN VERO CELLS

Incubation of HSV-1 with K21 for 3 minutes at room temperature

↓

HSV-1 particles were allowed to adhere and enter the cells for 30 minutes

↙ ↘

Trypsinized (+ Trypsin), washed thoroughly to remove viral particles that were bound to the outer cell surface but not entered into the cell

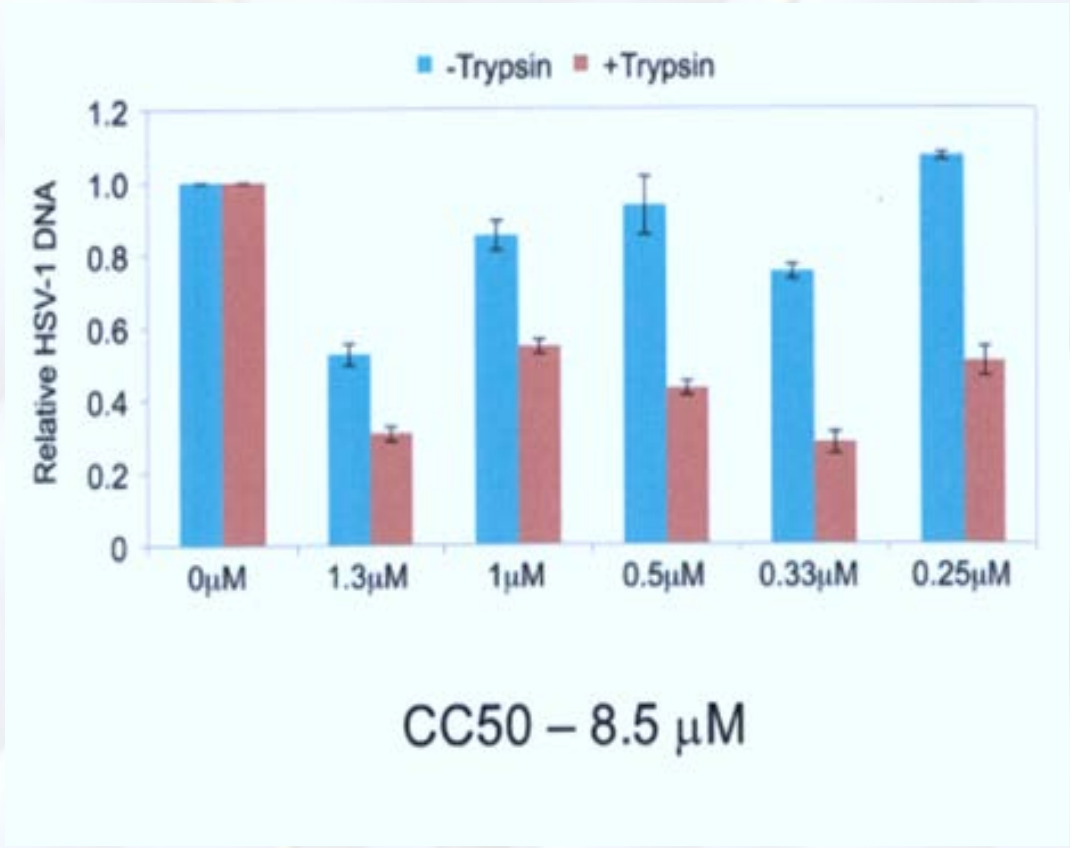
No trypsinization (- Trypsin) but washed thoroughly to remove unbound viral particles

↓

DNA Extraction

↓

Real time qPCR



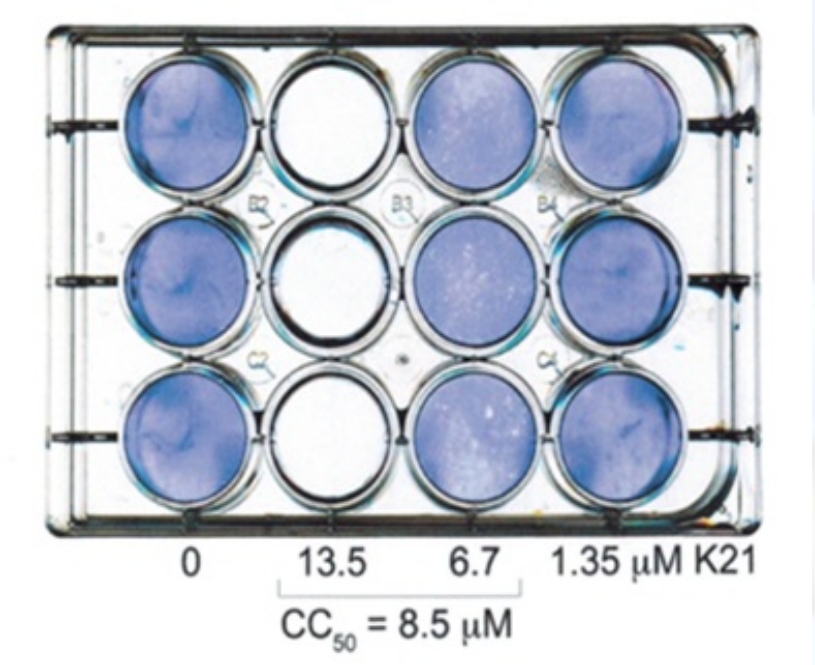
CC50 – 8.5 μM

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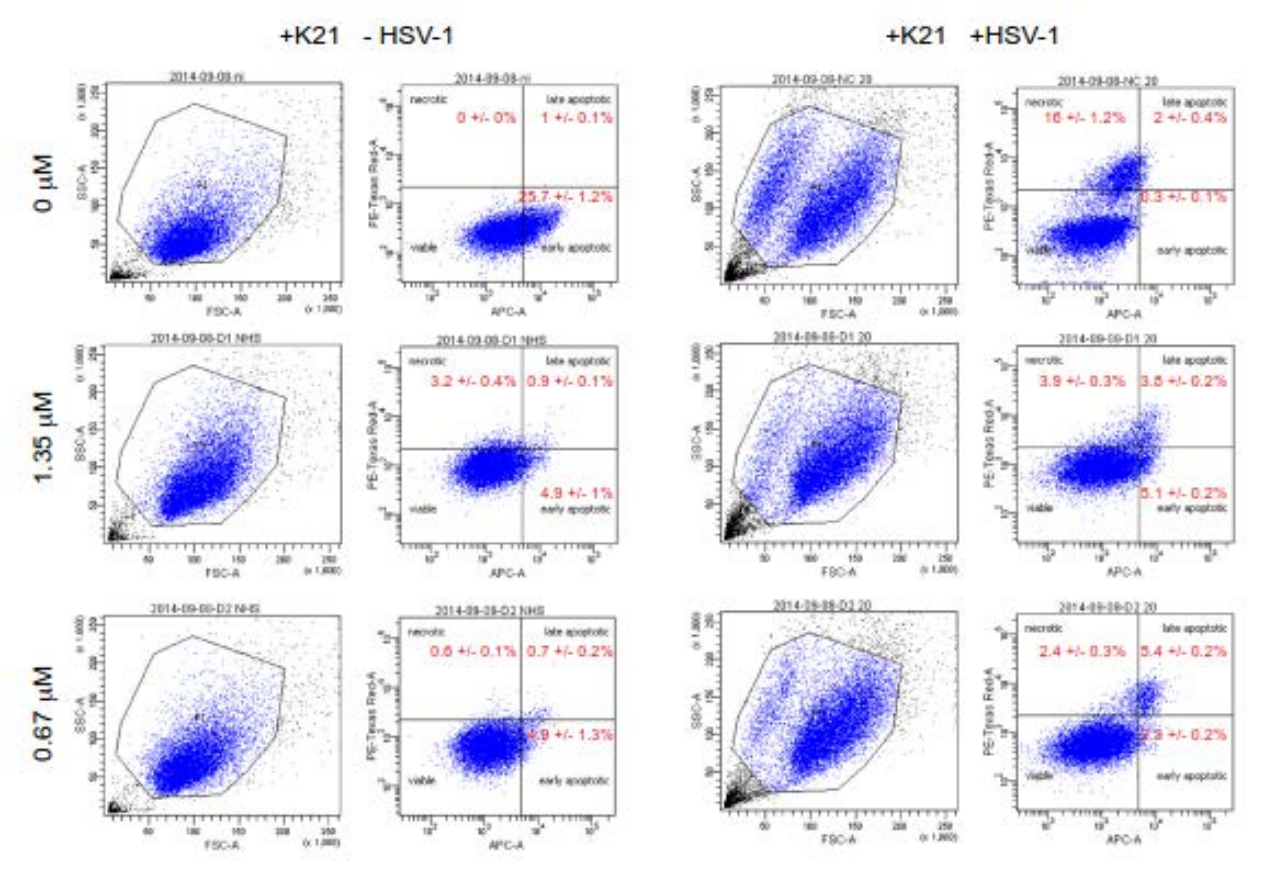
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CHALLENGES FOR THE USE OF K-21 IN VITRO USE

- **Solubility –**
- **K-21 dissolved in ethanol was completely insoluble in water. Hence was not suitable for *in vitro* use**
- **We managed to dissolve K-21 in water by diluting it through acetone and tested its effect on cytotoxicity in cell culture**
- **CC50 – 8.5µM**
- **In the Plaque assay (HSV-1) slide we see the effect of K-21 on infectious HSV-1 progeny formation. We infect Vero cells/HFFS in presence of K-21 or absence of it and then take the lysates from these cells and do second round if infection where we form plaques to count the progeny numbers**



IN VITRO INHIBITION OF HSV-1 BY FLOW CYTOMETRY

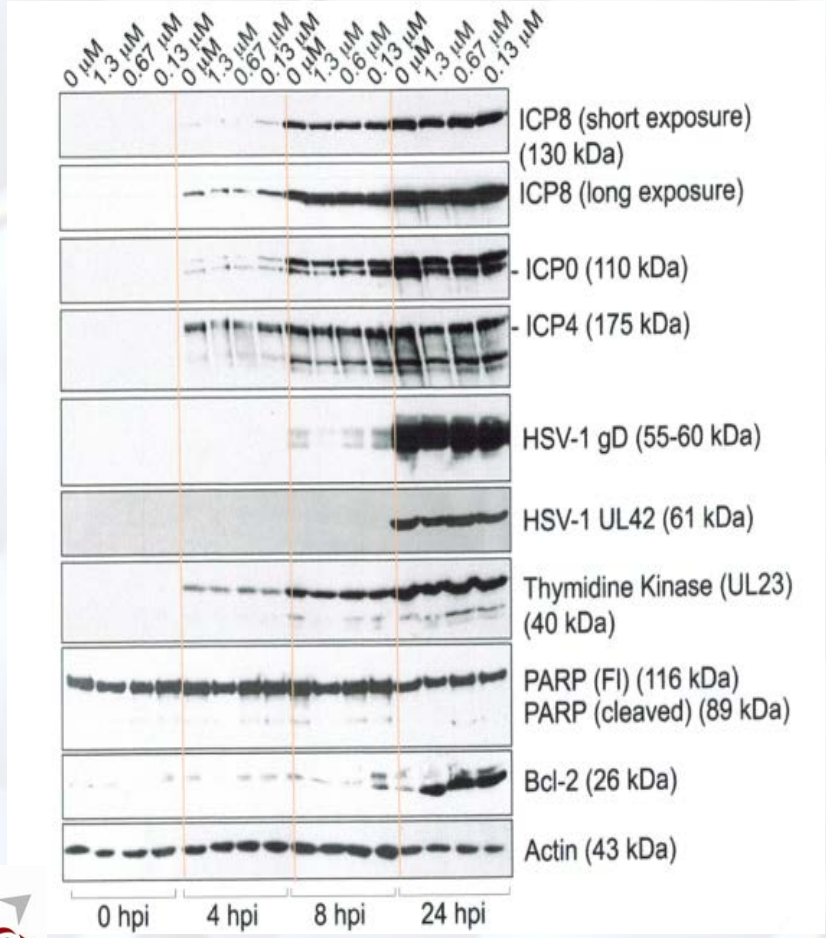


K-21 inhibits HSV-1 infection-induced cytopathic effects. Vero cells were infected with HSV-1 in presence of K-21 at different dilutions or were grown in the absence of HSV-1 infection but with same concentrations of K-21

24 hours post infection, cells were processed for flow cytometry to quantify number of apoptotic or necrotic cells. Data represents one of the triplicate experiments

By flow cytometry, we measure the cytotoxicity that arises because of HSV-1 infection. HSV-1 infection kills the host cell after the infection cycle. K-21 prevents this cell death because of the inhibition in infection

EFFECT OF K-21 ON HSV-1 IN VERO CELLS (EXPRESSION OF VIRAL PROTEINS)



K21 induces Bcl-2 expression, which possibly reduces the HSV-1 induced cytopathic effect

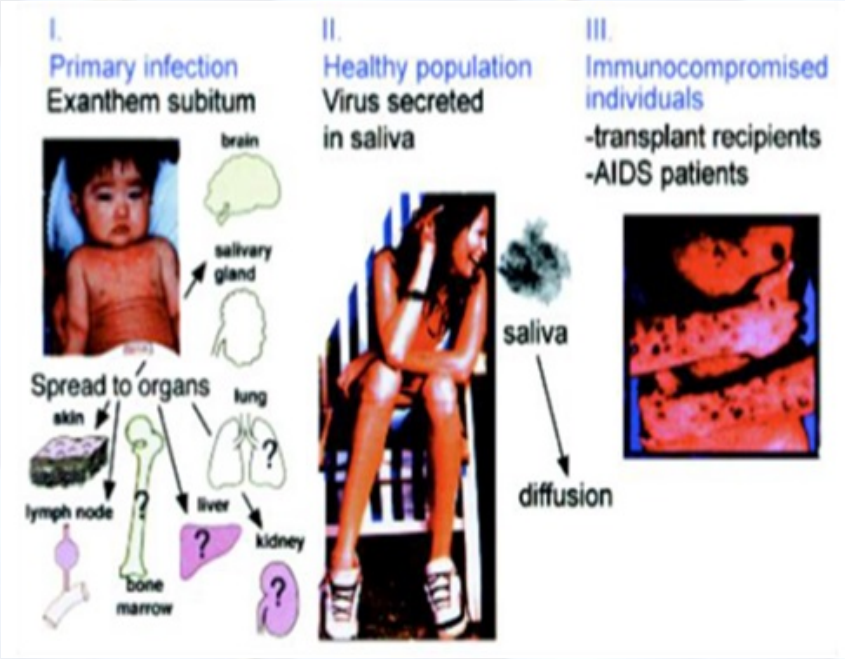
HSV-1 proteins like ICP0, ICP4, ICP8 are decreased in presence of K-21. However it does not have much effect on UL27 and TK. Bc12 is upregulated in HSV-1 infected cells treated with K-21 but not with untreated cells. This indicates that K-21 possibly induces premature senescence only in virus infected cells

HHV-6

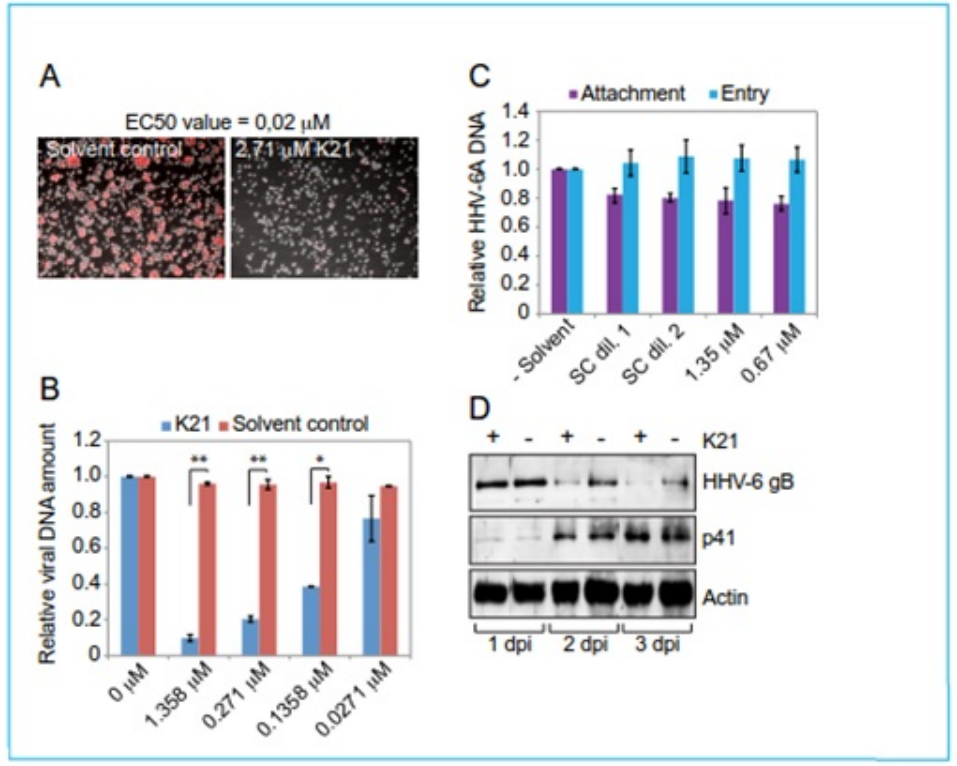
HHV-6 belongs to the genus Roseolaviruses (cause roseola infantum, and febrile illness in children). It is probably the most neurotropic virus known so far

- **Almost all children are exposed to this virus by the time they are 6-12 months old and more than ≥95% of healthy individuals have HHV-6 DNA detectable in their saliva. ≥95% the adults have HHV-6 IgG antibody**

- **90% of pregnant women have antibodies to HHV-6 and is transferred to the child through placenta, is detectable in infants during the first few months after birth**



EFFECTIVE INHIBITION OF HHV-6A INFECTION BY K-21



K21 inhibits HHV-6A infection. (A) HSB-2 cells were infected with HHV-6A that express mCherry protein in infected cells. Infected cells were treated with either solvent control or K21. At 72 h post infection, cells were imaged using epifluorescence microscope. (B) Total genomic DNA was extracted from a parallel set of experiment and HHV-6 DNA amount in infected cells were quantified by qPCR. (C) Effect of K21 on HHV-6A entry and attachment was studied by qPCR. SC, solvent control. (D) Effect of K21 on HHV-6 replication and growth was studied by immunoblotting

EFFECTIVE INHIBITION OF HHV-7 INFECTION BY K-21

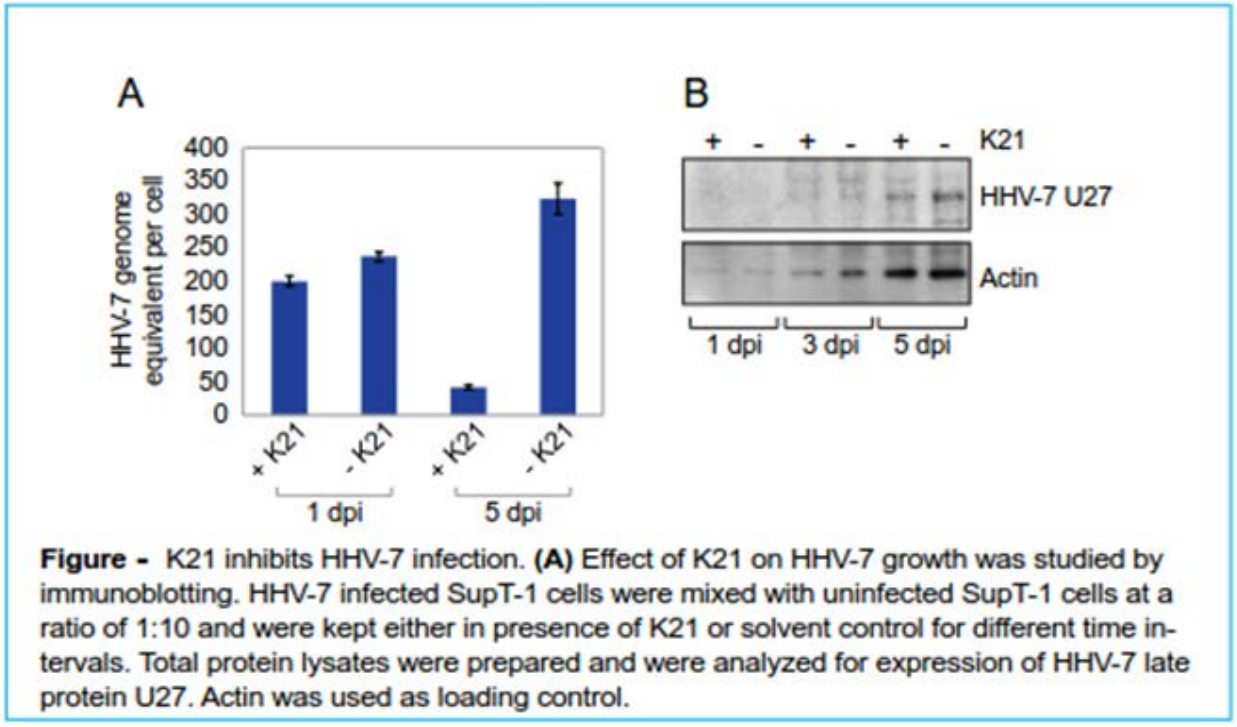


Figure - K21 inhibits HHV-7 infection. **(A)** Effect of K21 on HHV-7 growth was studied by immunoblotting. HHV-7 infected SupT-1 cells were mixed with uninfected SupT-1 cells at a ratio of 1:10 and were kept either in presence of K21 or solvent control for different time intervals. Total protein lysates were prepared and were analyzed for expression of HHV-7 late protein U27. Actin was used as loading control.

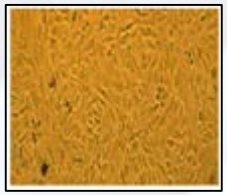
SupT-1 cells are also undergoing premature senescence like Vero cells in presence of K-21. The CPE is less but the infected cells are dying faster than the non-infected cells

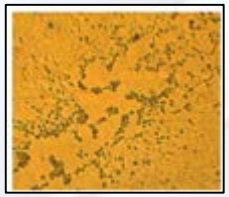
HHV-7 belongs to the genus Roseola Viruses and like HHV-6 it causes roseola infantum and febrile illnesses and epilepticus in 7% of the children with primary infection. Like HHV-6, HHV-7 antibody distribution is ≥96% and transmission is mostly by saliva and is ubiquitous. It is neurotropic virus. HHV-7 activates in vitro HHV-6B. HHV-7 contributes to limbic encephalitis in transplant patients undergoing HSCT

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RNA VIRUSES

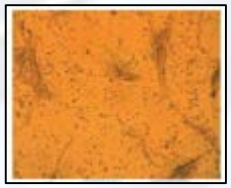


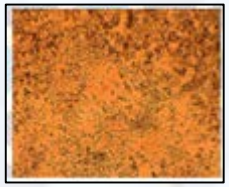


Feline calicivirus (FCV), ATCC VR-782

This virus is a non-enveloped, positive-stranded RNA member of the genus Vesivirus, and a common cause of respiratory infections in cats. Symptoms of infection in felines include nasal discharge and mouth ulcers. As a member of the *Calciviridae* viral family, FCV is closely related to human noroviruses, which cause acute gastroenteritis marked by nausea, vomiting, and diarrhea. Unlike human norovirus, however, a simple cell culture assay system is available for FCV. Therefore, feline calicivirus is the US EPA-approved surrogate microorganism for human norovirus label claims. Both FC V and human norovirus are able to remain viable on environmental surfaces for extended periods of time and are resistant to a number of disinfectant actives

Permissive Host Cell Line Selected for FCV: CRFK (Crandell-Rees Feline Kidney Cells), ATCC CCL-94

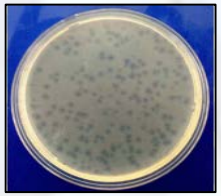




Influenza A (H1N1)

Influenza A virus is an enveloped, minus-stranded member of the family *Orthomyxoviridae,* and causative agent of the illness influenza (which is more widely recognized by the term “flu”). Influenza is more serious than other seasonal mild, respiratory tract infections (e.g. the common cold) with symptoms that can last for upwards of several weeks. Young children and the elderly are particularly susceptible to severe illness and death due to infection. Influenza is readily transmitted via infective aerosols direct contact with infective respiratory secretions. Potential transmission by contaminated environmental surfaces (fomites) has increasingly become of interest, and influenza virus is highly vulnerable to inactivation by drying and exposure to variety of disinfectant actives

Permissive Host Cell Line Selected for Influenza A (H1N1): MDCK (Madin Darby Canine Kidney Cells), ATCC CCL-34



Bacteriophage MS2

Bacteriophage MS2 is un-enveloped, positive strain RNA Virus of bacteriophage family Leviviridae. The host cells for MS2 is E.coli 15597. Because of its small size, icosahedral structure, and environmental resistance made MS2 for ideal use as Surrogate Virus in place of picornaviruses e.g. Polio Virus, Human Norovirus

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K-21 INHIBITS IN VITRO REPLICATION OF INFLUENZA A VIRUS

We used Influenza A Virus strain H1N1 (ATCC # VR. 1736)

1. Original Virus titer (in Log_{10}) = **5.80**
2. Virus inhibited by 0.025% K-21 (dissolved in acetone) = **≤4.10**
3. Virus recovered that did not get inhibited = **1.70**
4. Control i.e. No K-21 but solvent only that inhibited the Virus = **0.20**

Conclusion: The above data shows that –

(a) K-21 is a **POTENT INHIBITOR OF ENVELOPED RNA VIRUS** and

(b) that the concentration of K-21 that inhibited Influenza Virus was not toxic to cells and

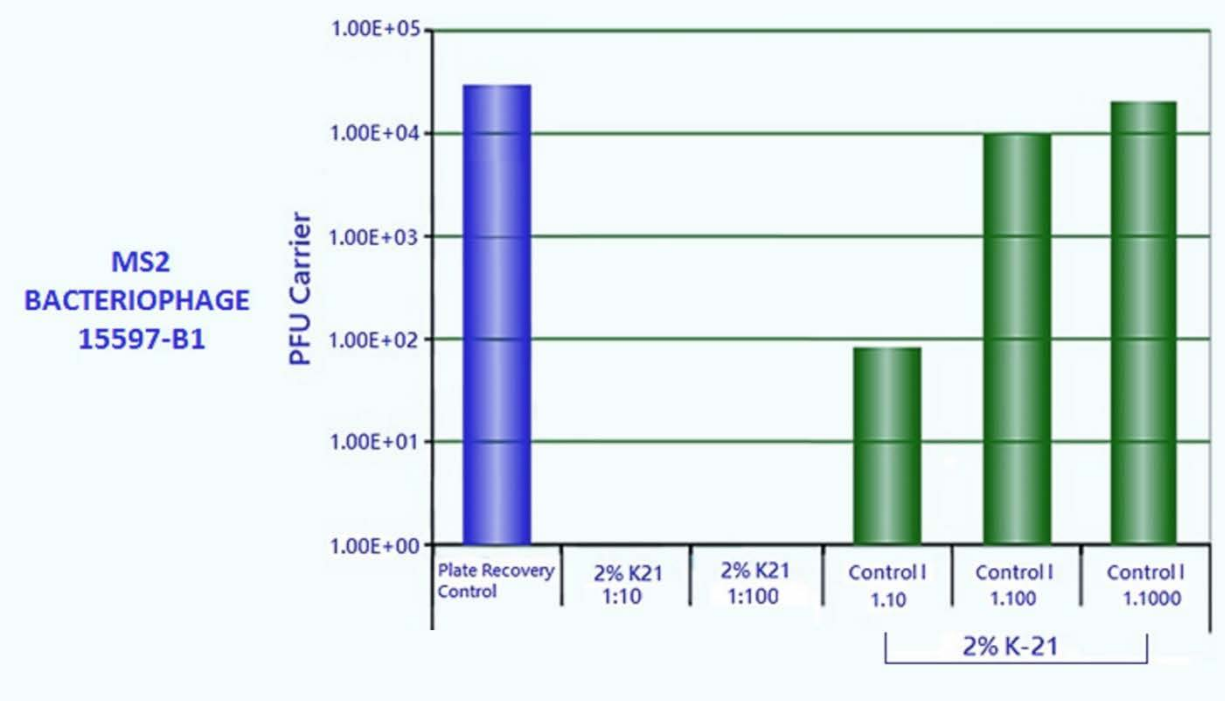
(c) The acetone alcohol had no significant effect on the Virus that could cause inhibition

Test was performed according to the EPA approved assay showing **INHIBITION BY K-21 IS ≥ 98.00 %**

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INHIBITION OF MS2 BACTERIOPHAGE BY K-21



Reduction in the Virus Titer Log_{10}

No K-21..... = ≥ 4.00
(2% K-21 with 1:10 and 1:00 dilution) .... = ≥ 3.15
Controls = No K-21 but ethylene glycol
1:10 = 2.54
1:100 = 0.4
1:1000 = 0.15
Conclusion: Ethylene glycol by itself is antiviral

EFFECT OF K-21 ON IN VITRO INHIBITION OF FELINE NOROVIRUS (CALICIVIRUS)

We used Calicivirus (Non-Enveloped RNA Virus) ATCC Strain #VR 782)

Experiment A

1.	Original Virus Titer (Log_{10})	= **5.55**
2.	Virus Titer in Presence of K-21 at **0.025%**	= **5.23**
3.	Log_{10} Virus Inhibited	= **0.32**
4.	Control i.e. Acetone + Alcohol	= **5.48**
	Inhibition of Virus by Control	= **≤0.08**
		=**52.68%**

Experiment B

1.	Original Virus Titer (Log_{10})	= **5.60**
2.	Virus Titer in Presence of K-21 at **0.1%** Made in Alcohol and Water ..	= **1.25**
3.	% Inhibition According to EPA Testing	= **94.38%**
4.	Inhibition in Log_{10}	= **4.35**
5.	Control	= **5.35**
	Inhibition by Control	= **0.25**

Since we used only one concentration of K-21 i.e. **0.1%** it was somewhat toxic to cells. Therefore, further experiments should be done using K-21 at lower concentrations

CONCLUSION:

K-21 at 0.10% inhibited significantly an un-enveloped RNA Virus (Norovirus) replication as conducted by EPA approved assay for germicidal spray, disinfectants. This concentration was toxic to cells

K-21 at 0.025 concentration did not inhibit the Norovirus significantly. Thus higher concentrations are needed to inhibit RNA non-enveloped viruses. QAC did not inhibit un-enveloped Polio Virus. This suggests that possible inhibition by K-21 of double stranded RNA Virus require higher concentration. Lower concentrations of K-21 could be supplemented with ethylene glycol to fully inhibit double stranded RNA virus replication. (Experiment under study)

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RESULTS:

1. K-21 in ethylene glycol reduced Bacteriophage MS2 viralcidal effects at 1:10 and 1:100 dilution by ≥99%

2. Ethylene glycol without K-21 also showed some viralcidal effects at 1:10 by ≤45% and 1:100 by ≤20%. Therefore, this solvent could be useful for K-21 to test on viruses or bacteria where K-21 at higher concentrations is toxic

3. The viralcidal effects of K-21 on Influenza A Virus, and Norovirus using a concentration of 0.025% reduced the viralcidal effect of Influenza A virus by ≥98% and ≤54% of feline norovirus, and this does was not toxic to test cells. However, when we used 1% K-21 dissolved in 0.1% water from 50% 0.1% alcohol it reduced the virus activity ≥99%, but since we used only one dilution i.e. 1:10 it was toxic to test cells. Further testing at lower concentrations will be done to see significant inhibition, where it is not toxic to the cells

4. K-21 at a dilution of 0.025% made from acetone inhibited significantly replication of HSV-1, HHV-6A, and HHV-7 and this dilution was not toxic to the cells

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CONCLUSIONS:

1. Contrary to QACs, having practically no viralcidal effect on non-enveloped Polio Virus, K-21 showed significant inhibitory viralcidal effects, both on Polio and feline norovirus

2. K-21 reduced Influenza A Virus titers significantly, and HSV-1, HHV-6, and HHV-7 (data under submission). These in vitro results can be used to test both for K-21 toxicity, and viral replication in animal models for HHV-6, HSV-1, and influenza A

3. Thus the data generated in our studies showed that K-21 is a potent antiviral and after safety studies can be useful for human trials to prevent various antimicrobial infections. Since we found that K-21 combined with ethylene glycol is viralcidal it would be useful in reducing cytotoxic effects where higher concentrations of K-21 are toxic

4. K-21 as of now is a potent viralcidal, and can be useful in the form of germicidal spray, disinfectant hand-gel and aerosol

5. We have data that K-21 has healed HSV-1 blisters and could be useful as a antiviral cream in treating cervical manifestations caused by HSV-2, and HHV-6 in mice where it induces neurological manifestations and possibly of other similar microbial infections

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K-21 APPLICATIONS FOR TOPICAL USE AND AS DISINFECTANT

- **K-21 in gel form has been shown to heal blisters, and reduce pain on lips due to Herpes Simplex Virus Infection *(Un-published)***
- **K-21 in the topical gel can be useful in healing lesions included by HSV-2, HHV-6 and HPV *(Not Yet Tested)***
- **K-21 could be used in Band-Aid to apply on skin injury to prevent any microbial infection and to improve healing *(Under Study)***
- **In conjunction with the Army the K-21 formulation would be tested to apply daily on soldier's skin to prevent blisters, ulcers pathogens, sun exposure, chemical exposure, alcohol damage, resists water and insect repellant**
- **K-21 formulation in the spray form would be an ideal potent disinfectant to clean laboratory benches, biosafety hoods, cleaning other hard surfaces, and any other surfaces as door knobs**
- **K-21 formulation shows killing of fungal spores. It may be useful to test against Anthrax Spores**
- **Since K-21 kills insects, we will be testing for anti-malaria activity *in vitro* and *in vivo***

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