In-Vitro HIV Study of African Herbal Therapies

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Research Study Progress Report

CONFIDENTIAL

Introduction:

Early in 2007 we began a project to characterize five mixtures of plants used in traditional African treatments for HIV/AIDS. Our goal was to determine whether the plant mixtures had any measurable effect on HIV-1 replication using typical lab strains of HIV and cellular based infectivity assays. Our initial studies showed that each of the five mixtures (A-E) had an effect on the replication of HIV-1. We then embarked on the current study to further characterize these plant mixtures. The specific AIMS of the proposed study were:

Aim 1. Do a detailed dose and time response for each mixture.

Aim 2. Do formal cell toxicity assay on each mixture.

We had also set as a secondary aim to determine the mechanism of activity of the plant mixtures as best we could in the time and with the resouces avaiable.

Summary of Results:

1. Water soluble extracts of each of the plant mixtures had an effect on HIV-1 virus. 5 gram (dry weight) portions of each plant mixture were placed in 50ml conical centrifuge tubes (100mg/ml). Distilled water was added to a volume of 50ml. The tubes were vortexed and were extracted overnight a 4°C. The residual insoluble material was removed by filtration through a 0.22 micron filter. The amount of soluble material was determined by drying one milliliter samples of each extract and weighing the residue. A portion of each water soluble fraction was also analyzed by SELDI mass spectrometry. A five microliter aliquot was mixed with 1 microliter of a saturated solution of sinapinic acid (SPA) in 50% Water, 50% acetonitrile and 0.1% trifluroacetic acid and placed on a gold surface. The sample was then dried and analyzed on the SELDI mass spectrometer.

- Mixture A 42mg/ml
- Mixture B 7mg/ml
- Mixture C 22mg/ml
- Mixture D 44mg/ml
- Mixture E 22mg/ml
- Mixture A-E 20mg/ml

These data show that a significant portion of the plant mixtures can be recovered as water soluble material. This is important since the extracts are administered as a water soluble formulation. The following SELDI spectrum shows what a profile of the plant mixtures looks like. The majority of the molecules present are <10,000 Da in size. The SELDI profile demonstrates that the mixtures are reasonably simple in composition (Fig. 1).

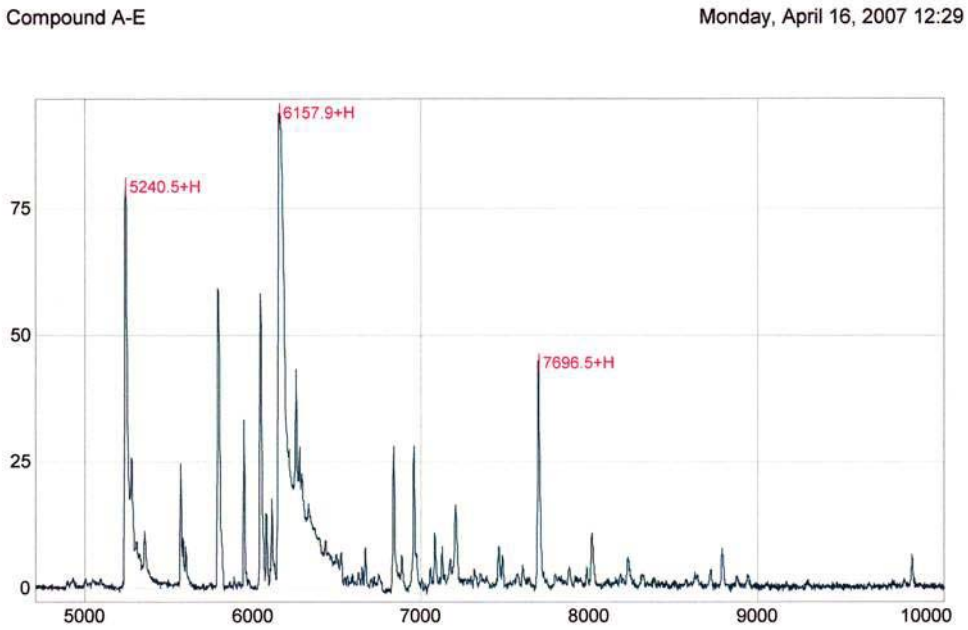

Figure 1. A portion of the water soluble fraction was analyzed by SELDI mass spectrometry. Note that the mass of compounds in the plant mixtures was <10,000 Da in each mixture. Shown is a representative spectrum of the addition of all mixtures A-E.

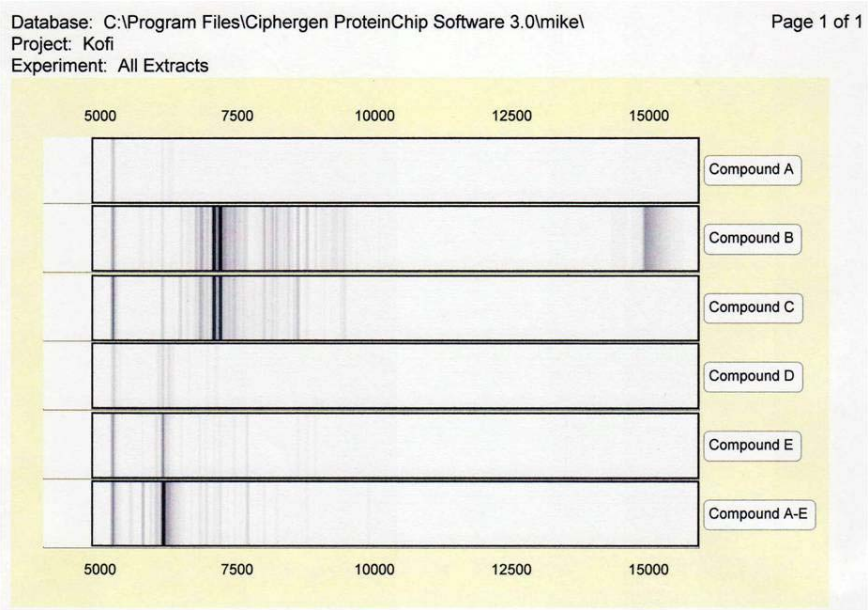

Figure 2. SELDI profiles shown in "gel" format. Please note that the mixture (A-E) was not simple the addition of the other compounds. The scale shown is in Da from 5,000 to 15,000 Da.

The SELDI profiles are shown in "gel" format below. Note that all of the mixtures have a relatively simple composition of molecules below 10,000 Da in size. Also note that the pattern of molecules present the mixture (A-E) is not simply a mixture of the other compounds. It appears that the extracts can react with each other to form new compounds or versions of the compounds. The conclusion from these experiments is that there is a portion of each plant mixture which can dissolve in water. The amount of water soluble material is significant (about 10-50 mg/ml).

To determine if the water-soluble fractions had any antiviral activity, we assayed their effect using the classical MAGI Cell infectivity assay. MAGI-Cells are genetically modified HeLa cells that contain a reporter gene cassette comprising the viral LTR, placed upstream of gene encoding *E.coli ß-gal*. Expression of the lac Z gene is dependent upon activation by the HIV Tat protein, which requires infection by HIV. Infected cells turn blue and can be counted using a microscope. We inoculated 6 well plates with 1 ng of HIV KFS virus. At the same time 200 microliters of extract was added to the cells and allowed to incubate 2 hours. The cells were washed 2 times and the extract was replaced by fresh medium. Cells were stained at 48 hours to determine the relative infectivity in the presence and absence of each plant extract.

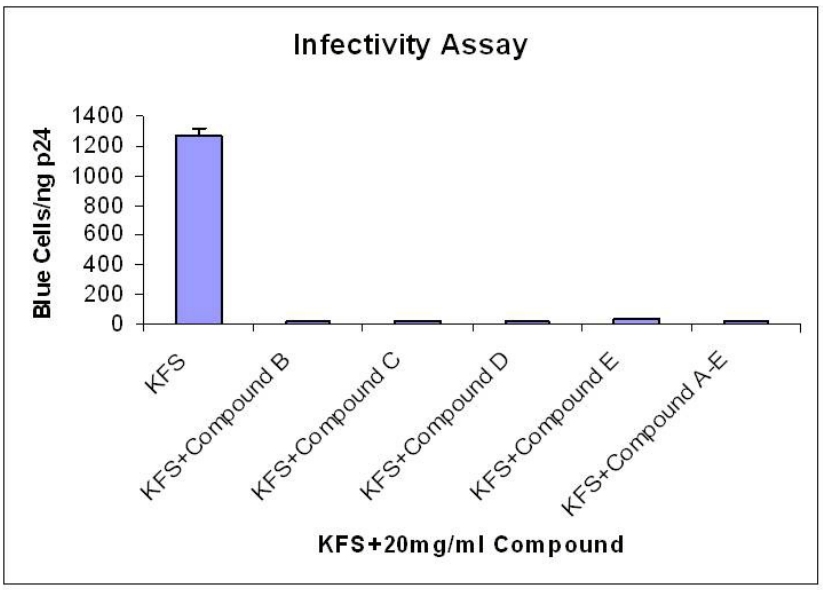

Figure 3. Each of the mixtures of plants was able to reduce the infectivity of 1ng of HIV NL4-3 virus to basically background levels. Note that mixture A appeared to have a deleterious effect on the cells used to score infectivity (MAGI cells).

In each of the cases, the material from the plant mixtures could significantly reduce the infectivity of HIV-1 (Fig. 3). This demonstrated that each of the mixtures had activity against

HIV-1. Our consideration at this point was to whether the agents were acting on the target cells or directly on the virus.

1. The extracts appear to work by directly inactivating the virus

To determine this we devised the following assay:

Virus was mixed with an individual compound C and a mixture of extracts A-E. The virus was allowed to interact with the extract for 30 minutes. The mixture was then fractionated by sedimentation through a sucrose cushion. The cushion allows the treated virus to pellet at the bottom of the tube, but retains the extract (which is of lower density). The viral pellet was then tested by MAGI assay. The results are summarized in Fig 4 below.

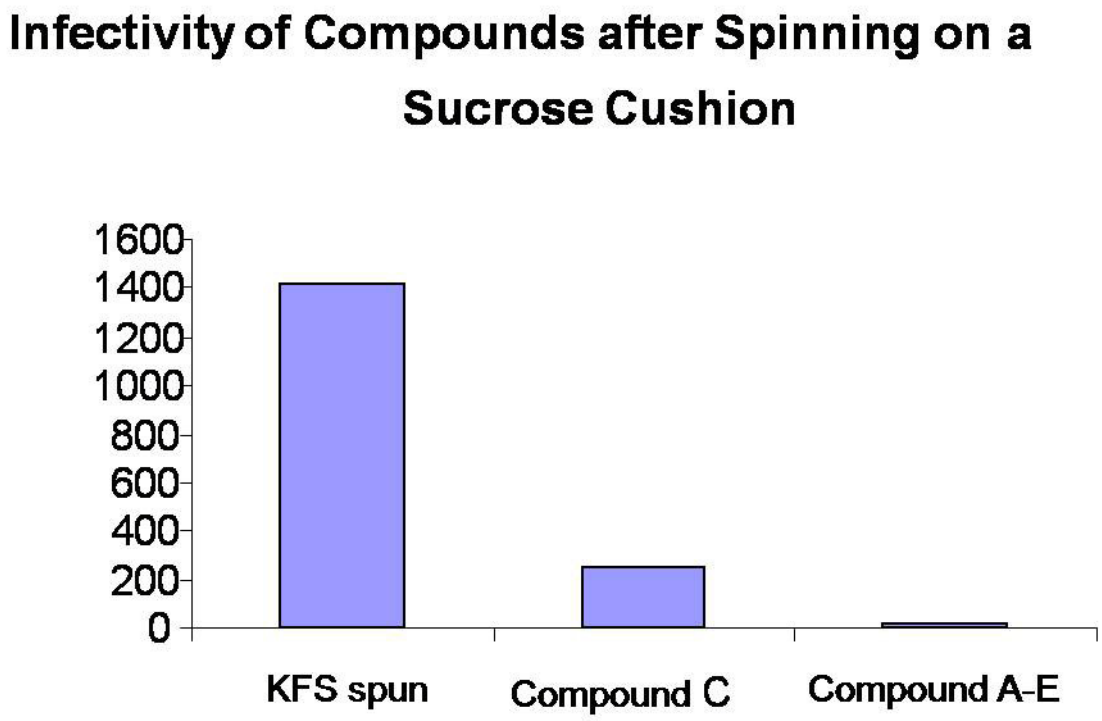

Figure 4. Virus exposed to Compound C or a mixture of all of the plant extracts (A-E) was treated to remove the mixture before mixing with target MAGI cells.

The results in Fig. 4 show that the plant mixtures were acting directly on the virus to block infectivity. As a more sophisticated test of the effects of the compounds, we did a detailed dose-response test. Each mixture A through E as well as all of the compounds combined A-E were tested at concentrations of 20, 50, 100, 200, 500 and 1000ng. The infectivity was measured by the beta galactosidase activity measured in a solution assay from extracts of infected cells. The results are shown in Fig. 5. All of the compounds had similar inactivation curves. There was significant antiviral activity at levels of 20ng. In other tests (data not shown) the compounds had at least some activity at picogram levels. The effect of the compounds on

virus appeared to immediate, as changing the time of exposure did not have a significant effect on the inactivation curves. We have tested times as short as 5 min to over 12 hours.

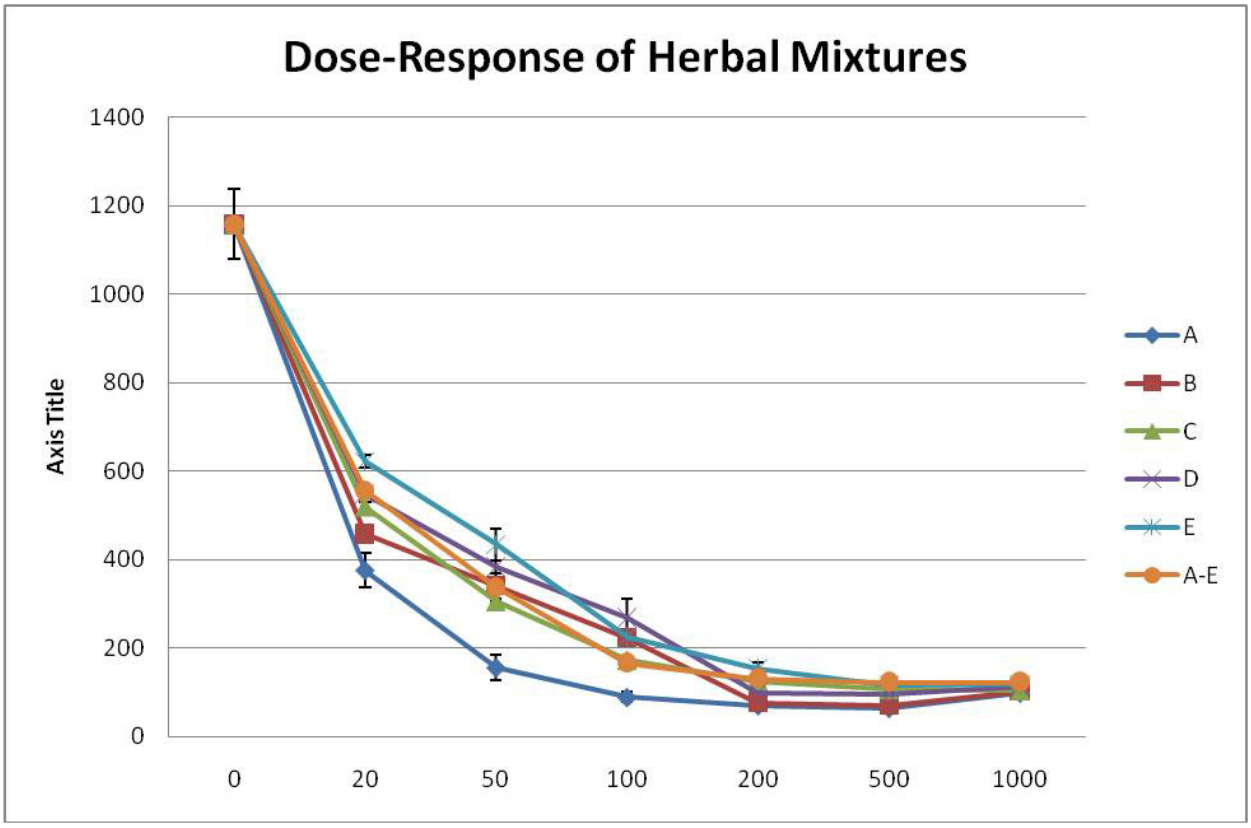

Figure 5. Each compound was tested at concentrations from 20ng to 1000ng. Note that at approximately 200ng the virus was almost totally inhibited. Also note that at the lowest concentration tested, 20 ng, there was significant antiviral activity.

3. The mixtures appear to have minimal toxicity testing by MTT assay.

The compounds all appeared to have the same type of activity with similar kinetics. Early in our testing we noted some toxicity with some of the compounds. Most notably, mixture A. During our study we came to realize that the water extracts contained a large amount of contaminating bacteria and yeast. We have since incorporated a multiple filtration approach to remove these contaminating organisms. Extracts processed in this way appeared to have minimal toxicity. However, we wanted to do formal toxicity testing to determine the relative effect of each compound. Therefore, we conducted MTT toxicity testing. MTT is a standard test to determine mitochondrial toxiticity (Mitochondrial Toxicity Test). The results are summarized in Fig. 6 below. Compounds B,D and E did not appear to have any measurable toxicity at any level to the limits of solubility. Compound C showed some toxicity at the highest level tested. Compound A was the most toxic having an LD50 of approximately 1.3mg/ml. These data are compared to Fig. 5 in which the virus was completely inhibited by a concentration of approximately 200ng/ml. There was also significant activity in as little as 10pg of material.

These results suggest that none of the compounds are extremely toxic. Especially in the therapeutic range.

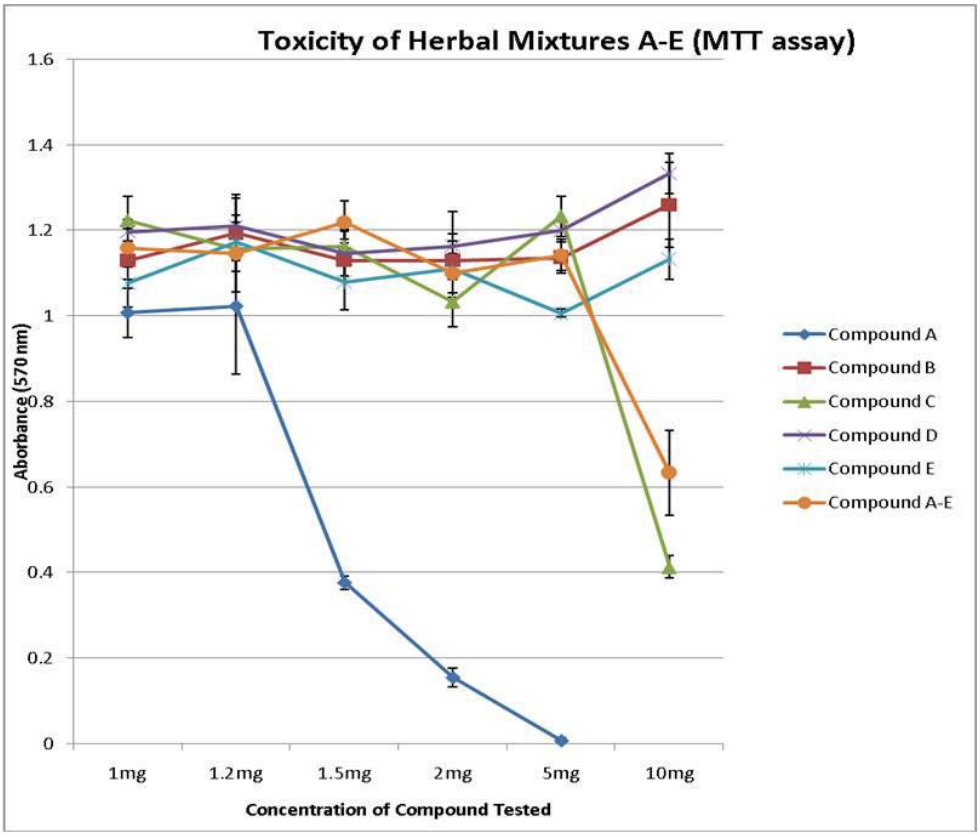

Figure 6. Cellular toxicity testing of each of the mixtures. Please note that a lower absorbance in this test is an indication of increasing cell death. Therefore, points with low absorbance are most toxic. This plot begins at 1mg/ml concentration. The concentration required to inhibit virus was significantly lower (in the ng to pg range).

4. The compounds appear to block the binding of gp120 to the cell surface.

Since the compunds appeared to directly affect virus, we wished to determine the specific mechanism of action. The first step in the virus cycle is binding of gp120 to its receptor CD4 on the cell surface. Therefore, we wished to test the compounds ability to block the binding of gp120 to T cells. To do this, we devised an assay in which we bind fluorescently labeled gp120 to Jurkat T cells. The gp120 will normally bind to the cell surface and cause the cell to fluoresce under confocal microscopy. This is demonstrated in Fig 7, A,B, and C. When the fluorescently labeled gp120 is first treated with 20 ng/ml of the compounds prior to binding, we found that the binding is almost competly blocked. Compare fig. 7 A-C with D-F. This suggests that the compounds work by blocking the binding of the viral protein gp120 with its cellular receptor CD4. This makes some sense in that plant mixtures can contain lectins, which are know to bind to glycoproteins (like gp120). Therefore, we believe that the plant mixtures each contain a

substance that acts as an “entry inhibitor” in a similar fashion to anti-retroviral drugs such as Fuzeon.

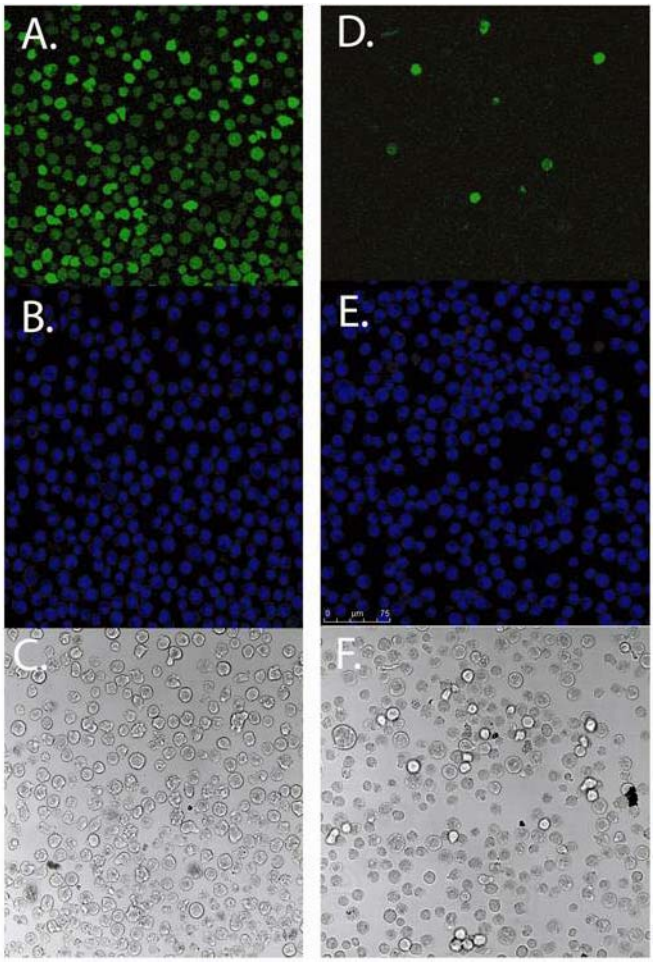

Figure 7. Addition of the compounds to fluorecscently labeled gp120 blocks its ability to bind to Jurkat T cells. In A,B and C fluorescent gp120 was mixed with Jurkat T cells and allowed to bind. The cells were then washed and viewed by confocal microscopy. The views are A: fluorescent, B: DRAQ5 nuclear stain, and C: Phase contrast. Panels D, E, and F show the same experiment where the fluorescent gp120 was pre-treated with the compounds prior to binding. Note that after 5 min of treatment the compounds almost completely block the binding of gp120 to cells. In this view the test was done with a mixture A-E but similar results were obtained using individual compounds.

Discussion

We have established several fundamental facts regarding the plant mixtures we originally obtained from ProMetra. First, a water soluble extract of the plants contains an activity that acts to prevent the infection of cells by HIV-1 virus. This activity appears to directly work on the virus since treatment of virus results in reduction of infectivity even if the virus is separated from the plant mixtures prior to infecting cells. Second, the toxicity of the compounds appears

to be minimal (or non-existent) at concentrations that can result in 100% inactivation of the virus. This suggests that active ingredient very potently blocks the ability of HIV-1 to infect cells without causing significant cellular toxicity. Both of these properties make the extracts interesting from the standpoint of developing an anti-retroviral compound. Presumably the active ingredient of the extract is only a fraction of the total mass that is present. Therefore, purified material is likely to be even more potent. Finally, we have established that the mechanism of action of inhibition is the blocking of binding of gp120 to the CD4 receptor. This activity appears to be non-specific to gp120 because virus pseudotyped with VSV-g protein is also inactivated. It also suggests that the compounds may have activity against other viruses with glycoprotein attachment proteins. We would like to test this in other viral systems (such as Herpes virus).

Although these results are exciting, and they suggest that at the very least the plant extracts contain antiviral activity, there is still an aspect to this treatment that is not explained by these data. We know from experience with fusion or entry inhibitors that, while they can be useful adjuncts to other retroviral therapy, they do not prevent viral latency once the infection is established. An interesting aspect of the herbal therapy is the reports that treated individuals do not appear to undergo viral rebound. That is, that once the therapy has been delivered the patients can discontinue treatment without experiencing relapse of the disease. This observation cannot be explained by the findings presented in this report. We suggest that this property of the mixtures also needs to be validated and documented. Clearing of latent reservoirs of viral infection is one of the most vexing problems with current anti-retroviral treatments. If the plant compounds have this activity it would be worth a great deal of resources to discover the mechanism of action. Therefore, we believe that characterization of the antiviral activity of the compounds is important. But, we also need to address the issue of latency and how this treatment could be circumventing this problem.

Future directions

AIM 1 – use gp120 affinity chromatography to isolate the active substances from the plant mixtures.

The finding that the antiviral activity of the plant mixtures is through binding of gp120 suggests a method for the purification of this activity. Biotinylated gp120 could be used to create an affinity column for the purification of the binding activity. The plant extracts would be flowed over such an affinity column and then eluted using various conditions. The eluted fractions would then be tested for antiviral activity and concentrated in preparation for structural study. The first step would be to determine the molecular weight using SELDI as we did for the original compounds. We would anticipate that the active substance should be the same in each mixture. If this is so, we need only isolate one compound from the plant extracts. It is possible that the molecular weights will be different in each plant mixture. In this case we will need to characterize more than one substance. But, the basic experimental approach should work for all of the plant mixtures.

AIM 2 – Identify the molecular structure of the active ingredient(s) of the plant mixtures.

Using the approaches in Aim 1 we should be able to obtain enough purified material to do mass spectrometry analysis. We will use gel analysis, biochemical testing and LC/MS/MS analysis to do a preliminary characterization of the isolated compounds. In addition to our HIV lab we also run the Mass spectrometry lab for our school. Therefore, we will be able to conduct initial testing in our own lab. Plant substances can be quite complex and easily need more complex analysis. The University of Georgia Complex Carbohydrate Center (CCRC) has local expertise in identifying plant compounds using mass spectrometry and NMR. If necessary, we will seek out this local resource to aid in the identification of the active ingredients. The CCRC has already expressed interest in collaborating on HIV related projects with our lab. Participation in this project would bring the necessary expertise to the project.

Parallel with the identification we will confirm the activity of the isolated compounds and work toward scaling up production. We may also wish to apply for an application to the NIH for their RAID program. This program provides contract assistance with identification of compounds, testing for efficacy, and mass production. The goal of this AIM is to identify the active ingredient(s) in the plant mixtures and devise a scheme to produce the material on a larger scale. A secondary aim is to confirm the activity and toxicity profile of the isolated compounds.

Summary

We feel that we have been able to make tremendous progress on this project. We have completed the initial aims of the original proposal. We have also made significant progress toward characterizing the activity of the compounds (which was not an original aim). The activity of the compounds is encouraging enough to warrant further study. At the least this would result in a promising candidate for another fusion inhibitor antiviral. It appears that this activity could also be effective against other viruses. With continued work we can determine this. We hope that our results justify your generous support and hope that we can continue to work on this interesting project.