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In-Vitro Cytotoxicity Screening of Plant Extracts

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The current year of activity included two main projects. The CEM/TART/1A2 anti-HIV screen was established to enable in-house evaluation of ICBG extracts for anti-HIV activity. This is a whole virus replication assay that follows HIV induced killing of human lymphocytes. HIV 1 tat.rev was grown and stocks prepared for cryogenic storage. TART and 1A2 cell line growth was quantified in the absence and presence of HIV infection. The cytoprotection protocol developed by Kiser and colleagues at NCI was evaluated using AZT as a positive control. The assay was found to be too variable and inconsistent for our intended use. Alternative protocols were developed. Trypan blue exclusion was found to be a sensitive and robust measure of cell viability during HIV infection. Its advantage is its simplicity, requiring only a microscope, a hemocytometer and a counter. Its disadvantage is that it is labor intensive. In addition to development of the HIV assay, this year's activity evaluated fractions of cytotoxic extracts with anti-cancer potential. Extract 799 displayed minor DNA cleavage activity in whole cells, and powerful cell cycle effects. Fractions 1986 and 1999 were found to be the most cytotoxic fractions and 1986 was found to have in vitro activity after incubation with purified human topoisomerase II.
Foreword

The current year of activity in this portion of AP6 included two main projects. The CEM TART/1A2 anti-HIV screen was established to enable in-house evaluation of ICBG extracts for anti-HIV activity. This is a whole virus replication assay that follows HIV induced killing of human lymphocytes. HIV 1 tat’ rev’ was grown and stocks prepared for cryogenic storage. TART and 1A2 cell line growth was quantified in the absence and presence of HIV infection. The cytoprotection protocol developed by Kiser and colleagues at NCI was evaluated using AZT as a positive control. The assay was found to be too variable and inconsistent our intended use. Alternative protocols or quantifying drug protection of cells from HIV killing were developed. Trypan blue exclusion was found to be an extremely sensitive and robust measure of cell viability during HIV infection. The advantage of this approach is its simplicity, requiring only a microscope, a hemocytometer and a counter. Its disadvantage is that it is labor intensive. In addition to development of the HIV assay, this year’s activity evaluated fractionations of the particularly cytotoxic and pharmacologically active extracts. Extract 799 displayed minor DNA cleavage activity in whole cells, and powerful cell cycle effects. Fractions 1986 and 1999 were found to be the most cytotoxic fractions and fraction 1986 was found to have in vitro activity after incubation with purified human topoisomerase II. Extracts of Picrolima nitida were also evaluated in purified enzyme systems and stimulation of topoisomerase I DNA cleavage was noted.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Front Cover</td>
<td>1</td>
</tr>
<tr>
<td>Standard Form 298</td>
<td>2</td>
</tr>
<tr>
<td>Foreword</td>
<td>3</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>4</td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Body</td>
<td>5-9</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>9</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>10</td>
</tr>
<tr>
<td>Conclusions</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>10</td>
</tr>
<tr>
<td>Appendices</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION:

API of the ICBG "Drug Development and Conservation of Biodiversity in West and Central Africa," provides data on the variety, distribution, abundance and dynamics of West and Central African flora. AP 2 focuses on determination of the chemical constituents of important West African plants. APs 3-5 are focused on the traditional uses and economic benefits derived from the flora. The research conducted under Program Project 6 is primarily concerned with development of African medicinal flora for their non-antiparasitic activities, particularly, anticancer and anti-HIV assessment. Screening extracts from locally used plants can capitalize on traditional knowledge in order to provide information about the potential alternative uses of known plants. Random screening of West African Flora for anticancer activity can provide information on the utility of species not recognized by traditional healers for diseases not regularly usually treated at that level.

BODY:

As described in our approved statement of work, our approach is to prioritize cytotoxic extracts based on screens that can detect cancer relevant mechanisms of tumor cell killing. We first test extracts for the ability to kill human cancer cells in culture. We then utilize mammalian cell based screens to detect molecules in the extracts that interfere with DNA metabolism or the cell cycle. In the mechanism screens cytotoxic extracts/molecules are tested for enhanced cytotoxicity in mutant cell lines that lack various cancer predisposition/tumor suppressor gene functions (caretaker functions). Cytotoxic extracts/molecules showing no DNA directed activity are tested by flow cytometry for the ability to disturb progression of normal cells through the cell cycle (tumor suppressor gatekeeper functions). These assays were described in last year's report. Follow up on this work is presented in Part II, below.

Also as discussed in last year's report, AP6 of the ICBG initiated evaluation of traditional medicines for treatment of HIV/AIDS. The overall objective of this work is to test the hypothesis that phytomedicines employed by local healers of Nigeria and Cameroon for the treatment of AIDS will have anti-HIV 1 activity in a whole-virus assay. This is described in Part I, immediately below.

Part I It is estimated that over 22 million people in sub-Saharan Africa are infected with HIV. This amounts to 70% of the disease incidence worldwide. Incidence of infection in Nigeria is thought to be over 5% of the adult population. There is evidence that AIDS existed in Central Africa for decades before it was widely recognized as an epidemic in the West. This implies that local healers have, in fact, been treating AIDS for decades. These traditional treatment strategies have been based on hundreds, or even thousands, of years of practical experience with the use of local flora to treat infectious diseases. AIDS is a complex and life threatening disease, the proximal cause of death most often being super-infection due to the victims weakened immune system. In Africa tuberculosis is a major proximal cause of death. Nevertheless, it is likely that many of the therapies currently being employed by traditional practitioners have direct anti-HIV activity. The people of Nigeria and Cameroon have a rich and varied tradition of plant use for treatment of infectious diseases. Experience of our ICBG has shown that extracts of botanicals used locally for malaria or other parasitic infections show very high "hit" rates (greater than 50%) in Western scientific assays of activity. This precedent provides reason to expect that plants used in Nigeria and Cameroon for AIDS or other viral infections will also show quantifiable anti-HIV activity in Western scientific assays, and that the active components may be isolated and identified using the strategies of modern drug discovery.

To test extracts of plants used in Nigeria and Cameroon for the treatment of AIDS, and other microbial infectious diseases, we established the whole virus "CEM-TART" assay. In this system replication defective HIV-1 is replicated in specially engineered human T-cells. Inhibition of virus production is monitored by ELISA analysis or by cytoprotection using MTT type colorimetric techniques. Such inhibition, at non-cytotoxic extract concentrations, may indicate potent useful anti-HIV activity. The system to be employed here was developed by Chen and co-workers (1) and optimized for screening by Kiser and colleagues (2). In this year's work we registered with the AIDS Research and Reagent Program
at NIH and received the CEMTART (CEM-TART), 1A2 cell lines and the defective (MC99IIIBATat-Rev, also called vIIIIBATatRey) virus and protocols developed at the NIH. We were able to establish the protocol developed by Kiser and colleagues and evaluate it for our use. Again, the assay measures inhibition of virus-dependent cell killing indicates anti-HIV activity. The assay therefore allows detection of anti-HIV components in complex mixtures (2). Because this is a whole virus production assay it is capable of detecting components active via a wide spectrum anti-viral mechanisms.

This preliminary work allowed an AIDS FIRCA application to be submitted January, 2001. After scientific review the application was awarded a very positive 157 priority score. The work presented here was the basis of this application. The additional support was necessary because the umbrella ICBG has ethnobotanical based discovery of anti-parasitics as its emphasis, and funds in AP6 are extremely limited. A collateral benefit of the FIRCA award will be the ability to put this assay in place in Nigeria, and the establishment of an enduring and self-sustaining drug screening and tissue culture capability in Nigeria. It is anticipated that this will serve as a core facility of the International Center for Ethnomedicine and Drug Development (Inter CEDD) at Nsukka. The Inter CEDD is supported in part by the BDCPN (Bioresources Development and Conservation Programme Nigeria). This project will complement ongoing support of BDCP by our ICBG, and will solidify the skills and scientific capabilities of the participating scientists on site. Establishment of the CEM-TART whole virus assay will be the mechanism used to establish this capacity. It will also complement and broaden the ICBG's anti-parasitic drug discovery focus. The project will build upon ICBG funded tissue culture workshops, which have introduced basic tissue culture techniques to the region.

Anti-HIV screening performed to-date has been accomplished under a contract between the ICBG (Program Project 6) and Southern Research Institute, Birmingham, Alabama, USA, as part of a panel of pathogenic microbes. Unfortunately, the cost is extremely high and, as mentioned above, the ICBG is not dedicated to discovery of anti-viral agents and has no funding dedicated to this task. Thirty five ethanol extracts or plants used in Nigeria in the treatment of AIDS were tested for anti-HIV activity at Southern Research Institute (SRI). Using the CEMSS strain (a human T-cell line that dies upon HIV production) the extracts of *Eupatorium sp.* and *Pachypodanthium sp.* provided 50% increase in CEMSS viability, with therapeutic indices of 2.5 or greater. Six additional extracts were active at the 25% cutoff, and are also considered by SRI as candidates for fractionation. This precedent provides reason to expect that plants used in Nigeria and Cameroon for AIDS or other viral infections will also show quantifiable anti-HIV activity in Western scientific assays, and that the active components may be isolated and identified using the strategies of modern drug discovery.

![Fractional Survival vs Concentration](image)

**Figure 1.** Figure 1 shows results of the cytoprotection assay developed Kiser and co-workers. Plotted is 1A2 cell fractional survival 10 days after infection with HIV I tat’ rev’. Cultures were treated in quadruplicate with increasing concentrations of AZT. Significant cytoprotection was evident at AZT concentrations of 0.05 to 5 ug/ml. The concentration of 50 ug/ml AZT was cytotoxic itself, resulting in the
parabolic survival curve. The error bars show the standard deviations determined for the quadruplicate data in the assay. While this assay is functional, its intra-assay precision is poor. We consistently observed wide variation in replicate wells, and so developed a more reproducible assay.

Trypan blue exclusion was used as an alternative means of quantifying CEM-TART and CEM-1A2 cell growth. The T-cell leukemia sub line 1A2 was found to be more sensitive than the TART line to cell killing by HIV infection and so it was selected as the primary cell line for this assay. Figure 2 shows total cell population over 11 days. Total number of cells in non-infected and infected cultures was decreased after day 7 of incubation unless cultures were refed. Refeeding does not spare the infected cultures from further population decrease. Total cell count is given as cells X 10^5/ml.

![Graph](image)

**Figure 2.**

Figure 3 shows the assay quantified as living (defined as trypan blue excluding) cells X 10^5/ml.

![Graph](image)

**Figure 3.**

Figure 4 shows the protective effect of AZT on HIV infected 1A2 cells following growth with no refed. The data shown are the fraction of live cells in the cultures, AZT was administered only on day 1. This is a very robust assay and yields an easily quantifiable signal when cultures are protected from HIV killing.
Figure 4.

Part II  Several extracts identified under last year's work as having potential anti-cancer activity were fractionated and assessed to continue the process of bio-assay guided fractionation. Extract 799, which was considerably cytotoxic had some DNA directed activity, and caused unusual cell cycle effects was subjected to such fractionation. 17 fractions of extract 799 were tested for cytotoxic activity. This is the easiest way to follow activity. Of those fractions 1986 and 1999 were the most active. Figure 5 a and b show cytotoxicity data for these samples in HCT 166 cells.

Figure 5a, b.

Each of the fractions was also tested in pure enzyme systems in-vitro for the ability to induce DNA damage directly, or for stimulation of topoisomerase dependent DNA damage. Fraction 1986 was found to have an unusual effect on DNA migration in agarose gel after incubation with purified human topoisomerase II. This effect appears to be specific because it is not seen in a similar assay containing topoisomerase I. Figure 6 shows agarose gel analysis of some of these fractions after incubation with plasmid DNA and purified human topoisomerase II (lanes are number from left to right).
Figure 6. Top II-SU 1982-1998, Lane 1, DNA; Lane 2, DNA + top II; Lane 3, 100μM etoposide; Lane 4, 1982; Lane 5, 1983; Lane 6, 1984; Lane 7, 1985; Lane 8, 1986; Lane 9, 1987; Lane 10, 1988; Lane 11, 1989; Lane 12, 1990; Lane 13, 1991; Lane 14, 1992; Lane 15, 1993; Lane 16, 1994; Lane 17, 1995; Lane 18, 1996; Lane 19, 1997; Lane 20, 1998.

Figure 7 shows a similar follow up on the extract form Picrolima nitida, data provide some evidence for topoisomerase I dependent cleavage of DNA in fractions 369, 370, and 847.

Figure 7. Lanes 1, 12, and 20 have only DNA in them. Lane 2 has DNA plus topo I. Lane 3 is the positive control with DNA, topo I and 4.5 M 9-amino Camptothecin. Lane 4 has DNA, topo I, and DMSO in it (negative control). Lanes 5 through 9 have DNA, topo I, and extracts 367, 369, 370 846, 847, and 848, respectively. Lane 11 has DNA and topo II. Lane 13 has DNA topo II and DMSO (negative control). Lane 14 is the positive control with DNA, topo II, and 50 mM Etoposide. Lanes 15 through 19 have DNA, topo II, and extracts 367, 369, 370, 846, 847, and 848 respectively.

While activity was detected in these purified enzyme assays for 799, 369, 370 and 847 the activity was not as high as expected. The actual concentration metabolites in the fractionated material may have been diminished, and this is of concern and is being followed up.

KEY RESEARCH ACCOMPLISHMENTS:

- A robust, simple and reproducible cytoprotection screen for anti-HIV activity was established.
- Extract with potential anti-cancer activity were fractionated and the active fractions identified.
- Extracts of Picrolima nitida containing activity against human topoisomerase I were identified.
REPORTABLE OUTCOMES:

- The majority of these data were presented at the International Conference on Traditional Medicine in HIV/AIDS and Malaria, in Abuja, Nigeria, December 2000, and were also included in the ICBG annual progress report. Manuscripts summarizing these data are in preparation.
- An AIDS Fogarty International Research Collaboration award was applied for, and received a 157 priority score, funding id pending.
- This research has supplied rotation projects and contributed to the training of two graduate (Ph.D.) students at the University of Utah.

CONCLUSIONS:

Traditional medicines have been the source of several very important anticancer medicines (e.g., the vinca alkaloids, the podophyllotoxins and the camptothecins). Traditional medicines are also the major source of relief in combating AIDS in Africa. The work in progress here has identified several medicinal plants, used traditionally in West Africa, as containing constituents with significant anticancer and anti-HIV potential. Significant progress has been made towards identification of the molecular mechanism of some lead compounds. These extracts are being fractionated and the active components isolated via chromatography and bioactivity guided isolation. These molecules possess the potential to combat some of the most serious diseases confronting mankind today, and as such possess significant economic value if that potential is realized.

REFERENCES:
