Memoranda/Mémorandums

In vitro screening of traditional medicines for anti-HIV activity: Memorandum from a WHO meeting*

Many plant products are being used by patients with acquired immunodeficiency syndrome (AIDS) in some countries without any scientific proof that they possess anti-HIV (human immunodeficiency virus) activity. Traditional healers are now offering their remedies for scientific evaluation, and a few studies provide information on the inhibitory activity against HIV of plants such as Viola yedoensis, Arctium lappa, Epimedium grandiflorum, Glycyrrhiza uralensis *and* Castanospermum australe.

Natural products can be selected for biological screening based on ethnomedical use, random collection or a chemotaxonomic approach (i.e., screening of species of the same botanical family for similar compounds), but the follow-up and selection of plants based on literature leads would seem to be the most cost-effective way of identifying plants with anti-HIV activity. No single in vitro *screening methodology for anti-HIV activity is ideal and confirmatory assays in multiple systems are needed to examine completely the potential use of a compound.*

To promote further research in traditional medicine and AIDS, appropriate institutions will be identified where the different activities for the scientific evaluation of plants and their extracts for possible treatment of AIDS can be carried out.

* This Memorandum is based on the report (document No. WHO/GPA/BMR 89.5) of an informal WHO Consultation on Traditional Medicines and AIDS: *In vitro* screening for Anti-HIV Activities, which was held in Geneva on 6–8 February 1989. The participants at the meeting were W.W. Anokbonggo, Department of Pharmacology and Therapeutics, Makerere Medical School, Kampala, Uganda; R.S. Chang, Department of Medical Microbiology, University of California, Davis, CA, USA; N. Farnsworth (*Chairman*), Program for Collaborative Research in Pharmaceutical Sciences, University of Illinois Medical Center, Chicago, IL, USA; J.J. McGowan, Developmental Therapeutics Branch, National Institutes of Health, Bethesda, MD, USA; J.D. Msonthi (*Co-Rapporteur*), Department of Chemistry, University of Malawi, Zomba, Malawi; L. Weibo (*Rapporteur*), Muhimbili Medical Center, Traditional Medicine Research Unit, Dar-es-Salaam, United Republic of Tanzania; R. Ruprecht, Harvard Medical School, Dana-Farber Cancer Institute, Boston, MA, USA; and N. Yamamoto, Department of Virology and Parasitology, Yamaguchi, University School of Medicine, Yamaguchi, Japan. *WHO Secretariat*: C.O. Akerele and Y. Maruyama, Traditional Medicine Programme; J.F. Dunne, Pharmaceuticals; D. Griffin, Special Programme of Research, Development and Research Training in Human Reproduction; J. Esparza, A. Jurado, J. Mann, S. Osmanov, H. Osore, and H. Tamashiro, Global Programme on AIDS, World Health Organization, Geneva, Switzerland.

A French translation of this article will appear in a later issue of the *Bulletin*. Requests for reprints should be sent to Traditional Medicine, World Health Organization, 1211 Geneva 27, Switzerland.

Reprint No. **5021**

Introduction

Traditional medicines are being used empirically in many countries for the treatment of acquired immunodeficiency syndrome (AIDS). Evaluation of these treatments in persons infected with human immunodeficiency virus (HIV) is a new challenge, especially since 5–6 million people are estimated to be infected, and all possible resources must be made available for the benefit of the affected populations. In this context, there is a need to evaluate those elements of traditional medicine, particularly medicinal plants and other natural products, that might yield effective and affordable therapeutic agents. This will require a systematic approach.

Against this background, WHO's Global Programme on AIDS (GPA) and Traditional Medicine Programme (TRM) convened an informal Consultation in Geneva, from 6 to 8 February 1989, with the following objectives:

— to review the status of research in traditional medicine as applied to HIV infection and AIDS;

— to review current activities in the area of medicinal plants and other natural products in relation to *in vitro* and *in vivo* preclinical evaluation for anti-retroviral or anti-reverse-transcriptase activity; and

— to identify opportunities for collaborative work and make appropriate recommendations.

Ongoing activities

A variety of plant products are being used by AIDS patients without any experimental evidence of anti-HIV activity. They include garlic (*Allium sativum*), shiitake mushrooms (*Lentinus edodes*), papaya (*Carica papaya*), ginseng (*Panax* species), *Aloe vera*, Ukrain (*Chelidonium majus*), immunact (a Peruvian plant root), Japanese pine cone extract, various flower essences, Easter lily bulbs, Fu-zheng (a Chinese traditional principle of treatment), and Padma 28 (a Tibetan formula of several plants). A number of other marine, fungal and animal products have also been used.

Traditional medicines are employed for the treatment of AIDS in all WHO regions. In African countries, many patients with a hospital diagnosis of AIDS seek alternative treatment among traditional practitioners when they see no change in their condition. In some cases, follow-up studies are effected by physicians in AIDS clinics and counselling centres. Generally, traditional practitioners voluntarily come forward to offer their remedies for scientific evaluation, especially when they see that patients on such therapies continue to be maintained in a reasonable state. Observations have shown that certain plants appear to be common to most of the infusions or concoctions given to patients. Some of these have biological activities which qualify them as candidates for further study for anti-HIV activity. For example, *Diospyros usambarensis* (*Ebenaceae*) has been found to possess fungicidal and cytotoxic properties.

Traditional Chinese Medicine is being used in AIDS patients in the United Republic of Tanzania, with the collaboration of Chinese and Tanzanian scientists. The research protocol for these studies stipulates that every year, for three years, 200 AIDS patients at different stages of the disease would be treated, with the same number of patients to be used as controls. A basic therapeutic recipe is prescribed which, during the trial, could be modified depending on the clinical manifestations. Some of the preparations used in the basic recipes include *Polyporus umbellatus, Cordyceps sinensis* and *Paeonia obovata*. A special outpatient clinic has been set up for follow-up studies of discharged patients. Immunological parameters to determine the T-lymphocyte subsets have been established and are being monitored. So far, 17 seropositive patients have been treated and some symptomatic improvement claimed.

Only a few experimental studies to discover anti-HIV agents from medicinal plants and other natural products are in progress. A major programme of this type is being carried out at the National Cancer Institute in the USA. The programme will screen about 4500 plant samples per year during the next five years for *in vitro* anti-HIV activity, based on a random selection of plants. No traditional medicine background information has been used in this programme.

It has recently been reported that 11 of 27 medicinal herbs used in Chinese traditional medicines as anti-infectives showed *in vitro* inhibitory activity against HIV. These plants were: *Arctium lappa, Epimedium grandiflorum, Lonicera japonica, Woodwardia unigemmata, Viola yedoensis, Senecio scandens, Andrographis paniculata, Coptis chinesis, Prunella vulgaris, Lithospermum erythrorhizon* and *Alternanthera philoxeroides*. The active principles of *Viola yedoensis* and *Prunella vulgaris* appear to be sulfonated polysaccharides (*1, 2*).

Other studies have provided additional information on natural products with experimental anti-HIV activity, including glycyrrhizin (from *Glycyrrhiza uralensis*) (*3*). In Japan, glycyrrhizin has been studied in AIDS patients. It was claimed that, when given orally to asymptomatic HIV carriers, glycyrrhizin delayed the progression of symptoms related to HIV infection. It was also claimed that relatively large doses of glycyrrhizin administered to AIDS patients caused a disappearance of HIV antigenaemia and an improvement in several haematological and immunological parameters. In another claim, the simultaneous administration of glycyrrhizin appeared to decrease the adverse effects of zidovudine.

An aqueous extract of the marine red alga *Schizymenia pacifica* was shown to inhibit reverse transcriptase and the activity was attributed to a sulfated polysaccharide (*4*). Apparently, many sulfated polysaccharides (e.g., heparin, dextran sulfate) inhibit HIV activity, but the non-sulfated polysaccharides (e.g., chondroitin, alginic acid, keratin and hyaluronic acid) do not.

Castanospermine, an indolizidine alkaloid extracted from the seeds of *Castanospermum australe*, blocks glycoprotein processing via inhibition of glucosidase I located in the endoplasmic reticulum. This alkaloid has been reported to have *in vitro* anti-HIV activity and has been shown to be active *in vivo* when administered orally to mice (*5*).

From the information available it appears that new initiatives would be fruitful in the search for anti-HIV compounds in plants and other natural products, based on their uses in traditional medicine. Further, it was recognized that there is a need for collection and analysis of information based on ethnomedical and experimental reports, which include *in vitro* and *in vivo* bioassay test models and chemical data.

A deficiency of the ethnomedical approach is the

lack of specific information on plants that may have anti-HIV activity. The following symptoms might be discussed with traditional healers to secure relevant information on their methods of treatment: skin lesions (Kaposi's sarcoma), chronic fever, diarrhoea, cough, haemoptysis and genital ulcers.

Methodology

Selection of plants for antiviral screening

Any approach to be used for identifying and evaluating specific plants for antiviral activity is dependent on a number of factors, e.g., simplicity, speed, cost, reproducibility, lack of interference by ubiquitous substances found in plants, and availability of plant material (including the provision of sufficient quantities of active plants for isolation studies).

Usually only a small amount of plant material is required for most *in vitro* screens, i.e., 100–200 g dry weight (or less) of the appropriate plant part. Proper documentation of the samples selected by a qualified botanist must be made, and the specimens must be properly dried. Care should be taken to avoid collection of threatened or endangered species. Normally, two types of extracts for each sample should be made; one with a non-polar (or intermediate polar) solvent and one with a more polar solvent, e.g., ethyl acetate or chloroform and methanol or ethanol, respectively.

Four basic methods are available for selecting plants for a screening programme to seek anti-HIV levels: (*a*) follow-up of ethnomedical information, (*b*) random collection of plants followed by bioassay, (*c*) selection of plants already reported in the literature to have properties that would suggest inhibitory activity against HIV, and (*d*) chemotaxonomic approaches. All factors considered, approaches (*a*) and (*c*) would seem to be the most cost-effective for identifying plants with anti-HIV activity.

Selection based on ethnomedical uses. Several terms are used to indicate the use of plants by indigenous peoples, including ethnobotany, ethnomedicine, folklore, and traditional medicine. Information derived by qualified observers in the field, in which the observer actually sees the plant being used, is the most convincing. Some observers simply question a traditional practitioner for information and record the claims for various plants. Most observers do not appear to have had a background in pharmacology or medicine, which raises questions about the validity of many ethnomedical claims found in the literature. For example, a claim that a plant is useful as a "contraceptive" could mean that the plant is used to "prevent conception" (rarely is it stated if this is for use by men or women) or to "prevent birth" (as an abortifacient, for example). Setting up a bioassay to demonstrate prevention of conception would be different from setting one up to show an abortifacient effect. Information derived from an observer who actually sees the use of the plant for a specific disease is obviously more valid than information based on unverified claims. Similarly, information from a recognized medical system such as Traditional Chinese Medicine, which is based on a written history of 3000 years, is probably more valid than undocumented or anecdotal information.

In spite of these apparent problems and the lack of uniformity in the available information on the ethonomedical uses of plants, a careful analysis of this information should lead to an indication of what plants are most likely to show a positive response in a specific bioassay system. Of 121 drugs currently used globally which are obtained from higher plants, 74% were discovered by scientists investigating the plants on the basis of ethnomedical claims (*6*).

However, the correlation of many ethnomedical claims with a disease condition through bioassays that are verifiable in the laboratory can be difficult. Such correlations would have to be further studied and defined, as experimental data become available. The obtaining of information from indigenous practitioners for information, through the network of WHO Collaborating Centres for Traditional Medicine, would seem to be a logical approach.

Random collection followed by mass screening. This approach is based on the belief that active compounds for any given disease will eventually be found if sufficient samples are randomly selected and tested. The U.S. National Cancer Institute, using this approach from 1956 to 1981, screened 32 000 species of flowering plants from various parts of the world for anti-tumour activity; about 2–8% of species showed reproducible antitumour activity when evaluated by at least one *in vitro* cytotoxicity system and one or more *in vivo* antitumour systems.

A large number of randomly selected plants have been screened for a battery of antiviral effects. Some of these were pursued chemically, and shown to have antiviral activity (*7*).

Random collection, followed by biological screening, has the advantage of costing less per sample than collection after location of a specific plant that for some other reason seems promising. In order to be random in nature, however, it would be necessary to determine in advance the total number of species to be collected and to ensure that all plant taxa are proportionally represented in the total collections. There is reasonable assurance that if enough samples are screened, active compounds will eventually be realized.

Follow-up of existing literature leads. A significant number of papers exist in the scientific literature in which plant extracts are reported as being tested against one or more viruses, usually *in vitro*. Very few of the active leads found in these reports have been followed through to a conclusion by isolating and characterizing the active principle(s). It would not be difficult to conduct a systematic search of the literature to identify all papers reporting on the antiviral testing of plant extracts and their active principles and analyse the reports appropriately. Indeed, there may be biological parameters to be considered (other than the direct cytopathogenic effects on the HIV virus) that would be useful as predictors of anti-AIDS activity, such as RNA synthesis inhibition, protein synthesis inhibition, reverse transcriptase inhibition, viral translation inhibition and others.

Chemotaxonomic approach. Botanically related plants tend to have similar as well as identical secondary metabolites. Thus, if it is known that a specific plant contains anti-HIV activity or secondary metabolites with anti-HIV activity, related plants can be identified that will have the same or related active principles based on phylogenetic schemes. If a useful anti-HIV secondary metabolite is found in a given species, but only in low concentrations, chemotaxonomy could well identify related species that might contain a higher concentration of the active compound.

Procedures for in vitro *screening of natural products for anti-HIV activity*

New drugs are identified in random drug screening or targeted drug development by having some method by which to assay for a particular effect of the drug or extract on the virus. Typically, in a standardized assay, different structural entities are tested to determine if they can inhibit HIV replication *in vitro*. A number of different assays are now being used to determine potential anti-HIV activity (Table 1). Compounds from a wide range of sources are being screened with emphasis on those compounds with unique structural features or evidence of biological activity. The success of these systems for identifying potential drugs to treat HIV infections is indicated by the number of drugs discovered. The challenge remains as to how to use this base of information to recommend tests for evaluating traditional therapies. These tests can be used to scientifically support the rationale of using a given therapy and to set the priority for study of a given drug.

The proposed scheme (Table 2) recognizes the difference between western and traditional healers and provides a framework for scientific evaluation of a therapeutic agent which might be used in the treatment of HIV infections. The scheme given is not specific and for that reason certain aspects are discussed in detail below.

Table 1: **Examples of biochemical and cell culture based assays that are used to determine anti-HIV activity**

Biochemical or cell culture assay	Viral parameter being measured
Polymerization/viral replication	Reverse transcriptase
Ribonuclease/viral replication	Ribonuclease H
Proteolysis/viral replication	Protease
CD4 binding/cell fusion	GP 120, viral envelope mediated fusion
Protein aggregation/viral replication or virion assembly	p24 protein interaction
GTPase/viral replication	nef
Phosphorylation/viral replication	nef

Authentication of extracts provided for analysis. Communication between the botanist, chemist, biologist, pharmacologist, ethnobotanist and traditional healers must be at an optimum level to ensure that the remedies selected for acquisition and testing are authenticated. The information needed includes but is not limited to the following:

— taxonomic identification of the species of plants in the extract;
— photographic documentation of the plant species;
— detailed characterization of the organs of the plants to be used;
— detailed characterization of the method and time of collection;
— characterization of other conditions used to prepare the extract as a traditional medicine.

The extract should be prepared to the specifications provided by the traditional practitioner and evaluated directly. The way it is used in traditional practice must be taken into consideration when evaluating high or low polar solvents for extraction of the traditional medicine. Other more sophisticated extractions or fractionation procedures should be considered as appropriate.

In vitro *assays of antiviral activity.* A wide range of biochemical and cell culture based assays are currently available. It is recognized that no one assay is ideal and that confirmatory assays in multiple systems are needed to examine completely the potential use of any compound. The proposed steps (Table 2) are based on use of a single assay followed by more detailed confirmatory assays, should an active compound(s) be identified.

No one single test system can be identified as the best; the cost, simplicity and reproducibility are key factors which should determine the selection of the assay system. However, a minimum of a single T-cell culture assay system (e.g., H9, ATH8, MT-2) should

Table 2: **Key steps in the evaluation of traditional medicines for their potential use in the treatment of HIV infection**

1. Provide authenticated extract for analysis.
2. Use single *in vitro* assay system to detect activity and determine potential toxicity.

   Stop if toxicity is greater than anti-HIV effect.

3. Partially purify the extract, and consider reviewing.
4. Exclude interferon-inducers.
5. Confirm antiviral activity and toxicity in a variety of cell systems.
6. Assay the antiviral activity and cytotoxicity of fractions in an approriate anti-HIV or animal cell culture system.

   Stop if toxicity is greater than antiviral effect.

7. If active fraction(s) is identified, then:
   (a) start chemical identification of active compound(s);
   (b) perform biochemical assays of the active fraction;
   (c) carry out *in vivo* efficacy and toxicity studies in an appropriate animal model system.
8. Depending on information obtained in 7, prioritize compounds for potential clinical evaluation.

be used as a first-line screen of traditional medicines.

HIV isolates from patients and from laboratory-adapted strains have different activities. However, convenience, cost and reproducibility must outweigh other considerations regarding the virus isolate to be chosen at this time. It is recommended that a low viral inoculum be used to allow for multiple rounds of viral production over a time period of at least seven days.

Some traditional medicines may need to be metabolized by the host cell. Therefore, the product should be added to the cells at least 2 hours prior to their infection with HIV. Longer times or pretreatment (24 h) might provide more useful information and would help identify extremely cytotoxic agents prior to infection with HIV. Dilutions of the candidate drug should be less than one log in difference. Threefold dilutions of the extract above and below the traditional concentration of ingestion are recommended. The range of dilutions should be determined by analysis of the data with time, although initially it is anticipated that at least 12 dilutions would be made per extract. The end-point for evaluating the antiviral effect could vary with the amount of cell survival, syncytium formation, p24 expression, reverse transcriptase levels in the supernatant, or fluorescence of viral antigens.

The key to the success of any therapeutic agent is lack of cytotoxicity. Therefore, careful analysis of cytotoxicity should be made. Tests should examine how long the cells will survive and how active they will replicate in the presence of the drug.

It is recognized that the antiviral effect must outweigh the potential toxicity. Clearly, some extracts will have toxic components in higher concentrations than a potential therapeutic fraction of the mixture; these extracts, at this phase of evaluation, should not be eliminated but given a lower priority for further evaluation.

Assay of "immunoenhancing" activity. In traditional medicine, many extracts are referred to as having an enhancing effect. Enhancement may be due to the anti-infective effect of the extract, an immunostimulatory effect, or some other effect on the body's functioning. Because of the large number of traditional medicines that have an enhancing effect and our present incomplete knowledge of the delicate interplay of HIV and the human immune response, the Consultation did not provide any definitive recommendation for assays of those compounds that have an enhancing effect. Indeed, the immune response may trigger the expression of HIV encoded genes to produce new viral particles.

Many higher plants are known to contain compounds with a broad antiviral spectrum. Some of these compounds are believed to be interferon-inducers that inactivate a variety of viruses intracellularly. Because interferon is being evaluated separately in the treatment of human viral diseases, including AIDS, it is recommended that extracts which inhibit the growth of HIV through interferon should be given a lower priority for further investigation. Extracts which do not induce interferon should be further evaluated for activity against HIV. Other immunological tests are available but not seen as practical for routine examination of potential extracts for use in the treatment of HIV infections.

Further evaluation of promising products. Confirmation of the therapeutic activity of a given product should be obtained in multiple cell assay systems, with HIV and other retroviruses. The confirmatory tests are left to the preference of the individual investigators. The use of other retroviruses is recommended for the following reasons: it will determine the specificity against retroviruses, set priorities for the selection of compounds to be studied in animal models, reduce exposure to HIV, and reduce the costs for evaluation of the extract as it is separated.

Separation and purification leading to chemical identification should be the goal. However, it is recommended that at least some partial purification process be utilized before further evaluation; assaying the fractions for antiviral activity is essential before proceeding to identify a therapeutic entity.

All three steps, chemical identification, efficacy and toxicity studies in appropriate animal models, and studies based on mechanism of action should proceed concurrently to provide the information necessary for further clinical development, synthetic modification or direct use of the compound(s) identified.

Legal and ethical considerations

Plant-derived compounds or their derivatives form the basis of a large number of established drugs and it has been estimated that the active ingredients of approximately 25% of the approved drugs prescribed in the USA have been developed from such compounds (*8*). Information leading to the discovery of many active drugs has often been obtained through the important historical legacy of folklore uses of plant preparations in many countries (*6*). In many cases, a single plant product was the initial lead that resulted in the development of broad classes of compounds.

Unfortunately the traditional healers and communities that supply the vital information have been neglected by the investigating scientists and have not been rewarded when useful compounds were discovered. During scientific investigation of traditional remedies, all efforts should be made to ensure that the persons and communities involved in the discovery of anti-HIV drugs (including traditional practitioners who supply information that may lead to new discoveries) are appropriately rewarded.

Institutions likely to be involved in a WHO-funded programme should have a policy on how potential income arising from discoveries might be distributed.

Conclusions and recommendations

It is recognized that medicinal plants provide many useful drugs for the alleviation of human illnesses and that about 75% of these were discovered because of the use of the plants in traditional medicine (*6*). Since systematic studies designed to discover anti-HIV drugs from plants and other natural products have been few in number, and since there is an urgent need for a wide variety of effective anti-HIV drugs, the Consultation believes that plants used in traditional medicine, if properly selected and evaluated, will produce active anti-HIV drugs and therefore recommends the following:

- WHO should identify appropriate institutions where the different activities needed for the scientific evaluation of traditional medicine relative to AIDS treatment, such as collection of plants, extraction, *in vitro* and *in vivo* screening, and structure elucidation can be carried out.
- Maximum effort should be made to utilize the existing network of WHO Collaborating Centres on AIDS and the WHO Collaborating Centres for Traditional Medicine.
- Research in the area of traditional medicine should be considered within the framework of the national programmes for the prevention and control of AIDS, and should result in the strengthening of the existing activities and institutions.
- The Global Programme on AIDS, in collaboration with the Traditional Medicine Programme, should establish a mechanism for the definition of priorities, preparation of protocols, and promotion and support of relevant research based on the approved strategies of the programme.
- National AIDS control programmes should collaborate with WHO in the identification of traditional medicinal remedies that would merit further scientific evaluation.
- The present search for a remedy for AIDS is everywhere marked by urgency. In industrialized countries, this activity is usually carried on within the research-based pharmaceutical industry or supported by governments. In developing countries, the resources of traditional medicine are being explored in human studies that are usually uncontrolled. The Consultation recognized that in those countries where remedies for AIDS are being used by the traditional practitioners, the effect of these interventions should be carefully monitored, both to detect any serious side-effects associated with treatment and to ensure that promising therapies are identified and subjected to further investigation and development.

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