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The *Croton megalobotrys* Müll Arg. traditional medicine in HIV/AIDS management: Documentation of patient use, *in vitro* activation of latent HIV-1 provirus, and isolation of active phorbol esters

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ABSTRACT

Ethnopharmacological relevance: Current HIV therapies do not act on latent cellular HIV reservoirs; hence they are not curative. While experimental latency reversal agents (LRAs) can promote HIV expression in these cells, thereby exposing them to immune recognition, existing LRAs exhibit limited clinical efficacy and high toxicity. We previously described a traditional 3-step medicinal plant regimen used for HIV/AIDS management in Northern Botswana that inhibits HIV replication in vitro. Here we describe use of one component of the regimen that additionally contains novel phorbol esters possessing HIV latency-reversal properties.

Aim of the study: We sought to document experiences of traditional medicine users, assess the ability of traditional medicine components to reverse HIV latency in vitro, and identify pure compounds that conferred these activities.

Materials and methods: Experiences of two HIV-positive traditional medicine users (patients) were documented using qualitative interview techniques. Latency reversal activity was assessed using a cell-based model (J-Lat, clone 9.2). Crude plant extracts were fractionated by open column chromatography and reverse-phase HPLC. Compound structures were elucidated using NMR spectroscopy and mass spectrometry.

Results: Patients using the 3-step regimen reported improved health over several years despite no reported use of standard HIV therapies. Crude extracts from *Croton megalobotrys* Müll Arg. ("Mukungulu"), the third component of the 3-step regimen, induced HIV expression in J-lat cells to levels comparable to the known LRA prostratin. Co-incubation with known LRAs and pharmacological inhibitors indicated that the active agent(s) in *C. megalobotrys* were likely to be protein kinase C (PKC) activator(s). Consistent with these results, two novel phorbol esters (Namushen 1 and 2) were isolated as abundant components of *C. megalobotrys* and were sufficient to confer HIV latency reversal in vitro.

Conclusion: We have identified novel LRAs of the phorbol ester class from a medicinal plant used in HIV/AIDS management. These data, combined with self-reported health effects and previously-described in vitro anti-HIV activities of this traditional 3-step regimen, support the utility of longitudinal observational studies of patients undergoing this regimen to quantify its effects on plasma viral loads and HIV reservoir size in vivo.

Abbreviations: AIDS, acquired immunodeficiency syndrome; ARVs, anti-retroviral drugs; cART, combination anti-retroviral therapy; HIV, human immunodeficiency virus; LRAs, latency reversal agents; PKC, protein kinase C; PLWHA, Persons living with HIV/AIDS

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1. Introduction

While combination antiretroviral therapy (cART) has successfully converted HIV to a manageable chronic condition in high-income countries, access to effective HIV therapies in Sub-Saharan Africa remains uneven (UNAIDS, 2013). Furthermore, HIV can persist within latent CD4+ T-cell reservoirs, long-lived cells that harbor an integrated, transcriptionally silent copy of the HIV genome that can spontaneously reactivate to produce infectious viral particles (Finzi et al., 1997). As cART does not act on HIV reservoirs, treatment must be maintained for life. Thus, strategies to achieve cART-free HIV remission or an eventual HIV cure are being pursued.

One strategy to target HIV reservoirs involves the use of latency reversal agents (LRAs) to activate HIV expression in latently infected cells, which can then be eliminated through viral cytopathic effects or host immune responses (Deeks, 2012). These agents could conceivably reduce the viral reservoir to the point of achieving long-term HIV remission in the absence of cART, or possibly eliminate it entirely. To date, various LRAs, in particular compounds belonging to the histone deacetylase (HDAC) inhibitor and protein kinase C (PKC) activator classes, have been shown to disrupt HIV latency in vitro and *ex vivo* in patient-derived CD4+ T cells and, in some cases, produce transient increases in plasma viremia when administered in vivo (Xing and Siliciano, 2013; Rasmussen and Lewin, 2016). However, none have reduced HIV reservoir size in vivo, and many are highly toxic and poorly tolerated (Xing and Siliciano, 2013; Rasmussen and Lewin, 2016). Thus, new agents that effectively target HIV reservoirs are needed.

One class of promising LRAs at the preclinical stage includes the phorbol esters, exemplified by prostratin (Fig. 1A). Prostratin blocks viral entry (and thus HIV replication) by inducing downregulation of the HIV entry receptors CD4 and CXCR4 (Gulakowski et al., 1997; Kulkosky et al., 2001; Hezareh et al., 2004) and activates viral gene transcription in cells latently infected with HIV by stimulating PKC signaling, resulting in nuclear translocation of NF-κB and initiation of HIV-1 proviral transcription (McKernan et al., 2012; Trushin et al., 2005; Williams et al., 2004; Korin et al., 2002; Kulkosky et al., 2001; Gulakowski et al., 1997). More recently, prostratin's latency reversal activity was found to synergize with LRAs of other functional classes, such as HDAC inhibitors (Reuse et al., 2009; Laird et al., 2015; Darcis et al., 2015), raising the possibility that combination LRA therapies may target a larger proportion of HIV reservoirs in vivo at lower, and thus less toxic, doses. Few PKC activators have been assessed in the clinic, with only preliminary results reported to date (Gutiérrez et al., 2016; Rasmussen and Lewin, 2016).

Despite increasing accessibility of cART, traditional medicine use in Africa remains common amongst individuals with moderate and advanced HIV infection (Nlooto and Naidoo, 2014; Gyasi et al., 2012; Lubinga et al., 2012; Namuddu et al., 2011). While the use of traditional medicines raises concerns regarding safety, efficacy, drug-drug interactions, and risk to public health due to HIV transmission if plasma viral loads are not fully suppressed (UNESCO, 2013), it also offers potential opportunities to identify viable and culturally acceptable treatments that could complement cART. We have thus adopted an innovative collaborative model where traditional healers are included as equal partners within the research team.

This collaboration allowed us to document a three-step treatment regimen using medicinal plant preparations from *Cassia sieberiana* D.C. ("Mororwe"), *Vitex doniana* (Sweet) ("Mofofo") and *Croton megalobotrys* Müll Arg. ("Mukungulu") and demonstrate that these preparations contained components that displayed progressively more potent anti-HIV activity in vitro (Tietjen et al., 2016). However, our past studies did not incorporate the views and experiences of users of this traditional medicine regimen, which has been encouraged by the World Health Organization (WHO) to improve early-stage studies to evaluate the efficacy of traditional medicine approaches, including herbal treatments (WHO, 2000).

Prior work by El Mekkawy et al. (2000) examined *Croton tiglium,* a species within the same genus as *Croton megalobotrys*, and identified a series of phorbol esters, including phorbol 12-myristate 13-acetate (PMA; Fig. 1B), that potently inhibited HIV-induced cytopathic effects on MT-4 cells and also activated PKC signaling in vitro. Based on these observations, we investigated whether "Mukungulu" extract contained similar compounds, which could explain its ability to inhibit HIV replication (Tietjen et al., 2016) and might also possess latency reversal activities.

This paper documents the experience of two HIV-infected individuals who have undergone multiple rounds of treatment with the above-mentioned three-step herbal regimen consisting of *C. sieberiana*, *V. doniana* and *C. megalobotrys* over a prolonged (> 12 year) period. These qualitative data are combined with detailed in vitro assessments of these agents, leading us to identify two novel phorbol esters, named Namushen 1 and 2, that display HIV latency reversal activities that are comparable to prostratin and PMA. Our observations provide a first characterization of the self-reported effects of phorbol ester-containing traditional medicines for HIV/AIDS management, and support the need for longitudinal observational studies of patients to quantify this regimen's effects on plasma viral loads and reservoir size in vivo.

2. Materials and methods

2.1. Focus group discussions, case reports and ethical considerations

In February 2016, our collaborator, the traditional healer Sundana Simonambango (SS) facilitated initial contacts between five persons living with HIV/AIDS (PLWHAs) and the research team, which led to a focus group discussion on 24th June 2016 in Maun, Ngamiland District, Botswana. Subsequent unstructured conversations with two consenting

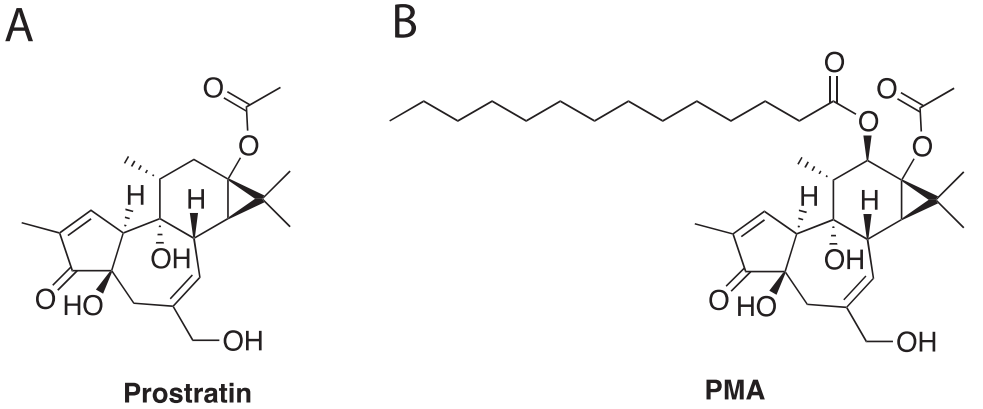

Fig. 1. Structures of (**A**) prostratin and (**B**) phorbol 12-myristate 13-acetate (PMA).