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Effect of Antioxidant (Turmeric, Turmerin and Curcumin) on Human Immunodeficiency Virus

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Abstract: Oxidative stress is implicated in HIV-infection. It has been suggested that plant antioxidants may offer protection from viral replication and cell death associated with oxidative stress in patients with HIV/AIDS. Because of inherent antioxidant properties of turmeric (T) and its derivatives, water-soluble extract turmerin (Tm) and lipid soluble curcumin (Cu), their potential efficacy as anti-HIV drugs were examined. Cell viability and p-24 antigen release by CEMss-T cells (1 x 10^5 cells/ml) infected with HIV-III_B strain, used as an acute model of infection, were tested in the presence of 3'azido-3'deoxythmidine (AZT). Proliferative responses of human mononuclear cells derived from HIV patients (chronic model) stimulated with phyohemagglutinin (PHA), concanavalin A (ConA), and pokeweed mitogen (PWM) were also examined in the presence of AZT and Tm. In the infection assay, T, Tm and Cu individually did not reduce p-24 antigen release or improve cell viability. AZT (5μM) + Tm (800 ng/ml) inhibited infection by 37 % and increased cell numbers by 30%; whereas, Tm (80 ng/ml) inhibited infection by 26% and increased cell number by 60%. In the proliferation assay, lymphocytes from HIV-infected patients showed better inhibition of mitogen responsiveness to Tm (800 ng/ml) when compared to AZT at 5 μM or Tm at 80 ng/ml. Turmerin inhibited HIV-infected T-cell proliferation and, in combination with AZT, decreased T-cell infection and increased cell viability. These data provide evidence suggesting that efficacious anti-HIV therapy may be possible using lower, less toxic doses of AZT in the presence of turmerin.

Keywords: Turmeric, turmerin, curcumin, p-24 antigen, proliferation.

Introduction

Turmeric (T) is a yellow powder, derived from the plant *Curcuma longa*. It has been consumed in amounts up to one g/day for thousands of years in countries such as India. Previous pharmacological studies have demonstrated its anti-tumor, anti-inflammatory, anti-infectious and antioxidant activity with very low toxicity [1-4]. Turmeric consists of a water-soluble component, turmerin (Tm; mol weight 24,000 daltons), and lipid-soluble component Curcumin (Cu; mol weight 369.89 daltons). Curcumin (diferuloylmethane), the yellow pigments in T that is widely used as a food coloring (curry) and preservative has been extensively studied. In particular, Cu inhibited purified human immunodeficiency virus type 1 (HIV-1) integrase [5, 6], HIV-1 and HIV-2 proteases [7], and HIV-1 long terminal repeat - directed gene expression of acutely or chronically infected HIV-1 cells [8]. Cu also inhibited lipopolysaccharide (LPS)-induced activation of nuclear factor kappa B, a factor involved in the activation and replication of HIV-1 [9]. However, Cu has been tested in clinical trials with not much success [10]. Whereas much investigation has focused on Cu, the anti-HIV activity of T and Tm has not been well characterized to date.

Oxidative stress (OS) may play a role in viral replication, decreased immune cell proliferation, and increased sensitivity to drug toxicity. An increase in free radical production and lipid peroxidation has also been described in HIV infected patients [11, 12]. During HIV-infection, free radical damage may be produced, not only by a direct production of oxygen radicals by phagocytes, but also by a tumor necrosis factor (TNF)-mediated generation in target cells. Antioxidants have demonstrated protective capacity for TNF cytotoxicity. TNF-induced free radicals can increase the replication of HIV-1 and destroy T-cells [13]. The superoxide (O_2^-) significantly enhanced cell-to-cell transmission of HIV-1 [14]. Evidence has accumulated suggesting that HIV-infected patients are under chronic OS [15]. A redox imbalance caused by an over-production of pro-oxidants or a decrease in antioxidants seems to play an important role in the normal physiological function. Following activation, lymphocytes produced increased levels of OS. Lymphocytes from such individuals were more prone to undergo apoptosis or cell death in-vitro [16]. Antioxidant supplementation significantly improved some measures of oxidative defense [17]. Thus, using antioxidants may be an alternate means of treating HIV-patients.

The anti-retroviral drug AZT is a dideoxynucleoside analog which, upon activation by the target cell, reduces viral infectivity and enhances immune functions in HIV-infected patients [18]. It inhibits retroviral reverse transcriptase as well as cellular DNA polymerase ß and γ [19,20]. AZT delays disease progression in HIV-infected patients but cause unpredictable side effects [21,22]. Ideally, reduction in AZT dosage requirements through combination with some other pharmacologically-active agent would lessen toxicity while maintaining viral inhibition. In addition, combination therapy facilitates drug synergism and delay development of resistance [23-25].

The rationale for testing turmeric and its derivatives was based on their potent antioxidant properties and their potential use as an adjuvant to AZT as modulators of OS. Previously, we have shown that T and

Cu protect kidney epithelial cells from H_2O_2 –induced injury while Tm had no protective effect [26]. In the present study, we have shown that Tm (800 ng/ml) in combination with AZT (μM) maximally inhibited HIV-infection in CEM-T cell-line model and Tm (800 ng/ml) maximally inhibited the activation of HIV-infected human mononuclear cells to T or B cell mitogens. Whether consumption of exogenous plant antioxidants [27] like T, Cu and Tm can be useful in inhibiting viral activation and the death of immune cells in HIV/AIDS through their antioxidant properties is not addressed in this study and will certainly warrant further investigation.

Materials and Methods

Anti-retroviral Nucleosides

AZT was purchased from Wellcome Beckenham (Kent, UK) and re-suspended in sterile distilled water as stock solutions and serially diluted before experiments. Concentration of AZT added was 5μM.

T, Tm and Cu

Turmeric powder was obtained from Raja Foods (Lincolnwood, IL). Curcumin was obtained from Aldrich Chemicals (St Louis, MO). Turmerin was obtained by boiling 1% turmeric solution for 30 minutes, centrifuging at 1200 x g for 10 minutes and passing the supernatant through 0.22 μ filter. The protein yield of the filtered solution was 80 μg/ml.

Study Population

For the present study, blood was obtained from 10 HIV-1 seropositive participants.

Lymphocyte Preparation (Chronic T-cell model of HIV infection)

Fresh peripheral blood mononuclear cells (PBMC) were obtained from HIV-1 seropositive subjects (positive p-24), the blood was diluted 1:1 with sterile phosphate buffered saline (PBS), underlayed with lymphocyte separation medium (LSM, Oreganon, Teknika, West Chester, PA). The sample was centrifuged at 800Xg for 25 minutes, the PBMC layer was removed and washed twice with PBS, and the cell pellet was resuspended in medium 1640. These cells were cultured in round-bottomed, 96-well plates (Falcon) in RPMI 1640 medium (Gibco) with 10% human AB serum and antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin) at a concentration of 1×10^6 cells/ml (viability ≥ 95%) with each well receiving 100μl of the cells. Cells were incubated with media alone or with final 20μg/ml phytohemagglutinin (PHA), 10 μg/ml Concanavalin A (Con A) or 1 ng/ml Pokeweed mitogen (PWM) (Sigma). In the first three wells, cells were incubated with 100μl of media serving as media controls. In the subsequent wells the plates were incubated with 1) 100μl of mitogen (mitogen controls), 2) 100 μl of mitogen+10 μl of Tm (800ng/ml), 3) 100 μl of mitogen+10μl of Tm (80ng/ml). 4) 100 μl of

mitogen+10µl of AZT (5 µM). The controls for AZT, Tm (800ng/ml) and Tm (80ng/ml) were cells that only had the drug with no mitogen. All lymphocyte proliferation assays were done in triplicate in 5% CO_2. After 3 days of incubation at 37°C, the cells were labeled with 20µCi/ml (25 µl/well) of 3[H] thymidine (specific activity 5 mM Radiochemical Inc, Amersham, USA) in RPMI. Cells were harvested (PHD cell harvester Brandel, Inc.) and incorporated label was determined by scintillation counting. The results were expressed as mean counts per minute (cpm) ± SD.

Virus

HIV-1 was obtained from the culture supernatant of HTLV-III_B-producing H9 cells. During the exponential phase of growth, cell-free supernatant was harvested, standardized for reverse transcriptase activity, and frozen in aliquots at -70^0C.

CEM preparation (Acute T-cell model of HIV infection)

A human non-adherent CD4+ T cell line, CEM-ss cells were obtained from (NIH AIDS Research and Reference Reagent Program) and 5 x10^5 cells were infected with HTLV-III_B (1 reverse transcriptase unit/10 cells) at 37^0C for 2 hrs. Non-adsorbed virus was then removed by washing 3 times with phosphate buffered saline. Cells were cultured on a 24-plate and incubated in the presence and absence of drugs. Cells were incubated with 100 µl of Turmeric (100µg/ml or 10µg/ml), Curcumin (100µg/ml or 10µg/ml), or Turmerin (800 ng/ml or 80ng/ml). HIV-1 production was monitored at 3- and 7-day time intervals by measuring the level of p-24 antigen (24 kDa HIV-1 gag gene product) in culture supernatants. The culture supernatant was tested for the presence of HIV-1 p24 antigen, using the HIV AGA enzyme immunoassay kit from Abbott Laboratories (Abott Park, IL) according to manufacturer instructions. A standard curve (Optical Density at 492nm as a function of p24 antigen concentration) was obtained using p24 antigen provided by the supplier. An optical density value of 1 corresponds to 200 pgs of HIV-1 p-24 antigen. All our results had a cut-off value of 0.1 optical density (OD) units. These experiments were repeated at least twice. After 7 days, cell viability and cell number was determined by the trypan blue exclusion method.

Results

Selective inhibition of p-24 antigen and increased cell viability by turmerin

CD4+-T-cell line infected with HTLV-III_B strain was used to study inhibition of HIV infectivity. Cells exposed to T (100µg/ml or 10µg/ml), Cu (100µg/ml or 10µg/ml) or Tm (800ng/ml or 80 ng/ml) alone or in combination with AZT were tested for p24 antigen production (Table 1) and cell viability was determined (Table 2).

Table 1. Percentage increase or decrease of infection (p-24 antigen release)

Drug Treatment	(-) AZT	(+) AZT
T (100µg/ml)	5.2	21.1
T (10 µg/ml)	201.7	10.5
Cu (100µg/ml)	46.6	63.2
Cu (10µg/ml)	184.5	5.3
Tm (800ng/ml)	63.8	-36.8
Tm (80ng/ml)	115.5	-26.3

% increase in infection = 100 x ([T/Cu/Tm treatment OD minus Control OD])/(Control OD) where the Control OD is the Optical density of the cells with media alone (100%).

Table 2. Percentage increase or decrease in viability from control (Trypan Blue exclusion)

Drug Treatment	(-) AZT	(+) AZT
T (100µg/ml)	-50.0	-20.0
T (10 µg/ml)	-38.9	50.0
Cu (100µg/ml)	-22.2	-10.0
Cu (10µg/ml)	-11.1	50.0
Tm (800ng/ml)	-11.1	30.0
Tm (80ng/ml)	-16.7	60.0

% increase in viability = 100 x [(T/Cu/Tm treatment cell number minus Control cell number])/(Control cell number) is where the control cell number is the cell number with media alone (100%).

Turmeric (100µg/ml), the parent compound, had the lowest percentage increase for infection (5.2%) in the absence of AZT while AZT + Tm (800ng/ml) showed the lowest percentage increase for infection which corresponds to an inhibition of 36.8% (Table 1). With respect to viability, T (800ng/ml) and Cu (10 µg/ml) showed the highest percentage increase for viability which corresponds to an inhibition of 11.1% in the absence of AZT while Tm (80 ng/ml) showed highest percentage increase for viability (60%) in the presence of AZT (Table 2).

Proliferative response of PHA, Con A, PWM activated HIV-infected Human PBMC

Table 3 is the compilation of proliferative response of PHA, Con A and PWM in HIV-infected human PBMC data from ten patients. Figure 1 is an example of data from patient 1 showing that AZT or Tm at 800 ng/ml and 80 ng/ml inhibited proliferation when compared to cells treated alone with T cell mitogens PHA and Con A or B cell mitogen PWM. Tm 800 ng/ml showed maximum inhibition of proliferation when compared to AZT or Tm .In particular for patient #1, stimulation index (ratio of cpm of stimulated cells versus cpm of unstimulated cells [media control]) for Tm 800 ng/ml + PHA was 16.6, for Tm 800 ng/ml + Con A was 2.3 and for Tm 800 ng/ml + PWM was 3.8. A trypan blue exclusion assay showing

Table 3a. Total CPM (Mean ± SD) in PBMC of HIV+ patients treated with PHA.

Pt#	Media	PHA	PHA+AZT 5μM	PHA+Tm80ng/ml	PHA+Tm 800ng/ml
1	1,048±69	58,377±6,534	30,373±561	40,388±6,041	17,431±870
2	235±98	28,727±1,815	15,050±3,597	32,170±1,496	N.D.
3	1,603±417	323,061±43,362	229,133±11,376	367,124±35,117	N.D.
4	1,307±313	44,758±9,154	33,552±4,370	41,579±4,969	25,476±3,745
5	474±140	284,041±12,721	N.D.	129,729±2,465	22,643±3,754
6	973±152	56,5021±1,602	N.D.	227,385±16,031	30,503±5,964
7	847±190	43,725±3,197	N.D.	38,530±2,443	1,218±130
8	767±92	28,988±97	N.D.	26,305±1,206	2,245±655
9	1,690±615	24,191±394	N.D.	20,145±470	3,612±970
10	1,422±64	59,369±971	N.D.	46,999±2,112	3,207±390

Table 3b. Total CPM (mean ± SD) in PBMC of HIV+ patients treated with Con A.

Pt#	Media	Con A	Con A+AZT 5μM	Con A+Tm80ng/ml	Con A+Tm 800ng/ml
1	1,048±69	24,962±1,286	8,849±833	7,862±1,584	2,364±305
2	235±98	1,099±747	499±101	233±120	N.D.
3	1,603±417	261,903±16,282	145,582±7,796	147,782±1,759	N.D.
4	1,307±313	25,130±148	17,163±5,742	15,157±4,345	5,530±1,192
5	474±140	132,930±16,300	N.D.	37,777±6,429	9,530±1,198
6	973±152	265,056±26,172	N.D.	130,535±20,361	29,437±11,618
7	847±190	26,001±2,772	N.D.	17,535±2,736	372±72
8	767±92	14,638±154	N.D.	12,244±165	1,560±599
9	1,690±615	8,236±54	N.D.	7,463±1,171	3,714±552
10	1,422±64	20,456±1,120	N.D.	14,164±1,654	1,921±471

Table 3c. Total CPM (mean ± SD) in PBMC of HIV+ patients treated with PWM.

Pt#	Media	PWM	PWM+AZT 5μM	PWM+Tm80ng/ml	PWM+Tm 800ng/ml
1	1,048±69	12,056±276	5,851±1,203	10,427±3,763	3,967±663
2	235±98	4,503±1,365	2,396±538	4,886±654	N.D.
3	1,603±417	82,535±18,509	50,016±8,365	72,954±6,993	N.D.
4	1,307±313	14,148±5,056	9,795±1,322	15,485±1,544	9,736±624
5	474±140	123,251±2,597	N.D.	63,382±6,358	11,847±1,809
6	973±152	202,760±21,170	N.D.	81,732±2,815	4,816±192
7	847±190	22,593±3,419	N.D.	17,625±992	14,529±884
8	767±92	5,166±165	N.D.	4,772±259	1,361±83
9	1,690±615	3,662±187	N.D.	4,816±192	3,454±600
10	1,422±64	16,382±694	N.D.	14,529±884	1,376±260

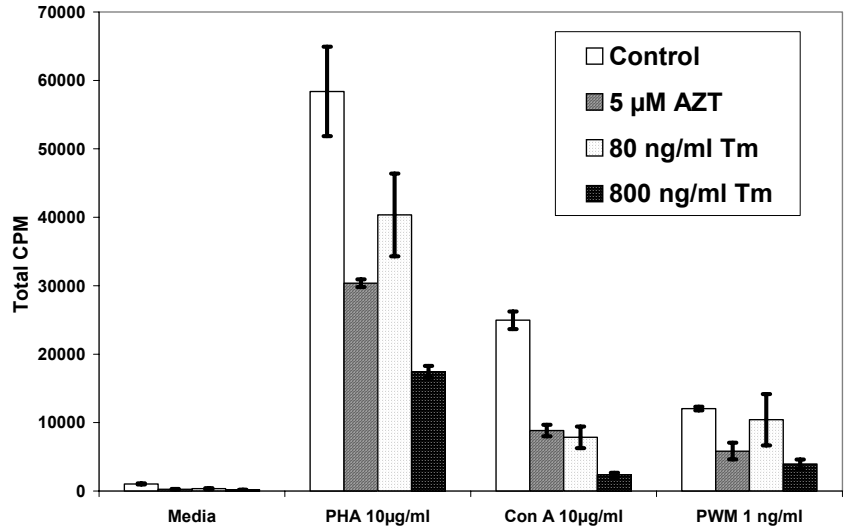

Figure 1. Comparison of peripheral blood mononuclear cells isolated from HIV+ patient and incubated with PHA, Con A and PWM in combination with Tm (800ng/ml; 80 ng/ml) or AZT (5µM) for 72 hrs. The cells were pulsed with radiolabeled thymidine for 18 hrs and then harvested for counting by liquid scintillation. Responses are expressed as net cpm and dose of drug is expressed as ng/ml and µg/ml. Values represent the mean of at least triplicate readings and the standard deviation (S.D.).

similar viability in drug-exposed and control cultures (data not shown) confirmed that inhibition by Tm did not depend on generalized drug toxicity

Discussion

Loss or reduction of T-cell proliferative capacity to in-vitro stimulation is one of the qualitative changes observed in the functional performance of PBMC from HIV-infected subjects [28-34]. T-cell proliferative capacity is an important independent predictor of progression to HIV disease [35, 36] and can also serve to monitor immunological improvement after therapy [37, 38]. Decreased proliferation of T-cells from HIV-infected individuals has been previously measured in response to in-vitro stimulation with CD3 monoclonal antibody, pokeweed mitogen, alloantigens, and recall antigens [39-43]. The response to phytohemagglutinin (PHA) remains unaffected in the early phases but is significantly reduced later in infection [44, 45]. In our series, there was high proliferation to PHA. This implies that the patients we are dealing with are in the early phases of HIV-infection.

The mechanism of action of turmeric and turmerin on HIV infection has yet to be determined. The inhibition of mitogen stimulated PBMC and CEM-ss cells suggest that the drug treatment is critical to the virus infected chronic T cell infection as well as to the virus adsorbed acute T cell infection. That T and Cu inhibit virus adsorbed acutely infected CEM-ss T cells at fairly high concentrations while a low dose

of Tm would suffice suggests that the bulk of ingested drug as a treatment therapy with Tm will be less than with T and Cu.

Our results in HIV-infection studies exhibited a clear advantage of using Tm at 800 ng/ml in combination with AZT to maximally decrease the infection when compared to turmeric and curcumin. The viability results showed that Tm at 80 ng/ml maximally increases viability when compared with turmeric and curcumin. The interesting point to note is that the higher concentration of Tm 800ng/ml increases viability only by 30% when compared with 60% with Tm at 80 ng/ml indicating that higher concentrations of turmerin may be toxic. With respect to mitogen proliferation (patient 1 and Figure 1), it is clear that Tm maximally inhibited proliferation for PHA, Con A and PWM as noted by decreased stimulation indices. T cell proliferation data clearly shows that in 8/10 patients Tm at 800ng/ml and 80 ng/ml or AZT individually inhibit proliferation of mitogen stimulated cells. Two out of ten patients (#2 and #3) show that Tm is not inhibiting PHA stimulation. In addition, Tm in patient # 2 is not inhibiting PWM stimulation. Inhibition of mitogen proliferation of some patient cells is not inhibited in the presence of Tm alone suggests that Tm lacks the effectiveness in preventing infected T cell proliferation.

T-cell activation is known to be required for the induction of HIV mRNA transcription, viral replication, and spread of infection to newly activated CD4+ T cells [46]. This implies that by downmodulating mitogen responsiveness, AZT used alone or in combination with turmerin could confer an unresponsive status to the HIV-infected T-cells, thereby decreasing capacity for viral replication. If so, AZT in combination with Tm could hypothetically inhibit the increases in the viral load and inhibit infection of virgin cells - a worthwhile achievement in the treatment of asymptomatic HIV+ patients in whom viral spread has not occurred. Also, our proliferation data shows that turmerin will not be effective in a cohort of patients because it did not reduce T-cell proliferation.

These findings provide evidence that toxicity of AZT may be reduced by virtue of being able to use lower doses of the drug in combination with turmerin. Additional work is in progress to determine the optimum dosage at which AZT concentration can be decreased in combination with turmerin to reduce its toxicity.

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