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The anti-HIV activity of the phytochemical α-terthienyl

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Summary

The plant trithiophene, α-terthienyl (αT), was evaluated for activity against the human immunodeficiency virus (HIV-1). Antiviral activity specifically required long wavelength light (UVA, 320–400 nm). The compound had little or no activity in visible light or in the dark. The anti-HIV effect was UVA-dose dependent and was proportional to the concentration of αT, according to several parameters of virus infectivity and replication. The efficacy was decreased to some extent by the presence of bovine serum in the reactions; but under optimal conditions 0.1 μg/ml. αT (3×10^{-7} M) could inactivate 10^4–10^5 infectious particles. In contrast poliovirus and Coxsackievirus infectivity were relatively resistant to αT + UVA.

Anti-HIV activity; α-Terthienyl; Phytochemical

Introduction

Thiophenes are sulfur-containing heterocyclic compounds that are distributed widely among species of the Asteraceae (Compositae) family, including many species with known medicinal uses (Bohlmann et al., 1973; Towers, 1984; Arnason et al., 1990). The many biological activities of these compounds are invariably dependent upon the presence of long wavelength ultraviolet light (UVA), which is an important component of sunlight (Kagan, 1991; Hudson and Towers, 1991).

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We have been particularly interested in the simple unsubstituted trithiophene, α-terthienyl, which can be isolated from many species of *Tagetes*, and which possesses impressive phototoxicity against a wide range of organisms (Towers and Hudson, 1987; Hudson and Towers, 1991). The principal target for this compound appears to be membrane fatty acids, particularly unsaturated ones, which are oxidized by the singlet oxygen that is produced by irradiating α-terthienyl (αT) with UVA (McRae et al., 1985; Evans et al., 1986).

In accordance with this concept, we found that αT (+ UVA) rapidly and efficiently inactivated viruses with membranes, but not bacteriophages (Hudson et al., 1986, 1987). However, the inactivated virus still retained its integrity, and it was able to penetrate susceptible cells, although it did not replicate (Hudson et al., 1986).

In the absence of UVA, αT has relatively little cytotoxicity, and it has been safely administered to rodents (Rampone et al., 1986; Hudson and Towers, 1991). In view of these attractive properties of αT, we have considered possible therapeutic applications, e.g., in the treatment of microbial and viral infections, and in the decontamination of blood products. Both of these applications are relevant to HIV-1 infections, and for this reason we decided to evaluate αT as an anti-HIV agent.

Furthermore, since self-administration of various plant extracts is becoming more popular among HIV-infected individuals, it is important to evaluate beneficial and detrimental components of such extracts, as well as the effects of light on their biological activities (Abrams, 1990; Kagan, 1991; Hudson and Towers, 1991).

Materials and Methods

αT

The compound was synthesized as described by Garcia et al. (1984), and was stored in dark vials at 4°C. Stock solutions were prepared at 1.0 mg/ml in 95% ethanol. These could be stored in dark vials at 4°C for many months with no apparent loss in antiviral activity. The ethanolic solution was diluted into cell culture medium or buffered salt solution just prior to use.

Viruses and cells

The LAV strain of HIV-1 was obtained from Dr. L. Montagnier. The virus was propagated and assayed (as $TCID_{50}$ U/ml) in CEM cells. The cells were grown in RPMI 1640 supplemented with 10% v/v fetal bovine serum, 25 μg/ml gentamicin, 2 mM glutamine.

Poliovirus type 1 and Coxsackievirus B6 were obtained from the B.C. Provincial Laboratory (courtesy of Dr. P. Middleton). They were propagated and assayed, by plaque formation, in Vero cells, in Dulbecco MEM supplemented by 10% v/v fetal bovine serum, 25 μg/ml gentamicin, and 0.5 μg/ml econazole (Cilag-Chemie, Zurich).

Photochemical treatments

The standard regimen for HIV-1 was as follows: stock virus (10^5–10^6 $TCID_{50}$ HIV-1/ml), in the form of clarified supernatant from infected CEM cultures, was diluted into complete medium to give 10^3 $TCID_{50}$ in 0.5 ml/well of a 24-well tissue culture tray (Falcon). To this was added 0.5 ml αT at the desired concentration in complete medium. These reaction mixtures were then kept in the dark (i.e., enclosed in aluminum foil) for 20 min followed by exposure to a portable UVA lamp for 30 min. The incident radiation was 5 Watts/m^2, as measured by a UVA photometer. The plastic trays were impermeable to wavelengths below 320 nm. In the early experiments the trays were set in ice water; but this was subsequently found to be unnecessary since the radiant energy was not enough to increase the ambient temperature within the biosafety cabinet. Immediately following the radiation, 5×10^5 CEM cells, in 1.0 ml medium, were added to each well, and the trays were placed in a dark 37°C incubator with 5% CO_2–95% air. Cultures were examined daily, after 3 days p.i., under a microscope for cpe (foci). At 4/5 and 7/8 days p.i. half of each supernatant was removed and replaced by fresh medium.

Controls included similar reaction mixtures kept in the dark (enclosed in aluminum foil), and virus or cells alone ± UVA. All reactions were carried out in replicates of 2–4 wells. Variations in individual experiments are indicated in the text.

In the case of poliovirus and Coxsackievirus, the stock virus (respectively 2×10^8 and 3×10^7 Pfu/ml in culture supernatant) were diluted into phosphate buffered saline for the reactions with αT. Irradiation was provided by a bank of 6 black-light blue (UVA) lamps in a temperature controlled environmental chamber. The temperature was normally set at 20°C, although the results were the same at 4°C. The incident radiation was 5 Watts/m^2, the same as the system used for HIV-1. Following treatment, infectious virus was titrated by plaque formation on Vero cells.

In the experiments that involved visible light, a fluorescent lamp was used at a distance that gave the same amount of incident radiation to the reaction mixtures as the UVA lamps, i.e., 5 Watts/m^2.

Assessment of cytopathic effects

In earlier experiments the relative numbers of foci of infection (syncytia) per microscopic field were enumerated. However, a more reliable indicator of progression of cpe and hence virus growth was obtained by estimating the percentage of cells showing virus cpe. This value thus included the expanding primary foci of infection, plus all the secondary foci. These percentages were estimated to the nearest 5% of cells in the field. Generally there was good agreement between replicate cultures and between the estimates of two observers (± 10%). For the standard dose of virus inoculum (10^3 $TCID_{50}$), cpe were barely discernible at 3 days p.i.; but approximately 10% of the cells were involved at 4 days p.i. This value increased to 100% by 8–10 days p.i. provided the media were replenished adequately.

Occasionally, some of the replicate cultures (derived from photochemically-treated virus) showed cpe, due to one or a few residual infectious virus, while others did not (due to complete inactivation of virus). In these instances, the cpe-positive cultures always progressed eventually to 100%, whereas cpe-negative cultures remained negative.

Assays for p24

The relative amount of p24 protein synthesized was determined, on duplicate cultures, by the Abbot ELISA system. Absorbance readings were converted to ng/ml of the p24 protein by comparison to Abbot standards.

Fluorescent antibody staining

Aliquots of infected cells were centrifuged, washed with phosphate-buffered saline, and air-dried on glass slides. The cells were subjected to normal two-step fluorescent antibody staining, with a high titre human HIV-1 positive serum (positive for all HIV-1 proteins on a Western blot), and fluorescein-labelled anti-human globulin (affinipure goat anti-human IgG H + L; Jackson Immunoresearch Laboratories, PA, USA).

Results

The effects of different light wavelengths

α-Terthienyl has a strong absorbance peak at 350 nm, and is photoactivated by black light blue lamps with maximum emission at about 360 nm. The data in Table 1 indicate that αT has a potent anti-HIV effect when irradiated with UVA. As little as 0.01 μg/ml (about 0.03 μM) caused partial inactivation of the virus (10^3 $TCID_{50}$) while 0.1 and 1.0 μg/ml completely abolished virus infectivity; i.e., there were no cpe in the inoculated cultures and no p24 protein was produced.

When virus + αT mixtures were incubated in the dark there was little or no

TABLE 1

Anti-HIV effect of αT

Concentration of αT (μg/ml)	UVA		Visible		Dark	
	cpe[a]	p24[b]	cpe	p24	cpe	p24
0	4 + (100)	100	4 + (100)	100	4 + (100)	100
0.01	±[c] (20)	17	3 + (80)	100	3 + (80)	100
0.10	− (0)	0	3 + (100)	100	3 + (80)	100
1.0	− (0)	0	±[c] (7)	13	3 + (80)	100

[a] cpe score: + to 4+, increasing percentage of cells showing viral cpe, at 6 days p.i. Numbers in brackets refer to relative numbers of foci per microscopic field.

[b] p24: amount of HIV-1 p24 produced (6 days p.i.) as percentage of untreated virus.

[c] ± some cultures; +, some −.

Irradiation in all cases was for 30 min.

effect (Table 1), and visible light irradiation (from fluorescent lamps) of the mixtures produced significant inhibition, but only at the highest concentration, 1.0 μg/ml αT.

This result is in accordance with other known biological activities of αT, which require UVA (Towers, 1984; Arnason et al., 1990; Kagan, 1991; Hudson and Towers, 1991). We therefore decided to carry out further experiments with UVA irradiation only, in comparison with dark controls.

Effects of αT concentration on HIV-1 parameters

Fig. 1 illustrates the time course of virus growth in cultures inoculated with treated (αT + UVA) and untreated HIV-1, as well as data for p24 protein production and fluorescent antibody-staining cells at 6 days p.i.

At the lowest concentration of αT, 0.02 μg/ml, the HIV-1 was only partly inactivated, such that the resulting inoculated cultures lagged behind the virus controls in terms of extent of cpe and production of p24.

At 0.1 μg/ml αT (+ UVA), no cpe were observed until 6 days p.i., when one culture showed cpe and a reduced amount of p24 (triangles with asterisks). This culture progressed to 100% cpe after 8 days.

At the highest concentration of αT, 0.5 μg/ml, there was no indication of viral cpe or p24 synthesis. Thus, the viral inoculum had been completely inactivated. In contrast, all virus preparations treated with αT in the dark gave rise to cultures that were indistinguishable from those inoculated with untreated virus (not shown).

Fig. 2 shows that the p24 test could also be used to monitor the growth of virus. At 4 days p.i., levels of p24 protein were detectable, and these rose

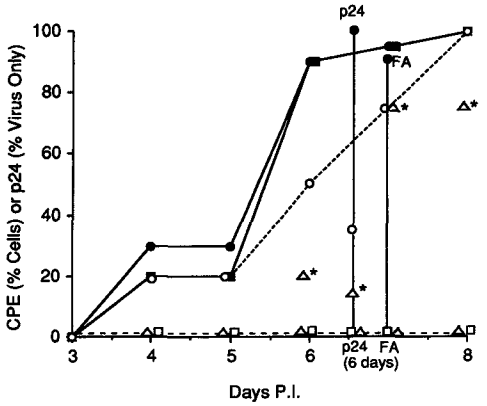

Fig. 1. The effect of α-terthienyl (αT) and UVA treatment on viral cpe, p24 synthesis, and FA-positive cells. Aliquots of virus (10^3 $TCID_{50}$) were treated with αT (0.02–0.5 μg/ml) ± UVA, and then added to replicate CEM cultures. All cultures were examined daily for cpe, and at 6 days p.i. supernatants or cells were assayed respectively for p24 protein (100% value, 984 ng/ml) and fluorescent-antibody-staining cells. Symbols: ●, no αT; ○, 0.02 μg/ml αT; △, 0.1 μg/ml αT; △*, one culture was positive for virus; □, 0.5 μg/ml αT; ■, 0.5 μg/ml αT in the dark. Values for 0.1 and 0.02 μg/ml αT in the dark were the same as for no αT.

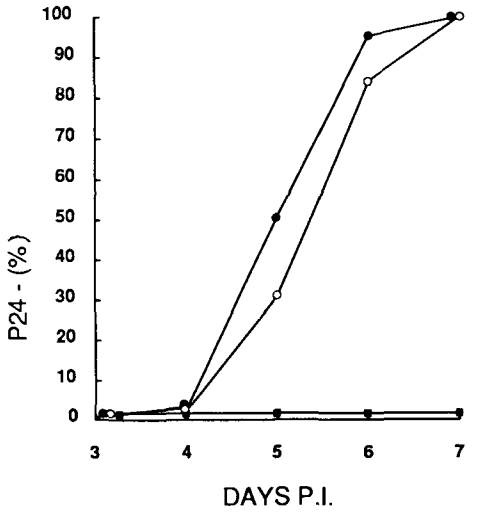

Fig. 2. Kinetics of synthesis of p24 antigen in cultures infected with treated and untreated virus. 100% value = 2,137 ng/ml. Symbols: ●, HIV only; ○, HIV + αT (0.1 μg/ml) in the dark; ■, HIV + αT (0.1 μg/ml) + UVA. Half of each culture supernatant was removed each day for the p24 assay. The 10-day p.i. values were less than the corresponding 7-day values.

rapidly between 5 and 7 days p.i. After 7 days, the levels began to decrease, presumably due to gradual degradation in the absence of further synthesis. At no time however was p24 detected in cultures that had been exposed to αT + UVA treated virus (squares, Fig. 2).

There have been reports that certain phytochemicals, e.g., hypericin, can inhibit reverse transcriptase (Schinazi et al., 1990). We therefore evaluated αT for possible effects on the p24 antigen assay. However, only a slight reduction in p24 assayed was obtained following prolonged incubation with the compound (up to 0.5 μg/ml) ± UVA (data not shown). Therefore we concluded that the p24 assay does provide a reliable quantitative assessment of HIV-1 replication in infected cells exposed to αT.

Effect of irradiation time

Aliquots of HIV-1 were irradiated with the standard dose of UVA for variable lengths of time, ± αT at 0.1 μg/ml. Dark controls were also used. Fig. 3 shows the result in terms of p24 production and fluorescent antibody-staining cells (measured at 5 days p.i.). Some inactivation was evident with 5 min irradiation. 10 min and 20 min irradiation showed proportionally more, while 30 min irradiation resulted in complete inactivation of the virus.

The p24 levels generally decreased more than the number of FA-positive cells, probably reflecting reduced amounts of p24 in the individual FA + cells. These results were supported by cpe assessments (not shown).

Also in Fig. 3 is shown the effect of UVA alone (squares), without αT. Up to

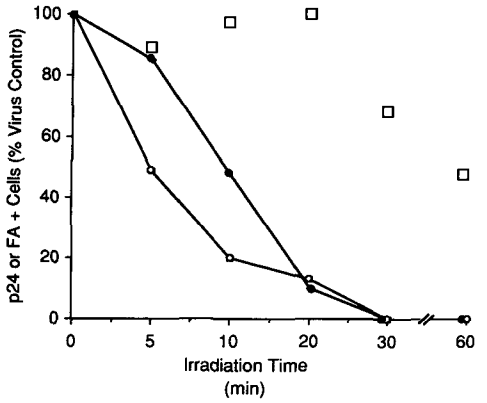

Fig. 3. Effect of UVA radiation time. Standard conditions were used, with 0.1 μg/ml αT, ± UVA for varying lengths of time, before inoculating the treated viruses into CEM cultures. Symbols: ○, p24 as percentage of untreated virus, 5 days p.i. (100% value = 390 ng/ml); ●, number of cells staining positive for HIV-antigens, as percentage of untreated virus, 5 days p.i.; □, p24, 5 days p.i. UVA only no αT.

20 min irradiation produced essentially no decrease in p24 yield, although higher doses gave rise to observable decreases.

Virus concentration and the effect of serum on αT efficacy

In order to determine the potency of αT, a standard concentration of the compound, 0.1 μg/ml, was mixed with different amounts of HIV-1, and irradiated with UVA as usual. Normally these reaction mixtures are made up in complete medium, containing 10% bovine serum, to conserve viability of the virus. It is conceivable however that serum components could influence the antiviral efficacy of αT. This possibility was evaluated by irradiating parallel mixtures of virus + αT in the absence of serum. Following treatment the serum level was restored prior to inoculation into cell cultures as usual. In all cases the resulting cultures were assessed for cpe (daily) and for p24 production. Results for the 5 days p.i. time points are given in Fig. 4.

In the case of 10^3 $TCID_{50}$ of HIV-1, infectivity was completely abolished by αT + UVA in the presence or absence of serum (maximum serum level in the − serum samples, due to residues from stock virus, would have been approximately 0.01% by volume). Thus no p24 protein was produced, and no cpe were observed. In contrast, similar samples without αT produced 90 ng/ml p24 and by 7 days p.i. the cultures showed 100% cpe (data not presented).

For 10^4 $TCID_{50}$ virus there was a clear distinction between + and − serum (Fig. 4). In this instance αT + serum resulted in a 90% decrease in p24 production i.e., 40 ng/ml compared with 400 ng/ml in the absence of αT (± serum), whereas for αT minus serum p24 was undetectable. The cpe scores (not shown) agreed with these results.

A similar result was obtained for 10^5 $TCID_{50}$ virus (Fig. 4). In the presence of serum, αT reduced the p24 level to 16.6% (295 ng/ml) of the corresponding

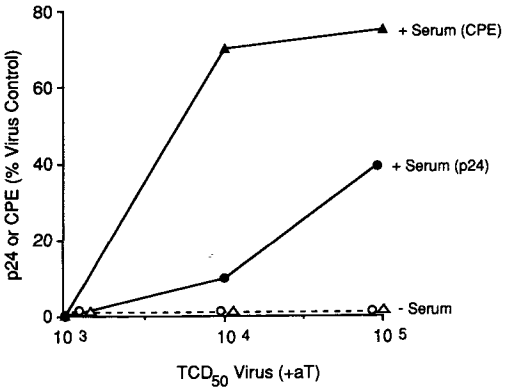

Fig. 4. Effect of serum on anti-HIV efficacy of αT. Various amounts of HIV (10^3–10^5 $TCID_{50}$) were treated with 0.1 μg/ml αT (+ UVA) in the presence or absence of 10% v/v fetal bovine serum. These treated viruses were inoculated into CEM cultures, and viral cpe and p24 production (5 days p.i.) were measured as usual. Values are expressed as percentages of the corresponding virus controls (no αT). Symbols: ▲,△, cpe scores at 5 days p.i. ± serum; ●,○, p24 protein at 5 days p.i. ± serum.

level without αT; but in the absence of serum, αT virtually completely inactivated the virus, such that little or no p24 was produced, and no cpe were observed. Thus 0.1 μg/ml αT (+ UVA) in the absence of serum essentially abolished 10^5 $TCID_{50}$ of HIV-1.

Effect of αT on enteroviruses

The principal target for the biological activities of αT is thought to be membrane constituents (McRae et al., 1985; Hudson and Towers, 1991). We decided to test αT, at several concentrations, ± UVA, and in the absence of serum, for activity against poliovirus type 1 and Coxsackievirus B6. Both viruses showed a small, UVA-dependant and αT concentration-dependant, decrease in infectivity (maximum 40%) which was not considered significant (data not shown).

Discussion

We had previously shown that αT (+ UVA) was a very potent anti-viral compound (Hudson et al., 1986; Hudson, 1990). This present study indicated that HIV-1 was also very susceptible to the compound, and that the effect was almost totally dependent on UVA radiation. In visible light or in the dark HIV-1 was essentially resistant. The fact that the two non-membrane containing viruses, poliovirus type 1, and Coxsackievirus B6, were largely resistant to αT + UVA, supports the concept of a membrane target and hence a similar mechanism of action to that proposed for other organisms: i.e., UVA-mediated production of singlet oxygen, which then reacts with unsaturated fatty acids (McRae et al., 1985).

The possibility of alternative targets can not be ruled out however, and several studies have shown that αT is capable of adversely affecting DNA at > 5 μg/ml (Wang et al., 1991) and proteins (Hudson, 1989). In this study too, αT ± UVA did show small though significant interactions with p24 protein, the two enteroviruses (with resulting slight decreases in infectivity), and with one or more components of bovine serum.

The interaction with serum components is of interest from the point of view of therapeutic applications. Some photodynamic dyes, such as porphyrins and cyanins, can bind to serum proteins or lipoproteins, and in the case of porphyrins the interaction with high density lipoproteins actually facilitates the penetration of the agent into cells (Allison et al., 1990; Sieber et al., 1988). In addition some unsaturated compounds in serum could conceivably quench singlet oxygen and thereby reduce the effectiveness of an antiviral agent, although Kanofsky (1990) has pointed out that this could be advantageous by minimising unwanted side effects. Serum evidently decreased the anti-HIV efficacy of αT + UVA to some extent in this study, and it would be interesting to determine which component(s) in the serum is (are) responsible for this.

Several photodynamic agents have been proposed for use in the decontamination of blood products, where a number of viruses, including retroviruses, constitute potential hazards. Porphyrin and cyanin dyes are examples that have been evaluated in model systems (Neyndorff et al., 1990; Matthews et al., 1988; Sieber et al., 1990). Furocoumarins, in particular 8-methoxypsoralen (8-MOP), have also been advocated for this and similar applications (Edelson, 1988; Hudson and Towers, 1991). This type of compound also requires UVA for its biological manifestations (including antiviral activities, Hudson, 1989; Watson et al., 1990), although because of its multiple targets 8-MOP does have undesirable side effects, such as mutagenesis and oncogenesis, and the potential to activate HIV gene expression at certain UVA doses (Gupta and Anderson, 1987; Zmudzka and Beer, 1990; Dall'Acqua and Martelli, 1991). Nevertheless 8-MOP + UVA is in use as a treatment mode, the so-called photopheresis, for some blood disorders (Edelson et al., 1987), and has recently been used in AIDS patients (Bisaccia et al., 1990).

αT and related compounds are the most potent antiviral phytochemicals that we have encountered so far in our search for potential therapeutic plant materials (Hudson, 1990; Hudson and Towers, 1991). Furthermore, αT is innocuous in the absence of UVA, and this enhances its attraction as a candidate for potential applications. Nevertheless, the compound does show concentration-dependant cytotoxicity (in UVA only) toward a tumor-cell line (Hudson et al., 1989). Therefore it will be necessary to evaluate this in more detail in comparison with the antiviral effect.

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