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Substituted naphthalenones as a new structural class of HIV-1 reverse transcriptase inhibitors

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Summary

A novel substituted naphthalenone (TGG-II-23A) has been found that inhibits HIV-1 infection of CEM-SS cells at concentrations that are not cytotoxic. Time of addition experiments indicate that TGG-II-23A functions at a stage of the HIV-1 life cycle at or near reverse transcription. Cell free assays confirmed that TGG-II-23A inhibits HIV-1 reverse transcriptase. Similar to other non-nucleoside inhibitors, TGG-II-23A was specific for HIV-1 and failed to inhibit the replication of HIV-2. The binding site of TGG-II-23A appears to be in close proximity to that of the TIBO-like inhibitors, since a TIBO-resistant HIV-1 was also resistant to TGG-II-23A treatment. TGG-II-23A is a mixed non-competitive inhibitor that exhibits the same template:primer selectivity as other non-nucleoside inhibitors. TGG-II-23A therefore represents a new structural entry into the TIBO/Nevirapine class of inhibitors of HIV-1 reverse transcriptase.

Naphthalenones; HIV-1; Reverse transcriptase inhibitors

Introduction

A variety of synthetic nucleoside analogs, including AZT, DDC, DDI, and

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D4T have proven effective against human immunodeficiency virus (HIV) replication. These compounds block HIV-1 and HIV-2 infection through the inhibition of the reverse transcriptase (RT) (De Clercq, 1992a). In addition, four classes of non-nucleoside RT inhibitors have been reported that specifically inhibit the RT of HIV-1 and have no effect on HIV-2 infection. They include TIBO (tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-ones and -thiones) (Pauwels et al., 1990; Debyser et al., 1991), BI-RG-587 (dipyridodiazepinone) (Merluzzi, 1990; Wu et al., 1991; Tramontano and Cheng, 1992), L-697,639 and L-697,661 (pyridinones) (Goldman et al., 1991), and BHAP (bis[heteroaryl]piperazine) (Romero et al., 1991). All of these non-nucleoside analogs appear to interact with RT at a similar location, since resistance to any one of these compounds results in various degrees of cross-resistance against all of the compounds (Nunberg et al., 1991; Richman et al., 1991; Mellors et al., 1992; DeVreese et al., 1992; Condra et al., 1992; Larder, 1992; Balzarini et al., 1993). However, other pyrimidine analogs, HEPT (1-[(2-hydroxyethoxy)methyl]-6-phenylthio-thymine) (Baba et al., 1989, 1991) and TSAO [2′,5′-bis-*O*-(tert-butyldimethyl-silyl)-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)]-thymine), were also selectively effective against HIV-1 and TSAO appears to bind to a site on HIV-1 RT not shared with any of the other non-nucleoside analogs (Balzarini et al., 1992a,b, 1993).

Here we report the discovery of a novel substituted naphthalenone, TGG-II-23A, that also possesses a selective anti-HIV-1 activity that results from non-competitive inhibition of the viral RT.

Materials and Methods

Cells and viruses. The CEM-SS and MT-2 human T cell lines, the RF strain of HIV-1, the CBL-20 strain of HIV-2, and the proviral clone pNL4-3 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The HIV-2 strain EHO was a gift from J. McClure. A strain of HIV-1 RF resistant to TIBO derivative R82150 was isolated in our laboratories by serial passage of HIV-1 RF in the presence of increasing concentrations of drug (final concentration 2 μM). The resulting virus (HIV-1 RF-TIBO^R) was >33-fold resistant to TIBO derivative R82150 and contained the previously reported Tyr to Cys change at RT amino acid residue 181 (Nunberg et al., 1991; Richman et al., 1991) and a new mutation Val 108 Ile (unpublished result), but not the Leu 100 Ile substitution (Mellors et al., 1993).

Chemicals and enzymes. The detailed synthesis of TGG-II-23A (1,4-dimethyl-1-[5,5-dimethyl-2-oxazolinyl]-naphthalen-2-one)and its analogs have been previously reported (Meyers and Gant, 1992). The TIBO derivative, R-82150 ((+)-(5*S*)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)-imidazo-(4,5,1-jk)(1,4)-benzodiazepin-2(1H)-thione) was purchased from Pharmatech

International, W. Orange, NJ. OKT4A monoclonal antibody was contributed by Ortho Diagnostics, Raritan, NJ. DDI and the protease inhibitor, L-687,908 (Vacca et al., 1991), were synthesized at Bristol-Myers Squibb.

HIV-1 (NL4-3) RT was expressed in *E. coli*. Briefly, standard PCR methods were used to clone the coding regions for the p66 and p51 components of RT. These sequences were placed under the transcriptional control of the T7 RNA polymerase promoter in plasmids containing either the ColE1 or P15A origins of replication. Expression of heterodimeric RT was achieved by IPTG induction of *E. coli* strain BL21 (DE3) that was co-transformed with both plasmids.

The TIBO-resistant RT containing the Y181C substitution was cloned using similar PCR methods from the resistant isolate, HIV-1 RF-TIBO^R (see above). It was expressed in a single vector system based on the plasmid pRSET A (Invitrogen). The recombinant RTs were purified from *E. coli* lysates as previously described (Condra et al., 1992).

Antiviral assay. The inhibitory effect of compounds on HIV-1 replication was measured by the XTT dye reduction method (Weislow et al., 1989). Briefly, CEM-SS cells (1×10^5 cells/ml) were infected with HIV-1 RF, HIV-2, or HIV-1 RF-TIBO^R and incubated at 37°C in the presence of serial dilutions of compounds. 6 days later, XTT (Polysciences, Warrington, PA) and *N*-methyl-phenazonium methosulfate (PMS) were added to each well and plates were incubated at 37°C for 4 h to allow for XTT formazan production. Cell viability was quantified by light absorbance at 450 nm using a reference wavelength of 650 nm. Data was expressed as a percentage of formazan produced in drug test wells compared to formazan produced in wells of untreated control cells. The EC_{50} was calculated as the concentration of drug that increased the percentage of formazan production in virus-infected cells to 50% of that produced by uninfected cells.

Time of addition assay. CEM-SS cells (5×10^5 cells/ml) were infected with HIV-1 RF at a multiplicity of infection > 1. Following a 1 h adsorption period, cells were washed 3 times and incubated at 37°C. Compound or medium alone was added to duplicate cultures at various times after infection. 48 h after infection culture supernatants were collected and the levels of p24 determined by HIV-1 p24 ELISA (NEN Research Products, Dupont, Wilmington, DE).

Reverse transcriptase assays. Experiments were performed at 37°C using an assay mixture (50 μl) consisting of 50 mM Tris, 125 mM glutamate, 1 mM DTT, 2 mM $MgCl_2$ and 0.04% CHAPS at pH 8.25. For determining IC_{50} concentrations, template:primer and nucleotide components consisted of either 0.8 μg/ml poly(rC):oligo(dG) and 450 nM [8,5′-^{3}H]dGTP, or 0.3 μg/ml poly(rA):oligo(dT) and 125 nM [methyl-5 ^{3}H]dTTP. Reactions were started by addition of nucleotide. After 1 h the reaction was stopped by addition of 50 μl of 20% (w/v) trichloroacetic acid and 4% (w/v) sodium pyrophosphate. The

samples were cooled at 4°C for 10 min before they were harvested onto glass fiber filters using a Skatron cell harvester. Dried filters were counted in an LKB 1205 Betaplate Liquid Scintillation Counter. Values of IC_{50} were calculated by fitting the data from 2 to 4 separate experiments to a logistic sigmoid using the program Ultrafit.

For steady state kinetic studies, the poly(rC):oligo(dG) concentration was 2.5 μg/ml; dGTP was varied from 60 nM to 2500 nM, and the concentration of TGG-II-23A was between 0 and 300 μM. Reactions were started by addition of nucleotide. At the appropriate intervals, a 50 μl aliquot was removed from the reaction mixture and added to 10 μl of 0.5 M EDTA. Acid precipitation and harvesting were as described above. Initial rates were calculated from linear plots of cpm incorporated versus time with < 10% of substrate converted to products. Counting efficiency for nucleotide was determined in the same system to convert cpm to molar concentrations. Kinetic parameters were determined by direct fitting of initial rates to the Michaelis-Menton equation using the program Ultrafit.

Results and Discussion

Screening of compounds in cell culture assays is routinely used to identify novel antivirals. When the substituted naphthalenone, TGG-II-23A, was evaluated for its inhibitory effect on the replication of HIV-1 RF in CEM-SS cells, it showed a 50% reduction in viral growth (EC_{50}) at 27 μM, whereas the 50% cytotoxic concentration (CC_{50}) was 550 μM (Fig. 1). The anti-HIV-1

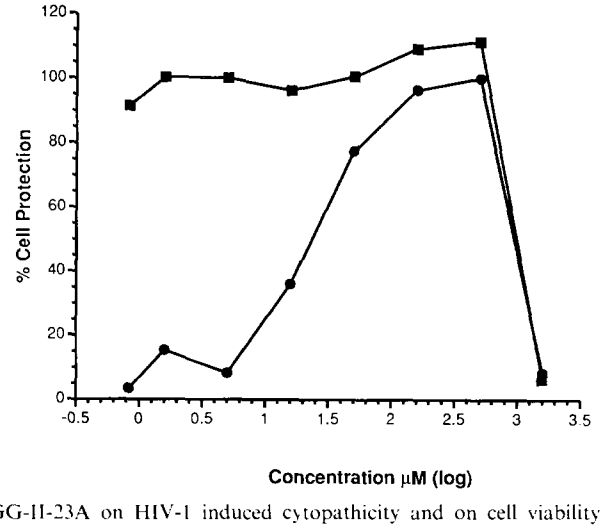
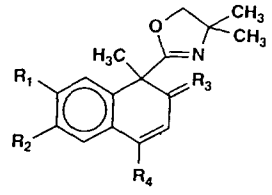


Fig. 1. Effect of TGG-II-23A on HIV-1 induced cytopathicity and on cell viability in CEM-SS cells. Viability of virus infected (circles) and uninfected control CEM-SS cells (squares) was measured by the XTT method.

TABLE 1

Assay of TGG-II-23A analogs against HIV-1



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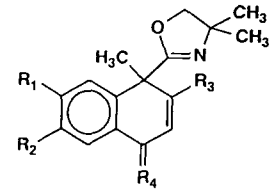

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Compound	Double bond disposition	R_1	R_2	R_3	R_4	EC_{50} (μM)	CC_{50} (μM)
TGG-II-23A	X	H	H	O	H,CH_3	26	550
TGG-II-23B	Y	H	H	H,OCH_3	CH_2	140	476
TGG-II-33A	Y	H	H	H,OCH_3	O	>320	320
TGG-II-39	Y	H	H	H,H	H,H	>434	434
TGG-II-44A	X	CH_3O	CH_3	O	H,CH_3	20	392
TGG-II-44B	Y	CH_3O	CH_3	H,OCH_3	CH_2	>81	81
TGG-II-45A	X	H	H	O	H,$CH(CH_3)_2$	Weak	348
TGG-II-45B	X	H	H	O	H,Ph	>850	850
TGG-II-45C	X	H	H	O	H	40	348

Results represent the average from two experiments.

activity of TGG-II-23A appeared to be specific for HIV since concentrations of TGG-II-23A up to 370 μM failed to inhibit the replication of HSV-1 in Vero cells and Influenza A virus infection of MDCK cells (data not shown).

Eight analogs related to TGG-II-23A were subsequently examined in the HIV-1 XTT cell culture assay. Results (Table 1) showed that two other analogs (TGG-II-44A and TGG-II-45C) had nearly equivalent potency. In comparing TGG-II-23A to TGG-II-44A, it appears that substitution of the aromatic ring has little effect on the anti-HIV activity, although an increase in cytotoxicity was noted. The 4-methyl group of TGG-II-23A seems to be important for antiviral activity because C-4 substituted analogs showed decreased anti-HIV activity with increasing steric divergence (TGG-II-45A and -45B). However, reduction of the methyl group to hydrogen at the C-4 position (TGG-II-45C) failed to significantly alter the antiviral potency. TGG-II-23B, the precursor of TGG-II-23A, also had reduced antiviral activity. This activity may result from the hydrolysis of TGG-II-23B during assay to generate TGG-II-23A. However, further modification of TGG-II-23B (-44B, -33A and -39) resulted in compounds that were inactive in the HIV assay.

To determine the stage of HIV-1 infection inhibited by TGG-II-23A, a time of addition experiment was performed. CEM-SS cells were infected with HIV-1 at a high m.o.i. to facilitate a synchronous viral growth cycle, with TGG-II-

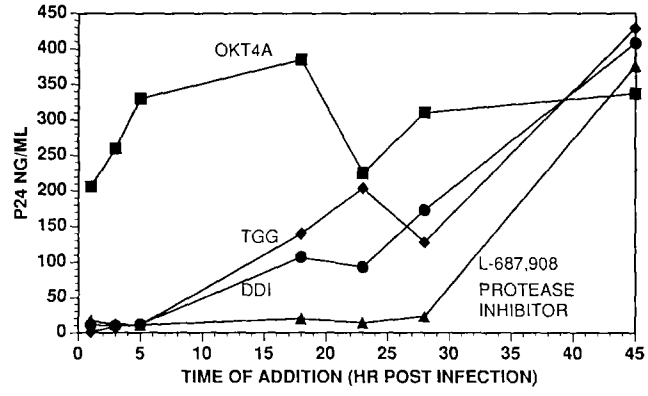

Fig. 2. Time of addition experiment. CEM-SS cells were infected with HIV-1 RF using a multiplicity of infection of >1. Test compounds were added at the times indicated. Viral p24 antigen production was determined at 48 h post-infection.

23A added at different times after infection. Similar studies utilizing this technique have successfully determined the stage of HIV replication blocked by other HIV inhibitors (De Clercq et al., 1992b). Depending on the stage of viral replication inhibited, the addition of the test compound could be delayed without loss of activity. Three known HIV antivirals were included as standards to define early, middle, and late events. Results (Fig. 2) showed that MAb OKT4A (an inhibitor of HIV-1 binding to CD4 receptors) was effective only when added at time 0; DDI (RT inhibitor) could be added to infected cells up to 5 h post-infection, while L-687,908 (protease inhibitor that interferes with viral assembly and maturation) was still effective if added as late as 28 h after infection. Evaluation of TGG-II-A showed that the compound was effective when added up to 5–6 h after infection (Fig. 2). These results indicated that TGG-II-A did not block early or late events, but appeared to inhibit HIV-1 at a time interval similar to that observed with the RT inhibitor DDI.

Since several viral functions occur within this middle time interval, a series of

TABLE 2

The effect of TGG-II-23A and TIBO on wild-type and mutant RTs using different template:primer pairs

Enzyme	Template:primer	IC_{50} (μM)	
		TGG-II-23A	TIBO
HIV-1 (NL4-3)	rC:dG	136	0.58
	rA:dT	1250	2.22
HIV-1 (RF-Y181C)	rC:dG	12800	3.83
	rA:dT	10900	9.83

Values are accurate to $\pm$ 20%.

TABLE 3

The anti-HIV-1 specificity of TGG-II-23A

Compound	EC_{50} (μM)			CC_{50} (μM)
	HIV-1 RF	HIV-2 CBL-20	HIV-1 RF TIBO^R	CEM-SS cells
TGG-II-23A	27	>1000	>1000	550
R82150	<0.03	>100	>100	50
AZT	0.02	<0.01	0.04	>10

The EC_{50} and CC_{50} values were determined on CEM-SS cells using the XTT dye reduction as described in Materials and Methods. EC_{50} of TGG-II-23A = 27 $\pm$ 7.5 μM and CC_{50} = 550 $\pm$ 240 μM. Results of HIV-2 CBL-20 and HIV-1 RF-TIBO^R represent the average from two experiments. The anti-HIV-1 specificity of TGG-II-23A has been confirmed with two other strains of virus: The EC_{50} of the compound against HIV-1 (from pNL4-3) infection of MT-2 cells is 13 μM; however, the compound is inactive against the HIV-2 EHO infection of CEM-SS cells at a concentration of 800 μM.

biochemical and cellular assays were performed to ascertain the precise viral target of TGG-II-23A. TGG-II-23A did not appear to inhibit the regulatory proteins TAT and REV, since no effect was observed on the transactivation of HIV-1 LTR-luciferase by TAT protein (Sodroski et al., 1984, 1985) or the in vitro binding of REV protein to its target RRE-RNA (Heaphy et al., 1990) (data not shown). However, inhibition (IC_{50} = 136 μM) was observed when TGG-II-23A was assayed against purified HIV-1 RT using a poly(rC):oligo(dG) template:primer (Table 2). TGG-II-23A appears to have weak potency compared to the 585 nM IC_{50} obtained with the TIBO-derivative R-81250 included as a control. The R-81250 result is in good agreement with the published IC_{50} of 300 nM against HIV-1 RT using the same template:primer (Debyser et al., 1991).

To determine whether TGG-II-23A shows the same specificity as other non-nucleoside inhibitors, we tested the effect of TGG-II-23A on the acute infection of HIV-2 in CEM-SS cells. Results (Table 3) show that the TGG-II-23A was inactive against both CBL-20 and EHO strains of HIV-2, indicating that they appear to have the same specificity of other non-nucleoside inhibitors. TGG-II-23A was subsequently assayed against the TIBO-resistant isolate, HIV-1 RF-TIBO^R, to ascertain whether TGG-II-23A binds to HIV-1 RT at a site similar to that of the TIBO compounds. Results (Table 3) showed that concentrations of TGG-II-23A up to 1 mM also failed to block the replication of the R-81250 resistant HIV-1 in CEM-SS cells.

The preceding experiments strongly suggest that TGG-II-23A is a new member of the HIV-1 specific non-nucleoside class of HIV RT inhibitors but with less potency. The most convincing evidence is that a mutant RT derived from HIV-1 RF-TIBO^R showed >90-fold reduction in sensitivity to TGG-II-23A (Table 2). In fact, compared to the wild-type enzyme, the mutant RT is even more resistant to TGG-II-23A than to TIBO, which showed only a 7-fold level of resistance. Since the TIBO result is in line with previous reports

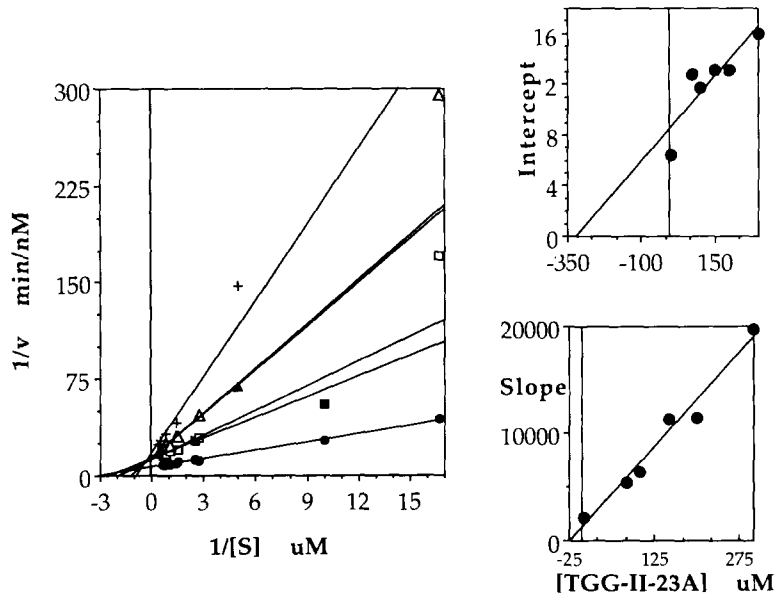

Fig. 3. A double reciprocal plot of inhibition of RT by TGG-II-23A using saturating concentrations of poly(rC):oligo(dG) template:primer with dGTP as the variable substrate. Concentrations of TGG-II-23A were 0 (solid circle), 75 (solid square), 100 (open square), 150 (solid triangle), 200 (open triangle), and 300 (+) μM.

(Richman et al., 1991; Sardana et al., 1992), this result suggests that an interaction with Tyr 181 in RT is very important for inhibition by TGG-II-23A. In addition, this group of inhibitors has also been shown to possess greater potency when the enzyme is assayed with poly(rC):oligo(dG) template:primer compared to a poly(rA):oligo(dT) template:primer (Debyser et al., 1991; Tramontano and Cheng, 1992; Goldman et al., 1991). This enhancement of activity was also observed with TGG-II-23A (Table 2).

More evidence of the similarity of TGG-II-23A and HIV-1 specific non-nucleoside type inhibitors is provided by steady state kinetic studies. Each of these compounds binds to both the free enzyme and enzyme-substrate complex. While TIBO (Debyser et al., 1991) and Nevirapine (Merluzzi et al., 1990; Kopp et al., 1991) show inhibition that is non-competitive with respect to dGTP, TGG-II-23A exhibits mixed inhibition (Fig. 3). Although the mechanisms are very similar, this difference implies that TIBO and Nevirapine bind equally well to the enzyme and enzyme-substrate complexes, but TGG-II-23A binds less tightly to the enzyme-substrate complex than to free enzyme. The inhibition constants, determined from replots of slopes and intercepts from Fig. 3, reflect this change in affinity. TGG-II-23A interacts with the RT:template-primer complex with a K_i of 23 μM, with the K_i increasing to 320 μM for the ternary complex of RT:template-primer:dGTP. The K_i of 23 μM is within the range of

concentrations where TGG-II-23A is active against HIV in cell culture. The higher IC_{50} from in vitro testing against the enzyme is caused by partial saturation of the enzyme with substrates in this assay.

Taken together the data indicate that TGG-II-23 represents another structural entry into the class of non-nucleoside RT inhibitors, and its binding sites on HIV-1 RT may be similar to that of TIBO derivatives.

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