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COMPETITIVE INHIBITION OF HIV-1 PROTEASE BY WARFARIN DERIVATIVES

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Summary: The oral anticoagulant warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)-benzopyran-2-one) is a structurally novel low micromolar competitive inhibitor of HIV-1 protease in vitro. It was recently reported that warfarin inhibits HIV-1 infection in U-1 monocytes and viral production in ACH-2 lymphocytes (Bourinbaiar, A. S. et al., (1993) *AIDS* **7**, 129-130.). Our results demonstrate that warfarin and a series of structurally related analogs inhibit the viral protease, the most potent analog having an IC_{50} = 1.9 μM. Kinetic analysis reveals inhibition by warfarin occurs in a competitive manner, with K_i = 3.3 μM. While it is unclear whether the cellular inhibition previously reported is due to inhibition of HIV-1 protease, the warfarin analogs are a novel class of nonpeptide HIV-1 protease inhibitors.

Warfarin, 4-hydroxy-3-(3-oxo-1-phenylbutyl)-benzopyran-2-one, is one of several 4-hydroxy-coumarin anticoagulants widely used in the treatment of thrombo-embolic disease (1). The anticoagulant properties of warfarin are due to its inhibition of vitamin K reductase and vitamin K 2,3-epoxide reductase (2). The reduced form of the vitamin is an essential cofactor for posttranslational modification of clotting Factors II, VII, IX and X and anticoagulant protein C (3).

Bourinbaiar and coworkers recently reported that warfarin and other coumarin derivatives inhibit cell-free and cell-mediated HIV-1 viral replication in MOLT-4 lymphocytes in the absence of cellular toxicity (4, 5). The authors proposed that the compounds may act by inhibiting host cell proteases. The studies presented here examine inhibition of the viral protease in vitro by this compound and by a series of structural analogs. The retroviral protease of human immunodeficiency virus type 1 (HIV-1) is responsible for the processing of viral gag and gag/pol polyproteins into active viral enzymes and structural proteins; the proteolysis is essential for maturation of infectious virions (6, 7). A point mutation in the HIV-1 genome that codes for an inactive protease was shown to result in noninfectious virions (8), establishing the effectiveness of HIV-1 protease as a target for AIDS chemotherapy.

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Nine warfarin analogs were found to have inhibitory activity in vitro ($IC_{50} < 40$ μM), and kinetic analysis revealed that warfarin inhibits in a competitive manner. While highly potent peptide and peptidomimetic HIV-1 protease inhibitors have been developed (for reviews, see (9-12)), they generally have the poor therapeutic characteristics of low oral bioavailability and rapid clearance (13, 14). Warfarin is more than 95% absorbed after oral administration and its pharmacology has been well characterized (1, 15). This small nonpeptide inhibitor, though possessing weak potency relative to the peptide inhibitors, is a promising compound for medicinal chemistry efforts in the development of a nonpeptide HIV-1 protease inhibitor with anti-HIV activity and good pharmacological characteristics.

MATERIALS AND METHODS

Materials

Recombinant HIV-1 protease (>96% purity) and HIV protease substrate III (the undecapeptide H-His-Lys-Ala-Arg-Val-Leu-(*p*-Nitrophenylalanine)-Glu-Ala-Norleucine-Ser-NH_2, >97% purity) were purchased from Bachem Bioscience Inc., King of Prussia, PA, USA.

Methods

Protease Inhibition Assays. For determination of IC_{50} values, HIV-1 protease, 5.0 nM final, was added to a solution containing inhibitor, 40 μM substrate III and 1.0% Me_2SO in assay buffer: 1.0 mM dithiothreitol, 0.1% polyethylene glycol (m.w. 8000), 80 mM sodium acetate, 160 mM NaCl, 1.0 mM EDTA, pH 4.7 at 37°C (total volume, 100 μl). Polyethylene glycol was used in the assay in place of glycerol since the former has been reported to be a more effective stabilizing agent of the protease (16). The final inhibitor concentrations used were 0, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 μM. The solution was mixed, incubated for 5 minutes and the reaction quenched by addition of trifluoroacetic acid, 2% final. The (leu-p-nitro-phe) bond of the substrate is cleaved by the enzyme and substrate and products were separated by reverse-phase HPLC. Absorbance was measured at 220 nm, peak areas determined, and % conversion to product calculated.

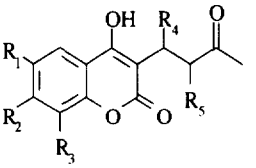
Determination of Ki was performed in a similar manner, with the following modifications. The substrate concentrations used were 12.5, 16.7, 25, 50, 100 μM. In order to measure accurate initial velocities, the incubation time was varied with substrate concentration as follows: 12.5 μM, 1.5 min.; 16.7 μM, 1.75 min.; 25 μM, 2.0 min.; 50 μM, 4.0 min.; 100 μM, 6.0 min. This resulted in consistent conversion values (products / substrate + products) of 25-30% in the absence of inhibitor.

Estimation of Maximal Inhibitor Solubility. Each inhibitor was diluted from a 10 mM stock in Me_2SO into assay buffer to final concentrations of 10-100 μM. Ultraviolet absorbance of the inhibitor was measured at a wavelength at which its extinction coefficient is less than 20 mM^{-1} cm^{-1}. The solutions were then centrifuged (tabletop centrifuge) for 5 minutes and the absorbance of the supernatant measured. For inhibitors that were completely soluble up to 100 μM, absorbance before and after centrifugation was the same, and there was a linear relationship between absorbance and inhibitor concentration. For partially soluble inhibitors, the maximal solubility was estimated as the lowest concentration at which there was a significant decline (>10 %) in absorbance due to centrifugation.

RESULTS AND DISCUSSION

The inhibition of HIV-1 protease by warfarin derivatives was measured, IC_{50} values calculated and the results summarized in Table 1. An IC_{50} determination was performed twice on each active compound, and all the compounds assayed were racemic mixtures. Nine of the thirteen compounds assayed were found to be active (IC_{50}<40 μM). The maximal solubility of each compound was also determined. While several compounds were found to be only partially

Table 1. Inhibitory Activity and Solubility of Warfarin Analogs



Number	R1	R2	R3	R4	R5	IC50 Avg., μM	Est. Max. Sol., μM
Warfarin	H	H	H	Ph	H	18, n=2	>100
W1	H	CH_3	CH_3	Ph	H	1.9, n=2	60
W2	CH_3	H	CH_3	Ph	H	> 200, n=1	60
W3	CH_3	H	H	*p*-(CH_3O)-Ph	H	> 200, n=1	> 100
W4	CH_3	H	H	*p*-(Cl)-Ph	H	35, n=2	70
W5	H	H	$CH(CH_3)_2$	*p*-(CH_3O)-Ph	H	25, n=2	> 100
W6	H	H	CH_3	H	Ph	22, n=2	90
W7	CH_3	H	H	H	Ph	160, n=2	70
W8	H	CH_3	H	H	Ph	> 200, n=2	< 20
W9	H	H	Ph	H	Ph	15, n=2	< 20
W10	H	H	CH_3	H	*p*-(Cl)-Ph	25, n=2	60
W11	H	H	CH_3	H	*p*-(CH_3O)-Ph	36, n=2	20
W12	H	H	H	H	*o*-(CH_3O)-Ph	9.1, n=2	< 20

soluble in their range of partial inhibition, four low μM inhibitors are highly soluble (warfarin, W1, W5 and W6).

Warfarin is a low micromolar inhibitor of HIV-1 protease. Substitution on the benzopyran-2-one ring of warfarin greatly modulates inhibitory activity, since the IC_{50} of 6,8-dimethyl-warfarin (W2) is >200 μM while 7,8-dimethyl-warfarin (W1) is ten-fold more potent than the parent compound. This is further exemplified by comparison of W3 to W5, where a single replacement of the 6-methyl on the benzopyran-2-one ring with an 8-isopropyl group changes the compound from IC_{50}>200 μM to IC_{50}=25 μM. The only 6-methyl warfarin derivative in the series found to have significant inhibitory activity is W4.

The inhibitory activity of several analogs with a 2-phenyl (in place of 1-phenyl) substituted 3-oxo-butyl group were also studied. The potency of these compounds is also dependent on substitution on the benzopyran-2-one ring, where the 8-methyl (W6) is most active and the 6-methyl (W7) and 7-methyl (W8) exhibit little or no inhibition. Replacement of the 8-methyl with an 8-phenyl (W9) on the benzopyran-2-one ring does not measurable affect inhibitory activity. The activity of W6 (4-hydroxy-8-methyl-3-(3-oxo-2-phenylbutyl)-benzopyran-2-one) is also not measurably changed by substitution of the 2-phenyl group with a 2-(*p*-chloro-phenyl), W10, or with a 2-(*p*-methoxy-phenyl), W11. W12, which is unsubstituted on the 4-hydroxy-benzopyran-2-one ring and with a 2-(*o*-methoxy-phenyl) substituted 3-oxo-butyl group, is a potent inhibitor. In summary, the structure-activity relationship demonstrates that inhibition is greatly dependent on substitution at the 6, 7 and 8 positions on the 4-hydroxy-

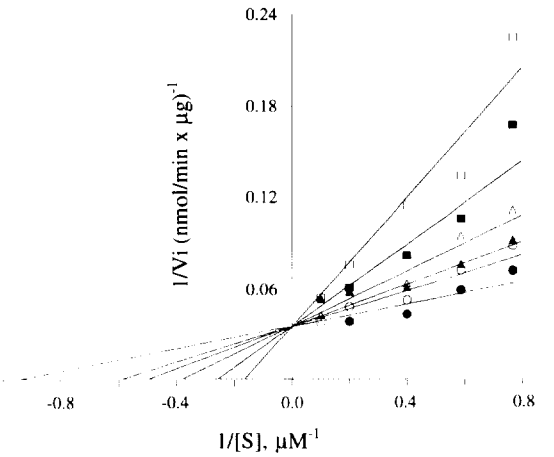


FIGURE 1. Double reciprocal plot for inhibition of HIV-1 protease by C1. Inhibitor concentration, µM: (□) 16, (■) 9.0, (Δ) 5.0, (▲) 3.0, (○) 2.0, (●) 0.0. The data are shown fit to a competitive inhibition model by a nonlinear least-squares algorithm, with K_m = 11+/-2.2 (std. error) µM, k_{cat} = 10.6+/-0.57 sec^{-1} and K_i = 3.3+/-0.76 µM.

benzopyran-2-one ring where 8-methyl, 8-isopropyl and 8-phenyl increase potency. It also demonstrates that inhibitory activity is not significantly dependent on the phenyl substituent on the 3-oxobutyl group, or on its position. This structure-activity relationship was generated in the absence of any synthetic chemistry efforts, and provides information on a series of small nonpeptide HIV-1 protease inhibitors.

The mode of warfarin inhibition of HIV-1 protease was examined, and the results shown as a double reciprocal plot in Figure 1. The data were initially fit to a mixed noncompetitive inhibition model, described by the equation $V_i = V_{max}[S]/([S](1 + [I]/K_{i2}) + K_m(1 + [I]/K_{i1}))$, using a nonlinear least-squares algorithm. The value determined for K_{i2} was insignificant, indicating no inhibition of V_{max} by this inhibitor. Warfarin is therefore a competitive inhibitor, and the data in Figure 1 are shown fit to the equation for competitive inhibition ($V_i = V_{max}[S]/([S] + K_m(1 + [I]/K_i))$, yielding a K_i = 3.3+/-0.76 µM. The K_m values was determined to be 11+/-2.2 (std. error) µM, consistent with that determined by Friedman and coworkers (17) using the same substrate. The k_{cat} was calculated to be 10.6+/-0.57 sec^{-1}, which is somewhat lower than values reported by Richards and coworkers (20 sec^{-1}) at the same pH using a series of similar substrates. This is expected since the previous experiments were performed at a higher [NaCl] of 1.0 M, which is known to increase the protease activity. This kinetic analysis reveals that warfarin inhibits the protease in a competitive manner, and thus interacts directly at the enzyme active site.

Other 4-hydroxy-benzopyran-2-ones have been found by our group to inhibit the protease in vitro with similar potency (18). Computer modeling of the structure of protease-(4-hydroxy-benzopyran-2-one) complexes along with synthesis and inhibition studies of analogs has also been performed (19). In work independent from our group, P. Tomich and K. Watenpaugh recently reported in vitro HIV-1 protease inhibition by warfarin analogs (20). There are two

advantages to warfarin derivatives over other nonpeptide compounds in the development of HIV-1 protease inhibitors for AIDS chemotherapy. The first is that the extensive study of the pharmacology of warfarin and its analogs as widely used oral anticoagulants (1, 15) would facilitate any future clinical studies of compounds from this series as antiviral agents. Secondly, warfarin itself has been shown to possess anti-HIV-1 activity in cell assays in the absence of toxicity (4, 5). Though the mechanism has not yet been elucidated, the antiviral activity measured in cells may be due to inhibition of HIV-1 protease and thus more potent in vitro inhibitors may lead to better cellular inhibitory activity.

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