

Bile pigments as HIV-1 protease inhibitors and their effects on HIV-1 viral maturation and infectivity *in vitro*

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Using recently developed molecular-shape description algorithms, we searched the Available Chemical Directory for known compounds similar in shape to the potent HIV-1 protease inhibitor Merck L-700,417; 15 compounds most similar in shape to the inhibitor were selected for testing *in vitro*. Four of these inhibited the protease at 100 μM or less and the most active of the four were the naturally occurring pigments biliverdin and bilirubin. Biliverdin and bilirubin inhibited recombinant HIV-1 protease *in vitro* at pH 7.8 with K_i values of approx. 1 μM , and also inhibited HIV-2 and simian immunodeficiency virus proteases. The related pyrrolic pigments stercobilin, urobilin, biliverdin dimethyl ester and xanthobilirubic acid showed similar

inhibitory activity at low micromolar concentrations. Biliverdin, bilirubin and xanthobilirubic acid did not inhibit viral poly-protein processing in cultured cells, but they reduced viral infectivity significantly. At 100 μM , xanthobilirubic acid affected viral assembly, resulting in a 50% decrease in the generation of infectious particles. In contrast, at the same concentrations biliverdin and bilirubin exerted little or no effect on viral assembly but blocked infection of HeLaT4 cells by 50%. These results suggest that bile pigments might be a new class of potential lead compounds for developing protease inhibitors and they raise the question of whether hyperbilirubinaemia can influence the course of HIV infection.

INTRODUCTION

Extensive efforts are currently being spent on the discovery and development of compounds that block replication of the human immunodeficiency virus (HIV) and that can be used for treatment of AIDS [1]. One vulnerable target is the virally encoded HIV protease. HIV proteases are dimeric aspartyl enzymes that are essential for viral maturation. Their inactivation results in the formation of immature, non-infectious, viral particles [2–4]. Since the determination of the three-dimensional structure of HIV protease and the elucidation of its catalytic mechanism, many mechanism-based and structure-based inhibitors have been designed [5–11]. These include extensively modified peptides [12], symmetrical inhibitors [13] and cyclic urea analogues [14]. However, extended exposure of the virus to protease inhibitors in tissue culture or in clinical trials has led to the evolution of resistant viral strains and a decreased efficacy of inhibition [15] and there is still a pressing need for drugs that can be used in combination with existing protease inhibitors to augment their antiviral effects.

An important determinant in the binding of a drug to its target is its molecular shape. Computer programs have been developed that use interatomic connectivities and intramolecular distances to generate 'shape profiles' that can be used to compare the shapes of different molecules rapidly [16]. Using the potent peptide-based HIV protease inhibitor Merck L-700,417 [17] (**1**, Figure 1) as a template, we used these programs to search a large database of available chemicals for novel lead compounds with potential HIV protease activity. Of the 15 most promising compounds generated by this search, two showed significant biological activity in preliminary tests. Surprisingly, both of these were bile pigments: biliverdin and bilirubin (**2** and **3**, Figure 1), which are naturally occurring tetrapyrroles biosynthesized in

humans at a rate of about 300 mg/day by the normal metabolism of haem. This unexpected finding, coupled with recent reports that biliverdin has antiviral activity [18,19], prompted us to examine in more detail the antiproteolytic and antiviral properties of these common endogenous pigments and some related compounds. Our findings are documented here. They reveal that both biliverdin and bilirubin competitively inhibit human and simian immunodeficiency virus (SIV) proteases at low micromolar concentrations *in vitro*. When tested in cell culture the pigments did not block HIV viral maturation but did block the cellular entry of infectious viral particles at micromolar concentrations.

EXPERIMENTAL

Computational methods

The reference compound used for the shape similarity search was the Merck protease inhibitor L-700,417 [17]. Coordinates for this were extracted from the Brookhaven Protein Database (reference code 4PHV) [20]. The compound database used for the similarity search [16] was the FCD-3D database (now called the Available Chemical Directory) (Version 89.2 from Molecular Design Ltd. Information Systems, San Leandro, CA, U.S.A.). This database contained 50 000 compounds. The computer program CONCORD (Tripos Associates, St. Louis, MO, U.S.A.) [21] was used to produce the molecular conformations that were used in constructing distance–shape profiles. Searches were conducted on a Silicon Graphics Iris workstation (Silicon Graphics Computer Systems, Mountain View, CA, U.S.A.). Both covalent and three-dimensional similarities were considered. Similarity searches were rapid, requiring only a few minutes of computer time.

Abbreviation used: SIV, simian immunodeficiency virus.

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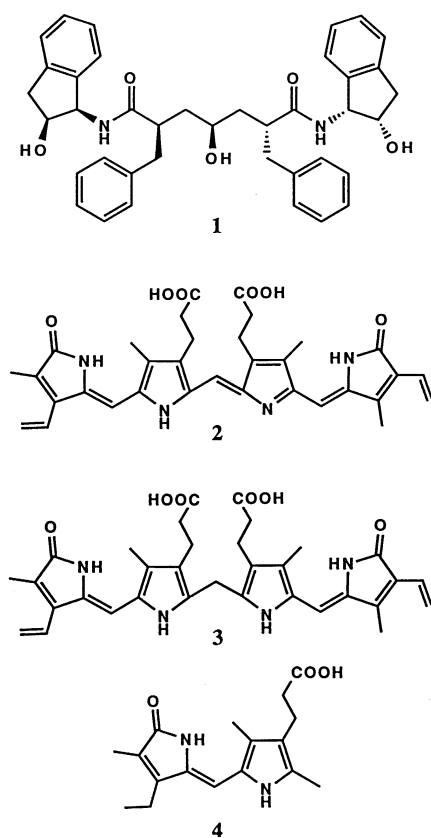


Figure 1 Constitutional structures of L-700,417 inhibitor (1), biliverdin (2), bilirubin (3) and xanthobilirubic acid (4)

Purification of bile pigments

Bilirubin (Porphyrin Products, Logan, UT, U.S.A.) was purified and crystallized as previously described [22]. Biliverdin and biliverdin hydrochloride from different vendors were found by TLC to contain substantial impurities, as previously reported [23]. Commercial biliverdin was therefore purified by a modification of the method described by McDonagh [23,24]. Crude biliverdin (115 mg; Sigma Chemical Company, St. Louis, MO, U.S.A.), dissolved in chloroform/methanol (1:1, v/v) was adsorbed on a silica gel flash chromatography column [22 mm external diam. \times 200 mm; Merck, grade 60 (Aldrich Chemical Company, St. Louis, MO, U.S.A.)] prepared in chloroform/methanol (9:1, v/v). Pigments were eluted with 200 ml of chloroform/methanol (9:1, v/v), followed by 200 ml chloroform/methanol/acetic acid (9:1:0.1, by vol) and finally, chloroform/methanol/acetic acid (9:2:0.1, by vol). Yellow-green or green eluates were discarded and the main mobile blue-green fraction was collected and evaporated to dryness under decreased pressure. The residue was dissolved in 4 ml of 0.1 M sodium hydroxide and the solution filtered through a glass microfibre filter, which was then washed with a small volume of 0.1 M sodium hydroxide; the combined filtrate and washings were treated with acetic acid (three or four drops). The green precipitate was collected by centrifugation, washed three times with water and freeze-dried to give 20 mg of biliverdin (λ_{\max} 377 nm, ϵ 51 500 M⁻¹·cm⁻¹ and λ_{\max} 666 nm, ϵ 15 200 M⁻¹·cm⁻¹ in methanol). The isomeric composition of the product, determined by HPLC [25] after reduction with sodium borohydride to bilirubin [23], was 79% IX α , 16% XIII α and 5% III α .

Stercobilin, urobilin and biliverdin dimethyl ester (Porphyrin Products, Logan, UT, U.S.A.) were used as supplied without further purification or analysis. Xanthobilirubic acid was a gift from Dr. D. A. Lightner (University of Nevada, Reno, NV, U.S.A.).

Expression and purification of HIV and SIV proteases

HIV-1, HIV-2 and SIV proteases were expressed and purified to homogeneity as previously described [26–28]. Concentrations of active HIV-1 and HIV-2 proteases were determined by active-site titration with the peptidomimetic inhibitor U-85548 (a gift from Dr. A. Tomasselli, Upjohn Company, Kalamazoo, MI, U.S.A.), Val-Ser-Gln-Asn-Leu- Ψ -[CH(OH)CH₂]-Val-Ile-Val [29].

Assay *in vitro* of HIV and SIV protease inhibition

Fluorescence measurements were performed on a Fluoroskan II (LabSystems, Marlboro, MA, U.S.A.). HIV-1, HIV-2 and SIV proteases were assayed against the fluorescent substrate amino-benzoyl-Thr-Ile-Nle-Phe(*p*-NO₂)-Gln-Arg-NH₂ (a gift from Dr. Jorge P. Li, Sandoz Agro, Palo Alto, CA, U.S.A.). Stock solutions of substrate (1–10 mM) and inhibitor (50 μ M to 1 mM) were freshly prepared in DMSO. Inhibitor was added to assay buffer [50 mM Hepes (pH 7.8)/1 mM dithiothreitol/0.2 M NaCl/20% (v/v) glycerol/0.1% CHAPS] containing the appropriate protease enzyme (0.4–0.8 ng), and the mixture was preincubated for 1 min at 37 °C before initiating the reaction by the addition of substrate. The final concentration of DMSO in the assay was 5% (v/v). Baseline measurements were performed with 5% DMSO in the absence of inhibitor. A freshly prepared 10 μ M solution of bilirubin in the assay buffer at pH 5.5 was optically clear (λ_{\max} 442 nm, shoulder 490 nm) and showed no change in absorbance when kept at room temperature in the dark for 25 min. However, when the solution was shaken vigorously for a few seconds the absorbance decreased by 6% at 442 nm and increased slightly on the long-wavelength edge of the band, indicating aggregation of pigment. At pH 7.8 the absorption spectrum of a 10 μ M solution of bilirubin in the assay buffer showed λ_{\max} 448 nm, with no long-wavelength shoulder, and was stable for at least 25 min at room temperature in the dark. Other control experiments indicated that the solubility of biliverdin in the assay buffer is 10 μ M or less at pH 5.5, but more than 100 μ M at pH 7.8.

Inhibition constants (K_i values) were calculated by two different methods. In method 1, K_i values were estimated by applying the following equation:

$$IC_{50} = (1 + [S]/K_m)K_i$$

where IC_{50} is the concentration of inhibitor required to reduce protease activity by 50%, $[S]$ is the substrate concentration used in the assay and K_m is the Michaelis–Menten constant of the enzyme at the recorded pH. In method 2, K_i values were calculated by fitting the initial enzyme rates to the Michaelis–Menten equation and kinetic constants were calculated with a nonlinear regression program (KaleidaGraph 2.0; Synergy Software, Reading, PA, U.S.A.).

Viral polyprotein processing assay

A stable cell line (CH-1) has been established that produces all of the HIV-1 HXB2 proteins with the exception of envelope gp160 [30]. This cell line was used to determine the effects of biliverdin and bilirubin on viral polyprotein maturation. Solutions of biliverdin and bilirubin were prepared in DME-H 21 medium

(University of California San Francisco Cell Culture Facility) containing penicillin (100 i.u./ml), streptomycin sulphate (100 mg/ml), xanthine (0.25 mg/ml), hypoxanthine (14 μ g/ml), mycophenolic acid (25 μ g/ml), 2% (v/v) fetal bovine serum (Gemini Bioproducts, Calabasas, CA, U.S.A.) and 0.5% (v/v) DMSO. Cells were incubated with the respective bile pigment for 24 h at 37 °C. Inhibition of polyprotein processing was measured by monitoring changes in the ratio of mature p24 and precursor p55 proteins encapsidated. Viral capsid purification and analyses were performed as described previously [27,31].

Infectivity assays

Viral capsids were produced by transfection of human kidney 293-T cells by the calcium phosphate procedure [32] with the following modifications. HIV-gpt DNA (10 μ g) [31] was used per 10 cm dish of approx. 30% confluent cells, and 60 μ g of HXB2-env [31] was included with the HIV-gpt to generate infectious particles. Inhibitor was added to the DME-H 21 culture medium (see above) in DMSO (final concentration 0.0005%, v/v) either 14 h after transfection or after the generation of infectious particles. In the former, the supernatant was collected 48 h after transfection and used to infect HeLaT4 cells. Viral titres were determined by drug selection on monolayers of HeLaT4 cells as described previously [31].

RESULTS

Lead discovery

One hundred compounds whose structures were most similar to the reference compound Merck L-700,417, based on both atomic connectivity (two-dimensional) and intramolecular distance (three-dimensional), were selected from the database. These compounds were reviewed for chemical stability, price, availability, potential toxicity and for molecular features such as hydrogen bonding and hydrophobicity. Clearly unsuitable compounds were rejected. In this way the original list was reduced to 15 compounds (Table 1), which were selected for testing *in vitro*.

Of these 15, only four (biliverdin, bilirubin, secalononic acid D and alphazurin A) inhibited HIV-1 protease with an IC_{50} of less than 100 μ M. In the initial screening assay, performed at pH 5.5 [8,33], biliverdin showed the most inhibitory activity (IC_{50} 3 μ M), with bilirubin a close second (IC_{50} 15 μ M). However, assays at pH 5.5 showed poor reproducibility, probably because of the low solubility of biliverdin and bilirubin at that pH and the tendency of the pigments to form metastable supersaturated solutions.

Kinetic parameters

K_i values for the bile pigments were calculated from assays performed at pH 7.8, yielding the values shown in Figure 2(a). Both bilirubin and biliverdin inhibited SIV protease and the two HIV protease enzymes with K_i values of less than 5 μ M. Bilirubin was marginally more effective than biliverdin. The effect of pH on the K_i values for biliverdin and bilirubin is shown in Figure 2(b). K_i values decreased significantly for both biliverdin and bilirubin when the pH was increased from 6.4 to 7.4, and decreased marginally from pH 7.4 to 7.8. A Dixon plot for bilirubin is shown in Figure 2(c) in which the intersecting family of linear curves is consistent with partial competitive inhibition [8,33], with a K_i of 2.0 ± 2 μ M. A similar mode of inhibition was observed for biliverdin.

Open-chain tetrapyrroles other than biliverdin and bilirubin also inhibited HIV proteolytic activity at low micromolar concentrations (Table 2). Of these, it should be noted that the commercial stercobilin and urobilin preparations used were probably mixtures of diastereoisomers. Interestingly, xanthobilirubic acid (Figure 1), which is more soluble than biliverdin and bilirubin and whose structure is roughly one-half of a bilirubin molecule, was also a competitive inhibitor of the HIV proteases with a K_i of 5 μ M (calculated by method 2, above).

Bile pigment effects on viral maturation and infectivity

The effects of biliverdin, bilirubin and xanthobilirubic acid on protease activity in intact cells were studied by measuring the relative production of mature p24 and precursor p55 proteins in a CH-1 cell line. For replication, these cells required at least 2%

Table 1 Compounds most similar to L-700,417, based on connectivity and intramolecular distances, and measured IC_{50} values for inhibition of HIV-1 protease *in vitro*

Similarity searches were done by computer as described in the Experimental section. Protease activity was determined fluorimetrically with a fluorescent substrate. Inhibitor was added to assay buffer (pH 5.5) containing the protease; after a 1 min preincubation at 37 °C the reaction was initiated by the addition of substrate. For details see the Experimental section.

Compound	Rank on distance-based list	Rank on connectivity-based list	IC_{50} (μ M)*
Isomaltose octa-acetate	1	8	Inactive
Glyceryl tris(4-trimellitrate anhydride)	—	1	Inactive
Pentamethylenebis(triphenylphosphonium bromide)	2	—	Inactive
Alphazurin A	3	2	100
Secalononic acid D	—	3	38
4'-Methylumbelliferyl 2,3,5-tri- <i>O</i> -benzoyl- α -L-arabinofuranoside	4	4	Inactive
Rutin	5	—	Inactive
2',3',5'-Tri- <i>O</i> -benzoylguanosine	6	—	Inactive
Neohesperidin dihydrochalcone	7	—	Inactive
Bilirubin	—	7	15†
Tetraethyl-1,2-bis(2-amino-5-methylphenoxy)ethane- <i>N,N,N,N'</i> -tetra-acetate	8	5	Inactive
Dihydroergotamine methanesulphonate salt	9	—	Inactive
Didansyl-L-histidine	—	9	Inactive
Ergotamine maleate	10	—	Inactive
Biliverdin dihydrochloride	11	6	3†

* Inactive compounds showed no inhibition at 100 μ M.

† Determined with unpurified compounds from Sigma.

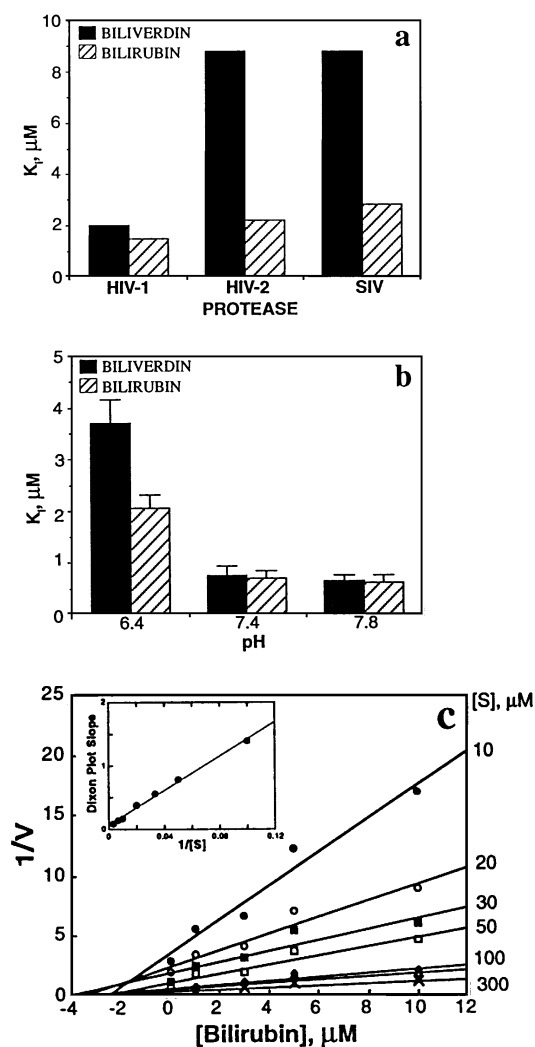


Figure 2 Inhibitory effects of biliverdin and bilirubin on HIV and SIV proteases

(a) Inhibition of HIV and SIV proteases by biliverdin and bilirubin *in vitro* at pH 7.8. K_i values were calculated as described for method 1 in the Experimental section. All results represent the average of two independent experiments performed in triplicate. (b) Effects of pH on the K_i values for biliverdin and bilirubin against HIV-1. K_i values were calculated as described for method 2 in the Experimental section. All results represent means \pm S.D. for at least three separate experiments. (c) Dixon plot of the inhibition of HIV-1 protease peptide hydrolysis by bilirubin at pH 7.4: [S] denotes the substrate concentration used in the assay. A plot (inset) of the slopes from the Dixon plot against the reciprocal of the substrate concentration indicates a K_i of $2.0 \pm 0.2 \mu\text{M}$.

(v/v) fetal bovine serum in the medium, resulting in an albumin concentration of $64 \mu\text{M}$. Neither biliverdin nor bilirubin had any detectable effect on polyprotein processing when added to the medium at concentrations up to $50 \mu\text{M}$. Higher concentrations seemed to be toxic to the cells used in this study (results not shown). The lower toxicity of xanthobilirubic acid permitted analysis at concentrations up to $300 \mu\text{M}$. Once again, no significant effects on polyprotein processing were observed, suggesting that this type of inhibitor does not prevent proteolytic cleavage of the viral polyprotein at the concentrations used.

To examine the effects of biliverdin, bilirubin and xanthobilirubic acid on viral infectivity, infectious viral particles were generated by co-transfecting the wild type HIV-gpt plasmid with

Table 2 Inhibitory effects of bile pigments and derivatives on HIV-1 protease activity *in vitro*

Protease activity was determined fluorimetrically with a fluorescent substrate. Inhibitor was added to assay buffer (pH 7.8) containing the protease and after a 1 min preincubation at 37°C the reaction was initiated by the addition of substrate. For details see the Experimental section. K_i values were calculated by method 1 in the Experimental section. Results represent the averages of at least two independent experiments, each performed in duplicate.

Compound	K_i (μM)
Biliverdin	1.0
Bilirubin	0.8
Stercobilin	4.0
Urobilin	6.0
Biliverdin dimethyl ester	1.8
Xanthobilirubic acid	2.5

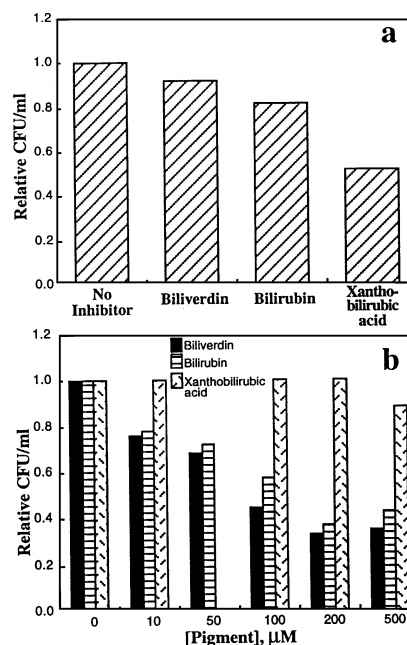


Figure 3 Effects of biliverdin, bilirubin and xanthobilirubic acid on HIV-1 viral infectivity

Virions were produced by co-transfection of 293-T cells with HIV-gpt and HIV-env DNAs. (a) Inhibitory effects of the pigments on viral assembly. Pigment was added 14 h after transfection, and culture supernatants were collected at 48 h after transfection and titrated on HeLaT4 cells to determine the number of infectious units. The number of infectious particles is expressed as colony-forming units per ml of supernatant. (b) Inhibitory effects of the pigments on viral entry. Culture supernatants were collected 48 h after transfection before the addition of pigment to the viral capsids and envelope protein. After 1 h the infectious units were titrated on HeLaT4 cells.

the HIV-1 gp160-expressing plasmid, HXB2-env, into 293-T cells. Pigments were added 14 h after transfection and samples analysed 48 h later to yield the results shown in Figure 3(a). Biliverdin and bilirubin, at $100 \mu\text{M}$, exhibited little effect on viral infectivity (8% and 18% reduction in viral titre respectively). In contrast, the number of infectious particles was halved at this concentration of xanthobilirubic acid. To determine whether the pigments affected viral entry into HeLaT4 cells, infectious viral

particles were generated and collected 48 h after transfection. These viral particles were then incubated with inhibitor for 1 h before infecting HeLaT4 cells. A dose-dependent reduction in infectivity was observed with biliverdin and bilirubin, whereas xanthobilirubic acid exhibited little or no significant effect on viral infectivity up to a concentration of 500 μM (Figure 3b).

DISCUSSION

Computerized searching of a database of 50000 commercially available chemicals for compounds similar in shape to the potent HIV protease inhibitor Merck L700,417 yielded two naturally occurring compounds, biliverdin and bilirubin [34], that inhibited HIV protease *in vitro* with IC_{50} values of 15 μM or less. Biliverdin has previously been reported to inhibit replication of human herpes virus type 6 and to inhibit the infectivity of HIV-1 *in vitro* at low micromolar concentrations [18,19]. The commercial biliverdin preparations used in those studies might have been of low purity [23,24]. Bilirubin is known to be cytotoxic and to inhibit many enzymes *in vitro* [24,35] and is neurotoxic *in vivo* when it gains access to the central nervous system [34,35]. Recent studies indicate that it might impair osteoblast proliferation in patients with chronic cholestatic liver disease [36] and that it can function as an endogenous antioxidant [37–39].

In the present studies both biliverdin and bilirubin were found to inhibit HIV-1 protease activity competitively *in vitro* at pH 7.8 with K_i values of approx. 1 μM (Table 2). The activity of bilirubin towards HIV-2 protease and SIV protease was similar (Figure 2a). The dipyrinone model compound xanthobilirubic acid also inhibited HIV-1 protease with a K_i of 2.5 μM under the same conditions. However, when tested in a whole-cell system containing 64 μM serum albumin, polyprotein processing was not significantly affected by xanthobilirubic acid at concentrations up to 300 μM and neither biliverdin nor bilirubin had any effect on viral polyprotein processing at concentrations up to 50 μM . The lack of activity of biliverdin and bilirubin could have been caused by binding to intracellular proteins such as glutathione S-transferases. More probably it resulted from the obligatory presence of serum albumin in the culture medium. Both biliverdin and bilirubin bind stoichiometrically to serum albumin with high affinity [40,41] and this might have prevented their cellular uptake. At concentrations above 50 μM , exceeding the high-affinity albumin-binding capacity, the pigments were toxic to the CH-1 cells and their effects on viral polyprotein processing could not therefore be measured.

Mori et al. [18] reported that biliverdin (but not bilirubin) inhibits the cytopathic effects of HIV-1 in MT-4 cells *in vitro* in a dose-dependent manner; they speculated that the anti-HIV activity of biliverdin is caused by direct inactivation of virions and inhibition of the binding of virus to target cells. Similarly, Nakagami et al. [19] reported that biliverdin could interact with MT-4 cells *in vitro*, thereby blocking an early stage of human herpes virus infection. Our results indicate that both biliverdin and bilirubin reduce HIV infectivity *in vitro* in a dose-dependent manner by blocking viral entry into HeLaT4 target cells. In contrast, xanthobilirubic acid seemed to decrease infectivity by interfering with viral assembly after cleavage of the structural proteins from the polyprotein precursor, rather than by interacting with the envelope protein or with the HeLaT4 receptor for the HIV-1 envelope protein. Thus our infectivity results indicate that xanthobilirubic acid disrupts viral maturation, whereas biliverdin and bilirubin block viral entry into cells *in vitro*.

Despite their apparent similarities when drawn in planar representations (Figure 1), biliverdin and bilirubin have markedly different biological and physicochemical properties. Bilirubin is

lipophilic, soluble in chloroform and almost insoluble in methanol, whereas biliverdin is very sparingly soluble in chloroform, is not lipophilic and dissolves readily in methanol [24]. These differences stem from conformational differences between the two pigments. In solution and in the protein-bound state bilirubin adopts a folded conformation shaped like the ridge-tile of a roof and stabilized by intramolecular hydrogen bonding between its carboxy groups and the pyrromethenone lactam and -NH groups [42,43]. Planar conformations, such as those depicted in Figure 1, or extended linear conformations, such as those generated by the CONCORD program used in the shape similarity search, are impossible or energetically highly unfavourable for bilirubin. In contrast, biliverdin prefers to adopt helical porphyrin-like conformations [42,43], as seen in the crystal structure of biliverdin complexed to apo-myoglobin [44], or linear extended forms more like those generated by the CONCORD program [42,45]. In view of the marked differences in three-dimensional structure and hydrophobicity between bilirubin and biliverdin, it is remarkable that both compounds showed similar inhibitory activity towards HIV protease *in vitro* and that xanthobilirubic acid, which corresponds structurally to one half of a bilirubin molecule and is much less lipophilic than bilirubin, also showed activity. To investigate the mechanism of inhibition and the way in which these inhibitors bind to the HIV protease enzyme we have attempted to grow crystals of inhibitor–protease complexes suitable for X-ray structure analysis. Green bipyramidal crystals of biliverdin complexed with SIV protease and yellow crystals of xanthobilirubic acid complexed with HIV-1 protease have been obtained and an X-ray analysis of these is in progress. Data at 2.4 Å resolution have been obtained for the biliverdin–protease crystals, but disordered density in the flap region of the protease has so far limited the analysis (E. Rutenber and R. Stroud, unpublished work).

Similarity shape searching is a potentially useful method for discovering new therapeutic lead compounds when other active drugs are known. Because molecular databases, especially connectivity databases, are readily available, and because the search times are much shorter than those associated with evaluations of molecular interactions [46,47], the shape-searching methods provide a rapid route to the selection and identification of potential enzyme inhibitors. The two lead compounds, biliverdin and bilirubin, emerging from this search for HIV protease inhibitors were effective *in vitro* at micromolar concentrations. However, disappointingly, they failed to show any effect on viral polyprotein processing *in vitro* in the CH-1 cell system at concentrations up to 50 μM . (This result might merely reflect the high affinities of biliverdin and bilirubin for the fetal calf serum albumin required in the cell culture medium.) Many compounds are now known that inhibit HIV protease at subnanomolar concentrations, and biliverdin and bilirubin are relatively weak inhibitors. However, some of the most clinically promising inhibitors have been developed by modification of lead compounds with activities in the micromolar range. Without further information on their non-covalent molecular interactions with HIV protease and their specificity, it is too soon to predict whether biliverdin, bilirubin or xanthobilirubic acid are useful leads.

Although our search was for potential HIV protease inhibitors, both biliverdin and bilirubin turned out to decrease viral infectivity significantly in the HeLaT4 cell assay *in vitro* at concentrations above 10 μM . In humans and other mammals, biliverdin is only a fleeting metabolic intermediate and biliverdinaemia is extremely uncommon. However, unconjugated hyperbilirubinaemia is not. Although the normal concentration of bilirubin in plasma is less than 17 μM , plasma bilirubin

concentrations in Gilbert's syndrome, which occurs in approx. 7% of adults, are generally in the range 20–80 μM and increase on fasting [34,48]. Hyperbilirubinaemia also occurs in newborn babies. In about 20–30% of newborns, accumulation of bilirubin in the body during the first postnatal week is so pronounced that it causes jaundice; plasma concentrations of 200–250 μM are not uncommon [35,49]. Our finding that the infectivity of infectious viral particles *in vitro* is decreased by exposure to bilirubin raises the question of whether unconjugated hyperbilirubinaemia can influence the course of HIV infection.

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