REVIEW

Dermatologische Klinik und Poliklinik[1], Technische Universität München, Dermatologische Klinik und Poliklinik[2], Ludwig-Maximilians-Universität München, and Institut für Pharmazeutische Chemie[3], Philipps-Universität Marburg, Germany

Inhibitors of aspartic proteases in human diseases: molecular modeling comes of age

L. Hoegl[1], H. C. Korting[2] and G. Klebe[3]

The family of aspartic proteases such as cathepsin D, gastricin, pepsin, renin, HIV protease and others have been the subject of molecular modeling in the field of drug design in the last years. The first aspartic protease inhibitor was reported thirty years ago as a renin inhibitor. The success of HIV protease inhibitors in preventing progression to AIDS was based on the transition state analogs of renin inhibitors. Taking these three decades into consideration, an astonishing variety of chemical classes, *in vitro* and *in vivo* activities and species specificities of inhibitors of aspartic proteases have been reported. Especially inhibitors of renin, HIV protease and secreted aspartic protease of *Candida albicans* are covered.

1. Introduction

In the last three decades, the family of aspartic proteases has been the subject of intensive research in molecular genetics, protein chemistry and clinical treatment. Several enzymes belong to the aspartic proteases: the mammalian enzymes chymosin, gastricin, pepsin, renin and cathepsin D and E; the secreted aspartic protease (SAP) of Candida species and other fungal proteases like penicillopepsin, endothiapepsin and rhizopuspepsin; and the retroviral proteases, especially HIV protease [1].

All mammalian and fungal aspartic proteases are characterized by a single polypeptide chain which is folded into two domain chains with a large cleft in between. In contrast, the retroviral proteases consist of two identical polypeptide chains, which display structural homology to the domains of the mammalian and fungal single-chain enzymes. The classification as aspartic protease was designated corresponding to two aspartic residues that are responsible for the catalytic mechanism. These aspartic acid moieties are located in the active site cleft of the aspartic proteases which forms a long deep groove between the NH_2- and COOH-terminal lobes. This cleft is capable to accommodate molecules as active site inhibitors. The subsites can accommodate at least seven to nine amino acid residues of a substrate. The difference in specificity and activity of the members of aspartic proteases are attributed to structural differences in these subsites [2]. As an example, a detailed sketch of renin, HIV protease and SAP is shown in Fig. 1.

In 1968, the first aspartic protease inhibitor was reported to inhibit renin. In the following years, a series of inhibitors mimicking the substrate was synthesized [3]. Once the HIV protease structure got available, the pertinent experience gained in the synthesis of renin inhibitors stimulated the research directed toward HIV protease during the last years. So far, the HIV protease inhibitors are the only aspartic protease inhibitors already in clinical use. The rapid and successful synthesis of these inhibitors was promoted by the knowledge of the three-dimensional structure of the HIV protease making high selectivity possible [4].

Besides renin and HIV protease inhibitors, no other inhibitors of other aspartic proteases were evaluated in clinical trials for several reasons. In the case of chymosin its biological role is questionable in humans. Each of the other enzymes serves for specific functions in several tissues making it difficult to synthesize specific tissue related inhibitors [2]. In a double-blind randomised clinical trial, a pentapeptide inhibiting pepsin had no significant advantage over placebo in the treatment of duodenal ulcers with respect to ulcer healing and clinical symptoms. It has been concluded that the inhibition of pepsin in human gastric juice does not have a major influence on the healing of duodenal ulcers [5].

Cathepsin D has been shown to be a marker of poor prognosis when found at high levels in primary breast tumors. It has been suggested that cathepsin D increases the invasive potential of the tumor cells and increases the probability of metastases. In specific studies, however, no correlation was found between cathepsin D secretion and invasive behavior. *In vitro* inhibition of cathepsin D with pepstatin had no effect on the cells in culture. Therefore, high levels of cathepsin D were only considered as a secondary effect of the high levels of cathepsin D in the stromal components of breast tissue caused by infiltrating inflammatory cells [6]. Azaryan et al. described an aspartic protease in chromaffine granules of brain tissue which catalyzes the cleavage of proenkephalin [7]. They hypothesized that inhibition of this enzyme could be a new approach in the pharmacological intervention of opioid peptide metabolism, but specific inhibitors were not evaluated.

Overall, even though inhibition of aspartic proteases would in principle allow for a high specificity in therapy only SAP inhibition has become a new target in drug modelling besides inhibition of renin and HIV protease so far. In the following, the structures of inhibitors of these three aspartic proteases are discussed in detail.

2. Renin inhibitors

2.1. Molecular and biochemical properties of renin

The renin-angiotensin system (RAS) regulates the physiological control of blood pressure and fluid volume. The rate-limiting step in this system is the aspartic protease renin. This enzyme is secreted by the juxtaglomerular cells in the kidney in response to decreased perfusion in the kidney or a fall in plasma sodium concentration.

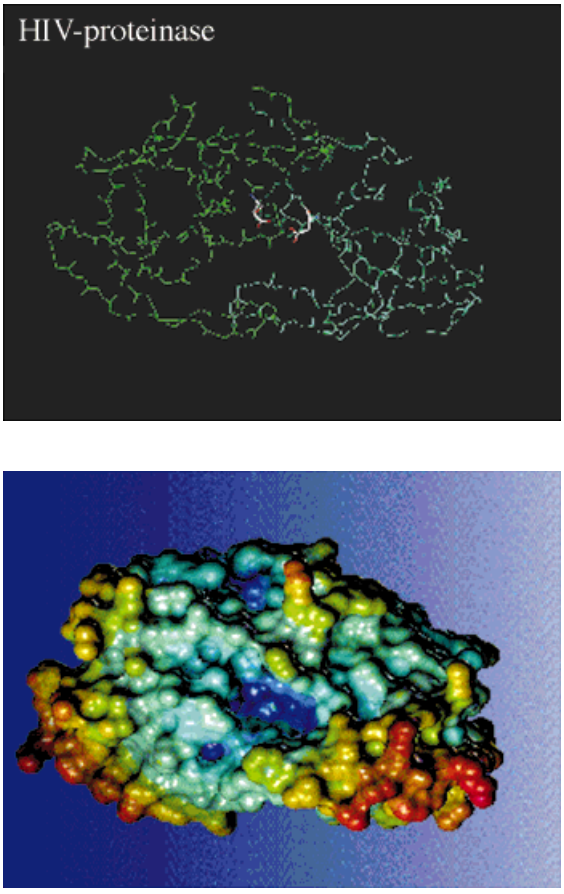
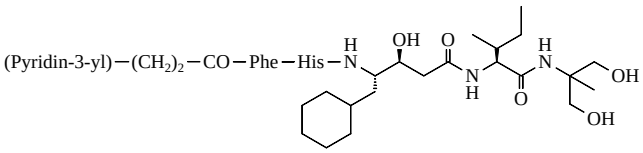


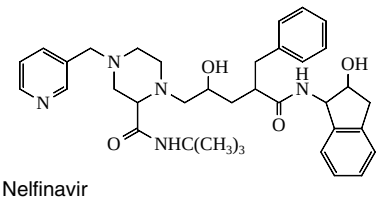
Fig. 1: Structure of HIV protease, renin, and SAP. In the upper part, the geometry of the backbone chain is shown (green), the two catalytic Asp are high-lighted in atom-type coding. In the bottom part, the proteins are displayed in the same orientation together with their solvent-accessible surfaces. A color coding has been superimposed onto the surfaces according to the partial charges of the surface residues (red positive, blue negative partial charge). The active site can be localized in all three cases in the center as a pronounced cavity and is highlighted by the blue color (indicating accumulated negative charges). The images have been produced by the software tool Sybyl using the Molcad subroutine

Renin has a molecular mass of 37 to 40 kDa. According to X-ray crystallographic studies of aspartic proteases, renin has a bilobal structure with a pronounced cleft between the two lobes where the two catalytic aspartyl residues are located close together [8]. In contrast to other aspartic proteases, the pH optimum of renin is 6.0 and, thus, significantly higher than that of the other members of this enzyme family. Renin has a high specificity against its only known natural substrate, i.e. angiotensinogen [9]. Angiotensinogen is a circulating globular glycoprotein synthesized primarily by the liver with the specific peptide sequence $His^{(P5)}-Pro^{(P4)}-Phe^{(P3)}-His^{(P2)}-Leu^{(P1)}-Val^{(P1')}-Ile^{(P2')}-His^{(P3')}$. Its enzymatic cleavage by renin to angiotensin I (AI) is the rate-limiting step in the intrinsic series of RAS [9]. AI has little *in vivo* activity and is rapidly transformed by angiotensin-converting enzyme (ACE) to the octapeptide angiotensin II (AII), the effector hormone of the RAS and a downregulator of renin release. AII is one of the most potent vasoconstrictors and exerts numerous hemodynamic effects that lead to increased intravascular volume, sodium retention and elevated blood pressure [10].

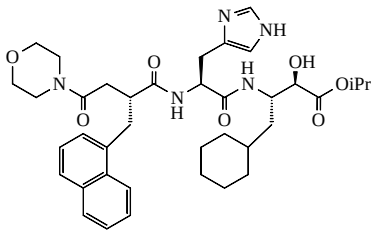
SR 43 385



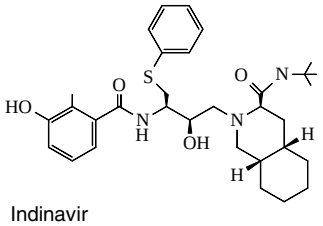
Nelfinavir



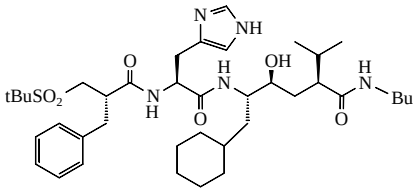
KRI-1314



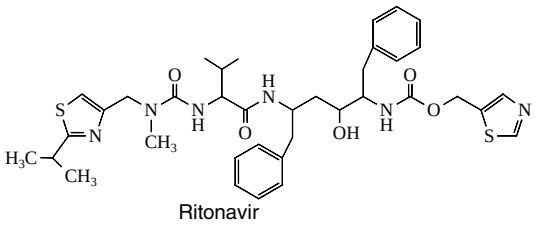
Indinavir



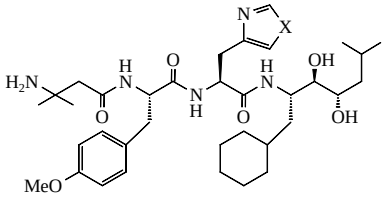
CGP 38 560



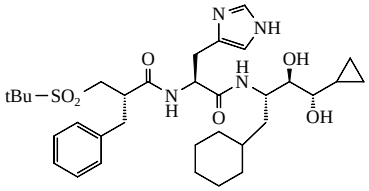
Ritonavir



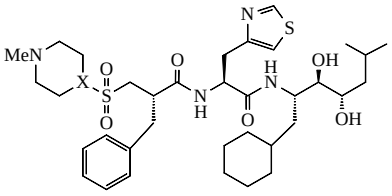
Enalkiren



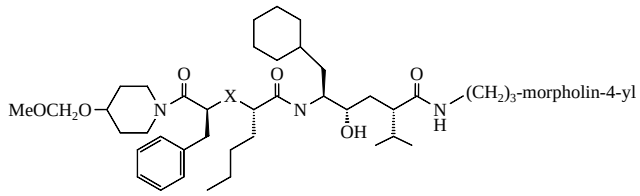
Remikren



Zamikren



A-74 273



2.2. Structures and chemical classes

Antihypertensive drugs could be based on the obstruction of the RAS by inhibition of renin or ACE or antagonism of the AII receptor. Four types of renin inhibitors have been studied: renin antibodies (antisera, monoclonal antibodies, Fab fragments), synthetic derivatives of the prosegment of renin precursor, pepstatin analogs and angiotensinogen analogs [9]. The fourth class of renin inhibitors, the angiotensinogen substrate analogs, are reviewed in the following.

The cleavage site of angiotensinogen in human and non-human primates is at the $Leu^{10}-Val^{11}$ peptide bond while the cleavage site in other species like dogs or rodents is $Leu^{10}-Leu^{11}$ [11]. When renin interacts with angiotensinogen, the two aspartic acid residues perform complementary functions during catalysis, one being unprotonated and the other protonated [12]. The unprotonated aspartic carboxylate deprotonates a water molecule, the protonated aspartic acid transfers a hydrogen to the carbonyl oxygen of the scissile amid bond to form a tetrahedral transition state intermediate.

Therefore, one strategy for making renin inhibitors was to replace the fragile peptide bond by a structural unit at which the cleavage reaction cannot occur. This class of renin inhibitors are called transition state analogs.

Among the earliest examples realized for such a transition state mimic in the field of renin inhibition were hydroxyethylene derivates developed by Szelke. The hydroxy group mimics partially served as the hydrated carbonyl of the tetrahedral diol intermediate, the side-chains of the adjacent Leu^{10} and Val^{11} are correctly positioned into the specificity pockets of renin. The hydroxyethylene portion was incorporated into the sequence of angiotensinogen to replace the natural Leu^{10} and Val^{11} residues [3].

The goal to synthesize more specific and more potent renin inhibitors was achieved by replacing the scissile bond between $Leu^{10}-Leu^{11}$ in human angiotensinogen by a sta-

tin residue as for example realized in the inhibitor pepstatin. A very high binding affinity and specificity for renin is observed due to the ability of the statine residue to operate as a transition state analog. Further examples for statine-based inhibitors are SR 43845, ES-6864 and ES-8891 for which the structures are given in Fig. 2 as for other renin inhibitors [10].

Further studies optimized the P1 residue replacing the original Leu[10] in order to produce other transition-state analogs [9]. The norstatine-derived inhibitors KRI-1314 and terlakiren incorporated an isopropyl ester at the P1′-site Val[11] side-chain. In hydroxyethylene isosteres such as CGP 38560 and ditekiren an ethyl group is substituted at the P1′-site of Val[11].

The best studied ligands belong to inhibitors incorporating a C-terminal glycol: enalkiren, remikren and zankiren [10]. These compounds are discussed in detail in the following sections refering to *in vitro* and *in vivo* data. Other inhibitors from this group are PD 132002 and PD 134672. Phosphate or fluorine-containing compounds replace the C-terminal carbonyl in statine- and norstatine-derived renin inhibitors. In FK 906 a C-terminal isopropyl group and in GR 70982 a C-terminal alkyl group substituted by a heterocycle are incorporated.

A different approach towards developing bioavailable renin inhibitors was the design of compounds with reduced peptide character. These compounds were for example synthesized by replacing of the amide bond between the P2- and P3-sites such as in A-74273. Rosenberg described further inhibitors such as macrocyclic renin inhibitors and examples not derived from angiotensinogen [10].

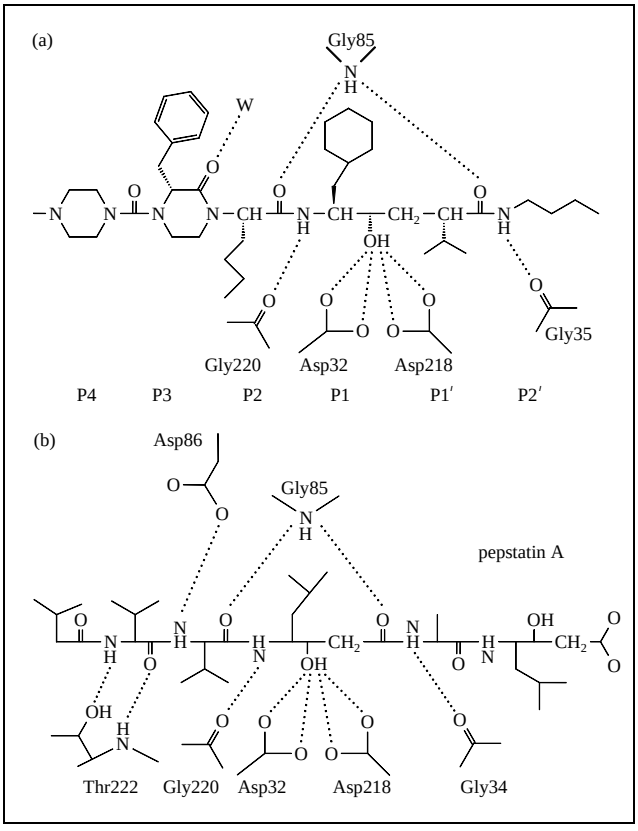


Fig. 2: Hydrogen bonding diagrams from A70450 bound to SAP2 (above) and pepstatin bound to SAP2 (below). Distances of less than 3.5 A between electronegative atoms are indicated by dotted lines, modified [64]

2.3. In vitro activities

As described in the previous section, renin inhibitors block the renin-catalyzed hydrolysis of angiotensinogen by competiting with the natural substrate for the catalytic site. As a result of renin inhibition, any formation of AI and AII is inhibited and their plasma concentrations are reduced.

Several methods have been applied to evaluate the *in vitro* effects of renin inhibitors in order to investigate the consequences of an inhibition of the renin-mediated conversion to AI, generally quantified by radioimmunoassay [13]. According to the studies in human plasma at pH 7.4, enalkiren shows a 50% inhibitory concentration (IC_{50}) of 14 μM, remikren of 0.8 μM and zankiren of 1.1 μM. IC_{50} values of these and other renin inhibitors in various species are listed in Table 1 [10].

Table 1: The potency of renin inhibitors of different classes against renin from various species, modified [10]

Inhibitor	Human IC_{50} (μM)[a]	Monkey IC_{50} (μM)[a]	Rat IC_{50} (μM)[a]	Dog IC_{50} (μM)[a]
SR 43845 (Statine)	<0.01	<0.01	130	not reported
KRI-1314 (Norstatine)	4.7	51	31000	79
CGP 38560 (Hydroxyethylene)	0.7	0.7	1000	7
Enalkiren (C-terminal glycol)	14	2.3	>10000	43
Remikren (C-terminal glycol)	0.8	1.0	3600	107
Zankiren (C-terminal glycol)	1.1	0.24	1400	110
A-74273 (Atom deletion)	3.1	3.6	1600	43

[a] Plasma renin, pH 7.0–7.4

2.4. Activity of renin inhibitors against other aspartic proteases and their species specificity

Inhibition of enzymes other than renin could lead to side-effects questioning the potential advantages of renin inhibitors over ACE inhibitors. As shown in Table 2, the C-terminal heterocycles (CGP 38560) as the most potent transition-state analogs possess IC_{50} values 10- to 100-fold higher against pepsin and cathepsin D [10, 14].

Since these compounds were derived from the sequence of human angiotensinogen, they are potent inhibitors of human and monkey renin. Activities in these two species are generally quite similar (see Table 1) [10]. However, these inhibitors bind only weakly to rat renin while their potency against rat and dog renin varies with structure. This species specificity is a result of the location of the scissible bond between Leu[10] and Val[11] in the primate substrate, whereas it is located between Leu[10] and Val[11] in nonprimate angiotensinogens. Furthermore, the specificity can be attributed to differences in the three-dimensional structures of individual renins. This finding clearly indicates the limited value of renin inhibitor testing in various animal models.

2.5. Pharmacokinetics

The mechanisms of renin inhibitors uptake from the intestine and their elimination pathway are poorly understood. Most of the renin inhibitors have molecular weights of

500 Da and more. Compounds of this size tend to be taken up by the liver and excreted into the bile. This type of excretion has been demonstrated for enalkiren, CGP38560, ditekiren and PD134672. Studies have confirmed that administration of renin inhibitors immediately leads to a dose-dependent increase in renin secretion. However, most of the renin inhibitors are cleaved or rapidly metabolized, accordingly blood pressure recovers within minutes after terminating intravenous drug administration. Therefore, renin inhibitors have to be kept at a high concentration for a long period in order to inhibit circulating and newly released renin. A detailed discussion of the pharmacokinetic and pharmacodynamic properties has been given by Rosenberg for several inhibitors [10]. The most intensely studied compounds are discussed in the following.

After intravenous administration, enalkiren exhibits a biphase elimination with a distribution and elimination half-life of 15 ± 6 min and 1.6 ± 0.43 h, respectively. Peroral application in rats has shown that it is poorly absorbed (<1%). However, although exhibiting moderate portal drug levels, enalkiren was subject to high liver extraction [15].

Remikren has a high systemic plasma clearance and a long terminal half-life of 7 h. After oral administration in animal studies, the major elimination pathway is extensive liver metabolism followed directly by biliary excretion of both the metabolite and the unmodified compound, with minor urinary elimination of the original compound. The bioavailability has been determined to be <1%, mainly attributed to the high first-pass effect rather than to poor absorption [16, 17].

The enhanced lipophilicity of zankiren improves absorption upon peroral application, and the reduced conjugate formation limits hepatic elimination. Zankiren was the first peptide-based renin inhibitor to demonstrate detectable oral absorption in any species. The bioavailabilities were at 8%, 24%, 32% and 53% in the monkey, rat, ferret and dog, after 10 mg/kg doses of zankiren [18].

2.6. In vivo activities

In vivo activity has most often been demonstrated in primates because of the high enzyme homology with human renin. Greenlee et al. have summarized various *in vivo* models used to study renin inhibitors [19]. It is difficult to compare the reported *in vivo* activities, because inhibitors were tested in a variety of different primate species and, in addition, the response to renin inhibition depends on the degree of sodium depletion [20].

From a pathophysiological viewpoint the hypothesis has been put forward that blocking the hydrolytic cleavage of angiotensinogen by renin inhibitors would result in a decrease in the serum levels of AI and AII and subsequently in a reduction of systemic blood pressure. It is of special interest with respect to *in vivo* studies of renin inhibitors to determine the correlation of hypotensive response and parameters such as plasma renin activity (PRA) and AI or AII levels. As reported by Fischli et al., levels of PRA are higher and of longer duration than the observed blood pressure effects [21]. Furthermore, after a total suppression of PRA a decrease of blood pressure was observed. This separation between suppression of PRA and the blood pressure-reducing response is a matter of debate. It has been suggested that renin inhibitors interact with a second, extravascular tissue-based RAS system [22]. The presence of renin has been demonstrated in brain, vasculature, heart, adrenal gland, and other tissues.

Enalkiren, remikren and zankiren have been studied extensively in both normotensive and hypertensive patients. Infusions of enalkiren lead to a progressive decrease in blood pressure, PRA, plasma AII and aldosterone levels. In spite of the relatively short elimination half-life, enalkiren has been shown to produce prolonged (>6 h), dose-dependent (0.03 mg/kg and 0.1 mg/kg intravenously) suppression of PRA, AII and systolic and diastolic blood pressure with no change in cardiac response and pulse rate. The reduction in blood pressure was enhanced by salt depletion and pretreatment or concomitant diuretic treatment [15]. Boger and Crowly conducted a study to evaluate the effects of enalkiren administered for one week at daily doses of 1.2 mg/kg and 0.3 mg/kg [22]. This study showed that the drug was well tolerated while it led to a prolonged blood pressure reduction. Although enalkiren has been shown to be an effective antihypertensive drug, its clinical applicability is limited because of its poor oral bioavailability.

Remikren at doses of 1 mg/kg intravenously and 10 mg/kg perorally was shown to be significantly more effective than enalkiren reducing arterial pressure in sodium-depleted normotensive squirrel monkeys. It is as effective as cilazapril, a new, longacting ACE inhibitor. In hypertensive patients, a single 600 mg peroral dose reduced PRA and plasma AII levels significantly for at least 8 h. Both, systolic and diastolic blood pressure are significantly lowered for at least 20 h. In 8-day studies, hypertensive patients were exposed to daily doses of remikren 300 mg or 600 mg. PRA and diastolic blood pressure were significantly reduced for the first 24 h and peristed for the duration of the study [16]. In contrast, others reported that the diastolic blood pressure was not significantly lowered and blood pressure effects were no longer observed after the 8th day [17]. Even though peroral remikren causes doserelated declines of PRA and plasma AII levels in normotensive men, no change in blood pressure was observed. These studies also demonstrated that remikren is clinically effective only in those patients who require a therapeutic hypotensive effect.

Various doses of intravenous zankiren caused dose-dependent decreases in PRA and blood pressure in sodium-depleted hypertensive patients. A clinically important antihypertensive activity occured after a single dose of 25 mg zankiren. A significant reduction in mean arterial pressure was observed throughout the 6 h post dosing, with no effect 24 h after administration [18].

2.7. Tachyphylaxis and adverse effects

No evidence of pharmacological tolerance or tachyphylaxis was demonstrated during an 8-day administration of several renin inhibitors according to a dose-related decrease of PRA and plasma AII levels [10].

All studied renin inhibitors were very well tolerated. Only some patients reported minor adverse effects such as dizziness, malaise, palpitations and rash. Decreased blood pressure was not correlated with changes in cardiac reponse or pulse rate. No direct inotropic or chronotropic effects on the heart were observed, and their hypotensive effects caused no reflex tachycardia as observed with ACE inhibitors. These results suggested that the reduction of blood pressure by renin inhibitors is the result of vasodilation and direct modulation of renal hemodynamics with increases in glomerular filtration rate, sodium extraction and renal blood flow [10]. Unexpectedly, hyponatriemic and hyperkalemic states in patients did not increase in fre-

quency and no rebound phenomenon of hypertension after discontinuation of drug administration was observed.
Other *in vivo* effects were reported in some patients after administration of renin inhibitors. Corneal application of the inhibitor enalkiren decreases the intraocular pressure in unanesthesized rabbits and anesthesized monkeys [23]. Boger and Crowly described one patient who developed signs of psoriasis following treatment with enalkiren [22].
Despite a high *in vitro* potency of many renin inhibitors, *in vivo* studies point toward a limited effectiveness. Main problems are attributed to an oral bioavailability below 1% and a short life-time. Only moderate hypotensive effects were observed following peroral administration [9, 10]. Furthermore, for many renin inhibitors no proper dose-response relationship could be established. The correlation between the suppression of PRA, plasma AII levels and the decrease in blood pressure was not significant. It has been supposed that the amide bond $Phe^{(P3)}-His^{(P2)}$ gets cleaved immediately after being exposed to chymotrypsin.
Even though the first substrate-based renin inhibitors were reported in 1968 [3], there are currently no marketed renin inhibitors available. In contrast to the development of HIV protease inhibitors, most of the renin inhibitors were synthesized before the crystal structure of renin became available for drug modeling. Nevertheless, the reduced profile of side-effects of renin inhibitors compared to ACE inhibitors still bears the chance to represent an improved antihypertensive principle. However, synergistic effects can be expected from a combined therapy with ACE inhibitors. Furthermore, renin inhibition might be of clinical use in a number of other clinical scenarios like stroke, myocardial infarction, scleroderma, renal trauma and acute closure of renal artery grafts.

3. HIV protease inhibitors

3.1. Molecular and biochemical properties of HIV protease

As mentioned above, HIV protease was the first enzyme essential for the life cycle of the virus for which a crystal structure became available in 1989 [24]. The molecular structure is shown in Fig. 1 [25].
Each of the monomers is related to the other by two-fold rotation according to crystallography [26]. HIV protease is the smallest of the retroviral proteases and is much smaller than mammalian and fungal aspartic proteases with approximately 325 residues [25]. HIV protease has a molecular weight of 12 kDa and is composed of two homodimers each with 99 amino acids [27].
The catalytically essential sequence Asp-Thr-Gly composed of the acids 25 to 27 occurs in a loop that forms a number of hydrogen bonds with the symmetry-related loop of the opposite monomer [25]. In contrast to monomeric mammalian and fungal aspartic proteases, HIV protease binds inhibitors along the edge of the flap region of each monomer. Thus, HIV protease forms characteristic hydrogen bonds which are distinct from those of other aspartic proteases.
HIV protease mediates the cleavage of HIV polypeptide precursors during maturation of the newly replicating virus. These polypeptides are encoded by three main genes of HIV: env, gag and pol. As non-functional precursor polypeptides they are cleaved by posttranslational proteolysis to functional proteins.
The env gene encodes for envelope proteins. In contrast to pol and gag genes, the envelope precursor glycoprotein gp 160 is cleaved from the precursor by a host cell protease and not by the HIV protease.
HIV protease cleaves itself autocatalytically from the gag-pol precursor protein and subsequently it dimerizes to the fully active enzyme [28]. The pol gene encodes for the viral enzymes reverse transcriptase/ribonuclease H and integrase. These enzymes are then carried in virions for further replication of the virus [29]. The gag gene encodes for the structural core proteins which are synthesized as a single precusor protein Pr 55.
Pr 55 is carried in a plaque at the plasma membrane of infected cells and during budding it is cleaved by HIV protease to the proteins p17, p24 and p15 [30]. The matrix protein p17 is part of the inner surface of the viral envelope. The capside protein p24 forms the central core capsid. P15 is cleaved into the nucleocapsid proteins p7 and p6. This process results in the mature, infectious form of HIV mediated by the HIV protease.

3.2. Structures and chemical classes

Eight potential cleavage sites between P1 and P1′ can be detected in the gag-pol precursor protein on which HIV protease can operate: Tyr-Pro, Leu-Ala, Met-Met, Phe-Leu, Phe-Pro, Pro-Phe, Phe-Tyr and Leu-Phe [31]. The development of different protease inhibitors was based on these cleavage sites. Protease inhibitors with high specificity such as hydroxyethylamine and hydroxyethylene derivates correspond to the sequence Tyr-Pro and Phe-Pro, protease inhibitors originally targeted for renin mimic the sequence Leu-Ala and C_2-symmetrical inhibitors correspond to the Met-Met unit.
As in the case of renin, HIV protease inhibitors are short peptide-like ligands which mimic substrates for HIV protease. They compete with the natural substrate for the active site. Although budding is not inhibited, the gag proteins fail to advance from the plaque stage. Free non-infectious virions are produced and the gag and pol genes cannot be processed into their functional units. Therefore, the produced new virions are unable to infect other cells [31].
The first lead structures for HIV protease inhibitors were based on existing renin inhibitors comprising statine-based transition state analogs [32]. Original renin inhibitors were modified such that the scissile Leu-Leu bond present in the sequence of angiotensinogen was modified to a Leu-Ala bond. As described above, a nonhydrolyzable surrogate has been introduced to replace the amide bond usually cleaved by HIV protease. It mimicks the tetrahedral transition state. Statine and norstatine analogs such as KNI-227 or inhibitors incorporating a tetrahedral phosphinic acid or an α,α-difluoroketone exhibit only moderate protease inhibition.
The scissile bond analogs derived from hydroxyethylamine and hydroxyethylene yielded inhibitors highly potent *in vitro* and *in vivo.* Examples are saquinavir (Invirase™) [33–35], VX-478/141W94 [36], indinavir (Crixivan™) [37], L-687, 908, U-81749 and UK-88947 [32].
The knowledge of the 3D structure of HIV protease being a C_2-symmetric dimer has promoted the design of new compounds showing good steric complementarity to the binding site and correspondingly to is C_2-symmetry. An example for an HIV protease inhibitor with high *in vitro* and *in vivo* potency of the C_2-symmetric type is ritonavir (Norvir™) [38, 39], additional ones are A-74704, A-77003, A-80987 and L-700,414 [32]. Furthermore, inhibitors to hamper protein dimerization were synthesized ex-

Table 2: The potency of renin inhibitors of different classes against other aspartic proteases than renin, modified [10]

Inhibitor	Porcine pepsin IC_{50} (μM)	Human gastricin IC_{50} (μM)	Bovine cathepsin D, IC_{50} (μM)
SR 43845 (Statine)	> 20	not reported	5
KRI-1314 (Norstatine)	>100	not reported	80
CGP 38560 (Hydroxyethylene)	5[a]	3	0.6[a]
Enalkiren (C-terminal glycol)	> 10	>10	>10[a]
Remikren (C-terminal glycol)	240	not reported	35
Zankiren (C-terminal glycol)	> 10[a]	>10	10[a]
A-74273 (Atom deletion)	> 10[a]	>10	>10[a]

[a] Human enzyme

ploiting the fact that HIV protease can only function in its homodimeric form. Therefore, inhibitors with the capability to prevent self-association or promote the dimer dissociation were evaluated. *In vitro* studies, however, demonstrated only poor inhibition of HIV protease by these compounds [27].

The availability of the X-ray structure of HIV protease allowed its use for molecular modeling to design molecules that fit into the active site pocket [40]. Non-peptidic compounds were developed such as the pyron-based coumarin derivatives, e.g. U-96988 [41], or cyclic ureas, e.g. DPM 323, 412 and 450 [42]. These compounds show poor *in vivo* effects due to their reduced bioavailability. In contrast, the nonpeptidic inhibitor nelfinavir (Viracept™) [43] showed high affinity *in vitro* and *in vivo*.

3.3. In vitro activities

It has been demonstrated that HIV protease inhibitors are active both in acutely and chronically HIV-infected cells in contrast to nucleoside and non-nucleoside analogs of the reverse transcriptase being only active in acutely HIV-infected cells [44]. Therefore, the use of different HIV inhibitors allows a selective blocking of different steps in the viral replicative cycle. The antiviral activity was also observed in primary human lymphoid and monocytic cell lines and against a variety of HIV strains including clinical isolates with zidovudine resistance [45, 46]. Acute *in vitro* cytotoxicity of the lead protease inhibitors has only been reported at micromolar concentrations, suggesting that these agents have a high therapeutic index.

The range of IC_{50} values for several chemical classes of HIV protease inhibitors are listed in Table 3 [29]. Saquinavir exhibited the highest antiviral activity in HIV infected cells of different types testing syncytia formation and viral infectivity in target cells [35]. In a long-term study it could be shown that HIV infected cells incubated with saquinavir were completely free of HIV after 87 days. The IC_{50} values reported for saquinavir, VX-478, ritonavir and nelfinavir were 3.5, 10, 27 and 22 in nM respectively [46]. For indinavir no values have been reported.

Studies of combination treatment of one HIV protease inhibitor with a nucleoside and/or non-nucleoside analog inhibitor of reverse transcriptase in cell cultures demonstrated remarkable synergistic effects with respect to infectivity and development of drug resistance [47, 48].

Table 3: Chemical classes of HIV protease inhibitors and range of antiviral activity *in vitro* as expressed by the IC_{50}, modified [29]

Chemical class	Compound	IC_{50}
Statine/Norstatine derivates	KNI-227	10^{-6} M
Hydroxyethylamine isosteres	Saquinavir VX-478	10^{-9} M
Hydroxyethylene isosteres	Indinavir L-687, 908 U-81749 UK-88947	10^{-8} M–10^{-6} M
C2-symmetric inhibitors	Ritonavir A-74704 A-77003 A-80987 L-700, 414	10^{-8} M–10^{-7} M
Nonpeptidic inhibitors	Nelfinavir DPM 323 DPM 412 DPM 450 U-96988	10^{-8} M–10^{-7} M

3.4. Activity of HIV protease inhibitors against other aspartic proteases

Tests revealed that all HIV protease inhibitors applied at concentrations up to 10^5 times higher than those required for HIV protease inhibition produced less than 50% inhibition of human aspartic proteases such as renin, pepsin, gastricin and other classes like elastases, collagenases or prolidases [35].

3.5. Pharmacokinetics

Pharmacokinetic data of HIV protease inhibitors are listed in Table 4 [46]. Peptide-based drugs possess reduced bioavailability, notably squinavir. Poor uptake from the intestine, a short serum half-life attributed to a first-pass hepatic metabolism caused by the cytochrome P450 isoenzyme CYP3A4 and rapid liver clearance have been reported in this context. In contrast, the C_2-symmetric inhibitor ritonavir and some other drugs are supposed to act as inhibitors of metabolization of squinavir resulting in higher serum levels and longer half-life.

3.6. In vivo activities

Within the last years, HIV protease inhibitors improved the prognosis of HIV infection remarkably [49, 50]. However, most HIV protease inhibitors with high *in vitro* specificity showed only low *in vivo* activities. This discrepancy has been attributed to low bioavailability, serum

Table 4: Pharmacokinetics of HIV protease inhibitors, modified [46]

Compound	Bioavailability (%)	Protein binding (%)
Saquinavir	4	>98
VX-478	Not reported	93
Indinavir	30[a]	60
Ritonavir	60–70	98–99
Nelfinavir	20–80[a]	>99

[a] In monkeys

protein binding and other pharmacokinetic characteristics as reported in the section above.

There are several reviews in which the design and results of phase III studies were reported in detail [46]. The aim of all these studies was to assess the potency of each HIV protease inhibitor to raise the CD4 cell count, to lower the viral RNA level in the serum and to prevent opportunistic infections. Favorable effects were observed with all five HIV protease inhibitors marketed so far, applied as single treatment or in combination. The daily doses of HIV protease inhibitors currently recommended are as follows: saquinavir 1800 mg, indinavir 2400 mg, ritonavir 1200 mg, nelfinavir 2250 mg and VX-478 1800 mg.

AIDS Clinical Trials Group study 229 (ACTG 229) was the largest study with an HIV protease inhibitor over a period of 48 weeks including 295 patients having CD4 cell counts of less than 300/μl [51]. Efficacy markers responded most significantly upon triple combination therapy with daily doses of 1800 mg saquinavir, 600 mg zidovudine and 2.25 mg zalcitabine in contrast to double combinations. A mean rise in CD4 cell count of 33/μl above baseline and a mean virus load reduction of 0.51 $\log_{10}$ copies/ml were observed.

Other studies with each of the five HIV protease inhibitors showed the best results according to the efficacy markers upon combination therapy comprimising three or four compounds. Treatment included one or two HIV protease inhibitors and one or two nucleoside or non-nucleoside analogs [46]. CD4 cell counts were raised to up to 150/μl and virus load reduced down to 2.5 $\log_{10}$ copies/ml under baseline in several studies with different combination regimes dependent on duration of treatment, antiviral pretreatment, baseline of CD4 cell count and virus load. Therefore, corresponding to somewhat puzzling *in vivo* results, general recommandations for rational treatment regimes with HIV protease inhibitors and combinations with nucleoside and non-nucleoside analogs are missing. This has to be considered taking into account side effects and accumulation of resistance patterns [49].

3.7. Adverse activities

Activity and safety data from phase I/II studies indicate that HIV protease inhibitors are well tolerated due to their high specificity against HIV protease. Most common adverse reactions to saquinavir treatment were mild gastrointestinal disturbances and raised liver transaminases in less than 5% of patients [46]. Adverse events caused by ritonavir were reported to be diarrhoea, raised transaminases and triglycerides, in the case of indinavir urolithiasis in up to 5% of patients and hyperbilirubinemia in about 15% of patients. Approximately 40% of patients suffered from fatigue and poor concentration and loose stools during treatment with nelfinavir [48].

3.8. Resistance

The high replication rate of HIV and the absence of mechanisms to correct replication errors produce large numbers of new HIV variants in HIV infected patients. It has been shown that the presence of antiretroviral drugs favoured the development of HIV variants with reduced drug sensitivity or resistance ($\geq$100 fold increase in IC50) [52]. Mutations reported in the context of HIV protease inhibitors mostly occur in close neighborhood to the active site of HIV protease, thus effectively reducing binding affinity in the resistant variants [24, 45].

Some of the 99 amino acids in each polypeptide of the homodimer have been shown to exhibit specific genotypic mutations upon the selection strain of a particular HIV protease inhibitor [37, 53]. Patterns of resistance mutations can be distinguished by their position. Primary mutations occur at the active site of the enzyme. They appear to occur at incipient stages of a treatment resulting in mutations specific and unique for each protease inhibitor. Secondary mutations are found beyond the active site and are observed following the primary mutations. These mutations are in common to all protease inhibitors.

Specific primary mutations for saquinavir are related to the codons 48 and 90 [54], for indinavir and ritonavir to codons 82 and 84 and for nelfinavir to codons 32, 82 and 84 [55, 56]. Secondary mutations are common for all protease inhibitors, involving codons 36, 46 and 71, and, in addition, codons 20, 46, 54 and 63 with saquinavir, indinavir and ritonavir.

Therefore, an initial therapy with only one HIV protease inhibitor limits the chances for further therapy with additional HIV protease inhibitors by initiating cross-resistance based on previous primary mutations. In addition, secondary mutations caused by the application of the first protease inhibitor restrict the condition to a situation where fewer mutations are required to produce high-level resistance to a subsequently applied protease inhibitor even though initial primary cross-resistance was not observed [53]. Reintroduction of the first applied HIV protease inhibitor in later phases of treatment resulted in reappearance of the resistant variants within few weeks typical for the HIV protease inhibitor [37, 55].

However, therapeutic failures with HIV protease inhibitors were not only related to resistance, but also to other pharmacological problems such as poor bioavailability reflected by low serum levels of the inhibitor. Furthermore, resistance of HIV variants to HIV protease inhibitors exists in the predominant population of HIV in up to 3% of previously untreated HIV infected patients [56].

4. Inhibitors of the secreted aspartic protease (SAP) of *Candida albicans*

4.1. Molecular and biochemical properties of SAP

Inhibition of SAP is a new approach in the treatment of candidosis. SAP is a complex family with at least eight different encoding genes. In comparison to other aspartic proteases, the sequence of SAP2 shares 22 to 27% identity with other fungal proteases such as rhizopuspepsin, penicillopepsin and endothiapepsin and the mammalian protases pepsin, chymosin, renin and cathepsin D [57].

Inhibition of SAP is of special interest in the treatment of candidosis for several reasons. The level of SAP production is correlated with the frequency of clinical signs of candidosis. Strains representing SAP-deficient mutants of *Candida albicans* showed reduced virulence. Furthermore, SAP degrades a broad range of substrates and thus enhances not only attachment, but also colonization and penetration of *Candida* species. It inactivates host immune defense (e.g. by immunoglobulin degradation, alteration of phagocytic cell or complement functions). In particular, intracellular functions are compromised when *Candida* is engulfed [58]. Therefore, inhibition of SAP should result in a reduction of the virulence potential of *Candida* species, especially *Candida albicans* in which SAP was mainly observed.

4.2. Structure and chemical classes

SAP inhibitors were synthesized at a time when solely structural studies of SAP2 from *Candida albicans* were available [57]. Several additional analogs were synthesized, varying either end of the ligands. A70450 was proposed to be the most potent SAP inhibitor apart from A79912 [59]. Other inhibitors of SAP were reported, however detailed characterizations are missing, and a testing of their in vivo efficacy was not reported [60–63].

The chemical composition of the P1, P1′ and P2′ units of the most potent SAP inhibitor A70450 are identical to those of the renin inhibitor CGP38560, belonging to the hydroxyethylene peptide bond isosteres of transition state mimics. At the protein binding site the hydroxy group of the ligand is located between Asp32 and Asp218, similar to the situation in pepsin with the active site residues Asp32 and Asp215 (Fig. 3) [64]. A unique feature of the structure of A70450 compared to other aspartic protease inhibitors is the incorporation of the phenylmethylpiperazine in the peptide chain ring. This moiety occupying a large pocket at the binding site it is believed that this occupancy is critical for the high inhibition potency of A70450 [59].

4.3. In vitro activities

Capobianco et al. developed a sensitive, rapid assay system for SAP activity based on fluorogenic substrates that allowed the investigation of the biochemical properties of SAP, in particular the inhibitor potency of specific ligands [65].

One virulence factor of SAP is its ability to degrade the heavy chains of immunoglobins, i.e. IgA. *In vitro* studies showed that IgA was fully protected by A70450 from degradation by SAP [57]. It is also known that SAP cleaves cytoskeletal proteins such as vimentin of mammalian cells. Incubation of SAP2 with vimentin resulted in a specific cleavage which was completely inhibited by A70450. Representing another virulence factor, SAP alters the cell morphology of skin fibroblasts in culture and reduces their growth. The addition of A70450 restored normal growth and morphology in cells incubated with SAP. Differences of IC_{50} values of the compounds tested by Abad-Zapatero et al. are listed in Table 5 [59].

4.4. Pharmacokinetics

The pharmacokinetics of A70450 and other SAP inhibitors was determined in mice. Peak serum levels of active inhibitors are dose-dependent following intraperitoneal administration, ranging from 2.5 to 15 μM for A70450, and serum levels remained detectable for 24 h after an initial rapid decline over the first few hours following administration. Therefore, serum levels of A70540 should be sufficiently high to inhibit SAP, because levels of 0.25 μM can successfully inhibit SAP *in vitro* [57].

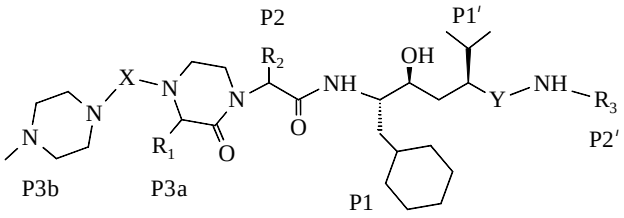
4.5. Species specificities

Species specificity of A70450 and several additional analogs were tested by Abad-Zapatero et al. [59]. All compounds had reduced activity against human renin and cathepsin D as shown in Table 5. A70450 binds to SAP2 with a potency about 20-fold stronger than that of pepstatin; the IC_{50} values for renin and cathepsin D inhibition are by a factor of about 5 to 500 higher [64]. The potential of SAP inhibitors to affect other aspartic proteases was not tested.

4.6. In vivo activities

It is known from the literature on the *in vivo* activity of pepstatin that it proves weak in the mouse model with respect to a protection against *Candida albicans.* Groups of 10 mice in the standard mouse protection model (Table 6) and 5 mice in the modified mouse protection model (Table 7) were infected intravenously with 10 LD_{50} (table 6) and 1 LD_{50} (Table 7) of Candida albicans [57]. Examining the effects of A70450 and A79912 in the mouse model, CFU/kidney did not decline and no increases in survival time or even cures could be detected,

Table 5: Inhibitors of SAP tested by Abad-Zapatero et al. [59]



Cmpd.	X	R_1	R_2	Y	R_3	C. alb. IC_{50} (nM)	Renin IC_{50} (nM)	Cat. D IC_{50} (nM)
1 (A-70450)	C=O	(R)-CH_2Ph	(S)-$(CH_2)_3CH_3$	C=O	$-(CH_2)_3CH_3$	1.4	7.1	770
2	C=O	(R)-CH_2Ph	(R)-$(CH_2)_3CH_3$	C=O	$-(CH_2)_3CH_3$	25	na	na
3	C=O	(S)-CH_2Ph	(S)-$(CH_2)_3CH_3$	C=O	$-(CH_2)_3CH_3$	2.0	>100*	na
4	C=O	(R)-CH_2Ph	(S)-$(CH_2)_3CH_3$	CH_2	$-(CH_2)_3CH_3$	490	na	na
5	SO_2	(R)-CH_2Ph	(S)-$(CH_2)_3CH_3$	C=O	$-(CH_2)_3CH_3$	2.7	1.5	150
6	C=O	(R)-CH_2Ph	(S)-$(CH_2)_3CH_3$	C=O	$-(CH_2)_3N(CH_2CH_2)_2O$	3.8	110	22000
7	C=O	(R)-CH_2Ph	(S)-$(CH_2)_3CH_3$	C=O	$-(CH_2)_2N(CH_3)_2$	6.2	>100	58000
8	C=O	(R)-CH_2Ph	(S)-$(CH_2)_3CH_3$	C=O	$-(CH_2)_3N(CH_3)_2$	3.8	140	16000

The figure on top shows the subsites P3–P2′ of the inhibitors. Chemical structures at the R_1–R_3 subsites and the positions X and Y of the inhibitors and IC_{50} (nM) against *Candida albicans* SAP (SAP), renin and cathepsin D (cath. D) are listed

Table 6: Effect of SAP inhibitors A70450 and A79912 compared to fluconazole and no treatment in the standard mouse protection model, modified [57]

Compound	Dose	CFU/kidney	MST	Cure	Survival
A70450	20	5.27	9.9	0	0
A79912	20	4.94	11.3	0	0
Fluconazole	10	3.02	20.8	0	70
Untreated	–	4.6	12.0	0	20

Dose: Doses in mg/kg; CFU/kidney: Valus of CFU/kidney in log; MST: Mean survival time in days; Cure: % of culture negative kidneys at day 30; Survival: % of survival at day 30

Table 7: Effect of SAP inhibitors A70450 and A79912 compared to fluconazole and no treatment in the modified mouse protection model, modified [57]

Compounds	log CFU/kidney at day 3		log CFU/kidney at day 7	
	20 mg/kg dose	2 mg/kg dose	20 mg/kg dose	2 mg/kg dose
A70450	3.23	3.04	3.25	3.26
A79912	3.12	3.49	3.50	3.78
Fluconazole	2.37	–	1.93	–
Pepstatin A	3.17	3.3	4.09	3.2

regardless of the dose of the applied SAP inhibitor (see Tables 6 and 7) [57].

Goldman et al. modified the test methods in order to optimize bioavailability and guarantee adaquate pH conditions for SAP inhibitors [57]. However, lack of SAP inhibition was the result of low specificity. They supposed that the inhibitors did not target the appropriate proteases and discussed the possibility of inadequate inhibition of all members of the SAP family, at least those SAP's most important for virulence. Data indicate that SAP1, SAP2 and SAP3 are equally sensitive to both A70450 and A79912, but that their activity against SAP4-8 is questionable [59, 64].

Biosynthesis of SAP is regulated by SAP genes and depends on growth states, morphological, or phenotypic transitions, stages of infection and tissue sites during infection. Knowledge of such regulation steps would greatly improve the possibility to synthesize SAP inhibitors for specific indications. Correspondingly, inhibition of individual SAP with a particular function in a defined stage of pathogenesis of candidosis might provide a unique armamentarium to combat this pathogen showing increasing importance in human disease.

Acknowledgement: The authors are grateful to Andrea Schafferhans (Univ. Marburg) for her kind help in producing the colored images.

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Received December 3, 1998
Accepted December 20, 1998

Prof. Hans C. Korting, M.D.
Klinikum Innenstadt der LMU
Dermatologische Klinik und Poliklinik
Frauenlobstr. 9–11
D-80337 München