Targeting virulence: A new paradigm for antifungals

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In the recent past, we have found ourselves in need of truly novel antifungal drugs as drug resistance in fungi has been evolving. Moreover, effective therapy has become particularly important as the number of immuno-compromised patients with life-threatening fungal infections increases. Fortunately, during the last few years, virulence factors of fungi and their inhibitors have, at least to some extent, been discovered and characterized. This should provide new options for the development of potential antifungal therapeutics. Inhibitors of the secreted aspartic proteinase of *Candida albicans* might turn out to be particularly rewarding.

Introduction

At the beginning of the new millennium antimicrobial chemotherapy at large has been overshadowed by the fact that antimicrobial resistance is on the increase questioning the clinical use of well-established classes of antimicrobials while the pharmaceutical industry rather tends to step out of the field than into it. This was attributed to 'the failure of current discovery paradigms to deliver promising new drug leads' [1]. One has in fact at the same time started to address 'bacterial virulence as a target for antimicrobial chemotherapy' [2]. Correspondingly, it is a central thesis of the present paper to postulate that targeting virulence should also be a new paradigm for antifungals. At this point it might be wise to address briefly both the definition of introducing a new paradigm or paradigm shift as well as virulence, and in particular virulence factors. As Kuhn has described in his seminal work entitled 'The structure of scientific revolutions' [3] a 'paradigm shift' or even a 'scientific revolution' takes place if a current theory generally accepted by the scientific community no longer is considered adequate to describe all relevant facets of a scientific problem. With the development of new antimicrobial agents at large the point is that in the era of emergent multi-resistance it is no longer considered adequate only to target 'essential' processes', rather one shall at least also address the 'virulence process' [2]. The simple idea behind, in fact, is no longer to try to kill the micro-organism acting as a pathogen by all means, but rather to hinder it to cause any harm to the host. This in fact may offer a 'kinder and gentler' approach to antimicrobial chemotherapy [2]. This new approach has been condensed by the catchy question: 'To kill or not to kill?' [4]. According to the common understanding virulence factors are attributes of pathogens which are in general not needed for survival *in vitro*, while they are important with respect to causing disease in a host. According to the concept of Jones and Falkow [5] causing overt disease by invading a host means for the micro-organism in the first place to find a portal of entry, then to overcome primary and secondary defence lines and last spread within the host or move on to a new one. In this view a virulence factor is 'a component that specifically functions in the pathway of virulence' [2]. Obviously, a virulence factor worth considering as an antimicrobial target should fulfil 'molecular Koch's postulates' [6] insofar as genetic disruption should be linked to attenuation. At present a major approach to identify pertinent targets is based on the new opportunities provided by genetics. The tools in the context range from so-called *in vivo* expression technology [7] up to transcriptional profiling [8]. However, with antibacterial chemotherapy the approach of targeting virulence is still questionable as a true clinical proof of concept so far is missing [2]. As described later this is different with virulence factor oriented antimycotic therapy – at least in principle. As with many concepts

☆ *Disclosure*: Dr C. Borelli, Prof Dr H.C. Korting and collaborators have recently submitted a patent application addressing inhibitors of secreted aspartic proteinases of *Candida albicans* to be used as active pharmaceutical ingredients.

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1359-6446/06/$ - see front matter © 2009 Published by Elsevier Ltd. doi:10.1016/j.drudis.2008.11.013

also the concept of virulence factor oriented antimicrobial chemotherapy has its limitations, one being the fact that the inherent specificity makes people expect 'a relatively narrow spectrum of action' [1]. Fortunately, there also seem to be approaches to overcome this problem, one possibly being the so-called 'real-time' diagnostics [1].

Efficient treatment for fungal infections has become more and more important as the frequency of fungal disease, particularly life-threatening fungal disease, increases due to the progress in the treatment of critically ill intensive care unit patients and haemato-oncological patients. Systemic mycosis is, in particular, seen in critically ill patients whose immune status has deteriorated. Accordingly, they are particularly prone to develop colonization and infection with opportunistic organisms such as yeasts, for example *Candida albicans* and moulds such as *Aspergillus flavus*. The role of an effective immune system in the prevention of fungal disease has become particularly obvious from the lessons learned in the early days of HIV infection. Originally, up to 90% of those patients suffered from mucosal candidosis [9]. In systemic fungal disease, white blood cells play an essential role: severe granulocytopenic fungal disease caused by yeasts can become life-threatening [10]. It is not, however, only yeasts that are relevant. *A. flavus* is a prime example of a mould that can also be life-threatening if the immune system is compromised: pertinent infections exhibit a lethality rate of at least 50% [11]. Fortunately, over the last few decades, potent antifungals have given the opportunity to fight even life-threatening fungal disease effectively. This has, however, produced issues of their own. On the one hand, there is a possibility of development of fungal drug resistance, on the other hand, virulence factors of the pathogens can be altered. A prime example is the following: Wu *et al*. demonstrated that secreted aspartic proteinase (Sap), a prime virulence factor of *C. albicans*, is upregulated in HIV patients treated with fluconazole at subinhibitory concentrations [12]. In fact, Navarathna *et al*. have been able to show in animal experiments that the virulence of *C. albicans* strains is increased upon exposure to subinhibitory concentrations of fluconazole [13].

Fortunately, current drug treatment does not only pose problems, but can also provide new opportunities. Upon the introduction of potent inhibitors of HIV protease for the treatment of HIV infection, it was soon observed that this type of antiviral treatment favourably influenced the frequency of mucosal candidosis [14]. In fact, this is not, or at least not completely, due to partial or total reconstitution of the immune status as originally presumed, but rather due to a direct inhibitory activity of these compounds against proteases from *C. albicans*, namely secretic aspartic proteinases as demonstrated by Korting *et al*. [15].

Against this background, it is tempting to speculate that systematic development of active pharmaceutical ingredients addressing virulence factors as a prime target could provide entirely novel therapeutic options for the treatment and/or prevention of localized or systemic fungal disease.

Current approaches to antifungal therapy

Drugs currently used for the treatment of localized or systemic fungal diseases comprise several groups, in particular, polyenes, azoles, allylamines, flucytosine and griseofulvin.

The polyenes, in particular amphotericin B and nystatin, irreversibly bind to ergosterol, a major component of the fungal cytoplasmatic membrane. This results in disruption of membrane integrity and ultimately cell death [16]. Amphotericin B can be administered both systemically and topically and has a broad spectrum of activity against a variety of fungi. Its systemic use, however, is restricted due to dose-related toxicity, in particular nephrotoxicity. The introduction of particular lipidic formulations, including a liposome formulation, has increased the risk benefit ratio. Due to increased tolerance, higher doses can be considered to make treatment more efficacious.

Ergosterol is also affected by the azoles. They are capable of inhibiting the synthesis of ergosterol through interaction with the cytochrome P450-dependent enzyme, lanosterol-delta-14-demethylase (Figure 1). While imidazoles can only be applied topically, as a result of systemic toxicity or a lack of peroral bioavailibility, ketoconazole being an exception, the triazoles, such as itraconazole and fluconazole, can also be administered systemically. Fluconazole is still a valid therapeutic option for various types of established or suspected fungal diseases of internal organs. Moreover, it can be used for several types of localized fungal diseases, including oral candidosis. This, in particular, applies to localized candidosis in HIV-infected patients. In this group of patients, however, development of fluconazole resistance has become a problem [17,18], which adds to other drug-related problems, such as interactions with other drugs. Systemic use of azoles can moreover be linked to problems of hepatotoxicity which has limited the use of some, for example ketaconazole for this reason is no longer used for onychomycosis.

Allylamines, such as terbinafine, bind to squalene epoxidase and, thus, also inhibit ergosterol biosynthesis. Terbinafine accumulates in hair, nail and skin and is one of the mainstays in the treatment of dermatophytosis. It can be applied both systemically

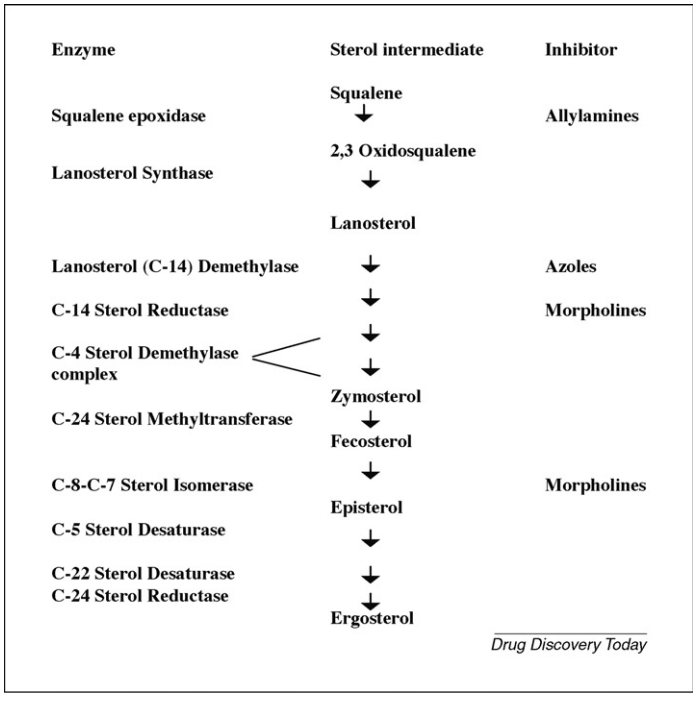

FIGURE 1

Ergosterol biosynthesis pathway and clinically useful inhibitors.

and topically. Systemic terbinafine today clearly is the treatment of choice for onychomycosis [19] not open to topical treatment, topical terbinafine in a particular formulation, that is the so-called film-forming solution, has made single-shot treatment of the most frequent type of dermatophytosis, namely tinea pedis of the interdigital type [20], feasible. The superiority of terbinafine over alternatives for the treatment of dermatophytoses seems to be definitely linked to its high antimicrobial activity against relevant fungal organisms *in vitro*: minimum inhibitory concentrations are by several orders of magnitude lower as compared to azoles, moreover it is not only fungistatic, but fungicidal.

Similar to the allylamines, the morpholines, currently represented by amorolfine, block two enzymes in the ergosterol biosynthetic pathway: these are the sterol C_8–C_7 isomerase and the C-14 sterol reductase (Figure 1). This results in depletion of ergosterol. Amorolfine is mainly active against dermatophytes and yeasts, but its only feasible route of administration is topical [21]. The prime indication clearly is onychomycosis which can be treated using a lacquer.

Flucytosine inhibits fungal protein synthesis on the DNA/RNA level, but is not recommended for monotherapy, due to well-defined problems with both primary and secondary resistance. The spectrum of activity is very limited (*Candida* species, *Cryptococcus neoformans*, some moulds). In general flucytosine is given in combination with amphotericin B. Clinical indications are cryptococcal meningitis and selected life-threatening *Candida* syndromes, such as endocarditis, meningitis and hepatosplenic disease [22].

Griseofulvin interferes with intracellular microtubule production, which inhibits fungal mitosis. Griseofulvin only exerts activity against dermatophytes; it can be given systemically for the long-term therapy of mycoses of the hair, nail and skin. Griseofulvin has been linked to a variety of unwanted effects while cure rates in major indications seem to be less high than claimed earlier. Today tinea capitis is the major indication.

New agents in antifungal therapy focus on the production/integrity of the fungal cell wall: the candins, including echinocandin, pneumocandin and papulacandin, inhibit β-1,3-glucan-synthase, which is not compatible with the formation of an intact cell wall. They are active against several *Candida* spp., but inactive against *C. neoformans*. Although the target does not appear to be closely related to any other target in the mammalian host, moderate reversible haemolysis is a concern.

The mycins, comprising nikkomycin, pramidicin and benanomicin are another group of antimycotic agents, which act on the fungal cell wall. Whereas nikkomycin inhibits the enzyme chitin-synthase [23], pramidicin and benanomicin bind to mannoproteins in the fungal cell wall and, thus, compromise its integrity [24]. They have no major end-organ toxicity, side-effects are discoloration of urine and elevation of hepatic transaminases.

In summary, one can conclude that during the past 50 years an entire armamentarium of potent antifungal drugs for both systemic and localized fungal disease has been established. Fungal disease, however, can still be a challenge. Certainly, it is wise to continue with conventional approaches to development of new antifungal drugs. In this context, interference with the production of major elements of cell membrane or cell wall should still be in focus. Facing increasing problems in terms of drug resistance, however, it would appear to be wise also to consider totally new options. In this context, virulence factors in particular deserve interest as alternatives to conventional drug targets.

Virulence factors of fungi

A virulence factor, produced by a pathogen, is essential for causing disease in a host and refers to its degree of pathogenicity. The word virulent, which is the corresponding adjective to virulence, derives from the Latin word virulentus, which means 'full of poison'.

Obviously, many virulence factors exist and some are of such obvious importance, that they are often taken for granted. These are, for example, the ability to grow at 37°C and physiological pH or a size compatible with alveolar deposition with fungi acquired by inhalation. We will, in this review, concentrate on virulence factors of clinically important fungi.

Proteases

Saps are hydrolytic enzymes and major virulence factors of *C. albicans*. They are encoded by a gene family with 10 members (Sap1–Sap10). The molecular masses of the proteins Sap1 to Sap10 range from 35 to 50 kDa. Most of them possess N-glycosylation sites, Sap9 and 10 contain, in addition, C-terminal consensus sequences typical for glycosylphosphatidylinositol (GPI) proteins [25,26]. Other different enzymatic characteristics are the optimum pH (low with Sap1–3, higher with Sap4–6) and the net charge: While Sap1–3 are negatively charged on the whole, Sap5 is positive [27]. Until recently the exact structure has only been known, however, for Sap2 and Sap3: The crystal structure of Sap2 complexed with pepstatin A has been known since 1993 [28], whereas the crystal structure of Sap3 and its complex with pepstatin A was first presented in 2007 [29] (Figure 2). The secondary structures of Sap2 and 3 as well as 1 and 5 as described recently [27] are very similar and largely composed of beta sheets. Nevertheless, there are differences between all those structures which are responsible for their particular substrate specificities.

The knowledge of the exact structure of individual Saps is considered to be important for the development of specific inhibitors.

The Saps can be attached to the cell membrane, incorporated into the cell wall via GPI anchors (Sap9, Sap10), or released into the extracellular space.

The main roles of these proteinases are to provide nutrition for the cells, to aid penetration and invasion and to evade immune responses. *In vivo* and *in vitro* studies demonstrated that different Sap genes are activated depending on the type of infection (oral, vaginal) and on the time-point of the infection process [30–32].

Whereas Sap1, Sap2 and Sap3 have been shown to contribute significantly to tissue damage and invasion of oral epithelium and cutaneous epidermis, Saps 4, 5 and 6 seem to be important for systemic infections. This was demonstrated by using Sap-deficient mutants [33].

Sap 2 degrades extracellular matrix and host surface proteins, such as keratin, collagen, vimentin and mucin, but also several host defense proteins such as secretory IgA and salivary lactoferrin. In addition it can degrade alpha2-macroglobulin [34] and cystatin A [35] and can also cleave human endothelin-1 precursor (a vasoconstrictive peptide) altering vascular homeostasis [36].

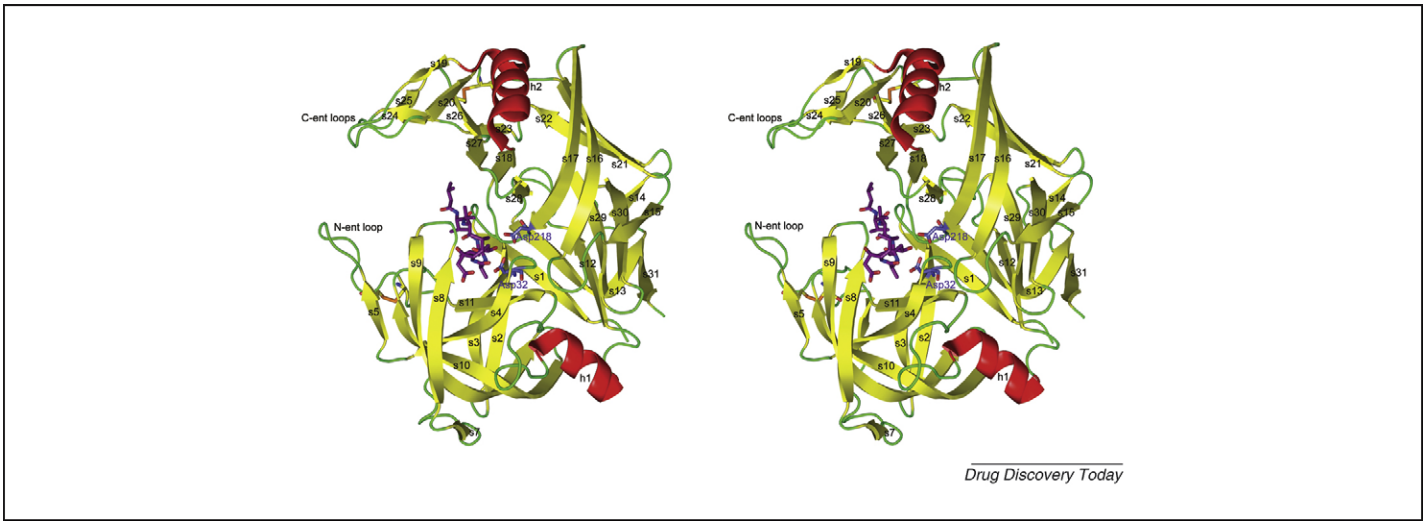

FIGURE 2

Structure of secreted aspartic proteinase 3 (Sap3) complexed with pepstatin A. Stereo overall view of Sap3 shown in *ribbon* structure. The side chains of the two catalytic aspartates are shown in blue, the two-disulphide bridges (Cys47–Cys59, Cys256–Cys294) are displayed in orange. Sap3 structurally appears as a kidney-shaped bilobed globular protein mainly consisting of β-strands. The structure clearly divides into an N-terminal (bottom) and a C-terminal (top) domain. In the active center of Sap3 pepstatin A is bound. The figure was generated with Pymol. From: Borelli *et al.* [29], with permission.

Sap 9 and Sap10 play a role in adhesion, cell separation and cell surface integrity, while almost nothing is known about Sap 7 and Sap 8 [37].

Phospholipases

Another important virulence factor is the secreted phospholipase B in *C. neoformans*. The germ exhibits three phospholipase activities, which are phospholipase B, lysophospholipase and lysophospholipase transacylase. Secreted phospholipase B plays a role in the survival of *C. neoformans* in macrophages, in the destruction of lung tissue and phagocytic activity. In addition to this it is involved in the pathogenesis of *C. albicans*- and *A. fumigatus*-related disease [38,39]. In particular, phospholipase B1 (PLB1) in *C. albicans* has been shown to be required for virulence [40].

Catalases

The list of putative virulence factors or to put it differently, molecules produced during growth of the mycelia for *Aspergillus fumigatus*, in fact the 'most prevalent airborne fungal pathogen in developed countries' [41], has long comprised 'toxic molecules' on the one hand, namely ribonuclease, haemolysin and secondary metabolites such gliotoxin, and 'enzymes' on the other. While aspartic proteinase and phospholipases have to be named in the context, catalases might be the most important. When invading the host *A. fumigatus* is confronted with phagocytic cells attacking them via reactive oxygen species. To detoxify hydrogen peroxide catalases are needed. *A. fumigatus* commands three of them, one being produced by conidia and two by mycelia. One of the catalases, a 749-amino-acid polypeptide, is very similar on structural grounds to a corresponding enzyme to be found with *Aspergillus nidulans*. Based on delicate investigations it has been postulated that it is in fact the mycelia catalases which act as virulence factors [42].

Calcineurin

Calcineurin, a calcium-regulated signaling enzyme, seems to be essential for the virulence of *C. albicans*, especially for survival in serum [43]. This is interesting as calcineurin is also a virulence factor of other fungal organisms: in *C. neoformans,* for example, it is important for the survival at 37°C. Most recently a calcineurin effector protein, CrzA, was characterized in detail and found to regulate conidial germination and hyphal growth. In fact, being considered crucial in pathogenesis it has been called an 'attractive fungus-specific antifungal target' for the treatment of invasive aspergillosis [44].

Melanin

In several fungal species, melanin has been shown to play an important role with respect to virulence: *Wangiella dermatitidis* and *Sporothrix schenkii* produce dihydroxynaphthalene–melanin (DHN–melanin), which protects the fungal hyphae from the host immune system, for example, by scavenging reactive oxygen species and is also able to prevent phagocytosis to some degree [45]. New findings indicate that melanin also contributes to the structural rigidity of cell walls [46]. Dihydroxyphenylalanine–melanin (DOPA–melanin) in *C. neoformans* is postulated to inhibit TNF-alpha production and proliferation of lymphocytes [47]. Additionally, melanized cells seem to be more resistant to enzymatic or microbial lysis and less susceptible to killing by reactive oxygen species (ROS). In fact, the capability of dihydroxiphenylalanine (DOPA) and dihydroxinaphthalene (DHN) melanins of free radical quenching was even called an 'important factor in virulence' [48].

Lipid signaling molecules

Lipid signaling molecules play a role in virulence in several clinically important germs. In *C. neoformans* the sphingolipid–diacylglycerol pathway seems to be involved in the development of the virulence of the organism. Inositol phosphoryl ceramide (IPC) synthase (PC1) transfers an inositol phosphate group from phosphatidyl–inositol (PI) to the C1 hydroxyl of phytoceramide forming IPC and diacylglycerol [49,50] (Figure 3). Diacylglycerol (DAG) has been shown to induce the transcription of antiphagocytic

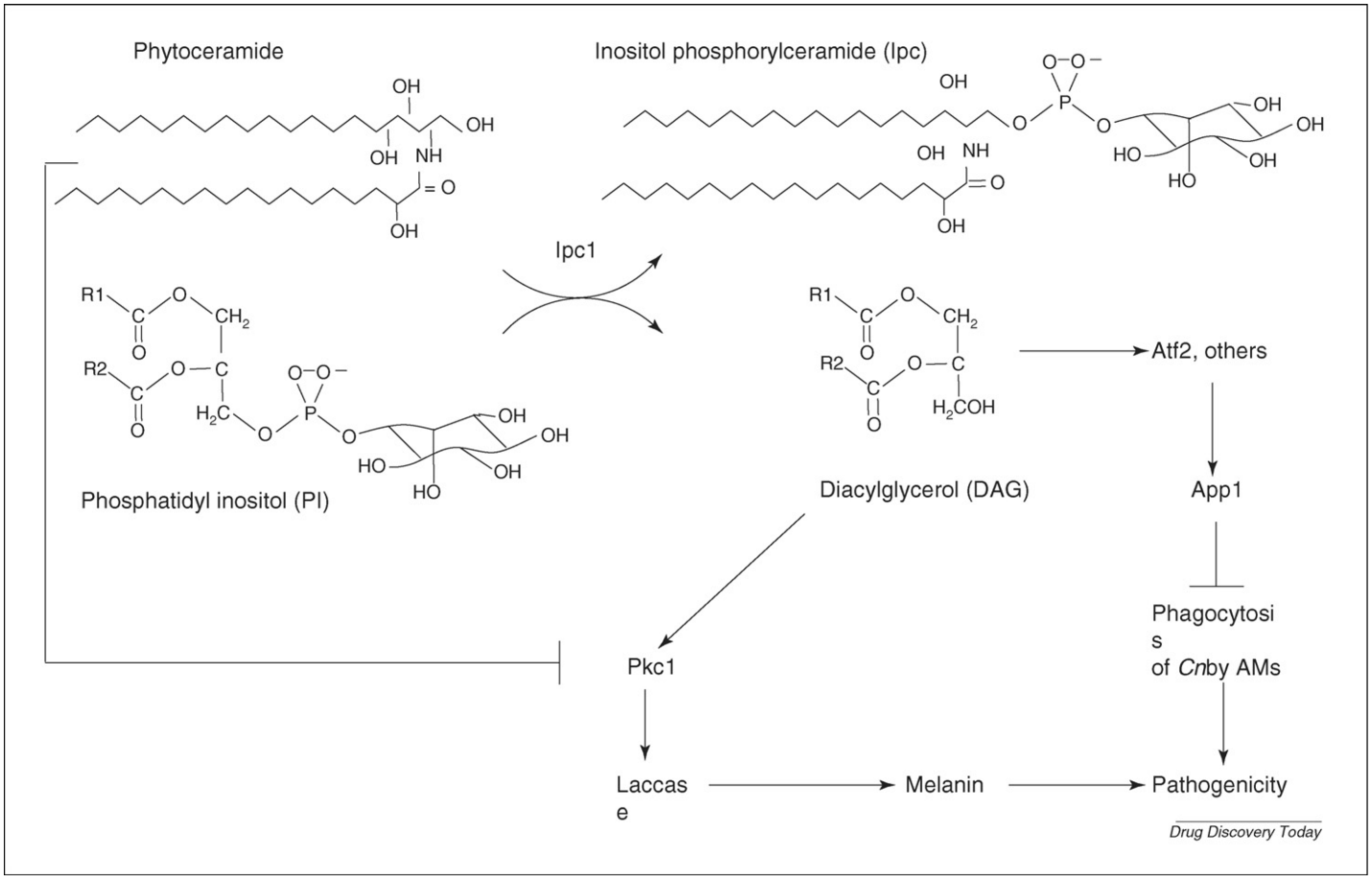

FIGURE 3

IPC1-diacylglycerol signaling. From: Shea and del Poeta [53], with permission.

protein 1 (App1), a putative virulence factor, which inhibits the phagocytosis of fungal cells by alveolar macrophages [51].

Thus, DAG activates the protein kinase C Pkc1, which promotes cell-wall stability and increases melanin production [52,53].

The oxylipin farnesol was found to be increased in *C. albicans* pre-treated with fluconazole in subinhibitory concentrations [13]: Using a mouse model, Navarathna *et al*. could show that mice infected with drug-treated *C. albicans* died earlier and had a greater lethality rate than the mice in control groups infected with untreated *C. albicans*. Farnesol influences the regulation of specific genes involved in hyphal development, drug resistance and iron acquisition and increases the resistance to oxidative stress in *C. albicans*. Thus, farnesol can increase the virulence of *C. albicans*. Interestingly, Semighini *et al*. demonstrated that the growth of *Aspergillus nidulans* was dramatically limited when the fungi *A. nidulans* and *C. albicans* were cultured together, but this growth inhibition was only found in the presence of albumin, which has non-specific lipid-binding properties. Apparently, farnesol also possesses antifungal activity and can induce apoptosis-like cell death [54]. This opens new possibilities of using farnesol as an antifungal agent. Unfortunately, it is a well-known contact allergen [55].

Besides the virulence factors mentioned above, *C. albicans* also commands the following virulence factors: host recognition biomolecules (adhesins); hyphae formation and 'phenotypic switching', which is accompanied by changes in antigen expression, colony morphology and tissue affinities and, thus, allows adaptation to environmental conditions.

Although several virulence factors of fungi have already been detected many more are yet to be discovered. The discovery of further virulence factors would give us the option of further new potential targets for the development of antifungal drugs.

Candidates in the development of new antifungal drugs addressing virulence factors

Pepstatin A is a specific aspartic proteinase inhibitor (Table 1).

In the adherence assay based on human keratinocytes grown *in vitro* it was capable of reducing Candida adhesion to the host cells in a dose-dependent manner [9]. Unfortunately, it cannot be used clinically, at least not systemically, because of its metabolism in the liver and rapid clearance from blood [56]. Yet since the introduction of new anti-HIV drugs of the proteinase inhibitor type, new possibilities for the inhibition of Sap were discovered: HIV-infected patients, treated with HIV proteinase inhibitors, developed fewer fungal infections including clinically apparent oral candidosis.

Zingman described in a case report an HIV-infected patient with oral candidosis, refractory to treatment with fluconazole, itraconazole, amphotericin B and nystatin. After initiation of didanosine (an antiretroviral agent) treatment plus saquinavir (an HIV

TABLE 1

Presence of relevant virulence factors and their inhibitors in relevant fungi.

Virulence factors	Germs	Inhibitors	Refs
Proteinases	*C. albicans*	Pepstatin A, saquinavir, indinavir Human domain antibodies	Korting *et al.* [15] De Bernardis *et al.* [61]
Phospholipases	*C. albicans, C. neoformans, Aspergillus flavus*	Alexidine dihydrochloride, 1,12 bis-(tributylphosphonium)-dodecane dibromide	Ganendren *et al.* [63]
Calcineurin	*C. albicans, C. neoformans*	Tacrolimus, cyclosporin A	Liu *et al.* [65]
Inositol phosphoryl ceramide synthase (IPC1)	*C. neoformans, Candida* spp., *Aspergillus* spp.	Aureobasidin A, khafrefungin	Takesako *et al.* [66]
Elastase	*Trichophyton mentagrophytes, Candida* spp.	Aliphatic aldehydes	Battinelli *et al.* [67]
Hyphal formation	*Candida* spp., *C. neoformans*	Saponins	Zhang *et al.* [68]

proteinase inhibitor) the infection was finally resolved [57]. Similar results were obtained in a retrospective study: treatment with HIV proteinase inhibitors had a positive effect on the frequency and/or severity of mucosal infections in HIV-infected patients [14]. These therapeutic successes were first explained as the result of an improvement in the patient's immune status, but in 1999, Korting *et al*. demonstrated that the HIV proteinase inhibitors saquinavir and indinavir exerted a direct, dose-dependant inhibition of Sap *in vitro* (Figure 4a,b). In the same year Cauda *et al*. demonstrated in a case-control study [58] that HIV proteinase inhibitors directly prevent recurrence of oral candidosis in HIV-infected patients.

Saps of *C. albicans* and HIV proteinase belong to the same class of aspartic proteinases and are inhibited by the classical ligand, pepstatin A. Saquinavir and indinavir have similar inhibitory effects on Saps *in vitro* as pepstatin A. As Sap is important for the pathogenesis of mucosal candidosis, inhibition of this proteinase by saquinavir and indinavir should reduce the virulence of *C. albicans*. Therefore, the development of saquinavir and indinavir as potential anticandidal agents might be justified. Interestingly, saquinavir seems not to inhibit the Sap of *Candida parapsilosis*, whereas ritonavir – another HIV proteinase inhibitor – does reduce its Sap activity [59]. Similar results were obtained by Pichová *et al*. in 2001, who compared the effect of different peptidomimetic Sap inhibitors, derived from the structure of pepstatin A, addressing the Candida species *C. albicans, C. tropicalis, C. parapsilosis* and *C. lusitaniae* [60]. Their findings indicate that Saps of different *Candida* spp. display diverse substrate specificities. This knowledge is important for the possible future development and use of Sap inhibitors.

Another promising approach is the development of antibodies against virulence factors of *C. albicans*: De Bernardis *et al*. showed that human domain antibodies against Sap2 and the 65-kDA mannoprotein inhibit the adherence of *C. albicans* to the epithelial cells of rat vagina and that they exert a protective activity against experimental vaginal candidosis [61].

In the middle of the nineties of the last century Saps and here in particular Sap2 had become subject of an industrial drug development project leading to a potent congener of pepstatin, named A-70450. The compound was found active in the nanomolar range [62]. However, the compound also inhibited renin, a human aspartic proteinase, at a concentration not much higher than the inhibitory concentration for Sap (7.1 vs. 1.4). The industrial project was stopped early, still in the pre-clinics. This was probably primarily due to the fact that in those days the pharmaceutical industry was not yet ready for a paradigm shift: A target like *Candida albicans* Sap seemingly did not fit into the then current concept of broad-spectrum antifungal and in general antimicrobial chemotherapeutic agents.

In 2003 Ganendren *et al*. described agents with antifungal activity by inhibiting phospholipases *in vitro* [63]. These were commercially available compounds with structural similarities to phospholipid substrates: Alexidine dihydrochloride and 1,12 bis-(tributylphosphonium)-dodecane dibromide. Alexidine dihydrochloride is non-toxic to humans and is already used as a bactericidal agent for the treatment of gingivitis. 1,12 bis-(tributylphosphonium)-dodecane dibromide has low haemolytic activity and is known as an ion-pairing reagent. They could be attractive molecules for further development as antifungal agents.

Tacrolimus (FK 506) and cyclosporin A are natural products, which can be isolated from *Streptomyces tsukubaensis* and *Tolypocladium inflatum* Gams. They are potent immunosuppressive substances used after organ transplantation to avoid rejection reactions. They are, however, also able to inhibit virulence factors: by forming a complex with intracellular proteins, called immunophilins, present in mammalian and fungal cells, they inhibit the virulence factor calcineurin [64,65]. Figure 5 presents the complex between calcineurin (CN), cyclosporin A (CsA) and the immunophilin cyclophilin A (CyPA). This leads to the decay of *C. neoformans* cells growing *in vitro* at 36°C. Unfortunately, immunosuppression overweighs antifungal action *in vivo*.

As inositol phosphoryl ceramide synthase is a key enzyme of the sphingolipid pathway (see above), it is an interesting candidate for the development of antifungal agents. Inhibitors of IPC1 already exist, for example aureobasidin A and khafrefungin. Aureobasidin A is a cyclic nonadepsi peptide produced by *Aureobasidium pullulans*. It has antifungal activity against *Candida* spp., *C. neoformans* and some *Aspergillus* spp., but not *A. fumigatus* [66]. They might hold great promise for the development as antifungal agents.

Olive extracts are traditionally used in a variety of troubles, including skin infections. The antifungal activity of some aliphatic aldehydes from olive fruit (hexanal, nonanal, (E)-2-hexenal, (E)-2-heptenal, (E)-2-octenal, (E)-2-nonenal) against *Trichophyton mentagrophytes, Microsporum canis* and *Candida* spp. has been shown *in vitro*. The ability of these substances to inhibit elastase, a virulence

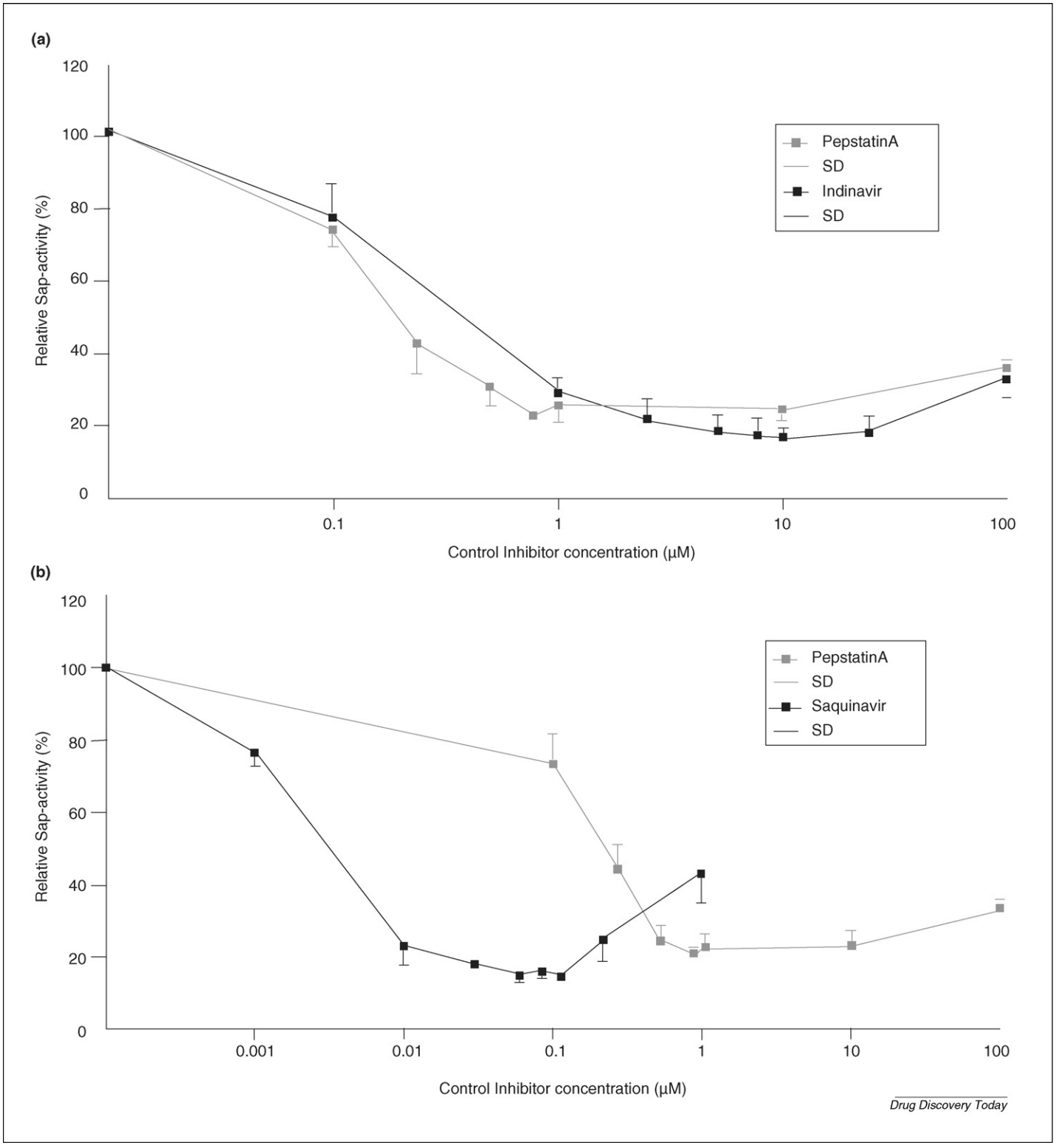

FIGURE 4

(a, b) Percent activity of Saps as a function of inhibitor (saquinavir/indinavir and pepstatin A) concentrations. Each point represents the mean ± standard deviation (S.D.) for three duplicate determinations for five *C. albicans* isolates. The declining activity of saquinavir with high concentrations has not been fully explained yet. From: Korting *et al.* [15], with permission.

factor essential for dermatophytes with respect to skin colonization, clearly deserves interest [67].

The saponins also are natural products, known to have antifungal and antibacterial activity. Zhang *et al*. showed that two steroid saponins, isolated from *Tribulus terrestris L*. are active against *Candida species* and C. *neoformans in vitro* [68]. These two steroid saponins were identified as tigogenin-3-*O*-beta-D-xylopyranosyl (1–2)-[beta-D-xylopyranosyl (1–3)]-beta-D-

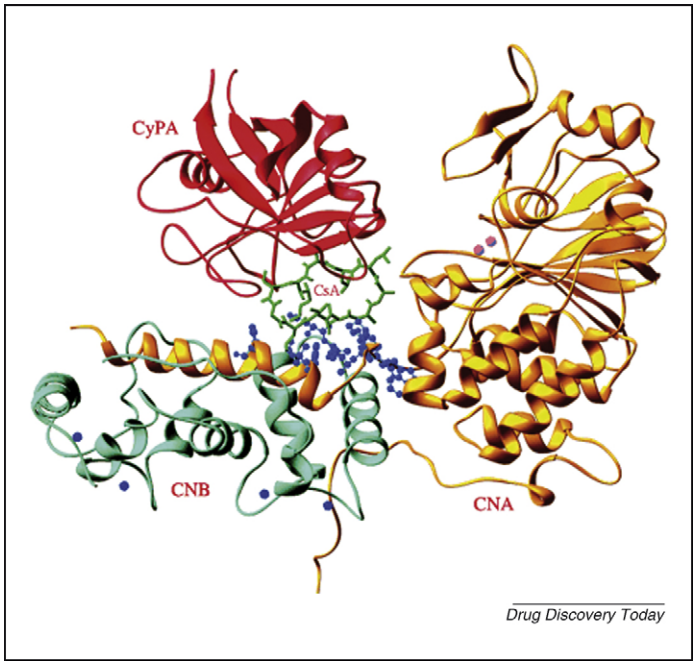

FIGURE 5

Complex between calcineurin (CN), cyclosporin A (CsA) and the immunophilin cyclophilin A (CyPA): ribbon representation of CyPA–CsA–CN. Color codes are catalytic subunit of CN (CNA), gold; regulatory subunit of CN (CNB), cyan; CsA, green; CyPA, red; Zn^{2+} and Fe^{3+}, pink; and calcium, blue. The residues from CN involved in binding of CyPA-CsA are shown as blue balls. From: Huai *et al.* [64], with permission.

glucopyranosyl (1–4)-[alpha-L-rhamnopyranosyl (1–2)]-beta-D-galactopyranoside (=TTS-12) and igogenin-3-*O*-beta-D-glucopyranosyl (1–2)-[beta-D-xylopyranosyl (1–3)]-beta-D-glucopyranosyl (1–4)-beta-D-galactopyranoside (=TTS 15). TTS-12, in particular, inhibits hyphal formation, an important virulence factor of *C. albicans* also being capable of destroying the cell membrane.

Since the turn of the century microbial genome sequencing has become a major tool for obtaining further insight into pathogenesis of infectious diseases [69]. Knowing the genome of relevant pathogens could indeed allow new approaches to target-based drug discovery replacing the conventional 'whole-cell-based procedures' [70]. In the context quorum sensing as a major mechanism of the regulation of gene expression might well come into the focus [71].

Conclusion

Today the obvious need for completely new antimycotic agents is clear, as the rapid increase of severe systemic infections and the spread of resistant micro-organisms are indisputable facts. The present antimycotic agents interfere with the production and integrity of the cell wall, ergosterol biosynthesis and membrane function, nucleic acid biosynthesis and function as well as mitosis.

Virulence factors of fungi clearly hold promise to be new potential drug targets. There are many possibilities for developing agents which can act as inhibitors. Possibly, the clinical potential will already become clear within a few years.

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