Review of Literature
2. Review of literature

2.1. Historical perspective

Medical mycology is the study of mycoses of man and of their etiological agents. Mycoses are the diseases caused by fungi among 50,000 to 500,000 species of moulds that are widespread in nature (soil, plant, water, air etc), approximately 200 species are recognized as pathogens responsible for diseases in humans and animals. These fungal diseases may vary from the generalized superficial skin infections such as dermatophytosis to more specialized mycoses such as coccidioidomycosis. Mycotic infections are encountered unequivocally and ubiquitously all over the world with varied manifestations [164].

Fungal infections afflict all strata of society, irrespective of human race, caste, creed, culture or religion etc. In tropical countries like India, fungal diseases are more prevalent, especially because of high temperature; humidity, and socio-economic conditions. The pathogenic fungi, like moulds and yeasts, grow best in the environments that are warm and humid and where organic matter is abundant [5]. Aspergillosis was one of the first fungal diseases of man or animals recognized. Mayer and Emmert [151] described an infection in the lungs of a Jay (Corvus glandarius). Florentine botanist Micheli was the first who coined the name Aspergillus in his book Nova Plantarum Genera [160]. Micheli noted nine species each of which he gave a sentence-long Latin name (the binomial system of nomenclature developed by Linnaeus did not appear until 1753). Later, in 1809, Link [131] delineated several distinct types that he had isolated from decaying vegetation including Aspergillus flavus, A. candidus, and A. glaucus. The term “aspergillosis” was first used by Fresenius [84] in his work on a fungus infection of the air sac of a bird – The Bustard (Otis tardaga) from the Frankfort Zoo. He named the isolate as Aspergillus fumigatus. Human infection due to this fungus was recognized by Bennet in 1842 [22] and Sluyter described Aspergillus pneumomycosis in 1847. By analyzing his description, the fungus in Bennett's case does not appear to be an Aspergillus, so that the report of Sluyter probably represents the first case of aspergillosis [238].

Virchow published a report of bronchial and pulmonary disease, together with such an accurate description of the etiologic agents as to allow them to
be recognizable as an *A. fumigatus*, in 1856 [253]. This presentation of 4 cases is a classic paper of descriptive pathology. Except for the association of the pulmonary form with "pigeon feeder's" disease by Virchow and later by Dieulafoy *et al.* [70], in the following years very few cases of the disease were reported. In 1897 Renon [210] published a book containing an excellent review of the field and also of the association of the disease with certain occupations in addition to pigeon handlers, such as wig cleaner, and concluded that moldy grain was the source of the conidia. By 1910 almost all forms of aspergillosis in animals had been delineated including mycotic abortion and fatal respiratory disease [13].

2.2. Population at the risk of aspergillosis

Aspergillosis was included as an AIDS-defining opportunistic infection in the initial case of AIDS, reported by the Centers for Disease Control in 1982. Aspergillosis was rarely reported in HIV patient prior to 1990; since then there has been a surge of reports predominantly from North America and Europe [67,162]. This dramatic change in *Aspergillus* epidemiology is not only explained by the rapid expansion of populations at risk, but also by the growing awareness for aspergillosis among clinicians, the availability of non-invasive diagnostic tools and better microbiological laboratory techniques, the implementation of new diagnostic criteria, and the widespread use of limited-spectrum triazoles for prophylactic purposes. For a number of reasons, one can expect that fungal infections in general, and aspergillosis in particular, will further increase over the coming decades:

- The number of solid organ transplantations increases by 1.5 % annually, including high-risk procedures such as liver- and lung transplantation.
- Hematopoietic stem cell transplantation - either autologous or allogeneic - is now a common procedure for the treatment of a vast number of malignancies and immunological disorders.
- Non-myeloablative, so-called mini-transplants are being pioneered for a wide range of cancers; the main objective is to induce a degree of graft-versus-malignancy effect, which may further increase the risk of invasive aspergillosis.
- Cord blood transplants will be done more frequently, but may be complicated by longer periods of granulocytopenia.
A range of immunosuppressive monoclonal antibodies is due to be licensed soon and may shift the spectrum of patients at risk towards non-classical populations (e.g. rheumatoid arthritis, Crohn's disease, etc.).

The use of purine analogues is not any longer confined to the treatment of low-grade lymphoid malignancies; agents such as fludarabine are now also used in cytotoxic regimens for acute leukemia and myelodysplastic syndromes.

The increased use of high-dose corticosteroids in autoimmune disorders and chronic lung diseases has created a new population at risk. AIDS-patients, especially those who fail antiretroviral therapy still develop aspergillosis.

2.3. Clinical manifestation of aspergillosis

Aspergillus fumigatus is a saprophytic fungus which survives and grows over a large variety of organic remains and whose most common ecological niche is on the ground. It is one of the most ubiquitous fungus, due to the ease of dispersion of its conidia [127]. The small size of conidia, from 2 to 3 μm, means that they can remain in suspension in the environment for a long period of time, and can reach the pulmonary alveoli, since human is constantly exposed to inhaling them [1]. It is calculated that a person could inhale several hundred conidia of A. fumigatus per day. In immunocompetent individuals the inhalation of these conidia rarely has serious adverse effects, since they are efficiently eliminated by innate and acquired immune mechanisms [266]. However, when it concerns an immunocompromised host, such as patients who have undergone transplants, patients with various types of leukemia or people infected by HIV, these mechanisms may not be sufficient for the elimination of all the conidia. Although inhalation is the most important transmission route for the acquisition of aspergillosis, the existence of other routes in the hospital environment must not be discounted [259]. For most patients, the main portal of entry and site of infection for A. fumigatus is the respiratory tract. Although other sites of infections have been described in the normal or immunocompromised host, such as the skin, peritoneum, kidneys, bones, eyes, and gastrointestinal tract. Inhalation of conidia of A. fumigatus can give rise to a number of different clinical forms of aspergillosis.
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depending on the immunological status of the host [55]. In non-compromised individuals, *A. fumigatus* can act as potent allergen or cause localized infection of lungs or sinuses. In neutropenic patients there is much vulnerability of establishment of the fungus in the bronchi and dissemination to other organs. This etiologic condition is usually life threatening, even if diagnosed during antemortem cases. It must, however, be emphasized that with early diagnosis and treatment a small but significant number of patients may be cured [258]. *Aspergillus* species may be associated with asthma, pulmonary infiltrative disease, or intracavitary mycetoma or aspergilloma [192]. Hinson *et al.* [101] suggested a classification of three types of aspergillosis with pulmonary infiltrates. These included (i) saprophytic (aspergilloma), (ii) septicemic or pyemic and, (iii) allergic; in the "saprophytic type" the *Aspergillus* colonizes a preexisting lung condition such as brochiectatic cyst (mycetoma), an infarction, neoplasm tuberculosis, pneumonia lung abscess, or bronchitis. According to Rippon [213] there are basically three categories of disease involving aspergilli. These are (i) allergic aspergillosis, (ii) colonizing aspergillosis and, (iii) invasive aspergillosis. In addition, aspergilli can exist as saprobes in bronchi or on body surfaces without eliciting any pathology. Aspergillosis can be both primary and secondary. Allergic and colonizing aspergillosis are often primary conditions, whereas, invasive and especially disseminated aspergillosis are secondary to other disease [190].

The pathogenic spectrum of aspergillosis is complicated, confusing and represents a whole group of diseases. The clinical diseases are being discussed as categories that, most simply, allow separation and characterization of the particular syndrome

2.3.1. Pulmonary-allergic aspergillosis

2.3.1.1. Extrinsic asthma

Asthmatic allergy to conidia of the various *Aspergillus* species is well known and defined disease. It does not vary significantly from allergy to other types of dander and pollen or conidia. The conidia seldom germinate in the bronchial passages. The clinical picture is that of segmental shadowing, most frequently in the upper lobes. It has a tendency to recur in the same segment [98,191]
2.3.1.2. Extrinsic allergic alveolitis

This is a manifestation of allergy to *Aspergillus* conidia in the nonatopic individual. It usually occurs in individuals who have repeated exposure to organic dust that is heavily laden with conidia and mycelial debris. One well-known example of this is the so-called malt worker's lung, which occurs in brewery workers and is associated with moldy (usually *A. clavatus*) barley [212].

2.3.1.3. Allergic bronchopulmonary aspergillosis

First described in 1952 by Hinson *et al.* [101] also called mucomembranous *Aspergillus* bronchitis; this form of aspergillosis may develop as an exaggeration of the above disease, in chronic or extrinsic asthma, late onset asthma, or as some other manifestation of atopic diathesis. The disease is categorized by low grade fever, episodic wheezing, expectoration of brown mucous plugs, transient pulmonary infiltrates, eosinophilia of blood and sputum, markedly elevated serum IgE, positive immediate and late skin tests and precipitating antibody to *Aspergillus* in the serum [90].

2.3.2. Pulmonary-colonizing aspergillosis (aspergilloma)

Aspergilloma is the development of "fungus ball", which may result from chronic allergic aspergillosis [69] or from colonization of preformed cavities caused by other diseases. The former condition is associated with "eosinophillic" pneumonia and bronchiectasis. The patients usually have a history consistent with chronic allergic aspergillosis, and an aspergilloma forms in an ectatic bronchus. Clinically the symptoms are those of the allergic disease, but bouts of severe hemoptysis are more regularly seen. Aspergilloma or fungus balls have been reported in other areas of the body as well. The most commonly involved extra pulmonary sites are the various nasal sinuses. In rare instances aspergilloma may develop in the urinary bladder, this represents dissemination of pulmonary disease in patients with acute myelocytic leukemia [227].

2.3.3. Pulmonary invasive aspergillosis

This is the commonest form of aspergillosis in the immunocompromised patients. Invasive aspergillosis has often occurred in patients with malignant hematological conditions [105,269] and in patients
with renal, liver and heart allografts [7,32,36,169,231]. Much less commonly the disease appears in patients with disseminated solid tumors or collagen disease such as dermatomyositis or systemic lupus erythematosus. Invasive aspergillosis is common in patients with the rare genetic defects of neutrophil polymorphonuclear leucocyte function, such as granulomatous disease of childhood [262]. The lung is the most frequent site of invasive aspergillosis in patients with hematological neoplasia and in organ transplant recipients.

2.3.4. Disseminated aspergillosis

A relatively recent and as yet rare disease, this appears to be a product of the antibiotic-steroid-cytotoxin era. The first case of disseminated disease was reported by Link in 1939 [132]. The lung, brain and leptomeninges were involved. Young et al. [269] reported that the lung was the sole site of infection in 70% of patients.

2.3.5. Central nervous system (CNS) aspergillosis

Aspergillosis of the CNS is rare. About 80 cases of cerebral aspergillosis have been reported so far and these include only a few cases of solitary abscess. Cerebral abscesses due to Aspergillus are usually hematogenous in origin and spread from a primary focus in the lung. Intracranial involvement may be secondary to an adjacent focus in the ear, nose, paranasal sinus or orbit. However, in primary aspergillosis, the exact pathway by which the Aspergillus reaches the brain cannot always be established [163].

2.3.6. Cutaneous aspergillosis

This is a rare disease and is usually secondary to dissemination but may be primary under certain immunosuppressed condition of host [252,39]. Cases have also been described, in which no underlying factors could be discerned and in which the condition is primary infection. In case of primary infection the lesions consist of multiple nodules, and the skin is thick, edematous, and has a purplish discoloration.

2.3.7. Naso-orbital aspergillosis

Aspergillosis of the paranasal air sinuses is a rare entity. It is endemic in Sudan and a few states in South America. It affects healthy and immunocompromised individuals alike. It presents in two forms the invasive and non-invasive aspergillosis. In this disorder primarily the infection occurs in
the nasal sinuses that may eventually involve the orbit of the eye [130]. The lesions most often involved the ethmoid sinuses, although disease in all cavities was recorded.

### 2.3.8.iatrogenic aspergillosis

As described before aspergilli are constantly present in the environment and grow on all types of organic debris. For this reason they may often contaminate hospital rooms and supplies [4,112]. They may thus inadvertently gain entrance to susceptible patients by many portals. *A. fumigatus* growing in the procedure room on leaking dialysis bags may culminate to fatal disseminated disease following dialysis [221].

#### 2.4. Antifungal agents

To combat fungal infections there are several antifungal agents available in the market. These antifungals are available in the form of various formulations as given in Table 1.

**Table 1. Antifungal agents and their activity against *Aspergillus***

<table>
<thead>
<tr>
<th>Drug Class, Drug Name (brand/investigational name)</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyene</strong></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B deoxycholate (Fungizone)a</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Amphotericin B lipidcomplex (Abelcet)a</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Amphotericin B colloidal dispersion (Amphotec) a</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Liposomal amphotericin B (Ambisome)a</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Liposomal nystatin (Nyotran)</td>
<td>Intravenous</td>
</tr>
<tr>
<td><strong>Triazole</strong></td>
<td></td>
</tr>
<tr>
<td>Itraconazole (Sporanox)a</td>
<td>Oral, intravenous</td>
</tr>
<tr>
<td>Voriconazole (VFend)a</td>
<td>Oral, intravenous</td>
</tr>
<tr>
<td>Posaconazole (SCH 56592)</td>
<td>Oral</td>
</tr>
<tr>
<td>Ravuconazole(BMS-207147;ER-30346)</td>
<td>Oral</td>
</tr>
<tr>
<td><strong>Echinocandin</strong></td>
<td></td>
</tr>
<tr>
<td>Caspofungin (Cancidas)a</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Anidulafungin (VER-002; LY303366)</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Micafungin (FK463)</td>
<td>Intravenous</td>
</tr>
</tbody>
</table>

a = Licensed for clinical use in United States.

#### 2.4.1. Polyenes

#### 2.4.1.1. Amphotericin B

Amphotericin B (AmB) is clearly not a newer antifungal since its initial approval for use in 1958; it has remained the reference standard for treatment of invasive aspergillosis as well as the standard of comparison for all newer antifungal agents [240]. However, the fact that amphotericin B remains at such a post is not by virtue of its effectiveness but rather because alternatives were lacking, until recently [120], its mode of action is not completely
AmB binds to membrane sterols, creating transmembrane channels, which result in an increased permeability of monovalent cations [24]. AmB has two major drawbacks. First, because of its insolubility in water, it has to be solubilized in an aqueous milieu to become biologically active. To overcome this problem, the first AmB formulation, Fungizone (marketed by Bristol-Myers Squibb), was a mixture of AmB with the detergent deoxycholate in the ratio 3:7. The second drawback is the toxicity of the molecule to the patient [54,185,187]. Severe side effects, particularly nephrotoxicity are common. Minimum inhibitory concentrations (MICs) of AmB in vitro vary from 0.5 to 2 μg/ml depending upon the formulation tested [75,103]. Several strategies, relying primarily on modifications of the delivery system, have been used to improve the therapeutic effectiveness of AmB and at the same time reduce its toxicity. The most promising approach has involved the modification of the physical state of AmB in different AmB lipid formulations [29,53,57,133,134]. Three lipid formulations of AmB are now commercially available [100,243]. For patients who require treatment with AmB for proven or probable systemic fungal disease but have preexisting renal dysfunction (serum creatinine >2.5 mg/dL), most infectious disease expert advocate a lipid formulation of AmB as initial therapy. Lipid formulation of AmB is 10 to 20 – fold higher in cost per dose. In addition, the optimum daily or total dose of these lipid compounds has not been established.

2.4.1.2. Nystatin

Nystatin, a tetraene diene macrolide, was the first polyene antifungal. It was discovered in 1949 in a soil sample from Virginia and named after the state of New York [97]. Nystatin was licensed for use in 1951 against superficial Candida infections [8]; however, problems with solubility and toxicity with parenteral use limited nystatin to topical use [10]. Recent liposomal reformulation has reduced toxicity and preserved antifungal activity in vitro [154,155]. The new liposomal nystatin (Nyotran; Antigenics) is a multi-lamellar liposomal formulation of dimyristoyl phosphatidyl choline, dimyristol phosphatidyl glycerol, and nystatin (7:3:1) [41].
2.4.2. Azales

2.4.2.1. Itraconazole

First publicly described in 1983 [25,249] and available for treatment of Aspergillus infection in 1990, itraconazole (Sporanox; Ortho-Biotech) adopted a triazole nucleus with higher specificity for the fungal cytochrome P-450 enzyme system over the older imidazoles [256]. Itraconazole's fungicidal activity is not as efficient as that of amphotericin B, because inhibition of sterol synthesis takes longer than directly creating channels in the cell membrane [145]. Historically there have been several constraints with itraconazole: no parenteral formulation, erratic oral absorption in high-risk patients, and significant drug interactions. As a potent inhibitor of the fungal cytochrome P-450 3A4 enzyme, itraconazole also has some affinity for the human enzyme and therefore has important drug interactions. Prior or concurrent use of rifampin, phenytoin, carbamazepine, and phenobarbital should be avoided.

Any drug handled by this cytochrome pathway with normally low bioavailability, extensive first-pass metabolism, or a narrow therapeutic window may be especially vulnerable [113]. Itraconazole's insolubility has led to hampered oral absorption when administered in capsules. To overcome problems with variable absorption, itraconazole has now been solubilized in cyclodextrin, with substantial improvement as an oral solution [61,15]. Cyclodextrins are naturally occurring dough-nut-shaped glucose oligomers produced by enzymatic degradation of starch that have been chemically modified for medical use. The external face of the molecule is hydrophilic to facilitate solubilization of the complex in water and shield a lipophilic guest molecule [242]. Although there is a theoretical concern of polyene-azole antagonism due to ergosterol mechanisms of action, this regimen appeared safe in a recent survey of clinical practice of 93 patients treated with this sequential therapy [186]. This reserves itraconazole for maintenance therapy once the patient's condition improves or as initial therapy for less-immunosuppressed patients. In some studies, itraconazole is a useful alternative therapy to amphotericin B, with comparable response rates [68,241]. The introduction of intravenous itraconazole now offers a new option for patients intolerant of amphotericin B.
2.4.2.2. Voriconazole (UK-109, 496)

Voriconazole (VFend; Pfizer Pharmaceuticals) is a new second-generation triazole synthetic derivative of fluconazole. First described in 1995, it was developed as part of a program to enhance the potency and spectrum of activity of fluconazole. The addition of a methyl group to fluconazole's propyl backbone and the substitution of a triazole moiety with a fluoropyrimidine group increased the affinity for the target enzyme in \(A. fumigatus\) by 1 order of magnitude [225]. Both fungicidal [111] and fungistatic activities [77] against \(Aspergillus\) have been demonstrated. In addition to its action on the 14-a -demethylase, like other azoles, voriconazole also inhibits 24-methylene dehydrolanosterol demethylation, explaining why it is active against a mold such as \(Aspergillus\) when fluconazole is not [111]. Independent of its activity on the cytochrome P-450 enzymes, voriconazole is also the only antifungal under investigation that inhibits \(Aspergillus\) conidiation and pigmentation [250]. One large review of \textit{in vitro} studies showed that voriconazole had activity superior to itraconazole and amphotericin B against \(A. fumigatus\), although itraconazole was superior for \(A. nidulans\) and \(A. terreus\), and all had equivalent activity against \(A. niger\) [76]. One study compared data for 205 \(Aspergillus\) isolates from patients in clinical trials and showed that voriconazole and itraconazole were more potent \textit{in vitro} against \(Aspergillus\) than was amphotericin B, with itraconazole having the lowest MICs [247]. Although voriconazole therapy has not been compared with other treatment modalities, such as itraconazole or echinocandins or combination therapy, in a randomized trial, the superiority of voriconazole demonstrated over the reference standard, initial therapy with amphotericin B, makes it the current first choice of primary therapy for invasive aspergillosis, with a few caveats.

2.4.2.3. Posaconazole (SCH 56592)

Posaconazole (Schering-Plough Research Institute) is a second-generation triazole and is closely related to itraconazole. It is fungicidal \textit{in vitro}, and its activity is slightly superior at 48 h to that of itraconazole and voriconazole yet inferior to that of amphotericin B [146]. At present it is available as an oral formulation, but a new carrier system is in development.
Posaconazole has been effective as salvage therapy against a broad spectrum of invasive fungal infections [205]. Forty-one percent of patients with invasive aspergillosis that was refractory or who had intolerance to standard antifungal therapy had a complete or partial response to posaconazole. In a pilot study of chronic granulomatous disease (CGD) patients with invasive mold infections refractory to voriconazole, posaconazole was safe and effective [233].

2.4.2.4. Ravuconazole (BMS-207147, ER-30346)

Ravuconazole (Bristol-Myers Squibb) is structurally similar to fluconazole and voriconazole, containing a thiazole instead of a second triazole. It is often fungicidal [166,11] and has 47%–74% bioavailability, with linear pharmacokinetics. The drug is well tolerated, with headache a main side effect, and urine studies suggest no cytochrome P-450 isoenzyme induction [74].

2.4.3. Echinocandins

An entirely new class of antifungals, the echinocandins and the amino-containing pneumocandin analogues, are cyclic hexapeptide agents that interfere with cell wall biosynthesis by noncompetitive inhibition of 1,3-D-glucan synthase, an enzyme present in fungi but absent in mammalian cells. This 1,3-β-glucan, an essential cell wall polysaccharide, forms a fibril of 3 helically entwined linear polysaccharides and provides structural integrity for the fungal cell wall [16].

2.4.3.1. Caspofungin (MK-0991, L-743, 872)

Caspofungin (Cancidas; Merck) is a fungicidal water-soluble semi synthetic derivative of the natural product pneumocandin B0 [51]. Caspofungin is also non hemolytic for human and mouse red blood cells, and because 1,3-β-glucan is a selective target present only in fungal cell walls and not in mammalian cells, this eliminates drug mechanism–based toxicity. Caspofungin was approved by the US FDA in February 2001 and is indicated for patients with refractory aspergillosis or intolerance to other therapies. Caspofungin also showed an additive interaction with monocytes and monocyte-derived macrophages, but not neutrophils, on Aspergillus hyphal growth. The mechanism of action is through effector cells damaging hyphae, allowing better activity of the antifungal. A recent update on all 90 patients
enrolled in a trial revealed that 45% had a complete or partial response, and the drug was generally well tolerated [139].

2.4.3.2. Micafungin (FK463)

Micafungin (Fujisawa Healthcare) is an echinocandin lipopeptide compound [161,96], like all echinocandins it is fungistatic in vitro against Aspergillus. A recent study of micafungin combined with existing antifungal agents in pediatric and adult bone marrow transplant recipients with invasive aspergillosis revealed an overall complete or partial response in 39% of patients, including improvement in 40% of allogeneic transplant recipients [207].

2.4.3.3. Anidulafungin (VER-002, LY-303366)

Anidulafungin (Vicuron) is a semisynthetic terphenyl-substituted antifungal derived from echinocandin B, a lipopeptide fungal product [272]. It has shown fungistatic or fungicidal activity in different settings [195].

2.4.4. Allylamines

2.4.4.1. Terbinafine

The allylamine class of antifungals [196], which includes terbinafine, inhibits the enzyme squalene epoxidase in the fungal biosynthesis of ergosterol and is currently indicated for the treatment of superficial dermatophyte and yeast infections. In vitro studies have shown terbinafine to have fungicidal action [193] against Aspergillus similar to that of amphotericin B or itraconazole [223]. Limited reports show some efficacy for invasive aspergillosis, and combination therapy remains a possibility.

2.5. Why limited antifungals are available?

The adequate treatment of mycotic infections is difficult since fungi are eukaryotic organisms with a structure and metabolism that is similar to those of eukaryotic host. For this reason the antifungal agents presently available for treatment of fungal infection can destroy the fungal pathogen as well as damage the host cell.

2.6. Composition of the cell wall and its effect on pathogenesis

Fungus cell wall is highly dynamic structure and despite decades of research elucidating its composition, very little is known about the processes involved in its assembly and subsequent rearrangement during cell growth. The cell wall is composed of a complex of macromolecules that protects the
cell and resists the high turgor pressure of the enclosed protoplast, thereby giving the cell its shape. The main structural components of the A. fumigatus cell wall are polysaccharides that can be divided into two groups, depending on their solubility in hot alkali. The alkali soluble fraction is composed mainly of α (1, 3)-glucans with some galactomannan [254]. The alkali insoluble fraction of the A. fumigatus cell wall, which is the fraction believed to be responsible for fungal cell wall rigidity, has been analyzed in detail [82].

Some components of the wall are directly associated with the colonization of the host tissue and others with the damage to these tissues. In Fig. 1 molecules associated with the virulence of conidium and hyphae are shown. The most abundant polysaccharide in the cell wall is β (1-3)-glucan, which forms a skeleton over it, and becomes an anchor for polysaccharides such as galactomannan and chitin [271]. β (1-3)-glucan has different biological activities, triggering the activation of complement and the production of inflammatory mediators such as leukotrienes and TNFα [107]. Further, β (1-3)-glucan is deposited in the organs over a long period, being metabolized slowly by phagocytes, as opposed to a more rapid metabolism of mannan. It is detected in patients with deep mycosis and it reflects the inflammatory reactions and the immune response of the host. The detection of this component by means of a Limulus G test is used in Japan for the diagnosis of fungal infections, and its kinetics correlate very well with that of galactomannan in patients with invasive aspergillosis [189].

The enzyme glucan synthase is a transmembrane protein complex formed by several proteins: Rho1p, Fks1p and other two proteins, a homolog to a 100 kDa membrane ATPase and another homolog to an ABC (ATP-binding cassette) glucan bacterial transporter of 160 kDa. There are four rho genes (denominated rho1 to rho 4), of which the most studied is rho1, which encodes a protein of 21.5 kDa for the binding and hydrolysis motifs of GTP. The fks1 gene encodes a transmembrane protein, which is the catalytic subunit of β (1-3)-glucan-synthetase and its protein has a molecular weight of 218 kDa [17, 79]. This protein has been detected in the apex of the conidial germ tube where β (1-3)-glucan is actively being synthesized. It has been
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Fig:1. Putative molecules of the conidium and hypha related with virulence (Rementaria et al. 2005) [209]
demonstrated that reduction in the expression of this gene is sufficient to inhibit normal growth [170]. Fks1p is an enzyme essential for the growth of *A. fumigatus* and has been the basis of the development of a new family of antifungals, the echinocandins, whose antifungal mechanism of action is to interfere with the synthesis of glucan by competitive inhibition. Also, in the wall, other enzymes are found to a lesser extent, capable of participating in the extracellular elongation and modification of this polysaccharide and thus in the growth of the fungus. Three glucanosyltransferases enzymes exist for the elongation of glucan (Gel), Gel1p, Gel2p and Gel3p, which are encoded by the *gel1*, *gel2* and *gel3* genes [171]. All of these belong to the family of glycoside hydrolases and contain theanchoring motif to membrane of the glycosyl-phosphatidyl-inositol (GPI). Gel1p appears to be required in the assembly and morphology necessary for the cell wall [40]. These enzymes could be used as targets for the development of new antifungals. Recently a β-(1-3)-endoglucanase of 74 kDa, belonging to a new family of β-(1-3)-glucanases, has been isolated in *A. fumigatus* [172]. This enzyme is encoded by the *engl1* gene, but it has been observed that its disruption does not lead to any phenotype different from the parent strain, thus it does not appear to play an essential role in the constituent cell growth.

Galactomannan has been detected in the walls of hyphae and conidia of *A. fumigatus*. Some authors indicate that, in the conidia, this galactomannan possesses substitutions with sialic acid motifs which could be related to its binding to fibronectin and laminin [260]. Galactomannan can be detected in the supernatants of the cultures of this fungus and is the principal exoantigen released during tissue invasion [127]. Galactomannan is the most useful diagnostic marker, which can be detected in serum, urine, cerebro-spinal fluid, etc., of patients with invasive aspergillosis, although its concentration can fluctuate. Two commercialized tests have been developed for its detection in patients with invasive aspergillosis, Pastorex Aspergillus (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France), based on latex particle agglutination, and Platelia Aspergillus® (Bio-Rad, Marnes-La-Coquette, France), based on the ELISA technique. This latter technique is much more sensitive than the first in detecting this marker [65], and although it can give false positives [85],
it is very useful in the diagnosis of invasive aspergillosis in neutropenic patients [109,159,167,188,222].

The \textit{afmp1} gene encodes an \textit{A. fumigatus} antigenic cell wall galactomannan protein (Afmp1p), which possesses 284 amino acids and a predicted molecular mass of 31.4 kDa. It contains in its sequence a region rich in serine/threonine for O-glycosylation, a signal peptide and a signal GPI, which in many proteins is used for anchoring to the eukaryotic membrane. It seems to have important functions in cell recognition and adhesion, acting like a receptor for the transport of ions and nutrients. Specific antibodies against Afmp1 are only developed in patients infected with \textit{A. fumigatus}. This makes this protein useful in the serological diagnosis of aspergilloma and invasive aspergillosis [271]. Likewise, Afmp1p can be useful as a potential immunomodulatory glycopeptide by its use in nasal immunization systems, which could stimulate the production of specific IgAs. Since \textit{A. fumigatus} is acquired by inhalation, these specific IgAs could prevent the adherence of the fungus to the lung, increase the stimulation of the complement pathway and facilitate its phagocytosis, thus preventing infection. A new protein of this super family of antigenic mannoproteins has recently been discovered. The \textit{afmp2} gene encodes the Afmp2p protein, which has 510 amino acid residues and a molecular mass of 51.5 kDa. It has a N-terminal signal peptide, a putative GPI C-terminal and regions of O-glycosylation. This protein appears to be involved in the assembly of the cell wall, also being secreted in the culture supernatants of this microorganism. As in the case of Afm1p, this protein is immunogenic and antibodies are developed against it. The fact that this is an abundant secretory protein, its minimal cross reactivity with Afmp1p and the presence of antibodies against Afmp2p in patients with infections due to \textit{A. fumigatus}, suggests that it is a good candidate to complement Afmp1p in the sero-diagnosis of these infections [52] and perhaps enabling the improvement of the commercialized systems.

Chitin is the third polysaccharide component of the wall and several chitin synthases (Chs) have been detected in \textit{A. fumigatus}. Several classes of these enzymes (class I to VI) are reported in the literature. The family of \textit{chs} genes in \textit{A. fumigatus} includes at least seven different genes, called \textit{chsA} (class I), \textit{chsB} (class II), \textit{chsC} (class III), \textit{chsD} (class VI), \textit{chsE} (class V), \textit{chsF}
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(chlclass IV) and chsG (class Ill). Of all of them, the only genes in which disruption leads to an altered phenotype in A. fumigatus are chsE and chsG. The chsE- mutant has reduced levels of chitin in the mycelium, swellings throughout the length of the hypha and reduced conidiation [12,157,158].

The strains with chsG- mutations showed a reduced activity of the chitin synthetase enzyme, reduced radial growth and highly branched hyphae, suggesting that the function of the enzyme is in the apex of the hypha. The chsE-ischsG- double mutation is not lethal for this fungus, and the strains that possess it show some characteristics, which are the addition of characteristics of each mutation, and should indicate that these enzymes are related to different, non-interconnected morphogenic routes. The data indicates a participation of the Chs in the virulence of the fungus, perhaps due to stabilization of the cell wall with chitin, and makes those genes and enzymes possible antifungal targets. For example, the mortality rate of mice infected by chsG- or chsC-ischsG- mutant strains was reduced [157]. The mutants in the Chs are viable and pathogenic despite the phenotype changes since the fungus has compensation systems for the loss of these Chs activities and replace the chitin lost by other polysaccharide components. For example, the chsE-IchsG- double mutants have a cell wall highly enriched with α-glucan.

These data would make studying of the acs genes which encode the α (1-3)-glucansynthetase enzymes interesting. Some data show that the disruption of chitin synthesis with inhibitors such as mycomycin are associated with a greater sensitivity and synergic effects with echinocandins and itraconazole, perhaps due to a greater permeability across the wall of these components. A new possibility for treatment would be to study these synergic effects between inhibitors of the cell wall, which may be incapable by themselves to destroy the fungus [158].

2.7. Why cell wall?

A thorough analysis of the biochemical organization and biogenesis of the cell wall of the opportunistic pathogen A. fumigatus is essential to obtain a thorough understanding of the growth of the filamentous fungus, both in vitro and in human host. All exchanges between the fungal cell and its environment rely upon a functional and permeable cell wall. In A. fumigatus, as in other pathogenic fungi, the cell wall is continuously in contact with the host cells and
proteins, and acts as a sieve and a reservoir for molecules such as antigens and enzymes having an active role during infection [127]. This includes alteration and modulation of host immune responses. Moreover, the cell wall is a physically rigid layer, which protects the fungal cell from its environment, especially in the lung where the mycelial and conidial cell wall have a major role against the lung phagocyte killing mechanisms. Cell-wall biosynthetic enzymes have been long recognized as being essential for the fungal life and unique specific drug targets, as a fungus cannot survive without a wall or even if its cell wall is markedly altered from the native form. Fungal cell wall polysaccharides do not have a counterpart in humans suggesting that inhibitors of the fungal polysaccharide biosynthetic pathway should be potent and specific antifungals without marked secondary side effects in humans [18].

2.8. New approaches in antifungal drug development

In recent time, promising novel antifungal agents have been discovered in an effort to enhance the new and unique antifungal compounds. Currently available, highly diverse chemical collections, including large chemical files, natural product libraries and combinatorial chemical libraries, undoubtedly contain important new lead molecules for development of novel antifungals. Many of these compounds have known and defined mechanisms of action against fungal cell and in some cases, aided the identification of new selective targets in fungi [142].

The currently used antifungal agents are prone to resistance and suffer from pharmacological limitations, harmful drug-drug interactions, limited activity spectrum and/or high general cytotoxicity [3]. Therefore, the search for new antifungal components with a novel mode of action is imperative. Recent advances in genetics and genome-based technologies will allow for the identification and validation of new antifungal drug targets to design novel target-based screening strategies.

2.8.1. Inhibitors of fungal cell wall biosynthesis

The fungal cell wall has many vital functions, including physical protection and osmotic stabilization of the protoplast; support for a number of enzymes; regulation of cell shape; prevention of passage of certain large molecules; mediation of adherence and agglutination; and protection from cell
lysis by other microorganisms and by host phagocytes. The fungal cell wall is composed mainly of carbohydrate, some of which are covalently linked to protein. Glucan polymers of (1,3)- and (1,6)-β-linked D-glucose residues and α-mannan, highly glycosylated, secreted mannoproteins each comprise almost 50% of the dry weight of the cell wall. Within the potential complexity of the carbohydrates structures of the fungal cell wall are a number of molecular targets that provide opportunities for antifungal drug discovery. Ideally, molecular targets unique to fungi and essential for viability represent the best candidates. Mannans do not appear to be unique because similar O- and N-glycosylated proteins are found in all eukaryotes. However, the pramidicins and benanomycins are a unique group of antifungal agents that form Ca$^{2+}$-dependent, insoluble complexes with yeast mannans, resulting in subsequent alterations of plasma membrane permeability. Major cell wall targets unique to fungi for which potentially useful antifungal compounds have been identified are chitin synthesis and glucan synthesis. Polyoxins and nikkomycins, analogs of UDP-N-acetyl-D-glucosamine (the natural substrate), are competitive inhibitors of chitin synthase. Compounds of the echinocandins lipopeptide class and the papulacandin glycolipid class are non-competitive inhibitors of (1,3)-β-D-glucan synthase [256]

2.8.2. Progress in the use and delivery of existing antifungal molecules

Mostly, members of azoles cause problem of resistance in fungal pathogens due to excessive use and some of them lack parenteral availability and variable oral absorption make azoles unreliable agents in certain conditions. So, despite recent advances AmB remains the drug of choice for serious fungal infections but it has toxic effect. Thus, currently available antifungal agents are neither broad enough in activity, nor consistently effective and are toxic. The increase in serious fungal infections among greater number of immunocompromised patients warrants the continued effort in developing new antifungal agents or improving the delivery of existing ones. The simplest of these methods is inclusion of these molecules in liposome. In addition to conventional amphotericin B, three fundamentally different lipid-associated formulations have been developed that offer the advantage of an increased daily dose of the parent drug, better delivery to the primary reticuloendothelial organs (the lungs, liver, and spleen) [72,204], and reduced
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toxicity. The US FDA approved amphotericin B lipid complex (Abelcet; Enzon) in December 1995, amphotericin B colloidal dispersion (Amphocil; AstraZeneca or Amphotec; Intermune Pharmaceuticals) in December 1996, and liposomal amphotericin B (AmBisome; Fujisawa Healthcare) in August 1997 [265].

Amphotericin B lipid complex is a tightly packed ribbon-like structure of a bilayered membrane formed by combining dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, and amphotericin B in a ratio of 7:3:3. Amphotericin B colloidal dispersion is composed of disk-like structures of cholesterol sulfate complexed with amphotericin B in an equimolar ratio. Liposomal amphotericin B, the only true liposomal product, consists of small uniformly sized unilamellar vesicles of a lipid bilayer of hydrogenated soy phosphatidylcholine–distcaryl phosphatidylglycerol–cholesterol–amphotericin B in the ratio 2:0.8:1:0.4 [28]. The lipid formulations have unique pharmacokinetics generally have a slower onset of action and are less active than amphotericin B in time-kill studies, presumably because of the required dissociation of free amphotericin B from the lipid vehicle [206]. It is postulated that activated monocytes/macrophages take up drug-laden lipid formulations and transport them to the site of infection, where phospholipases release the free drug.

2.8.3. Selective inhibitors of fungal protein synthesis

Protein synthesis is an attractive target for antibacterials, but that action is hampered in antifungals because of the eukaryotic nature of fungi and thus similarity between fungal and mammalian protein synthesis. Most previous targets used for antifungals exploit differences in the 2 cells, for example ergosterol, 1,3 β-glucan, or chitin [73]. The new sordarin class affects the translocation step of the elongation cycle of protein synthesis [58] by inhibiting elongation factor 2, an essential factor for fungal protein synthesis, and halting the addition of amino acid residues to the growing peptide chain. The sordarins have minimal in vitro activity against Aspergillus [99] and in a disseminated murine model have shown an irregular response and poor survival efficacy. With such a limited protective effect against invasive aspergillosis, this class of antifungals was pursued for clinical use against other fungal infections, but combination therapy against invasive
aspergillosis remains to be explored. Further investigations for this class of antifungal use are presently suspended [148].

2.8.4. Inhibitors of tubulins (novel target)

Microtubules are key components of normal functioning cells and disruption of their activity offers an effective antifungal target. Some of these targets have already been exploited, especially in β-tubulin, but many target sites in other microtubule proteins remain to be exposed and developed. The conserved nature of the target is not a serious constraint and selectivity can be achieved for targeting regions where a single amino acid difference between organisms is functionally important. Rhizoxin, taxol and griseofulvin are the well-known agents having antitubulin activity [37].

2.8.5. Sphingolipid biosynthesis inhibitors

Inositol phosphorylceramide (IPC) synthase is a common and essential enzyme in fungi and plants, which catalyzes the transfer of phosphoinositol to the C-1 hydroxy of ceramide to produce IPC. This reaction is a key step in fungal sphingolipid biosynthesis, therefore the enzyme is a potential target for the development of nontoxic therapeutic antifungal agents. Natural products aureobasidin A (AbA), khafrefungin, and galbonolide A, with a desired biological activity, have been reported. AbA, a cyclic depsipeptide containing 8 amino acids and a hydroxyl acid, is a broad spectrum antifungal with strong activity against many pathogenic fungi such as Candida spp., Cryptococcus neoformans, and some Aspergillus spp. Khafrefungin, an aldonic acid ester with a C22 long alkyl chain, has antifungal activity against C. albicans, C. neoformans, and Saccharomyces cerevisiae. Galbonolide A is a 14-membered macrolide with fungicidal activity against clinically important strains, and is especially potent against C. neoformans. These classes of natural products are potent and specific antifungal agents [228].

2.8.6. Need of immunotherapeutics

The usefulness of the azoles has significantly diminished in recent years as a result of the increasing incidence of resistance. The increase in infection, combined with the reduced efficacy of the currently available drugs, highlights the need for new antifungal drugs and the immunotherapeutics that may suit better. Since antibody forms the natural route of defense use of
these or genetically engineered similar molecules or the peptide sequences derived from antibody stand a good chance of developing into new drugs.

2.8.7. Immunotherapy for treatment of aspergillosis

2.8.7.1. Augmentation of neutrophil number

2.8.7.1.1. Colony-stimulating factors

Colony-stimulating factors (CSFs) are used to accelerate myelopoiesis in neutropenic patients. Prophylaxis with a CSF can reduce the incidence of neutropenic fever by as much as 50%, which in some studies translated into a reduction in hospitalization and use of antibiotics [184]. In one randomized study in patients receiving chemotherapy for acute myelogenous leukemia, prophylaxis with granulocyte-macrophage colony-stimulating factor (GM-CSF) led to a lower frequency of fatal fungal infections compared with placebo (1.9 vs.19%, respectively) and reduced overall early mortality [88]. However, CSFs have not produced a survival advantage in the remainder of studies. CSFs also augment phagocyte function. Granulocyte colony stimulating factor (G-CSF), GM-CSF, and macrophage colony-stimulating factor increase the fungicidal activity of phagocytes in vitro against Candida and Aspergillus species [214,215]. G-CSF influences survival, proliferation, and differentiation of all cells in the neutrophil lineage and augments the function of mature neutrophils. Macrophage colony stimulating factor increases phagocytosis, chemotaxis, and secondary cytokine production in monocytes and macrophages. GM-CSF stimulates various neutrophil effector functions and prolongs neutrophil survival in vitro, accelerates the proliferation of the monocytes macrophage system, and is a potent activator of monocytes and macrophages [176]. Thus, GM-CSF may have a theoretical advantage against pathogens such as Aspergillus species, for which host defense is dependent on both neutrophil and macrophage function. The clinical database on CSFs as adjunctive therapy for fungal infections is inadequate to assess efficacy. The American Society of Clinical Oncology appropriately advises that a CSF should be considered in serious infections, such as invasive fungal infections [184].

2.8.7.1.2. Granulocyte transfusions

The rationale for granulocyte transfusions is to provide supportive therapy for neutropenic patients with a life-threatening infection by
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augmenting the number of circulating neutrophils until neutrophil recovery occurs. Today, the impetus to reevaluate granulocyte transfusions stems largely from improvements made in donor mobilization using G-CSF and corticosteroids [23]. The use of community donors for granulo-cytapheresis was safe in a phase I/II study, thus increasing the pool of potential donors. Granulocyte transfusions for patients with prolonged neutropenia and life-threatening infections refractory to conventional therapy has been advocated [203]. In allogeneic transplants in which the donor and recipient are cytomegalovirus seronegative, using CMV-seronegative granulocyte donors has been advised [178]. The Transfusion Medicine and Hemostasis network of the National Heart Lung and Blood Institute is planning a randomized study of adjunctive granulocyte transfusions in neutropenic patients with severe bacterial and fungal infections.

2.8.7.1.3. IFN-γ

Several cytokines (e.g. interleukin 12 [IL-12], IL-15, IL-18, chemokines) [153,251,264] hold promise as adjunctive therapeutics for invasive fungal infections. IFN-γ augments innate and Th1-dependent immunity, both of which contribute to host defense against Aspergillus infection. IFN-γ augmented human neutrophil oxidative response and killing of Aspergillus hyphae in vitro, and acted additively with G-CSF [215]. It prevented corticosteroid-mediated suppression of neutrophil killing of hyphae [216] and enhanced killing of Aspergillus hyphae by human monocytes. Administration of rIFN-γ to chronic granulomatous disease (CGD) patients augmented ex-vivo neutrophil-mediated damage of Aspergillus hyphae, presumably through non-NADP reduced oxidase-dependent pathways [211]. In animal models, augmentation of the Th1/Th2 cytokine balance either through administration or depletion of cytokines conferred protection in experimental aspergillosis [66,48]. Though preliminary results suggest that rIFN-γ is safe in allogeneic hematopoietic stem cell transplantation (HSCT) recipients [226], the safety of rIFN-γ cannot be predicted based on this limited database, and therefore merits evaluation in a clinical trial with safety as the primary endpoint.

2.8.7.2. Innate pathogen recognition pathways

2.8.7.2.1. Toll-like receptors
Toll-like receptors (TLRs) recognize common pattern motifs on microbes, and initiate T-cell and dendritic cell (DC) maturation. *Aspergillus* conidia, but not hyphae, stimulate macrophages to produce the pro-inflammatory cytokines tumor necrosis factor-α and IL-10 in a TLR4-dependent fashion [177]. In contrast, *Aspergillus* hyphae, but not conidia, stimulated production of the anti-inflammatory cytokine IL-10 through TLR2-dependent mechanisms. This switch from a pro-inflammatory to anti-inflammatory signals during germination may help *Aspergillus* evade host defense. Others found that both TLR2 and TLR4 recognize *Aspergillus* hyphae, stimulate pro-inflammatory cytokines in effector cells, and stimulate neutrophil recruitment [156,143]. Local delivery of CpG oligodeoxynucleotides (which signal through TLR 9) and the AspF16 *Aspergillus* allergen resulted in activation of airway DCs capable of inducing Th1 priming and resistance to the fungus in mice [26].

Thymosin α1 a naturally occurring thymic peptide, induced maturation and IL-12 production in DCs pulsed with *Aspergillus*, an effect mediated by distinct TLRs [217]. Thymosin α1, augmented Th1 immunity against *Aspergillus*, accelerated myeloid recovery in neutropenic mice, and was protective against *Aspergillus* challenge in murine hematopoietic stem cell transplantation HSCT recipients. Recognition of *Aspergillus* motifs and activation of neutrophils is coordinated by distinct members of the TLR family, each likely activating specialized antifungal effector functions and inflammatory responses [20]. Indeed, liposomal amphotericin B, in addition to its intrinsic antifungal activity, may activate antifungal resistance by activating TLR-4 in neutrophils [19]. These studies provide a rationale to stimulate or inhibit specific classes of TLRs to enhance innate and antigen specific immunity to fungi.

2.8.7.2.2. Pentraxins

Pentraxin-3 is an innate pathogen recognition protein that binds to specific motifs on *A. fumigatus* and other pathogens. Pentraxin-3 -deficient mice had defective recognition of conidia by alveolar macrophages and DCs, inappropriate induction of type 2 cytokine responses, and were highly susceptible to *Aspergillus* infection [86]. Administration of pentraxin 3 protected against *Aspergillus* challenge in murine hematopoietic stem cell
transplantation (HSCT) recipients and potentiated the protective effect of sub therapeutic amphotericin B.

2.8.7.3. Vaccines

Vaccine development is a priority for several fungal pathogens and requires knowledge about host-pathogen interactions [219]. The importance of cell-mediated immunity against Aspergillus infection has become well established in mice [66]. Immunization of immunocompetent mice with an Aspergillus crude culture filtrate resulted in memory responses mediated by antigen-specific, Th-1 -committed CD4+ T cells [47]. Adoptive transfer of these cells conferred protection to neutropenic mice - establishing a "proof of principle " regarding the cellular immunity as a target for immune augmentation in invasive aspergillosis. Cellular adoptive immunotherapy may also include active vaccination with DCs. DCs pulsed with C. albicans or A. fumigatus activated CD4+ Th1 cell responses on adoptive transfer into immunocompetent mice. The infusion of fungus-pulsed DCs accelerated the recovery of antifungal Th1 responses in mouse allogeneic HSCT recipients and conferred protection against aspergillosis [27]. DCs are also key players in containing and dampening inflammatory responses by tolerization through the induction of regulatory T cells. These studies in mice demonstrate the functional plasticity of DCs in response to fungi that can be exploited in vaccine development [194].

Recently Torosantucci et al. [245] generated a novel glyco-conjugate vaccine against fungal pathogens and conjugated laminarin (Lam), a well characterized but poorly immunogenic β-glucan preparation from the brown alga Laminaria digitata, with the dipetheria toxoid CRM197, a carrier protein used in some glyco-conjugate bacterial vaccines. This Lam-CRM conjugate proved to be immunogenic and protective as immunoprophylactic vaccine against both systemic and mucosal (vaginal) infections by C. albicans. Lam-CRM-vaccinated mice also were protected from a lethal challenge with conidia of A. fumigatus, and their serum bound to and markedly inhibited the growth of A. fumigatus hyphae. Thus, this novel conjugate vaccine can efficiently immunize and protect against two major fungal pathogens by mechanisms that may include direct antifungal properties of anti-β-glucan antibodies.
2.8.7.4. Genomics

Fungal genomics offers an opportunity to develop novel antifungal agents that are complementary to traditional drug-screening methods focused on existing targets. DNA microarray chips for A. fumigatus are now being made available to investigators through The Institute for Genomic Research. Genomics will be an important tool in understanding the molecular biology of Aspergillus and will facilitate the identification of novel virulence genes. Emerging experimental analysis tools, such as chemogenomics, fitness profiling, transcript profiling, and proteomics will further enhance the analysis of genome wide functional studies [110]. This knowledge may be exploited to identify novel targets for drug discovery. Moreover, characterizing gene profiles of host cells (e.g., phagocytes, endothelial cells) to Aspergillus will throw new light on the broad range of host responses to this pathogen, and potentially pave the way to new immune augmentation strategies.

2.8.7.5. Monoclonal antibodies in therapy

The potential therapeutic application of monoclonal antibody created a tremendous interest in the medical and pharmaceutical community. A recent survey suggested that over a quarter of all biotech drugs in development are MAbs. Within this group more than 30 chimeric, humanized, or fully human antibodies account for more than 30 products being routinely used or investigated in the clinic for various indications. The most promising and advanced therapeutic strategies include inhibition of alloimmune and autoimmune reactivity, antitumor therapy, antiplatelet therapy, and antiviral therapy.

Two MAbs, which were recently approved by US-FDA for the treatment of non-Hodgkin's lymphoma (Rituxan) and breast cancer (Herceptin), already produce annual sales in the US$100 million to US$500 million range. Therapeutics MAbs, which approved in USA and Europe, are given in Table 2. The ability of monoclonal antibodies to bind specifically to antigen and to block antigen function or synthesis has led to their increasing importance as laboratory reagents, diagnostics, and therapeutics. The mode of action of MAb is totally different from conventional antifungal drugs. The modular structures of antibodies have also made it possible to construct smaller antibody fragments, such as the Fab and single chain Fv (scFv) that retain the
antigen-binding properties of the immunoglobulin from which they were derived. Besides having different pharmacokinetic properties which can be useful, [268] Fab and scFv can be expressed in functional form in *E. coli*, allowing rapid protein engineering to reduce immunogenicity, increase affinity, or alter specificity [91]. Single chain Fv has proven particularly useful, since they can be encoded in a single gene and yield a single polypeptide chain. Compared to heterodimeric IgG or dimeric Fab, scFv facilitates construction of fusion proteins, such as immunotoxins and viral or nonviral gene therapy vectors [80]. MAbs are classically obtained from mice with a purified antigen (hybridoma technology). The variable region genes of MAbs can be used to construct recombinant Fab or scFv antibody fragments.

**Table 2. Therapeutic MAbs approved in the United States and Euopean Union**

<table>
<thead>
<tr>
<th>Sponsor Company</th>
<th>Generic name</th>
<th>MAb type</th>
<th>Therapeutic category</th>
</tr>
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<tbody>
<tr>
<td>Johnson &amp; Johnson</td>
<td>Muromonab-CD3</td>
<td>Murine</td>
<td>Immunological</td>
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<tr>
<td>Centocor</td>
<td>Abciximab</td>
<td>Chimeric</td>
<td>Hemostasis</td>
</tr>
<tr>
<td>Biogen IDEC</td>
<td>Rituximab</td>
<td>Chimeric</td>
<td>Antineoplastic</td>
</tr>
<tr>
<td>Protein Design Labs</td>
<td>Daclizumab</td>
<td>Humanized</td>
<td>Immunological</td>
</tr>
<tr>
<td>Novartis</td>
<td>Basiliximab</td>
<td>Chimeric</td>
<td>Immunological</td>
</tr>
<tr>
<td>MedImmune</td>
<td>Palivizumab</td>
<td>Humanized</td>
<td>Anti-infective</td>
</tr>
<tr>
<td>Centocor</td>
<td>Infliximab</td>
<td>Chimeric</td>
<td>Immunological</td>
</tr>
<tr>
<td>Genentech</td>
<td>Trastuzumab</td>
<td>Humanized</td>
<td>Antineoplastic</td>
</tr>
<tr>
<td>Wyeth</td>
<td>Gemtuzumab ozogamicin</td>
<td>Humanized</td>
<td>Antineoplastic</td>
</tr>
<tr>
<td>Millenium/ILEX</td>
<td>Alemtuzumab</td>
<td>Humanized</td>
<td>Antineoplastic</td>
</tr>
<tr>
<td>Biogen IDEC</td>
<td>Ibritumonab tiuxetan</td>
<td>Murine</td>
<td>Antineoplastic</td>
</tr>
<tr>
<td>Abbott</td>
<td>Adalimumab</td>
<td>Human</td>
<td>Immunological</td>
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<td>Omalizumab</td>
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<tr>
<td>Genentech</td>
<td>Bevacizumab</td>
<td>Humanized</td>
<td>Antineoplastic</td>
</tr>
</tbody>
</table>

Many fungicidal monoclonal antibodies have been produced against *C. albicans* and *C. neoformans*. Monoclonal antibodies that mimic the image of yeast killer toxin [149] and bind with the β-glucan of killer toxin receptor, and another MAb-C7 [32] raised against the main target (>200 kDa) of salivary slgA in the cell wall of *C. albicans*, are reported to be candidacidal. Recently a humanized MAb (Mycograb®) directed against HSP90 [198] has entered clinical trials for the treatment of candidiasis. Recently candidacidal activity of
a monoclonal antibody MAb-G5 that binds with glycosyl moieties of proteins of *C. albicans* has been reported [115]. Very few monoclonal antibodies have been used in immunotherapy against experimental aspergillosis; recently efficacy of KT MAbs against *A. fumigatus* was investigated in a mouse model of T-cell-depleted allogeneic bone marrow transplantation (BMT) with invasive pulmonary aspergillosis. Treatment with KT MAbs protected the mice from infection, as judged by the long-term survival and decreased pathology associated with inhibition of fungal growth and hyphal development in the lungs [49].

It has been reported previously that monoclonal antibodies could be attached to the distal ends of chains of long-circulating liposomes prepared by coating the liposome surface with PEG [183]. Monoclonal antibody 34A recognizes surface glycoproteins that are expressed on the luminal surface of the pulmonary capillary vessel wall in the mouse lung. Binding of this immuno liposome to the lung is very rapid and is not captured by reticulo-endothelial system (RES). Combination of this MAb and AmB (34A-PEG-L-AmB) produced a marked reduction in the number of *A. fumigatus* organisms in the lungs. Pharmacokinetic studies showed the presence of high AmB concentrations in the plasma of mice treated with PEG-L-AmB (40.8 mg/ml) and in the lungs of mice treated with 34A-PEG-L-AmB (42.3 mg/g). It showed that 34A-PEG-L-AmB, a long circulating immunoliposomal AmB, is a promising form of AmB against invasive pulmonary aspergillosis.

### 2.8.7.6. Proteomics

As proteins are the principal targets of drug discovery, the evolution of proteomics techniques is of major importance to the drug development process. While the complexity of structure and function in the proteome presents a significant challenge, the pharmaceutical industry and its biotech and academic partners are expending a tremendous amount of resources to decipher and utilize proteomic data to make drug development more efficient and successful. The practice of proteomics ranges from the identification of thousands of proteins in a particular model system, to the detailed analysis of the 3D structure, possible modifications/isoforms, and function of a single protein. Screening of proteins on the gene level is not without caveats. A gene can lead to the production of numerous protein species through alternative
splicing products. Post-translational modifications of each protein, such as phosphorylations or proteolytic cleavage, often regulate protein activity, while the addition of complex tags, such as oligosaccharides or lipid anchors, are used for cell compartment localization. Such post-translational modifications give rise to many additional protein products originating from a single gene or from a single transcript, thus increasing the complexity of identifying a disease-related protein. All of these explorations can be equally valuable depending upon the issue addressed and the phase of drug development.

2.8.7.7. Target identification

High-throughput proteomics, identifying potentially hundreds to thousands of protein expression changes in model systems following perturbation by drug treatment or disease, lends itself particularly well to target identification in drug discovery. In addition to the hope that proteomics technologies can help achieve higher drug development success rates, the recent emphasis on developing disease-modifying compounds makes proteomic analyses of disease etiology and progression of critical importance. Researchers at Pfizer, Inc., using protein identification by two-dimensional gel electrophoresis (2-DE) and mass spectrometry, profiled protein expression changes in a rat model of endothelial-induced cardiac hypertrophy and myocardial infarction [138]. This approach helped drug companies build large databases of possible protein targets in heart disease.

2.8.7.8. Biomarker identification

In addition to using proteomic tools to identify an array of proteins that are modified in the diseased state, a significant focus of proteomic activities in drug discovery has turned to the identification of biomarkers in easily accessible biological fluids. The importance of the development of such markers is evident when one considers the influence of such a tool on all stages of drug development. Not only can a biomarker aid in the understanding of the disease process and progression and what molecular pathways are involved, but also this biomarker can then serve as a monitoring tool in later stages of development. For instance, a change in the status of this marker may be useful in determining the efficacy of various drug candidates in the process of lead optimization, and then can also be used in the selection of
appropriate animal models for pre-clinical studies as well as in patient profiling for clinical trials.

2.8.7.9. Identifying protein modifications

Beyond the identification of proteins involved in disease progression, it is often critical to explore protein modifications to elucidate the changes in molecular pathways involved in disease. In a study of lymphocytes with normal or Scott syndrome (inherited bleeding disorder) phenotype, changes in the tyrosine phosphorylation status of Ig-chain precursor, fascin, and actin associated proteins were identified by immunoprecipitation of tyrosine-phosphorylated proteins followed by separation of the proteins by 2-DE [106]. Using an alternative method for immunoprecipitation of phosphorylated proteins, scientists investigated the phosphorylation of caspase-9 (which inhibits apoptosis) via the ERK-MAPK pathway, which may help to resolve the mechanism of tumor induction during constitutive activation of this pathway. Another protein modification that is clearly modified in many disease states is glycosylation; in fact, many proteins known to be involved in disease processes or that are already being used as biomarkers or possible therapeutic candidates are glycoproteins. The result of these phosphorylation and glycosylation studies highlights the importance of identifying potential modifications in proteins of interest, as these modifications may be indicative of the disease state or may prove to be valuable ways to develop biomarker assays.

2.8.7.10. Exploring protein-protein interactions

The study of molecular pathways in diseased and normal states necessitates the development of tools to study protein-protein interactions. Methods used to study protein interaction include two-hybrid systems, phage display, Biaocore® surface plasmon resonance (SPR) technology, and the more recent techniques of reverse transfection and tandem affinity purification (TAP). Reverse transfection involves creating an array of the cDNA of interest on a glass slide, followed by transfection of cells that attach to the slide. The protein is then generally visualized with fluorescent antibodies to assess its localization. This technique can be combined with a two-hybrid approach to assess protein interactions [34]. Advances in mass spectrometry have also facilitated the study of protein-protein complexes. Fourier transform ion
cyclotron resonance mass spectrometry (FTICR-MS) provides high mass resolution and accuracy of whole proteins and protein-protein complexes via the circulation of ions in superconducting magnets; isotopes can be clearly resolved in high charge states.

2.8.7.11. Target validation

It is important to establish that a potential protein target is present in the disease-relevant cell or human tissue, the next phase in development is to begin to validate that target by modulating the protein’s activity in a model system to determine the outcome on disease phenotype. One of the most powerful tools in target validation, though, has been the use of models where the protein target of interest has been completely "knocked out". While knockout mice have been very useful in this area, they are costly and time-consuming models. Alternative systems to the knockout animal are becoming available to facilitate target validation; one of the most exciting of these is RNA interference (RNAi), a technique where protein expression is “silenced” at the post-translational level. Protein and antibody microarrays are also being used for screening of protein interaction and function. Protein “biochips” or microarrays are ideal for studying receptor-ligand interactions, enzyme activity, and antibody-antigen interaction with rapid throughput. These tools can be useful for both understanding molecular pathways in which the target protein participates, as well as screening model systems for changes that occur in disease states or following drug treatment (i.e. enzyme activity testing) [104]. Tools for screening interactions with entire “proteome sets” of both known and unknown proteins are also available in microarray format.

The results of recent proteomics pursuits have demonstrated the potential value that proteomics has to offer in drug development. Proteomics techniques are providing precise and fairly rapid methods to screen both target proteins and potential therapeutics compounds. In-house, drug companies have mainly focused on proteins profiling for target identification, to develop efficacy and toxicity biomarkers, and to create valuable protein databases for access in future projects.
2.8.7.12. Engineered killer mimotopes: New synthetic peptides for antimicrobial therapy

A novel approach to produce synthetic antibiotic peptides (killer mimotopes), similar to those described for the conversion of epitopes into peptide mimotopes, may allow their use as surrogate vaccines. Synthetic peptides pertaining to the complementarity determining regions (CDRs) of a recombinant antiidiotypic antibody (PaKTscFv), which mimic the wide spectrum of microbicidal activity of a killer toxin produced by the yeast *Pichia anomala* (PaKT), have proven to act as structural or functional mimotopes of PaKT. This activity appeared to be mediated by interaction with specific cell wall killer toxin receptors (KTRs), mainly constituted by β glucans. Killer mimotopes have shown *in vitro* an impressive microbicidal activity against *C. albicans*. They were adopted as a model of PaKT- and PaKTscFv susceptible microorganisms. Optimization through alanine scanning led to the generation of an engineered decapeptide (KP) of a CDR-L1 pertaining antibody fragment with an enhanced *in vitro* microbicidal activity. It had a potent therapeutic effect against experimental vaginal and systemic candidiasis in normal and immunodeficient mice caused by fluconazole susceptible and resistant yeast isolates. KP exerted a microbicidal activity in vitro against multidrug-resistant eukaryotic and prokaryotic pathogenic microorganisms, which was neutralized by interaction with laminarin (β 1,3-glucans). Recently therapeutic activity of this killer peptide has been reported against experimental paracoccidioidomycosis. To our knowledge, KP represents the prototype of an engineered peptide fragment derived from a microbicidal recombinant antiidiotypic antibody. It is capable of exerting antimicrobial activity *in vitro* and a therapeutic effect *in vivo* presumably acting through interaction with the β glucan KTR component in the cell walls of pathogenic microorganisms especially in fungus [142].

2.8.8. Proteome analysis of *A. fumigatus*

The field of fungal proteomics is gathering pace as a result of the simultaneous occurrence of fungal genome sequence availability and advances in high sensitivity protein mass spectrometry. Many *Aspergillus* species have merited attention from a biotechnological standpoint, as a consequence of a secreted proteome rich in industrially useful enzymes [175].
Until recently, however, little information was available with respect to either the technical requirements for protein extraction or the proteome content of most *Aspergillus* species. However, dissection of the secreted proteome (secretome) of *Aspergillus flavus* has demonstrated the ability of this organism to adapt to altered conditions such as the presence of various carbon sources (e.g., rutin) [152]. With respect to the human pathogenic fungus, *A. fumigatus*, Bruneau et al. adopted a proteomic approach to identify glycosylphosphatidylinositol-anchored proteins, which are involved in the organization of the fungal cell wall [33]. More recently, identification of nonribosomal peptide synthetases in *A. fumigatus* using a combined proteomic and molecular approach [208] has been described. The recent completion of the sequencing of the *A. fumigatus* genome and the availability of the *in silico* annotated genome at (www.cadre.man.ac.uk) [137] should enable detailed molecular dissection of the biology of this organism. Moreover, both unannotated and annotated genome data can be interrogated to increase our knowledge of the *A. fumigatus* proteome and the organismal response to altered environmental conditions. In January 2006 an Irish group identified 54 intracellular proteins from 2D-gel electrophoresis through MALDI-MS [38]. Still there are more to know in case of *A. fumigatus* proteome analysis.