Perspective

Environmental fungicides and triazole resistance in *Aspergillus*

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Abstract

Fungal diseases are problematic in both human health and agriculture. Treatment options are limited and resistance may emerge. The relatively recent recognition of triazole resistance in *Aspergillus fumigatus* has prompted questioning of the origin of resistance. While multiple mechanisms are described in clinical isolates from triazole-treated patients, some *de novo* resistance is also recognised, especially attributable to TR$_{34}$/L98H. Such strains probably arose in the environment, and, indeed, multiple studies have now demonstrated TR$_{34}$/L98H triazole resistance strains of *A. fumigatus* from soil. Docking and other *in vitro* studies are consistent with environmental resistance induction through exposure to certain triazole fungicides, notably difenoconazole, propiconazole, epoxiconazole, bromuconazole and tebuconazole. This article addresses the potential implications of this issue for both human health and food security.
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The triazole class of drugs is the mainstay of both human antifungal therapy and agricultural antifungal treatments (Fig. 1). Several recent publications have linked triazole resistance in the environmentally derived human pathogen *Aspergillus fumigatus* with agricultural fungicide use.[1] The European Centre for Disease Prevention and Control (ECDC) recently convened an expert committee on this topic which reviewed the issue in detail and offered several recommendations.[2] A major concern is that this situation may represent an early stage in the evolution of widespread antifungal drug resistance, paralleling the rise in antibiotic resistance, which has been driven by the use of compounds with a common mode of action in livestock and in humans.[1−4] These issues are summarised here.

The triazole mode of action is via inhibition of the fungal ergosterol biosynthetic pathway, specifically by inhibition of lanosterol 14 alpha-demethylase, and triazoles are otherwise known as demethylase inhibitors (DMIs).[5] Azoles have also been shown to inhibit the activity of other related cytochrome P450 enzymes, such as human 14 alpha-demethylases, and steroid biosynthesis genes, such as CYP17A. The antifungal efficacy of azoles may be the result of different roles of sterols in mammals and fungi: in mammalian cells cholesterol is limited to a role in membrane structure, whereas in fungi ergosterol also effectively functions as an oxygen sensor.[6] Sterols in both kingdoms complex with sphingolipids in the membrane to form rigid lipid rafts that anchor GPI-linked proteins to the membrane and harbour other important protein subclasses.[7,8] Plant and human fungal pathogens share the same ergosterol biosynthesis pathway, although the pathway appears to have been duplicated in certain organisms.

Fungal plant pathogens cause extensive loss of crops in every area of human agriculture. Fungicide options in crop treatment are considerably wider than in medicine, with a number of alternative chemistries available such as QoI (complex III target), benomyl (tubulin) or complex II inhibitors such as bixafen, fluapyroxad and isopyrazam.

Globally, around 200 000 people develop invasive aspergillosis annually,[9] and at least 10 times that number are at risk (1.5 – 10% of highly immunocompromised patients) (Fig. 2). Those infected with an TR$_{34}$/L98H multiazole-resistant isolate have an 88% mortality,[10] in contrast to those infected with susceptible isolates, where the mortality is 30 – 50% of those treated.[11]

Chronic pulmonary aspergillosis (CPA) follows tuberculosis in around 10% of people (United Kingdom) without immune deficits, as well as other lung problems such as sarcoidosis, chronic obstructive pulmonary disease (COPD) and lung cancer survivors, after pneumothorax[12] (Fig. 2). About 1.2 million people are estimated to be living with CPA following TB, and overall about 3 million, with ~450 000 deaths each year.[13] Antifungal therapy is up to 60% effective for CPA, but it must be given long term (years) to arrest progression of the disease.[14,15]

Aspergillus can exacerbate asthma either with allergic bronchopulmonary aspergillosis (ABPA) or severe asthma with sensitisation (SAFS). ABPA affects ~2.5% of adult asthmatics (estimated at ~4.8 million of ~200 million worldwide) (Fig. 2), and SAFS probably affects 1 – 10% of adult asthmatics with severe asthma (3.5 – 15 million). ABPA and SAFS patients improve with itraconazole, voriconazole or posaconazole, with 60 – 80% responding to therapy.[16,17]

There are only three triazole antifungals licensed for the treatment of aspergillosis. Itraconazole (Sporonox, Janssen Pharmaceutica) was the first to become available in 1991 and was the first orally active drug for aspergillosis.[18] Prior to this there was only amphotericin B, which is only active intravenously. So the

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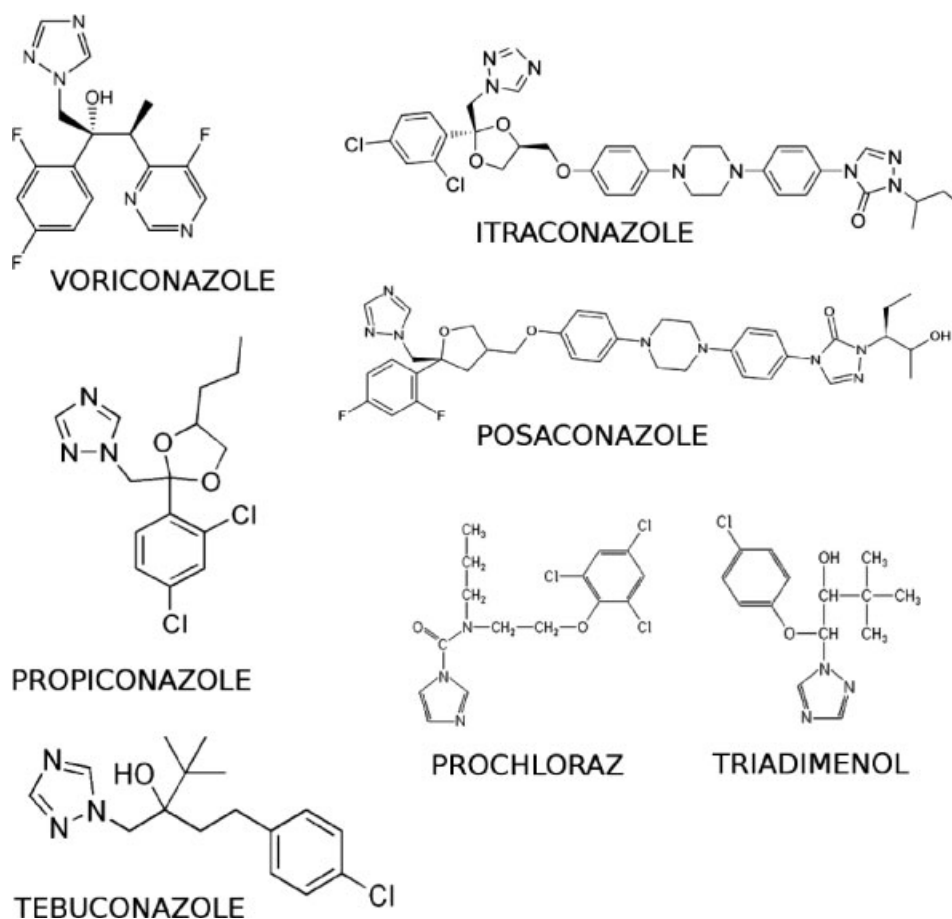
Figure 1. Structures of important triazoles in human (itraconazole, voriconazole and posaconazole) and agricultural use (propiconazole, tebuconazole, prochloraz and triadimenol).

advent of oral therapy for life-threatening invasive aspergillosis was revolutionary and not readily accepted by a skeptical medical fraternity, especially because of poor oral absorption in some patients, usually those at most risk of dying, lots of drug–drug interactions and modest (30–40%) response rates. In 2002, voriconazole (Vfend, Pfizer) was approved following a very challenging pair of randomised controlled studies that demonstrated superiority over amphotericin B.[19] The combination of intravenous and oral voriconazole, with a 20% better overall response rate, propelled voriconazole to first-line therapy for invasive aspergillosis in almost all clinical guidelines and markets.[11,20] In 2006, posaconazole (Noxifil, Merck) was approved for the prevention of fungal infection (especially aspergillosis) in leukaemia and bone marrow transplant patients,[21,22] and for second-line therapy.[23] Just as voriconazole improved survival for treatment, so posaconazole improved survival by preventing infection in high-risk patients. Posaconazole is only available orally, and poor bioavailability limits its utility in many patients currently.

Of the 25+ azole (demethylation inhibitor) fungicides that have been marketed over the last 30 years, only a few bear a structural similarity to the three clinically licensed azoles. Their eradicant and protectant properties, with broad-spectrum systemic action, have earned many azoles a key role in controlling certain plant diseases, ensuring high yields and quality produce (especially cereals and soybeans). Over 25% of total fungicide sales are azoles, and most are triazoles. Examples of agricultural

triazoles include prothioconazole, myclobutanil, penconazole, fenbuconazole, epoxiconazole, tebuconazole, cyproconazole, metconazole, propiconazole, difenoconazole and flusilazole, and imidazoles include imazalil, prochloraz, triflumizole, pyrifenox and triforine. Triazoles are important fungicides for arable crops, controlling rusts and mildews, *Septoria* leaf blotch, *Rhynchosporium* and *Fusarium*. Seed treatments of wheat and barley are predominantly triazoles. Various other triazoles are used to control fruit rot and the like. High numbers of applications (i.e. up to 6 times annually) are especially necessary on soft fruit and fruit trees, herbaceous plants and hops. Both tebuconazole and propiconazole are complementary for wood preservation and are primary components of copper organic wood preservatives (with copper carbonate) for treatment of timbers.

Itraconazole resistance was first described in 1997 in two isolates of *A. fumigatus* isolates from California, cultured in 1988/1989.[24] Since 2003 a rising number of isolates of *A. fumigatus* have been isolated, primarily in northern Europe.[25] A particular double mutation resistance mechanism called TR$_{34}$/L98H was found in many isolates from the Netherlands, denoting the combination of a promoter-based tandem repeat of 39 base pairs upstream of the Erg11 or 14 alpha-demethylase gene and a histidine for lysine substitution in position 98 of the same gene, the key target of all azole antifungals.[26] In the Netherlands the prevalence of azole resistance varied between 0.8 and 9.4% in different hospitals.[10] In Denmark 4.5% of *A. fumigatus* isolates obtained from 133 cystic fibrosis (CF) patients were triazole resistant,
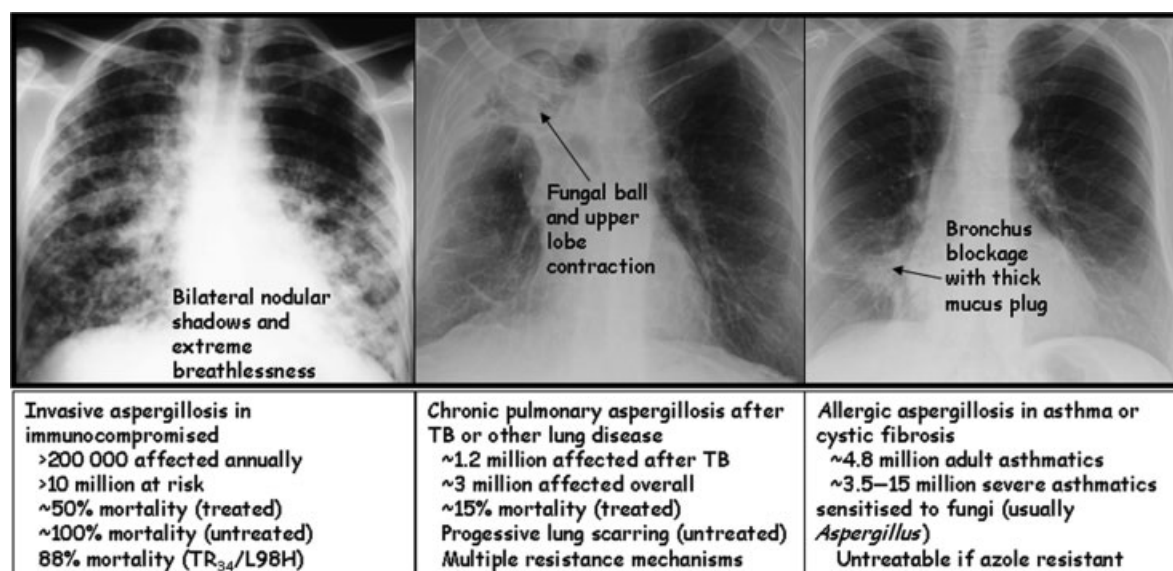
Figure 2. Examples of three different human forms of aspergillosis of the lungs.

most harbouring the TR$_{34}$/L98H mutation,[27] and in France 8% of CF patients had resistant isolates, 50% carrying the TR$_{34}$/L98H mutation.[28] A 5.8% resistance rate was found in a worldwide survey of 62 medical centres in 2008/2009, with most resistant isolates (80% TR$_{34}$/L98H) coming from Hangzhou in China.[29,30] Isolates harbouring the TR$_{34}$/L98H mutation have now been found in India[31] and can be detected directly by molecular methods, without culture.[32] Concurrently, many other target mutations have been found, and modelling indicates how these many mutations may confer resistance to one or more triazoles.[33–35] It seems unlikely that medical azole use is responsible for such widespread environmental azole resistance. For example, itraconazole is used in the United Kingdom in kilogramme amounts yearly, compared with tonnes of agricultural azoles, and cannot be excreted from the human body in active form. Ketoconazole-containing shampoo and fluconazole for thrush are unlikely to contribute, as both agents have negligible activity against *Aspergillus* spp. Other topical azoles are used in miniscule (g) amounts. Veterinary use is similarly modest compared with agricultural use. Although resistance appears to be largely related to itraconazole, this is likely to be due to the fact that multiple mutations appear to confer itraconazole resistance, few isolates of *A. fumigatus* are killed by itraconazole compared with voriconazole and posaconazole and the separation between susceptible and resistant minimum inhibitory concentrations has been defined for longer and is clearer. Many tonnes of agricultural azoles are applied to a large geographical area. For example, in the United Kingdom over 8 million hectares are treated annually, suggesting that exposure of environmental fungi to azoles may be relatively low per square metre, but low azole fungicide concentrations are probably more conducive to resistance emergence.

Isolates harbouring the TR$_{34}$/L98H mutation have also been found in the environment, in both city and hospital environments and agricultural settings.[36–38] In Belgium the air contained 1.8% of itraconazole-resistant *Aspergillus*.[39] Recently voriconazole-resistant isolates, which may be susceptible to itraconazole and posaconazole, have been found in patients and in the environment in the Netherlands, each bearing a different combination resistance marker denoted TR$_{46}$/Y121F/T289A.[40]

Some triazole fungicides have a similar 3D structure to the three medical triazoles (Fig. 1). A range of agricultural azoles have been shown to be active against *A. fumigatus* and to bind CYP51A effectively in computational models. It is noticeable that agricultural azoles that inhibit *A. fumigatus* are structurally similar to the three medical triazoles and appear to bind CYP51A in a similar manner (Figure 3). In particular, difenoconazole, propiconazole, epoxiconazole, bromuconazole and tebuconazole are structurally very similar to the medical triazoles and active against *A. fumigatus in vitro*. TR$_{34}$/L98H isolates are resistant to these very same five triazole fungicides, and *in silico* modelling suggests that the TR$_{34}$/L98H resistance mechanism may prevent proper alignment of these triazole fungicides to AfCYP51A, although L98H is not in the active site of the enzyme.[41] Similar computational models show nearly identical drug binding in the *Mycosphaerella graminicola* CYP51 protein.[42] In this study, known *M. graminicola* azole-resistant CYP51 mutants were shown to have reduced azole binding capability. These studies support the suggestion that *A. fumigatus* is likely to develop resistance to agricultural azoles in selective situations, and that mutations leading to resistance to agricultural azoles may also give rise to resistance to medical azoles. It is important to note that the agricultural azoles show different levels of activity against *A. fumigatus*: imazalil, prochloraz and metconazole show similar potency; difenoconazole, bromuconazole, tebuconazole and propiconazole show ∼4–20-fold lower activity, depending on the isolate tested; epoxiconazole and prothioconazole show 4–40-fold lower activity; triflumizole, fenarimol, nuarimol, triadimefon, triadimenol, bitertanol, penconazole, cyproconazole and myclobutanil are essentially inactive.[41]

In reality the situation is more complex. The *M. graminicola* CYP51 gene is in fact an orthologue of the Af CYP51B gene, in which resistance mutations are not known to be associated with resistance in *A. fumigatus*. It has never been demonstrated that *A. fumigatus* receives sufficient azole challenge in the environment to select for drug resistance. Additionally, azoles are usually applied to plants in mixtures with other classes of antifungal that might prevent emergence of single drug resistance mechanisms. Nevertheless, azole resistance is readily observable in many plant
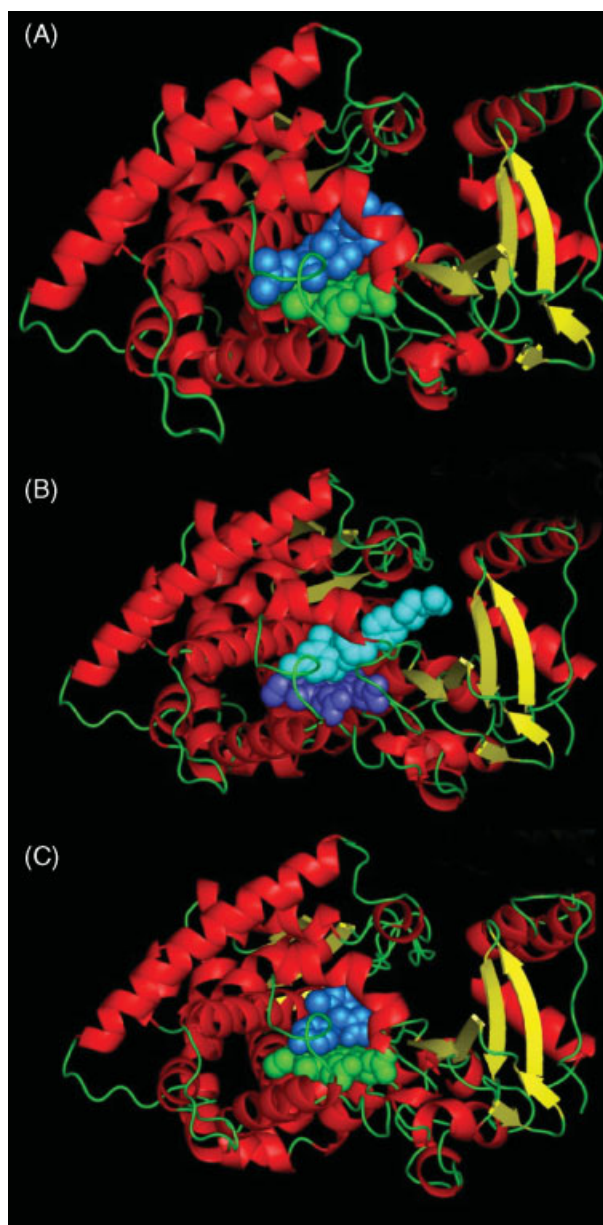
Figure 3. Computational models of (A) lanosterol, (B) itraconazole and (C) epoxiconazole bound to the *An fumigatus* CYP51A protein. The natural substrate for the enzyme, lanosterol (blue spheres), is shown in contact with the heme molecule (green spheres) in the putative active site of the enzyme. Itraconazole (panel B) is shown in light blue conjugated to the heme (purple spheres), and epoxiconazole is shown in blue (panel C) conjugated with the heme (green spheres). Models were constructed using Modeller v.9 (Andrej Sali, San Francisco, CA), and ligands were docked using Autodock Vina (The Scripps Research Institute. La Jolla, CA). Molecular dynamics was performed using AMBER (University of California, San Francisco, CA) or GROMOS (Swiss Federal Institute of Technology, Zürich, Switzerland).

pathogenic fungi; indeed, either reduced sensitivity or overt resistance to triazoles has been reported in *Magnaporthe grisea* (rice), *Mycosphaerella graminicola* (wheat), *Botrytis cinerea* (strawberry), *Rhynchosporium secalis* (barley), *Pyrenopeziza brassicae* (oilseed rape), *Venturia inaequalis* (apple) and *Monilinia fructicola* (stone fruit).

Although some triazoles were approved in Europe as fungicides in the 1980s, approval of propiconazole (1980),

tebuconazole (1986), difenoconazole (1988), bromuconazole (1990) and epoxiconazole (1993) introduction into routine use was often much later (1990–1996 in the Netherlands). In any case, the use of these triazoles preceded the first culture of TR$_{34}$/L98H resistance clinical isolate in 1998. Neither of the isolates obtained in the United States in 1988/1989 was TR$_{34}$/L98H,[24] and nor were most of the early itraconazole-resistant isolates reported.[43]

While there are alternative intravenous antifungals for invasive aspergillosis [amphotericin B and the echinocandins micafungin (Astellas) and caspofungin (Merck)], only the triazoles are available for oral use. Patients with CPA, ABPA and SAFS who develop triazole resistance become effectively untreatable.

So what are the implications for farmers, the agrochemical community and regulators? Direct confirmation that spraying fields with specific azole fungicides leads to triazole resistance in *A. fumigatus* (especially the TR$_{34}$/L98H mutation) is lacking. However, the circumstantial evidence is strong and at least as compelling as that related to antibiotics in animal feeds and certain patterns of bacterial resistance. Clearly, fungi do not employ the same plasmid-based resistance mechanisms as bacteria, and therefore spread of resistance is not likely to follow a similar promiscuous pattern but rather be confined to instances of resistance arising on a genome-specific basis. The immediate ban of the implicated fungicide triazoles would have a major negative impact on food yields from certain crops, with potentially profound food supply and economic consequences. In fact, loss of azole is predicted to result in loss of food self-sufficiency for many EU countries (http://www.ecpa.eu/article/agriculture-today/assessment-economic-importance-azoles-european-agriculture-wheat-case-study). However, even moderate increases in azole resistance in clinically important fungi would result in a significant impact on morbidity, mortality and economic cost of disease. It is possible that withdrawing triazole fungicides from some crop spraying rotations may have no discernable impact on crop yield, but more research and modelling is required to identify further the cause or the sources of the development of azole resistance in both the clinical setting and the environment. Other possible scenarios might include withdrawing triazole fungicides from the spraying of some crops but retaining their use in seed dressing, where they are disseminated much less through the environment. Use of alternatives to triazoles in wood preservation is another option, so that long-term localised triazole 'reservoirs' (in fence posts stuck in the ground) are removed. Also, the use of azoles that are not active against clinically important fungi could reduce selection pressure.

The management of azole resistance in crops has a very strong user and science base. Several organisations exist to monitor and study resistance [e.g. in the United Kingdom: FRAC (http://www.frac.info/frac/index.htm) and ADAS (http://www.adas.co.uk/); in the United States: the EPA (http://www.epa.gov/)] and have been quick to introduce strategies to reduce risks of phytopathogen fungicide resistance. Although it has never been straightforward, the farming and research communities working together with the agrochemical industry have been able to continue using azoles as an effective antifungal class for many years, in spite of continuous identification of azole-resistant pathogens in the field.[44] Models of field fungicide resistance dynamics are available that could be used to limit the development of fungicide resistance.[45,46] One challenge to these strategies in Europe has been the introduction of EU Regulation 1107/2009 (http://ec.europa.eu/food/plant/protection/index_en.htm), which

limits the use of fungicides that may have undesirable effects on humans. This legislation is still in an implementation phase and its effects are not yet known, but it does not consider risks of drug resistance in human pathogens. A consensus between the agricultural fungicide community and the medical fungicide community is urgently needed in order to design strategies that could reduce the risks of generating drug-resistant human pathogens in the environment. The ECDC report urgently recommends both increased surveillance for clinical azole-resistant *A. fumigatus*, continuing environmental testing for azole-resistant pathogens and field trials to test the impact of azole use on the development of resistance in clinically important fungi. In this context, closer collaboration between the agricultural fungicide community and clinical mycologists is essential.

Additional information about *Aspergillus* and aspergillosis can be found on the encyclopedic *Aspergillus* website (www.aspergillus.org.uk), and about human fungal infections generally on the LIFE website (www.LIFE-Worldwide.org).

Conflicts of interest

Both authors were members of the ECDC expert committee on triazole resistance. Paul Bowyer has received grant support from the Fungal Infection Trust, BBSRC, National Institute of Health Research, the European Union Framework 7 and AstraZeneca and holds founder shares in Alergenetica S.L. Dr Denning holds founder shares in F2G Ltd, a University of Manchester spin-out company, and has current grant support from the National Institute of Allergy and Infectious Diseases, the National Institute of Health Research, the European Union and AstraZeneca. He acts as an advisor/consultant to Myconostica (now part of the Lab21 group), as well as to other companies over the last 5 years, including Pfizer, Merck, Astellas T2 Biosystems and Gilead. He has been paid for talks on behalf of Merck, Astellas, GSK, Novartis, Merck, Dainippon and Pfizer.

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