APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2007, p. 5011–5019 Vol. 73, No. 15
0099-2240/07/$08.00+0 doi:10.1128/AEM.00285-07
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

# Control of *Mycosphaerella graminicola* on Wheat Seedlings by Medical Drugs Known To Modulate the Activity of ATP-Binding Cassette Transporters▽

Ramin Roohparvar,[1,2] Aurelie Huser,[1,3] Lute-Harm Zwiers,[1,4] and Maarten A. De Waard[1]*

*Laboratory of Phytopathology, Wageningen University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands[1]; Department of Cereal Research, Seed and Plant Improvement Institute, Agricultural Research and Education Organization, P.O. Box 31585-4119, Karaj, Iran[2]; Department of Molecular Plant Microbe Interactions, Molecular Plant Development, Max Planck Institute of Plant Breeding Research, Carl von Linne Weg 10, 50829 Köln, Germany[3]; and Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, P.O. Box 85167, 3508 AD Utrecht, The Netherlands[4]*

Received 5 February 2007/Accepted 23 May 2007

**Medical drugs known to modulate the activity of human ATP-binding cassette (ABC) transporter proteins (modulators) were tested for the ability to potentiate the activity of the azole fungicide cyproconazole against in vitro growth of *Mycosphaerella graminicola* and to control disease development due to this pathogen on wheat seedlings. In vitro modulation of cyproconazole activity could be demonstrated in paper disk bioassays. Some of the active modulators (amitriptyline, flavanone, and phenothiazines) increased the accumulation of cyproconazole in *M. graminicola*, suggesting that they reversed cyproconazole efflux. However, synergism between cyproconazole and modulators against *M. graminicola* on wheat seedlings could not be shown. Despite their low in vitro toxicity to *M. graminicola*, some modulators (amitriptyline, loperamide, and promazine) did show significant intrinsic disease control activity in preventive and curative foliar spray tests with wheat seedlings. The results suggest that these compounds have indirect disease control activity based on modulation of fungal ABC transporters essential for virulence and constitute a new class of disease control agents.**

Plant-pathogenic fungi possess various mechanisms to cope with the activity of natural toxic compounds that may occur in their living environments. These compounds can be either antibiotics produced by other microorganisms or plant defense compounds present in host plants, such as phytoalexins and phytoanticipins. The mechanisms may involve the evolution of insensitive target sites, compartmentalization, and metabolism of toxic compounds into nontoxic products. Another mechanism operating in many organisms involves reduced accumulation of toxic compounds at the target site due to secretion by ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters (8, 33). These transporters are membrane pumps that can transport a wide variety of natural products, including plant antimicrobials. Transport mediated by these transport systems into the outer environment can significantly reduce the intracellular concentration of toxicants and protect organisms with a sensitive target site against toxic activity of toxic compounds. The validity of this mechanism of natural insensitivity to natural toxic products was first demonstrated in studies with a mutant of *Staphylococcus aureus* that lacks the multidrug pump NorA (18). The same mechanism has been described for the ABC transporters BcAtrB and GpAbc1 from the plant pathogens *Botrytis cinerea* and *Gibberella pulicaris*, which function as virulence factors of these pathogens by providing protection against the plant defense products resveratrol in grapevine and rishitin in potato, respectively (11, 28). The importance of ABC transporters in virulence has also been established for ABC transporter mutants of *Magnaporthe grisea* and *Mycosphaerella graminicola* on rice and wheat, respectively. However, the plant defense compounds acting as substrates for these transporters have not yet been identified (32, 34). MFS transporters can also mediate natural insensitivity to plant defense compounds, as shown for Bcmfs1 from *B. cinerea* and MgMfs1 from *M. graminicola* (15, 27).

A second physiological function of ABC and MFS transporters from plant pathogens is the secretion of endogenous toxic products that are relevant for saprophytic survival and virulence on host plants. The known examples are MFS transporters of *Cercospora kikuchii*, *Cochliobolus carbonum*, and *Fusarium sporothichioides* that mediate the secretion of cercosporin, HT toxin, and trichothecene, respectively (1, 4, 23). These transporters also function in self-protection against the toxins. ABC transporters with a similar role in the virulence of plant pathogens have not been reported, but it is expected that they do exist.

ABC and MFS transporters belong to the largest protein families identified. The redundancy of the transporters and their broad and overlapping substrate specificities explain why these proteins are also able to transport xenobiotics over biological membranes. The xenobiotics transported include medical drugs, antibiotics, antimycotics, and agricultural fungicides (2). In this respect, ABC transporters have become particularly known for their role in multidrug resistance (MDR), which is the simultaneous development of resistance against multiple unrelated chemicals. This phenomenon was first demonstrated in cancer cells after prolonged treatment with anticancer drugs. MDR can be based on overexpression of specific ABC transporters that prevent accumulation of the drugs in cancer

* Corresponding author. Mailing address: Laboratory of Phytopathology, Wageningen University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands. Phone: 31 317 48 31 23. Fax: 31 317 48 34 12. E-mail: maarten.dewaard@wur.nl.

▽ Published ahead of print on 1 June 2007.

TABLE 1. Putative functions of ABC transporters from plant pathogens and exploitation of modulators of these proteins in disease control

| Function of ABC transporters | Effect of modulators |
|---|---|
| Prevention of accumulation of plant defense products in fungal cells during pathogenesis | Increased accumulation of plant defense products in fungal cells resulting in reduced fungal growth and colonization of plant tissue |
| Secretion of fungal toxins with a role in virulence | Suicidal activity on the producing fungus; reduced virulence on host plants |
| Reduction of sensitivity of plant pathogens to fungicides with a sensitive target site | Synergistic activity between modulators and fungicides resulting in a wider spectrum of antifungal activity of fungicide |
| MDR of plant pathogens | Reversal of MDR to wild-type sensitivity of multiple chemicals |

cells to effective concentrations at their target sites (13). At present, MDR is also a serious threat to effective control of bacterial and fungal diseases in mammals and a potential risk in chemical control of agricultural pests and diseases (9).

The important role of ABC transporters in MDR of cancer cells has led to wide interest in pharmacological agents that inhibit the activity of ABC transporters. Such agents are described in medical literature as modulators or reversal agents since they may reduce MDR in oncology (26). The wide interest in these agents has resulted in the discovery of hundreds of compounds that inhibit ABC transporter activity. Some characteristics of these compounds involve amphiphilic properties, the presence of aromatic rings, and a positive charge at neutral pH (39). In oncology, modulators are classified as first-generation modulators (compounds already used in clinics for other indications), second-generation modulators (analogues of these drugs), and third-generation modulators (drugs with original structures developed for the purpose of MDR reversal) (26). Many natural toxic compounds, such as plant alkaloids and flavonoids, have also been described as multidrug pump inhibitors (14, 21). Isoflavones have been described as potentiators of antibacterial activity of natural toxic plant compounds, indicating that they enhance activity of (endogenous) natural antimicrobial compounds (8, 22). Recently, the synthetic flavonoid derivative 4′-hydroxyflavone was identified as an inhibitor of fungicide efflux in the plant pathogen *Pyrenophora tritici-repens* which is capable of restoring fungicide activity against fungicide-resistant isolates to normal wild-type sensitivity (25). In view of the considerations described above, modulators of fungal ABC transporters can be regarded as promising lead compounds for control of disease due to plant pathogens. The putative ways in which these compounds can be exploited are summarized in Table 1.

In this study we selected from the literature a number of modulators representing diverse chemical groups. These compounds were analyzed to determine their in vitro modulation of azole fungicide activity against isolates of *M. graminicola* that differ in sensitivity to azoles. This fungus is considered the major threat for wheat crops in Europe and causes serious losses in both bread and durum wheat. Disease management is based mainly on the use of resistant wheat cultivars and chemical control, particularly with azole fungicides. Compounds which enhanced the activity of the azole fungicide cyproconazole in vitro were tested further to determine their effects on accumulation of azole fungicides in mycelium and to determine their synergistic activities in control of the pathogen. Some of the modulators selected displayed disease control activity on their own. The activities of these products were analyzed further.

## MATERIALS AND METHODS

**Fungal strains, cultural conditions, and preparation of biomass suspensions.** The *M. graminicola* strains used in this study were field isolates IPO323 and S190, originating from The Netherlands and Germany, respectively (19, 31). Strains IPO323 and S190 are field isolates with relatively high and low sensitivities to azole fungicides, respectively. Strain IPO323C1 was isolated in the laboratory by selection for resistance to cyproconazole. This strain has an MDR phenotype with unrelated compounds, such as cycloheximide and rhodamine 6G (42). Yeast-like cells and mycelia of these strains were grown in liquid yeast extract-sucrose medium and Czapek Dox mycological peptone, respectively, as described previously (42). Cells used for inoculation experiments were harvested by centrifugation at 3,000 × *g* for 10 min at 10°C, washed once in sterile MilliQ water, and resuspended in 0.15% Tween 20 at a density of $10^7$ cells $ml^{-1}$. Cell suspensions used for accumulation experiments were washed and resuspended in 50 mM potassium phosphate buffer (pH 6.0) containing 10 g $liter^{-1}$ glucose at a density of 6 mg (wet weight) $ml^{-1}$. Mycelial suspensions used for accumulation experiments were prepared by filtering cultures with a 0.85-mm-pore-size sieve and collecting the mycelia on a 0.055-mm-pore-size sieve. The collected mycelia were washed and resuspended in the same buffer that was used for cell suspensions.

**Toxicity bioassays.** Paper disks (diameter, 6 mm; Whatman) were saturated in methanolic solutions of modulators (3,000 mg $liter^{-1}$) and used in paper disk bioassays. The disks were dried, placed on plates (diameter, 9 cm) with potato dextrose agar (PDA; 20 ml) seeded with *M. graminicola* cells ($10^7$ cells $ml^{-1}$), and amended with cyproconazole at sublethal concentrations. Six paper disks per modulator were used in each plate. The diameters of inhibition zones around the disks were measured after incubation at 20°C for 14 days. The experiment was carried out in duplicate and repeated twice. In agar growth bioassays plates (diameter, 9 cm) with PDA (20 ml) amended with modulators or fungicides at various concentrations were inoculated in triplicate with 5-μl drops of an *M. graminicola* cell suspension ($4 \times 10^5$ cells $ml^{-1}$) and incubated at 20°C for 10 days. Then MICs that fully inhibited growth were determined.

**Crossed-paper-strip bioassay.** Filter paper strips (0.7 by 8 cm) were saturated in methanolic solutions of modulators (3,000 mg $liter^{-1}$) and cyproconazole (1 and 10 mg $liter^{-1}$). The strips were dried and transferred to plates (diameter, 9 cm) containing PDA (15 ml) and seeded with *M. graminicola* cells ($10^7$ cells $ml^{-1}$). Plates seeded with strain IPO323 contained a strip treated with 1 mg $liter^{-1}$ cyproconazole, and plates seeded with strains S190 and IPO323C1 contained a strip treated with 10 mg $liter^{-1}$ cyproconazole. The growth patterns along the paper strips were visually assessed after incubation at 20°C for 14 days.

**Foliar spray experiments.** The disease control activities of modulators alone and in combination with cyproconazole were tested in preventive foliar spray experiments with wheat seedlings (~20 seedlings) grown in pots (6 by 6 cm). Cultivars Obelisk and Vivant were used in experiments with strains IPO323 and S190, respectively. Foliar spraying was carried out in a spray cabinet equipped with a turntable. Wheat seedlings (8 days old) were sprayed with modulators alone (0, 30, 100, 300, and 1,000 mg $liter^{-1}$) and with modulators mixed with cyproconazole (0.1 mg $liter^{-1}$) for 2 min at a pressure of $0.8 \times 10^5$ Pa, until runoff. Control seedlings were sprayed with 0.15% Tween 20. The seedlings were dried overnight and subsequently sprayed with cell suspensions of *M. graminicola*

TABLE 2. Compounds described in literature as modulators of drug efflux from cancer cells

| Category | Examples of compounds[a] | Pharmacological properties | Reference(s) |
|---|---|---|---|
| Calcium channel blockers | **Verapamil**, nifedipine, and related compounds from different structural classes | Coronary vasodilator | 12 |
| Calmodulin antagonists | Phenothiazines like **chlorpromazine**, **promazine**, and **thioridazine** | Antipsychotic drug | 12, 20 |
| Alkaloids | **Camptothecin**, vinblastine, vincristine, and related products | Cytotoxic activity | 12, 24 |
| Steroids and hormonal analogs | Progesteron, **diethylstilbestrol** | Hormonal activity | 12, 29 |
| Miscellaneous hydrophobic cationic compounds | **Quinidine**, **reserpine** | Antiarrhythmic activity | 12 |
| Natural polyphenols and synthetic derivatives | **Chrysin**, **epicathechin**, **genistein**, **naringenin**, **quercetine**, **resorcinol**, **rutin** | Secondary plant metabolites; important constituents of human daily food | 6, 17, 35, 38 |
| Flavonoids | **Flavanone**, flavone, flavonol, isoflavones, flavolignan | Secondary plant metabolites; important constituents of human daily food | 21 |
| Cyclosporine derivatives | **Cyclosporine A** | Immunosuppressive activity | 29 |
| Rifamycin derivatives | **Rifamycin B** | Antibacterial drug | 7, 10 |
| Opioids | **Loperamide** | Antidiarrheal drug | 37 |
| Tricyclic serotonin reuptake inhibitors | **Amitriptyline**, **imapramin** | Antipsychotic drug | 36 |

[a] Compounds in bold were used in the present study.

strains at a density of $10^7$ cells $ml^{-1}$ in 0.1% Tween 20. Inoculated plants were placed on water-soaked cloths in sealed containers with Perspex lids at 18°C in climate rooms in the dark. Control plants and plants treated with modulators were placed in separate boxes in order to avoid the effects of possible vapor-phase activity. After 2 days of incubation a 16-h daylight period was applied. Emerging second leaves were clipped every 4 to 5 days to facilitate disease assessment and light penetration. Virulence was assessed visually by evaluation of necrotic leaf areas (10 leaves per treatment) and the abundance of pycnidia in necrotic lesions at 16 and 21 days postinoculation (dpi). An estimate of the expected interaction between cyproconazole (0.1 mg $liter^{-1}$) and modulators (30 and 300 mg $liter^{-1}$) was calculated using the equation of Colby (5): $E = X_p Y_q / 100$, where $E$ is the expected disease expressed as a percentage of the control for the mixture of compounds A and B (at concentrations $p$ and $q$) and $X_p$ and $Y_q$ are the observed disease levels expressed as a percentage of the control with a single compound (compound A at concentration $p$ or compound B at concentration $q$). A deviation from the expected response indicates synergism or antagonism. Curative foliar spray experiments were performed by application of compounds in a way similar to the way described above but 1 day after fungal inoculation. The experiments were carried out in triplicate.

**Accumulation of cyproconazole.** Cell and mycelial suspensions (55 ml) were incubated in flasks (300 ml) at 25°C and 140 rpm for 30 min. At zero time [$^{14}$C]cyproconazole (Syngenta, Basel, Switzerland) was added to an external concentration of 100 μM (1.5 MBq $mmol^{-1}$). Modulators were added 30 min after the addition of cyproconazole to external concentrations of 100 and 300 μM. Amitriptyline, chlorpromazine, flavanone, and promazine at a concentration of 300 μM are equivalent to 94, 96, 47, and 106 mg $liter^{-1}$, respectively. Cells and mycelia were harvested at intervals by vacuum filtration of samples (5 ml) and washed five times with 5 ml of phosphate buffer (pH 6.0), and the radioactivity in the biomass was measured with a Beckman LS6000TA liquid scintillation counter. Accumulation of [$^{14}$C]cyproconazole was expressed as nmol mg (dry weight) of $biomass^{-1}$ (42).

## RESULTS

**Selection of experimental modulators.** In a classical overview of drugs that alter MDR in cancer cells, Ford and Hait (12) categorized modulators as calcium channel blockers, calmodulin antagonists, *Vinca* alkaloids, steroids, hormonal analogs, and miscellaneous hydrophobic cationic compounds (Table 2). Since that study numerous other modulators have been described, and a number of them are also listed in Table 2. Modulators used in the present study are indicated. Calcium channel blockers and cyclosporins were not included since similar experiments with *B. cinerea* were not successful (16). A relatively large number of phenothiazines were selected because some of these compounds showed strong synergistic activity with the azole fungicide oxpoconazole against *B. cinerea* in vitro (16). Most of the polyphenol and flavonoid modulators listed in Table 2 are natural products that occur abundantly in plants, including food crop species. This suggests that the mammalian toxicity of these compounds is low. Modulation of fungal ABC transporters by plant polyphenols and flavonoids may imply that these compounds can enhance the activity of the plants' own natural antimicrobial compounds. Such a mechanism might contribute to the basal insensitivity of a nonhost plant to plant pathogens. In order to test this hypothesis, relatively large numbers of polyphenol and flavonoid compounds were tested. The flavonoid 2-(4-ethoxy-phenyl)-chromen-4-one was described as an azole efflux inhibitor of *P. tritici-repentis* (25). Since this compound is not commercially available, we tested the structural analogue 5,7-dimethoxy-2-phenyl-chromen-4-one.

**Interaction between cyproconazole and modulators in paper disk bioassays.** The experimental modulators with fungitoxic activity against most of the *M. graminicola* strains tested were amitriptyline, the phenothiazines chlorpromazine, promazine, and thioridazine, diethylstilbestrol, and flavanone (Table 3). Phenothiazines had relatively high fungitoxic activity. The activity of amitriptyline seemed to be weaker against strain S190 than against wild-type strain IPO323. For most of these compounds the inhibition zones in plates with cyproconazole were larger than those in plates without the fungicide, suggesting that the compounds did potentiate cyproconazole activity. The phenothiazines had a relatively strong effect against all *M. graminicola* strains tested, suggesting that these compounds are interesting candidates for further research. The experimental modulators without fungitoxic activity under the test conditions used included plant alkaloids, polyphenols, and flavonoids (Table 3). Most of these compounds did not potentiate the activity of cyproconazole; the only exceptions were imipramine and loperamide in tests with strain IPO323.

TABLE 3. Activities of putative modulators of ABC transporter activity in paper disk bioassays in the absence and presence of cyproconazole for growth of *M. graminicola* strains IPO323 (wild type), IPO323C1 (cyproconazole-resistant laboratory mutant), and S190 (field isolate with relatively low cyproconazole sensitivity)

| Modulator | Diam of growth inhibition zone (mm)[a] | | | | | |
|---|---|---|---|---|---|---|
| | Strain IPO323 | | Strain IPO323C1 | | Strain S190 | |
| | Without cyproconazole | With cyproconazole | Without cyproconazole | With cyproconazole | Without cyproconazole | With cyproconazole |
| Control | 0 | 0 | 0 | 0 | 0 | 0 |
| Amitriptyline | 2.2 ± 0.7 | 2.2 ± 0.7 | 1.0 ± 0.6 | 2.0 ± 0.6[b] | 0 | 1.4 ± 1.4[b] |
| Camptothecin | 0 | 0 | 0 | 0 | 0 | 0 |
| Chlorpromazine | 4.3 ± 1.1 | 5.3 ± 0.4[b] | 5.0 ± 1.2 | 8.5 ± 2.7[b] | 3.8 ± 0.4 | 5.3 ± 0.4[b] |
| Chrysin | 0 | 0 | 0 | 0 | 0 | 0 |
| Cyclosporine | 0 | 0 | 0 | 0 | 0 | 0 |
| Diethylstilbestrol | 1.4 ± 0.5 | 2.2 ± 0.4[b] | 0 | 2.8 ± 0.4[b] | 1.0 ± 0.6 | 1.6 ± 0.8 |
| Epicathechin | 0 | 0 | 0 | 0 | 0 | 0 |
| Flavanone | 1.0 ± 0 | 2.0 ± 0[b] | 1.4 ± 0.5 | 2.0 ± 0.6[b] | 2.2 ± 0.4 | 3.4 ± 0.5[b] |
| Imipramine | 0 | 0.5 ± 0.5 | 0 | 0 | 0 | 0 |
| Loperamide | 0 | 0.3 ± 0.4 | 0 | 0 | 0 | 0 |
| Naringenin | 0 | 0 | 0 | 0 | 0 | 0 |
| Promazine | 1.8 ± 1.1 | 4.0 ± 1.2[b] | 1.8 ± 0.4 | 2.3 ± 0.4 | 0.8 ± 0.4 | 2.0 ± 0[b] |
| Quercetin | 0 | 0 | 0 | 0 | 0 | 0 |
| Quinidine | 0 | 0 | 0 | 0 | 0 | 0 |
| Reserpine | 0 | 0 | 0 | 0 | 0 | 0 |
| Resorcinol | 0 | 0 | 0 | 0 | 0 | 0 |
| Rutin | 0 | 0 | 0 | 0 | 0 | 0 |
| Thioridazine | 4.5 ± 0.5 | 6.5 ± 0.5[b] | 4.0 ± 0.7 | 7.3 ± 0.8[b] | 5.8 ± 0.8 | 6.5 ± 0.5 |
| Verapamil | 0 | 0 | 0 | 0 | 0 | 0 |
| 5,7-Dimethoxy-2-chromen-4-one | 0 | 0 | 0 | 0 | 0 | 0 |

[a] The values (means ± standard deviations) are the diameters of the zones of growth inhibition around disks impregnated with modulators (3,000 mg liter$^{-1}$). The cyproconazole concentration was 0.001 mg liter$^{-1}$ in agar plates seeded with IPO323 and 0.01 mg liter$^{-1}$ in plates seeded with IPO323C1 and S190.
[b] The values for plates with and without cyproconazole differ significantly ($P = 0.05$).

**Interaction between cyproconazole and modulators in crossed-paper-strip bioassays.** All compounds were screened for modulating activity with cyproconazole in crossed-paper-strip bioassays with *M. graminicola* IPO323, S190, and IPO323C1. The phenothiazines chlorpromazine, promazine, and thioridazine clearly synergized the activity of cyproconazole. The modulating activities of these compounds with strain IPO323 are shown in Fig. 1. Diethylstilbestrol, flavanone, loperamide, naringenin, and quinidine showed weak synergistic interactions with cyproconazole only for growth of strain IPO323C1. Most other interactions tested were independent; the only exception was the interaction with resorcinol, which antagonized the activity of cyproconazole with all strains tested, especially strain S190.

**MICs of modulators in agar growth tests.** The MICs of eight experimental modulators with synergistic activity in mixtures with cyproconazole in paper disk bioassays were determined in agar growth tests. Rutin, which did not display any synergism, and cyproconazole itself were included as controls. Table 4 shows that the toxicities of the modulators towards *M. graminicola* IPO323 and S190 are low compared to that of cyproconazole. For strain IPO323, the ratio of the MIC of the modulator to the MIC of cyproconazole was 1.000 or even higher for most of the modulators tested. A relatively low value was found only for diethylstilbestrol (ratio, 100). The MICs of two of the three phenothiazines tested (promazine and thioridazine) correlated with the MICs of cyproconazole for strain IPO323 and S190.

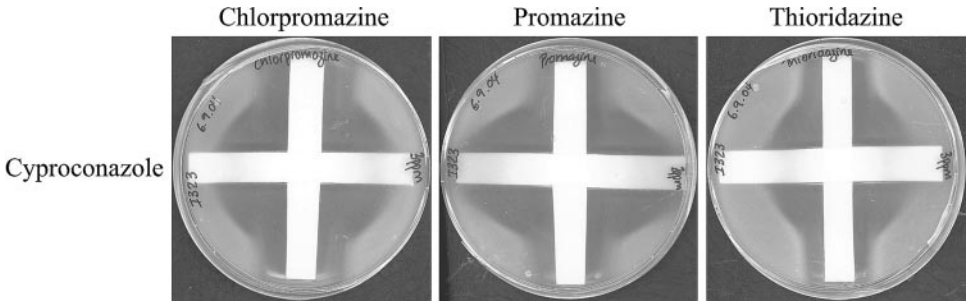


FIG. 1. Synergistic activity between cyproconazole and the putative modulators of ABC transporter activity chlorpromazine, promazine, and thioridazine against growth of *M. graminicola* strain IPO323 in crossed-paper-strip experiments. The horizontal strips were impregnated with cyproconazole (1 mg liter$^{-1}$ in methanol), and the vertical strips were impregnated with the modulators (3,000 mg liter$^{-1}$ in methanol).

TABLE 4. MICs of modulators and cyproconazole for growth of *M. graminicola* strains IPO323 and S190 in agar growth tests

| Compound | MIC (mg liter$^{-1}$) | |
|---|---|---|
| | Strain IPO323 | Strain S190 |
| Modulators | | |
| Amitriptyline | >300 | >300 |
| Chlorpromazine | 300 | 300 |
| Diethylstilbestrol | 30 | 30 |
| Flavanone | 300 | 300 |
| Loperamide | >300 | >300 |
| Promazine | 300 | >300 |
| Quinidine | >300 | >300 |
| Rutin | >300 | >300 |
| Thioridazine | 100 | 300 |
| Azole fungicide | | |
| Cyproconazole | 0.3 | 1.0 |

**Disease control activities of modulators.** The disease control activities of some of the experimental modulators listed in Table 4 were tested using *M. graminicola* strains IPO323 and S190 and cultivar Obelisk and Vivant wheat seedlings, respectively, in preventive foliar spray experiments. In control treatments, the first symptoms became visible at 8 dpi as small chlorotic spots near the tips of the leaves. In time, the lesions expanded longitudinally across the leaves and developed into necrotic lesions covered with pycnidia. At 21 dpi, the percentages of the leaf area with these symptoms for cultivars Obelisk and Vivant were 64% and 87%, respectively. The disease control activities of eight modulators were studied (Table 5). All compounds showed significant disease control activity in treatments with 30 and 300 mg liter$^{-1}$ ($P < 0.05$). Promazine was the most active compound against strain IPO323 on wheat cultivar Obelisk since the disease control activity was 97% of the control. The disease control activities for the other compounds varied from 23 to 85% (Table 5). The disease control activities of the modulators against strain S190 on wheat cultivar Vivant were also obvious. Amitriptyline and quinidine were the most active compounds, showing up to 78% disease control activity (Table 5). Treatment of plants with modulators at a concentration of 1,000 mg liter$^{-1}$ or higher caused phytotoxic symptoms, visible as necrotic lesions at 10 dpi (results not shown). Visible necrotic symptoms were not observed at a concentration of 300 mg liter$^{-1}$ or lower.

Experimental modulators with relatively high disease control activity in the preventive foliar spray tests (amitriptyline, loperamide, and promazine) were studied further in curative foliar spray tests by applying foliar sprays with the compounds 1 day after inoculation of the wheat seedlings. Disease development was assessed at 16 and 21 dpi (Fig. 2). Figure 2 shows that all compounds tested had a significant effect on disease development, especially at 16 dpi. At 21 dpi, the disease control activity was still obvious but less than that at 16 dpi, indicating that the activity of modulators is transient in time.

**Disease control activities of mixtures of modulators and cyproconazole.** Interactions between experimental modulators and cyproconazole in disease control were studied using mixtures of modulators at 30 and 300 mg liter$^{-1}$. The cyproconazole concentration in the mixtures was set at 0.1 mg liter$^{-1}$ since preliminary experiments demonstrated that this concentration controlled about 50% of the disease in foliar spray experiments, which is the optimal percentage to study synergism with other compounds (Table 6). The experiments were performed simultaneously with the experiments to assess the disease control activity of modulators using preventive foliar spray (Table 5). An overview of the observed and expected necrotic leaf areas in disease control experiments with *M. graminicola* strains IPO323 and S190 on wheat cultivars Obelisk

TABLE 5. Activities of compounds described in literature as modulators of ABC transporters in the control of *M. graminicola* on wheat seedlings in preventive foliar spray experiments

| *M. graminicola* strain | Wheat cultivar | Modulator | Necrotic area of leaves treated with modulator at the following concn, expressed as % of control[a]: | | Disease control by modulator at the following concn, expressed as % of control[b]: | |
|---|---|---|---|---|---|---|
| | | | 30 mg liter$^{-1}$ | 300 mg liter$^{-1}$ | 30 mg liter$^{-1}$ | 300 mg liter$^{-1}$ |
| IPO323 | Obelisk | Amitriptyline | 34.1 ± 8.9 | 62.2 ± 5.2 | 65.9 | 37.8 |
| | | Chlorpromazine | 59.3 ± 4.4 | 43.7 ± 5.2 | 40.7 | 56.3 |
| | | Diethylstilbestrol | 32.6 ± 10.0 | 23.7 ± 8.1 | 67.4 | 76.3 |
| | | Flavanone | 62.2 ± 11.8 | 63.7 ± 3.7 | 37.8 | 36.3 |
| | | Loperamide | 46.7 ± 5.2 | 14.1 ± 8.9 | 53.3 | 85.9 |
| | | Promazine | 15.6 ± 5.2 | 3.0 ± 2.2 | 84.4 | 97.0 |
| | | Quinidine | 76.3 ± 8.1 | 77.8 ± 4.4 | 23.7 | 22.8 |
| | | Thioridazine | 31.1 ± 8.1 | 17.0 ± 4.4 | 68.9 | 83.0 |
| S190 | Vivant | Amitriptyline | 31.0 ± 9.5 | 24.3 ± 5.2 | 69.0 | 75.7 |
| | | Chlorpromazine | 62.0 ± 11.2 | 24.0 ± 7.2 | 38.0 | 76.0 |
| | | Diethylstilbestrol | 73.0 ± 15.3 | 47.9 ± 5.4 | 27.0 | 52.1 |
| | | Flavanone | 52.5 ± 11.0 | 36.3 ± 8.1 | 47.5 | 63.7 |
| | | Loperamide | 51.1 ± 4.6 | 58.2 ± 8.0 | 48.9 | 41.8 |
| | | Promazine | 44.3 ± 7.5 | 27.8 ± 3.3 | 55.7 | 72.2 |
| | | Quinidine | 38.9 ± 7.4 | 21.6 ± 5.1 | 61.1 | 78.4 |
| | | Thioridazine | 49.3 ± 4.3 | 68.0 ± 11.6 | 50.7 | 32.0 |

[a] The necrotic area of leaves represents the leaf area with disease symptoms. The necrotic areas (means ± standard deviations) for the control treatment with strains IPO323 and S190 were 64.0% ± 6.6% and 87.1% ± 13.2%, respectively (defined as 100%).

[b] The disease control by modulators represents the healthy leaf area in treatments.

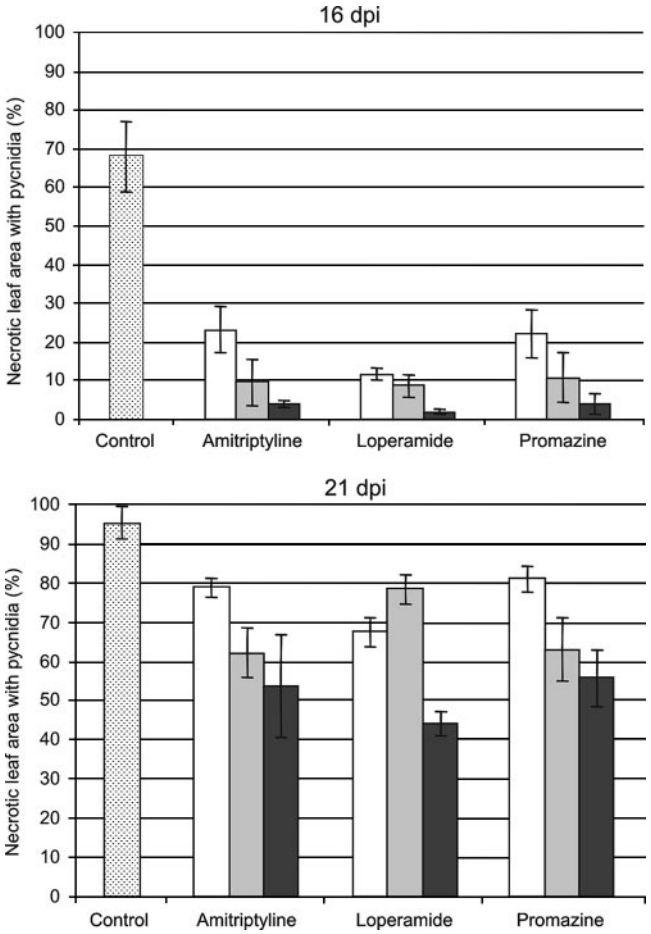


FIG. 2. Activities of amitriptyline, loperamide, and promazine in control of *M. graminicola* IPO323 on cultivar Obelisk wheat seedlings in curative foliar spray tests. The concentrations of the compounds tested were 1 mg liter$^{-1}$ (open bars), 10 mg liter$^{-1}$ (gray bars), and 100 mg liter$^{-1}$ (black bars). Disease was assessed at 16 and 21 dpi. The bars indicate the mean percentages of necrotic leaf area with pycnidia, and the error bars indicate standard deviations.

and Vivant indicated that the observed necrotic areas on leaves treated with mixtures were similar to or larger than the expected necrotic leaf areas, suggesting that that there were no synergistic activities for the interactions tested (Table 6).

**Effect of modulators on accumulation of cyproconazole.** Accumulation of cyproconazole in the absence of experimental modulators in both yeast-like cells and mycelium of *M. graminicola* IPO323 and S190 was slightly transient in time (Fig. 3). The levels accumulated by strain S190 were higher than the levels accumulated by strain IPO323. Addition of all modulators tested caused an instantaneous increase in cyproconazole accumulation. For most modulators the increase in fungicide accumulation was transient. The exception was chlorpromazine added to yeast-like cells. In this case, the level of cyproconazole that accumulated remained at an almost constant elevated value. Modulators added to mycelial and cell suspensions at a final concentration of 100 μM had similar effects on accumulation of cyproconazole, but the effects were less pronounced (results not shown). Loperamide, quinidine, and thioridazine (300 μM) did not significantly enhance accumulation of cyproconazole in either cells or mycelium (results not shown).

## DISCUSSION

Several modulators described in the literature as compounds that alter MDR in cancer cells were also able to increase the activity of the azole fungicide cyproconazole against *M. graminicola*. This activity could be demonstrated for amitriptyline, diethylstilbestrol, flavanone, and the phenothiazines chlorpromazine, promazine, and thioridazine in paper disk bioassays with three strains of the pathogen that differ in sensitivity to cyproconazole. The modulating activity of the phenothiazines was also apparent in crossed-paper-strip experiments. These results corroborate the synergistic activities of chlorpromazine and cyproconazole against *M. graminicola*, as reported previously (30). Chlorpromazine can also modulate the activity of azole fungicides against *B. cinerea*, particularly an azole-secreting, ABC transporter BcatrD overexpression mutant (16). Additional studies demonstrated that amitriptyline, chlorpromazine, flavanone, and promazine had instantaneous effects on the levels of cyproconazole accumulated in yeast-like cells and mycelium of *M. graminicola*, suggesting that cyproconazole efflux by fungal drug transporters was inhibited. Chlorpromazine had a relatively strong effect since its reversal of efflux activity remained almost constant over time. This characteristic of chlorpromazine may be related to its relatively strong modulating activity in the in vitro assays. The modulating activity may be due to affinity of the modulators to binding sites of ABC transporter proteins which results in inhibition of cyproconazole transport (40).

Several ABC transporters of *M. graminicola* that can provide protection against azole fungicides have been described (41). Hence, it might be that reversal of the activity of one or more of these ABC transporters in *M. graminicola* by the phenothiazines or other compounds tested is responsible for the synergism with cyproconazole. The MFS transporter MgMfs1 has also been described as a potent transporter of azole fungicides (27). However, the compounds tested are not described in the literature as modulators of MFS transporters, and therefore modulation of MgMfs1 by phenothiazines is probably not responsible for the synergism observed.

Various models have been described to explain reversal of drug efflux activity mediated by ABC transporters (3). A proposed mechanism of action is direct binding of the modulator to a binding site(s) on the transporter protein, which results in blocking transport in either a competitive or noncompetitive mode (40).

Foliar spray experiments with mixtures of cyproconazole and modulators demonstrated that the expected disease control activity calculated as described by Colby (5) was merely additive or even antagonistic. Thus, none of the modulators tested showed synergism with cyproconazole in planta, not even the modulators exhibiting synergism in vitro. This situation contrasts with the modulating activity reported for a 4′-hydroxyflavone derivative for resistance to azoles and other fungicides in *P. tritici-repens* (25), but it is not uncommon for MDR modulators in clinical situations (26). There are several reasons that could explain the lack of in planta modulating activity: (i) the in planta-mediated degree of natural insensitivity or

TABLE 6. Interactions between cyproconazole and modulators in control of *M. graminicola* on wheat seedlings[a]

| Modulator in mixture with cyproconazole (0.1 mg liter$^{-1}$)[b] | Necrotic leaf areas of wheat seedlings sprayed with mixtures of cyproconazole and modulators | | | |
|---|---|---|---|---|
| | Cultivar Obelisk inoculated with *M. graminicola* IPO323 | | Cultivar Vivant inoculated with *M. graminicola* S190 | |
| | Observed necrotic leaf area[c] | Expected necrotic leaf area[d] | Observed necrotic leaf area[c] | Expected necrotic leaf area[d] |
| Amitriptyline (30 mg liter$^{-1}$) | 68.5 ± 5.2 | 14.7 | 28.1 ± 9.4 | 18.3 |
| Amitriptyline (300 mg liter$^{-1}$) | 32.6 ± 5.3 | 26.7 | 16.6 ± 5.6 | 14.4 |
| Chlorpromazine (30 mg liter$^{-1}$) | 50.2 ± 5.0 | 25.4 | 43.4 ± 9.0 | 36.6 |
| Chlorpromazine (300 mg liter$^{-1}$) | 20.6 ± 8.9 | 18.8 | 24.9 ± 14.0 | 14.2 |
| Diethylstilbestrol (30 mg liter$^{-1}$) | 34.0 ± 2.2 | 13.8 | 36.1 ± 9.6 | 43.1 |
| Diethylstilbestrol (300 mg liter$^{-1}$) | 18.7 ± 6.7 | 10.2 | 62.6 ± 9.4 | 28.2 |
| Flavanone (30 mg liter$^{-1}$) | 59.2 ± 10.0 | 26.7 | 31.5 ± 7.7 | 31.0 |
| Flavanone (300 mg liter$^{-1}$) | 50.0 ± 11.8 | 27.4 | 22.8 ± 4.2 | 21.4 |
| Loperamide (30 mg liter$^{-1}$) | 17.0 ± 3.7 | 20.1 | 51.5 ± 6.3 | 30.1 |
| Loperamide (300 mg liter$^{-1}$) | 20.1 ± 5.2 | 6.1 | 38.3 ± 4.1 | 34.3 |
| Promazine (30 mg liter$^{-1}$) | 3.0 ± 3.0 | 6.7 | 25.1 ± 5.2 | 26.1 |
| Promazine (300 mg liter$^{-1}$) | 11.0 ± 5.2 | 1.3 | 20.7 ± 3.4 | 16.4 |
| Quinidine (30 mg liter$^{-1}$) | 70.0 ± 8.8 | 32.8 | 34.1 ± 4.7 | 23.0 |
| Quinidine (300 mg liter$^{-1}$) | 62.0 ± 8.0 | 33.4 | 13.4 ± 5.6 | 12.7 |
| Thioridazine (30 mg liter$^{-1}$) | 27.7 ± 7.0 | 13.3 | 45.9 ± 2.7 | 29.1 |
| Thioridazine (300 mg liter$^{-1}$) | 11.0 ± 8.7 | 7.3 | 43.3 ± 6.4 | 40.1 |

[a] The results were obtained in experiments performed simultaneously with the disease control experiments with individual compounds described in Table 5.
[b] The necrotic areas of leaves sprayed with cyproconazole (0.1 mg liter$^{-1}$) expressed as percentages (averages ± standard deviations) of the control in the experiments with strains IPO323 and S190 were 43.0% ± 4.8% and 59.0% ± 8.0, respectively.
[c] The values are the necrotic areas of leaves (averages ± standard deviations) expressed as percentages of the water controls.
[d] The expected necrotic leaf area was calculated as described by Colby (5), using values for necrotic leaf areas obtained for single treatments with cyproconazole and modulators.

resistance of *M. graminicola* through ABC transporters is too low to show an interaction; (ii) the mixtures of fungicide and modulators may have had a phytotoxic or senescent effect on wheat, promoting its susceptibility to the pathogen; and (iii) cyproconazole is a systemic fungicide, while the modulators probably have only a residual effect on the leaf surface. These different properties may result in a rapid spatial separation of the two compounds in plant tissues, and once the pathogen has invaded the host via the stomatal cavities, it is exposed only to the fungicide. For these reasons similar experiments with an MDR strain of *M. graminicola* with high levels of resistance and a systemic modulator without phytotoxicity are recommended. However, field isolates with such a phenotype are not available. Laboratory mutants that possess MDR phenotypes (e.g., strain IPO323C1) are impaired in virulence on wheat, and systemic modulators are not known.

All experimental modulators tested individually controlled *M. graminicola* on wheat seedlings in preventive foliar spray experiments (Table 5). A curative foliar spray test (16 dpi) with amitriptyline, loperamide, and promazine demonstrated that at relatively low concentrations disease control is especially evident during the initial phase of disease development (Fig. 2). The reduced disease control activity observed later (21 dpi) can probably be ascribed to physical and metabolic breakdown of the compounds. The disease control efficacy of the compounds may be explained in different ways. One possibility is that the presence of the modulators leads to reversal of ABC transporters that act as pathogenicity factors, such as MgAtr4 (32). Thus, as shown in Table 1, modulator disease control activity can be ascribed to increased accumulation of plant defense products in the pathogen or reduced secretion of fungal toxins. This may apply particularly to modulators such as amitriptyline, loperamide, and promazine, which did not possess in vitro toxicity to *M. graminicola* in agar growth tests (Table 4). Compounds active in such a way can be regarded as disease control agents with an indirect mode of action. This hypothesis is difficult to verify since a clear role of fungal toxins and plant defense compounds in the interaction of *M. graminicola* and wheat has not been elucidated. Most of the other modulators that possessed disease control activity have low or moderate direct toxicity to *M. graminicola* in vitro (chlorpromazine, diethylstilbestrol, flavanone, promazine, and thioridazine). This may imply that the disease control activity of these compounds may be a consequence of both modulation of ABC transporter activity and direct activity against *M. graminicola*. For instance, the MICs of diethylstilbestrol and cyproconazole for in vitro growth were 30 and 0.3 mg liter$^{-1}$, respectively, indicating that the toxicity ratio of the two compounds is 100 (Table 5). If the same ratio applied for disease control activity on wheat seedlings, approximately 50% disease control would be expected with diethylstilbestrol at a concentration of 10 mg liter$^{-1}$ (50% disease control by cyproconazole is obtained at 0.1 mg liter$^{-1}$) (Table 6). Indeed, disease control by diethylstilbestrol occurs with concentrations that are this order of magnitude, and for this reason disease control by this compound is probably due to direct toxicity to the pathogen. For compounds with an MIC of 300 mg liter$^{-1}$ (chlorpromazine, flavanone, and promazine) similar reasoning is less obvious since their toxicity ratio with cyproconazole (1,000) probably cannot be fully explained by direct toxicity. Amitriptyline and loperamide belonged to the category of compounds with the highest MICs determined (>300 mg liter$^{-1}$). Still, these compounds displayed relatively high disease control activity in both preventive and curative disease control tests at low concentrations. For these reasons it is likely that the disease control

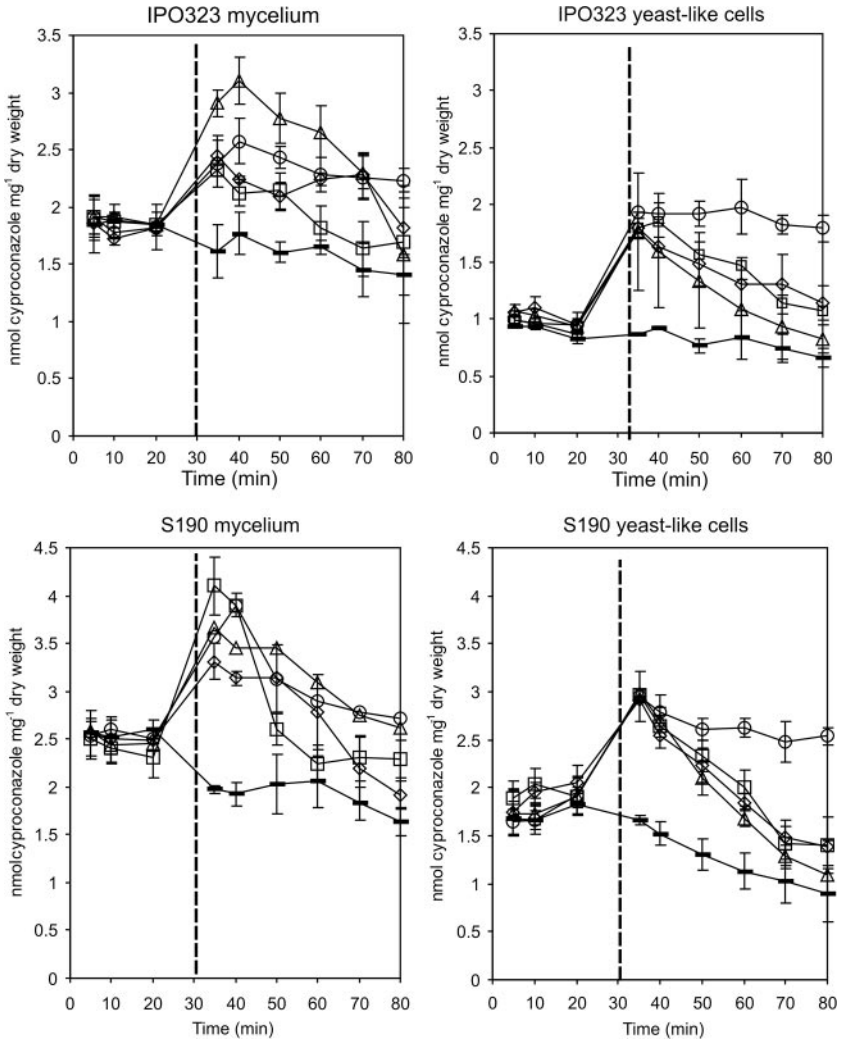


FIG. 3. Effects of modulators on accumulation of [$^{14}$C]cyproconazole (100 μM) by mycelium and yeast-like cells of *M. graminicola* IPO323 and S190. The modulators (300 μM) amitriptyline (□), chlorpromazine (○), flavanone (△), and promazine (◇) were added 30 min after the addition of cyproconazole (dashed line). —, control treatment. The error bars indicate the standard deviations of the means.

activity of amitriptyline, loperamide, and promazine is due to an indirect mode of action and not due to direct toxicity.

Remarkably, modulators with the highest disease control activity against *M. graminicola* (amitriptyline, loperamide, and promazine) also potentiate the activity of cyproconazole in paper disk bioassays. As described above, these two characteristics of modulators are not necessarily due to reversal of activity of the same ABC transporter(s). This reasoning implies that the modulators inhibit the activity of multiple ABC transporters and indicates that the compounds have no reversal selectivity. Such a property would make the in vitro selection of new reversal agents in bioassays as described in this paper easier. The modulators tested did not display phytotoxicity at a concentration of 100 mg liter$^{-1}$ in foliar spray tests. This indicates that selective toxicity for different groups of organisms is feasible.

In conclusion, the results reported in this paper describe the first steps in the discovery and development of modulators of ABC transporter activity which potentiate the activity of azole fungicides towards plant pathogens and which may possess indirect disease control activity. The modulators with disease control activity are amitriptyline, loperamide, and promazine, which are known chemical drugs used for control of human diseases. The second step in the development of disease control agents could be the synthesis and screening of structural analogues of these compounds for improved biological activity, systemic activity in plants, and selective activity against plant pathogens and other classes of organisms. As a third step modulators with a new chemical structure can be developed. A similar sequence of events has been described for the development of modulators for clinical use (26).

## ACKNOWLEDGMENTS

We acknowledge P. J. G. M. de Wit for critically reading the manuscript and J. G. M. van Nistelrooij for skillful assistance with the accumulation experiments.

We acknowledge the Agricultural Research and Education Organization (AREO) of Iran and the Iranian Ministry of Science, Research, and Technology for financial support of Ramin Roohparvar.

## REFERENCES


1. **Alexander, N. J., S. P. McCormick, and T. M. Hohn.** 1999. TRI12, a trichothecene efflux pump from *Fusarium sporotrichioides*: gene isolation and expression in yeast. Mol. Gen. Genet. **261:**977–984.
2. **Ambudkar, S. V., S. Dey, C. A. Hrycyna, M. Ramachandra, I. Pastan, and M. M. Gottesman.** 1999. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu. Rev. Pharmacol. Toxicol. **39:**361–398.
3. **Baird, R. D., and S. B. Kaye.** 2003. Drug resistance reversal—are we getting closer? Eur. J. Cancer **39:**2450–2461.
4. **Callahan, T. M., M. S. Rose, M. J. Meade, M. Ehrenshaft, and R. G. Upchurch.** 1999. CFP, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. Mol. Plant-Microbe Interact. **12:**901–910.
5. **Colby, S. R.** 1967. Calculating synergistic and antagonistic responses of herbicide combinations. Weeds **15:**2–22.
6. **Conseil, G., A. Decottignies, J.-M. Jault, G. Comte, D. Barron, A. Goffeau, and A. Di Pietro.** 2000. Prenyl-flavonoids as potent inhibitors of the Pdr5 multidrug transporter from *Saccharomyces cerevisiae*. Biochemistry **39:**6910–6917.
7. **Courtois, A., L. Payen, L. Vernhet, E. G. E. de-Vries, A. Guillouzo, and O. Fardel.** 1999. Inhibition of multidrug resistance-associated protein (MRP) activity by rifampicin in human multidrug-resistant lung tumor cells. Cancer Lett. **139:**97–104.
8. **De Waard, M. A.** 1997. Significance of ABC transporters in fungicide sensitivity and resistance. Pestic. Sci. **51:**271–275.
9. **De Waard, M. A., A. C. Andrade, K. Hayashi, H. Schoonbeek, I. Stergiopoulos, and L.-H. Zwiers.** 2006. Impact of fungal drug transporters on fungicide sensitivity, multidrug resistance and virulence. Pest Manag. Sci. **62:**195–207.
10. **Fardel, O., V. Lecureur, P. Loyer, and A. Guillouzzo.** 1995. Rifampicin enhances anti-cancer drug accumulation and activity in multidrug-resistant cells. Biochem. Pharmacol. **49:**1255–1260.
11. **Fleissner, A., C. Sopalla, and K.-M. Weltring.** 2002. An ATP-binding cassette multidrug-resistance transporter is necessary for tolerance of *Gibberella pulicaris* to phytoalexins and virulence on potato tubers. Mol. Plant-Microbe Interact. **15:**102–108.
12. **Ford, J. M., and W. N. Hait.** 1990. Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev. **42:**155–199.
13. **Gottesman, M. M., and I. Pastan.** 1988. The multidrug transporter, a double-edged sword. J. Biol. Chem. **263:**12163–12166.
14. **Gus, N. R., F. R. Stermitz, J. B. Johnson, T. D. Beeson, S. Willen, J. Hsiang, and K. Lewis.** 2001. Flavonolignan and flavone inhibitors of a *Staphylococcus aureus* multidrug resistance pump: structure-activity relationships. J. Med. Chem. **44:**261–268.
15. **Hayashi, K., H. Schoonbeek, and M. A. De Waard.** 2002. Bcmfs1, a novel major facilitator superfamily transporter from *Botrytis cinerea*, provides tolerance towards the natural toxic compounds camptothecin and cercosporin and towards fungicides. Appl. Environ. Microbiol. **68:**4996–5004.
16. **Hayashi, K., H. Schoonbeek, and M. A. De Waard.** 2003. Modulators of membrane drug transporters potentiate the activity of the DMI fungicide oxpoconazole against *Botrytis cinerea*. Pest Manag. Sci. **59:**294–302.
17. **Hooijberg, J. H., H. J. Broxterman, M. Heijn, D. L. A. Fles, J. Lankelma, and H. M. Pinedo.** 1997. Modulation by (iso)flavonoids of the ATPase activity of the multidrug resistance protein. FEBS Lett. **413:**344–348.
18. **Hsieh, P. C., S. A. Siegel, B. Rogers, D. Davis, and K. Lewis.** 1998. Bacteria lacking a multidrug pump: a sensitive tool for drug discovery. Proc. Natl. Acad. Sci. USA **95:**6602–6606.
19. **Kema, G. H. J., and C. H. Van Silfhout.** 1997. Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. III. Comparative seedling and adult plant experiments. Phytopathology **87:**266–272.
20. **Kolaczkowski, M., K. Michalak, and N. Motohashi.** 2003. Phenothiazines as potent modulators of yeast multidrug resistance. Int. J. Antimicrob. Agents **22:**279–283.
21. **Lania-Pietrzak, B., K. Michalak, A. B. Hendrich, D. Mosiadz, G. Grynkiewicz, N. Motohashi, and Y. Shirataki.** 2005. Modulation of MRP1 protein transport by plant and synthetically modified flavonoids. Life Sci. **77:**1879–1891.
22. **Morel, C., F. R. Stermitz, G. Tegos, and K. Lewis.** 2003. Isoflavones as potentiators of antibacterial activity. J. Agric. Food Chem. **51:**5677–5679.
23. **Pitkin, J. W., D. G. Panaccione, and J. D. Walton.** 1996. A putative cyclic peptide efflux pump encoded by the TOXA gene of the plant-pathogenic fungus *Cochliobolus carbonum*. Microbiology **142:**1557–1565.
24. **Pizzolato, J. F., and L. B. Saltz.** 2003. The camptothecins. Lancet **361:**2235–2242.
25. **Reimann, S., and H. B. Deising.** 2005. Inhibition of efflux transporter-mediated fungicide resistance in *Pyrenophora tritici-repentis* by a derivative of 4′-hydroxyflavone and enhancement of fungicide activity. Appl. Environ. Microbiol. **71:**3269–3275.
26. **Robert, J., and C. Jarry.** 2003. Multidrug resistance reversal agents. J. Med. Chem. **46:**4805–4817.
27. **Roohparvar, R., M. A. De Waard, G. H. J. Kema, and L.-H. Zwiers.** 2007. MgMfs1, a major facilitator superfamily transporter from the fungal wheat pathogen *Mycosphaerella graminicola*, is a strong protectant against natural toxic compounds and fungicides. Fungal Genet. Biol. **44:**378–388.
28. **Schoonbeek, H., G. Del Sorbo, and M. A. De Waard.** 2001. The ABC transporter BcatrB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. Mol. Plant-Microbe Interact. **14:**562–571.
29. **Sparreboom, A., R. Danesi, Y. Ando, J. Chan, and W. D. Figg.** 2003. Pharmacogenomics of ABC transporters and its role in cancer therapy. Drug Resist. Updates **6:**71–84.
30. **Stergiopoulos, I.** 2003. The role of ATP-binding cassette (ABC) transporters in pathogenesis and multidrug resistance of the wheat pathogen *Mycosphaerella graminicola*. Ph.D. thesis. Department of Plant Sciences, Wageningen University and Research Center, Wageningen, The Netherlands.
31. **Stergiopoulos, I., J. G. M. Van Nistelrooy, G. H. J. Kema, and M. A. De Waard.** 2003. Multiple mechanisms account for variation in base-line sensitivity to azole fungicides in field isolates of *Mycosphaerella graminicola*. Pest Manag. Sci. **59:**1333–1343.
32. **Stergiopoulos, I., L.-H. Zwiers, and M. A. De Waard.** 2003. The ABC transporter MgAtr4 is a virulence factor of *Mycosphaerella graminicola* that affects colonisation of substomatal cavities in wheat leaves. Mol. Plant-Microbe Interact. **16:**689–698.
33. **Stergiopoulos, I., L.-H. Zwiers, and M. A. De Waard.** 2002. Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP-binding cassette and major facilitator superfamily. Eur. J. Plant Pathol. **108:**719–734.
34. **Urban, M., T. Bhargava, and J. E. Hamer.** 1999. An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. EMBO J. **18:**512–521.
35. **Vaidyanathan, J. B., and T. Walle.** 2003. Cellular uptake and efflux of the tea flavonoid (−)-epicatechin-3-gallate in the human intestinal cell line Caco-2. J. Pharmacol. Exp. Ther. **307:**745–752.
36. **Varga, A., H. Nugel, R. Baehr, U. Marx, A. Hever, J. Nacsa, I. Ocsovszky, and J. Molnar.** 1996. Reversal of multidrug resistance by amitriptyline in vitro. Anticancer Res. **16:**209–211.
37. **Wandel, C., R. Kim, M. Wood, and A. Wood.** 2002. Interaction of morphine, fentanyl, sufentenyl, alfentanyl, and loperamide with the efflux drug transporter P-glycoprotein. Anesthesiology **96:**913–920.
38. **Wu, C.-P., A. M. Calcagno, S. B. Hladky, S. V. Ambudkar, and M. A. Barrand.** 2005. Modulatory effects of plant phenols on human multidrug-resistance proteins 1, 4 and 5 (ABCC1, 4 and 5). FEBS J. **272:**4725–4740.
39. **Zamora, J. M., H. L. Pearse, and W. T. Beck.** 1988. Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. Mol. Pharmacol. **33:**454–462.
40. **Zloh, M., G. W. Kaatz, and S. Gibbons.** 2004. Inhibitors of multidrug resistance (MDR) have affinity for MDR substrates. Bioorg. Med. Chem. Lett. **14:**881–885.
41. **Zwiers, L.-H., I. Stergiopoulos, M. M. C. Gielkens, S. D. Goodall, and M. A. De Waard.** 2003. ABC transporters of the wheat pathogen *Mycosphaerella graminicola* function as protectants against biotic and xenobiotic toxic compounds. Mol. Genet. Genomics **269:**499–507.
42. **Zwiers, L.-H., I. Stergiopoulos, J. G. M. Van Nistelrooy, and M. A. De Waard.** 2002. ABC transporters and azole susceptibility in laboratory strains of the wheat pathogen *Mycosphaerella graminicola*. Antimicrob. Agents Chemother. **46:**3900–3906.