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Metabolism of imazalil by wild-type and DMI-resistant isolates of Penicillium italicum

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Abstract

Metabolism of imazalil (1-[2-(2,4-dichlorophenyl)-2-(2- propenyloxy)ethyl]-1*H*-imidazole) in *Penicillium italicum* isolates with a wild-type sensitivity and with various degrees of resistance to sterol demethylation inhibitors was studied in liquid cultures. The metabolite 1-[2(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (R42243) was detected in the culture filtrate after prolonged incubation. The metabolism occurred in the propenyl side chain of imazalil probably through epoxidation and hydratation. This is the first report of such a conversion of imazalil in fungi. R42243 was much less toxic to *P. italicum* than imazalil. Therefore, the metabolism can be regarded as a detoxification step. Both wild-type and resistant isolates metabolized imazalil, but metabolism by resistant isolates was faster than by the wild-type isolate. This is probably caused by a relatively strong inhibition of growth of the wild-type isolate by the fungicide. Results indicate that the detoxification of imazalil does not operate as a mechanism of resistance. This conclusion was confirmed by the fact that resistant isolates showed cross-resistance to miconazole and R42243, which had a similar structure as imazalil except for the propenyl side chain.

Additional keywords: DMIs, detoxification, mechanism of resistance, metabolism, miconazole.

Introduction

Differential metabolism can either be responsible for selective fungitoxic action between target and non-target fungi or for acquired resistance in a specific target organism (Lyr, 1987; Dekker, 1987). This statement also holds true for fungicides which inhibit sterol 14α-demethylation (DMIs) in ergosterol biosynthesis. For instance, detoxification of triforine was supposed to be responsible for insensitivity of *Aspergillus niger, Colletotrichum atramentarium* and *Stemphylium radicinum* (Gasztonyi and Josepovits, 1975). Insensitivity to triadimefon of *Stemphylium radicinum* and *Saccharomyces cerevisiae* (Gasztonyi and Josepovits, 1984), *A. niger* (Deas and Clifford, 1981) and some others fungi (Fuchs, 1988) can be due to a reduced rate of transformation into triadimenol of which certain stereoisomers have a relatively higher fungitoxicity. So far, resistance based on metabolism of DMIs has only been observed in resistant strains of *Cladosporium cucumerinum* (Fuchs and De Vries, 1984) and

Nectria haematococca (Kalamarakis et al., 1986). The resistant strains of the fungi showed a slower rate of transformation of triadimefon to more toxic isomers of triadimenol than the sensitive strains.

The aim of the present work was to investigate whether *Penicillium italicum* isolates with a wild-type sensitivity and with resistance to DMIs metabolize imazalil and if so, whether the metabolism operates as a mechanism of resistance.

Materials and methods

Fungal strains, culture methods and toxicity tests. Wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17}, I_{33} and J_4 of *P. italicum* were used in the experiments (De Waard et al., 1982; De Waard and Van Nistelrooy, 1990). The fungus was maintained on malt extract agar medium. Toxicity of imazalil, 1-[2(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (R42243) and miconazole (gifts from Janssen Pharmaceutica, Beerse, Belgium) to various isolates was tested on malt extract agar medium pH 7.0 according to the method described previously (Guan et al. 1989).

Metabolism of imazalil. Metabolism of [^{14}C]imazalil (gift from Janssen Pharmaceutica, Beerse, Belgium) was studied in liquid malt medium pH 7 (Guan et al., 1989). Liquid cultures were prepared by inoculating the medium (100 ml) in flasks (300 ml) with 1 ml spore suspension (10^8 conidia ml^{-1}) collected from 7 to 10 days old agar plates. The flasks were incubated at 26 °C in an orbital shaker at 200 rpm for 10 h. [^{14}C]imazalil (sp. act. 0.56 and 0.028 GBq mmol^{-1} in methanol) was added to the mycelial suspension to final concentrations of 0.005 and 0.1 μg ml^{-1}, respectively. The mycelial suspensions were further incubated in duplicate for 16 and 24 h, respectively. Similar experiments were carried out with non-labelled imazalil. Fungicide-free culture filtrates (24-h-old) of all isolates were further incubated with imazalil (0.005 μg ml^{-1}) for another 24 h in order to check their ability to metabolize imazalil.

Extraction of imazalil and metabolite. For each experiment, mycelium was harvested from liquid cultures (100 ml) by filtration on a Büchner funnel under vacuum. Mycelium was extracted twice with methanol (100 ml) by shaking on a reciprocal shaker for 10 and 5 h, respectively. The methanol extracts were combined and water in the extracts was removed by adding 2 g of anhydrous sodium sulphate. The methanol extracts were evaporated under vacuum at 40 °C and the residues dissolved in chloroform (2 ml). Culture filtrates (100 ml) were adjusted to pH 11 with 10 M sodium hydroxide and extracted three times with equal volumes of chloroform. Water in the combined chloroform extracts was removed by adding 5 gram of anhydrous sodium sulphate and the extracts were evaporated under vacuum at 40 °C. The residues were dissolved in chloroform (2 ml). Samples of 20 μl (duplicate) from mycelial extracts and culture filtrates were taken for determining radioactivity in a liquid scintillation counter (Beckman LS 2800). The chloroform was dried down under nitrogen and the residues were stored at −20 °C.

Thin layer chromatography. Residues of extracts of mycelium and culture filtrates were dissolved in chloroform (100 μl) and spotted on TCL plates (Silicagel F254, 0.25

mm thick, Merck, Germany). The plates were developed in a solvent system of diethylether/chloroform/methanol/ammonium hydroxide (100:85:15:1, v/v/v/v). Two authentic metabolites of imazalil detected in animals, 1-[2(2,4-dichlorohenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (R42243) and 1-[2(2,4-dichlorophenyl)-2-(hydroxy)ethyl]-1*H*-imidazole (R14821) (gifts from Janssen Pharmaceutica, Beerse, Belgium) were used as references. Radioactive areas on the plates were located with a thin layer scanner (Berthold, Wildbad, Germany). The identification of imazalil and a metabolite was tentatively made by comparison of R_f values with those of reference compounds which could be detected under UV light. Silicagel containing radioactivity was scraped off and counted in a liquid scintillation system.

In similar experiments, metabolism of non-labelled imazalil was studied. The metabolite on TLC plates was located under UV light. Silicagel containing the metabolite was scraped from the plates and extracted twice with chloroform (10 ml). The combined chloroform extracts were taken to dryness under nitrogen. The residue was purified to some extent by repeating the TLC procedure twice. The final residue containing the metabolite was stored in a glass tube filled with nitrogen at −20 °C till mass spectrometric analysis.

Radio-HPLC analysis. The radio-HPLC equipment consisted of two Waters 6000 A pumps, a Waters 680 gradient controller, a Waters WISP 710 automatic injector and a Rheodyne 2 ml loop injector for manual injections. A stainless steel column (30 × 0.46 cm), packed with Hypersil C-18 (5 μm, Shandon) by a balanced density slurry procedure using a Haskel DSTV 122-C pump at 7×10^7 Pa, was used for separation. Residues of extracts of mycelium dissolved in dimethyl sulfoxide (500 μl) and culture filtrates (without further processing) were used for radio-HPLC analysis. The amount of radioactivity injected ranged from 834 to 11 000 dpm. The column was eluted with a linear gradient from 70% solvent A (0.1 M ammonium acetate pH 8 and 30% solvent B (1.0 M ammonium acetate pH 8/methanol/acetonitrile, 10/45/45, v/v/v) to 15% solvent A and 85% solvent B over a 40 min period. The latter solvent composition was held for 5 min before a short gradient to 100% solvent B was applied for 1 min. Solvent flow rate was 1 ml min^{-1}. UV detection was carried out at 230 nm by a Varian Varichrom spectrophotometric detector. On-line radioactivity detection was carried out with a Berthold Radioactivity Monitor LB 504, equipped with a 800 μl flow-through cell. The elutes were mixed with Pico-Fluor 30 (Packard) as scintillation cocktail, delivered by a FMI LB 5031 pump at a flow rate of 4 ml min^{-1}. Imazalil and a metabolite were identified by comparing their retention times with those of reference data by means of the UV detector.

Mass spectrometry. Mass spectrometric analysis was performed by introducing a few micrograms of the samples with the direct insertion probe into a VG MN7070F mass spectrometer working at a resolution of $M/\Delta M = 1000$ and using 50 eV electron impact ionization.

Results

Incubation with [^{14}C]imazalil and extraction of radioactivity. Metabolism of imazalil in *P. italicum* was studied at 0.005 and 0.1 μg imazalil ml^{-1}. The lowest

Table 1. Metabolism of [^{14}C]imazalil by wild-type and DMI-resistant isolates of *Penicillium italicum* in a liquid malt extract medium pH 7 during 24 h of incubation.

Isolate	Imazalil (μg ml^{-1})	Growth[1]	Recovery of radioactivity[2]				
			total	Mycelium		Culture filtrate	
				imazalil	metabolite	imazalil	metabolite
W_5	0.005	75	89.1	42.7	0	28.2	18.2
$E_{300\text{-}3}$	0.005	100	93.5	45.0	0	31.2	17.3
H_{17}	0.005	100	79.4	37.7	0	10.8	30.9
I_{33}	0.005	100	97.3	35.7	0	28.0	33.6
J_4	0.005	100	90.4	30.2	0	29.2	31.0
W_5	0.1	0	91.4	40.3	0	51.1	0
$E_{300\text{-}3}$	0.1	25	86.3	39.5	0	46.8	0
H_{17}	0.1	100	97.9	37.4	0	36.5	24.0
I_{33}	0.1	100	98.0	33.1	0	34.6	30.3
J_4	0.1	100	94.1	35.2	0	30.5	28.4

[1] Percentage of control.
[2] Percentage of total radioactivity added.

concentration inhibited growth of the wild-type isolate W_5 for about 25%. The highest concentration severely inhibited growth of isolates W_5 and low-resistant isolate $E_{300\text{-}3}$ but still allowed growth of medium- and high- resistant isolates H_{17}, I_{33} and J_4 (Table 1). Radioactivity recovered after 24 h of incubation with 0.005 and 0.1 μg imazalil ml^{-1} was in the order of 86 to 98% of total radioactivity added. Radioactivity recovered after 16 h of incubation was in the same order of magnitude (results not shown).

Thin layer chromatography (TLC). Scans of radioactivity on TLC plates with extracts from mycelium and culture filtrates of all isolates obtained after 16 h of incubation with 0.005 μg imazalil ml^{-1} revealed only one peak of which the R_f value (0.67) was identical to imazalil (results not shown). The same result was found for mycelial extracts of all isolates incubated with 0.005 μg imazalil ml^{-1} for 24 h (Table 1). Scans of radioactivity on TLC plates with extracts of culture filtrates of wild-type isolate W_5 incubated with 0.005 μg imazalil ml^{-1} for 24 h showed two peaks. One had a R_f value (0.67) identical to imazalil and the other one had the same R_f value (0.37) as the authentic metabolite R42243 (Fig. 1). Similar results were observed with resistant isolates. However, the amount of metabolite detected in extracts of isolates H_{17}, I_{33} and J_4 was higher than in those of isolates W_5 and $E_{300\text{-}3}$ (Table 1). When the mycelia were incubated with 0.1 μg imazalil ml^{-1} the metabolite was only detected in the culture filtrates of medium- and high-resistant isolates H_{17}, I_{33} and J_4, respectively (Table 1). At a lower concentration (0.005 μg ml^{-1}) mycelium of medium- and high-resistant isolates H_{17}, I_{33} and J_4 accumulated a slightly lower amount of imazalil than mycelium of the wild-type and low- resistant isolates. At a higher concentration (0.1 μg ml^{-1}) this difference seemed to be smaller (Table 1). Incubation

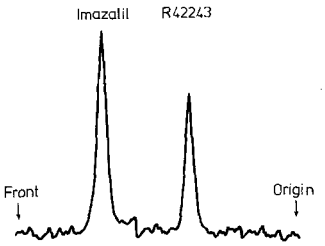

Fig. 1. TLC radioscans of extracts of culture filtrates of *Penicillium italicum* W_5 incubated for 24 h with [^{14}C]imazalil (0.005 μg ml^{-1}). TLC solvent, diethylether/chloroform/methanol/ammonium hydroxide (100:85:15:1, v/v/v/v).

of 24-h-old culture filtrates of all isolates with 0.005 μg imazalil ml^{-1} for another 24 h did not result in production of any metabolites (results not shown).

Radio-HPLC. Mycelial suspensions were incubated with 0.1 μg imazalil ml^{-1} for 24 h. Analysis of the mycelial extracts of isolates W_5 and J_4 showed only a single peak with a retention time of 44.3 min, which is identical to the one of imazalil. Analysis of the culture filtrate of isolate W_5 gave the same result. Analysis of the culture filtrate of isolate J_4 showed two peaks with retention times of 44.3 and 26.8 min, respectively. The retention time of the later compound is identical to the one of imazalil metabolite R42243. Relative peak size area of both compounds were 54.3 and 45.7%, respectively.

Mass spectrometry. Both reference R42243 and the isolated metabolite of imazalil were subjected to mass spectrometric analysis. The mass spectrum of the metabolite was identical to the spectrum of R42243 (Fig. 3). The spectra showed very weak molecular ion peaks at m/e 330 and 332 which is in agreement with the formula $C_{14}H_{16}Cl_2N_2O_3$ of R42243. Peaks caused by chloride-containing fragments are m/e 299 (loss of methoxyl moiety), m/e 295 (loss of one chlorine atom), m/e 249 (loss of methylene imidazole) and m/e 175 (dichlorobenzyl alcohol moiety). The 299, 249 and 175 peaks are accompanied by satellite peaks in an intensity ratio characteristic for the presence of two chlorine atoms in the corresponding fragments. Other peaks are m/e 82 (methyl imidazole), m/e 75 (dihydroxypropyl) and m/e 57 (loss of water from m/e 75).

Cross resistance. In order to study whether metabolism of imazalil in the propenyl side chain was important for resistance, toxicity of miconazole and R42243 to various isolates was tested. Results indicate that the DMI-resistant isolates of *P. italicum* were

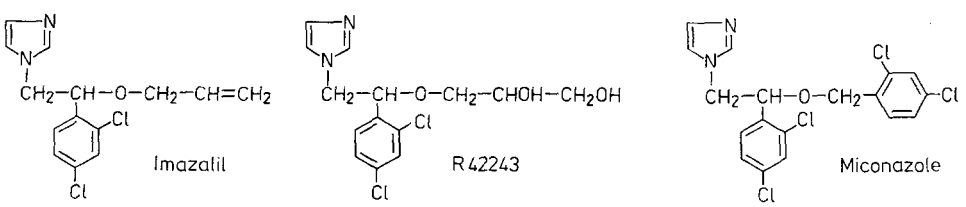

Fig. 2. Chemical structures of imazalil, 1-[2(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy) ethyl]-1*H*-imidazole (R42243) and miconazole.

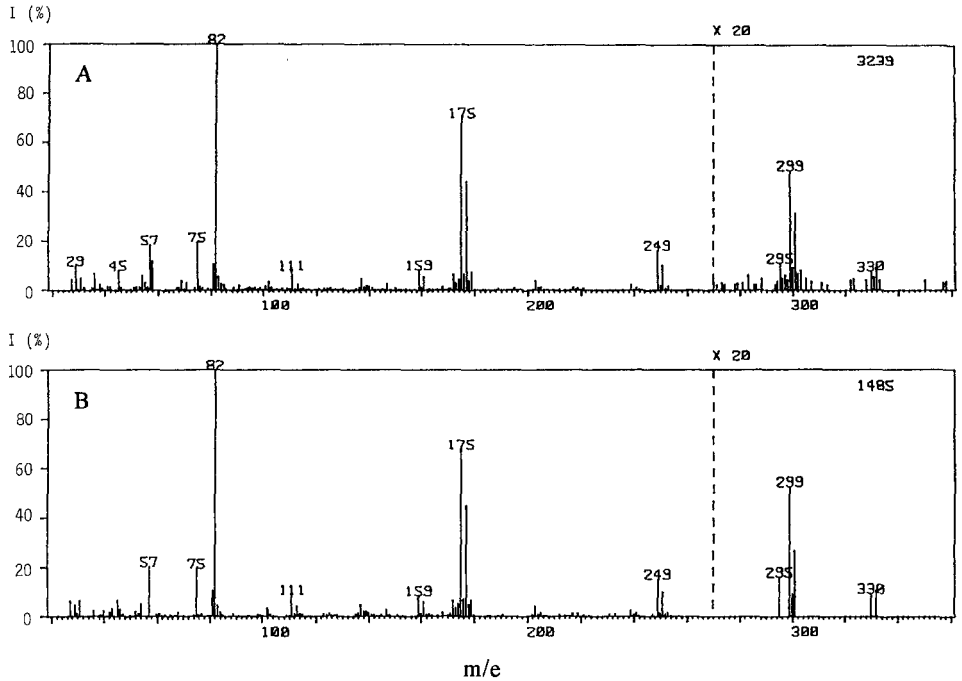

Fig. 3. Mass spectra of metabolite of imazalil isolated from culture filtrate of *Penicillium italicum* (A) and of authentic R42243 (B).

Table 2. Inhibition of imazalil, R42243 and miconazole on radial growth of wild-type and DMI-resistant isolates of *Penicillium italicum* on malt extract medium at pH 7.

Isolate	EC_{50} values ($\mu g\ ml^{-1}$)[1]		
	imazalil	R42243	miconazole
W_5	0.005	32	0.05
$E_{300\text{-}3}$	0.015 (3)[2]	130 (4)	0.27 (6)
H_{17}	0.21 (45)	330 (10)	0.70 (15)
I_{33}	0.27 (59)	600 (19)	1.20 (26)
J_4	0.19 (41)	590 (18)	1.70 (37)

[1] Concentration which inhibits radial growth by 50%.
[2] Between brackets: degree of resistance (EC_{50} resistant isolate : EC_{50} wild-type isolate).

also cross-resistant to miconazole and R42243 although the toxicity of the latter compound was relatively low (Table 2).

Discussion

After 24 h of incubation of *P. italicum* with imazalil (0.005 $\mu g\ ml^{-1}$) one metabolite could be detected (Fig. 1). The identity of the metabolite was confirmed to be R42243

by TLC, radio-HPLC and mass spectrometry. It has been reported that R42243 is one of the first metabolites of imazalil formed in animals. This was ascribed as epoxidation and hydratation processes in the propenyl side chain (Heykants, 1978). The same metabolic mechanism may be involved in *P. italicum*. The present paper is the first report that a fungus has such a capacity. The low toxicity of R42243 (Table 2) indicates that the metabolism is a detoxification step. The fact that R42243 was only detected in the incubation medium and the culture filtrate did not have the potency to metabolize imazalil suggest that imazalil was metabolized intercellularly and that the metabolite was secreted into the culture medium. No metabolite was found upon incubation of the wild-type and low-resistant isolates with a relatively high concentration of imazalil (0.1 $\mu g\ ml^{-1}$). This is caused by inhibition of mycelial growth. Metabolism of imazalil has also been studied in plants, such as in apple (Cano et al., 1987) and cucumber (Vonk and Dekhuijzen, 1979). In cucumber leaves, imazalil was metabolized to non-identified polar products. However, the metabolic process occurred at a much slower rate possibly through hydrolysis of the ether bond in the molecule (Vonk and Dekhuijzen, 1979).

Accumulation of imazalil by medium- and high-resistant isolates was lower than in the wild-type isolate (Table 1). This suggests that accumulation of imazalil may be involved in the mechanism of resistance. This suggestion is in line with previous reports (De Waard and van Nistelrooy, 1988).

At least three arguments support the statement that detoxification of imazalil does not operate as mechanism of resistance. Firstly, the metabolite could only be detected after prolonged incubation (24 h) while resistance to imazalil already became apparent within 8 h (Guan et al., 1989). Secondly, if resistance was based on metabolism of the propenyl side chain of imazalil, no cross-resistance should be observed to the related DMI-fungicide miconazole and to metabolite R42243 which have identical structures as imazalil except for the propenyl side chain (Fig. 2). This was however, not the case (Table 2). In addition, cross resistance is also present to structurally non-related DMIs (De Waard et al., 1982; De Waard and Van Nistelrooy, 1990). Thirdly, the rate of metabolism of imazalil in the various isolates was concentration-dependent. The lower detoxification rate by isolates W_5 (wild-type) and $E_{300\text{-}3}$ (low resistant) is probably the consequence of less biomass caused by growth inhibition during the incubation period (Table 1).

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