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Original Article

Action Mechanism of Diethofencarb to a Benzimidazole-Resistant Mutant in *Neurospora crassa*

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In the presence of 0.5 μg/ml diethofencarb, conidia of a benzimidazole-resistant mutant of *Neurospora crassa* germinated, with distorted, swollen germ tubes. Diethofencarb induced scattered nucleus and inhibited mitotic nuclear division in the resistant strain. The morphological abnormality was quite similar to the one observed in the wild-type strain treated with carbendazim (MBC). Diethofencarb, however, did not morphologically affect the germ tubes and nuclei of a wild-type strain. Diethofencarb formed a complex *in vitro* with a protein present in the mycelial extracts of the resistant mutant. The binding protein of the resistant strain was retained on a DEAE-Sephadex A-50 and eluted with 0.5 M KCl. The molecular weight of the binding protein was estimated 105,000 by gel filtration on Sephacryl S-200 chromatography. The data well coincide with those on the MBC-binding protein in the wild-type strain, suggesting that diethofencarb was selectively toxic to the benzimidazole-resistant strain by binding to the tubulin.

INTRODUCTION

Benzimidazole fungicides such as benomyl, carbendazim (MBC), thiophanate-methyl have contributed to the protection of agricultural crops from pathogen attack, but in recent years many kinds of plant pathogens have built up resistance to benzimidazole, causing a serious problem.[1] Leroux & Gredt[2-4] have found that benzimidazole-resistant isolates of *Botrytis cinerea* and *Penicillium expansum* showed an increased sensitivity to *N*-phenylcarbamate herbicides. Diethofencarb, a derivative of *N*-phenylcarbamates, exhibited remarkable antifungal activity to some benzimidazole-resistant strains but not to wild-type (benzimidazole-sensitive) strains.[5] The compound was systemic in plants, curative as well as preventive, but not herbicidal.[5] Thus it has provided a new tool to cope with fungicide resistance in the field.[6]

Benzimidazoles are reported to inhibit fungal mitosis by binding to tubulin.[7,8] In many cases, resistance to benzimidazoles is caused by mutation of β-tubulin gene.[9-12] We have previously proved a genetic mechanism of negatively correlated cross-resistance between benzimidazoles and *N*-phenylcarbamates using *Neurospora crassa* as a model fungus: Some benzimidazole-resistant strains of *N. crassa* showed an increased sensitivity to *N*-phenylcarbamates, and amino acid change at position 198 of β-tubulin conferred benzimidazole resistance and *N*-phenylcarbamate sensitivity.[13] The action mechanism of diethofencarb in benzimidazole-resistant mutants of *N. crassa* still remains obscure, however, the present investigation was aimed to elucidate the mode of action of diethofencarb, with its focus on cytological and biochemical studies in order to understand why diethofencarb is much more toxic to benzimidazole-resistant *N. crassa* than

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to sensitive one.

MATERIALS AND METHODS

1. *Strains*

Strain 74-OR31-14a (*al-2*, *pan-2* and *cot-1* a; FGSC4934) of *N. crassa* was used as wild type and F914a as benzimidazole resistant.[13] They were cultured in Vogel's minimal medium containing panthotenic acid at a concentration of 100 μg/ml.[14]

2. *Chemicals*

Methyl benzimidazole-2-yl carbamate (MBC) was prepared by reacting of *O*-phenylenediamine with dimethyl 2-methylisothiourea-1,3-dicarboxylate. Diethofencarb was synthesized by the method reported previously.[15] ^{14}C-diethofencarb (spec. act. 1.82 GBq/mmol) was uniformly labeled at the phenyl ring. The radiochemical purity was higher than 99%, based on TLC analysis [*n*-hexane–acetone (4/1, v/v) and toluene–ethyl acetate (5/2, v/v)]. Stock solution of [phenyl-^{14}C] diethofencarb was prepared in benzene/*n*-hexane solution (1 : 1) at a concentration of 1.31 mg/ml.

3. *Microscopic Observation*

Conidia of *N. crassa* were inoculated in Vogel's medium containing a chemical and cultured statically at 28°C for 18 hr. Germinated conidia were collected by centrifugation (1000×*g*), kept in ethanol for 10 min and suspended in distilled water. They were then treated with 4′, 6′-diamidino-2-phenylindole (DAPI) at a concentration of 1 μg/ml for 5 min and the germ tubes and nuclei were morphologically observed with a Nikon fluorescence microscope.

4. *Diethofencarb-Binding Activity of Mycelial Extracts from Wild-Type and Benzimidazole-Resistant Strains*

Conidia of the wild-type and benzimidazole-resistant strains of *N. crassa* were suspended in sterilized water and filtered through cheese cloth. They were then inoculated into 100 ml of liquid nutrient medium in a 500-ml Sakaguchi flask at a final cell density of 5×10^5/ml. Both strains were cultured at 28°C on a reciprocal shaker at 115 rpm for 20 hr. Mycelia (10 g on a fresh weight basis) were collected and ground in liquid nitrogen, and the powder was transferred into PIPES buffer I [0.1 M PIPES-Na, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 mM GTP, and 1 : 100 solution P (87 mg of *p*-methylphenylsulfonyl fluoride and 1.5 mg of pepstatin A in 5 ml of ethanol)] as described by Kilmartin.[8] [Phenyl-^{14}C] diethofencarb was added to the mycelial extract and incubated at 4°C for 1 hr. The incubated mixture was then centrifuged at 50,000×*g* for 45 min, and the supernatant was applied to a Sephadex G-25 equilibrated with PIPES buffer II (0.1 M PIPES-Na, pH 6.9, 0.2 M KCl, 0.2 mM $MgCl_2$, 0.1 mM GTP, and 1 : 1000 solution P) to remove unbound diethofencarb. Radioactivity and protein contents in each fraction were measured, the latter with a BCA protein assay reagent (PIERCE).

5. *Purification of Diethofencarb-Binding Protein*

The mycelial extract solution was centrifuged at 50,000×*g* for 45 min, incubated with [phenyl-^{14}C] diethofencarb at 4°C for 1 hr and applied onto a DEAE-Sephadex A-50 column equilibrated with buffer II. The column was washed with 3-bed volumes of PIPES buffer II and subsequently eluted with PIPES buffer II containing 0.5 KCl and 1 M KCl. Radioactive fractions from the column were concentrated and desalted with a Centriprep-10 (Amicon). The resulting solution was again incubated with [phenyl-^{14}C] diethofencarb at 4°C for 1 hr and the molecular weight of the diethofencarb-protein complex was determined by gel filtration with a Sephacryl S-200 (1.5 ×100 cm) equilibrated with PIPES buffer II. The molecular weight was calibrated with a gel filtration calibration kit (Pharmacia)

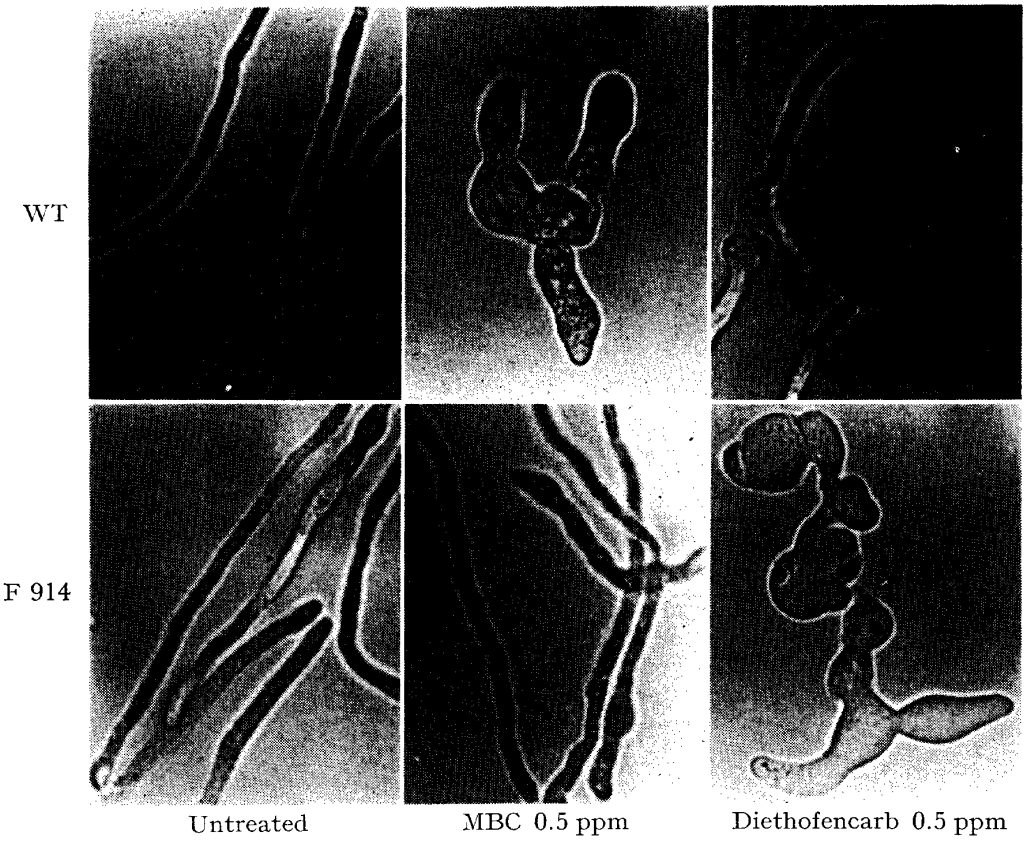
6. *Radioanalysis*

Aliquots of the fractions were added to low potassium glass vials (Wheaton, USA) containing 10 ml of Emulsifier Scintillator 299 (Packard, USA). Radioactivity in the fractions was measured with a Tri-Carb 460 CD liquid scintillation spectrometer (Packard, USA).

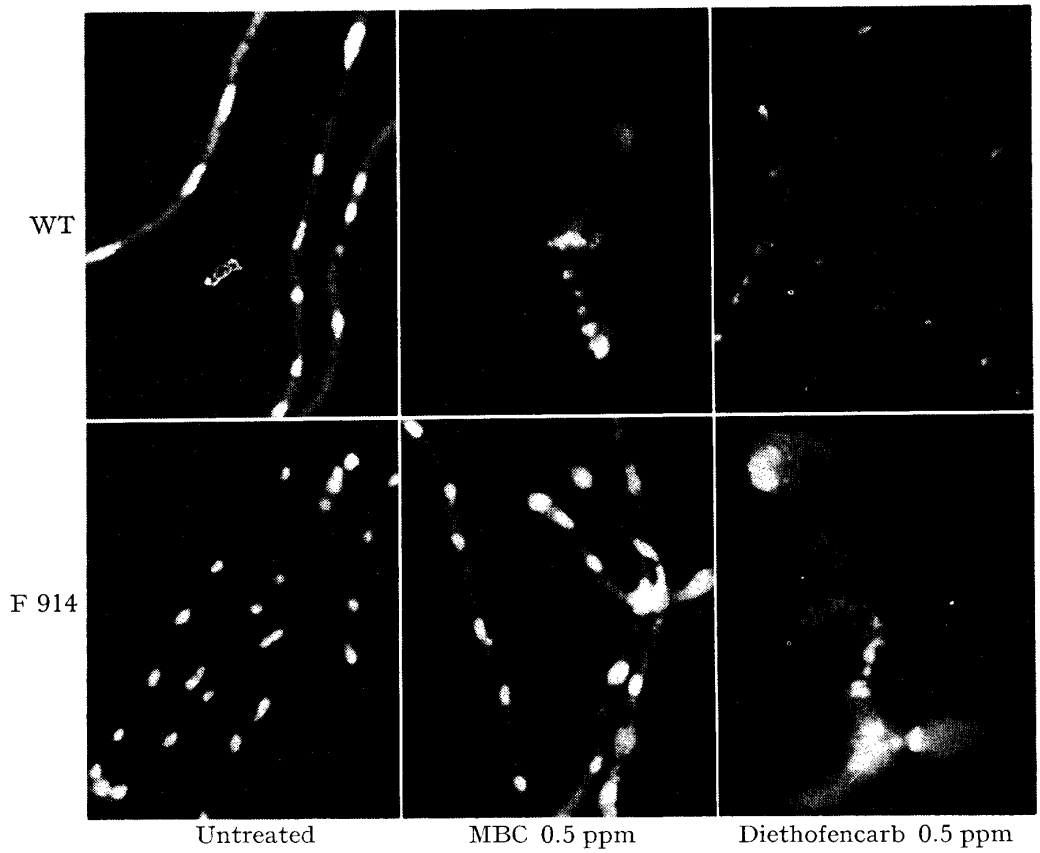
RESULTS

1. *Cytological Effects of Diethofencarb on the Benzimidazole-Resistant Strain*

We have previously reported that diethofen-



(a)



(b)

Fig. 1 (a) Effects of diethofencarb and MBC on fungal morphology of *N. crassa*.
(b) Effects of diethofencarb and MBC on nuclear morphology of *N. crassa*.

carb was selectively fungitoxic to benzimidazole-resistant strain F914, and defined the mutation as a single amino acid replacement from ^{198}glutamic acid to glycine in β-tubulin.[13] Diethofencarb did not inhibit the conidial germination of F914 even at a concentration of 100 μg/ml, but the conidia exposed to 0.5 μg/ml diethofencarb formed multiple germination tubes. The germ tubes were distorted and swollen, but further extension of the hyphae was not observed (Fig. 1(a)). Such morphological abnormality did not occur in the wild-type strain treated with diethofencarb. On the other hand, MBC caused distortion and swelling of germ tubes in the wild-type strain, but not in the resistant strain at the concentration of 0.5 μg/ml. Morphological changes in the resistant strain caused by diethofencarb were quite similar to those caused by MBC in the wild-type strain.

The nuclei of the same samples were also examined morphologically after staining with DAPI. Diethofencarb inhibited nuclear division in the benzimidazole-resistant strain at the concentration of 0.5 μg/ml. Chromatins in the conidia were scattered, and there were no round-shaped nuclei as found in the control cells (Fig. 1(b)). Diethofencarb did not affect the nuclear morphology of the wild-type strain, and normal phases of mitosis were found in the treated hyphae. On the other hand, MBC disturbed the mitosis of the wild-type strain at the concentration of 0.5 μg/ml, but did not exert any effect on the structures of nuclei in the resistant strain. Morphological abnormality in nuclei was not distinguishable between the diethofencarb-treated resistant and the MBC-treated wild-type strain.

2. *Selective Binding of Diethofencarb to Extracts from the Benzimidazole-Resistant Strain*

It is suggested that selective fungitoxicity of diethofencarb to benzimidazole-resistant strains may be due to the specific binding to the cell components. We compared the cell extracts from the wild-type and resistant strains in diethofencarb-binding activity, using [phenyl-^{14}C] diethofencarb as a maker. Extracts from the mycelia of the wild-type and resistant strains were incubated with [phenyl-^{14}C] diethofencarb. The 50,000$\times g$ supernatant of each strain was applied to the Sephadex G-25 to remove unbound diethofencarb. Diethofencarb was bound in the 50,000$\times g$ supernatant from the resistant strain, whereas binding was hardly observed in the supernatant from the wild type (Table 1). Binding activity appeared to be correlated with the *in vivo* diethofencarb sensitivity of the strains. The pellet obtained by centrifugation (50,000$\times g$, 45 min) showed little binding to diethofencarb, and no difference was observed between the wild type and the resistant strain.

Table 1 Diethofencarb-binding activity in mycelial extracts of a benzimidazole-resistant strain.

Strains	MIC (μg/ml)	Protein conc. (mg/ml)	Bound diethofencarb[a] (dpm)
wild-type	>100	29.5	171
resistant F914	0.1	26.1	30,229

[a] The supernatant of a mycelial extract was applied to a Sephadex G-25, and radioactivity in the resultant solution was measured (detail shown in Materials and Methods).

3. *Purification of Diethofencarb-Binding Protein*

The diethofencarb-binding component was further characterized by chromatography on a DEAE-Sephadex A-50 anion exchanger. As described earlier, cytological effects of diethofencarb on the benzimidazole-resistant strain were quite similar to those of MBC on the wild-type strain. For this reason, the diethofencarb-binding protein is assumed to be identical with fungal tubulin. A 50,000$\times g$ supernatant of the resistant strain incubated with [phenyl-^{14}C] diethofencarb was run on a DEAE-Sephadex A-50 column, which has proven to be useful in purification of tubulin from other organisms.[8,16] When the column was washed by 3-bed volumes of equilibrated PIPES buffer II (0.2 M KCl), significant radioactivity was observed (Fig. 2). The radioactive fractions (fraction Nos. 12–16) were then applied to the Sephadex G-25 to remove unbound diethofencarb. There was no significant radioactivity in the solution desalted with the Se-

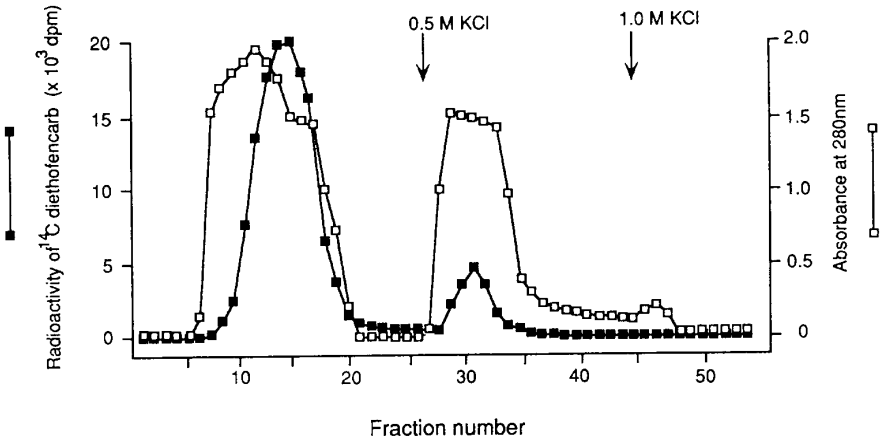


Fig. 2 DEAE-Sephadex A-50 column chromatography of mycelial extracts of a benzimidazole-resistant strain of *N. crassa*.

Mycelial extracts (50,000×*g* supernatant) incubated with [phenyl-^{14}C] diethofencarb were applied onto a DEAE-Sephadex A-50 column and eluted with 0.5 M KCl and 1.0 M KCl. The graphs show elution patterns of radioactivity and absorbances at 280 nm.

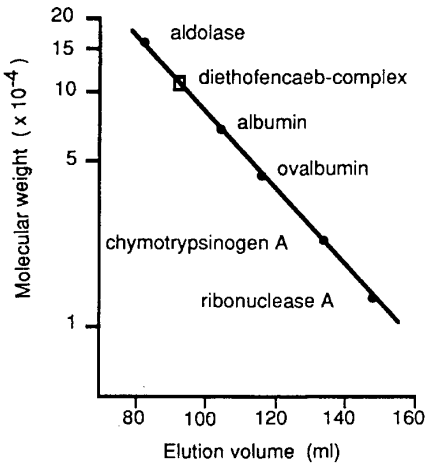


Fig. 3 Molecular-weight determination of the diethofencarb-protein complex.

Radioactive fractions of DEAE-Sephadex A-50 were gel-filtrated through a calibrated Sephacryl S-200 column.

phadex G-25, which indicated free diethofencarb was eluted from the column at this step. Fractions eluted by the PIPES buffer II with 0.5 M KCl exhibited considerable radioactivity, but fractions eluted at 1.0 M KCl contained little radioactivity (Fig. 2).

To estimate the molecular weight of diethofencarb-binding protein, radioactive fractions (fraction Nos. 31–33) obtained by DEAE-Sephadex A-50 chromatography were collected and concentrated with a Centricon C-10. The resulting desalted fractions were incubated with [phenyl-^{14}C] diethofencarb again and run on a Sephacryl S-200 column. The bulk of th bound radioactivity was eluted in a single peak which corresponded well to 105,000 daltons protein (Fig. 3). This is very close to the value found for a MBC-tubulin complex.[7] The data strongly suggested that diethofencarb acted as a selective fungicide to the benzimidazole-resistant strain by binding to the tubulin.

DISCUSSION

To elucidate the action mechanism of diethofencarb in the benzimidazole-resistant strain, we studied the effects of diethofencarb on a benzimidazole-resistant strain, F914, of *N. crassa*. The strain increased the sensitivity to diethofencarb. The mutation has been genetically defined as single amino acid replacement from glutamic acid to glycine at position 198 in β-tubulin.[13]

The present investigation indicated a negative correlation between MBC and diethofencarb in the effects on fungal morphology and mitosis. It is well known that MBC inhibits mitosis in fungi by binding to the fungal tubulin.[7,8] Abnormal morphology of germ tubes and disintegrated configuration of the chromatins occurred in both the benzimidazole-resistant strain treated with diethofencarb and the wild-type strain treated with MBC. Similar observations are reported on *B. cinerea* treated with diethofencarb and MDPC,[5,17] which suggests that diethofencarb

interferes with the formation or functions of microtubules in resistant strains in a selective manner. This assumption is supported by biochemical studies on cell components that bind diethofencarb.

Diethofencarb-binding activity was found in the cell extracts from the resistant strain but not in those from the wild type. This diethofencarb-protein complex was retained on DEAE-Sephadex A-50 column, and its molecular weight was estimated to be around 105,000. The MBC-tubulin complex in the wild type of *Aspergillus nidulans* was retained on a DEAE-Sephadex A-50 anion exchanger and its molecular weight was estimated at 110,000.[7] On the basis of these results it can be concluded that the diethofencarb binding protein is identical with fungal tubulin. In binding studies using ^{14}C MBC on the wild-type and benzimidazole-resistant strains, only extracts of the sensitive types showed binding activity, which suggests that affinity at the target site of benzimidazoles determines the antifungal activity of benzimidazoles.[7,8,18,19]

This is the first report on the selective fungitoxicity of diethofencarb to a benzimidazole-resistant strain by binding the tubulin. Recently we isolated and characterized several mutants different in the sensitivity to benzimidazoles and *N*-phenylcarbamates, and determined the substitutions of amino acids in β-tubulin in these mutants. Biochemical analysis of these mutants will help study the binding mode of these compounds to tubulin.

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要　　約

アカパンカビにおけるジエトフェンカルブのベンズイミダゾール耐性株に対する選択的殺菌作用機構

藤村　真，鎌倉高志，山口　勇

ベンズイミダゾール耐性菌に選択的殺菌活性を示すジエトフェンカルブは，アカパンカビの分生胞子の発芽を阻害しなかったが，0.5 μg/ml の濃度において，ベンズイミダゾール耐性株の発芽管の膨潤および異常分岐を引き起こした．また，ジエトフェンカルブは，耐性株に選択的に核の形態の異常を引き起こし，核分裂を阻害していることが DAPI による核染色により観察された．これらのジエトフェンカルブの耐性株に対する発芽管および核の形態異常は，野生株に対する MBC の作用ときわめて類似していた．さらに，^{14}C–ジエトフェンカルブを用いて，ジエトフェンカルブ結合蛋白質の検索を行なったところ，耐性株の細胞破砕液の遠心 (50,000×g) の上清の高分子分画に特異的な結合蛋白質の存在を示唆する高い放射能活性が認められた．このジエトフェンカルブ結合蛋白質は，DEAE Sephadex A-50 に吸着され，0.5 M KCl で溶出された．その分子量は，Sephacryl S-200 によるゲル濾過により約 105,000 であると推定された．これらの結果は，ベンズイミダゾール感受性菌の MBC 結合蛋白質 (チューブリン) と一致していることから，ジエトフェンカルブは，ベンズイミダゾール耐性株のチューブリンに特異的に結合して，微小管の形成を阻害することにより，選択的殺菌活性を示すと考えられた．