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Antifungal Activity of Methyl 2-Benzimidazole Carbamate

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Antifungal properties of methyl 2-benzimidazole carbamate (BMC) were investigated using 16 fungi. Cytotoxicity test of BMC revealed that the morphology of HeLa cells was considerably deformed even at the concentrations as low as 0.1 ppm. Minimum inhibitory concentration (MIC) values of BMC for 7 fungi among the 16 tested ones were lower than 1.95×10^{-4} μg/ml, while *Aspergillus flavus* showed an MIC value higher than 1.0 μg/ml. Tolerance induction against BMC was successful only for *Paecilomyces farinosus* LAR10, contrary to the expectation that tolerance would be induced for the fungi having high MIC values such as *Aspergillus niger* ATCC 9642 and *A. flavus* ATCC 9643. Spore germination of *A. niger* ATCC 9642 was suppressed by BMC. However the mycelial growth of the fungus once germinated was not retarded at all by BMC up to 8 MIC. Addition of lanosterol provided a remedy for the reduced germination rate of *A. niger* ATCC 9642 spores.

KEYWORDS: Cytotoxicity, Methyl-2-benzimidazole carbamate, MIC, Tolerance

Many fungal spores, such as *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Alternaria* sp. and *Mucor* sp. float around in air and they are pathogens which provoke opportunity infections. *Aspergillus* sp. gives rise to asthmatic disease. *Penicillium* sp. causes fungal corneitis, pencillosis and otitis externa. *Cladosporium* sp. brings on pigment blastomycosis and pigment mycosis. *Alternaria* sp. leads to fungal infection, skin infection and osteomyelitis, while *Mucor* sp. is the cause of mucormycosis and meatus acusticus externus infection. *Paecilomyces* sp. is responsible for endocarditis and mycotic keratitis.

Antifungal agents have been developed not only to cope with the pathogenic fungi but also to improve the human living environment. Materials possessing antifungal activities find a broad application for clothes, household appliances, lavatory products and construction materials. Medical implements also require antimicrobial properties.

Methyl 2-benzimidazole carbamate (BMC) has been compounded with paints or with adhesives to prevent the materials from microbial contamination (Chung, 1997).

In this study, cytotoxicity of BMC was investigated by observing the cell viability of HeLa cells. Susceptibility of 16 fungi to BMC was examined by measuring the respective MIC values, and the possibility of tolerance induction against BMC was studied. Antifungal activity of BMC was explored for *Aspergillus niger*, whose BMC-tolerance was not induced, either in the spore stage or in the mycelial growth period.

Materials and Methods

Antifungal agent. BMC (Aldrich) dissolved in dimethyl sulfoxide was subjected to the antifungal activity tests.

Microorganisms. The following fungal strains were used: *Aspergillus* spp. (*A. niger* AN-2, *A. flavus* AF-2, *A. niger* ATCC 9642, *A. fumigatus* IFO 30870, *A. flavus* ATCC 9643), *Fusarium* spp. (*F. graminearum* F98-20, *F. solani* F6079, *F. sambucinum*, *F. moniliform*), *Penicillium* spp. (*P. hirsutum* PE-1, *P. italicum* PE-2, *P. expansum* PE-3, *P. pinophilum* ATCC 9644), *Rhizopus stolonifer* RI97-2, *Paecilomyces farinosus* LAR 10 and *Trichoderma viride* ATCC 32098. Susceptibility tests were performed using fresh overnight subcultures of the strains.

Cytotoxicity test. Human epithelial cells (cell line HeLa) were used in this study. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction assay was performed according to the method reported by Ciapetii *et al.* (1993) and Dang *et al.* (1996).

Broth micro-dilution test. A antifungal susceptibility was assessed by determining the minimum inhibitory concentration (MIC) of the antifungal agents by using the broth micro-dilution method in accordance with the procedures described by the National Committee for Clinical Laboratory Standards (1995). The medium used was RPMI 1640 (Sigma); it was buffered with 4-morpholinepropanesulfonic acid sodium salt (MOPS, Sigma) to a final molarity of 0.165 and adjusted to pH 7.0. The medium (90 μl) and BMC (10 μl) were introduced to each well of a sterile 96-well microtitre plate, and then spore suspension (1×10^7 spore/ml) was added to each well. After incubating at 28°C for 72 hours, the lowest BMC concentration inhibiting visible growth was recorded.

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Agar disk diffusion test. After fungal spore suspension (1×10^7 spores/ml) was inoculated on potato dextrose agar (PDA, Sigma), 0.05, 0.1, 0.5, 1 or 2% of BMC was loaded onto each filter paper disk. The plates were incubated at 28°C for 72 hours. Antifungal activity was determined by the diameter of the clear inhibition zone around the disk.

BMC resistance induction of *P. farinosus* LAR10. To induce resistance to BMC, *P. farinosus* was grown on potato dextrose broth (PDB, Sigma) at 28°C for 5 days. To the mycelial suspension of the sensitive strain, freshly prepared BMC was added at various concentrations and incubated at 28°C for 7 days.

Effect of BMC on the life cycle of *A. niger* ATCC 9642. To determine the inhibition mechanism of BMC on the formation of spores and mycelia, spore suspension was inoculated in the thin slab of PDA containing 1 MIC and 2 MIC of BMC. After 0.5, 1, 2 and 4 days, agar slabs with fully grown mycelia were stained in lactofuchsin and the morphological abnomalities caused by the BMC were examined under the light microscope.

To investigate the antifungal effects on the spore and mycelial stages, 1 m*l* of spore suspension was incubated in 50 m*l* PDB in the presence of 1 MIC, 2 MIC, 4 MIC or 8 MIC of BMC, at 28°C, 140 rpm. At the intervals of 1 day, the mycelia were harvested and the dry weight was measured.

Effect of lanosterol. Spore suspension of *A. niger* ATCC 9642, lanosterol (1~1000 ppm) and 8 MIC of BMC were added to 50 m*l* PDB, and then incubated at 28°C for 3 days. The control cultures, in the absence of both BMC and lanosterol, were processed similarly for comparison.

Results and Discussion

Cytotocicity of BMC. Benzimidazole homologues for antifungal application including benomyl, carbendazim, thiabendazole, and thiophanate-methyl have been used as antifungal agents in the agricultural field for a long time. The chemical structure of BMC, one of the benzimidazole homologues, is shown in Fig. 1.

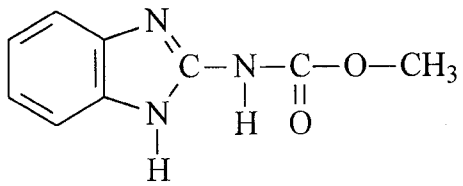

Fig. 1. Chemical structure of methyl 2-benzimidazole carbamate.

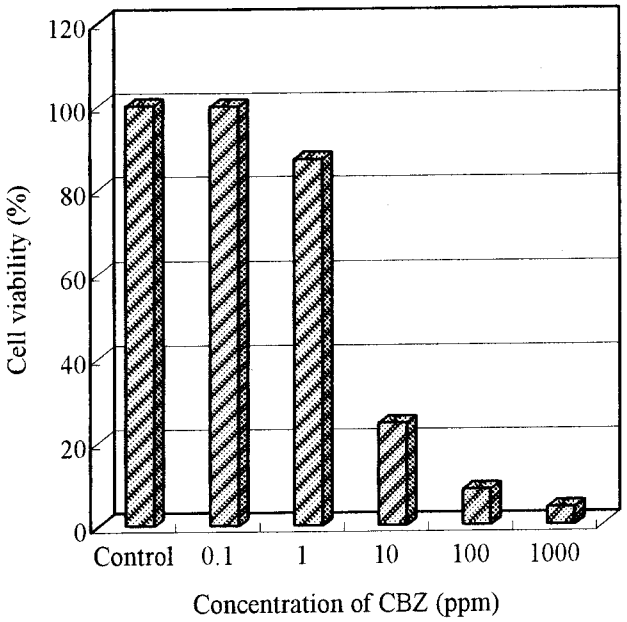

Fig. 2. Cell viability of HeLa cells after incubation for 48 h. MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) reduction assay was performed according to the method reported by Ciapetii *et al.* (1993) and Dang *et al.* (1996).

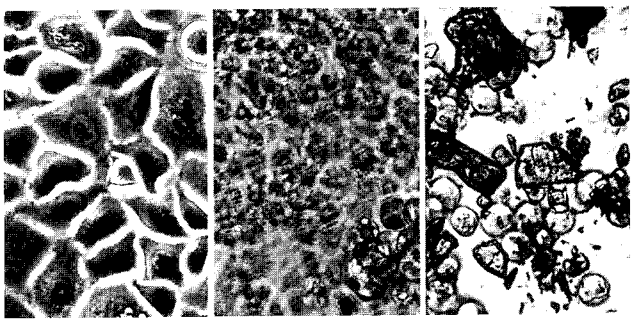

Fig. 3. Electron micrographs (100×) of HeLa cells treated with methyl 2-benzimidazole carbamate (BMC) for 48 h. (A) control, (B) treated with 0.1 ppm of BMC, (C) treated with 1,000 ppm of BMC.

Figure 2 demonstrates the cytotoxicity of BMC on HeLa cells. The cell viability was decreased to 99.8, 87.1, 24.5, 8.5 and 4.2% as the concentration of BMC increased to 0.1, 1, 10, 100 and 1,000 ppm, respectively. The morphology of the HeLa cells after the cytotoxicity test is exhibited in Fig. 3. The HeLa cell was originally big in size with a well-developed nucleus. The HeLa cells treated with 0.1 ppm of BMC showed almost identical cell viability but was much smaller in size compared to the pristine HeLa cells. BMC at 1,000 ppm deformed the HeLa cell morphology tremendously. Therefore BMC should be carefully used (Andriole, 1993, 2000; Kim, 1998). MIC values of BMC for 16 fungi are summarized

Table 1. MICs for BMC by broth microdilution test

Microorganism	MIC (μg/ml)
Aspergillus flavus AF-2	>1
A. flavus ATCC 9643	>1
A. fumigatus IFO 30870	$<1.95\times10^{-4}$
A. niger AN-2	0.5
A. niger ATCC 9642	0.5
Fusarium graminearum F98-20	0.1
F. moniliform	>0.1
F. sambusinum	$<1.95\times10^{-4}$
F. solani F6079	$<1.95\times10^{-4}$
Paecilomyces farinosus LAR 10	>0.1
Penicillium expansum PE-3	$<1.95\times10^{-4}$
P. hirsutum PE-1	$<1.95\times10^{-4}$
P. italicum PE-2	0.1
P. pinophilum ATCC 9644	7.8×10^{-4}
Rhizopus stolonifer RI97-2	$<1.95\times10^{-4}$
Trichoderma viride ATCC 32098	$<1.95\times10^{-4}$

MIC : Minimum inhibitory concentration.
BMC : methyl 2-benzimidazole carbamate.

in Table 1. MIC for 7 fungi was as low as 1.95×10^{-4} μg/ml. In marked contrast, *A. flavus* AF-2 and *A. flavus* ATCC 9643 showed MIC values higher than 1.0 μg/ml. MIC values of other antifungal agents have been reported to be in the range of 0.1~16 μg/ml. Manavatha *et al.* (1998) observed MIC values of itraconazole, miconazole and ketoconazole to be 0.125~16 μg/ml, 0.125~16 μg/ml and 0.125~2 μg/ml respectively against *A. fumigatus* by the broth microdilution test. Paul *et al.* (1998) examined MIC values of itraconazole and voriconazole for 150 strains of *A. fumigatus* using the agar dilution test, and obtained MIC values of 0.25 μg/ml and 0.5 μg/ml, respectively. Rath (1998) reported that MIC values of itraconazole for *A. fumigatus*, *A. flavus*, *A. niger*, *A. nidulans* and *A. terreus* were in the range of 0.03~0.5 μg/ml.

Induction of tolerance. Induction of tolerance against BMC was examined for the 16 fungi in Table 1. Contrary to the expectation that the tolerance against BMC would be induced for fungi having high MIC values such as *F. graminearum* F98-20, *A. niger* AN-2, *A. flavus* AF-2, *P. italicum* PE-3, *R. stolonifer* RI97-2, *A. flavus* ATCC 9643 and *F. moniliform*, the tolerance was induced only for *P. farinosus* LAR10.

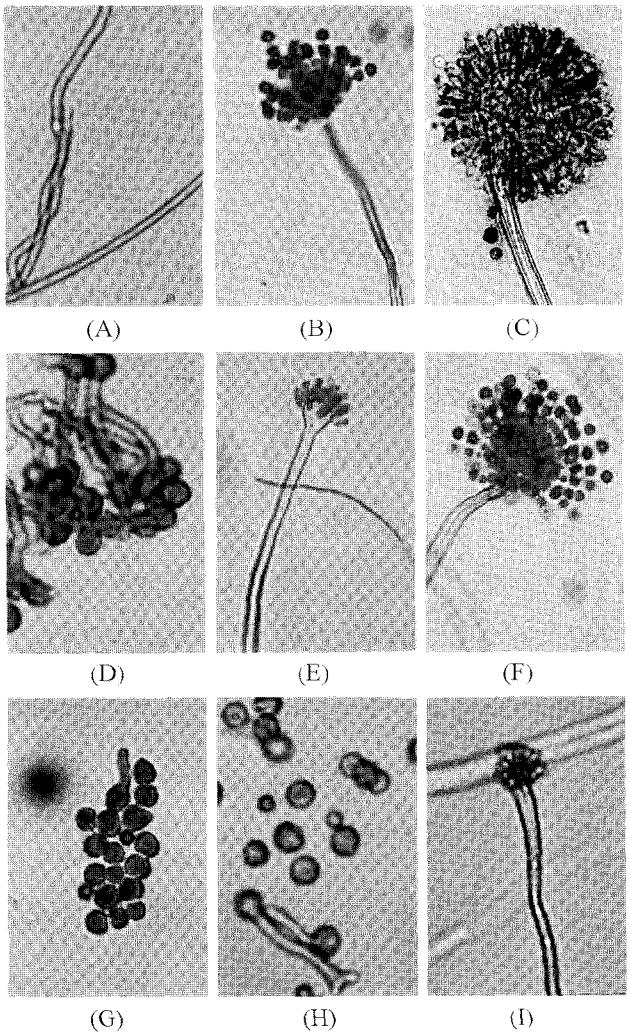

Fig. 4. Effect of methyl 2-benzimidazole carbamate (BMC) on the morphology of *A. niger* ATCC 9642. (A)-(C): control, (D)-(F): added with 1 MIC of BMC, (G)-(I): added with 2 MIC of BMC. MIC; Minimum inhibitory concentration. Exposure time (A), (D), (G): 12 h, (B), (E), (H): 2 days, (C), (F), (I): 4 days.

Table 2 discloses that MIC values of BMC for BMC-tolerance induced *P. farinosus* was about 50,000 times higher than that for pristine *P. farinosus*. Growth of the BMC-tolerance induced *P. farinosus* was not discernably

Table 2. Susceptibility of CBZ resistance induced *Paecilomyces farinosus* LAR 10 to BMC

Strain	Broth microdilution test	Agar disk diffusion test				
	MIC (μg/ml)	Diameter of growth inhibition zone (mm) according to BMC concentration (%)				
		2	1	0.5	0.1	0.05
Pristine *P. farinosus* LAR10	>0.1	48	48	48	46	31
BMC resistance induced *P. farinosus* LAR10	$>5.12\times10^{3}$	0	0	0	0	0

MIC : Minimum inhibitory concentration.
BMC : methyl 2-benzimidazole carbamate.

inhibited even in the presence of 2% of BMC. Taggart *et al.* (1999) observed that the BMC-tolerance was also induced for *Rhyncohsporium secalis* and that the fungus showed resistance against other benzimidazole homologues as well.

Effect of BMC on the germination of *A. niger*. Mycelia of *A. niger* ATCC 9642 was formed after 12 hours of cultivation (Fig. 4-A). A conidiophore bearing dense conidia was developed after 2 days (Fig. 4-B) and was fully grown after 4 days (Fig. 4-C). When 1 MIC of BMC was added, the mycelia became shorter (Fig. 4-D) and smaller conidiophore with loose conidia population was formed (Fig. 4-E, Fig. 4-F). Germination tubes did not develop until 12 hours as shown in Fig. 4-G, when 2 MIC of BMC was added.

Figure 5 reveals that addition of BMC to the spores of *A. niger* ATCC 9642 strongly inhibited the growth of the fungus. In marked contrast, when BMC was added at the mycelial stage, the growth of the fungus was not much affected by BMC up to 8 MIC (Fig. 6). These results are in line with its morphology in Fig. 4 in that the BMC suppressed the spore germination of the fungus but the growth of the mycelia once formed was not retarded by BMC. Therefore it can be said that BMC exhibits its antifungal activity on *A. niger* ATCC 9642 by suppressing its spore germination rather than by inhibiting mycelial growth.

Fungus usually contains ergosterol as a cell membrane sterol (Theodore *et al.*, 1998). The growth rate of *A. niger* ATCC 9642 was measured when 8 MIC of BMC was

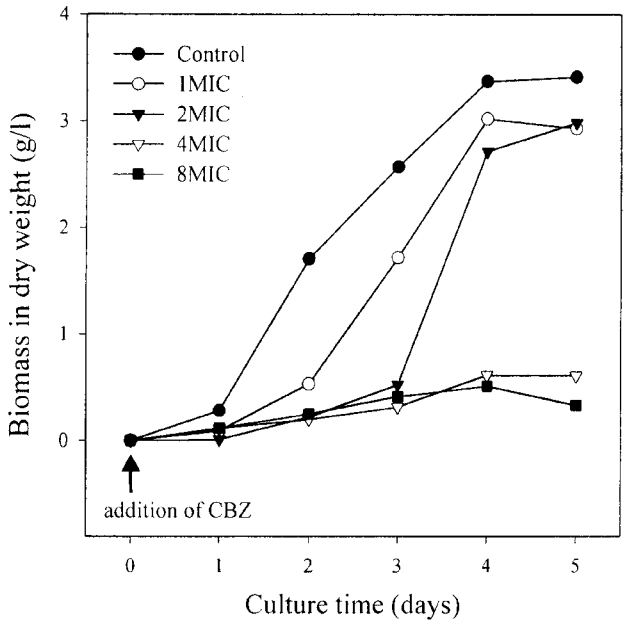

Fig. 5. Growth behavior of *Aspergillus niger* ATCC 9642 when methyl 2-benzimidazole carbamate (BMC) was added to the culture medium at the spore stage. MIC; Minimum inhibitory concentration. Spore suspension (1 m*l*) was incubated while stirring at 140 rpm in 50 m*l* PDB in the presence of BMC, at 28°C.

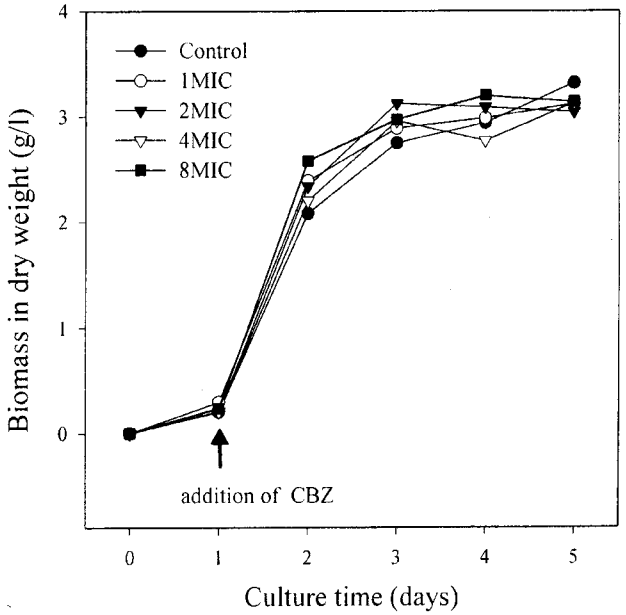

Fig. 6. Growth behavior of *Aspergillus niger* ATCC 9642 when methyl 2-benzimidazole carbamate (BMC) was added to the culture medium at the mycelial stage. MIC; Minimum inhibitory concentration. Mycelial suspension (1 m*l*) was incubated while stirring at 140 rpm in 50 m*l* PDB in the presence of BMC, at 28°C.

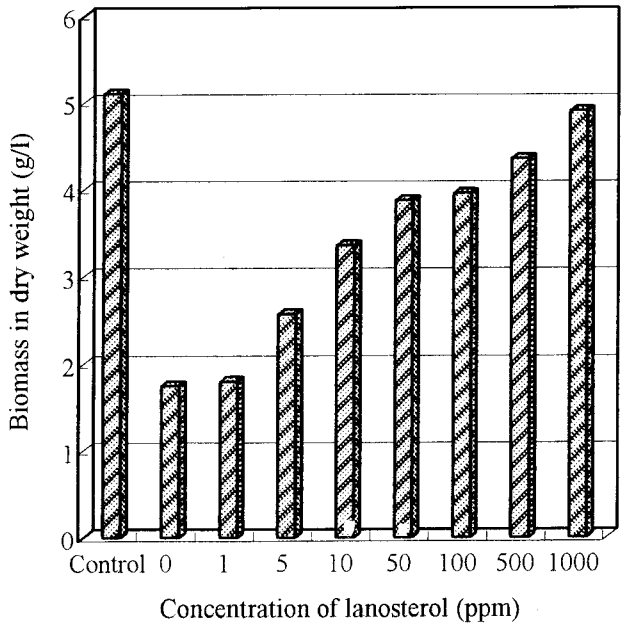

Fig. 7. Effect of lanosterol on the growth of *Aspergillus niger* ATCC 9642 in the presence of methyl 2-benzimidazole carbamate (BMC) at 8 MIC. MIC; Minimum inhibitory concentration. Spore suspension of *A. niger* ATCC 9642, lanosterol (1~1,000 ppm) and 8 MIC of BMC were added to 50 m*l* PDB, and then incubated at 28°C for 3 days.

added to the spores together with lanosterol at different concentrations. The biomass on dry basis, after 72 hours of cultivation, is shown in Fig. 7. According to Fig. 7, the biomass produced in the presence of BMC at 8 MIC was one-third of the biomass in the absence of BMC (control experiment). However, the biomass increased gradually by the increase of lanosterol concentration. When 1,000 ppm of lanosterol was added, the strain grew even in the presence of 8 MIC of BMC at almost the same rate as that in the control experiment.

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