

# Differential Effect of Imidazole Antibiotics on Untransformed and Virus-transformed Rat Cell Lines<sup>1</sup>

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## ABSTRACT

The imidazole antimycotics clotrimazole and miconazole were tested on untransformed rat cell line 3Y1-B clone 1-6 (3Y1) and six transformed cell lines, which were independently isolated from 3Y1 or 3Y1-B clone 1 after infection with adenovirus 12 (AD-12) or with SV40, to determine their sensitivities to these drugs. The relative plating efficiency of three cell lines (T3, W4, and W5) transformed with AD-12 was reduced to  $10^{-1}$  of the initial value by clotrimazole (2 to 4  $\mu\text{g}/\text{ml}$ ), whereas that of the parental cell line 3Y1 was reduced to  $10^{-1}$  of the initial value by clotrimazole (20 to 25  $\mu\text{g}/\text{ml}$ ). By contrast, the differential effect of miconazole on 3Y1 and AD-12-transformed cell lines was found to be a factor of 2. The sensitivity of the SV40-transformed cell lines to these drugs was between the untransformed 3Y1 and the AD-12-transformed cell lines. The cellular sensitivity of untransformed 3Y1 cells to clotrimazole was significantly enhanced when exposed to various doses of the unsaturated fatty acid, linoleic acid. The untransformed and transformed cell lines showed sensitivities similar to the cytotoxic activity of sterol-binding antimycotics, amphotericin B and filipin.

## INTRODUCTION

To obtain specific growth control of transformed cells, various studies have been done in search of drugs that can detect biochemical changes in transformed cells that are absent in normal cells. Transformed cells show altered sensitivity *in vitro* to various agents such as the sterol-binding polyene antimycotic, amphotericin B (3, 18, 19). In particular, we have been interested in altered membranous lipid composition of transformed cells (for review, see Refs. 13 and 28). Our previous study referred to altered sterol metabolism of sterol synthesis-deficient hamster cells in connection with specific growth control by polyene antibiotics (1, 9). In this study, we focus on imidazole antimycotics that interact with fatty acids in fungal membranes (16, 20, 21, 24-26) to see whether cellular sensitivity of virus-transformed cells differs from that of untransformed cells when exposed to imidazole antimycotics or polyene antibiotics.

## MATERIALS AND METHODS

**Cell Lines Used.** The cell lines used in this study are listed in Table 1. Both 3Y1-B clone 1 and clone 1-6 (3Y1) cell lines showed almost the same properties with respect to growth behavior and sensitivity to

various drugs. The clones showed normal type of growth as described previously (15).

**Cell Culture and Colony Formation.** All cell lines were grown in monolayer in minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan) containing 10% newborn calf serum (Flow Laboratories, Stanmore, New South Wales, Australia), 0.1% Bactopeptone (Difco Laboratories, Detroit, Mich.) and Penicillin G (100 units/ml) under our routine culture conditions (1, 10). For assay of colony formation, the cells (300 cells/dish) were first plated in a 60-mm dish in the absence of drugs at 37° for 18 hr, after which time they were exposed to drugs and incubated for an additional 10 days. Colony number was counted after staining with Giemsa (9).

**Growth Curve.** Drug effect on growth curve of each cell line was followed when  $3 \times 10^4$  cells were initially inoculated into a 35-mm dish. After the incubation for 1 day, various doses of imidazole antimycotics were added. At indicated times, cell viability was tested directly by trypan blue exclusion on detached cells as well as on dish-adherent cells. The dish-adherent cells were suspended by treatment with trypsin.

**Chemicals.** Linoleic acid was purchased from P-L Biochemicals, Inc., Milwaukee, Wis., and it was dissolved in ethanol at 10 mg/ml. Pure samples of imidazole antimycotics, clotrimazole and miconazole, were kindly donated from Japan Bayer Co., Tokyo, Japan, and Mochida Pharmaceutical Co., Tokyo, Japan, respectively. Both imidazole compounds were dissolved in ethanol at 2 mg/ml (miconazole) and at 10 mg/ml (clotrimazole). As polyene antibiotics, pure amphotericin B and filipin without sodium deoxycholate were prepared by dissolving in dimethyl sulfoxide before each test as described previously (1). All control experiments were done by adding the same amount of ethanol or dimethyl sulfoxide.

## RESULTS

**Effect of Clotrimazole on Untransformed and Virus-transformed Cell Lines.** Dose response to clotrimazole was compared between transformed and untransformed rat cell lines by colony-forming ability. Untransformed 3Y1 cells are more resistant to clotrimazole than are the 3 AD-12<sup>2</sup>-transformed cell lines, T3, W4, and W5. Colony formation of the parental cell line 3Y1 was reduced to  $10^{-1}$  of the initial value in the presence of clotrimazole (20 to 25  $\mu\text{g}/\text{ml}$ ) whereas the colony formation of AD-12-transformed cell lines, T3, W4, and W5, was reduced to  $10^{-1}$  in the presence of clotrimazole (1 to 4  $\mu\text{g}/\text{ml}$ ) (Chart 1a). Three AD-12-transformed cell lines thus showed apparently higher sensitivity to the imidazole antimycotic than did 3Y1; in particular, W5 cells were found to be most sensitive to the cytotoxic action of clotrimazole. In contrast, colony formation of the 3 SV40-transformed cell lines (lines 9, C-65, and C-66) was blocked by 90% of the initial values in the presence of clotrimazole (10 to 15  $\mu\text{g}/\text{ml}$ ) (Chart 1b). As compared with the dose response of 3Y1 cells to the imidazole, lines 9, C-65,

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<sup>2</sup> The abbreviation used is: AD-12, adenovirus 12.

Table 1  
 Cell lines used in this study

| Cell line             | Parental cell line | Derivations  |
|-----------------------|--------------------|--|
| 3Y1-B clone 1         |                    | Untransformed rat cell line originally isolated from Fischer rat embryo (15) |
| 3Y1-B clone 1-6 (3Y1) |                    | Subclone derived from 3Y1-B clone 1 (6)                                      |
| T3                    | 3Y1-B clone 1      | Transformed by AD-12 (6, 27)   |
| No. 9                 | 3Y1-B clone 1      | Transformed by SV40 virus (strain SV40-1) (6)                                |
| W4                    | 3Y1                | Transformed by AD-12 (15)  |
| W5                    | 3Y1                | Transformed by AD-12 (15)  |
| C-65                  | 3Y1                | Transformed by SV40 (strain SV68C) (this study)                              |
| C-66                  | 3Y1                | Transformed by SV40 (strain SV68C) (this study)                              |

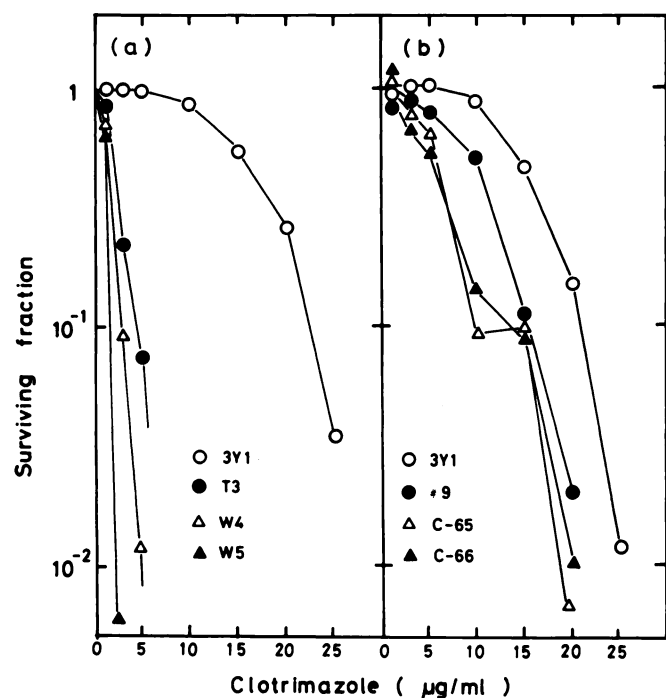


Chart 1. Dose response of colony formation of AD-12-transformed clones (a) and SV40-transformed clones (b) to clotrimazole. Exponentially growing cells (300 cells) or 3Y1, T3, W4, and W5 in a and 3Y1, No. 9, C-65, and C-66 in b were plated. Ten days later colony formation assays were carried out in the presence of various doses of clotrimazole. Points, average value of duplicate trials. The initial number of colonies in the absence of any drug was 150 (3Y1), 89 (T3), 98 (W4), 120 (W5) in a and 148 (3Y1), 131 (line 9), 125 (C-65), and 118 (C-66) in b. The surviving fraction was obtained by normalizing the ratio of colonies that appeared in the presence of clotrimazole to those in the absence of the drug.

and C-66 were slightly more sensitive to the cytotoxic action of clotrimazole (Chart 1b). The AD-12-transformed clones were found to be more sensitive to clotrimazole than were the SV40-transformed clones. In addition, the sensitivity of 3Y1-B clone 1 from which 3Y1 was derived (Table 1) was confirmed to be almost identical to that of 3Y1.<sup>3</sup>

The effect of clotrimazole on growth curves of 3Y1, T3, and line 9 cells was examined when  $3 \times 10^4$  cells/ml were initially inoculated. One day later, cells were exposed to clotrimazole (0, 3, 7, and 10  $\mu\text{g}/\text{ml}$ ), followed by the counting of viable cell number by trypan blue dye exclusion at the indicated times

<sup>3</sup> M. Kaneko and M. Kuwano, Unpublished data.

(Chart 2). The cell growth of 3Y1 was affected only slightly, if at all, by increasing the dose of clotrimazole up to 10  $\mu\text{g}/\text{ml}$ . By contrast, 7- or 10- $\mu\text{g}/\text{ml}$  amounts of the drug significantly inhibited growth of AD-12-transformed cell lines; growth of SV40-transformed cells was also affected to some extent. In particular, growth of an AD-12-transformed cell line T3 was almost completely blocked by clotrimazole (7 or 10  $\mu\text{g}/\text{ml}$ ) (Chart 2).

**Potential of Clotrimazole-induced Cytotoxic Effect by Linoleate against Untransformed 3Y1.** To determine whether unsaturated fatty acid alters the cellular sensitivity to an imidazole antimycotic, clotrimazole, we added linoleic acid to cultured medium for 3Y1 cells. The dose response of 3Y1 cells to clotrimazole was assayed when the cells were treated without or with linoleic acid (20, 40, and 60  $\mu\text{g}/\text{ml}$ ), and colonies appearing after 10 days were scored. As seen in Chart 3, increasing the concentration of linoleic acid enhanced the sensitivity of 3Y1 cells to clotrimazole. The survival fraction of 3Y1 was reduced to  $10^{-1}$  of the initial value by 16- to 18- $\mu\text{g}/\text{ml}$  amounts of the imidazole, whereas 2- to 3- $\mu\text{g}/\text{ml}$  doses of the imidazole reduced the survival fraction to  $10^{-1}$  of 3Y1 cells which were cultured with linoleic acid (60  $\mu\text{g}/\text{ml}$ ) (Chart 3). The dose-response curve of 3Y1 cells with linoleic acid (60  $\mu\text{g}/\text{ml}$ ) was very similar to that of T3. By contrast, the sensitivity of T3 cells to cytotoxic action of clotrimazole was enhanced approximately 2-fold when T3 cells were exposed to linoleic acid (60  $\mu\text{g}/\text{ml}$ ) (Chart 3). Incubation of 3Y1 or T3 cells with linoleic acid (60  $\mu\text{g}/\text{ml}$ ) alone decreased the number of the colony formation to 40 to 50% of that in the absence of the fatty acid.

**Dose-Response Curves of Untransformed and Transformed Cell Lines to Miconazole, Amphotericin B, and Filipin.** Untransformed 3Y1 cells appeared to show different sensitivity to clotrimazole from transformed cell lines. To determine whether transformed and untransformed cell lines show differential responses to other imidazole antimycotics, miconazole was tested for the effect on the colony formation. All 3 AD-12-transformed cell lines showed higher sensitivity to the cytotoxic

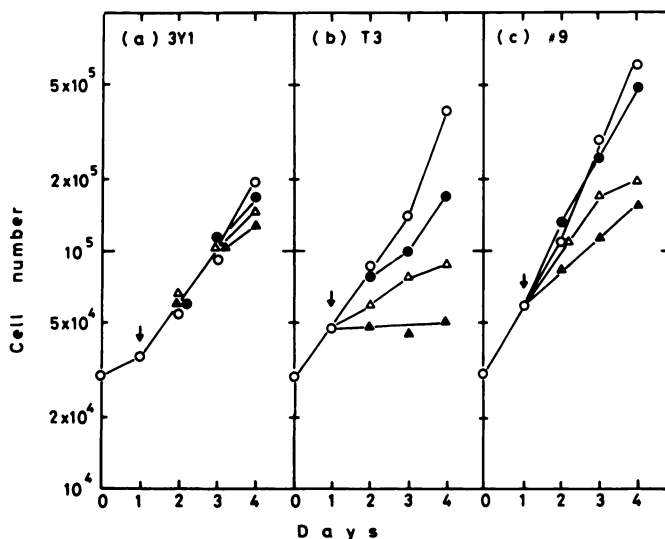


Chart 2. Effect of clotrimazole on growth curves of 3Y1 (a), T3 (b), and line 9 (c) clones. Each clone ( $3 \times 10^4$  cells/ml) was plated, and 1 day later 3 (●), 7 (Δ), and 10 (▲)  $\mu\text{g}$  clotrimazole per ml were added to each plate. For the control (○), no drug was added. Points, average of duplicate experiments. At time indicated by arrow (1 day), clotrimazole was added to each plate. Viable cells were scored by trypan blue dye exclusion.

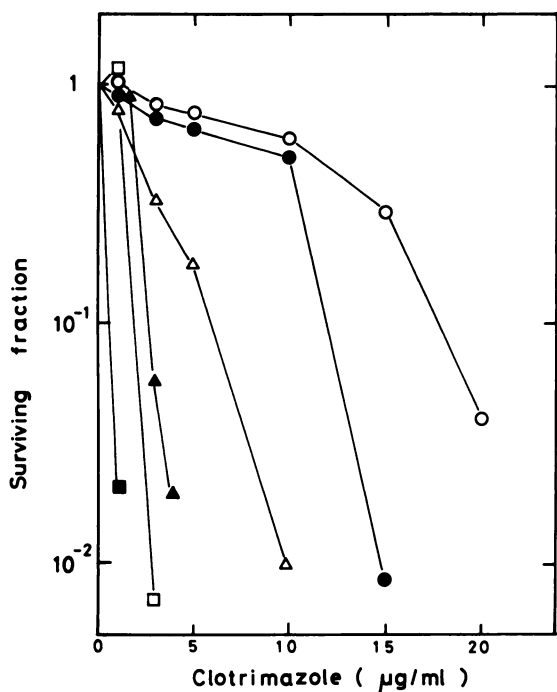


Chart 3. Effect of linoleic acid on colony formation of 3Y1 in the presence of clotrimazole. 3Y1 cells (300 cells) were plated, and 1 day later various doses of clotrimazole without (○) or with 20 (●), 40 (△), 60 (▲) µg linoleic acid per ml were added to each plate; 10 days later, colony formation was assayed. T3 cells treated without (□) or with linoleic acid (60 µg/ml) (■) were tested for their dose response to clotrimazole. The initial number of 3Y1 was 133 (none), 117 (20 µg linoleic acid per ml), 99 (40 µg linoleic acid per ml), and 51 (60 µg linoleic acid per ml), respectively, and the number of T3 was 120 (none) and 56 (60 µg/ml), respectively. The surviving fraction of 3Y1 and T3 cells was obtained by normalizing the ratio of colonies that appeared under each condition to those in the absence of drug or in the presence of linoleic acid alone.

activity of miconazole than did their parental untransformed 3Y1 cells (Chart 4). The colony formation of 3Y1 cells was decreased to  $10^{-1}$  of the initial values by miconazole (30 µg/ml), whereas that of 3 AD-12-transformed cell lines (T3, W4, and W5) was decreased to  $10^{-1}$  in the presence of about 15-µg/ml amounts of the drug (Chart 4). The difference in the sensitivity between untransformed and transformed cell lines was a factor of about 2. As compared with the dose of clotrimazole to inhibit colony formation of our rat cell lines, a higher dose (about 150%) of miconazole was required to inhibit the colony formation to similar extent (compare Charts 1 and 4).

One might argue whether untransformed and transformed cell lines show differential response to other antimycotic agents such as sterol-binding polyene antibiotics. The sensitivity of both transformed and untransformed cell lines to the antimycotic polyene antibiotics amphotericin B (heptaene) and filipin (pentaene) was also tested by colony formation (Chart 5). The cellular sensitivity to polyene antibiotics appears to be mediated through free cholesterol via phospholipids (9, 10). No significant difference was observed in the sensitivity to both polyenes among transformed and untransformed cell lines.

**DISCUSSION**

Clotrimazole is an imidazole antimycotic agent that interacts with membranous lipids in *Candida albicans* or *Saccharomyces cerevisiae* (16, 17, 20, 21, 24-26). In this study, clotrimazole preferentially inhibited growth of AD-12- or SV40-transformed

cell lines compared to their parental untransformed clone, 3Y1. AD-12-transformed cell lines showed 5- to 10-fold higher sensitivity to the imidazole than did 3Y1, while SV40-transformed cell lines showed 2-fold (or less) higher sensitivity than did

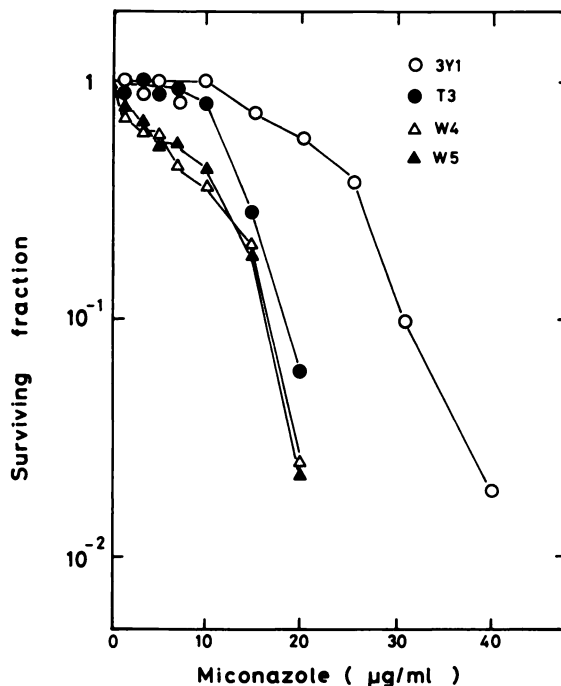


Chart 4. Dose response of untransformed 3Y1 and AD-12-transformed cell lines to miconazole assaying by colony formation. 3Y1 and AD-12-transformed clones T3, W4, W5 were assayed for their dose response to miconazole by colony formation assays as described in Chart 1. Points, average value of duplicate trials. Number of colonies in the absence of miconazole was 178 (3Y1), 148 (T3), 158 (W4), and 121 (W5).

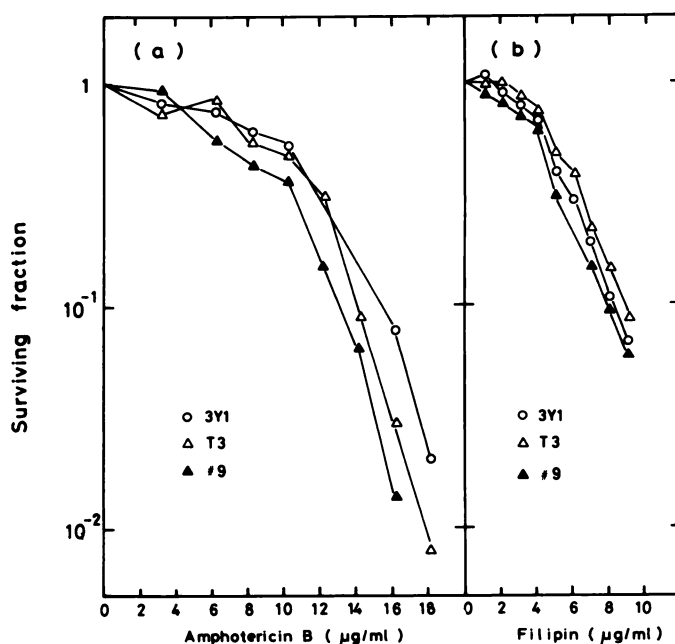


Chart 5. Dose response of 3Y1, AD-12- or SV40-transformed cell lines to amphotericin B (a) and filipin (b) by colony formation. Colony formation was assayed as described in Chart 1. Colony numbers of 3Y1, T3, and line 9 are 91, 97, and 135 in the absence of drug. Points, average value of duplicate trials. The surviving fraction was obtained by normalizing the ratio of colonies that appeared in the presence of polyene to those in the absence of drug.

3Y1. The colony formation and growth curves supported these conclusions. As compared with the differential effect of clotrimazole on untransformed and AD-12-transformed cell lines, the effect by miconazole is much less (Charts 1 and 4). Although they are both imidazole antimycotics, the biological effect of miconazole (20) might be different from that of clotrimazole (21). Besides interaction with fatty acid, miconazole was shown to inhibit sterol biosynthesis in *C. albicans* (22). Further study is necessary to elucidate action site(s) for both imidazole antimycotics in fungi as well as in animal cells.

Cellular membrane constituents such as glycolipids or glycoproteins have been reported to differ among untransformed and transformed cell lines (7, 23). From the standpoint of specific growth control of transformed cells, we are particularly interested in alterations of lipid components during cellular transformation. Lipid-bilayer membranes made up of various sterols and phospholipids appear to be altered during the transformation (28). Transformation of mouse 3T3 cells by SV40 was reported to result in a specific membrane modification which altered the sensitivity of these cells to sterol-binding antibiotic amphotericin B (3, 18, 19). However, in our assay system, amphotericin B and filipin failed to discriminate AD-12- or SV40-transformed cell lines from the parental untransformed 3Y1. The cellular sensitivity to polyene antibiotics is thought to be mediated through free sterol content via phospholipid (9), and sterol contents of these 3Y1, T3, and line 9 cells were found to be similar.<sup>4</sup> The discrepancy of cellular sensitivity to polyene might be due to differences in cell lines used for each study.

AD-12-transformed 3Y1 cell lines acquired higher sensitivity to miconazole as well as clotrimazole. These imidazole compounds were suggested to specifically interact with unsaturated fatty acids or phospholipids rather than with sterol molecules (17, 24–26). Addition of linoleic acid to culture medium for 3Y1 cells significantly enhanced the cellular sensitivity to clotrimazole, while the sensitivity of T3 cells to the drug was enhanced only 2-fold by culturing with linoleic acid (see Chart 3). Since cellular uptake of fatty acid is very active in cultured mammalian cells supplemented with fatty acids (5, 29), 3Y1 cells but not their transformed counterpart T3, might be modified as a result of lipid supplementation. AD-12-transformed cells might be already occupied with unsaturated fatty acid, but this assumption is highly speculative until further analysis on membrane lipid composition is done.

On the other hand, the presence of linoleic acid altered the cellular morphology as well as the fatty acid composition of the normal 3T3 cell, which resulted in a cell similar to SV40-transformed 3T3 cells (12). An interesting observation by Holley *et al.* (11) showed that the addition of linoleic acid to quiescent cells led to reinitiation of DNA synthesis and growth. Relevant work by Hale *et al.* (8) reported modification of the lipid composition of Rous sarcoma-infected cells and that 1,2-amino-1-butanol supplementation was found to cause phenotypic conversion from normal cells into transformed cells. Many studies indicate that the alteration of membrane lipids occurs to accompany the transformation by oncogenic viruses, such as SV40 or RNA tumor viruses (4, 13, 14, 30). It might be expected that AD-12 transformation cause dramatic change in lipid components because AD-12-transformed cell lines con-

sistently have the highest sensitivity to clotrimazole. This notion deserves further study to determine whether cellular lipids are altered in transformed cells and also whether cells transformed by chemical carcinogens or mutagens alter the cellular susceptibility to the imidazole antimycotics. If altered lipid metabolism is generally correlated with malignant transformation, any drug to recognize specifically such biochemical change in membranous lipid composition should be useful to obtain a specific growth control of malignant tumors.

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