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MCI-186 Inhibits Tumor Growth Through Suppression of EGFR Phosphorylation and Cell Cycle Arrest

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Abstract. *Background: It has been suggested that radicals stimulate tumor cell growth. We examined if the hydroxyl radical scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), affects tumor growth in vitro. Materials and Methods: Human hepatocarcinoma HepG2, mesothelioma MSTO-211H, gastric carcinoma TMK-1 and breast carcinoma MCF-7 were used for cell proliferation assay. Cell cycle analysis was performed using propidium iodide for fluorescence activated cell sorter. By Western blotting, EGF receptor (EGFR) phosphorylation and EGFR expression were analyzed. Results: Growth inhibition was observed from 10 μM to 300 μM of MCI-186 in a dose-dependent manner. Cell cycle analysis revealed that MCI-186 arrested the cell cycle at the G0/G1-phase. MCI-186 inhibited EGF-stimulated cell growth. The phosphorylation level of EGFR was decreased by MCI-186, but the EGFR level was unchanged. Conclusion: From the data obtained, we suggest that tumor inhibition by MCI-186 was due, at least in part, to the modulation of EGFR signaling and cell cycle arrest.*

High levels of reactive oxygen species (ROS) are known to induce not only cell death (1), but also DNA damage and genomic instability (2, 3), which in turn leads to tumorigenesis and its advancement. ROS levels are significantly higher in certain tumor cells when compared with non-tumor cells (4), suggesting that sustained production of ROS may contribute to tumor progression. In addition, low levels of ROS also enhance cell proliferation (5, 6). Other studies have shown that antioxidants, which scavenge intracellular ROS, suppress colony formation (7) and proliferation of transformed cells (8).

Mitogen-activated and stress-activated protein kinases transduce signals from the plasma membrane into the nucleus to modulate expression of genes involved in tumor progression. Extra-cellular signals, such as those from cellular stress and growth factors, regulate the cell decision to re-enter the cell cycle, or undergo cell cycle arrest (9). EGF signals are essential for proliferation and are physiologically well regulated. On the contrary, in tumor cells they are abnormally activated and induce tumor progression (10). ROS have been known to function as important second messengers for growth factors, including EGF (11). Catalase is well known as one of the most powerful antioxidants. In A431 human epidermoid carcinoma cells, catalase decreased EGF receptor signaling (11). Therefore, the suppression of ROS signals could be a potential strategy for the prevention of tumor advancement by blocking aberrant EGF receptor signaling.

MCI-186 [3-methyl-1-phenyl-2-pyrazolin-5-one] (Figure 1) is a hydroxyl radical scavenger whose antioxidant property is successfully used in treating patients with cerebral infarction (12). In the present study, we focused on the effect of MCI-186 on human tumor cell growth and have found that MCI-186 inhibited cell growth at the G0/G1-phase and that this inhibition occurred partly through suppression of EGFR phosphorylation.

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Materials and Methods

Cell lines and reagents. MCI-186 was kindly provided by Mitsubishi Pharma Corporation, Japan. Ebselen [2-phenyl-1, 2-benzisoselenazol-3 (SH)-one] (Cayman Chemical, Ann. Arbor, MI, USA) was dissolved in ethanol and used at a final concentration of 5 mg/ml. Mannitol (0.5 M, Sigma Chemical, St. Louis, MO, USA) was dissolved in culture medium. Nocodazole [methyl (5-[2-

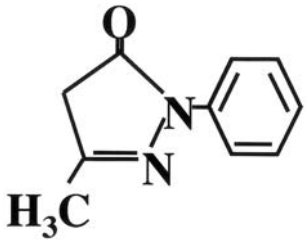

Figure 1. *Chemical structure of 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186).*

thiemylcarbonyl]-1H-benzimidazol-2-yl) carbamate], aphidicolin and DAPI were bought from Sigma Aldrich (St.Louis, MO, USA). Polyclonal anti-EGFR antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-phosphotyrosine monoclonal antibody (py69) was from Seikagaku Corporation, Japan. The human hepatocarcinoma cell line HepG2 and human lung mesothelioma cell line MSTO-211H were purchased from the American Type Culture Collection. HepG2 was maintained in a culture medium of low glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum. MSTO-211H was maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 1 mM sodium pyruvate. The human gastric carcinoma cell line TMK-1, derived from a poorly-differentiated adenocarcinoma, was a kind gift from Prof. Yokozaki (Hiroshima University, Japan). Breast carcinoma MCF-7 was from the Institute of Development, Aging and Cancer, Tohoku University, Japan, and maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum.

Cell growth assessment. Growing cells were seeded at a density of 3 x 10^4 in a 24-well culture dish (TPP, Switzerland). Following overnight incubation in a CO_2 incubator, the medium was changed to 1 ml fresh medium containing MCI-186 (0 μM to 300 μM), ebselen, mannitol or to a medium not containing antioxidants. After a 48-h incubation, the cells were trypsinized and harvested. Cell numbers were counted in triplicates for each group using trypan blue stain to determine dead cells. The data was recorded as the average of three independent experiments. The tumor growth inhibition rate was calculated as a percentage of viable cell numbers compared to the control group.

DAPI staining. Thirty thousand HepG2 cells were cultured in an 8-well Lab-Tek Chamber Slide (Nalgen Nunc International, IL, USA) overnight followed by 48-h incubation in the presence of MCI-186 (0 μM to 100 μM). After staining with DAPI (1μg/ml in methanol solution), the slide was sealed with glycerol-PBS (1:1) and the presence of apoptotic cells was observed under fluorescence microscopy.

Synchronization and cell cycle assessment. To synchronize the cell cycle at the G1/S boundary, HepG2 cells were exposed to 10 μM of aphidicolin for 15 h. For synchronizing at the G2/M-phase, they were treated with 1 μM of nocodazole for 24 h. After synchronization, the cells were cultured for 24 h with or without MCI-186 and collected by trypsinization. The cells were then washed with PBS, centrifuged (200 x g, 4˚C, 10 min) and fixed with ice-cold 70% ethanol at 20˚C for 4 h. After staining with 50 μg /ml propidium iodide with 500 μg/ml RNase at room temperature for 30 min, the cells were subjected to FACS, to analyze cell cycles, using FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Western blotting. HepG2 cells were subjected to starvation by incubating in a medium containing 0.1% FBS for 10 h. After starvation, the cells were pre-incubated for 30 min with either 30 or 300 μM of MCI-186 or without MCI-186. Thereafter, the cells were stimulated with 5ng/ml EGF for 15 min. To demonstrate serum-derived EGFR activation, the cells were starved for 16 h and pre-incubated with MCI-186 for 30 min, then incubated with 10% FBS for 15 min. The cells were then washed with PBS twice, immediately scraped with 1 x SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% W/V SDS, 10% glycerol, 50 mM DTT] and kept on ice. After centrifugation (8000 x g, 15 min, 4˚C), the supernatants were boiled for 5 min and chilled on ice. Protein concentrations were determined by BCA method and equal amounts of samples were loaded onto 8% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) blotting membranes. The membranes were then immersed in blocking buffer (5% non-fat dried milk in TBS) at room temperature for 1 h and then incubated with primary antibodies with gentle agitation overnight at 4˚C. After washing, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Detection of protein-bound antibodies was conducted using the ECL plus detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA), according to the manufacturer's instructions. Protein expression levels were analyzed with Kodak Digital Science EDAS 290, Version 3.5.

EGF stimulation and growth assessment. HepG2 cells were plated at a density of 3 x 10^4 cells per culture plate in a 35-mm dish (TPP, Switzerland) with 10% FBS-DMEM and cultured for 9 h. The medium was then changed for a fresh one containing EGF (5ng/ml) plus MCI-186 or EGF alone. The cells were cultured for 48 h and cell numbers were determined.

Statistical analysis. All results were expressed as mean ± SEM. Group differences in continuous variables were determined through analysis using the Student's *t*-test. A p value of less than 0.05 was considered statistically significant.

Results

MCI-186 inhibits tumor growth dose-dependently. First, we examined the effect of MCI-186 on the cell growth of tumor cell lines. (Figure 2). We found that MCI-186 inhibited all the tumor cells (HepG2, MSTO-211H, TMK-1 and MCF-7 cells) tested in a dose-dependent manner. Growth inhibition rates in HepG2 by MCI-186 concentrations of 10 μM, 30 μM, 100 μM and 300 μM were 22.9±4.25%, 25.7±6.9%, 35.8±4% and 45.7±3%, respectively ($*p<0.01$ *vs* control). Similar dose-dependent growth inhibition by MCI-186 was observed in MSTO-211H, TMK-1 and MCF-7 (Figure 2). In contrast to tumor cells, the cell growth of a human normal fibroblast cell line WI38 was not affected by MCI-186 (data not shown). Trypan blue staining did not show dead cells by MCI-186 at concentrations of 10 μM – 300 μM (Figure 2).

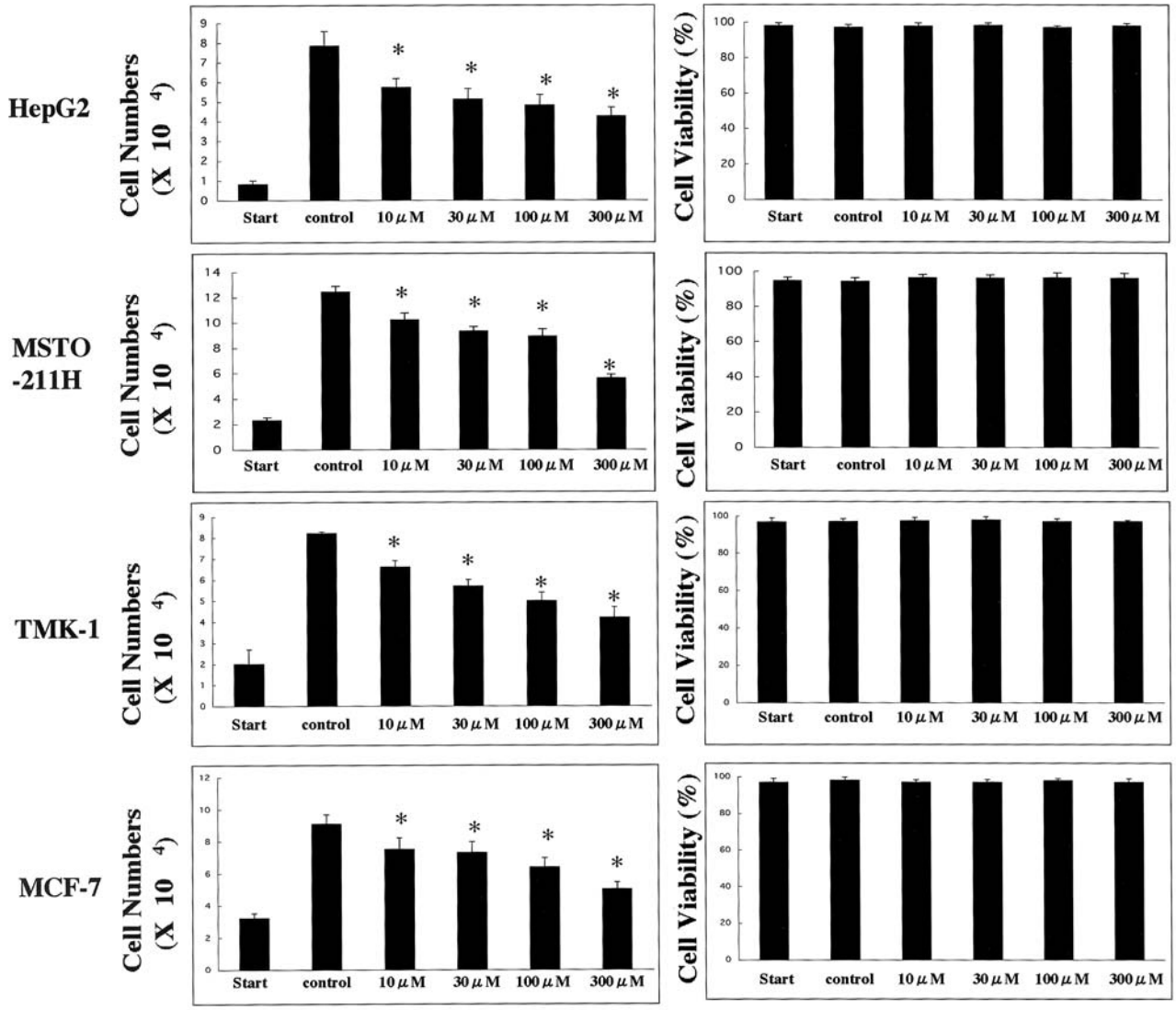

Figure 2. *Effect of MCI-186 on human tumor cell lines. Human tumor cell lines, HepG2, MSTO-211H, TMK-1 and MCF-7 were treated with MCI-186 (10 μM-300 μM; control not treated with MCI-186) and the effect on cell growth was determined after 48 h by counting cell numbers. Cell numbers were counted in triplicate for each group, and three independent experiments were performed. Growth inhibition was dose-dependent in all the tumor cell lines. The inhibition percentages were as follows: HepG2, 10 μM 22.9±4.24%, 30 μM 25.7±6.9%, 100 μM 35.8 ±4% and 300 μM 45.7±3.9%; in MSTO-211H, 10 μM 17.3±0.7%, 30 μM 23±1.5%, 100 μM 31.3±1.8% and 300 μM 50±3.2%; in TMK-1, 10 μM 19.4±2.8%, 30 μM 31.2±2.9%, 100 μM 40±4.6% and 300 μM 48.8±3.1%; and in MCF-7, 10 μM 17±0.72%, 30 μM 20±0.68, 100 μM 30±0.57% and 300 μM 45.4±0.45%. Cell viability was determined using trypan blue staining. No significant change in cell viability was seen. All results are represented as an average of 3 experiments and are expressed as mean ± SEM. *p<0.01 vs control (one-tail test).*

The effect on cell viability was further confirmed by DAPI staining in HepG2 cells (Figure 3). When HepG2 cells were treated with H_2O_2, condensed chromatin bodies were observed after DAPI staining (Figure 3 d), whereas apoptotic bodies were not apparent in the HepG2 cells treated with MCI-186 (Figure 3 a, b, c). These results suggest that inhibition of cell growth was not due to the cytotoxic or apoptotic activity of MCI-186.

MCI-186 inhibits tumor cell growth at G0/G1-phase. Since we did not observe any cytotoxic effects of MCI-186, its inhibitory effect on cell numbers is probably due to cell cycle arrest at a specific phase. To confirm this possibility, we analyzed the effects of MCI-186 on the cell cycle in HepG2 cells. When cells were cultured in normal medium without treatment, the cell population at G0/G1- and S-phases accounted for 50.04±2.21% and 31.08±1.12%,

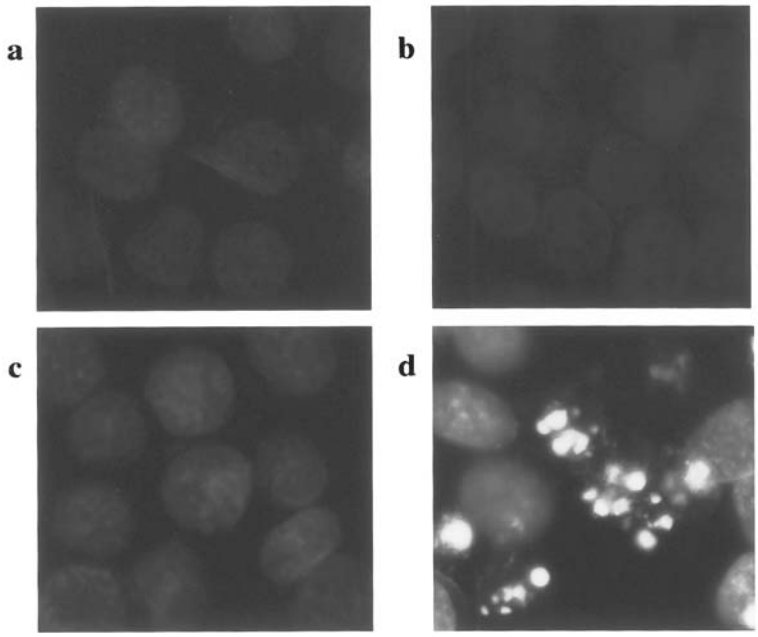

Figure 3. *Absence of MCI-186-induced apoptosis in HepG2 cells. DAPI staining was used to demonstrate apoptotic cell death. Cells were incubated with or without MCI-186 (30 μM-100 μM; control not treated with MCI-186), for 48 h. After incubation, cells were stained with DAPI (1μg/ml, in methanol). As the positive control for apoptosis, cells were treated with 300 μM H_20_2 for 4 h, which showed chromatin condensation. Apoptotic cell death was not detected in the other test groups. Investigation was conducted using fluorescent microscope (Nikon). a)control, b)MCI-186 30 μM, c) MCI-186 100 μM, d) H_2O_2.*

respectively, and 13.51±0.36% at G2/M-phase (Figure 4 a). After synchronization of the cell cycle at the G1/S with aphidicolin, the G0/G1 population increased to 81±1.6% and went down to 58.88±0.8% when aphidicolin was removed (Figure 4 c). When 100 μM MCI-186 was added, however, the majority of the cells (81.35 ± 0.06%) remained in G0/G1 (Figure 4 d) even after the influence of aphidicolin had been removed. This result suggests that MCI-186 prevented cells from re-entering into the S-phase. Cell cycle inhibition by MCI-186 at G0/G1 was also observed with 30 μM concentration of MCI-186. In contrast, when cells were synchronized at the G2/M-phase using nocodazole and released, the cell population, accumulated at the G0/G1-phase, was not affected by MCI-186 (Figure 4 e, f. g). Taken together, these results indicate that MCI-186 prevents cells from entering into the S-phase at G0/G1.

Other antioxidants did not affect the tumor cell growth. In order to examine whether the cell growth inhibition by MCI-186 was simply due to the radical scavenging ability of MCI-186, we compared the effects of an antioxidant, ebselen, and another hydroxyl radical scavenger, mannitol, to those of MCI-186. Since other studies showed that ebselen exerts an apoptotic action over a dose of 50 μM in HepG2 cells (13), we chose doses up to 30 μM. Ebselen showed neither cell growth inhibition (Figure 5 a), nor cell cycle arrest (data not shown). Similarly, mannitol (3-30 mM) did not show any effects on cell proliferation within the dose, which does not affect apoptosis (Figure 5 b). These results suggest that the mode of action of MCI-186 as an antioxidant was not the same as that of ebselen and mannitol.

MCI-186 inhibits EGF-stimulated cell growth and EGFR phosphorylation. As a next step, to investigate the mechanism of tumor growth inhibition by MCI-186, we examined if MCI-186 affects growth factor stimulated cell growth. Since EGF is one of the key growth factors responsible for tumor progression, we examined the effects of MCI-186 on cell signals evoked by EGF. The cell growth stimulated by EGF was inhibited up to 35±5.3% with 30 μM, 45.1±3.1% with 100 μM and 55.5 ± 3.8% with 300 μM MCI-186, respectively (Figure 6 a). Ligand binding to the EGF receptor causes phosphorylation at the tyrosine residue of intracellular domain. Therefore, subsequent studies were performed to examine the effects of MCI-186 on EGFR phosphorylation. As shown in Figure 6b, MCI-186 treatment apparently suppressed the phosphorylation levels of the EGF receptor dose-

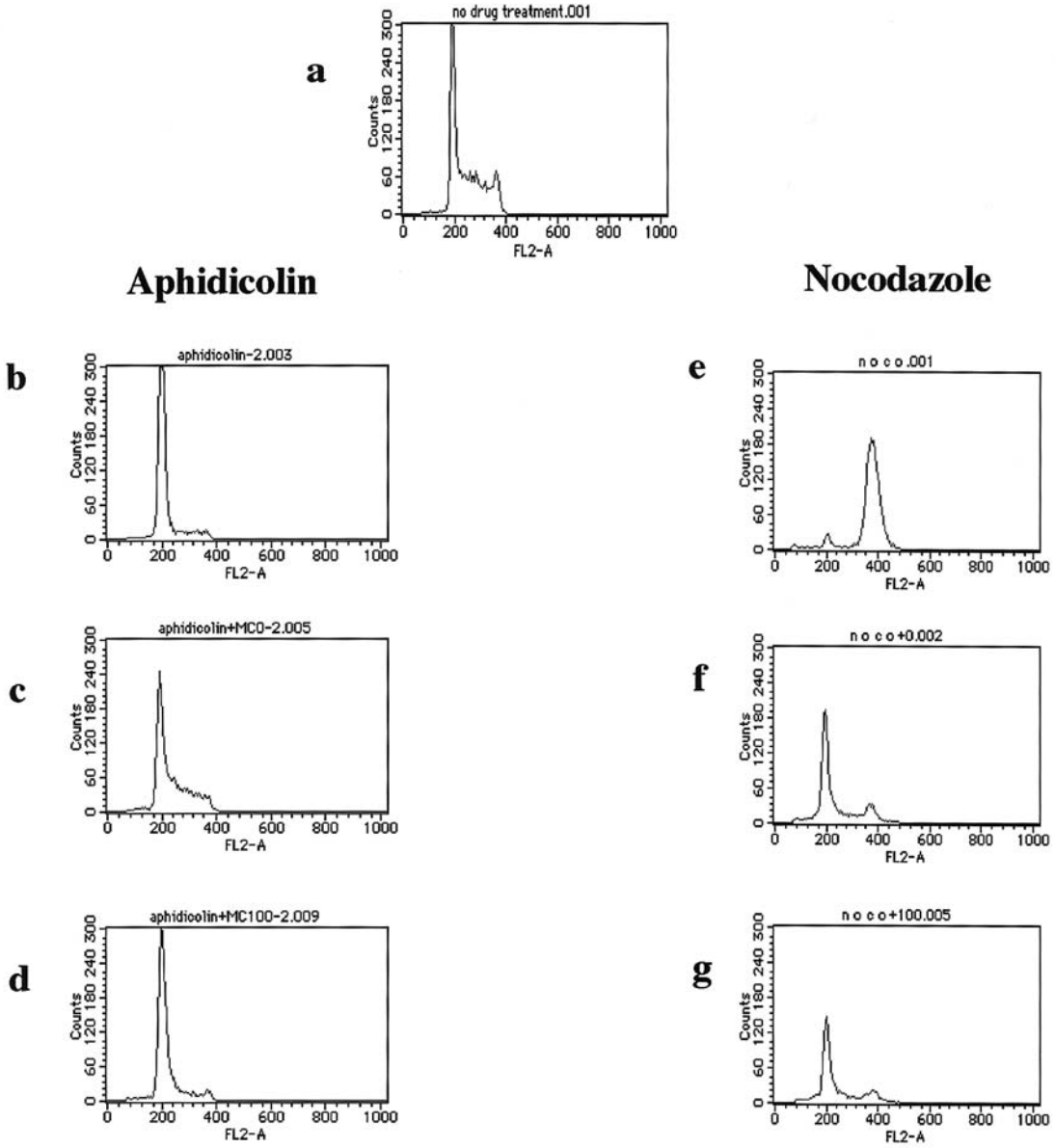

Figure 4. *Blocking of HepG2 cell cycle at the G0/G1-phase by MCI-186. The cell cycle was synchronized at the late G1/S-phase by culturing with 10 μM aphidicolin for 15 h and at G2/M by culturing with 1 μM nocodazole for 24 h. Afterwards, culturing was continued for 24 h with or without MCI-186. a) no drug treatment (control), b) aphidicolin, c) aphidicolin and MCI-186 0 μM, d) aphidicolin and MCI-186 100 μM, e) nocodazole, f) nocodazole and MCI-186 0 μM, g) nocodazole and MCI-186 100 μM. (Data for 30 μM was the same as that for 100 μM).*

dependently, whereas MCI-186 did not affect the expression of total EGFR. Similarly, MCI-186 inhibited EGFR phosphorylation in MCF-7 and MSTO-211H, and the total EGFR level was unchanged (data not shown). MCI-186 inhibited not only EGF stimulation, but also serum-driven EGFR phosphorylation (Figure 6c). Thus, these results suggest that the cell growth inhibition by MCI-186 may occur in part through suppression of EGF receptor phosphorylation.

Discussion

MCI-186 is a specific hydroxyl radical scavenger exhibiting its antiradical action by preventing cerebral dysfunction following ischemia-reperfusion (14-16), preserving the myocardium (17, 18), inhibiting 15-HPETE-induced cytotoxicity *in vitro* (19) and inhibiting lipid peroxidation (20). In the present study, we showed evidence of an *in vitro* inhibitory effect of MCI-186 on tumor cell growth. This

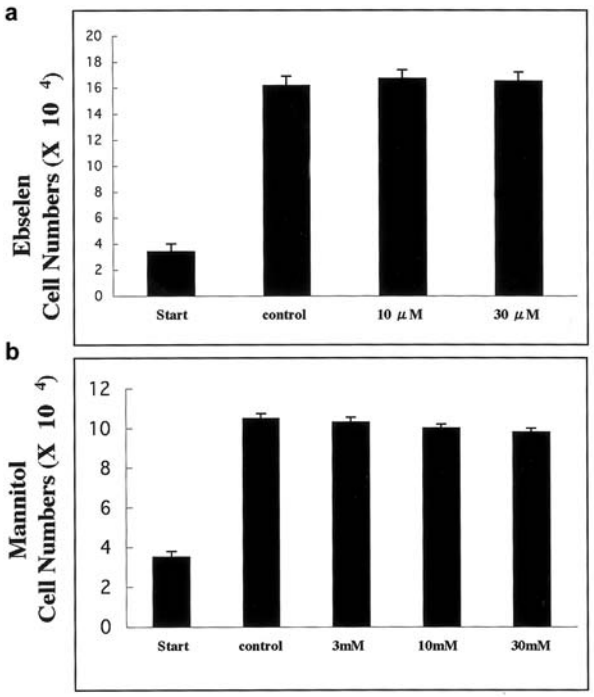

Figure 5. *Other antioxidants did not affect the tumor cell growth. Studies with other radical scavengers influencing cell growth (mannitol and ebselen) were investigated. Tumor cells were cultured with or without the agents for 48 h and cell numbers were counted. Both the antioxidants did not show any effect on tumor cell growth. a) Ebselen; b)Mannitol. Cell numbers are shown as mean ± SEM for three independent experiments.*

inhibition occurred partly through (i) suppression of the EGFR signaling pathway and (ii) induction of cell cycle arrest at the G0/G1-phase. Moreover, growth inhibition by MCI-186 affected all the tumor cell lines used for testing, but not normal human fibroblast cells. The results demonstrated here strongly suggest the antitumor potential of MCI-186.

Our results showed that MCI-186 inhibited both EGF-stimulated and serum-induced cell proliferation and decreased phosphorylation of EGFR in both these conditions. Thus, the inhibiting effects on proliferation by MCI-186 may directly or indirectly be linked to EGF signaling. At present, we are unable to propose the exact mechanism of inhibition of EGFR phosphorylation by MCI-186.

The EGFR signal is important for survival signaling (21), cell migration (22), angiogenesis (23, 24) and cell proliferation. Since aberrant signaling *via* EGF receptors is associated with tumor expansion, EGFR signals have been a target for cancer therapeutics (25, 26). Harceptin and Iressa, which down-regulate EGFR signals, are being extensively used clinically in anticancer therapy. MCI-186's action of inhibiting EGFR phosphorylation with cell growth arrest at G0/G1-phase in a non-apoptotic pathway is very similar to the action of Iressa (27). Although Iressa was active at lower doses than MCI-186 in Janne's study, MCI-186 could be an alternative choice as an antitumor agent for EGF signal targeting. Moreover, our recent data has demonstrated that MCI-186 and another commonly used anticancer drug, mytomycin C, complementarily inhibit human tumor cell lines (manuscript preparation).

In clinical usage, the serum concentration Cmax of MCI-186 is approximately 6 μM after *i.v.* administration at 30mg/30min. In this study, we observed tumor growth inhibition at 10 μM and suppression of EGF phosphorylation at 30 μM. Therefore, by using clinical methods to elevate the local dose of drug concentration, such as the infusion pump, more effective concentrations of MCI-186 could be achieved for tumor suppression.

We did not detect any growth inhibition with other antioxidants such as ebselen and mannitol. This implies that certain effects on cell functions by antioxidants are not the same among different drugs. This concept is consistent with previous reports. To give an example, the induction of eNOS by H_2O_2 in endothelial cells was not affected by mannitol, but it was inhibited by ebselen (28). Another report showed that ebselen was much more efficient than BSO (buthionine sulfoximine) in depleting intracellular GSH (13). We can infer that the antioxidation targets of MCI-186 were not the same as those of other antioxidants.

It is known that all solid tumors are hypoxic and cellular responses to external stimuli, including growth factors, are not the same between normal and hypoxic conditions (29). Therefore, the effects of MCI-186 on tumor cells observed in this study may not be the same in hypoxic cell cultures. This point needs further investigation.

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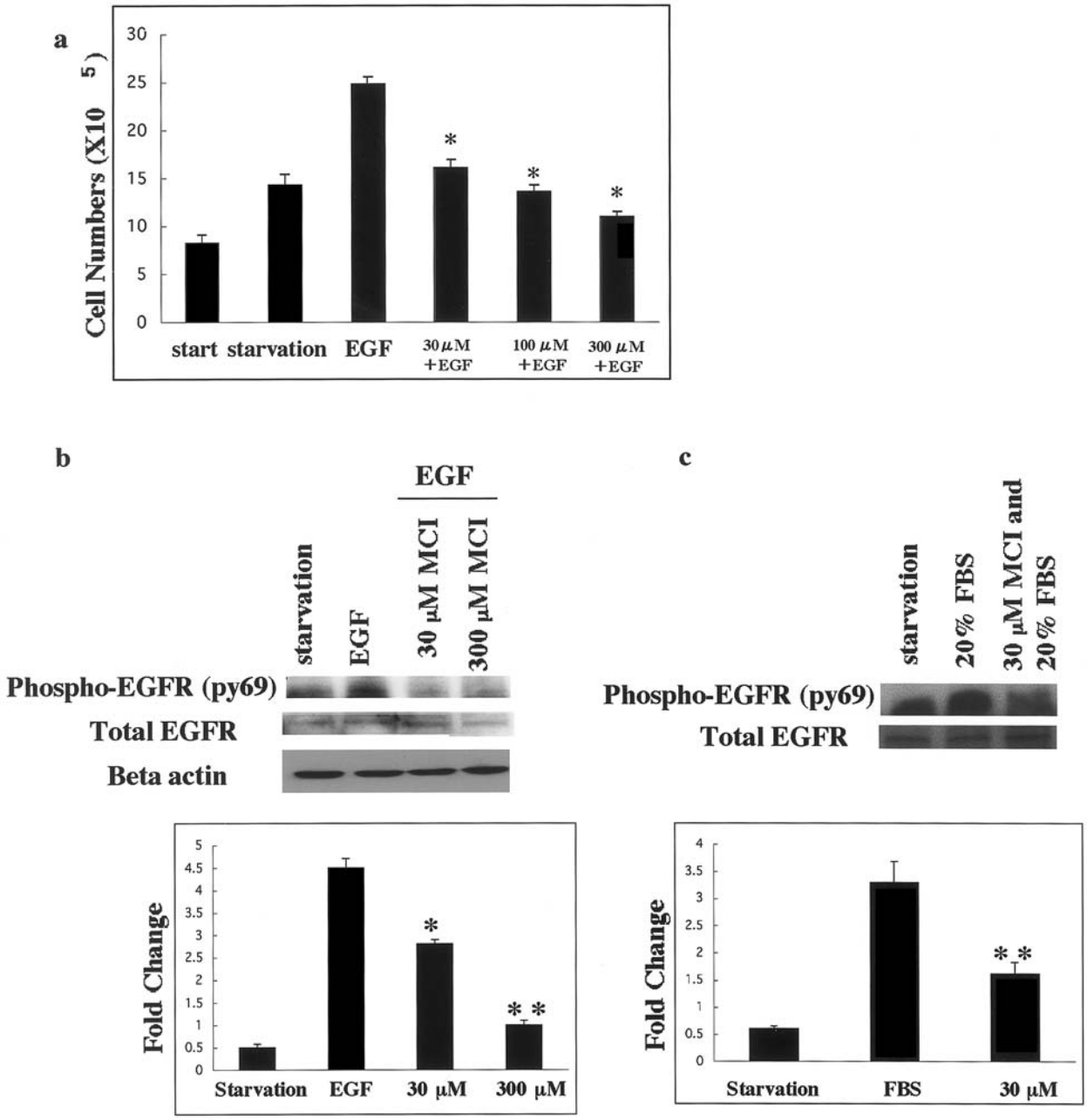

Figure 6. *MCI-186 inhibition of EGF-induced cell proliferation and inhibition of phosphorylation. a) HepG2 cells were cultured in a 0.1% FBS medium containing 5 ng/ml EGF only, 30 μM MCI-186 and 5 ng/ml EGF, 100 μM MCI-186 and 5ng/ml EGF or 300 μM MCI-186 and 5 ng/ml EGF. EGF-induced cell proliferation was inhibited by MCI-186 dose-dependently (6a). The experiment was repeated three times and calculated as mean ± SEM.*p<0.01 vs 5 ng/ml EGF only. b) Western blotting analysis of phosphorylation of EGFR after exposure to EGF with or without MCI-186. After 10-h starvation, cells were pre-incubated with 30 μM or 300 μM MCI-186 for 30 min and stimulated with 5 ng/ml EGF for 15 min. PVDF membrane was incubated with anti-phosphotyrosin monoclonal antibody (py69) for detection of phosphorylated EGFR. After starvation, the phosphorylation level was reduced and increased on EGF treatment. MCI-186, however, inhibited phosphorylation, which was triggered by EGF treatment, dose-dependently. Beta-actin expression shows equal protein volume. After detection of phosphorylation of EGFR, the membrane was re-blotted and incubated with anti-EGFR antibody. The expression levels were unaltered both in starvation and also in MCI-186-treated groups. Fold change was shown by dividing the phospho-EGF value by total EGF value using densitometric analysis. The bars in Figure 6b represent the mean of three independent trials. *p<0.05 vs 5 ng/ml EGF, **p<0.01 vs 5 ng/ml EGF. c) Phosphorylation of EGFR after exposure to FBS with or without MCI-186. After 16-h starvation, cells were pre-incubated with 30 μM of MCI-186 for 30 min and stimulated with 10% FBS for 15 min. PVDF membrane was incubated with py69 for phosphorylation of EGFR and then re-blotted with anti-EGFR antibody. Fold change was shown by dividing the phospho-EGF value by total EGF value using densitometric analysis. The bars in Figure 6c represent the mean of three independent trials. **p<0.01 vs 10%FBS.*

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