Anti-cancer Effects of MW-03, a Novel Indole Compound, by Inducing 15-Hydroxyprostaglandin Dehydrogenase and Cellular Growth Inhibition in the LS174T Human Colon Cancer Cell Line

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Increases in the expression of prostaglandin E2 (PGE2) are widely known to be involved in aberrant growth in the early stage of colon cancer development. We herein demonstrated that the novel indole compound MW-03 reduced PGE2-induced cAMP formation by catalyzation to an inactive metabolite by inducing 15-hydroxyprostaglandin dehydrogenase through the activation of peroxisome proliferator-activated receptor-γ. MW-03 also inhibited colon cancer cell growth by arresting the cell cycle at the S phase. Although the target of MW-03 for cell cycle inhibition has not yet been identified, these dual anti-cancer effects of MW-03 itself and/or its leading compound(s) on colon cancer cells may reduce colon cancer development and, thus, have potential as a novel treatment for the early stage of this disease.

Key words indole compound; MW-03; prostaglandin E2; 15-hydroxyprostaglandin dehydrogenase; peroxisome proliferator-activated receptor-γ; colon cancer

One major prostanoid, prostaglandin E2 (PGE2) is widely known to be up-regulated by the action of increased levels of cyclooxygenase-2 (COX-2) in colorectal cancer.1,2) Thus, the enhanced expression of COX-2 and biosynthesis of PGE2 are responsible for the malignancy of diseases.3,4) Since the over-expression of COX-2 and PGE2 is frequently observed in the early stage of colorectal cancer development, they are considered to be the biomarkers of this stage.3,4)

Cruciferous vegetables, such as cauliflower, cabbage, and broccoli, are regarded as good food sources for reducing the risk of cancer including colon cancer.4,5) Natural compounds that contain indole structures are relatively rich in cruciferous vegetables.4,5) The consumption of cruciferous vegetables has been recommended by the National Research Council in the United States because of the protective mechanisms of phytochemicals including derivatives of indoles, e.g. indole-3-carbinol and diindolylmethane.4,5) These indole derivatives have been shown to induce cancer cell death such as apoptosis; although many mechanisms are suggested and reported,4,5) the exact mechanisms underlying currently remain unclear. These cell death mechanisms are not only exerted by phytochemical indoles, but also by synthesized indoles such as one popular non-steroidal anti-inflammatory drug (NSAID), indomethacin.6,7)

Some indole compounds have been used as tools for researching prostanoid receptors and/or their signaling pathways. Among them, indomethacin has been used as an E-type prostanoid 2 (EP2) prostanoid receptor antagonist.8) D-type prostanoid receptor antagonist,9,10) chemoattractant receptor-homologous molecule expressed on T helper 2 cell receptor agonist/antagonist,11) and peroxisome proliferator-activated receptor-γ (PPARγ) agonist/antagonist,12,13) by itself and/or its leading compounds with cyclooxygenase inhibition-independent activities.14) A previous study reported that indomethacin induced 15-hydroxyprostaglandin dehydrogenase (15-PGDH), an enzyme that catalyzes prostanoids such as PGE2 to inactive, the metabolites of 15-keto-prostanoids, such as 15-keto-PGE2 in a rate-limited manner.15) We also demonstrated that the indole compound AWT-489 acted as an antagonist of DP receptors.16)

Based on the anti-colon cancer activity of indomethacin and indole compounds are potential tools for prostanoid receptors research, the novel indole-skeleton containing compound from the Chiba Chemical Library, MW-03, was used for exploring in this study. We herein show using the LS174T human colon cancer cell line, MW-03 exerted anti-colon cancer effects by two distinct mechanisms. The first effect was enhancing the metabolism of PGE2 to inactive form by inducing 15-PGDH. The second effect was cellular growth inhibition by the arrest of cells at the S phase. Since LS174T cells do not express COX-2, so as PGE2,17,18) these effects of MW-03 may function independently of cyclooxygenase inhibition as a NSAID. Thus, MW-03 itself and/or its derivatives may be applicable to novel anti-colon cancer therapeutic(s) with double effective anti-colon cancer mechanisms in the near future.
MW-03 was synthesized and purified (purity: 99.0%) by the Department of Chemistry, Graduate School of Sciences, Chiba University (Dr. Takayoshi Arai’s laboratory) as reported previously.\(^{(19)}\)

**Cell Culture, Cell Counts, and Materials**

LSI74T human colon cancer cells were maintained in Minimum Essential Medium-α (α-MEM; Invitrogen, Carlsbad, CA, U.S.A.) containing 5% fetal bovine serum (FBS; Thermo Scientific, Waltham, MA, U.S.A.), 100 IU/mL penicillin (Meiji Seika, Japan), and 100 µg/mL streptomycin (Meiji Seika) at 37°C. HEK-293 Epstein–Barr virus (EBNA) cells stably expressing the human EP2 receptor or DP receptor, HEK-EP2 cells or HEK-DP cells, respectively, were as described previously,\(^{(16,20)}\) which were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM; Nacalai Tesque, Japan) containing 10% FBS, 250 µg/mL hygromycin B (Enzo Life Sciences, Farmingdale, NY, U.S.A.) and 100 µg/mL gentamicin (Life Technologies, Carlsbad, CA, U.S.A.) as described previously.\(^{(19)}\) The numbers of cells was counted under a Nikon eclipse TS100 microscope (Nikon, Tokyo, Japan) following trypsinization. Unless stated otherwise, all materials were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**cAMP Assay**

LSI74T cells, HEK-EP2 cells, and HEK-DP cells were cultivated in 6-well plates and, prior to experiments, culture media were switched to Opti-MEM (Thermo Scientific) containing the corresponding antibiotics as stated above for 16h. In the case of LSI74T cells, cells were treated for 25 min with 0.1 mg/mL isobutylmethyl-xanthine (Sigma, St. Louis, MO, U.S.A.) following to a pretreatment with the indicated concentrations of MW-03 for 16h. Cells were then treated with vehicle or the indicated concentrations of PGE\(_2\) (Cayman Chemical, Ann Arbor, MI, U.S.A.) at 37°C for 1 h or 0.01 µg/mL cholera toxin (CTX, List Biological Laboratories, Campbell, CA, U.S.A.) for 4h. In the case of HEK cells stably expressing EP2 or DP receptors, cells were treated for 25 min with 0.1 mg/mL isobutylmethyl-xanthine followed by a pretreatment with vehicle or the indicated concentrations of MW-03 for 15 min, and then treated with vehicle or 1 nM PGE\(_2\) (HEK-EP2 cells) or 1 nM prostaglandin D\(_2\) (PGD\(_2\); Cayman Chemical) (HEK-DP cells) at 37°C for 60 min. After the termination of experiments by removing the medium and placing plates on ice, the cAMP that formed was measured as described previously,\(^{(19)}\) which was estimated from a standard curve obtained using non-radio labeled cAMP.

**Western Blotting**

LSI74T cells were cultured in 6-well plates or 10 cm dishes and, prior to immunoblot experiments, culture media were switched to Opti-MEM at 37°C for 16h containing 100 IU/mL penicillin and 100 µg/mL streptomycin. LSI74T cells were then treated with vehicle or the indicated concentrations of MW-03 for 16h. In the case of PPAR\(_γ\) short-interfering RNA (siRNA) experiments, LSI74T cells were transiently transfected using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA, U.S.A.) and 15 µmol/1 PPAR\(_γ\) siRNA (sc-29455, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or RNAiMAX Reagent with water (control) for 5h, and medium was then replaced with Opti-MEM containing 100 IU/mL penicillin and 100 µg/mL streptomycin at 37°C for 48h followed by a treatment with vehicle or 3 µM MW-03 for 16h. Cells were scraped into lysis buffer consisting of 50 mM Tris–HCl (pH 8.0), 5 mM ethylene diamine (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 10 mM sodium fluoride, 10 mM disodium pyrophosphate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 µg/mL leupeptin, and 10 µg/mL aprotinin as described previously.\(^{(21)}\) In the case of protein in medium, whole medium was corrected and protein concentrations were calculated using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Approximately 25 to 50 µg of protein samples were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes as reported previously.\(^{(21)}\) After one hour of blocking with 5% nonfat milk, membranes were incubated with a 1:1000 dilution of an anti-EP2 receptor antibody (#101750, Cayman Chemical), anti-DP receptor antibody (#101640, Cayman Chemical), anti-PPAR\(_γ\) antibody (sc-7273, Santa Cruz Biotechnology), anti-15-PGDH antibody (#160615, Cayman Chemical), or anti-β-tubulin antibody (T4026, Sigma) with 5% bovine serum albumin (BSA; Sigma) for 16h, followed by a 1:4000 dilution of appropriate secondary antibodies conjugated with horseradish peroxidase following to washouts as described previously.\(^{(21)}\) In order to ensure the equal loading of proteins, membranes were stripped and re-probed with the anti-β-tubulin antibody under the conditions described above.

**In Silico Analysis**

Construction of the three-dimensional structure of PPAR\(_γ\) and a docking simulation of MW-03 to PPAR\(_γ\) were performed with MOE (version 2016.08, CCG Inc., Montreal, Canada) based on the Brookhaven Protein Databank 2YFE.

**Flow Cytometry**

LSI74T cells were cultivated in 6-well plates and, prior to experiments, culture media were switched to Opti-MEM at 37°C for 16h containing 100 IU/mL penicillin and 100 µg/mL streptomycin. LSI74T cells were then treated with vehicle or the indicated concentrations of MW-03 for 16h. Cells were trypsinized, washed, and fixed using 1.5% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 30min. Fixed cells were treated with 50 µg/mL propidium iodide (Sigma) containing 200 µg/mL RNase (Promega, Madison, WI, U.S.A.) in 0.1% Tween 20 in PBS at 37°C for 30min. A total of 5×10\(^5\) cells/sample were analyzed using the Guava easyCyto flow cytometer (Millipore, Darmstadt, Germany).

**RESULTS AND DISCUSSION**

**Effects of MW-03 on PGE\(_2\)-Induced cAMP Formation**

The newly synthesized indole compound MW-03 is shown in Fig. 1.\(^{(19)}\) We previously reported that a popular indole compound of NSAIDs, indomethacin, reduced the PGE\(_2\)-stimulated formation of cAMP in LSI74T cells with an IC\(_{50}\) of approximately 21 µM.\(^{(18)}\) The effects of MW-03 on cAMP formation stimulated by PGE\(_2\) or CTX were evaluated using LSI74T cells. As shown in Fig. 1B, the pretreatment with MW-03 inhibited 1 µM PGE\(_2\)-stimulated cAMP formation in a concentration-dependent manner with an IC\(_{50}\) of 1.93±1.69 µM. However, the pretreatment with MW-03 had a negligible effect on CTX-stimulated cAMP formation, except for the 10 µM MW-03 pretreatment, and MW-03 itself did not exert any marked effect on basal cAMP formation in LSI74T cells. Thus, in terms of the inhibition of PGE\(_2\)-stimulated cAMP
formation, MW-03 was approximately 10-fold more potent than indomethacin. A treatment with indomethacin has also been shown to decrease the expression of endogenous EP2 receptors in LS174T cells. The inhibitory effects of MW-03 on PGE2-stimulated cAMP formation may be due to its inhibitory effect on EP2 receptor expression in LS174T cells. In order to test this possibility, LS174T cells were treated with 0.1 to 10 µM of MW-03 for 16h and the expression of EP2 receptors was then examined using an immunoblot analysis. As shown in Fig. 1C, MW-03 did not significantly reduce the expression of EP2 receptors, except for the 10 µM treatment. LS174T cells also express DP receptors; therefore, the effects of MW-03 were examined. DP receptor expression was not significantly altered by the treatment with MW-03, as shown in Fig. 1D. Since 10 µM MW-03 reduced CTX-induced cAMP formation, as shown in Fig. 1B, the inhibitory effects of 10 µM MW-03 on PGE2-stimulated cAMP formation were not due to reductions in EP2 receptor expression; they may be attributed to cytotoxicity or other effects induced by higher concentrations of MW-03. On the other hand, we and other groups previously reported that indomethacin and its modified compounds act as antagonists of human EP2 or DP receptors. Thus, another possibility is that the inhibitory effects of MW-03 on PGE2-stimulated cAMP formation are due to the antagonistic effects of MW-03 on EP2 and/or DP receptors, which are expressed in LS174T cells. In order to test this possibility, HEK-293 cells stably expressing either human EP2 receptors or DP receptors were pretreated with MW-03 for 15min and then treated with vehicle (black circles), or 1 nM PGE2 (E, white circles), or 1 nM PGD2 (F, gray circles) for 1h. The amounts of cAMP that formed were assessed as described in Materials and Methods. Data are normalized to 1 µM PGE2 (B: white circles) or 0.01 mg/mL CTX (B: gray circles) not pretreated with MW-03 as 100%, and were the mean ± S.E.M. of more than three independent experiments each performed in duplicate. *p<0.05, ANOVA, significantly different from that not pretreated with MW-03 (0 µM). †p<0.05, ANOVA, significantly different from that treated with CTX and the same MW-03 concentration pretreated in (B). Photographs were from representative experiments and histograms represent the ratio of EP2 receptors (C) or DP receptors (D) to β-tubulin not pretreated with MW-03 (0 µM) as 100%. *p<0.05, ANOVA, significantly different from that not pretreated with MW-03 (0 µM) (C).
As shown in Figs. 1E and F, the pretreatment with MW-03 neither reduced PGE$_2$-stimulated nor PGD$_2$-stimulated cAMP formation in HEK-EP2 cells or HEK-DP cells, respectively, at any of the concentrations tested. Moreover, MW-03 did not exert agonist effects in HEK-EP2 or HEK-DP cells. Collectively, the results shown in Fig. 1 suggested that MW-03 reduced PGE$_2$-stimulated cAMP formation independent of its function as an EP2 or DP receptor antagonist and/or reducing the expression of EP2 or DP receptors.

Previous studies reported that LS174T cells do not express or produce COX-2 or PGE$_2$, respectively. Additionally, the bulky structure of MW-03, as shown in Fig. 1A, may not act on COX-1, and, thus, a plausible reason why the MW-03 pretreatment reduced PGE$_2$-stimulated cAMP, as shown in Fig. 1B, is that exogenously administered PGE$_2$ may be metabolized by cells and inactivated quickly. A candidate enzyme is 15-PGDH, which oxidizes the 15-hydroxyl moiety of prostanoid to carbonyl, to 15-keto-PGE$_2$, and 15-keto-PGE$_2$ is considered to be abrogated its binding to the receptors. Since indomethacin at a concentration of 10$\mu$M has been shown to induce the expression of 15-PGDH in LS174T cells, the effects of MW-03 on the expression of 15-PGDH were examined. As shown in Fig. 2A, the treatment with 0.1 to 3$\mu$M MW-03 for 16h increased the expression of 15-PGDH in a concentration-dependent manner, with 1 and 3$\mu$M MW-03 significantly enhanced its expression. In order to confirm the functional effects of MW-03-induced 15-PGDH on cAMP formation, LS174T cells were pretreated with vehicle or 3$\mu$M MW-03 for 16h and then treated with 1 nM to 10$\mu$M PGE$_2$ for 1h, and this was followed by an evaluation of PGE$_2$-stimulated cAMP formation. Figure 2B shows that concentration-dependent cAMP formation curves by PGE$_2$ were significantly shifted to the right and/or reduced by the pretreatment with 3$\mu$M MW-03 (control EC$_{50}$: 2.54±0.88$\mu$M; MW-03 EC$_{50}$: 12.5±7.90$\mu$M). Thus, the inhibitory effects of MW-03 on PGE$_2$-stimulated cAMP formation do not appear to be due to antagonistic effects and/or reductions in the expression of EP2 or DP receptors, but by the induction of 15-PGDH to inactivate PGE$_2$, plausibly by oxidation to 15-keto-PGE$_2$.

The activity and expression of 15-PGDH have been reported to be modified by NSAIDs and/or agonists of PPAR$_\gamma$. Indomethacin is also known to bind directly to and activate PPAR$_\gamma$. Thus, in an attempt to identify the site(s) of action and/or target(s) of MW-03, we examined the effects of MW-03 on the expression of PPAR$_\gamma$ in LS174T cells because the ligands of PPAR$_\gamma$ have frequently been shown to modify
the expression of PPARγ by themselves,25) since promoter of PPARγ contains PPARγ binding site to set up positive feed back loops.26) As shown in Fig. 2C, the treatment with 0.1 to 1 µM MW-03 for 16 h increased the expression of PPARγ in a concentration-dependent manner and 3 µM MW-03 also significantly enhanced its expression. In order to investigate the possibility of the direct binding of MW-03 to PPARγ, we performed a docking simulation of MW-03 to PPARγ. As shown in Fig. 2D, MW-03 bound the Amorfrutin (a typical PPARγ activator) binding site of PPARγ.27) Therefore, we presumed that MW-03 has sufficient potential to bind directly to and activate PPARγ.

In order to confirm that the MW-03-induced expression of 15-PGDH is mediated through PPARγ, the effects of PPARγ siRNA on 15-PGDH expression were examined using LS174T cells. As shown in Fig. 3A, the expression of 15-PGDH in the cellular fraction (left, top panel) was concomitantly and significantly reduced with the expression of PPARγ (left, middle panel) by the siRNA of PPARγ to basal levels, but not by control siRNA. Similarly, the protein level of 15-PGDH secreted in the medium by LS174T cells treated with MW-03 was also reduced to basal levels when the siRNA of PPARγ was transfected (right, top panel). Collectively, these results indicate that MW-03 induces 15-PGDH through the activation of PPARγ by directly binding to LS174T cells.

**Effects of MW-03 on Cell Growth**

Indomethacin is known to play cellular growth inhibitory roles in human colon cancer cell lines, such as HT-29 cells, SW640 cells, and or HCT116 cells, regardless of their expression of cyclooxygenase.28) As described in the Introduction part, a number of indole compounds have been shown to induce cell death such as apoptosis by numerous mechanisms.4,5) Thus, the effects of MW-03 on cellular growth were evaluated in LS174T cells treated with 0.1 to 10 µM of MW-03 for 48 h. As shown in Fig. 3B, the treatments with MW-03 inhibited cellular growth in a concentration-dependent manner in LS174T cells. In order to examine whether MW-03 inhibited cellular growth in a concentration-dependent manner in LS174T cells, in order to examine whether MW-03 inhibited cellular growth in a concentration-dependent manner in LS174T cells. In order to examine whether MW-03 inhibited cellular growth in a concentration-dependent manner in LS174T cells. In order to examine whether MW-03 inhibited cellular growth in a concentration-dependent manner in LS174T cells. In order to examine whether MW-03 inhibited cellular growth in a concentration-dependent manner in LS174T cells. In order to examine whether MW-03 inhibited cellular growth in a concentration-dependent manner in LS174T cells.

![Fig. 3. Effects of PPARγ siRNA on the MW-03-Induced Expression of PPARγ and 15-PGDH, Secreted 15-PGDH in Medium (A), or MW-03-Induced Cell Growth Inhibition (B, C)].(LS174T cells were transfected with control or PPARγ siRNA for 48 h. Cells were then treated with 3 µM MW-03 for 16 h and subjected to an immunoblot analysis for PPARγ and 15-PGDH (A, left panels), or secreted 15-PGDH in the culture medium (A, right panels). LS174T human colon cancer cells were treated with dimethylsulfoxide (0 µM) or the indicated concentrations of MW-03 for 48 h, and then trypsinized and counted (B). After transfection with control or PPARγ siRNA for 48 h, cells were treated with 3 µM MW-03 for another 48 h and cell counts were then performed as described in Materials and Methods (C). Photographs were from representative experiments and histograms represent the ratio of PPARγ or 15-PGDH to β-tubulin that not pretreated with MW-03 (0 µM) as 100% (A), or control vehicle-treated cell numbers as 100% (C). Number of cells not treated with MW-03 as 100% (B). *p<0.05, ANOVA, significantly different from that not pretreated with MW-03 (0 µM) (A, B). †p<0.05, ANOVA, significantly different from control cells treated with MW-03 to the PPARγ siRNA cells treated with MW-03.)
no significant effects were observed on MW-03-induced cellular growth inhibition by the blockade of PPARγ with siRNA. As shown in Figs. 1B and 3A, MW-03 itself barely induce cAMP, and the expression of 15-PGDH was almost completely reduced by using siRNA for PPARγ to the basal levels in both the cellular and medium fractions. Also, as shown in Fig. 3C, unlike the expression of 15-PGDH, the MW-03-induced cell growth inhibition was not blocked by siRNA of PPARγ. Thus, the PGE2-stimulated cAMP accumulation as well as MW-03-induced PPARγ activation did not appear to be involved in the cellular growth inhibitory effects of MW-03.

Of particular interest, indomethacin is reported to induce S-phase arrest in plant cells, and partially in HT-29 human colon cancer cells though mechanism was not described. Therefore, in an attempt to identify alternate/additional targets of MW-03 in LS174T cells, a cell-cycle analysis were performed with MW-03-treated LS174T cells using a flow cytometer. As shown in Fig. 4A, when treated with MW-03, the cell-cycle was concentration-dependently arrested at the S phase with reductions in the population of cells in the G1/G0 phases. Thus, MW-03 may exert the additional effect of arresting the cell-cycle at the S phase, which is independent on its effects on PPARγ in LS174T cells.

CONCLUSION

MW-03, a novel indole compound, exerted anti-colon cancer effects by two distinct mechanisms, as shown in Fig. 4B. The first effect was enhancing the metabolism of PGE2 to its inactive form, 15-keto-PGE2 by inducing 15-PGDH. The second effect was cellular growth inhibition by arresting cells at the S phase as like indomethacin. Moreover, the effective concentrations of MW-03 used in the present study were at least 10-fold lower than that of indomethacin. Further experiments are needed in order to elucidate, for example, whether MW-03 exerts the same effects as NSAIDs. Moreover, since a number of cell death mechanisms have been suggested for each indole compound, as yet unknown mechanism(s) and target(s), particularly for the second effect of MW-03, need to be investigated in more detail in not only colon cancer

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Fig. 4. Effects of MW-03 on the Cell Cycle in LS174T Cells (A) and a Scheme of Two Distinct Anti-cancer Effects of MW-03 (B)

LS174T cells were treated with the indicated concentrations of MW-03 for 48 h and cell-cycle phases were detected by a flow cytometer analysis as described in Materials and Methods (A). A scheme of two possible anti-cancer effects of MW-03 in LS174T cells (B). *p<0.05, ANOVA, significantly increased from that not pretreated with MW-03 (untreated). †p<0.05, ANOVA, significantly decreased from that not pretreated with MW-03 (untreated). AC: adenylate cyclase.
cells but also in other cancer cells or in normal colon cells. However, we consider those two distinct and unique effects of MW-03, or its derivatives, on colon cancer cells to be a great beneficial therapeutic tool for colon cancer treatment, or even other PGE2-regulated cancer such as lung cancer, in the future.

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Conflict of Interest The authors declare no conflict of interest.

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