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A novel indole compound, AWT-489, inhibits prostaglandin D_2-induced CD55 expression by acting on DP prostanoid receptors as an antagonist in LS174T human colon cancer cells

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ABSTRACT

Indoles are composed of a common core structure, the indole ring, and are widely used as pharmaceuticals and their precursors. In this study, a newly composed relatively small indole compound, AWT-489 was examined to find a novel specific antagonist for DP receptors; the cognate receptors for prostaglandin D_2 (PGD_2), to prevent colon cancer malignancy. Here we showed that AWT-489 antagonized DP receptor-mediated cyclic AMP formation, and expression of CD55, an inhibitor of the complement system that correlates with poor survival in patients with colorectal cancer, in LS174T human colon cancer cells. Interestingly, unlike a popular indole compound, indomethacin, AWT-489 did not act on the cyclooxygenases as a non-steroidal anti-inflammatory drug. Moreover, AWT-489 exhibited a better inhibitory effect than that of the well-used DP receptor antagonist, BWA868C when a dose close to the physiological concentration of PGD_2 was used. These results suggest that AWT-489 can act as a novel human DP receptor antagonist to reduce the expression of CD55 in LS174T human colon cancer cells. We believe that AWT-489 has potential as a lead compound for designing a new DP receptor antagonist that may help improve PGD_2-related diseases, especially colon cancer in the near future.

Introduction

Prostaglandin D_2, DP prostanoid receptor, and colon cancer

Non-steroidal anti-inflammatory drugs (NSAIDs)[1] have been used to treat and/or prevent the development of colorectal cancer [1]. Cyclooxygenase (COX)-2 is an inducible and important factor for prostanoid synthesis in colorectal tumorigenesis [1]. The pro-tumorigenic mechanisms of COX-2 are considered to be mediated by its major metabolite prostaglandin E_2 (PGE_2), the production of which is increased in human colorectal tumors [1]. The effects of NSAIDs on colorectal cancer are considered to be mediated through the inhibition of COX-2 activity, which reduces the production of prostanoids, such as PGE_2. Therefore, prostanoids, especially PGE_2, have effects on cancer malignancy by promoting cellular survival and proliferation due to activation of their signaling pathways, for example, β-catenin-dependent transcriptional activation via their cognate receptors, e.g. EP2 and EP4 prostanoid receptors [2,3]. While PGE_2 is known to be involved in colorectal cancer malignancy, the roles of prostaglandin D_2 (PGD_2) on cancer development are obscure. Thus, the well-known sleep inducer, PGD_2, is considered as both a positive and negative regulator on cancer malignancy. For example, activating the PGD_2 cognate DP receptor induced the secretion of high molecular weight glycoproteins with clustered oligosaccharides, mucins, in LS174T human colon cancer cells [4]. Interestingly, the expression of some mucins such as MUC1 was shown to be increased in colon cancer, which has been correlated with a worse prognosis [5] Therefore, some mucins have been investigated as therapeutic targets [6]. Moreover, an increase in the synthesis of PGD_2 and DP receptor expression has been observed in healthy individuals with a prior history of ulcerative colitis, and these elevations may contribute to the maintenance of colonic tissue homeostasis as well as an increased risk of colorectal cancer [7]. Furthermore, not only PGE_2, but also PGD_2 stimulations were reported to induce a complement inhibitor decay-accelerating factor, also known as CD55, via a cyclic AMP (cAMP)/protein kinase A-dependent mechanism in human colon cancer LS174T cells [8]. Since CD55 plays several important roles on modulating immunosuppression and tumorigenesis, PGD_2-mediated CD55 induction may enhance the

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[1] *Abbreviation used:* NSAID, Non-steroidal anti-inflammatory drugs; EIA, enzyme immune assay; COX, cyclooxygenase; MUC, mucins.

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malignancy of cancer cells by impairing regulation of complement activation such as antibody-mediated cytotoxicity [8]. On the other hand, knockout of PGD_2 synthase in the model mice of human familial adenomatous polyposis (FAP), which often proceed colorectal cancer, led to development of 50% more intestinal adenomas [9]. As FAP model mice with high expression of human PGD_2 synthase had 80% fewer intestinal adenomas, PGD_2 synthase and/or PGD_2 may control an inhibitory effect on intestinal tumors such as colorectal cancer [9]. In addition, knockout of DP receptors in mice had no effect on the formation of colon carcinogen azoxymethane-induced aberrant crypt foci [10]; therefore, the involvement of PGD_2/DP receptor signaling in colorectal cancer remains unclear.

Indole compounds and prostanoid receptors

We recently demonstrated that one popular NSAID, indomethacin, had a potential antagonize effect on human EP2 receptors [11]. This activity as a human EP2 receptor antagonist is one of the cyclooxygenase-inhibition independent effects of indomethacin. Thus, indomethacin has also been shown to exhibit agonistic activity on chemoattractant receptor-homologous molecule expressed on T helper 2 cells (CRTH2) receptors, also known as DP2 receptors [12]. Moreover, DP receptor antagonists are designed by chemically modified indomethacin [13,14]. Since indomethacin has the potential to act on prostanoid receptors besides the COX inhibitor as a NSAID, we investigated newly synthesized indole compounds [15], and their effects on prostanoid receptors for exploring the additional remedy/method for anti-colon cancer treatment. Here, we showed that a novel and relatively small indole compound, AWT-489 had an antagonistic effect on DP receptors in human colon cancer LS174T cells. Thus, AWT-489 significantly inhibited PGD_2-induced cAMP formation as well as CD55 expression without affecting human EP2 receptors or COX-1/COX-2. Since the widely used DP receptor antagonist, BWA868C exhibited a slight agonistic effect on DP receptors, AWT-489 appears to be a better antagonist than BWA868C to reduce the CD55 expression in LS174T human colon cancer cells when the cells were treated with PGD_2 at physiological range of concentrations. These results suggest the potential for designing new DP receptor antagonist(s) based on AWT-489 as a lead compound for reducing the CD55-mediated colon cancer malignancy. Thus, we believe, the new compound(s) developed from AWT-489 may help to improve colon cancer tumorigenesis in the near future.

Materials and methods

AWT-489: AWT-489 was synthesized by Dr. Takayoshi Arai's laboratory in the Department of Chemistry, Graduate School of Science, Chiba University as reported previously [15].

Cell culture and materials: LS174T human colon cancer cells were used since they express negligible levels of COX-2 [11]; therefore, endogenous prostanoids production is very little. LS174T cells were maintained at 37 °C in Minimum Essential Medium-alpha (α-MEM; Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum (FBS; Thermo Scientific, Walthman, MO), 100 IU/ml penicillin (Meiji Seika, Japan) and 100 μg/ml streptomycin (Meiji Seika, Japan). HEK-293 cells stably expressing the human DP (HEK-DP cells) or human EP2 (HEK-EP2 cells) prostanoid receptor subtypes were generated and provided by the laboratory of Dr. John W. Regan. HEK-DP cells and HEK-EP2 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) containing 10% FBS, 250 μg/ml geneticin (Phyto Technology Laboratories, Shawnee Mission, KS), 200 μg/ml hygromycin B (Enzo Life Sciences, Farmingdale, NY), and 100 μg/ml gentamicin (Life Technologies, Carlsbad, CA). All materials were obtained from Wako Pure Chemical (Osaka, Japan) unless stated otherwise.

cAMP assay: LS174T cells, HEK-DP cells, and/or HEK-EP2 cells were cultured in 6-well plates, and were switched from α-MEM (LS174T cells) or DMEM (HEK-DP cells or HEK-EP2 cells) to Opti-MEM (Invitrogen, Carlsbad, CA) containing 100 IU/ml penicillin and 100 μg/ml streptomycin for LS174T cells or 250 μg/ml geneticin, 200 μg/ml hygromycin B, and 100 μg/ml gentamicin for HEK-DP cells and HEK-EP2 cells 16 h prior to the experiments. Cells were treated with 0.1 mg/ml isobutylmethyl-xanthine (Sigma, St. Louis, MO) for 25 min followed by either vehicle (0.1% dimethyl sulfoxide; Me_2SO), or indicated concentrations of AWT-489 or BWA868C for 15 min (pretreatment) or 60 min (treatment) at 37 °C. In the cases of treatment with PGD_2, PGE_2, or BW245C, cells were then treated with either vehicle (0.1% Me_2SO) or the indicated concentrations of PGD_2 (Cayman, Ann Arbor, MI), PGE_2 (Cayman, Ann Arbor, MI), or BW245C (Cayman, Ann Arbor, MI) for 60 min at 37 °C. Experiments were terminated by removing the medium and cells were placed on ice. The amount of cAMP formed was measured and calculated from a standard curve prepared using non-radiolabeled cAMP as described previously [16]. The amount of cAMP formed by 100 nM PGD_2 and 1 nM PGD_2 for 60 min in HEK-DP cells was approximately 30 pmol or 15 pmol/5×10^4 cells, respectively. The amount formed by 1 nM PGE_2 for 60 min in HEK-EP2 cells was approximately 15 pmol/5×10^4 cells. The amount formed in LS174T cells by 1 μM PGD_2 or 100 nM BW245C for 60 min was approximately 45 pmol/5×10^4 cells each.

Whole cell radioligand binding assay: HEK-DP cells or LS174T cells were cultured in 10 cm dishes, and were switched from DMEM (HEK-DP cells) or α-MEM (LS174T cells) to Opti-MEM containing 250 μg/ml geneticin, 200 μg/ml hygromycin B, and 100 μg/ml gentamicin for HEK-DP cells or 100 IU/ml penicillin and 100 μg/ml streptomycin for LS174T cells 16 h prior to the experiments. Cells were then trypsinized, centrifuged at 500 *g* for 2 min, and re-suspended at a concentration of 10^6 cells/100 ml (HEK-DP cells) or 5×10^6 cells/100 ml (LS174T cells) in ice-cold HEPES buffer consisting of 10 mM HEPES (pH 7.4; Kishida Chemicals, Osaka, Japan), 1 mM EDTA, and 10 mM $MnCl_2$ (Sigma, St. Louis, MO). [^{3}H]PGD_2 binding was performed using 100 μl of sample added to a final volume of 200 μl containing 0.8 nM [^{3}H]PGD_2 (GE Healthcare, Waukesha, WI) or 0.8 nM [^{3}H]PGD_2 plus the indicated concentrations of AWT-489. Samples were incubated for 120 min at 4 °C and filtered through Whatman GF/C glass filters (Whatman, Maidstone, UK) to terminate the incubation. Filters were then washed 3–5 times with ice-cold HEPES buffer, and radioactivity was measured by liquid scintillation counting.

Reverse transcriptional polymerase chain reaction (*RT-PCR*): LS174T cells were cultured in 6-well plates, and were switched from α-MEM to Opti-MEM containing 100 IU/ml penicillin and 100 μg/ml streptomycin 16 h prior to the experiments. Cells were treated with either vehicle (0.1% Me_2SO), 10 nM PGD_2, or 1 nM BW245C for the periods indicated at 37 °C, or treated with either vehicle (0.1% Me_2SO) or the indicated concentrations of PGD_2, or BW245C for 4 h at 37 °C. In the case of experiments using inhibitors, cells were pretreated with either vehicle (0.1% Me_2SO), 10 μM AWT-489, or 10 nM BWA868C for 15 min followed by treatment either with vehicle (0.1% Me_2SO), 10 nM PGD_2, or 1 nM BW245C for 4 h at 37 °C. RNA was prepared using ISOGEN II (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription was carried out using AMV reverse transcriptase (Promega, Madison, WI) and approximately 3 μg of RNA/sample that had been pretreated with DNase I (Promega, Madison, WI). This was followed by a polymerase chain reaction with an initial incubation at 94 °C for 1 min, followed by 25 cycles (CD55) or 35 cycles (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) of 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 60 s. The