Colorectal cancer (CRC) is a deadly disease regardless of sex, and a few therapeutic approaches have been fully developed at advanced stages, even if some strategies have durable clinical benefits, such as immunotherapy and chemotherapy. *Ganoderma lucidum* has been recognized as an organism that suppresses tumors and inflammation; however, the molecular mechanisms induced by a triterpenoid in *Ganoderma lucidum*, Lucidumol A, have not yet been fully explored in CRC and inflammatory responses. To this end, we extracted Lucidumol A from *Ganoderma lucidum* and analyzed its anticancer effect and anti-inflammatory potential in CRC cell lines and RAW264.7 macrophage-derived cell lines, respectively. A series of *in vitro* experiments including cell survival, wound healing, and migration assays were performed to determine the role of Lucidumol A in the CRC cell line. We also analyzed inflammatory responses using qRT-PCR, Western Blot, and ELISA in RAW 264.7 macrophage-derived cell lines exposed to various concentrations of Lucidumol A. Lucidumol A efficiently suppressed the metastatic potential of CRC at very low concentrations. Furthermore, significant anti-inflammatory activities were observed in Lucidumol A-treated RAW264.7 cells through modulation of inflammation-associated marker genes and cytokines. In conclusion, Lucidumol A plays an important role in *Ganoderma lucidum*-dependent tumor suppression and anti-inflammation, suggesting different strategies to treat CRC patients, and other diseases evoked by proinflammatory cytokines, despite the need to explore further its mechanism of action.

1. Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide. In 2020, CRC was ranked as the third most common cancer in the US, in terms of new cases and mortality in both sexes, and the number of new cases will be expected to increase in developed countries [1, 2]. Targeted approaches to block the pathways affected by oncogenic driver mutations have been used to treat CRC patients. However, these approaches are only limited to the patients harboring oncogenic driver mutations such as EGFR and KRAS, which account for a small subset of total CRC cases.
Moreover, responses against oxidative stresses and inflammatory stimuli in cancer microenvironmental cells, including monocytes, play an important role in cancer progression and metastasis [3–5]. Furthermore, multiple strategies using immune checkpoint blockade have been tried, but have suffered from several uncharacterized resistant mechanisms [6, 7]. Thus, new approaches or combinatorial strategies to enhance the survival rate against this deadly disease are needed.

_Ganoderma lucidum_ has become an increasingly attractive medicinal fungus species with high therapeutic potential in various diseases, including cancer. Recent biochemical and cell biological approaches revealed that this substance consists of various bioactive molecules such as terpenoids, steroids, and phenolic compounds, some of which exhibited pharmacological activities, suggesting that _Ganoderma lucidum_ extract is likely to have high phyto-medical potential [8–10]. Indeed, the whole genome sequencing analysis of _Ganoderma lucidum_ revealed that most genes are involved in secondary metabolism and regulation, which plays an important role in _Ganoderma lucidum_-induced human health through the production of triterpenoids and polysaccharides [11, 12]. In particular, the _Ganoderma lucidum_ polysaccharides are the main bioactive components, functioning as an immunomodulator and exhibiting tumor-suppressive effects [8, 13].

Triterpenes are a subtype of terpenes very popular in plants and are known to be bioactive molecules [14, 15]. More than 150 triterpenoids, consisting of five different structural classes, have been isolated from _Ganoderma lucidum_. Amongst them, Lucidumol A was identified as a lanostane-type triterpene [16]. Several lines of evidence suggest that the major components of _Ganoderma lucidum_—both triterpenoids and polysaccharides—have tumor-suppressive effects. This allows the fungus to be considered an attractive anticancer phytomedicine substance by suppressing stress kinase pathways, NF-kB pathways, and P53-dependent tumor-suppressive pathways. Moreover, Lucidumol A has exhibited antitumor activities in breast cancer and leukemia by targeting antiapoptotic Bcl-2 protein [10,17]. However, the contribution of this pathway by each specific substance is relatively less studied. Moreover, little is known about the biological role of Lucidumol A in CRC progression. Here we report a novel anticancer effect of Lucidumol A in colorectal cancer and how Lucidumol A improves inflammatory responses in macrophages.

## 2. Materials and Methods

### 2.1. Lucidumol A Preparation

The fruiting bodies of _Ganoderma lucidum_ were purchased from Kyong-dong Korean Traditional Market, Seoul, in August 2018. Dr. K. Bae, Chung-Nam National University, South Korea, previously identified the mushroom. A voucher specimen (no. GL001) was deposited at the Department of Life Science, Korea Polar Research Institute.

### 2.2. Extraction and Isolation

The fruiting bodies of _Ganoderma lucidum_ (1.5 kg) were extracted with methanol (MeOH, 3 × 2 L) at room temperature. The solvent was concentrated _in vacuo_ to yield 27.0 g of crude extract, subsequently suspended in 300 ml of distilled water and successively with solvent-partitioned with hexane (300 ml), ethyl acetate (300 ml), and n-butanol (300 ml). After that, the EtOAc extracts (7.0 g) were subject to silica gel column and 8 mm chromatography (CC; 230–400 mesh, 2.5 kg) by a gradient solvent system of hexane EtOAc (50:1 to 0:100), to afford 17 fractions (E1 - E17). Fraction E1 (3.0 g) was subject to silica gel CC (230–400 mesh, 500 g), using CHCl3: MeOH (39:1 to 6:4) as the solvent system, yielding nine subfractions (E1S1 - E1S9). Subfraction E1S4 (675.0 mg) was separated by a Sephadex LH-20 gel column and eluted with H2O:MeOH (60:40 to 0:100), to afford 13 subfractions (E1S4L1 - E1S4L13). Subfraction E1S4L1 (174.0 mg) was subjected to semipreparative HPLC (MeOH:H2O = 30:70 to 100:0), to yield Lucidumol A (11.0 mg, t<sub>1/2</sub> 85.9 min). Finally, H NMR spectroscopy was performed to determine the purity of Lucidumol A, and Lucidumol A was identified by comparison with literature data (Figure 1) [18].

### 2.3. Cell Culture

Both RAW 264.7 macrophage-derived cell lines and HCT116 colorectal cell lines were initially purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea) and maintained in high glucose DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated FBS, 3 mM Glutamine, antibiotics (100 U/mL penicillin and 100 U/mL streptomycins) (Invitrogen, Grand Island, NY, USA). Mycoplasma contamination was regularly monitored using a specific detection kit by the manufacturer's protocol (Invitrogen).

#### 2.3.1. Measurement of Proinflammatory Cytokine Production

5 × 10<sup>5</sup> cells of the RAW 264.7 cell were seeded in 24-well plates and treated with various concentrations of Lucidumol A (0, 6.25, 12.5, 25, and 50 μM) for 1 h and then stimulated with 0.5 μg/mL LPS for 24 h. The proinflammatory cytokines were measured by enzyme-linked immunosorbent assay (ELISA) in the collected media following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

#### 2.4. Wound-Healing Assay

The indicated cells were subcultured in a 6-well plate for 24 h and the wound was addressed by gently scraping the surface with a sterile p200 pipette tip. Subsequently, the wound-healing ability was monitored under a microscope. All scratch assays were performed in triplicate.

#### 2.5. Migration Assay

The experimental procedures were conducted following the manufacturer's protocol (Calbiochem). Briefly, the cells were placed in the reduced serum medium (1% FBS) for 16 h. Subsequently, the cells were treated with Lucidumol A as shown in the figure, followed by placement in a migration chamber. The complete media containing 10% FBS was placed into the lower chamber as a chemoattractant. After 24–48 h, the migrated and invasive
cells were quantified by Cell Stain Solution (400 μL) photographed.

2.6. Cytotoxicity Assay. An MTS assay monitored cell cytotoxicity in RAW 264.7 and HCT116 cells. The cells were plated into a 96-well plate. 10% MTS solution was added to the cell culture medium for 2 h, and the absorbance was measured using a microplate reader (Infinite 200 pro, TECAN) at 490 nm to determine the cell viability.

2.7. Morphological Analysis. The HCT116 cells were seeded in 12-well plates and subsequently exposed to Lucidumol A at different times and concentrations. The morphological change was observed under an inverted phase contrast microscope (Nescope, Seattle, WA, USA) after 24 h of treatment.

2.8. Quantitative Real-Time Polymerase Chain Reaction Analysis (RT-PCR). Total RNA was extracted using TRIzol Reagent Invitrogen (Cat# 15596–018) following the manufacturer’s instruction. Briefly, the pellet was lysed by TRIzol solution, and chloroform was used for phase separation followed by standard ethanol precipitation performed to obtain total RNA. 1 μg of total RNA was subject to generate cDNA using a high-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). RT-PCR was performed by a standard protocol of SYBR Green-based system in a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA). The primer sequences used are indicated in Table S1.

2.9. Apoptotic Analysis Using Flow Cytometry. Flow cytometry assay was performed to evaluate apoptosis induced by the Lucidumol A in HCT116 cells. First, the cells were plated into a 6-well plate for 24 h. Then, Lucidumol A was added at concentrations of 6.25, 12.5, 25, and 50 μM for an additional 24 h. Subsequently, the cells were harvested and fixed using 70% ethanol, then staining the samples using Annexin V/FITC and PI (BD Biosciences, San Jose, CA, USA). Finally, the samples were analyzed using a flow cytometer (Beckman Coulter Inc., Brea, CA, USA).

2.10. Immunoblot Analysis. The harvested cells were lysed using a buffer containing 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (CComplete™, Roche), 1 mM Na3VO4, 50 mM NaF and 10 mM EDTA. The cell lysate was centrifuged at 15,000 × g for 30 min at 4°C to obtain a supernatant containing protein and quantified using the Bradford method. The 40 μg of the quantified protein was electrophoresed on 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (BIO-RAD, Hercules, CA, USA). The primary antibodies for iNOS (1/1000 dilution), COX-2 (1/1000 dilution), and GAPDH (1/1000 dilution) were incubated at 4°C for 16 h followed by incubating a secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG (1/5000 dilution). The membrane was visualized with ECL solution (Millipore, Billerica, MA, USA).

2.11. Nitric Oxide Production. Before treatment of Lucidumol A, RAW 264.7 macrophages were stimulated with LPS from E. coli (500 ng/mL, Sigma-Aldrich, CA, USA) for 24 h and NO production was measured in the culture solution. Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride, and 5% phosphoric acid, Promega) was added in a ratio of 1:1 to the 96-well plate and reacted for 10 min. Absorbance was measured at 540 nm using a microplate reader (Infinite 200 pro, TECAN).

2.12. Statistical Analysis. The data shown in the current study are representative results with technical replicate. The statistical analysis was performed by paired or unpaired Student’s t-test. Only p value < 0.05 was considered significant.

3. Results

3.1. Lucidumol A Induces Cell Cytotoxicity in Colorectal Cancer Cells. To analyze the subcellular role of Lucidumol A, we employed the HCT116 colorectal cancer cell line and performed the assays for cellular cytotoxicity. First, we determined the optimal concentration of Lucidumol A by analyzing relative cell growth in various concentrations of Lucidumol A and found that cell growth started to be inhibited at 12.5 μM and gradually decreased at higher concentrations (Figures 1(a) and 1(b)). Also, morphological analysis under a light microscope consistently showed that the density of the cells reduced at around 12.5 μM concentration, and the morphological change by stress condition at 25 μM (Figure 1(c)). Similarly, a flow cytometric analysis using Annexin-v and PE consistently showed that cell death was induced in response to Lucidumol A treatment, suggesting that Lucidumol A evoked cytotoxic effects in colorectal cancer cells (Figure 2(a)). Collectively, these data suggest that the physiological concentration of Lucidumol A has an inhibiting role in colorectal cancer growth.

Many studies have revealed that the early apoptotic pathway is tightly controlled by multiple proteins such as B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated x (Bax) [19, 20]. These proteins play a crucial role in regulating the response to numerous extrinsic or intrinsic apoptotic signals and stimulate apoptosis for the maintenance of cell integrity. In the present study, to analyze the molecular mechanism underlying Lucidumol A-dependent cellular cytotoxicity, we analyzed the expression of the Bcl-2 family known as crucial apoptotic regulating factors. The qRT-PCR analysis showed ant apoptotic expression in the Bcl-2 family; both BCL-2 and MCL-1 mRNA expression decreased. In contrast, a proapoptotic BCL-2 member, Bax, was found to be enhanced in response to Lucidumol A treatment, suggesting that the cytotoxic effect evoked by Lucidumol A is mediated by the differential regulation of the Bcl-2 family (Figures 2(b)–2(d)).
3.2. Lucidumol A Suppresses Metastatic Potential in Colorectal Cancer Cells. Next, we analyzed the metastatic potential of the colorectal cancer cell to observe the role of Lucidumol A as an anticancer agent in colorectal cancer cells. First, the wound-healing analysis showed that the scratched HCT116 cells recovered at even a low concentration of Lucidumol A (3.125 μM) (Figure 3(a)). Moreover, we consistently observed that Lucidumol A suppressed migratory ability, suggesting that Lucidumol A suppresses the metastatic potential of colorectal cancer cells without cytotoxicity (Figure 3(b)).

3.3. Lucidumol A Enhances the Subcellular Anti-Inflammatory State. Several lines of evidence have suggested that Gano- derma lucidum has an anti-inflammatory effect in cancer, which motivated us to analyze the anti-inflammatory role of Lucidumol A in cancer microenvironmental cells employing RAW264.7 macrophage cell line [10]. First, the cell survival rate was determined to find the optimal experimental condition in RAW264.7 cells. Serially diluted Lucidumol A was incubated in the presence or absence of LPS, and we found that it seemed to be less toxic up to 50 μM of Lucidumol A (Figure 4(a)). Given the information about the...
sweetspotofLucidumolA,wenextmeasuredthesubcellular
nitricoxidelevelregulatedbyLucidumolAandfoundthatit
gradually decreased in response to LPS treatment
(Figure 4(b)). Te expression of the COX-2 and iNOS
mRNAs was analyzed by qRT-PCR and found to be sup-
pressed in the cells exposed to Lucidumol A (Figures 4(c)
and 4(d)). Moreover, both COX-2 and iNOS proteins
seemedtobeconsistentlysuppressedinthesamecontext,as
seen in Western blot analyses (Figure 4(e)). Lastly, the
proinflammatory cytokines were also evaluated in the same
experiment condition. Te qRT-PCR analyses showed that
TNF-α and IL-6 mRNAs were suppressed by Lucidumol A
treatment (Figure 5(a) and 5(b)). To confrm the previous
effect, we analyzed the production of TNF-α and IL-6 using
ELISA assays. Consistent suppression was observed in re-
spoon to Lucidumol A (Figures 5(c) and 5(d)). These data
strongly suggest that Lucidumol A has a potent anti-
inflammatory capacity.

4. Discussion

Despite the comparative studies on the biological roles of
Ganoderma lucidum, its underlying mechanism at the
molecular level has been less investigated. Moreover, little is
known about its extract, Lucidumol A. Here we identify the
anticancer role of Lucidumol A, the extract from Gano-
derma lucidum, in colorectal cancer, and the regulation of
the anti-infammatory state, which was consistent with the
reported role of Ganoderma lucidum.

Ganoderma lucidum contains over 400 bioactive com-
pounds, which have shown medicinal benefts including
anticancer and anti-inflammatory effects. Also, its biological
Figure 3: Decreased metastatic potential of HCT116 cells by Lucidumol A. The cells were exposed to Lucidumol A at indicated concentrations and wound-healing assay (a) and migration assay (b) were performed. All the expressions shown in this figure were performed in three independent experiments (n = 3). Bars mean ± S.D. and the p value were obtained by Student’s t-test (n = 3, *p < 0.001; **p < 0.05).

Figure 4: Continued.
Figure 4: Lucidumol A attenuates LPS-induced proinflammatory responses. (a) The cytotoxic effect of LPS and Lucidumol A in RAW264.7 cells. (b) Nitric oxide assay showing decreased nitric oxide production in RAW264.7 cells in response to Lucidumol A and LPS treatment. (c–e) The suppressed expression of COX-2 and iNOS in response to Lucidumol A. The cells were exposed to LPS (0.5 μg/ml) in the presence of various concentrations of Lucidumol A as indicated in each figure. Subsequently, the mRNA levels of COX-2 and iNOS genes were analyzed by a standard qRT-PCR analysis (c, d) and protein expression by Western blot analysis using anti-COX-2 and iNOS antibodies. The Western blot analysis was performed with three independent experiments and the summarized values after normalized by GAPDH were shown on the right two panels (e). All the expressions shown in this figure were performed in three independent experiments (n = 3). Bars mean ± S.D. and the p value were obtained by Student’s t-test (n = 3, * p < 0.001; ** p < 0.05).

Figure 5: Lucidumol A modulates the expression of proinflammatory cytokines. qRT-PCR analysis shows decreased TNF-α (a) and IL-6 mRNA (b). (C, D) ELISA assay revealed Lucidumol A-dependent TNF-α (c) and IL-6 (d) production in RAW264.7 cells. Bars indicate ± S.D. and the p value were obtained by Student’s t-test (n = 3, * p < 0.001; ** p < 0.05; *** p < 0.005).
activity may help cancer immunotherapy by regulating several types of cancer-related immune compositions, including NK cells, T cells, and macrophages. These biological compounds that have pharmacological potential consist of two major substances, triterpenoids, and polysaccharides. In particular, several triterpenoid derivatives have been reported to have antitumorigenic effects by regulating critical oncogenic signaling pathways such as MAPK and NF-kB pathways in several solid tumors. Also, Lucidumol A, a triterpenoid of *Ganoderma lucidum* inhibits invasive breast cancer cell progression and cell cycle arrest in leukemia. However little was known about its roles in CRC progression [10, 13, 16].

First, we identified the decreased cell growth rate and increased cell death rate of the colorectal cancer cells exposed to various concentrations of Lucidumol A, in addition to the suppressed metastatic potential in the same experimental condition. These results suggest that differential regulation of the Bcl-2 family (Figures 1 and 2) mediates these functions. Previous reports claimed that a lanostane-type triterpene found in *Ganoderma lucidum* induced cell cytotoxicity and decreased metastatic potential of lung and prostate cancer; this is consistent with current findings even though the role of Lucidumol A has not been elucidated [21, 22]. Interestingly, the antimetastatic effect of Lucidumol A was also exhibited at a lower concentration (3.125 μM) than that of cell cytotoxicity (12.5 μM), suggesting that the modulation of those pathways could be mediated by different subcellular pathways, in addition, to suggesting a physiologically relevant range of this compound’s application to human disease. Collectively, Lucidumol A has a strong tumor-suppressive effect in colorectal cancer progression by suppressing cell proliferation and migratory ability and increasing cell death.

Reactive oxygen species produced by metabolic processes in the surrounding cell regulate cancer progression through cross-talks to cancer cells by induction of subcellular stress pathways, such as oxidative stress and ER stress [3, 23]. However, it has also been known that these stresses could evoke cancer progression by mediating oncogenic mutations as well as enhancing metastatic potential in multiple cancers [5, 24, 25]. Furthermore, the hydroxy groups covalently connected to a carbon atom in triterpenes are frequently oxidized, likely enabling the triterpenoids to have such antioxidant pathways. Indeed, Lucidumol C and D have been reported to be antioxidant molecules that consequently suppress cancer progression [26, 27]. Moreover, our data suggest that Lucidumol A suppressed the expression of anti-inflammatory molecules such as COX-2 and iNOS (Figures 3–5). Therefore, we believe Lucidumol A could regulate tumor progression through the controlled inflammatory response in the surrounding cells such as monocytes including macrophages. However, the current conclusion could be limited in providing the mode of action of how Lucidumol A could control tumor progression and inflammatory response. Therefore, the conclusion should be further confirmed in multiple cell lines and robust evidence must be provided through future studies.

5. Conclusions

The present study concluded that a newly characterized triterpenoid, Lucidumol A extracted from *Ganoderma lucidum* suppresses colorectal cancer cell progression by inducing cell cytotoxicity and reducing metastatic potential, as well as enhancing the anti-inflammatory capacity of the surrounding cells, providing supporting evidence for *Ganoderma lucidum* as an anticancer therapy.

Data Availability

The datasets generated during and/or analyzed during the current study are available.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors’ Contributions

S–S. S. and Y–J. J. conceptualized the study; H–J. C., J. W. L., M. H. K., H. J. K., M. J. S., S. I., S. H. P., and H. E. L. created methodology; H–J. C., J. W. L., M. H. K., J. B. S., S–H. K., U. J. Y., and S–S. S. formally analyzed the data; U. J. Y., Y–J. J., and S–S. S. investigated the data; U. J. Y., Y–J. J., and S–S. S. wrote and prepared the original draft; Y–J. J. and S–S. S. supervised the study; Y–J. J. and S–S. S. administrated the project. All authors approved the final version of the manuscript.

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Supplementary Materials

Table S1 shows the list of primers used in this study. (Supplementary Materials)

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