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# Trisindoline synthesis and anticancer activity

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ABSTRACT

Expression of a *Rhodococcus*-derived oxygenase gene in *Escherichia coli* yielded indigo metabolites with cytotoxic activity against cancer cells. Bioactivity-guided fractionation of these indigo metabolites led to the isolation of trisindoline as the agent responsible for the observed *in vitro* cytotoxic activity against cancer cells. While the cytotoxicity of etoposide, a common anticancer drug, was dramatically decreased in multidrug-resistant (MDR) cancer cells compared with treatment of parental cells, trisindoline was found to have similar cytotoxicity effects on both parental and MDR cell lines. In addition, the cytotoxic effects of trisindoline were resistant to *P*-glycoprotein overexpression, one of the most common mechanisms of drug resistance in cancer cells, supporting its use to kill MDR cancer cells.



Following the discovery of indigo production by *Escherichia coli* harboring cloned naphthalene dioxygenase genes [1], bacterial oxygenases capable of producing indigo attracted research interest because of their potential to generate environmentally friendly indigo dyes to the textile industry. The oxygenase pathway for indigo production generally involves an initial oxidation of indole (2,3-benzopyrrole) at C-3 to produce indoxyl or isatin. In the presence of oxygen, the blue dye indigo is formed through the dimerization of indoxyls, while a condensation of indoxyl and isatin leads to the formation of indirubin [2]. Indirubin and its derivatives were later found to have potential uses as anticancer drugs as they have been reported to inhibit the activities of cyclin-dependent kinases, key controllers of cell cycle progression in eukaryotic cells [3–5]. These findings have prompted research into the possible use of indigo-producing oxygenases in the anti-cancer pharmaceutical arena [2,6–8].

Recently, we characterized a novel indigo-producing oxygenase gene, designated *ipoA*, from a *Rhodococcus* strain using a combination of random and saturation mutagenesis [9]. *E. coli* harboring a plasmid with the *ipoA* gene (pKEB1880) has the ability to produce indigo when grown in liquid Luria–Bertani (LB) medium. Interestingly, a single amino acid substitution of serine for alanine at position 58 results in a greater than 1.5-fold increase in the production of indigo (50.7 ± 4.9 μg/mL compared with 30.9 ± 0.7 μg/mL). Furthermore, preliminary data revealed that the dried ethyl acetate extracts of *E. coli* cultures expressing the mutant IpoA enzyme had cytotoxic effects on some cancer cells, leading us to further investigate and characterize indigo metabolites with anticancer activities.

## Materials and methods

*Production of indigoid compounds. Escherichia coli* strain DH5α expressing the mutant IpoA (A58S) enzyme [9] were selected by plating cells on an LB agar plate containing ampicillin (100 μg/mL) and incubating at 37 °C overnight. A single blue colony of the transformed *E. coli* was inoculated in 200 mL LB medium and incubated for 24 h at 30 °C with shaking (180 rpm). Four liters of the *E. coli* culture were collected, extracted twice with equal volumes of ethyl acetate, and concentrated. The concentrate was then dried under nitrogen and the molecular mass was determined by subtracting the pre-weighed vial weight from the vial weight after nitrogen drying.

*Purification and analysis of metabolites.* The dried residue of the indigoid compounds was dissolved in 30 mL dichloromethane and chromatographed through silica gel (230–400 mesh, Merck Co., Ltd, USA, 190 g, packed into a 3.8 cm i.d. × 38 cm high) using dichloromethane with a 10% increase in the ethyl acetate step gradient for fractionation. Each fraction was further purified by thin layer chromatography (TLC) on a $SiO_2$-precoated TLC plate $GF_{254}$ in the solvent system of dichloromethane:ethylacetate (10:1). When necessary, TLC-separated spots were scraped from the plate,

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eluted with dichloromethane, and further separated on a silica gel column (1.6 cm i.d. × 25 cm high) using the same solvent system as for TLC. Electron-impact mass spectra (EIMS) were recorded on a Varian-1200L spectrometer (70 eV, Varian). Nuclear magnetic resonance (NMR) spectra were measured on a Varian Unity 300 (300.145 MHz) spectrometer, with the chemical shifts represented as parts per million (ppm, $\delta$), using tetramethylsilane (TMS) as the internal standard.

*Cancer cell culture.* All cell cultures were maintained using RPMI 1640 cell growth medium (Invitrogen, USA) supplemented with 5% fetal bovine serum (FBS) (Invitrogen) and grown at 37 °C in a humidified atmosphere containing 5% $CO_2$. The human uterine sarcoma cell lines, MES-SA and MES-SA/DX5, were purchased from the American Type Culture Collection, and the colorectal adenocarcinoma cell line, HCT15, was provided by the National Cancer Institute (NCI). The HCT15/CL02 cell lines were established from HCT15 cells by continuous and stepwise exposure of the cells to doxorubicin in the Korea Research Institute of Chemical Technology as described previously [10].

*In vitro cytotoxicity assays.* All of the experimental procedures followed the NCI's protocols with some minor changes based on the sulforhodamine B (SRB) method as described previously [11,12]. Stock solutions of trisindoline (6000 μM), etoposide (6000 μM, Sigma, St. Louis, MO), and paclitaxel (200 μM, Sigma) were prepared in DMSO and diluted in culture media by at least 200-fold for cell cytotoxicity assays. For each experiment, cell toxicity was calculated by comparing the number of cells surviving at time zero (*Tz*) with those surviving at the end of the 72-h experiment, referred to as DT for cells incubated with serial dilutions of the drug, or CC for control cells incubated without drug. If DT is greater than or equal to *Tz*, the net percent of cell growth inhibition was calculated as $(DT - Tz)/(CC - Tz) \times 100$. If DT is less than *Tz*, the net percent of cell killing activity was calculated as $(DT - Tz)/Tz \times 100$. All data represent the average values of at least three wells per experiment. All values are expressed as the mean ± the standard deviation. Data were analyzed by a one-way analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparisons using Sigma Stat software (Jandel Co., USA). For each of the comparisons of cell growth, the differences are considered statistically significant when $p$ is less than 0.05.

To assess the effects of verapamil (VER, Sigma) on drug cytotoxicity, the cancer cells were incubated with serial dilutions of trisindoline or paclitaxel, as described above, in the presence or absence of VER (10 μM) for 72 h. The calculation of the fractions of surviving cells was identical to the calculation performed for the cytotoxicity assay except that the control cells were treated with VER (10 μM) alone without any cytotoxic agent.

## Results and discussion

### *Purification and identification of the cytotoxic indigo metabolite, trisindoline*

Extraction and concentration of a 4-L culture of a recombinant *E. coli* clone, expressing the engineered IpoA enzyme, yielded a total of 1.9 g of a crude metabolite mixture. These metabolites were initially fractionated on a silica gel column and identified by TLC as follows: indole (320 mg, $Rf = 0.57$), indigo (4 mg, blue, $Rf = 0.45$), indirubin (6 mg, red, $Rf = 0.25$), *iso*-indigo (3 mg, brown, $Rf = 0.15$), and four fractions designated as FR-A (52 mg), FR-B (88 mg), FR-C (167 mg), and FR-D (38 mg). FR-A was found to have cytotoxic effects on the human HCT15 colorectal adenocarcinoma cells and was further purified by column chromatography into three components: FR-A1, FR-A2, and FR-A3. FR-A2 (24 mg) was identified as the metabolite that was responsible for the observed *in vitro* cytotoxic activity against cancer cells.

EIMS and $^1H$ and $^{13}C$ NMR spectroscopy were conducted to determine the structure of the FR-A2 metabolite. EIMS analysis of FR-A2 revealed a molecular ion peak at $m/z$ 363 and a base peak (M-29) due to fission of carbon monoxide and a hydrogen atom. $^1H$ NMR spectral assignments of FR-A2 were as follows:$\delta$: 10.2 (2H, br s), 9.58 (1H, br s), 7.46 (2H, d, $J = 8.0$ Hz), 7.38 (2H, d, $J = 8.2$ Hz), 7.32 (1H, d, d, $J = 7.5$ Hz), 7.09–6.93 (6H, m), 6.82 (2H, $J = 7.9$, 7.2 Hz). In addition, the $^{13}C$ NMR spectrum of trisindoline showed one amide carbonyl carbon ($\delta_C$ 183.4) and one quaternary carbon ($\delta_C$ 55.7) (Table 1). All of the spectral data of FR-A2 were identical with those reported for trisindoline [13].

### *Cytotoxicity of trisindoline on human cancer cells*

The bioactivity-guided fractionation of ethyl acetate extracts of the *E. coli* culture expressing an engineered IpoA enzyme allowed us to identify trisindoline as an active component with *in vitro* cytotoxic activity after incubation with human cancer cells. To investigate this activity in more detail, trisindoline cytotoxicity against cancer cells was evaluated and compared with that of etoposide, a chemotherapeutic agent that is commonly used to treat ovarian and testicular cancers. For these studies, we used two human cancer cell lines, HCT15, isolated from a colorectal adenocarcinoma, and MES-SA, isolated from a uterine sarcoma, along with their multidrug-resistant (MDR) sublines, HCT15/CL02 and MES-SA/DX5, respectively. As summarized in Table 2, $IC_{50}$ values increased 1.6- and 29-fold for trisindoline and etoposide in MES-SA/DX5, respectively. In contrast, trisindoline cytotoxicity increased slightly in the HCT15/CL02 MDR cell line, while etoposide cytotoxicity decreased dramatically in the same cell line. Overall, trisindoline was found to be more effective in killing MDR cancer cells compared with etoposide, which was more cytotoxic to the parental cancer cells.

### *Effect of VER on trisindoline cytotoxicity*

The most frequently occurring mechanism of MDR in cancer cells is the overexpression of *P*-glycoprotein (*P*-gp), the 170-kDa transmembrane glycoprotein product encoded by the *mdr1* gene. *P*-gp is known to function as an energy-dependent efflux pump and works to increase the efflux of cytotoxic drugs from cancer cells, thus lowering intracellular drug concentrations [14]. We have previously confirmed *mdr1* gene expression in the cells tested in this study using RT-PCR, and found that while the parental MES-SA cells had no detectable *mdr1* gene expression, the MES-SA/DX5, HCT15, and HCT15/CL02 cells did express the *mdr1* gene (unpublished data). Since the resistance of MES-SA/DX5 cells to trisindoline was approximately 1.6-fold higher than that of its parental cells, we determined whether trisindoline affects the functional activity of *P*-gp.

Since enhancement of a compound's cytotoxicity by verapamil (VER), a well-characterized *P*-gp-mediated MDR reversal agent, is considered evidence that the compound is a *P*-gp substrate [12], we tested the effects of VER treatment on trisindoline's cytotoxicity against cancer cells. As shown in Fig. 1, VER has no effect on trisindoline's cytotoxicity on any of the tested cancer cell lines (Fig. 1A and C). In contrast, VER enhanced the cytotoxicity of paclitaxel (Fig. 1B and D), a well-known *P*-gp substrate, approximately 32-, 3-, and 35-fold in MES-SA/DX5, HCT15, and HCT15/CL02 cells, respectively. These results strongly suggest that trisindoline is not a substrate of *P*-gp, supporting its use to kill MDR cancer cells.

In this study, we expressed and purified trisindoline from a mixture of indigo metabolites and subsequently observed that this compound is effective in killing parental cancer cell lines, and importantly, in killing their MDR counterparts. Considering that drug resistance is one of the most significant impediments to suc-

**Table 1**
$^{13}C$ NMR (75 MHz) chemical shifts ($\delta_C$) for the FR-A2 metabolite

| Position | $CD_3OD$ | Acetone $d_6$ | Position | $CD_3OD$ | Acetone $d_6$ |
|---|---|---|---|---|---|
| 2 | 183.4 | 180.9 | 4′,4″ | 123.4 | 123.6 |
| 3 | 55.7 | 55.2 | 5′,5″ | 120.5 | 120.6 |
| 3a | 137.5 | 137.2 | 6′,6″ | 123.3 | 123.5 |
| 4 | 127.3 | 127.5 | 7′,7″ | 113.3 | 113.6 |
| 5 | 124.1 | 123.8 | 7a′,7a″ | 139.6 | 139.7 |
| 6 | 129.9 | 130.0 | | | |
| 7 | 111.8 | 111.7 | | | |
| 7a | 143.3 | 143.7 | | | |
| 2′,2″ | 126.6 | 126.7 | | | |
| 3′,3″ | 116.7 | 117.6 | | | |
| 3a′,3a″ | 128.2 | 128.6 | | | |

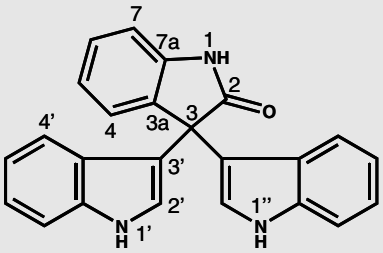


**Table 2**
Comparison of *in vitro* cytotoxicities of trisindoline and etoposide on human cancer cell growth

| Human cancer cells | | $IC_{50}$ (μM)[a] of | |
|---|---|---|---|
| | | Trisindoline | Etoposide |
| Uterine sarcoma | MES-SA | 3.51 ± 0.03 | 0.28 ± 0.02 |
| | MES-SA/DX5 | 5.50 ± 0.53 | 8.14 ± 0.89 |
| Colorectal adenocarcinoma | HCT15 | 6.63 ± 0.43 | 1.42 ± 0.02 |
| | HCT15/CL02 | 5.11 ± 0.44 | 11.68 ± 2.38 |

[a] Concentration of drug that causes 50% inhibition of cell growth. Values represent the averages from at least three independent experiments.

cessful cancer chemotherapy, our work provides insight into designing novel anticancer drugs with potent cytotoxic activities against a wide range of cancer cells. To the best of our knowledge, this is the first report of the synthesis of trisindoline and the characterization of its anticancer activity.

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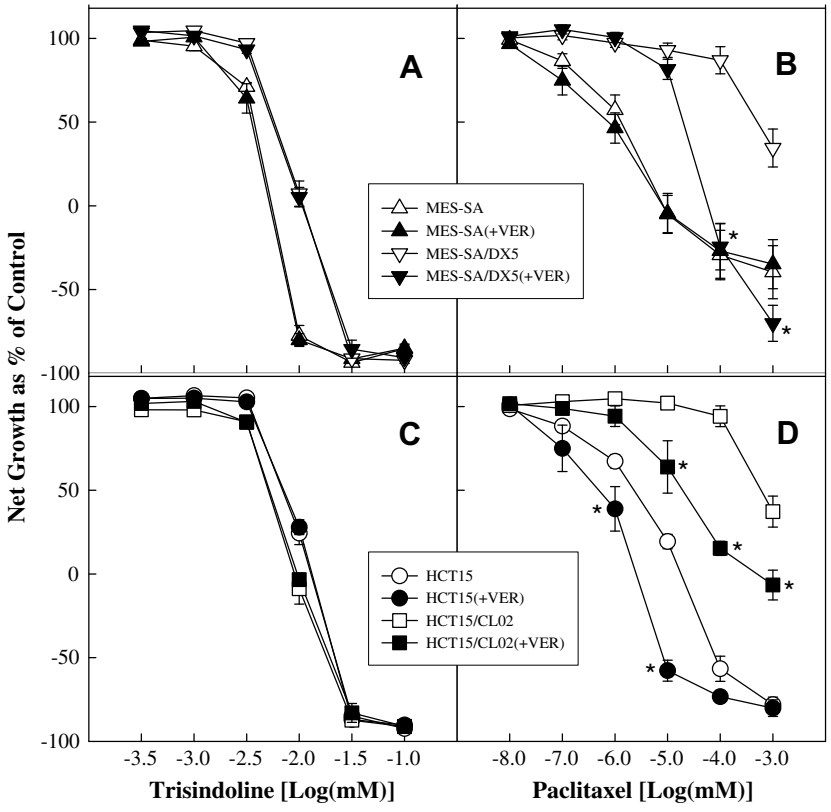


**Fig. 1.** *In vitro* cytotoxicities of trisindoline and paclitaxel on parental and MDR cancer cells in the presence or absence of VER. Cell survival fractions were assessed by SRB assay following 72 h of drug exposure. Each data point represents the mean of at least three experiments, and the error bars represent the standard error associated with the mean. Asterisks indicate significant differences from the data in the absence of VER ($p < 0.05$).

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