RESEARCH ARTICLE

Diosmin Enhances the Anti-migration Activity of Curcumin Analog PGV-1 on Colorectal Cancer Cells

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Abstract

BACKGROUND: Diosmin enhances the cytotoxicity of Pentagamavunone-1 (PGV-1) in cancer cells. PGV-1 and diosmin are predicted to target several matrix metalloproteinases (MMPs) in metastatic cancer, including colorectal cancer, but the anti-migration potency of their combination has not established yet. This study evaluates the anti-migration effect of PGV-1 and diosmin combination in colorectal cancer.

METHODS: The cytotoxicity assay using Cell Counting Kit 8 (CCK-8) method in WiDr colorectal cancer cells was carried out to determine the concentration for anti-migration experiments. The wound healing assay was used to observe the anti-migration activity by measuring the cell-free area. Gelatin zymography was employed to detect the MMP activity indicating by the clear band density. The interaction between PGV-1 or diosmin and MMP proteins was predicted by molecular dockings.

RESULTS: PGV-1 was cytotoxic (IC_{50} 17 μM), while diosmin up to 100 μM did not affect cell viability. Both 10 μM PGV-1 as well as 50 and 100 μM diosmin slowed down the closure of cell-free area. A 100 μM diosmin was significantly enhance the anti-migratory activity of 50 and 100 μM PGV-1. The activity of MMP-9 and MMP-2 was also lower in the presence of diosmin compared to than that of PGV-1 alone. PGV-1 or diosmin was also able to interact with MMP proteins with a lower energy compared to than that of the native ligands.

CONCLUSION: Diosmin enhances the anti-migration activity of PGV-1 in WiDr cells, possibly by affecting MMPs' activity. This study is an evidence that diosmin is a potential co-chemotherapy candidate for PGV-1, that can be utilized to overcome metastatis in colorectal cancer.

KEYWORDS: cancer, citrus flavonoid, co-chemotherapy, diosmin, matrix metalloproteinases (MMPs), migration, Pentagamavunone-1, WiDr cancer cell

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Introduction

Colon cancer is one of the most frequently diagnosed cancer cases globally in 2020.(1) The 5-fluorouracil (5-FU) as the first line therapy shows the phenomenon of resistance in patients undergoing chemotherapy.(2) Therefore, efforts to find new candidates for an effective and safe anticancer agent are urgently needed. In addition to resistance, the complexity of the molecular pathogenesis of cancer cells which includes their ability to migrate and invade other tissues causing metastases affects the effectiveness of therapy.(3) Metastases cause worsening of prognosis and high mortality, including in breast cancer and colon cancer.

(4) Therapy that targets cell migration is needed to overcome this problem.

One candidate for a potential anticancer agent is the compound curcumin analog Pentagamavunon-1 (PGV-1) synthesized by the Faculty of Pharmacy, Universitas Gadjah Mada.(5,6) This compound has been shown to be able to inhibit the growth of various types of cancer cells both *in vitro* and *in vivo* (7), including colon or colorectal cancer (8-10). Compared to anticancer drugs that have been used clinically, PGV-1 is able to irreversibly maintain its anticancer effects, and selectively target cancer cells but not normal cells.(7,9-12) Thus PGV-1 is not only effective, but also safe.

In addition to inhibiting the migration of WiDr colon cancer cells, PGV-1 also suppresses the expression and activity of matrix metalloproteinase (MMP)-9.(9) MMP is a catalytic enzyme that degrades extracellular matrix (ECM) components in tissues that allows cell migration and invasion, neo-angiogenesis, and ultimately cancer metastasis.(13) The decreases in MMP-2 and MMP-9 expression in cell model and anti-metastasis ability in mouse xenograft models are also demonstrated by PGV-1.(14) Given its ability to alter MMP expression, it is conceivable that this mechanism play roles in PGV-1's anti-migration action.

Even though it shows great potency and selectivity and can be used orally (10), the dose required by PGV-1 is still higher compared to existing anticancer drugs, as indicated by the value of 50% inhibitory concentration (IC_{50}), for example ranging from 0.1-18 μM on WiDr colorectal cancer cells.(6,8,9,15) Therefore, PGV-1 can be combined with other compounds that are less cytotoxic so that the dose required by PGV-1 can be reduced but still effective. This strategy of combination chemotherapy (co-chemotherapy) can simultaneously slow down the occurrence of drug resistance.(16)

Several natural compounds have been shown to increase the effectiveness of PGV-1 in cancer cells with metastatic properties. As an example, synergism with PGV-1 in 4T1 breast cancer cells is demonstrated by diosmin, which is abundant in orange peel.(17) Diosmin also in concert increases the cytotoxicity of PGV-1 in WiDr colon cancer cells that have the potential to metastases.(15) Based on bioinformatics studies, PGV-1 and diosmin are predicted to target MMP-1 collagenase in breast cancer (17) and MMP-1 and MMP-7 gelatinase in colon cancer (15). Thus, evidence of increased anti-migration activity of PGV-1 by diosmin in metastatic cancer cells is needed to strengthen the basis for developing a combination of PGV-1 with natural compounds as a cancer therapy strategy.

Previous studies have shown that diosmin synergistically enhances PGV-1's anticancer activity in metastatic/potentially metastatic cancer cells through antiproliferation, induction of mitotic catastrophe, and induction of senescence.(15,17) Those studies predict that several ECM degrading enzymes, namely MMPs, which are one of the important proteins in the metastatic process, become the target proteins of PGV-1 and diosmin in both types of cancer. However, the anti-migration activity of the combination of PGV-1 and diosmin has not been proven. This study aims to validate the potency of diosmin in increasing the anti-migration activity of PGV-1 in colorectal cancer, which includes anti-migration assay with the wound healing assay, measuring MMPs' expression level with gelatin zymography, and predicting PGV-1 and diosmin interaction with MMPs (MMP-1, -2, -3, -7, and -9) with an *in silico* approach.

Methods

Compounds

The tested compound PGV-1 with a purity of 95% was synthesized as previously described (6,18), while diosmin was obtained from Selleck (Cat. #S2292, Selleckchem, Houston, Texas, US).

Cell Culture

WiDr cell line used as a model for colorectal cancer cells with metastatic potency was a collection of Laboratory of Functional Genomics and Medicine, Nara Institute of Science and Technology (NAIST), Japan. The cells were maintained as previously described (9,19) in RPMI 1640 media (Cat. #30264-85, Nacalai Tesque, Kyoto, Japan) with a supplementation of 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), L-L-alanyl-L-glutamine (Cat. #04260-64, Nacalai Tesque), and antibiotics (Cat. #26253-84, Nacalai Tesque). The cells were kept at humidified atmosphere with 5% CO_2 at 37°C in Sanyo Incusafe Dual Stack CO_2 Incubator (MCO-181C, Sanyo, Osaka, Japan). Passaging cells were carried out by trypsinization using 0.1% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) (Cat. #32777-44, Nacalai Tesque) in phosphate buffered saline (PBS) (Cat. #166-2355, Fujifilm Wako, Osaka, Japan). The protocol of this study was approved by the Medical and Health Research Ethics Committee (MHERC) of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Indonesia (No. KE/FK/1333/EC/2023).

Cytotoxicity Assay

The cytotoxicity assay was carried out with CCK-8 (Cat. #347-07621, Dojindo, Osaka, Japan) to determine the optimal concentration for the follow up experiment. After seeding WiDr cells on 96-wellplates (5×10^3 cells/well) for overnight, the cells were treated with PGV-1 or diosmin for 24 h. PGV-1 was diluted in dimethyl sulfoxide (DMSO) (Cat. #D8418, Sigma-Aldrich) as stock solution at 100 mM and then serially diluted in media to reach concentrations of 1-25 μM. As suggested by the manufacturer, diosmin was diluted in DMSO as a stock solution at 10 mM and then serially diluted in media to obtain concentrations of 10-100 μM. At the end of treatment, the cells were washed with PBS, and a new complete medium with 10% CCK-8 solution was added to each well.(11) The cell viability was measured based on the absorbance at 450 nm (Multi-mode plate reader TriStar LB942) after 2- to 4-h incubation (20) and the IC_{50} was calculated.(21)

Wound Healing Assay

The anti-migration activity was observed by the wound healing assay using Culture-Inserts 2 Well (Cat. #80209, Ibidi, Gräfelfing, Germany) as previously described.(20) Cells were seeded on each well of the insert on 24-wellplates (8×10^4 cells/70 μL) and incubated at 37°C with 5% CO_2 aeration to allow the cells to attach. The medium was replaced by the starvation medium containing 0.5% FBS for overnight followed by the addition of mitomycin C (Cat. #20898-21, Nacalai Tesque) at the final concentration of 10 μg/mL in the complete medium. After 2-h pretreatment with mitomycin C to prevent cell proliferation, the insert was removed, the cells were washed with PBS, and 500 μL complete media containing PGV-1, diosmin, or their combination at certain concentrations based on the cytotoxicity assay were added. The concentration for PGV-1 was around ¼ and ½ of IC_{50}, while diosmin was employed based on the highest tested concentration: ¼, ½, and the highest 100 μM. The cell migration to the cell-free area (wound) was observed and documented by Nikon Diaphot 300 microscope (Nikon, Tokyo, Japan) with Sony Nex-5 camera (Sony, Tokyo, Japan) at 0-, 24-, 48-, and 72-h for the quantification in ImageJ (National Institutes of Health, Bethesda, MA, USA).(9,11) Each treatment was carried in two inserts for one experiment and a minimal of three areas per insert were documented for the analysis.

Gelatin Zymography Assay

The expression of MMPs was observed based on the gelatinolytic activity using zymography. Cells (1×10^5/well) were grown in a 24-wellplate for 24-h followed by medium replacement with the starvation one. After the overnight starvation, 500 μL of the starvation media containing PGV-1, diosmin, or their combination at the dedicated concentrations were added. The same concentration as the wound healing assay was used. At the end of 24-h treatment, the culture media were collected (two wells for each treatment were pooled), centrifuged at 400 g for 5 min at 4°C, and the culture supernatants were transferred into new tubes and kept at -20°C. Meanwhile, the cells were washed with cold PBS twice and cold freshly-prepared cell lysis buffer 75 μL/well (1% NP-40, 100 mM NaCl in 25 mM Tris-HCl pH 7.5 containing proteinase inhibitor cocktail (Cat. #04080-11, Nacalai Tesque) and phenylmethylsulfonyl fluoride (Cat. #164-12181, Wako, Osaka, Japan)) were added. The cell lysates (two wells for each treatment were pooled, the same as the cell media) were then processed to obtain the protein cell lysates as described previously.(22) Briefly, after obtaining the lysates following the centrifugation 15,000 g for 20 min at 4°C, the protein concentration was determined by Pierce 660 nm Protein Assay (Cat. #22660, Thermo Fisher, Scientific, Waltham, MA, USA).(20) The protein concentration then was used to normalize the loaded volume of culture supernatant for zymography.

The culture supernatants were mixed with a sample buffer (prepared as a 5× stock consists of 50% glycerol, 10% sodium dodecyl sulfate (SDS), and 0.05 % bromophenol blue in 0.31 M Tris-HCl pH 6.8). The normalized volume to the total protein content was resolved in the 8% SDS-polyacrylamide gel containing 0.1% gelatin (Cat. #G-9391, Sigma-Aldrich) in final with a maximum volume of 30 μL per well.(20) The electrophoresis was run at 30 mA/gel (Atto AE-6530M) until the marker (Cat. #17044601, GE Healthcare, Chicago, IL, USA) reach the end of the gel. After incubating the gel in a gel renaturing solution for 30 min with a gentle shaking at room temperature (RT), the gel was rinsed generously with double distilled water, following by incubation in the gel developing solution for another 30 min at RT. The developing solution was changed into a new one and the incubation was continued for 16-h at 37°C. The gel then was stained in Coomassie Brilliant Blue (CBB) R-250 (Cat. #09408-52, Nacalai Tesque) solution for 1-h at RT and de-stained until clear bands indicating the gelatinolytic activity appeared. The composition of renaturing, developing, CBB staining, and de-staining solutions were adopted and optimized from previous reports.(11,20,22) The zymogram was documented and the clear bands representing the expression level of MMPs were analyzed with ImageJ.(9)

Molecular Docking

Molecular docking simulated the binding interaction between PGV-1 and diosmin toward MMPs. Molecular Operating Environment (MOE) software (version 2010.10) with the default setting was employed for molecular docking simulation following previous procedure.(18,23) MMP-1 (PDB ID: 966C), MMP-2 (PDB ID: 1HOV), MMP-3 (PDB ID: 2D1O), MMP-7 (PDB ID: 2Y6C), and MMP-9 (PDB ID: 2OW1) were retrieved as protein model considering the present of native ligand for catalytic binding site identification. Chemical structure of PGV-1 and diosmin were prepared in MOE, then proceeded for energy minimization and conformation. Triangle matcher and London dG were employed as placement settings and scoring methods, respectively. The force field method was applied to refine the docking results through 10 retention settings. Conformation with lowest docking score was used for binding interaction analysis.

Data Analysis

Each of the cell-based assays were independently performed twice with at least triplicate for each experiment and the representative results are presented in average±standard error (SE) as indicated in the figure's caption. GraphPad Prism 9 for macOS (version 9.5.0) (GraphPad Software, La Jolla, CA USA) was used for the statistical analysis. The normal data distribution was confirmed by Saphiro-Wilk test, followed by one-way ANOVA analysis with multiple comparisons. The statistical significance was set at 95% confidence level. The raw data in which quantitative analysis placed are available upon request.

Results

The Effect of PGV-1 and Diosmin in WiDr Colorectal Cancer Cells

The strong cytotoxicity activity of PGV-1 and the non-cytotoxicity of diosmin, as well as their synergistic combination in WiDr cells, have been confirmed previously. However, it is important to once again measure it in order to obtain the most appropriate concentration for the anti-migration experiment. Low concentrations of PGV-1 (1-5 μM) did not affect the WiDr cell viability, however, start from 10 μM, PGV-1 caused significant decrease in the cell viability (Figure 1A). Conversely, until the highest tested concentration at 100 μM diosmin did not affect the cell viability (Figure 1B). The obtained IC_{50} value of PGV-1 was 17 μM, while the IC_{50} of diosmin could not be determined since none of the tested concentration, even up to the highest one, was able to decrease the cell viability to 50%. Nevertheless, by an extrapolation, a value of 768 μM was acquired. Hence, diosmin was practically not cytotoxic in WiDr cells.

The Effect of PGV-1 and Diosmin Combination on WiDr Cell Migration

Based on the single cytotoxicity assay, concentrations of around ¼ and ½ IC_{50}, 5 and 10 μM for PGV-1, and 25, 50, and 100 μM for diosmin were applied, respectively, in the anti-migration combination assay. As hypothesized, the synergist combination of PGV-1 and diosmin would also reflected by their synergistic anti-migration activity. WiDr cells started to migrate rapidly at 24-h in the wound healing assay and by 48-h the cell-free area was closed nearly completely (Figure 2). PGV-1 was able to slow the cell migration and inhibit the closure of the cell-free area with 10 μM was more efficiently than 5 μM. Interestingly, in spite of its non-cytotoxicity, diosmin was also able to inhibit the cell migration in a dose-dependent manner. Confirming our hypothesis, the combination of PGV-1 and diosmin at all tested concentration was able to inhibit WiDr cell migration, indicated by the existence of the cell-free area at 72-h while the wound area in the group without treatment was obviously closed completely.

The quantification of the cell-free area was executed to further reinforce the microscopic observation as shown in Figure 3. The cell-free area of treated cells at 24-h was all higher than that of control cell without treatment with significant differences in 100 μM diosmin and almost all PGV-1 and diosmin combination (Figure 3A). After 48-h, the ability of PGV-1, diosmin, and its combination in inhibiting WiDr cell migration became more apparent. In particular, for example, the cell-free area of 10 μM PGV-1 was around 5%, but in combination with diosmin 25, 50, and 100 μM, it resulted a significant anti-migration effect at around 20% to 30% cell-free area with $p<0.001$ (Figure 3B). The same pattern was also seen at 72-h (Figure 3C), in which combination of PGV-1 with 100 μM diosmin was significantly maintained the cell-free area at around 25% ($p<0.0001$). Hence, the effectivity of diosmin in enhancing the anti-migratory activity of PGV-1 was evident.

The Effect of PGV-1 and Diosmin Combination on The Expression of MMP-9 and 2 in WiDr Cells

The initial hypothesis of this study is ignited by several MMP proteins being the predictive targets of PGV-1 and diosmin in colorectal cancer. Thus, it is plausible to examine

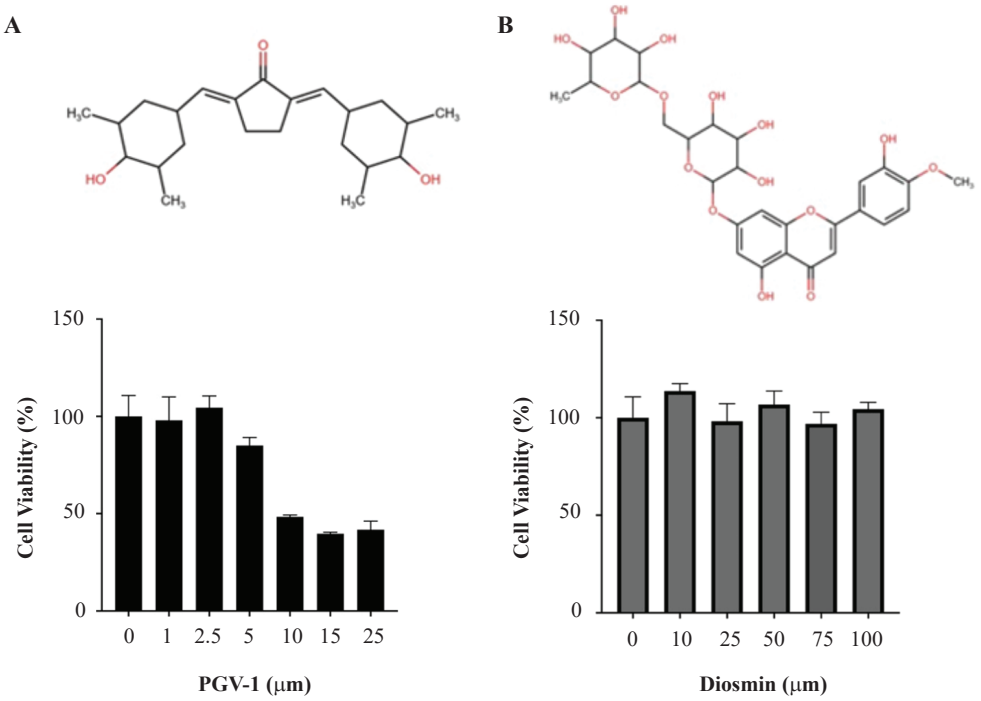

Figure 1. The WiDr cell viability under the treatment of PGV-1 (A) and diosmin (B). The cells were treated with the compound at a serial concentration as indicated for 24-h and assayed with CCK-8. The assays were independently performed twice with at least triplicate for each experiment and the representative results are depicted. The cell viability is presented as average and the error bar represents SE (n=3). The chemical structure of the compound is depicted above each graph.

the activity of MMPs. The gelatin zymography assay would provide a beneficial tools in measuring the activity of MMP-9 and MMP-2 as indicated by the gelatinolytic activity. In this assay, unfortunately, only the latent (pro) form of MMP-9 and MMP-2 could be detected at the size of around 97 and 66 kD, respectively (Figure 4A). Nevertheless, after measuring the band density, a clear reduction in MMP-9 and MMP-2 activity was able to retrieve (Figure 4B). Comparing to that of untreated cells, almost all treated cells were significantly show decrease in MMP-9 and 2 activity, with MMP-2 being more profound (right panel). In agreement with the anti-migration activity based on the wound healing assay above, the addition of diosmin at all concentrations to 5 μM PGV-1 supressed the activity of MMP-9 and MMP-2 significantly (Figure 4B). The similar tendency for MMP-9 was also noticed in the combination of 5 μM PGV-1 and

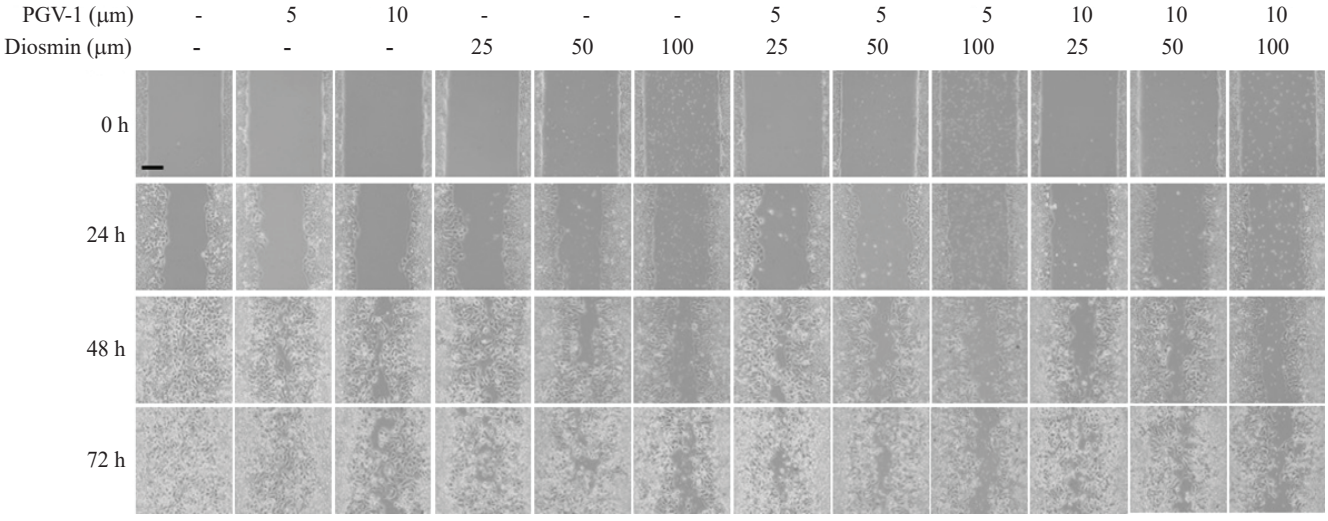

Figure 2. The effect of PGV-1 and diosmin combination on WiDr cell migration. The scratch wound healing was performed by using Ibidi insert. Followed the overnight medium starvation and mitomycin-C pretreatment to prevent cell proliferation, the cells were treated with PGV-1, diosmin, or their combination in the medium with a full serum supplementation. The cell migration was observed at the dedicated time point up to 72-h and the representative images are presented. The assays were carried out in a two independent experiment and the representative results from one experiment are depicted at the same magnification. Scale bar: 100 μm.

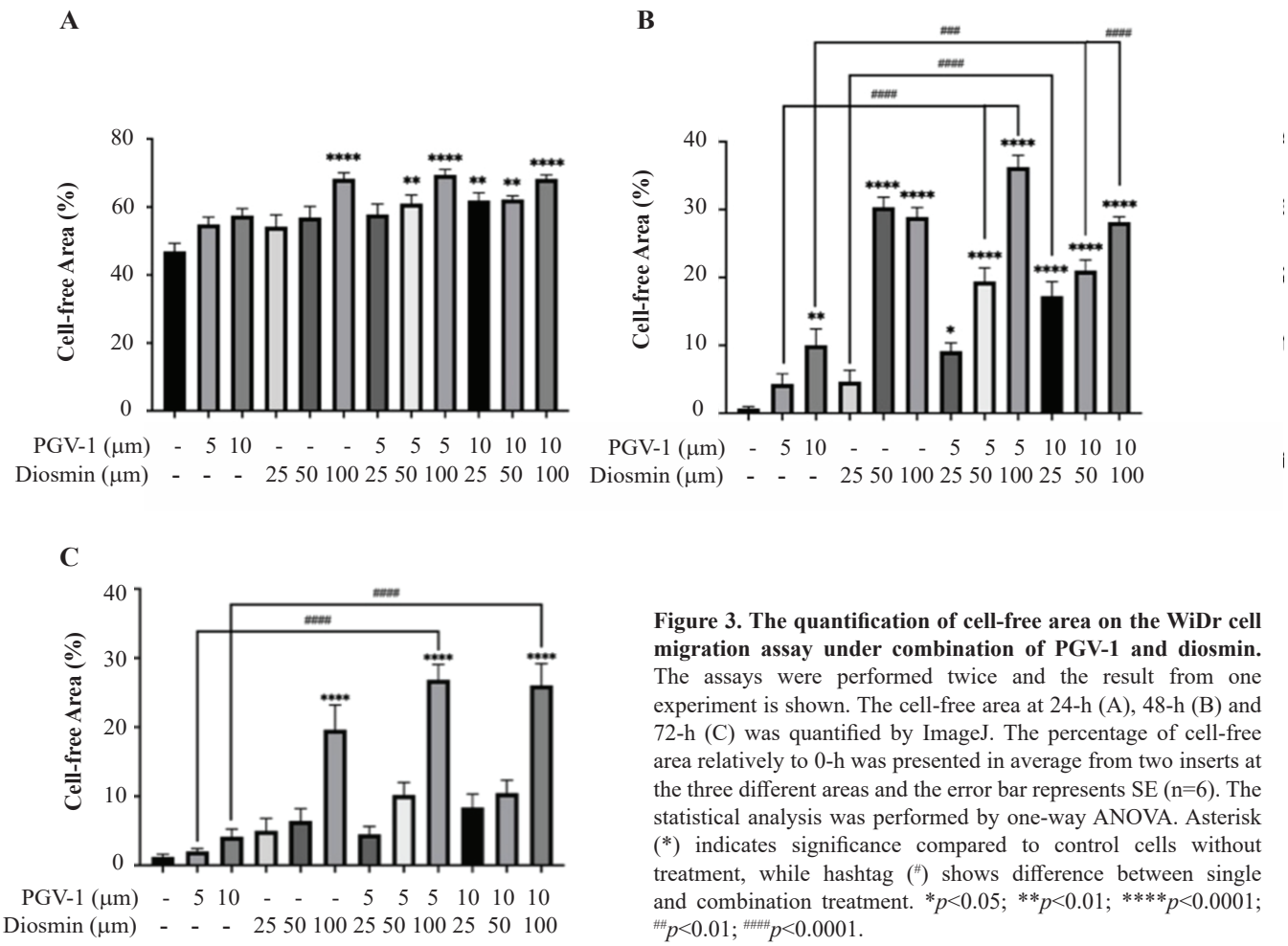

Figure 3. The quantification of cell-free area on the WiDr cell migration assay under combination of PGV-1 and diosmin. The assays were performed twice and the result from one experiment is shown. The cell-free area at 24-h (A), 48-h (B) and 72-h (C) was quantified by ImageJ. The percentage of cell-free area relatively to 0-h was presented in average from two inserts at the three different areas and the error bar represents SE (n=6). The statistical analysis was performed by one-way ANOVA. Asterisk (*) indicates significance compared to control cells without treatment, while hashtag (#) shows difference between single and combination treatment. *p<0.05; **p<0.01; ****p<0.0001; ##p<0.01; ####p<0.0001.

100 μM diosmin (p<0.0001) (left panel). We attested that when combined, diosmin improved PGV-1's capacity to quench MMP-9 and MMP-2 activity in WiDr cells.

The Molecular Interaction Prediction between PGV-1 or Diosmin and MMP Proteins

MMPs contributed as the main facilitator of cell invasion and metastasis by degradation of ECM. We speculated that PGV-1 and/or diosmin affect MMPs' activity through direct binding interaction. To confirm our hypothesis, we conducted molecular study by simulating the affinity of our compounds toward several MMPs such as MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9 focusing on the catalytic binding site. For the validation, we performed re-docking study using native ligands referred to selective and/or non-selective MMP inhibitors (Table 1). All native ligands showed RMSD≤2 Å suggesting that our molecular docking model was suitable for the virtual screening (Table 2). In all MMPs, diosmin possessed lower docking scores, followed by PGV-1 and native ligand (Table 1), implying that diosmin and PGV-1 possessed more potent binding affinity than the native ligand. Diosmin and PGV-1 formed more essential hydrogen bond with several amino acids in catalytic binding site such as Pro238, His120, and His229. Histidine and proline in MMPs formed triad which chelated Zn for activation of MMPs catalytic.(24) Taken together, we postulated that PGV-1 or diosmin could interact with MMP-1, 2, 3, 7, and 9 in a competitively fashioned manner with the native ligand, thus lessen their proteinase activities.

Discussion

Here we report the cytotoxicity of PGV-1 in WiDr cells with an IC_{50} value of 17 μM (~6 μg/mL) at 24-h that is similar with previously reported as much as 12 μM (8) or 18 μM (6). The broad difference of PGV-1's IC_{50} values in WiDr cells among these studies with our earlier work (15) with a value of 0.1 μM probably caused by the difference of the cell viability assay, in which the cell exclusion dye with a direct counting was used instead of the enzymatic colorimetry. Accordingly, the IC_{50} value of diosmin is greater than 100 μM in previous

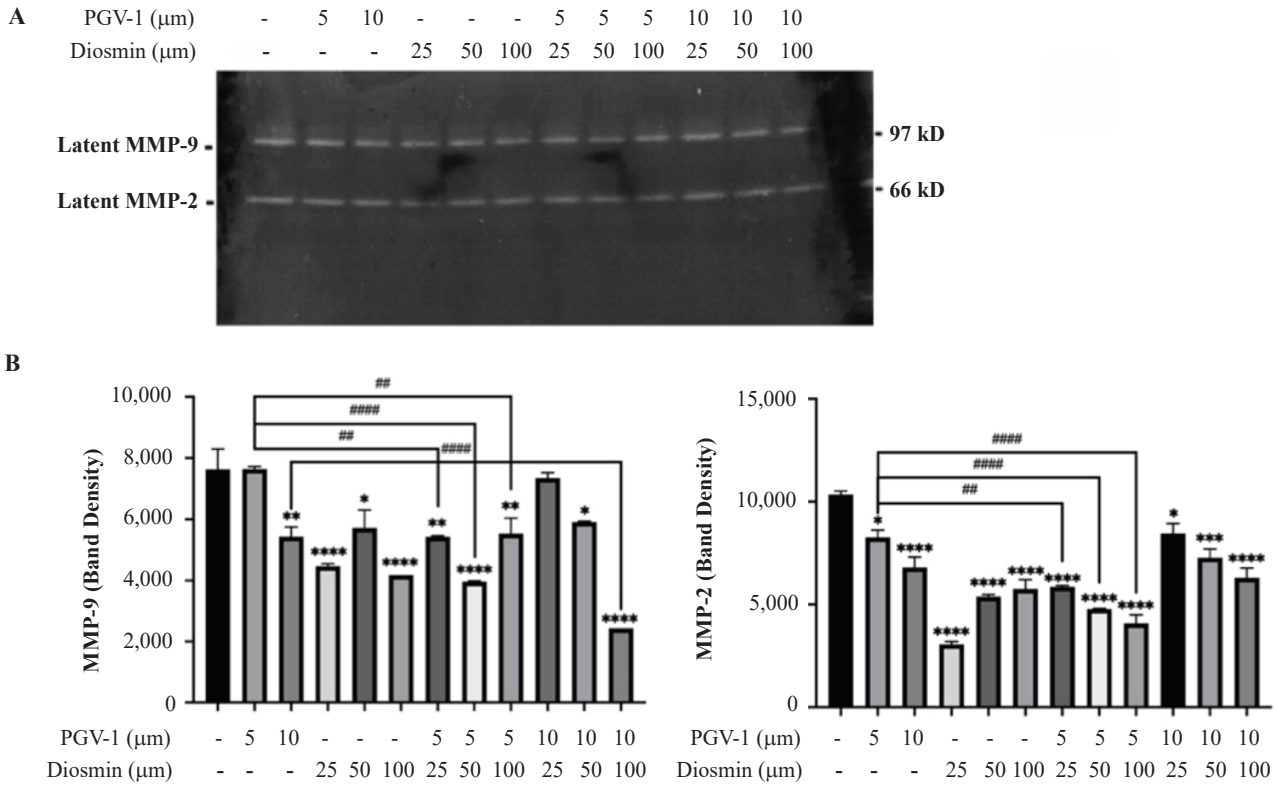

Figure 4. The effect of PGV-1 and diosmin combination on MMPs' expression based on the gelatinolytic activities. The cells were treated in the starvation medium for 24-h and the culture medium was collected. The cells were also harvested and proceeded for the total protein measurement. The culture media at a volume normalized to the total protein contents were resolved onto a gelatin zymography gel (A). The zymogram was analyzed by densitometer and the results are presented in the bar graph (B). The assays were performed twice and the result from one experiment is shown. The band density is shown as mean±SE from a triplicate measurement. The statistical analysis was performed by one-way ANOVA. Asterisk (*) indicates significance compared to control cells without treatment, while hashtag (#) shows difference between single and combination treatment. *p<0.05; **p<0.01; ****p<0.0001; ##p<0.01; ####p<0.0001.

report (15) and so does in this current investigation with an extrapolated value of 768 µM (~467 µg/mL), that is not cytotoxic. Unlike the combination study of PGV-1 with other natural compound that focusing in measuring the synergism by calculating the combination index (CI), such as the one that recently reported (25), this study heavily concentrates toward the anti-migration only. However, such experiments may helpful, *i.e.*, the cytotoxic combination assay at 24-, 48-, and 72-h [8], then CI calculation is applied to obtain the best concentration combination.

According to our initial theory, PGV-1 and diosmin's synergistic anti-migration function would likewise be a reflection of their synergistic combination. Indeed, here we demonstrated that starting from 24-h and at least up to

Table 1. Docking score of PGV-1 and diosmin towards MMP proteins.

Compound	Docking Score (Gibbs Energy, kcal/mol)				
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9
Native ligand					
RS-104966	-11.20	-	-	-	-
SC-74020	-	-14.57	-	-	-
SM-25453	-	-	-14.01	-	-
Compound II	-	-	-	-18.37	-
MS-560	-	-	-	-	-11.36
PGV-1	-14.56*	-15.02*	-14.90*	-8.19	-16.71*
Diosmin	-17.93*	-18.21*	-18.74*	-15.61*	-21.75*

*lower energy compared to the native ligand.

Table 2. RMSD value of the native ligand.

Compound	RMSD (Å)				
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9
Native ligand	0.653	1.078	1.083	1.084	0.820

72-h the addition of diosmin as little as 25 μM is adequate to increase the anti-migration property of PGV-1 in WiDr cells. This anti-migration property is hand in hand with the decrease in MMP-9 and MMP-2 proteolytic activity. We could not detect the active form of MMP-9 and MMP-2 in zymogram, but the molecular interaction of the tested compounds with MMP proteins was predicted by using the active form, thus indicating that PGV-1 and diosmin may also block the enzymes' activity in degrading ECM. Further confirmation by measuring the transcript levels of MMP-9 and MMP-2 is also valuable.(20)

ECM is a complex network composed of several macromolecules, including collagen, arranged according to the specific needs of each cell-tissue.(26) The components of the ECM are continuously being deposited, degraded, or altered, a process that altogether known as "remodelling". The ECM is degraded by degrading enzymes MMPs, as these MMPs are substrates of collagens and other components of ECM.(26) Tumors cause changes in the ECM composition and density, and are associated with changes toward both stiffness and degradation.(27) Nearly every cell in the tumor microenvironment (TME) interacts with the cells, which may allow them to modify elements of ECM in a way that promotes tumor growth. The stiffness and breakdown of ECM lead to angiogenesis, migration, and proliferation of cancer cells. Cancer cell invasion is facilitated by ECM stiffness in a mechanism connected to transforming growth factor-β (TGF-β) that may create a bridge in the basement membrane and ECM degradation MMP pathway, which could open a passage in the TME. The ability of MMPs, specifically MMP-2 and MMP-9, to destroy ECM components plays a role in the growth and metastasis of colon cancer.(28)

In this report we showed that diosmin is able to enhance the anti-migration activity of PGV-1 in the wound healing assay as reflected by the significant decrease in the wound (cell-free) area. Align with that, the gelatinolytic activity of ECM-degrading enzymes, MMP-2 and MMP-9, is also reduced significantly as indicated by a reduction in the clear band of the zymogram by the PGV-1 and diosmin combination. We mimic the ECM by incorporating gelatin, a denaturing form of collagen, into the polyacrylamide gel. Furthermore, diosmin predictively binds to MMP proteins, including MMP-2 and MMP-9, with a higher binding affinity than that of PGV-1 or native ligands. This implies that the enhancement of the anti-migration activity of PGV-1 by diosmin is indispensable to the inhibition of MMP-2 and MMP-9's activity and it is possibly caused by the direct binding of diosmin to MMPs.

At this point, the exact mechanism how diosmin enhances the anti-migration activity of PGV-1 in WiDr colorectal cancer cells remains unclear. It is interesting to examine the interaction of PGV-1 with MMP proteins with the presence of diosmin, and *vice versa*. Those kind of observation can be rendered in the computational setting. However, a pull-down assay experiment (29) would also provide a clearer evidence. The follow-up investigations on the molecular mechanism and determining the precise combination concentration are crucial, but this current report contributes in establishing diosmin as one of the suitable combination agent for PGV-1 for treating colorectal cancer, especially when there is a chance of metastases.

Conclusion

When combined, diosmin can increase the anti-migration capacity of PGV-1 in WiDr cells, potentially through influencing the production and function of MMP-9 and MMP-2. As a result, this study provides more evidence that diosmin is a prominent co-chemotherapy candidate for PGV-1, which can be used to treat colorectal cancer particularly with metastatic risk.

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Authors Contribution

The study was conceptualized and planned by MI, EM, and CO. RYU was responsible for PGV-1 synthesis. MI and RYU performed the data acquisition/collection, calculated the experimental data, and performed the analysis, for the *in vitro* and *in silico* experiments, respectively. NPH aided in the data analysis and figure preparation. MI drafted the manuscript and prepared the figures. EM and CO assisted MI in interpreting the results. All authors contributed to the critical revision of the manuscript.

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